UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA

A novel approach for therapeutic angiogenesis: low doses of ionizing radiation

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Orientadores: Professora Doutora Susana Constantino Rosa Santos
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Tese especialmente elaborada para obtenção do grau de Doutor em **Medicina**
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As opiniões expressas nesta publicação
são da exclusiva responsabilidade do seu autor.

This thesis is dedicated to my wife, Isabel, and my children, Laura and Augusto, in the hope that they will understand, one day, my many absences. Consciously, I sacrificed for this profession time that I could have dedicated to you, but I believe that it was for a good reason. Science and Medicine, in particular, is a calling for those who are willing to give their lives to the work, without reservations, like secular priests, devoted to the research and development of new technologies to be used in the diagnosis and cure of the suffering. I honestly hope that one day you will be proud of my work and of myself.

As a medical doctor, I live and feel medicine.

As a researcher, I fulfilled my first mission.

May God help you to fulfill yours.

“To serve and to heal through education research and humane care.”

JOHN P. MCGOVERN

PREFACE

The present PhD thesis comprises seven chapters, preceded by a summary, both in Portuguese and English and an abbreviation list.

Chapter I consists of a general introduction, based on literature review, which facilitates the comprehension of the research findings described in the following chapters. Five main topics are depicted along this introduction. In the first topic, an overview of peripheral arterial disease (PAD) is addressed, discussing PAD's clinical manifestations, pathophysiological circumstances and therapeutic options. Special emphasis is given to the failure of the currently available medical-surgical therapies taking a significant number of "non-option patients" to a major amputation, the only treatment option. In the second topic, a comprehensive description of angiogenesis, its regulators and role in the disease is presented. The third topic is focused on vasculogenesis and special attention is paid to endothelial progenitor cells (EPCs) and mesenchymal stromal cells (MSCs). The fourth topic focused on therapeutic angiogenesis, describing numerous clinical trials that have been performed in the field of gene and cell therapies. In the fifth topic, a brief overview of ionizing radiation is presented, highlighting the effects of high and low doses of ionizing radiation in angiogenesis.

Following the introduction, the purposes of this PhD thesis are presented in **Chapter II**. Contents of **Chapters III to VI** comprise three research articles and one ongoing clinical trial, which is based on the experimental work performed along this PhD project.

Chapter VII comprises the concluding remarks and future perspectives.

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> "O fim duma viagem é apenas o começo doutra. É preciso ver o que não foi visto, ver outra vez o que se viu já, ver na Primavera o que se vira no Verão, ver de dia o que se viu de noite, com sol onde primeiramente a chuva caía, ver a seara verde, o fruto maduro, a pedra que mudou de lugar, a sombra que aqui não estava. É preciso voltar aos passos que foram dados, para os repetir, e traçar caminhos novos ao lado deles. É preciso recomeçar a viagem. Sempre. O viajante volta já."
>
> JOSÉ SARAMAGO

Uma tese de doutoramento é o recomeço de uma longa viagem. Um momento de retiro e meditação. Uma oportunidade singular para revisitar e clarificar ideias, para construir o enredo de um projeto científico desafiante, com muitos percalços, em que várias pessoas, de uma forma ou de outra, convergiram para me dar o ânimo e a força sem os quais nenhuma tarefa consegue obter êxito. Embora uma tese seja, pela sua finalidade académica, um trabalho individual, há contributos que não podem e não devem deixar de ser realçados. Por essa razão, desejo expressar os meus sinceros agradecimentos:

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RESUMO

A doença arterial periférica (PAD, *peripheral arterial disease*) de etiologia aterosclerótica, afeta 3 a 10% da população em geral e cerca de 15 a 20% dos indivíduos com mais de 70 anos de idade. A isquemia crítica dos membros inferiores (CLI, *critical limb ischemia*) representa a manifestação clínica mais grave da PAD e é caracterizada por dor em repouso, com ou sem perda tecidual (úlceras e/ou gangrena). A CLI resulta de uma perturbação grave, quer da macrocirculação quer da microcirculação e está associada a elevadas taxas de morbimortalidade: cerca de 30% dos doentes irão sofrer uma amputação no primeiro ano após o diagnóstico, enquanto outros 25% irão morrer de causa cardiovascular.

O tratamento da CLI deverá tomar em consideração não só os efeitos da aterosclerose a nível da circulação periférica, mas também a sua natureza sistémica. Deste modo, a sua terapêutica deve incluir o controlo dos fatores de risco para a doença cardiovascular, controlo da dor, gestão de úlceras isquémicas, assim como a revascularização do membro afetado. Apesar da cirurgia de revascularização ser o pilar fundamental do tratamento e salvação do membro, muitas vezes não é exequível, devido à presença de comorbilidades médicas significativas, ou devido à ausência de critérios anatómicos que viabilizem a intervenção, estando concomitantemente associada a elevadas taxas de morbi-mortalidade. Os doentes com isquemia grave, sem condições para a realização de uma cirurgia de revascularização ou com uma revascularização prévia mal sucedida, têm opções muito limitadas, sendo a amputação muitas vezes a única alternativa terapêutica. Assim, é cada vez maior a necessidade de criar e desenvolver novas opções terapêuticas, que permitam a salvação de membro nos doentes com CLI.

Estudos em modelos animais e diversos ensaios clínicos indicam que vários dos fatores de risco para a PAD, em particular a idade e a diabetes (DM), estão associados a níveis diminuídos de fatores de crescimento pró-angiogénicos, assim como, a deficiências a nível das células progenitoras (EPCs, *endothelial progenitor cells*). O défice destes fatores poderá contribuir para o estabelecimento e progressão da PAD.

A angiogénese terapêutica é um conceito que se baseia no pressuposto de que o crescimento de novos vasos sanguíneos pode ser estimulado pela administração direta ou indireta de fatores de crescimento e/ou de células estaminais multipotentes. Embora os resultados dos ensaios clínicos iniciais tenham sido promissores,

estudos mais recentes randomizados e controlados têm mostrado resultados menos consistentes. As explicações para estes resultados incluem limitações associadas: (i) aos modelos animais utilizados na investigação básica; (ii) à seleção de doentes e a questões relacionadas com as diferentes etapas de preparação e administração de genes ou células estaminais (por exemplo, utilização de um único agente pró-angiogénico), e (iii) finalmente, à incapacidade das células endoteliais (ECs, *endothelial cells*) residentes responderem ao estímulo pró-angiogénico, processo designado por disfunção endotelial.

Atualmente, a vertente celular da angiogénese terapêutica tem ganho preponderância. De entre as células candidatas à melhor escolha para a terapia celular, as células estaminais mesenquimatosas (MSCs, *mesenchymal stromal cells)*, devido à sua eficaz expansão *ex vivo*, atividade parácrina pró-angiogénica/ vasculogénica, transdiferenciação e propriedades imunossupressoras associadas a baixa imunogenicidade, são consideradas uma das opções mais promissoras. De entre as fontes potenciais de MSCs, o cordão umbilical apresenta inúmeras vantagens, que vão desde a colheita indolor e eticamente aceite, à elevada vitalidade e baixa imunogenecidade das células colhidas.

Em colaboração com a ECBio (Amadora, Portugal), empresa de biotecnologia, investigámos o potencial angiogénico das MSCs derivadas do cordão umbilical humano, patenteadas e denominadas UCX®. Recorrendo a um modelo de isquemia da pata posterior do ratinho C57BL/6, demonstrámos que as UCX® administradas por via intramuscular aumentam a perfusão sanguínea avaliada por Laser Doppler, estimulando a angiogénese (densidade capilar determinada por imunohistoquímica CD31) e a arteriogénese (densidade colateral determinada por diafanização). Estes resultados foram conseguidos através da descoberta de um novo mecanismo no qual se verifica que as células UCX® induzem a expressão de vários genes pró-angiogénicos nas ECs, isoladas a partir do músculo isquémico.
As propriedades fenotípicas, imunomoduladoras e angiogénicas desta população específica de MSCs, derivadas do cordão umbilical, não são afetadas pela criopreservação e subsequente descongelação, tanto *in vitro* como *in vivo*.
Com este trabalho, demonstrámos que no modelo de isquemia da pata posterior do ratinho, as UCX® estimulam o potencial angiogénico das ECs. Este estudo sugere também que o processo de produção das UCX®, patenteado pela ECBio, não interfere com as propriedades intrínsecas das UCX®. No entanto, o uso de UCX® em doentes com CLI está condicionado pela falta de trabalhos que garantam a sua segurança e a ausência de efeitos adversos significativos.

Nesta investigação, a angiogénese terapêutica foi estudada muito para além da sua vertente celular, numa perspetiva inovadora baseada na utilização de baixas doses de radiação ionizante (LDIR, *low-dose ionizing radiation*). Recentemente, demonstrámos que LDIR (<0.8 Gy) induzem um fenótipo pró-angiogénico em ECs *in vitro*, modulando a disfunção endotelial. Do mesmo modo, demostrámos que as LDIR promovem a neovascularização *in vivo* durante o desenvolvimento embrionário e na regeneração da barbatana caudal do peixe zebra. Com base nestes resultados, um dos objetivos deste trabalho foi o de avaliar se as LDIR poderiam ser utilizadas como uma nova opção no arsenal terapêutico da CLI.

Com este objetivo, recorremos novamente ao modelo de isquemia da pata posterior do ratinho C57BL/6. Após a indução da isquemia, os ratinhos foram irradiados ou não com 0.3 Gy, durante 4 dias consecutivos. Os resultados das nossas experiências sugerem que as LDIR podem ter uso clínico no tratamento da PAD através da (i) estimulação, mediada pelo recetor 2 do fator de crescimento endotelial vascular (VEGFR2, *vascular endothelial growth factor receptor 2*), da expressão de vários genes pró-angiogénicos nas ECs, aumentando a densidade capilar e (ii) mobilização e recrutamento de EPCs, via aumento das concentrações séricas do fator de crescimento endotelial vascular, fator de crescimento placentário e fator estimulador de crescimento de colónias de granulócitos, levando a um aumento da densidade colateral que por sua vez melhora a perfusão sanguínea do membro isquémico.

Uma vez que o tratamento com radiação ionizante envolve o risco de alterações genéticas e carcinogénese, os possíveis efeitos tóxicos das LDIR foram avaliados e constatou-se que, após 52 semanas, as LDIR não induzem toxicidade significativa em ratinhos C57BL/6. No intuito de se identificar a menor dose de radiação ionizante capaz de induzir angiogénese terapêutica, foram avaliadas diferentes doses e diferentes frações de dose. Os nossos resultados mostram que uma dose de 0.3 Gy administrada durante 4 dias consecutivos, constitui a menor dose capaz de induzir um aumento significativo da taxa de reperfusão no modelo de isquemia da pata posterior do ratinho.

De acordo com estes resultados, propusemo-nos realizar um ensaio clínico exploratório – *Low-dose ionizing radiation modulates the expression of pro-angiogenic genes in Critical Limb Ischemia Patients*, com o qual pretendemos analisar os efeitos pró-angiogénicos destas LDIR em doentes com CLI, cuja única opção terapêutica é a amputação do membro isquémico.

Até à data foram recrutados 16 doentes, dos quais apenas 12, correspondendo a 13 membros, foram considerados para análise. Uma vez que o número de amputações previsto para análise do objetivo primário ainda não foi atingido, o ensaio clínico encontra-se atualmente em fase de recrutamento.
A análise dos resultados preliminares mostra uma sobrexpressão dos fatores pró-angiogénicos: fator de crescimento dos hepatócitos, angiopoietina 2 e *VEGFR2* nas ECs isoladas dos membros irradiados, quando comparados com os controlos. Pelo contrário, o recetor 1 do fator de crescimento endotelial vascular (*VEGFR1*), um competidor do *VEGFR2*, tem a sua expressão diminuída. Estes resultados sugerem que as LDIR induzem um efeito pró-angiogénico através da modulação de vários fatores pró-angiogénicos nas ECs. Estes resultados são corroborados histologicamente por uma tendência que se verifica no sentido de um aumento da densidade capilar e, angiograficamente, por um aumento significativo da densidade vascular (P = 0,03), trinta dias nos músculos irradiados quando comparados com os controlos. Nos doentes não amputados, é interessante salientar que, após seis meses de seguimento, o músculo isquémico irradiado foi o único que mostrou um aumento da densidade vascular quando comparado com os controlos.
Conforme esperado, em virtude da população selecionada, trinta dias pós-intervenção não se verificaram diferenças significativas no que diz respeito aos parâmetros de avaliação clínica.

De acordo com estes resultados, e tomando em consideração que 40% dos doentes com CLI não são candidatos a um procedimento de revascularização, as LDIR podem ser consideradas como uma nova abordagem no seu manuseamento terapêutico. O sucesso deste primeiro ensaio clínico levará ao desenvolvimento de futuros ensaios clínicos com o objetivo de propor uma ferramenta terapêutica nova e efetiva com impacto real no tratamento da DAP.

Palavras-chave: *Angiogénese terapêutica, Doença arterial periférica, Isquemia crítica dos membros inferiores, Baixas doses de radiação ionizante, UCX®.*

ABSTRACT

Peripheral arterial disease (PAD) is a vascular occlusive disease accompanied by an insufficient angiogenic response, resulting in hypoperfusion of the affected extremities. When arterial lesions impair blood flow to such an extent that the nutritive requirements of the tissues cannot be met, limb pain is chronically present at rest, frequently with trophic and necrotic skin lesions. This condition corresponds to the most advanced clinical stage of PAD, known as critical limb ischemia (CLI).

Although just a minority of PAD patients progress to CLI, as PAD is highly prevalent among diabetics, smokers and people over 70 years, this condition remains very common, with 500 to 1000 new cases per million inhabitants every year, in the developed countries.

The goals of CLI treatment are ischemic pain relief, ulcer healing, improvement of muscle function and quality of life, limb loss prevention, and overall survival increase. The most important aspect of CLI is the poor prognosis, no matter what treatment is employed. Any kind of surgical/endovascular revascularization, the therapy of choice in CLI patients, should be done whenever technically possible. Attempts to manipulate and normalize the microcirculatory flow pharmacologically may enhance the results of revascularization or be one option in patients in whom revascularization is impossible or has failed. In patients with CLI not eligible for arterial revascularization, the “non-option” patients, most pharmacological agents have reduced or no real effect. Amputation is often recommended, even if it is associated to morbidity and mortality. The need for alternative treatment in CLI patients is therefore compelling, and therapeutic angiogenesis is an undoubtedly promising tool to treat these patients.

There are three major processes that contribute to neovascularization and are essential for the establishment of functional collateral networks, namely angiogenesis, arteriogenesis and vasculogenesis. While angiogenesis describes the process of growth of new blood vessels from pre-existing ones, arteriogenesis is characterized by the enlargement of arteriolar anastomoses to collateral vessels through growth and proliferation and vasculogenesis is *de novo* formation of blood vessels. These vessels can grow considerably, enough even to take over the role of a large artery when occluded.

Several strategies have been pursued to stimulate therapeutic angiogenesis, ranging from recombinant protein and gene transfer therapies to the use of progenitor cell therapies.

During this research, in collaboration with the biotechnology company ECBio (Amadora, Portugal), cell-based therapeutic angiogenesis was addressed, as we evaluated the possibility of UCX®, a specific population of human umbilical cord derived-mesenchymal stromal cells (UC-MSCs), to induce therapeutic angiogenesis. Using a C57BL/6 mouse model of hindlimb ischemia (HLI), we demonstrated that UCX® delivered via intramuscular injection enhance blood perfusion (evaluated by laser Doppler imaging) by stimulating angiogenesis (capillary density determined by CD31 immunohistochemistry) and arteriogenesis (collateral vessel density determined by diaphonization) in ischemic muscles. This is achieved through a new mechanism in which durable and simultaneous up-regulation of several pro-angiogenic genes in endothelial cells (ECs) are induced by UCX®.

Cryopreservation and subsequent thawing, both *in vitro* and *in vivo*, did not impair phenotype, immunomodulatory and angiogenic potencials of this specific UC-MSCs population.

These data demonstrate that UCX® improve the angiogenic potency of ECs in the murine ischemic limb, suggesting the potential of UCX® as a new therapeutic tool for CLI. This study also suggests that potency impairment related to cryopreservation in a given tissue source can be avoided by the production process. The results have positive implications for the development of an advanced therapy medicinal product. However, the use of UCX® in patients with CLI is conditioned by the lack of studies to ensure its safety and the absence of significant adverse effects.

The scope of our work, in the search for a new therapeutic approach that could overcome the current lack of available medical treatments for a large number of CLI patients, led us to consider therapeutic angiogenesis beyond its cellular component, in a unique perspective based on the use of low-dose ionizing radiation (LDIR).

Our previous research has demonstrated that LDIR (<0.8 Gy) induces a pro-angiogenic phenotype in ECs *in vitro,* modulating endothelial dysfunction, promoting survival, migration and preventing ECs apoptosis. Likewise, LDIR promotes neovascularization *in vivo* by inducing angiogenic sprouting in the transgenic fluorescent zebrafish Tg (fli1:EGFP) embryos and by increasing vessel density in adult fli1:EGFP zebrafish after caudal fin regeneration. Therefore, according to our previous results, LDIR induces angiogenesis *in vitro* and *in vivo*; however, there is no

evidence so far, that it enhances neovascularization in vascular occlusive disease, our overall objective.
After surgical induction of unilateral HLI, both hindlimbs of female C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days and limb perfusion, capillary density and collateral vessel formation were measured. We found that LDIR (4 x 0.3 Gy) improves limb perfusion by enhancing arteriogenesis through EPCs recruitment to sites of collateral vessel development, an effect dependent on exposure of the ischemic niche to LDIR, but not on the local recruitment of myeloid cells. Likewise, LDIR also favours angiogenesis through simultaneous activation of a repertoire of pro-angiogenic factors in mature ECs in a mechanism dependent of VEGFR signaling, with no short-term side effects and no effects on resting vasculature, opening a possibility to new therapeutic strategies in lower limb vascular insufficiency. The vasculature in an irradiated non-ischemic bed was not affected and after 52-wk of LDIR exposure no differences in the incidence of morbidity and mortality were noticed.
Moreover, it was found that a dose of 0.3 Gy administered during 4 consecutive days does not induce toxicity in C57BL/6 mice and this dose fraction was identified as the lowest dose that is still able to induce therapeutic angiogenesis.

The outcome of these *in vivo* experiments performed in a mice model suggests that LDIR may have a potential clinical use in the treatment of lower limb vascular insufficiency, emerging as a novel approach in the treatment of CLI patients. Accordingly, we designed an already approved and ongoing clinical trial – *Low-dose ionizing radiation modulates the expression of pro-angiogenic genes in Critical Limb Ischemia Patients* – and report our preliminary results.
To date, 16 “non-option” CLI patients were enrolled in the study, but only 12 patients, corresponding to 13 limbs, were considered for analysis. As the expected amputation number (12 major limb amputations) for analysis of the primary endpoint was not reached, the trial is still ongoing.

Concerning the primary endpoint, preliminary results suggest that LDIR induces a pro-angiogenic effect through the modulation of several pro-angiogenic factors in ECs collected from “non-option” CLI gastrocnemius muscles.
The primary endpoint is corroborated by the finding that LDIR is associated with an increase in capillary density and a significant increase (P = 0.03) in vessel density (VD), 30 days post-intervention. It is also interesting to report that in the non-amputated patients, 6 months after irradiation, only the one muscle exposed to LDIR showed a persistent and continuous increase of VD.

Regarding the surrogate clinical endpoints of ischemia and as expected, no significant differences were found between LDIR limbs and controls.

LDIR has a biological rationale widely investigated and discussed in the work developed by our research group. Therefore, based on these preliminary results and considering that up to 40% of CLI patients are not candidates to revascularization, LDIR may be considered as a novel approach for the management of these patients.

Keywords: *Therapeutic angiogenesis, Peripheral arterial disease, Critical limb ischemia, Low-dose ionizing radiation, UCX®.*

ABBREVIATIONS

ABI	ankle-brachial systolic index
Akt	Akt serine/threonine kinase 1
AI	arthritic index
AIA	adjuvant-induced arthritis model
ANG	angiopoietin
ATMP	advanced therapy medicinal product
AT-MSCs	adipose tissue-derived mesenchymal stromal cells
BM	bone marrow
BM-MNCs	bone marrow-derived mononuclear cells
BM-MSCs	bone marrow-derived mesenchymal stromal cells
CAD	coronary artery disease
CCUL	centro de investigação cardiovascular da universidade de Lisboa
CHLN	centro hospitalar lisboa norte
CLI	critical limb ischemia
CM	conditioned medium
$CoCl_2$	cobalt chloride
CRF	case report form
CVD	collateral vessels density
CXCR	c-x-c motif chemokine receptors
DLL	delta-like
DM	diabetes mellitus
DNA	deoxyribonucleic acid
EBM	endothelial basal medium
ECM	extracellular matrix
ECs	endothelial cells
EGM	endothelial growth medium
ELISA	enzyme linked immunosorbent assay
EOCs	endothelial outgrowth cells
EPCs	endothelial progenitor cells
ERK	extracellular signal regulated kinase
ESRD	end-stage renal disease
FACS	fluorescent activated cell sorter
FBS	foetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FMUL	faculdade de medicina da universidade de Lisboa
G-CSF	granulocyte colony-stimulating factor

GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	good manufacturing practices
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HLA	human leukocyte antigen
HLI	hindlimb ischemia
HMEC-L	lung human microvascular endothelial cells
HUVECs	human umbilical vein endothelial cells
IC	intermittent claudication
IL	interleukin
IQR	interquartile range
ISCT	international society for cellular therapy
KitL	kit ligand
LCM	laser capture microdissection microscope
LDL	low density lipoprotein
LDIR	low-dose ionizing radiation
LNT	linear no-threshold
MAPK	mitogen-activated protein kinases
MCP	monocyte chemotactic protein
MMPs	matrix metalloproteinases
MNCs	mononuclear cells
mo	month
MSCs	mesenchymal stromal cells
MV	mega-electron volt
n	number
NO	nitric oxide
NOS	nitric oxide synthase
NTE	non-DNA targeted effects
PAD	peripheral arterial disease
PB	peripheral blood
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCA	principal component analysis
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PIGF	placental growth factor
PI3K	phosphatidylinositol 3-kinase
POST-HLI	post-hindlimb ischemia
PRE-HLI	before hindlimb ischemia

PTK/ZK	PTK787/ZK222584
qRT-PCR	quantitative real-time polymerase chain reaction
rAAV	recombinant adenoassociated virus
rABI	post-treatment/baseline ankle-brachial systolic index ratio
RNA	ribonucleic acid
ROI	region of interest
RT	room temperature
SD	standard deviation
SDF	stromal-derived factor
S1P1	sphingosine 1 phosphate 1
TBI	toe-brachial systolic index
TGFβ	transforming growth factor β
TGFβR	transforming growth factor β receptor
TIE	tyrosine-protein kinase receptor of angiopoietin
TPS	treatment planning system
UC-MSCs	umbilical cord tissue-derived mesenchymal stromal cells
VCAM1	vascular cell adhesion molecule 1
VD	vessel density
VD pre-Int	vessel density pre-intervention
VD post-Int	vessel density post-intervention
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

TABLE OF CONTENTS

LIST OF TABLES & FIGURES

1. GENERAL INTRODUCTION

TABLES

FIGURES

3. RESEARCH ARTICLE: THERAPEUTIC ANGIOGENESIS INDUCED BY HUMAN UMBILICAL CORD TISSUE-DERIVED MESENCHYMAL STROMAL CELLS IN A MURINE MODEL OF HINDLIMB ISCHEMIA

TABLES

FIGURES

4. RESEARCH ARTICLE: UMBILICAL CORD TISSUE–DERIVED MESENCHYMAL STROMAL CELLS MAINTAIN IMMUNOMODULATORY AND ANGIOGENIC POTENCIES AFTER CRYOPRESERVATION AND SUBSEQUENT THAWING

FIGURES

5. RESEARCH ARTICLE: LOW-DOSE IONIZING RADIATION INDUCES THERAPEUTIC NEOVASCULARIZATION IN A PRE-CLINICAL MODEL OF HINDLIMB ISCHEMIA

TABLES

FIGURES

6. ONGOING CLINICAL TRAIL: LOW-DOSE IONIZING RADIATION MODULATES THE EXPRESSION OF PRO-ANGIOGENIC GENES IN CRITICAL LIMB ISCHEMIA PATIENTS

TABLES

FIGURES

1

GENERAL INTRODUCTION

1.1. PERIPHERAL ARTERIAL DISEASE

Peripheral arterial disease (PAD), also referred to as peripheral vascular disease or lower limb ischemia, is a global problem that can have potentially devastating consequences like loss of limbs or even death. PAD is mainly the result of atherosclerosis (PAD causes – Table 1). Surveys have shown that 3% to 10% of the general population experiences PAD and that 15% to 20% of individuals over 70 years old suffer from this disease in the contemporary developed communities[1]. There are three major factors responsible for the recent increase in PAD prevalence: (i) the general ageing of the population in developed countries; (ii) the rapid increase of diabetes mellitus (DM) among the younger generations, and (iii) the increasing number of patients who have undergone prior PAD revascularization and are potentially at risk for either graft or stent failure or disease progression.

Table 1. Causes of Peripheral Arterial Disease[a]

Causes of Arterial Occlusive Lesions in the Lower Extremity
Atherosclerosis – occlusive or aneurysmal
Embolic disease
Trauma
Persistent sciatic artery
Thromboangiitis obliterans – Buerger´s disease
Cystic adventicial disease
Fibromuscular disease
Popliteal artery entrapment
Arteritis
Congenital and acquired coarctation of aorta
Primary vascular tumors

[a] *Adapted*[2].

Patients diagnosed with PAD have a 3% higher mortality risk and a 6% higher risk to succumb from cardiovascular disease than the general population[3]. This risk is equivalent in men and women and remains high, even when the patient does not present clinical evidence of cardiovascular disease. The increased severity of the disease, determined by the ankle-brachial systolic pressure index (ABI), is accompanied by an increased risk of acute myocardial infarction, stroke, and cardiovascular ischemic death (Figure 1).

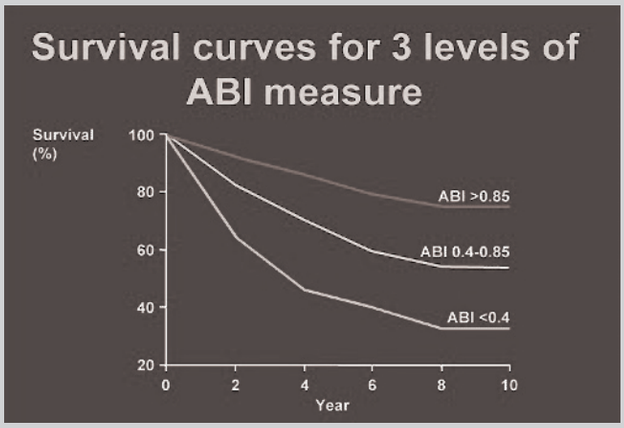

Figure 1. Survival Curves for 3 Levels of Ankle-Brachial Systolic Pressure Index Measures[a]

[a]Adapted[3].

The diagnosis of PAD, even in the absence of coronary artery disease (CAD) history, confers the same risk of cardiovascular death as a patient with a previous cardiovascular event[4]. As such, PAD (as well as carotid obstructive disease and aneurysmal disease) is considered an equivalent of CAD. In fact, the *American Heart Association,* the *National Heart, Lung, and Blood Institute* and the *National Cholesterol Education Program* recommend the same atherosclerotic risk reduction strategies in patients with PAD and CAD[5-7] .

Accordingly, the goals of PAD treatment should consider not only the effects of atherosclerosis within the peripheral circulation, but also its systemic nature. Controlling blood pressure and glucose serum levels, use of statins and antiplatelet therapy, physical exercise and stopping smoking can reduce the atherosclerotic burden.

Symptoms of intermittent claudication (IC), distance walked without pain, and quality of life can be improved by adopting a structured and supervised exercise program. Furthermore, drug therapy can be used in selected patients.

Patients with critical limb ischemia (CLI) require restoration of pulsatile blood flow to heal wounds, relieve ischemic pain, and provide limb salvage. In patients who are candidates for surgical and/or endovascular therapy, antiplatelet and anticoagulants may be used to promote graft patency, with the administration of beta blockers being associated to a reduced risk of cardiovascular complications in the perioperative period.

As the essay progresses, the research will look in detail at the underlying PAD causes and risk factors, clinical manifestations and natural history, treatment guidelines as well as new therapeutic developments with regard to PAD.

1.1.1. Atherosclerosis

Atherosclerosis and its related diseases are major causes of death and premature disability in the developed world today. Accordingly, atherosclerosis accounts for about 90% of PAD cases. It is a disease of the arterial wall that occurs specially at certain susceptible vascular beds in the major conduit arteries. It starts with lipid retention, oxidation, and modification, provoking chronic inflammation and ultimately triggering thrombosis, leading to arterial stenosis or occlusions[8].

The different clinical manifestations of atherosclerosis depend on the particular vascular bed involved and may include angina pectoris, acute myocardial infarction, stroke, IC, CLI, aortic aneurysm, mesenteric ischemia, renovascular hypertension, and ischemic nephropathy.

Atherosclerosis is a degenerative disease, that presents a continuous and increasingly complex evolution determined by the deposition of cholesterol-rich lipids and by the resulting inflammatory response at the level of the arterial wall plaques. Lesions begin in the arterial intima and progress to affect the entire arterial wall, including the media and the adventitia. Although not fully understood at the molecular level, the concept of progressive degeneration of the atherosclerotic plaque allows us to integrate the different stages of the histological and macrovascular development, simplifying the understanding of its clinical translation (see Table 2).

It is useful, although not scientifically rigorous, to consider risk factors (Table 3) as causes of atherosclerosis. Risk factors can be divided into constitutional (not changeable) and acquired or potentially manageable (changeable). Among the risk factors classified as not changeable are age (the risk of PAD increases by 1.5 to 2 times for every 10 years), sex, family history of atherosclerosis, and race. Changeable risk factors include smoking, physical inactivity, obesity, high blood pressure (hypertension), type 2 DM, and dyslipidemia, diet rich in saturated fats and cholesterol and low in antioxidants, hyperhomocysteinemia, and coagulation disorders.

Elevated levels of the low-density lipoprotein (LDL) fraction of cholesterol were first associated with atherosclerosis in studies of familial hypercholesterolemia. High levels of circulating LDL promote endothelial activation and accumulation of lipid particles in the sub-endothelial space. Moreover, oxidative modification of these particles is a potent stimulus for inflammation, smooth muscle cell migration, and cell death.

Hypertension results from a series of integrated mechanisms, including increased arterial wall stiffness and activation of the renin-angiotensin-aldosterone system. Angiotensin II stimulates collagen deposition and extracellular matrix (ECM)

Table 2. General Categories of Atherosclerotic Lesions[a]

Lesion Name	Lesion Description by Histopathology	Thrombosis
Nonatherosclerotic intimal lesions		
1. Intimal thickening	The normal accumulation of SMCs in the intima with the absence of lipid or macrophage foam cells	Thrombus is absent
2. Intimal xanthoma or fatty streaks	The normal accumulation of SMCs in the intima with the absence of lipid or macrophage foam cells Subendothelial accumulation of foam cells in intima without necrotic core or fibrous cap; animal and human data show that such lesions usually regress	Thrombus is absent
Progressive atherosclerotic lesions		
1a. Pathologic intimal thickening	SMCs in a proteoglycan-rich matrix with areas of extracellular lipid accumulation without necrosis	Thrombus is absent
1b. With erosion	Luminal thrombosis, plaque same as above	Thrombus most often mural and infrequently occlusive
2a. Fibrous cap atheroma	Well-formed necrotic core with overlying fibrous cap	Thrombus is absent
2b. With erosion	Luminal thrombosis; plaque same as above, no communication of thrombus with necrotic core	Thrombus most often mural and infrequently occlusive
3. TCFA	A thin fibrous cap infiltrated with macrophages and lymphocytes, rare SMCs, and an underlying necrotic core	Absent, with intraplaque hemorrhage/fibrin
a. With rupture	Fibroatheroma with cap disruption; luminal thrombus communicates with underlying necrotic core	Thrombus usually occlusive
4. Calcified nodule	Eruptive nodular calcification with underlying fibrocalcific plaque	Thrombus usually occlusive
5. Fibrocalcific plaque	Collagen-rich plaque usually with significant stenosis; contains large areas of calcification with few inflammatory cells; necrotic core may be present	Thrombus is absent

[a]*Adapted*[9].
Abbreviations: *SMC, smooth muscle cell; TCFA, thin-cap fibroatheroma.*

remodeling and induces vascular cell adhesion molecule 1 (VCAM1) and monocyte chemoattractant protein 1 expression by endothelial cells (ECs), all of which are important steps in the formation of atherosclerotic lesions. Arterial stiffness leads to turbulent blood flow, particularly at bifurcations, which amplifies the endothelial dysfunction.

Insulin resistance and type 2 DM are characterized by hyperglycemia and increased free fatty acids. In addition to the lipid abnormalities that are commonly present in patients with type 2 DM, high blood glucose levels induce the formation of advanced glycosylation products and oxygen reactive species, contributing to a state of oxidative stress that promotes endothelial activation, vasoconstriction, inflammation, and hypercoagulation.

Cigarette smoking has a pathogenic action in all phases of atherosclerosis, being responsible for endothelial activation and decreased nitric oxide (NO)-mediated vasoreactivity, increased expression of inflammatory cytokines and adhesion molecules, promotion of platelet aggregation, and impairment of fibrinolytic mechanisms.

Hyperhomocysteinemia is a debated risk factor for atherosclerosis that was first implicated in atheroma formation in studies of patients with defects in enzymes responsible for homocysteine metabolism. Its proposed mechanism of action comprises suppression of NO, promotion of oxidative stress, and enhancement of platelet activation and aggregation.

Table 3. Peripheral Arterial Disease – Risk factors[a]

Major Risk Factors	Minor Risk Factors
Age Diabetes Mellitus Smoking status C reactive protein Hyperlipidemia Hypertension Hyperhomocysteinemia Hyperfibrinogenemia	Sex Race Diet Hypercoagulable status Alcoholism Asymmetric dimethylarginine

[a]*Adapted*[2].

Clinical manifestations of atherosclerosis

The earliest atherosclerotic lesions are normally devoid of clinical repercussions. In the initial phases of development, plaque growth is usually eccentric and does not encroach on the arterial lumen. Furthermore, the affected vessels tend to enlarge in diameter as a compensatory mechanism. Only after it exceeds around 40% of the area surrounded by the internal elastic lamina, does atherosclerotic plaque lead to a significant reduction in luminal diameter.

This flow-limiting stenosis is responsible for the chronic, more “stable” symptoms of atherosclerotic disease, such as stress-triggered angina pectoris and lower limb claudication. Even so, vessel occlusion can occur over time without any manifestation of clinical consequences as progressive luminal narrowing with the resulting decrease in perfusion and tissue hypoxia stimulates development of collateral vessels. In contrast, most acute or unstable atherosclerotic syndromes arise from plaque instability and rupture of the fibrous cap with subsequent thrombus formation. This is the mechanism responsible for acute ischemia in various vascular beds, like the coronary circulation, the lower limbs, and the splanchnic vessels. Fragmentation of plaque and distal embolization of atherothrombotic debris is responsible for most ischemic events in the central nervous system, like transient ischemic attacks and stroke.

1.1.2. Pathophysiology of Peripheral Arterial Disease

Treating PAD requires gaining more insight into the pathophysiology of the disease (Table 4). Most of the existing knowledge comes from *in vitro* and *in vivo* laboratory tests. Greater understanding in this area, especially with regard to the pathophysiology of PAD in humans, is complex since it involves the macrovascular and microvascular systems and surrounding tissues. Therefore, even though the disease appears first in larger arteries, the subsequent downstream chronic ischemia produces structural and functional adaptations within the small resistance arteries.

From a pathophysiologic perspective, PAD can be categorized as functional or critical. Functional ischemia occurs when blood flow is normal at rest, but becomes insufficient during exercise. This condition is clinically presented as IC. Critical ischemia occurs when blood flow is insufficient, even at rest, and is typically characterized by the presence of trophic lesions in the feet or pain while resting. Multilevel obstructive disease is usually required for CLI. This is a very serious condition since the natural history of CLI shows that the end result can lead to the loss of the affected limb.

The body’s first response to ischemia is angiogenesis, also commonly known as capillary sprouting. This is accompanied by arteriogenesis promoting the expansion of pre-existing collaterals to ensure a rise in the blood flow levels to the ischemic limb[11,12]. However, when such mechanisms fail to provide sufficient blood flow to the affected limb, the body releases endothelium-derived relaxing factor[13] that causes arteriolar vasodilation which leads to increased blood flow to the critically ischemic extremity. Thus, in patients with CLI, arterioles become maximally vasodilated and

Table 4. Peripheral Arterial Disease Pathophysiology[a]

Macrovascular Changes	Microvascular Changes
Atherosclerosis	
Arterial stenosis	
Angiogenesis	
Arteriogenesis	Decreased nitric oxide production
Increased VEGF	Increased reactive oxygen species
Increased SDF-1	Increased peroxynitrite production
Increased CXCR4 expression	Increased platelet activation
Vasomotor paralysis	Increased leukocyte adhesion
Arterial remodelling	Microvascular thrombosis
Decreased wall thickness	Precapillary arteriole collapse
Decreased cross-sectional area	Impaired oxygen exchange
Decreased wall-to-lumen ratio	
Increased skin perfusion	
Edema	

[a]*Adapted[11].*
Abbreviations: *CXCR4, CXC chemokine receptor; SDF1, stromal cell-derived factor 1; VEGF, vascular endothelial growth factor.*

insensitive to provasodilatory stimuli[12]. McEwan and Ledingham[14] contended that, due to the chronic exposure of the arterioles to vasorelaxing factors, compensatory vasodilation results in arteriolar dysfunction. They called this phenomenon "vasomotor paralysis," which has also been identified as a contributing factor to the formation of edema in CLI[13].

Edema is a major concern in PAD patients. CLI patients tend to keep their limbs in a dependent position that offers them comfort. However, in combination with loss of vasomotor control, this leads to a further exacerbation of the edema. This has a negative effect, since edema will result in an increase in the hydrostatic pressure within the distal portion of the limb, which in turn causes compression of already compromised capillaries and impairs diffusion of oxygen and nutrients to the tissue[12].

To complicate matters, in addition to macrovascular changes, microvascular dysfunction also occurs. The regulatory system of microvascular flow includes modulation by humoral and circulatory factors released by blood cells and neurogenic mechanisms of extrinsic and intrinsic local mediators. As such, endothelial function is impaired in patients with PAD resulting in reduced integrity of the blood vessels (Figure 2). CLI is known to be linked to ECs swelling, increased endothelial

permeability, and, subsequently, formation of edema[15,16]. In turn, these changes lead to a rise in the release of vascular endothelial growth factor (VEGF)[15,17].

With the damage of endothelium, there is an imbalance in the NO levels, because of which the molecules that should remain in the blood are able to pass through the blood vessels walls and into the next body tissue. One of these proteins is C reactive protein produced by the liver and that causes inflammation[18]. With the inhibited action of NO, there is impairment of the endothelial signaling which leads to a widespread disease. This can be attributed to the fact that about 60 000 miles of blood vessels are maintained by the endothelium, making it the largest gland in the human body.

Towards the end of these microvascular changes, the consequence of endothelial dysfunction can also result in microthrombosis in the capillaries that exacerbate the formation of edema in the extremity[12]. The result of endothelial trauma is an increase in free radical production, unsuitable platelet activation, as well as leukocyte adhesion resulting in microthrombi formation[12]. This ultimately leads to impeded and less effective tissue oxygen exchange at capillary level.

In summary, endothelial dysfunction is characterized by a pro-inflammatory state with prothrombotic proprieties (Figure 2).

The final stage of the events resulting in the decreased capillary perfusion in CLI is yet to be established. The possible causes include precapillary arteriole collapse caused by aggregation of platelets and blood cells, arteriolar vasospasm, leukocyte adhesion, platelet aggregation, capillary occlusion by endothelial edema, collapse of capillaries through interstitial edema and microthrombosis.

Patients will benefit from blood flow restoration, which is necessary for limb salvage or wound healing to occur. Nevertheless, the simple reinstating of blood flow on a macrovascular level cannot reverse these imbalances. With this, only reactive hyperemia is initiated and an already complex problem may be further exacerbated with a cascade of events[12]. Thus, multiple factors must be considered when treating CLI patients on a case-by-case basis so that the optimal course of action can be sorted out.

Arterial structure may be somewhat affected by the prolonged and constant activation of the vasodilator compensation mechanism through the release of the endothelium-derived relaxing factors that inhibit protein synthesis. This may be beneficial in the early phases of CLI; nevertheless, in time, this mechanism may indirectly have a detrimental effect on artery function due to the inhibition of protein synthesis. Additionally, the reduction in blood flow that initially helps to maintain arterial wall structure, also contribute the atrophic changes noted in the arteries harvested from the ischemic vascular beds of patients with CLI[20].

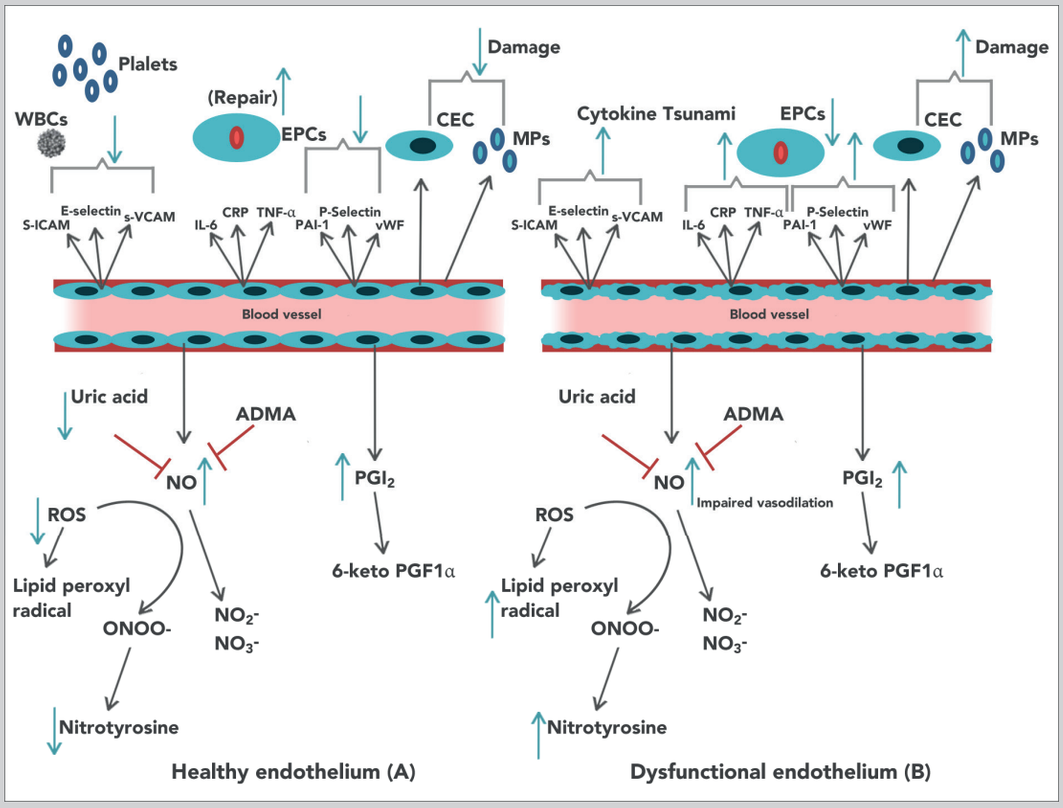

Figure 2. Healthy Endothelium and Dysfunctional Endothelium[a]

Differences between (A) healthy endothelium and (B) a dysfunctional one. Healthy endothelium presents: (i) a vasodilatory phenotype with high levels of vasodilators such as nitric oxide (NO) and prostacyclin (PGI2) and low levels of reactive oxygen species (ROS) and uric acid; (ii) an anticoagulative phenotype with low levels of plasminogen activator inhibitor 1 (PAI-1), von Willebrand factor (vWF), and P-selectin; (iii) reduced inflammation proprieties, as indicated by low levels of soluble vascular cell adhesion molecule (sVCAM), soluble intercellular adhesion molecule (sICAM), E-selectin, C-reactive protein (CRP), tumor necrosis factor α (TNFα), and interleukin 6 (IL6); (iv) the population of endothelial progenitor cells (EPCs) is high, whereas levels of endothelial microparticles (MPs) and circulating endothelial cells (CECs), indicative of endothelial stress or damage, are low. In contrast, the dysfunctional endothelium phenotypic characteristics include: (i) impaired vasodilation; (ii) increased oxidative stress/uric acid, lipid peroxide radical, nitrotyrosine and NO; (iii) procoagulant and proinflammatory phenotype with decreased vascular repair capacity and increased numbers of endothelial microparticles and ECs.

[a]*Adapted*[19].

Abbreviations: *6-keto $PGF_{1\alpha}$, 6-keto prostaglandin $F_{1\alpha}$ (a stable product of PGI_2); ADMA, asymmetric dimethyl arginine (an inhibitor of NO biosynthesis); EC, endothelial cell; NO_2, nitrite ion (a stable degradation product of NO); NO_3, nitrate ion (a stable degradation product of NO); ONOO, peroxynitrite (the product of superoxide-mediated inactivation of NO); WBC, white blood cell.*

These compensatory mechanisms are presumed to be suitable in the initial PAD stages, but physiologic compensation is no longer effective in patients who progress to CLI (Figure 3).

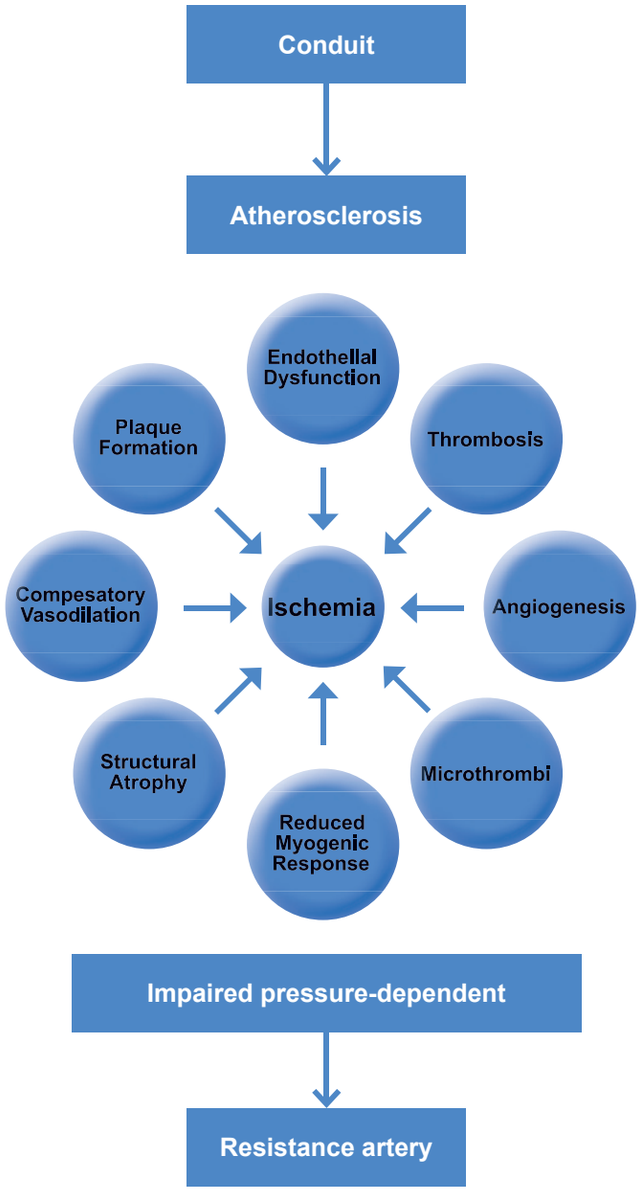

Figure 3. Postulated Overview of Physiological Adaptations of the Vasculature in Critical Limb Ischemia

1.1.3. Clinical Manifestations of Peripheral Arterial Disease

The most frequent symptom of PAD is IC, but the diagnosis of PAD is generally overlooked until limb-threatening ischemia is observed. With the progression of the disease, the patient may endure pain at rest or ischemic ulceration (CLI). Chronicity is implied by CLI and should be differentiated from acute limb ischemia (Table 5).

Table 5. Categories of Chronic Limb Ischemia[a]

Fontaine Classification		Rutherford Classification			
Grade	**Clinical Description**	**Grade**	**Category**	**Clinical Description**	**Objective Criteria**
I	Asymptomatic	**0**	**0**	Asymptomatic – no hemodynamically significant occlusive disease	Normal treadmill or reactive hyperemia test
IIa	Mild claudication	**I**	**1**	Mild claudication	Able to complete treadmill exercise (Five minutes at 2 mph on a 12% incline); arterial pressure after exercise > 50 mm Hg but at least 20 mm Hg lower than resting value
IIb	Moderate to severe claudication	**I**	**2**	Moderate claudication	Between categories 1 and 3
III	Ischemic rest pain	**I**	**3**	Severe claudication	Cannot complete standard treadmill exercise (Five minutes at 2 mph on a 12% incline) and arterial pressure after exercise < 50 mm Hg
IV	Minor to major tissue loss	**II**	**4**	Ischemic rest pain	Resting arterial pressure < 40 mm Hg, flat or barely pulsatile ankle or metatarsal PVR; TP < 30 mm Hg
		III	**5**	Minor tissue loss – nonhealing ulcer, focal gangrene with diffuse pedal ischemia	Resting arterial pressure < 60 mm Hg, ankle or metatarsal PVR flat or barely pulsatile; TP < 40 mm Hg
		III	**6**	Major tissue loss – extending above TM level, functional foot no longer salvageable	Same as category 5

[a]*Adapted*[2].
Abbreviations: *AP, Ankle pressure; PVR, pulse volume recording; TP, toe pressure; TM, transmetatarsal.*

Natural history of intermittent claudication

IC can be described as an exercise induced lower extremity pain in the calves (sometimes in the buttocks or thighs), which can be relieved after short periods of rest. Claudication results from arterial obstruction proximal to the affected muscle beds, which limits the normal exercise-induced increase in blood flow and produces transient muscle ischemia during exercise[2]. Symptoms of IC are noted only in a few PAD patients. The prevalence of asymptomatic PAD is not constant, but the available data suggest that for every patient with IC, there are three others who do not present symptoms[21].

It was documented in the Partners study that abnormal ABI with or without symptoms of IC was present in 13% of a total of 6979 patients over 50 years old screened for PAD[22]. Of these patients, their physicians had previously diagnosed only 24% with chronic lower limb ischemia.

IC is generally linked to ABI that ranges from 0.5 to 0.95 (Table 6), even though a patient may occasionally present mild IC with a normal ABI at rest, which decreases only with the stress of treadmill walking (generally indicating obstructive disease restricted to the aortoiliac system)[23]. At times, ABI is markedly reduced among patients without any limb-threatening symptoms, like claudication, or even asymptomatic.

Table 6. Interpretation of Ankle-Brachial Index[a]

Generally normal	0.91 – 1.3	
Mild-moderate disease	0.41 – 0.9	Pain in the foot, leg, or buttock may occur during exercise due to some narrowing of the arteries
Severe disease	≤ 0.40	Symptoms may occur even while resting; danger of limb loss
Rigid arteries	≥ 1.3	Calcified vessels; ultrasonography needed to check for peripheral artery disease instead of an ankle-brachial index test

[a]Adapted[24].

While atherosclerosis is a pathologically progressive disease, many studies identify claudication to be a surprisingly benign clinical entity, concerning lower extremity integrity[25,26]. In the Basle longitudinal study, angiographic disease progression was documented in 63% of patients, 5 years after the initial diagnosis[27]. Nevertheless, there was no evidence of activity-limiting claudication in 66% of those individuals who survived after 5 years following their diagnosis. Generally, it is suggested by studies from the last 4 decades that deterioration will occur in only about one-quarter of patients with IC, usually during the first year after diagnosis. After that, the annual deterioration is 2% to 3%[25,28]. Symptoms stabilization may occur after gait alteration that favours non-ischemic muscle groups, metabolic adaptation of ischemic muscle, or collateral development[25]. Recent studies suggest that it is rare that major amputations occur in these patients, with amputation being required in only 1% to 3.3% of patients during a 5-year period[25]. Additionally, it was noted in two large population based studies that major amputation was required in less than 2% of patients with PAD[29].

Many cohort analyses have reported risk factors, namely type 2 DM and smoking, related to the progression of IC[30]. After 10 years, revascularizations are required by less than 20% of patients the majority of them performed for claudication and not CLI. No clear criteria has been established that indicate the time revascularization should be carried out in patients with IC[31].

There are many reservations concerning invasive management of claudicants, such as the benign natural history pertaining to eventual limb threat and questions if early revascularization being better for such patients instead of waiting for CLI development. Only a randomized trial can answer such questions. Meanwhile, several authors have suggested that if all the other factors are equivalent, patients with DM and claudicants with reduced ABI should be offered invasive revascularization at a lower threshold, compared to other patients with claudication[32].

Natural history of critical limb ischemia

In the 1950's, the era of emerging vascular surgery, the initial classification of PAD by Fontaine was based exclusively on clinical symptoms. Fontaine´s stages III and IV correspond to CLI. Thereafter, with increased access to non-invasive hemodynamic measurements, a new concept emerged regarding the need to prove a causal relationship between PAD and the clinical findings indicative of CLI. Rutherford´s classification (1986) comprised objective hemodynamic criteria (see Table 5) that reflected an arterial insufficiency, severe enough to result in microcirculatory changes and trophic injuries, coursing with a high rate of mortality as well as of major amputation. Thus, CLI can be clinically diagnosed as pedal necrosis or rest pain with suitable documentation of circulatory impairment (similar to Fontaine stages III and IV)[2]. Table 7 illustrates the Transatlantic Intersociety Consensus Document II recommendations on a CLI definition as depicted in its original version.

Table 7. Transatlantic Intersociety Consensus Document II Recommended Clinical Definition and Diagnosis of Critical Limb Ischemia[a]

Recommendation 16: Clinical definition of critical limb ischemia
The term CLI should be used for all patients with chronic ischemic rest pain, ulcers or gangrene attributable to objectively proven arterial occlusive disease. The term CLI implies chronicity and is to be distinguished from acute limb ischemia.
Recommendation 19: **Diagnosis of critical limb ischemia**
CLI is a clinical diagnosis but should be supported by objective tests.
Recommendations are presented in a summarized version (reproduced above as they appeared in the original document).
Details and objective criteria can only be found in the full text as follows: "Ischemic rest pain most commonly occurs below an ankle pressure of 50 mm Hg or a toe pressure less than 30 mm Hg. […] For patients with ulcers or gangrene, the presence of CLI is suggested by an ankle pressure less than 70 mmHg or a toe systolic pressure less than 50 mm Hg. (It is important to understand that there is not complete consensus regarding the vascular hemodynamic parameters required to make the diagnosis of CLI.)"

Data reproduced as they appeared in the original document.
[a]Adapted[33].

Approximately 1% to 3% of patients with PAD are in CLI stage when first diagnosed and 5% to 10% of patients with claudication or asymptomatic PAD will progress to CLI within 5 years[33]. The former group generally includes patients with additional medical conditions that reduce peripheral perfusion, patients with sensory neuropathy and impaired pain sensation, and older sedentary patients with restricted mobility that present directly with limb-threatening disease. Several studies demonstrate that no PAD symptoms are present in half of CLI patients 6 months prior to the onset of CLI[10].

The rate of CLI inferred from major amputation rates and natural history of PAD has been assessed to be around 500 to 1000 in a million per year among the North American and European populations (150 000 patients per year in the United States of America)[33]. On the other hand, the occurrence of CLI in the general population, based on prospective population studies, is estimated to be 220 new cases in a million per year.

CLI prevalence in individuals between 60 and 90 years old is assessed to be 1% (range, 0.5%-1.2%), however, there is a wide variation in these rates between the vascular registries and population-based studies. For instance, in Sweden (2004), the prevalence of CLI was assessed to be 15 000 patients minimum. However, in the same period, only 1700 vascular interventions were registered[34].

Table 8. Natural History of Critical Limb Ischemia by Transatlantic Intersociety Consensus Document II[a]

At presentation: 20%–25% of patients undergo primary amputation, 50%–60% have vascular reconstruction (surgical and/or endovascular), and 25% are treated medically
One year later: 20%–25% of patients will have died, 25%–30% will have had major amputation, 20% will still be in CLI state, and 25% will be alive without major amputation and free from signs and symptoms of CLI

Data reproduced as they appeared in the original document.
[a]Adapted[33].

Population-based epidemiological studies match age-adjusted CLI prevalence among women and men over 50 years old; this finding compares with epidemiological studies on PAD prevalence based on the measurement of ABI[34-36]. CLI refers to a severe medical condition presenting a high risk of death, disability and amputation. In a certain way, it acts like a malignant disease. The Transatlantic Intersociety Consensus documents as well as review articles on the natural history of patients with CLI have been summarised in Table 8.

Considering the differences on the natural history of patients with CLI, including prognosis of the limb and of the patient, are thus difficult to compare studies in this context. Overall, CLI as defined by clinical and hemodynamic criteria remains a severe condition bearing poor prognosis, high medical costs, and a major impact in public health and patient's loss of functional capacity. Progress in best medical therapy of arterial disease and revascularization procedures will certainly improve the outcome of patients suffering of CLI.

1.1.4. Peripheral Arterial Disease Survival

PAD patients' survival is variable (see Figure 4). The stratification of survival can be done on the basis of symptoms as well as objective measures of peripheral occlusive disease through ABI. The mortality rates at 5-year, 10-year, and 15-year for patients with IC are about 30%, 50%, and 70%, respectively[37]. Several risk factors have been identified to be significant contributors to this high long-term cardiovascular mortality, which includes dialysis dependence, continued tobacco use and advanced age at diagnosis[38-41]. Most deaths are due to CAD, which is nearly universal in PAD population.

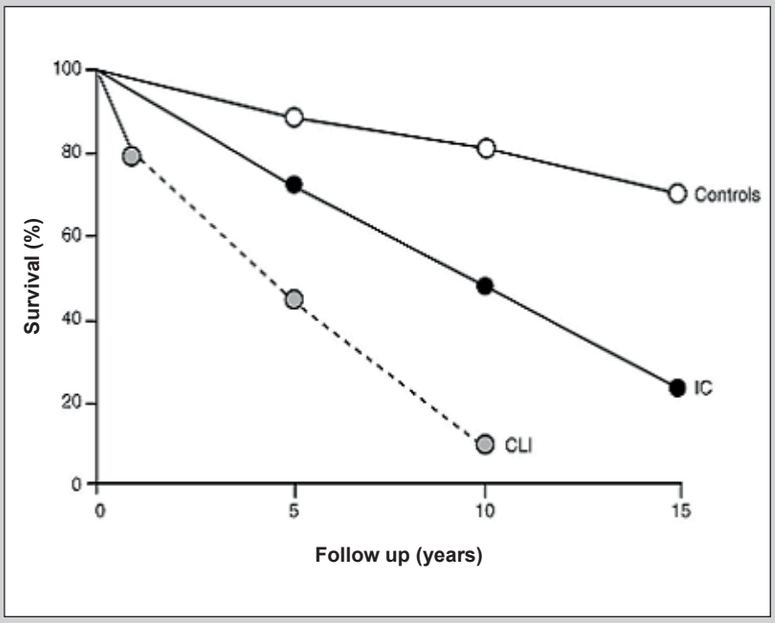

Figure 4. Peripheral Arterial Disease Patient's Survival Rate[a] – Clinical Stage

[a]*Adapted*[33].
Abbreviations: *CLI, critical limb ischemia; IC, intermittent claudication; PAD, peripheral arterial disease.*

Patients with CLI have poor survival rates (Figures 4 and 5). Therefore, much of their care can be regarded as palliative by nature. At 5 years, mortality rates for patients with rest pain goes up to 70% while at 10 years it goes up to 85%[42]. A five-year survival rate of 50% to 60% is generally noticed in many surgical series of CLI[43,44]. A vascular event is generally the main cause of death in about 80% of patients with PAD, with about 10% of them dying of stroke and over 60% from CAD[45].

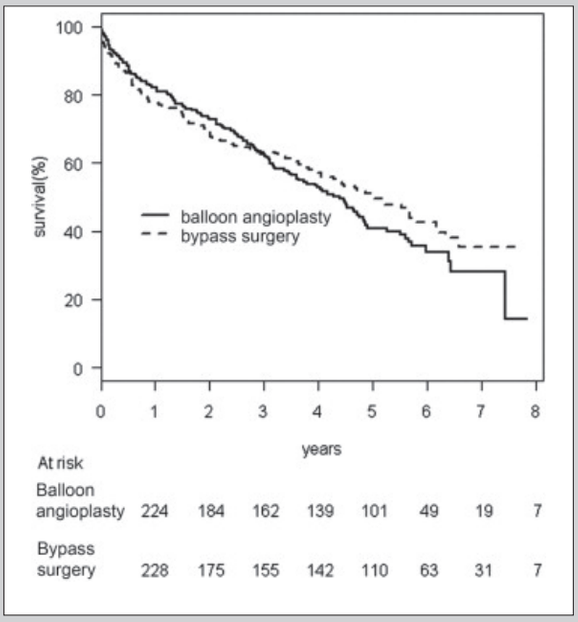

Figure 5. Peripheral Arterial Disease Patient's Survival Rate[a] - Treatment Option

[a]*Adapted*[46].

1.1.5. Treatment of Peripheral Arterial Disease

It is imperative that the treatment for PAD patients is directed towards limiting the repercussions of systemic atherosclerosis, like stroke or acute myocardial infarction. Thus, the use of certain therapies is recommended like antiplatelet agents, angiotensin-receptor blockers or angiotensin-converting enzyme inhibitors and statins (Figure 6).

Alternatively, treatment of limb-based symptoms should be prescribed for improving quality of life, exercise performance as well as functional capacity. The therapeutic options currently offered include various surgical/endovascular or medical modalities. Efficacy has been proven with cilostazol¸ revascularization with surgery/ angioplasty, and exercise training in a formal setting[47]. No documented efficacy for prostaglandins has been observed in the treatment of claudication. Quality of life as well as treadmill exercise performance has been observed to be enhanced with carnitine and its derivatives (propionyl-L-carnitine). Additionally, these drugs are recognized as having an excellent safety profile[47,48]. Wound care as well as other medical approaches should be provided to all patients presenting ischemic ulcers, so that healing can be achieved and pain reduced. There are no noteworthy medical therapies effective in enhancing perfusion to lower extremity in PAD patients[33,49,50].

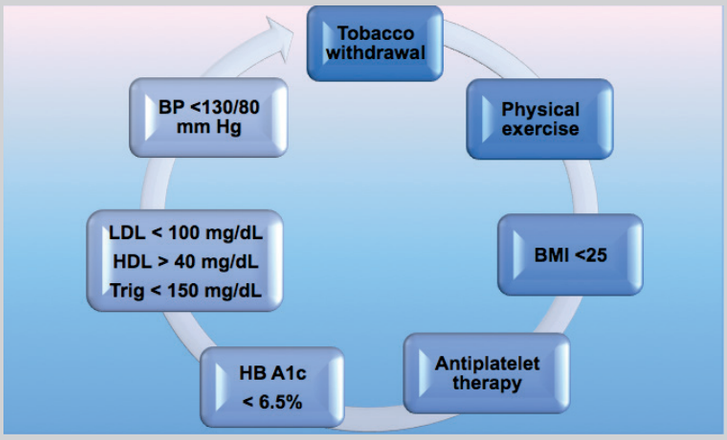

Figure 6. Recommendations for Treating Peripheral Arterial Disease

Abbreviations: *BP, arterial pressure; BMI, body mass index; HbA1c, haemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Trig, triglyceride.*

On the contrary, the gold-standard treatment and the real option for salvaging the limbs in CLI is revascularization. Lower limb revascularization can be carried out through conventional surgery, by percutaneous endovascular approach or by a hybrid approach (surgical and endovascular). The TASC II (Inter-Society Consensus for the Management of PAD) document aimed to define guidelines for the best therapeutic approach in lower limb revascularization, categorizing the arterial lesions in A, B, C and D, according to morphology, anatomical distribution and the overall probability of successful treatment of the lesion, either surgically or endovascularly[33]. Generally, type A lesions are segmental short lesions, while type D lesions correspond to more diffuse and complex lesions. Type B and C correspond to progressively more diffuse and complex lesions. This TASC II consensus suggests that the endovascular approach is the treatment of choice for type A lesions, and should also be the preferred approach for type B lesions. Minimal invasive percutaneous intervention should be recommended, whenever possible, to the elderly high-risk patients carrying numerous comorbidities[51]. Conventional surgery is the first therapeutic option for more complex type D lesions, and should be the preferred approach in the treatment of type C lesions. Open surgery should be the preferred therapeutic option for younger patients at lower cardiovascular risk and with longer life expectancy[51]. While waiting for more robust trials like the BEST-CLI (Best Endovascular vs. Best Surgical Therapy in Patients with Critical Limb Ischemia), this strategy is still corroborated by the long-standing BASIL (Bypass versus Angioplasty in Severe Ischemia of the Leg) study which showed that endovascular revascularization is associated with lower morbidity and decreased

length of hospitalization, but also lower primary and secondary patency rates when compared to conventional surgery. Therefore, surgical bypass remains the gold standard for revascularization of CLI patients who are eligible for surgery[52]. The 1-month mortality rate has been similar in the two groups. However, 2-year survival was better in the surgical-first strategy, in a post hoc analysis[52].

Based on these results, the American College of Cardiology/American Heart Association guidelines for the treatment of PAD recommend endovascular treatment as the initial option for selected patients who have an estimated life expectancy of ≤2 years or for those who have no autologous vein available, and bypass surgery should be the first choice in patients with an estimated mean life expectancy >2 years and who have an autologous vein available[53].

