João André Sargento Araújo de Freitas

ENDOTHELIAL PROGENITOR CELLS IN NEOANGIOGENESIS OF ACUTE ISCHEMIC STROKE

Tese de Doutoramento no âmbito do Programa de Doutoramento em Ciências da Saúde - ramo de Medicina, orientada pelo Professor Doutor Luís Augusto Salgueiro Cunha e pelo Professor Doutor Lino Silva Ferreira

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part A

Introduction

chapter I

ABSTRACT/RESUMO

Abstract

The treatment of acute ischemic stroke has significantly improved in the last years, with meaningful translation into clinical outcomes. Nonetheless, this evolution has mainly been driven by the development of efficient recanalization therapies that can only benefit a subset of selected patients, leaving the residual lesions deprived of targeted therapies. To address this issue, cell therapies have emerged as potential therapeutic approaches, through the administration of exogenous stem/progenitor cells or the modulation of endogenous cellular populations. An important source of progenitor cells for the treatment of stroke patients are Endothelial Progenitor Cells (EPCs). EPCs are derived from the bone marrow and can be mobilized to ischemic tissues, promoting the development of new vessels. These cells have shown promising effect in preclinical models and observational stroke studies. However, many doubts remain on their exact mechanism of action, integration within stroke pathophysiology and molecular programs.

In this project we used a multidisciplinary approach to understand the interplay between EPC activity and microRNA expression, stroke hemodynamics, blood-brain barrier (BBB) permeability and clinical outcome, ultimately aiming to progress in the inclusion of EPC into stroke treatment.

We included patients with 18-80 years of age and non-lacunar strokes within the territory of a Middle Cerebral Artery in a prospective longitudinal study. All patients were subject to periodical clinical assessments; magnetic resonance imaging (MRI) at baseline and seven days including quantification of BBB permeability through dynamic contrast enhancement; hemodynamic characterization using ultrasound at baseline, seven days and three months. EPCs of participants were isolated from peripheral venous blood at baseline and seven days and submitted to functional assays. The microRNA of a subset of EPCs (CD34+ cells) at admission were sequenced and analysed compared to clinical and neuroimaging data. Clinical outcome was defined according to the modified Rankin scale at three months.

The study population consisted of 45 patients, with a median age of 70.0 years (IQR: 10.0). Our results indicated that the functional properties of EPCs are associated with subacute BBB permeability, development of collateral circulation, subacute cerebral blood flow and clinical outcome. These associations were more evident at seven days than the first 24 hours. Genetic sequencing identified 24 miRNAs of CD34+ associated with good outcome, with a common effect on *adherens* junction pathway. These results established the positive association of EPCs with the angiogenic response after stroke, thus supporting the design of a randomized phase IIa trial to assess the efficacy of CD34+ cells in acute ischemic stroke.

In conclusion, the results obtained in this thesis indicate that EPCs have a substantial role in the pathophysiology of stroke, being associated with improved clinical outcomes and thus warranting their translation into randomized explanatory trials.

Resumo

O tratamento do Acidente Vascular Cerebral isquémico agudo evoluiu significativamente nos últimos anos, com tradução na melhoria de resultados clínicos. No entanto, esta progressão deveu-se essencialmente ao desenvolvimento de estratégias eficazes de recanalização, que conseguem beneficiar apenas um subgrupo de doentes selecionados, deixando a lesão residual sem medicação dirigida. Este vazio terapêutico impulsionou o estudo de terapias celulares, pela administração exógena de células progenitoras/estaminais ou pela modulação de populações celulares endógenas. Uma fonte importante de células progenitoras para o tratamento de AVC isquémico são as Células Progenitoras Endoteliais (EPC). EPCs são células derivadas da medula óssea com a capacidade de serem mobilizadas para tecidos isquémicos promovendo a formação de novos vasos. Modelos pré-clínicos e estudos observacionais afirmaram o seu potencial efeito positivo, contudo persistem dúvidas sobre o seu exato mecanismo de ação, integração na fisio-patologia cerebrovascular e programas moleculares.

Neste projeto utilizámos uma abordagem multidisciplinar para perceber a interação entre EPCs e sua expressão de microRNA com hemodinâmica vascular, permeabilidade da barreira hemato--encefálica e resultado clínico, procurando a integração de EPCs no tratamento do Acidente Vascular Cerebral.

Incluímos doentes entre 18 e 80 anos de idade com Acidente Vascular Cerebral não lacunar no território de uma Artéria Cerebral Média num estudo longitudinal prospetivo. Todos os doentes foram submetidos a avaliações clínicas periódicas; Ressonância Magnética nas primeiras 24 horas e aos sete dias incluindo quantificação da permeabilidade da Barreira Hemato-Encefálica por "*Dynamic Contrast Enhancement*"; caracterização hemodinâmica por técnicas ultrassonográficas nas primeiras 24 horas, sete dias e três meses. As EPCs dos participantes foram isoladas a partir de sangue venoso periférico com avaliação posterior das suas capacidades funcionais. Os microRNA das células CD34+ na admissão foram sequenciados, comparando com dados clínicos e neuroimagiológicos. O resultado clínico foi definido pela escala modificada de Rankin aos três meses.

A população de estudo incluiu 45 doentes com idade mediana de 70.0 anos (IQR: 10.0). Os nossos resultados indicaram que as capacidades funcionais das EPCs estão associadas à permeabilidade da Barreira Hemato-Encefálica e fluxo sanguíneo cerebral em fase subaguda, circulação colateral e resultado clínico. Estas associações foram mais evidentes sete dias após a instalação sintomática. A sequenciação genética das células CD34+ identificou 24 microRNAs associados a bom resultado clínico, partilhando a modulação da via das junções *adherens.* Estes resultados permitiram estabelecer a associação positiva entre EPCs e o desenvolvimento de neoangiogénese após Acidente Vascular Cerebral isquémico, servindo de suporte ao desenho de um ensaio randomizado de fase IIa para avaliar a eficácia das células CD34+.

Em conclusão, os resultados obtidos nesta tese indicaram o papel relevante das EPCs na fisiopatologia da doença cerebrovascular isquémica, mostrando a sua associação a melhores resultados clínicos e justificando a sua translação para ensaios randomizados explanatórios.

List of abbreviations

ACA	Anterior Cerebral Artery
AIF	Arterial Input Function
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB	Blood-Brain Barrier
CAC	Circulating Angiogenic Cell
CBF	Cerebral Blood Flow
CFU-EC	Colony Forming Unit – Endothelial Cell
CNS	Central Nervous System
CRP	C Reactive Protein
CSF	Cerebrospinal fluid
DALY	Disability-Adjusted Life Year
DAPI	4',6-diamidino-2-phenylindole
DCE	Dynamic Contrast Enhancement
EBM	Endothelial Growth Basal Medium
EDTA	ethylenediamine-tetra-acetic acid
EGM	Endothelial Growth Medium
EPC	Endothelial Progenitor Cell
FBS	Fetal Bovine Serum
GRE	Gradient Echo
ICA	Internal Carotid Artery
MCA	Middle Cerebral Artery
MNC	Mononuclear Cell
mRS	Modified Rankin scale
MSC	Mesenchymal Stem Cell
NIHSS	National Institute of Health Stroke Scale
NMDA	N-methyl-D-aspartate
oEPC	Outgrowth Endothelial Progenitor Cell
PCA	Posterior Cerebral Artery
PH	Parenchymal Hemorrhage
ROI	Region of Interest
SC	Stem Cell
TCCD	Transcranial Color Coded Doppler
TOAST	Trial of Org 10172 in Acute Stroke Treatment
USA	United States of America
VA	Vertebral Artery

THESIS OUTLINE

The thesis is divided in four parts:

Part A presents the abstract, lists the scientific publications arising from this thesis and introduces the scientific background that allows integration and interpretation of the present research. Here we have described the basic stroke concepts, with a particular focus on therapeutic gaps, fundamental notions on cell therapies, Blood-Brain Barrier and angiogenesis.

Part B summarizes the hypothesis, research aims and methodology used in this thesis.

Part C consists of the experimental work generated from the thesis and comprises three chapters, each corresponding to an original article. They are presented as the final accepted manuscript. Chapter VIII approaches the association between Endothelial Progenitor Cells, clinical outcome and Blood-Brain Barrier permeability; chapter IX addresses the impact of Endothelial Progenitor Cells on stroke hemodynamics in the acute, subacute and chronic phases; in chapter X the study protocol for STROKE34, a randomized phase IIa clinical trial is presented.

Part D describes the main conclusions of the present thesis as well as future opportunities opened by this work.

References of all chapters are listed together in chapter XIV valuing numerical order of appearance to facilitate consult.

DISSERTATION PURPOSE STATEMENT

Stroke is a potentially devastating and widely prevalent cause of both mortality and morbidity, representing one of the major global health concerns[1]. In 2013, the worldwide prevalence of stroke was 25.7 million with 31% of new strokes impinging people under 65 years of age[2,3]. Notwithstanding the devastating nature of severe fatal strokes, accounting for approximately one third of strokes, another third survive with life-long disability[4].

Pathologically, stroke involves a heterogeneous group of processes that can affect people at any age from fetal life until the elderly, with any genetic or environmental framework. In acute stroke management the only variably successful treatment strategy for salvaging ischemic tissue and improving functional outcome is reperfusion. In recent years, the dramatic evolution of advanced recanalization therapies has shown promise, however, their outcomes are still surpassed by the narrow therapeutic time window and quandaries of patient selection, rendering only up to 5 percent of patients treated, even in developed countries[4–8].

Neuroprotection has been an idealistic goal of innumerable treatments and albeit many successes in experimental models, no neuroprotective strategy has demonstrated unequivocal efficacy in clinical trials[9,10]. More recently, a novel therapeutic goal has been pursued, aiming at endogenous tissue repair and remodeling[11,12]. The pathophysiology of this process is highly heterogeneous in time and space and classical pharmacological strategies are widely considered to be of no further consequence in these events. This scientific disappointment has recently fostered special consideration to cell base therapies[13–15].

Stem and progenitor cells have the capacity of self-renewal and differentiation into one or more cell types, and have been reasoned with potential benefit not only for ischemic insult but also for multiple sclerosis, traumatic brain injury, spinal cord injury and some neurodegenerative conditions[15,16]. Amidst them, adult progenitor cells have received particular interest accounting for their intriguing physiological presence in the healthy, fully-developed human and their ability of mobilization into the injured tissue. However, investigators have struggled with the bulky task of cell consignment in the inhospitable ischemic environment of the infarct core, yielding strategies aiming to restore neural circuitries at least problematic. Nonetheless, recent scientific enthusiasm has emerged from the study of endothelial progenitor cells (EPCs), bone-marrow derived cells, circulating in peripheral blood that are capable of differentiating into mature endothelial cells to aid endothelial recovery and neoangiogenesis[17–20]. Some previous data suggests that increased number of EPCs in acute stroke might be associated with better outcomes, unaccounting for different genetic profiles of EPCs, therapeutic mechanisms, locations or hemodynamic evolution[21,22].

In this thesis, an integrated and multidisciplinary approach has been used, addressing some of the key clinical doubts on the effect of EPCs in angiogenesis and stroke, ultimately leading to build up their potential integration within treatment algorithms.

PUBLICATIONS ARISING FROM THIS THESIS

Endothelial progenitor cells enhance blood-brain barrier permeability in subacute stroke.

Sargento-Freitas J, Aday S, Nunes C, Cordeiro M, Gouveia A, Silva F, Machado C, Rodrigues B, Cordeiro G, Ferreira C, Amorim A, Sousa S, Gomes AC, Castelo-Branco M, Ferreira L and Cunha L.

Neurology; 2018 Jan 9;90(2):e127-e134.

Endothelial Progenitor Cells influence acute and subacute stroke hemodynamics.

Sargento-Freitas J, Aday S, Nunes C, Cordeiro M, Gouveia A, Silva F, Machado C, Rodrigues B, Santo G, Ferreira C, Castelo-Branco M, Ferreira L and Cunha L.

Journal of the Neurological Sciences; 2018 Feb 15;385:119-125.

STROKE34 study protocol: A randomized controlled phase IIa trial of intra-arterial CD34+ cells in acute ischemic stroke.

Sargento-Freitas J, Pereira A, Gomes A, Freire P, Matos T, Cardoso C, Silva F, Cordeiro G, Nunes C, Galego O, Carda J, Branco J, Lourenço V, Cunha L and Ferreira L.

Frontiers in Neurology, 2018 May 7; 9(302):1-6.

chapter V

BACKGROUND

A. Basic stroke concepts

i. Epidemiology

Stroke remains a major global health problem and its significance is likely to increase in the future due to ongoing demographic changes, including aging of the population and health transitions observed in developing countries[2,23]. Estimates from the *Global Burden of Diseases, Injuries, and Risk Factors Study* ranked stroke as the second most common cause of death and disability worldwide[3,24,25].

The evolution in burden of stroke and its incidence over the last five decades has indicated interesting but troublesome findings with large disparities. In fact, the age-standardized incidence rates of ischemic and hemorrhagic strokes have decreased significantly in high income countries (13% reduction for ischemic and 19% for hemorrhagic strokes)[26]. Even more expressive is the reduction in mortality (37% reduction for ischemic and 38% for hemorrhagic strokes) probably translating the revolution in stroke care[5,6,26]. By contrast, low and middle income countries have witnessed a significant increase in hemorrhagic stroke incidence (22%) and a non-significant increase for ischemic stroke (6%). An alarming evidence is that the rates of disability and mortality associated with stroke are at least 10 times greater in medically underserved regions of the world compared with the most developed nations[27]. For ischemic strokes, the highest mortality rates (124 to 174 per 100.000 person-years) were reported in Russia and Kazakhstan, with the lowest (at or below 25 per 100.000 person-years) observed in Western Europe, North and Central America[28]. Most of the causes of these disparities have been identified, mainly as a consequence of scarce primary care treatment to screen and control vascular risk factors, difficulty in access to basic diagnostic tools and treatments, together with the absence of poststroke rehabilitation, follow-up and secondary prevention[1,27,29].

Data from the north of the country rank Portugal as having an high stroke incidence, with an annual rate of 2.13 per 1.000 for first-ever-in-a-lifetime ischemic strokes[30]. The evolution across the last two decades seems to follow world-wide tendencies with a reduction of disabling strokes by 29% and fatal strokes by 46%; intracerebral hemorrhage decreased by 51% and the risk of disability after stroke by 11%[31]. These continuous reductions classified Portugal as having a low age-standardized death rate of cardiovascular diseases in the 2015 Global Burden of Disease study[25].

The unadjusted epidemiological rates show a somewhat different global perspective. In fact, the prevalence, mortality and Disability-Adjusted Life Year (DALY) lost has nearly doubled from 1990 to 2013 for both ischemic and hemorrhagic stroke[2,32]. This apparent inconsistency is clearly driven by the rapid global epidemiological change, particularly the increase in life expectancy, amplified by the steep increase in stroke incidence with age[4,23,33,34].

Possibly the most disturbing feature of cerebrovascular events is long-term disability. In 2013 there were almost 25.7 million stroke survivors (71% with ischemic stroke), and 113 million DALYs

lost due to stroke (58% due to ischemic stroke)[2]. Moreover, when analysing epidemiological rates across age strata a decrease in age-adjusted incidence is evident in participants age 65 years and older (absolute decrease of 1.35 per 1000 person-years) but not evident in participants younger than 65 years[35], reinforcing the need for effective primary prevention.

Altogether, epidemiological data show that in the past 25 years there was a significant decrease of age-adjusted cerebrovascular indexes in regions with very high income, but only a gradual decrease or no change in most world regions. These partially positive data from adjusted rates are dampened by the ongoing demographic progress of population aging implying continuous increase in total numbers and urging policymakers to focus on achieving specific global health targets.

ii. Physiopathology

Ischemic stroke is the quintessential manifestation of a vascular cerebral occlusion, induced by either in situ thrombosis or embolization of a clot from a proximal arterial or cardiac source. This pragmatic but simplistic definition hides an intricate chain of events, involving a plethora of organs, tissues and cell types, from the cardiovascular and hematological systems, through the entire nervous system and often directly or indirectly encompassing all other human tissues.

The first pathological event is vessel occlusion. It should be noted that in a broader stroke definition, it may occur without arterial occlusion: as in the case of global brain ischemia secondary to cardiac arrest, or focal ischemia in the context of a venous infarction. However, this thesis will focus only on the more recent definition of ischemic stroke as an episode of neurological dysfunction caused by focal cerebral, spinal, or retinal arterial infarction[36]. In this context, the pathological cascade is triggered by the mechanism underlying the arterial occlusion, which can simplistically occur due to migration of an embolus from a cardiac or proximal arterial source, in situ thrombosis or extracranial arterial steno-occlusive disease with downstream cerebral ischemia. In a practical attempt to systematize etiological mechanisms many classifications have been developed, all with advantages and limitations, nonetheless, one remains as the most widely used in clinical and investigational settings for adult patients: TOAST (Trial of ORG 10172 in Acute Stroke Treatment). Common to TOAST and other classifications (such as ASCOD and Causative Classification System) is the recognition of the three main causative determinants: cardioembolism, large artery atherosclerosis and small vessel disease[37–39]. A previous study from our group among 631 admitted for ischemic stroke during 2010 identified cardioembolism as the main local stroke etiology, causing 34.5% of strokes of which 91.3% were due to non-valvular atrial fibrillation (Figure V.1).

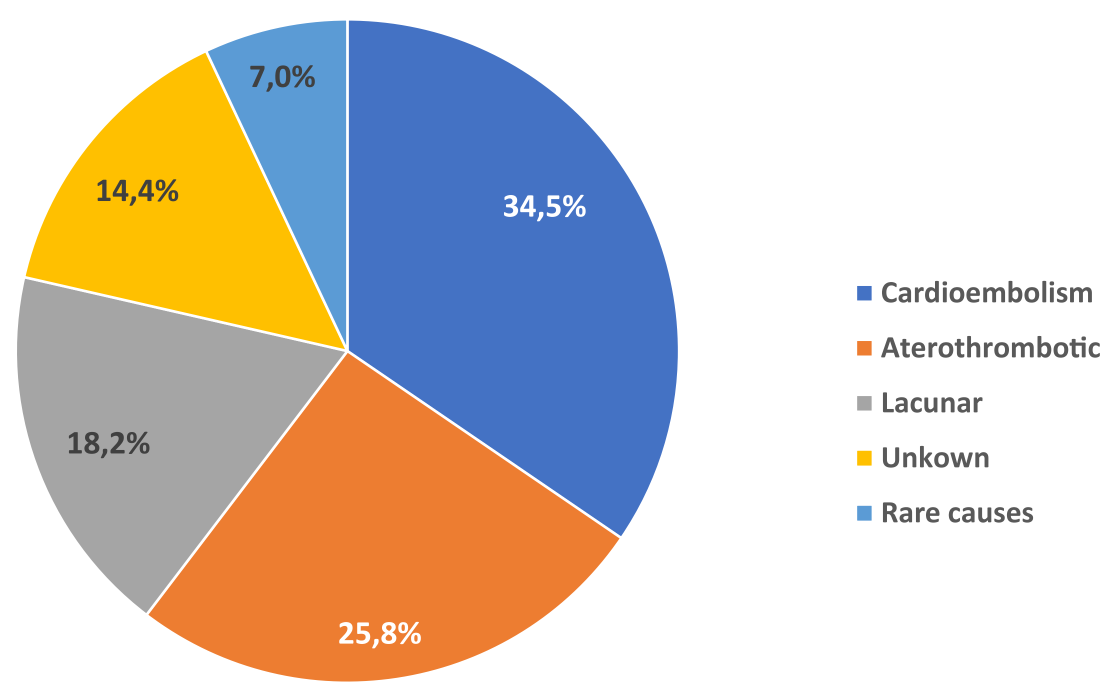


Figure V.1. Distribution of ischemic stroke etiologies according to TOAST classification from patients admitted in Centro Hospitalar e Universitário de Coimbra during the year of 2010.*Adapted with permission from Sargento-Freitas et al. 2013.*

Arterial occlusion offsets cerebral hypoperfusion but the level of flow reduction required to precipitate ischemia is difficult to define, and probably dependent on individual, anatomic and energetic local conditions. Perfusion studies with neuroimaging indicate that <30% blood flow may define the ischemic core; however it shows only a sensibility of 64% and 76% specificity for irreversible lesion suggesting that this threshold is neither necessary nor sufficient[40]. In fact, a state of zero flow is rarely (if ever) present in focal cerebrovascular lesions, reinforcing the complex multitude of factors influencing neuronal injury[41].

A myriad of pathological events follow arterial occlusion that were traditionally thought of in cascade, but are now known to overlap and mutually potentiate themselves. These include cellular bioenergetic failure, excitotoxicity, oxidative stress, blood-brain barrier dysfunction, microvascular injury, hemostatic activation, inflammation, and eventually tissue necrosis[42] (Figure V.2).

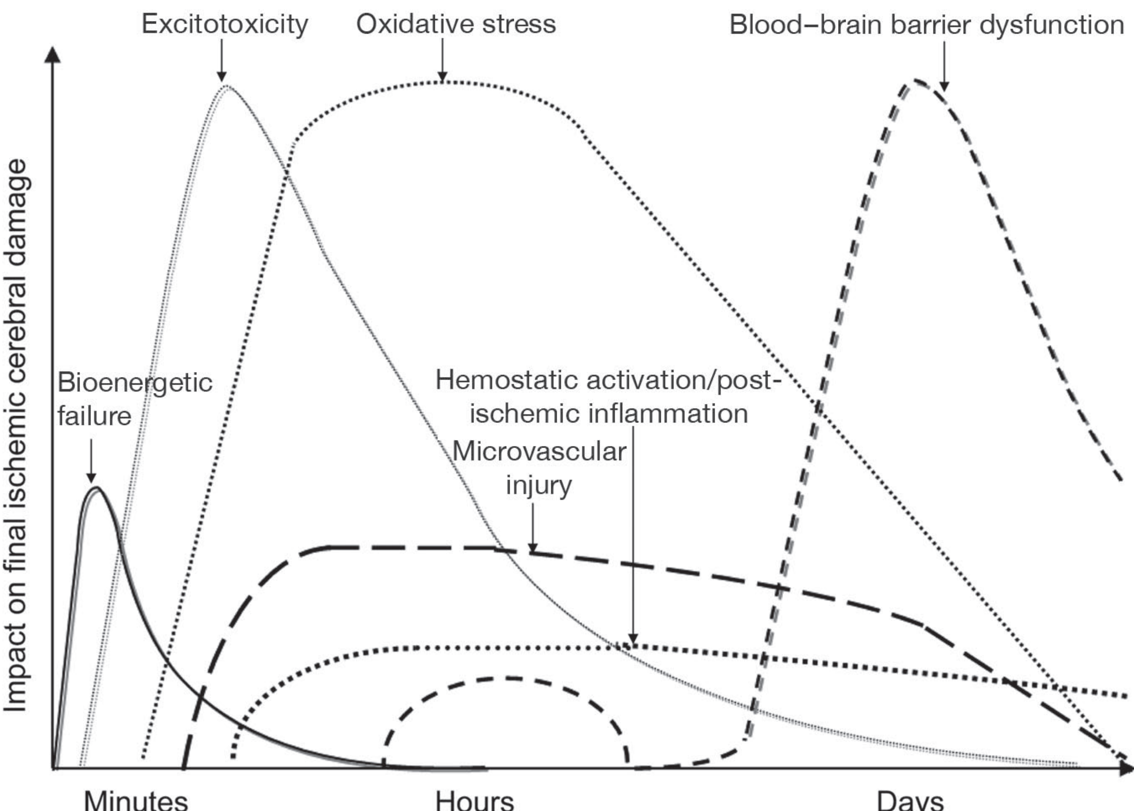


Figure V.2. Pathological mechanisms involved in ischemic stroke injury over time. *Reproduced with authorization from Saenger et al. 2010* [42]

One of the first cellular structures to suffer from deprivation of glucose and oxygen is the highly active and ATP-dependent sodium-potassium pump. Failure of this ion pump leads to depolarization and passive diffusion of Na+ ions inside the cells creating a concentration gradient that is accompanied by large fluid volume[42–44]. These events cause cytotoxic edema and restrict intracellular water diffusion that can be detected in neuroimaging exams. Depolarization activates somatodendritic as well as presynaptic voltage-dependent Ca2+ channels, releasing excitatory amino acids, especially glutamate, aggravated by the impairment of its presynaptic reuptake. Excitotoxic amino acids have deleterious effects directly (such as activation of NMDA and AMPA glutamate receptors) and indirectly, by promoting calcium influx thus activating intracellular enzymes (such as protein kinase C, phospholipase A2, phospholipase C, cyclooxygenase, nitric oxide synthase and calpain) causing potentially irreversible mitochondrial damage and cell death[45–47]. Metabolic cell impairment and excitotoxicity release oxygen radicals that will further perpetuate cellular lesion through lipid peroxidation membrane damage, additional dysregulation of cellular processes, genome damage and immune activation[48–50].

Immune system responds in parallel with Central Nervous System (CNS) cell injuries, and in its own turn, potentiating them. It is offset within a few hours after insult by two main mechanisms: expression of intercellular adhesion molecule–1 or vascular cell adhesion molecule–1 by the injured endothelial cells, which activates circulating immune cells to transmigrate to the brain, losing its immune privilege, and activation of CNS immunoeffector cells by the ongoing cellular

injury and oxygen radicals[51,52]. This often uncontrolled immune response has been recognized as a main pathological substrate of cell injury prolonging the insult.

Stroke hemodynamics and blood-brain barrier (BBB) properties exhibit a multiphasic evolution during the different temporal and pathological stages of stroke with antagonic effects. Their complexity, importance and relevance for the present study plan warrant an approach in dedicated sections of the thesis.

iii. Stroke hemodynamics

Ischemic stroke is a hemodynamic event in its essence. Notwithstanding the significant advances in the understanding of the molecular machinery involved in cellular and intercellular injury and resistance, the pathological hallmark remains a vascular occlusion and the only clinically efficient strategy in the acute stage is recanalization.

In response to the acute arterial occlusion the main hemodynamic responses relate to the development of collateral circulation in the attempt to preserve cerebral perfusion and autoregulation. The cerebral collateral circulation refers to the subsidiary network of vascular channels that stabilize cerebral blood flow upon the occlusion of the principal anatomic conduits[53]. The establishment of a good collateral circulation enables tissue viability even in the absence of recanalization. This has a number of additional positive effects, namely the increase in recanalization rates (both for intravenous and intra-arterial therapies), decreasing infarct growth and hemorrhagic transformation[54–59].

Cerebral autoregulation is simplistically defined as the capacity to maintain cerebral blood flow in spite of variations in systemic blood pressure. It involves a synergic action of all the constituents of the neurovascular unit (endothelial cells, pericytes astrocytes and neurons, in intimate connection with immune cells; described in greater detail in section V.D.) providing instantaneous hemodynamic adaptation to variations in blood pressure, both in case of spontaneous physiological fluctuations and in response to injury. In the context of ischemic stroke, autoregulation impairment is common in the symptomatic arterial territory and has been associated with unfavourable clinical outcome, larger lesions and hemorrhagic transformation[60–62]. Another possible clinical and hemodynamic manifestation of impaired vasomotor response is intracranial arterial steal phenomenon. Following descriptions of its importance in arteriovenous malformations, tumours and extracranial arterial pathologies (such as Subclavian Steel Syndrome) it was more recently described in the context of ischemic stroke[63]. In brief, the hemodynamic phenomenon relates to paradoxical decreases in flow velocity of vessels supplying ischemic brain during episodes of hypercapnia accompanied by velocity increase in nonaffected vessels[63,64]. The steal magnitude was linked to severity of neurological worsening in patients with acute stroke, leading to the "Reversed Robin Hood syndrome".

Arterial reocclusions were a common finding in the era of isolated intravenous thrombolysis, with reported rates of up to 61%, assuming the main cause of clinical deterioration following initial improvements[65,66]. The principal contributors to this phenomenon were the short half-life of alteplase, thrombus length, and the presence of severe proximal extracranial pathology[65,66]. These rates were dramatically reduced to below 5% with the instalment of thrombectomy as a treatment option, however, it still remains a matter of concern, particularly after partial recanalizations and intracranial stenosis.

Albeit the undisputed clinical merits of recanalization therapies, abruptly restoring flow to an irreversibly injured tissue may paradoxically exacerbate the insult, triggering a chain of hemodynamic and biological mechanisms[67,68]. It is usually mediated by a loss of cerebral autoregulation, with a sharp increase in blood pressure leading to tissue necrosis and inflammatory activation[69]. Symptomatic presentation may include headache, epileptic fits and neurological deterioration due to cerebral edema or hemorrhagic transformation.

Stroke remains an eminently hemodynamic event with most pathological events directly or indirectly linked to the vascular insult. Notwithstanding, its effects are limited in the timecourse of a disease that will frequently render lifelong consequences. Moreover, knowledge of the accompanying molecular machinery and cellular responses have greatly advanced, shedding new research avenues in the pursuit for an holistic therapeutic approach.

iv. Current treatment options: where we are and what is missing

The treatment of acute ischemic stroke has been substantially improved in the last years. In fact, clinical algorithms have evolved from a nihilist inspection of pathological events with a feeble hope on minor salicylates' effects, to advanced neuroimaging techniques and devices with overwhelming impact on selected patients. Nonetheless, even in the current era, there are still many patients left with severe sequela, mostly due to presentation outside an efficient treatment window for recanalization therapies or even failure of these techniques in a residual number of patients. A conceptual construct on the therapeutic approach to ischemic stroke may be divided into three stages: hyperacute, acute/subacute and chronic.

In hyperacute stroke management the only successful treatment strategy for salvaging ischemic tissue and improving functional outcome is reperfusion. Notwithstanding the increasing efficacy of contemporary recanalization therapies, the overall impact on the outcome of stroke patients is still surpassed by the relatively narrow therapeutic time window and quandaries of patient selection.

Acute/subacute stroke care also encompasses the comprehensive approach of stroke unit attendance, a holistic methodology aimed at providing optimal circumstances for recovery, including stabilized and personalized hemodynamics, dedicated management of complications and perma-

nent surveillance. As a whole, stroke unit care has been demonstrated to have a positive effect in clinical outcome, but still far from solving all unmet needs[70,71].

In the late subacute to chronic stages, functional recovery is a main focus. Personalized rehabilitation techniques have greatly evolved and can now deliver significant results, albeit their moderate effect size, particularly in patients with large residual lesions.

Stroke prevention is another field with important recent developments and of pivotal clinical importance. Guided by the accurate diagnosis of stroke etiology and vascular status of each specific patient a multi-targeted therapeutic plan is vital, including, but not only, thromboprophylaxis and management of risk factors adapted to the patients' specific needs at a given timepoint of evolution.

In summary, current therapies aim at minimizing ischemic lesion by early recanalization in appropriate candidates, enhancing the residual functional status left after the sequelar lesion and prevention of recurrent events. It should be noted, that under optimal circumstances, these combined approaches can have a dramatic effect in the overall clinical outcome. Furthermore, additional developments are expected in the near future with improvement of recanalization therapies and extension of their indications; newer rehabilitation technology, as well as better and more appropriately used prophylactic treatments. However, all stroke community still recognizes limitations, particularly by leaving the cellular lesion left to scar, devoid of any efficacious and directed therapy.

B. Cell therapies in Stroke

i. Overview on cell therapy concepts

In the overwhelming field of stem cell biology, the first and somewhat controversial concept is the definition of a stem cell (SC)[72]. The most accepted definition is that SCs are undifferentiated cells that retain the ability to continuously divide throughout life giving rise to unchanged descendants as well as progenitor and then specialized cells that are able to take the place of cells that die or are lost[73–76]. Two main properties thus define a SC: the capacity for self-renewal and to differentiate into specialized cells. This definition tries to categorize a continuum in cell biology outlined by genetic and epigenetic phenomena, ranging from undifferentiated, self-renewing cell populations to fully differentiated non-proliferative cells. A progenitor cell is generically defined as any diving cell with the capacity to differentiate, representing an intermediary state between SC and mature cells, either with undemonstrated or limited capacity for self-renewal[76]. To avoid misuse of these terms we will use the recommended label of stem/progenitor cell to collectively designate these cell populations[72].

The potency of stem/progenitor cells is related to their differentiation potential, in other words the capacity to differentiate into distinct cell types[77]. Albeit some different classifications, generally

the potency of a SC can be graded into three categories: totipotent, pluripotent and multipotent, with two additional potency categories attributed to progenitor cells: oligopotent and unipotent (Figure V.3).

- Totipotent: these cells are able to differentiate into embryonic and extraembryonic cell types (such as the placenta), thus capable to construct a whole, biologically viable organism. In human development, only the fertilized egg and the cells produced by its first few divisions in the zygote maintain totipotency[78].

- Pluripotent SC have the potential to differentiate into any cell in the body, without the capacity to form extraembryonic tissues. In human biology, after about seven days of development the zygote evolves into the blastocyst, containing a mass of cells that will eventually become the fetus, as well as trophoblastic tissue to constitute the placenta. Embryonic SC are derived from the inner cell mass of blastocysts and can be grown indefinitely in their undifferentiated state, being commonly referred as immortals. However they are also capable of differentiating into any cell type of the adult body, thus showing pluripotency[79]. Their therapeutic potential is overwhelming, offering substantial opportunities to provide large number of well-defined differentiated cells for drug discovery, toxicology, and regenerative medicine[80–82]. Nonetheless, research has been hampered by safety and immunological concerns, ethical and religious distresses[83].

  In 2006, Shinya Yamanaka's lab in Kyoto pioneered a way of inducing pluripotency by disdifferentiating adult fibroblast mainly through the introduction of four genes encoding transcription factors (Oct3/4, Sox2, c-Myc, and Klf4), hence losing tissue-specific features and gaining pluripotency[84]. The cells obtained were named induced pluripotent SC and have to some extent changed the way we see human biology and disease. Like embryonic SC they can differentiate into all organs and tissues, however, they are not embryonic nor adult SC, but rather reprogrammed cells with pluripotent capabilities[77,84,85]. Induced pluripotent SC have become a powerful method for the creation of patient- and disease-specific cell lines for cell therapy, drug discovery, and disease modelling[86–88].

- Multipotent SC have the ability to differentiate into a closely related family of cells. Examples of multipotent SC can be found in the fetus (fetal SC), cord blood (cord blood SC) and in the fully formed human (adult SC).

  Fetal SC can be isolated from fetal blood, bone marrow and other fetal tissues, including liver and kidney[89]. Considering their potential clinical applications they have several advantages over their adult counterparts, including better intrinsic homing and engraftment, greater potency and lower immunogenicity[89]. Moreover, fetal SC are less ethically contentious than embryonic stem cells[89,90]. Fetal SC may thus represent an intermediate cell type in the debate focusing on dichotomized adult versus embryonic SC, nonetheless they are still limited by restrictions in their access[89,91].

Cord Blood SC are present in large numbers in the cord blood and are genetically identical to the new-born. They are multipotent stem cells that have been shown to differentiate into haematopoietic and mesenchymal cell lineages[92,93]. This cell type has practical and ethical advantages, allowing the isolation of three important cell types such as endothelial progenitor Cells (EPC), hematopoietic and mesenchymal SC.

Adult SC are crucial for physiological tissue renewal and regeneration after injury, being defined as any cell found in a developed organism with the ability to divide creating another cell alike and to differentiate into a specific cell type[94]. Pluripotent adult SC are very rare and scarce in number, as most adult SC are lineage-restricted and are generally referred by their tissue origin[95]. Examples of adult SC with multipotency are hematopoietic SC, that can differentiate into the whole hematopoietic lineage (red blood cells, white blood cells, platelets)[73], or mesenchymal stem cells that can differentiate into multiple cell types, such as osteoblasts, myocytes, adipocytes and chondrocytes[96,97]. They can be found in numerous tissues, such as brain, bone marrow, blood, blood vessels, skeletal muscles, skin or liver, remaining in a quiescent state for years until activated by disease or tissue injury[77,98]. Despite their generally recognized tissue restriction, some adult SC (including Bone Marrow SC) have been shown to have developmental plasticity enabling differentiation across boundaries of lineage, tissue and germ layers[99–101].

– Oligopotency defines the capacity of differentiating into only a few cells, such as endothelial progenitor cells (EPC) that can give rise to both endothelial cells and functional vascular smooth muscle cells[17,102]; neural stem cells can give rise to neurons and glia of the nervous system[103–107]; lymphoid or myeloid stem cells.

– Unipotency describes cells with some capacity of self-renewal but restricted to differentiating into a specific cell type. Oligodendrocyte progenitor cells are an example of unipotency renewing adult Oligodendrocytes.

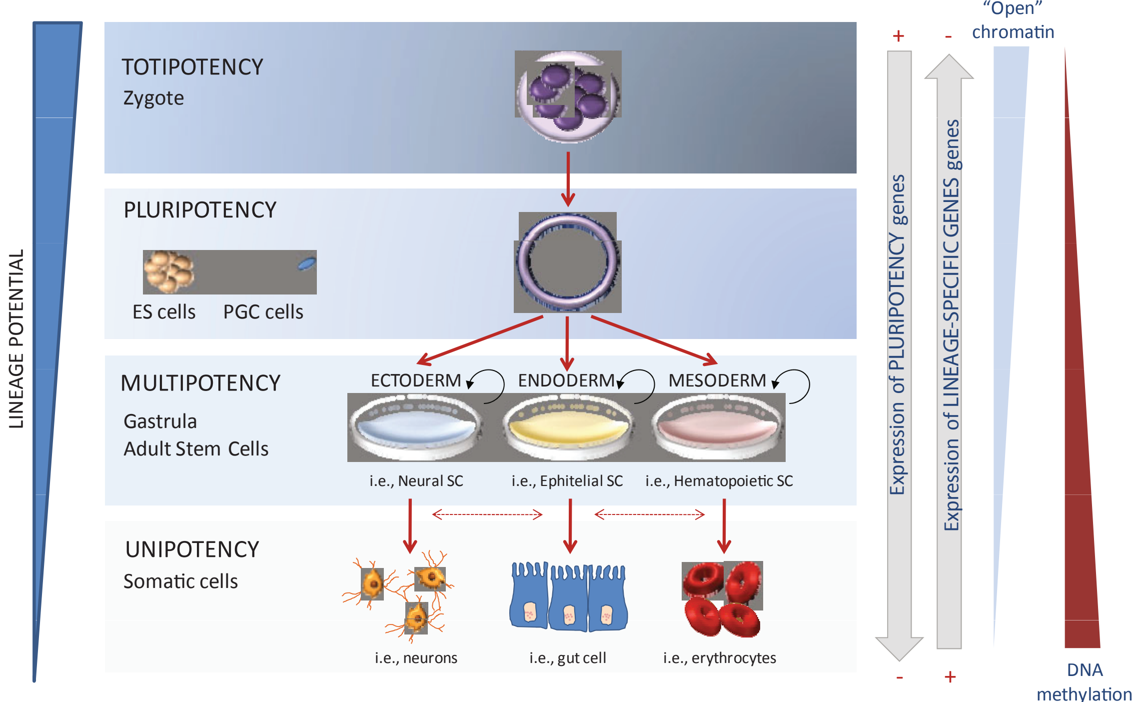


Figure V.3. Stem/progenitor cell potency in human development. *From top to bottom, the potency is decreasing, paralleled to the reduced expression of pluripotency genes and the inverse increase in expression of lineage-specific genes. Reproduced with authorization from Berdasco et al. 2011*[79].

In living organisms stem cells usually subsist in niches, the *in vivo* milieu that houses a specific cellular subset and a broad spectrum of extracellular substrates regulating SC survival, self-renewal, and differentiation[108]. As dynamic entities, the key function of SC niches is to indefinitely maintain a balanced proportion of quiescent and activated cells, ensuring a relatively constant number of progenitor cells.

The different potencies, types, availabilities, numbers and isolation methods of stem cells present interesting features allowing adaptation of biological properties to therapeutical targets and methods of delivery. However, they also impose high level of complexity and heterogeneity that has been partially responsible for some delay in clinical translation and doubts in medical, political and general population. Notwithstanding, cell therapy is undoubtly recognized as the (near) future in medicine, after careful and sustained overcome of hurdles in the definition of stem cell population, method and timing of delivery for each pathological substrate[78,109,110].

ii. Cell therapies in Medicine

Cell therapies play a central role in regenerative medicine and are used in current clinical practice on some medical fields[111,112]. The concept of engineering tissues considered irreversibly-affected has fuelled investigations to manipulate stem/progenitor cells into repairing and replacing damaged tissues or pathological descendants of normal turnover[108,113]. These theories have been fostered by the discovery of stem/progenitor cells residing in a multitude of tissues/organs (e.g., bone marrow, central nervous system, adipose tissue and others) or circulating in the blood, that can

remain quiescent for long periods of time until their activation for physiological tissue maintenance with cell turn-over or by tissue injury. Unfortunately, endogenous responses are not able to offer an holistic solution as continuous cell activation may alter the balance of a stem cell niche, which will ultimately limit the its regenerative potential. Nonetheless, tissues with higher endogenous regeneration activity can induce more rapid and efficient repair, capacities that suffer by an age-related decline in progenitor populations[114,115]. In the event of a pathological insult the limits of tissue-specific SC niches will commonly be revealed, be it by exhaustion or age-related failing of repair mechanisms and thus require external modulation to achieve functional reconstitution.

Two main approaches have been pursued for therapeutic goals: cell transplantation versus re-cruitment.

Stem/progenitor cell transplantation has probably been the most promising strategy in modern medicine[108]. The cellular origin defines three types of transplant: autologous, allogenic and syn-geneic.

In autologous stem/progenitor cell transplant the patient's own SCs are used, greatly reducing transplant rejection. It usually involves collection of adult SC at a certain point in time that are reinfused in a specific organ or to reconstitute a meanwhile therapeutically ablated biological structure[110,116]. In current clinical practice, this concept is already in use for the treatment of hematological diseases (leukemias, lymphomas and multiple myeloma) and as a final option for auto-immune disorders (such as multiple sclerosis) where adult SCs are used to reconstitute the patient's bone marrow after its ablation with high doses of immunosuppression[117–120]. Another application under development is its use for solid organ regeneration[13,116,121–127]. Albeit some promising preclinical and early-stages clinical trials, only one treatment has received formal approval by regulatory agencies for solid organ pathologies at the time of thesis publication: autologous limbal SC expanded cultures (Holoclar®) are formally approved and registered for clinical use in physical or chemical burns of the eye causing limbal SC deficiency[110].

In allogenic transplantation the donor is a genetically similar, but not identical individual. This is often the case of a sibling, but may also be a compatible, unrelated donor. Other possible sources are fetal or cord blood SC. The advantages implied are the possibility of retransplanting according to needs and possible use of the immune response against the transplanted cells (the graft-versus-cancer effect) as a therapeutic application in some forms of cancer.

Syngeneic transplant involves cell collection from a different individual that is genetically identical (twin), thus reducing graft-versus-host response.

Despite the progress achieved in the clinical translation of stem/progenitor cells, many technical limitations still exist such as the need for *ex vivo* safe and effective expansion methods and *in vivo*

delivery restrictions due to limited availability of stem/progenitor cell sources, immunogenicity and potential tumorogenicity, besides the inherently excessive manufacturing costs[108,128].

An alternative approach to cell transplantation is to potentiate stem/progenitor cell populations already present in a patient's body, which can be actively attracted to sites of injury[108,129,130]. The pharmacological activation of endogenous stem/progenitor cells from either the blood or a tissue-specific niche is a promising approach for therapeutic reconstitution of damaged organs. The recruitment process may be facilitated by external stimuli, such as the provision of cell homing factors, displaying a therapeutic window to harness the host's innate capacity for regeneration[131]. In the human adult brain two main neurogenic niches are observed, the ventricular-subventricular zone and the subgranular zone[98,107,132,133]. The accepted conceptual construct for the subventricular zone is that relatively quiescent SCs give rise to rapidly proliferating progenitors that migrate into the olfactory bulb to form granule cells and some periglomerular interneurons[134]. In addition, new neurons are also formed into the adult mammalian hippocampus where new dentate gyrus granule cells are regularly added[106,135]. The subgranular zone is a caudal extension of the subventricular zone, lying between the hippocampus and the corpus callosum, whose majority of cells migrate into the corpus callosum to become oligodendrocytes *in vivo*[136]. Albeit attractive, the concept of mobilization of endogenous stem/progenitor cells is hindered by the need for the baseline presence of viable progenitor cell population and the biological difficulty to isolate, mobilize and constrain these cells in the injured tissue. An additional limitation is the potential for the endogenous stem/progenitor cells to develop themselves the pathological condition they were set out to treat, which has been documented for degenerative conditions such as Parkinson's Disease[124].

iii. Cell therapies for the treatment of Ischemic Stroke

The treatment of ischemic stroke has dramatically evolved in the last years due to the development of effective strategies to promote vascular recanalization. In spite of these advances 15 to 50% of stroke survivors are still left with functionally significant sequelae[137–141], that are devoid of efficacious therapies in the current treatment arsenal. Upon the ischemic insult many different cell types essential for brain function are lost: not only neurons and their connections, but also astrocytes and other glial cells together with pericytes, endothelial cells and smooth muscle cells. In fact, after stroke a series of endogenous responses are triggered to restore function, including neurogenesis (from neural stem cells of the subventricular and subgranular zones) and also synaptogenesis, axonal sprouting, glial cell activation, angiogenesis and vascular remodeling[142]. These mechanisms depend on cellular resistance to ischemia and existent excitotoxicity, but are mostly dependent on the function of resident adult stem/progenitor cell niches[143,144]. Unfortunately, as stated in the previous chapter, these repair mechanisms are quickly exhausted in the setting of a major insult, in particular on aged patients. Cell therapies ultimately offer the possibility of optimizing, enhancing and enduring these endogenous mechanisms. Several questions arise when considering SC therapy for stroke (Table V.1).

Table V.1. Main unanswered research questions in cell therapy for ischemic stroke. SC: stem cells; iPS: induced Pluripotent Stem cells; EPC: Endothelial Progenitor Cells; BBB: Blood-Brain Barrier.

Basic question	Biological question	Possible answers
What?	What is the optimal cell type?	Pluripotent SC (Embryonic SC, iPS...) Multipotent SC (Mesenchymal SC, Amnion Epithelial SC...) Oligopotent SC (Neural SC...) Progenitors (EPC...)
When?	When is the optimal time for cell delivery?	Acute stage Subacute stage Chronic stage
Where?	Where should stem/progenitor cells be delivered for optimal effect?	Systemic circulation Regional vascular system Central nervous system
How?	What method should be used for cell delivery?	Intravenous Intra-arterial Intrathecal Stereotaxic
Whom?	Will all patients benefit? Who should we chose for treatment?	Young vs. old Recanalyzed vs. non-recanalyzed Lacunar vs. cortical-subcortical strokes BBB impairment

What stem/progenitor cell should be used?

Several cell types have been studied and tested for ischemic stroke, each with its own specific properties and clinical targets. The absolute answer to the question is complex, as it is intrinsically connected to the questions of when/how they are applied and ultimately the therapeutical target.

Multipotent SC, such as Mesenchymal SC (MSC) have been the most investigated cell type in ischemic stroke. The immunomodulatory and neuroprotective effect of MSCs have been tested with high cell numbers and systemic administration. This has become a popular cell-based therapy due to the relatively easy isolation of these cells (through bone marrow, adipose tissue, umbilical cord or dental pulp of the patient), notwithstanding the historical disappointment with the hundreds of other treatments designed with the same target[9,145]. Recent phase I and IIa clinical trials (MASTERS and InveST) have confirmed its safety, albeit failing to show therapeutic effect[145–147]. A recent phase I study of SB623 cells (bone marrow derived cells transiently transfected with a plasmid containing the human Notch-1A) used a stereotaxical cell delivery approach in chronic

stroke patients and showed safe and positive results, although no control group was included in the design[148]. A somewhat similar objective is intended with ALD-401 cells (bone marrow derived SCs with high activity aldehyde dehydrogenase) through carotid infusion, that has also failed to show a functional improvement at three months in a phase II trial treating patients 13-19 days post-stroke. Amnion epithelial SC is another type of multipotent SC with potential application in stroke. They are derived from the amniotic epithelium of the placenta which originates from the epiblast at approximately 8 days after fertilization[149]. Previous studies have demonstrated that these cells are adult cells expressing surface markers common to those of embryonic SCs, primordial germ cells and somatic stem cells[150]. They show unique characteristics, such as low level expression of major histocompatibility complex antigens, and a less restricted differentiation potential[149]. Moreover, they are less limited in ethical constraints and cell number availability as they are isolated from the maternal placenta that is usually discarded after birth. Animal models have suggested their effect in reducing infarct size, mainly through immunomodulation[151].

Neural SC have also been studied for ischemic stroke. Having neurogenesis, synaptogenesis and (in a more distant prospect) neuroprotection as a goal, these cells have mainly been designed for a stereotaxical approach. PISCES was a phase I trial that used this approach and showed clinical improvement from baseline, although no control group was included. Despite the high frequency of side effects due to the delivery method there were no long-term sequelae related to treatment[152].

To overcome the major hurdles of the previously described strategies, a somewhat different approach may include EPCs: these are bone marrow derived cells with the capacity of promoting the devolvement of new vessels by direct differentiation into endothelial and smooth muscle cells and also through paracrine mechanisms[17,21,153]. In stroke patients, a phase I clinical trial has demonstrated safety in their autotransplant, with no control group included to assess efficacy[154].

Endogenous SC activation form the subventricular and subgranular zones is a different therapeutic concept with potential use for neurological disorders. These cell populations have limited regenerative potential, but can be regulated by physiological triggers (such as hormones, growth factors, excitatory neurotransmission and learning), pharmacological agents and pathological insults[155–158]. Endogenous SC have been documented to migrate into the ischemic lesion to assist tissue recovery[129,133,143,159], however, the number of endogenous Neural SCs activated is so limited that their neuroprotective role has mostly been unsatisfying[156]. Moreover, there are several challenges in their targeting, especially the need to achieve specificity of delivery and responses[160]. All these hurdles have limited their isolated therapeutic use, although it has been recognised that modulating and improving survival of endogenous SC populations is an important paracrine effect of exogenous cell therapies[161].

When should stem/progenitor cell be used?

For practical and conceptual organization the cell-based therapies for stroke may be separated

into chronological order: early subacute, late subacute and chronic stages with variable durations based on persistency and severity of ischemic insult as well as patient's responses. Albeit relatively arbitrary, this division has its foundation in the pathological substrate of stroke at each stage.

For the hyperacute stage (first 6 to 24 hours) recanalization has been the hallmark and can now be efficiently achieved[5,6]; cell therapy is not expected to play a major therapeutic role, particularly considering the clear efficacy of recanalization therapies and the logistic limitation for cell therapy in this stage.

In the early subacute time window (approximately 1-5 days after stroke) many pathological targets come into play: excitotoxicity with cellular and BBB disruption, inflammation, edema and potentially reperfusion injury. As such, a cell therapy targeting this pathological cascade will inevitably have to modulate these local and systemic responses, as well survive in the very inhospitable environment of a recently injured ischemic tissue, ultimately aiming to achieve meaningful neuroprotection. In this domain, the tested strategies mainly involved using a large number of pluri- or multipotent stem cells[147,162–165].

In the late subacute time window (5-20 days after stroke), apoptosis persists due to the cellular insults set out by the acute insult and persistent inflammation. On the other hand, neovascularization is triggered as a physiological response to attenuate injury and accelerate recovery. At this timepoint, apart from the previously referred cell lines, EPCs present as an interesting alternative, enhancing the angiogenic response.

The chronic stage is also an heterogeneous and somewhat controversial stage in its limits, varying according to different criteria between few weeks to several decades after stroke[166]. Considering tissue recovery, a time window of 30 days-12 months is usually accepted, apprising different pathological substrates and therapeutic targets. At this stage the lesion is already well established, leaving neuroprotective, angiogenic or immunomodulatory strategies devoid of biological substrate. Therefore a direct cell infusion with more mature cell lines aiming at neuro- and synaptogenesis is likely to be more proficient. Early-stage clinical trials have used MSC and NSC, and albeit no control group was yet included, it seemed to be associated with functional improvement; in the PISCES trial there was one case of malignancy reported that the authors mentioned as unlikely related to treatment (melanoma in the ear pinna)[148,152].

Where and how should stem/progenitor cells be administered?

In the context of stroke, three different delivery methods are locations are possible: systemic, regional and local. A systemic administration through intravenous infusion is more appropriate for the acute stage, mostly modulating immune and excitotoxic responses with immature and potent stem cells[164]. This strategy is relatively easy and would allow a whole-body dissemination, ideal features for the acute stage. However, it is limited by the pulmonary pass that will inevitably

filter out many cells, and will inherently be inefficient in tissue delivery, requiring very large cell numbers to achieve biological effect, which will impair autotransplantation.

A regional delivery in the context of stroke comprises intra-arterial and intra-thecal approaches. These moderately invasive methods allow cell delivery both in the lesion itself and nearby areas, promoting recovery by themselves and by activation of resident stem/progenitor cell populations. Apart from their relative invasiveness (which can potentially be overcome with experience), a significant cell number is necessary and, in the case of intra-arterial delivery, it would still be required to pass the BBB, limiting this strategy to periods with open BBB.

Local or lesional delivery represents a challenge in stroke patients due to the inaccessibility of the injured brain. In current medicine it will involve using a stereotaxic approach, thus being able to obtain direct lesional access, at the expense of a very aggressive approach, implicating transcranial transport. This method is of presumed limited use in the acute and early subacute stages considering its invasiveness and the inhospitable environment at this stage, impairing the survival of cells with high metabolic demand. Therefore, existing phase I-II trials using stereotaxic delivery have investigated its use in chronic stroke patients (6-60 months after stroke) with more mature cell lines[148,152].

Whom is likely to benefit from stem/progenitor cell therapy after stroke?

It is difficult to determine the patients that will most likely benefit from cell therapies. Two synergistic approaches may be followed for this identification: cellular and clinical. At the cellular approach, the patients most likely to benefit are those with impaired biological responses to insults. In fact, cellular reconstitution is mainly dependent on the multiplication, migration and renewal of resident stem/progenitor cell niches[114,115]. In humans, time introduces selective and environmental constraints that gradually diminish the fitness and plasticity of adult stem cells[167]. The most significant consequence of age in brain SC niches consists in the alteration of their cytostructure and in reduction of the neuroblast population, with reduced proliferation and neurogenic capacity[168]. This effect is exponentiated by vascular impairment and chronic inflammation, enriching the niche's environment with toxic factors that aggravate SC exhaustion[169–171]. In addition to age, vascular risk factors are thus introduced as negative modulators of SC proficiency. In fact, several risk factors have been associated with reduced numbers of SCs, such as smoking, hypertension, hypercholesterolemia, obesity and diabetes[172–178]. Moreover, the severity of risk factors has also been correlated with decreased cell numbers and improved clinical control promotes partial reversibility of their effects[177,179,180]. It is therefore presumable that aged subjects with several vascular risk factors would be more likely to benefit from cell therapies, considering their impaired cellular response to insults. On the other side these patients may also be more therapeutic resistant due to the limitations of resident SC population. Altogether, current knowledge on cell biology does not allow an accurate identification of patients most likely to benefit, although future large scale clinical trials are expected to address these questions.

The clinical stroke features will also present a challenge for patient selection, although some traits will likely need to be present in prospect of therapeutic effect. Firstly, as it has been extensively documented, SC survival will fundamentally be impaired in the absence of blood flow; therefore, a recanalyzed vascular bed will be mandatory, and its oblivion will likely render any SC treatment inconsequent, as the lessons from hundreds of previous neuroprotectant agents that disregarded this condition taught the scientific community[145]. Another important clinical distinction will relate to lacunar infarctions. In these patients, using SC lineages designed as immunomodulators or angiogenic will probably be ineffective considering this specific pathophysiology. Nonetheless, neuro-, synaptogenesis and myelinisation may still be a target, which is confirmed by a recent study in a mouse model of lacunar stroke, that documented a positive effect of perilesional subacute transplantation of cultured bone marrow-mesenchymal stem cells, with confirmed differentiation into neural cells, facilitating neural reconstruction and improving function[181]. Apart from neuro-/synaptogenic strategies in subacute or chronic lesions, cortical-subcortical strokes will possibly benefit from strategies aiming at immunomodulation and vascular reconstitution. In fact, regarding the latter, it is assumable that in the absence of a capable vascular bed, downstream mechanisms of tissue recovery will have few chances of success.

An important clinical feature to take under consideration is the BBB permeability at the time of cell treatment. Clinical and animal studies report that BBB is permeable from the acute stage for up to five weeks, peaking in the first hours and from 72 hours to the second week due to different mechanisms[182,183]. This knowledge needs to be taken under consideration, disavowing the use of intravenous or intra-arterial delivery outside this time-window, as it would probably hinder lesional and perilesional cellular access.

Additional clinical circumstances that may preclude SC treatments are the presence of an active infection or medication with cytostatic drugs. The presence of active malignancy is traditionally excluded from clinical trials, and a cautious approach will extend for SC stroke application considering still unknown effects in the malignancy.

Similarly to other considerations on cell therapies, the clinical features of each specific stroke will have to be considered on the decision for cell therapies, particularly cell type, delivery method and timing.

iv. Endothelial Progenitor Cells

EPCs are bone-marrow derived cells that express a variety of surface markers shared by vascular endothelial cells and have the capacity to promote the formation of new vessels in ischemic areas[184]. Residual numbers of EPCs can be found in the peripheral blood; however, physiological and pathophysiological events requiring neovascularization may mobilize EPCs from the bone marrow through the release of several cytokines, growth factors, and hormones from the target tissue[185]. In fact, neovascularization seems to be a critical step in tissue recovery after ischemia, without

whom downstream recovery processes (such as cellular reconstitution and subsequent cellular and biochemical mechanisms) will be fundamentally impaired. Moreover, human autologous cell therapies using EPC-containing products (such as bone marrow or mobilized peripheral blood) are feasible and effective in the treatment of coronary and peripheral ischemic syndromes[186–188].

There are essentially two methods to isolate EPCs: (i) by selective adhesion to substrates and cell culture conditions and (ii) by selective cell surface markers (Table V.2).

Cell culture protocols are used to obtain subpopulations of EPCs with distinct characteristics: circulating angiogenic cells (CAC), colony-forming unit-endothelial cells (CFU-EC) and outgrowth endothelial progenitor Cells (OEC). Each sub-population is isolated using protocols that differ in the cell culture conditions (time, cell culture media, substrates, etc.). Short-term culture protocols (4-7 days) yield mainly myeloid/hematopoietic-like cells (CACs and CFU-ECs) whereas long-term protocols (14-21 days) yield cells with a more mature endothelial cell phenotype (oEPC)[153]. Cells obtained from short-term protocols "early-EPCs" usually express CD45 and typical myeloid markers such as CD14 and CD11b. Their strong angiogenic potential is mostly mediated by the paracrine release of potent growth factors that support angiogenesis. Nonetheless, formally naming these cells "EPCs" is not universally accepted due to their myeloid background, and have therefore been recently renamed as CACs. Of note, direct cell-to-cell contact with surrounding cellular environment may lead to the additional coexpressions, modulating cell transdifferentiation through the transfer of epigenetic material, including microRNAs, proteins and nucleic acids[189,190]. Long-term culture protocols give rise to outgrowing cells with more mature endothelial phenotypes and high proliferative capacity, referred as late or outgrowing EPCs[153,191]. The exact origin of these cells at the tissue level is not ascertained, but they have been demonstrated to have direct angiogenic capacities, and several authors postulate that they derive from the vascular wall as a proliferating shed of endothelial cells[153,192]. Another distinctive feature of oEPCs is their capacity to form vascular structures by themselves, whereas the short-term cultured cells require the interaction with existing endothelial cells to promote the formation of a vascular network[193]. However, it should be noted that not all subjects are capable of differentiating oEPCs, as it seems to be an ON/OFF phenomena, usually decreased with age and accumulation of vascular risk factors[153,194–196]. An additional cell population has been described as "endothelial colony-forming cells" (ECFCs), following a long-term culture protocol essentially similar to oEPC, only using collagen instead of fibronectin to coat culture wells, with presumed similar functional and biological characteristics[194,195].

Table V.2. Types of EPCs according to isolation protocol[153].

Isolation method		Cells obtained
Cell culture	Short term protocols	Circulating Angiogenic Cells
		Colony Forming Unit-Endothelial Cells
	Long term protocols	Outgrowth EPCs
Surface antigens	CD34+	"Classical" EPCs
	CD133+	CD34+CD133+ cells identify potent EPCs
	KDR+ (VEGF receptor)	CD34+KDR+ cells have vasoregenerative potential
	CD45-	Immature endothelial lineage
	CXCR4+	CD34+CD133+CXR4+ cells can be easily mobilizable
	CD31+	Cells with endothelial lineage derived from bone marrow

Surface antigens can also be used to isolate EPCs, without the necessity of *ex vivo* manipulation, facilitating diagnostic and therapeutic objectives. The classical definition of EPCs relies on the surface expression of CD34 (thus CD34+ cells). In fact, CD34- cells are unable to show proangiogenic activity[197]. Having in mind that CD34 is also expressed in mature endothelial cells, CD133 was later identified as a marker of immaturity, thus putative EPCs were defined as CD34+CD133+. Multiple research groups have now documented that CD34+ or CD133+ cells derived from either peripheral- cord-blood and bone marrow can express endothelial marker genes and form endothelial structures *in vitro* and *in vivo*[197–199]. Other proposed surface markers of EPCs are KDR+ (VEGF-receptor 2), further defining a more vasoregenerative and immature phenotype[200,201]; CD45- as a marker of more mature endothelial lineage[197,202]; CXCR4+ (homing receptor for hematopoietic cells) isolating cell with high migration and regenerative capacities[203]; or CD31+ (PECAM-1, present in endothelial cells and monocytes), marking high proangiogenic and vasculogenic properties[204].

The molecular program regulating EPC activity has been a field of active research in the attempt to understand and optimize their effect and survival. Several strategies have been pursued to study genome and more recently epigenetic regulation. In the field of epigenomics, microRNAs (miRNA) have gained attention as pivotal regulators of gene expression. They are 21–25 nucleotide single-stranded noncoding RNA molecules that regulate the expression of specific gene sets, modulating cellular pathways and functions. Recent studies have begun to unveil powerful and unexpected roles for miRNAs in numerous forms of ischemic diseases, providing a unique opportunity to translate this knowledge into the clinical setting as prognostic indicators and in the form of miRNA-based therapeutics[205,206]. MicroRNA profiles of early EPCs, late EPCs and human umbilical vein endothelial cells have been identified[207]. Notably, 722 microRNAs are expressed in the above three endothelial lineages, with some specific miRNAs expressed only in some of EPC cell types, indicating their role in regulating EPC functions[207]. MicroRNAs have been demonstrated to regulate the proliferation, mobilisation, senescence, differentiation, adhesion and tubule structure formation of EPCs and EPC-induced angiogenesis[208–212]. However, the miRNAs associated with improved clinical outcome and *in vivo* angiogenesis of stroke patients are still scarcely understood.

Numerous studies have documented EPCs as an important biological marker of endothelial function, vascular risk and particularly cerebrovascular events[18,153,196,213–215]. In fact, mounting evidence advocates that various stroke risk factors reduce the level of EPCs, such as hypertension, dyslipidaemia, smoking and diabetes[214,216–219].

In the setting of acute ischemic stroke there is limited but consistent published data associating EPCs with improved clinical outcome. The available results come from human observational studies of different research groups that isolated EPCs based on cell culture protocols and/or expression of surface antigens, reporting the presence of both higher cell number and enhanced angiogenic properties of EPCs in patients with better clinical outcomes[21,22,220–222]. These studies showed that the ischemic insult induces a transient increase in the number of circulating EPC, with peak angiogenic activity in the subacute stage (3-7 days)[22,220,223]. However, doubts remain on whether EPCs are effective players in improving outcomes or merely markers of a more proficient vascular status and response to ischemia[153]. Moreover, predating this thesis no study on stroke patients had yet addressed mechanism of action, hemodynamic impact nor epigenetic machinery responsible for the therapeutic effect of EPCs.

A few phase I and II clinical trials have been designed to assess the efficacy of EPCs in ischemic stroke (Table V.3). Most protocols have used an auto-transplant of immune-selected CD34+ cells isolated from the bone marrow. Studies on subacute stroke (between 5 and 9 days) used mainly an intravenous/intra-arterial delivery, whereas chronic stroke protocols (6-60months) delivered cells stereotaxically in the injured brain tissue to overcome the fully formed BBB. There is still very limited efficacy data, however no safety concerns have arisen from the proposed protocols.

Table V.3. Clinical trials of EPCs in ischemic stroke registered in European Medicines Agency and/or Food and Drug Administration.

Study name	Target condition	Route/phase/ number of participants	Status/results
Autologous Endothelial Progenitor Cells Transplantation for Chronic Ischemic Stroke	Chronic stroke (6-60 months)	Intravenous; phase I; 30 participants	Unknown (reported completion date was November/2017)
Autologous Bone Marrow Stromal Cell and Endothelial Progenitor Cell Transplantation in Ischemic Stroke	Subacute stroke (7 days)	Intravenous; phase I; 20 participants	Unknown (reported completion date was March/2017)
Efficacy Study of CD34 Stem Cell in Chronic Stroke Patients	Chronic stroke (6-60 months)	Stereotaxic; phase II; 30 participants	Unknown (reported completion date was December/2010)
Autologous Bone Marrow Stem Cells in Ischemic Stroke	Subacute stroke (7 days)	Intra-arterial; phase I; 5 participants	Completed; treatment was safe; no control group for efficacy outcome
Study of Purified Umbilical Cord Blood CD34+ Stem Cell on Chronic Ischemic Stroke	Chronic stroke (6-60 months)	Stereotaxic; phase I; 6 participants	Unknown (reported completion date was December/2013)
Autologous Bone Marrow Stem Cells in Middle Cerebral Artery Acute Stroke Treatment	Subacute stroke (5-9 days)	Intra-arterial; phase I-II; 20 participants	Completed; treatment was safe; no difference in clinical outcome

C. Angiogenesis

i. Basic Principles

The evolution of life into multicellular organisms forged the development of blood vessels allowing haematopoietic cells to supply oxygen and nutrients, dispose of waste and perform immune surveillance. The composition of an adequate network of vessels thus assumed a central role in life and apart from its vastly positive effects, have also been involved in many ill conditions both as protective agents as well as pathological promoters. Not surprisingly, angiogenesis research has boosted in the last two-three decades and it is speculated that its modulation may ultimately benefit more than 500 million people worldwide in the next decades[224].

In the developing embryo, an early vascular plexus stems from the mesoderm by the differentiation of angioblasts, assembling into a primitive vascular labyrinth of small capillaries — a process known as vasculogenesis[224,225]. These initial endothelial precursors, still devoid of vascular lumen, share their origin with haematopoietic progenitors, an intrinsic bond that will remain throughout life, critical for growing tissues as vehicles of nutrition and instructing trophic signals to promote organ morphogenesis. This common origin for blood and endothelial cells is further suggested by molecular links with shared expression of a number of different genes, such as CD34, CD133,

PECAM-1 (encoding platelet endothelial cell adhesion molecule-1), c-Kit and Sca-1[226]. Subsequently, angiogenesis takes place by vessel sprouting thus creating a vascular tube network with that will ultimately be remodelled into arteries and veins[227,228]. Pericytes and vascular smooth muscle cells will then be recruited to encircle the sprouting tubules of endothelial cells providing structural stability and regulating perfusion (arteriogenesis)[229].

Early after birth, angiogenesis still plays an active role supporting and regulating organ growth but in the full-fledged adult, most blood vessels remain quiescent and under physiological conditions, angiogenesis is mainly limited to the cycling ovary and in the placenta during pregnancy[226]. In fact, endothelial cells have long half-lives and are capable of self-generating maintenance signals such as VEGF, NOTCH, angiopoietin-1 (ANG-1) and fibroblast growth factors (FGFs) protecting them against insults[224]. Moreover, endothelial cells are equipped with oxygen sensors and hypoxia-inducible factors — such as prolyl hydroxylase domain 2 (PHD2) and hypoxia-inducible factor-2α (HIF-2α), respectively —allowing vessels to re-adjust their size to optimize blood flow[224]. However, pathological conditions such as tissue hypoxia or lymph vessel inflammation can reactivate intrinsic vascular responses such as angiogenesis, vasculogenesis and arteriogenesis that can ultimately coexist in the same injured tissue.