Another option to keep in mind is the so-called hybrid (surgical and endovascular) revascularization procedures. This blended approach was shown to result in less extensive procedures with decreased risks of perioperative complications, especially in older patients with several comorbidities[54,55].

If patients are selected correctly, either of the treatment modalities may lead to preservation of limb or life in more than 75% of patients in 1 year. Nevertheless, several CLI patients are poor candidates or simply lack revascularization options. It is thus evident that new treatment strategies are necessary for CLI patients who fail or cannot undergo revascularization interventions[50,56].

1.1.6. New Horizons: Therapeutic Angiogenesis

While thorough investigation has been done on medical therapies for PAD, the standard revascularization methods cannot be used for nearly about 20% to 30% of patients with CLI[57] like those who are chronically non-ambulatory, neurologically impaired or for those who lack graftable distal vessels. Amputation is often the last option for such patients. Even though there is a considerable increase in the number of endovascular or surgical procedures that are performed annually, there has not been a decrease in amputation rates[58].

More recent clinical trials as well as experimental animal studies show that the majority of the risk factors for PAD, particularly, type 2 DM and age are related to decreased levels of pro-angiogenic growth factors like insulin-like growth factor 1, fibroblast growth factor (FGF) and VEGF[59]. Alternatively, there is accumulating evidence that patients with type 2 DM have compromised mechanisms of wound healing, peripherally or at the bone marrow (BM) level, due to deficiencies in the BM-derived endothelial progenitor cells (EPCs)[60]. Thus, the establishment and

progression of arterial disease may be promoted by deficiency of angiogenic/vasculogenic factors and depressed angiogenic potential.

Alternative treatment approaches are required for the “non-option” patients. Based on recent evidence, many strategies to stimulate new vessel formation (ie, angiogenesis) are under investigation. The idea of “therapeutic angiogenesis” has been developed as an investigational method that promotes blood vessel remodelling and growth for improving blood flow to the lower extremity in order to lower adverse outcomes or symptoms in PAD patients not fit for revascularization procedures. The basis for this concept is the assumption that direct or indirect administration of pluripotent stem cells and/or growth factors can stimulate the growth of new blood vessels.

The current criterion in the treatment of PAD includes early diagnosis followed by referral to vascular surgery. The role of vascular surgeons in the general treatment of patients warrants them to be qualified to intervene in the control of the global cardiovascular risk rather than just treating the clinical syndromes of PAD. There is an association between the nonsurgical symptomatic treatment of IC and the small advantages in pain-free walking distance. In addition to decreasing pain and increasing the walking distance, claudication is also treated for improving the quality of life of the patient. While limb salvage is the objective of treating CLI, 20% to 30% of patients are considered “non-option” patients; thus, it is imperative that new pharmacologic alternatives are developed in this field, so that the number of major amputations performed annually can be decreased.

Finally, it is important to stress that therapeutic vasculogenesis and angiogenesis have moved up from the field of basic research and requests have been raised for several drugs based on these principles to be commercially approved.

1.2. ANGIOGENESIS

William Harvey (1628) published “Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus” wherein the cardiovascular system was better understood in addition to where the blood is pumped around the heart into the arteries and pumped back through the veins. A few years later, Marcello Malphighi and Caspar Aselius completed the circle with the identification of capillaries and lymphatic vessels[61].
Formation of blood vessels can be explained by two mechanisms: angiogenesis and vasculogenesis. At the time of embryogenesis, blood vessels develop from the mesodermal endothelial precursors, which gather into a primitive vascular labyrinth of small capillaries through a process called vasculogenesis, which refers to *de novo* blood vessel formation. There is progressive expansion of this vascular network through vessel sprouting, known as angiogenesis, which is further stabilized by covering smooth muscle cells and pericytes at the time of arteriogenesis (detailed in chapter 4)[62].
Tissues can also become vascularized by other mechanisms. For example, intussusception is the splitting of pre-existing vessels leading to the rise of daughter vessels. Further, vessel co-option can occur, wherein the existing vasculature is hijacked by tumor cells; or vascular mimicry wherein vessels are lined by tumor cells. Putative cancer stem-like cells can differentiate into ECs generating the tumor vasculature[63,64].

1.2.1. Early Blood Vascular Development and Maturation of Blood Vessels

The extra-embryonic yolk sac and intraembryonic tissue show the first evidence of blood vessel development, where aggregation and condensation of groups of splanchnic cells, that become hemangioblasts, occur leading to the formation of blood islands. This is where the differentiation of hemangioblasts, blood cell and ECs precursors, occurs (see Figure 7). The cells that are at the blood island perimeter convert into angioblasts, which are the ECs precursors. The ones that are at the centre form the hematopoietic precursors of all blood cells[65].
With the formation of the yolk sac, there is multiplication, differentiation and migration of angioblasts to distant sites, through which a primary vascular plexus, a primitive network of simple endothelial tubes, is formed. Accordingly, vasculogenesis can be defined as the process wherein blood vessels are formed *de novo* from the EPCs due to local signals like growth factors. At the time of this process, ECs differentiate, migrate, form a lumen and interconnect to form a primary vascular plexus, which then undergoes angiogenesis to produce an extensive vascular network[65-67].

This is the point where hypoxia facilitates sprouting angiogenesis, which up-regulates several genes attached to the maturation, patterning and formation of vessels, like angiopoietin (ANG) 2, VEGF and endothelial nitric oxide synthase (NOS). As a response to NO (a product of endothelial NOS), the existing vessels dilate and in response to VEGF, they become leaky, which indirectly controls intracellular adhesion molecule redistribution, including vascular-endothelial cadherin and platelet-endothelial cell adhesion molecule 1. With the dissolution of the ECM due to the activation of matrix metalloproteinases (MMPs) (eg, MMP2, MMP3, MMP9) and the suppression of the tissue inhibitors of metalloproteinases (tissue inhibitor of MMPs 2), the role of a provisional matrix is assumed by plasma proteins leaked from these nascent vessels[63].

With the dissolution of physical barriers, the migration of ECs is enabled, as a result, interactions are established between their matrix proteins and integrins, concurrently thriving in response to VEGF as well as other endothelial mitogens like ANG1 and ANG2, FGFs, and platelet derived growth factors (PDGF(s)) (in the presence of VEGF)[65,66]. ECs selection for sprouting is an extremely regulated process, wherein a crucial role is played by Notch signaling[68].

In the maturation of new vessels, mural cells are recruited and the surrounding matrix is expanded. There are four pathways involved in the regulation of this process: (i) PDGF β/PDGFβ receptor, (ii) ANG 1/tyrosine-protein kinase receptor of ANG (TIE) 2, (iii) sphingosine-1-phosphate-1 (S1P1)/endothelial differentiation G-protein coupled receptor 1, and (iv) transforming growth factor β (TGFβ) signaling. The recruitment of mural cells critically requires PDGFβ. In response to VEGF, ECs recruiting PDGFβ receptor-positive mural cells around vessel sprouts secrete PDGFβ, through which the vessels are stabilized by the inhibition of ECs migration and proliferation[69,70]. The recruitment and differentiation of pericytes involve S1P1/endothelial differentiation G-protein coupled receptor 1 as well as TGFβ[66,67,71]. Organ-specific specialization is the last step in the maturation process, wherein highly specialized properties are acquired by ECs so that functional needs can be provided within specific tissues. This process involves ECs differentiation to organ-specific capillary structures, homotypic and heterotypic junction formation and arterial-venous determination[65].

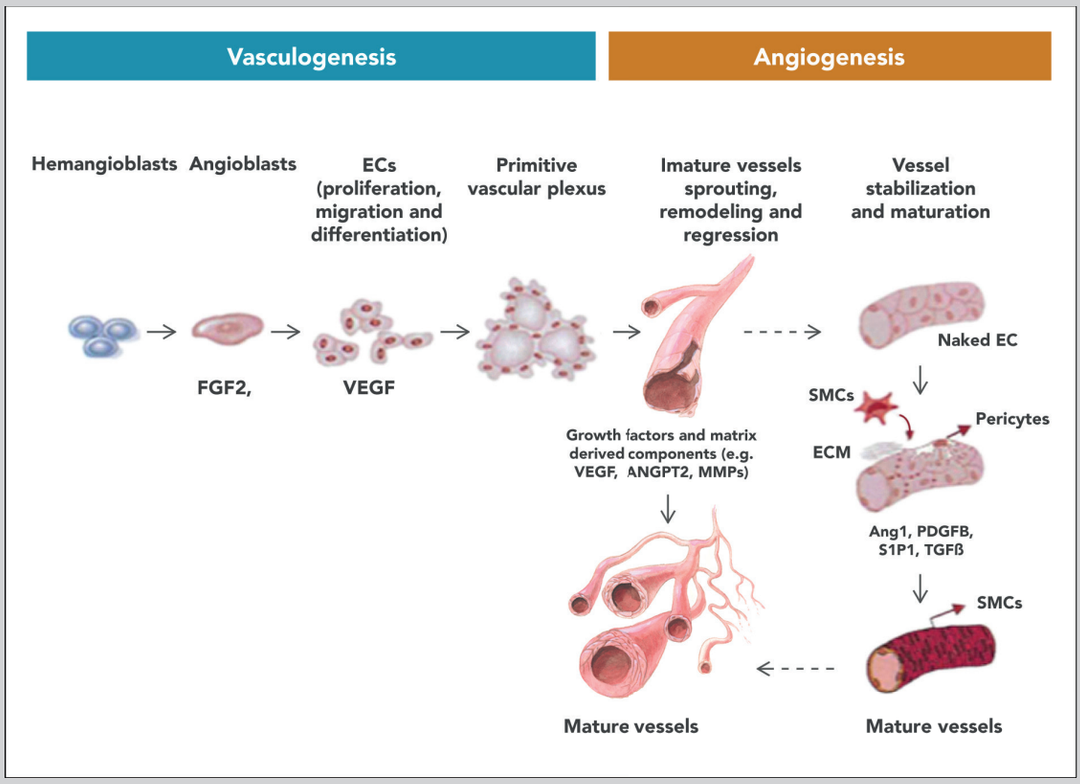

Figure 7. Vasculogenesis and Angiogenesis During Embryonic Development

Abbreviations: *Ang1, angiopoietin 1; ECs, endothelial cells; ECM, endothelial cell matrix; FGF, fibroblast growth factor; MMPs, matrix metalloproteinases; PDGFB, platelet-derived growth factor B; S1P1, sphingosine 1-phosphate 1; SMCs, smooth muscle cells; TGFβ, transforming growth factor β; VEGF, vascular endothelial growth factor.*

[a] *Adapted*[66].

1.2.2. Postnatal Neovascularization

It was generally accepted until 1997 that it was only during embryogenesis that vasculogenesis could occur. However, postnatal vasculogenesis has been confirmed to exist through evidence showing postnatal circulation of EPCs in peripheral blood (PB)[72] and its recruitment could be done from the BM along with its incorporation in the active neovascularization sites[73,74]. After this, the progenitor cells are incorporated into the endothelial lining through postnatal vasculogenesis[75]. EPCs recruitment and integration include differentiation into mature ECs, incorporation into the vasculature, migration to the interstitial space, active rest and chemoattraction[76].

1.2.3. Basic Mechanisms of Angiogenesis

For vessels to expand and branch, ECs must grow, migrate, and survive. The formation of a functioning vasculature requires the orchestrated interaction between ECs, the ECM, and surrounding cells. The ECM must disintegrate to allow ECs to migrate and expand and to permit the recruitment and proliferation of mural cells. For nascent vessels (all except capillaries) to function properly, they must be covered by mural cells, which provide maturity and stability. Those serial steps are tightly regulated by different factors, including vascular-specific growth factors, inflammatory cytokines, adhesion molecules, and NO (Figure 8)[77].

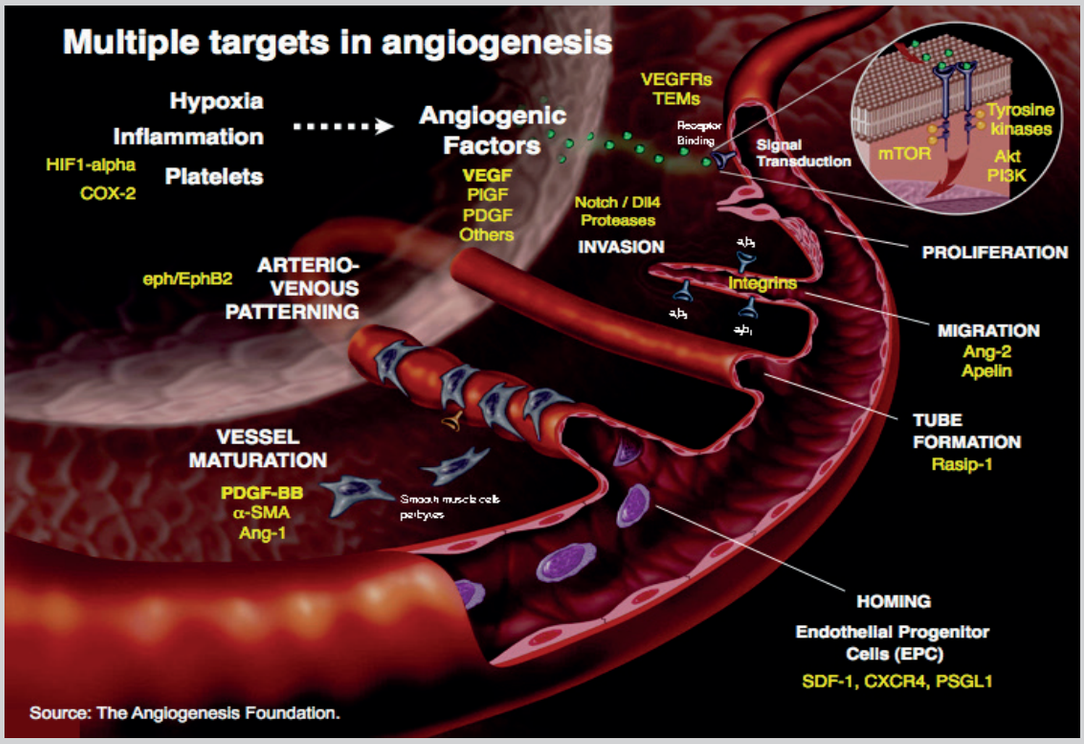

Figure 8. Targets in angiogenesis

In healthy individuals, a balance of growth factor signaling maintains ECs in a quiescent, or resting state. However in the presence of hypoxia or other endogenous signals, cells became activated inducing the release of signaling factors such as VEGF, ANG2, FGF, and other chemokines. As a result pericytes disconnect from the vessel (ANG2 signaling), and ECs are activated losing their intimate contact as the vessel dilates (vascular-endothelial cadherin signaling). Next a tip cell is selected (selection influenced by neuropilin, VEGF/VEGFR and NOTCH/DLL4 and JAGGED1 signaling), and the release of MMPs contribute to the degradation of the basement membrane and to the remodelling of the ECM. A VEGF gradient directs the sprout tip cell oriented migration (via semaphorins, ephrins, and integrins guidance signals). Proliferating stalk cells, following the tip cell, establish junctions with neighbouring ECs and release molecules such as EGFL7 (an EC chemoattractant expressed by proliferating ECs) that bind to extracellular membrane components and regulate vascular lumen formation. When 2 tip cells came together, establishing EC-EC junctions (VE-cadherin, ANG1) a continuous lumen is finally formed. After the establishing of a new basement membrane (tissue inhibitors of metalloproteinases), EC proliferation ceases, and pericytes are recruited to stabilize the new vessel (PDGFR/PDGFβ, ANG1). Once blood flow is established, the perfusion of oxygen and nutrient reduces angiogenic stimuli (VEGF expression) and inactivates EC oxygen sensors, re-establishing the quiescent state of the blood vessel.

[a]*Adapted from Angiogenesis Foundation Website (http://www.angio.org/learn/angiogenesis/).*

Abbreviations: *ANG, angiopoietin; COX-2, cyclooxygenase 2; CXCR4, chemokine receptor 4; ECs, endothelial cells; Eph, ephrin; HIF1, hypoxia inducible factor 1; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PlGF, placental growth factor; PSGL, P-selectin glycoprotein ligand 1; Rasip1, Ras-interacting protein 1; SMA, smooth muscle actin; TEMs, Tie-2 expressing monocytes; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.*

1.2.4. Vessel Branching, Maturation, and Quiescence

In the case of a healthy adult, half-lives are long for quiescent ECs that are protected against insults by the autocrine action of maintenance signals like VEGF, FGFs, ANG1 and Notch. Since oxygen is supplied by vessels, oxygen sensors are present in ECs along with hypoxia-inducible factors (HIF) such as HIF2α and prolyl-hydroxylase domain 2, through which the vessels can readjust their shape to optimize blood flow. A monolayer of phalanx cells is formed from quiescent ECs with a streamlined surface that is interconnected by junctional molecules like vascular-endothelial cadherin and claudins. Quiescent ECs are covered by pericytes, which suppress ECs proliferation and release cell-survival signals such as VEGF and ANG. A common basement membrane is produced by pericytes and ECs at rest[63].
When a quiescent vessel senses an angiogenic signal, released by hypoxia, inflammatory or tumor cell, cell-cell junctions and the basement membrane are remodeled by MMPs. Mural cells detach allowing an activated ECs tip cell to migrate in response to guidance signals. ECs become motile and invasive and protrude filopodia. Trailing behind tip cells, stalk cells extend fewer filopodia but establish a lumen and proliferate to support sprout elongation. Tip cells anastomose with cells from neighboring sprouts to build vessel loops. The initiation of blood flow, the establishment of a basement membrane, and the recruitment of mural cells stabilize new connections[78].
The balance between stalk (proliferating and forming the vessel lumen) and the number of tip cells (leading and guiding vessel sprout) is determined by the interplay between delta-like 4 (DLL4)/Notch/VEGFR. VEGFR expression is regulated by Notch, while DLL4 expression in ECs is controlled by VEGF signals. It is postulated by the vessel-branching model that generally stalk cells proliferate and the tip cells migrate[79]. DLL 4 expression in tip cells is upregulated by the activation of VEGFR2 in response to VEGF. In the neighboring ECs, Notch is activated by DLL4, which down regulates VEGFR2 and VEGFR3 inhibiting the tip cell phenotype; while VEGFR1, functioning as a decoy for VEGF, is upregulated, resulting in the formation of stalk cells materializing the proliferative core of the nascent sprout. As a result, stalk cells are less responsive to VEGF's sprouting activity, but show higher sensitivity to molecules like placental growth factor (PIGF). Accordingly, branching is restricted by DLL4 and Notch signaling, but perfused vessels are generated by the same signaling interaction[79]. Several studies demonstrated that by inducing genetic or pharmacologic inhibition of endothelial Notch activity, elevation of VEGF expression as well as the emergence of tip cells occurred[80]. On the contrary, vessel maturation is stimulated by the up-regulation of PDGFRβ in Notch-positive mural cells and

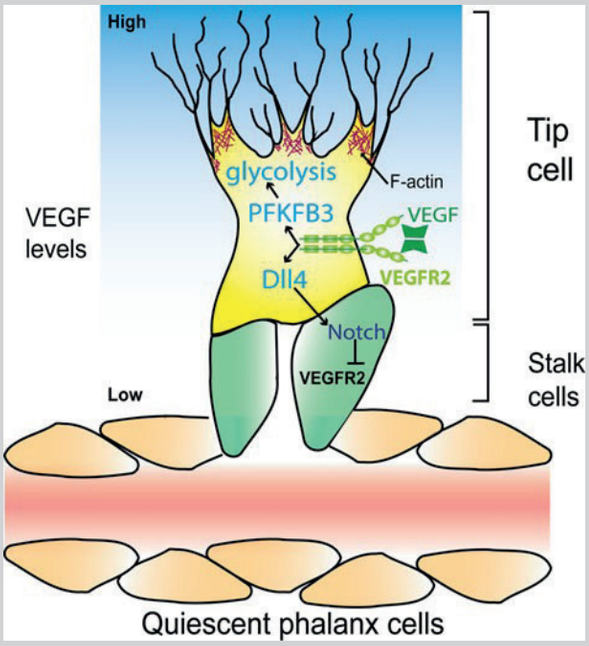

Figure 9. Schematic Overview of a Growing Vessel Sprout[a]

VEGF sensitivity and VEGFR2 biding resulting in DLL4 up-regulation determine tip cell (yellow) specification. In the neighbouring ECs, stalk (green) commitment occurs as a result of a Notch mediated inhibition mechanism that decreases the expression of VEGFR2 preventing ECs to adopt a tip cell phenotype and promoting stalk cell differentiation. Tip cells direct the sprout as a result of a VEGF gradient and stalk cells proliferate and elongate the sprout. Finally vessels are lined by quiescent "phalanx" cells (orange). VEGFR 2 signaling in tip cells rise the expression of PFKFB3 glycolytic enzyme stimulating glycolytic energy production. Glycolytic enzymes are concentrated in the lamellipodia and filopodia, colocalizing with actin filaments (F-actin). This compartmentalization of glycolysis with F-actin provides local, rapid adenosine triphosphate (ATP) production needed for actin-cytoskeletal remodelling required for migration.

[a] *Adapted*[83].

Abbreviations: *DLL4, delta-like-4; PFKFB3, 6-phosphofructo-2-kinase; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.*

DLL4 in ECs. Tip cell selection is further promoted by Jag1, another Notch ligand expressed by stalk cells, by interfering with the reciprocal DLL4 and Notch signaling between the stalk cell and the tip cell[81]. Nowadays, it is believed that tip and stalk cells change roles and this rearrangement is arbitrated by the differential dynamics of VE-cadherin junctions, which are regulated by Notch/VEGFR signaling[82].

1.2.5. Angiogenic Regulators

Functional angiogenesis requires a complex coordination of steps, which are regulated by a subtle balance between anti and pro-angiogenic factors. Tables 9 and 10 present a list of some of the well-described angiogenic stimulators and inhibitors, respectively. Some of these factors will be briefly detailed below.

Table 9. Critical Stimulators of Angiogenesis and Their Role in the Formation of Blood Vessels

Class	Factor	Biological Functions	Reference
Growth factors, Cytokines and Chemokines	Angiotropin	↑ECs migration Angiogenesis *in vivo*	84
	Angiopoietin 1 (ANG1)	↓ECs apoptosis ECs sprouting Vessel stabilization	70,85,86,87
	Angiopoietin 2 (ANG2)	↑ECs migration ↑ECs proliferation ECs spouting only in the presence of VEGF	70,85,86,87
	Epidermal growth factor	↑ECs proliferation ↑VEGF Angiogenesis *in vivo*	88
	Erythropoietin (EPO)	↑ECs proliferation Angiogenesis *in vivo*	89
	Fibroblast growth factors (FGFs) family	↑Plasminogen activators ↑ECs proliferation ↑ECs migration ↑$\alpha v\beta^{*}$ integrin ↓ECs apoptosis Angiogenesis *in vivo*	90,91,92,93,94
	Hepatocyte growth factor (HGF)	↑ECs proliferation ↑ECs migration Angiogenesis *in vivo*	95
	Insulin-like growth factor 1 (IGF1)	↑ECs proliferation ↓ECs apoptosis ↑VEGF ↑Plasminogen activators	96
	Interleukin 8 (IL8)	↑ECs proliferation ↑ECs migration ↓ECs apoptosis	97
	Platelet-derived growth factor (PDGF)	↑SMCs and pericytes proliferation ↑VEGF Vessel stabilization	85,92,98
	Transforming growth factor β (TGFβ)*	↑ECs proliferation ↕ECs migration ↓ECs apoptosis ↑PDGF and eNOS Tube formation Angiogenesis *in vivo*	71,92,99,100,101
	Vascular endothelial growth factor (VEGF)	↑Permeability ↑Plasminogen activators ↑ECs proliferation ↑ECs migration ↓ECs apoptosis Angiogenesis *in vivo*	85,87,102,103,104,105
Matrix proteins and adhesion molecules	Cysteine-rich protein 61 (CYR61)	↑ECs proliferation ↑ECs migration ↓ECs apoptosis Tube formation	106,107,108,109
	Integrins	ECs attachment ↕ ECs migration ↓ ECs apoptosis FGF induced angiogenesis	85,110
	Platelet endothelial cell adhesion molecule 1 (PECAM-1)	ECs aggregation ↑ECs migration Tube formation Vessel stabilization FGF induced angiogenesis	92
	Vascular endothelial-cadherin (VE-cadherin)	↓ECs apoptosis Vessel stabilization Angiogenesis *in vivo*	111
Proteases	Matrix metalloproteinases (MMPs)*	ECs matrix degradation	112
Others	Angiogenin	↑ECs proliferation	113
	Ephrin	↑ECs proliferation ↑ECs migration Vessel stabilization	114
	Nitric oxide	↑Permeability ↑ECs proliferation ↑ECs migration ↑FGF and VEGF	115

**Can show opposite effects depending on dose and environmental condition.*
Abbreviations: *ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; SMC, smooth muscle cell.*

Table 10. Major Endogenous Inhibitors of Angiogenesis and Their Role in the Formation of Blood Vessels

Class	Factor	Biological function	Reference
Growth factors, Cytokines and Chemokines	Angiopoietin 2* (ANG2)	↑Apoptosis Vessel destabilization	70,85,86,87
	Interleukin 12 (IL12)	↓FGF mediated angiogenesis	116
	Interferon-α, -β, -γ (IFNα, β, γ)	↓MMPs ↓FGF ↓IL8-mediated angiogenesis ↑Plasminogen activators	85,117
	Platelet factor 4 (PF-4)	↓FGF-mediated angiogenesis	85,117
	Transforming growth factor β (TGF-β)	↓ECs proliferation ↓ECs migration ↑ECs apoptosis ↑TIMPs ↑Plasminogen activators	71,92,99,100,101
Matrix proteins and adhesion molecules	Arresten	↓ECs proliferation ↓ECs migration ↑Tube formation	117
	Endostatin	↓ECs proliferation ↓MMPs	85, 117
	Thrombospondin 1 and 2 (TSP-1, TSP-2)	↓ECs migration ↑ECs apoptosis	92
Proteases	Matrix metalloproteinases (MMPs)*	Generate angiostatin	112
	Tissue inhibitors of metalloproteinases (TIMPS)	↓MMPs	85, 112
Others	Angiostatin	↓ECs proliferation ↓ECs migration ↑ECs apoptosis ↑Tube formation	85, 92, 117

**Can show opposite effects depending on dose and environmental condition.*
Abbreviations: *ECs, endothelial cells; FGF, fibroblast growth factor; MMP, Matrix metalloproteinases*

Vascular endothelial growth factor family

Harold Dvorak's group (1980's) found a protein secreted by tumor cells, which makes blood vessels leaky; this protein originally termed as *vascular permeability factor* is currently known as VEGF[118]. It can be defined as a multitasking cytokine stimulating vascular permeability, tubulogenesis, proliferation, migration, survival, and differentiation in ECs in addition to exerting pleotropic effects on various cell types and tissues[119].

The major growth factor regulating physiological and tumor angiogenesis among the VEGF family members (VEGFA, VEGFB, VEGFC, VEGFD, the Orf virus-encoded factor/VEGFE and placental growth factor/PIGF) is VEGFA, also commonly known as VEGF (see Figure 10)[120,121]. VEGF binds to vascular endothelial growth factor receptors (VEGFR) 1 and 2, in addition to co-receptors neuropilin 1 and neuropilin 2 initially described as semaphoring receptors[122]. VEGFC and VEGFD also recognize

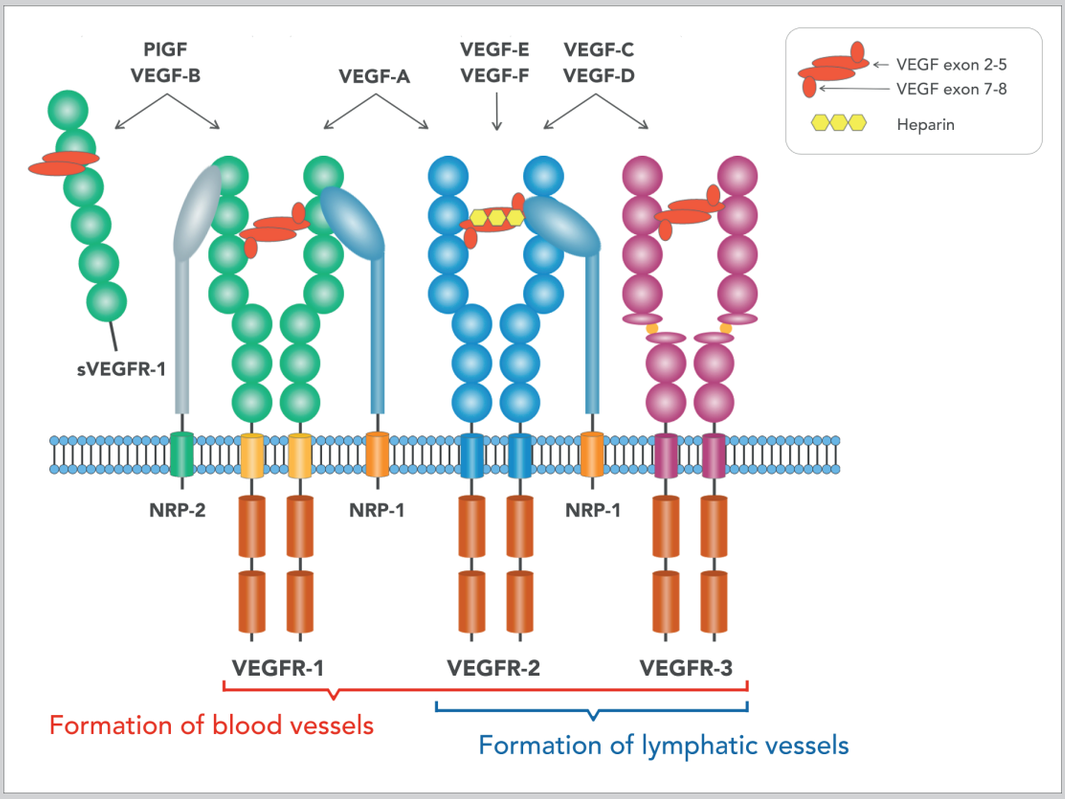

Figure 10. Schematic Representation of Vascular Endothelial Growth Factor Family Ligands and Their Receptors[a]

VEGF ligands and receptors. The receptors contain a cytoplasmic region containing a bipartite tyrosine kinase domain divided into 2 parts by an insert sequence, a single hydrophobic transmembrane domain and an extracellular region comprising 7 immunoglobulin-like domains.

[a]*Adapted*[123].

Abbreviations: *NRP, neuropilin; PIGF, placental growth factor; VEGF, vascular endothelial growth factor; VERFR, vascular endothelial growth factor receptor*

VEGFR2 with lower affinity and bind to a third receptor, VEGFR3 that has a central role in the lymphatic vasculature development.

The binding affinity of VEGFR1 for VEGFA is at least ten fold higher than that of VEGFR2. However, despite binding VEGF with high affinity, VEGFR1 presents weak tyrosine kinase phosphorylation activity following VEGF stimulation[124]. Experiments with mice lacking *Vegfr1* show that this receptor acts as a negative regulator of angiogenesis during embryonic development. *Vegfr1-/-* mice die *in utero* between E8.5-9.0 exhibiting a severe disorganization of the vasculature and uncontrolled ECs proliferation which results in the obstruction of vessel lumen and early lethality[125]. Based on the biochemical and genetic data it was proposed that VEGFR1 could be a negative regulator of the VEGF activity, acting as a “decoy” receptor to sequester VEGF, thus rendering it less available for interacting with VEGFR2[105].

In adult, VEGFR1 plays a role in activating VEGFR2 and thereby in angiogenesis, by binding of PlGF[126]. This mechanism gains importance in angiogenesis-associated pathologies, where PlGF has often been described as being upregulated. Furthermore, VEGFR1 is involved in the preparation of the metastatic niche, since VEGFR1-positive haematopoietic progenitor cells were shown to colonize tumor specific pre-metastatic sites prior to the arrival of tumor cells[127].

VEGFR2 has a higher tyrosine kinase activity and it is responsible for the major biological effects of VEGF. As other tyrosine kinase receptors, upon binding to its ligand (VEGF), VEGFR2 dimerizes and autophosphorylates in specific tyrosine residues promoting its activation. Upon activation, this receptor has the ability to phosphorylate specific tyrosine residues in proteins involved in intracellular signaling (Figure 11) that leads to survival and anti-apoptotic signals, such as phospholipase Cy, phosphatidylinositol 3-kinase (PI3K), Ras and Src[128]. Knockout *Vegfr2-/-* embryos die *in utero* between E8.5-9.0 as a result of profound defects in vasculogenesis and angiogenesis. *Vegfr2-/-* embryos fail to develop yolk-sac blood islands and organized blood vessels and show a reduced number of hematopoietic and ECs precursors[129]. These results suggested a pivotal role for VEGFR2 in vascular development and therefore it is considered the major mediator of the VEGF signaling during vasculogenesis and angiogenesis[124].

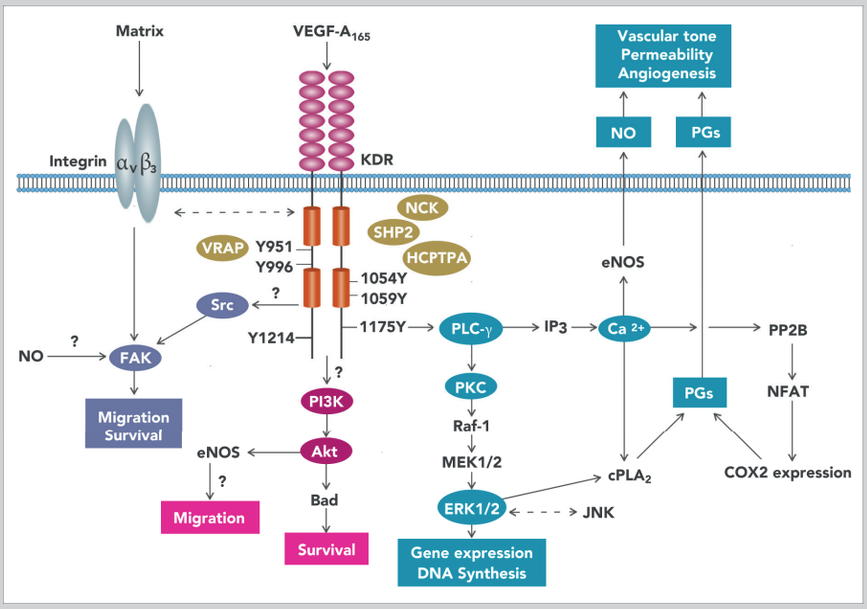

Figure 11. Vascular Endothelial Growth Factor signaling pathways[a]

Biologically relevant VEGF signaling is mediated via VEGFR2 (KDR). Activation of KDR occurs through ligand-induced dimerization and receptor autophosphorylation at multiple tyrosine residues in the intracellular domain. Though KDR associates with several other SH2 domain proteins (yellow ovals), VEGFR-associated protein (VRAP) and PLC-γ associate with Tyr-951 and Tyr-1175. VEGF-dependent EC survival is mediated in part via PI 3-kinase (PI3K)-mediated activation of the anti-apoptotic kinase Akt, though the mechanism of PI 3-kinase activation is unclear. Akt phosphorylates and inhibits the pro-apoptotic protein Bad, leading to inhibition of caspase activity. Akt also causes Ca2+-independent eNOS activation through phosphorylation, and this pathway may also be essential for migration. Increased tyrosine phosphorylation of FAK, mediated in part through Src, is a point of convergence for VEGF and integrin-mediated survival and migration signaling. Direct interactions between integrin αvβ3 and KDR may also play a role in survival functions of VEGF. A major mitogenic signaling mechanism for VEGF is the PLC-γ pathway resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate, generation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol, and subsequent mobilization of intracellular Ca2+ and PKC activation. PKC mediates activation of ERKs 1/2 via Raf-1 and MEK, and this pathway is a major mediator of both mitogenesis and cPLA2 activation leading to generation of COX-derived prostanoids (PGs), including PGI2 and PGE2. Ca2+ signaling is also important for eNOS activation and NO generation, and activates the serine/threonine phosphatase, calcineurin (protein serine/threonine phosphatase 2B), leading to activation of the transcription factor NFAT (nuclear factor of activated T-cells) and induction of COX-2, which mediates long-term prostanoid production. Functional signaling converges and branches at several points, emphasizing how linear signaling pathways are integrated to form signal transduction networks.

[a]*Adapted*[119].

Abbreviations: *VEFG, vascular endothelial growth factor; KDR, human kinase insert domain receptor; VRAP, VEGF associated receptor; PLC-γ, phosphoinositide phospholipase C; PI3K, phosphatidylinositol 3 kinase; Akt, protein kinase B; Ca2+, free calcium; eNOS, nitric oxide synthase; FAK, focal adhesion kinase; Src, sarcoma tyrosine kinase; IP3, inositol-3-phosphate; PKC, protein kinase C; ERK, extracellular signal-regulated kinases; MEK, mitogen activated-protein kinase; PLA2, phospholipase A2; COX, ciclooxygenase; Src, sarcoma tyrosine kinase; NO, nitric oxide; HCTPA, human cellular protein tyrosine phosphatase A.*

The platelet-derived growth factor family

Platelet derived growth factor (PDGF) is a heparin-binding family of polypeptide growth factors that comprises four members: PDGFA, PDGFB, PDGFC, and PDGFD, which form either homo or heterodimers (PDGF-AA, -AB, -BB, -CC, -DD). The PDGFs bind to the 3 different tyrosine kinase platelet-derived growth factor receptors (PDGFR): PDGFRαα, PDGFRββ and PDGFRαβ. When PDGFs bind to

their receptors, each half of the dimeric PDGF ligand recruits one receptor subunit to assemble the PDGFR dimer. Once dimerized, receptor subunits then cross-phosphorylate each other in specific tyrosine residues, activating the receptor, which in turn phosphorylates several target proteins in order to activate different signaling pathways[130] (Figure 12). PDGFRα and PDGFRβ are PDGF signaling receptors that induce angiogenesis through the up-regulation of VEGF production and the modulation of perivascular cell recruitment and proliferation[131]. In agreement, *Pdgfb -/- and Pdgfrβ -/-* knockout mice are embryonic lethal presenting severe hemorrhage and edema, due to the failure of immature vessels to attract pericytes[132]. PDGFC promotes vascular development in the embryo and in wound healing, as well as angiogenesis in avascular tissues, whereas PDGFD is involved in tumor neovascularization[133,134].

For the stabilization of ECs channels, PDGFB is released by ECs for chemoattracting PDGFβ pericytes[98,135]. Therefore, pericytes deficiency after PDGFB ablation leads to bleeding, microaneurysm formation, tortuosity and vessel leakage.

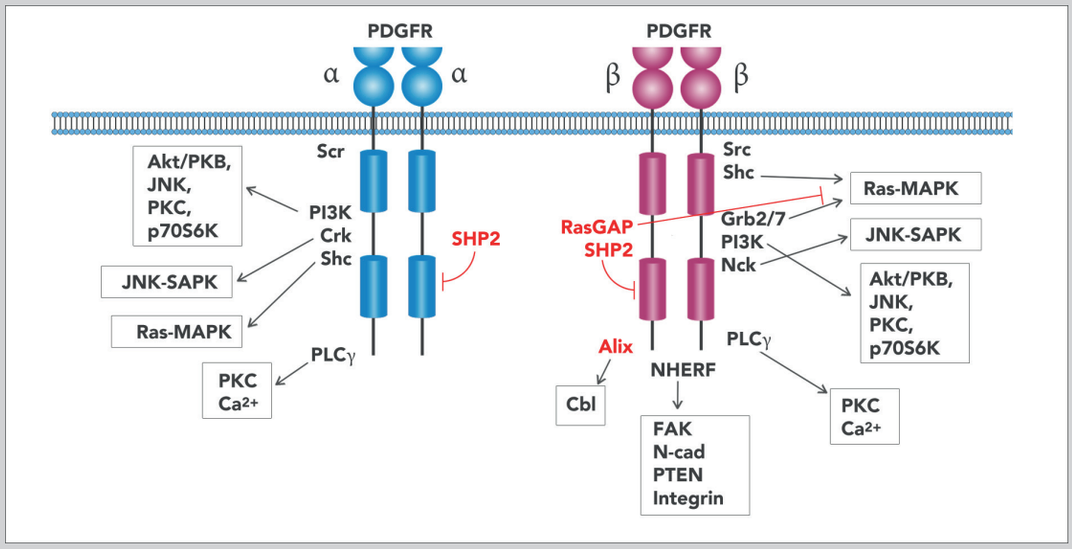

Figure 12. Platelet-Derived Growth Factor Receptor signal transductions[a]

The intracellular domains of PDGFRα and PDGFRβ and some of their direct interactors are illustrated. Arrows imply links to major signal transduction pathways and secondary effectors. Negative feedback signaling is indicated in red.

[a] *Adapted[136].*

Abbreviations: *Ca2+, free calcium; Cbl, casitas B-lineage lymphoma adaptor protein; Crk, Crk adaptor protein; FAK, focal adhesion kinase; Grb, growth factor receptor-bound protein; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NHERF, sodium-hydrogen exchanger regulatory factor; p70S6K, phosphoprotein 70 ribosomal protein S6 kinase; PI3K, phosphatidylinositol-3-kinase; PKB, protein kinase B; PKC, protein kinase C; PDGFR, platelet-derived growth factor receptor; PLC, phospholipase C; Ras, rat sarcoma gene; PTEN, protein tyrosine phosphatase; SAPK, stress activated protein kinase; Shc, Shc transforming protein ;SHP2, Src homology phosphatase 2; Src, sarcoma tyrosine kinase.*

Angiopoietins

The ANG family consists of four members, ANG1, ANG2, ANG3 and ANG4, which bind to specific tyrosine kinase receptors with immunoglobulin-like and EGF-like domains TIE1 and TIE2[92]. The most exhaustively studied angiopoietins are ANG1 and ANG2 (see Figure 13).

ANG1 is secreted mostly by pericytes and acts in a paracrine manner to activate TIE2 in ECs surface. ANG1 was shown to support ECs survival and to promote vascular stabilization, maintaining the vasculature in a quiescent state. Mice lacking ANG1 start do develop a primary vasculature, which fails to stabilize and remodel leading to embryonic lethality[137]. Therefore, ANG1 is essential for maturation and stabilization of the developing vasculature. Moreover, overexpression of ANG1 produces enlarged and leakage-resistant vessels in adult mice. It was also found that ANG1 acts synergistically with VEGF to promote angiogenesis[87].

On the other hand, ANG2 is specifically produced by ECs and stored in Weibel-Palade bodies, from which it can be rapidly released upon stimulation, acting in an autocrine manner[138]. ANG2 has been considered to have the opposite effect of ANG1, since it disrupts the connections between the endothelium and perivascular cells and promotes cell death and vascular regression by blocking ANG1-mediated TIE2 activation[139]. However, a number of studies on ANG2 function have suggested a more complex condition. Corneal pocket assays have shown that both ANG1 and ANG2 had similar effects acting synergistically with VEGF to promote the growth of new blood vessels, suggesting a proangiogenic role for ANG2[140]. Moreover, it was found that *in vitro* and at high concentrations ANG2 can also be pro-angiogenic, suggesting the possibility that there was a dose-dependent endothelial response[141]. Furthermore, it was observed that the action of ANG2 could depend on ECs differentiation state, since the activation of TIE2 by ANG2 was observed when ECs were cultivated on fibrin gel (a substrate that stimulates ECs differentiation)[142]. In microvascular ECs cultured in a three-dimensional collagen gel, ANG2 can also induce TIE2 activation and promote formation of capillary-like structures[143]. Importantly, it was found that *in vivo*, ANG2 is expressed during development at sites where blood vessel remodeling was occurring[139], as well as in highly vascularized tumors[144,145]. Finally, it was also demonstrated that *in vivo*, ANG2 could stimulate angiogenesis or capillary regression depending on the presence of VEGF[146]. In the presence of endogenous VEGF, ANG2 had a complex effect and efficiently induced increased blood vessel diameter, remodeling of the basal lamina, ECs proliferation, migration, and sprouting. If endogenous VEGF activity was inhibited, ANG2 effectively promoted capillary regression.

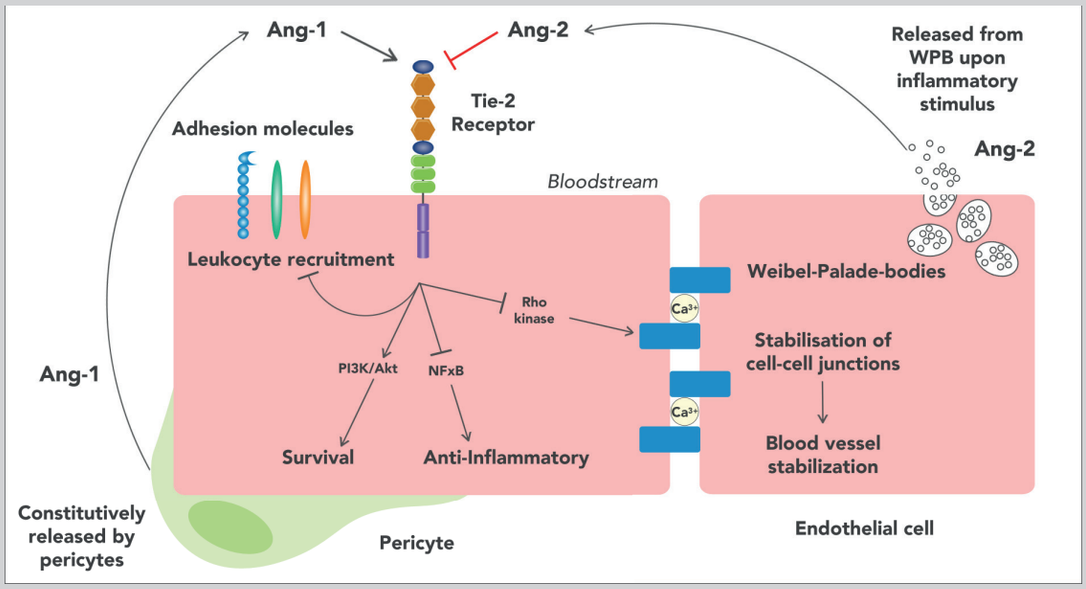

Figure 13. Angiopoietin Signaling in Angiogenesis[a]

ANG1 and 2 both bind to TIE2 in ECs. Upon ligand binding, TIE2 dimerizes and is autophosphorylated, promoting the activation of multiple downstream signaling molecules. Endothelial cell survival is stimulated through PI3/Akt and eNOS pathway, as proliferation and migration depend on the activation of PAK (p21-activated kinase) and mitogen-activated protein kinase/ERK signaling pathways. Moreover, ANG2 is also able to stimulate an integrin-mediated response, enhancing its canonical signaling pathway effects.

[a]*Adapted angiopoietin.de (http://www.angiopoietin.de/pageID_6911126.html).*

Abbreviations: *ANG, angiopoietin; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; NFκB, nuclear factor-κB; PI3K, phosphoinositide 3-kinase; TIE, tyrosine kinase with Ig and EGF homology; WPB, Weibel-Palade bodies.*

Its central role in the regulation of physiological and pathological angiogenesis makes the ANG/TIE signaling pathway a therapeutically attractive target for the treatment of vascular disease and cancer.

Transforming Growth Factor β

TGFβ refers to a family of cytokines comprising 3 isoforms; TGFβ1, TGFβ2, and TGFβ3. The secretion of all TGFβ isoforms is done in an inactive latent form that can be activated by shear forces, low pH heat, reactive oxygen species, thrombospondin 1, integrins and proteases. After their activation, TGFβ ligands bind to transforming growth factor β receptors (TGFRβ) that become activated. The intracellular Smad signaling pathway (Figure 14) is the one most activated, however, other signaling pathways such as PI3K, Rho-like GTPase, and mitogen-activated protein kinase pathways[147] are also activated.

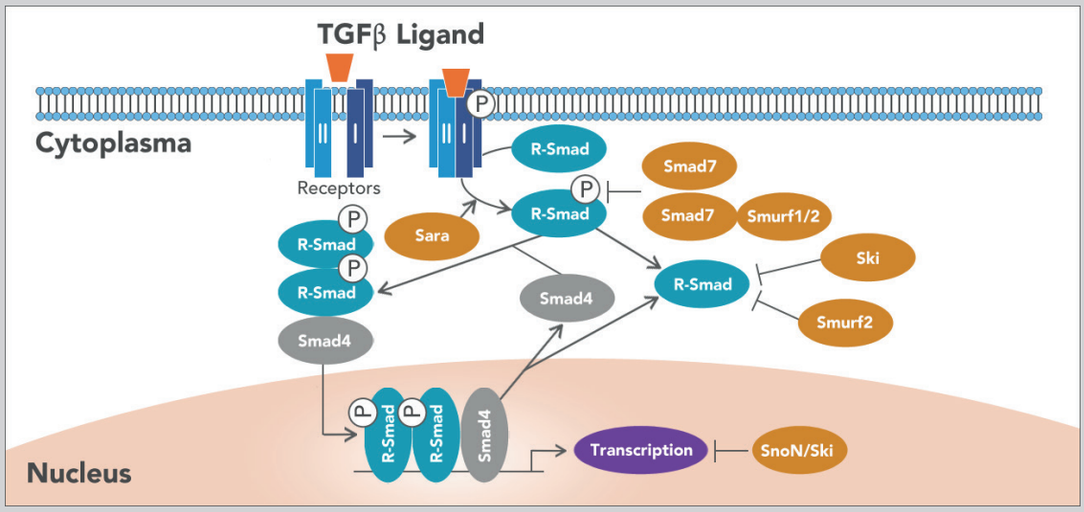

Figure 14. Transforming Growth Factor β Signaling Pathway[a]
Feedback loops shaping the cellular response have been identified, ie, via Smurf1/2 (ubiquitin E3 ligases), Smad7 (transforming growth factor receptor I antagonist), SARA (Smad anchor for receptor activation), or SnoN/Ski (oncoproteins).

[a]*Adapted*[158].
Abbreviations: *TGFβ, transforming growth factor β.*

TGFβ has many distinct functions depending on its targets and cellular contexts including angiogenesis, immune responses, bone remodelling, cell growth, differentiation and apoptosis[148,149].

The anti-inflammatory actions of TGFβ1 on ECs are well described. Moreover, it was found that low-dose ionizing radiation (LDIR) (0.3 Gy) induces a significant increase in TGFβ1 circulating levels and this effect may contribute to the anti-inflammatory effect mediated by LDIR[150].

In physiological conditions, TGFβ can influence the angiogenic process in different ways, depending on its concentration and the presence of other cytokines in the microenvironment. Therefore, it was shown *in vitro* that, at low concentrations, TGFβ potentiated the effect of VEGF and FGF2 in enhancing ECs invasion, but at high concentrations it has the opposite role[151,152]. Moreover, it was found that at low doses, TGFβ1 up-regulates expression of angiogenic factors and ECM degrading proteases and consequently contributes to the angiogenic switch, whereas at high doses TGFβ1 inhibits ECs growth, promotes the reorganization of the basement membrane and stimulates smooth muscle cells differentiation and recruitment[153].

Genetic studies in mice have shown that the loss of *Tgf*β signaling components results in frail vessels with decreased vessel wall integrity. Inactivation of the *Tgf*β1 caused lethality due to defects in the hematopoietic system and yolk sac vasculature[154,155].

In a tumoral context, TGFβ signaling has been shown to act as a strong activator of tumor growth and metastasis by acting directly in tumor cells and local environment. TGFβ contributes for immunosupression, modification of the ECM and induction of angiogenesis. TGFβ1 and β2 induce cancer cells to produce VEGF and plasminogen activator inhibitor (PAI1), promoting ECs proliferation and vascular remodeling[156,157]. Some studies demonstrated that hypoxia and TGFβ signaling pathways could synergize in the regulation of VEGF gene expression at the transcriptional level and cooperate in the induction of the promoter activity of VEGF[155]. Blocking of TGFβ action inhibits tumor viability, migration, and metastasis in mammary cancer, melanoma and prostate cancer. Reduction of TGFβ production and activity may be a promising target of therapeutic strategies to control tumor growth[100].

Fibroblast Growth Factor

A diverse and large growth factor signaling family is represented by FGFs as well as their tyrosine kinase fibroblast growth factor receptors (FGFR) (see Figure 15), which control a wide range of biological functions[90]. In this work, we are especially interested in FGF2 (also known as bFGF), a well-known pro-angiogenic factor involved in the maintenance and activation of vascular endothelium.

In normal conditions, FGF2 is expressed in low levels and most of it, when released, is trapped in the ECM. However, during wound healing or some pathological conditions like cancer, FGF2 levels its activity increase: its expression is upregulated and the levels of some proteases that release FGF2 from ECM also increase, allowing it to activate its receptors and exert its functions[159]. FGF2 binds to 4 membrane tyrosine kinase receptors (FGFR1, 2, 3 and 4) expressed by several cell types. FGFR1 is the main FGFR expressed in ECs, but small amounts of FGFR2 have also been found. FGFR3 and FGFR4 have never been reported in the endothelium[94,160].

When FGF2 binds to FGFR1, it dimerizes, autophosphorylates in specific tyrosine residues and phosphorylates other molecules, activating them. Stimulation of FGFR1 in ECs leads to proliferation, migration, protease production and tubular morphogenesis, whereas activation of FGFR2 increases only cell motility[161]. Although most of these effects are transduced through mitogen activated protein kinases (MAPK) activation[162], protein kinase C and PI3K activation are also required for FGF-induced ECs proliferation and migration[163,164]. Studies using knockout mice have demonstrated essential functions for FGFR1 and FGFR2 in early development. Mice lacking individual *Fgfs* revealed a variety of phenotypes, which range from early embryonic lethality to very mild defects, most likely reflecting the redundancy of the FGF family of ligands or their uniqueness of expression in

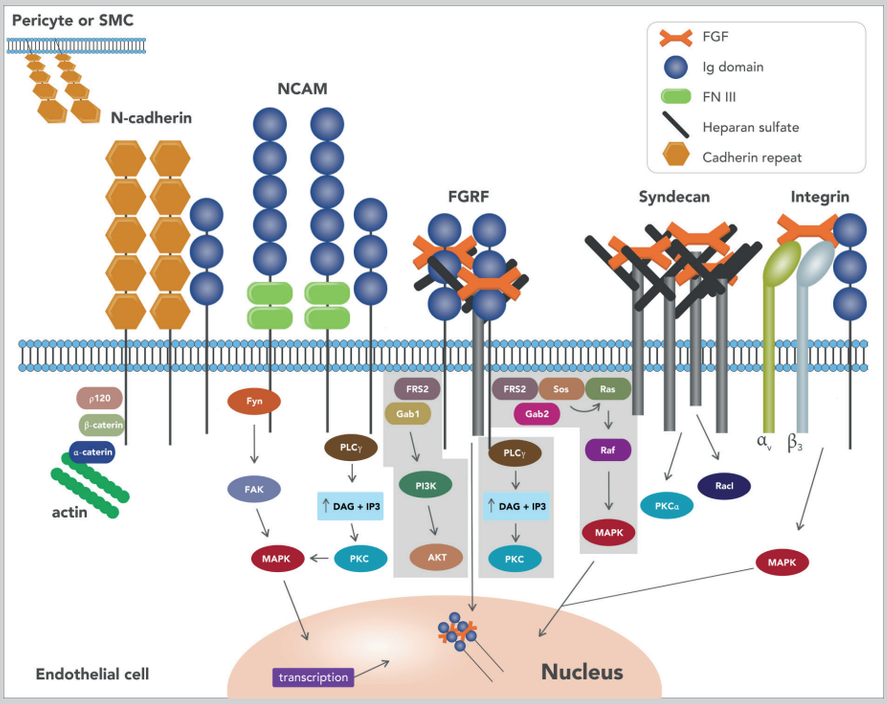

Figure 15. Fibroblast Growth Factor Canonical and Noncanonical Signaling Pathways[a]
The canonical signaling pathway is highlighted in grey.

[a]*Adapted*[165].
Abbreviations: *DAG, diacylglycerol; FGFR, fibroblast growth factor receptor; Ig, immunoglobulin; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C, PLC-γ, phospholipase C-γ.*

specific tissues[165]. Nevertheless, FGFs have been postulated to play a major role in wound healing, with particular focus on potential roles for FGF1, FGF2 and FGF7. Accordingly, topical application of FGF1 and FGF2 accelerates wound healing in a number of animal models[166]. Moreover, *Fgf2* and *Fgf1*/*Fgf2* knockout mice exhibit delay in the remodeling of damaged blood vessels during wound healing and tumor angiogenesis[93]. Additionally, tube formation stimulated by VEGF is totally abolished when neutralizing antibodies to FGF2 are added to the system, showing that in this particular setting, VEGF requires the presence of FGF2 to promote vessel assembly[167].

Hepatocyte Growth Factor

Hepatocyte growth factor (HGF) is secreted by different cell types in an inactive form, which after being cleaved, originates 2 independent and active subunits (α and β),

that in turn activate a transmembrane tyrosine kinase receptor named c-MET. After binding to HGF, c-MET is activated and phosphorylates intracellular signaling proteins, such as Ras, PI3K, PLCγ, Shp2 and Crk2 (Figure 16).

Although HGF is only expressed by cells of mesenchymal origin, c-Met is expressed by different cell types like pericytes, hematopoietic cells, hepatocytes, neural cells and ECs[168]. Consequently, the HGF/c-Met signaling pathway is involved in several physiological and pathological processes such as embryogenesis, wound healing, organ regeneration, inflammation, and tumor invasion. Since angiogenesis is a component of each of these processes, it was demonstrated that the *in vivo* biologic action of HGF might be due to its effect on both epithelial and vascular ECs.

HGF is an angiogenic factor for its ability to promote ECs growth, survival, and migration both *in vitro* and *in vivo*. It was found that HGF induces the repair of wounds in EC monolayer. Moreover, HGF stimulates the scatter of ECs grown on three-dimensional collagen gels, inducing an elongated phenotype[169]. In the rabbit cornea, highly purified HGF promotes neovascularization at sub-nanomolar concentrations[169]. It was also found that HGF stimulates ECs expression of urokinase. Urokinase bound to its specific cell surface receptor mediates focal, directed, extracellular proteolysis, which is required for ECs invasion and migration during the early stages of angiogenesis[170]. Moreover, it was found that combining HGF and VEGF results in a much more robust endothelial proliferative and chemotactic response, than either growth factor alone[171].

Early studies of the proangiogenic actions of HGF attributed the effects of HGF to the induction of VEGF. It was also observed that HGF increased the expression of keratinocyte-derived VEGF and suggested that HGF might induce angiogenesis by a paracrine mechanism[172,173]. However, other studies suggested that the proangiogenic effects of HGF were independent of VEGF[174]. A gene expression profiling study clearly demonstrated that HGF and VEGF signal through discrete pathways in vascular ECs, and moreover the combination of the two growth factors synergistically induces a number of genes involved in the regulation of the cell cycle[175].

Oncogenic activities of HGF have been proposed through its role in promoting angiogenesis in tumors. Data supporting this hypothesis came from examining the correlation of vessel density and HGF production in tumor samples and xenograft models[176,177,178].

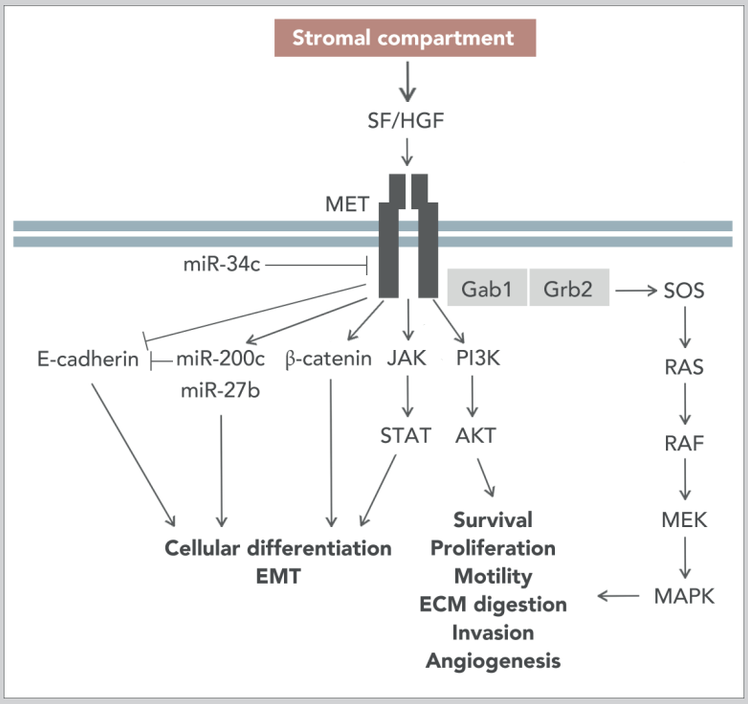

Figure 16. Hepatocyte Growth Factor/c-Met Signaling Pathway[a]

Signal transduction of HGF/c-Met is initiated by binding of HGF to c-Met. Inhibitors of the HGF/c-Met signaling pathway (competitive inhibitors, tyrosine kinase inhibitors, or downstream inhibitors) target 1 of the molecular events of HGF/c-Met signaling activation and transduction.

[a]*Adapted*[179].

Abbreviations: *FAK, Src/focal adhesion kinase; HGF, hepatocyte growth factor; PI3K, phosphoinositide 3-kinase; STAT, p120/signal transducer activator of transcription.*

1.2.6. Angiogenesis and Disease

ECs functions comprise the regulation of hypoxic tissues neovascularization, leukocyte trafficking, nutrient and electrolyte uptake¸ blood coagulation and regulation of vascular tone and barrier. Accordingly, Oszajca et al. contend that the presence of endothelial dysfunction (failure of the endothelium to perform its physiological functions) results in unavoidable development of diseases[201].

Endothelial dysfunction broadly refers to the denudation caused by the inability to replace desquamating and apoptosis of ECs. However, it also refers to the reduced synthesis of molecules that have a protective effect on the vasculature (ie, NO) as well as the expression of proteins like VCAM1 and intercellular adhesion molecule 1, through which the inflammatory cell adhesion on the endothelium surface (endothelium activation) is mediated. In addition to being the first step in the formation of atherosclerotic plaques, the inflammatory conditions induced

by ECs dysfunction are also perceived to be involved in cardiovascular disease progression[202-204].

Since pathological blood vessels affect so many medical disorders (Tables 11 and 12), the face of medicine will change with angiogenesis research in the coming decades, as it is estimated that more than 500 million people worldwide would benefit from anti or pro-angiogenic treatment[61].

Table 11. Diseases Characterized or Caused by Insufficient Angiogenesis or Vessel Regressions[a]

Organ	Disease in Mice or Humans
Numerous organs	Cancer and metastasis, infectious diseases, vasculitis and angiogenesis in autoimmune disorders such as systemic sclerosis, multiple sclerosis, Sjögren disease
Blood and lymph vessels	Vascular malformations, DiGeorge syndrome, hereditary hemorrhagic telangiectasia (Osler-Weber-Rendu), cavernous hemangioma, cutaneous hemangioma, lymphatic malformations, transplant arteriopathy and atherosclerosis
Adipose tissue	Obesity (angiogenesis induced by fat diet, weight loss by angiogenesis inhibitors, adipocytokines stimulate angiogenesis, lymph induces preadipocyte differentiation)
Skin	Psoriasis, warts, allergic dermatitis, scar keloids, pyogenic granulomas, blistering disease, Kaposi sarcoma in AIDS patients, systemic sclerosis
Eye	Persistent hyperplastic vitreous syndrome, diabetic retinopathy, retinopathy of prematurity, choroidal neovascularization
Lung	Primary pulmonary hypertension, asthma, nasal polyps, rhinitis, chronic airway inflammation, cystic fibrosis
Gastrointestinal tract	Inflammatory bowel disease, periodontal disease, ascites, peritoneal adhesions, liver cirrhosis
Reproductive system	Endometriosis, uterine bleeding, ovarian cysts, ovarian hyperstimulation
Bone, joints	Arthritis and synovitis, osteomyelitis, osteophyte formation, human immunodeficiency virus-induced bone marrow angiogenesis
Kidney	Diabetic nephropathy (early stage)

[a] *Adapted[205].*

Table 12. Diseases Characterized or Caused by Abnormal or Excessive Angiogenesis[a]

Organ	Disease in Mice or Humans
Nervous system	Alzheimer disease, amyotrophic lateral sclerosis, diabetic neuropathy, stroke
Blood and lymph vessels	Diabetic angiopathy, hypertension, atherosclerosis, restenosis, lymphedema
Gastrointestinal tract	Gastric and oral ulcerations, Crohn disease
Skin	Hair loss, skin purpura, telangiectasia, systemic sclerosis, lupus
Reproductive system	Preeclampsia, menorrhagia
Lung	Neonatal respiratory distress syndrome, pulmonary fibrosis, emphysema
Kidney	Nephropathy, glomerulosclerosis, tubulointerstitial fibrosis
Bone	Osteoporosis, impaired bone fracture healing
Heart	Ischemic heart disease, cardiac failure

[a] *Adapted[205].*

1.3. VASCULOGENESIS

1.3.1. Endothelial Progenitor Cells

The vascular endothelium establishes a dynamic barrier between blood and tissues, regulating several homeostatic processes and hemorreologic functions.

EPCs were discovered by Asahara et al.[180], and since then, several studies in different models of tissue injury and remodeling (lower limb ischemia¸ stroke, retinopathy, myocardial infarction and wound healing) have shown that they are key players to the neovascularization process. Even though, EPCs are primarily located in BM, in the alleged stem cell niche, one can also find EPCs in PB and as resident cells in certain organs of the body (Figure 17).

EPCs can be described as lineage-committed stem cells as they present stem cell properties like differentiation, clonagenicity and self-renewal. They are generally defined based on their surface markers, isolation methods and cell origin. Sukmawati et al.[181] summarized the various methods through which EPCs can be isolated: (i) colony formation assay; (ii) detection of acetylated LDL uptake and lectin-binding and (iii) flow cytometry.

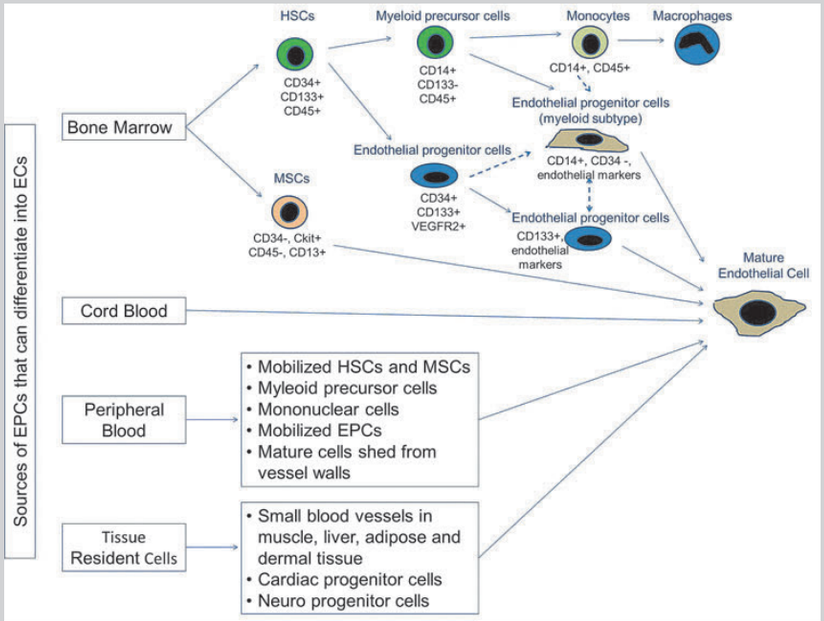

Figure 17. Origin and Differentiation of Endothelial Progenitor Cells[a]

EPCs sources: Hematopoietic stem cells (HSCs) resident in the bone marrow (BM) niche give rise to a population of cells that differentiate into myeloid progenitor or endothelial progenitor subtypes. These subsets differentiate into their respective mature cell type. Alternative pathways (dashed lines) to derive EPCs have been proposed, where EPCs arise directly from HSCs or via a myeloid progenitor/monocyte intermediate. Cell surface markers differentiate cells along the proposed pathway of EPC ontogeny.

[a]*Adapted[183].*

Presently, EPCs are characterized as having at least antigenic markers (by flow cytometry) of stem and hematopoietic lineage (Human: CD34 (mucosialin) and CD133 (early hematopoietic stem cell marker); Mouse: CD34, c-Kit or Sca-1), in addition with markers representative of their endothelial commitment (Human and mouse: VEGFR2) and finally along with clonal, functional and morphological characteristics. As opposed to the CD34 marker of hematopoietic and stem lineage, the CD133 marker is lost by mature ECs. Thus, Kim et al. select a combination of VEGFR2, CD133 and CD34 as the most promising markers in identifying EPCs[182].
There are two main categories from which the circulating EPCs are derived: the first is hematopoietic EPCs (derived from the statin cells in BM) and the second is non-hematopoietic EPCs (organ or tissue derived, including blood cells) also referred to as endothelial outgrowth cells (EOCs). Thus, early EPCs can be found with the following surface markers: VEGFR2, CD34, CD133 and CD45, and mature EPCs are characterized by loss of antigenic marker 133, with the maintenance of VEGFR2, CD34 along with von-Willebrand factor, CD31 and VE-cadherin. In comparison to early EPCs, late EPCs show higher levels of VEGFR2 expression. Consequently, late EPCs display better capacity to form tubular structures as well as better long-term survival in comparison to early EPCs. Nevertheless, early EPCs add to neovascularization through the secretion of greater amounts of angiogenic factors like IL8 and VEGF.
Even though vasculogenic potential is retained by EOCs, they seem to have less proliferative potential in addition to unavoidable gradual senescence compared to hematopoietic EPCs, and as such they are not the first choice for clinical use.
The availability of EPCs directs the process of postnatal vasculogenesis, which further develops in four stages (see Figure 18). Tissue injury results in BM EPCs mobilization and subsequently in their attachment to the target tissue. This complex process is regulated by different cytokines, receptors, ligands, enzymes, hypoxia and growth factors. As a result, EPCs migrate and invade the target tissues through differentiating into mature ECs under paracrine and juxtacrine control. During this process, EPCs interact with various physiological compartments like the target organ, blood vessels, PB and BM[184].
The proportion of circulating EPCs is considerably low in physiological conditions. EPCs are mainly located in the stem cell niche in BM, characterized by low oxygen pressure as well as a high concentration of stromal-derived factor (SDF) 1, which is a very potent chemoattractant for EPCs. Under hypoxic conditions, HIF is up-regulated and bounds to the VEGF promoter. VEGFR1 and VEGFR2 are expressed on ECs surface. EPCs mobilization is driven as a result of the overproduction of VEGF at the BM niche and at the ischemic location.

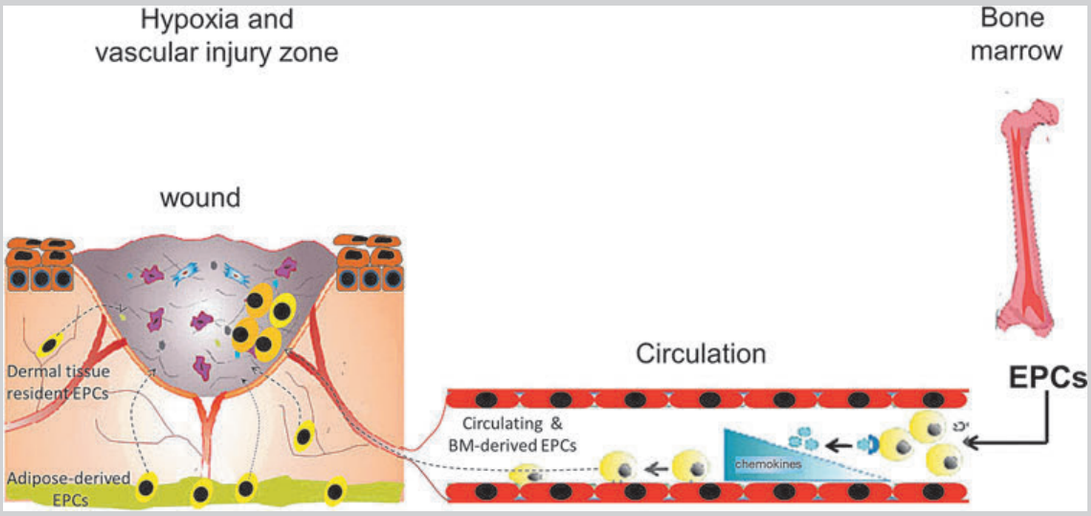

Figure 18. Endothelial Progenitor Cells Mobilise in Response to Hypoxia induced by Trauma or Vascular Injury[a]

Under normal physiological circumstances, EPCs exist in a stem cell niche in the bone marrow (BM). Peripheral tissue hypoxia inducing concentrations of EPC-mobilising chemokines and growth factors to a level higher then that seen in BM result in EPCs release and mobilization into PB. Circulating EPCs home to injury locations in response to chemokine signaling. Alongside, circulating progenitor cells, tissue-resident, and adipose-derived stem cells respond to the chemokine signaling and home to the active tissue-remodelling site.

[a]*Adapted*[183].

Tissue ischemia (hypoxia) also leads to the up-regulation of SDF1. Similar to VEGF, SDF1 is directly and strongly stimulated by HIF1α, According to Dimmeler et al, NOS activated by these factors leads to an increased production of NO, thereby regulating the enzymatic activity of MMPs, particularly MMP9[185]. The transformation of membrane bound Kit ligand (mKitL) into soluble ligand Kit (sKitL) is promoted by the activation of MMP9, which is then followed by the mobilization of stem cells and c-Kit positive progenitor cells into the BM vascular zone, resulting in the subsequent mobilization and release of EPCs to peripheral circulation[186].

Circulating EPCs respond and bind to tissues in active remodelling, a process modulated by several chemokines. Of these, cell-derived factor 4 receptor (CXCR4) and SDF1 are perceived as crucial mediators for EPCs recruitment. Other receptors and chemokine identified to be playing a crucial role in the homing and activation process of EPCs include: chemokine receptors 2 and 5 and CC-chemokine, CCL5 and CCR5, growth regulated oncogene and CXCR1, and IL8 and CXCR2[187]. After the activation of EPCs, they initiate a process of integrin-mediated adhesion to ECs along with subsequent trans-endothelial migration to tissue remodelling and vascular areas. Once EPCs cross the monolayer of ECs, they migrate through the basal membrane of the vessel as well as interstitial matrix, in a process again dependent on the

enzymatic activity of MMPs, specifically MMP9. Once EPCs enter the healing area, they promote neovascularization in different ways: (i) direct incorporation into the forming vessels; (ii) differentiating into mature ECs and (iii) production of paracrine and juxtacrine mediators (VEGF, SDF1, insulin-like growth factor 1, monocyte chemotactic protein 1 (MCP1), PDGF) that regulate the neovascularization process.

The expression profile of integrins by EPCs is quite dynamic, allowing the regulation of their differentiation, invasion, trans-endothelial migration, homing and mobilization. Integrins α4 and β3 are the major integrins regulating the fixation of EPCs in BM. Chavakis et al. identified β2 integrin as being mainly responsible for EPCs' fixation in angiogenic remodeling sites. The trans-endothelial migration of EPCs is also modulated by β2 integrin along with VEGF and MCP1[188].
In summary, many potential candidate cells have been evaluated as promoters of neovascularization, including whole BM cells, bone marrow-derived mononuclear cells (BM-MNCs), hematopoietic stem cells, EPCs and hemangiocytes. Each one of these candidate cells (sources) has potential advantages and limitations for clinical use that are summarized in Table 13.

Table 13. Advantages and Limitations of Different Types of Stem Cells[a]

Stem cell type	Limitations	Advantages
Embryonic stem cells	Ethical dilemmas, possible immune rejection after implantation, a small number of differentiated cardiomyocytes being generated, leading to teratocarcinomas; genetic instability	Differentiating into cells of all three germ layers
Pluripotent stem cells	Genetic instability, more research needed before using for cardiovascular repair/regeneration	Avoiding ethical concerns
Adult stem cells	Natural regeneration capacity of CSCs being too limited, acquisition and isolation difficulties, more research needed	Avoiding ethical concerns, lower risk of immune rejection
Mesenchymal stem cells	More research needed	Allowing for allogeneic grafting without the use of immunosuppressive agents, self-renewal, proliferating, and differentiating, promoting growth of adjacent cells, less susceptible to mutations, easy to collect
Hematopoietic stem cells	High maintenance, low frequencies, unknown signaling pathways	Proliferating and migrating to injury site in response to physiological/pathological stimuli, capable of myogenesis and angiogenesis
Endothelial progenitor cells	Extremely low numbers in PB and BM making ex vivo expansion difficult	Increasing its numbers in response to ischemia/cytokine stimuli and migrating to injury site and differentiating into new myocytes

[a] *Adapted*[189].

1.3.2. Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are among the possible sources for therapeutic angiogenesis cell approach (Table 13). MSCs are multipotent non-hematopoietic, fibroblast-like cells, forming a distinct entity to the mesenchyme, embryonic connective tissue, which is derived from the mesoderm and differentiates to form hematopoietic stem cells.

MSCs were first collected from BM and since then they have also been isolated from a variety of other sources: PB, umbilical cord blood/tissue, adipose tissue, lung, heart, placenta, periodontal ligament tissue, along with foetal and amniotic membranes. MSCs isolated from various adult and neonatal tissue sources have significantly different morphology and express a distinct pattern of cell surface markers[190]. Moreover, they exhibit different *ex vivo* differentiation capacity and pro-angiogenic properties[191]. These observations suggest that MSCs from different tissues may not be biologically equivalent and exhibit variable self-renewal and multipotential capabilities.

MSCs are ease to isolate, have good *ex vivo* expansion capacity and are regarded as a great promise for therapeutic angiogenesis due to their pro-angiogenic/ vasculogenic paracrine activity, transdifferentiation, immunosuppressive capacity and low immunogenicity. No unique cell surface marker unequivocally distinguishes MSCs from other stem cells, which makes a uniform definition difficult[192]. The International Society for Cellular Therapy (ISCT) proposed criteria that comprise (i) adherence to plastic in standard culture conditions; (ii) expression of the surface molecules CD73, CD90, and CD105 in the absence of CD34, CD45, HLA-DR, CD14 or CD11b, CD79a, or CD19 surface molecules as assessed by fluorescence-activated cell sorter analysis and (iii) a capacity for differentiation to osteoblasts, adipocytes, and chondrocytes *in vitro*. These criteria were established to standardize human MSCs isolation but do not apply uniformly to other species.

MSCs collected from different sources (Figure 19) have also been shown in experimental models to be effective in the treatment of hindlimb ischemia (HLI)[191]. MSCs can home to and survive in an ischemic environment. Through paracrine effects, they aid in the promotion of arteriogenesis and angiogenesis by releasing various angiogenic growth factors including VEGF, FGF, and SDF1α. Analysis of the MSCs secretome using a protein array screen revealed a large fraction of chemotactic cytokines, or chemokines. Genetic modification of MSCs has been shown to further increase the range of paracrine effects. As part of their multiple differentiation potential, MSCs can be induced to adopt endothelial properties in the presence of VEGF and/or bFGF, and to express endothelial-specific markers. These

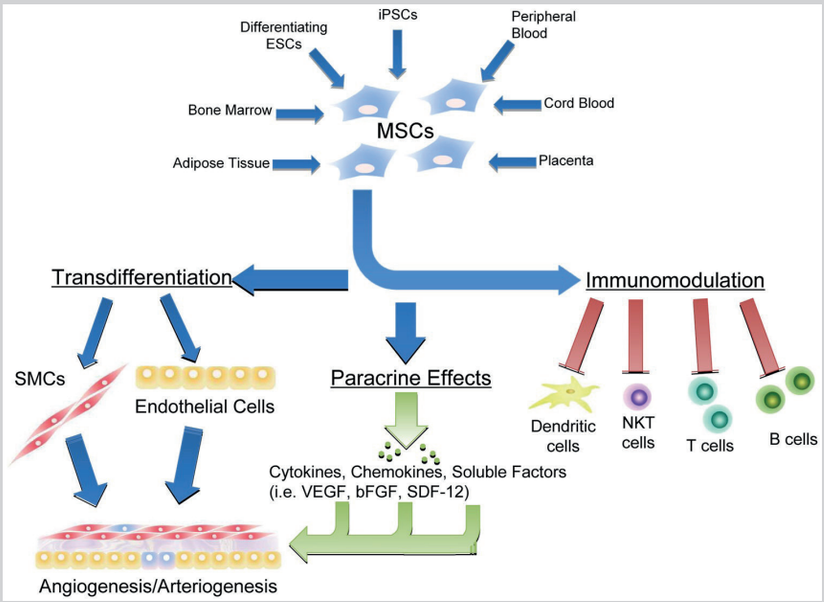

Figure 19. Biological Sources and Activity of Mesenchymal Stem Cellsy[a]

MSCs can be isolated from multiple sources (adipose tissue, bone marrow, cord blood/tissue, placenta) and exert therapeutic effects on multiple systems to contribute to PAD therapeutic.

[a]*Adapted*[191].

Abbreviations: *ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stem cells; SMCs, smooth muscle cells; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; SDF12, stromal cell derived factor 12; NKT cells, natural killer T cells.*

features enable MSCs to promote post-ischemic neovascularization and blood flow recovery in ischemic diseases secondary to PAD, although the specific mechanisms by which they do so still need to be fully characterized.

Furthermore, several studies show that MSCs have unique immunological characteristics that allow their persistence in a xenogeneic environment and confirmed their immunomodulatory properties[193]. Many mechanisms have been proposed for the immunomodulatory effects of MSCs. It is widely accepted that this immunomodulation is exerted mainly through a paracrine effect, via the secretion of soluble factors such as TGF1 and HGF, soluble human leukocyte antigen G (HLAG), interleukin (IL) 10, prostaglandin E_2, NO, galectin-1 and galectin-3, IL-6, indoleamine 2,3-dioxygenase, leukemia inhibitory factor, heme oxygenase 1, chemokine (C-C motif) ligand 2 (CCL2), and insulin-like growth factor-binding proteins[194]. Beyond soluble factors, CD200, CD276, and HLAE have recently been suggested to be involved in the immunoregulatory mechanisms of MSCs[194]. MSCs can induce immunosuppressive activity for a broad range of immune cells, including T lymphocytes, B lymphocytes, natural killer cells and dendritic cells. Thus,

this immunosuppressive effect may give MSCs unique advantages as a transplanted cell therapy[191].

In summary, MSCs exert vascular therapeutic effects via secretion of paracrine factors that may have anti-inflammatory and immunomodulatory effects. Immune-modulation is a unique feature for MSCs, making their combination with other stem cells subtypes very appealing to enhance allogeneic injection for inducing repair. Studies have shown that cells obtained from older patients with multiple risk factors have impaired functions: for this reason, therapeutic success in CLI patients could be increased by using MSCs from young donors, overcoming age and vascular disease–mediated deficiencies in EPCs number, function, and angiogenic cytokine production.

Umbilical cord tissue-derived mesenchymal stromal cells

The umbilical cord tissue is among the possible sources of MSCs[195]. Mesenchymal stem cells derived from umbilical cord tissue (UC-MSCs) have the similar surface phenotype, plastic adherence and multipotency as those of MSCs derived from other sources. Compared with the counterparts of other origins, UC-MSCs have attractive advantages: (i) a noninvasive collection procedure for autologous or allogeneic use and ethically acceptable; (ii) a lower risk of infection; (iii) a low risk of teratoma; (iv) easy to obtain a substantial number of UC-MSCs after several passages and extensive *ex vivo* expansion; (v) faster self-renewal when compared to BM-MSCs; (vi) low immunogenicity with a good immunosuppressive ability[196].

ECBio (Amadora, Portugal), a biotechnology company, has developed proprietary technology to consistently isolate, expand and cryopreserve a well-characterized population of human stem cells derived from umbilical cord tissue (Wharton's jelly) named as UCX® cells[197]. The technology used for UCX® isolation is different from other UC-MSCs isolation methods. Very briefly: (i) the introduction of a three-stage strategy for cell recovery was introduced in order to make the method 100% reliable; (ii) the isolation begins with peeling off the amniotic membrane which reduces the frequency of microbial contamination and augments the purity of the resulting UCX® population by eliminating epithelial progenitors; (iii) tissue incisions and/or tissue mincing/crushing steps are avoided since, they could hinder its application in good manufacturing practice (GMP) settings and jeopardize stem cell phenotype due to excessive mechanical manipulation and (iv) the use of an optimized ratio between tissue mass, digestion enzyme activity units, overall solution volume and void volume allowing UCX® cell-specific release and intact cord vessels that will reduce contamination with endothelial and sub-ECs from umbilical arteries and vein[197].

UCX® cells are a homogeneous population of stem cells that comply with the current definition of MSCs as established by ISCT[197,198].

Importantly, it was shown that UCX® cells may be an effective and promising new approach for treating both local and systemic manifestations of inflammatory arthritis[197]. It was observed that UCX® cells have the capacity to inhibit human T-cell proliferation and concomitantly promote the expansion of Tregs more efficiently than BM-MSCs. Accordingly, xenogeneic UCX® administration in both acute carrageenan-induced arthritis and chronic adjuvant-induced arthritis models for arthritic inflammation, can reduce paw edema *in vivo* more efficiently than BM-MSCs and showed faster remission of local and systemic arthritic manifestations, respectively. These data strongly suggest that UCX® are immunosuppressive and have low immunogenicity, relevant for allogeneic applications[197].

Furthermore, it was shown that UCX® preserve cardiac function and attenuate adverse tissue remodeling after intramyocardial transplantation in a murine myocardial infarction model[199]. The cardioprotective effect of UCX® is exerted through paracrine mechanisms that appear to enhance angiogenesis, limit the extent of the apoptosis in the heart, increase proliferation and activate the cardiomyogenic gene expression program in a pool of myocardial resident cardiac progenitor cells[199]. In this particular work, it was shown that UCX® increase capillary density in the infarcted myocardium, since CD31-expressing cells were more abundant within the infarcted wall of UCX®-transplanted hearts when compared to vehicle-injected hearts[199]. It was also previously demonstrated that UCX® accelerate wound healing[200]. Those studies strongly suggest that UCX® act in different cell types through paracrine mechanisms. In addition, it was found that conditioned medium of UCX® cultures induces fibroblast and keratinocyte migration and UCX® are chemotactic to $CD34^-/CD45^-$ BM-MSCs[200]. Furthermore, it was also shown that *in vitro* UCX®-conditioned medium induces angiogenesis by promoting the formation of capillary-like structures by HUVECs[197].

1.4. THERAPEUTIC ANGIOGENESIS

The quality of life of CLI patients, as shown by psychological testing, has been described to be similar to that of terminally ill cancer patients[206]. Poor prognosis, the increasing number of patients with CLI, and the significant number of patients not candidates for revascularization procedures have created a growing need for new therapies to induce angiogenesis, with greater emphasis being placed on gene and cell therapy. In advanced PAD, the focus of new treatment strategies has been on the restoration of the balance in favour of tissue survival with the use of exogenous cellular and molecular agents, for promoting the regeneration of diseased microvasculature[62,207-209].

Therapeutic angiogenesis refers to the iatrogenic induced formation of a capillary network in ischemic tissue by changing the local milieu, so that a proangiogenic environment can be provided. Physiologically, the angiogenic molecules are outweighed by the angiostatic mediators, which prevent angiogenesis from occurring. This equilibrium is disrupted by the introduction of a vector forcing the expression of an angiogenic factor. As already discussed, there are two mechanisms through which angiogenesis can occur; the first is vasculogenesis and the second is angiogenesis. The capillaries formed by these mechanisms are small, with a diameter of about 10 to 20 μm, and are insufficient to fully compensate for a large occluded transport artery.

Arteriogenesis, also known as collateralization, is the process by which pre-existing collateral vessels increase up to 25 times their diameter, thus increasing blood perfusion[210]. Through this mechanism no new vessels are formed. This process implies structural remodeling of existing vessels and is essentially modulated by changes in shear stress forces, meaning that arteriogenesis is not merely the result of a vasodilation that becomes permanent[211]. Vessels formed by this mechanism are about 20 to 100 μm in diameter and have a fully developed tunica media, which allows vasodilation and regulation of blood flow. As such, the vessels formed by arteriogenesis are structurally more robust and functionally have a greater capacity for blood transport, especially when compared to the capillaries formed through angiogenesis[212]. Following an arterial occlusion, there is an increase in fluid shear stress against the vessel wall and consequently ECs are activated expressing monokines and monocyte adhesion proteins. This process generates a chemotactic gradient for the recruitment of monocytes that release proteases to degrade extracellular structures. Activated ECs then upregulate bFGF, PDGF-B and TGF-β1, thereby inducing the regrowth of smooth muscle cells and vessel enlargement[213]. Finally, vascular smooth muscle cells acquire contractile components and interstitial

matrix components providing the developing arteries with viscoelastic properties (elastin and fibrillin-2) and structural strength (collagen and fibrillin-1)[213].
Although they may occur independently, angiogenesis and arteriogenesis may also occur in a synergistic way, in which case angiogenesis is usually the initial process and the result will always be better, when compared to the isolated occurrence of any of the processes.
Early clinical studies have investigated the effects of exogenous administration of recombinant proangiogenic proteins, but this approach was hampered by difficulties to achieve adequate levels within the ischemic areas due to the short half-life of these agents[214]. As a step forward, gene therapy with DNA plasmids encoding proangiogenic factors was developed to increase the duration of transgene expression compared to direct administration of proteins. Although these plasmids were well tolerated by the immune system, gene transfer rates were low with a limited duration of transgene expression. It is also important to refer that PAD is associated with ECs dysfunction and with an important impairment in the number and function of circulating EPCs. More recently, cell therapy has gained predominance.

1.4.1. Gene Therapy

Currently, gene therapy in cardiovascular disease can be systematically organized into three key approaches: (i) viral gene transfer: hemagglutinating virus of Japan (sendai virus), adeno-associated virus, adenovirus and retrovirus; (ii) liposomal gene transfer with the use of cationic liposomes; and (iii) naked plasmid DNA transfer[215]. The initial approach based on an isolated single therapy application has been replaced by a multiple application strategy (in a time frame of 4 to 8-week) that allows for continued conditioning of the target area for angiogenesis.
Presently, the best delivery method for human gene therapy is the recombinant adeno-associated virus (rAAV) vectors. Systemic or even local administration of rAAV vectors is associated with leakage and their half-life is generally limited to a few hours[216]. Additionally, rAAV induce only minimal immune responses in the host while providing higher levels of transgene expression compared to plasmid DNA as well as long-term gene expression profiles, when confronted to adenoviral vectors[217]. Moreover, it is typical for rAAV vectors to remain episomal, dissipating one of the major concerns with retroviruses – genomic integration resulting in oncogene activation.
Jeffrey Isner is credited with popularizing therapeutic angiogenesis with his group's first trials using the isoform VEGF165 on a plasmid. After Isner´s first study, clinical

trials have tested various angiogenic growth factors like HGF, FGF isoforms, VEGF2, and VEGF121. Table 14 summarizes trials that have been performed in the field of gene therapy along with their clinical outcomes.

Table 14. Results of Gene Therapy for Critical Limb Ischemia[a]

Author	Year	Study Level	Number	Vector	Product	Delivery	Clinical Outcome*
Isner	1996	Patient series/uncontrolled trial	1	Plasmid	pVEGF165	Catheter	
Baumgartner	1998	Patient series/uncontrolled trial	9	Plasmid	pVEGF165	IM	Positive
Isner	1998	Patient series/uncontrolled trial	6	Plasmid	phVEGF165	IM	Positive
Baumgartner	2000	Patient series/uncontrolled trial	62	Plasmid	pVEGF165	IM	Equivocal
Rajagopalan	2001	Patient series/uncontrolled trial	5	Adenovirus	VEGF121	IM	Equivocal
Comerota	2002	Patient series/uncontrolled trial	51	Plasmid	NV1FGF	IM	Positive
Makinen	2002	Controlled trial	36	Adenovirus	VEGF	Catheter	Positive
Rajagopalan	2003	Randomised/controlled trial	105	Adenovirus	VEGF121	IM	Negative
Mohler	2003	Patient series/uncontrolled trial	21	Adenovirus	VEGF121	IM	Equivocal
Shyu	2003	Patient series/uncontrolled trial	21	Adenovirus	VEGF165	IM	Positive
Morishita	2004	Patient series/uncontrolled trial	6	Plasmid	HGF	IM	Positive
Kim	2004	Patient series/uncontrolled trial	9	Plasmid	VEGF165	IM	Equivocal
Matyas	2005	Controlled trial	13	Adenovirus	FGF4	IM	Equivocal
Kusumanto	2006	Controlled trial	54	Plasmid	phVEGF165	IM	Equivocal
Marul	2007	Patient series/uncontrolled trial	7	Plasmid	FGF1	IM	Positive
Rajagopalan	2007	Controlled trial	34	Adenovirus	HIF1α	IM	Equivocal
Powell	2008	Controlled trial	104	Plasmid	HGF	IM	Equivocal
Nikol	2008	Controlled trial	125	Plasmid	NV1FGF	IM	Positive
Baumgartner	2009	Patient series/uncontrolled trial	6	Plasmid	FGF1	IM	Equivocal
Shigematsu	2010	Controlled trial	44	Plasmid	HGF	IM	Positive
Powell	2010	Controlled trial	27	Plasmid	HGF	IM	Equivocal
Gu	2011	Patient series/uncontrolled trial	21	Plasmid	HGF	IM	Positive
Henry	2011	Patient series/uncontrolled trial	12	Plasmid	HGF	IM	Positive
Morishita	2011	Patient series/uncontrolled trial	22	Plasmid	HGF	IM	Positive
Shigematsu	2011	Patient series/uncontrolled trial	10	Plasmid	HGF	IM	Positive
Belch	2011	Controlled trial	525	Plasmid	NV1FGF	IM	Negative
Niebuhr	2012	Controlled trial	72	Plasmid	NV1FGF	IM	Negative
Makino	2012	Patient series/uncontrolled trial	22	Plasmid	HGF	IM	Positive

* Positive clinical outcome defined as improved perfusion, reduced pain, or decreased amputation.

[a]*Adapted*[224,225].

Abbreviations: *FGF, fibroblast growth factor; HGF, hepatocyte growth factor; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; NV, non-viral.*

TRAFFIC trial

In the TheRapeutic Angiogenesis with Recombinant FGF2 for IC (TRAFFIC) trial, 190 claudicants were infused with 1 or 2 doses of intra-arterial rFGF2[218]. Among these patients, 174 reached 90-day outcome mark; peak-walking time was increased by 0.60 min with placebo, by 1.77 min with single-dose, and by 1.54 min with double-dose. Therefore, a significant increase in peak walking time at 90 days was observed following intra-arterial infusion of recombinant FGF2.

RAVE trial

The safety and efficacy of the intramuscular delivery of adenoviral VEGF121 was assessed in 105 patients in the Regional Angiogenesis with VEGF (RAVE) study (a phase II, double blind, placebo controlled study)[118]. Placebo, low-dose, and high-dose groups did not show any variation in the primary efficacy endpoint – change in peak walking time at 12 weeks. Secondary endpoints like quality-of-life measures, claudication onset time, and ABI were also equivalent in all groups at 12 and 26 weeks. However, the administration of adenoviral VEGF121 was, in these patients, associated to the development of peripheral edema, as a side effect.

TALISMAN 201 trial

The safety and efficacy of intramuscular administration of NV1FGF, a plasmid-based FGF1, versus placebo was evaluated in the Therapeutic Angiogenesis Leg ISchemia for the Management of Arteriopathy and Non-healing ulcer (TALISMAN). This study was a phase II of administration of NV1FGF including 125 CLI patients presenting non-healing ulcers[219]. Randomization was done in patients who received 8 intramuscular injections of vector or placebo on days 1, 15, 30, and 45. Similar improvements in ulcer healing were shown in patients who received NV1FGF or placebo (19.6% or 14.3%; $P = 0.514$). Nevertheless, the risk of major amputations (hazard ratio of 0.371; $P = 0.015$) and all amputations (hazard ratio of 0.498; $P = .015$) was significantly reduced (by twofold) by the use of NV1FGF.

TAMARIS trial

The trial described above provided the stimulus for the phase III trial. Therefore, the safety and efficacy of the NV1FGF (described above – TALISMAN 201 trial) in the reduction of death and amputation in CLI patients, not eligible for revascularization,

was evaluated in a randomized, double-blind, placebo controlled study – the safety and efficacy of the XRP0038/NV1FGF in CLI patients with skin lesions (TAMARIS) trial[220]. The study included 525 patients from 171 sites in 30 countries on 5 continents, who were administered either placebo or NV1FGF intramuscularly, every 2 weeks over a 6-week period. Unfortunately, the benefit of NV1FGF has not been demonstrated. Patients treated with NV1FGF presented a 37% rate of death or major amputation at 1 year, while the placebo treated patients showed a 33% rate. Given the highly impressive results of the phase II TALISMAN study, the TAMARIS findings were particularly disappointing.

WALK trial

The efficacy and safety study of Ad2/HIF1α/VP16 gene transfer among patients with IC (WALK) trial assessed if intramuscular administration of Ad2/HIF1α/VP16, which is an engineered recombinant type 2 adenovirus vector encoding constitutively active HIF1α, enhanced the walking time in patients with IC. Two hundred and eighty-nine patients were recruited for this randomized, placebo controlled study, received 20 intramuscular injections of HIF1α in both legs and were observed for 12 months to determine changes in peak walking time from baseline.
Patients treated with escalating doses of HIF1α (2×10^9, 2×10^{10}, and 2×10^{11} viral particles) or placebo were included in this study. No significant differences were observed in the quality-of-life measurements, ABI and claudication onset time between each HIF1α group and the placebo one[221].

HGF-STAT trial

The safety of intramuscular injection of HGF plasmid in improving limb perfusion in patients with CLI (HGF-STAT trial) was assessed in this placebo-controlled randomized multicenter phase II trial[222]. The study included 105 patients to receive HGF plasmid or placebo intramuscularly, scheduled as 0.4 mg at days 0, 14, and 28 (low dose); 4.0 mg at days 0, 14, and 28 (middle dose); or 4.0 mg at days 0, 14, and 28 (high dose). Eighty-six per cent of patients presented adverse events, most of them associated to previous comorbid conditions and no differences between groups were observed. At 6 months, there was an increase in the transcutaneous oxygen pressure in the high-dose group, in comparison to the middle-dose, low-dose, and placebo groups. Regarding secondary endpoints – major amputation, wound healing, pain relief, ABI – there were no differences between groups. Taken together, this study showed that HGF gene therapy was well tolerated and that the high dose

gene therapy group had a significant improvement in the transcutaneous oxygen pressure from baseline compared with placebo.
Based on the results of the HGF-STAT trial, a second follow-up phase II study was conducted to further define the safety and potential efficacy of a modified HGF gene delivery technique to improve limb perfusion in patients with CLI and no revascularization options. In this study (HGF-0205 trial[223]), patient randomization was 3:1 to HGF (n = 21) versus placebo (n = 6). No variations in serious adverse events or adverse events were observed. There was significant improvement in ABI from baseline at 6 months in the HGF-treated group compared to placebo. The HGF-treated group also showed a significant improvement in rest pain assessment by visual analogue scale from baseline at 6 months in comparison to placebo. Thirty-one per cent of patients in the HGF group showed complete ulcer healing at 12 months while no patients reported the same result in the placebo group. At 12 months, there was no difference between groups in major amputation of the treated limb (29% in HGF group vs 33% in placebo group) or mortality (19% in HGF group vs 17% in placebo group). Therefore, this trial has shown that HGF gene therapy using a specific delivery technique is safe, maintained limb perfusion, and decreased rest pain in patients with CLI compared with placebo.

VIROMED trial

This phase I clinical trial aimed at evaluating the preliminary efficacy, tolerability and safety of naked DNA therapy expressing 2 isoforms of HGF (pCK-HGF-X7) in 22 CLI patients. Over a period of three months there was a significant reduction in pain complaints as well as a significant increase not only in ABI but also in the transcutaneous oxygen pressure measured on the dorsum of the foot and anterior and posterior calf. Six out of nine patients showed improved ulcer healing[211].

Gene therapy: summary

Concerning gene therapy, the TRAFFIC trial (FGF), the RAVE trial (VEGF121), the TALISMAN 201 trial (NVFGF), the WALK trial (HIF1α), and HGF-STAT largely showed encouraging, although inconsistent results, probably due to their unreliable endpoints.
Gene therapy, in the context of cardiovascular disease, encompasses a very heterogeneous series of therapeutic approaches, with differences being growth factor selection, dose, route of administration and vector type (viral and non-viral)[226].

The future should rely on merged therapies undoubtedly concerning several genes encoding for various angiogenic growth factors and cofactors.

1.4.2 Cell Therapy

Cell delivery strategies, namely cell-based therapies, have been developed enabling the stable supply of growth factors/cytokines for angiogenesis of ischemic tissues. The mechanisms of cellular therapy in neovascularization remain vastly unknown. Even though cellular therapy may rely in the direct differentiation of graft into vessel cells, recent studies suggest that this direct incorporation is relatively rare[227]. On the contrary, a potent mix of pro-angiogenic factors secreted by the graft cells seems to be the primary responsible for neovascularization[227].

Cell-based therapy in PAD has primarily focused on progenitor cells derived from PB or BM; of late, clinical trials are including other cell types like MSCs derived from placenta or umbilical cord and adipose tissue[228-237].

Density gradient centrifugation (Ficoll method) or plasmapheresis are used for harvesting BM, both of which warrant access to certified good clinical practice facilities[238]. In order to circumvent this resource and labour-intensive process, newer bedside centrifugation systems have been developed[239]. Peripheral EPCs are generally mobilized using a variety of techniques. The most widely used cytokines for this purpose include granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF)[240,241]. On the other hand, EPCs have also been recruited by angiogenic growth factors like SDF, HIF, FGF¸ and VEGF. The administration of the ECs isolated using these methods can be done as a whole or further selection can be done for cell-surface antigens. Direct intramuscular and intra-arterial injections are the usual routes of delivery.

The superiority of no route of administration¸ dosing regimen, selection technique, culture or single cell source has been established till date; as a result, the ongoing studies of therapeutic vasculogenesis have become quite heterogeneous in research design as well as outcome measurements.

To date, the best route for stem cell administration, intramuscular or intra-arterial, remains a subject of debate. Intramuscular administration appears to be associated with a transient deposition of stem cells in the ischemic area, allowing them to promote paracrine effects along with some degree of incorporation into the neovasculature. On the contrary, intra-arterial administration of stem cells seems to direct them into peri-ischemic areas that are viable and where the supply of nutrients and oxygen is

sufficient enough to ensure cellular functions. Table 15 illustrates the various patient series as well as controlled studies conducted in this area and the stated successful outcomes.

Table 15. Results of Cell Therapy for Critical Limb Ischemia[a]

Author	Year	Study Level	Number	Product	Clinical Outcome*
Tateishi-Yuyama	2002	Randomised unblinded trial	45	BM-MNC	Positive
Esato	2002	Patient series/uncontrolled trial	8	BM-MNC	Positive
Saigawa	2004	Patient series/uncontrolled trial	8	BM-MNC	Positive
Higashi	2004	Patient series/uncontrolled trial	8	BM-MNC	Positive
Miyamoto	2004	Patient series/uncontrolled trial	12	BM-MNC	Positive
Nizankowski	2005	Patient series/uncontrolled trial	10	BM-MNC	Positive
Durdu	2006	Randomised unblinded trial	28	BM-MNC	Positive
Bartsch	2006	Patient series/uncontrolled trial	10	BM-MNC	Positive
Miyamoto	2006	Patient series/uncontrolled trial	8	BM-MNC	Positive
Arai	2006	Randomised/controlled trial	39	G-CSF	Positive
Kajiiguchi	2007	Patient series/uncontrolled trial	7	BM-MNC	Equivocal
Huang	2007	Controlled trial	74	BM-MNC	Positive
Hernandez	2007	Patient series/uncontrolled trial	12	BM-MNC	Positive
Gu	2008	Patient series/uncontrolled trial	16	BM-MNC	Positive
Chochola	2008	Patient series/uncontrolled trial	28	BM-MNC	Positive
Wester	2008		8	BM-MNC	Positive
VanTongeren	2008	Patient series/uncontrolled trial	27	BM-MNC	Positive
DeVriese	2008	Patient series/uncontrolled trial	16	BM-MNC	Equivocal
Kawamoto	2009	Patient series/uncontrolled trial	17	EPCs	Positive
Amann	2009	Patient series/uncontrolled trial	51	BM-MNC	Positive
Prohazka	2009	Patient series/uncontrolled trial	37	BM-MNC	Positive
Huang	2004	Patient series/uncontrolled trial	5	PB-MNC	Positive
Kawamura	2005	Patient series/uncontrolled trial	30	PB-MNC	Positive
Lenk	2005	Patient series/uncontrolled trial	7	PB-MNC	Positive
Huang	2005	Controlled trial	28	PB-MNC	Positive
Ishida	2005	Patient series/uncontrolled trial	6	PB-MNC	Positive
Kawamura	2006	Patient series/uncontrolled trial	75	PB-MNC	Positive
Huang	2007	Controlled trial	76	PB-MNC	Positive
Powell	2011	Controlled trial	86	BM-MNC	Positive
Walter	2011	Controlled trial	40	BM-MNC	Equivocal
Murphy	2011	Patient series/uncontrolled trial	29	BM-MNC	Positive
Lu	2011	Patient series/uncontrolled trial	41	BM-MNC or BM-MSC	BM-MSC +
Losordo	2012	Randomised/controlled	28	EPCs	Positive

*Positive clinical outcome defined as improved perfusion, reduced pain, or decreased amputation.

[a]*Adapted*[225].

Abbreviations: *BM-MNCs, bone marrow mononuclear cells; BM-MSC, bone marrow mesenchymal stem cell; BM-TRC, bone marrow tissue repair cells; G-CSF, granulocyte colony-stimulating factor; PB-MNC, peripheral blood mononuclear cells.*

Intramuscular Bone Marrow Mononuclear Cells

TACT trial

The Therapeutic Angiogenesis Using Cell Transplantation (TACT) study was the first large report on the use of BM-MNCs in limb ischemia. Intramuscular injection of autologous BM-MNCs led to a 3-year amputation-free rate of 60%. The leg pain scale and ulcer size showed significant improvement and after at least 3 years of therapy, pain-free walking distance was maintained, even though transcutaneous oxygen pressure and ABI did not change significantly[242]. Acetylcholine-mediated endothelium-dependent blood flow has been shown to increase for up to 4 weeks through the implantation of intramuscular BM-MNCs, which suggests that endothelial dysfunction could be reversed by stem cell therapy among patients with severe atherosclerotic CLI[243]. Functional neovascularization distal to the site of intramuscular BM-MNCs treatment was suggested by pathologic analysis[244].

BONMOT-1 trial

The Bone Marrow Outcomes Trial 1 (BONMOT1) assessed intramuscular injections of autologous BM cells among 51 patients with imminent major amputation due to severe CLI. The study showed a limb salvage rate of 59% at 6 months and 53% at last follow-up[239].

Patients with consecutive limb salvage showed an increase in the transcutaneous oxygen pressure and in perfusion measured with ABI at baseline and after 6 months; however, these parameters did not change in patients who underwent major amputation. Improvement was seen in mean Rutherford category from a baseline of 4.9 to 3.3 at 6 months among patients who did not required major amputation. Moreover, there was a 62% decrease in the use of analgesics[239].

BONMOT-1 and the subsequent placebo controlled, double blind study (BONMOT-CLI), involved the placement of the BM-MNCs injections along the axial line of the occluded native arteries, with the number of injections consistent with the length of the arterial occlusion.

RESTORE-CLI trial

In a randomized, double-blinded, placebo-controlled, phase II trial of 77 patients, ixmyelocel-T was used for treating CLI (RESTORE–CLI). Ixmyelocel-T is a patient-specific, expanded, multicellular therapy. In preclinical studies, it has been shown to promote angiogenesis being involved in tissue remodeling and immune modulation. Patients received one-time injections over 20 locations in a single leg and were

followed for 12 months. Ixmyelocel-T treatment resulted in a significantly prolonged first occurrence of treatment failure (*de novo* gangrene, doubling of total wound surface area from baseline, all-cause mortality, major amputation of injected leg). There was a 32% reduction in amputation-free survival (major amputation of injected leg; all-cause mortality), however this was not statistically significant[245]. No major safety issues related to ixmyelocel-T treatment were observed.

HARVEST trial

A multicenter, prospective, randomized, double-blind, placebo controlled trial for "non-option" CLI patients was conducted wherein the activity and safety of autologous BM aspirate concentrate was tested for the treatment of CLI due to PAD (HARVEST). The therapy involved BM aspirate concentrate, which was injected percutaneously in 40 injections to the affected limb. Patients were randomized to placebo and BM aspirate. Although, this study was not powered to demonstrate statistical significance, a trend toward improvement in ABI, Rutherford classification, quality of life, pain and amputation was observed on the BM aspirate concentrate group when compared to the control group[246].

Intra-arterial Bone Marrow Mononuclear Cells

PROVASA trial

The intra-arterial progenitor cell transplantation of BM-MNCs for induction of neovascularization in patients with PAD (PROVASA) study randomized 40 patients to intra-arterial delivery of the BM-MNCs or placebo[247]. This is the unique multicenter, randomized trial of intra-arterial BM-MNCs therapy among patients with CLI. The results showed that cell therapy was associated with significant improved wound healing in a dose dependent manner and considerable reductions in rest pain were observed when compared to placebo, even though the limb salvage rates and ABI did not improve[247].

Intra-arterial and Intramuscular Bone Marrow Mononuclear Cells

Combined intramuscular and intra-arterial BM-MNCs administration may have advantages compared to single-route administration. Franz et al. conducted a study that used a dual intramuscular and intra-arterial autologous BM-MNCs implantation strategy in 9 patients recommended for lower limb amputation. The procedure led to no considerable improvement in ABI. Three patients (33%) underwent major lower limb amputation, while rest pain improved in the remaining

6 patients. Non-amputees with open trophic lesions evolved with complete ulcer healing within 3 months[248].

In another study, exclusive intramuscular (n = 12) delivery of autologous BM-MNCs was compared to combine intramuscular plus intra-arterial (n = 15) delivery of autologous BM-MNCs, and the results showed no adverse reactions associated to cell injection[249]. In the combined group, limb amputation due to ongoing critical ischemia was required by two patients, while in the intramuscular group 7 patients required limb amputation (*P* = 0.17). A significant yet sustained improvement was seen in the remaining of patients in pain scores, mean ABI and pain-free walking distance at 6 weeks[249]. An earlier trial also showed similar findings[250].

Based on the current data, it appears that the concurrent intramuscular and intra-arterial administration of unselected BM-MNCs leads to several benefits that are similar to those provided by either of the administration routes applied individually[248-251].

G-CSF-mobilized peripheral mononuclear cells have been also investigated in PAD. Intra-arterial injection and intramuscular injection of this type of unselected G-CSF-mobilized peripheral cells led to a > 0.1-point improvement in ABI, in addition to a double increase in maximum walking distance in small clinical series[252-255]. In a previous trial, it was shown that intramuscular administration of G-CSF-mobilized selected CD34-positive cells in CLI patients resulted in endpoint improvement, however, cell yield was lower than what the study expected (as low as 10^5 cells)[256]. Accordingly, it was suggested in a recent study (autologous cell therapy-34 CLI trial) that the intramuscular injection of G-CSF-mobilized selected CD34 cells provide a dose-dependent improvement in freedom from amputation[257]. This was a 28-patient double-blind study, wherein patients were randomized for receiving placebo (cell diluent alone) (n = 12) or 10^5 (n = 7) or 10^6 (n = 9) autologous CD34-positive cells per 1 kg body weight. In the combined cell treatment groups, the amputation rates favored patients under active cell treatment, but future studies with stronger power are clearly needed to support these preliminary results.

Cell Therapy – Summary

As with gene therapy, in the field of cell therapy, several studies (including TACT, BONMOT-1, RESTORE CLI, HARVEST, and PROVASA) also showed trends towards positive results, although these again fell short of initial expectations.

Only two small studies (57 patients) were included in the Cochrane database. It was shown in both studies that a greater reduction in rest pain (*P* < 0 .001) was experienced in the active treatment groups, while the ABI increased along with a

statistical increase in pain-free walking distance (mean increase 306.4 vs 78.6 m; *P* = 0.007), in comparison to control groups. Nevertheless, amputation occurred in a small proportion of the treatment group in comparison to the control group (0% vs 36%, *P* = 0.007)[258].

A meta-analysis was conducted for the autologous cell therapy to treat patients with CLI, wherein 37 trials using autologous BM or G-CSF-mobilised PB cells were identified[259]; these trials included randomized and non-randomized and controlled and non-controlled trials. Autologous cell therapy was found to be effective in improving hard endpoints (ulcer healing and amputation), subjective symptoms and surrogate indexes of ischemia. On the other hand, G-CSF monotherapy was not related to substantial improvement of the same endpoints. The intramuscular route of administration as well as the use of BM-derived cells appeared to be more effective compared to intra-arterial administration as well as the use of PB cells. In conclusion, this meta-analysis suggests that intramuscular autologous BM cell therapy is a potentially effective, relatively safe and feasible therapeutic strategy for PAD patients not fit for traditional revascularization.

The need for further randomized controlled clinical trials has been stressed out in order to evaluate the real and sustained effect of cell therapy in clinical outcomes.

It is also important to stress that the efficacy of cell therapy seems to depend on the pathogenesis of PAD. Compared to vasculitic PAD, 1-year amputation rates and two-year survival rates are seemingly worse in atherosclerotic PAD[260]. Fewer overall therapeutic benefits have been observed, by the PROVASA study authors, in patients with advanced atherosclerotic CLI¸ in comparison to patients with vasculitic CLI[247,261]. Furthermore, compared to patients with vasculitic PAD, it is less likely for these benefits to be sustained for a longer time among patients with atherosclerotic PAD[262]. Such observations question the health of autologous stem cells in the chronic and advanced age cardiovascular disease.

1.4.3. Limited Success of Clinical Studies

Even though there have been some encouraging results, like those discussed above, therapeutic angiogenesis, as a research strategy has been considered only as partially successful. Despite the positive results that emerged from the initial studies in multiple animal models and the initial claims of success that derived from early, exploratory, small open-label trials, a clear clinical benefit for therapeutic angiogenesis has not yet been established in a single double-blind randomized placebo controlled trial[263].

Several explanations may help to clarify these findings. Thus, we can immediately consider that these results have been conditioned by limitations associated to: (i) the animal models commonly involved; (ii) selection of patients for clinical trials and issues related to the different stages of preparation to administration of genes or stem cells, and (iii) finally we cannot fail to take into account the low physiological reserve of the critical patient suffering from a vital disease to respond to the active intervention.

Accordingly, one of the reasons that may explain translational failure of therapeutic angiogenesis is the limitation of the preclinical PAD models. While it is consensual that, with respect to PAD and particularly CLI, the mouse HLI model is the one with the greatest translational clinical relevance, the reality is that it cannot reproduce the complexity of the human condition, and its limitations should always be taken into account whenever interpreting results.

A major difference could be that the animals that are tested are generally healthy and young with tissues having a higher regeneration capacity, when compared to older patients with chronic diseases like DM and systemic atherosclerosis[264]. It is true that diabetic and elderly animals have poor therapeutic vascular growth[265]. Compared with younger mice, older murine recover slower after femoral artery ligation, and this recovery will be complete only after the therapeutic intervention. Older mice seem to show diminished arteriogenesis in the ischemic hindlimb and as a result revascularization occurs mainly through angiogenesis[266]. However, the costs associated with using older mice in basic science are prohibitive.

Most of the models described in literature are models of acute HLI involving large arterial ligation leading to an abrupt decrease in perfusion pressure with the resulting changes throughout the vascular bed occurring rather fast, when compared to those observed clinically. As previously addressed, atherosclerotic PAD, even with overlapping exacerbation events, usually develops in a progressive manner, which allows a gradual macro and microvascular adaptation to the progressive decrease of perfusion pressures. Nonetheless, the hydraulic occluder model used in studies of chronic myocardial ischemia without infarction produce arterial occlusion at slower rates, but differs only slightly from the acute ligation models[11].

The variability in the number and distribution of collateral vessels observed in humans, even when comparing healthy individuals, is conditioned by genetic and environmental factors. Conversely, mice present similar intrastrain and interstrain collateral distribution. BALB/c and C57BL/6 are the two most commonly used mouse strains for HLIMs. These are two highly inbred strains with negligible genetic heterogeneity among animals, ensuring the reproducibility and reliability of different sequential experiments[266]. Regarding collateralization, C57BL/6 mice present a

greater functional reserve and greater intrastrain homogeneity. C57BL/6 mice (the most commonly used strain) have a very robust endogenous response to HLI, in such a way, that even after the ligation of a large inflow artery, like the femoral artery, lower limb perfusion levels may return to normal within 2 to 3 weeks[267].

Comorbidities, like, hypercholesterolemia¸ hypertension and type 2 DM commonly seen in CLI patients are absent in young, wild type mouse. Induction of inflammation and oxidative stress is a common sequel to these conditions, resulting in impaired arteriogenesis and endothelial dysfunction partly via a decreased outward vascular remodeling and flow-mediated dilatation[49]. Environmental or genetic modifications were made to wild-type mice to mimetize a state of endothelial dysfunction, categorized by a diminished and delayed response to angiogenic factors, excessive inflammation, decreased NO-dependent reactivity and impaired NO signaling[268]. These same factors/comorbidities (ie, DM) that are associated with deficient recover after arterial ligation are also implicated in the poor life and limb outcomes of PAD patients[269].

Regardless of whether the HLIM involves the use of wild type mice or environmentally or genetically engineered mice, the reality is that after the induction of ischemia the entire limb perfusion becomes dependent on the number and function of the collateral vessels, as well as on the connections established between these vessels and the microcirculation. Thus, the PAD model is unlikely to have inherent limitations.

Secondly, angiogenic factors and placebo were not compared in the first open-label studies; thus, physiologic salvage may have caused the reported improvements instead of being attributable to active treatment. Another key issue that potentially influences the study outcomes is the selection of patient population. The non-option CLI patients with very advanced disease, predominantly enrolled in the initial trials, may have a more acceptable risk-benefit ratio. However, in these trials active interventions may have been too late in the course of a progressive disease and at an irreversible stage[270]. As a result, various subsequent trials were conducted in claudicant stable patients with less advanced disease[118,221,271].

Thirdly, it should also be considered that the duration of administration and expression of the angiogenic growth factor might not be long enough to promote the development of stable and definitive neovessels. The end-result of cessation of VEGF expression following a short treatment (< 2 wk) in a transgenic animal model is regression of most neo-vessels. However, after longer expression (> 4 wk), a critical transition was quite clear, following which new vessels persisted for months after the withdrawal of VEGF[272]. Thus, in order for neovasculature to persist, longer VEGF expression is needed. This justifies why expected improvements may not come from the delivery of a protein and why it is preferable to have gene therapy.

However, these data take us to the next question: why are the results of clinical trials using gene transfer equally unsatisfactory?

Gene therapy still raises significant questions. Generally, gene transfer therapy is inversely related to host size because of limited tissue diffusion of the gene vectors as well as larger volumes of the transfected tissues[273].

The fourth explanation for the clinical failure is the choice of growth factor. The administration of a single pro-angiogenic growth factor will not be sufficient to produce long-lasting neovessels. The most potent pro-angiogenic stimulant is VEGF; however, new vessels formed in response to this growth factor are leaky and regress after discontinuation of the VEGF stimulus. To overcome this limitation, the initial administration of VEGF could be followed by the delivery of factors such as PDGF, PIGF, and ANG that could lead to the stabilization of the new vessels, through the recruitment of perivascular cells. Nevertheless, it is imperative to define precise time frames, since vessel sprouting is also inhibited by vessel stabilization.

Lastly, another key factor that should be considered is host endothelial dysfunction. Traditional cardiovascular risk factors for systemic atherosclerosis, and so for PAD, have been generally associated to decreased circulating EPCs numbers and function. Different studies report endogenous EPCs dysfunction in the setting of type 2 DM, smoking, dyslipidemia and hypertension[274-281], while negatively correlating it to the Framingham cardiovascular risk score. In fact, Schmidt-Lucke et al. have shown that low number of EPCs is associated with a future increased risk of adverse cardiovascular events[282]. According to Urbich et al., studies published in the literature show a great diversity of EPCs incorporation rates (1 to 50%), which seems to result primarily from the great heterogeneity of EPCs sources, isolation and culture protocols[283].

Reduced levels of EPCs and their dysfunction have been related to type 1 and type 2 DM; these dysfunctions include poor differentiation, incorporation, matrix adhesion, integrin profile, proliferation, and mobilization[284]. These observations can be explained by three mechanisms: reduced half-life of EPCs in circulation¸ increased consumption of EPCs at vascular repair sites and reduced mobilization of EPCs from the BM. Thus, it is imperative to give attention and to optimize EPCs applications in advanced cardiovascular states, with particular emphasis given at improving the mechanical and metabolic background of cell therapy. Among other measures, antioxidants and physical exercise tend to improve EPCs-mediated vasculogenesis, as they increase the concentration gradient of angiogenic factors in favour of ischemic tissues[285].

Future clinical trials need to focus necessarily on the definition of the best pro-angiogenic factor or to adopt a strategy involving a combination of several

pro-angiogenic factors, as well as to define the best dose, time and route of administration, and should be able to select patients who will most benefit from the treatment. At present, no reliable measure of blood flow at tissue level in ischemic limbs is known. This is a big challenge for therapeutic development to the extent that endpoints such as major amputation make it difficult to scrutinize the exploratory early trials. The development of new therapies for PAD will be greatly enhanced with a reliable surrogate endpoint that has the capability of assessing blood flow improvements and predict clinical and physiological improvement.

1.4.4. How to Overcome the Limitations of the Clinical Application of Endothelial Progenitor Cells

Ischemia result in a very hostile microenvironment, which is characterized by increased levels of oxygen free radicals, active inflammation and low to very low oxygen tension which may collectively compromise the function and survival of EPCs, which in turn compromises their therapeutic potential. Thus, clinical efficacy of cell and gene therapy can be improved with modulation of inflammation and ischemic microenvironment.

Certain therapeutic strategies have been developed to improve the EPCs function in the ischemic microenvironment (Figure 20): (i) drug-based preconditioning strategy prior to cell transplant into an ischemic area[286]: (ii) genetic modification of EPCs with integrin-linked kinase, insulin-like growth factor 1, and cytoprotective mediators, pharmacologic qualified drugs (estradiol, resveratrol), growth factors, natural compounds (fucoidan, tripterine) or signal related triggers for primed cells; (iii) combinatorial or co-transplantation cell therapy using two types of cells[287] that offers synergist effects; (iv) EPCs encapsulated in bioartifitial niches used along with ECM[288,289].

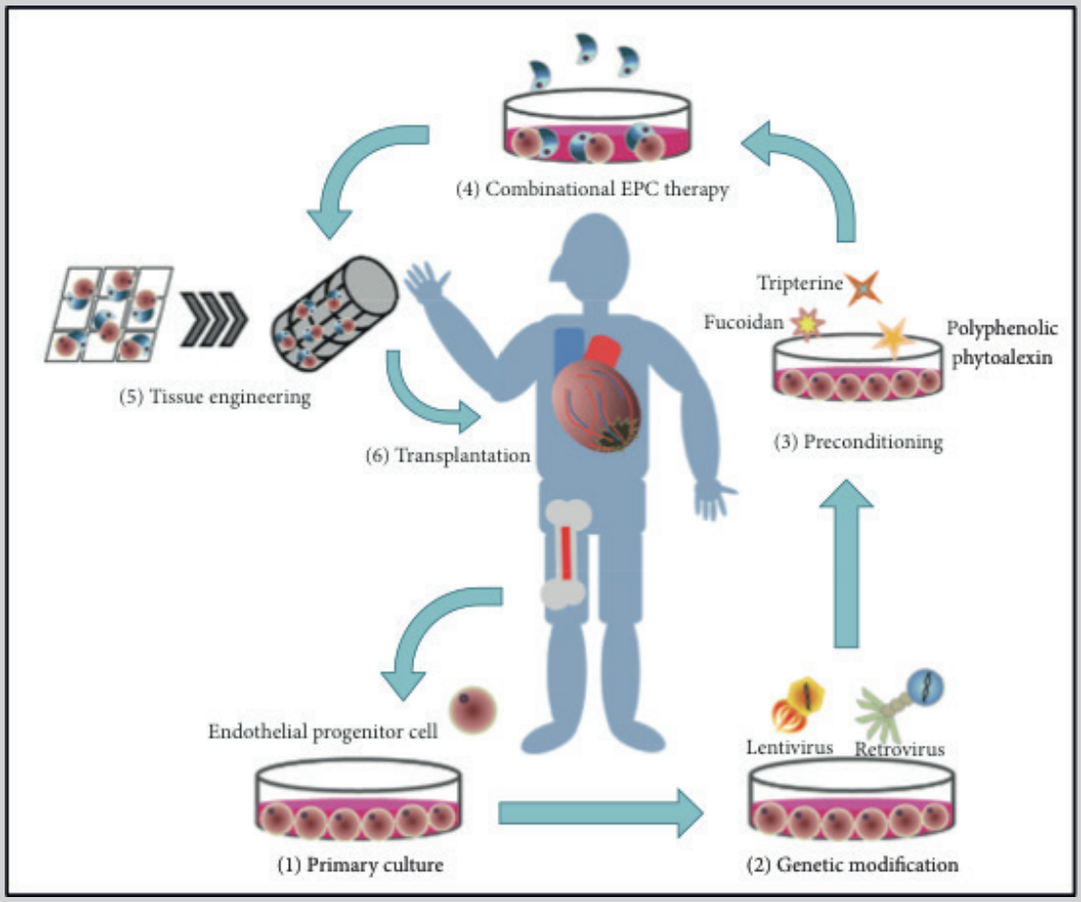

Figure 20. Promising Therapeutic Strategies for Endothelial Progenitor Cell therapy[a]

Cell-derived therapeutic strategies in cardiovascular disease include: (1) isolation of patient-derived EPCs from healthy tissue; ((2), (3), (4)) improvement in EPCs bioactivities via genetic modifications or pre-treatment with natural compound or co-cultured with other stem cells, such as mesenchymal stem cells (MSCs) and circulating progenitor cells (CPCs); and ((5), (6)) tissue engineering techniques using biomaterials protect cells from the harsh conditions of ischemic regions.

[a]*Adapted*[182].

1.4.5. Conclusions

In the late 1990's, the field of therapeutic angiogenesis became very popular with the description, in trials that were mostly uncontrolled, of a radical new paradigm for the treatment of non-option CLI patients. Nevertheless, the enthusiasm that the early studies generated was dulled by the discouraging results from the more recent and more robust trials on therapeutic angiogenesis (see Table 16). However, some studies, exclusively with CLI patients, have shown promising results, which, together with the poor prognosis of the disease, seem to justify and support further clinical investigation and trials in this area.

The extensive basic and clinical experience developed in the area of therapeutic angiogenesis has enabled us to identify several research directions for the future; for instance, (i) using novel genetic strategies to identify new ways to treat PAD, (ii) improving delivery and environmental host conditions to respond to stem cell therapy; (iii) improving delivery and identifying new vectors to enhance gene expression; (iv) recognizing the possible limits of gene transfer in humans; (v) the need for incorporating information from the field of systems biology and (vi) considering the way therapeutic angiogenesis can be assessed in the setting of clinical care for CLI patients.

Finally, in the near future, the hypothesis of resorting to therapeutic angiogenesis should be considered as an adjuvant to revascularization that allows the modulation of the more distal vessels of the peripheral circulation.

Table 16. Limited Success of Clinical Trials

Use of a single angiogenic factor
Development of unstable vessel growth
Rapid diffusion, poor biostability, and short half-lives after growth factor delivery
Excessive uncontrolled vascular formation in undesired locations
Incapacity from the resident ECS to respond

[a] *Adapted*[182].

1.5. IONIZING RADIATION

Radiotherapy, alone or in combination with chemotherapy and surgery, is a treatment of choice for malignancies characterized by autonomous growth and the ability to invade adjacent tissues to metastasize. Different types of radiation produce different biological effects and the rate at which radiation is received determines the magnitude of the effect. The primary factor that determines the biological effects of a given absorbed dose is the dose rate.

Ionizing radiation, consisting of electromagnet radiation (X-rays and y-rays), is the type of radiation used in onco-radiotherapy. Radiotherapy treatments can be applied in two ways; the first is brachytherapy and the second is external beam therapy. Brachytherapy, generally used in the treatment of prostate cancer, involves the use of surgically implanted radioactive pellets in the tumor, since the anatomical distribution of structures in this area would make it difficult to use external radiotherapy without causing significant damage to adjacent organs.

External beam radiation therapy is the most frequently used and involves the use of an external source of radiation that produces a beam directed at the patient based on a unique dosimetric plane (Figure 21). In general, this standard treatment requires the application of high-energy photons generated by mega-electron-volt (MV) linear particle accelerators[291].

Generally external radiotherapy is delivered through fractionated schemes, consisting of a daily small dose (typically of 1.8 to 2.0 Gy in adults and 1.5 to 1.7 Gy in children), repeated until a potentially curative tumor-specific dose has accumulated. The administration of radiation in fractionated doses is the method of choice, since it appears to provide greater protection to the healthy tissues surrounding the tumor area. Healthy cells, at intervals between each radiotherapy session, have cellular and genetic repair mechanisms that effectively restore radiation-induced lesions, when compared to tumor clonogens that more easily accumulate ionizing radiation-induced damages[292,293]. Furthermore, external photon beam radiotherapy is generally conducted with more than one shaped radiation beam, so that a uniform dose distribution can be achieved inside the target volume, restricting the dose in the normal neighboring tissues[293]. Nevertheless, these bordering healthy tissues are exposed to gradually increasing doses from 0 Gy to the maximal (Figure 21), and it is important to consider their biological effects, as it will be briefly detailed.

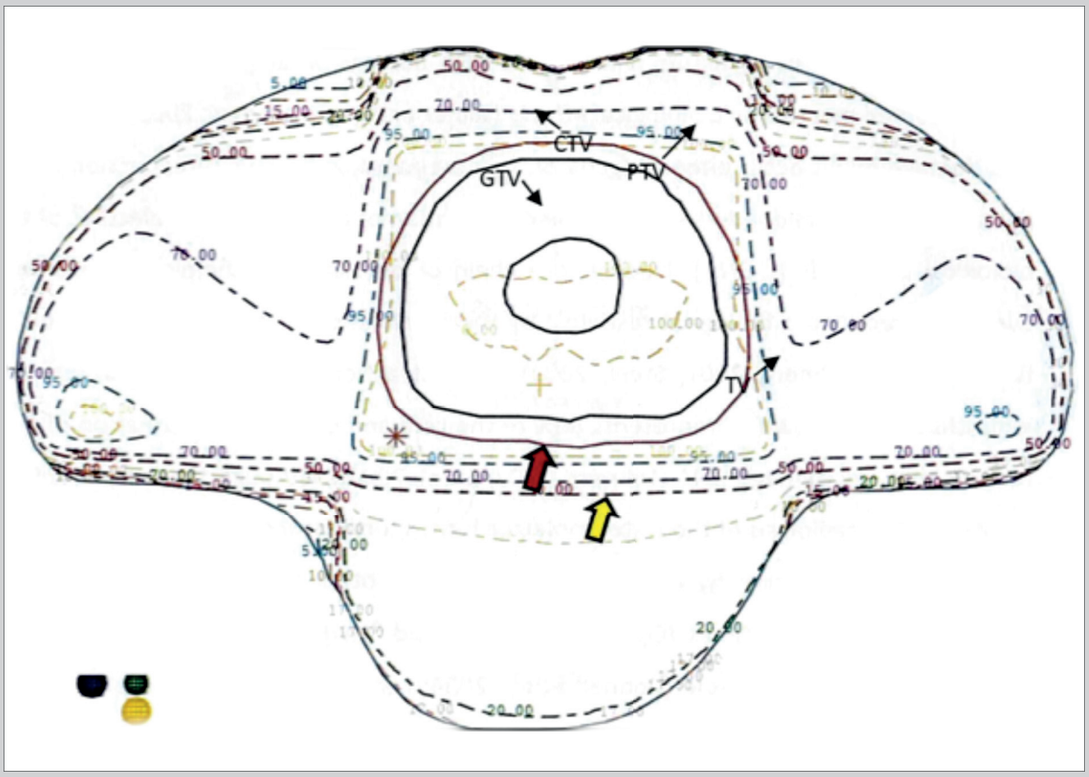

Figure 21. Isodose Curves in a Pelvic Axial Slice

Isodose (dashed) lines show the contour of the radiation levels in the anatomy and provide spatial information about the deposition of radiation. Gross tumor volume (GTV) corresponds to the detectable tumor volume, clinical target volume (CTV) indicates the sum of GTV with volumes with expected subclinical spread, and planning target volume (PTV) is based on CTV plus a safety margin for movement and deformation. Treatment volume (TV) corresponds to tissue that will receive the prescribed dose, thus the isodose curve of 100%. Red and yellow arrows indicate the isodose curves correspondent to the tissues that will receive 50% or 30% of the total daily dose (0.9-1.0 Gy or 0.5-0.6 Gy).