In the human adult the term ''angiogenesis'' is commonly used to reference the process of vessel growth but in the strictest sense is characterized by capillary sprouting, endothelial cell migration, proliferation, and luminogenesis to generate new capillaries[228,230]. Adult vasculogenesis is mainly mediated by the action of circulating cells such as EPCs that can be mobilized from the bone marrow after ischemic insults[20,22,108,231]. As mentioned in previous sections, these cells seem to play mostly a paracrine angiogenic function, but a subset of these cell also incorporate into the vasculature (inosculation)[153,191,230,232,233]. Adult arteriogenesis represents the remodelling of existing collateral underdeveloped channels with virtually no flow into a functional vessel. This process can stem from vessels that receded during growth or from neoangiogenesis after a vascular insult. As a result of shear fluid pressure from occlusions or severe stenosis, flow is redirected through these channels increasing diameter and becoming functionally relevant. This positive remodelling seems to be modulated by endothelial factors with the contribution of infiltrating macrophages, through a process of dynamic reconstruction of the extracellular matrix with degradation and resynthesis; vascular smooth muscle cell apoptosis as well as proliferation[230]. Altogether, the concepts of angiogenesis, vasculogenesis and arteriogenesis usually concur biologically in the adult to the broader concept of new vessel formation for demanding tissues: neoangiogenesis[234,235].

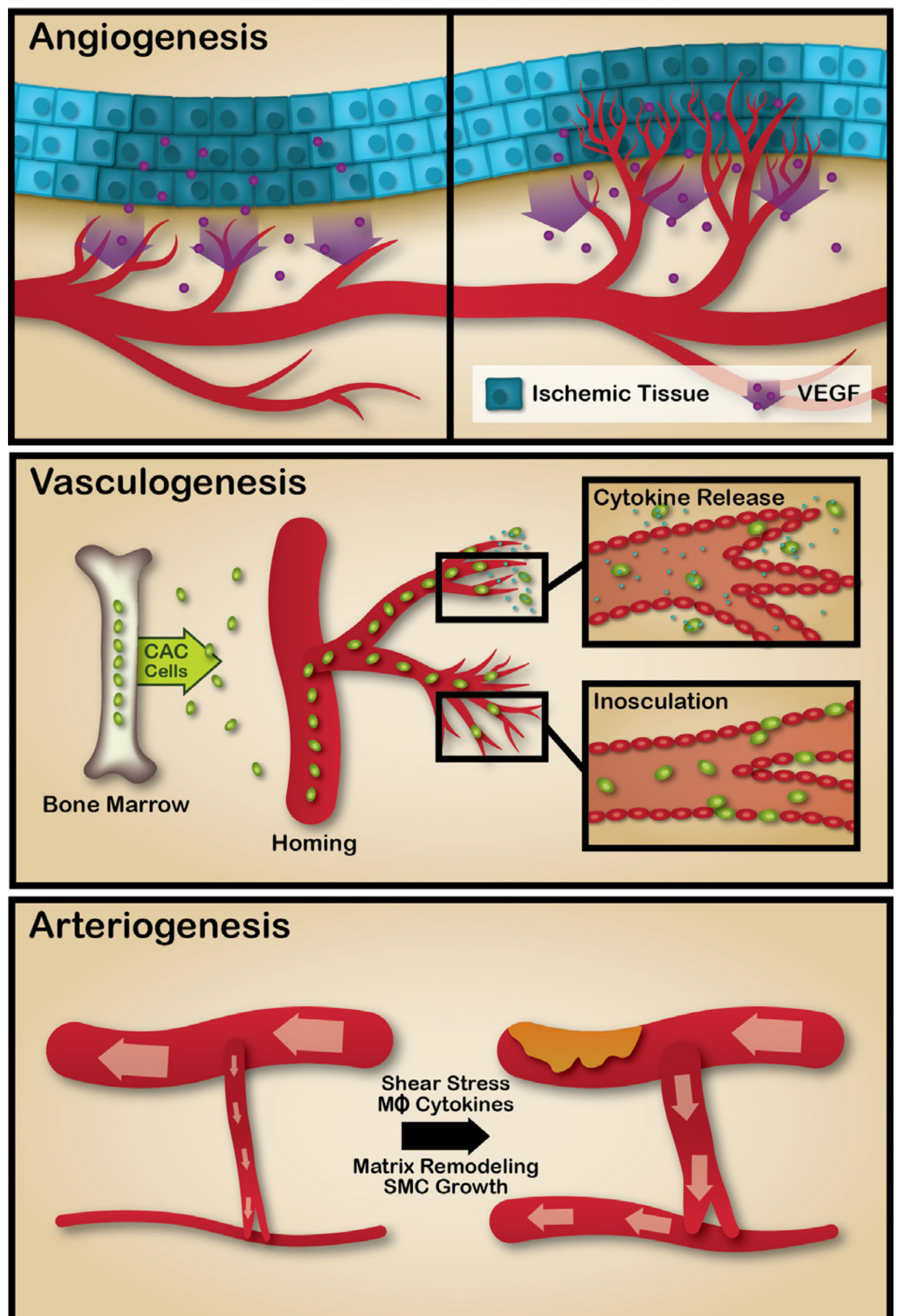


Figure V.4. Illustrative representation of the main biological events involved in the formation of new vessels after an ischemic insult. *In the top image, Angiogenesis is represented with capillary sprouting and endothelial cell migration; Vasculogenesis is presented in the middle image with bone marrow precursors promoting new vessels through paracrine and direct effects; in the bottom image Arteriogenesis takes place mediated by shear stress modulation. CAC: Circulating Angiogenic Cells. Reproduced with permission, from Cooke et al 2015* [230]

ii. Angiogenesis in pathological conditions

Angiogenesis has been implicated in more than 70 disorders so far, and the list in ever growing with multiple and often antagonic roles[236]. Firstly, the promotion of neoangiogenesis is thought to be a turning point in ischemic pathologies of multiple organs, namely ischemic stroke, myocardial infarction and peripheral artery disease, which as a whole represent over 30% of worldwide deaths[23]. The revascularization of ischemic tissues would therefore benefit millions, but therapeutic angiogenesis remains an unmet medical need. Other pathologies where the angiogenic potential

is insufficient for tissue demand include pre-eclampsia and cases of impaired wound healing[237,238]. A progressive age-dependent microvascular deterioration has also been implicated in Alzheimer's disease, nephropathy, osteoporosis, skin telangiectasias and hair loss[239–242].

On the other end of the spectrum are disorders where aberrant angiogenesis is itself the main conditioner of pathology such as proliferative retinopathies, age-related macular degeneration, endometriosis or uterine bleeding[243–246]. In these cases the formation of immature, leaky and histologically incomplete vessels in response to chronic stimuli impair the function of the organ and their frailty preconditions recurrent bleeding with pathological implications. In fact, anti-angiogenic therapies have become a field of very active research and clinical applications in Oftalmology[247,248].

With a different perspective, angiogenesis has also been the target of numerous other conditions where neovascularization of pathological tissues is a required step in disease development. This is especially relevant in the case of cancer, where the highly active cancer cells require a proportional vascular perfusion and promote neovascularization[249,250]. Such drugs as bevacizumab, ranibizumab, sunitinib, pazopanib and vandetanib have been approved for several metastatic cancers such as non-squamous non-small cell lung cancer, colorectal cancer, renal cell cancer, metastatic breast cancer, hepatocellular carcinoma or medullary thyroid cancer[228,251]. Moreover, auto-immune disorders have become a recent and promising target of anti-angiogenic agents, following on the demonstration of the key role of angiogenesis in their development[252,253]. Numerous compounds such as anti-angiogenic factors antibodies, peptides, estrogen metabolites, and even some disease-modifying anti-rheumatic drugs have been found to have anti-angiogenic properties[254].

Altogether, it is clear that angiogenesis has a widespread fundamental presence in healthy and pathological conditions (Table V.4). Inhibitors of angiogenesis are already in clinical use in some medical fields such as ophthalmology and cancer; however, much is yet to be clarified and improved regarding effective angiogenic modulators, and, above all, how to promote physiologically competent new vessels in the adult.

Table V.4. Pathological conditions in which angiogenesis is directly involved.

Biological effect	Disease
Protective effects	Ischemic stroke Myocardial infarction Nephropathy Osteoporosis Skin telangiectasias Alzheimer's disease Hair loss
Pathological effects	Age-related macular degeneration Endometriosis Excessive uterine bleeding
Pathological modulator	Aggressive cancer Auto-immune disorders

iii. Angiogenesis in ischemic stroke

Animal models indicate that minutes after ischemic stroke angiogenic genes are upregulated, producing both message and translated proteins[255]. The proliferation of endothelial cells is then documented as early as 12-24 hours after insult, leading to an increase of vessels in the peri-infarcted region at three days[256,257] (Figure V. 5). These are still immature vessels, mainly constituted by sprouting endothelial cells, devoid of BBB[258,259]. In the human brain active angiogenesis has been demonstrated from 3–4 days following the ischemic insult and an higher number of new vessels correlated with longer survival[255,260].

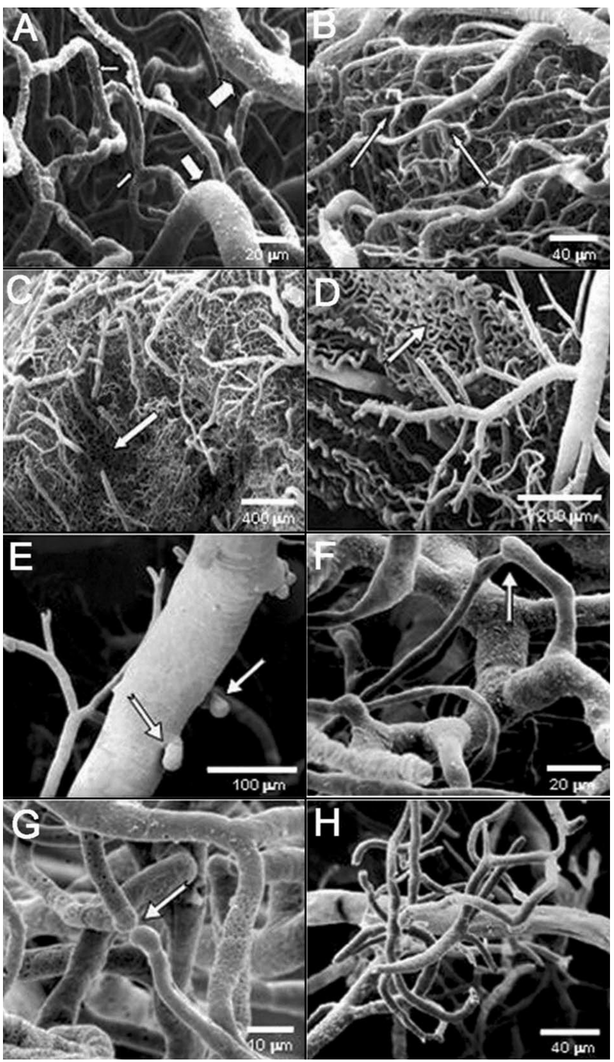


Figure V. 5. Endogenous angiogenesis occurs in the post-ischemic penumbra. *Scanning electron microscopy images of leptomeningeal (large arrows) and small penetrating arterioles (small arrows) in normal brain (A and B). (C) Areas of infarction where no blood vessels are visible (arrow), and (D) stressed microvessels 24 h after middle cerebral artery occlusion. (E and F) Vascular buds were visible three days after middle cerebral artery occlusion (arrows), and (G and H) connections or 'nests' of small microvessels associating with surrounding vessels (arrows). Size is shown in the inserted bars. Reproduced with permission from Navaratna et al. 2009* [255]

Despite the consistent demonstration that brain cells produce and secrete angiogenic peptides after focal cerebral ischemia, the purpose of this angiogenic response remains speculative[255]. Some authors propose a "clean-up hypothesis" whereby new-born vessels contribute to the clearing up of cellular debris[261,262]. This is supported by the increase in microvessel density adjacent to areas of pan-necrosis with increased numbers of macrophages, whereas no vascular changes were displayed in ischemic brain areas without macrophages. Opponents of this hypothesis state that this association is indicative that the formation of new vessels is required for a myriad of biological responses, including macrophage infiltration for removal of necrotic brain. Current thinking and existing evidence thus sustain that angiogenesis after stroke is mostly concentrated in penumbral regions, accompanying and promoting intense brain remodelling by providing the necessary molecular, anatomic and cellular support for recovering neural networks[255,260]. Notwithstanding significant recent clarifications, several questions still remain on how to modulate stroke angiogenesis: what is the best method to induce effective angiogenesis (cytokines, peptides, antibod-

ies, proteins or cells)? When is the ideal timepoint to start and stop the intervention to avoid pathological angiogenesis? How to select patients suitable for angiogenic therapies, particularly considering the differential responses in patients with distinct ages, vascular profiles or mechanisms of ischemic injury? As angiogenic therapies transpose the barrier into clinical application these queries will have to be addressed.

D. Blood-brain barrier in stroke

i. Blood-brain barrier composition and function

Normal brain function requires a strict preservation of neural environment within fine hemostatic parameters. To achieve this pivotal need the human body displays a dynamic barrier between the systemic circulation and the central nervous system milieu: the blood-brain barrier (BBB). The earlier reports of its existence date back to the late 19th century, when Paul Ehrlich described that injecting die into the blood circulation stained peripheral organs but not the spinal cord and the brain[263,264]. In the 1950s BBB was described as a lipid barrier, keeping water-soluble substances away from the Central Nervous System[265]. Subsequent ultrastructural studies in the following decades documented the specific role of cerebral endothelial cells, tight junctions and basement membrane, ensuing the documentation of transcellular transporters that allow glucose and particular electrolytes, vitamins and regulatory proteins to cross the barrier at rates 10–100 times faster than those predicted by the classic lipid membrane model and also brain-to-blood efflux transporters that have been described for nearly every substance class, including ions, amino acids, peptides and cytokines and have been demonstrated to be essential to maintain a functional neuronal environment[265]. Considering this recent clarification of the BBB has a complex, dynamic and adaptable structure, some authors even propose renaming it into a more appropriate "blood-brain interface", although BBB still remains the accepted denomination.

Histologically, the BBB has some variance according to anatomical location and function. The inner layer of the vascular BBB, the spinal cord barrier and the barriers of the cranial nerves are mainly formed by endothelial cells, whereas in the choroid plexus modified epithelial (ependymal) cells assume that function[266,267]. Moreover, circumventricular areas do not show a strict BBB and are separated from the rest of the brain by a barrier formed by tanycytes and from the adjacent cerebrospinal fluid by ependymal cells. The blood–retinal barrier consists of an inner barrier of endothelial cells and an outer barrier involving the retinal pigment epithelium[268]. Knowledge in some of these barriers is scarce, however, they share common features such as intercellular tight junctions as a central feature to its barrier function, local adaptation to the specific needs of the surrounding tissues and responsiveness to pathological insults[265] (Figure V.6).

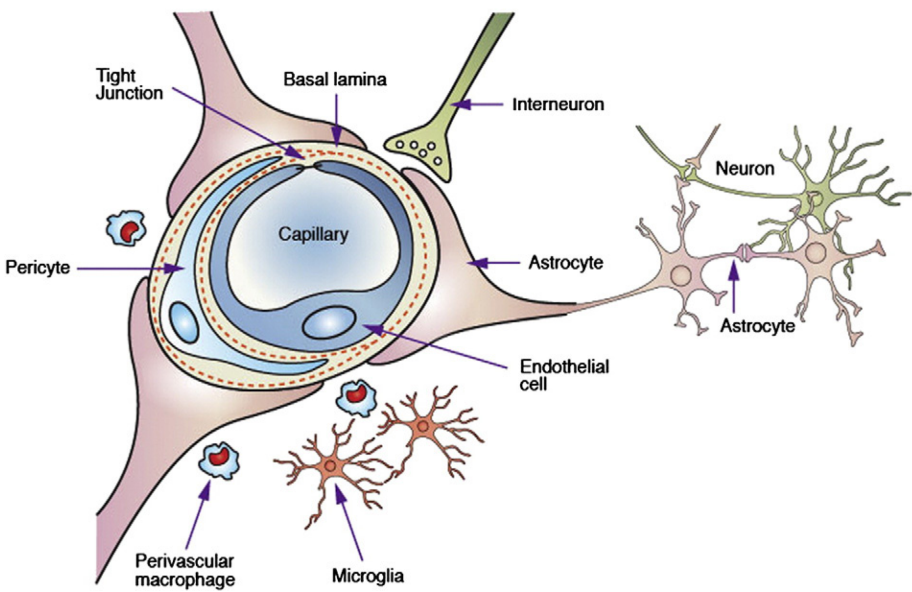


Figure V.6. Representation of the BBB, contextualizing with other components of the neurovascular unit. *Reproduced with permission from Chen et al. 2012* [269]

The most disseminated and best studied segment is the vascular BBB, encompassing brain endothelial cells ensheathed by a basement membrane separating them from pericytes and astrocytes, in intimate connection with neurons and immune cells. In the human brain these cells maintain a continuous crosstalk to guarantee most brain functions in a pivotal structure named collectively as the neurovascular unit[265,270,271] (Figure V.6). This crosstalk constantly refines the BBB permeability and function to serve brain needs and brain-body communication, enabling the capillary bed to adopt and adjust barrier characteristics, mainly through the action of astrocytes and pericytes[272–274]. This liaison is critical to comply with what seems to be a fundamental flaw in neural design: it shows unparalleled energy consumption in all body states, however, it holds no storage for nutrients, turning it directly dependent on immediate flow delivery, mainly of glucose and oxygen. This highlights the importance of flow delivery at the right time, place and amount, which is ultimately regulated by the neurovascular unit. The main segment of the neurovascular network where flow is modulated seems to be the capillaries and arterioles, which are closer to neurons than arteries, and considering their large surface area, minimal changes in their diameter can produce large changes in flow. *In vivo* studies suggest that up to 84% of flow change can be modulated at the capillary and perforating arteriolar level through the action of pericytes and smooth muscle cells, with additional but residual contribution from pial and large cerebral arteries[275]. Increasing knowledge of this structure has challenged classical perspectives on neural pathology, with a dichotomized responsibility of neurons in neurodegenerative conditions or vessels in traditional neurovascular disorders. In fact, evidence is mounting on the symbiotic relationship of the BBB and neurovascular unit with neurons, brain function and pathology, not simply as a passive consequence but as a key determinant of disease inception and evolution. With little controversy, healthy BBB and neurovascular unit warrant a healthier brain, however, besides the control of vascular risk factors no specific modulators of their function have yet been identified.

The human BBB also regulates the immunological environment in the CNS. The unique features

of brain endothelial cells, distinguishing them from their counterparts in the remaining organs, mediate this function, through continuous intercellular tight junctions and paucity of leukocyte adhesion molecules, creating an immunological privilege in the CNS, protecting it from potentially destructive inflammatory reactions, but still guarantying immune surveillance[276].

Altogether, the BBB is a complex and heterogeneous structure, displaying endothelial cells in the first row of a modulated-interface, through a selective environment for passive diffusion and active blood-to-brain and brain-to-blood transporters anchored by intercellular tight junctions and interplaying with pericytes, glia, neurons and immune cells ultimately assuming not only a mere discriminating barrier, but a real-time regulator of CNS environment and brain perfusion in health and disease.

ii. Mechanisms of BBB Injury in ischemic stroke

Disruption of the BBB is one of the first pathological steps in acute ischemic stroke and has received recent focus from the scientific community as a potential therapeutical target. Classical animal models of ischemia-reperfusion constructed the "open-closed-open" hypothesis where after an initial period of increased permeability there is a return to baseline and a second period of increase. However, these models showed very different and often conflicting timelines of BBB closure, from 3-4 hours up to 24 and 48 hours, being time-limited to the first days of injury[277–279]. The latest data from human and animal studies seem to indicate that the BBB remains permeable for up to five weeks following a multiphasic and heterogeneous evolution with distinct biological background and even antagonic clinical impact[182,264,280–283] (Figure V.7).

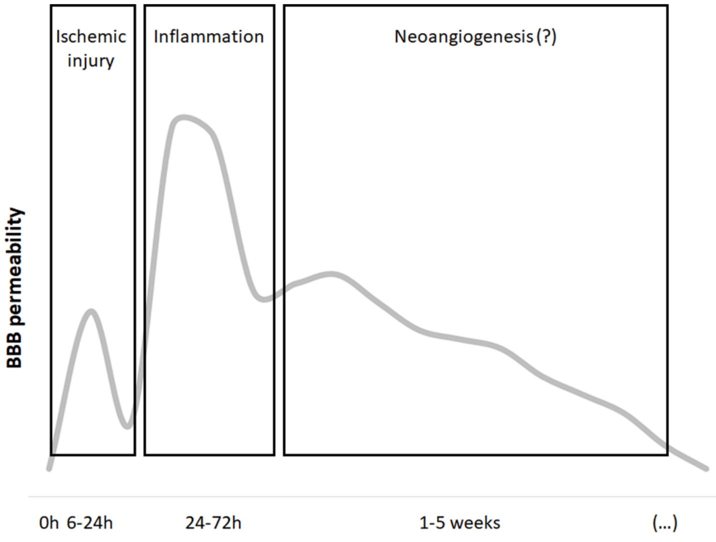


Figure V.7. Graphic representation of the multiphasic permeability of the BBB in the first hours (h) and weeks after an ischemic stroke. *Above, the main hypothesized biological substrates of BBB permeability are represented.*

After stroke onset, the early disruption of the BBB is directly mediated by the ischemic insult to its constituents and has been consistently associated with an increased risk of hemorrhagic transformation[284–287]. Animal models locate the initial injury site of the BBB in brain endothelial

cells and basal lamina, a process simultaneously mediated and aggravated by the activation of metalloproteinases (MMPs), released by leukocytes subsequent breakdown of the tight junction proteins[288,289]. The time and volume of ischemia intensifies the biological injury, which can be further accelerated by the effect of alteplase both at the endothelial cell level and in the adjacent proteins of basal lamina and neurovascular unit by activation of plasminogen and secretion of MMPs directly or indirectly through formation of free radicals[287,290,291]. In fact, these blunt mechanisms help to understand the association of alteplase with hemorrhagic transformation, a link that goes beyond its effect on thrombolysis and the consumptive coagulopathy caused by fibrinogen reduction[287,292–299]. Following the arterial occlusion, recanalization is the goal of all approved therapies in the acute stage, with a potentially dramatic positive effect on clinical outcome; however, it may also trigger a chain of pathological mechanisms leading to reperfusion injury[297,300]. This process is highly heterogeneous and dependent on the severity, duration, cellular resistance, etiology of the ischemic insult and recanalization method (intravenous, endovascular, combination of both or spontaneous)[291,300,301]. Hemodynamically, reperfusion injury is mediated by loss of autoregulation, commonly concurrent with a sharp increase in blood pressure that can lead to tissue necrosis and inflammatory activation[69,302]. This inflammatory response can be documented from six hours after stroke onset, with peak activity between 24 and 72 hours causing parallel disruption of the BBB[281,303]. More recent studies have documented a later and less pronounced opening of the BBB that can persist up to five weeks with somewhat uncertain clinical significance and biological background[182,259,280,304]. Histological studies of animal models have suggested an association of this later opening with neurovascular remodelling and angiogenesis[183,259,305]. In fact, as described in previous sections, the primordial step in process of neoangiogenesis is endothelial cell sprouting from existing vessels. This event leads to the formation of vessels that in their immature stages do not have a full BBB established, leading to an increased permeability that may ultimately have a positive effect, in contrary to other stages of BBB permeability. It is noteworthy, that before this thesis no human study had yet correlated late subacute BBB permeability and markers of angiogenesis.

iii. Methods to evaluate BBB permeability

The evaluation of BBB permeability was classically avoided in clinical studies due to its technical complexity and paucity of available methods. This led to the often erroneous and almost mystical understanding of BBB mechanism in health and disease. Now-a-days a plethora of methods exist, however each one has its own specifications and limitations that researchers and clinicians need to be aware for accurate interpretations (Table V.5).

Table V.5. Methods to evaluate BBB permeability. CSF: cerebrospinal fluid; HRP: horseradish peroxidase; PET: positron emission tomography; MRI: magnetic resonance imaging; DCE: dynamic contrast enhancement.

	Method
Ex vivo	CSF/serum ratio of albumin CSF/serum ratio of IgM CSF/serum ratio of IgG Evans blue dye Dextran amines Free fluorophores HRP Transthyretin S100B
In vitro	Fluorescent microscopy
In vivo	PET MRI (DCE)

The *ex vivo* BBB assessment is basically performed through the measurement of tracers in brain parenchyma, which can be made with endogenous or exogenous substances[282]. Among the endogenous substances are albumin, fibrinogen, IgM, and IgG that are too large to diffuse over the healthy BBB[282,306]. A natural disadvantage of this method is that it may miss subtle BBB changes, considering the large size of most endogenous tracers. Exogenous substances such as Evans blue dye, dextran amines, free fluorophores and horseradish peroxidase (HRP) can also be used but their particular chemical properties may interact with the BBB and/or serum albumin and therefore may not be generalizable to in vivo settings[307,308]. A common limitation to *ex vivo* assays is the impossibility of assessing BBB permeability continuously over time, allowing only one evaluation per specimen.

Fluorescent microscopy of surface microvessels provides an *in vivo* method for animal models with high spacial and temporal resolution using a number of available fluorescent tracers, such as indoline derivatives, sodium fluorescein, Lucifer-yellow. The main disadvantages rely on the need for anesthesia (which can alter BBB permeability) and the limitation of assessing only superficial vasculature due to poor tissue penetration and light scattering.

In human subjects, *ex vivo* methods have limited applicability (reduced to excised tumours or biopsied specimens in specific contexts), whereas microscopic *in vivo* assays are infeasible. To tackle these limitations two strategies have been adopted: indirect biochemical measurements of BBB integrity and advanced neuroimaging techniques with quantification of specific tracers. Indirect measurements of BBB function rely on quantifying the serum concentrations of CNS

proteins (such as the 14-kDa monomeric form of transthyretin or the astrocytic protein S100B) or calculating ratios of large plasma proteins, such as CSF/serum ratio of albumin, IgG or IgM[309–311]. These methods are attractive due to their feasibility, quantitative nature and ease of interpretation, however they have severe limitations in pathological settings as the results can be altered directly by the disease itself, in addition, these methods do not provide information on temporal nor spatial resolution.

Neuroimaging techniques have gained a central role in assessing BBB integrity due to their good temporal and spatial resolution. There are essentially two conceptual constructs for these assays: positron emission tomography (PET) brain quantification of radioactive exogenous products with known BBB permeability or performing serial MR/CT acquisitions after injecting a contrast agent and quantifying its extravasation rate: Dynamic Contrast Enhancement (DCE).

Quantification of radioactive tracers with PET is occasionally used in human studies, with validated applications for the use of ${}_{13}$N-Glutamate, ${}_{82}$Rubidium-chloride, and ${}_{68}$Gallium-ethylenediamine-tetra-acetic acid (EDTA). They provide very good spatial resolutions (well below 1mm), and good temporal resolution for a single exam, however their use is limited considering potential biological hazards with direct restrictions on the possibility of serial assessments[312–314].

Disruption of the BBB allows low-molecular weight MRI contrast agents to penetrate into the extravascular extracellular space of affected tissues leading to increased signal intensity in T1-weighted images[283]. DCE-MRI quantifies the rate of contrast extravasation of a specified brain area of the study subject. In brief, DCE-MRI consists of intravenous injection of contrast agent (usually gadolinium) followed by the repeated acquisition of T1-weighted images, providing measurements of signal enhancement as a function of time[280,283,304,315]. The enhancement kinetics can then be used to calculate quantitative information regarding BBB permeability. It should be noted that contrast agent extravasation is determined by several factors, including capillary permeability but also tissue perfusion, intrinsic characteristics of the tissue (such as the pre-contrast longitudinal relaxation time) and characteristics of the contrast agent (like the longitudinal and transverse relaxivities)[283,316,317]. Therefore, in order to accurately calculate permeability pharmacokinetic modelling was introduced in DCE-MRI calculations. Several parameters can then be extrapolated from the acquired and process images, of which two obtain particular clinical significance: the fractional interstitial volume (Ve, an estimate of the extravascular extracellular space), the volume transfer constant KTrans, i.e., the rate at which contrast agent is delivered to the extravascular extracellular space per volume of tissue and contrast agent concentration in the arterial blood plasma (Figure V.8). Ktrans has thus been widely disseminated as a marker of capillary permeability. It was first used to study BBB integrity but has since been extrapolated for many other tissues, mainly as a marker or immature angiogenesis in oncologic setting, assessing the effect of anti-angiogenic cancer drugs[283,318–320]. A further advantageous feature of DCE-MRI is the capacity to improve temporal resolution by selecting specific regions of interest (ROI) within the brain that can add value in its interpretation.

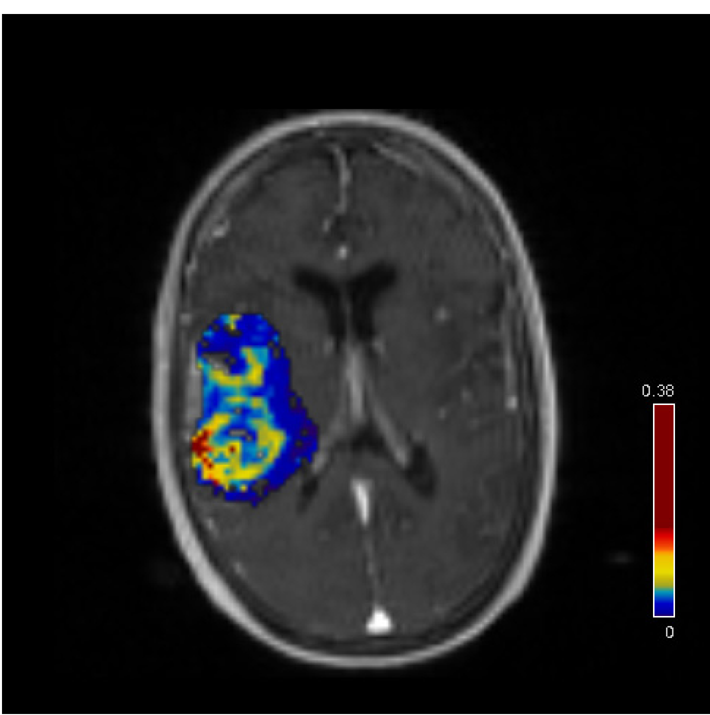


Figure V.8. MRI with DCE of a stroke patient. *Colour scale represents the Ktrans values obtained from a specific Region of Interest, manually defined to include an ischemic lesion within the territory of the right middle cerebral artery.*

part B

Research plan

chapter VI

HYPOTHESIS AND AIMS

This thesis was designed to address the following hypothesis:

- The number and/or functional properties of EPCs are associated with clinical outcome in acute ischemic stroke;
- The BBB permeability in the subacute stage after ischemic stroke is linked to cellular markers of neoangiogenesis;
- MicroRNAs available on EPCs may be used as a prognostic of clinical output after ischemic stroke;
- The evolution of patients' hemodynamic status after ischemic stroke is related with EPCs.

Aims:

- To evaluate the association of number and *in vitro* properties of EPCs in the acute and subacute stage of ischemic stroke with functional outcome at three months.
- To assess the link between BBB permeability at different time points, EPCs and functional outcome.
- To identify the microRNAs responsible for enhanced biological properties of EPCs and stroke recovery.
- To characterize the interplay between the evolution of stroke hemodynamics and EPCs.

MATERIALS AND METHODS

A. Study population

We conducted a prospective longitudinal study of consecutive patients admitted into our department for acute ischemic stroke during a period of 27 months. We included patients with ages between 18-80 years and non-lacunar infarctions within the territory supplied by a Middle Cerebral Artery (MCA) that could have full clinical, neuroimaging and cellular evaluation within 24 hours after the onset of symptoms. The full list of study inclusions and exclusions is presented in the Table VII.1.

Table VII.1. Inclusion and exclusion criteria for study participation.

Inclusion criteria	n (%)
Patients with acute ischemic stroke with ages between 18-80 years during the study period	893 (100%)
Non lacunar strokes	721 (80.7%)
Strokes attributable to infarction of the middle cerebral artery (any arterial segment)	678 (75.9%)
Exclusion criteria	
Date of presentation with unavailable MRI (available only on working days)	294 (32.9%)
Intra-arterial therapy	64 (7.2%)
Premorbid mRS>2	51 (5.7%)
Active infection	47 (5.3%)
Renal insufficiency	41 (4.6%)
Active malignancy	39 (4.4%)
Contra-indications to MRI	36 (4.0%)
Premorbid neurocognitive pathology	25 (2.8%)
Symptom onset >24 hours	14 (1.6%)
Recent surgery	12 (1.3%)
Coma	10 (1.1%)

B. Study design and variables analysed

All study participants completed a thorough and multidisciplinary evaluation of clinical, imagiological, *in vitro* and hemodynamic studies.

Upon study inclusion all patients were evaluated by vascular neurologist with report of anamnesis (including vascular risk factors, demographic variables and previous medication), previous functional status using the modified Rankin scale (mRS) and neurological status using the National Institute of Health Stroke Scale (NIHSS) (Figure VIII.1). Neurological status was reassessed at seven days post-stroke with mRS and NIHSS. The functional outcome was assessed in person by neurologist blinded to *in vitro*, BBB permeability and genetic data using mRS at three months. Neuroimaging analyses by MRI were performed at day zero and seven, including assessment of BBB permeability. Stroke hemodynamics were evaluated by ultrasound at days zero, seven and three months. EPC cell populations such as circulating angiogenic cells (CAC), outgrowth EPCs (oEPC) and colony forming unit endothelial cells (CFU-EC) were isolated from the peripheral venous blood at days zero and seven after stroke, and their functional properties assessed by migration,

wound-healing and angiogenesis assays. At day zero the $CD34^+$ cells were isolated from peripheral venous blood for flow cytometry and miRNA analyses, comparing a subset of patients with good vs. bad clinical outcome (mRS 0-2 vs. 3-6).