1.5.1. Unexpected Effects of Ionizing Radiation in Metastasis Development

Radiation therapy was classically seen to exert its therapeutic effect by promoting tumor cell death, however, more recent evidence seems to suggest that its therapeutic effects may extend far beyond onco-radiotherapy. The processes behind ionizing radiation and the ultimate outcome in tumor cells include interactions among tumor cells themselves and their tumor-related host cells like nerve cells, myofibroblasts, fibroblasts, macrophages, leukocytes, and ECs[294]. These effects are not limited to the irradiated cells but extend to the adjacent tissues as they have the ability of communicating through soluble factors and cell junctions[295].

The microenvironment is modulated by ionizing radiation[294]. Nevertheless, it is supported by experimental and clinical observations that metastatic behaviour of cancer cells may be promoted by ionizing radiation and tumor-promoting effects

may be exerted by the irradiated host microenvironment[294,296,297]. Von Essen et al.[297] identified several mechanisms involved in this prometastatic action: (i) cancer cell modification by direct action of ionizing radiation leading to radioresistant cells, (ii) indirect change of the metastatic site through abscopal effects, and (iii) modulation of tissues that harbour the primary tumour.

The focus of recent studies has been on the molecular and cellular mechanisms through which ionizing radiation induces invasion and metastasis. When cells are exposed to ionizing radiation, it induces a cascade of chemokines, cytokines and growth factors that activate multiple signal transduction pathways through which signals move in and out of the cell; this in turn modulates various cellular functions in the tumour microenvironment and the potential metastatic environment.

Ionizing radiation at a given dose and time frame stimulates key molecules that promote the activity of tumour-associated host cells supporting metastasis and invasion; for instance, nerve cells (producing efferent growth and invasion-promoting molecules), osteoblasts and osteoclasts (establishing bone metastases), myofibroblasts (inducing desmoplasia), leukocytes, and macrophages (causing inflammation) and ECs (inducing angiogenesis)[296].

Regarding the vascular field, ionizing radiation may exert an anti-angiogenic effect through its direct effect in promoting ECs apoptosis via the ceramide pathway[298], but may also exert a proangiogenic activity through signals released by the tumor cells[296,299,300]. Furthermore, the induction of ECs apoptosis during the initial hours after radiotherapy is followed by the stimulation, generating a biphasic response. This implies that completely different effects are induced by total and fraction dose. Ionizing radiation induces the production of pro-angiogenic factors by the tumour cells, like FGF2, MMP2, VEGF, IL1Rα, IL10, IL13, IL4, and IL5, contributing indirectly to the protection of the vasculature[299,300] (Figure 22). Likewise, ionizing radiation also has the capability of directly acting on ECs producing the same outcome, for instance, by up-regulation of VEGFR2[301], PDGFB¸ FGF2, proangiogenic CXC chemokines[302], IL8[303], platelet-endothelial cell adhesion molecule 1[304], αvβ3-integrin[299] and the NO pathway[305].

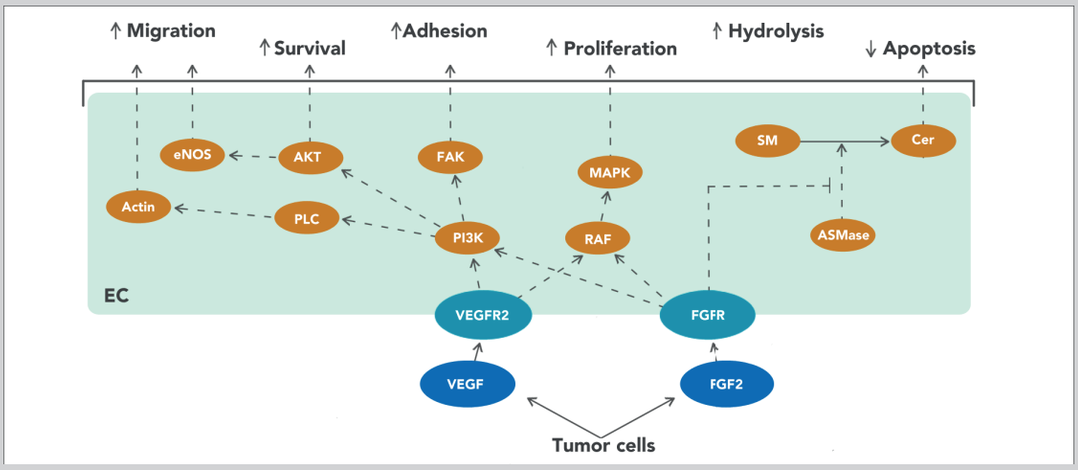

Figure 22 Schematic Representation of Some Major Pro-Angiogenic Signaling From Irradiated Cancer Cells to ECs

At certain conditions, IR can induce the production of proangiogenic molecules by the tumor, which may activate ECs, promoting an angiogenic response. The diagram shows only some of the possibilities.

[a] *Adapted*[295].

Abbreviations: *Cer, ceramide; eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; FGF, fibroblast growth factor; FGFR, FGF receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; SM, smooth muscle; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.*

1.5.2. The Putative Effects of Ionizing Radiation in Therapeutic Angiogenesis

Ionizing radiation has an angiogenic potential role in both the neoplastic and the "healthy" tissues[305-311]. Wound healing is accelerated by lower dose of ionizing radiation in addition to improving neovascularization by the promotion of ECs proliferation[312].

In ischemic conditions, ionizing radiation modifies cellular and chemical composition of the tissue microenvironment. It further promotes tissue regeneration, inducing the expression of genes in mast and stromal cells, for further promoting the incorporation of BM derived cells in ischemic tissue that promotes vascular stability in addition to the release of angiogenic factors via protease modulation[313].

In the ischemic microenvironment, neoangiogenesis is modulated by ionizing radiation. It promotes mast cell-dependent vascular regeneration by releasing VEGF and inducing the up-regulation of MMP-9 on stromal cells and ECs that lead to the release of KitL. The mobilization of EPCs and hematopoietic cells in circulation, which can be ultimately incorporated into ischemic tissues, is promoted by KitL that also acts as a potent factor for promoting migration and proliferation of mast

cells[191,314]. In the pathways that are partially dependent on MMP-9, VEGF release from mast cells is also increased by ionizing radiation, which further promotes their local accumulation. Local angiogenesis is directly stimulated by VEGF that may also induce the up-regulation of MMP-9. This process was conditioned in MMP-9 deficient mice, leading to a decline of mast cells in tissues along with a delayed vessel formation in the ischemic limb, as well as abolished in mice deficient in mast cell and in those where antibodies blocked VEGF. Otherwise, no differences were observed in perfusion, vessel density and necrotic area, when comparing irradiated and non-irradiated muscles removed from non-ischemic mice. These data suggest that noticeable changes in muscle microenvironment are not caused by ionizing radiation itselves. Nevertheless, ionizing radiation stimulates angiogenesis under ischemic conditions[315].

Moreover, tissues surrounding the tumor area exposed to doses of ionizing radiation lower than those delivered inside the tumor mass; this is because multiple radiation beams are used for delivering external radiotherapy to the tumor avoiding damage to the organs at risk (Figure 21). Vala et al.[316] illustrated *in vitro* that ECs cycle arrest or apoptosis is not caused by ionizing radiation doses lower than 0.8 Gy. It was observed by the authors that low-dose ionizing radiation (LDIR) promoted the phosphorylation of various cellular proteins such as VEGFR2. By activating VEGFR2, LDIR enhances ECs migration and prevents ECs death induced by bevacizumab, a VEGF-neutralising antibody. As expected, these effects were significantly abrogated with the treatment of ECs with a VEGFR2 tyrosine kinase inhibitor.

Interestingly, under hypoxic conditions, LDIR promotes VEGF expression. *In vivo*, Vala et al. showed that LDIR promotes angiogenic sprouting during zebrafish embryonic development as well as adult caudal fin regeneration. Subsequently, the authors found that angiogenesis is also promoted by LDIR in a murine Matrigel plug assay[316].

In summary, Vala et al.[316] showed LDIR acts in ECs inducing a proangiogenic phenotype both *in vitro* and *in vivo*; nevertheless, the induction of therapeutic angiogenesis by such LDIR has not yet been investigated.

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OBJECTIVES

2. OBJECTIVES

Peripheral arterial disease (PAD), a manifestation of systemic atherosclerosis, remains a significant health problem with rising prevalence in developed occidental countries. Critical limb ischemia (CLI), the “end-stage” of PAD, describes patients with chronic ischemic rest pain or with ischemic skin lesions, either ulcers or gangrene. CLI involves a severe disturbance of both macrocirculation and microcirculation. The limitations of surgical/endovascular revascularization and of pharmacological treatment are well recognized. Therefore, several patients still require amputation, as the only therapeutic approach, despite its associated morbidity and mortality rates.

Limb salvage has encouraged research into alternative therapies, such as therapeutic angiogenesis. The basic principle of therapeutic angiogenesis is the promotion of neovascularization through the administration of exogenous factors. Although the administration of proteins, genes and cells has been shown to be effective in animal models of ischemic disease, its clinical efficacy remains to be demonstrated, and as such, the field of therapeutic angiogenesis still has many barriers to overcome before becoming a true alternative for human medicine.

Through this work, we aimed:

1. To investigate if human mesenchymal stromal cells derived from umbilical cord tissue (UCX®) could promote therapeutic angiogenesis in an experimental model of hindlimb ischemia (HLI).

To address this aim, we developed a mouse model of HLI and five hours after surgical induction of unilateral HLI, UCX® or their vehicle (as a control) were administered in the ischemic muscle. Then, the effect of UCX® on blood perfusion, angiogenesis and arteriogenesis was assessed and the mechanism of action described. These data are presented in chapter III (Research article 1).

2. To evaluate the effects of cryopreservation and subsequent thawing on the therapeutic properties of UCX®, namely immunomodulatory and angiogenic potentials.

To address this aim, the effect of UCX® cryopreservation on cell phenotype and immunomodulatory and angiogenic potentials was analyzed. These data are presented in Chapter IV (Research article 2).

3. To investigate the use of low-dose ionizing radiation (LDIR) as an innovative and non-invasive strategy to stimulate therapeutic neovascularization using a model of experimentally induced HLI.

We have previously shown that LDIR induces angiogenesis *in vitro* and *in vivo,* but there is no evidence that it induces neovascularization in the setting of PAD.

To address this aim, we used a mouse model of HLI and after surgical induction of unilateral HLI, both hindlimbs were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days, and allowed to recover. The effect of LDIR on blood perfusion, angiogenesis and arteriogenesis was assessed and the mechanisms of action described. These data are presented in chapter V (Research article 3).

4. To evaluate the pro-angiogenic effects of LDIR in "non-option" CLI patients.

To address this aim, we developed an exploratory, investigator-blinded, randomized, sham-controlled clinical trial, where "non-option" CLI patients were irradiated with four daily fractions of 0.3 Gy, in consecutive days, or sham-irradiated. The effect of LDIR in the expression of pro-angiogenic genes in endothelial cells (ECs), angiogenesis, arteriogenesis and surrogate clinical endpoints of ischemia was assessed. The preliminary data are presented in chapter VI (Ongoing clinical trial).

3

RESEARCH ARTICLE:
THERAPEUTIC ANGIOGENESIS INDUCED BY HUMAN UMBILICAL CORD TISSUE-DERIVED MESENCHYMAL STROMAL CELLS IN A MURINE MODEL OF HINDLIMB ISCHEMIA

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3. RESEARCH ARTICLE: THERAPEUTIC ANGIOGENESIS INDUCED BY HUMAN UMBILICAL CORD TISSUE-DERIVED MESENCHYMAL STROMAL CELLS IN A MURINE MODEL OF HINDLIMB ISCHEMIA

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ABSTRACT

Background: Mesenchymal Stem Cells derived from human umbilical cord tissue, termed UCX®, have the potential to promote a full range of events leading to tissue regeneration and homeostasis. The main goal of this work was to investigate UCX® action in experimentally induced hindlimb ischemia (HLI).

Methods: UCX® obtained by using a proprietary technology developed by ECBio, were delivered via intramuscular injection to C57BL/6 females after unilateral HLI induction. Perfusion recovery, capillary and collateral density increase were evaluated by laser doppler, CD31 immunohistochemistry and diaphonization, respectively. The activation state of endothelial cells (ECs) was analysed after ECs isolation by laser capture microdissection microscope followed by RNA extraction, cDNA synthesis and quantitative RT-PCR analysis. The UCX-conditioned medium was analysed on Gallios imaging flow cytometer. The capacity of UCX® in promoting tubulogenesis and EC migration was assessed by matrigel tubule formation and wound healing assay, respectively.

Results: We demonstrated that UCX® enhance angiogenesis *in vitro* via a paracrine effect. Importantly, after HLI induction, UCX® improve blood perfusion by stimulating angiogenesis and arteriogenesis. This is achieved through a new mechanism in which durable and simultaneous up-regulation of transforming growth factor β2, angiopoietin 2, fibroblast growth factor 2, hepatocyte growth factor, in endothelial cells is modulated by UCX®.

Conclusion: In conclusion, our data demonstrate that UCX® improve the angiogenic potency of endothelial cells in the murine ischemic limb suggesting the potential of UCX® as a new therapeutic tool for Critical limb ischemia.

Keywords: UCX®; Mesenchymal stem cells; Angiogenesis; Arteriogenesis; Critical limb ischemia; Endothelial cells; Hindlimb ischemia

3.1. BACKGROUND

Critical limb ischemia (CLI) is a severe form of Peripheral Artery Disease (PAD) in which patients with occlusive arterial disease of the legs experience chronic ischemic rest pain, ulcer, or gangrene[1]. This syndrome is associated with severe prognosis, with 1-year mortality exceeding 25% and about 30% to 50% major limb amputation at 1 year from diagnosis[1]. The limitations of surgical/endovascular revascularization, due the distribution and diffuseness of arterial occlusions, are well recognized and amputation, despite its associated morbidity and mortality rates, is often recommended[2]. The goal of limb salvage has stimulated research into alternative methods, including therapeutic angiogenesis which can be achieved either by local administration of pro-angiogenic growth factors and gene or cell-based therapies[3].

Mesenchymal stem cells (MSCs) hold great promise as a therapy for PAD, mainly due to their paracrine activity and immunosuppressive capacity[4]. MSCs are known to home specifically to hypoxic tissues following injury[5] where they potentiate vascular growth through the release of proangiogenic factors[6]. It was previously reported that autologous, allogeneic and xenogeneic MSC administration induced therapeutic angiogenesis in animal models of HLI[7]. In a meta-analysis of cell therapy for PAD, it was found that autologous bone marrow MSCs delivery led to improved indices of ischemia and pain-free walking[8]. Although firstly harvested from the bone marrow, MSCs have since been identified in many other tissues, namely adipose tissue[9] and umbilical cord tissue[10]. UCX® in particular are MSCs obtained from the human umbilical cord Wharton's jelly that are isolated, expanded and cryopreserved according to a patented method (PCT/IB2008/054067; WO 2009044379) and produced according to advanced therapy medicinal product (ATMP) guidelines[11]. UCX® fulfil the MSC criteria as defined by the International Society for Cellular Therapy (ISCT)[12]. UCX® cells are advantageous in comparison to other sources due to the absence of invasiveness in their collection process, their faster self-renewal, higher cell yield and being more potent modulators of the immune system than bone marrow MSCs, making them more attractive for allogeneic cellular therapies.

Recently, it was demonstrated that UCX® paracrine activity represses T-cell activation and promotes the expansion of Tregs better than bone marrow MSCs[13]. Furthermore, by using an acute arthritis *in vivo* model it was found that UCX® can reduce paw edema more efficiently than bone marrow MSCs. The use of a chronic arthritis model showed that UCX® induce faster remission of local and systemic arthritic manifestations[13]. The UCX® tissue regeneration capacity suggested for rheumatoid arthritis was later corroborated for myocardial infarction. By using a mouse model of

myocardial infarction, it was shown that UCX® preserve cardiac function and attenuate adverse tissue remodelling after intra-myocardial transplantation[14]. Interestingly, it was demonstrated that this cardio-protective effect was exerted through paracrine mechanisms involving angiogenesis promotion[14]. Furthermore, i*n vitro* studies, performed with conditioned medium (CM) produced by UCX® grown in classical two-dimensional monolayer cultures, have demonstrated the potential to induce keratinocyte migration in the early stages of wound healing[15]. Moreover, UCX® were able to attract bone marrow MSCs *in vivo* with potential to promote the formation of granulation tissue, contraction by myofibroblasts, angiogenesis, vasculogenesis and epithelization[15]. These results strongly suggest that UCX® have the potential to promote a full range of events leading to tissue regeneration and homeostasis. Interestingly, it was shown recently that the later proliferative and remodelling stages of wound healing promoted by the CM of the two-dimensional monolayer cultures was improved by using three-dimensional culture derived CM[16]. An enhanced secretion of healing-inducing paracrine factors by UCX® including the extracellular cellular matrix metalloproteinase2 (MMP2) and matrix metalloproteinase9 (MMP9), collagen I, fibronectin, laminin, collagen IV and angiogenic factors such as vascular endothelial growth factor A (VEGFA), granulocyte-colony stimulating factor (GCSF), transforming growth factor β1 (TGFβ1), fibroblast growth factor 2 (FGF2), hepatocyte growth factor (HGF) and interleukin 6 (IL6) was observed in the CM of the three-dimensional monolayer cultures[16].

In this work, our data corroborate that UCX® enhance angiogenesis *in vitro* and *in vivo*, by modulating ECs. *In vitro*, we found that UCX® promote tubule formation and endothelial cell migration. Importantly, our results have demonstrated that UCX® in the setting of experimentally induced unilateral hindlimb ischemia (HLI) stimulate angiogenesis and collateral development and thereby improve blood perfusion in the ischemic limb demonstrating the potential of UCX® as new therapeutic tool for CLI. Furthermore, and to the best of our knowledge, this is the first study in an experimental model of HLI showing that, *in vivo* and in a sustained way human MSCs up-regulate the endothelial gene expression of several pro-angiogenic players (such as transforming growth factor β2 (*Tgfβ2*), angiopoietin 2 (*Ang-2*), *Fgf-2*, *Hgf*), shedding light on the potential mechanisms of action of this particular ATMP.

3.2. MATERIAL AND METHODS

3.2.1. Ethics and regulations

Umbilical cord donations, with written informed consents, as well as umbilical cord procurement, were made according to Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurements, testing, processing, preservation, storage and distribution of human tissues and cells. Human umbilical cord tissue-derived mesenchymal stem cells (UCX®) were isolated according to a patented proprietary technology developed by ECBio (Amadora, Portugal). All animal procedures were performed according to Directive 2010/63/EU. The procedures were approved by the institutional Animal Welfare Body and licensed by DGAV, the Portuguese competent authority for animal protection (license number 023861/2013).

3.2.2 Cell Culture

UCX® were cultured in static monolayers in α-MEM with 1 g/L glucose and 2 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA), hereafter designated Basal Medium, supplemented with 20% Foetal Bovine Serum (FBS; Gibco®, Madrid, Spain), in a humidified incubator at 37°C and 7% CO_2.
Human Umbilical Vein Endothelial Cells (HUVECs) (Sciencell, Carlsbad, CA, USA) were cultured in M199 media (Sigma-Aldrich, St. Louis, Missouri, EUA), hereafter designated Endothelial Basal Medium (EBM) supplemented with 10% FBS (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA), 50 µg/ml ECGS (Sigma-Aldrich), 100 µg/ml Heparin (Sigma-Aldrich) and 1% Penicillin-Streptomycin (10,000 U/mL, Sigma-Aldrich), hereafter designated Endothelial Growth Medium (EGM). Cells were grown in flasks coated with 0.2% gelatin (Sigma-Aldrich) until 70% confluence and used up to passage 6.

3.2.3. Preparation of conditioned media

UCX® were seeded in basal medium supplemented with 5% FBS and grown until 90% confluence. After washing cells with PBS, basal medium (without serum) was added for 24 hr. Thereafter, basal medium was replaced and conditioned for 48 hours at 37°C and 7% CO_2 after which it was collected, centrifuged at 300 *g* for 10 minutes, to

remove cell debris, filtered (0.22 mm pore size, Millipore), and concentrated using 5 kDa cut-off spin concentrators (Agilent Technologies, Santa Clara, CA, USA) as per manufacturer's recommendations. Control conditioned medium samples underwent the same procedure as described above, but in the absence of cells.

3.2.4. Quantification of secreted factors

The quantification of HGF, VEGFA, TGFβ1, IL8, PDGFA and FGF2 in the conditioned medium was performed using a commercially available kit (FlowCytomix™; eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Samples were acquired on a Gallios imaging flow cytometer (Beckman Coulter, Brea, CA, USA) and the results obtained using FlowCytomix Pro 3.0 Software.

3.2.5. Tubule formation assay

The tubule formation assay was performed using the thick gel method of preparation. Briefly, after thawing overnight, the matrigel (Corning, Corning, NY, USA) was plated into a pre-cooled 48-well plate (Nunc™, Thermo Fisher Scientific) (180 µl per well) using a chilled pipet tip. After polymerization of matrigel at 37°C, 5% CO_2, for 45 minutes, HUVECs were inoculated at a density of 4.5×10^4 cells/cm^2 on top of the Matrigel in 350 µl of EBM. Cells were then incubated 1 hour at 37°C, 5% CO_2. 1×10^6 UCX® were resuspended in 350 µl BM and loaded on a 1 µm insert (Brand, Wertheim, Germany) and carefully placed in a 48-well plate and incubated for 16 hours at 37°C, 5% CO_2. Controls with no UCX® included HUVECs in (i) EBM, (ii) EGM and (iii) EBM supplemented with FGF-2 (50 ng/ml). After incubation, inserts were removed and all wells were photographed (10x amplification) using a Nikon Eclipse Ti-U inverted microscope with a conjugated Nikon DS-Qi1Mc camera (Nikon, Tokyo, Japan). Tubule formation was quantified on 4 random fields per replicate, using the Angiogenesis analyser from Image J.

3.2.6. Wound healing assay

HUVECs were plated to confluence in a 24-well plate (previously coated with gelatine 0.2 %) with EBM supplemented with 5% FBS. After culturing overnight, wounds were created in the monolayer by scrapping the plate with a sterile pipette tip. After that, 1×10^6 UCX®, resuspended in EBM supplemented with 5% FBS and loaded on

a 1 μm insert (Brand, Wertheim, Germany), were carefully placed in a 24-well plate and incubated for 9 hours at 37°C, 5% CO_2. In controls, the same procedure was performed in the absence of UCX®. Photographs were taken in Primovert (Zeiss) on a 4x magnification and 3 independent wound areas were measured with ImageJ immediately after wounding and 9 hours later.

3.2.7. Mice

Twenty–two-week-old C57BL/6 female mice, purchased from Charles River Laboratories, Spain, were used in all experiments. The animals were anesthetized with Ketamine-Medetomidine Cocktail (75 mg/kg BW and 1 mg/kg BW, respectively) intraperitoneally for the surgical procedure as well as for the other analysis procedures. The anaesthesia was partially reverted with Atipamezole (5 mg/kg BW). Postoperatively, analgesia was performed (buprenorphine 100 μl/15-30 g BW q 8-12h) and the animals were closely monitored.

3.2.8. HLI Model

A surgical procedure was performed to induce unilateral HLI in the mice. Briefly, an incision in the skin overlying the thigh of the right hindlimb of each mouse was made and the distal external iliac artery and the femoral artery and veins were ligated and excised. The vein was ligated both to increase the severity of the ischemia as well as to increase the technical reproducibility of the model, as isolation of the femoral artery alone often results in tearing of the vein resulting in hemorrhage.

3.2.9. UCX® administration

A dose of $2x10^5$ UCX® was injected intramuscularly, 5 hours post-ischemia induction on the gastrocnemius muscle (right hindlimb) in a volume of 50 μl per animal, divided into two injections of 25μl. As a control, UCX® vehicle (PBS) was administered at the same conditions.

3.2.10. Laser Doppler perfusion imaging

The laser doppler perfusion imager (MoorLDI-V6.0, Moor Instruments Ltd, Axminster, UK) was used to assess limb perfusion. Hair was removed one day before laser doppler analysis using an electrical shaver followed by depilatory cream. Blood flow was measured both in the ischemic leg and the contralateral one, before HLI induction (PRE-HLI), immediately post-HLI (POST-HLI), and at day 7, 14 and 21 post-HLI (d7 POST-HLI, d14 POST-HLI and d21 POST-HLI, respectively). Color-coded images of tissue perfusion were recorded and poor or no perfusion was displayed as dark blue, and the highest perfusion level was displayed as red. Mean flux values were calculated using the Moor LDI V6.0 image processing software. To account for variables such as temperature and ambient light, blood perfusion is expressed as the ratio of ischemic to non-ischemic limb. The mice were placed on a 37°C heating pad to reduce heat loss during measurements.

3.2.11. Immunohistochemistry and Capillary density analysis

Mice were sacrificed at day 90 post-HLI (to assure capillary stabilization after the ischemic injury). The gastrocnemius muscles of both legs were harvested, placed in transverse orientation on a small cork disc with the help of 10% tragacanth, snap frozen in liquid nitrogen cooled isopentane and stored at -80ºC until sectioned. Seven-micrometer sections were labeled with CD31 monoclonal antibody (Pharmingen). After fixation in acetone for 10 minutes, hydrogen peroxidase (0.3% diluted in methanol) was added for 30 minutes, at room temperature (RT) and followed by 2 washes in PBS for a total of 10 minutes. Blocking solution (5% rabbit serum in PBS) was applied for 30 minutes, at RT, and the slides were then incubated for 1 hour RT with rat monoclonal antibody against mouse CD31 at 1:500, diluted in 1% BSA in PBS. After 3 washes in PBS for a total of 30 minutes, a secondary biotinylated rabbit anti-rat IgG antibody was added at 1:200 in 1% BSA in PBS and 5% rabbit serum for 30 minutes, at RT. Washes were performed as before and labeled avidin-conjugated peroxidase complex (Vectastain ABC kit; Vector Laboratories) was used for color development according to the manufacturer's recommendations for 30 minutes, at RT. After rinsing in PBS (3 times for 5 minutes), DAB peroxidase substrate kit (Dako) was added for 5 minutes to localize the immune complexes. The sections were counterstained with Hematoxylin (Merck) for 10 seconds and mounted with Entelan (Merck). Omission of the first antibody was used as a negative control. Analysis of tissue samples was conducted using Leica DM2500 upright brightfield microscope

(Leica Microsystems). Capillary densities, i.e. number of capillaries per number of myocytes, were measured in 2 different sections of 4 distinct anatomic areas of each specimen using the ImageJ Software.

3.2.12. Contrast Agent Perfusion and Diaphonisation

Nineteen days post-ischemia induction (to assure vascular stabilization after the ischemic injury), mice were deeply anesthetized and the torso and limbs were shaved. A medial thoracotomy was performed to expose the heart and a needle (26 Gauge), attached to an automatic injector, was introduced in the left ventricle. An incision was performed in the right atrium to allow venous drainage. Mice were primarily perfused with heparinized serum (3000 IU/L) until the blood was completely removed from circulation. A vasodilatation mixture of adenosine (1 mg/L) and papaverine (4 mg/L) was subsequently administered, right before the contrast agent, for 2 minutes. The contrast used was a mixture of barium sulfate (50%) and gelatin (5%). This solution was kept warm until the injection time to avoid thickening. Contrast agent was perfused manually until the feet blanched. Right after the injection the mice were transferred to a cold chamber, so that the contrast agent became solidified. All the solutions were injected with a perfusion rate of 0.7 mL/minute. Then, the skin of each mouse was removed from the lower body and diaphonisation was performed by using a modified version of Spalteholz technique. Briefly, the mice were fixated, decalcified, whitened, washed, dehydrated by freeze substitution and placed into a vacuum pump with Spalteholz solution (benzyl benzoate and methyl salicylate) until transparency was acquired.

3.2.13. Collateral vessels quantification

Mice were kept in Spalteholz solution during image acquisition, in order to achieve a homogenous density between the tissues and the media, with minimal absorption or reflection of light. Mice entire limbs were photographed in a magnifier with a light source. After acquisition, images were aligned and stitched together using Adobe Photoshop CS6®, and entire limb photographs were obtained. In order to exclude the femoral artery and all venous structures from this quantification, only the collateral vessels were manually segmented by highlighting them using Adobe Photoshop CS6®. We considered as collateral vessels all the vessels with a diameter between 20 and 300 μm. For every mouse, an anatomically determinable region comprising

the ligation site was selected and defined as a region of interest (ROI). Collateral vessels density (CVD) was quantified in equivalent ROIs corresponding to 20% of the total limb area. The CVD was calculated as the ratio between the vascular and the ROI areas.
To quantify CVD and to exclude any animal-specific anatomical, perfusion or diaphonisation procedure variations, the CVD of the non-ischemic limb obtained for each mouse was assumed to correspond to 100%. According to this assumption, the CVD percentage in the ischemic limb was calculated relatively to the non-ischemic one. The percentage of CVD increase was determined as the difference between the CVD percentage among the ischemic and non-ischemic limbs. Means of CVD percentages and standard deviations were calculated for each experimental group. All density measurements were performed using ImageJ® software.

3.2.14. Laser capture microdissection of capillaries

Mice were sacrificed at day 70 post-HLI. Twelve-micrometer sections of the gastrocnemius muscles were labeled with CD31 monoclonal antibody (Pharmingen). The sections were stored at –80°C until microdissection. The immunohistochemistry protocol described above was modified to improve RNA preservation by using high salt buffer, 2M NaCl in PBS 1x (at 4ºC) in all incubation and washing steps [17]. Briefly, slides were placed in acetone, for 5 minutes, air-dry, rehydrated with 2M NaCl /PBS (4ºC) and incubated, overnight, at 4ºC with rat monoclonal antibody against mouse CD31, at 1:500, in 2M NaCl /PBS. After 2 washes in 2M NaCl /PBS for a total of 6 minutes, a secondary biotinylated rabbit anti-rat IgG antibody was added at 1:200 in 2M NaCl /PBS and 5% rabbit serum for 30 minutes, at 4ºC. Washes were performed as before and labeled avidin-conjugated peroxidase complex (Vectastain ABC kit; Vector Laboratories) was used for color development according to the manufacturer's recommendations for 30 minutes, at 4ºC. After rinsing, DAB peroxidase substrate kit (Vector Laboratories) was added for 5 minutes to localize the immune complexes. Sections were dehydrated in ice cold 90% ethanol followed by 100% ethanol and allowed to dry. 10000 capillaries were microdissected using a Zeiss PALM MicroBeam Laser Microdissection System (Carl Zeiss Microscopy, Germany) equipped with a pulsed solid-state 355 nm laser. Dissected capillaries were catapulted into a microfuge tube adhesive-cap.

3.2.15. RNA extraction, cDNA synthesis, pre-amplification and RT-PCR

Total RNA from the microdissected capillaries was isolated using an RNeasy Micro Kit (QIAGEN), including DNase treatment to remove potential genomic DNA contamination. For synthesis and preamplification of cDNA, RT^2 PreAMP cDNA Synthesis kit (QIAGEN) was used with two rounds of pre-amplification using the following primers: *Fgf2*_F (5'-ACTCCAGTTGGTATGTGGCACTGA-3'); *Fgf2*_R (5'-AACAGTATGGCCTTCTGTCCAGGT-3'); *Tgfb2*_F (5'-GCTTTGGATGCGGCC TATTGCTTT-3'); *Tgfb2*_R (5'-CTCCAGCACAGAAGTTGGCATTGT-3'); *Ang2*_F (5'-ATCCAACACCGAGAAGATGGCAGT-3'); *Ang2*_R (5'-AACTCATTGCCCAGCC AGTACTCT-3'); *Hgf*_F (5'-GCATTCAAGGCCAAGGAGAAGGTT-3'); *Hgf*_R (5'-TCATGCTTGTGAGGGTACTGCGAA-3'); *18s*_F (5'-GCCCTATCAACTTT CGATGGTAGT-3'); *18s*_R (5'-CCGGAATCGAACCCTGATT-3'). RT-PCR was performed according to the manufacturer's protocol using Power SYBR® Green (Invitrogen) and an Applied Biosystems 7500 Fast Real-Time PCR for the same targets described above. The housekeeping gene used to normalize was 18S. RT-PCR program consisted of an initial denaturation step, at 95ºC, for 10 minutes followed by 50 cycles, at 95ºC, for 15 seconds and at 60ºC, for 1 minute. The relative quantification was performed according to the comparative method ($2^{-\Delta\Delta Ct}$; Applied Biosystems User Bulletin no. 2P/N 4303859), with the non-ischemic muscle as internal calibrator. The formula used is $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\ (sample) - \Delta Ct\ (calibrator)]}$, where ΔCt (sample) =Ct (sample) –Ct (reference gene). For the internal calibrator $\Delta\Delta Ct=0$ and $2^0=1$. For the remaining samples the value of $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the calibrator. The ΔCt value for each sample is the average of triplicates.

3.2.16. Statistical analysis

For the wound healing assay, the values assume normal distribution and unequal variances and therefore an independent two-tailed t-test was performed. For Doppler analysis, an independent two-tailed t-test was also performed at days 7, 14 and 21. The values assume normal distribution. Only at day 7, we cannot assume equal variances. For capillary and collateral analysis, the values also assume normal distribution and equal variances and therefore an independent two-tailed t-test was also performed. For Master Junctions and Segment Length, One Way Independent ANOVA was developed as data followed a normal distribution, though equality of variances could not be assumed for the first. Therefore, a Games Howell Corrected Hoc Test was used to identify differences between groups. For Meshes Area, as

normality could not be assumed and so a Kruskal-Wallis non-parametric test was developed followed by its paired analysis to identify differences between groups. The effect size and power was determined by using the G-Power software in all type of analyses. P<0,05 was interpreted to denote statistical significance.

3.3 RESULTS

3.3.1. UCX® promote tubulogenesis and endothelial cell migration

We first evaluated the effect of UCX® on the capillary structure formation. A matrigel tubule formation assay was used as an *in vitro* model. HUVECs were seeded onto Matrigel in Endothelial Basal Medium (EBM) and co-cultured with UCX®. As a control HUVECs were seeded in EBM, where we do not expect to visualize capillary-like structures formed by HUVECS. For that reason, two positive controls were added and HUVECs were seeded both in Endothelial Growth Medium (EGM) that contains several pro-angiogenic factors such as VEGF and FGF2 and in EBM supplemented with FGF2 (EBM-FGF2). As shown in Fig. 1A and quantified in Figure 1B, UCX® induce tubule formation by HUVECs and these structures consistently showed a significant increase in the number of master junctions, total mesh area and total segment length when compared to those from HUVECs cultured in EBM or EGM. With the exception of the total mesh area, all the measured parameters were also significantly different between HUVECs cultured with UCX® in EBM and HUVECs cultured in EBM supplemented with FGF2. Next, we assessed the migratory capacity of HUVECs seeded in EBM supplemented with 5% of serum co-cultured with UCX® without cell to cell contact. With this objective, a wound-healing assay was performed and as a control HUVECs were seeded in the same culture conditions but in the absence of UCX®. As shown in Figure 2A and 2B, the wound area decreased more rapidly in the presence of UCX® than in the control condition, suggesting that UCX® promote the migration of HUVECs via a paracrine secretion. Taken together, these results clearly show that, in vitro, UCX® induce angiogenic processes in endothelial cells.

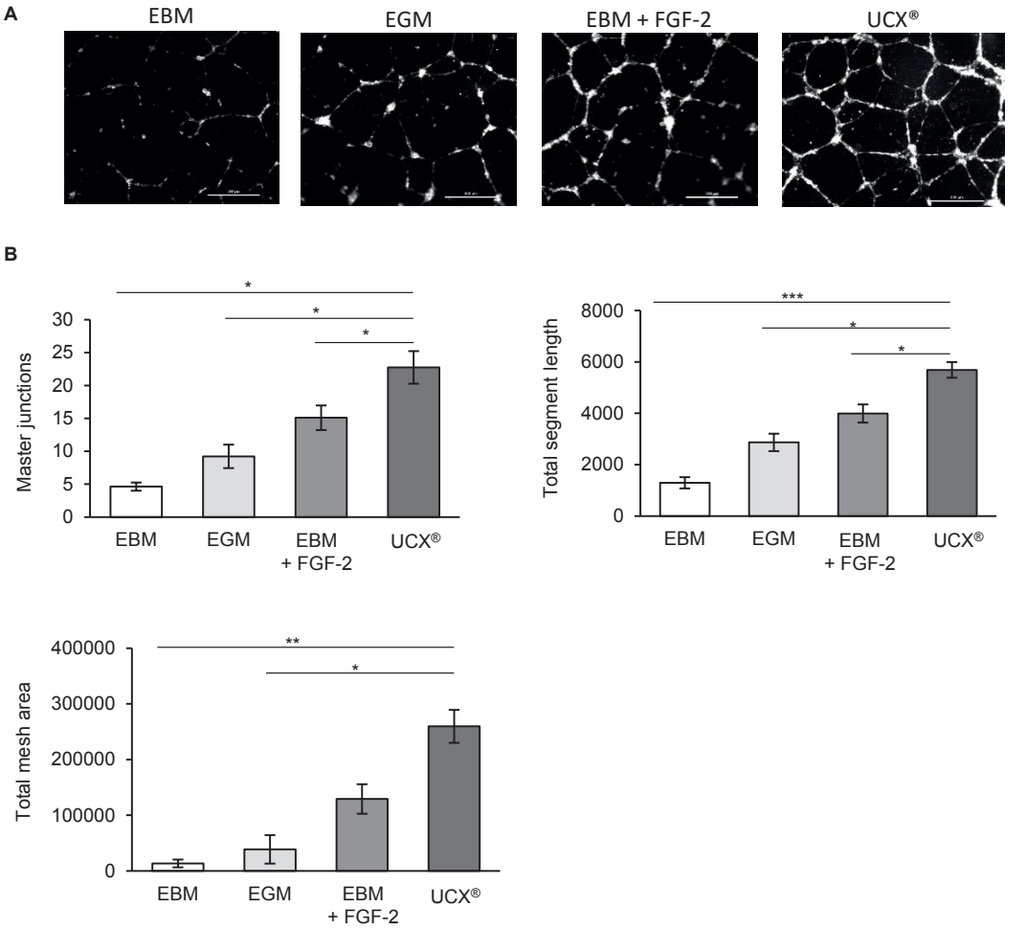

Figure 1. UCX® promote tubulogenesis.

Matrigel assay was performed by seeding HUVECs in Endothelial Basal Medium (EBM), Endothelial growth medium (EGM), EBM supplemented with FGF-2 (EBM + FGF2) or EBM co-cultured with UCX®. (A) Representative images from the different experimental conditions are shown (B) Quantitative evaluation demonstrated significantly enhanced master junctions, total mesh area and total segment length in capillary-like structures formed in HUVECs co-cultured with UCX® when compared to the other experimental conditions. For Master Junctions, following Games Howell post hoc the mean differences between UCX® and the other experimental conditions were: EBM (mean dif. =24,27; p=0,01); EGM (mean dif.=19,67; p=0.03) and EBM + FGF2 (mean dif.= 13,8; p=0.034). The effect size to Basal was 2.08 and power 0.98. For Meshes Area, following pairwise comparisons, the equivalent to a post hoc Kruskal-Wallis, significant differences between UCX® and EBM or UC®X and EGM were observed with KW=22.66, p<0.01 or KW=18.77, p=0.01, respectively. For Segment Length, following Games Howell post hoc, UCX® show significant difference when compared to the other groups: EBM (mean dif. = 4920; p<0.001); EGM (Mean Dif. = 3346; p=0,01) and EBM + FGF-2 (Mean Dif.= 2223; p=0,016). Effect size to Basal was 3.85 and power 1. Scale bar, 500 mm.

Abbreviations: *EBM, endothelial basal medium; EGM, endothelial growth medium; FGF, fibroblast growth factor*

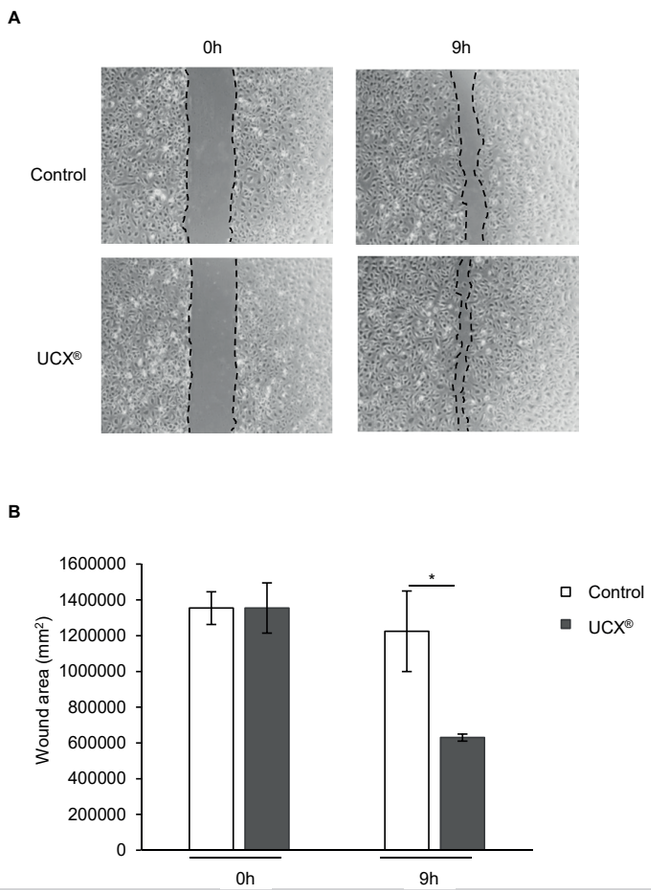

Figure 2. UCX® stimulate endothelial cell migration.

HUVECs were seeded to confluence in EBM supplemented with 5% FBS and cultured for 16 hours after wounding. Immediately after wound, HUVECs were co-cultured with UCX® or not (control) for 9 hours. (A) Representative images are shown. The left and right panels concern to HUVEC monolayer immediately after or 9 hours post-wounding, respectively. (B) The wound area was measured immediately after and 9 hours post-wounding by using ImageJ software (3 independent measurements were performed for each experimental group; t (2.03) = 4.66; *P < 0.05); effect size was 3.82 and power 0.93).

Abbreviations: *EBM, endothelial basal medium; EGM, endothelial growth medium; FGF, fibroblast growth factor*

3.3.2. UCX® secrete pro-angiogenic factors

Since it was already demonstrated that the beneficial effects promoted by UCX® result from a paracrine secretion, we decided to evaluate the secretion of six representative trophic factors critical for angiogenesis in the conditioned medium of a UCX® monolayer seeded in basal medium for 48 hr. As a control, the same trophic factors were measured in basal medium that has not been in contact with cells. The expression of HGF, VEGFA, TGFβ1, interleukin 8 (IL8), platelet-derived growth factorAA (PDGFAA) and FGF-2 were analysed. Our results show a significant increase of HGF, TGFβ1, I-8 and PDGFAA in the conditioned medium of UCX® when

compared to the control (Table 1), demonstrating that UCX® secrete pro-angiogenic factors to the extracellular medium.

3.3.3. UCX® increase perfusion recovery after HLI

In order to evaluate the therapeutic potential of UCX® in a HLI mouse model, the perfusion recovery was assessed overtime after ischemia induction and UCX® administration. Five hours after surgical induction of unilateral HLI, UCX® or their vehicle (as a control) were administered in the ischemic muscle and perfusion was measured overtime. As shown in Fig. 3A and quantified in Fig. 3B, a dramatic reduction in blood flow was observed in the ischemic limb immediately after surgery, in comparison to the contralateral limb, followed by a gradual normalization of ischemic blood flow over time. Importantly, UCX® markedly improved blood flow recovery at 7, 14 or 21 days post-HLI, comparing with control mice, demonstrating that UCX® administration contributes towards the functional recovery of ischemic tissues.

Table 1. Pro-angiogenic factors present in the conditioned medium from UCX® or basal medium

	UCX® CM (pg/ml) Mean±SEM	Basal CM (pg/ml) Mean±SEM	t-test
HGF	2,64 ± 0,718	0,001 ± 0	0,0144
VEGFA	2,025 ± 1,043	0,001 ± 0	0,1099
TGFβ1	0,983 ± 0,122	0,001 ± 0	0,0005
IL8	0,765 ± 0,069	0,001 ± 0	0,0001
PDGFAA	0,123 ± 0,045	0,001 ± 0	0,0412
FGF2	0,0317 ± 0,009	0,02 ± 0	0,2874

CM: conditioned medium

Abbreviations: *CM, conditioned medium.*

3.3.4. UCX® increase capillary and collateral vessel density after HLI

As blood flow recovery depends on both angiogenesis and arteriogenesis we examined whether UCX® would affect capillary and collateral vessel densities in hindlimb muscles. Five hours after surgical induction of unilateral HLI, UCX® or their vehicle (as a control) were administered in the ischemic muscle and mice were sacrificed 90 days post-HLI induction. Capillary density was assessed through

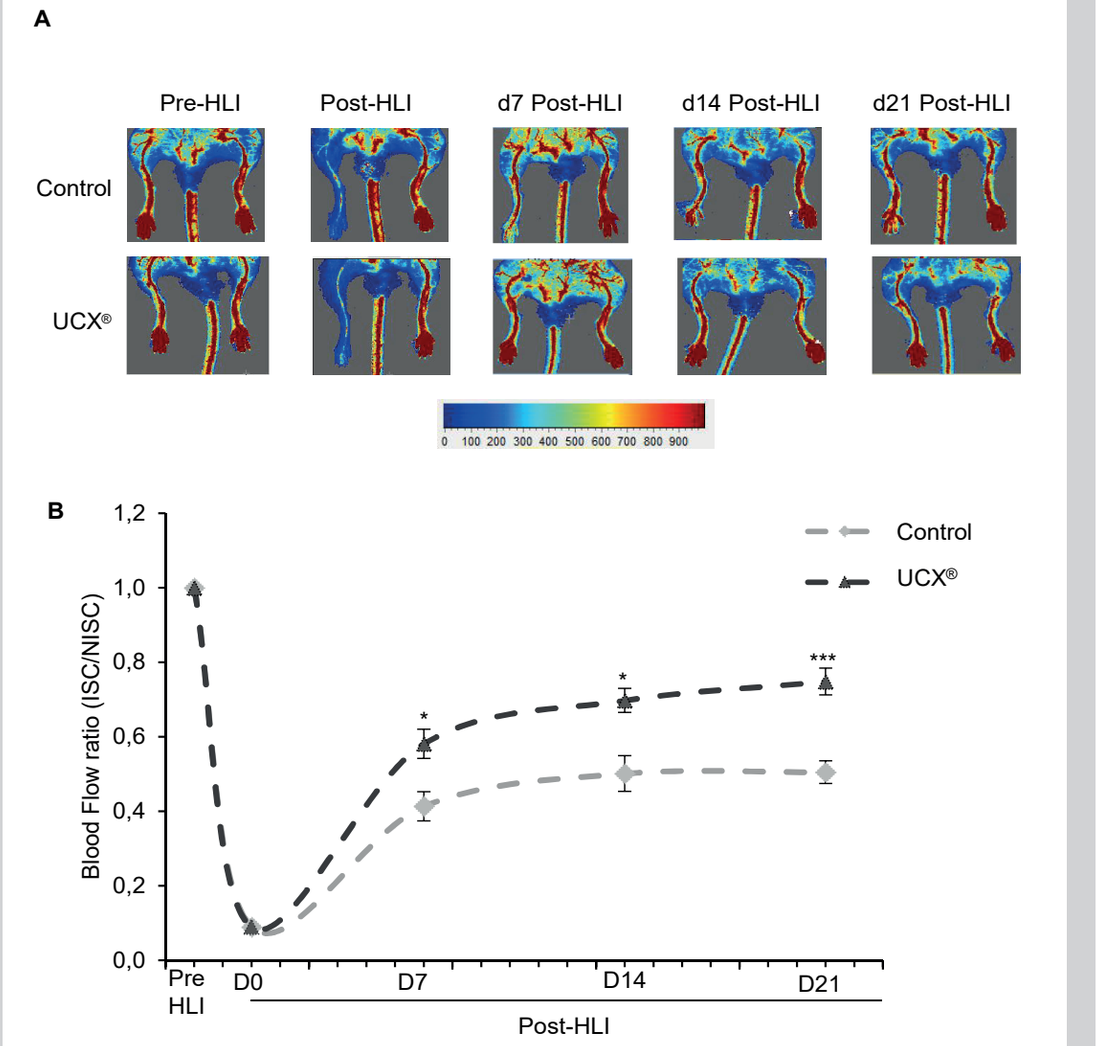

Figure 3. UCX® increase perfusion recovery.

UCX® or their vehicle (as a control) were administered in the ischemic gastrocnemius muscle 5 hours after HLI induction. **(A)** Representative laser doppler flow images before (PRE-HLI), immediately after (d0 POST-HLI) and at 7, 14 and 21 days post-HLI induction (d7 POST-HLI, d14 POST-HLI, d21 POST-HLI). (**B**) Quantitative evaluation of blood flow expressed as a ratio of ISC to NISC limb demonstrated significantly enhanced limb blood perfusion in UCX®-treated mice at 7, 14 and 21 days post-HLI. (n=16 for each experimental group; D7: t (22.69) = 4.26; ***P < 0.001; effect size was 1.51 and power 0.98; D14: t (30) = 4.7; ***P < 0.001; effect size was 1.66 and power 0.99; D21: t (30) = 7.22; ***P < 0.001; effect size was 2.56 and power 0.99).

Abbreviations: *HLI, hindlimb ischemia; ISC, ischemic; NISC, non-ischemic.*

quantification of CD31-positive capillaries on histological sections of gastrocnemius muscle. As expected, the capillary density was greater in the ischemic versus the non-ischemic hindlimb in both experimental groups (Figure 4B). Notably, the level of capillary density in the ischemic hindlimb treated with UCX® was significantly higher than the one observed in the control ischemic hindlimb, as shown in Figure 4A and quantified in Figure 4B. In order to evaluate the collateral vessel density (CVD), mice were diaphonized and an equivalent region of interest (ROI), corresponding to 20%

of the limb area, was selected for CVD quantification (Figure 4C). A significantly higher CVD increase was observed in UCX®-treated mice when compared to the control (Figure 4D). Taken together, our results show that UCX® significantly augment capillary density and CVD increase after ischemic injury.

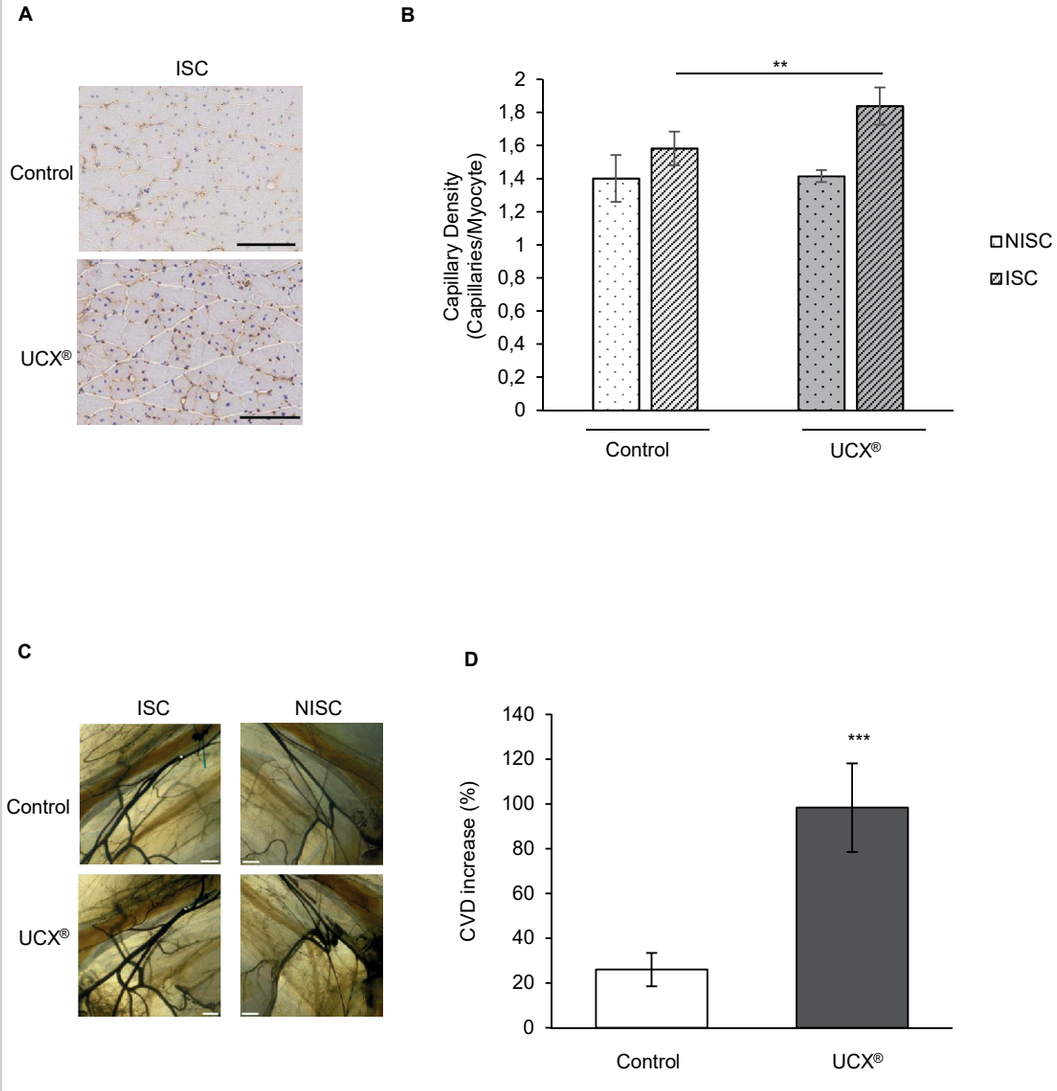

Figure 4. UCX® increase capillary and collateral densities.

UCX® or their vehicle (as a control) were administered in the ischemic gastrocnemius muscle 5 hours after HLI induction. (**A**) Representative sections from control and UCX®-treated ischemic gastrocnemius muscles at 90 days post-HLI. Capillaries and myocytes were identified by CD31 immunohistochemistry and haematoxylin, respectively. Scale bar, 125mm. (**B**) Quantitative analysis, at 90 days post-HLI, revealed increased capillary density (capillaries/myocyte) in UCX®-treated ischemic gastrocnemius muscles compared to control ischemic ones. (n=3 and n=7 for control and UCX®, respectively; t (8) = 4.44; ***P* = 0.0057; effect size was 3.064 and power 0.97). (**C**) Illustrative images of selected Regions of Interest (ROI) for control and UCX®-treated mice. ISC and NISC limbs are shown. Scale bar, 1mm (**D**) Data are represented as the percentage of Collateral Vessel Density (CVD) increase of the ISC limb relatively to the NISC one. At 90 days post-HLI, UCX®-treated mice presented significantly higher CVD increase (%) when compared to control mice (n=5 for each experimental group; t (8) = 7.63; ****P*= 0.000062; effect size was 4.82 and power 0.99).

Abbreviations: *ISC, ischemic; NISC, non-ischemic.*

3.3.5. UCX® up-regulate the endothelial *Tgfβ2, Ang2, Fgf2* and *Hgf* expression in response to HLI

To elucidate the mechanism of action of UCX® in our HLI model we investigated if UCX® could change the endothelial gene expression. For this purpose, five hours after surgical induction of unilateral HLI, UCX® or their vehicle (as a control) were administered in the ischemic muscle and mice were sacrificed at day 70 post-HLI induction. The gastrocnemius muscle sections were stained for CD31 and visualized using a Laser Capture Microdissection microscope. CD31-positive cells were dissected, isolated and analyzed by quantitative RT-PCR for the expression of several pro-angiogenic factors. Our results show that transcripts for *Tgfβ2, Ang2, Fgf2* and *Hgf* were clearly up-regulated in endothelial cells isolated from muscle of the ischemic limb, comparing with those found in ECs from the contralateral limb. This is observed exclusively in mice treated with UCX®. Control mice show the opposite trend, down-regulating the expression of the angiogenic genes in endothelium from the ischemic limb, when compared with the contralateral limb (Figure 5). These results suggest that the mechanism of action of UCX® in HLI mouse model involves the up-regulation of several angiogenic genes in the ECs present in the ischemic muscle.

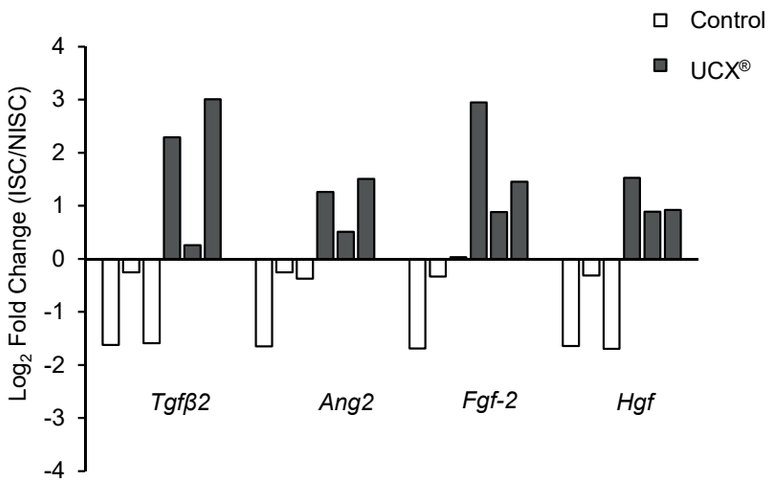

Figure 5. UCX® up-regulate the expression of *Tgfβ2, Ang2, Fgf2* and *Hgf* in endothelial cells isolated from ischemic gastrocnemius muscles.

UCX® or their vehicle (as a control) were administered in the ischemic gastrocnemius muscle 5 hours after HLI induction. At 70 days post-HLI, the expression of pro-angiogenic factors and their receptors was evaluated by qRT-PCR exclusively on endothelial cells isolated from gastrocnemius muscles. Each bar represents the relative gene expression in one animal. White and gray bars represent control and UCX® treated mice, respectively. Values were normalized to 18S to obtain relative expression levels. Results expressed as log2 fold changes between ISC and NISC samples demonstrated relative abundance of the transcripts in UCX® treated mice; in contrast, a down-regulation is observed in control mice.

3.4 DISCUSSION

CLI is a manifestation of Peripheral Arterial Disease that describes patients with chronic ischemic rest pain, ulcers or gangrene[1]. Available therapies are limited and patients may require amputation. The goal of limb salvage has stimulated research into alternative methods, including therapeutic angiogenesis. Stem cell therapy holds great potential for therapeutic angiogenesis, but its clinical translation has been slow due to (i) lengthy procedures performed *ex vivo* in which stem cells are manipulated and often lose viability, differentiate or change their characteristics, and (ii) high costs involved. In addition, the clinical use of bone marrow MCSs has demonstrated some caveats, mainly due to low yields which further decrease with donor's age and medical condition, leading to variable and/or limited cell doses.

Among the other possible sources for MSCs, there is the umbilical cord tissue[10], from which UCX® are derived. UCX® have already demonstrated the potential to lead to tissue regeneration and homeostasis, promoting faster remission of local and systemic manifestations of inflammatory arthritis[13]; preserving cardiac function after intramyocardial transplantation in a myocardial infarction murine model[14]; and accelerating wound healing[15]. Those studies strongly suggest that UCX® act in different cell types through paracrine mechanisms. In addition, it was found that conditioned medium of UCX® cultures induces fibroblast and keratinocyte migration and UCX® are chemotactic to $CD34^-/CD45^-$ bone marrow MSCs[15]. Further, it was also shown that *in vitro* UCX®-conditioned medium induces angiogenesis by promoting the formation of capillary-like structures by HUVECs[13]. Herein, we corroborated those findings showing that UCX® promotes tubulogenesis and ECs migration. We observed that UCX® when co-cultured with HUVECs induce tubule formation by HUVECs to a higher extent than the known pro-angiogenic factor FGF2, and also promote migration. As already demonstrated for other cell types, our data strongly suggest that UCX® act in ECs through paracrine mechanisms since a significant increase of pro-angiogenic factors such as HGF, TGFβ1, IL8 and PDGFAA was observed in the conditioned medium of UCX® when compared to the control.

In order to investigate if UCX® could promote therapeutic angiogenesis in a HLI context, we developed an experimental model of unilateral HLI and two important parameters were assessed: angiogenesis and arteriogenesis. Our data indicate that UCX® administration significantly induces blood perfusion recovery in the limb after HLI induction. Accordingly, a significantly higher capillary density and CVD increase was found in the ischemic muscles after UCX® treatment. These evaluations were performed at day 90 post-HLI suggesting that the effect induced

by UCX® in angiogenesis and arteriogenesis is maintained over time. Besides, the non-ischemic muscles are unaffected by UCX® since capillary density and CVD are similar in non-ischemic gastrocnemius muscles both from UCX®-treated and control mice, indicating that UCX® might have a local action. These data suggest that UCX® could secrete cytokines/chemokines and paracrinally interfere with the ischemic microenvironment contributing to a therapeutic angiogenesis. More importantly, a new mechanisms of action of UCX® is demonstrated for the first time: UCX® modulate the expression of pro-angiogenic players in ECs. We found that 70 days post-HLI and UCX® treatment, ECs isolated from UCX®-treated microenvironment present an up-regulation in their expression levels for *Tgfβ2, Ang2, Fgf2* and *Hgf* when isolated from the ischemic gastrocnemius muscle in comparison with the contralateral one. This effect is UCX®-specific as the opposite is verified in control mice. According to these findings, we show that UCX® simultaneously up-regulate the expression of several angiogenic factors in ECs and by this mechanism we may hypothesize that UCX® improve their endogenous angiogenic potency in an ischemic context. This could be very relevant when compared to other strategies where only a single angiogenic factor is administered, especially if we understand angiogenesis as a complex process that involves multiple cytokines. It is also very interesting to note that the same factors modulated by UCX® have been extensively studied in therapeutic angiogenesis. Specifically, in gene and protein therapy, FGF2 was used in a rabbit model of HLI[18], where it was shown to promote the development of collateral vessels. In clinical trials, the efficacy of FGF2 was also evaluated in clinical trials such as FIRST (FGF Initiating Revascularization Trial)[19] and TRAFFIC (Therapeutic Angiogenesis with Recombinant FGF2 for Intermittent Claudication)[20]; however, it did not show a sustained success[21]. Concerning HGF, it was also used in clinical trials through gene transfer to treat CLI, where its safety was shown[22]. More rigorous controlled trials (randomized and placebo-controlled studies with larger numbers of patients) are currently ongoing. Given the fact that CLI is a common complication of diabetes mellitus, future studies should assess the UCX® efficacy in a diabetic experimental model since CLI may adversely affect EPC function and thus limit therapeutic efficacy. Recent data have further shown that the secreted proangiogenic protein osteopontin, significantly downregulated in diabetic EPCs, could increase the secretion of angiogenic proteins from EPCs increasing their therapeutic efficacy[23]. Importantly, these findings show that the function of EPCs could be modulated and raise interestingly questions for future investigation: Which molecules could eventually improve the therapeutic efficacy of UCX® in an ischemic microenvironment? Could different ischemic microenvironments modulate differently the function of UCX®; if so, by which mechanisms? Should UCX® be

modulated by the administration of selected molecules according to the pathologies associated with CLI?

3.5 CONCLUSION

In conclusion, here we propose a model of enhanced and sustained angiogenesis induction by using UCX® as a promising therapeutic approach for CLI. UCX® significantly induce blood perfusion, capillary density and collateral development. This could be at least in part achieved by a new mechanism since we show that UCX® upregulate the simultaneous expression of several pro-angiogenic factors in endothelial cells that could improve their angiogenic potency.

DECLARATIONS

Ethics approval and consent to participate

All animal procedures were carried out with the permission of the local animal ethical committee in accordance with the Directive 2010/63/EU. The procedures were approved by the institutional Animal Welfare Body and licensed by DGAV, the Portuguese competent authority for animal protection (license number 023861/2013)

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

HC and PC are shareholders of ECBio; JMS, MF, MT are employees of ECBio. RB was an employee of ECBio when this work was performed. The other authors declare that they have no competing interests.

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Authors' contributions

PC and HC devised the overall preclinical development strategy of UCX® for Peripheral Arterial Disease and CLI and sourced for funding of the research. SC conceived and designed the experiments, in discussion with FP, MS, RB, PC and HC. SC wrote the manuscript and FP, MS, RB, PC and HC critically revised it. ARP performed laser doppler perfusion imaging, immunohistochemistry and capillary density analysis, analyzed the data and revised the manuscript; TM performed collateral vessel quantification and gene expression analysis, analysed the data and revised the manuscript;

ARP and TM performed endothelial culture and wound healing assay and analysed the data. AM. JON performed diaphonisation. AM performed the surgical HLI and UCX® administration in vivo, performed contrast agent perfusion before diaphonization and participated in data analysis, interpretation and critically revised the manuscript. MS and RB designed the experiments on tubule formation and preparation of conditioned medium. MT and MF performed UCX® culture and tubule formation assays, prepared conditioned medium and performed the quantification of secreted factor and revised the manuscript. All the authors read and approved the final version of the manuscript.

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RESEARCH ARTICLE:
UMBILICAL CORD TISSUE–DERIVED MESENCHYMAL STROMAL CELLS MAINTAIN IMMUNOMODULATORY AND ANGIOGENIC POTENCIES AFTER CRYOPRESERVATION AND SUBSEQUENT THAWING

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4. RESEARCH ARTICLE: UMBILICAL CORD TISSUE–DERIVED MESENCHYMAL STROMAL CELLS MAINTAIN IMMUNOMODULATORY AND ANGIOGENIC POTENCIES AFTER CRYOPRESERVATION AND SUBSEQUENT THAWING

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ABSTRACT

Background aims: The effect of cryopreservation on mesenchymal stromal cells (MSCs) therapeutic properties has become highly controversial. However, data thus far have indiscriminately involved the assessment of different types of MSCs with distinct production processes. This study assumed that MSC-based products are affected differently depending on the tissue source and manufacturing process and analyzed the effect of cryopreservation on a specific population of umbilical cord tissue–derived MSCs (UC-MSCs), UCX®.

Methods: Cell phenotype was assessed by flow cytometry through the evaluation of the expression of relevant surface markers such as CD14, CD19, CD31, CD34, CD44, CD45, CD90, CD105, CD146, CD200, CD273, CD274 and HLA-DR. Immunomodulatory activity was analyzed *in vitro* through the ability to inhibit activated T cells and *in vivo* by the ability to reverse the signs of inflammation in an adjuvant-induced arthritis (AIA) model. Angiogenic potential was evaluated *in vitro* using a human umbilical vein endothelial cell–based angiogenesis assay, and *in vivo* using a mouse model for hindlimb ischemia.

Results: Phenotype and immunomodulatory and angiogenic potencies of this specific UC-MSCs population were not impaired by cryopreservation and subsequent thawing, both *in vitro* and *in vivo*.

Discussion: This study suggests that potency impairment related to cryopreservation in a given tissue source can be avoided by the production process.The results have positive implications for the development of advanced-therapy medicinal products.

Keywords: *Advanced-therapy medicinal product; Cryopreservation; Mesenchymal stromal cells; Potency; Thawing.*

4.1. INTRODUCTION

Mesenchymal stromal cells (MSCs) are promising active substances for advanced therapy medicinal products (ATMPs), mainly because of their immunomodulatory, anti-inflammatory, pro-angiogenic and regenerative properties. Currently, there are

approximately 700 clinical trial entries using “mesenchymal cells” in the search tool at the ClinicalTrials.gov website, consisting of trials promoted by both academic institutions and industry alike for a wide variety of indications. Umbilical cord tissue–derived expanded MSCs, UCX®, consists of a specific population of MSCs from the umbilical cord tissue (UC-MSCs), isolated according to a patented technology that is the basis for several allogeneic ATMPs under development[1,2]. Preclinical studies using UCX® have demonstrated the safety and quality of the cells[2], as well as their efficacy in animal models for several immune-related and cardiovascular diseases. Specifically, UCX® have demonstrated an anti-inflammatory effect in both acute and chronic arthritis models[3], leading to a drastic reduction of local and systemic arthritic manifestations. They have promoted recovery of cardiac function upon intra-myocardial administration in an acute myocardial infarction murine model, mostly due to a paracrine activity that activated pro-angiogenesis and anti-apoptotic mechanisms[4]. In addition, an UCX® pro-angiogenic effect has recently been demonstrated in a murine model of hindlimb ischemia[5], whereas the paracrine effects of UCX® cells have also been noted in a wounded skin *in vivo* model, where they have promoted healing via a mechanism that could implicate the recruitment of other systemic/endogenous MSCs[6].

Given their recognized potential for cellular therapy and regenerative medicine, it is necessary to cryopreserve and bank MSCs to ensure their immediate availability. A remarkable amount of research and resources have been expended toward optimizing the cryopreservation/thawing processes, as well as developing Good Manufacturing Practices (GMP) to ensure that MSCs retain their safety and therapeutic characteristics after cryopreservation. Nevertheless, the results are controversial. Reports have indicated that cryopreservation of bone marrow-derived MSCs (BM-MSCs) impaired their immunosuppressive properties due to a heat-shock response[7] and resulted in their faster complement-mediated elimination after blood exposure[8]. Similar claims have been made for adipose tissue-derived MSCs (AT-MSCs), where administration of freshly thawed cryopreserved AT-MSCs was associated with significant adverse effects in contrast to administration of cultured AT-MSCs, which resulted in improvement in renal function without adverse effects[9]. However, the belief that cryopreservation negatively affects the performance of MSCs has recently been challenged. One study has shown that xenogen-free, GMP-grade BM-MSCs can undergo cryopreservation without altering their characteristics and immune-modulating potential[10]. More recently, an *in vivo* analysis comparing freshly thawed and cultured BM-MSCs in a mouse model of allergic airway inflammation also demonstrated no difference between freshly thawed and cultured cells[11]. Most importantly, in a phase II clinical study using BM-MSCs in acute graft-versus-host

disease in which either cultured or freshly thawed cells were infused, there was no report of clinical differences observed between the two groups[12].
UCX® cells are specifically meant to serve as a basis for off-the-shelf cryopreserved products, and the challenge is to ensure that cryopreserved cells retain the therapeutic potential of their freshly isolated counterparts. In previous preclinical studies, UCX® were not freshly thawed immediately before *in vivo* use, meaning they were typically administered immediately after a culture step[2–6]. In the present study, we set out to test whether the phenotype and therapeutic potency of our specific UC-MSC population is affected by our cryopreservation and/or thawing procedures, comparing cultured cells with freshly thawed cells in terms of their immunophenotype as well as their immunomodulatory and angiogenic potencies, both *in vitro* and *in vivo*.

4.2. METHODS

4.2.1. Umbilical cord samples

This study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee at the Cascais Hospital Dr. José de Almeida. Umbilical cord donations were obtained with written informed consent according to Directive 2004/ 23/EC of the European Parliament (transposed to the Portuguese Law 12/2009 of March 26).

4.2.2. Isolation of UCX® cells

Human UCX cells were isolated according to patented proprietary method developed by ECBio as described in Soares *et al.*[1]. Briefly, human umbilical cords, obtained after full-term natural or cesarian delivery births, were depleted of blood, cut in transversal sections and digested with animal component free collagenase NB4 (Serva) and TrypLE Select (Gibco, Life Technologies). After overnight incubation at 37°C under 7% CO2 in a humidified incubator, non-adherent cells were removed, and fresh medium was added, α-Minimum Essential basal medium with 1 g/L glucose (Biochrom), 2 mmol/L glutamine (Sigma-Aldrich) complemented with 20% fetal bovine serum (FBS) (Gibco). Change of culture medium was performed twice weekly. Cells were passaged when 80-90% confluence was observed.

4.2.3. Cryopreservation and thawing of UCX® cells

UCX® cells used in this study were cultured up to passage 7 and tested between passage 5 and 7. In near confluent cultures, cells were detached using TrypLE Select (Gibco). Approximately 3 × 106 cells were centrifuged at 200*g*, 4°C for 10 min and cryopreserved in 10% dimethylsulfoxide (WAK-Chemie) and 90% FBS (Gibco). Cryopreservation was performed in a Controlled Rate Freezer (CRF) (IceCube14S, Sylab) using a freezing profile described by Freimark *et al.*[13]. During thawing, cryovials were removed from the liquid N2 container and placed immediately in the water bath at 37°C until cell suspension was almost entirely thawed and diluted with MSC medium, counted and plated in a culture flask (Nunc, Thermo Fisher Scientific). Cell suspension was then either (i) washed and suspended in the vehicle for further assays directly after thawing (termed here "freshly thawed") or (ii) incubated at 37°C, 7% CO2 to be kept in culture for a minimum of 5 days or up to two passages before administration (termed here "cultured cells").

4.2.4. Viability and apoptosis assays

Cell viability and recovery (after thawing) were evaluated using a hemocytometer with the trypan blue (Sigma-Aldrich) exclusion method and observed under an Olympus CK30 inverted microscope (Olympus). The cell recovery rates were calculated by the ratio between the number of viable cells counted after thawing and the total cell amount cryopreserved.

Apoptosis was measured by Annexin V. As a positive control, apoptosis was induced by incubating cells with apoptosis-inducing medium (α-MEM, 20% FBS, 2 mmol/L H2O2). After 2 h, cells were washed, detached and stained with a PE-labeled Annexin V antibody (BioLegend) for apoptosis detection. As a control, the mouse isotype IgG1k PE antibody was used (BioLegend). The cells were analyzed by flow cytometry on a Gallios Flow cytometer (Beckman Coulter) and the results analyzed with Kaluza software (Beckman Coulter).

4.2.5. Immune phenotypic analyses

To analyze cell-surface expression markers, cells were detached, counted and labeled with the following anti-human antibody conjugates: CD44— APC; CD73—APC; CD90—PE; CD14—PerCp/Cy5.5; CD45—PerCp/Cy5.5; CD31—fluorescein

isothiocyanate (FITC); CD34—FITC; CD19—Pacific Blue; HLA-DR—Pacific Blue; CD200—Alexa Fluor 647; CD273—PE and CD274—BrilliantViolet 421 (all from BioLegend) and also CD105—PE (eBioscience) and CD146—PerCP/Cy5.5 (BD). The mouse isotype antibodies used as the respective controls were as follows: Pacific Blue IgG1; Pacific Blue IgG2a; PerCp/Cy5.5 IgG1k; PerCp/Cy5.5 IgG2a; PE IgG1k; APC IgG1k; FITC IgG1k; Alexa Fluor 647 IgG1k and BrilliantViolet 421 IgG2b (all from BioLegend).Ten thousand labeled cells were acquired using a Gallios Flow cytometer (Beckman Coulter), and results were analyzed with Kaluza software (Beckman Coulter).

4.2.6. Immunosuppression assay

The immunosuppression assay was performed in 96-well microtiter plates using RPMI (Gibco) supplemented with 5% HEPES (Gibco), 5% Pen-Strep (Gibco), 5% NaPyr (Gibco) and 5% human serum obtained from a specific donor. In each assay, peripheral blood mononuclear cells were obtained from two donors, cultured at 2 × 105 cells per well and stimulated with anti-CD3 (eBioscience), anti-CD28 (eBioscience) and IL-2 (eBioscience). Suppressor cells (cultured and freshly thawed UCX®) were irradiated with 50 Gy (Gammacell ELAN 3000, Best Theratronics) before addition to the culture at 2 × 105 cells (1:1), 2 × 104 cells (1:10) or 4 × 103 cells (1:50) per well. Triplicate cultures were performed for each condition. Cultures were incubated at 37°C in 5% CO2 for 4 days, pulsed with [3H]thymidine (1 microCi per well, Amersham Biosciences, Piscataway) for 16 h, and the cells were harvested onto filter mats using a Tomtec 96-well cell harvester (Perkin Elmer). Radioactivity incorporated into the dividing cells was determined using a scintillation counter (Microbeta Trilux Scintillation and Luminescence Counter 145 LSC, Perkin Elmer).

4.2.7. Human umbilical vein endothelial cell–based tube formation assay

Human umbilical vein endothelial cells (HUVECs; Sciencell) were used, on a Matrigel tube formation assay to assess the UCX® angiogenesis effect. HUVEC were grown in flasks coated with 1% gelatin (Sigma-Aldrich) until 70% confluence. Cells were cultured in endothelial growth medium: M199 medium (Sigma-Aldrich) supplemented with 10% FBS (Gibco), 50 µg/ mL ECGS (Sigma-Aldrich), 100 µg/mL Heparin (Sigma-Aldrich) and 1% penicillin-streptomycin (10000 U/mL, Sigma-Aldrich). HUVEC plated on Matrigel-coated wells rapidly form a dense capillary network.

Growth factor– reduced Matrigel (Corning) was allowed to polymerize for 30 min at 37°C after coating 48-well plates (200 µL/ well) (Nunc). HUVECs up to P10 were trypsinized and seeded on top of the Matrigel (4.5 × 104 cells per well) in 350 µL of endothelial basal medium (EBM, Lonza) with 1% penicillin-streptomycin (10 000 U/ mL). Cells were then incubated 1 h at 37°C, 5% CO2. UCX® angiogenesis effect was monitored by the loading of 1 × 106 cells either cultured (previously trypsinized and re-suspended in 350 µL basal medium) or freshly thawed (centrifuged and re-suspended in 350 µL basal medium) ona 1-µm insert (Brand). Insert was then carefully placed in a 48-well plate and incubated for 16 h at 37°C, 5% CO2. Controls with no UCX® included HUVECs in (i) EBM and (ii) EBM with vascular endothelial growth factor (VEGF; 100 ng/ mL). After incubation, inserts were removed, and all wells were photographed (10 × amplification) using a Nikon Eclipse Ti-U inverted microscope with a conjugated Nikon DS-Qi1Mc camera (Nikon). The experiment was repeated twice using internal triplicates. Tube formation was quantified on four random fields per replicate, using the Angiogenesis analyzer from Image J software, version 1.49 (National Institutes of Health).

4.2.8. Therapeutic evaluation in animal models

All animal experiments were carried out with the permission of the local animal ethical committee in accordance with the EU Directive (2010/63/EU), Portuguese law (DL 113/2013) and all relevant legislations. The experimental protocol was approved by Direcção Geral de Alimentação e Veterinária (DGAV). Animals were obtained from Charles River Laboratories (Santa Perpetua de Mogoda, Spain).

4.2.9. Chronic AIA model

Adult male Wistar rats, weighing 365–480 g, were randomly divided into three groups of six animals each. Animals were acclimatized before the experiments and housed in plastic cages under standard laboratory conditions, fed commercial chow and acidified drinking water *ad libitum*. As a rule, inflammation was induced by a single intradermal injection of 0.10 mL of a 10-mg/mL suspension of *Mycobacterium butyricum* (killed and dried) in Incomplete Freund's Adjuvant (Difco Laboratories), homogenized by ultrasound.

Treatment was initiated at day 7 after induction. One group of induced animals received phosphate-buffered saline (PBS) by intra-articular route of administration

(sham group). This group was kept under the same conditions as treated animals. One group of induced animals received cultured UCX® (1.7 × 106), and the other induced group received freshly thawed UCX® (1.7 × 106). In both groups, a total of three, every-other-day, intra-articular administrations were performed. The experiment lasted for 64 days. The therapeutic effect of UCX® cells was evaluated by physical changes in body weight and in volume of right and left paws measured by a water displacement method, using a plethysmometer (Ugo Basile). Animals were scored for clinical arthritis as previously described, with some modifications [3]. At pre-selected times, animals were blindly graded on the sum of the following grades: 0 = normal; 1 = for each inflamed paw; 1 = tail lesion; 1 = joint rigidity or deformity; 1 = wounded paw; 1 = infected paw; 1 = necrotic paw. The sum of the parameters was calculated as an arthritic index (AI) with a maximum possible score of 9. The experiment was performed twice with consistent observations, using two UCX® isolates (different donors).

4.2.10. Hindlimb ischemia model

Twelve-week-old C57BL/6 female mice were used in all experiments. Animals were randomly divided into three groups. A total of 12 animals were used per group in two independent experiments. For this experiment only the UCX® isolate (donor) that had presented better blood perfusion performances from a previous comparative study among donor isolates[5] was used. The animals were anesthetized with ketamine-medetomidine cocktail (75 mg/kg body weight and 1 mg/kg body weight, respectively) intraperitoneally for the surgical procedure as well as for the other analysis procedures. The anesthesia was partially reverted with atipamezole (5 mg/kg body weight). Postoperatively, analgesia was administered (buprenorphine 100 µL/15–30 g body weight every 8–12 h), and the animals were closely monitored. A surgical procedure was performed to induce unilateral hindlimb ischemia in the mice. Briefly, an incision in the skin overlying the thigh of the right hindlimb of each mouse was made, and the distal external iliac artery and the femoral artery and veins were ligated and excised. The vein was ligated both to increase the severity of the ischemia as well as to increase the technical reproducibility of the model, as isolation of the femoral artery alone often results in tearing of the vein resulting in hemorrhage. The contralateral limb served as an internal control for each mouse. A dose of 2 × 105 UCX® cells was injected intra-muscularly, 5 h post-ischemia induction on the gastrocnemius muscle (right hindlimb) in a volume of 50 µL per animal, divided into two injections of 25 µL laser Doppler perfusion imaging.

4.2.11. Laser Doppler perfusion imaging

The laser Doppler perfusion imager (MoorLDI-V6.0, Moor Instruments) was used to assess limb perfusion. Hair was removed 1 day before laser Doppler analysis using an electrical shaver followed by depilatory cream.The mice were placed on a 37°C heating pad to reduce heat loss during measurements. Blood flow was measured both in the ischemic and the contralateral legs, before hindlimb ischemia induction, immediately post-ischemia induction and on days 7, 14 and 21 after the ischemia induction. Consecutive measurements, for each time point described, were performed by scanning over the same region of the hindlimb. Blood flow was displayed by coloured pixels from minimal (dark blue) to maximal (red). Mean flux values were calculated using the Moor LDIV6.0 image processing software. To account for variables such as temperature and ambient light, blood perfusion is expressed as the ratio of ischemic to non-ischemic limb.

4.3. RESULTS

4.3.1. Recovery, viability and apoptosis after cryopreservation

For an off-the-shelf cellular product, the phenotypic characteristics must be guaranteed and the number of recovered viable cells after cryopreservation needs to be high and stable, ideally for several years.

UCX® cells are MSCs, and as such they are characterized by the presence or absence of specific surface markers, as defined by the International Society for Cellular Therapies[14].To answer determine whether cryopreservation and subsequent thawing could alter the immunophenotype of these cells, a panel of 10 representative surface markers was studied, including CD90, CD105, CD44, CD73, CD31, CD34, CD14, CD45, CD19 and HLA-DR. The results showed that no significant differences in surface marker expression were caused by our cryopreservation step, indicating that cryopreservation did not alter the immunophenotype of the cells (Figure 1).

Concerning product stability, we were able to recover 82 ± 23% of UCX® cryopreserved for less than 1 year and 91 ± 17% when cryopreserved for more than 3 years (Figure 2A), demonstrating that the cryopreserved product is stable at least over 3 years and that the post-thawing recovery of viable cells is consistently high. The viability of the cell population was also studied for the cryopreserved product and compared to cells in culture (Figure 2B). Viability of freshly thawed cells was 90 ± 9% for cells cryopreserved for less than 1 year and 86 ± 7% for cells cryopreserved for longer

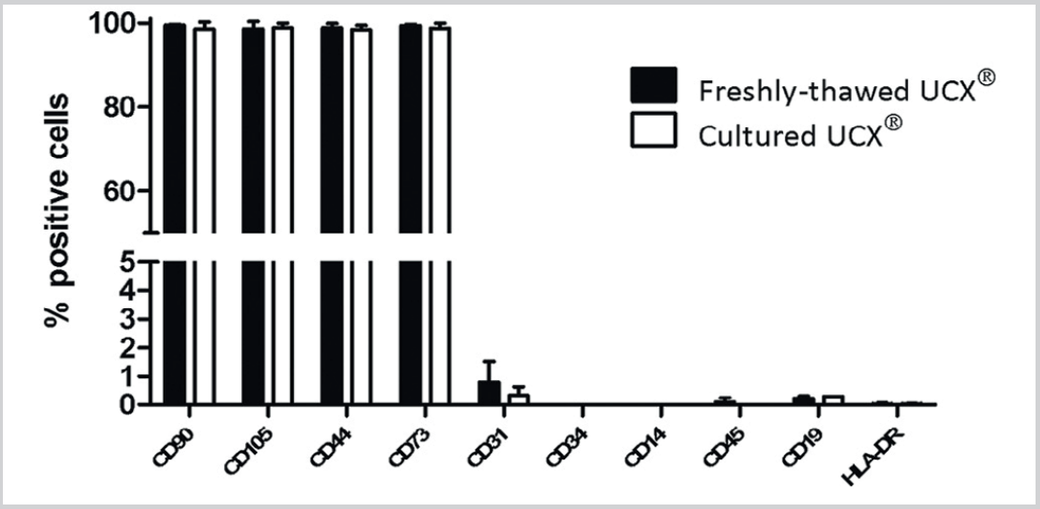

Figure 1. MSCs immunophenotypic markers.

Flow cytometric analysis comparing International Society for Cellular Therapies minimal MSCs markers with additional CD44 and CD31 markers for cultured (n = 3) and freshly thawed UCX® (n = 3). No significant differences were found between the two populations. Results are represented as mean ± SD.

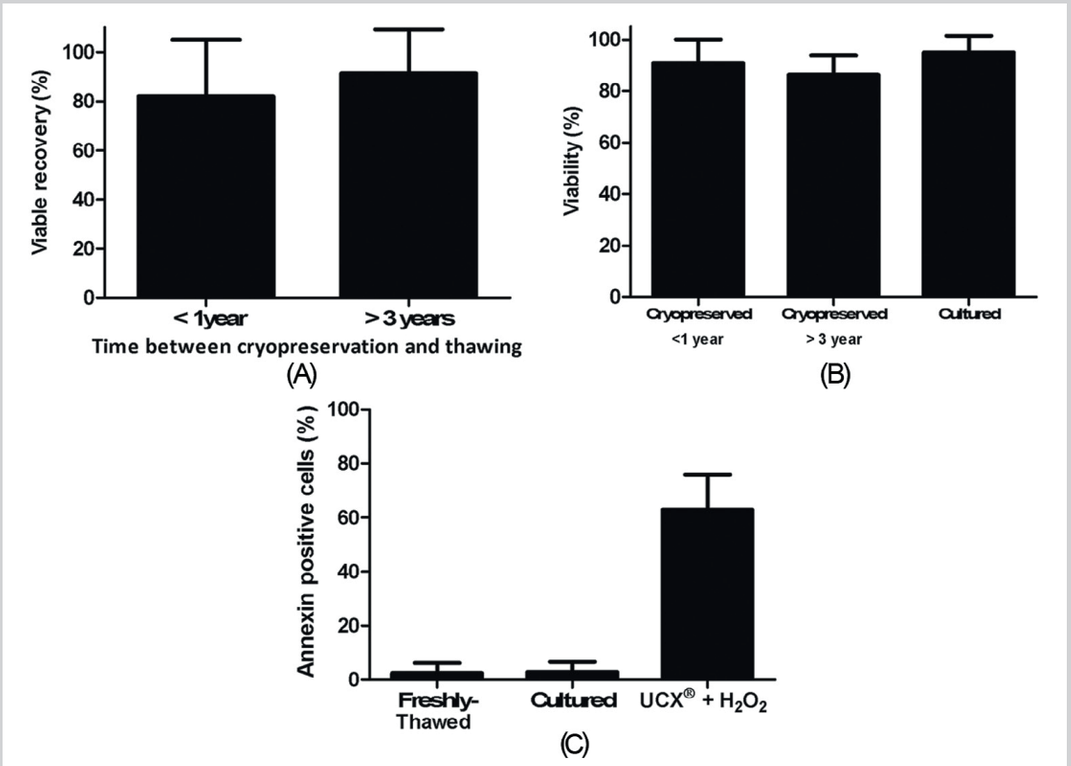

Figure 2. Recovery, viability and apoptosis of freshly thawed UCX®.

(A) The number of viable cells that were recovered from each cryovial (considering the number of cells initially cryopreserved) was analyzed by trypan blue exclusion. Samples were divided into cells cryopreserved for less than 1 year (n = 11) and cells cryopreserved for more than 3 years (n =5). No statistical differences were observed by *t*-test. (B) Viability was measured by trypan blue exclusion and compared between cryopreserved less than 1 year (n = 12), cryopreserved more than 3 years (n =5) and cultured populations (n = 12). No statistical differences were observed among the groups using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. (C) The presence of apoptotic cells in cultured and freshly thawed populations was measured by annexin V staining and flow cytometric analysis. As a positive control, cultured cells were incubated with H2O2 (2 mmol/L) for 2 h. The number of apoptotic cells in both freshly thawed and cultured UCX was extremely low, and no statistical differences were found between the two groups using one-way ANOVA followed by Tukey's test. All results were represented as mean ± SD.

than 3 years. Not surprisingly, viability of cultured cells was slightly higher than freshly thawed cells (95 ±6%), although this difference was not significant ($P > 0.05$, One-way analysis of variance followed by a Tukey's Multiple Comparison Test). Although cells were viable and recovered well, the stress caused by cryopreservation followed by thawing might induce apoptosis or early cell death, undetected by trypan blue exclusion assay, which could bring on undesirable effects upon infusion. Apoptosis of freshly thawed and cultured cells was therefore measured via Annexin V expression. Although cells exposed to 2 mmol/L H^2O^2 for 2 h (positive control) showed high levels of apoptosis (63%) as expected, both UCX® cells in culture and freshly thawed showed only negligible levels of Annexin V positivity (2.9% and 2.5%, respectively), demonstrating that the number of apoptotic cells is negligible in both cultured and freshly thawed cell populations (Figure 2C).

4.3.2. Immunomodulatory potency of UCX® is not affected by freeze-thawing

MSCs, including UCX®, are known for their immunomodulatory capacities[2,3,15]. CD200 is a surface protein known to play a role in the fetal-maternal tolerance. Not surprisingly, CD200 is highly expressed in UC-MSCs. CD274 and CD273 are co-stimulatory molecules involved in downregulating lymphocyte activation that are constitutively expressed by UC-MSCs. In turn, CD146 is a cell adhesion molecule that is also important in immunomodulation. As a small representative group of functional markers, we asked whether expression of CD200, CD274, CD273 and CD146 was affected by cryopreservation as a whole. Paired analysis using flow cytometry to analyze the expression of cultured and freshly thawed cells from the same donor revealed that, although small changes were individually observed, no significant difference was observed in the expression of the functional markers tested (Figure 3A). These data suggest that the immunomodulatory function of freshly thawed cells might be no different from that of cells in culture as expression of functional markers are not significantly affected.

One immunomodulatory mechanism promoted by MSCs is the suppression of activated T cells, which we believe to be a valid test for the potential immune-suppressive function attributed to these cells. This mechanism can be quantified *in vitro* by assessing the proliferation of PBMCs activated by anti-CD3, anti-CD28 and interleukin (IL)-2 and incubated with irradiated UCX® cells for 4 days. Cells from three umbilical cord donors were used and their potency tested by applying three ratios of MSCs:PBMCs (Figure 3). Flow cytometric analysis of immunoregulatory markers CD200, CD273, CD274 and CD146 were studied using a paired analysis

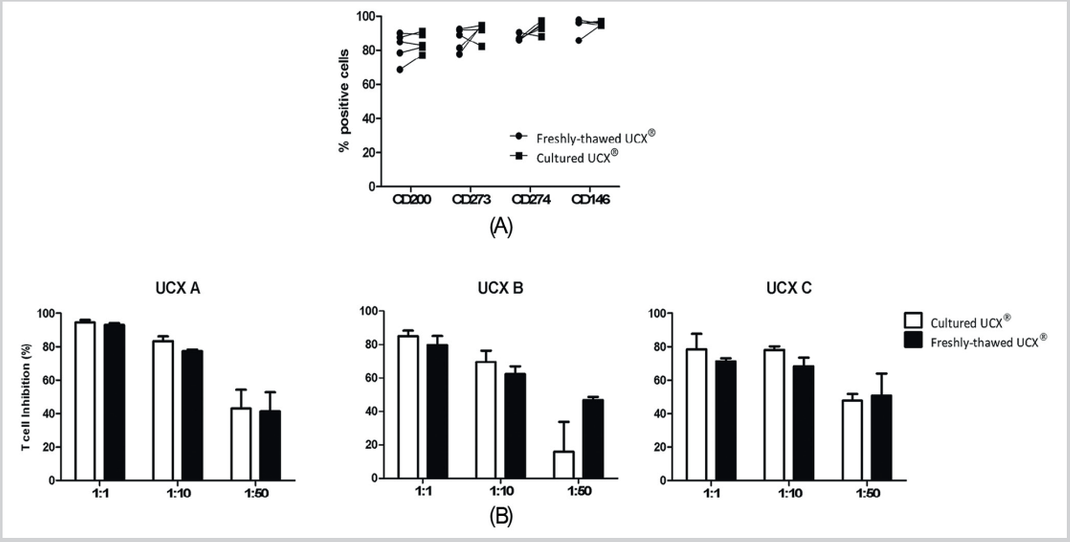

Figure 3. *In vitro* immunomodulatory potency.
(A) Flow cytometric analysis of immunoregulatory markers CD200, CD273, CD274 and CD146 were studied using a paired analysis between freshly thawed and cultured UCX®. Expression of these markers is consistently high, and no significant differences were observed between freshly thawed and cultured UCX® using a paired *t*-test for each marker. (B) T-cell suppression assay was performed using three UCX® isolates, corresponding to three donors (A, B and C). In turn, for each assay, PBMCs were obtained from two donors, and triplicate cultures were performed for each condition. Suppression of lymphocyte proliferation in the presence of irradiated cultured or freshly thawed UCX® was achieved by co-incubation of PBMCs activated with anti-CD3, anti-CD28 and IL-2, with irradiated MSCs at three MSC:PBMC ratios (1:, 1:10 and 1:50). All UCX® were able to suppress activated T cells in a dose-dependent manner. Results are represented as mean ± SD.

between freshly thawed and cultured UCX®. Expression of these markers was consistently high, and no significant differences were observed between freshly thawed and cultured UCX® using a paired *t*-test for each marker. Suppression of activated T cells was observed with all UCX® samples in a dose-response manner, that is, suppression was higher in the 1:1 ratio and lowest when 1:50 ratio was used, confirming the immunosuppression capabilities of UCX®. In one sample (UCX B), at the lowest ratio of 1:50, freshly thawed UCX® were more immunosuppressive than cultured UCX®. In all other ratios and samples, no significant differences were observed in the suppression of activated T cells between freshly thawed and cultured UCX®. Hence, in an *in vitro* potency assay, cryopreservation had no deleterious effect on the immunosuppressive capacity of UCX® to suppress T-cell activation.

Having established that freshly thawed UCX® maintained their functional markers and their ability to suppress activated T cells, we further tested whether freshly thawed UCX® cells showed impaired immunomodulatory therapeutic benefits *in vivo*. For this purpose, a chronic AIA rat model was used over the course of 64 days. Arthritis was induced in the subplantar area of right-hind paws where three intra-articular injections of either sham (PBS) or cells (freshly thawed or cultured) were administered every other day, starting at day 7 post-disease induction.

Animals were observed weekly, and readouts such as body weight, volume of left and right hind paws and arthritic index (AI) were monitored over time. The results confirmed that the administration of cells was highly effective in ameliorating local inflammatory signals, as evidenced by right and left paw volume (Figure 4A,B), as well as systemic manifestations of the disease reflected in a clear reduction in the AI (Figure 4C). Weight loss recovery started early and faster for UCX®-treated animals compared with PBS-treated animals (sham) (Figure 4D). Most relevant, the results showed that there was no significant difference between treatments performed with either cultured or freshly thawed cells for any readout measured. Taken together, these results clearly demonstrate that cryopreservation does not alter the immunomodulatory potency of UCX® used immediately after thawing.

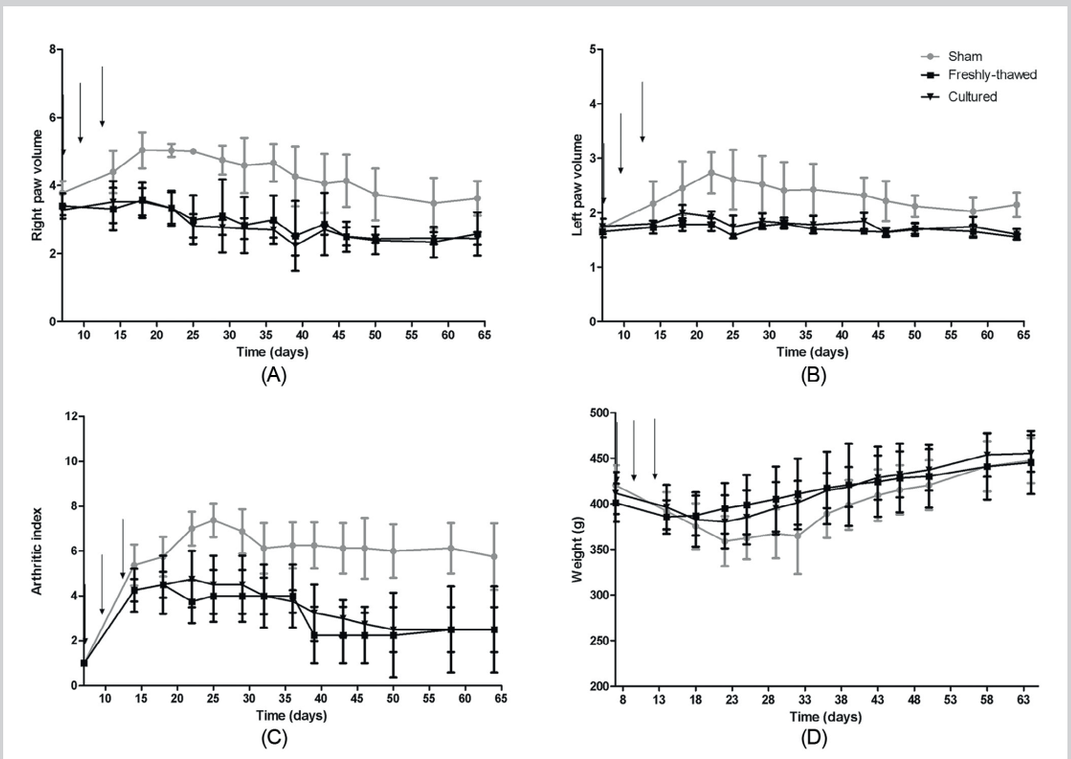

Figure 4. *In vivo* immunomodulatory potency assessed by a rat model of chronic AIA.
Cultured and freshly thawed UCX® (1.7×10^6) were administered three times every other day (arrows) via intra-articular injection 7 days post–disease induction. Control animals (gray) received a sham injection of vehicle (PBS) (n = 6). Animals were monitored over 64 days, during which time the right paw volume (A) and left paw volume (B), arthritic index (AI) (C) and body weight (D) were recorded. In the experimental groups where the administration of either cultured (n = 6) or freshly thawed cells (n = 6) was performed, a consistently and significantly lower AI, as well as right and left paw volumes, were observed (one-way analysis of variance followed by a Dunnett's post-test; $P < 0.0001$). The experiment was performed twice, using two UCX® isolates (different donors). Results are represented as mean ± SD.

4.3.3. The angiogenic potency of UCX® cells is not affected by cryopreservation

MSCs are known for their ability to induce angiogenesis and/or vasculogenesis, particularly when placed in ischemic environments. This was previously demonstrated for UCX® in both an acute myocardial infarction model in mice[4] and a murine model of hindlimb ischemia (HLI)[5]. Herein, the angiogenic potency of MSCs has been demonstrated *in vitro* using a Matrigel/HUVEC tube formation assay. Typically in such assays, MSC-conditioned medium is prepared and used to induce HUVECs to form tubes in a Matrigel bed. However, to directly assess the impact of UCX® physical presence on HUVECs activity, we used a system in which UCX® cells were co-cultured in a Boyden chamber, in an insert on top of a Matrigel containing HUVECs. After 16 h of co-culture, photographs of the Matrigel beds were taken and subjected to Image J's Angiogenesis analyzer for quantification of (i) the number of master junctions (branching points), (ii) total segment length (tube length) and (iii) total mesh area.Tube formation was induced by adding VEGF (100 ng/mL) to basal medium (positive control) or by co-culturing freshly thawed or cultured UCX® in basal medium. Results demonstrated that UCX® cells are strong inducers of cellular angiogenesis shown by an increase in all three criteria analyzed compared with basal medium control and with the positive control (VEGF) (Figure 5). Most importantly, no significant difference was observed between cultured and freshly

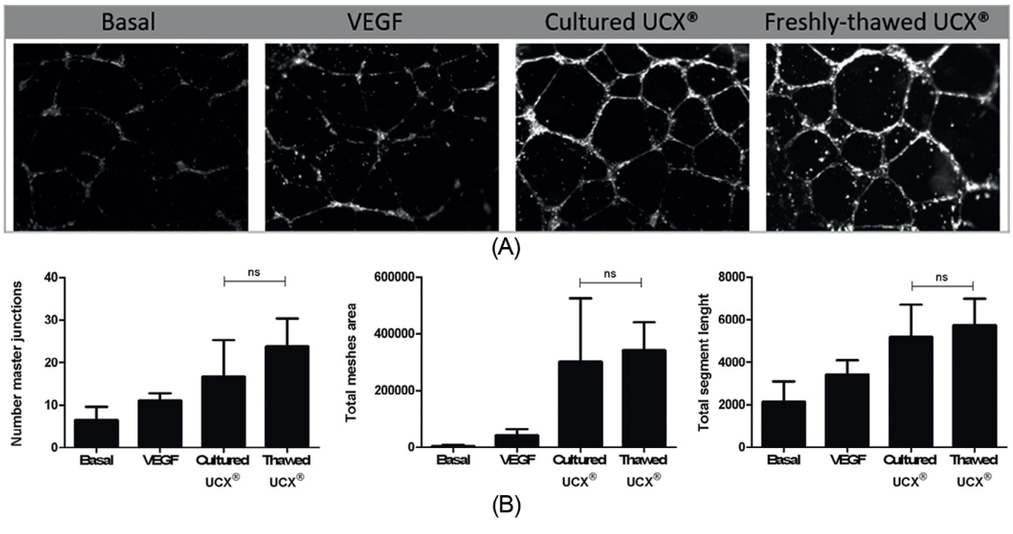

Figure 5. *In vitro* pro-angiogenesis effect.
An HUVEC-Matrigel tube formation assay was used to assess the angiogenic potency of UCX®. Cells were placed in an insert on top of a Matrigel coated well with HUVECs for 16 h. Controls included HUVECs in basal media (Basal) and in basal media with VEGF (100 ng/mL). The experiment was repeated twice using internal triplicates. Wells were photographed (A) and tube formation was quantified on four random fields per replicate, using the angiogenesis analyser from Image J software, version 0.49 (National Institutes of Health) (B). Statistical differences between cultured and freshly thawed UCX® were not found (one-way analysis of variance with Tukey's post-test; $P < 0.05$). Values are represented as mean ± SD

thawed UCX®, indicating that cryopreservation and thawing do not alter their pro-angiogenic potential. The effect of cryopreservation on the angiogenic potential of UCX® was further evaluated *in vivo*. UCX® cells have been shown to promote treatment of critical limb ischemia (CLI) through the induction of collateral vessel formation and increase in capillary density in a mouse model of HLI. Here, the same model was used to test whether cryopreservation and thawing had any effect on the therapeutic angiogenic potential of UCX®, evaluated by analyzing the recovery of blood perfusion in the ischemic limb.

HLI was achieved by ligation and excision of the distal external iliac artery and the femoral artery and veins in C57BL/6 mice. Five hours after ischemia induction, either cultured or freshly thawed UCX® was injected into the gastrocnemius muscle. Laser Doppler analysis was performed on days 0, 7, 14 and 21 post-ischemia to assess limb perfusion using the contralateral leg as control. Both experimental groups (cultured and freshly thawed UCX®) presented comparable levels of blood flow at days 7, 14 and 21 that were significantly higher compared with the control group (Figure 6), demonstrating that UCX® cells promoted perfusion recovery. Moreover, there was

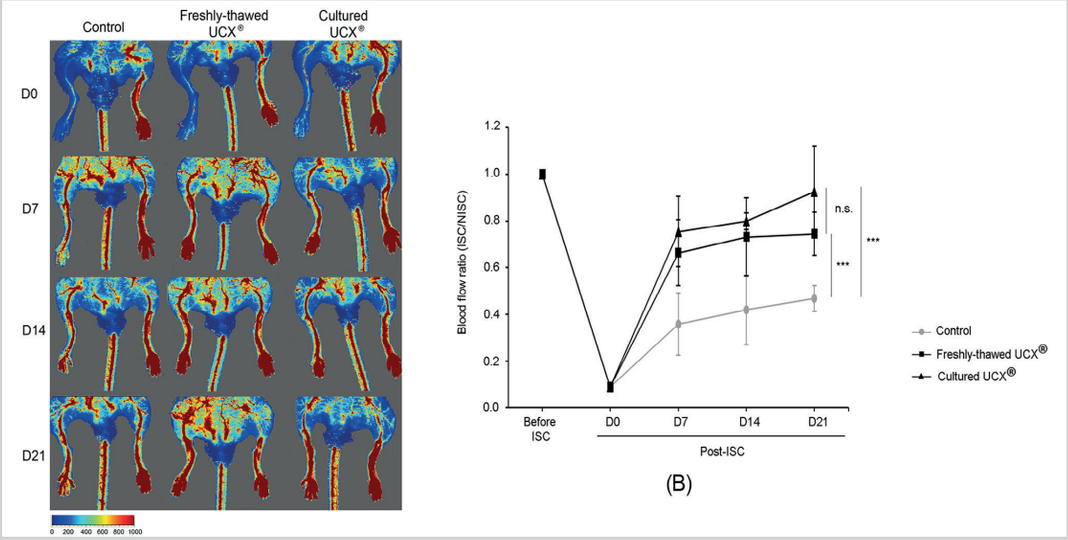

Figure 6 UCX® pro-angiogenic potency in a mouse model for hindlimb ischemia.

C57BL/6 mice after hindlimb ischemia induction were treated with vehicle (control) or with 2×10^5 of cultured or freshly thawed UCX® cells in the gastrocnemius muscle and allowed to recover. Twelve animals per group were used in independent experiments. (A) Representative laser Doppler flow images immediately after (D0) and at days 7, 14 and 21 post–hindlimb ischemia induction (POST-ISC; D7, D14 and D21). In color-coded images, normal perfusion is depicted in red; a marked reduction in blood flow in the ischemic hindlimb is depicted in blue. Recovery of blood flow in the ischemic hindlimb of mice treated with either cultured or freshly thawed UCX® is increased at D7, D14 and D21 POST-ISC compared with control mice. (B) Quantitative evaluation of blood flow expressed as a ratio of ischemic (ISC) to non-ischemic (NISC) limb was performed before ISC, immediately after ISC (D0) and at D7, D14 and D21 POST-ISC. A significant increase in ISC/NISC limb blood perfusion in mice treated with cultured or freshly thawed UCX® is observed compared with the control group at days 7, 14 and 21. (Values assumed normal distribution, and independent two-tailed *t*-test was used.) At day 7, equal variance was assumed between control versus freshly thawed UCX®, $*P = 0.019$, and control versus cultured UCX®, $**P = 0.008$. At day 14, equal variance was assumed between control versus freshly thawed UCX®, $*P = 0.031$, and unequal variance was assumed between control versus cultured UCX® $*P = 0.012$. At day 21, equal variance was assumed between control versus freshly thawed UCX®, $***P = 0.002$ and control versus cultured UCX® $***P = 0.004$). However, there was no significant difference between the two experimental groups at days 7, 14 and 21. Results are represented as mean ± SD.

no significant difference in perfusion recovery between treatments performed with cultured or freshly thawed UCX®, demonstrating that the angiogenic potency of these cells is not affected by cryopreservation and/or subsequent thawing *in vivo*.

4.4. DISCUSSION

Cryopreservation is a critical manufacturing step for the success of any off-the-shelf, cell-based ATMP. If cryopreservation and/or subsequent thawing alter the characteristics and potency of the ATMP, then the viability of the product and its therapeutic benefits could be compromised. Here, we have unequivocally demonstrated that after cryopreservation and thawing, a specific population of MSCs derived from UC-MSCs maintains its surface markers, shows high viable recoveries and is equally capable of immunomodulation and inducing angiogenesis both *in vitro* and *in vivo*. The disparities of these findings with some previous reports claiming that cryopreservation and thawing negatively affect the performance of MSCs may be explained by differences in tissue source and manipulation during the manufacturing process, including the cryopreservation and thawing procedures[15–17]. Previous studies used BM and AT-derived MSCs[7–9], whereas the present study reports to UC-MSCs. In particular, whereas BM-MSCs need priming to improve their immunomodulatory effects, UC-MSCs have demonstrated no need for priming[14,15]. In 2012, the first group published that cryopreserved MSCs had compromised immunosuppressive properties and suggested that this was due to a heat-shock response, leading to impaired interferon-γ licensing[7]. Our findings show that cryopreservation has no deleterious effect on the immunomodulatory potential of non-primed UC-MSCs, as seen *in vitro* by the proliferation suppression of PBMCs activated by anti-CD3, anti-CD28 and IL-2. Although the immune functionality testing *in vitro* of UCX® cells, as performed in this study, was only partial, and the impact on cell biochemical responsiveness to inflammatory cues such as interferon-γ, tumor necrosis factor-α, IL1 or toll-like receptor agonists were not evaluated, our results support the thesis that UC-MSC immunosuppression activity is independent of priming mechanisms[14,15] and that it is not affected by cryopreservation. These observations were fully supported *in vivo* via local administration of UCX® in an AIA model. Post-thaw functionality after systemic administration (as performed in previous studies by this group) was not evaluated in this study.

Some other reports suggest that cryopreservation is innocuous and that the controversy on MSCs activity found in previous reports may be due to differences in the manufacturing procedures, namely those involving mechanical manipulation,

expansion, freezing, thawing and reconstitution. One such study using xenogen-free, GMP-grade BM-MSCs, showed that cryopreservation did not alter the cells' immunomodulating potential *in vitro*[10].The process of thawing, for example, may lead to a series of reactive cell physiological effects, which temporarily impairs the pharmaceutical fitness of MSCs. This so called freezer burn effect, known to induce early apoptosis and promote early clearance and attenuation of MSCs engraftment, was nonetheless found to be reversible after 24 to 48 h in culture[7,8,17]. In this study, we obtained no evidence of increased apoptosis or changes in surface markers in the freshly thawed population compared with the cultured population, and although clearance and engraftment was not directly compared *in vivo*, the two conditions showed no differences in therapeutic activity/potency *in vivo*. This indicates that UCX® cells do not require a recovery period and may be infused immediately after thawing. Besides cell source, process-related factors that might have contributed to this outcome concern the drastic reduction of tissue mechanical manipulation of our specific isolation process compared with other methods found in the literature, namely those involving tissue mincing, grinding, excessive sub-sectioning or complex excisions with the aim of extracting the umbilical vessels and/or blood clots[1,18-26]. It is well known that excessive tissue manipulation induces cell differentiation, which is undesirable if maintaining the precursor cell phenotype is the goal[27-29]. Also, our process development involved a thorough optimization of the tissue preparation process and digestion parameters that resulted in a neglectful contamination of epithelial, endothelial and sub-endothelial cells, thus promoting the phenotypic homogeneity of the final population [1]. Overall, the results show that our specific UC-MSCs population maintains strong immunomodulatory capacity and angiogenic potential, regardless of cryopreservation. By demonstrating that UCX® do not require priming or a recovery step after thawing, this study validates UCX® as a viable base technology for the production of allogeneic, off-the-shelf, cryopreserved MSC-based ATMPs with great therapeutic potential.

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Disclosure of interests: PEC and HJC are share-holders and RNB, JMS, MF and MT were employed at ECBio S.A., while developing this work.The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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5

RESEARCH ARTICLE :
LOW-DOSE IONIZING RADIATION INDUCES THERAPEUTIC NEOVASCULARIZATION IN A PRE-CLINICAL MODEL OF HINDLIMB ISCHEMIA

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5. RESEARCH ARTICLE: LOW-DOSE IONIZING RADIATION INDUCES THERAPEUTIC NEOVASCULARIZATION IN A PRE-CLINICAL MODEL OF HINDLIMB ISCHEMIA

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ABSTRACT

Aims: We have previously shown that low-dose ionizing radiation (LDIR) induces angiogenesis but there is no evidence that it induces neovascularization in the setting of peripheral arterial disease. Here, we investigated the use of LDIR as an innovative and non-invasive strategy to stimulate therapeutic neovascularization using a model of experimentally induced hindlimb ischemia (HLI).

Methods and results: After surgical induction of unilateral HLI, both hindlimbs of female C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days and allowed to recovered. We demonstrate that LDIR, significantly improved blood perfusion in the murine ischemic limb by stimulating neovascularization, as assessed by laser Doppler flow, capillary density and collateral vessel formation. LDIR significantly increased the circulating levels of VEGF, PlGF and G-CSF, as well as the number of circulating endothelial progenitor cells (EPCs) mediating their incorporation to ischemic muscles. These effects were dependent upon LDIR exposition on the ischemic niche (thigh and shank regions). In irradiated ischemic muscles, these effects were independent of the recruitment of monocytes and macrophages. Importantly, LDIR induced a durable and simultaneous up-regulation of a repertoire of pro-angiogenic factors and their receptors in endothelial cells (ECs), as evident in ECs isolated from the irradiated gastrocnemius muscles by laser capture microdissection. This specific mechanism was mediated via vascular endothelial growth factor (VEGF) receptor signaling, since VEGF receptor inhibition abrogated the LDIR-mediated gene up-regulation and impeded the increase in capillary density. Finally, the vasculature in an irradiated non-ischemic bed was not affected and after 52-wk of LDIR exposure no differences in the incidence of morbidity and mortality were seen.

Conclusions: These findings disclose an innovative, non-invasive strategy to induce therapeutic neovascularization in a mouse model of hindlimb ischemia, emerging as a novel approach in the treatment of critical limb ischemia patients.

5.1. INTRODUCTION

Critical limb ischemia (CLI) is the end stage of peripheral arterial disease, and severe obstruction of blood flow to the affected extremity results in ischemic rest pain, ulcers or gangrene. Surgical revascularization remains the cornerstone of therapy for limb salvage but ~30% of CLI patients require amputation in the first year, procedure associated with high morbidity and mortality. Therapeutic angiogenesis became a promising treatment for limb preservation through the revascularization of ischemic tissues by local administration of pro-angiogenic growth factor[1-3]. Several clinical trials showed that therapeutic angiogenesis could be extended to CLI patients[4]. However, the initial enthusiasm was tempered by the less successful more recent, randomized and placebo-controlled studies with larger numbers of patients [4]. Several factors could contribute to this: (i) formation of a functional vascular network requires the concurrent use of multiple angiogenic factors, and not a monotherapy-based approach; (ii) instability of currently used factors to achieve long-term benefits; and (iii) dysfunction of endothelial cells (ECs) that may not respond[5,6]. To solve this, cell-based therapeutic strategies were developed. Although clinical trials showed that autologous bone marrow-derived mononuclear cells, including endothelial progenitor cells (EPCs), increased collateral vessel formation and had clinical benefits[4], there are still major challenges that include determination of optimal cell phenotype, preparation protocols, dosing, route and frequency of administration. Moreover, endothelial dysfunction is associated with a scarce viable and functional EPC population[7].

We previously showed that low-dose ionizing radiation (LDIR) (< 0.8 Gy) induces a pro-angiogenic phenotype in ECs *in vitro,* and promote angiogenesis *in vivo* during regeneration[8]. Herein we aimed at testing an innovative non-invasive strategy, using LDIR to induce therapeutic neovascularization in CLI. Using a model of experimentally induced hindlimb ischemia (HLI), we show that LDIR improves limb reperfusion by enhancing collateral formation through EPC recruitment to sites of arteriogenesis. The effects of LDIR depend on exposure of the ischemic niche, but not on the local recruitment of myeloid cells. Moreover, LDIR induces capillary density in the gastrocnemius muscle by simultaneous activation of a repertoire of pro-angiogenic factors in a mechanism dependent of the vascular endothelial growth factor (VEGF) receptor signaling. No effects on resting vasculature were observed, disclosing the possibility of using LDIR as a non-invasive and effective therapeutic tool in lower limb vascular insufficiency.

5.2. METHODS

Expanded method descriptions are available in Supplementary material.

5.3. STUDY APPROVAL

All animal procedures were performed according to Directive 2010/63/EU. The procedures were approved by the institutional Animal Welfare Body, licensed by DGAV, the Portuguese competent authority for animal protection (license number 023861/2013).

5.3.1. *In vitro* experiments

Lung human microvascular endothelial cells (HMEC-L) were purchased from Lonza and cultured according to manufacturer's instructions. Cells were used at passages 4-6. Affymetrix GeneChip HuGene 1.0 ST Arrays were used.

5.3.2. *In vivo* experiments

Twenty–two-week-old female C57BL/6 mice, purchased from Charles River Laboratories, Spain, were used in all experiments. Nine-week-old female C57Bl/6-Tg(CAG-EGFP)10sb/J mice were used as a donor in bone marrow transplantation model (Instituto Gulbenkian de Ciência). Unilateral HLI was induced by surgery. Ionizing radiation was delivered using a linear accelerator operating at a dose rate of 500 MU/min. In most experiments the dose of 0.3 Gy was administered for four consecutive days, starting 12 hours after ischemia induction. Blood flow was assessed by laser Doppler perfusion imaging. Capillary and collateral densities were assessed after immunohistochemistry and diaphonization, respectively. Capillaries were microdissected using a Zeiss PALM MicroBeam Laser Microdissection System. The immune cell infiltrate and EPCs were assessed by FACS. In plasma, cytokines were assessed by ELISA. RNA extraction, cDNA synthesis and qRT-PCR was performed using the primers described in the supplementary material online**.** After 52 weeks post-HLI, body weights were recorded, urine, blood and different organs collected and analysed.

5.3.3. Statistics

Experimental results are shown as the mean ± SEM. Data were analysed with SPSS 20.0 software for windows. Statistical test employed are detailed described in figure legends. For GeneChip data analysis, probe sets showing differential expression were determined using one-way Analysis of Variance (ANOVA).

5.4. RESULTS

5.4.1. LDIR increases perfusion recovery and capillary and collateral densities

We used a previously established mouse model of HLI[9] to assess the effect of LDIR in the restoration of blood flow to ischemic muscle. After surgical induction of unilateral HLI, both hindlimbs were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days (as illustrated in Figure 1A) and perfusion was measured overtime. As shown in Figure 1B and quantified in Figure 1C, a dramatic reduction in blood flow was observed in the ischemic limb immediately after surgery, in comparison to the contralateral limb, and as expected, a gradual improvement in perfusion was seen overtime. Strikingly, a significant improvement in blood flow recovery was seen in the LDIR group, at days 15 and 45 post-HLI, comparing with sham-irradiated mice. This demonstrates a benefit of LDIR in the setting of HLI.

Lower numbers of fractions (1 x 0.3 Gy; 2 x 0.3 Gy or 3 x 0.3 Gy) or lower dose per fraction (4 x 0.1 Gy) were also evaluated but failed to show an effect (Supplementary material, Figure S1A and B). We also evaluated the effect of the same fraction over 7 days (7 x 0.3 Gy), but no benefit was seen comparing within the 4-day (Supplementary material, Figure S1C).

Subsequently, we quantified capillary density and collateral vessel development in hindlimb muscles, since blood flow recovery depends on both angiogenesis and arteriogenesis. Consistently, HLI increases capillary density *per se*, assessed through quantification of CD31-positive capillaries on histological sections of gastrocnemius muscle. Importantly, this effect is further amplified after LDIR exposure and a significant increase in capillary density is observed in irradiated ischemic muscle versus the sham-irradiated ischemic ones at days 15 and 45 post-HLI (Figure 1D and 1E). Of note, while the capillary density did not significantly increase between days 15 and 45 in the sham-irradiated ischemic muscles, a significant increase is observed for the irradiated ones.

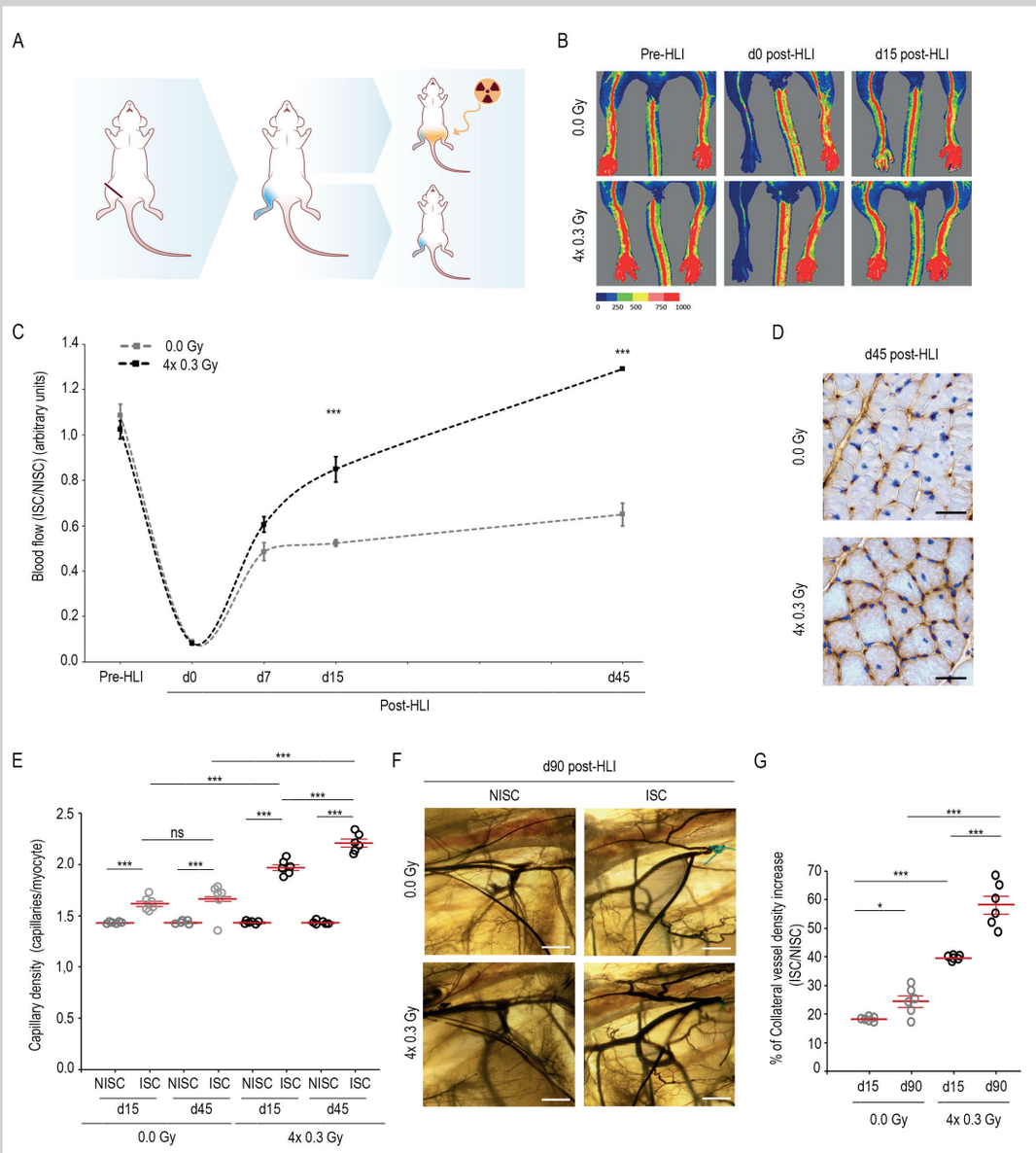

Figure 1. LDIR increases perfusion recovery, capillary and collateral densities

After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days and allowed to recover. (**A**) A schematic illustration of our experimental design. After unilateral HLI (represented by a thick brown line), the flow to the ischemic limb is dramatically decreased (in blue). Both hindlimbs are irradiated (in orange) or sham-irradiated. (**B**) Representative laser Doppler flow images pre-HLI, and at days 0 (d0) and 15 (d15) post-HLI induction. (**C**) Quantitative evaluation of blood flow expressed as a ratio of ISC to NISC limb demonstrated significantly enhanced limb blood perfusion in irradiated mice vs sham-irradiated ones both at days 15 (d15) and 45 (d45) post-HLI. Between-group changes were assessed by two-way repeated measurements ANOVA followed by Bonferroni post-hoc test (n=12 mice per group). Means ± SEM are shown. (**D**) Representative sections from sham-irradiated and irradiated ischemic gastrocnemius muscles at day 45 post-HLI. Capillaries and myocytes were identified by CD31 immunohistochemistry and haematoxylin, respectively. Scale bar, 150mm. (**E**) Quantitative analysis revealed increased capillary density (capillaries/myocyte) in irradiated ischemic gastrocnemius muscles compared to sham-irradiated ischemic ones at days 15 and 45 post-HLI. Mixed ANOVA followed by Bonferroni post-hoc test was conducted with a within-subject factor of ISC and between-subject factors of day and irradiation (n=6 mice per group). (**F**) Illustrative images of selected regions of interest (ROI) for sham-irradiated and irradiated mice. ISC and NISC limbs at day 90 post-HLI are shown. Scale bar, 300mm. (**G**) Data are represented as the percentage of collateral vessel density (CVD) increase of the ISC limb relatively to the NISC one. At days 15 and 90 post-HLI, irradiated mice presented significantly higher CVD increase (%) versus sham-irradiated mice. Two-way ANOVA was conducted followed by Bonferroni post-hoc test with a between-subject factors of day and irradiation (n=6 mice per group). (**E**, **G**) Individual data and means ± SEM (in red) are shown. **P* < 0.05; *** *P* < 0.001.

Abbreviations: *ns, non-significant. HLI, hindlimb ischemia; ISC, ischemic; NISC, non-ischemic; Pre-HLI, before hindlimb ischemia.*

The collateral vessel density (CVD) was evaluated, at days 15 and 90 post-HLI. Mice were diaphonized and an equivalent ROI, corresponding to 20% of the limb area, was selected for CVD quantification (Figure 1F). A greater increase in CVD was observed for the ischemic limbs of LDIR mice, versus the sham-irradiated ones (Figure 1G). Noteworthy, no difference in these parameters was seen in non-ischemic muscle, LDIR and sham-irradiated, showing that irradiation *per se* does not have an effect on resting vasculature.

5.4.2. LDIR modulates the expression of endothelial genes involved in an angiogenic response *in vitro*

Previously, using human lung microvascular ECs (HMVEC-L) we showed that 0.3 Gy leads to rapid phosphorylation of the VEGF receptor 2 (VEGFR2)[8], a key signal transduction mediator in the angiogenic process. Consequently, signaling pathways such as PI3K/AKT and ERK/MAPK are activated and gene expression modulated. To assess the effect of LDIR on the EC gene expression profile, HMVEC-L were exposed to a single dose of 0.3 Gy, RNA was extracted at 4 hours post-LDIR and a global gene expression analysis was performed; sham-irradiated HMVEC-L were used as control. Principal component analysis (PCA) revealed a separation of all HMVEC-L based on the irradiation status (Figure 2A). Two thousand three hundred and seventy-four genes were differentially expressed in LDIR vs control HMVEC-L, at a cutoff corresponding to a *P* value < 0.03 (Figure 2B).

Particular attention was paid to growth factors and receptors associated with angiogenesis, VEGF receptor 1 (VEGFR1) and VEGFR2, angiopoietin-2 (ANG2), transforming growth factor beta (TGF□), platelet derived growth factor (PDGF) and fibroblast growth factor2 (FGF2), and their expression was validated by quantitative RT-PCR. Hepatocyte growth factor (HGF) and its receptor, MET, were also validated, as the use of HGF has been proposed in the setting of therapeutic angiogenesis, and clinical trials with HGF gene therapy are ongoing[4]. HMVEC-L were irradiated with a single dose of 0.3 Gy and screened at 4, 8 and 12 hours post-LDIR. Gene expression increased at 4 hours post-LDIR, compared with sham-irradiated HMVEC-L, with exception of *Tgfb2* whose increase was significant at 8 hours post-LDIR. Expression levels of all genes returned to baseline at 12 hours post-LDIR (Figure 2C). We tried to modulate gene expression by increasing total dose (or number of fractions), using daily 0.3 *Gy* fractions during 2, 3 and 4 consecutive days; and cells were screened at 4, 8 and 12 hours after the last irradiation. Regardless of total dose, gene expression pattern and magnitude were similar to that observed after the single irradiation dose experiment (Figure 2D-F).