C. Isolation of EPC sub-populations.

Cell isolation was performed from 18 mL of peripheral venous blood collected at day zero and according to previously validated protocols[17,21,22,153,220,321]. Mononuclear cells (MNC) were isolated from peripheral blood by density gradient centrifugation using Lymphoprep™ density gradient medium from Axis-Shield (Alere Technologies AS, Oslo, Norway). Three different types of EPCs were analyzed: circulating angiogenic cells (CACs), outgrowth endothelial progenitor cells (oEPCs) and colony forming unit-endothelial cells (CFU-ECs). For CACs, MNCs were plated into 2 $\mu g/cm^2$ fibronectin-coated plates (24-well plates; 1.9×10^6 cells/well) and cultured in Endothelial Growth Medium-2 Microvascular (EGM-2 MV; Lonza, Gaithersburg, MD, USA) containing 5% fetal bovine serum (FBS) during five days at 37°C and 5 %CO_2. Adherent cells were detached using trypsin (Lonza, Gaithersburg, MD, USA), counted and used for functional assays.

For oEPCs and CFU-ECs, 10×10^6 MNCs suspended in Endothelial Growth Medium-2 (EGM-2; Lonza, Gaithersburg, MD, USA) with 10% FBS were seeded into one well of 2 $\mu g/cm^2$ fibronectin-coated 24-well plate. After 48 hours at 37°C and 5 %CO_2, 3×10^6 nonadherent cells were collected and replated into three fibronectin-coated 24-well plates. At day 5, colony-forming units were counted manually in four random fields (20× magnification) using an inverted microscope (Carl Zeiss, Oberkochen, Germany). The cells were detached using trypsin (Lonza, Gaithersburg, MD, USA) and used for functional studies. The remaining cells were cultured for 14-21 days at 37°C and 5 %CO_2 to obtain oEPCs. Medium for oEPCs and CFU-ECs was changed every 48 hours.

D. Functional tests of EPCs.

The angiogenic capacity of oEPCs and CFU-ECs were determined by the sprout formation on Matrigel (using an IBIDI µ-slide angiogenesis kit; IBIDI GmbH, Munich, Germany). The total tube length and number of branching points (i.e. points featuring more than two connections) were manually measured in four random fields (20× magnification) by using AxioVision software (Carl Zeiss, Oberkochen, Germany) 24 hours after plating.

The wound healing capacity of oEPCs and CFU-ECs was evaluated using the *in vitro* wound-healing (scratch) assay. In brief, cells were plated in 2 $\mu g/cm^2$ fibronectin-coated 96-well plates and cultured until they form a monolayer. Then, the wounds were created by scratching the cell layer with a 200 µL pipette tip. The area was photographed at time zero and after 24 hours at 37°C and 5% CO_2. Migratory capacity was quantified as the percentage of wound closure after 24 hours.

Migration assay was performed as described previously by Vasa and colleagues[322]. Briefly, isolated

CACs were detached using trypsin (Lonza, Gaithersburg, MD, USA) and resuspended in endothelial basal medium (EBM-2; Lonza, Gaithersburg, MD, USA), and 2×10^4 CACs were placed in the upper chamber of a modified Boyden chamber (2 µg/cm^2 fibronectin-coated; BD Biosciences, San Diego, CA, USA). The chamber was placed in a 24-well culture dish containing EBM-2 and human recombinant VEGF (50 ng/mL; Peprotech, Rocky Hill, NJ, USA). After 24 hours incubation at 37°C and 5 %CO_2, the lower side of the filter was washed with PBS and fixed with 4% paraformaldehyde (EMS, Hatfield, PA, USA). For quantification, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA). Cells migrating into the lower chamber were counted manually in 3 random microscopic fields (20× magnification) using an inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

E. FACS analysis.

After magnetic separation, CD34+ cells were resuspended in PBS supplemented with 5% (v/v) FBS. For each replicate, 3 donors were pooled together (~0.3×10^5 cells per condition) and stained with antigen-specific antibodies: anti-human CD31-FITC (eBioscience, Thermo Fisher Scientific, San Diego, CA, USA), KDR/Flk1-PE (R&D Systems, Minneapolis, MN, USA), CD34-APC (Miltenyi Biotec, Germany), and CD45-FITC (Miltenyi Biotec, Germany). Cells were analyzed without fixation and unstained cells were used as controls. Propidium Iodide (Thermo Fisher Scientific, San Diego, CA, USA) was used to exclude dead cells. Novocyte Flow Cytometer (Acea Biosciences Inc., San Diego, CA, USA) and NovoExpress Software (Acea Biosciences Inc., San Diego, CA, USA) were used for the acquisition and analysis of the data.

F. Neuroimaging analyses.

We estimated the BBB permeability based on gradient echo (GRE) images where we acquired a 3D GRE sequence with the following settings: TR/TE 5.9/1.5 ms, FOV 240 mm, matrix 128×128, slice thickness 5 mm, 32 slices, flip-angle 20°, for 20 times with a temporal resolution of about 11s. The patients were fasting for at least four hours before imaging. Magnetic Resonance Imaging was performed using a 3.0 Tesla scanner (3T MAGNETOM Trio, A Tim System, Siemens Medical Solutions, Erlangen, Germany) with a standard phased array 12-channel head matrix coil. The subjects were positioned on the scanner table with the head at the center of the head-coil, and provided with headphones and paddings to minimize head movements during the scan. Structural and functional sequences were acquired, namely, MPRAGE_p2_1mm-iso, 1H-Spectroscopy svs_529, t1_tse_rs_tra. Then, a paramagnetic contrast agent - Gadobutrol (Gadovist 1,0 mmol/ml, Bayer Pharma AG, Berlin, Germany) - was injected after the third sequence, using a previously applied intravenous catheter into the antecubital vein, with a power-injector (Optistar™ MR Contrast Delivery System, Mallinckrodt Pharmaceuticals, St. Louis, United States) with programed flow rate and volume (constant rate of 3.5 mL/s). The dose was 0.1mmol/kg body-weight (i.e., most of the times approximately 7 mL to 9 mL). The rate and volume of saline-flush injection was 3.5 mL/s and 20 mL, respectively.

In order to estimate the T1 map, we obtained three variable flip-angle gradient echo sequences (TR 5.9 ms; TE 2.11 ms; FA 2º, 5º, or 10º; 128 × 128 matrix, 1.875 × 1.845-mm in-plane pixel size, 240 × 240-mm field of view, resulting in 5-mm slice thickness and 1950 Hz/pixel bandwidth). The perfusion sequence was the same with a flip-angle of 20º and 20 continuous measurements. The evaluation of permeability was performed by a trained biomedical engineer and an experienced neuroradiologist using Tissue 4D v1.0, on a Syngo workstation MRB19 (Siemens Medical Solutions, Erlangen, Germany). After motion correction and registration, we manually drew a ROI including the entire ischemic region (as defined by diffusion weighted imaging). Whenever parenchymal hemorrhagic transformations were visible within the ischemic lesion (PH1 or PH2 according to the ECASS criteria, as identified in GRE images), these areas were excluded from the ROI. A second ROI was drawn in the brain parenchyma contralateral to the ischemic area with similar shape, volume and anatomical position and used as control for the analysis.

Pharmacokinetic modeling was performed automatically for each ROI using the Tofts Model in order to obtain the volume transfer coefficient (Ktrans). The arterial input function (AIF) used was the fast model provided by Tissue 4D. When setting the AIF we aimed for the best fit for the time curve of contrast agent concentration. For statistical analysis we used the median of Ktrans values obtained for each patient.

Haemorrhagic transformation was graded in the GRE sequences of the MRI on day seven according to the ECASS criteria. Only PH2 haemorrhages were considered for analysis (i.e. presence of blood in over 30% of the infarct area and substantial space effect).

Infarct volume was calculated by manual planimetric method, tracing the perimeter of the area with abnormal signal intensity in the FLAIR sequences obtained at day seven. For the assessment of infarct growth we calculated the difference between infarct volume at seven days and early ischemic lesion (calculated in the diffusion-weighted imaging sequences of the MRI performed at day zero by manual planimetric method).

G. Hemodynamic evaluation.

The hemodynamic evolution was assessed through serial cervical and transcranial neurosonological exams at admission (day zero), six hours, seven days and three months after stroke (11MHz sector-probe and 3MHz linear-probe respectively; General Electric Logiq7). All exams were performed with patients lying in a supine position after at least 10 minutes rest, collecting data on flow diversion, recanalization and CBF. Flow velocities were assessed bilaterally using transcranial colour coded Doppler (TCCD) with angle correction in anterior, middle and posterior cerebral arteries (ACA, MCA and PCA respectively). Flow diversion was defined as a high-velocity, low-resistance flow signal in the ACA-A1 or PCA (P1–P2 segments) ipsilateral to the occluded MCA; the ACA or PCA flow velocity had to be equal to or higher than the nonaffected MCA[323]. Recanalization was defined as grades four or five by the Thrombolysis in Brain Ischemia scale in the TCCD performed

six hours after symptom onset. Cerebral blood flow (CBF) was quantified as the sum of flow volumes in both internal carotid arteries (ICA) and vertebral arteries (VA) (Figure IX.2). Intravascular flow volumes were determined by the ultrasound equipment's software using the angle-corrected mean flow velocity and the vessel's luminal diameter perpendicular to its longitudinal axis at the same location as the flow measurement[324]. ICAs were assessed at least 2 cm after bifurcation and VAs in their V2 segment, at the C4-C5 intertransverse area. All arteries were measured in a straight segment for three times and the calculated mean taken for CBF analysis.

H. Routine blood analysis.

For characterization of routine analytical parameters we evaluated the glucose, C reactive protein (CRP) levels at admission. Moreover, we also assessed CRP at a second timepoint (48 hours to seven days) from the blood analysis performed routinely as part of the medical care of patients. For this last value, whenever more than one measurement was available, we considered the value collected closest to seven days.

I. miRNA isolation and analysis.

Peripheral blood $CD34^+$ hematopoietic stem cells were isolated from stroke patients on day zero, as described in previous sections. After isolation, cells were centrifuged and 300 µL of RNAprotect Cell Reagent (Qiagen) was added onto the cell pellet. Cells were kept at -80ºC until RNA isolation. Cells isolated from six patients with adequate $CD34^+$ cell count were used for miRNA sequencing. We analysed three patients with good outcome (i.e., mRS at three months ≤2 and high subacute BBB permeability) vs. three other with bad outcome (mRS at three months >2 and low subacute BBB permeability). Total RNA isolation and miRNA sequencing was performed by Seqmatic LLC (Fremont, CA). Briefly, TRIzol reagent (Thermo Fisher) was used to extract RNA from the cell samples. Total RNA was resuspended in 21 µL of nuclease-free water. Libraries were generated using total RNA prepared by SeqMatic. The SeqMatic Tailormix miRNA library prep kit V2 was used to create libraries that are compatible with Illumina sequencing. Each sample was validated for yield and quality using a BioAnalyzer High Sensitivity DNA assay. Libraries were normalized and pooled in equal molar ratios. The pool was gel purified and the purified product was quantified using the SeqMatic TailorMix HT1 qPCR assay. A single end read 50 bp sequencing run was performed using the Illumina MiSeq instrument. Reads were demultiplexed by barcode and output into FASTQ format for subsequent analysis. Raw sequence data was trimmed of adapters and low quality sequences using Trimmomatic. Only reads with a minimum Qscore of 15 and length of 16 nt were kept for downstream processing. Clean reads were mapped to human reference genome hg38/Grch38 using Bowtie. RNA species was determined using Ensembl genome annotation version 84 and counted using HTSeq-count. A replicated differential expression analysis was performed on the miRNA expression data. The following comparisons were performed: "Good" *vs.* "Bad". The data was deposited on the Gene Expression Omnibus (GEO Accession Number: GSE85299). 24 miRNA were found between "good" vs. "bad" groups when the microarray analysis was per-

formed using averages. However, the analyses based on the “average” of the groups do not take into consideration the differences among donors. In order to prevent misinterpretation of the data, after this first analysis, authors went back to raw data and focused on the count numbers of each differentially expressed miRNA for each donor. After this analysis, a miRNA was considered as “robust” when it was 1) down-regulated/up-regulated for all patients in the group of good outcome, 2) not down-regulated/up-regulated for any of the samples in the bad outcome group. This approach was chosen to reduce the noise in the analysis and to focus on just miRNAs differentially expressed in all of the donors in a group of patients (i.e. “good” vs. “bad” outcome groups). For example, for the miRNA hsa-miR-6802-5p, the count numbers for good patients are: 0, 113, and 33. For the bad patients, the count numbers are: 0, 0, 2. According to the replicated differential expression analysis, hsa-miR-6802-5p is significantly upregulated in “good” vs. “bad” outcome group. However, this analysis does not count the variability among individual patients in each group. Count numbers for individual patients show that the first patient in “good” outcome group does not express this specific miRNA (count number is zero). The second analysis based on the count numbers of the individual patients in a group aims at finding the most robust miRNAs coming from miRNA seq. According to this analysis, hsa-miR-6802-5p was not accepted as a “robust” miRNA since it was not expressed by one of the patients in “good” outcome group.

J. Missing data management.

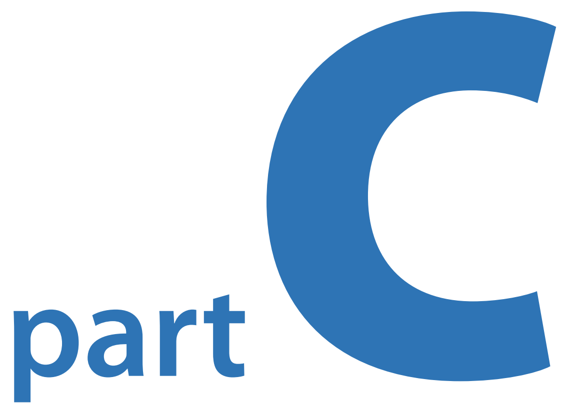
No patient was lost to follow-up, therefore we had no missing data regarding clinical variables. Due to death, 3 patients did not perform the Magnetic Resonance imaging at seven days and in one patient we could not collect blood for *in vitro* studies at seven days. Cases with missing data were excluded listwise for each analysis.

K. Study size calculation.

The sample size was difficult to estimate considering the multiple dimensions and outcomes analysed. Moreover, the previous published studies with the variables of interest (vasculogenic capacity, infarct volume, clinical outcome) reported effect sizes ranging from medium to high (d=0.4-0.8). We chose to introduce strict inclusion criteria to homogenize the population, basing our total sample size estimates on finding large effects (d=0.8). A between-group comparison with a 0.05 one-sided alpha significance level and 80% power would require a total sample population of 42 patients. We inflated the numbers for 45 patients in order to account for potential dropouts and technique failures in MRI analysis.

L. Quantitative analysis.

Quantitative variables were tested for normality using the Shapiro-Wilk test. Baseline NIHSS showed a normal distribution; all other variables did not have a normal distribution. Considering no association/correlation was studied between two normally-distributed variables, all univariate studies were performed using non-parametric tests. All results are presented as median (inter-quartile range).



Experimental work

ENDOTHELIAL PROGENITOR CELLS ENHANCE BLOOD-BRAIN BARRIER PERMEABILITY IN SUBACUTE STROKE

Endothelial progenitor cells enhance blood-brain barrier permeability in subacute stroke

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Abstract

Objective: To study the association between Endothelial Progenitor Cells (EPCs), subacute blood-brain barrier (BBB) permeability and clinical outcome after ischemic stroke, determining the micro RNAs (miRNA) of EPCs responsible for good clinical outcome.

Methods: We included consecutive patients with nonlacunar acute ischemic strokes in the territory of a middle cerebral artery and ages between 18-80 years. Clinical outcome was defined as modified Rankin scale at three months. Neuroimaging was performed at day zero and seven by MRI, including assessment of BBB permeability by dynamic contrast enhancement. EPCs were isolated from peripheral venous blood, quantified and submitted to *in vitro* functional tests, including migratory and angiogenic assays. Stroke hemodynamics were evaluated serially by ultrasound. Statistical significance was set at $p<0.05$.

Results: We included 45 patients, mean age was 70.0±10.0 years. The *in vitro* functional properties of EPCs were associated with BBB permeability, particularly at day seven. The number of each EPC subset at both timepoints was not associated with BBB permeability. Permeability of BBB at day seven was independently associated with improved clinical outcome (OR: 0.897; 95%CI: 0.816-0.986; p=0.025). The EPCs ($CD34^+$ cell subset) of patients with good clinical outcome showed 24 differentially expressed miRNAs, with a common effect on adherens junction pathway.

Conclusion: The functional properties of EPCs are associated with enhanced subacute permeability of BBB and improved clinical outcome after acute ischemic stroke.

A. Introduction

The therapeutic approach to acute ischemic stroke has dramatically evolved in the last few years. However, this improvement has been mostly due to the promotion of recanalization, leaving tissue recovery still devoid of efficacious strategies. Endothelial Progenitor Cells (EPCs) have the capacity to promote *in vivo* angiogenesis and vascular repair after ischemic events[17,153]. In the context of ischemic stroke in humans, an increase in their number during the first weeks has been associated with good clinical outcome[220] and smaller lesion volumes[21]. Unfortunately, their underlying therapeutic mechanism and molecular program are largely unknown.

After an acute ischemic stroke, blood-brain barrier (BBB) permeability shows a multiphasic response with distinct pathophysiological substrates and clinical implications[300,325]. In the first hours, the increase in BBB permeability, has been associated with haemorrhagic transformation and poor clinical outcome[300,325]. In the 24-72 hours after stroke, a second opening of the BBB is mediated by inflammatory mechanisms[291,326]. Later increase in permeability in the subacute phase is likely related to new, immature blood vessels as demonstrated by animal studies[259,327]. Yet, it remains relatively unknown the dynamics of the BBB permeability in humans in the subacute phase of stroke and its association with the properties of EPCs.

Herein, we evaluated the association between EPCs, clinical outcome and BBB permeability in the acute and subacute stages. We also studied the microRNA (miRNA) expression program of a subset of EPCs ($CD34^+$ cells) associated with a good clinical outcome.

B. Materials And Methods

Study population

We conducted a prospective longitudinal study of consecutive patients admitted into our department for acute ischemic stroke during a period of 27 months. We included patients with ages between 18-80 years and non-lacunar infarctions within the territory supplied by a Middle Cerebral Artery (MCA) that could have full clinical, neuroimaging and cellular evaluation within 24 hours after the onset of symptoms. The full list of study inclusions and exclusions is presented in the Table VII.1.

Study design and variables analysed

Clinical outcome was assessed in person by neurologist blinded to *in vitro*, BBB permeability and genetic data using modified Rankin scale (mRS) at three months. Neuroimaging analyses by MRI were performed at day zero and seven, including assessment of BBB permeability (Figure VIII.1). Stroke hemodynamics were evaluated by ultrasound at days zero, seven and three months. EPC cell populations such as circulating angiogenic cells (CAC), outgrowth EPCs (oEPC) and colony

forming unit endothelial cells (CFU-EC) were isolated from the peripheral venous blood at days zero and seven after stroke, and their functional properties assessed by migration, wound-healing and angiogenesis assays. At day zero the CD34⁺ cells were isolated from peripheral venous blood for flow cytometry and miRNA analyses, comparing a subset of patients with good vs. bad clinical outcome (mRS 0-2 vs. 3-6). Detailed methods on neuroimaging, hemodynamic and *in vitro* tests are presented as Supplementary Information (Chapter VII).

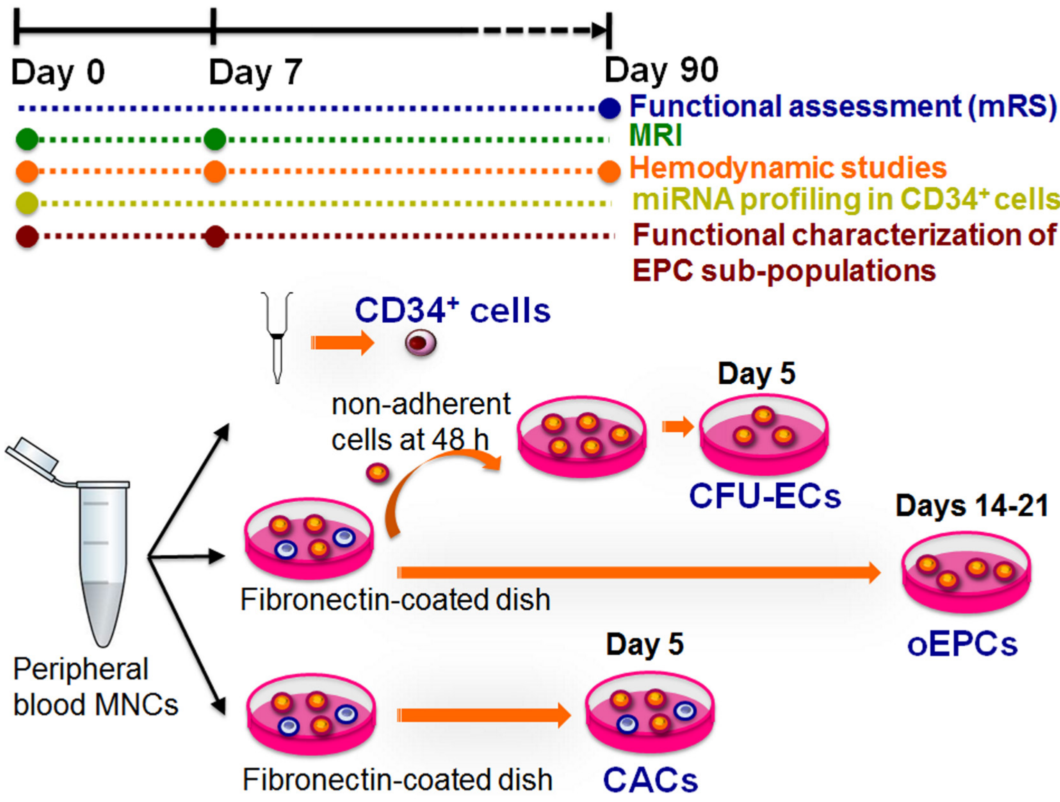

Figure VIII.1. Schematic representation of the study protocol. *Timeline of the analyses performed (represented by bullets) and protocols to isolate the endothelial progenitor cell (EPC) subpopulations described in this study. CACs = circulating angiogenic cells; CFU-ECs = colony-forming units–endothelial cells; miRNA = microRNA; MNCs = mononuclear cells; mRS = modified Rankin Scale; oEPCs = outgrowth endothelial progenitor cells.*

Standard Protocol Approvals, Registrations, and Patient consents

This study was approved by the local ethics committee (Ref. 130-CE-2011). All patients signed written informed consent.

Statistical analysis

Univariate associations of number and functional properties of EPCs with baseline clinical features and BBB permeability were assessed using Mann-Whitney U-test, Spearman correlation or Kruskal-Wallis test as indicated by the variables' characteristics. Association with functional outcome was evaluated by ordinal regression (mRS as dependent variable, with higher values meaning decreased functional status) adjusting for the baseline variables associated with good outcome (age, sex,

baseline NIHSS, early recanalization and intravenous thrombolysis). For the association of BBB with clinical outcome a second model was performed including infarct volume as a covariate. miRNA sequencing data was analysed using Bioconductor DESeq package. All univariate analysis were corrected for multiple comparisons by false discovery rate, and the corrected p-values are presented. Statistical significance was set for p<0.05 in two-tailed analyses.

C. Results

Clinical features

During the study period, a total of 678 patients were admitted to our department due to a non-lacunar ischemic stroke in the MCA territory. After exclusion criteria (Table VII.1), 45 patients were included in the study (53.3% male, median age 70.0 years) and monitored using clinical, neuroimaging and cellular analyses (Figure VIII.1). The baseline clinical features of the study population and their association with functional outcome are presented in Table VIII.1. The number of CACs, oEPCs and CFU-EC at days zero and seven were not associated with any of the baseline characteristics of the study population (Tables e-2 and e-3). Three months after stroke, 22 patients (48.9%) had good clinical outcome (mRS between 0 and 2) and 5 patients (11.1%) were dead. The associations of number and functional properties of each EPC subset with mRS at three months and BBB permeability in the ischemic area are shown in Figure VIII.2, Tables e-4 and e-5. There were no statistically significant associations between EPC and BBB permeability contralateral to the stroke area (Table e-6), infarct volume at day 7 or infarct growth from day 0 to day 7 (Table e-7). No arterial reocclusions were documented in the study participants.

BBB permeability

The median Ktrans at day 0 was 0.014 (IQR: 0.011) and 0.012 (IQR: 0.01) at day 7. The number of each EPC sub-population was not associated with BBB permeability (Table e-4). The univariate comparison of BBB with baseline clinical features showed a statistically significant association between BBB at day 0 with age and C - reactive protein measured from 48 hours to 7 days (Table e-8). In addition, BBB permeability at day zero was not associated with functional outcome (OR: 0.900; 95%CI: 0.803-1.009; p=0.071), even after inclusion of stroke volume in the regression model (OR: 0.892; 95%CI: 0.792-1.004; p=0.058). Importantly, the *in vitro* functional properties of CACs, oEPCs and CFU-EC were associated with BBB permeability, particularly at day seven (Figure VIII.2, and Table e-4). Moreover, BBB permeability at day seven was independently associated with low mRS at three months (OR: 0.897; 95%CI: 0.816-0.986; p=0.025) (Figure VIII.3). The association remained statistically significant after inclusion of stroke volume in the model (OR: 0.855; 95%CI: 0.763-0.956; p=0.006).

Table VIII.1. Baseline variables in the total population. IQR: Interquartile Range; NIHSS: National Institute of Health Stroke Scale; SD: Standard Deviation; mRS: modified Rankin Scale. Variables with statistically significant differences between groups are marked with * if p<0.05 or ** for p>0.001. All p-values presented are corrected for multiple comparisons.

Variable	Total population (n=45)	Good outcome	Bad outcome
Age, years, median (IQR)	70.0 (10.0)	67.50 (11.00)	74.00 (8.00)
Male	24 (53.3%)	10 (45.5%)	14 (60.9%)
Weight, median (IQR)	75.0 (10.5)	75.75 (11.50)	74.39 (13.30)
Height, median (IQR)	165.0 (10.0)	165.00 (7.00)	165.00 (10.00)
Hypertension	32 (71.1%)	13 (59.1%)	19 (82.6%)
Diabetes Mellitus	9 (20.0%)	5 (22.7%)	4 (17.4%)
Atrial fibrillation	24 (53.3%)	11 (50%)	13 (56.5%)
Dyslipidemia	33 (73.3%)	16 (72.7%)	17 (73.9%)
Hyperuricemia	12 (26.7%)	6 (27.3%)	6 (26.1%)
Cardiac Insufficiency	4 (8.9%)	2 (9.1%)	2 (8.7%)
Coronariopathy	6 (13.3%)	4 (18.2%)	2 (8.7%)
Previous stroke	2 (4.4%)	0 (0%)	2 (8.7%)
Smoking	4 (8.9%)	1 (4.5%)	3 (13.0%)
Peripheral arterial disease	4 (8.9%)	2 (9.1%)	2 (8.7%)
Obesity	17 (37.8%)	8 (36.4%)	9 (39.1%)
Previous statin	14 (31.1%)	6 (27.3%)	8 (34.8%)
Previous antiplatelet	10 (22.2)	5 (22.7%)	5 (21.7%)
Intravenous thrombolysis	26 (57.8%)	13 (59.1%)	13 (56.5%)
NIHSS at admission, mean (SD)**	14.51 (5.31)	11.82 (4.72)	17.09 (4.60)
Previous mRS, median (IQR)	0 (0)	0 (1.0)	1.0 (0)
Glucose at admission, median (IQR)	125.50 (21.0)	122.50 (28.75)	118.50 (33.50)
Type 2 Parenchymal Haemorrhage	5 (11.1%)	1 (4.5%)	4 (17.4%)
Early Recanalization*	18 (40.0%)	4 (17.4%)	14 (63.6%)
Infarct volume at 7 days, mL, median (IQR)*	30.74 (74.05)	9.41 (30.76)	52.35 (73.37)
Infarct progression, mL, median (IQR)	2.02 (29.49)	0.9 (25.23)	15.61 (35.6)
Subcortical infarct pattern	10 (22.2%)	7 (31.8%)	3 (13%)
Ktrans at day 0, median (IQR)	0.014 (0.011)	0.016 (0.012)	0.01 (0.01)
Ktrans at day 7, median (IQR)	0.012 (0.01)	0.015 (0.017)	0.009 (0.005)
Stroke etiology			
Cardioembolism	25 (55.6%)	11 (50.0%)	14 (60.9%)
Atherothrombosis	6 (13.3%)	3 (13.6%)	3 (13.0%)
Other causes	4 (8.9%)	2 (9.1%)	2 (8.7%)
Undetermined	10 (22.2%)	6 (27.3%)	4 /17.4%)

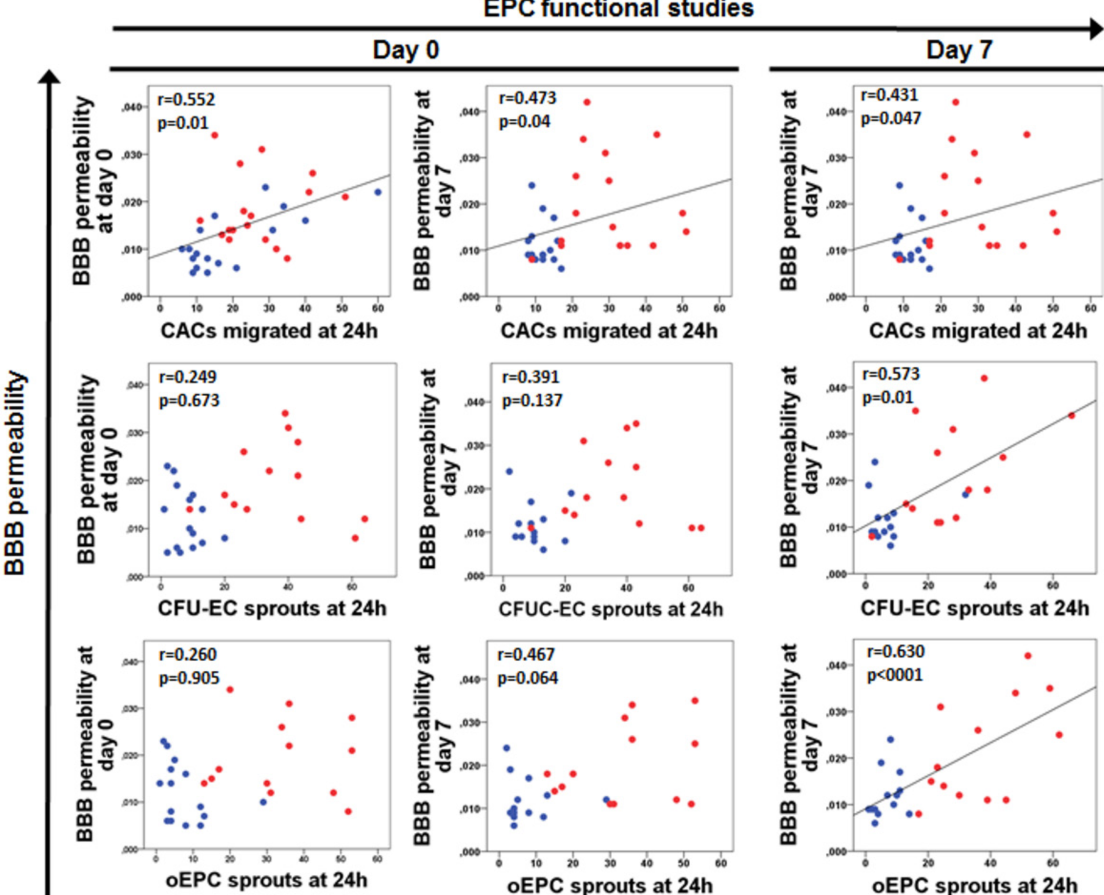

Figure VIII.2. Association among blood–brain barrier (BBB) permeability, endothelial progenitor cell (EPC) functional properties, and clinical outcome. *Scatterplots show the correlations between functional properties of EPCs at days 0 and 7 with BBB permeability at days 0 and 7. Data related to patients with poor and good clinical outcome are presented in blue and red, respectively. Spearman correlation coefficient with respective statistical significance is indicated in each graph; an intersection line was drawn whenever statistically significant. CACs = circulating angiogenic cells; CFU-EC = colony-forming unit–endothelial cells; oEPC = outgrowth endothelial progenitor cell.*

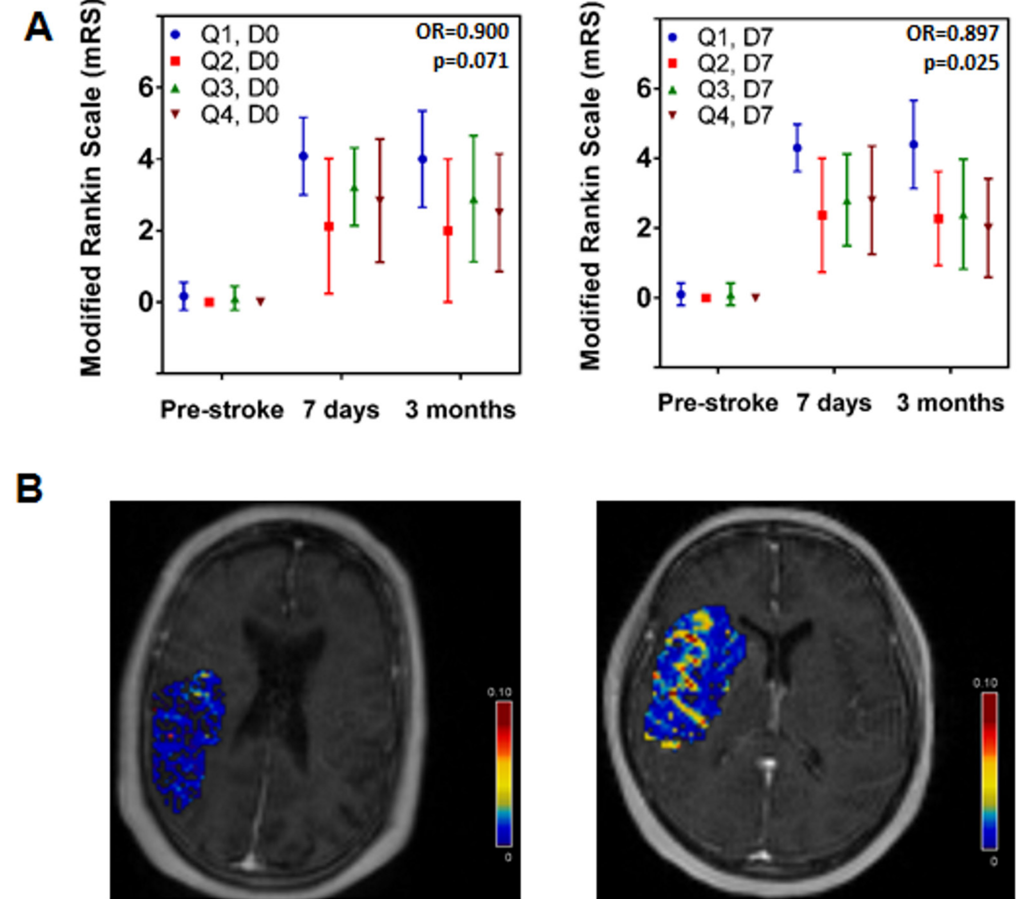

Figure VIII.3. Blood–brain barrier (BBB) permeability and clinical outcome. *(A) Evolution of the patients' modified Rankin Scale score throughout the study (prestroke, 7 days, and 3 months), with the results clustered with BBB permeability at days 0 and 7. The BBB permeability is represented in quartiles with quartile 1 indicating patients with the lowest BBB permeability and quartile 4 the highest. (B) Representative magnetic resonance permeability (Ktrans) maps at day 7 after stroke of patients with poor (left) and good (right) clinical outcomes. Low Ktrans is visible on the left image and high on the right. OR = odds ratio.*

MicroRNA profiling of CD34$^+$ cells

CD34$^+$ cells at day zero from patients with good outcome showed 24 miRNAs differentially regulated ($p<0.05$) relative to patients with bad outcome (median mRS at three months in the two groups was 1.0 vs. 5.0; median seven days Ktrans 0.031 vs. 0.019; median age 67.0 vs. 71.0 years respectively) (Figure VIII.4A). Among these miRNAs, 9 were differentially expressed only in the patients with good outcome (Figure VIII.4B). In Figure e-1, DIANA mirPath analysis shows that the differentially downregulated miRNAs modulate pathways such as Hippo signalling as well as ECM-receptor interactions and adherens junctions[328]. Adherens junctions pathway is targeted by all investigated miRNAs. The analysis based on the predicted gene targets revealed ErbB signalling, cell adhesion molecules and axon guidance pathways as the most shared pathways (shared by three miRNAs) among six upregulated miRNAs (Figure e-2).

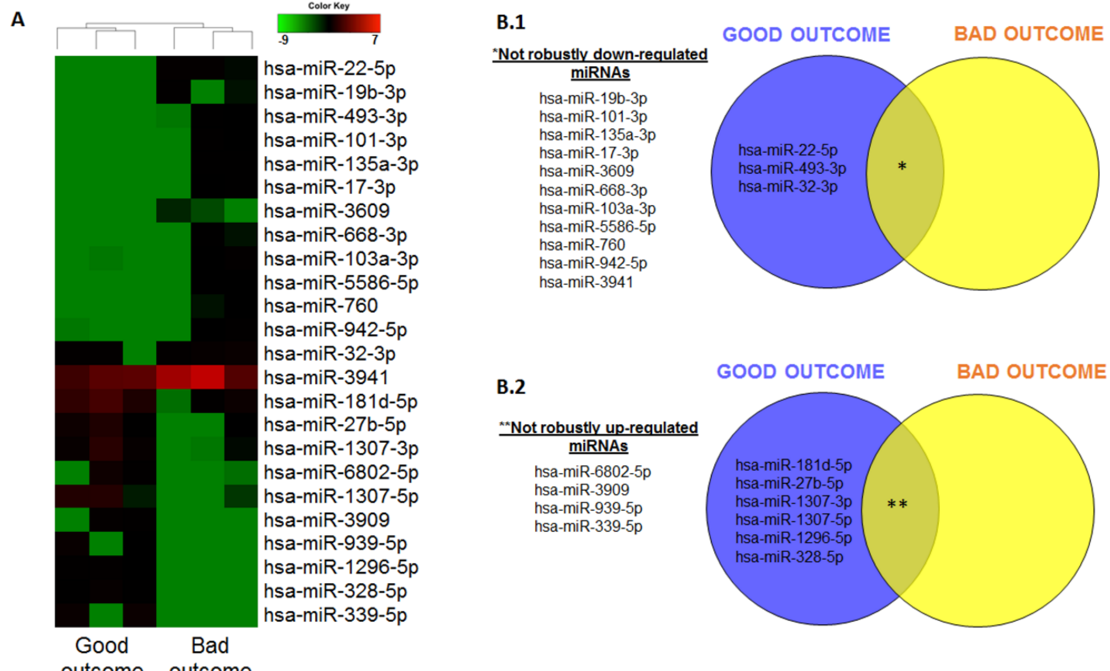

Figure VIII.4. MicroRNAs (miRNAs) associated with clinical outcome. *miRNA profiling in CD34+ cells collected at time zero from patients with good (n = 3) and poor (n = 3) clinical outcome. (A) Heatmap for log2 normalized counts for miRNA with p < 0.05 and fold change >2 in good vs poor clinical outcome groups. The analysis was performed using average count numbers of miRNAs in both groups. Clustering analysis was performed using pheatmap in R. The colour key scale represents the relative downregulation and upregulation with green and red, respectively. (B) Venn diagrams for significantly downregulated/upregulated miRNAs. The analysis was performed using individual count numbers of individual samples for the miRNAs given in the heatmap. An miRNA was considered as robust when it was (B.a) down-regulated/upregulated for all patients in the good outcome group, and (B.b) not downregulated/upregulated for any of the samples in the poor outcome group. The diagrams were prepared using VENNY software and presented in the figure.*

In FACS analysis the CD34+ cells isolated from patients at day 0 showed high coexpression of other EPC cell markers such as CD31 and CD45, with low expression of KDR (Figure e-3).

D. Discussion

In this work, we show that the functional properties of EPCs are associated with subacute (day seven after stroke) BBB permeability and good clinical outcome in patients with ischemic stroke. Previous studies, performed in animals, indicated a multiphasic response of the BBB with variable intensity, in accordance to the sequential pathological cascade of events after stroke: ischemic insult, reperfusion injury, subacute inflammatory response and finally, angiogenesis[291]. In the hyperacute stage, permeability of the BBB is due to the ischemic insult and has consistently been associated with parenchymal hemorrhage, even after intra-arterial therapies[300]. Reperfusion is recognized as having a largely positive effect in the symptomatic vascular territory; however, it may also trigger a chain of pathological events leading to hyperperfusion injury. The extent of this phase is highly heterogeneous and seemingly dependent upon the severity, time and form of ischemic insult[291]. Reperfusion injury is mediated by loss of autoregulation, commonly concurrent with a sharp increase in blood-pressure, potentially leading to tissue necrosis and inflammatory activation[329]. This inflammatory response is visible as early as 6 hours after the ischemic insult and often leads by itself to a subsequent BBB breakdown 24-72 hours after stroke, when it reaches

its peak activity (our results in Table e-8 reinforce these previous findings showing the association between BBB permeability in day 0 and C-Reactive Protein measured between 48 hours and 7 days)[303,326]. A final stage of BBB permeability has been described, starting within the first week and persisting for up to five weeks, however the biological substrate involved is scarcely understood, and its effect on functional outcome was still unaddressed[182]. Animal models that included histological assessments have suggested an association of this later BBB permeability with neurovascular remodelling and angiogenesis[183,259,305]. It is also known that $CD34^{+}$ cells, one of the surface markers of EPCs, induce angiogenesis in the ischemic zone of mouse models, during the subacute stage[198]. Yet, in humans, it is unclear whether there is subacute permeability of BBB after stroke and whether that permeability is correlated with angiogenesis. Our results indicate an association of subacute BBB permeability with the migratory and angiogenic capacities of EPCs especially in day 7, unrelated with type 2 parenchymal haemorrhage nor systemic inflammatory markers at admission or during the first week (Figure VIII.2, Tables e-2, e-3 and e-6). Our results further show a positive effect on functional outcome of BBB permeability in the subacute stage after ischemic stroke, which is also associated with migratory and angiogenic potential of EPCs, reinforcing their involvement in post-stroke angiogenesis. It should be noted that our data suggests that the positive effect of EPCs and subacute BBB permeability appears to translate into clinical outcome after the first week, as demonstrated in Figure VIII.3A, where no meaningful association is observed in 7 days mRS and Table e-7, where no associations are documented with subacute infarct volume. Moreover, no statistically significant association was registered when comparing the number and functional properties of EPCs with the BBB permeability of brain parenchyma contralateral to the acute lesion indicating a direct effect and mobilization to the ischemic area.

In the context of this work, we have selected different EPC sub-populations, with distinct biological roles, to correlate with clinical outcome and BBB permeability. It is known that recruitment of CACs contribute to both angiogenesis and arteriogenesis in a paracrine way, while oEPCs and CFU-ECs are directly involved in neovascularization[330]. In addition, in the context of ischemic stroke, different subsets of EPCs have already been associated with improved clinical and imaging outcomes[21,220]. Our study indicates that the capacity of EPCs to migrate and promote *in vitro* angiogenesis have more effect on clinical outcome and *in vivo* angiogenesis than cell number in the acute or subacute stage. Moreover, despite the distinct biological properties previously described for each EPC subset (CACs, oEPCs and CFU-ECs) our study showed that all populations are associated with clinical outcome and subacute BBB permeability, indicating a possible contributing part in post stroke angiogenesis[153]. It is noteworthy that the association of EPCs' functional properties with clinical outcome and BBB permeability is particularly consistent and robust at day seven (Figure VIII.2), reinforcing the importance of this subacute stage in the beneficial effect of neoangiogenesis after stroke.

Previous studies have indicated CD34 as a surface marker of EPCs[17,153,198]. However, the therapeutic efficacy of CD34+ cells has been shown to be modulated by their miRNA expression program in other clinical settings[331]. MicroRNAs are non-coding RNAs that play a pivotal role

in post-transcriptional gene control, regulating pathways, biological networks and fine-tuning many biological processes regulating tissue repair[332]. In the setting of this work, we have profiled miRNA expression of CD34+ cells to identify potential miRNAs responsible for promoting effective angiogenesis and good clinical outcome. We have used CD34+ cells and not EPC sub-populations isolated by cell culture assays to improve cell homogeneity, to prevent artefacts of cell culture in the molecular program of the cells, and because CD34+ cells have shown to induce angiogenesis in the ischemic zone of mouse models[17,153,198]. Twenty-four miRNAs were differentially regulated ($p<0.05$) between patients with good and bad outcome. Among these 24 miRNAs, nine were differentially expressed only in the patients with good outcome. Six of these nine miRNAs have been reported to be involved in cell migration and angiogenesis. The downregulation of hsa-miR-22-5p and has-miR-32-3p in CD34+ cells isolated from patients with good outcome agrees with the fact that both miRs induce cell senescence, and its upregulation in EPCs or endothelial cells leads to a decreased in vitro migration and impaired angiogenesis[333,334]. Similarly, the downregulation of miR-493 in CD34+ cells from patients with good clinical outcome are in line with previous results showing that down-regulation of miR-493 promoted endothelial cell migration, tube formation and survival in vitro and increased angiogenesis, reduced the infarct volume and ameliorated neurological deficits in stroke animal models[335]. Among the upregulated miRNAs, three of them have been demonstrated to be involved in angiogenesis and cell migration. Up-regulation of miR-27b[336] or miR-181d[337] has been reported to enhance angiogenesis while up-regulation of miR-328 to enhance cell migration[338].

EPC migration seems to be enhanced in patients with good clinical outcomes. DIANA miRPath (v.3) analysis revealed *NCKAP1*, which is important in lamellipodia formation and cell motility, as the only intersected gene target (gene targeted by all selected miRNAs) for the 3 robustly down-regulated miRNAs (miR-32-3p, miR-22-5p, miR-493-3p) in good vs. bad clinical outcome groups. Additionally, adherens junctions pathway was the only the pathway with statistically significant results for all these three microRNAs. Adherens junctions have diverse functions in stem/progenitor cells including anchorage to the niche, control of proliferation and differentiation[339] and have an important role in angiogenesis[340]. Moreover, the upregulation of miR-27b has been shown to promote *in vitro* angiogenesis[341].

Our study has inherent limitations that should be taken under consideration when interpreting its results. Firstly, it was a single centre study; nevertheless, the demographics of the participants suggest the findings are extensive to other acute stroke cohorts. Furthermore, assessing two time points (first 24 hours and seven days) may be limited in the complex pathological time course after stroke. Particularly, analysing the final infarct volume and BBB permeability at 3 months could have added value into the interpretation of the findings. Nonetheless, the time points analysed have been suggested as pathologically important in preclinical studies and are both potential treatment windows in stroke patients[342]. Moreover, animal studies suggest that at seven days, most of the biological substrate of BBB opening is related to vascular remodelling and angiogenesis, without other confounding effects[259]. Other limitations are related to the lack

of histological confirmation of angiogenesis (inherent to the human-based design of the study) and absence of confirmation of the miRNA findings with mRNA expression studies (restricted due to the low cell number available).

Our study shows that the functional properties of EPCs are associated with subacute permeability of the BBB and good clinical outcome after ischemic stroke, identifying the miRNAs modulating an improved outcome.

Table e-1. Inclusion and exclusion criteria for the study (presented in Table VII.1. of the thesis). Table e-2. Univariate association between baseline variables of the total population and the number of CACs, OECs and CFU-ECs at day zero. The values shown represent the statistical coefficient of the respective test used (Mann-Whitney U-test, Spearman correlation or Kruskal-Wallis) and corresponding p-value; all p-values presented are corrected for multiple comparisons.

Variable	CACs at day 0	p	oEPCs at day 0	p	CFU-ECs at day 0	p
Age	-0.145	0.653	-0.184	0.242	-0.261	0.48
Male	153.0	1.0	211.5	0.830	194.5	0.975
Weight	-0.018	0.937	0.099	0.531	0.159	0.725
Height	0.051	0.931	0.070	0.661	0.020	0.966
Hypertension	197.0	0.908	155.5	0.495	168.0	0.963
Diabetes Mellitus	146.0	0.963	115.5	0.511	132.5	0.942
Atrial fibrillation	188.5	0.710	174.5	0.251	205.5	1.0
Dyslipidemia	162.0	0.715	173.5	0.856	173.5	1.0
Hyperuricemia	127.5	0.715	177.0	0.933	167.5	1.0
Cardiac Insufficiency	63.5	0.789	68.0	0.732	36.0	0.516
Coronariopathy	79.0	0.693	55.5	0.059	58.5	0.563
Previous stroke	22.0	0.600	1.0	0.107	16.0	1.0
Smoking	48.0	0.716	66.0	0.668	68.0	0.997
Peripheral arterial disease	35.0	0.495	61.5	0.532	43.5	0.611
Obesity	178.5	0.572	157.0	0.186	158.5	0.597
Previous statin	192.0	0.897	110.5	0.053	129.5	0.681
Previous antiplatelet	154.0	0.892	135.0	0.974	107.5	0.774
Intravenous thrombolysis	187.0	0.630	149.5	0.091	174.0	0.777
NIHSS at admission	-0.159	0.606	0.085	0.594	0.116	0.928
Previous mRS	-0.253	0.588	0.194	0.218	0.091	0.999
Glucose at admission	0.101	0.782	-0.205	0.199	0.001	0.994
C-reactive protein at admission	-0.2	0.960	0.087	0.586	0.295	0.58
C-reactive protein 48 hours-7 days	-0.176	0.635	0.287	0.066	0.331	0.96
Type 2 parenchymal haemorrhage	47.0	0.620	68.5	0.748	52.0	0.758
Early recanalization	148.0	0.600	155.5	0.144	207.5	1.0
Subacute recanalization (6 hours-7 days)	159.0	0.630	141.5	0.146	143.0	0.543
Infarct volume (7 days)	-0.045	0.902	-0.050	0.761	0.021	1.0
Infarct progression	-0.145	0.646	-0.059	0.720	-0.082	1.0
Subcortical infarct pattern	-32.8	0.776	-13.3	0.651	49.1	0.825
Stroke etiology: Cardioembolism	1.115	0.928	4.249	0.236	1.075	0.979
Atherothrombosis						
Other causes						
Undetermined						

Table e-3. Univariate association between baseline variables of the total population and the number of CACs, OECs and CFU-ECs at day seven. The values shown represent the statistical coefficient of the respective test used (Mann-Whitney U-test, Spearman correlation or Kruskal-Wallis) and corresponding p-value; all p-values presented are corrected for multiple comparisons.

Variable	CACs at day 7	p	oEPCs at day 7	p	CFU-ECs at day 7	p
Age	0.154	0.736	-0.077	1.0	0.210	0.737
Sex	218.0	1.0	168.0	1.0	185.5	1.0
Weight	-0.223	0.548	-0.066	0.977	-0.282	1.0
Height	0.026	1.0	-0.151	1.0	-0.141	0.908
Hypertension	192.0	1.0	121.0	1.0	144.0	0.957
Diabetes Mellitus	113.5	0.6	88.5	1.0	108.0	0.879
Atrial fibrillation	183.0	0.563	190.0	0.944	199.0	0.978
Dyslipidemia	168.5	0.946	166.0	1.0	142.5	0.846
Hyperuricemia	180.0	1.0	115.0	1.0	125.5	0.627
Cardiac Insufficiency	38.5	0.45	40.0	1.0	53.0	0.902
Coronariopathy	58.0	0.55	94.0	0.953	94.0	0.953
Previous stroke	10.5	0.456	19.5	1.0	17.0	0.92
Smoking	59.0	0.782	47.5	1.0	45.5	0.958
Peripheral arterial disease	78.0	0.967	64.5	0.959	65.0	0.981
Obesity	155.0	0.54	166.5	1.0	142.0	0.715
Previous statins	177.0	0.99	144.0	1.0	132.5	0.885
Previous antiplatelets	107.0	0.609	109.5	0.959	108.5	0.964
Intravenous thrombolysis	221.5	1.0	152.5	1.0	184.5	0.865
NIHSS at admission	-0.331	0.42	0.123	0.964	0.097	0.922
Previous mRS	-0.042	1.0	0.087	1.0	0.198	0.657
Glucose at admission	0019	0.968	-0.201	1.0	-0.254	1.0
C-reactive protein at admission	0.036	0.98	-0.074	1.0	0.133	0.826
C-reactive protein 48 hours-7 days	-0.154	0.795	0.195	1.0	0.217	0.668
Type 2 parenchymal haemor-rhage	69.0	0.796	42.0	0.997	67.0	0.947
Early recanalization	117.0	0.15	183.0	0.998	182.5	0.985
Subacute recanalization (6 hours-7 days)	212.5	1.0	147.5	1.0	113.0	0.78
Infarct volume (7 days)	0.004	0.978	0.030	0.951	0.192	0.676
Infarct progression	-0.156	0.696	-0.131	0.997	-0.270	1.0
Subcortical infarct pattern	10.7	1.0	-0.452	1.0	-38.8	0.744
Stroke etiology: Cardioembolism	1.265	1.0	6.989	1.0	0.862	0.895
Atherothrombosis						
Other causes						
Undetermined						

Table e-4. Association of study variables with blood-brain barrier (BBB) permeability at day zero, day seven and functional outcome at three months. Associations with BBB permeability were assessed by univariate comparison; associations with functional outcome at three months were adjusted for age, sex, early recanalization, baseline NIHSS and intravenous thrombolysis. The p-values presented in univariate analysis are corrected for multiple comparisons. Statistically significant results are marked with "*".

Variable		BBB permeability at day 0		BBB permeability at day 7		Functional outcome at 3 months	
		Correlation coefficient	p	Correlation coefficient	p	OR (95%CI)	p
Day 0	CAC number	-0.020	0.912	0.197	0.372	0.992 (0.987-0.997)	0.003*
	oEPC number	-0.027	0.983	0.076	0.729	0.999 (0.992-1.007)	0.879
	CFU-EC number	-0.067	0.9	-0.238	0.329	1.003 (0.993-1.012)	0.571
	CAC migration	0.552	0.01*	0.473	0.04*	0.983 (0.938-1.030)	0.468
	oEPC migration	0.267	0.545	0.281	0.311	0.970 (0.940-1.003)	0.074
	CFU-EC migration	0.240	0.476	0.302	0.253	0.942 (0.906-0.979	0.003*
	oEPC sprout number	0.260	0.905	0.467	0.064	0.904 (0.855-0.957)	<0.001*
	CFU-EC sprout number	0.249	0.673	0.391	0.137	0.855 (0.830-0.943)	<0.001*
	oEPC sprout length	-0.101	0.871	0.145	0.566	1.000 (0.999-1.001)	0.061
	CFU-EC sprout length	0.210	0.472	0.170	0.508	0.997 (0.996-0.999)	0.005*
Day 7	CAC number	-	-	0.337	0.120	0.997 (0.990-1.003)	0.326
	oEPC number	-	-	0.062	0.754	1.000 (0.991-1.009)	0.945
	CFU-EC number	-	-	-0.107	0.654	0.994 (0.985-1.002)	0.157
	CAC migration	-	-	0.431	0.047*	0.953 (0.899-1.012)	0.109
	oEPC migration	-	-	0.439	0.1	0.850 (0.777-0.929)	<0.001*
	CFU-EC migration	-	-	0.446	0.105	0.894 (0.848-0.943)	<0.001*
	oEPC sprout number	-	-	0.630	<0.001*	0.895 (0.845-0.947)	<0.001*
	CFU-EC sprout number	-	-	0.573	0.01*	0.901 (0.845-0.960)	0.001*
	oEPC sprout length	-	-	0.354	0.1	0.999 (0.999-0.999)	0.044*
	CFU-EC sprout length	-	-	0.343	0.1	0.998 (0.997-0.999)	0.008*

Table e-5. Univariate association between the number and functional properties of CACs, OECs and CFU-ECs at day zero and seven with good/bad functional outcome at 3 months. Each comparison is presented by median (interquartile range) according to clinical outcome; all p-values presented are corrected for multiple comparisons.

Variable		Good outcome	Bad outcome	p
Day 0	CAC number	195.0 (249.0)	123.75 (172.50)	0.025*
	oEPC number	41.25 (89.38)	53.75 (78.75)	0.683
	CFU-EC number	60.88 (121.88)	69.38 (75.94)	0.64
	CAC migration	23.0 (12.0)	13.0 (13.0)	0.002*
	oEPC migration	59.7 (42.8)	16.5 (45.0)	0.018*
	CFU-EC migration	56.99 (18.6)	26.25 (33.28)	<0.001*
	oEPC sprout number	34.0 (28.0)	4.0 (9.0)	<0.001*
	CFU-EC sprout number	40.0 (18.0)	9.0 (7.5)	<0.001*
	oEPC sprout length	148.05 (312.51)	26.11 (59.34)	0.002*
	CFU-EC sprout length	141.31 (186.66)	42.94 (52.54)	0.002*
Day 7	NIHSS at 7 days	4.0 (7.0)	17.0 (9.0)	<0.001*
	CAC number	163.33 (215.0)	150.0 (115.31)	0.124
	oEPC number	40.0 (96.25)	48.75 (62.5)	0.924
	CFU-EC number	75.0 (85.5)	88.62 (118.13)	0.653
	CAC migration	29.5 (15.25)	12.0 (6.25)	<0.001*
	oEPC migration	72.77 (24.41)	21.23 (18.74)	<0.001*
	CFU-EC migration	77.51 (32.98)	19.22 (25.38)	<0.001*
	oEPC sprout number	36.0 (29.0)	4.5 (8.5)	<0.001*
	CFU-EC sprout number	28.0 (23.0)	5.0 (6.25)	<0.001*
	oEPC sprout length	138.35 (287.31)	26.79 (54.81)	<0.001*
	CFU-EC sprout length	87.49 (213.54)	37.03 (58.91)	0.003*

Table e-6. Association of study variables and C reactive protein with blood-brain barrier (BBB) permeability contralateral to stroke at day zero and day seven. All tests were assessed by univariate comparison (Spearman correlation); the p-values presented are corrected for multiple comparisons.

Variable		BBB permeability contralateral to stroke at day 0		BBB permeability contralateral to stroke at day 7	
		Correlation coefficient	p	Correlation coefficient	p
Day 0	CAC number	-0.214	0.598	-0.143	1.0
	oEPC number	-0.09	0.784	0.042	0.981
	CFU-EC number	-0.116	0.729	-0.046	1.0
	CAC migration	0.256	0.517	0.296	0.693
	oEPC migration	-0.151	0.763	-0.108	1.0
	CFU-EC migration	-0.197	0.653	0.168	1.0
	oEPC sprout number	-0.135	0.82	0.083	0.981
	CFU-EC sprout number	-0.017	0.923	-0.04	0.875
	oEPC sprout length	-0.357	0.418	-0.207	1.0
	CFU-EC sprout length	-0.039	0.915	-0.162	0.902
	C-reactive protein at admission	0.017	0.955	0.166	1.0
Day 7	CAC number	-0.039	0.927	0.035	0.913
	oEPC number	0.197	0.613	0.089	0.943
	CFU-EC number	-0.099	0.796	-0.038	0.953
	CAC migration	-0.066	0.821	0.107	0.926
	oEPC migration	-0.345	0.554	-0.029	0.893
	CFU-EC migration	-0.168	0.806	0.188	0.956
	oEPC sprout number	-0.083	0.835	0.196	1.0
	CFU-EC sprout number	-0.204	0.623	0.134	0.958
	oEPC sprout length	-0.231	0.619	-0.071	0.973
	CFU-EC sprout length	-0.356	0.308	-0.207	1.0
	C-reactive protein 48 hours-7days	-0.39	0.176	-0.295	1.0

Table e-7. Association of study variables with infarct volume at 7 days and infarct growth in the first week. All analysis were performed by linear regression, adjusting for age, sex, early recanalization, baseline NIHSS and intravenous thrombolysis.

Variable		Infarct volume at day 7		Infarct growth in first week	
		B (Standard Error)	p	B (Standard Error)	p
Day 0	CAC number	20.17 (59.05)	0.735	10.93 (39.01)	0.781
	oEPC number	-65.97 (110.41)	0.556	-32.50 (72.98)	0.659
	CFU-EC number	-47.45 (127.27)	0.712	-21.01 (84.03)	0.804
	CAC migration	-21.13 (664.62)	0.975	-259.1 (436.6)	0.557
	oEPC migration	-21.13 (664.62)	0.975	-473.7 (318.47)	0.155
	CFU-EC migration	-814.19 (468.71)	0.098	-390.5 (245.9)	0.128
	oEPC sprout number	-773.85 (689.02)	0.272	-132.99 (440.12)	0.765
	CFU-EC sprout number	-803.73 (661.2)	0.236	-366.67 (425.25)	0.398
	oEPC sprout length	-0.38 (7.04)	0.957	2.35 (4.37)	0.596
	CFU-EC sprout length	-1.71 (7.86)	0.830	-1.95 (4.97)	0.699
	BBB permeability	168.2 (687.07)	0.808	-5.69 (453.68)	0.99
Day 7	CAC number	111.26 (85.06)	0.199	30.36 (57.25)	0.599
	oEPC number	-19.94 (146.17)	0.892	-69.06 (94.07)	0.468
	CFU-EC number	-21.12 (127.75)	0.870	-90.18 (81.34)	0.276
	CAC migration	-959.01 (812.95)	0.246	-272.9 (544.96)	0.62
	oEPC migration	-373.03 (607.46)	0.550	-274.26 (303.68)	0.383
	CFU-EC migration	-526.36 (421.9)	0.228	-318.62 (264.54)	0.244
	oEPC sprout number	-817.46 (613.47)	0.195	-523.03 (373.72)	0.174
	CFU-EC sprout number	-876.29 (778.25)	0.271	-473.91 (478.51)	0.332
	oEPC sprout length	-2.93 (3.93)	0.397	-0,762 (2.1)	0.72
	CFU-EC sprout length	-876.29 (778.25)	0.271	-2.16 (5.14)	0.679
	BBB permeability	314.69 (876.85)	0.722	-150.29 (498.93)	0.765

Table e-8. Univariate association between baseline variables of the total population and BBB permeability at days zero and seven. The values shown represent the statistical coefficient of the respective test used (Mann-Whitney U-test, Spearman correlation or Kruskal-Wallis) and corresponding p-value; all p-values presented are corrected for multiple comparisons; statistically significant results are marked with "*".

Variable	BBB permeability at day 0	p	BBB permeability at day 7	p
Age	-0.526	0.03*	-0.170	0.814
Sex	135.0	0.957	110.5	1.0
Weight	0.065	0.975	-0.174	0.852
Height	-0.015	0.966	0.099	1.0
Hypertension	99.5	0.954	97.5	1.0
Diabetes Mellitus	70.5	1.0	53.5	1.0
Atrial fibrillation	132.5	0.480	118.0	1.0
Dyslipidemia	76.0	0.939	101.5	1.0
Hyperuricemia	77.0	0.405	57.5	1.0
Cardiac Insufficiency	24.0	0.975	56.0	1.0
Coronariopathy	68.5	1.0	64.5	1.0
Previous stroke	25.5	0.424	22.5	1.0
Smoking	9.5	0.525	12.0	0.954
Peripheral arterial disease	13.5	0.990	16.0	1.0
Obesity	114.5	0.378	110.5	1.0
Previous statins	76.0	1.0	83.0	1.0
Previous antiplatelets	101.0	0.350	72.5	0.912
Intravenous thrombolysis	77.0	0.386	95.0	0.894
NIHSS at admission	-0.284	0.477	-0.471	0.105
Previous mRS	-0.238	0.947	-0.105	1.0
Glucose at admission	0.026	0.884	0.054	1.0
C-reactive protein at admission	0.011	0.949	0.009	1.0
C-reactive protein 48 hours-7 days	-0.520	0.03*	-0.526	0.060
Type 2 parenchymal haemorrhage	21.0	0.403	18.0	0.735
Early recanalization	113.5	0.785	82.0	0.840
Subacute recanalization (6 hours-7 days)	110.0	1.0	112.5	1.0
Infarct volume (7 days), in L	-0.125	1.0	-0.072	1.0
Infarct progression	0.051	0.944	-0.041	1.0
Subcortical infarct pattern	90.0	0.875	78.5	0.977
Stroke etiology: Cardioembolism	1.333	0.940	0.201	1.0
Atherothrombosis				
Other causes				
Undetermined				

Table e-9. Results for the 24 differentially expressed miRNAs detected by miRNA sequencing. The list is sorted on Log2_FC.