5.4.3. LDIR induces the expression of pro-angiogenic genes in ECs isolated from irradiated ischemic gastrocnemius muscles

LDIR modulates the expression of angiogenic genes in a resting endothelial *in vitro* monoculture, not exposed to injury. Next, we evaluated the gene expression levels in ECs isolated from gastrocnemius muscle of mice subjected to HLI and exposed to LDIR in daily fractions of 0.3 Gy for 4 days or sham-irradiated. Forty-five days post-LDIR, mice were killed and gastrocnemius muscle sections stained for CD31 and visualized using a laser capture microdissection microscope (LCM). CD31-positive cells from ischemic and non-ischemic gastrocnemius muscles were dissected and isolated. First, we validated that these CD31-positive cells consisted primarily of ECs and not myeloid cells nor perivascular cells. We assessed the gene expression of surface and transcription markers *Pecam1* encoding CD31, *Erg* and *Etv2* that are specific for ECs; *Itgam* encoding CD11b and *Spi1* encoding PU-1 for myeloid cells and *Des* encoding Desmin, *Pdgfrb* and *Acta2* encoding smooth muscle alpha-actin for perivascular cells. The CD31+ cells isolated by LCM expressed high levels of endothelial-specific transcripts but negligible amounts (more than 10000 times less) of myeloid or perivascular-specific transcripts (Supplementary material, Figure S2). Next, the same ECs were assessed by quantitative RT-PCR for the expression of *Vegfr2, Vegfr1, Fgf2, Angpt2, Pdgfc, Tgfb2, Hgf* and *Met*. Transcripts for all these genes were clearly up-regulated in ECs isolated from muscle of the ischemic limb, comparing with the contralateral limb, exclusively in mice exposed to LDIR (Figure 3A). Sham-irradiated mice show the opposite, down-regulating the expression of the angiogenic genes repertoire in endothelium from the ischemic limb, comparing with the contralateral limb. Then, we questioned whether the increase of the capillary density conferred by LDIR and observed in the ischemic gastrocnemius muscle could be correlated with the increase of the expression levels of these pro-angiogenic genes. To address this, we used the adductor muscle that does not present an increase of the capillary density at day 45 post-HLI neither in response to ischemia *per se*, nor after LDIR exposure (Supplementary material, Figure S3A). Thus, the adductor muscle provides a control subjected to HLI and irradiation without increase in the capillary density. We isolated the ECs from the adductor muscles and the levels of *Vegfr2, Vegfr1, Fgf2, Angpt2, Pdgfc, Tgfb2, Hgf* and *Met* mRNA were measured. We confirmed that the transcripts are down-regulated in the irradiated ischemic limb when compared with the contralateral one (Supplementary material, Figure S3B). We have previously demonstrated that VEGFR tyrosine kinase inhibition impairs the LDIR-induced pro-angiogenic response[8]. To assess the functional and clinical relevance of VEGF signaling induced by LDIR in the setting of

HLI, VEGFR tyrosine kinase inhibition was achieved through oral gavage of PTK/ZK (100 mg/kg), after HLI and 2 hours before each LDIR exposure. VEGFR inhibition abrogated the LDIR-mediated gene up-regulation of pro-angiogenic factors and receptors (Figure 3B). Moreover, the capillary density induced by LDIR, but not by HLI, was denied by treatment with PTK/ZK (Figure 3C). Conversely, the collateral density induced after LDIR exposure was not affected PTK/ZK (Figure 3D).
Thus, 0.3 Gy administered during four consecutive days might act through VEGFR-signaling for angiogenesis, but not arteriogenesis in the ischemic gastrocnemius limb.

5.4.4. LDIR does not mobilize myeloid cells to the ischemic tissue

Given that, in response to ischemia, the myeloid cells play an important role in the collateral formation[10] we questioned whether LDIR controlled the migration of myeloid cells in the ischemic tissue. Note that this process was already found for higher doses of ionizing radiation[11] (daily therapeutic doses ex: 2.0 Gy). Thus, after unilateral HLI, both hindlimbs were sham-irradiated or irradiated with four daily fractions of 0.3 Gy or 2.0 Gy as additional control. The myeloid infiltration was assessed day 4 post-HLI in ischemic and non-ischemic adductor muscles by flow cytometry (Figure 4A). As expected, the number of CD45+ cells increased significantly in response to HLI. Strikingly, exposure with 0.3 Gy or 2.0 Gy inhibited the CD45+ cell accumulation (Figure 4B). Then we went on to assess which myeloid cells were modulated in the ischemic muscles. Monocyte and macrophage numbers increased significantly after HLI in sham-irradiated ischemic muscles. A similar response was observed in ischemic muscles upon 2.0 Gy exposure. In stark contrast, low 0.3 Gy irradiation dose inhibited this monocyte and macrophage accumulation. This shows that different doses of ionizing radiation differently modulate myeloid cell infiltration in response to ischemia (Figure 4C and D). Moreover, while neutrophil numbers increased substantially in response to HLI, both 0.3 Gy and 2 Gy irradiation dosages impaired this accumulation (Figure 4E). We also assessed whether at later time point myeloid cells could account for the increase in collateral density in response to LDIR. Fifteen days post-HLI the numbers of CD45+ cells, monocytes, macrophages and neutrophils were similar in the ischemic muscles in 0.3 Gy; 2 Gy or sham-irradiated groups (Supplementary material, Figure S4).

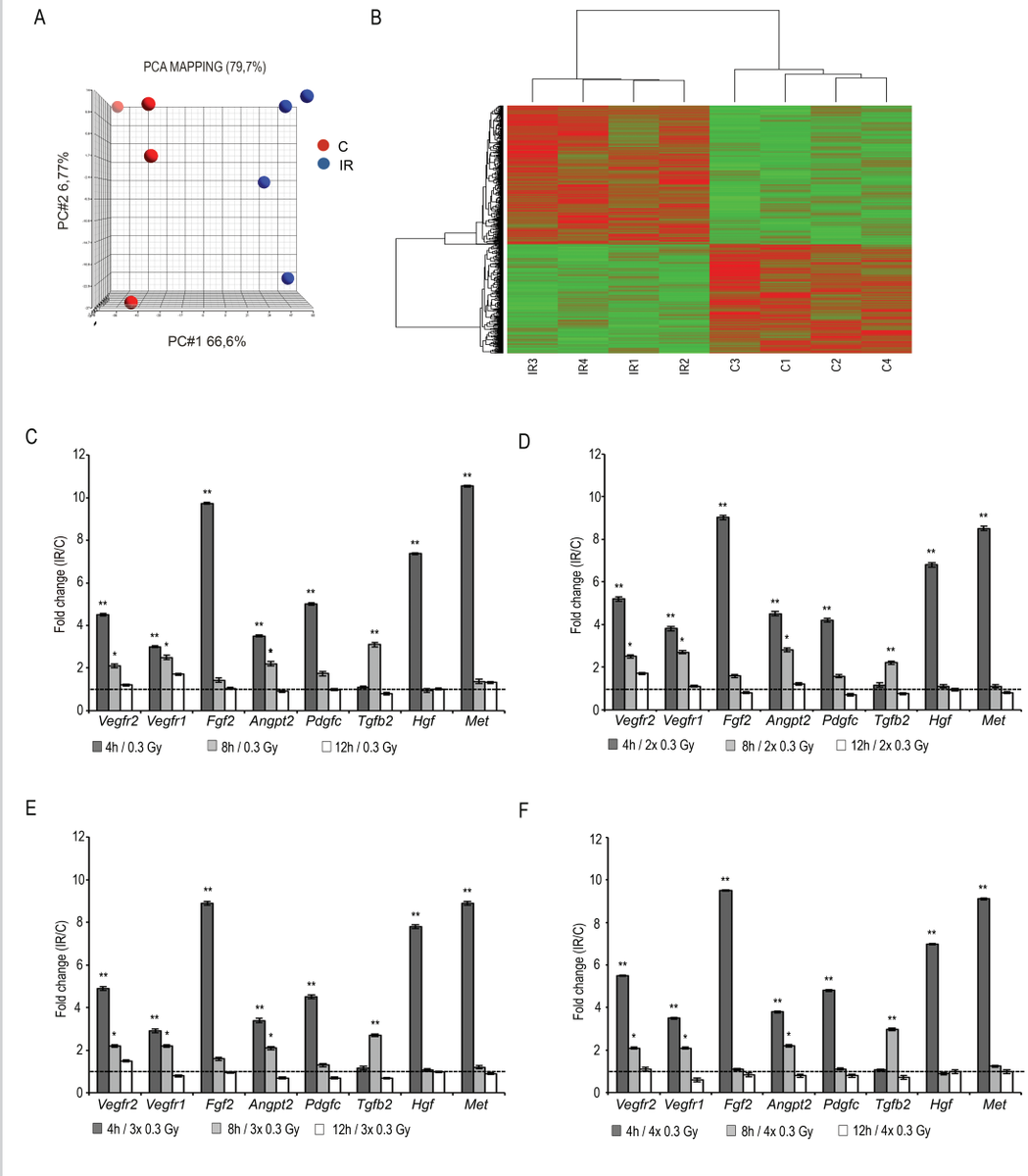

Figure 2. LDIR modulates the expression of endothelial genes involved in an angiogenic response

Four RNA samples of irradiated (0.3 Gy) or sham-irradiated HMVEC-L were processed for hybridization to Affymetrix Human Gene 1.0 ST arrays. (**A**) Three-dimensional PCA plot. The red and blue points represent control (sham-irradiated) and irradiated samples, respectively, indicating a separation of samples based on the ionizing radiation stimulus. (**B**) Heatmap for the 2374 genes differentially expressed in LDIR vs control (one-way analysis of variance (ANOVA test) $P < 0.03$)). Columns and rows and represent biological replicates and individual genes, respectively. Red and green indicate genes up- or down-regulated compared to control cells (sham-irradiated), respectively. (**C-F**) HMVEC-L sham-irradiated or irradiated with 0.3 Gy (**C**) once; (**D**) twice, (**E**) three or (**F**) four consecutive days. (**C-F**) Data (means ± SEM) represent the fold change in gene expression relative to the internal calibrator (sham-irradiated) in triplicate measurements and are representative of four independent experiments. Data demonstrated a significant increase in the relative expression of *Vegfr2, Vegfr1*, *Fgf2*, *Angpt2*, *Pdgfc*, *Hgf* and *Met*, at 4 hours, and of *Tgfb2* at 8 hours post-irradiation, when compared to sham-irradiated cells (dashed line). Values assumed normal distribution, equal variance and independent two-tailed t-test was used; $*P < 0.05$; $** P < 0.002$.

Abbreviations: *C, sham-irradiated; IR, irradiated.*

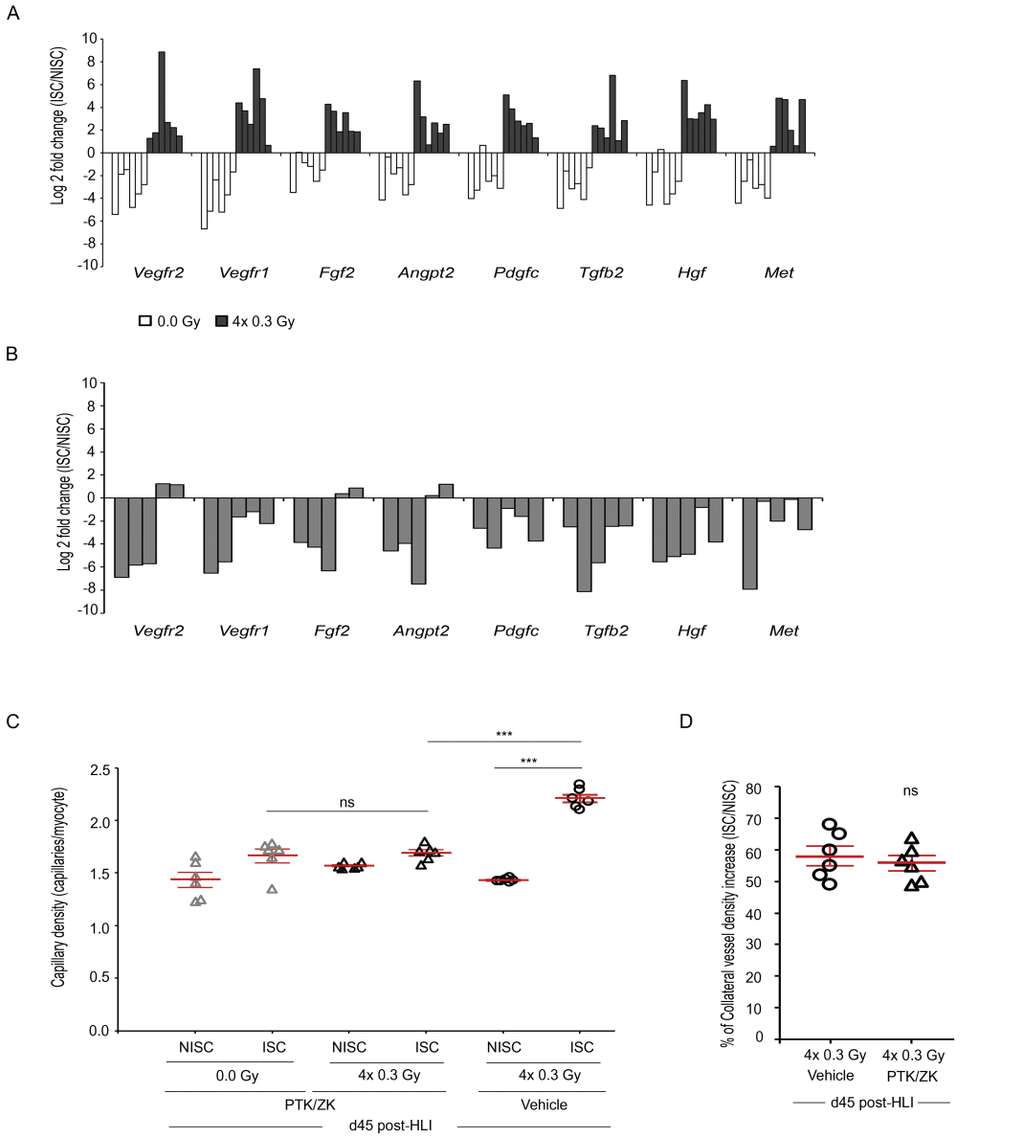

Figure 3. LDIR upregulates the expression of angiogenic genes in ECs isolated from irradiated ischemic gastrocnemius muscles

After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days and allowed to recover. (**A** and **B**) At day 45 post-HLI, the expression of pro-angiogenic factors and their receptors was evaluated by qRT-PCR exclusively on ECs. Gastrocnemius muscle sections were stained for CD31. Individual endothelial CD31+ cells were visualized, dissected and isolated using a laser capture microdissection microscope. (**A**) Each bar represents the relative gene expression in one animal. White and gray bars represent sham-irradiated and irradiated mice, respectively. Values were normalized to 18S to obtain relative expression levels. Results expressed as log2 fold changes between ISC and NISC samples demonstrated relative abundance of the transcripts in irradiated mice; in contrast, a down-regulation is observed in sham-irradiated mice. (**B-D**) Two hours before each irradiation, ischemic mice were pretreated with PTK/ZK (100mg/Kg). (**B**) Light grey bars represent irradiated mice pretreated with PTK/ZK. A down-regulation in relative gene expression is found in irradiated mice treated with PTK/ZK. (**C**) Quantitative analysis, at day 45 post-HLI, revealed no difference in capillary density (capillaries/myocyte) between ISC irradiated vs ISC sham-irradiated gastrocnemius muscles, both treated with PTK/ZK. As expected a significant increase is observed between ISC irradiated and PTK/ZK treated vs ISC irradiated and treated with the control vehicle. Mixed ANOVA followed by Bonferroni post-hoc test was conducted with a within-subject factor of ISC and between-subject factors of irradiation and PTK/ZK treatment. (**D**) Data are represented as the percentage of collateral vessel density (CVD) increase of the ISC limb relatively to the NISC one. At day 45 post-HLI, no difference was observed in CVD between irradiated mice treated with PTK/ZK vs irradiated mice treated with the control vehicle. Independent two-tailed t-test was used. (**C** and **D**) Individual data and means ± SEM (in red) are shown from n=6 mice per group; *** $P < 0.001$.

Abbreviations: *ns, non-significant. ISC, ischemic; NISC, non-ischemic.*

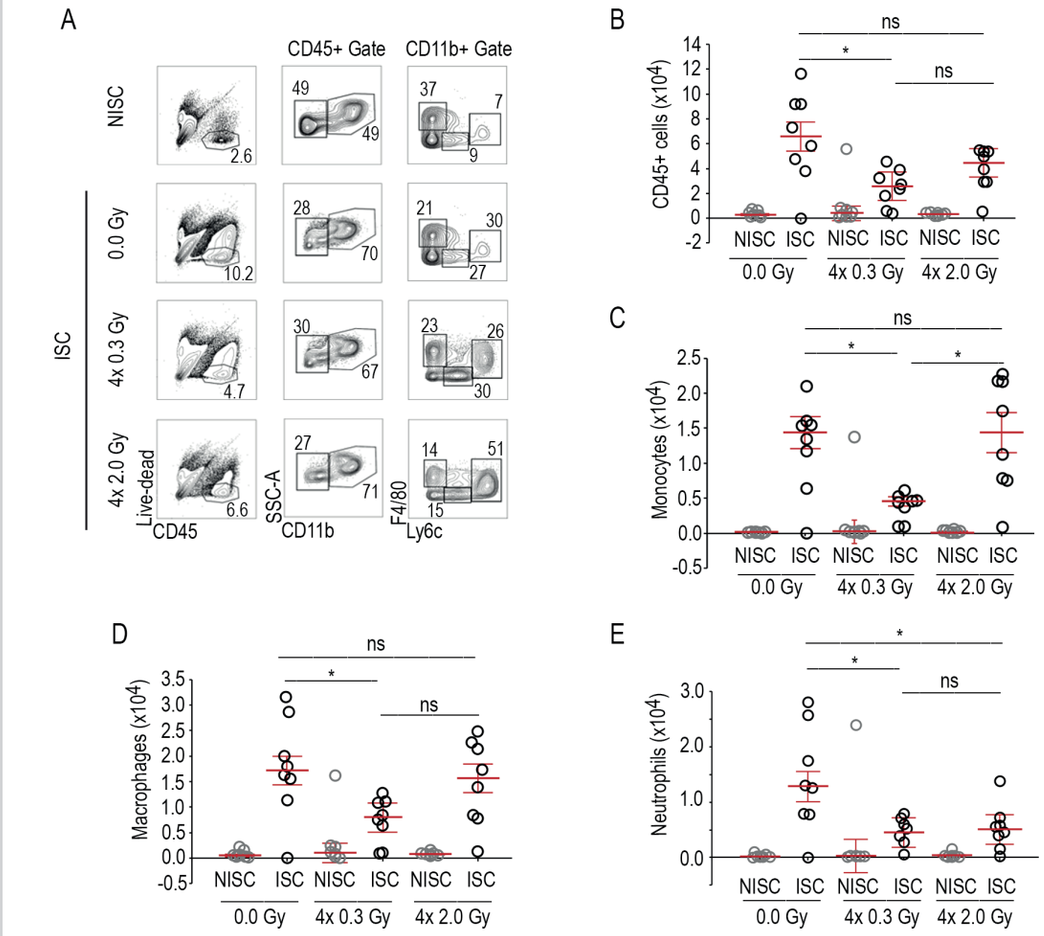

Figure 4. Profiles of leukocytes mobilized to ischemic muscles upon LDIR

After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy or 2.0 Gy in consecutive days. At day 4 post-HLI, analysis of CD45$^+$ immune cells that infiltrate ischemic adductor muscles was assessed. (**A**) Representative analysis of hematopoietic CD45+ cells present in ischemic muscle as assessed by flow cytometry. Analysis of the accumulation of myeloid CD11b+ cells, and in particular macrophages (CD45+CD11b+F4/80+ cells), monocytes (CD45+CD11b+LY6C+F4/80int cells), and neutrophils (CD45+CD11b+Ly6Cint). The graphs show numbers of (**B**) total CD45+ cells; (**C**) monocytes; (**D**) macrophages and (**E**) neutrophils, isolated from ISC and NISC adductor muscles and represent the data derived from two independent experiments. Mixed ANOVA followed by Bonferroni post-hoc test was conducted with a within-subject factor of ISC and between-subject factor of irradiation. Individual data and means ± SEM are shown (in red) from n=8 mice per group; *$P < 0.05$.

Abbreviations: *ns, non-significant. ISC, ischemic; NISC, non-ischemic.*

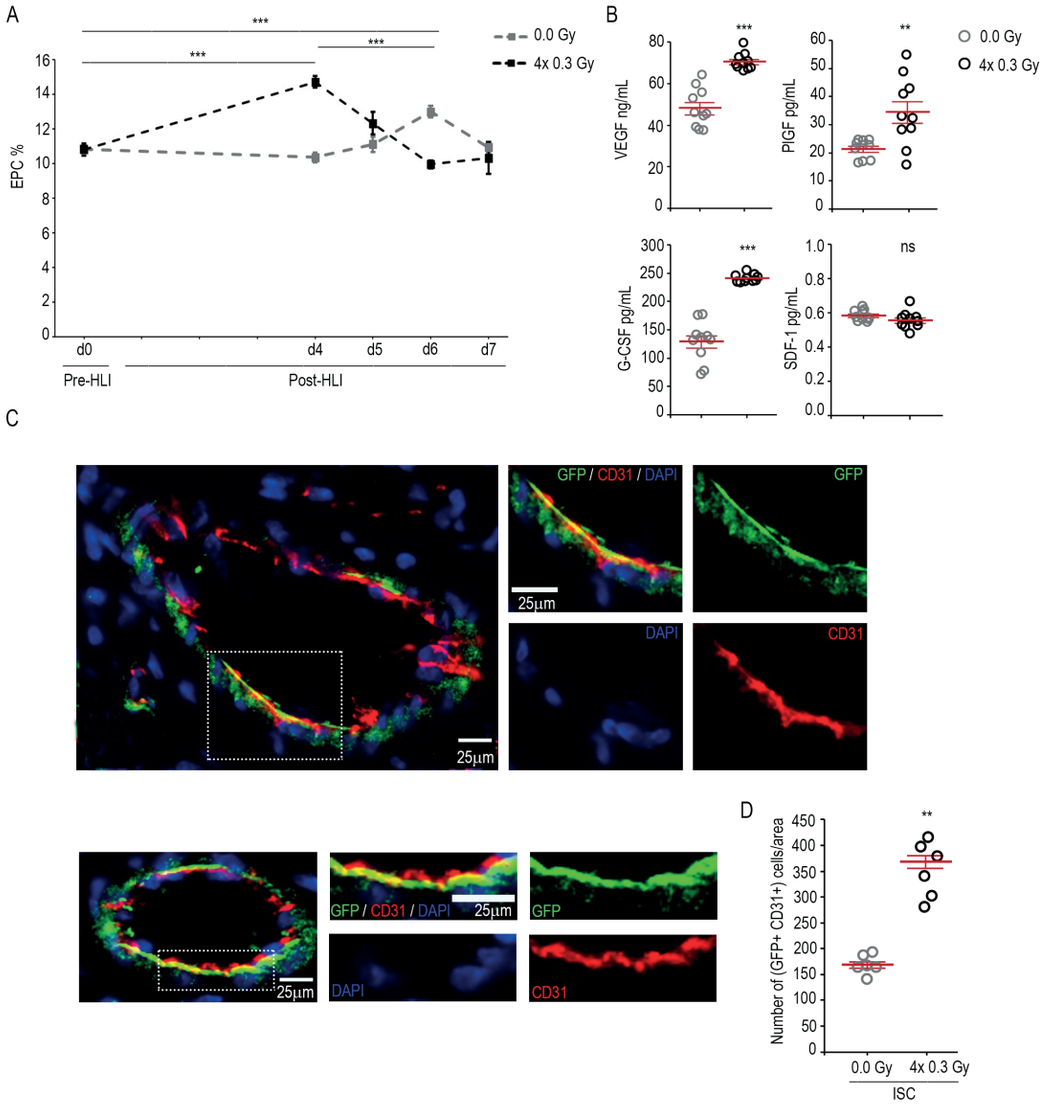

Figure 5. Post-HLI induction, LDIR increases the number of circulating EPCs, the levels of VEGF, PIFG and G-CSF and mediate EPCs recruitment in ischemia

After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days. (**A**) Quantitative analysis of EPCs in peripheral blood demonstrated a significant increase of the percentage of EPCs in irradiated mice, at day 4 post-HLI and in sham-irradiated mice, at day 6 post-HLI when compared to the percentage before HLI induction (d0 pre-HLI). Interestingly, the increase conferred by LDIR at day 4 in response to HLI is significantly higher to the one found at day 6 post-HLI in sham-irradiated mice. Two-way ANOVA was conducted followed by Bonferroni post-hoc test with a between-subject factors of day and irradiation; n=6 mice per group. Means ± SEM are shown. (**B**) The concentrations of VEGF, PIGF, G-CSF, and SDF-1□ were measured in the plasma, at day 4 post-HLI. LDIR significantly increases the VEGF, PIGF and G-CSF concentrations after HLI induction vs sham-irradiation (n=10 mice per group; values assumed normal distribution and equal or unequal (G-CSF) variance and independent two-tailed t-test was used). (**C** and **D**) Eight weeks after bone marrow transplantation, HLI was performed in C57BL/6 mice. Mice were sham-irradiated or irradiated with 0.3 Gy during four consecutive days and at day 15 post-HLI the adductor muscles were collected. (**C**) At left, two representative images of incorporated EPCs identified by double-fluorescent labeling (green/red) in collateral vessels. Transplanted GFP□-actin cells were identified by green fluorescence; vasculature by red fluorescence (CD31 staining) and nucleus by blue fluorescence (DAPI). Scale bar, 25 µm. At right, single and merged channels after digital zoomed of the boxed areas are shown. (**D**) Quantitative analysis revealed a significant increase of EPCs incorporation (identified as GFP+/CD31+ cells) into irradiated ischemic muscles compared to sham-irradiated ischemic ones (n = 6 mice per group; values assumed normal distribution, unequal variance and independent two-tailed t-test was used). (**B**, **D**) Individual data and means ± SEM (in red) are shown; ** $P < 0.01$; *** $P < 0.001$.

Abbreviations: *ns, non-significant. ISC, ischemic; Pre-HLI, before hindlimb ischemia.*

Table 1. Clinical, hematological, biochemical and histopathological data from sham-irradiated and LDIR mice

End Point	0.0 Gy	4 x 0.3 Gy
BW gain, % (n=140; mean ± SD)	14.2 ± 3.5	21.4 ± 5.3
Mortality (n=140)	2*/140	1†/140
Blood cell counts (n=24)	WNR[A]	WNR
Clinical chemistry (n=24)	WNR	WNR
Coagulation tests (n=24)	WNR	WNR
Urinalysis (n=24)	WNR	WNR
Bone Marrow cytology: abnormalities (n=6)	0/6	0/6
Histopathological Analysis (liver; kidney; lung; bone marrow; spleen; thymus) (n=6)	0/6	0/6

* found dead, from unknown cause.

† sacrificed due to abscess formation after surgical procedure (HLI).

[A] WNR, within normal range.

5.4.5. LDIR enhances collateral formation through EPC recruitment in a process that is dependent of the ischemic niche irradiation

We next assessed the effect of LDIR on EPC mobilization from the bone marrow into the circulation. Total blood was collected at days 4, 5, 6 and 7 post-HLI and EPCs were identified by flow cytometry as mononuclear cells being VEGFR2+/ Sca-1+/ CD117+ cells. Consistent with the literature[12], significant increase of the percentage of EPCs occurred at day 6 in sham-irradiated mice in response to HLI (Figure 5A). Surprisingly, the percentage of circulating EPCs significantly increased already at day 4 post-HLI in irradiated mice, and to a level noticeably higher than in sham-irradiated mice at day 6 (Figure 5A). This suggests LDIR synergized with ischemia to increase EPCs in peripheral blood, a process that is not inhibited after PTK/ZK treatment (Supplementary material, Figure S5A).

In addition, we confirmed that LDIR increases the circulating EPCs percentage in a process dependent of ischemia induction (Supplementary material, Figure S6). As EPCs mobilization involves a complex network of migratory factors[2,13,14], we assessed whether LDIR modulates cytokine or chemokine concentrations synergistically with ischemia that could generate gradients that guided EPCs to areas of ischemia or/and locally induce arteriogenesis. We previously showed that *in vitro* and under hypoxia-mimicking conditions LDIR enhances VEGF expression in ECs [8]. Using a similar approach, we found that in the presence of cobalt chloride (CoCl2), which mimics hypoxic conditions, ECs significantly increased *Pgf* mRNA (encoding

placental growth factor (PlGF)) expression, in a way that synergized with exposure to 0.3 Gy (Supplementary material, Figure S7). However, hypoxia and irradiation do not always synergize to regulate expression of migration factors. While *Cxcl12* mRNA (encoding stroma-derived factor-1α (SDF-1a)) expression was increased by hypoxia but not by LDIR, conversely, *Csf3* mRNA (encoding granulocyte-colony stimulating factor (G-CSF)) expression was induced by LDIR but not by hypoxia (Supplementary material, Figure S7). These findings were confirmed *in vivo* by ELISA. VEGF, PlGF and G-CSF concentrations in the plasma were significantly increased in the plasma of irradiated mice at day 4 post-HLI when compared with sham-irradiated ones (Figure 5B). In contrast and consistent with our *in vitro* data, the levels of SDF-1 are not modulated by LDIR at least at day 4 post-HLI. Moreover, the levels of VEGF, PlGF and G-CSF were not changed in irradiated mice treated with PTK/ZK after HLI induction (Supplementary material, Figure S5B).

To confirm that this process is dependent of the effect of LDIR on the ischemic/hypoxic niche, we irradiated mice outside the ischemic niche. Since technically, it is not possible to irradiate only the non-ischemic hindlimb assuring that the contralateral ischemic one was not exposed to LDIR, we irradiated the upper (above hip) part of the mouse body (Supplementary material, Figure S8A). Upper body LDIR exposition was not sufficient to increase the proportion of circulating EPCs (Supplementary material, Figure S8B). Consistently, the VEGF, PlGF, G-CSF and SDF-1a levels did not increase upon LDIR exposure (Supplementary material, Figure S8C). Importantly, the increase by LDIR of collateral density is not achieved (Supplementary material, Figure S8D). This strongly suggests that the exposure of the ischemic niche to LDIR is critical for the increase of cytokines, mobilization of EPCs and collateral formation.

Next, we aimed to show that LDIR-induced circulating EPCs are functionally relevant for enhancement of their recruitment and incorporation into ischemic tissues. A bone marrow transplantation using C57Bl/6-Tg(CAG-EGFP)10sb/J donor was performed in C57Bl/6 mice and 8 weeks after, HLI was induced. Mice were sham-irradiated or irradiated with 0.3 Gy during four consecutive days and at day 15 post-HLI the adductor muscles were collected. Our results show that at day 15 post-HLI EPCs are recruited in the large vessels as identified by double-fluorescent labeling (green and red), in response to HLI (Figure 5C). To confirm the wide-field data, confocal images of the same ten-micron-thick section were acquired to show that green and red fluorescent signals belong to the same cells (Supplementary material, Figure S9). Importantly, a quantitative evaluation of the histological sections revealed a significantly increased number of GFP+/CD31+ cells per area into irradiated ischemic thigh muscles when compared with the sham-irradiated ones (Figure 5D).

3.4.6. LDIR exposure is not associated with increased morbidity or mortality

To check for a possible effect of LDIR in the health status of the animals, a 52-week study was performed in a group of sham-irradiated and LDIR mice (n=140), throughout which mice were assessed for clinical signs of disease. There was no increased incidence of morbidity or mortality in the LDIR mice, compared with sham-irradiated, and there was no difference in body weight gain (at weeks 24, 36, 48 and 52 post-HLI). Fifty-two weeks post-HLI mice were killed and no significant difference was observed in organ weight, serum biochemistry (n=24), urinalysis or hematological parameters (Table 1); and the histological analysis revealed no neoplastic lesions or major changes in the liver, lung, spleen, thymus or bone marrow of these mice (n=6) (Supplementary material, Figure S10).

5.5 DISCUSSION

Therapeutic neovascularization aims to stimulate new blood vessel growth. Current strategies using proteins, genes or stem cells have demonstrated efficacy in animal models however, clinical translation remains challenging. We show that LDIR synergized with HLI and significantly enhance blood perfusion, capillary density in gastrocnemius muscle and collateral vessel development, tilting the angiogenic balance towards an even more pro-angiogenic phenotype, and suggesting that LDIR may favor the functional recovery of ischemic tissues. In contrast, resting vasculature, not subjected to ischemia, are unaffected by LDIR since capillary density and CVD are similar in non-ischemic muscles exposed or not to LDIR. This is in agreement with our previous work, where inter-ray capillary density remained unchanged after LDIR of non-amputated zebrafish caudal fin[8]. Our data also show that the maximal efficacy in perfusion recovery, capillary and collateral densities involves the administration of 1.2 Gy in four daily fractions of 0.3 Gy per fraction. A global gene expression analysis revealed that 2374 genes were modulated by LDIR and from those, 1344, many of which with a role in angiogenesis, were upregulated in LDIR versus control HMVEC-L. As soon as 4 hours after exposure to 0.3 Gy the expression of the majority of the pro-angiogenic molecules were increased, and returned to baseline12 hours post-LDIR. This acute short-term effect of LDIR on ECs is independent from dose fractionation since cells exposed to 0.3 Gy administered 2, 3 or 4 consecutive days presented similar gene expression pattern and magnitude. The evaluation of expression of angiogenic genes in ECs isolated

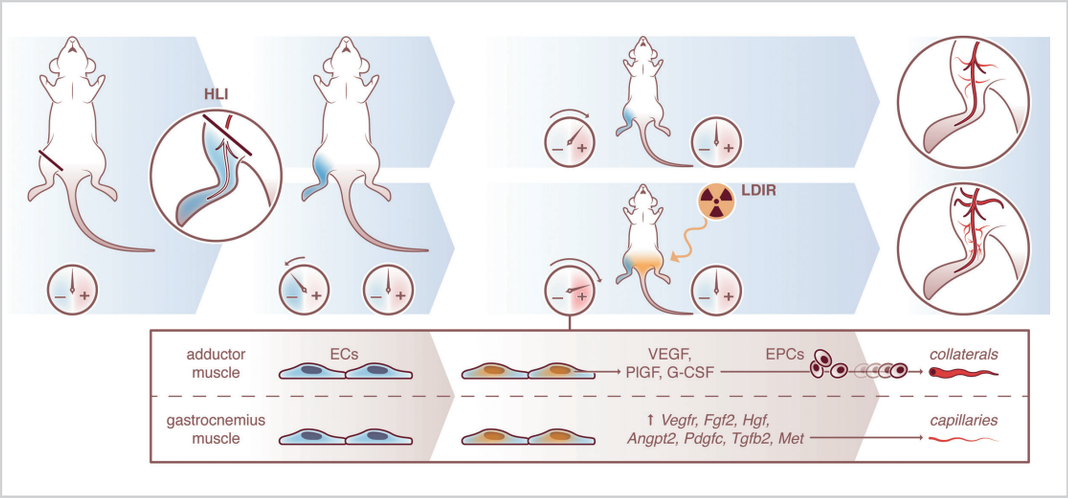

Figure 6. Proposed model of LDIR effect after HLI

After unilateral HLI (represented by a thick brown line), the flow to the ischemic limb is dramatically decreased (in blue) and vascular function is compromised as represented by a reduction in the dial. After LDIR exposure of both hindlimbs (in orange), we propose a synergistic effect occurs with the ischemic insult, tilting the dial towards an improvement of the vascular function by stimulating neovascularization. Then, ECs in an hypoxic microenvironment secrete VEGF, PlGF and G-CSF leading to the recruitment of EPCs and consequently to collateral density increase. Simultaneously, the up-regulation of several pro-angiogenic target genes namely *Vegfr (Vegfr-2 and -1)*, *Fgf2*, *Angpt2*, *Pdgfc, Tgfb2*, *Hgf* and *Met* in ECs induces capillary density. The vascular function of nonischemic hindlimbs was not affected by LDIR exposure as represented by the dial in an equilibrium status before, during and after ischemia recovery. (© Diogo Guerra. 2016).

Abbreviations: *ECs, endothelial cells; VEGF, vascular endothelial growth factor; PIGF, placental growth factor; G-CSF, granulocyte-colony stimulating factor; EPCS, endothelial progenitor cells; Vegfr, vascular endothelial growth factor receptor; Fgf, fibroblast growth factor; Hgf, hepatocyte growth factor; Pdgf, platelet-derived growth factor; Tgf, transforming growth factor; Met, receptor Hgf*

from gastrocnemius muscle of mice subjected to HLI revealed that LDIR modulates the expression of angiogenic genes in the endothelium and, thus, suggested a link for the long-term advantage in blood perfusion, capillary density and collaterals in HLI. LDIR induced a sustained and prolonged pro-angiogenic response in ECs, still evident 45 days after irradiation. Because this contrasts with the transient *in vitro* response, one may hypothesize either that endothelium itself could be differently modulated by LDIR in a hypoxic microenvironment created by ischemia; some cells (ex: adipocytes) could contribute to perpetuate the effect(s) of irradiation in ways that *in vitro* cultures cannot mimic.

There is evidence that ionizing radiation can affect a variety of inflammatory processes and the composition of responding immune cells[15]. However, this highly depends on the dose, for low doses (e.g. ≤ 1Gy) promote anti-inflammatory responses[16], while high doses (e.g. ≥ 2Gy) exert pro-inflammatory effects[17]. Therapeutic applicability was further demonstrated on inflammatory disease as symptomatic improvement of rheumatoid arthritis was observed when mice were irradiated with 0.5 Gy in five fractions within 1 week [18]. The hematopoietic infiltrate was monitored from inflamed and ischemic tissues to assess a potential role of immune cells upon LDIR. Ischemia

per se induced about 20-folds increase in the immune CD45+ cell infiltrate recruited to the injured muscle at day 4 post-HLI. Exposure with 4x 0.3 Gy significantly inhibited the CD45+ cell accumulation with particular effects on monocytes, macrophages and neutrophils. In contrast, with 4x 2.0 Gy the total CD45+ accumulation in ischemic muscle was still reduced, and although numbers of monocytes and macrophages were restored, neutrophils were not. This is consistent with the fact that high irradiation doses have opposing effects on certain myeloid subsets, for they activate macrophages[19] while they are reported to induce rapid, but transient, neutropenia[20]. Importantly, effect of irradiation was short-lasted. Fifteen days post-HLI the profiles of myeloid cells that infiltrated non-irradiated and irradiated ischemic muscles were similar. Altogether these data pointed for a mechanism of LDIR-induced arteriogenesis independent of local myeloid cell recruitment.

In the setting of HLI we showed that LDIR boosts the induction of a sustained VEGFR-mediated pro-angiogenic program in ECs from ischemic gastrocnemius muscle. These results corroborate our previous findings[8] and suggest a new mechanism: LDIR under HLI induces capillary density. Consistently, the capillary density induced by LDIR, but not by HLI, was abrogated by treatment with PTK/ZK. Conversely, the enhancement promoted in collateral density by LDIR was not affected by PTK/ZK, suggesting that this process is regulated by a mechanism independent of the VEGF receptor signaling.

Consistent with our previous results showing that in hypoxic mimicking conditions, LDIR increases the expression of VEGF in ECs[8], here we found that the expression of *Pgf* and *Csf3* is also increased in ECs. Importantly, these results were confirmed *in vivo* as VEGF, PlGF and G-CSF concentrations significantly increase in the plasma at day 4, upon LDIR exposure. Of note, beside VEGF, the up-regulation of other cytokines (such as PlGF and G-CSF), might explain why circulating EPCs are insensitive to PTK/ZK inhibition. It is plausible that the effects of VEGF, PlGF and G-CSF are redundant on EPCs, and may explain why PTK/ZK does not affect the enhancement promoted in collateral density by LDIR. These cytokines were reported as being involved in the guidance of EPC to ischemic tissue[2,13,14]. In line with this, we observed EPC mobilization and recruitment to the ischemic tissue upon LDIR. In the absence of ischemia, LDIR *per se* does not induce that effect and notably the irradiation of the ischemic tissue is critical for the mobilization of EPCs and collateral formation. Of note, in our transplantation model, all hematopoietic cells are GFP+ (including circulating and extravasated/tissue leucocytes, erythrocytes and platelets), but EPC, forming the inner lining of blood vessels, exhibit both green (GFP+) and red (CD31+). Thus, our results suggest that LDIR increases these growth factor concentrations synergistically with HLI and given the fact that this happens only if

the ischemic tissue is irradiated, we hypothesize that a hypoxic niche is critical for this process. Although we cannot exclude that other cells could modulate the levels of these cytokines upon LDIR, our results strongly suggest the involvement of ECs. We propose a model of enhanced and sustained angiogenesis induction by *in situ* LDIR administration as a promising therapeutic approach for ischemic diseases (*Figure 6*). LDIR applied as one daily irradiation of 0.3 Gy, administered for four consecutive days, synergistically act with the ischemic insult, exacerbating the local pro-angiogenic response. Our results suggest that this is achieved through (i) increased capillary density accompanied by an up-regulation of several pro-angiogenic target genes in ECs localized in gastrocnemius muscles, a process that is dependent of VEGF signaling and (ii) the mobilization and recruitment of EPCs by increasing the concentrations of VEGF, PlGF and G-CSF that may explain the collateral density increase in the ischemic limb leading to blood perfusion improvement.

One important concern when addressing ionizing radiation is its toxic effect. According to the linear no-threshold (LNT) hypothesis, the dose-response is linear and no threshold exists where damage begins to show. Recent advances in radiobiology challenge the validity of the LNT suggesting that it overestimates radiation risks[21]. We performed a 52-week and LDIR had no significant impact in the morbidity and mortality of the mice, although the possibility of LDIR long-term toxicity cannot be ruled out. Importantly, the LDIR proposed herein is usually absorbed by healthy tissues during radiotherapy, in areas where no adverse effects were found during the follow-up of the patient for several years.

The angiogenic potential of ionizing radiation has already been shown[22]. We and others have shown that LDIR favors angiogenesis by promoting EC proliferation and migration, accelerating wound healing[8,22]. However, there is no consensus about the doses described as pro-angiogenic, as different radiation schemes are used. Herein, ionizing radiation was delivered through a linear accelerator producing photon beams, currently used in the clinical practice. The use of conventional radiotherapy dose (2-10 Gy, administered once, Caesium-137 source) has been shown to induce neovascularization in HLI through VEGF release from mast cells and MMP9-mediated progenitor cell mobilization; however potential adverse effects were seen[11] and so, to the best of our knowledge, to date the use of those high doses has not been proposed for therapeutic neovascularization.

Our data supports the use of LDIR in enhancing ischemia-induced neovascularization *in vivo*, which is achieved through the increase of cytokines, mobilization and recruitment of EPC to the ischemic tissue and simultaneous activation of a repertoire of pro-angiogenic factors and resulting in enhanced recovery of blood flow. LDIR

may therefore have a clinical significant impact in the treatment of peripheral arterial disease that represents a growing health problem worldwide, with high economic burden and limited therapeutic options. We currently have an ongoing exploratory clinical trial to determine the clinical and molecular effects in “non-option” CLI patients. The success of this clinical trial will lead to the development of new trials to propose a novel and effective therapeutic tool with worldwide impact to peripheral arterial disease.

5.6 FUNDING

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Conflict of Interest

None declared.

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5.7. SUPPLEMENTARY MATERIAL AND METHODS

5.7.1. Cell Culture

Lung human microvascular endothelial cells (HMEC-L) were purchased from Lonza and cultured according to manufacturer's instructions. While different batches of primary cells were used over the course of the work, cells from the same batch were used in individual experiments (e.g. control vs. an experimental condition). Cells were used at passages 4 to 6.

5.7.2. Mice and Reagents

Twenty–two-week-old C57BL/6 female mice, purchased from Charles River Laboratories, Spain, were used in all HLI experiments; and nine-week-old C57Bl/6-Tg(CAG-EGFP)10sb/J female mice (Instituto Gulbenkian de Ciência) were used as a donor mice in for bone marrow transplantation. Briefly, for the HLI procedure and *in vivo* imaging, animals were anesthetized with ketamine-medetomidine (75mg/Kg BW and 1 mg/Kg BW, respectively) and the anaesthesia was reverted with atipamezole (5mg/kg BW). Postoperatively, analgesia was administered (buprenorphine 100µl/15-30g BW q8-12 hours) and the animals were closely monitored. Mice were euthanized by cervical dislocation. PTK787/ZK222584 (PTK/ZK) (100 mg/Kg) was kindly provided by Novartis Pharma AG, Basel, Switzerland. PTK/ZK, polyethylene glycol-300 was used as vehicle (Sigma) and administered by oral gavage. The animals were randomly assigned to each experimental group.

5.7.3. HLI Model

A surgical procedure was performed to induce unilateral HLI in the mice. Briefly, an incision in the skin overlying the thigh of the right hindlimb of each mouse was made and the distal external iliac artery and the femoral artery and veins were ligated and excised. The vein was ligated both to increase the severity of the ischemia as well as to increase the technical reproducibility of the model, as isolation of the femoral artery alone often results in tearing of the vein resulting in hemorrhage.

5.7.4. Irradiation

The radiotherapy plan was devised on a dedicated 3D treatment planning system (XiO, Elekta) using an isocentric dose distribution of two opposite fields (0°, 180°) at 6 MV photon energy, normalized to a reference point. Ionizing radiation was delivered, at room temperature, using a linear accelerator that produces X-rays photon beam (Synergy S, Elekta) operating at a dose rate of 500 MU/min.
Mice were transferred to an acrylic phantom in order to achieve the adequate thickness to improve homogeneity during the radiation therapy. A computed tomography scan (Somatom Sensation, Siemens) was performed and a volumetric acquisition was carried out; acquired images were reconstructed with axial slices width of 2 mm, and cross sectional data was transferred to the image processing system work station for contouring the planning target volume.
A 0.6 cm^3 PTW farmer ionizing chamber, connected to UNIDOS electrometer, was used to validate the IR doses calculated by the treatment planning system (TPS), according to the IAEA TRS-398 protocol. We obtained, in average, differences lower than 2% between the experimental and the XiO TPS dose values. The irradiation field included mice entire legs (pelvic girdle down to the feet) or the upper (above hip) part of the mouse body with a dose of 0.3 Gy administered for 4 consecutive days, starting 12 hours after ischemia induction. Control mice were sham-irradiated (0.0 Gy) following the same procedure for the irradiated experimental groups. The irradiation procedure was performed in a non-blinded manner. During this protocol the mice were anesthetized.

5.7.5. Laser Doppler perfusion imaging

The laser Doppler perfusion imager (MoorLDI-V6.0, Moor Instruments Ltd, Axminster, UK) was used to assess limb perfusion. Hair was removed one day before laser Doppler analysis using an electrical shaver followed by depilatory cream. Blood flow was measured both in the ischemic and non-ischemic legs, before HLI induction (Pre-HLI), immediately post-HLI (Post-HLI), and at days 7, 15 and 45 post-HLI (d7 Post-HLI, d15 Post-HLI and d45 Post-HLI, respectively). According to the analysis performed immediately post-HLI, mice are randomized assuring an equal reduction of blood flow in the different experimental groups. Color-coded images of tissue perfusion were recorded and poor or no perfusion was displayed as darkblue, and the highest perfusion level was displayed as red. Mean flux values were calculated using the Moor LDI V5.3 image processing software. To account for

variables such as temperature and ambient light, blood perfusion is expressed as the ratio of ischemic to non-ischemic limb. The mice were placed on a 37°C heating pad to reduce heat loss during measurements.

5.7.6. Immunohistochemistry and Capillary density analysis

Mice were sacrificed at days 15 and 45 post-HLI. The gastrocnemius or adductor muscles of both legs were harvested, placed in transverse orientation on a small cork disc with the help of 10% tragacanth, snap frozen in liquid nitrogen cooled isopentane and stored at -80ºC until sectioned. Seven-micrometer sections were labeled with CD31 monoclonal antibody (Pharmingen). After fixation in acetone, for 10 minutes, hydrogen peroxidase (0.3% diluted in methanol) was added, for 30 minutes, at room temperature, followed by 2 washes in PBS, for 10 minutes. Blocking solution (5% rabbit serum in PBS) was applied, for 30 minutes, at room temperature, and then slides were incubated, for 1 hour, at room temperature, with rat monoclonal antibody against mouse CD31 at 1:500, diluted in PBS-1% BSA. After 3 washes in PBS, for 30 minutes, a secondary biotinylated rabbit anti-rat IgG antibody was added at 1:200 in PBS-1% BSA plus 5% rabbit serum, for 30 minutes, at room temperature. Washes were performed as before and labeled avidin-conjugated peroxidase complex (Vectastain ABC kit; Vector Laboratories) was used for color development according to the manufacturer's recommendations, for 30 minutes, at room temperature. After rinsing in PBS (3 times, for 5 minutes), DAB peroxidase substrate kit (Vector Laboratories) was added, for 5 minutes to localize the immune complexes. The sections were counterstained with Hematoxylin (Bio-Optica), for 10 seconds, and mounted with Entelan (Merck). Omission of the primary antibody was run in parallel as a negative control.
Analysis of tissue samples was conducted using Leica DM2500 upright brightfield microscope (Leica Microsystems). Capillary densities, ie number of capillaries per number of muscle fibers, were measured in 2 different sections of 4 distinct anatomic areas of each specimen using the ImageJ® software.

5.7.7. Contrast Agent Perfusion and Diaphonization

Fifteen, forty-five and ninety days post-HLI induction, when mice were well stabilized from the ischemic injury, they were deeply anesthetized and the torso and limbs were shaved. A medial thoracotomy was performed to expose the heart and a needle

(26 Gauge), attached to an automatic injector, was introduced in the left ventricle. An incision was performed in the right atrium to allow venous drainage. Mice were primarily perfused with heparinized serum (3000 IU/L) until the blood was completely removed from circulation. A vasodilatation mixture of adenosine (3.7 mmol/L) and papaverine (11.8 mmol/L) was subsequently administered, right before the contrast agent, for 2 minutes. The contrast used was a mixture of barium sulfate (50%) and gelatin (5%). This solution was kept warm until the injection time to avoid thickening. Contrast agent was perfused manually until the feet blanched. Right after the injection the mice were transferred to a cold chamber, so that the contrast agent became solidified. All the solutions were injected with a perfusion rate of 0.01 cm^3/s. Then, the skin of each mouse was removed from the lower body and diaphonization was performed by using a modified version of Spalteholz technique. Briefly, the mice were fixated, decalcified, whitened, washed, dehydrated by freeze substitution and placed into a vacuum pump with Spalteholz solution (benzyl benzoate and methyl salicylate) until transparency was acquired. Diaphonization allows the visualization of effective lumen diameter, vessel angulation and emergence position.

5.7.8. Collateral vessels quantification

Mice were kept in Spalteholz solution during image acquisition, in order to achieve a homogenous density between the tissues and the media, with minimal absorption or reflection of light. Mice entire limbs were photographed in a magnifier with a light source. After acquisition, images were aligned and stitched together using Adobe Photoshop CS6® and entire limb photographs were obtained. Collateral vessels were manually segmented by highlighting them using Adobe Photoshop CS6®. We considered collateral vessels according to Longland's definition, a defined stem, mid-zone and re-entrant. According to this definition, collateral vessels with a diameter between 20 and 300 mm were included in our quantification excluding femoral, saphenous and popliteal arteries and all venous structures. Since arteriogenesis occurs primarily around the occluded blood vessel segment, the region of interest (ROI) in every mouse was in the same anatomic region in the adductor, surrounding and bellow the surgical occlusion. Collateral vessel density (CVD) was quantified in equivalent ROIs corresponding to 20% of the total limb area. The CVD was calculated as the ratio between the vascular area and the ROI areas. All density measurements were performed using ImageJ® software. To exclude variations in the anatomy, perfusion or diaphonization procedures, the CVD value of the non-ischemic limb for each mouse was assumed to correspond to 100%. According to this assumption, the CVD percentage in the ischemic limb was calculated relatively

to the non-ischemic one. The percentage of CVD increase was determined as the difference between the CVD percentage among the ischemic and non-ischemic limbs.

5.7.9. Laser capture microdissection of capillaries

Mice were sacrificed at day 45 post-HLI. Twelve-micrometer sections of the gastrocnemius or adductor muscles were labeled with CD31 monoclonal antibody (Pharmingen). The sections were stored at –80°C until microdissection. The immunohistochemistry protocol described above was modified to improve RNA preservation [1] by using high salt buffer, 2 mol/L NaCl in PBS (at 4ºC) in all incubation and washing steps. After immunohistochemistry, sections were dehydrated in ice cold 90% ethanol followed by 100% ethanol and allowed to dry. Ten thousand capillaries were microdissected using a Zeiss PALM MicroBeam Laser Microdissection System (Carl Zeiss Microscopy, Germany) equipped with a pulsed solid-state 355 nm laser. Dissected capillaries were catapulted into a microfuge tube adhesive-cap.

5.7.10. Isolation of immune cell infiltrate from muscle and FACS analysis

Adductor muscles were excised after perfusing the mice with 30ml PBS. Muscles were cut in small pieces and digested for 30 minutes with collagenase I (1.5 mg/ml, Worthington) and DNase I (10 mg/ml, Sigma) diluted in DMEM. The digested product was filtered through a 70 mm mesh cell-strainers (Becton Dickinson) using a plunger to disrupt undigested tissue and washed with RPMI supplemented with serum. To isolate the leukocyte fraction, the cells were resuspended in 40% Percoll, overlayed on 80% Percoll and spun for 25 minutes at 2400 rpm without brake. The interphase containing leukocytes was recovered, washed and erythrocytes were osmotically lysed in red blood cell lysis buffer (BioLegend). For cell surface staining, single-cell suspensions were incubated, in presence 5% normal mouse serum, for 30 minutes with saturating concentrations of combination of the following monoclonal antibodies that were purchased from BD Biosciences, eBiosciences or Biolegend: FITC anti-CD3 (145-2C11), PE anti-CD69 (H1-2F3), PerCP-Cy5.5 anti-Ly6G (1A8), PE-Cy7 anti-F4/80 (BM8), APC anti-CD11b (M1/70), APC-Cy7 anti-CD19 (1D3), brilliant violet 510 anti-CD45 (30-F11), brilliant violet 605 anti-Ly6C (HK1.4), brilliant violet 711 anti-4 (N418). Zombie violet dye (BioLegend) was then added to the final suspension for 10 minutes, at 4ºC in order to stain and exclude dead cells. Cells were then immediately analysed by flow cytometry on LSR Fortessa

(BD Biosciences). All graphical output was performed using FlowJo 10 (Tree Star, Costa Mesa, CA).

5.7.11. Endothelial progenitor cells and FACS analysis

Terminal peripheral blood collection was performed transcardially, to an EDTA-coated tube, and erythrocytes were osmotically lysed in red blood cell lysis buffer (BioLegend). Then, cells were incubated, in presence of PBS - 5% BSA, for 30 minutes with saturating concentrations of combination of the following monoclonal antibodies purchased from Invitrogen, eBioscience or BD Pharmingen: TOPRO 3, biotin anti-Ter119, PE-Cy7 anti-CD117, FITC anti-Sca-1, PE anti-VERGFR2. For Ter119, a secondary eFluor 450 streptavidin was used. Cells were then immediately washed with PBS and analysed by flow cytometry on LSR Fortessa (BD Bioscience). All graphical output was performed using FlowJo 10 (Tree Star, Costa Mesa, CA).
Endothelial progenitor cells (EPCs) were quantified within the monocytic cell population, by a dual expression of VEGFR2 and Sca-1 in the CD117 gate.

5.7.12. Circulating Cytokine Quantification

Peripheral blood was obtained by cardiac puncture at day 4 post-HLI induction. Blood was centrifuged 30 minutes and plasma separated from the cellular fraction and stored at -20ºC. In plasma, vascular endothelial growth factor (VEGF), granulocyte-colony stimulating factor (G-CSF), placenta growth factor (PlGF) and stroma-derived factor-1α (SDF1α) were measured via ELISA (R&D Systems) according to manufacturer's instructions.

5.7.13. Bone Marrow Transplantation Model and Immunofluorescence

Bone marrow cells were obtained by flushing the tibia and femur of eight-week-old C57BL/6 female mice C57Bl/6-Tg(CAG-EGFP)10sb/J (donor mice). Mononuclear cells were isolated by density centrifugation and filtered in a 70 μm cell strainer (BD). The transplantation was done in ten-week-old C57BL/6 female mice (recipient mice), lethally irradiated with 900 rads (Gammacell® 3000Elan). After irradiation, the recipient mice received unfractionated bone marrow cells ($5x10^6$) from the donor mice by tail vein injection. Eight weeks after, HLI was induced and mice

were sacrificed at day 15 post-HLI. The adductor muscles of both hindlimbs were harvested, placed in transverse orientation on a small cork disc with the help of 10% tragacanth, snap frozen in liquid nitrogen cooled isopentane and stored at -80ºC until sectioned. Ten-micrometer sections were labeled with CD31 monoclonal antibody (Pharmingen) and purified rabbit anti-GFP (Torrey Pines BioLabs Inc). After fixation in acetone, for 10 minutes, 0.5%Triton x-100 was added, for 10 minutes, at room temperature followed by 2 washes in PBS, for 10 minutes. Blocking solution (10% goat (DakoCytomation) plus 10% donkey (Sigma-Aldrich) serum in PBS-1% BSA) was applied, for 30 minutes, at room temperature, and the slides were then incubated for 1 hour, at room temperature, with a rat monoclonal antibody against mouse CD31 and rabbit anti-GFP in PBS-1% BSA. After 3 washes in PBS-0.1%Tween, for 15 minutes, secondary antibodies goat anti-rat Alexa fluor 594 (Invitrogen) and goat anti-donkey Alexa fluor 488 (Invitrogen) were added in PBS-1% BSA, for 1hour, at room temperature. Washes were performed as before. Sections were incubated with Dapi (Sigma-Aldrich), for 10 minutes, washed in PBS, for 5 minutes, mounted with vectashield mounting medium (Vector Laboratories) and seal with nail polish. Omission of the primary antibodies was run in parallel as a negative control. Analysis of tissue samples was conducted using a Motorized Widefield Fluorescence Microscope (Zeiss Axiovert 200M) with a 20x/0.8 objective or confocal microscope (Zeiss LSM 880) with a 63x/1.4 oil immersion objective, with the pinhole set to 1 Airy unit (optical slice thickness = 0.7 micron). The number of EPCs, defined as GFP+ CD31+, was measured on an area of 4.30 mm^2 into 5 different sections of the adductor muscle for each specimen. All measurements were performed using ImageJ® software.

5.7.14. RNA extraction, cDNA synthesis and pre-amplification

Total RNA from the microdissected capillaries or HMEC-L was isolated using an RNeasy Micro or Mini Kit (QIAGEN) including DNase treatment, respectively. For synthesis and preamplification of cDNA RT^2 PreAMP cDNA Synthesis kit (QIAGEN) was used with two rounds of pre-amplification using the following murine primers:

*Vegfr1*_F	(5'-TTGAGGAGCTTTCACCGAACTCCA-3')
*Vegfr1*_R	(5'-TATCTTCATGGAGGCCTTGGGCTT-3')
V*egfr2*_F	(5'-AGGCCCATTGAGTCCAACTACACA-3')
V*egfr2*_R	(5'-AGACCATGTGGCTCTGTTTCTCCA-3')
*Fgf2*_F	(5'-ACTCCAGTTGGTATGTGGCACTGA-3')
*Fgf2*_R	(5'-AACAGTATGGCCTTCTGTCCAGGT-3')

*Tgfb2*_F (5’-GCTTTGGATGCGGCCTATTGCTTT-3’)
*Tgfb2*_R (5’-CTCCAGCACAGAAGTTGGCATTGT-3’)
*Ang2*_F (5’-ATCCAACACCGAGAAGATGGCAGT-3’)
*Ang2*_R (5’-AACTCATTGCCCAGCCAGTACTCT-3’)
*Pdgf-c*_F (5’-ATGCCACAAGTCACAGAAACCACG-3’)
*Pdgf-c*_R (5’-AAGGCAGTCACAGCATTGTTGAGC-3’)
*Hgf*_F (5’-GCATTCAAGGCCAAGGAGAAGGTT-3’)
*Hgf*_R (5’-TCATGCTTGTGAGGGTACTGCGAA-3’)
*C-met*_F (5’-ACGTTGAAATGCACAGTTGGTCCC-3’)
*C-met*_R (5’-TTGCGTCGTCTCTCGACTGTTTGA-3’)
*Pecam*_F (5’-CCCATCACTTACCACCTTTATG-3’)
*Pecam*_R (5’-TGTCTCGGTGGGCTTAT-3’)
*Etv2*_F (5’-CACCGATCACACCAATGAA-3’)
*Etv2*_R (5’-GTACGTCTTCGTGAGGTAAAG-3’)
*Erg1*_F (5’-CCAAACTGGAGGAGATGATG-3’)
*Erg 1*_R (5’-GTGCTGCTGCTGCTATTA-3’)
*Spi1*_F (5’- GCGCTGGCACCTTTTTGTAT -3’)
*Spi1*_R (5’- CAATAATTTTACTTGTCTTTAGTGGTTA -3’)
*Itgam*_F (5’-TCTACTACCCATCTGGCTTATC-3’)
*Itgam*_R (5’-TGGACTCAGCAGGCTTTA-3’)
*Des*_F (5’-GCCACCTACCGGAAGCTACT-3’)
*Des*_R (5’-GCAGAGAAGGTCTGGATAGGAA-3’)
*Pdgfr*_F (5’-GTGGTGAACTTCCAATGGACG-3’)
*Pdgfr*_R (5’-GTCTGTCACTGGCTCCACCAG-3’)
*Acta2*_F (5’-CCAGCACCATGAAGATCAAG-3’)
*Acta2*_R (5’-TGGAAGGTAGACAGCGAAGC -3’)
*18s*_F (5’-GCCCTATCAACTTTCGATGGTAGT-3’)
*18s*_R (5’-CCGGAATCGAACCCTGATT-3’)

Real-time PCR was performed according to the manufacturer’s protocol using Power SYBR® Green (Applied Biosystems) and an Applied Biosystems 7500 Fast Real-Time PCR for the same targets described above. Human primer sequences are as follows:

*Vegfr1*_F (5’- CCCTCGCCGGAAGTTGTAT -3’)
*Vegfr1*_R (5’- GTCAAATAGCGAGCAGATTTCTCA -3’)
V*egfr2*_F (5’- ATTCCTCCCCCGCATCA -3’)
V*egfr2*_R (5’- GCTCGTTGGCGCACTCTT -3’)
*Fgf2*_F (5’- GCAGTGGCTCATGCCTATATT -3’)
*Fgf2*_R (5’- GGTTTCACCAGGTTGGTCTT -3’)

*Tgfb2*_F	(5'- GCTTTGGATGCGGCCTATTGCTTT -3')
*Tgfb2*_R	(5'- CTCCAGCACAGAAGTTGGCATTGT -3')
*Ang2*_F	(5'- AGGACACACCACGAATGGCATCTA -3')
*Ang2*_R	(5'- TGAATAATTGTCCACCCGCCTCCT -3')
*Pdgf-c*_F	(5'- AGGTCTTCAATCGTGGAAAGAA -3')
*Pdgf-c*_R	(5'- CAGAACCCAGCTAGTGGAATAC -3')
*Hgf*_F	(5'- GGTAAAGGACGCAGCTACAA -3')
*Hgf*_R	(5'- AGCTGTGTTCGTGTGGTATC -3')
*C-met*_F	(5'-CTGGTTCCTGGGCACCGAAAGATAAA-3')
*C-met*_R	(5'-CCATTGCTCCTCTGCACCAAGGTAAA-3')
*Pgf*_F	(5'-CATGCAGCTCCTAAAGATCC-3')
*Pgf*_R	(5'-CTTTCCGGCTTCATCTTCTC-3')
*Cxcl12*_F	(5'-TCTCAACACTCCAAACTGTG-3')
*Cxcl12*_R	(5'-TCTCCAGGTACTCCTGAATC-3')
*Csf3*_ F	(5'-GATGGAAGAACTGGGAATGG-3')
*Csf3*_R	(5'-AAGCTCTGCAGATGGGA-3')
*18s*_F	(5'-GCCCTATCAACTTTCGATGGTAGT-3')
*18s*_R	(5'-CCGGAATCGAACCCTGATT-3')

The housekeeping gene used to normalize was 18S. The RT-PCR program consisted of an initial denaturation step, at 95ºC, for 10 minutes followed by 50 cycles, at 95ºC, for 15 seconds and, at 60ºC, for 1 minute. The relative quantification was performed according to the comparative method ($2^{-\Delta\Delta Ct}$; Applied Biosystems User Bulletin no. 2P/N 4303859), with non-ischemic muscle as internal calibrator. The formula used is $2^{-\Delta\Delta Ct} = 2^{-[\Delta Ct\ (sample) - \Delta Ct\ (calibrator)]}$, where ΔCt (sample) = Ct (sample) – Ct (reference gene). For the internal calibrator, $\Delta\Delta Ct = 0$ and $2^0 = 1$. For the remaining samples, the value of $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the calibrator. ΔCt value for each sample is the average of triplicates.

5.7.15. Clinical Pathology and Histological Analyses

Fifty-two weeks post-HLI, body weight was recorded and mice were sacrificed with anesthetic overdose. Blood was collected by cardiac puncture and assessed for complete blood cell count, serum biochemistry (urea, creatinine, alanine aminotransferase, aspartate amino transferase, alkaline phosphatase, phosphorus and albumin) and coagulation tests (prothrombin time, activated partial thromboplastin time, thrombin time and fibrinogen assay). Urine was collected by urinary bladder

puncture for urinalyses.
Necropsy was performed and several organs and tissues were collected for routine cytological and histopathology analysis: bone marrow smears were performed from flushed bone marrow (femur), and lung, liver, kidney, thymus, spleen, lymph nodes and long bone (femur) were collected, formalin-fixed and paraffin embedded. Hematoxylin and eosin-stained 3 µm sections were analyzed by a pathologist blinded to experimental groups.

5.7.16. RNA Isolation, Target Synthesis and Hybridization to Affymetrix GeneChips

Total RNA was extracted using the RNeasy Micro Kit (QIAGEN) including DNase treatment. Concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA).
RNA was processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip HuGene 1.0 ST Arrays, according to the manufacturer's Whole Transcript Sense Target Labeling Assay. Briefly, 100 ng of total RNA containing spiked in Poly-A RNA controls (GeneChip Expression GeneChip Eukaryotic Poly-A RNA Control Kit; Affymetrix) was used in a reverse transcription reaction (GeneChip® WT cDNA Synthesis Kit; Affymetrix) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an in vitro transcription (IVT) reaction to generate cRNA (GeneChip® WT cDNA Amplification Kit; Affymetrix). 15 mg of this cRNA was used for a second cycle of first-strand cDNA synthesis (GeneChip® WT cDNA Synthesis Kit; Affymetrix). 5.5 mg of single stranded cDNA was fragmented and end-labeled (GeneChip® WT Terminal Labeling Kit; Affymetrix). Size distribution of the fragmented and end-labeled cDNA, respectively, was assessed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay. 5 mg of end-labeled, fragmented cDNA was used in a 100 ml hybridization cocktail containing added hybridization controls. 80 ml of mixture was hybridized on arrays for 17 hours, at 45ºC. Standard post hybridization wash and double-stain protocols (FS450_0007; GeneChip HWS kit, Affymetrix) were used on an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an Affymetrix GeneChip scanner 3000 7G.