	Base Mean	log fold change	P-value
hsa-miR-22-5p	41.697	-8.696	<0.001
hsa-miR-19b-3p	21.665	-6.977	<0.001
hsa-miR-493-3p	7.857	-6.281	0.003
hsa-miR-101-3p	9.735	-6.041	0.005
hsa-miR-135a-3p	7.479	-5.603	0.011
hsa-miR-17-3p	7.392	-5.585	0.011
hsa-miR-3609	7.798	-5.367	0.017
hsa-miR-668-3p	6.712	-5.363	0.016
hsa-miR-103a-3p	9.741	-5.227	0.009
hsa-miR-5586-5p	5.477	-5.007	0.027
hsa-miR-760	5.081	-4.863	0.033
hsa-miR-942-5p	7.722	-4.585	0.026
hsa-miR-32-3p	54.805	-2.798	0.028
hsa-miR-3941	1313.354	-2.248	0.025
hsa-miR-181d-5p	128.212	2.226	0.021
hsa-miR-27b-5p	32.160	3.034	0.047
hsa-miR-1307-3p	39.788	3.761	0.003
hsa-miR-6802-5p	14.214	4.128	0.040
hsa-miR-1307-5p	42.480	4.496	0.002
hsa-miR-3909	8.664	4.858	0.031
hsa-miR-939-5p	9.909	5.024	0.025
hsa-miR-1296-5p	8.653	5.665	0.006
hsa-miR-328-5p	10.772	6.007	0.003
hsa-miR-339-5p	26.578	6.405	0.002

Table e-10. Individual count numbers of statistically significant miRNAs for different patients. G: Good outcome group; B: bad outcome group.

miRNA name	G1	G2	G3	B1	B2	B3
hsa-miR-22-5p	0	0	0	46	46	13
hsa-miR-19b-3p	0	0	0	31	0	12
hsa-miR-493-3p	0	0	0	1	21	19
hsa-miR-101-3p	0	0	0	0	33	18
hsa-miR-135a-3p	0	0	0	0	21	21
hsa-miR-17-3p	0	0	0	0	20	22
hsa-miR-3609	0	0	0	10	6	0
hsa-miR-668-3p	0	0	0	0	23	12
hsa-miR-103a-3p	0	1	0	0	20	39
hsa-miR-5586-5p	0	0	0	0	15	16
hsa-miR-760	0	0	0	0	12	18
hsa-miR-942-5p	1	0	0	0	15	32
hsa-miR-32-3p	37	39	0	40	56	79
hsa-miR-3941	435	624	650	1117	1342	584
hsa-miR-222-3p	9442	11722	3517	524	1171	1784
hsa-miR-148a-3p	2131	2906	495	46	184	633
hsa-miR-181a-2-3p	578	1252	341	16	99	138
hsa-miR-181d-5p	343	491	214	2	34	90
hsa-miR-27b-5p	104	228	27	0	0	19
hsa-miR-1307-3p	69	295	62	0	1	13
hsa-miR-6802-5p	0	113	33	0	0	2
hsa-miR-1307-5p	246	258	11	0	0	8
hsa-miR-3909	0	42	29	0	0	0
hsa-miR-939-5p	75	0	14	0	0	0
hsa-miR-1296-5p	30	37	17	0	0	0
hsa-miR-328-5p	27	62	21	0	0	0
hsa-miR-339-5p	82	0	91	0	0	0

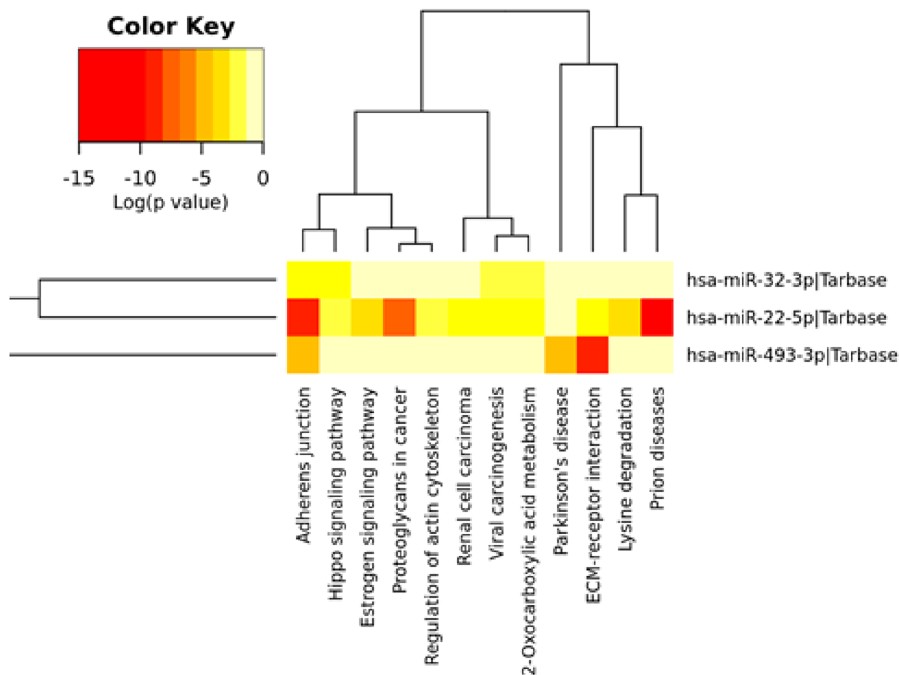

Figure e-1. miRNAs versus pathways heatmap (clustering based on significance levels) for downregulated miRNAs in good vs. bad clinical outcome groups. *The heatmap was prepared using DIANA mirPath v 3.0. Darker colours represent lower significance values. The dendrograms on both axes depict hierarchical clustering results for miRNAs and pathways, respectively. On the miRNA axis, miRNAs with similar pathway targeting patterns are clustered together. The results show that adherens junctions pathway is clearly targeted by all investigated miRNAs. P-value threshold: 0.05; Analysis: Pathway union.*

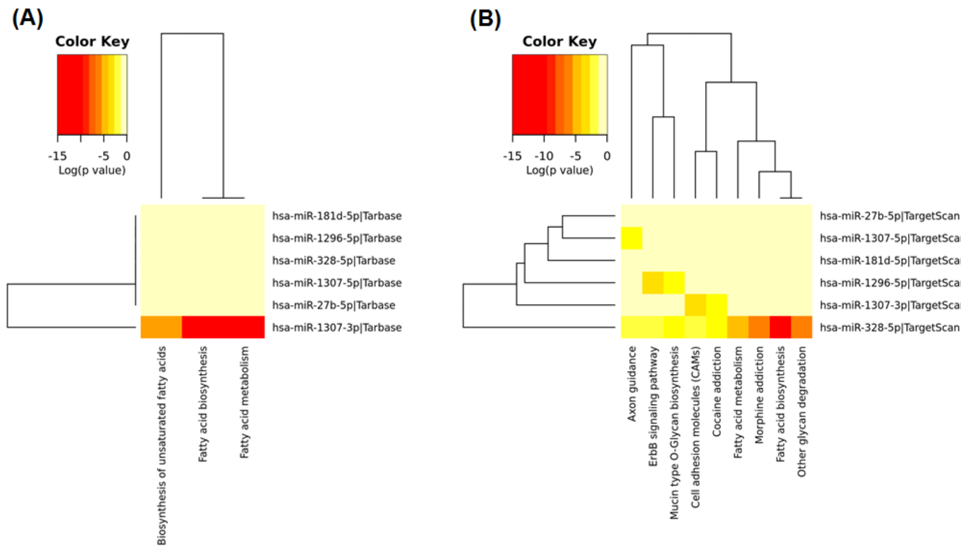

Figure e-2. miRNAs versus pathways heatmaps (clustering based on significance levels) for upregulated miRNAs in good vs. bad clinical outcome groups. *The heatmaps were prepared using DIANA mirPath v 3.0. Darker colours represent lower significance values. The dendrograms on both axes depict hierarchical clustering results for miRNAs and pathways, respectively. On the miRNA axis, miRNAs with similar pathway targeting patterns are clustered together. (A) miRNA/pathway heatmaps were first prepared using experimentally supported targets (Tarbase). However, no gene targets were determined for three miRNAs (miR-1296-5p, miR-181d-5p and miR-328-5p). (B) The upregulated miRNAs were further analysed for predicted gene targets (TargetScan). P-value threshold: 0.05; Analysis: Pathway union.*

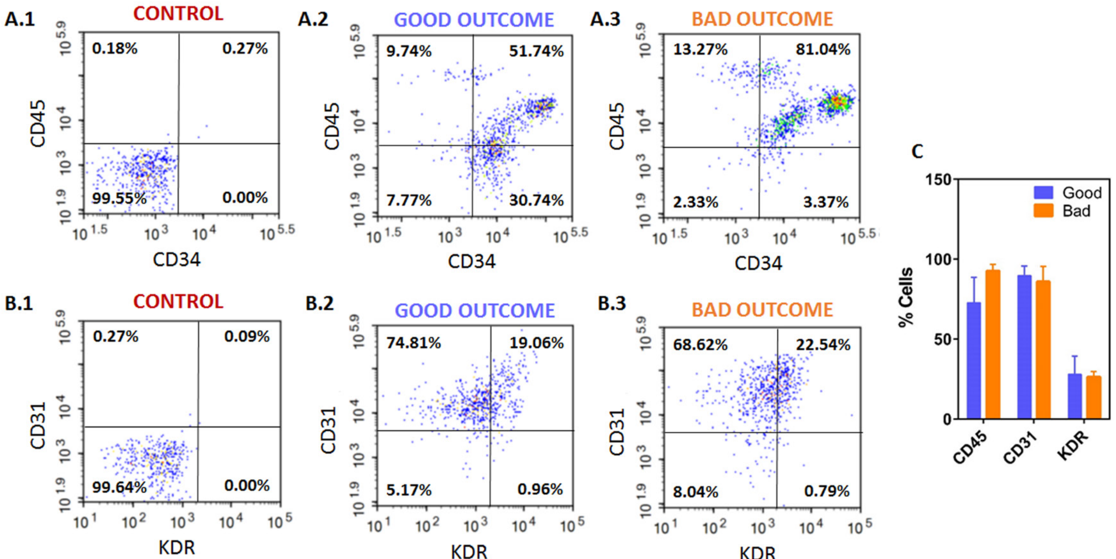

Figure e-3. Expression of different cell surface markers in CD34+ cells from patients with good and bad clinical outcome. *The expression of CD34 and CD45 (A) or CD31 and KDR (B) on CD34+ cells. Scatter plots show the expression of these markers for unstained control group (A.1 and B.1), patients with good outcome (A.2 and B.2) and patients with bad outcome (A.3 and B.3). (C) Quantification of the CD34+ cell surface marker expressions in good vs. bad clinical outcome groups (n=8 donors; 2 technical replicates for good clinical outcome and n=9 donors; 3 technical replicates for bad outcome groups).*

ENDOTHELIAL PROGENITOR CELLS INFLUENCE ACUTE AND SUBACUTE STROKE HEMODYNAMICS

Endothelial Progenitor Cells influence acute and subacute stroke hemodynamics

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Abstract

Background: Endothelial Progenitor Cells (EPCs) are a circulating stem cell population with *in vivo* capacity of promoting angiogenesis after ischemic events. Despite the promising preclinical data, their potential integration with reperfusion therapies and hemodynamic evolution of stroke patients is still unknown. Our aim was to determine the association of EPCs with acute, subacute and chronic hemodynamic features.

Methods: In this prospective study, we included consecutive patients with ages between 18-80 years and non-lacunar ischemic stroke within the territory of a middle cerebral artery. All patients were subject to hemodynamic evaluation by ultrasound at baseline, seven days and three months. We quantified cerebral blood flow (CBF) and assessed early recanalization and collateral flow. Hemorrhagic transformation was graded in Magnetic Resonance imaging performed at seven days. EPCs were isolated from peripheral venous blood collected in the first 24 hours and seven days, counted and submitted to functional *in vitro* tests.

Results: We included 45 patients with a median age of 70±10 years. The angiogenic and migratory capacities of EPCs were associated with increased collateral flow in the acute stage and day seven CBF, without statistically significant associations with recanalization nor haemorrhagic transformation. The number of EPCs was not associated with any hemodynamic variable.

Conclusions: The functional properties of EPCs are associated with acute and subacute stroke hemodynamics, with no effect on haemorrhagic transformation.

1. Introduction

The treatment algorithm for acute ischemic stroke has had significant recent updates due to new effective strategies to promote recanalization. However, after the first few hours no therapy has demonstrated meaningful impact on clinical recovery. Endothelial Progenitor Cells (EPCs) are circulating cells that have emerged as a promising treatment strategy, with demonstrated in vivo capacity of promoting neovascularization and neurological improvement after stroke[153,343]. Nonetheless, their timing of action and integration within stroke hemodynamics are still unknown, which can be critical for optimization of their clinical effect.

Acute stroke hemodynamics have been recognized as one of the main determinants of clinical evolution[59,344]. Several pathophysiological mechanisms take place in the regulation of vascular responses with distinct implications and at different timepoints. In the hyperacute stage the main determinant of evolution is early recanalization, with a marked clinical impact[302,345]. Collateral pial circulation and cerebral blood flow (CBF) are also essential early hemodynamic mechanisms, installed in the attempt to extend the preservation of cerebral tissue after the vascular insult[329]. After the acute stage, and depending on timing of recanalization and resistance to ischemia, a loss of autoregulation may precipitate hyperperfusion injury which may ultimately lead to hemorrhagic transformation[61]. Altogether, these are potentially modifiable hemodynamic responses, crucial to the clinical evolution after stroke.

The potential integration of EPC transplantation within the treating algorithm of stroke will have to take the hemodynamic response into account. As such, in this study we used a multidimensional approach to assess the interplay between EPCs and the several aspects of the patients' hemodynamic state.

2. Methods

2.1. Study population

We included consecutive acute ischemic stroke patients admitted in our department during a period of 27 months (June/2012 to August/2013 and June/2014 to July/2016) in a prospective observational cohort study. All patients with ages between 18-80 years and clinically defined non-lacunar strokes within the territory supplied by the Middle Cerebral Artery (MCA) that could have full clinical, neuroimaging and cellular evaluation within 24 hours after the onset of symptoms were included (Supplementary Figure IX.1 presents the flow chart for study participation, including exclusion criteria). All patients or legal representatives signed written informed consent for study participation. The study design was approved by the local ethics committee (Ref. 130-CE-2011).

At hospital presentation we collected demographic variables, vascular risk factors and quantified stroke severity using the National Institute of Health Stroke Scale (NIHSS)[346]. Functional outcome

was graded in person at three months according to the modified Rankin scale (mRS) by vascular neurologist blinded to CBF and *in vitro* data.

2.2. Study design

At day zero (first 24 hours after symptom onset) we collected patients' blood for cellular isolation and performed clinical and hemodynamic evaluation. At day 7 ± 2 days patients underwent MRI, repeated hemodynamic evaluation and blood collection for cellular studies. At 3 months ± one week participants had repeated clinical and hemodynamic evaluation. The primary objectives were to determine the associations between the number and functional properties of EPCs with CBF, hemorrhagic transformation, recanalization and flow diversion after ischemic stroke. We also aimed to understand the role of EPCs and CBF within clinical and demographic features as secondary objectives.

2.3. Isolation of EPC sub-populations

We isolated EPCs from 18 mL of peripheral venous blood collected in the first day and at day seven after stroke onset in accordance to previously validated protocols[153,343]. Mononuclear cells (MNC) were isolated from peripheral blood by density gradient centrifugation using Lymphoprep™ density gradient medium.

Three different types of EPCs were analysed: circulating angiogenic cells (CACs), outgrowth endothelial progenitor cells (oEPCs) and colony forming unit-endothelial cells (CFU-ECs). For CACs, MNCs were plated into 2 μg/cm^2 fibronectin-coated plates (24-well plates; 1.9×10^6 cells/well) and cultured in Endothelial Growth Medium-2 Microvascular (EGM-2 MV) containing 5% fetal bovine serum (FBS) during five days. Adherent cells were detached using trypsin, counted and used for functional assays.

For oEPCs and CFU-ECs, 10×10^6 MNCs in Endothelial Growth Medium-2 (EGM-2) with 10% FBS were seeded into one well of 2 μg/cm^2 fibronectin-coated 24-well plate. After 48 hours, nonadherent cells were collected and 3×10^6 cells were replated into three fibronectin-coated 24-well plates. At day 5, colony-forming units were counted manually in four random fields (20× magnification). The CFU-ECs were detached using trypsin and used for functional studies[20]. The adherent cells at 48 hours continued cell culture for 14-21 days[22] to obtain oEPCs. Medium for oEPCs and CFU-ECs was changed every 48 hours. All incubation periods were performed at 37°C and 5%CO_2.

2.4. Functional tests of EPCs

The wound healing capacity of oEPCs and CFU-ECs was evaluated using the *in vitro* wound-healing (scratch) assay. In brief, cells were plated in 2 μg/cm^2 fibronectin-coated 96-well plates and cultured until they form a monolayer. Then, the wounds were created by scratching the cell layer

with a 200 μL pipette tip. Migratory capacity was quantified as the percentage of wound closure after 24 hours (Figure IX.1).

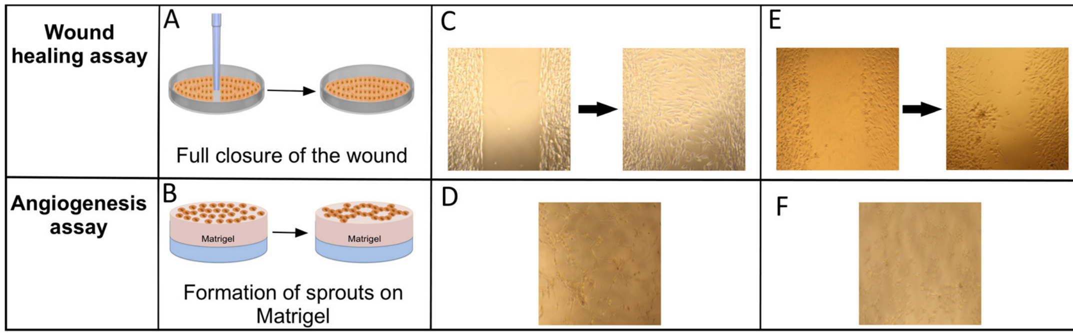

Figure IX.1. Representation of in vitro wound healing and angiogenesis assays (A and B respectively). *Examples with enhanced (C and D) and poor properties (E and F) in each test are shown.*

The angiogenic capacity of oEPCs and CFU-ECs were determined by the sprout formation on Matrigel (using an IBIDI μ-slide angiogenesis kit). The total tube length and number of branching points (i.e. points featuring more than two connections) were manually measured in four random fields (20× magnification) 24 hours after plating (Figure IX.1).

The migratory capacity of CACs was determined by transwell migration. In short, 2×10^4 CACs were placed in the upper chamber of a modified Boyden chamber (2 μg/cm^2 fibronectin-coated). The chamber was placed in a 24-well culture dish containing EBM-2 and human recombinant VEGF (50 ng/mL) and incubated for 24 hours. The lower side of the filter was then washed with PBS and fixed with 4% paraformaldehyde. For quantification, cell nuclei were stained with 4',6-diamidino-2-phenylindole. Cells migrating into the lower chamber were counted manually in 3 random microscopic fields (20× magnification).

2.5. Hemodynamic evaluation

The hemodynamic evolution was assessed through serial cervical and transcranial neurosonological exams at admission (day zero), six hours, seven days and three months after stroke (3MHz sector-probe and 11MHz linear-probe respectively; General Electric Logiq7). All exams were performed with patients lying in a supine position after at least 10 minutes rest, collecting data on flow diversion, recanalization and CBF. Flow velocities were assessed bilaterally using transcranial colour coded Doppler (TCCD) with angle correction in anterior, middle and posterior cerebral arteries (ACA, MCA and PCA respectively). Flow diversion was defined as a high-velocity, low-resistance flow signal in the ACA-A1 or PCA (P1–P2 segments) ipsilateral to the occluded MCA; the ACA or PCA flow velocity had to be equal to or higher than the nonaffected MCA[323]. Recanalization was defined as grades four or five by the Thrombolysis in Brain Ischemia scale in the TCCD performed six hours after symptom onset. Cerebral blood flow (CBF) was quantified as the sum of flow volumes in both internal carotid arteries (ICA) and vertebral arteries (VA) (Figure IX.2A). Intravascular flow volumes were determined by the ultrasound equipment's software using the angle-corrected mean flow velocity and the vessel's luminal diameter perpendicular to its longitudinal axis at the same

location as the flow measurement[324]. ICAs were assessed at least 2 cm after bifurcation and VAs in their V2 segment, at the C4-C5 intertransverse area. All arteries were measured in a straight segment for three times and the calculated mean taken for CBF analysis.

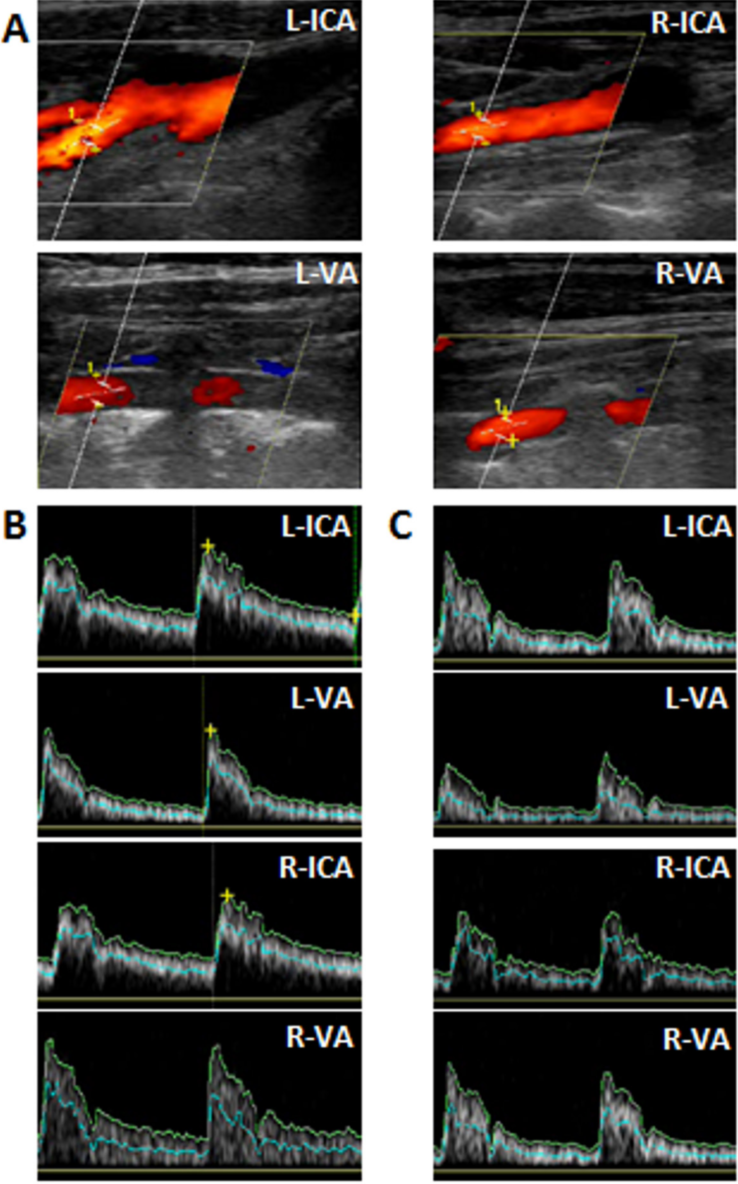

Figure IX.2. Quantification of Cerebral Blood Flow (CBF). *In (A) the identification of both Internal Carotid Arteries (ICA) and Vertebral Arteries (VA) is shown in continuous colour Doppler images. The parallelogram shown in yellow indicates the colour box steer in the right-to-left/caudal-cephalic orientation of flow. The vessel diameter used for the quantification of CBF is measured for each artery and indicated as the number "1". (B) and (C) represent the blood flow velocity analysis (in cm/sec) during two cardiac cycles of two patients: patient represented in (B) has milder NIHSS at admission, flow diversion and higher CBF than the patient in (C). The blue line in each flow analysis represents the mean flow velocity (used for CBF quantification) and the yellow line indicates the maximum flow velocity.*

2.6. Neuroimaging

Hemorrhagic transformation was classified in the gradient echo sequences of the MRI performed on day seven. Only type 2 parenchymal hemorrhages (PH2) were considered as hemorrhagic transformation[347].

2.7. Statistical analysis

Normality of all study variables was assessed by the Shapiro-Wilk test. The association of patients' demographics and vascular risk factors with CBF at admission, seven days and three months was evaluated using independent samples t-test, Pearson or Spearman correlation as indicated by the variables' characteristics. We determined the independent association of EPCs cell number and functional properties with CBF at different timepoints using multivariable linear regression. To evaluate the association of EPC cell number and functional properties with hemorrhagic transformation, recanalization and flow diversion we used binary logistic multivariate regressions. We then assessed the association of CBF, hemorrhagic transformation, recanalization and flow diversion with mRS at three months through uni- and multivariable ordinal regression. All univariate analysis were corrected for multiple comparisons by false discovery rate, and the corrected p-values are presented. All multivariable models included the variables with $p<0.1$ in univariate association with CBF at any timepoint (NIHSS and flow diversion) as well as intravenous thrombolysis, due to its recognized clinical and hemodynamic effect. Statistical significance was set for $p<0.05$.

The sample size calculation was based on estimating large treatment effects (d=0.8) due to stringent inclusion criteria, a between-group comparison with a 0.05 one-sided alpha significance level and 80% power, that would require a total sample population of 42 patients. We included 45 patients to account for potential dropouts and technical failures in MRI.

2.8. Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

3. Results

We included a total of 45 patients in our study population, median age 70 years, (interquartile range: 10) and 24 (53.3%) male. At three months 5 (11.1%) were dead and 22 patients (48.9%) showed good clinical outcome (mRS between 0 and 2). Univariate associations of baseline characteristics with CBF at days zero are presented in Table IX.1 (in Supplementary Table IX.1 we present the univariate associations of baseline variables with CBF at day seven and three months). At day zero, CBF was associated with the presence of flow diversion, with no statistically significant associations at day seven nor day 90. Figure IX.2 (B and C) represents the flow velocities in two patients with distinct CBF and clinical outcome.

Table IX.1. Univariate association of baseline variables with Cerebral Blood Flow (CBF) at day zero. In the first rows the mean CBF of patients with and without dichotomous variables is presented. In the bottom rows the correlation coefficients between CBF and continuous variables are presented. All p-values presented are corrected for multiple comparisons; *: p<0.05.

	CBF at day 0		p
	With risk factor	Without risk factor	
Male gender	481.3 (119.6)	438.0 (167.9)	0.717
Hypertension	448.7 (129.3)	489.1 (178.4)	0.739
Diabetes Mellitus	516.1 (118.0)	446.4 (148.8)	0.550
Atrial fibrillation	457.3 (171.0)	464.2 (113.0)	0.964
Dyslipidemia	443.2 (126.4)	507.0 (182.9)	0.616
Hyperuricemia	433.8 (115.6)	470.7 (154.5)	0.670
Heart failure	493.3 (208.7)	457.4 (139.9)	0.881
Ischemic heart disease	505.8 (161.0)	453.5 (142.9)	0.704
Previous stroke	385.0 (131.5)	464.2 (145.6)	0.717
Smoking	478.5 (158.7)	458.8 (145.2)	0.925
Peripheral artery disease	523.5 (151.5)	454.3 (144.4)	0.736
Obesity	418.2 (113.7)	487.3 (157.2)	0.546
Previous statin	414.9 (101.4)	482.0 (157.8)	0.565
Previous antiplatelet	445.7 (119.0)	464.5 (134.0)	0.908
Intravenous thrombolysis	499.8 (144.7)	404.0 (127.8)	0.213
Hemorrhagic transformation	451.6 (135.4)	461.8 (147.4)	0.884
Recanalization	509.9 (134.9)	426.5 (143.6)	0.325
Flow diversion	519.3 (136.6)	375.9 (112.5)	0.022*
	Correlation coefficient		
NIHSS at admission	-0.414		0.055
Glucose at admission	0.057		0.928
Age	-0.024		0.919
Weight	-0.191		0.523

The multivariable associations of CBF at different timepoints with number and functional properties of EPCs are presented in Table IX.2. At day zero, CBF was associated with the migratory properties of CACs. CBF at day seven was associated with the angiogenic properties of day zero CFU-ECs and day seven oEPCs. At three months no statistically significant associations were identified between CBF and EPC properties. No cellular variable was associated with recanalization nor hemorrhagic transformation on multivariable analyses (Table IX.3). Flow diversion showed an independent association with oEPC sprout number, CFU-EC migration and sprout length at day zero (Figure IX.3).

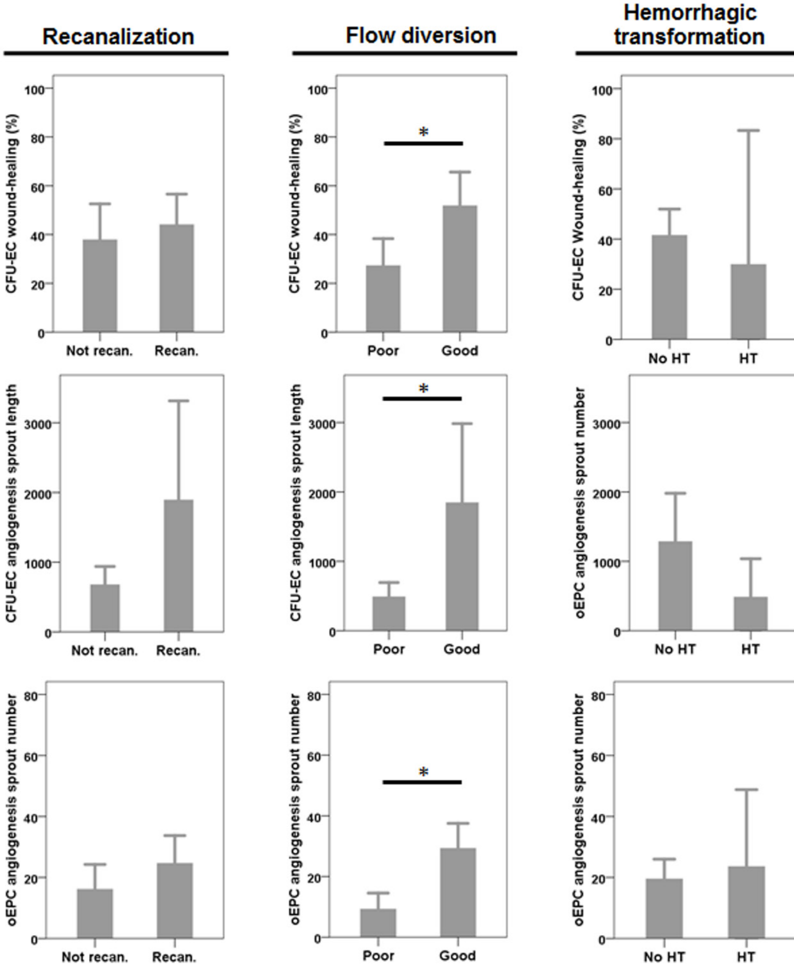

Figure IX.3. Association of the functional properties of EPCs at day zero with recanalization (Recan.), flow diversion and hemorrhagic transformation (HT). *Values are presented as mean and 95% confidence interval. *p < 0.05. Flow diversion is presented as "good" or "poor".*

Table IX.2. Association of cellular variables at days zero and seven with CBF at day zero, day seven and 3 months. All results were obtained by multivariable regression adjusted for baseline NIHSS, intravenous thrombolysis and flow diversion. SE: Standard Error; *: p<0.05.

Variable		CBF at day 0		CBF at day 7		CBF at 3 months	
		B (SE)	p	B (SE)	p	B (SE)	p
Day 0	CAC number	0.189 (0.124)	0.134	0.273 (0.156)	0.09	0.189 (0.143)	0.197
	oEPC number	0.101 (0.267)	0.707	0.481 (0.329)	0.154	0.161 (0.287)	0.579
	CFU-EC number	0.079 (0.292)	0.787	-0.333 (0.382)	0.39	-0.2 (0.313)	0.528
	CAC migration	-4.076 (1.496)	0.01*	-0.011 (2.097)	0.996	0.953 (2.088)	0.651
	oEPC migration	0.381 (1.084)	0.729	1.35 (1.431)	0.359	0.59 (1.182)	0.625
	CFU-EC migration	1.954 (0.957)	0.052	0.37 (1.419)	0.797	-0.341 (1.229)	0.784
	oEPC sprout number	-0.852 (1.662)	0.612	3.125 (1.968)	0.127	3.518 (1.922)	0.081
	CFU-EC sprout number	-1.778 (1.755)	0.32	4.913 (1.836)	0.014*	1.699 (2.022)	0.411
	oEPC sprout length	-0.04 (0.014)	0.789	0.016 (0.017)	0.34	0.028 (0.016)	0.096
	CFU-EC sprout length	-0.01 (0.017)	0.554	-0.024 (0.02)	0.235	0.013 (0.018)	0.476
Day 7	CAC number			0.061 (0.257)	0.816	0.144 (0.219)	0.516
	oEPC number			0.886 (0.414)	0.041*	0.423 (0.316)	0.191
	CFU-EC number			0.396 (0.35)	0.267	0.636 (0.32)	0.057
	CAC migration			1.244 (2.433)	0.612	1.506 (1.903)	0.435
	oEPC migration			0.641 (1.492)	0.674	2.294 (2.148)	0.311
	CFU-EC migration			1.6 (1.014)	0.133	1.68 (1.248)	0.2
	oEPC sprout number			3.433 (1.486)	0.03*	1.12 (1.471)	0.455
	CFU-EC sprout number			2.404 (1.881)	0.214	-1.056 (1.806)	0.565
	oEPC sprout length			0.021 (0.007)	0.009*	0.009 (0.007)	0.259
	CFU-EC sprout length			0.033 (0.02)	0.108	0.024 (0.018)	0.211

Table IX.3. Association of EPC cell number and functional properties with recanalization, flow diversion and hemorrhagic transformation. Results were obtained by multivariable regression adjusted for baseline NIHSS, intravenous thrombolysis and flow diversion. *: p<0.05.

Variable		Recanalization		Flow diversion		Hemorrhagic transformation	
		OR(95%CI)	p	OR(95%CI)	p	OR(95%CI)	p
Day 0	CAC number	1.004 (0.998-1.01)	0.163	1.001 (0.997-1.006)	0.544	0.992 (0.979-1.005)	0.213
	oEPC number	1.006 (0.996-1.016)	0.225	0.989 (0.976-1.002)	0.11	1.001 (0.984-1.018)	0.893
	CFU-EC number	1.004 (0.993-1.016)	0.454	1.003 (0.992-1.013)	0.616	0.997 (0.978-1.016)	0.723
	CAC migration	0.992 (0.934-1.052)	0.779	1.066 (0.994-1.143)	0.073	0.88 (0.746-1.038)	0.13
	oEPC migration	0.986 (0.945-1.029)	0.531	1.04 (1.0-1.081)	0.053	1.036 (0.975-1.101)	0.249
	CFU-EC migration	0.983 (0.945-1.023)	0.404	1.049 (1.003-1.096)	0.036*	0.969 (0.908-1.033)	0.333
	oEPC sprout number	0.965 (0.901-1.034)	0.312	1.101 (1.022-1.187)	0.012*	1.021 (0.93-1.121)	0.663
	CFU-EC sprout number	0.966 (0.904-1.033)	0.313	1.289 (0.991-1.672)	0.059	0.995 (0.906-1.092)	0.909
	oEPC sprout length	1.0 (1.0-1.001)	0.549	1.0 (1.0-1.001)	0.46	1.001 (1.0-1.002)	0.11
	CFU-EC sprout length	1.0 (0.999-1.001)	0.417	1.004 (1.0-1.008)	0.037*	0.998 (0.994-1.002)	0.369
Day 7	CAC number					0.995 (0.982-1.007)	0.404
	oEPC number					0.984 (0.956-1.013)	0.269
	CFU-EC number					1.0 (0.983-1.017)	0.996
	CAC migration					0.966 (0.864-1.081)	0.551
	oEPC migration					1.002 (0.936-1.072)	0.955
	CFU-EC migration					0.992 (0.94-1.048)	0.782
	oEPC sprout number					0.95 (0.837-1.079)	0.432
	CFU-EC sprout number					0.797 (0.511-1.245)	0.319
	oEPC sprout length					1.0 (0.999-1.001)	0.662
	CFU-EC sprout length					0.998 (0.994-1.003)	0.998

We also evaluated the association of each hemodynamic variable with functional outcome at three months (Table IX.4). Recanalization and flow diversion were independently associated with a better clinical outcome.

Table IX.4. Associations of hemodynamic variables with functional outcome at three months (multivariate ordinal regressions are adjusted for NIHSS, intravenous thrombolysis and flow diversion). The p-values presented in univariate analysis are corrected for multiple comparisons. mRS: modified Rankin scale; CBF: Cerebral Blood Flow; OR: Odds Ratio; CI: Confidence Interval.

Variable	mRS at 3 months Univariate OR (95%CI)	p	mRS at 3 months Multivariable OR (95%CI)	p
CBF at day 0	-0.005 (-0.009 to -0.001)	0.020*	0.001 (-0.004 to 0.006)	0.607
CBF at day 7	-0.004 (-0.008 to 0.000)	0.084	-0.003 (-0.008 to 0.001)	0.159
CBF at 3 months	-0.004 (-0.009 to 0.001)	0.109	-0.005 (-0.011 to 0.001)	0.093
Recanalization	-2.670 (-3.994 to -1.347)	<0.001*	-1.718 (-3.150 to -0.286)	0.019*
Hemorrhagic transformation	1.207 (-0.466 to 2.881)	0.157	1.546 (-0.336 to 3.428)	0.107
Flow diversion	-3.751 (-5.319 to -2.184)	<0.001*	-3.416 (-5.005 to -1.827)	<0.001*

4. Discussion

The main finding of our study is the association of EPC properties such as angiogenesis and migration with hemodynamic properties, namely increased flow diversion at day zero and CBF at day seven. Our results further show no statistical association between EPC properties and recanalization or hemorrhagic transformation.

The associations between the angiogenic properties of oEPCs and CFU-ECs with subacute CBF suggests the involvement of these cells in the formation of new blood vessels. Yet, it is important to note that this association was not visible in all functional tests, nor for all EPC lineages. In fact, previous studies in mice have demonstrated a weak but significant association between angiogenesis and CBF, which is more pronounced after seven days[348] and prolongs at least up to 21 days[349]. This subacute association of angiogenesis with CBF is reinforced by recent findings associating blood-brain barrier permeability of stroke patients at seven days with good clinical outcome and more proficient EPCs[343], and are in line with our current results, where no association was found between EPCs and CBF at three months. Moreover, the interpretation of our data within these previous reports, support the notion that the hemodynamic impact of the EPCs can only be partially explained by the effect of angiogenesis on CBF[349], as could be expected considering the anatomically restricted area of neoangiogenesis in comparison to the more widespread evaluation implicated in CBF. An alternative mechanism relates to the possibility that the presence of EPCs with enhanced functional properties might be a marker of a more proficient vascular response to the

ischemic insult and not a local consequence of their action, although the concomitant association with flow diversion and the dampening of effect at three months suggests a more direct relation.

Flow diversion in the acute stage was associated with CBF at day zero, functional properties of day zero EPCs and functional outcome at three months. The link between angiogenesis and collateral circulation has been suggested in previous animal model studies[258,350], but had never been studied in humans. The biological mechanisms implied in the development of collateral circulation in the context of an ischemic insult seem to vary over time. In the acute and subacute stages two processes appear to promote collaterals: shear fluid stress precipitated by the pressure gradient caused by the acute thrombus and angiogenesis, as hypoxia triggers endothelial cells to sprout and form capillary networks[351]. In this last critical step, EPCs take a pivotal role through their paracrine (mainly through CAC subpopulation) and direct angiogenic properties (mostly oEPCs and CFU-ECs). As stated earlier, the association of flow diversion in the first hours after stroke with the functional properties of EPCs may also suggest that both mechanisms (EPCs and flow diversion) may coexist as markers of an efficient response to stroke.

A potential offset of promoting a network of new vessels is hemorrhagic transformation. In fact, previous studies on animal models have yielded conflicting results considering the relation between angiogenesis and angiogenic factors (such as VEGF) with hemorrhagic transformation[349,352]. Nonetheless, this seems to be a time-dependent response: in the first hours after stroke VEGF promotes acute BBB disruption with hemorrhagic transformation, whereas in the subacute stage the enhancement of effective neoangiogenesis improves CBF and neurological recovery[349,352]. Our study supports the concept that in stroke patients enhanced functional properties of EPCs promote flow diversion with no effect on hemorrhagic transformation.

No effect was identified by EPCs on early arterial recanalization. Although no previous study had assessed the association of EPCs with recanalization in ischemic stroke, early works on EPCs suggested the rationale for an effect on recanalization, albeit being more likely on venous thrombi, due to the organization of fibrin meshwork and cellular composition of the venous thrombi[353].

Our study has inherent limitations that should be taken under consideration in its interpretation. Firstly it was a single centre study with a relatively reduced sample size, however, the strict inclusion criteria and features of the study population suggest external generalizability of the findings. Moreover, cellular assessment at two timepoints may be limited within the intricate pathology of stroke. Nonetheless, these timepoints have been suggested as pathologically important in preclinical studies and are both potential treatment windows in stroke patients. Moreover, performing comprehensive and serial evaluations of hemodynamic and neurological status allowed a rational and clinically meaningful interpretation of results.

5. Concusions

The functional properties of EPCs are associated with enhanced flow diversion and subacute CBF, with no impact on recanalization nor hemorrhagic transformation. These results promote the need and help the design of interventional trials on the efficacy of EPCs in acute stroke, reinforcing the integration of these cellular therapies within current therapies.

Supplementary Table IX.1. Univariate association of baseline variables with Cerebral Blood Flow (CBF) at day seven and three months. In the first rows the mean CBF of patients with and without dichotomous variables is presented, followed by the corresponding p-values. In the bottom rows the correlation coefficients between CBF and continuous variables are presented. All p-values are corrected for multiple testing.

	CBF at day 7		p	CBF at 3 months		p
	With risk factor	Without risk factor		With risk factor	Without risk factor	
Male gender	505.69 (98.41)	436.4 67.4)	0.875	484.0 (98.4)	436.4 67.8)	1.0
Hypertension	487.7 (123.2)	577.1 99.0)	0.594	464.2 (130.0)	458.4 49.0)	0.997
Diabetes Mellitus	510.1 (151.4)	515.4 55.4)	0.976	423.2 (111.1)	475.7 41.3)	0.880
Atrial fibrillation	477.1 (133.4)	553.4 65.0)	0.572	438.2 (144.8)	484.9 24.0)	0.977
Dyslipidemia	515.2 (159.0)	512.0 43.1)	0.954	455.6 (119.1)	481.3 79.4)	0.958
Hyperuricemia	485.7 (127.2)	526.3 62.8)	0.748	399.8 (120.4)	483.8 34.6)	0.589
Heart failure	442.3 (86.0)	523.0 57.3)	0.792	511.5 (181.7)	459.2 34.4)	1.0
Ischemic heart disease	557.8 (184.0)	505.8 47.7)	0.829	499.8 (106.4)	456.0 39.3)	0.931
Previous stroke	416.0 (124.5)	520.0 53.5)	0.712	351.0 (0.0)	465.5 35.2)	0.902
Smoking	499.3 (217.9)	515.6 49.9)	0.949	466.0 (38.5)	461.9 40.7)	1.0
Peripheral artery disease	636.0 (192.3)	499.5 43.6)	0.667	599.7 (82.9)	449.3 32.1)	0.693
Obesity	528.8 (141.3)	507.3 59.9)	0.953	434.0 (100.9)	478.9 50.8)	0.851
Previous statin	529.5 (178.9)	507.8 43.3)	0.903	471.7 (119.5)	458.4 42.4)	0.974
Previous antiplatelet	501.3 (179.5)	517.3 48.8)	0.933	463.0 (187.4)	462.1 25.4)	0.988
Intravenous thrombolysis	540.7 (165.6)	470.9 121.4)	0.660	502.6 (138.4)	408.5 12.3)	0.858
Hemorrhagic transformation	413.0 (151.2)	530.1 148.8)	0.611	447.0 (227.7)	464.2 23.8)	0.942
Recanalization	531.7 (157.5)	500.9 151.1)	0.807	472.9 (111.0)	453.2 54.2)	0.985
Flow diversion	533.3 (161.8)	483.0 135.6)	0.744	467.8 (113.7)	448.4 83.6)	0.915
	Correlation coefficient			Correlation coefficient		
NIHSS at admission	-0.333		0.968	-0.306		0.543
Glucose at admission	-0.213		0.567	-0.121		0.976
Age	-0.302		0.759	-0.275		0.484
Weight	-0.126		0.775	-0.078		1.0

Supplementary Figure IX.1. Flow chart for study inclusion.

678 patients with non-lacunar acute ischemic stroke in the MCA territory and 18-80 years of age

633 patients excluded:

Baseline assessments inaccessible in the first 24h: 308 (48.7%)
Comorbidities affecting cellular studies: 139 (22.0%)
Intra-arterial therapies: 64 (10.1%)
Previous mRS>2: 51 (8.1%)
Contra-indication to MRI: 36 (5.7%)
Comorbidities affecting neurological assessments: 35 (5.5%)

45 patients included

STROKE34 STUDY PROTOCOL

STROKE34 study protocol: A randomized controlled phase IIa trial of intra-arterial CD34+ cells in acute ischemic stroke

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Abstract

Rationale/aim: Despite the increasing efficacy of recanalization therapies for acute ischemic stroke a large number of patients are left with long term functional impairment, devoid of efficacious treatments. CD34+ cells comprise a subset of bone marrow derived mononuclear cells with the capacity to promote angiogenesis in ischemic lesions and have shown promising results in observational and in vitro studies. In this study we aim to assess the efficacy of an autotransplant of CD34+ cells in acute ischemic stroke.

Sample size estimates: 30 patients will be randomized for a power of 90% and alpha of 0.05 to detect a difference in 3 months infarct volume.

Methods and design: We will screen 18-80 years old patients with acute ischemic stroke due to occlusion of a middle cerebral artery for randomization. Persistent arterial occlusions, contraindications to Magnetic Resonance Imaging (MRI), premorbid dependency or other severe diseases will be excluded. Treatment will involve bone marrow aspiration, selection of CD34+ cells and their administration intra-arterially in the symptomatic middle cerebral artery by angiography. Patients will be randomized for treatment at 7 (±2) days, 20 (±5 days) or sham procedure, 10 in each group.

Study outcomes: The primary outcome will be infarct volume in MRI performed at 3 months. Secondary outcomes will include adverse events and multidimensional functional and neurological measures.

Discussion/conclusion: STROKE34 is a PROBE design phase IIa clinical trial to assess the efficacy of intra-arterial administration of CD34+ cells 7 and 20 days after acute ischemic stroke.

Trial registration (EU Clinical Trials Register): 2017-002456-88.

A. Introduction and rationale

Current treatment algorithms for ischemic stroke have their primary focus on promoting vascular reperfusion, leaving tissue recovery devoid of directed and efficient therapies. In fact, despite the increasing efficacy of recanalization methods, up to half of patients who survive ischemic stroke develop long term functional impairment. To overcome this therapeutic need, cell therapies have emerged as a potential method of promoting neurological recovery[139,146,147,152,354]. However many quandaries still limit their translation into clinical practice, especially in therapies aiming at neuro and synaptogenesis[164]. Recently, CD34+ cells have shown promising results in observational and *in vitro* studies, urging research translation into randomized trials[21,343]. They comprise a subset of bone marrow derived mononuclear cells representing the main source of endogenous Endothelial Progenitor Cells (EPC), with the capacity to promote angiogenesis in ischemic injuries. A phase I clinical trial demonstrated the safety and feasibility of administering intra-arterially autologous, bone marrow-derived CD34+ cells[154]. We aim to design a phase IIa clinical trial to evaluate the efficacy of the intra-arterial administration of CD34+ at two timepoints: 7±2 and 20±5 days.

B. Methods and analysis

i. Design

STROKE34 is a phase IIa superiority, unicenter, randomized, blinded outcome assessment, sham-controlled clinical trial evaluating the efficacy of intra-arterially administered CD34+ cells 7±2 and 20±5 days after ischemic stroke (PROBE design).

ii. Patient population

We will screen for inclusion consecutive patients with non-lacunar acute ischemic stroke infarctions within the territory supplied by a Middle Cerebral Artery (MCA). In Table X.1 we present the study's inclusion and exclusion criteria. Demonstration of arterial recanalization at the time of inclusion will be determined by angiography (modified TICI grades 2b or 3) or transcranial colour coded Doppler (TIBI grades 4 or 5)[345,355].

Table X.1. Inclusion and exclusion criteria for the study.

Inclusion criteria	Exclusion criteria
• Age 18-80 years;	• Patients found delirious, comatose, demented or having any mental impairment other than the neurological deficits related to the index stroke that in the investigator's opinion renders the subject incapable to participate in the study;
• Acute hemispheric ischemic stroke attributable to injury within the territory supplied by the Middle Cerebral Artery;	• Presence of high-grade (>70%) internal carotid artery stenosis or occlusion ipsilateral to the current stroke.
• Symptomatic arterial territory is recanalyzed;	• Presence of high-grade (>70%) internal carotid artery stenosis or occlusion ipsilateral to the current stroke.
• Onset of an acute ischemic stroke that can have full clinical, imagiological and bone marrow collection within seven days after onset. Onset is defined as the time that the subject was last seen in a normal state, or bedtime for unwitnessed strokes occurring during sleep;	• Inflammatory disease present at baseline (chronic systemic inflammatory disease active at the time of inclusion or acute inflammatory disease such as an infection);
• Readily accessible peripheral venous access blood sampling;	• Active malignancy, or recent surgery (within the previous 3 months);
• Ability to understand the requirements of the study and be willing to provide written informed consent, as evidenced by signature on an informed consent document, agreeing to perform the required assessments. In the event of incapacitated subjects, informed consent will be sought from a legally acceptable representative.	• Premorbid neurological deficits and functional limitations assessed by a premorbid Modified Rankin Scale (mRS) score >2;
	• Presence of a severe co-existing disease that may interfere with the conduct of the study, irrespective of stroke outcome;
	• Known pregnancy. Females of childbearing potential will be screened at baseline with urine pregnancy test and positive results will be excluded (the choice of excluding pregnancies is due to the relative contra-indication to MRI in these patients);
	• Contra-indication to MRI.
	• Allergy to contrast agents.

iii. Randomization

Subjects will be randomized, stratified for age, gender, baseline NIHSS (at randomization), laterality and performance of recanalization therapies (intravenous or intra-arterial) into intervention at day 7±2; intervention at day 20±5; sham procedure at day 7±2; sham at day 20±5, in a 2:2:1:1 ratio, using a computerized stratified randomization program (Figure X.1).

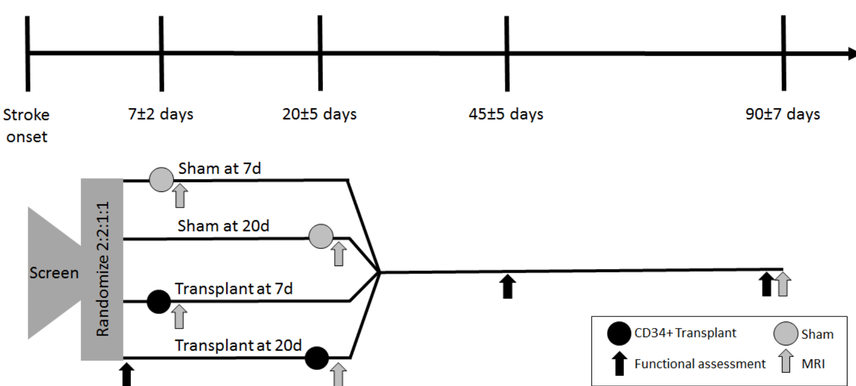

Figure X.1. Trial design flow chart. *MRI: Magnetic Resonance Imaging; d: days.*

iv. Intervention

First, a single-pull 10-ml aspirate of bone marrow from the iliac crest will be collected by experienced Haematologist under aseptic conditions, using only local anaesthesia at puncture site. CD34+ cells will then be isolated by magnetic cell sorting using CliniMACS Plus® (Miltenyi Biotec) without cellular manipulation and suspended in 10-100mL of saline solution. 24-48 hours after collection, 1x106 up to 1x108 cells suspended in human albumin solution will be administered intra-arterially in the symptomatic middle cerebral artery (Figure X.2). Before infusion, all solutions will be screened for infections according to national human tissue transplantation requirements (national legislation 12/2009): Syphilis, HIV 1 and 2, Hepatitis B and C in all patients, as well as human T lymphotropic virus 1 and 2 whenever indicated.

In the angio-suite, catheterization of the symptomatic MCA by neuroradiologist will follow routine practice. In short, all patients will initially be submitted to local anaesthesia of the groin for femoral artery puncture and intravenous administration of 5000 units of Unfractionated Heparin. Then, a guide catheter selected according anatomical features of the patient will be navigated up to the initial segment of the symptomatic internal carotid artery. At that point a microcatheter will navigate up to the beginning of the M1-MCA, where the solution containing the CD34+ cells will be slowly infused during approximately 10 minutes. The expected total duration of the procedure will be 30 minutes. All procedures will be accompanied by anaesthesiologist. Sedation will not be used routinely, but will be possible according to the anaesthesiologist's choice. No other drug will be used routinely during the procedure, but the treating physicians will be free to use any other treatment considered necessary according to clinical condition. In case the interventional-

ist encounters a previously undiagnosed high grade stenosis (>70%), vascular occlusion or any vascular malformation in the symptomatic arteries considered to be potentially hazardous in case of catheterization the treatment will be aborted.

Patients in the sham group will initially be subject to local anaesthesia of the iliac crest by Haematologist (without bone marrow aspiration) and brought to the angio-suite 24-48 hours later. All study participants will be blindfolded during the angiographic procedure and submitted to local anaesthesia of the groin. Patients in the sham group will not be subject to arterial puncture and will remain in the angio-suite for approximately 30 minutes simulating cellular infusion. No information about treatment allocation will be transferred to the recovery staff at stroke unit. Subsequent medical caregivers will also be blinded to treatment allocation. The study physicians present during the angiographic procedure will have no further contact with the patient during the study.

Until clinical stabilization, the patients will remain in the hospital and perform a control MRI 48 hours after treatment, followed by orientation by a dedicated rehabilitation center.

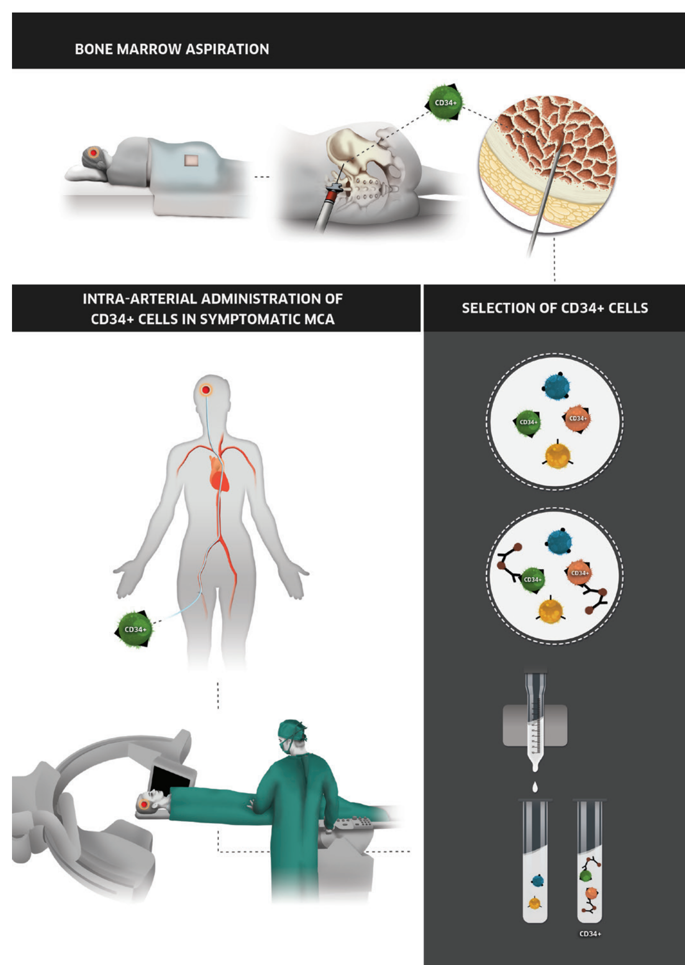

Figure X.2. Illustrative representation of autotransplant of CD34+ cells. *In the top of the image bone marrow aspiration by haematologist is represented. The second step (bottom right) is the isolation of CD34+ cells, followed by the intra-arterial administration of the CD34+ in the symptomatic Middle Cerebral Artery (bottom left). MCA: Middle Cerebral Artery.*

v. Allocation concealment

Stroke unit staff, patients, and outcome assessors are blinded to treatment allocation for the duration of the study. Unblinding will only take place whenever the treating physician decides that clinical management will critically depend on knowledge of treating allocation. Unblinding will be monitored and audited.

vi. Outcomes assessed

The primary outcome will be ischemic stroke volume, measured by automated planimetric method on FLAIR imaging sequences of MRI performed three months after index event. The secondary outcomes are presented in Table X.2. Neuroimaging assessment will include analysis of the three MRI performed during the study: before treatment, 48 hours after treatment and at 3 months. The neuroradiologist that will evaluate lesion volumes and physicians that will evaluate the secondary outcomes will not be involved in the initial management of the patient nor intervention and will be blinded for treatment allocation.

Table X.2. Study outcomes assessed.

Primary outcome
• Ischemic stroke volume on MRI performed at three months • Secondary outcomes • Hemorrhagic transformation (defined as type 2 parenchymal hemorrhage) • Evolution of ischemic stroke volume from pre-treatment MRI to control MRI (48 hours after intervention). • Any stroke (ischemic stroke, intracerebral hemorrhage and subarachnoid hemorrahge) • Death of any cause • Functional outcome at three months: modified Rankin Scale (mRS) • Impact of stroke throughout the first three months: Stroke Impact Scale (SIS) • Cognitive performance at three months: Montreal Cognitive Assessment scale (MoCA) • Functional Independence at three months: Barthel Scale • Upper limb Capacity at three months: Stroke Upper Limb Capacity Scale (SULCS) • Mood at three months: Hospital Anxiety and Depression Scale (HADS) • Quality of life at three months: EuroQol Scale (EQ-5D) • Gait speed at three months (10 meters test) • Temporo-spacial gait organization at three months (GAITRIte speed) • Deglutition at three months: Functional Oral Intake Scale (FOIS) • Language: Aphasia Rapid Test (ART)

vii. Data Monitoring Board

An independent Data and Safety and Monitoring Board (DSMB) will monitor any serious adverse events. It will be constituted by Neurologists and Internal Medicine physicians not involved in any part of the study design or execution. In the event of any safety concern, namely rate of hemorrhagic transformation, cancer, procedural complications or any other unexpected serious adverse event regarding the intervention group, the DSMB will make a recommendation to continue, stop, or modify the trial.

viii. Ethics

A signed written informed consent form will be sought by co-investigator stroke physicians and mandatory for participation in the trial. In the form all study procedures, potential risks and benefits are explained in simple terms. The study physicians will be responsible to explain and make sure the protocol is correctly understood by participants. All study subjects will be free to withdraw consent at any time. In case the patient is handicapped and not able to write down the required information, a legally acceptable representative will be allowed to fill out the informed consent form. Participants will be given a study number which will be kept throughout all the analysis, maintaining confidentiality. All study participants will have a prespecified insurance to cover any adverse event of study participation.

Possible safety concerns rely on MRI acquisition, bone marrow aspiration and angiographic catheterization (that will all follow routine protocols), as well as the autotransplant of CD34+ (that has been suggested to be safe in a phase I trial). Clinical follow-up after study completion will be the discretion of the treating physician.

An additional ethical concern will be to maintain blinding of the participants throughout the trial. Apart from the above mentioned design, all study and hospital staff personnel will receive training specific to this trial.

The study will be conducted in accordance with the principles of Good Clinical Practice, the Medical Research Involving Human Subjects act and the Declaration of Helsinki. The study has been approved by the funding body (COMPETE 2020, Ref: 3386). All protocol changes such as modifications in eligibility criteria, outcome measures, analyses or study procedures will be resubmitted.

ix. Sample size estimates

No human randomized trials are available for effect estimate on intra-arterial administration of CD34+ for acute ischemic stroke. Moreover, animal studies are not ideal considering the specificity and inherent heterogeneity of human subjects. As such, for sample size calculation, we have used data from human observational studies on EPCs[21,220]. These studies indicated a large effect

of EPCs in final ischemic lesion volume, with reported treatment effect sizes ranging from 1.2 to 2.0[21,220]. For our sample size calculation of a unicenter pivotal phase IIa trial we used an effect size of 1.2 for 90% power and a significance level of 5% to identify a difference between independent means of final infarct volume. A total of 30 patients was estimated for the trial: 10 patients treated at day 7±2, 10 at day 20±5 and 10 in the control group (5 sham procedures at each timepoint to ensure blinding).

x. Expected results

The authors expect that the autotransplant of CD34+ will be associated with lower infarct volumes at three months. Observational and *in vitro* data do not allow a rational expectation regarding the optimal timewindow for CD34+ administration (7+-2days vs. 20+-5 days). Considering the validated cellular protocol using autotransplantation and angiographic delivery using routine catheterization techniques we expect to confirm the overall safety of this procedure, with no serious adverse events.

xi. Dissemination and data availability

The results will be disseminated through peer-reviewed publications, presentation at relevant scientific conferences and the general public.

The datasets generated during the study will be available on reasonable request.

xii. Study organization and funding

STROKE34 is part of StrokeTherapy (POCI-01-0247-FEDER-003386), executed by Stemlab, S.A. in Co-Promotion with Centro de Reabilitação do Centro - Rovisco Pais and Universidade de Coimbra, in partnership with Centro Hospitalar e Universitário de Coimbra. The trial is funded by the Operational Program Portugal 2020 and the European Regional Development Fund through COMPETE 2020.

C. Discussion

The potential integration of stem cells in the treatment algorithm of acute ischemic stroke is still limited by several uncertainties, with most data coming from observational studies. STROKE34 is a randomized trial comparing different timepoints of CD34+ autotransplant: subacute (7 days) and late subacute stages (20 days). CD34+ have been confirmed to have a contributing role in the development of angiogenesis in vivo and in vitro. Angiogenesis plays a pivotal role in post-stroke recovery, however, animal models have raised the possibility of hemorrhagic transformation as a potential complication of promoting new, immature vessels[286]. Nonetheless, this link shows an apparent time-dependent response with acute disruption of the blood-brain barrier and hemor-

rhagic transformation in the first hours after stroke, whereas enhancing effective neoangiogenesis with improved outcomes in the subacute stage[343,356]. These facts reinforce the need to define an optimized therapeutic window for the design of future phase III trials.

Three methods are potential candidates for delivery of CD34+ cells: surgical cranial implantation, intravenous and intra-arterial. Considering the invasiveness and potential hazards of surgical implantation as well as the significant absorption by non-target organs with intravenous delivery, intra-arterial administration seems to be the most promising candidate. This method is further supported by the increasing use of intra-arterial access for the treatment of acute stroke, and the safety and feasibility demonstrated in phase I study[154].

Another critical point in the design of this trial is the choice of therapeutic time window. Angiogenesis after stroke has been demonstrated from 3-4 days up to five weeks after injury[259,305,357]. However, it is still unknown when CD34+ transplantation would be clinically effective. Early administration would imply exposing the highly active and metabolically demanding CD34+ cells to an inhospitable ischemic environment and would potentially promote ineffective angiogenesis and hemorrhagic transformation. Moreover, within the first 24 hours bone marrow aspiration would be contra-indicated in patients submitted to intravenous thrombolysis. Nonetheless, it is uncertain if later time windows would still have any clinical meaning, and the need for a permeable blood-brain barrier will ultimately limit cell delivery beyond the first five weeks[304]. Altogether, a wide time frame is still potentially usable (from the first days up to five weeks) with presumably different biological effects and clinical implications. In this trial we hope to answer if an earlier time window (5-9 days) is preferable compared to a later window (15-25 days).

A potential limitation of this study design is the small sample size planned. However, we have defined the primary endpoint as a neuroimaging variable (3 months infarct volume), to allow the inclusion of a smaller number of patients in this exploratory phase IIa trial, and have included comprehensive functional assessments as secondary outcomes.

D. Acknowledgements

The authors would like to thank Dr. Nélia Gouveia (from NOVA Clinical Research Unit, Lisbon, Portugal) for the help in design of the clinical trial.

E. Funding

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F. Authors' contributions

JSF wrote the article. JSF, LF, FS, GC and LC defined the inclusion criteria and clinical design. LF, AG, TM and CC defined the therapeutic product. CN and OG defined the neuroimaging protocol. JC defined the hematological protocol. AP, PF, JB and VL defined the rehabilitation program and functional assessments.

G. Competing interests

The authors declare that this study protocol was designed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

part D

Concluding remarks

chapter XI

CONCLUSIONS

Throughout this thesis several aspects on role of EPCs in acute ischemic stroke were approached, analysed and discussed. The main conclusions of this project are the following:

- The functional properties of EPCs are associated with clinical outcome in ischemic stroke patients. Our work demonstrated that the capacity of EPCs to migrate and create new vessels *in vitro* have more biological impact than their number. This association is particularly evident for the cell population in the subacute stage (seven days after stroke). These associations confirm previous results of *in vitro* experiments, animal models and observational clinical studies, reinforcing the role of EPCs in ischemic stroke.

- EPCs are associated with increased permeability of the BBB in the subacute stage. Our study was the first to show in stroke patients that differently from the recognized negative clinical impact of acute BBB permeability, subacute permeability of the BBB is associated with better functional status at three months. The link between EPCs, subacute BBB permeability and clinical outcome was independent from systemic inflammatory markers and anatomically restricted to the infarct tissue, reinforcing some preliminary animal model data indicating that neoangiogenesis promoted by EPCs is probably the main biological substrate of subacute BBB permeability in ischemic stroke.

- We identified 24 microRNAs of CD34+ cells of ischemic stroke patients associated with clinical outcome and enhanced subacute BBB permeability. The analysis based on the predicted gene targets revealed ErbB signalling, cell adhesion molecules and axon guidance pathways as the most shared pathways. The *adherens junctions* pathway is targeted by all identified miRNAs. These genes and pathways have demonstrated regulatory roles in endothelial cell migration, tube formation, *in vitro* survival and increased angiogenesis. These miRNAs of CD34+ cells associated with improved function have potential applications as prognostic tools and even therapeutic developments for cell therapies.

- The angiogenic and migratory capacities of EPCs are associated with increased collateral flow in the acute stage and day seven cerebral blood flow, without statistically significant associations with recanalization nor haemorrhagic transformation. No association was visible with hemodynamic features assessed in the chronic stage (3 months). Put together, these hemodynamic data emphasize the role of EPCs in the development of physiological responses to the ischemic injury during the acute and subacute stages, reinforcing the notion that effective angiogenesis is not associated with reperfusion lesion in the clinical setting.

- The observational findings of this project allowed and spurred the design of a phase IIa clinical trial assessing the effect of bone-marrow derived CD34+ in patients with ischemic stroke. Considering the subacute associations, two timelines were deemed plausible: early and late subacute stages after stroke, the period when angiogenesis is most likely to influence outcome. Moreover, in light of its anatomically restricted effect, an intra-arterial delivery is expected to produce tangible effects.

FUTURE PERSPECTIVES

The ultimate goal of potentially introducing EPCs into everyday clinical practice is still somewhat distant. However, the investigational path seems to show solid progression in the understanding of their action and gradual translation into human studies. The near research future must inevitably pursue careful methodological approaches to avoid faulty designs aimed at answering inadequate research questions. It is unlikely that one therapeutic strategy at any timepoint using any delivery strategy will solve all the unmet therapeutic needs in stroke. In fact, past the pragmatic hyperacute stage of ischemic stroke where recanalization is paramount, the intrinsically complicated physiopathology will require a paralleled, meticulously planned and targeted therapy.

Neoangiogenesis is now well established as an important physiological response to ischemia, with onset within the first week and persisting up to several weeks. Moreover, EPCs appear to be associated with this mechanism through direct and paracrine contributions. Nonetheless, it is not yet ascertained what is the optimal timeline to modulate this response:

a) Early intervention with the objective of anticipating neoangiogenesis? This conceptual construct may be appealing but may also trigger the formation of immature vessels during reperfusion, potentially increasing the risk of hemorrhagic transformation.
b) Subacute intervention, during the peak activity of neoangiogenesis? This may be ideal for patients with impaired angiogenesis, but may also be subject to a ceiling effect in many patients with already proficient responses.
c) Late subacute to chronic stages to prolong the neoangiogenic response? At this stage the angiogenic response may in fact reach an exhaustion state, but, on the other hand, its enhancement may prove futile due to the constitution of a definitive lesion.

An additional hurdle in clinical translation is the fact that functional capacities of stem/progenitor cells seem to be a major determinant in their effect, apart from cell number. Albeit its biological interest, this finding poses a therapeutic dilemma as enhancing the capacities of stem/progenitor cells *ex vivo* is challenging and time consuming, possibly limiting its use in an efficient therapeutic window.

Identification of candidates more likely to benefit from treatment with EPCs will also be a critical step. It is conceptually plausible and supported by our findings that in many patients the endogenous cellular response might be enough to optimize the angiogenic process after injury, whereas in other patients it would require external modulation. This poses the need to identify patients with suboptimal neoangiogenesis or alternatively those that would not require these treatments. Multimodal MRI with DCE appears to be an adequate investigational tool, but cut-offs and more generalizable measures need to be defined for wide-spread use. Genetic profiling of miRNAs seem to show promise, allowing a determination of molecular signatures associated cellular proficiency. A possible application is the development of screening tools to readily assess cellular properties based on the presence of the miRNAs identified in this project and thus tailor each patient's specific cell therapy needs.

It is conceivable that one isolated cell type at one specific timepoint will not be able to respond to the needs of all stroke patients, with foreseeable requirements of early immunomodulatory and neuroprotective strategies, besides late neuro- and synaptogenesis. Anyhow, our project reinforces neoangiogenesis and EPCs as pivotal players of tissue and functional recovery. Future studies will possibly benefit from encompassing data of different cell types, delivery methods and timelines, tailoring each patient specific biological requirements.

The definitive answer on the efficacy of EPCs in ischemic stroke will only be obtained through phase III clinical trials. Conversely, detailed preliminary data must be clarified to accomplish a well-designed trial with an optimal therapeutic strategy in dosing and delivery, for the right patient, at the right time. Our project added considerable observational information and will now continue into a phase IIa randomized assessment. The final response on the potential use of EPCs in stroke is not yet complete, but it is closer.