5.7.17. GeneChip Data Analysis

Scanned arrays were analyzed first with Affymetrix Expression Console software for quality control. Subsequent analysis was carried out using Partek Genomics Suite 6.4. Here the 8 arrays were normalized and modeled using Robust Multichip Averaging RMA. Probe sets showing differential expression were determined using 1-way Analysis of Variance (ANOVA) with a p-value cut-off of 0.03. Expression values of these 2374 well-annotated genes were imported into Chipster 3.7.2. Arrays and genes were clustered using Pearson correlation as a distance measure and average linkage for constructing a dendrogram, subsequently visualized as a heatmap. Microarray data are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE73341. Reviewer access: (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=afybsswgdfmlrsp&acc=GSE73341).

References

1. Brown AL, Smith DW. Improved RNA preservation for immunolabeling and laser microdissection. *RNA* 2009;**15**:2364-2374.

5.8 SUPPLEMENTARY FIGURES

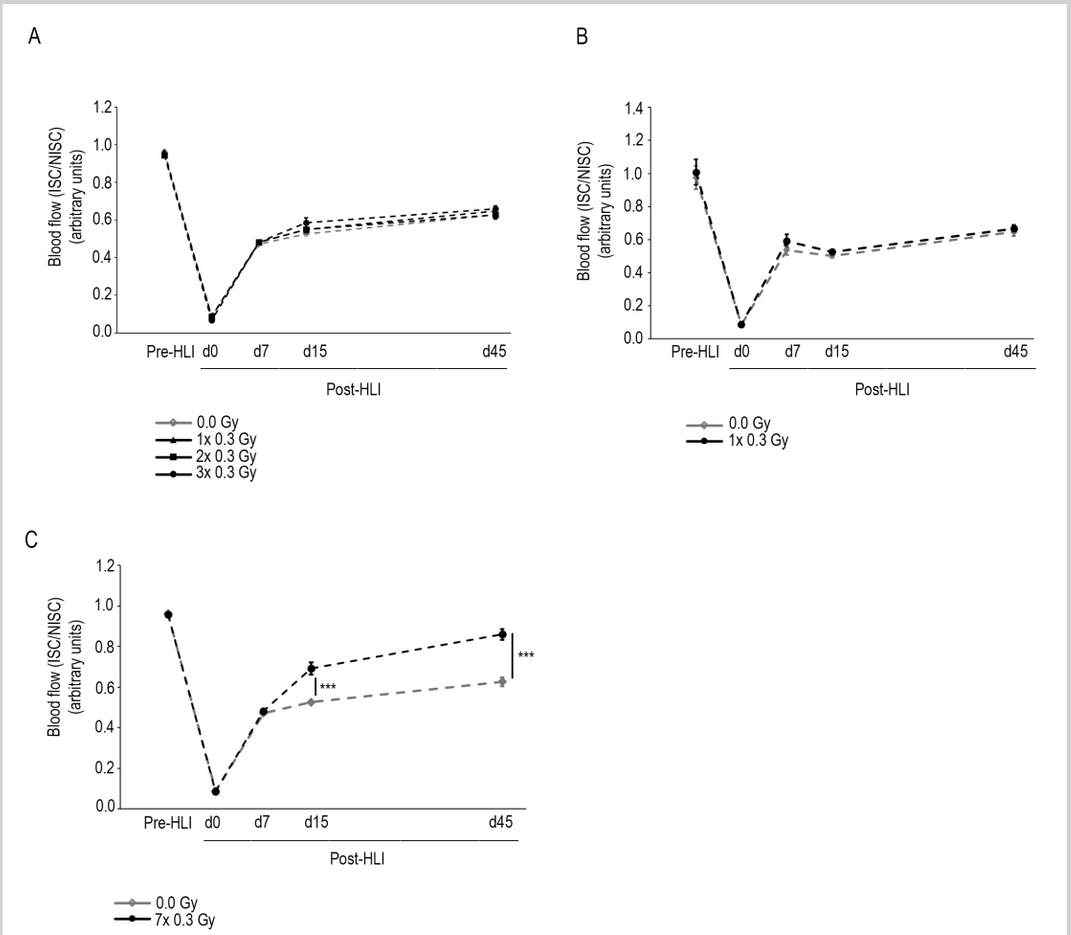

Figure S1. Perfusion recovery after exposure to different doses of IR

After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with (**A**) 0.3 Gy, once, twice or three consecutive days (**B**) 0.1 Gy for four consecutive days (**C**) 0.3 Gy for seven days. Quantitative evaluation of blood flow expressed as a ratio of ISC to NISC limb was assessed before (Pre-HLI) and at days 0, 7, 15 and 45 post-HLI induction. At days 15 and 45 post-HLI, blood perfusion is significantly induced in mice exposed to 0.3 Gy during seven days. Between-group changes were assessed by two-away repeated measurements ANOVA followed by Bonferroni post-hoc test (n=6 mice per group). Means ± SEM are shown. *** $P < 0.001$.

Abbreviations: *ISC, ischemic; NISC, non-ischemic; Pre-HLI, before hindlimb ischemia.*

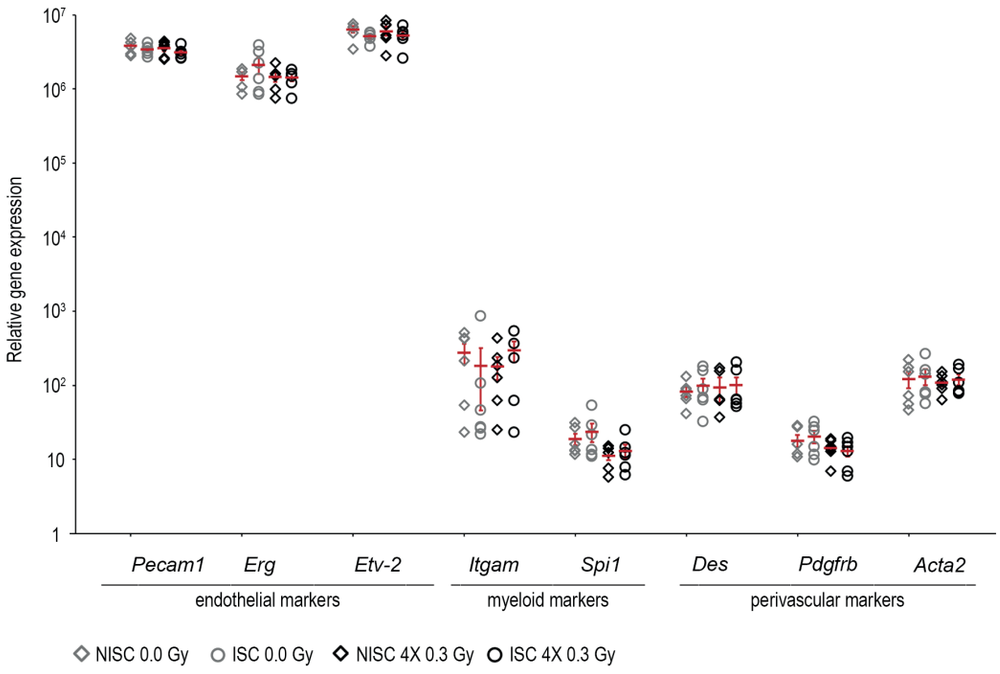

Figure S2. The CD31+ cells isolated from laser capture microdissection microscope express consisted primarily of ECs and no myeloid nor perivascular cells

After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days and allowed to recover. At day 45 post-HLI, gastrocnemius muscles were harvested sectioned, and stained for CD31. Individual endothelial CD31+ cells were and visualized, dissected and isolated using a laser capture microdissection microscope. To ensure that only ECs were isolated, the expression of markers specific for endothelium, myeloid or perivascular cells was assessed at the gene levels by qRT-PCR exclusively on ECs. The cell markers *Pecam1* encoding CD31, *Erg* and *Etv2* that are specific for ECs; *Itgam* encoding CD11b and *Spi1* encoding PU-1 for myeloid cells; and *Des* encoding Desmin, *Pdgfrb* and *Acta2* encoding smooth muscle alpha-actin for perivascular cells were assessed. The relative mRNA levels were determined by real time qRT-PCR relative to the level of 18S RNA expression. Each symbol represents data obtained from isolated individual ECs pooled from one mouse and means ± SEM (in red) are shown from n=6 mice per group.

Abbreviations: *ns, non-significant. ISC, ischemic; NISC, non-ischemic.*

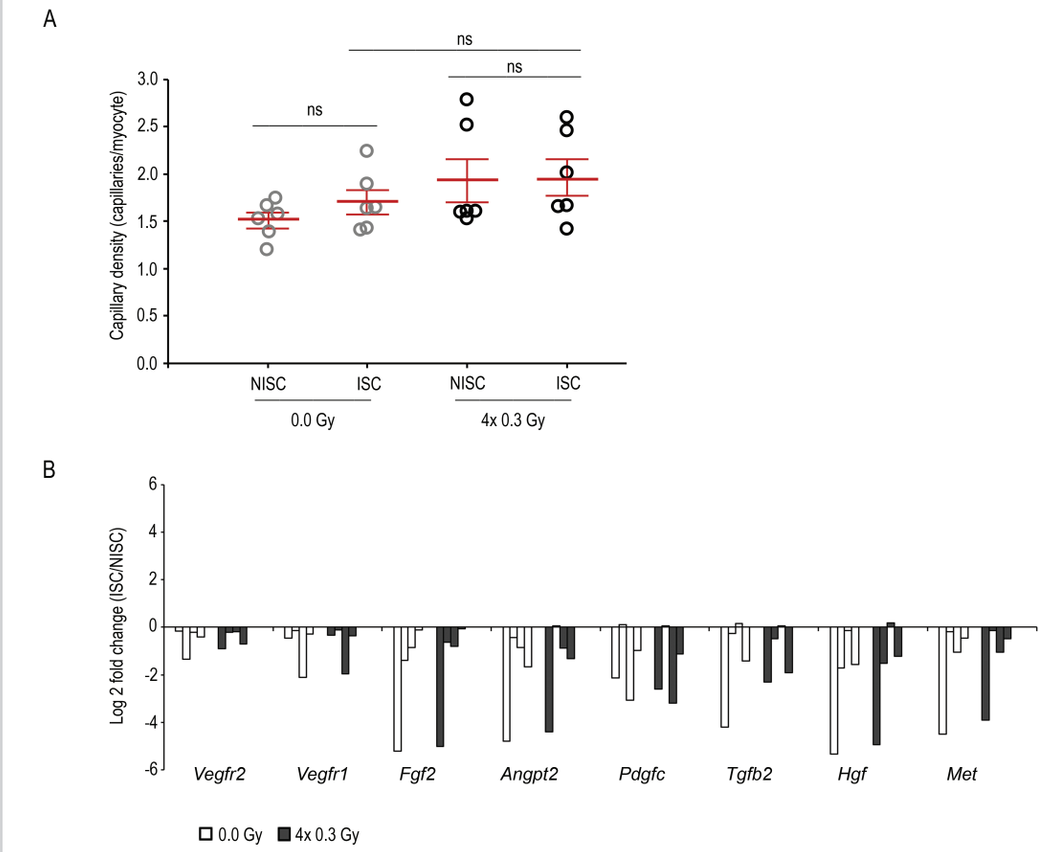

Figure S3. Capillary density is not increased in the adductor muscles neither in response to ischemia nor upon LDIR and ECs do not present an up-regulation of pro-angiogenic genes

After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days and allowed to recover. (**A**) At day 45 post-HLI, quantitative analysis does not show an increase in capillary density (capillaries/myocyte) in irradiated ischemic adductor muscles compared to sham-irradiated ischemic ones. Furthermore, capillary density is not increased in adductor muscles in response to ischemia *per se*. Mixed ANOVA followed by Bonferroni post-hoc test was conducted with a within-subject factor of ISC and between-subject factor of irradiation. Individual data and means ± SEM (in red) are shown from n=6 mice per group; ns, non-significant. (**B**) At day 45 post-HLI, the expression of pro-angiogenic factors and their receptors was evaluated by qRT-PCR exclusively on ECs isolated from the adductor muscles. Each bar represents the relative gene expression in one animal. White and gray bars represent sham-irradiated and irradiated mice, respectively. Values were normalized to 18S to obtain relative expression levels. Results expressed as log2 fold changes between ISC and NISC samples demonstrated a down-regulation of the transcripts in both experimental groups.

Abbreviations: *ISC, ischemic; NISC, non-ischemic.*

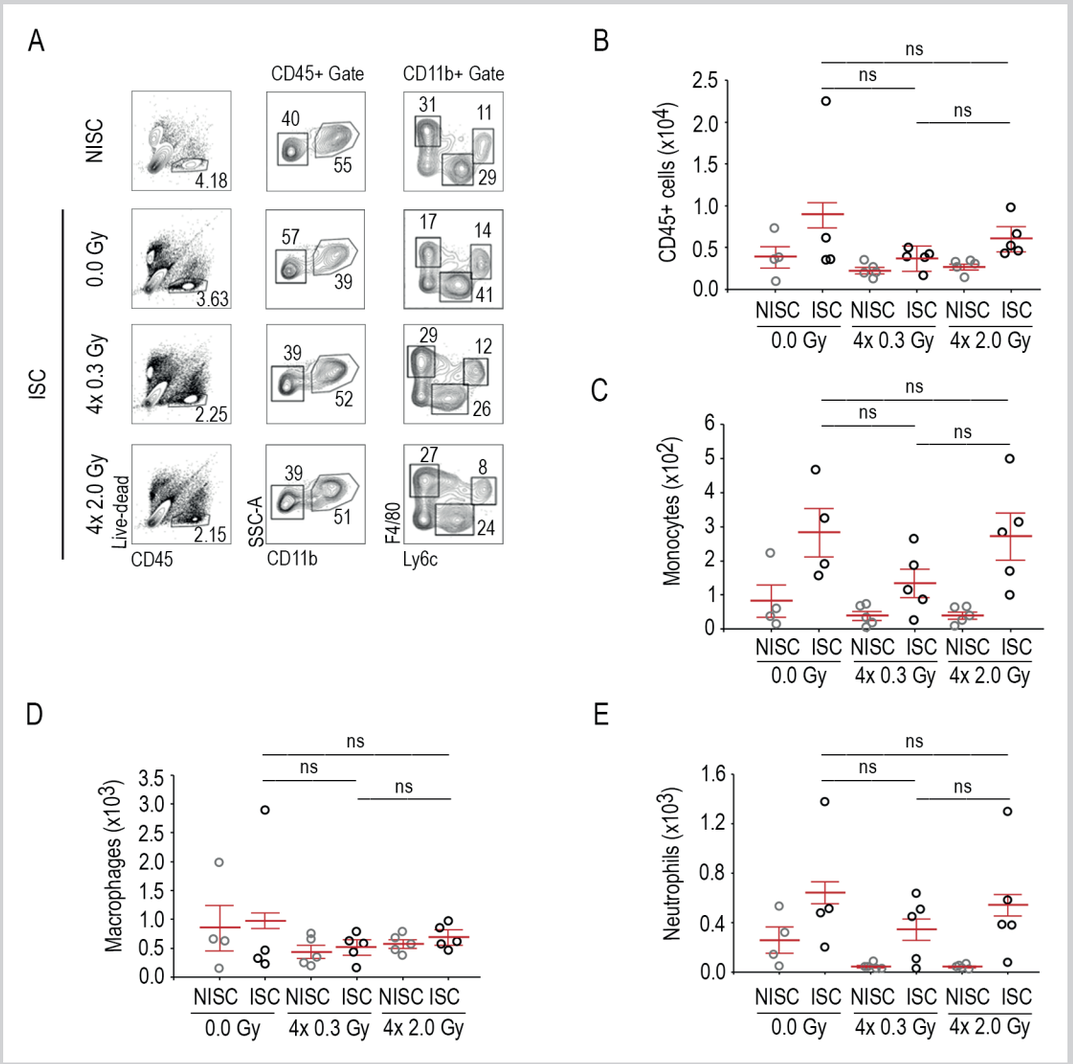

Figure S4. Profiles of leukocytes mobilized to ischemic muscles upon LDIR

After surgical induction of unilateral HLI, both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy or 2.0 Gy in consecutive days. At day 15 post-HLI, analysis of $CD45^+$ immune cells that infiltrate ischemic adductor muscles was assessed. (**A**) Representative analysis of hematopoietic CD45+ cells present in ischemic muscle as assessed by flow cytometry. Analysis of the accumulation of myeloid CD11b+ cells, and in particular macrophages (CD45+CD11b+F4/80+ cells), monocytes (CD45+CD11b+LY6C+F4/80int cells), and neutrophils (CD45+CD11b+Ly6Cint). The graphs show numbers of (**B**) total CD45+ cells; (**C**) monocytes; (**D**) macrophages and (**E**) neutrophils, isolated from ISC and NISC adductor muscles. Mixed ANOVA followed by Bonferroni post-hoc test was conducted with a within-subject factor of ISC and between-subject factor of irradiation. Individual data and means ± SEM (in red) are shown from n=4 mice per group.

Abbreviations: *ns, non-significant. ISC, ischemic; NISC, non-ischemic.*

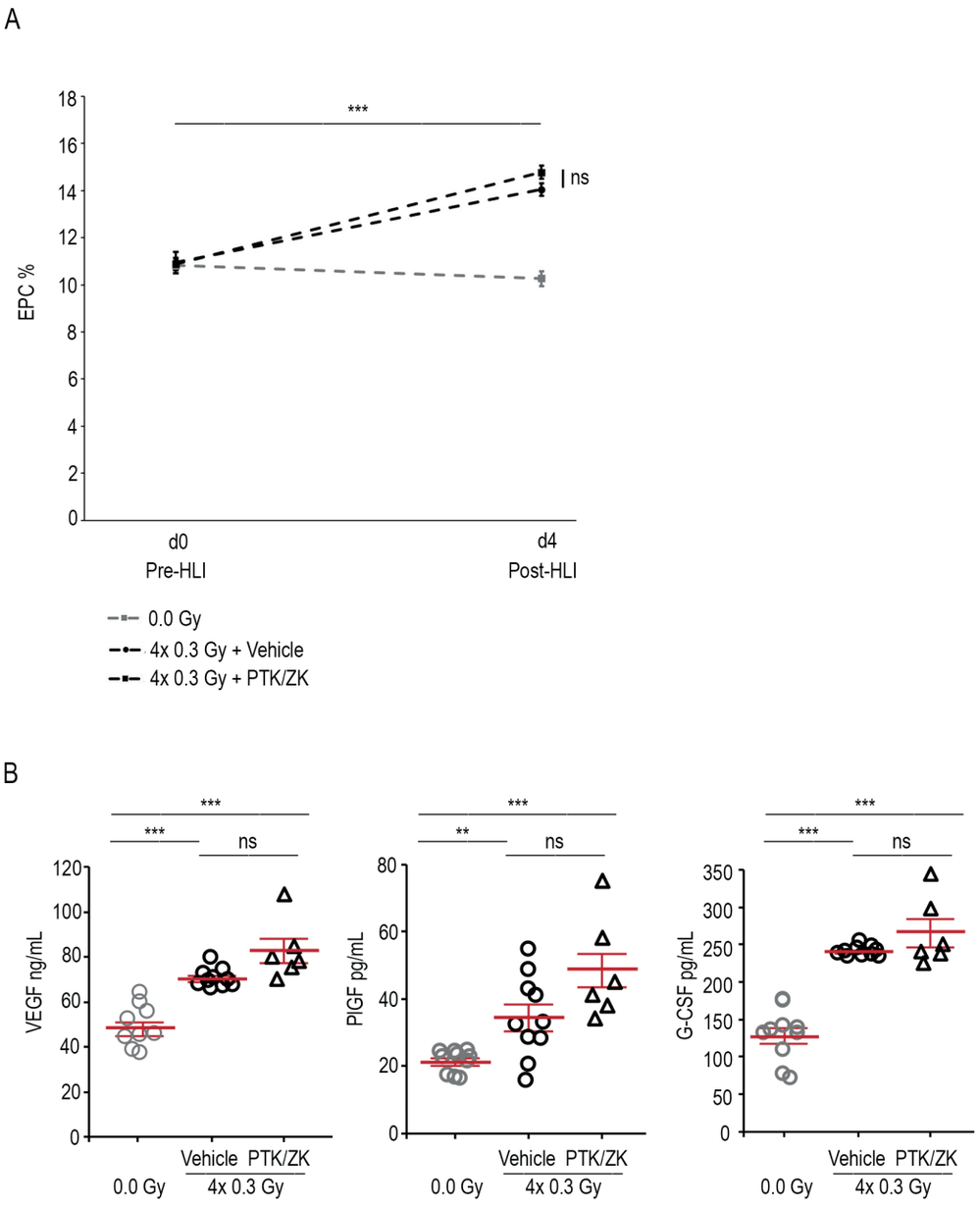

Figure S5. PTK/ZK treatment does not change the effect of LDIR in increasing the number of circulating EPCs or the levels of VEGF, PlGF and GCSF in plasma after HLI induction

After surgical induction of unilateral HLI both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days and allowed to recover. Two hours before each irradiation, ischemic mice were pretreated with PTK/ZK (100mg/Kg) or with the control vehicle. (**A**) Quantitative analysis of EPCs in peripheral blood demonstrated no differences at day 4 in irradiated mice pretreated with PTK/ZK vs irradiated mice pretreated with the control vehicle. As expected a significant increase of EPC at day 4 post-HLI is observed in both experimental groups when compared to day 0 pre-HLI or when compared to sham-irradiated mice at day 4. Two-way ANOVA was conducted followed by Bonferroni post-hoc test with a between-subject factors of day, irradiation and PTK/ZK treatment (n=6 mice per group). Means ± SEM are shown. (**B**) The concentrations of VEGF, PlGF and G-CSF were measured in the plasma, at day 4 post-HLI. The levels of VEGF, PlGF and G-CSF are not changed in irradiated mice pretreated with PTK/ZK treatment vs irradiated mice pretreated with the control vehicle. Two-way ANOVA was conducted followed by Bonferroni post-hoc test with a between-subject factors of irradiation and PTK/ZK pretreatment. Individual data and means ± SEM are shown (in red) from n=10 or 6 mice per group.

Abbreviations: *ns, non-significant. ISC, ischemic; NISC, non-ischemic; Pre-HLI, before hindlimb ischemia.*

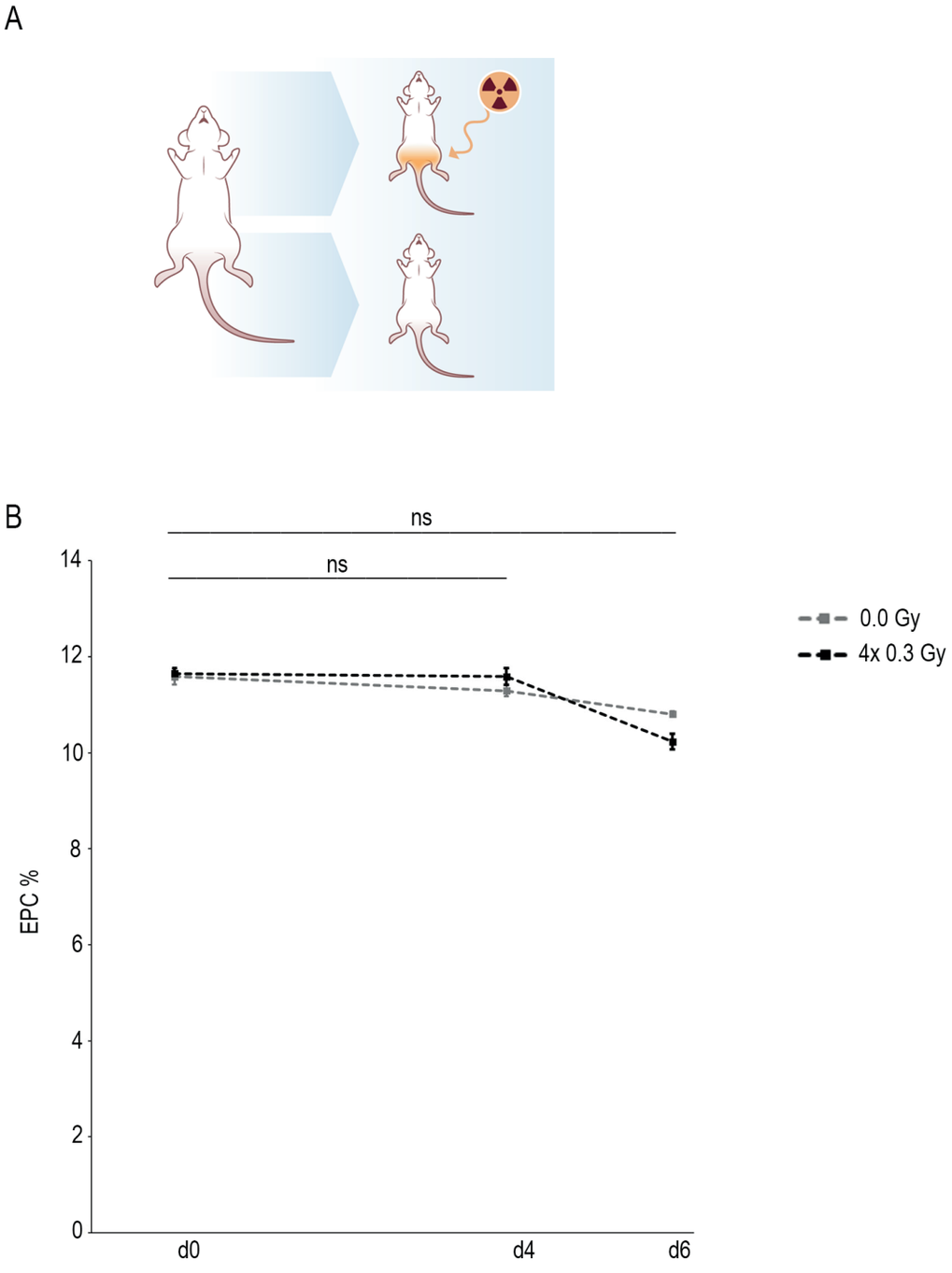

Figure S6. In the absence of HLI, LDIR *per se* does not increase the number of circulating EPC

Both hindlimbs of C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy, in consecutive days. (**A**) A schematic illustration of our experimental design. Both hindlimbs are irradiated (in orange) or sham-irradiated. (**B**) Quantitative analysis of EPCs in peripheral blood demonstrated no differences at day 4 in irradiated mice and at day 6 in sham-irradiated mice when each group is compared to the percentage at day 0, before irradiation. Two-way ANOVA was conducted followed by Bonferroni post-hoc test with a between-subject factors of day and irradiation. Means ± SEM are shown from n=6 mice per group.

Abbreviations: *ns, non-significant.*

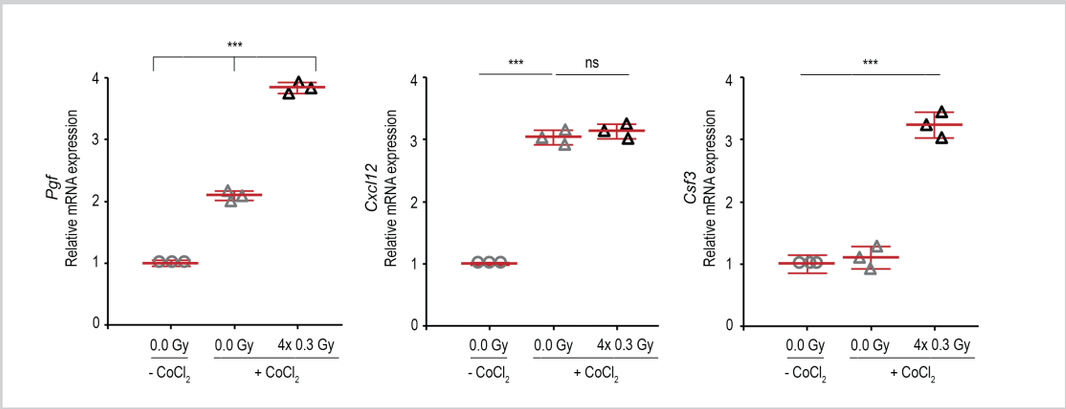

Figure S7. LDIR enhances hypoxia-induced Pgf and Csf3 expression

Cells were cultured with or without $CoCl_2$ (150 µM) in normoxia to mimic hypoxic conditions and immediately exposed to 0.3 Gy. Eight hours post-irradiation, *Pgf* and *Cxcl12* mRNA was quantified by qRT-PCR and 16 hours after *Csf3*. LDIR significantly increase *Pgf* and *Csf3* mRNA expression. Two-way ANOVA was conducted followed by Bonferroni post-hoc test with a between-subject factors of irradiation and $CoCl_2$ treatment. Individual data represent the fold change in gene expression relative to the internal calibrator (−CoCl2) in three independent experiments. Individual data and means ± SEM are shown (in red); ***$P < 0.001$.

Abbreviations: ns, non-significant.

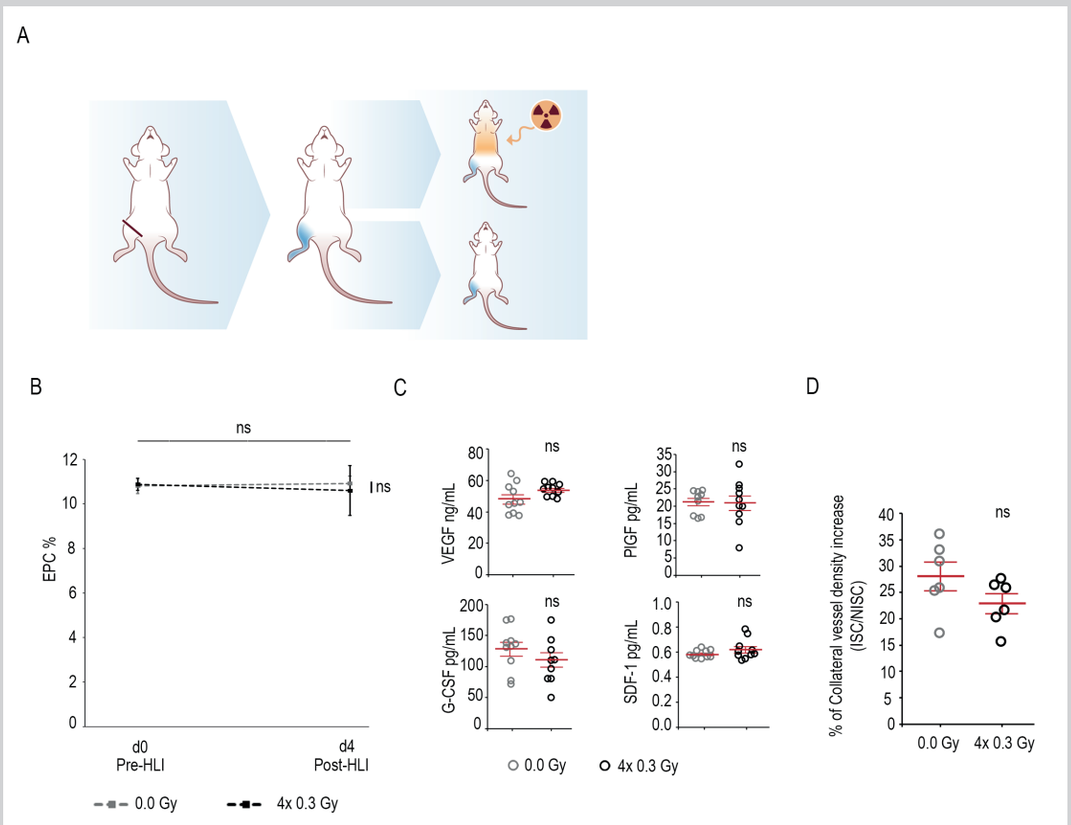

Figure S8. The exposure of the ischemic niche to LDIR is critical for the increase of cytokines, mobilization of EPCs and collateral formation

After surgical induction of unilateral HLI, C57BL/6 mice were irradiated but not in the ischemic niche with four daily fractions of 0.3 Gy, in consecutive days. As a control C57BL/6 mice were sham-irradiated. (**A**) A schematic illustration of our experimental design. After unilateral HLI (represented by a thick brown line), the flow to the ischemic limb is dramatically decreased (in blue). Mice are irradiated (in orange) or sham-irradiated but both hindlimbs are not exposed to LDIR. (**B**) Quantitative analysis of EPCs in peripheral blood demonstrated no differences at day 4 in irradiated mice and in sham-irradiated mice when compared to the percentage at day d0 pre-HLI. Two-way ANOVA was conducted followed by Bonferroni post-hoc test with a between-subject factors of day and irradiation (n=6 mice per group). Means ± SEM are shown. (**C**) The concentrations of VEGF, PlGF, G-CSF, and SDF-1alfa were measured in the plasma, at day 4 post-HLI. The VEGF, PlGF, G-CSF and SDF-1 concentrations are not changed by LDIR after HLI induction (*n* = 10 mice per group; for VEGF, PlGF, G-CSF values assumed normal distribution and equal variance and independent two-tailed t-test was used; for SDF-1 values not assumed normal distribution, Mann-Whitney test was used). (**D**) Data are represented as the percentage of collateral vessel density (CVD) increase of the ISC limb relatively to the NISC one. At day 45 post-HLI, no difference in CVD upon LDIR was observed. (n=6 mice per group; values assumed normal distribution, equal variance and independent two-tailed t-test was used). (**C, D**) Individual data and means ± SEM (in red) are shown.

Abbreviations: *ns, non-significant. Pre-HLI, before hindlimb ischemia; ISC, ischemic; NISC, non-ischemic.*

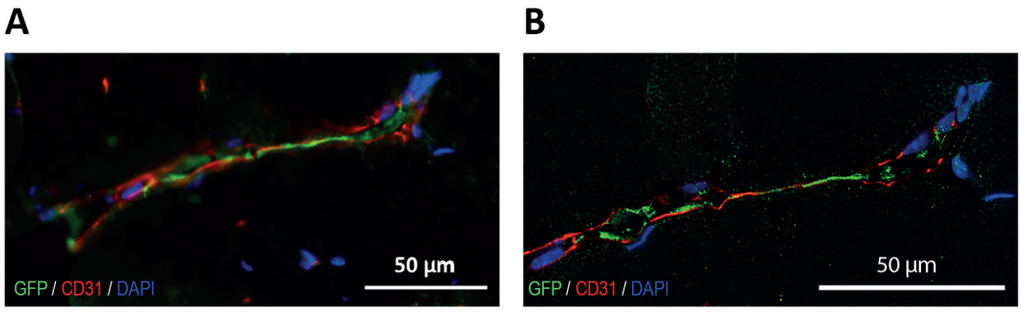

Figure S9. Confocal and widefield images of the same ten-micron-thick section show that green and red fluorescent signals belong to the same cells

(**A**) corresponds to a widefield image acquired with a 20x/0.8 objective on a Zeiss Axiovert 200M microscope, with the same acquisition settings that were used for the images in Figure 5C. (**B**) corresponds to the same section imaged on a Zeiss LSM 880 confocal microscope with a 63x/1.4 oil immersion objective, with the pinhole set to 1 Airy unit (optical slice thickness = 0.7 micron). Notice that even when imaging a single plane, cells show both green and red signals.

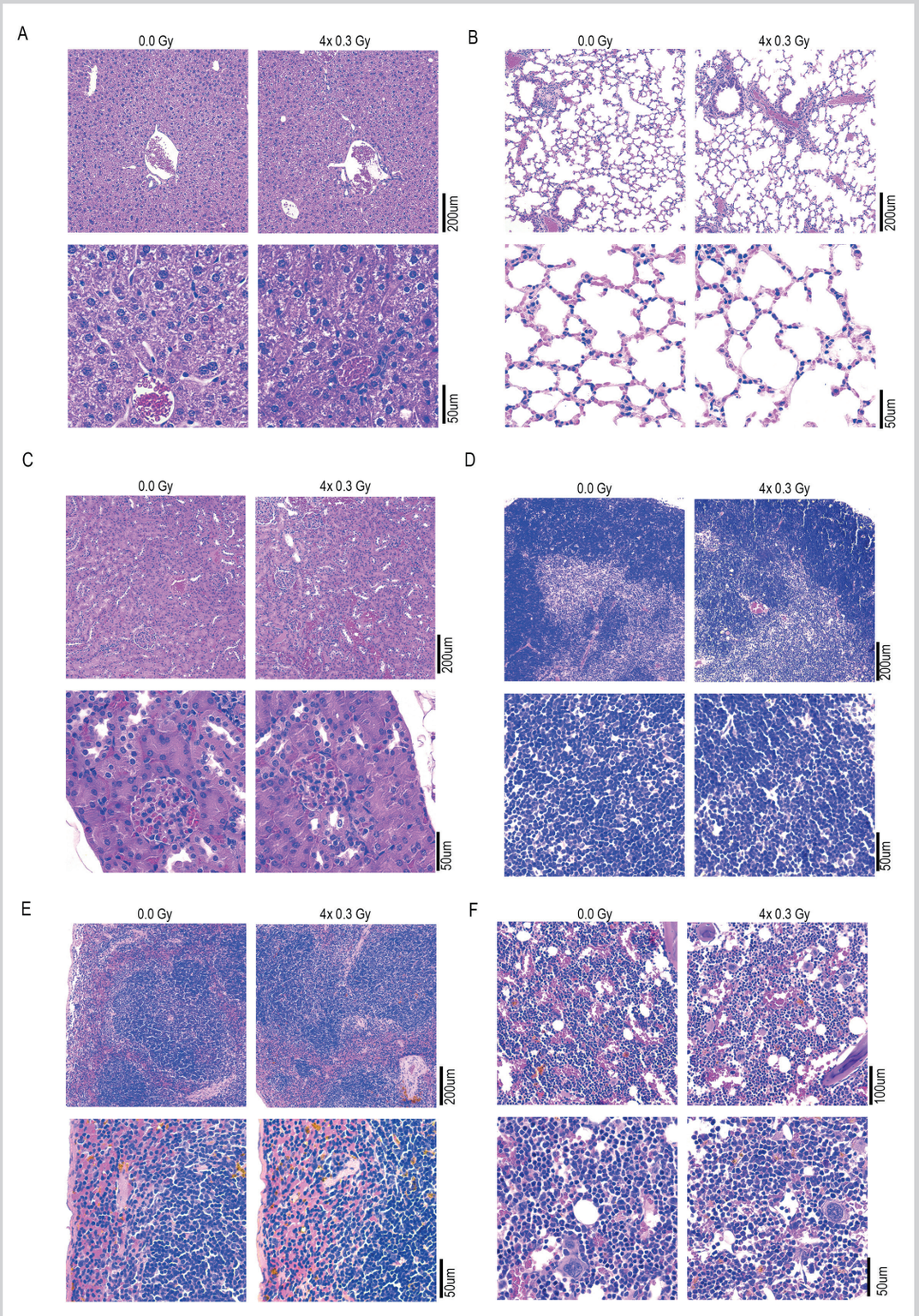

Figure S10. Representative microphotographs of selected organs and tissues from sham-irradiated mice and mice irradiated with LDIR

No significant changes were observed for liver (A), lung (B) and kidney (C); and no signs of immunotoxicity, altered cell distribution/density or neoplasia were seen in lympho-hematopoietic organs, namely thymus (D), spleen (E) and bone marrow (F). Hematoxylin and eosin stain; original objective magnification 5x (upper panels) and 40x (lower panels).

6

CLINICAL TRIAL:
LOW-DOSE IONIZING RADIATION MODULATES THE EXPRESSION OF PRO-ANGIOGENIC GENES IN CRITICAL LIMB ISCHEMIA PATIENTS

Ongoing

6. ONGOING CLINICAL TRIAL: LOW-DOSE IONIZING RADIATION MODULATES THE EXPRESSION OF PRO-ANGIOGENIC GENES IN CRITICAL LIMB ISCHEMIA PATIENTS

ABSTRACT

Approximately 16 million patients worldwide (1 in 20 people over the age of 50) suffer from peripheral arterial disease (PAD). Narrowing or occlusion of vessels supplying blood to the lower limbs, most often due to atherosclerosis, characterizes PAD. Symptoms of PAD include intermittent claudication that may progress to critical limb ischemia (CLI) manifested by rest pain, tissue loss and gangrene, which eventually may require amputation.

Low-dose ionizing radiation (LDIR) has been reported as inducer of angiogenesis. In the setting of experimentally induced hindlimb ischemia (HLI), we found that 0.3 Gy, delivered during 4 consecutive days, stimulates angiogenesis and collateral vessels development and thereby improves blood perfusion in the ischemic limb. The outcome of these *in vivo* experiments performed in a mice model suggests that LDIR may have clinical utility in the management of lower limb arterial insufficiency.

Here, we describe an single-centre, investigator-blinded, randomized, sham-controlled clinical trial, having as primary endpoint the evaluation of the effects of LDIR exposure in the expression of pro-angiogenic genes in endothelial cells (ECs) isolated from muscles of CLI patients.

6.1. INTRODUCTION

PAD occurs in around 7% of people over 40 years old and sharply increases with age to affect between 12 to 20% of Americans over 70 years of age[1-3]. CLI represents the most advanced form of PAD and is characterized by ischemic rest pain, with or without tissue loss (ulcers and/or gangrene)[4]. CLI causes a severe disturbance of both macro and microcirculation. It is estimated that between 5 to 10% of PAD patients over 50 years of age will develop CLI within 5 years[4]. CLI is associated to a significant morbidity and mortality: up to 30% of patients will require an amputation within 1 year post-diagnosis and another 25% will die from cardiovascular events[1,4]. Treatment of CLI includes risk factor modification (quitting smoking, exercise, managing diet, control of exercise, diabetes (DM), hyperlipidemia, hypertension, and use of antiplatelet medications), ischemic pain relieve, management of ischemic ulcers, and revascularization either by open bypass surgery or by endovascular intervention[4]. Although revascularization is a mainstay of treatment, it is often not feasible due to medical comorbidities or lack of anatomic options, and is associated to a significant rate of complications[5-7]. In addition, in CLI patients with tissue loss, survival and limb-related outcomes are poor, when compared to CLI patients with rest pain only[4]. Patients with severe limb ischemia, who are unable to undergo revascularization or with a failed revascularization, have limited options and an amputation is often required[8]. As it is estimated that there are at least between 150,000 to 300,000 new cases of CLI every year[4], there remains a significant need for less invasive therapeutic options in patients with CLI.

Angiogenesis and arteriogenesis. Angiogenesis is a physiologic process required for the menstrual cycle and wound healing in adults. It also plays a role in some pathologic processes, ie, tumor growth, rheumatoid arthritis, DM, and cardiovascular disease. Sprouting angiogenesis is the growth of new capillary vessels from pre-existing ones. The angiogenic process is complex and controlled by a balance of activators and inhibitors. Angiogenesis activators stimulate the proliferation and migration of cells to the vascular wall and can inhibit apoptosis, as well.

Moreover, arteriogenesis is the process of arteriolar enlargement and the formation of collateral vessels that connect arteries pre-occlusion to the diseased intra-ischemic arteries[9].

Both processes play an important role in the pathophysiology of vascular obstructive disease. By stimulating these mechanisms, one might be able to provide an alternative treatment strategy for patients with lower limb ischemia and coronary artery disease.

Therapeutic angiogenesis. Angiogenesis can be promoted by local administration of pro-angiogenic growth factors. While several clinical trials revealed encouraging results, more recent and randomized controlled clinical trials have produced less consistent results. Some explanations to account for these findings include: (i) use of a single angiogenic factor; (ii) development of unstable vessels; (iii) rapid diffusion, poor bio-stability and short half-lives after growth factor delivery; (iv) excessive uncontrolled vascular formation in undesired locations; and (v) incapacity from the resident ECs to respond[9].

Recently, we demonstrated that LDIR promotes therapeutic neovascularization in a pre-clinical model of HLI[10]. Using a model of experimentally induced HLI, we demonstrated that LDIR significantly increases the capillary density and collateral development and thereby improve blood perfusion in the ischemic limb. Molecularly, we found that LDIR induced a durable and simultaneous up-regulation of a repertoire of pro-angiogenic factors and their receptors in ECs isolated from the gastrocnemius ischemic muscles[10]. Moreover, LDIR protects ECs from apoptosis[11]. Taken together, these findings disclose an innovative and non-invasive strategy to induce therapeutic neovascularization in a mouse model of HLI, emerging as a novel approach in the treatment of CLI patients.

Need for an alternative therapy – an exploratory clinical trial. In patients with CLI, the prognosis is generally poor. Revascularization, either by endovascular means or by open surgery, combined with the best possible risk factor control does not achieve limb salvage in a considerable rate of these patients, and approximately one-fourth of them remain unsuitable for or fail endovascular or surgical therapy.

Pharmacological treatment alone has shown poor or no efficacy and therapeutic needs clearly seem to be unmet.
LDIR may have a clinical significant impact in the treatment of CLI. We currently have an ongoing exploratory clinical trial to determine the molecular and clinical effects of LDIR in “non-option” CLI patients. The success of this clinical trial will lead to the development of new trials to propose a novel and effective therapeutic tool with a worldwide impact to PAD.

6.2. OBJECTIVES

6.2.1. Primary objective

The primary objective of this study is to assess if:
(i) LDIR induces the expression of pro-angiogenic genes in the ECs isolated from ischemic muscle.

6.2.2. Secondary objectives

The secondary objectives of this study are to determine if:
(i) LDIR stimulates angiogenesis and arteriogenesis;
(ii) Document the safety and feasibility of LDIR exposure;
(iii) Evaluate the potential therapeutic benefits in patients with CLI.

6.3. OUTCOMES

6.3.1. Primary outcomes

Taking into account the primary objective, ECs were isolated from sham and irradiated limbs (collected during amputation) by using a Zeiss PALM MicroBeam Laser Microdissection System (LCM), followed by RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR) analysis. We expect to find a significant increase in the expression level of multiple pro-angiogenic genes in ECs removed from irradiated muscles, when compared to those removed from sham-irradiated muscles (vascular endothelial growth factor receptor 1 (*VEGFR1*); vascular endothelial growth factor receptor 2 (*VEGFR2*); fibroblast growth factor (*FGF2*); transforming growth

factor β (*TGF*β*2*); angiopoietin 2 (*ANG2*); platelet-derived growth factor (*PDGF*); hepatocyte growth factor (*HGF*); and hepatocyte growth factor receptor (*MET*)).

6.3.2. Secondary outcomes

Taking into account the secondary objectives, the secondary outcomes include:
(i) the measurement of capillary and vessel densities. The capillary density, expressed as the ratio of capillaries to myocytes, was determined in similar sections of calf muscles (collected at amputation) by CD31 immunohistochemistry and eosin counterstain. Analysis of tissue samples was conducted using Leica DM2500 upright brightfield microscope (Leica Microsystems). Capillary densities, ie, number of capillaries per number of muscle fibers, were measured in 2 different sections of 4 distinct anatomic areas of each specimen using the ImageJ software.
Lower limb digital subtraction angiography was performed using standard monoplane fluoroscopy equipment (Infinix, Toshiba Medical Systems) and the injection of 60 cm^3 of Visipaque® 320 at a flow rate of 6 cm^3/s administered using the injector Accutorn HP 200 (Medtron AG). Vessel density (VD) was quantified in angiography images where vessels were manually segmented by highlighting them using Adobe Photoshop CS6®. For each limb, an anatomically determinable angiographic region comprising the 100% isodose curve was selected and defined as the region of interest (ROI). VD was quantified in equivalent ROI as the ratio between the vascular and ROI areas. Measurements were performed at baseline and after 1 and 6 months of treatment.
(i) Document the safety and feasibility of LDIR exposure;
(ii) Therapeutic benefits, classified as abolition of rest pain and/or healing of the ischemic ulcer and/or downgrading in the Rutherford lower limb ischemia classification. With regard to this objective, non-invasive and invasive studies are intended to provide comprehensive data regarding both the clinical response of the ischemic limb and anatomic and functional evidence of collateral vessel development.

6.4. METHODS

6.4.1. Participants

The trial is taking place in the Vascular Surgery Department of Hospital de Santa Maria, Centro Hospital Lisboa Norte (CHLN), Lisboa, Portugal in collaboration with

the Department of Radiotherapy and Medical Physics Unit of Hospital de Santa Maria, CHLN, Lisboa, Portugal and Laboratory of Clinical Pharmacology and Therapeutics, Faculdade de Medicina da Universidade de Lisboa (FMUL), Lisboa, Portugal. This is an academic tertiary Hospital and a referral center for CLI patients. Gene expression analyses of pro-angiogenic factors are done in the Angiogenesis Unit, in Centro Cardiovascular da Universidade de Lisboa (CCUL), Lisboa, Portugal. Patients are being selected for this protocol if they have rest pain and/or non-healing ischemic ulcers (categories 4,5 and 6 of the Rutherford classification) and if they are considered non-satisfactory candidates for nonsurgical or surgical/endovascular revascularization – the “non-option” patients.

The consequences of PAD in the patients’ subset that was selected to study are sufficiently enough to predict a meaningful assessment of the results of LDIR therapy. Rest pain has an unrelenting course; ie, once the diagnosis has been established, it will not resolve spontaneously. For rest pain, a minimum of 4 weeks of dependence on narcotics is required for patient selection. For non-healing ulcers, patients are being included only if a minimum of 4 weeks of conservative measures had failed to improve the appearance of the ulcer. Published and/or clinical experience suggests that spontaneous improvement in either rest pain and/or ischemic ulcer following this duration of conservative therapy is unlikely[12].

In this ongoing clinical trial, a total of 12 patients will be recruited in order to achieve the primary endpoint. Six patients exposed to 0.3 Gy of LDIR, delivered for 4 consecutive days; 6 sham patients exposed to 0.0 Gy during 4 consecutive days. Sham patients have the discomfort of moving to the radiotherapy department, but free of associated risks. It is important to mention that so far, a higher number of patients were included, since 6 of them have not required amputation and for that reason the primary objective was not achieved in those patients. Therefore, this clinical trial is ongoing, although 16 patients were already enrolled. In the preliminary results section, a detailed description of each patient will be done.

6.4.2. Inclusion Criteria

Both men and women, between 45 and 80 years old are eligible. Symptoms include rest pain typical of arterial insufficiency, with or without established ischemic ulcers. A minimum of 4 weeks of rest pain with dependence on narcotics and with no improvement will be required. Signs include non-healing lesions of the lower extremities, due to arterial insufficiency.

Non-invasive findings will be as follows:

(i) resting ankle-brachial systolic index (ABI) in the affected limb must be <0.4, on two consecutive examinations performed at least 1 week apart. For patients with non-compressible ankle arteries (due to calcified deposits with or without associated DM), the great toe index (TBI) must be <0.4;

(ii) rest pain and/or ischemic ulcers may preclude exercise testing in most patients. Every patient, however, will be asked to exercise whenever possible.

Diagnostic angiography must demonstrate occlusion in the affected limb of one or more of the following vessels: superficial femoral, popliteal, and/or one or more of the infra-popliteal arteries.

Patients are included after dermatological evaluation and exclusion of skin neoplastic or pre-neoplastic lesions.

Patients are included only if they agree to and are judged appropriate to discontinue concomitant prostaglandin therapy and/or therapy with vasodilators, pentoxifylline, and/or hyperbaric oxygen. Patients may continue to receive aspirin and coumadin, provided that these therapies have been used by the patient for a minimum of 6 months before entry into the study.

6.4.3. Exclusion Criteria

Exclusion criteria include:

(i) aortic or lower-extremity arterial surgery, angioplasty, or lumbar sympathectomy within 3 months;

(ii) radiographic and radioisotopic evidence of concomitant osteomyelitis in the ischemic extremity;

(iii) any concomitant disease process with a life expectancy of less then 1 year or severe enough as to compromise clinical follow-up examinations;

(iv) significant history of alcohol or drug abuse within the past 3 months;

(v) previous or current history of neoplasm;

(vi) clinically significant abnormality in liver function or other laboratory tests, including prostate-specific antigen and carcinoembryonic antigen, and/or signs by chest radiograph, abdominal CT scan, mammography in the case of women or prostate examination in the case of men, of malignant disease;

(vii) clinical evidence of type I DM, diabetic retinopathy, and/or other ophthalmologic complications of DM;

(viii) refusal or inability to give informed consent.

6.5. INTERVENTIONS

This exploratory randomized, investigator-blinded, sham-controlled trial is being conducted in accordance to the Declaration of Helsinki principles and approved by the appropriate institutional review boards. Informed consent needs to be obtained from all patients. A clinical committee independent from the trial investigators examined the patients and monitored them on a daily basis following therapeutic procedures, to analyze any of the modifications criteria and mainly to decide the amputation timing. This committee is composed of three assistant vascular surgeons, members of the Vascular Surgery Department of Hospital de Santa Maria, CHLN, Lisboa, Portugal. After having obtained written informed consent, patients are randomly allocated, in equal proportions, to one of the two groups: active and sham treatment.
Randomization is being performed at the laboratory of Clinical Pharmacology and Therapeutics, FMUL, Lisboa, Portugal by independent researchers. For each patient, a numbered envelope is drawn from a set of sealed opaque envelopes containing the allocation on a card. An independent staff member records the assignment in a computer database. Shortly, before each radiotherapy intervention, the radiotherapy technician is informed about the patient's group assignment. Subsequent contact between the patient and the radiotherapy technician is minimized. Patients randomized to active or sham treatment are blinded with regard to their assignments. A research nurse, blinded to the treatment assignments, contacts the patients and carries out subsequent outcome assessments.

6.5.1. Irradiation and sham protocol

The radiation treatment consists of one treatment per day, for four consecutive days. Each treatment uses external photon beams delivering 0.3 Gy to the active patients or 0.0 Gy to sham patients. The cumulative dose delivered to the active treatment patients will be 1.2 Gy and for the sham patients 0.0 Gy. The process of planning radiation therapy has several steps, so that radiation will only be delivered to the designated areas. The steps are (i) CT simulation, (ii) treatment planning, and (iii) treatment delivery. The (i) CT simulation is performed with a computed tomography simulator (CT Sim, Somatom Sensation, Siemens), where the patient is positioned the same way as in the treatment room, fiducial marks are placed on patient skin, a CT scan of the area of the patient´s body to be treated is carried out, patient skin may be marked with several tattoos that are used to make sure patient is accurately positioned at each treatment session, a 3D reconstruction of the image acquisition is carried out and then transferred to the computerized treatment planning system

(TPS XiO, Elekta); during the (ii) treatment planning the target, lower limb (middle third of the leg), and critical structures in its vicinity will be localized and contoured, a 3D planning using 6 MV photon beams is carried out in order to determine beam geometry and shielding to delivery an homogeneous dose to the lower limb (target), while sparing the tissue in its vicinity, the treatment planning is then transferred to treatment equipment through a record and verify system (Mosaiq, Elekta); the (iii) treatment will be delivered in the linear accelerator with 6 MV photon beam (Synergy, Elekta) at a dose rate of 500 MU/min.

6.5.2. Patients follow up

The clinical committee evaluated all patients on a case-by-case basis. Patients were followed up on a daily basis within the first 3-4 weeks after the irradiation protocol. Ischemic ulcers were documented by color photography. Resting ABI and/or TBI were calculated by the quotient of absolute ankle or toe pressure to brachial pressure. Intra-arterial digital subtraction angiography was performed within 1 week before and 1 month after the treatment. Standard monoplane fluoroscopy equipment (Infinix, Toshiba Medical Systems) was applied in the diagnosis of intra-arterial digital subtraction angiography. Angiography was performed with selective catheterization of the primitive iliac arteries of both lower limbs by puncture of the common femoral artery (usually right). The angiographic study of the lower limbs was performed individually for each lower limb (random order), by steps with limb division into five segments (hip, thigh, knee, leg and foot) and successive advancement accompanying the progression of iodinated contrast. Each studied segment has radiation doses and number of images per second specific and appropriate to the intended evaluation in each segment (hip: 5 f/s, 300 µR; thigh 3 f/s, 300 µR; knee 2 f/s, 200 µR; leg 2 f/s, 200 µR; foot 1 f/s, 100 µR), and remains the same among patients. The contrast medium used was Visipaque® 320 (Iodixanol 320 mg/mL) administered with the high-performance injector Accutorn HP 200 (Medtron AG). The contrast volume administered (60 cm^3 for each lower limb) and flow rate (6 cm^3/s) was equal between patients and in both pre and post-intervention procedures.

This study is being conducted in patients proposed for major limb amputation within 1 month of LDIR exposure. Non-amputated patients performed an additional angiography 6 months after treatment.

A vascular surgeon, member of the clinical trial team, performs the amputation. At this moment, as a result of precise identification of the irradiation isodose curves on the anatomy, intraoperative harvesting of the irradiated gastrocnemious muscle is done at precise locations. For each patient, three samples were collected from

the middle third of the leg (100% isodose curve) and three muscle samples were collected from the upper third of the leg (0% isodose curve).
Although the study ends on the amputation moment, all patients, amputees or not, are being assessed by the Vascular Surgery Department staff at 3, 6 and 12 months and annually thereafter. Assessment of the peripheral circulation of patients is being performed in the non-amputees at 3, 6 and 12 months. This consists of a complete non-invasive vascular work-up, which includes measurement of systolic blood pressure, measurement of ABI and imaging studies that consisted of conventional intra-arterial digital subtraction angiography. All patients are followed-up for life keeping in mind eventual radiotherapy induced neoplasm.

6.5.3. LCM and gene expression analysis

Muscle biopsies were placed on a small cork disc with the help of 10% tragacanth, snap frozen in liquid nitrogen cooled isopentane and stored at -80ºC until sectioned. Twelve-micrometer sections were labeled with CD31 monoclonal antibody (Pharmingen). The immunohistochemistry protocol was done in order to preserve RNA by using high salt buffer, 2M NaCl in PBS 1x, at 4ºC, in all incubation and washing steps. Sections were dehydrated in ice cold 90% ethanol followed by 100% ethanol and allowed to dry. Ten thousand capillaries were microdissected using a Zeiss PALM MicroBeam Laser Microdissection System (Carl Zeiss Microscopy, Germany) equipped with a pulsed solid-state 355 nm laser. Dissected capillaries were catapulted into a microfuge tube adhesive-cap. Total RNA from the microdissected capillaries was isolated using an RNeasy Micro Kit (QIAGEN), including DNase treatment to remove potential genomic DNA contamination. For synthesis and preamplification of cDNA, RT^2 PreAMP cDNA Synthesis kit (QIAGEN) was used with 3 rounds of pre-amplification for the following targets: *VEGFR1*, *VEGFR2*, *FGF2*, *TGF*β*2*, *ANG2*, *HGF*, *c-Met*, *PDGF* and 18S (housekeeping gene). RT-PCR was performed according to the manufacturer's protocol using Power SYBR® Green (Invitrogen) and a 7500 Fast Real-Time PCR (Applied Biosystems) for the same targets described above. The real time PCR program consists of an initial denaturation step at 95ºC for 10 min followed by 50 cycles at 95ºC for 15 s and at 60ºC for 1 min. The relative quantification was performed according to the comparative method (2 -DDCt; Applied Biosystems User Bulletin no. 2P/N 4303859), with sham muscle as internal calibrator. The formula that will be used 2 -DDCt =2 -[DCt(sample)-DCt(calibrator)], where DCt(sample)=Ct(sample) -Ct(reference gene). For the internal calibrator, DDCt=0 and 2 0=1. For the remaining samples, the value of 2 -DDCt indicates the

fold change in gene expression relative to the calibrator. DCt value for each sample is the average of triplicates.

6.5.4. Modifications

Patients will be withdrawn from the study for clinical reasons or investigator or handling physician opinion or withdrawal of participant consent. It is important to refer that in this ongoing clinical trial, four patients were already excluded from the study after their enrolment, as detailed in the preliminary results section.
CLI: Rapid progression of disease with no control of rest pain or progression of the trophic lesions to gangrene and sepsis are reasons for withdrawing the patient from the protocol. The clinical committee document revealing improvements in the health status (changing – downgrading - category in the Rutherford classification) is a reason to postpone amputation.
Irradiation: The clinical committee will monitor for the following LDIR-related adverse effects daily through patient examination, blood tests and chart review: hematopoietic (aplastic anemia), gastrointestinal (nausea, vomiting, loss of appetite and abdominal pain), neurological/vascular (dizziness, headache and decreased level of consciousness) and cutaneous ionizing radiation-induced side effects.
Treatment emergent adverse reaction will be forwarded to the Portuguese Pharmacovigilance System.

6.6. RANDOMIZATION

6.6.1. Sequence generation/allocation

Patients are randomly assigned to either control or experimental group with 1:1 allocation as per a computer generated randomization schedules, independent from trial investigators, based on the Laboratory of Clinical Pharmacology and Therapeutics, FMUL, Lisboa, Portugal.

6.6.2. Implementation

At the inclusion moment, investigators receive information concerning the participant’s allocation.

6.6.3. Missing Data

No statistical techniques will be implemented to replace missing values.

6.6.4. Outliers

If necessary, analysis will be performed excluding identified outliers to confirm results and for reliability purposes.

6.6.5. Significance level

A 0.05 significance level will be assumed for all statistical calculations.

6.7. PRELIMINARY RESULTS

Potentially eligible patients with “non-option” CLI were referred for inclusion in the present clinical trial by practicing vascular surgeons of the Vascular Surgery Department of CHLN, Lisboa, Portugal. Trial design is summarized in Figure 1.
Between December 2015 and February 2017, 16 patients aged between 48 and 78 years with intractable CLI were enrolled in the study. From the 16 recruited patients, four patients withdrew before completion of the study protocol: 2 suffered significant clinical worsening and needed amputation before irradiation could be initiated, 1 did not tolerate the irradiation sessions due to severe rest pain and 1 withdrew from the study voluntarily. Twelve patients were considered for analysis (10 male; 2 female). Median age was 66.5 years (interquartile range, 65 – 73.5). The most prevalent cardiovascular risk factor was hypertension (66.7%), followed by DM (50%), cigarette smoking (41.7%) and end-stage renal failure (25%). Thirteen limbs underwent the study protocol: 2 limbs (15.4%) presented with Rutherford class 4, 9 limbs (69.2%) presented with Rutherford class 5 and 2 (15.4%) with Rutherford class 6. Median ABI on initial evaluation was 0.31 (interquartile range, 0.25 – 0.4). Baseline patient and limb characteristics are summarized in Table 1.

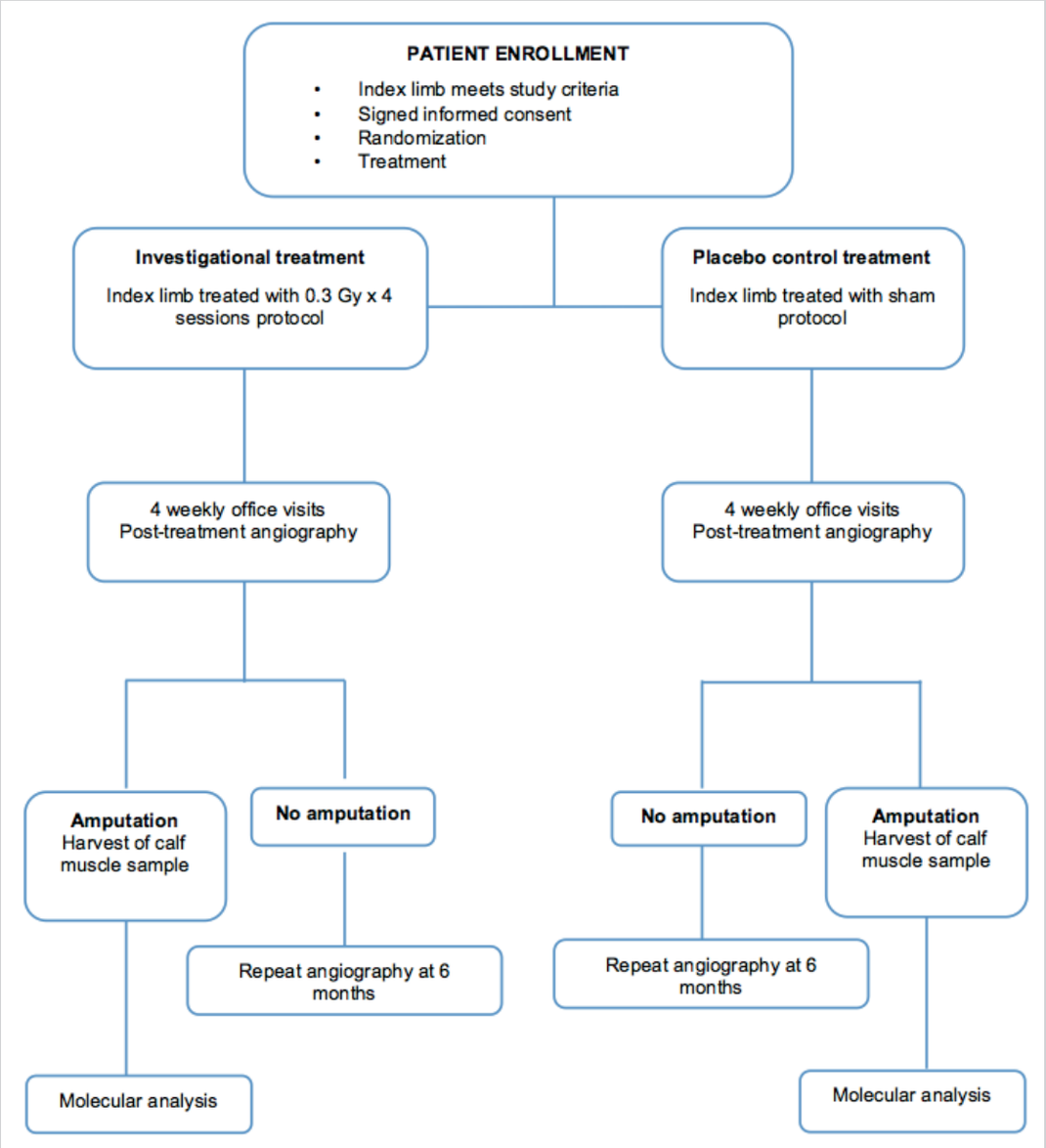

Figure 1. Trial design

Table 1. Baseline patient and limb characteristics

Patient characteristics (n = 12)	
Median age – years (IQR)	67 (65 – 73,5)
Male sex – n (%)	10 (83.3)
Comorbidities	
Hypertension – n (%)	8 (66.7)
Diabetes – n (%)	6 (50)
Smoking – n (%)	5 (41.7)
ESRD – n (%)	3 (25)
Limb characteristics (n = 13)	
Rutherford class – n (%)	
4	2 (15.4)
5	9 (69.2)
6	2 (15.4)
Median ABI (IQR)	0.31 (0.25 – 0.4)
Median Wagner Ulcer Grade (IQR)	1 (1 – 2)
Median Pain scale (IQR)	8 (4 – 9)

Continuous variables are presented as median with interquartile range (IQR; 25^{th} – 75^{th} percentiles). For categorical variables, absolute and relatives frequencies were calculated. Relative frequencies are expressed as percentages (%).

Abbreviations**:** *n, Number; IQR, Interquartile Range; ESRD, End Stage Renal Disease; ABI, Ankle-Brachial Index.*

Five limbs were randomized to receive LDIR and the other 8 underwent the sham protocol. Seven limbs (53.8%) underwent amputation at the end of the protocol. For the other 6 limbs, amputation was deemed unnecessary based on clinical grounds. Median time between the final irradiation session and limb amputation was 14 days (interquartile range, 7 – 28). Four of the amputated limbs and 1 of the non-amputated limbs had received LDIR protocol. Patient flow and limb characteristics by study arm are represented in Figure 2 and Table 2, respectively.

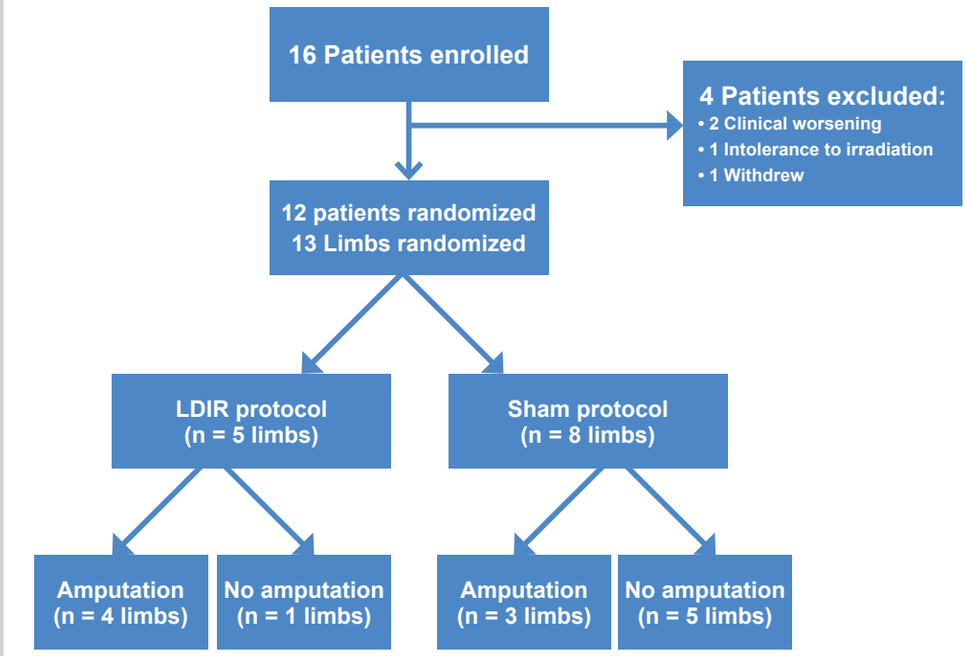

Figure 2. Patient Flow

Abbreviations: *LDIR, Low-dose ionizing radiation.*

All the non-amputated limbs were evaluated in four post-treatment weekly clinical visits, according to the study protocol. Amputated limbs were evaluated in as many post-treatment clinical visits as possible, until limb amputation was deemed necessary. Amputated patients completed on average 1.7 post-treatment clinical visits.

Pre-treatment angiographies were available for all limbs and post-treatment angiographies were available for all, except for 2 limbs: 1 due to clinical deterioration necessitating emergent amputation and 1 due to previous revascularization surgery precluding ipsilateral access.

The clinical trial is still ongoing since the primary endpoint should be achieved with 12 amputated patients. Presently, we have unblinded the initial results for preliminary data analysis.

Table 2. Baseline patient and limb characteristics by study arm

Patient characteristics (n = 12)	**Irradiated (n = 5)**	**Sham (n = 7)**	
Median age – years (IQR)	67 (66 – 78)	66 (65 – 69)	*P = 0.62*
Male sex – n (%)	4 (80.0)	6 (85.7)	*P = 0.68*
Comorbidities			
Diabetes – n (%)	4 (80.0)	2 (28.6)	*P = 0.12*
Hypertension – n (%)	3 (60.0)	5 (71.4)	*P = 0.57*
Smoking – n (%)	2 (40.0)	3 (42.9)	*P = 0.68*
ESRD – n (%)	2 (40.0)	1 (14.3)	*P = 0.36*
Limb characteristics (n=13)	**Irradiated (n=5)**	**Sham (n=8)**	
Rutherford class – n (%)			
4	1 (20.0)	1 (12.5)	*P = 0.64*
5/6	4 (80.0)	7 (87.5)	
Median ABI (IQR)	0.25 (0.2 – 0.4)	0.36 (0.3 – 0.4)	*P = 0.50*
Median Wagner Ulcer Grade (IQR)	2 (1 – 2)	1 (1 – 2)	*P = 0.64*
Median Pain scale (IQR)	8 (4 – 8)	7.5 (5.5 – 9.5)	*P = 0.76*

Continuous variables are presented as median with interquartile range (IQR; 25^{th} – 75^{th} percentiles). For categorical variables, absolute and relatives frequencies were calculated. Relative frequencies are expressed as percentages (%). Categorical and continuous (or ordinal) variables are compared between groups using Fisher exact test and Mann-Whitney test, respectively. A significance level of $P < 0.05$ was assumed for all tests.

Abbreviations: *n, Number; IQR, Interquartile Range; ESRD, End Stage Renal Disease; ABI, Ankle-Brachial Index.*

6.7.1. Effects of LDIR on the expression of pro-angiogenic genes in ECs isolated from ischemic muscle

Irradiated and sham-irradiated gastrocnemius muscle samples were obtained from all amputated limbs. Moreover, non-irradiated samples, herein defined as control samples (0% isodose curve), were obtained for each amputated limb and used as an internal calibrator in the evaluation of pro-angiogenic gene expression in ECs. Muscle sections were stained for CD31 and labeled capillaries were isolated using LCM. Expression of angiogenic genes in ECs was assessed by qRT-PCR.

Due to the limited number of samples, these data were not powered to demonstrate statistical significance, but did demonstrate favorable trends for irradiated limbs versus sham-irradiated ones. Transcript levels for *HGF*, *ANG2* and *VEGFR2* trended toward up-regulation in ECs isolated from irradiated samples, when comparing with the control ones, exclusively in limbs exposed to LDIR. Interestingly, sham-

irradiated limbs demonstrated a trend toward up-regulation in *VEGFR1* expression levels, when compared to irradiated limbs.

The expression levels of the pro-angiogenic factors *FGF2*, *PDGF* and *TGFB2* do not seem different between both study groups (Figure 3). The levels of c-*MET* expression were not detected in all samples.

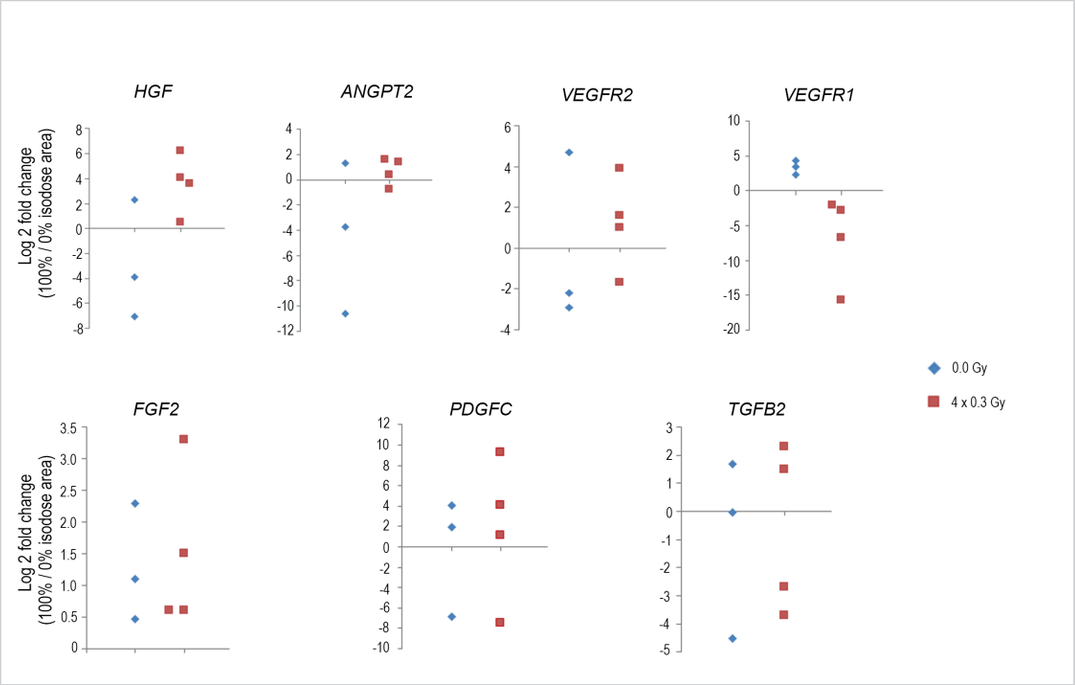

Figure 3. LDIR showed a trend to upregulate the expression of *HGF*, *ANG2* and *VEGFR2* and to downregulate the expression of *VEGFR1*.

Each point represents the relative gene expression in one limb. Blue and red points represent sham-irradiated and irradiated muscles, respectively. Results expressed as log2 fold changes between 100% isodose and 0% isodose samples demonstrated that the expression of *HGF*, *ANG2* and *VEGFR2* was increased in irradiated muscles compared to sham-irradiated, whilst expression of *VEGFR1* was decreased. No difference in expression of *FGF2*, *PDGF* and *TGFB2* was observed in both study groups.

6.7.2. Effects of LDIR on angiogenesis and arteriogenesis

The capillary density, ie, number of capillaries to myocytes, was assessed through quantification of CD31-positive capillaries on histological sections of the gastrocnemius muscle, collected at the moment of the amputation.

Although the limited number of samples did not allow for statistical analysis, our data demonstrated a trend toward a capillary density increase in irradiated limbs, when compared to sham-irradiated (figure 4).

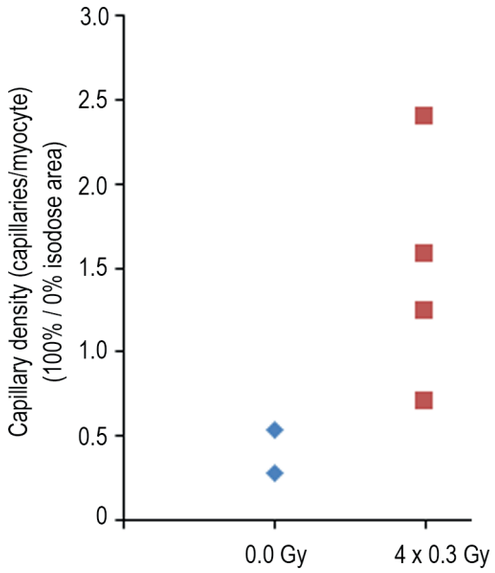

Figure 4. LDIR showed a trend to increase capillary density.
Quantitative analysis expressed as capillary density (capillaries/myocyte) between 100% isodose and 0% isodose samples demonstrated a trend toward an increase in LDIR-irradiated limbs when compared to sham-irradiated ones. Individual data represented by blue and red points represent sham-irradiated and irradiated limbs, respectively.

Angiography images were acquired one month after LDIR exposure, before amputation, to determine VD. With the objective of determining the VD, regions of interest (ROI) were selected for every limb in equivalent anatomic regions pre and post-LDIR exposure, surrounding the area corresponding to the 100% isodose irradiation curve. In order to determine VD, using Adobe Photoshop CS6®, vessels were highlighted excluding trunk vessels and density was calculated in relation to the total area of the ROI, as illustrated in Figure 5. The ratio between post and pre-intervention VD was determined as a measure of change in VD.

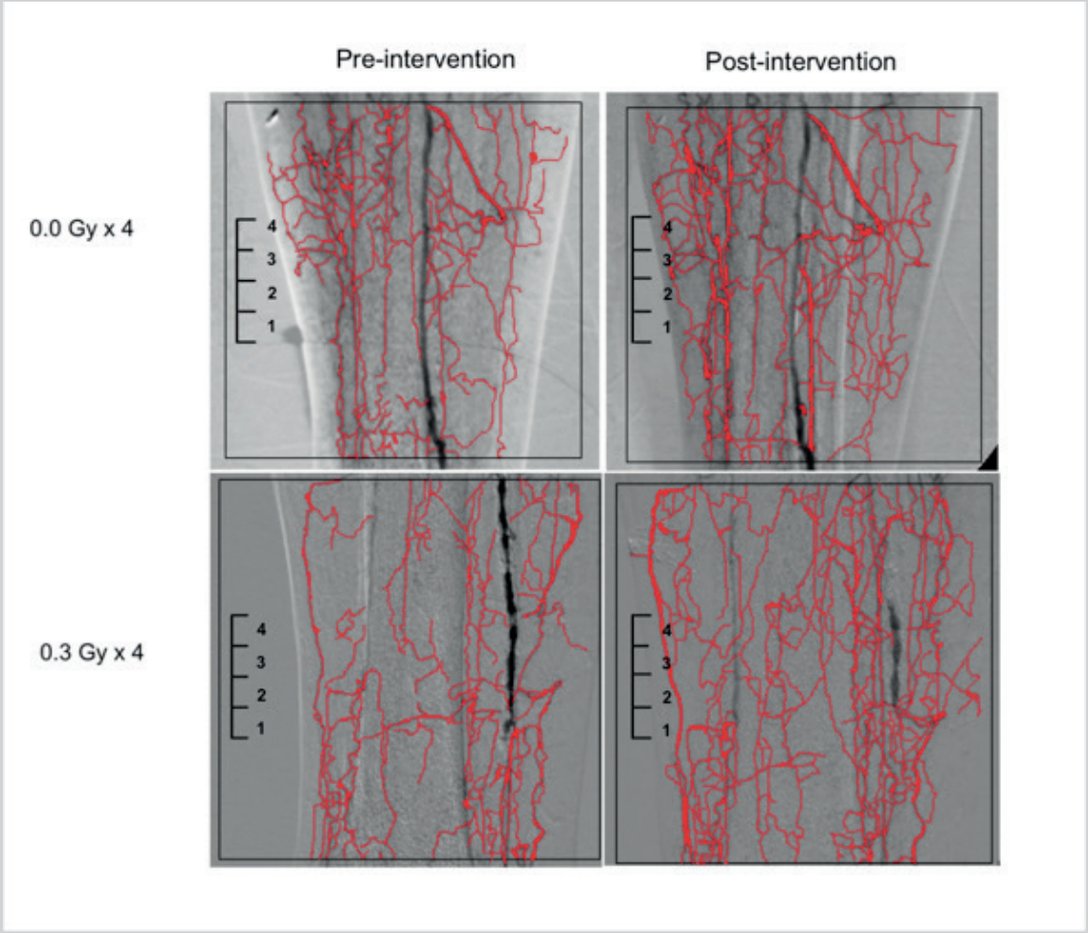

Figure 5. Illustrative angiographic images of selected ROIs from sham-irradiated (0.0 Gy x 4) and irradiated (0.3Gy x 4) lower limbs pre-intervention and 1 month post-intervention are shown. Scale bar 1:1 cm.

Table 3. Numeric data from irradiated and sham-irradiated limbs

Sham irradiated				Irradiated			
CRF	**VD pre-Int**	**VD post-Int**	**VD post-Int/ pre- Int**	**CRF**	**VD pre-Int**	**VD post-Int**	**VD post-Int/ pre-Int**
10	0,06	0,06	1,10	2	0,04	0,05	1,17
11	0,09	0,09	1,06	6	0,06	0,09	1,57
13	0,10	0,13	1,29	8	0,07	0,12	1,70
16	0,15	0,08	0,54	14	0,07	0,14	1,89

Abbreviations: *CRF, Case Report Form; VD pre-Int, Vessel density pre-intervention; VD post-Int, Vessel density post-intervention.*

Angiographies from 3 limbs were deemed of unsuitable image quality and excluded from the analysis. Pre and post-intervention angiographies from 8 limbs (4 irradiated and 4 sham-irradiated) were available for comparison (Table 3).
The limbs exposed to 0.3 Gy during four consecutive days demonstrated a significant VD increase, when compared to the sham-irradiated limbs (*P* = 0.032) (Figure 6).

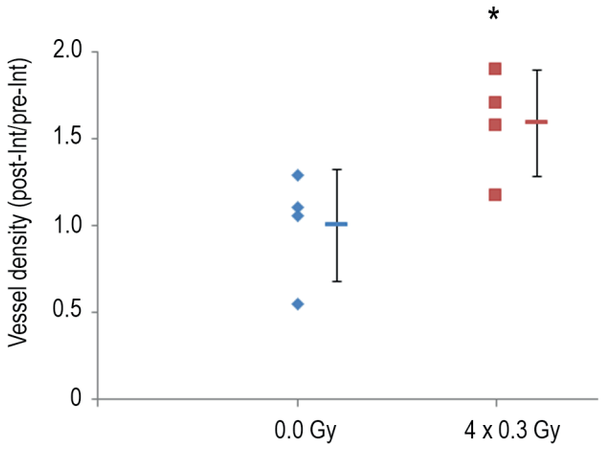

Figure 6. Vessel density (VD) is significantly increased in limbs exposed to LDIR.
Data are represented as the ratio of VD post-intervention to VD pre-intervention for each experimental group. One month post-amputation, irradiated muscles presented a significantly higher VD increase vs. sham-irradiated muscles. Individual data represented by blue and red points represent sham-irradiated and irradiated muscles, respectively and means ± SD (in black) are shown from n=4 muscles per group. **P* <0.05. Two-way ANOVA was conducted followed by Bonferroni post-hoc test with a between-subject factors of day and irradiation

Abbreviations: *VD, Vessel Density; SD, Standard Deviation.*

Since 5 limbs were not amputated as would be expected after LDIR exposure, these limbs were re-evaluated by angiography at 6 months post-intervention. Absolute VD and VD ratio were determined. Although the irradiated group has only one patient yet, our results suggest that only the irradiated limb presents an increase in VD ratio over time (Figure 7).

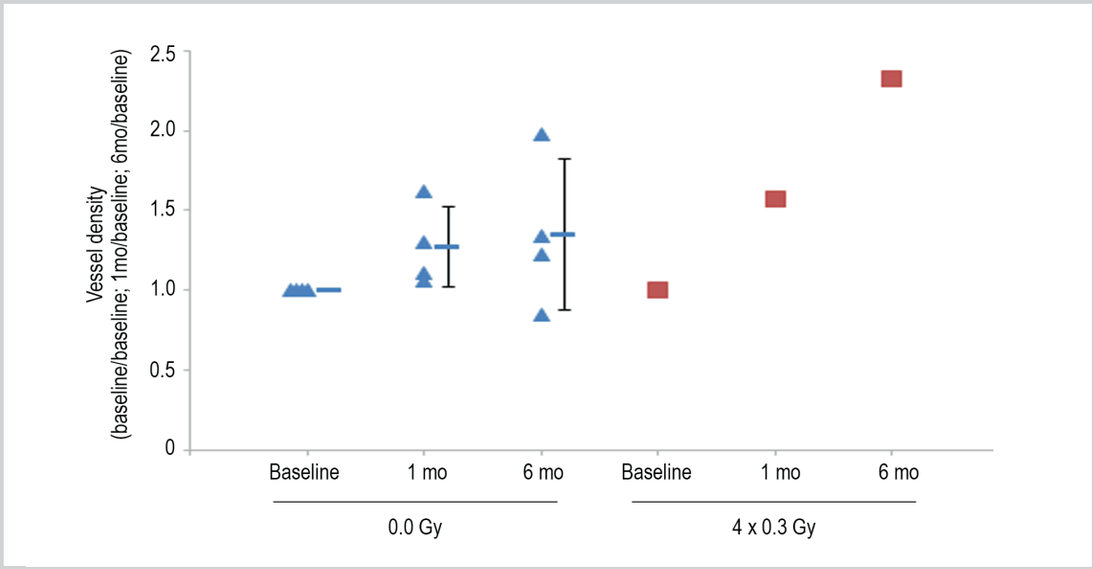

Figure 7. Vessel density (VD) for non-amputated limbs over time.
Data are represented as the ratio of VD baseline, VD at 1 mo post-intervention or VD at 6 mo post intervention relatively to baseline. Individual data represented by blue points represent sham-irradiated muscles and means ± SD (in black) are shown from 3 muscles. Individual data represented by red points represent irradiated muscle (n=1). Only the one muscle exposed to LDIR showed a persistent and continuous increase of VD.

Abbreviations: *VD, Vessel Density; SD, Standard Deviation; mo, month.*

6.7.3. Effects of LDIR on surrogate clinical endpoints of ischemia

With regard to clinical outcomes, no patients in any group achieved abolition of rest pain or complete wound healing. Post-treatment ABI was significantly higher in the overall cohort, when compared with initial ABI ($P < 0.05$). There was no statistically significant difference in ulcer and pain severity. Overall, 35.9% of limbs had clinical improvement according to the recommended scale for gauging changes in clinical status (defined by a downgrading on Rutherford classification or a plus 0.1 improvement in ABI); 30.8% of patients had no difference in clinical status and 15.4% of patients suffered deterioration in at least one of the previous criteria.

When comparing the irradiated and sham-irradiated groups, there was no significant difference in the rate of clinical improvement as gauged by the recommended scale (Table 4). There were, however, no cases of deterioration in the irradiated group, as opposed to a 25% rate of deterioration in the sham-irradiated group, although this difference failed to reach statistical significance. There was also a 2-degree improvement in 16.7% of irradiated limbs, when compared with the sham-irradiated group, but again with no statistically significant difference. Post-treatment ABI,

median score on the pain scale and median ulcer grade on the Wagner scale were also not significantly different between groups. In order to assess the effect of treatment on ABI, the ratio between post-treatment and baseline ABI (rABI) was calculated. Median rABI was higher in the irradiated group, although this difference failed to reach statistical significance.

Table 4. Clinical outcomes by study arm

Clinical outcomes	**Irradiated (n = 5)**	**Sham (n = 8)**	
Recommended scale – n (%)			
-1	0 (0)	2 (25)	*P = 0.54*
0	2 (40)	2 (25)	
+1	2 (40)	4 (50)	
+2	1 (20)	0 (0)	
Median post-intervention ABI (IQR)	0.38 (0.31 – 0.61)	0.44 (0.34 – 0.52)	*P = 0.94*
Median rABI (IQR)	1.5 (1.5 – 2.25)	1.38 (1.19 – 1.58)	*P = 0.30*
Median Wagner Ulcer Grade (IQR)	4 (2 – 4)	1 (1 – 2)	*P = 0.26*
Median Pain scale (IQR)	5 (4 – 8)	7.5 (5 – 9.5)	*P = 0.50*

Continuous variables are presented as median with interquartile range (IQR; 25^{th} – 75^{th} percentiles). For categorical variables, absolute and relatives frequencies were calculated. Relative frequencies are expressed as percentages (%). Categorical and continuous (or ordinal) variables are compared between groups using Fisher exact test and Mann-Whitney test, respectively. Wilcoxon signed-rank test was used when comparing variables before and after intervention. A significance level of P < .05 was assumed for all tests.

Abbreviations: *n, Number; IQR, interquartile Range; ABI, Ankle-Brachial Index; rABI, post-treatment ABI/baseline ABI.*

6.7.4. Adverse Effects

No adverse effects directly related to the irradiation process were observed.

6.8. DISCUSSION

In pre-clinical models, LDIR has shown to enhance neovascularization through endothelial progenitor cells (EPCs) recruitment to ischemic niches, and simultaneous activation of a repertoire of pro-angiogenic factors in a mechanism dependent of VEGFR signaling[10]. In this exploratory clinical trial, we tested the hypothesis that delivery of LDIR would induce the expression of pro-angiogenic genes in ECs collected from ischemic gastrocnemius muscles from “non-option” CLI patients.

The first aspect to consider when analyzing these preliminary results is the fact that this trial is single centered enrolling "non-option" CLI patients, for whom no therapeutic alternative was available other than limb amputation. In fact, un-reconstructable CLI carries a grim prognosis with an amputation rate of up to 40% at 6 months according to TASC II guidelines[13]. Thus, one has to admit that the active intervention – LDIR – may have been used too late in the course of a progressive disease and at an irreversible stage[14], precluding the evaluation of surrogate clinical endpoints of ischemia. Therefore, LDIR therapy was not expected to reduce 1-month amputation rates and 1-month all-cause mortality, or other clinical endpoints of ischemia (ABI, Recommended Scale score, Pain scale, Wagner ulcer grade).

This clinical trial is ongoing and according to the results already obtained it is not possible to demonstrate statistical significance, but our data demonstrate favorable trends for irradiated limbs versus sham-irradiated ones. Concerning the primary endpoint, results have demonstrated a trend toward increased expression of pro-angiogenic factors *HGF*, *ANG2* and *VEGFR2* in irradiated limbs compared to sham-irradiated ones. Furthermore, increased expression of *VEGFR2* was accompanied by down-regulation of *VEGFR1* in irradiated limbs. These data are very interesting, since *VEGFR1*, acting as a decoy for vascular endothelial growth factor (*VEGF*), assumes the role of a negative regulator of *VEGF* activity at the time of angiogenesis[15]. Maynard et al. demonstrated that in adults increased levels of *VEGFR1* harvesting soluble *VEGF* induce ECs dysfunction[16]. These results are in line with previous findings in animal models and support, in humans, the potential pro-angiogenic effect of LDIR through the up-regulation of several pro-angiogenic factors[10].

Besides interfering with ECs activation responses, LDIR also showed to modulate the capillary density, assessed by CD31 immunohistochemistry and the VD, assessed by angiography. At 30 days post-intervention, LDIR is consistently associated with an increase in capillary density. As our previous experimental research suggests[10], this increase may be the result of the up-regulation of the mentioned pro-angiogenic genes. Regarding VD, using post versus pre-intervention ratio as a marker of treatment effect on VD, irradiated limbs showed a statistically significant increase in VD, when compared to sham-irradiated ones ($P = 0.03$). According to our previous data, these new vessels probably originate from mobilized EPCs and, as such, are a result of vasculogenesis[10].

Another important aspect to consider is the fact that, when analyzing non-amputees through angiography at 6 months, the only limb that presented a persistent increase in VD was the irradiated one, suggesting that the effect of LDIR persists over time.

Concerning safety, preliminary results have shown no adverse effects directly

related to LDIR therapy. When addressing ionizing radiation in a peripheral vascular setting, the two main concerns are radiation arteritis and neoplastic disease. In particular, the occurrence of radiation arteritis could offset any potential gains in arresting disease progression and reversing CLI in the long run. It is known that ionizing radiation and cardiovascular risk factors are synergic agents in the development of an accelerated form of atherosclerosis[17,18]. However, radiation arteritis is described only for conventional therapeutic doses of ionizing radiation. On the contrary, LDIR has been used for several decades in the treatment of chronic inflammatory and degenerative benign diseases, namely in single doses of 0.5 or 1.0 Gy[19,20]. Even though the underlying mechanisms are not clear, today it is widely accepted that LDIR may change an inflammatory condition to an anti-inflammatory one[19,21]. Among the clinical indications for LDIR, one can include: skin diseases like psoriasis, necrotizing abscesses, insertion tendonitis, degenerative joint disorders like exacerbated refractory and painful osteoarthritis, impingement syndrome of the shoulder, epicondylopathia humeri, endocrine orbitopathy, painful heel spur or the prevention of heterotopic ossification[22]. Longer follow-up should elicit more definitive conclusions on the long-term safety of this intervention.

Given these preliminary results, some limitations to this trial can already be disclosed: (i) aging and the high prevalence of cardiovascular risk factors in our study population are known to be associated with reduced angiogenic potential; (ii) the small size of the study population, with only a fraction of the initially proposed patient cohort being available for analysis of all outcomes; (iii) the short follow-up period also hinders the ability to draw on more definitive conclusions, specially when addressing clinical endpoints. In addition, further study is needed to determine whether LDIR is beneficial in intermittent claudication or to other patients not eligible for this trial or even as an adjunct to revascularization interventions.

LDIR has a biological rationale widely investigated and discussed in the work developed by our research group[10,11]. Therefore, based on these preliminary results and taking into account that up to 40% of CLI patients are not candidates to revascularization, LDIR may be considered as a novel approach for the management of these patients, and one may even consider that ethically it should be mandatory, whenever possible, to offer this approach to the “non-option” CLI patients.

For the future, we propose to make the study multicentric in order to facilitate the recruitment of patients. Further research is needed to investigate the variability of response to LDIR and its clinical significance extending patient follow-up.

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7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

PAD is a highly prevalent, increasingly recognized and debilitating condition, estimated to affect more than 25 million individuals in western countries[1]. CLI, the end-stage of PAD, is a clinical syndrome in which patients with peripheral obstructive arteriopathy present chronic rest pain and/or trophic lesions in the lower limbs (ulcer or gangrene)[2]. It is estimated that 1 to 3% of patients with PAD develop CLI, and in the United States this number is projected to be of around 160,000 new cases annually[3]. CLI patients commonly present multiple comorbidities, which carry high mortality rates and unbearable costs for the national health systems[4].

Besides the adoption of the best medical treatment, which entails strict control of risk factors for systemic atherosclerosis, the gold standard treatment and the only option for limb salvage in CLI is revascularization. Lower limb revascularization can be carried out either by conventional surgery, percutaneous endovascular intervention or by a hybrid procedure (surgical and endovascular). Taking into account PAD´s complexity, one patient one anatomical disease pattern, the published guidelines and above all factors, the availability of an autologous vein conduit for bypass surgery, a patient-tailored approach is probably the best way for success in treating these patients. Choosing the proper therapeutic option may lead to limb salvage and survival rates above 75% in the first year post-diagnosis. However, in spite of the latest technological advances and the generalization of differentiated postoperative care in the treatment of patients with CLI, mortality in the first year remains close to 25%, with more 30% of patients requiring amputation in the first year post-diagnosis[2]. The 5-year survival rate for CLI patients is stated to be lower than 30%[5,6].

The high surgical risk due to multiple comorbidities, the distribution and the extent of the arterial lesions, mean that approximately 20% to 30% of CLI patients are considered non-candidates for lower limb revascularization procedures. In these patients, amputation is usually the only available therapeutic option. Moreover, individuals with chronic renal failure, as well as those with DM that develop CLI, hold a very poor limb salvage prognosis and it is reasoned by a number of surgeons that primary amputation should be the first therapeutic option for this group of patients[7,8].

In summary, the increasing number of CLI patients, high risk and poor clinical CLI prognosis, failed previous revascularization procedures and the significant number of “non-option” CLI patients, have created a growing need for new therapies to induce neovascularization, as therapeutic angiogenesis, with greater emphasis being placed on gene and cell therapy, focusing on the use of autologous and/or allogeneic molecular and cellular agents to promote neovascularization of compromised ischemic tissues.

Even though preclinical studies and early clinical trials regarding therapeutic angiogenesis presented promising results, single-dose protein administration did not show lasting effects, probably because of the short half-lives of the proteins administered. As a result, gene therapy with DNA plasmids encoding pro-angiogenic factors has been developed in order to increase the duration of transgenic expression as compared to direct administration of proteins. Although these plasmids are well tolerated by the immune system, they usually result in low gene transfer rates with a limited duration of transgenic expression. In conclusion, all strategies aimed at increasing the expression of a unique pro-angiogenic factor have resulted in clinical failure. More recently, cell-based therapies have gained preponderance. However, several questions remain to be properly clarified: (i) acquisition of adequate stem cells; (ii) *ex vivo* expansion efficiency; (iii) optimal administration approach and dose; (iv) *in vivo* differentiation efficiency and functional integration. Among the potential candidate cells, including whole BM cells, BM-MNCs, hematopoietic stem cells, EPCs and hemangiocytes, MSCs due to their (i) good *ex vivo* expansion capacity; (ii) pro-angiogenic/vasculogenic paracrine activity; (iii) transdifferentiation and immunosuppressive capacities and (iv) low immunogenicity, are considered one of the most promising options for cell-based therapies. Bearing in mind the several MSCs sources, the human UC-MSCs are considered a ethical noncontroversial good choice of MSCs for clinical application, because of their non-invasive “painless” collection[9], high cell vitality[10], low immunogenicity[11], and high paracrine potential for accelerating injury tissue repair processes[12].

During this research work, in collaboration with the biotechnology company ECBio, cell-based therapeutic angiogenesis was addressed, as we evaluated the possibility of UCX®, a specific population of human UC-MSCs, to induce therapeutic angiogenesis in a murine HLI model. ECBio developed proprietary technology to consistently isolate, expand and cryopreserve UCX®.

Some of the intrinsic proprieties of UCX®, have been already studied in basic science models of chronic inflammation and cardiovascular disease, positioning them as excellent tools in the management of immune disorders and for tissue repair. Santos et al.[13] have shown that UCX® are immunosuppressive and have low immunogenicity, both very relevant features for allogeneic applications. The authors showed that when compared to BM-MSCs, UCX® more effectively inhibit T cell proliferation and promote Tregs expansion. Accordingly, xenogeneic UCX® administration in both acute carrageenan-induced arthritis and chronic adjuvant-induced arthritis models for arthritic inflammation, can reduce paw edema *in vivo* more efficiently than BM-MSCs and showed faster remission of local and systemic arthritic manifestations, respectively[13].

Furthermore, it was shown that conditioned medium of UCX® cell cultures: (i) induces angiogenesis by promoting the formation of capillary-like structures by HUVECs[13]; (ii) accelerates wound healing and induces fibroblast and keratinocyte migration and (iii) is chemotactic to $CD34^-/CD45^-$ BM-MSCs[14]. Additionally, it was demonstrated in a murine model of myocardial infarction, that UCX® have cardioprotective effects expressed by: (i) promotion of angiogenesis as evidenced by an increase in capillary density; (ii) inhibition of apoptosis in the heart; (iii) activation of the cardiomyogenic gene-expression program in a pool of myocardial resident cardiac progenitor cells[15]. These studies also suggest that UCX® act in different cell types through paracrine mechanisms[13,15]. These unique features of UCX® places them as a potential candidate for cellular therapy in CLI, the overall goal of our work with UCX® cells.

Our current research established that UCX® enhance angiogenesis *in vitro* and *in vivo*, by modulating ECs. We observed and to some extend corroborated that UCX® promote tubulogenesis and ECs migration. When co-cultured with HUVECs, UCX® induce migration and tubule formation by HUVECs to a higher extent than the known pro-angiogenic factor FGF2. Our data suggest that UCX® act in ECs through paracrine mechanisms, since a significant increase of pro-angiogenic factors such as HGF, TGFβ1, IL8 and PDGFAA was observed in the conditioned medium of UCX®, when compared to the control.

Using 22 weeks old female C57BL/6 mice (Charles River, Wilmington, MA, USA), we assessed if UCX® could promote therapeutic angiogenesis in a HLI model. In this experimental setting, the risk of rejection was considered minimal, since UCX® cells were previously xeno-implanted in immunocompetent mice, rats, rabbits and sheep without inducing any significant immunological reaction[13,15].

Intra-muscular injection has been the most commonly administration route used for cellular therapy both in preclinical and clinical studies[16]. Our previous research work established that the intra-muscular injections were safe and again the best administration route for UCX® in a dose of 2×10^5 (unpublished data). Accordingly, perfusion recovery was evaluated over time using laser Doppler imaging, capillary density was measured in the gastrocnemius muscle by CD31 immunohistochemistry, and CVD was measured in the adductor muscle using the diaphonization technique. Our results showed an increment in perfusion recovery in mice treated with $2x10^5$ UCX® at 14, 19 and 24 days post-ischemia, when compared to untreated ones. Moreover, an increase in capillary density and CVD was noted in mice treated with $2x10^5$ UCX®.

These experiments were performed on the 90^{th} day after HLI, suggesting that the effects of UCX® as angiogenesis and arteriogenesis promoters persisted over time. Additionally, we also found that nonischemic muscles were unaffected by UCX®,

since capillary density and CDV are similar when comparing nonischemic muscles of UCX®-treated and control mice, indicating that UCX® might have a local action. These data suggest that UCX® act paracrinally secreting cytokines/chemokines that alter the ischemic microenvironment stimulating therapeutic angiogenesis.

Having established that UCX® promote perfusion recovery, angiogenesis and arteriogenesis, the next step of this work was to determine the mechanisms by which UCX® display these effects; and a new mechanism of action was shown for the first time: UCX® modulate the expression of several pro-angiogenic players in ECs.

The study showed that 70 days after the induction of ischemia and treatment with UCX®, ECs isolated from the ischemic gastrocnemius muscles presented the expression of several proangiogenic factors – *Hgf*, *Fgf2*, *Ang2*, and *Tgβ2* – upregulated, in comparison to the non-ischemic contra-lateral ones. This effect is UCX®-specific as the opposite is verified in control mice. In conclusion and supported by the findings that UCX® simultaneously up regulate the expression of several angiogenic factors in ECs, we may hypothesize that through this mechanism UCX® improve their endogenous angiogenic potency in an ischemic context.

As previously mentioned, with the aim of verifying possible therapeutic effects, UCX® have been experimentally tested in several “clinical settings”. However, to carry out the translational application, a recurrent question with MSCs (and UCX®) is whether the manufacturing process, primarily cryopreservation and thawing, interferes with safety and therapeutic proprieties of stem cells.

Cryopreservation is a process that maintains biological samples in a state of suspended animation at cryogenic temperature for any considerable period and is used to preserve the fine structure of cells[17,18].

Some reports demonstrate that cryopreservation negatively affects the immunosuppressive properties of BM-MSCs in a reversible manner. This is associated with a heat-shock stress response initiated during the thawing process, triggering the instant blood-mediated inflammatory reaction, resulting in faster complement-mediated elimination after blood exposure[19]. Temporary repression of non-constitutively expressed genes during the stress response allows the cells to prioritize cell survival, before recovering their functional properties[20]. A cell culture recovery period is able to restore the immunosuppressive properties of MSCs, including transcriptional indoleamine 2,3-dioxygenase responsiveness to interferon γ and down-regulation of heat-shock proteins expression[20].

However, the belief that cryopreservation negatively affects the performance of MSCs has recently been challenged. Lützkendorf et al. showed that xenogen-free, GMP-grade BM-MSCs cryopreservation had no impact on viability and consensus

criteria of MSCs. In co-culture with PBMCs, MSCs showed low immunogenicity and suppressed mitogen-stimulated proliferation of PBMCs irrespective of cryopreservation. Cytogenetic aberrations were not observed consistently in fresh and cryopreserved products, and no signs of malignant transformation occurred in functional assays[21]. More recently, Cruz et al. *in vivo* analysis verified the potent xenogeneic effects of human BM-MSCs in an immunocompetent mouse model of allergic airways inflammation and established that thawed MSCs are as effective as fresh MSCs[22]. Most importantly, in a phase II clinical study using bone marrow-MSCs in acute graft-versus-host disease in which either cultured or freshly thawed cells were infused, there was no report of clinical differences observed between the two groups[19].

The paradoxes between reports, some suggesting that freshly thawed previously cryopreserved MSCs may not have the same effectiveness or breadth of anti-inflammatory activities as do continuously cultured MSCs, may be explained by differences in tissue source and manipulation during the manufacturing process[23]. Previous studies used BM and adipose tissue-derived MSCs[20,24], whereas the present work reports to UC-MSCs. Results showed that UCX® were less immunogenic and showed higher immunosuppression activity than BM-MSCs. Further, UCX® did not need prior activation or priming to exert their immunomodulatory effects[23].

Our findings showed that cryopreservation had no deleterious effect on the immunomodulatory potential of non-primed UC-MSCs, as seen *in vitro* by the proliferation suppression of PBMCs activated by anti-CD3, anti-CD28 and IL-2, and also no differences were documented in the expression of some functional markers, CD200, CD274, CD273 and CD146 after cryopreservation. Although the immune functionality testing *in vitro* of UCX® cells was only partial, and the impact on cell biochemical responsiveness to inflammatory cues such as interferon γ, TNFα, IL-1 or toll-like receptor agonists were not evaluated, our results support the thesis that UC-MSC immunosuppression activity is independent of priming mechanisms[14,15] and that it is not affected by cryopreservation. Having established that freshly thawed UCX® maintained their functional markers and their ability to suppress activated T cells, we further tested whether freshly thawed UCX® cells showed impaired immunomodulatory therapeutic benefits *in vivo*. For this purpose, a chronic adjuvant-induced arthritis rat model was used over the course of 64 days. The results showed that there was no significant difference between treatments performed with either cultured or freshly thawed cells in any readout measured. In this study, we obtained no evidence of increased apoptosis or changes in surface markers in the freshly thawed population, when compared with the cultured population, and although clearance and engraftment were not directly compared

in vivo, the two conditions showed no differences in therapeutic activity/potency *in vivo*.

Regarding angiogenic potential, the previous results were obtained using UCX® cultured during five days, before the administration in the ischemic hindlimb. In addition, we also compared the effect obtained in perfusion recovery of UCX® in: (i) UCX® administered immediately after thawing and (ii) UCX® administered after 24 hours in culture. Our results suggest that at days 14 and 21 post-ischemia no significant differences were obtained between them. The different experimental conditions present similar levels of blood flow that are significantly different, when compared to the levels of untreated mice. However, at day 7 post-ischemia, UCX® administered after 24 hours in culture did not show significant differences, when compared to untreated mice, in contrast to UCX® cultured during five days before the administration or UCX® administered immediately after thawing. In order to explain these results, we may hypothesize that UCX® thawed and placed in culture during 24 hours are not yet adapted to *in vitro* culture conditions and are not proliferating, in contrast to UCX® cultured during 5 days before administration. Therefore, according to the *in vitro* culture condition, UCX® may present different molecular expression patterns that will differently influence the ischemic microenvironment, contributing to a different response in the first few days after administration. After that period of adaptation to an *in viv*o microenvironment, UCX® that survive could conduct similar biological responses. Concerning the condition where UCX® were administered immediately after thawing, it is possible that the present results are a consequence of the fact that the cells did not have to pass through the adaptation to different conditions in a short time span. This indicates that UCX® cells do not require a recovery period and may be infused immediately after thawing.

Besides cell source, process-related factors that might have contributed to this outcome concern the drastic reduction of tissue mechanical manipulation due to ECBio's specific isolation process. Additionally, ECBio procedure development involved a thorough optimization of the tissue preparation process and digestion parameters that resulted in a neglectful contamination of epithelial, endothelial and subendothelial cells, thus promoting the phenotypic homogeneity of the final population.

The evidence established in this work supports the use of UCX® in ischemic cardiovascular disease. Even though cell-based therapies present a favorable safety profile with a low adverse event rate and a substantial amount of evidence supports the safety of human MSCs in several diseases[25], the use of UCX® in patients with CLI is conditioned by the lack of studies to ensure their safety and the absence of significant adverse effects.

The scope of our work, in search of a new therapeutic approach that could overcome the current lack of available medical treatments for a large number of CLI patients, led us to consider therapeutic angiogenesis beyond its cellular component, in a unique perspective based on the use of LDIR.

Ionizing radiation, in doses that range from 2 up to 8 Gy has been established to promote angiogenesis through the up-regulation of the expression, by tumor cells, of pro-angiogenic factors like IL5, IL4, IL3, TGFβ, IL1Ra, VEGF, FGF, and IL10[26-31]. Since radiotherapy is a widely used therapy of choice for several malignant neoplasms, research has focused on understanding the mechanisms by which therapeutic doses of ionizing radiation activate the tumor vasculature, as well as the role of the irradiated microenvironment in the tumor invasion and metastasis[30-38].

Our research has demonstrated for the first time that LDIR (< 0.8 Gy) induces a pro-angiogenic phenotype in ECs *in vitro,* modulating endothelial dysfunction, promoting survival, migration and preventing ECs apoptosis[39]. Likewise, we showed that LDIR promotes neovascularization *in vivo* by inducing angiogenic sprouting in the transgenic fluorescent zebrafish Tg (fli1:EGFP) embryos and by increasing vessel density in adult fli1:EGFP zebrafish after caudal fin regeneration[39].

These findings were supported by the data obtained from an *in vitro* microarray study, where several transcripts encoding for proteins required for angiogenesis were induced upon LDIR delivery. A global gene expression analysis revealed that 2374 genes were modulated by LDIR and from those, 1344, many of which with a role in angiogenesis, were up regulated in LDIR *versus* non-irradiated HMVEC-L.

The genes that represent the best candidates for a proangiogenic response and whose expression is significantly altered by LDIR were selected – VEGFR1 and VEGFR2, ANG2, TGFβ, PDGF and FGF2. HGF and its receptor, c-MET, were also validated, as the use of HGF has been proposed in the setting of therapeutic angiogenesis, and clinical trials with HGF gene therapy are ongoing. Their expression was validated by qRT-PCR and Western blot using irradiated and non-irradiated HMVEC-L. As soon as 4 hours after exposure to 0.3 Gy, the expression of the majority of the selected pro-angiogenic molecules were increased, and returned to baseline 12 hours post-LDIR. This short-term effect of LDIR on ECs is independent from dose fractionation (and consequently total dose), since cells exposed to 0.3 Gy administered for 2, 3, or 4 consecutive days presented similar gene expression pattern and magnitude.

Additionally, it was found that LDIR activates the endothelium through the phosphorylation of VEGFR2[39], a critical player in the angiogenic process. The activation of VEGFR2 leads to a fast triggering of different cellular proteins and consequently to the *de novo* mRNA and protein expression of mediators involved

in the angiogenic response[40,41]. Consequently, signaling pathways such as PI3K/Akt and ERK/MAPK are activated and gene expression modulated.

Using a murine model of HLI, we aimed at testing an innovative non-invasive strategy, using LDIR to induce therapeutic angiogenesis *in vivo*. We showed that LDIR provides a kinetic advantage, synergizing with HLI and inducing faster recovery from ischemia with significant improvements in perfusion, capillary, and collateral densities, tilting the angiogenic balance towards an even more pro-angiogenic phenotype. In contrast, no effects of LDIR were documented in the resting vasculature, not subjected to ischemia. A common sequel of obstructive arteriopathy is induction of inflammation and oxidative stress, leading to endothelial dysfunction and impaired arteriogenesis, in part through a decrease in both flow-mediated dilatation and outward vascular remodeling[42]. All the concerns involving inflammation and endothelial dysfunction after ischemia and LDIR were also addressed during our research work.

In the experimental model, the acute ischemic stimulus *per se*, created by the femoral ligation and excision, triggers a regenerative response by itself[43]. After surgical induction of unilateral HLI, 22 weeks old female C57BL/6 mice were sham-irradiated or irradiated with four daily fractions of 0.3 Gy in consecutive days and allowed to recover. Unilateral excision of the femoral artery allowed the contralateral limb to be used as a control comparator, although changes in gait or recruitment of spinal reflexes mean that the contralateral muscle is not fully equivalent to unprocessed controls[44,45].

In comparison to the contralateral limb, a dramatic reduction in blood flow was observed in the ischemic limb immediately after surgery and, as expected, a gradual improvement in perfusion was seen overtime. Strikingly, a significant improvement in blood flow recovery was seen in the LDIR group 15 days post-HLI that persisted at 45 days post-HLI, when comparing with control mice. This demonstrates a benefit of LDIR in perfusion recovery, in the setting of HLI ($P < 0.001$).

Even though the advances in radiobiology during the past two decades challenge the validity of the LNT theory suggesting that it overestimates radiation risks[46], or the more recent hormesis hypothesis, the next steep in our work, keeping in mind translation to human medicine, was to determine the lowest dose of ionizing radiation able to induce perfusion recovery. Using the same HLI mouse model, mice were irradiated with: (i) lower number of fractions (1 x 0.3 Gy; 2 x 0.3 Gy; or 3 x 0.3 Gy); (ii) lower dose per fraction (4 x 0.1 Gy); or (iii) higher number of fractions (7 x 0.3 Gy). Sham-irradiated mice were used as a control. No significant enhancements were noticed in muscles irradiated with a lower number of fractions (1 x 0.3 Gy; 2 x 0.3 Gy; or 3 x 0.3 Gy) or with lower dose per fraction (4 x 0.1 Gy), when compared to sham-irradiated muscles. However, we found that blood perfusion recovery was

significantly increased at days 15 and 45 post-ischemia in the ischemic limb of irradiated mice with a higher number of fractions (7 x 0.3 Gy), when compared to sham-irradiated mice. Therefore, our results suggest that temporal dynamics for blood flow recovery, angiogenesis or arteriogenesis may be modulated by different doses of LDIR. These observations accord with previous findings that different doses of LDIR may conduct to different biological effects, since they differently modulate intracellular signaling[39].

Calling upon the same HLI model, histological and molecular analysis of tissue samples was the next step of our research. Although this evaluation required sacrifice of the animals, assessment of capillary density in the gastrocnemius muscle was implemented at several time points. Nevertheless, results are shown at the 15-day post ischemia and, importantly, at day 45-post ischemia, after histological complete muscle regeneration. Capillary density, the number of capillaries per number of muscle fibers, was quantified by staining muscle sections for an endothelial marker (CD31). Interestingly, when comparing irradiated (4 x 0.3 Gy) and sham-irradiated ischemic gastrocnemius muscles at day 45-post ischemia, there was a significant increase in capillary density ($P < 0.001$), suggesting a synergy between LDIR and HLI.

Collateral growth represents the expansive growth of pre-existing vessels[47] and is therefore of utmost importance in ischemic tissue recovery[48,49]. Again, exposing the mice to LDIR (0.3 Gy) or sham-irradiation, during 4 consecutive days after ischemia induction, we evaluated if mice would regenerate with higher CVD. Through the injection of a contrast agent and the use of the diaphonization technique, we were able to visualize in detail the arterial branching and quantify the CVD. The ROI was selected in every mouse in equivalent gastrocnemius anatomic regions, surrounding the ligation site at the thigh and corresponding to 20% of the total limb area. These regions represent the best location to measure collateral vessel growth in response to ischemia, since it was reported that arteriogenesis occurs primarily around the occluded vessel segment[50]. In the present work, we have shown that at 15 and 90 days post ischemia LDIR promotes collateral vessels growth in the adductor muscles of the thigh ($P < 0.001$), when comparing ischemic irradiated muscles to sham-irradiated ones.

After establishing that LDIR acts synergistically with HLI inducing a faster recovery from ischemia, the next step in our research was to determine the mechanism(s) responsible for this kinetic cooperation.

In order to evaluate the expression of angiogenic genes, using LCM microscope, CD31 positive cells were isolated from ischemic, LDIR-irradiated and sham-irradiated gastrocnemius muscles. First, assessing the expression levels of endothelial-specific

transcripts Pecam1 encoding CD31, the Erg and Etv2, we confirmed that these CD 31 positive cells were primarily ECs with negligible amounts of myeloid or perivascular cells. According to our *in vitro* results, several proangiogenic targets (*Vegfr2, Vegfr1, Fgf2, Angpt2, Pdgfc, Tgfb2, Hgf* and *Met*) were shown overexpressed exclusively in the ischemic irradiated gastrocnemius muscles, when compared to the sham-irradiated controls. These findings imply a link between the long-term advantage of irradiated ECs in blood perfusion, capillary density, and collaterals in HLI. LDIR induced a sustained and prolonged pro-angiogenic response in ECs, still evident 45 days after irradiation. Because this contrasts with the transient *in vitro* response, one may hypothesize either that endothelium itself could be differently modulated by LDIR in a hypoxic microenvironment created by ischemia, or that some cells (e.g. adipocytes) could contribute to perpetuate the effect(s) of irradiation in ways that *in vitro* cultures cannot mimic.

Importantly, no effect was observed in the adductor muscles capillary density or gene expression profile, neither in response to ischemia *per se* or after LDIR exposure. Moreover, we showed that the increase in capillary density and the up-regulation in relative gene expression found in irradiated gastrocnemius muscle is abrogated or inhibited by treatment with PTK/ZK, a VEGFR2 tyrosine kinase inhibitor. On the contrary, PTK/ZK has no effect on the increase of gastrocnemius capillary density modulated exclusively by HLI. This is in accordance with the fact that blockage of VEGFR2 activation prevents the ionizing radiation-mediated angiogenic response, as previously reported[51]. Conversely, the CVD induced after LDIR exposure was not affected by PTK/ZK. These results suggest that LDIR induces capillary density and pro-angiogenic gene expression in a mechanism dependent of VEGFR2 activation, but this same mechanism does not explain arteriogenesis.

The ability of EPCs to repair ischemic injuries requires them to first be mobilized from BM in order to be able to migrate into the ischemic region, where EPCs can then differentiate into mature ECs. According to our results, LDIR acts synergistically with HLI, promoting an earlier and greater increase in the number of serum EPCs identified by flow cytometry as VEGFR2/Sca1/CD117 positive mononuclear cells.

Several cytokines were reported as being involved in the guidance of EPCs to ischemic tissue[52-54]. Our published results showed that in hypoxic mimicking conditions LDIR increases ECs VEGF expression[39]. In this work we also showed, not only *in vitro* but also *in vivo*, that *Pgf* and *Csf3* expression is upregulated in ECs and a significant increase in plasma levels of VEGF, PIGF and G-CSF was observed at day 4 after 4 x 0.3 Gy LDIR exposure. The up-regulation of these cytokines, and not only VEGF, might explain why PB EPCs levels are not modulated by PTK/ZK inhibition. Our results showed that PB levels of SDF1 (an important cytokine for EPC

mobilization) are not modulated by LDIR (0.3 Gy), contrary to the work of Lerman et al[55], which showed that a single dose of 5 Gy IR up-regulates SDF-1 through both HIF-1-dependent and independent pathways. Again, our results suggest that the effects of IR are dose-dependent.

In order to evaluate the functional relevance of these LDIR-induced circulating EPCs, we used a transplantation model, in which all hematopoietic cells were GFP^+ (including circulating and extravasated/tissue, leucocytes, erythrocytes, and platelets), but EPCs, forming the inner lining of blood vessels, exhibited both green (GFP^+) and red ($CD31^+$). A quantitative evaluation of the histological sections revealed a significant increased number of $GFP^+/CD31^+$ cells per area in irradiated thigh muscles, when compared to the sham-irradiated ones.

LDIR *per se* does not increase these growth factors concentrations and, notably, the irradiation of the ischemic tissue is critical for the mobilization of EPCs and collateral formation. The relationship between ionizing radiation and the immune system is complex and multifactorial and is conditioned by the dose and quality of the radiation, as well as the type of cell studied. The effects of LDIR on the vasculature are non-linear and cannot be extrapolated from high doses. High-dose irradiation entails both protective and harmful effects, but very low doses (0.025–0.05 Gy), given at a low dose rate, were shown to be protective[56]. In general doses higher than 2 Gy produce pro-inflammatory effects[57]. On the other hand, doses lower than 1 Gy are responsible for the modulation of several inflammatory processes resulting in unequivocal anti-inflammatory properties[58]. The usefulness of radiation therapy, based on the anti-inflammatory properties of ionizing radiation, has been long known. Therapeutic applicability was demonstrated on inflammatory disease as symptomatic improvement of rheumatoid arthritis was observed when mice were irradiated with 0.5 Gy in five fractions within one week[59]. Accordingly, symptomatic improvement was also demonstrated in a mouse model of induced arthritis with irradiation doses of 1 Gy with 5 fractions and 0.5 Gy with 5 fractions, in a mechanism dependent of reduced inducible NOS activity and increased hemoxygenase 1 levels[60].

In our experimental HLI model, the hematopoietic infiltrate was monitored from inflamed and ischemic tissues to assess a potential role of immune cells upon LDIR. Ischemia *per se* induced an increase of about 20 times in the immune $CD45^+$ cell infiltrate recruited to the injured muscle. Exposure to LDIR significantly inhibited the $CD45^+$ cell accumulation with particular effects on monocytes, macrophages and neutrophils. When 2.0 Gy were administered during 4 consecutive days, the total $CD45^+$ accumulation in ischemic muscle was still reduced when compared to sham-irradiated mice, although numbers of monocytes and macrophages (but not of neutrophils) were restored. This is consistent with the fact that high irradiation

doses have opposing effects on certain myeloid subsets, for example they activate macrophages[61] while are reported to induce rapid, but transient, neutropenia[62].
Importantly, the effect of irradiation was short-lasted. Fifteen days post-HLI, the profiles of myeloid cells that infiltrated sham-irradiated and irradiated ischemic muscles were similar. Altogether, these data pointed for a mechanism of LDIR-induced angiogenesis independent of local myeloid cell recruitment and suggest, like previously mentioned, that LDIR may even have anti-inflammatory properties.
One important concern when addressing ionizing radiation is its toxic effects. According to the currently challenged LNT hypothesis, the dose-response is linear and no threshold exists where damage begins to show. Higher doses of IR (2 to 10 Gy) have been used to induce revascularization in several studies[63-65] and, in fact, radiation therapy still continues to be an accepted treatment for benign diseases[66]. However, there is no consensus on the doses described as pro-angiogenic, as different radiation sources are used. The use of conventional radiotherapy doses (2-10 Gy, administered once, Caesium-137 source) has been shown to induce neovascularization in HLI through VEGF release from mast cells and MMP-9-mediated progenitor cell mobilization; however, potential adverse effects were referred[63]. To the best of our knowledge, up to date the use of those high doses has not been proposed for therapeutic angiogenesis.
Here, ionizing radiation was delivered through a linear accelerator producing X-rays photon beam, currently used in the clinical practice. In order to assess long-term toxicity of LDIR we used the same HLI model and LDIR regimen described previously. No difference was seen in weight gain (at weeks 24, 36, 48 and 52 post-HLI) between sham-irradiated and LDIR animals. At week 52 post-HLI, mice were sacrificed and morphological, biochemical and histological parameters recorded. No morbidity or mortality was observed. Urinalysis and blood count, serum biochemistry and coagulation tests results were within normal range for the C57BL/6 mouse strain, for both groups. BM smear and histological sections of skin, lung, spleen, muscle, bone marrow and lymph nodes were analyzed and the few lesions identified were common to all experimental groups, mostly related to ageing and the strain of the mice used[67], and not associated with LDIR. Thus far, and although the possibility of LDIR long-term toxicity cannot be completely ruled out, as genomic instability and non-targeted bystander effects are delayed effects of ionizing radiation, no LDIR-associated toxicological effects were observed. Furthermore, the beneficial effects of such a therapy in a disease with limited life expectancy, as CLI, can overcome its putative negligible or minor toxic effect.
In summary, using a model of experimentally induced HLI, mice were exposed to LDIR and limb perfusion, capillary density and collateral vessel formation were

measured. We show that LDIR (4 x 0.3 Gy) improves limb perfusion by enhancing arteriogenesis through EPCs recruitment to sites of collateral vessel development, an effect dependent on exposure of the ischemic niche to LDIR, but not on the local recruitment of myeloid cells. Likewise, LDIR also favors angiogenesis through simultaneous activation of a repertoire of pro-angiogenic factors in mature ECs in a mechanism dependent of VEGFR signaling, with no short-term side effects and no effects on resting vasculature, opening a possibility to new therapeutic strategies in lower limb vascular insufficiency.

The mechanisms described in our research allow us to overcome some of the most pressing limitations currently pointed to therapeutic angiogenesis and also to think of LDIR as a way of improving microcirculation in association with macrovascular revascularization.

Since the effects of LDIR in CLI patients are unknown, the next step of our work was the development of an exploratory, proof of concept, clinical trial – “Low-dose ionizing radiation modulates the expression of pro-angiogenic genes in Critical Limb Ischemia Patients” – in order to determine the pro-angiogenic effects of this LDIR in CLI patients. The primary objective of the trial was to access if LDIR induces the expression of pro-angiogenic genes in ECs isolated from ischemic muscle. The secondary objectives include: (i) assess whether LDIR stimulates angiogenesis and arteriogenesis; (ii) document the safety and feasibility of LDIR administration and (iii) analyze potential therapeutic benefits in patients with CLI.

Up to date, 16 “non-option” CLI patients were enrolled in the study. Since four patients withdrew/were excluded before completion of the trial protocol, currently only 12 patients, corresponding to 13 limbs, were considered for analysis. As the expected amputation number (12 major limb amputations) for analysis of the primary endpoint was not reached, the trial is still ongoing.

Presently, we have unblinded the initial results for preliminary data analysis. Up to date, five limbs were randomized to receive LDIR and the other eight underwent the sham protocol. Patient and limb baseline demographic and clinical characteristics were similar between groups. Seven limbs underwent amputation at the end of the protocol and for the other six limbs, amputation was deemed unnecessary on clinical grounds. Four of the amputated limbs and one of the non-amputated limbs had received LDIR.

Concerning the primary endpoint, preliminary results show an up-regulation of the pro-angiogenic factors *HGF*, *ANG2* and *VEGFR2* in ECs isolated from ischemic LDIR-irradiated limbs, when compared to the sham-irradiated ones. Also, *VEGFR1*, a known competitor of *VEGFR2* for soluble *VEGF,* is downregulated in this population cohort. These results suggest that LDIR induces a pro-angiogenic effect through the

modulation of several pro-angiogenic factors in ECs collected from “non-option” CLI gastrocnemius muscles.

The primary endpoint is corroborated clinically by the finding that LDIR is associated with an increase in capillary density and a significant increase (P = 0.03) in VD at 1 month post-amputation in muscles irradiated with LDIR, when compared to sham-irradiated ones. It is also interesting to report that in the non-amputated patients, 6 months after irradiation, only the one muscle exposed to LDIR showed a persistent and continuous increase of VD.

Regarding the surrogate clinical endpoints of ischemia, and as expected, no significant differences were found between LDIR limbs and sham-irradiated ones. This is a recurrent finding in several clinical trials in the field of therapeutic angiogenesis[68]. Old patients with multiple cardiovascular risk factors, end-stage CLI patients possibly at an irreversible stage of the disease, short follow-up and clinical endpoints with low specificity may be some of the reasons that justify the absence of clinical response in this study.

LDIR has a biological rationale widely investigated and discussed in the work developed by our research group[39,69]. Therefore, based on these preliminary results and taking into account that up to 40% of CLI patients are not candidate to revascularization, LDIR may be considered as a novel approach for the management of the condition of these patients.

The success of this first clinical trial will lead to the future development of new trials, with the goal of proposing a novel and effective therapeutic tool with a worldwide impact to CLI disease.

7.1. FUTURE PERSPECTIVES

Preclinical and clinical trials on gene and cell therapy of the recent two decades established the proof of principle that therapeutic angiogenesis can be regarded as an alternative for ‘no-option’ patients suffering from vascular disorders, including CLI. However, the remarkable efficiency of therapeutic angiogenesis observed in preclinical models has not yet been translated into clinic trials.

Regarding cell therapy, our research showed that UCX® may be looked at as a promising therapeutic approach for CLI. UCX® significantly induce blood perfusion, capillary density and collateral development. This could be at least in part achieved by a new mechanism, since we show that UCX® up-regulate the simultaneous expression of several pro-angiogenic factors in ECs that could improve their

angiogenic potency. Again, this could be very relevant, when compared to other strategies where only a single angiogenic factor is administered, especially if we understand angiogenesis as a complex process that involves multiple cytokines.

Concerning the manufacturing procedure, in addition to dispensing the need to histocompatible tissue matching, our results show that UCX® have other advantages. UCX® maintains strong immunomodulatory capacity and angiogenic potential, regardless of cryopreservation and do not require priming or a recovery period after thawing, validating them as a viable base technology for the production of allogeneic, off-the-shelf, cryopreserved MSC-based ATMPs with great therapeutic potential. It is of utmost importance to evaluate the safety of UCX® for clinical application.

LDIR may hopefully become a new tool in regenerative medicine, since it activates, simultaneously, several pro-angiogenic factors and also increases EPCs mobilization, improving their function, proliferation and survival features. These mechanisms could be extremely relevant, given the complex and fine-tuned process that represents the formation of a functional vessel network, requiring the constant interplay of several angiogenic factors.

As a future perspective, since the prevalence of DM is increasing, and since DM is one of the main risk factors for CLI, it would be very important to evaluate the efficacy of LDIR in a diabetic mouse model of HLI, as endothelial dysfunction is a main feature of DM.

Finally, the success of the ongoing clinical trial will give us more impute regarding LDIR and CLI as an important clinical discovery worldwide, with major impact in contemporary clinical practice. Increasing the size of the study population, the selection of patients in the less advanced stages and the extension of the follow-up period will allow us to adjust the indication for the use of LDIR in patients with PAD, in order to maximize its therapeutic potential.

Another important aspect to evaluate in the future is the use of LDIR as an adjuvant to revascularization procedures, in order to interfere with microcirculation as an adjunct to the macrocirculation revascularization.

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