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chapter XIV

REFERENCES

1. Mendis S, Davis S, Norrving B. Organizational update: the world health organization global status report on noncommunicable diseases 2014; one more landmark step in the combat against stroke and vascular disease. *Stroke* 2015; 46: e121–e122.
2. Feigin VL, Krishnamurthi R V., Parmar P, et al. Update on the Global Burden of Ischemic and Hemorrhagic Stroke in 1990-2013: The GBD 2013 Study. *NeuroepidemiologyNeuroepidemiology* 2015; 45: 161–176.
3. Feigin VL, Forouzanfar MH, Krishnamurthi R, et al. Global and regional burden of stroke during 1990-2010: findings from the Global Burden of Disease Study 2010. *Lancet (London, England)* 2014; 383: 245–54.
4. Mendis S, Puska P, Norrving B. *Global Atlas on cardiovascular disease prevention and control*. World Health Organization, 2011.
5. Powers WJ, Derdeyn CP, Biller J, et al. *2015 AHA/ASA Focused Update of the 2013 Guidelines for the Early Management of Patients With Acute Ischemic Stroke Regarding Endovascular Treatment*. 2015. Epub ahead of print 2015. DOI: 10.1161/STR.0000000000000074.
6. Wahlgren N, Moreira T, Michel P, et al. Mechanical thrombectomy in acute ischemic stroke: Consensus statement by ESO-Karolinska Stroke Update 2014/2015, supported by ESO, ESMINT, ESNR and EAN. *Int J Stroke* 2016; 11: 134–147.
7. Schwamm LH, Ali SF, Reeves MJ, et al. Temporal trends in patient characteristics and treatment with intravenous thrombolysis among acute ischemic stroke patients at Get with the Guidelines-Stroke hospitals. *Circ Cardiovasc Qual Outcomes* 2013; 6: 543–549.
8. Adeoye O, Hornung R, Khatri P, et al. Recombinant tissue-type plasminogen activator use for ischemic stroke in the united states: A doubling of treatment rates over the course of 5 years. *Stroke* 2011; 42: 1952–1955.
9. Sacchetti ML. Is it time to definitely abandon neuroprotection in acute ischemic stroke? *Stroke*, 2008, pp. 1659–1660.
10. Tymianski M. Novel approaches to neuroprotection trials in acute ischemic stroke. *Stroke* 2013; 44: 2942–2950.
11. Lindvall O, Kokaia Z. Stem cell research in stroke: How far from the clinic? *Stroke* 2011; 42: 2369–2375.
12. Bacigaluppi M, Pluchino S, Jametti LP, et al. Delayed post-ischaemic neuroprotection following systemic neural stem cell transplantation involves multiple mechanisms. *Brain* 2009; 132: 2239–2251.
13. Greenberg DA. Stem cells and stroke recovery: Introduction. In: *Stroke*. 2007, p. 809.
14. Faiz M, Sachewsky N, Gasc??n S, et al. Adult Neural Stem Cells from the Subventricular Zone Give Rise to Reactive Astrocytes in the Cortex after Stroke. *Cell Stem Cell* 2015; 17: 624–634.
15. Lindvall O, Kokaia Z. Stem cells for the treatment of neurological disorders. *Nature* 2006; 441: 1094–1096.
16. Walker PA, Harting MT, Shah SK, et al. Progenitor Cell Therapy for the Treatment of Central Nervous System Injury: A Review of the State of Current Clinical Trials. *Stem Cells Int* 2010; 2010: 1–8.
17. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for

angiogenesis. *Science* 1997; 275: 964–7.
18. Ghani U, Shuaib A, Salam A, et al. Endothelial progenitor cells during cerebrovascular disease. *Stroke* 2005; 36: 151–153.
19. Jung K-H, Roh J-K. Circulating endothelial progenitor cells in cerebrovascular disease. *J Clin Neurol* 2008; 4: 139–47.
20. Hill JM, Zalos G, Halcox JPJ, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003; 348: 593–600.
21. Bogoslovsky T, Chaudhry A, Latour L, et al. Endothelial progenitor cells correlate with lesion volume and growth in acute stroke. *Neurology* 2010; 75: 2059–2062.
22. Navarro-Sobrino M, Rosell A, Hernandez-Guillamon M, et al. Mobilization, endothelial differentiation and functional capacity of endothelial progenitor cells after ischemic stroke. *Microvasc Res* 2010; 80: 317–323.
23. Who. Global burden of stroke. *atlas Hear Dis stroke* 2004; 15: 50–51.
24. L Murray CJ, Vos T, Lozano R, et al. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012; 2197–2223.
25. Roth GA, Johnson C, Abajobir A, et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. *Journal of the American College of Cardiology*, 2017. Epub ahead of print 2017. DOI: 10.1016/j.jacc.2017.04.052.
26. Krishnamurthi R V., Feigin VL, Forouzanfar MH, et al. Global and regional burden of first-ever ischaemic and haemorrhagic stroke during 1990-2010: Findings from the Global Burden of Disease Study 2010. *Lancet Glob Heal*; 1. Epub ahead of print 2013. DOI: 10.1016/S2214-109X(13)70089-5.
27. Norrving B, Kissela B. The global burden of stroke and need for a continuum of care. *Neurology* 2013; 80: S5-12.
28. Barker-Collo S, Bennett DA, Krishnamurthi R V, et al. Sex Differences in Stroke Incidence, Prevalence, Mortality and Disability-Adjusted Life Years: Results from the Global Burden of Disease Study 2013. *Neuroepidemiology* 2015; 45: 203–214.
29. Feigin VL, Lawes CM, Bennett DA, et al. Worldwide stroke incidence and early case fatality reported in 56 population-based studies: a systematic review. *The Lancet Neurology* 2009; 8: 355–369.
30. Correia M, Magalhães R, Silva MR, et al. Stroke Types in Rural and Urban Northern Portugal: Incidence and 7-Year Survival in a Community-Based Study. *Cerebrovasc Dis Extra* 2013; 3: 137–149.
31. Correia M, Magalhães R, Felgueiras R, et al. Changes in stroke incidence, outcome, and associated factors in Porto between 1998 and 2011. *Int J Stroke* 2017; 12: 169–179.
32. Sousa RM, Ferri CP, Acosta D, et al. Contribution of chronic diseases to disability in elderly people in\ncountries with low and middle incomes: a 10/66 Dementia Research Group\npopulation-based survey. *Lancet* 2009; 374: 1821–1830.
33. World Health Organization. World Health Statistics 2014. In: *World Health Statistics 2014*. 2014, p. 175.

34. Feigin VL, Mensah GA, Norrving B, et al. Atlas of the Global Burden of Stroke (1990-2013): The GBD 2013 Study. *Neuroepidemiology* 2015; 45: 230–236.
35. Koton S, Schneider ALC, Rosamond WD, et al. Stroke Incidence and Mortality Trends in US Communities, 1987 to 2011. *JAMA* 2014; 312: 259.
36. Sacco RL, Kasner SE, Broderick JP, et al. An updated definition of stroke for the 21st century: A statement for healthcare professionals from the American heart association/American stroke association. *Stroke* 2013; 44: 2064–2089.
37. Adams HP, Bendixen BH, Kappelle LJ, et al. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. *Stroke* 1993; 24: 35–41.
38. Ay H, Furie KL, Singhal A, et al. An evidence-based causative classification system for acute ischemic stroke. *Ann Neurol* 2005; 58: 688–697.
39. Amarenco P, Bogousslavsky J, Caplan LR, et al. The ASCOD phenotyping of ischemic stroke (Updated ASCO Phenotyping). *Cerebrovascular Diseases* 2013; 36: 1–5.
40. Lin L, Bivard A, Krishnamurthy V, et al. Whole-Brain CT Perfusion to Quantify Acute Ischemic Penumbra and Core. *Radiology* 2016; 279: 876–887.
41. Grotta JC, Helgason C. Ischemic stroke pathophysiology. *J Stroke Cerebrovasc Dis* 1999; 8: 114–116.
42. Saenger AK, Christenson RH. Stroke biomarkers: Progress and challenges for diagnosis, prognosis, differentiation, and treatment. *Clinical Chemistry* 2010; 56: 21–33.
43. Deb P, Sharma S, Hassan KM. Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis. *Pathophysiology* 2010; 17: 197–218.
44. Levine SR. Pathophysiology and therapeutic targets for ischemic stroke. *Clin Cardiol* 2004; 27: II12-24.
45. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: An integrated view. *Trends in Neurosciences* 1999; 22: 391–397.
46. Lo EH, Dalkara T, Moskowitz MA. Neurological diseases: Mechanisms, challenges and opportunities in stroke. *Nat Rev Neurosci* 2003; 4: 399–414.
47. Kanekar SG, Zacharia T, Roller R. Imaging of stroke: Part 2, pathophysiology at the molecular and cellular levels and corresponding imaging changes. *American Journal of Roentgenology* 2012; 198: 63–74.
48. Durukan A, Tatlisumak T. Acute ischemic stroke: Overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. *Pharmacol Biochem Behav* 2007; 87: 179–197.
49. Pradeep H, Diya JB, Shashikumar S, et al. Oxidative stress – assassin behind the ischemic stroke. *Folia Neuropathol* 2012; 3: 219–230.
50. Zhao H, Han Z, Ji X, et al. Epigenetic Regulation of Oxidative Stress in Ischemic Stroke. *Aging Dis* 2016; 7: 295–306.
51. Tanne D, Haim M, Boyko V, et al. Soluble intercellular adhesion molecule-1 and risk of future ischemic stroke: A nested case-control study from the Bezafibrate Infarction Prevention (BIP)

study cohort. *Stroke* 2002; 33: 2182–2186.

52. Schilling M, Besselmann M, Leonhard C, et al. Microglial activation precedes and predominates over macrophage infiltration in transient focal cerebral ischemia: A study in green fluorescent protein transgenic bone marrow chimeric mice. *Exp Neurol* 2003; 183: 25–33.
53. Bang OY, Goyal M, Liebeskind DS. Collateral Circulation in Ischemic Stroke: Assessment Tools and Therapeutic Strategies. *Stroke* 2015; 46: 3302–3309.
54. Christoforidis GA, Mohammad Y, Kehagias D, et al. Angiographic assessment of pial collaterals as a prognostic indicator following intra-arterial thrombolysis for acute ischemic stroke. *Am J Neuroradiol* 2005; 26: 1789–1797.
55. Shi ZS, Liebeskind DS, Xiang B, et al. Predictors of functional dependence despite successful revascularization in large-vessel occlusion strokes. *Stroke* 2014; 45: 1977–1984.
56. Bang OY, Saver JL, Kim SJ, et al. Collateral flow predicts response to endovascular therapy for acute ischemic stroke. *Stroke* 2011; 42: 693–9.
57. Bang OY, Saver JL, Kim SJ, et al. Collateral flow averts hemorrhagic transformation after endovascular therapy for acute ischemic stroke. *Stroke* 2011; 42: 2235–2239.
58. Liebeskind DS, Tomsick TA, Foster LD, et al. Collaterals at angiography and outcomes in the interventional management of stroke (IMS) III trial. *Stroke* 2014; 45: 759–764.
59. Ribo M, Flores A, Rubiera M, et al. Extending the time window for endovascular procedures according to collateral pial circulation. *Stroke* 2011; 42: 3465–3469.
60. Reinhard M, Roth M, Guschlbauer B, et al. Dynamic cerebral autoregulation in acute ischemic stroke assessed from spontaneous blood pressure fluctuations. *Stroke* 2005; 36: 1684–1689.
61. Castro P, Serrador JM, Rocha I, et al. Efficacy of cerebral autoregulation in early ischemic stroke predicts smaller infarcts and better outcome. *Front Neurol*; 8. Epub ahead of print 2017. DOI: 10.3389/fneur.2017.00113.
62. Castro P, Azevedo E, Serrador J, et al. Hemorrhagic transformation and cerebral edema in acute ischemic stroke: Link to cerebral autoregulation. *J Neurol Sci* 2017; 372: 256–261.
63. Alexandrov A V, Sharma VK, Lao AY, et al. Reversed Robin Hood syndrome in acute ischemic stroke patients. *Stroke* 2007; 38: 3045–3048.
64. Alexandrov A V., Nguyen HT, Rubiera M, et al. Prevalence and risk factors associated with reversed robin hood syndrome in acute ischemic stroke. *Stroke* 2009; 40: 2738–2742.
65. Rubiera M, Alvarez-Sabín J, Ribo M, et al. Predictors of early arterial reocclusion after tissue plasminogen activator-induced recanalization in acute ischemic stroke. *Stroke* 2005; 36: 1452–1456.
66. Alexandrov A V., Grotta JC. Arterial reocclusion in stroke patients treated with intravenous tissue plasminogen activator. *Neurology* 2002; 59: 862–867.
67. Nour M, Scalzo F, Liebeskind DS. Ischemia-reperfusion injury in stroke. *Interv Neurol* 2013; 1: 185–99.
68. Mishra NK, Christensen S, Wouters A, et al. Reperfusion of Very Low Cerebral Blood Volume Lesion Predicts Parenchymal Hematoma after Endovascular Therapy. *Stroke* 2015; 46: 1245–1249.
69. Eltzschig HK, Eckle T. Ischemia and reperfusion-from mechanism to translation. *Nature Medi-*

cine 2011; 17: 1391–1401.

70. Indredavik B, Bakke F, Slørdahl SA, et al. Stroke unit treatment improves long-term quality of life: a randomized controlled trial. *Stroke* 1998; 29: 895–9.
71. Unit Trialists' Collaboration S. How Do Stroke Units Improve Patient Outcomes? : A Collaborative Systematic Review of the Randomized Trials. *Stroke* 1997; 28: 2139–2144.
72. Tajbakhsh S. Stem cell: what's in a name? *Nat Reports Stem Cells*. Epub ahead of print 2009. DOI: 10.1038/stemcells.2009.90.
73. Avasthi S, Ms RNS, Ms AS. Stem Cell : Past , Present and Future- A Review Article. *Internet J Med Updat* 2008; 3: 22–30.
74. Rando TA. Stem cells, ageing and the quest for immortality. *Nature* 2006; 441: 1080–1086.
75. Goodell MA, Rando TA. Stem cells and healthy aging. *Science (80-)* 2015; 350: 1199–1204.
76. Smith A. A glossary for stem-cell biology. *Nature* 2006; 441: 1060.
77. Bindu A H, B S. Potency of Various Types of Stem Cells and their Transplantation. *J Stem Cell Res Ther*; 1. Epub ahead of print 2011. DOI: 10.4172/2157-7633.1000115.
78. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. *Cell* 2000; 100: 157–168.
79. Berdasco M, Esteller M. DNA methylation in stem cell renewal and multipotency. *Stem Cell Res Ther* 2011; 2: 42.
80. Draper JS, Moore HD, Ruban LN, et al. Culture and Characterization of Human Embryonic Stem Cells. *Stem Cells Dev* 2004; 13: 325–336.
81. Hoffman LM, Carpenter MK. Characterization and culture of human embryonic stem cells. *Nat Biotechnol* 2005; 23: 699–708.
82. Angeles A, Ferrari F, Xi R, et al. Hallmarks of pluripotency TL - 525. *Nature*; 525 VN-. Epub ahead of print 2015. DOI: 10.1038/nature15515.
83. Kalichman MW, Schwartz PH. Guidelines for Embryonic Stem Cell Research Oversight (ESCRO) Committees. In: *Human Stem Cell Manual*. 2007, pp. 437–447.
84. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; 126: 663–676.
85. Sánchez Alvarado A, Yamanaka S. Rethinking differentiation: Stem cells, regeneration, and plasticity. *Cell* 2014; 157: 110–119.
86. Puri MC, Nagy A. Concise review: Embryonic stem cells versus induced pluripotent stem cells: the game is on. *Stem Cells* 2012; 30: 10–4.
87. Avior Y, Sagi I, Benvenisty N. Pluripotent stem cells in disease modelling and drug discovery. *Nat Rev Mol Cell Biol* 2016; 17: 170–182.
88. Abud EM, Ramirez RN, Martinez ES, et al. iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron* 2017; 94: 278–293.e9.
89. O'Donoghue K, Fisk NM. Fetal stem cells. *Best Practice and Research: Clinical Obstetrics and Gynaecology* 2004; 18: 853–875.
90. Guillot P V., O'Donoghue K, Kurata H, et al. Fetal stem cells: Betwixt and between. *Seminars in Reproductive Medicine* 2006; 24: 340–347.
91. Pappa KI, Anagnou NP. Novel sources of fetal stem cells: where do they fit on the develop-

mental continuum? *Regen Med* 2009; 4: 423–433.

92. Boltze J, Reich DM, Hau S, et al. Assessment of neuroprotective effects of human umbilical cord blood mononuclear cell subpopulations in vitro and in vivo. *Cell Transplant* 2012; 21: 723–737.
93. Lee OK, Kuo TK, Chen W-M, et al. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* 2004; 103: 1669–1675.
94. Li L, Clevers H. Coexistence of Quiescent and Active Adult Stem Cells in Mammals. *Science (80-)* 2010; 327: 542–545.
95. Wagers AJ, Weissman IL. Plasticity of adult stem cells. *Cell* 2004; 116: 639–648.
96. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; 284: 143–7.
97. Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* 2008; 8: 726–736.
98. Fuentealba LC, Obernier K, Alvarez-Buylla A. Adult neural stem cells bridge their niche. *Cell Stem Cell* 2012; 10: 698–708.
99. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science (80-)* 1999; 284: 143–147.
100. Zannettino ACW, Paton S, Kortesidis A, et al. Human mulipotential mesenchymal/stromal stem cells are derived from a discrete subpopulation of STRO-1bright/CD34 /CD45(-)/glycophorin-A-bone marrow cells. *Haematologica* 2007; 92: 1707–8.
101. Yue R, Zhou BO, Shimada IS, et al. Leptin Receptor Promotes Adipogenesis and Reduces Osteogenesis by Regulating Mesenchymal Stromal Cells in Adult Bone Marrow. *Cell Stem Cell* 2016; 18: 782–796.
102. Griese DP, Ehsan A, Melo LG, et al. Isolation and Transplantation of Autologous Circulating Endothelial Cells into Denuded Vessels and Prosthetic Grafts: Implications for Cell-Based Vascular Therapy. *Circulation* 2003; 108: 2710–2715.
103. Bazán E, Alonso FJM, Redondo C, et al. In vitro and in vivo characterization of neural stem cells. *Histol Histopathol* 2004; 19: 1261–1275.
104. Rietze RL, Valcanis H, Brooker GF, et al. Purification of a pluripotent neural stem cell from the adult mouse brain. *Nature* 2001; 412: 736–739.
105. Cai J, Limke TL, Ginis I, et al. Identifying and tracking neural stem cells. *Blood Cells, Molecules, and Diseases* 2003; 31: 18–27.
106. Gage FH. Mammalian Neural Stem Cells. *Science (80-)* 2000; 287: 1433–1438.
107. Kornblum HI. Introduction to neural stem cells. *Stroke* 2007; 38: 810–6.
108. Chen FM, Wu LA, Zhang M, et al. Homing of endogenous stem/progenitor cells for in situ tissue regeneration: Promises, strategies, and translational perspectives. *Biomaterials* 2011; 32: 3189–3209.
109. Trainor N, Pietak A, Smith T. Rethinking clinical delivery of adult stem cell therapies. *Nat Biotechnol* 2014; 32: 729–735.
110. Trounson A, McDonald C. Stem Cell Therapies in Clinical Trials: Progress and Challenges. *Cell Stem Cell* 2015; 17: 11–22.

111. Copelan EA. Hematopoietic stem-cell transplantation. *N Engl J Med* 2006; 354: 1813–1826.
112. Barrett AJ. Essential biology of stem cell transplantation. In: *Hematopoietic Stem Cell Transplantation in Clinical Practice*. 2009, pp. 9–21.
113. Daley GQ, Scadden DT. Prospects for Stem Cell-Based Therapy. *Cell* 2008; 132: 544–548.
114. Lutolf MP, Gilbert PM, Blau HM. Designing materials to direct stem-cell fate. *Nature* 2009; 462: 433–441.
115. Murphy WL, McDevitt TC, Engler AJ. Materials as stem cell regulators. *Nat Mater* 2014; 13: 547–557.
116. Mandai M, Watanabe A, Kurimoto Y, et al. Autologous Induced Stem-Cell–Derived Retinal Cells for Macular Degeneration. *N Engl J Med* 2017; 376: 1038–1046.
117. Abrahamsson S V., Angelini DF, Dubinsky AN, et al. Non-myeloablative autologous haematopoietic stem cell transplantation expands regulatory cells and depletes IL-17 producing mucosal-associated invariant T cells in multiple sclerosis. *Brain* 2013; 136: 2888–2903.
118. Burman J, Iacobaeus E, Svenningsson A, et al. Autologous haematopoietic stem cell transplantation for aggressive multiple sclerosis: the Swedish experience. *J Neurol Neurosurg Psychiatry* 2014; 85: 1116–1121.
119. Muraro PA, Martin R, Mancardi GL, et al. Autologous haematopoietic stem cell transplantation for treatment of multiple sclerosis. *Nat Rev Neurol* 2017; 13: 391–405.
120. Alexander T, Arnold R, Hiepe F, et al. Resetting the immune system with immunoablation and autologous haematopoietic stem cell transplantation in autoimmune diseases. *Clin Exp Rheumatol* 2016; 34: 53–57.
121. Jj M, a P, P B, et al. The Intrathecal Infusion of Mesenchymal Stem Cells into Healthy Rabbits is Safe and Devoid of Neurological or Clinical Complications. *J Stem Cell Res Ther* 2011; 1: 1–5.
122. Garbern JC, Lee RT. Cardiac stem cell therapy and the promise of heart regeneration. *Cell Stem Cell* 2013; 12: 689–698.
123. Segers VFM, Lee RT. Stem-cell therapy for cardiac disease. *Nature* 2008; 451: 937–942.
124. Kurowska Z, Englund E, Widner H, et al. Signs of degeneration in 12-22-year old grafts of mesencephalic dopamine neurons in patients with Parkinson's disease. *J Parkinsons Dis* 2011; 1: 83–92.
125. Tadesse T, Gearing M, Senitzer D, et al. Analysis of graft survival in a trial of stem cell transplant in ALS. *Ann Clin Transl Neurol* 2014; 1: 900–908.
126. Barker RA, Parmar M, Kirkeby A, et al. Are Stem Cell-Based Therapies for Parkinson's Disease Ready for the Clinic in 2016? IMPENDING CLINICAL STEM CELL TRIAL RAISES QUESTIONS. *J Parkinsons Dis* 2016; 6: 57–63.
127. Reissis D, Tang QO, Cooper NC, et al. Current clinical evidence for the use of mesenchymal stem cells in articular cartilage repair. *Expert Opin Biol Ther* 2016; 16: 535–557.
128. Weissman I. Stem cell therapies could change medicine... If they get the chance. *Cell Stem Cell* 2012; 10: 663–665.
129. Borlongan C V., Hess DC. New hope for stroke patients: Mobilization of endogenous stem cells. *CMAJ* 2006; 174: 954–955.

130. Yu H, Talib M, Khanh Vu TH, et al. Mobilizing Endogenous Stem Cells for Retinal Repair. In: *Translating Regenerative Medicine to the Clinic*. 2015, pp. 297–308.
131. Discher DE, Mooney DJ, Zandstra PW. Growth Factors, Matrices, and Forces Combine and Control Stem Cells. *Science (80-)* 2009; 324: 1673–1677.
132. Göritz C, Frisén J. Neural stem cells and neurogenesis in the adult. *Cell Stem Cell* 2012; 10: 657–659.
133. Bellenchi GC, Volpicelli F, Piscopo V, et al. Adult neural stem cells: An endogenous tool to repair brain injury? *Journal of Neurochemistry* 2013; 124: 159–167.
134. Doetsch F, García-Verdugo A, Alvarez-Buylla J. Cellular Composition and Three-Dimensional Organization of the Subventricular Germinal Zone in the Adult Mammalian Brain. *J Neurosci* 1997; 17(13): 5046–61.
135. Gage FH, Kempermann G, Palmer TD, et al. Multipotent progenitor cells in the adult dentate gyrus. *J Neurobiol* 1998; 36: 249–66.
136. Seri B, Herrera DG, Gritti A, et al. Composition and organization of the SCZ: A large germinal layer containing neural stem Cells in the adult mammalian brain. *Cereb Cortex*; 16. Epub ahead of print 2006. DOI: 10.1093/cercor/bhk027.
137. Jovin TG, Chamorro A, Cobo E, et al. Thrombectomy within 8 Hours after Symptom Onset in Ischemic Stroke. *N Engl J Med* 2015; 372: 2296–2306.
138. Saver JL, Goyal M, Bonafe A, et al. Stent-Retriever Thrombectomy after Intravenous t-PA vs. t-PA Alone in Stroke. *N Engl J Med* 2015; 372: 2285–2295.
139. Berkhemer OA, Fransen PSS, Beumer D, et al. A Randomized Trial of Intraarterial Treatment for Acute Ischemic Stroke. *N Engl J Med* 2014; 372: 141217070022009.
140. Goyal M, Demchuk AM, Menon BK, et al. Randomized Assessment of Rapid Endovascular Treatment of Ischemic Stroke. *N Engl J Med* 2015; 372: 1019–1030.
141. Campbell BC V, Mitchell PJ, Kleinig TJ, et al. Endovascular therapy for ischemic stroke with perfusion-imaging selection (EXTEND-IA). *N Engl J Med* 2015; 372: 1009–18.
142. Zhang J, Chopp M. Cell-based therapy for ischemic stroke. *Expert Opin Biol Ther* 2013; 13: 1229–1240.
143. Grégoire C-A, Goldenstein BL, Floriddia EM, et al. Endogenous neural stem cell responses to stroke and spinal cord injury. *Glia* 2015; 63: 1469–1482.
144. Jablonska A, Lukomska B. Stroke induced brain changes: Implications for stem cell transplantation. *Acta Neurobiologiae Experimentalis* 2011; 71: 74–85.
145. O'Collins VE, Macleod MR, Donnan GA, et al. 1,026 Experimental treatments in acute stroke. *Ann Neurol* 2006; 59: 467–477.
146. Hess DC, Wechsler LR, Clark WM, et al. Safety and efficacy of multipotent adult progenitor cells in acute ischaemic stroke (MASTERS): a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Neurol* 2017; 16: 360–368.
147. Prasad K, Sharma A, Garg A, et al. Intravenous autologous bone marrow mononuclear stem cell therapy for ischemic stroke: a multicentric, randomized trial. *Stroke* 2014; 45: 3618–24.
148. Steinberg GK, Kondziolka D, Wechsler LR, et al. Clinical outcomes of transplanted modified

bone marrow-derived mesenchymal stem cells in stroke: A phase 1/2a study. *Stroke* 2016; 47: 1817–1824.

149. Miki T, Strom SC. Amnion-derived pluripotent/ multipotent stem cells. *Stem Cell Rev* 2006; 2: 133–42.
150. Castillo-Melendez M, Yawno T, Jenkin G, et al. Stem cell therapy to protect and repair the developing brain: A review of mechanisms of action of cord blood and amnion epithelial derived cells. *Frontiers in Neuroscience*. Epub ahead of print 2013. DOI: 10.3389/fnins.2013.00194.
151. Liu T, Wu J, Huang Q, et al. Human amniotic epithelial cells ameliorate behavioral dysfunction and reduce infarct size in the rat middle cerebral artery occlusion model. *Shock* 2008; 29: 603–611.
152. Kalladka D, Sinden J, Pollock K, et al. Human neural stem cells in patients with chronic ischaemic stroke (PISCES): a phase 1, first-in-man study. *Lancet* 2016; 388: 787–796.
153. Fadini GP, Losordo D, Dimmeler S. Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. *Circulation Research* 2012; 110: 624–637.
154. Banerjee S, Bentley P, Hamady M, et al. Intra-Arterial Immunoselected CD34+ Stem Cells for Acute Ischemic Stroke. *Stem Cells Transl Med* 2014; 3: 1322–1330.
155. Jin K, Minami M, Lan JQ, et al. Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. *Proc Natl Acad Sci* 2001; 98: 4710–4715.
156. Xu W, Zheng J, Gao L, et al. Neuroprotective Effects of Stem Cells in Ischemic Stroke. *Stem Cells International*; 2017. Epub ahead of print 2017. DOI: 10.1155/2017/4653936.
157. Chen J, Venkat P, Zacharek A, et al. Neurorestorative therapy for stroke. *Front Hum Neurosci* 2014; 8: 382.
158. Grade S, Bernardino L, Malva JO. Oligodendrogenesis from neural stem cells: Perspectives for remyelinating strategies. *International Journal of Developmental Neuroscience* 2013; 31: 692–700.
159. Sharp FR, Liu J, Bernabeu R. Neurogenesis following brain ischemia. *Dev Brain Res* 2002; 134: 23–30.
160. Lane SW, Williams DA, Watt FM. Modulating the stem cell niche for tissue regeneration. *Nat Biotechnol* 2014; 32: 795–803.
161. Jeong CH, Kim SM, Lim JY, et al. Mesenchymal stem cells expressing brain-derived neurotrophic factor enhance endogenous neurogenesis in an ischemic stroke model. *Biomed Res Int*; 2014. Epub ahead of print 2014. DOI: 10.1155/2014/129145.
162. Seifert HA, Hall AA, Chapman CB, et al. A transient decrease in spleen size following stroke corresponds to splenocyte release into systemic circulation. *Journal of Neuroimmune Pharmacology* 2012; 7: 1017–1024.
163. Sahota P, Vahidy F, Nguyen C, et al. Changes in spleen size in patients with acute ischemic stroke: a pilot observational study. *Int J Stroke* 2013; 8: 60–7.
164. Kenmuir CL, Wechsler LR. Update on cell therapy for stroke. *Stroke Vasc Neurol* 2017; 2: 59–64.
165. Honmou O, Houkin K, Matsunaga T, et al. Intravenous administration of auto serum-expanded autologous mesenchymal stem cells in stroke. *Brain* 2011; 134: 1790–1807.

166. Kornienko VN, Pronin IN. *Diagnostic neuroradiology*. 2009. Epub ahead of print 2009. DOI: 10.1007/978-3-540-75653-8.
167. Asumda FZ. Age-associated changes in the ecological niche: implications for mesenchymal stem cell aging. *Stem Cell Res Ther* 2013; 4: 47.
168. Kerever A, Yamada T, Suzuki Y, et al. Fractone aging in the subventricular zone of the lateral ventricle. *J Chem Neuroanat* 2015; 66–67: 52–60.
169. Farkas E, Luiten PGM. Cerebral microvascular pathology in aging and Alzheimer's disease. *Prog Neurobiol* 2001; 64: 575–611.
170. Oh J, Lee YD, Wagers AJ. Stem cell aging: Mechanisms, regulators and therapeutic opportunities. *Nature Medicine* 2014; 20: 870–880.
171. Molofsky A V., Slutsky SG, Joseph NM, et al. Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. *Nature* 2006; 443: 448–452.
172. Kondo T, Hayashi M, Takeshita K, et al. Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. *Arterioscler Thromb Vasc Biol* 2004; 24: 1442–1447.
173. Yue W-S, Wang M, Yan G-H, et al. Smoking is associated with depletion of circulating endothelial progenitor cells and elevated pulmonary artery systolic pressure in patients with coronary artery disease. *Am J Cardiol* 2010; 106: 1248–1254.
174. Pirro M, Schillaci G, Menecali C, et al. Reduced number of circulating endothelial progenitors and HOXA9 expression in CD34+ cells of hypertensive patients. *J Hypertens* 2007; 25: 2093–2099.
175. Giannotti G, Doerries C, Mocharla PS, et al. Impaired endothelial repair capacity of early endothelial progenitor cells in prehypertension: Relation to endothelial dysfunction. *Hypertension* 2010; 55: 1389–1397.
176. Rossi F, Bertone C, Montanile F, et al. HDL cholesterol is a strong determinant of endothelial progenitor cells in hypercholesterolemic subjects. *Microvasc Res* 2010; 80: 274–279.
177. Heida N-M, Müller J-P, Cheng I-F, et al. Effects of obesity and weight loss on the functional properties of early outgrowth endothelial progenitor cells. *J Am Coll Cardiol* 2010; 55: 357–67.
178. Egan CG, Lavery R, Caporali F, et al. Generalised reduction of putative endothelial progenitors and CXCR4-positive peripheral blood cells in type 2 diabetes. *Diabetologia* 2008; 51: 1296–1305.
179. Fadini GP, Avogaro A. Potential manipulation of endothelial progenitor cells in diabetes and its complications. *Diabetes, Obesity and Metabolism* 2010; 12: 570–583.
180. Vasa M, Fichtlscherer S, Adler K, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation* 2001; 103: 2885–2890.
181. Uchida H, Niizuma K, Kushida Y, et al. Human Muse Cells Reconstruct Neuronal Circuitry in Subacute Lacunar Stroke Model. *Stroke* 2017; 48: 428–435.
182. Strbian D, Durukan A, Pitkonen M, et al. The blood-brain barrier is continuously open for several weeks following transient focal cerebral ischemia. *Neuroscience* 2008; 153: 175–181.
183. Schoch HJ, Fischer S, Marti HH. Hypoxia-induced vascular endothelial growth factor expression causes vascular leakage in the brain. *Brain* 2002; 125: 2549–57.

184. Yoder MC. Human endothelial progenitor cells. *Cold Spring Harb Perspect Med* 2012; 2: a006692.
185. Jujo K, Ii M, Losordo DW. Endothelial progenitor cells in neovascularization of infarcted myocardium. *Journal of Molecular and Cellular Cardiology* 2008; 45: 530–544.
186. Dimmeler S, Burchfield J, Zeiher AM. Cell-based therapy of myocardial infarction. *Arteriosclerosis, Thrombosis, and Vascular Biology* 2008; 28: 208–216.
187. Fadini GP, Agostini C, Avogaro A. Autologous stem cell therapy for peripheral arterial disease. Meta-analysis and systematic review of the literature. *Atherosclerosis* 2010; 209: 10–17.
188. Lipinski MJ, Biondi-Zoccai GGL, Abbate A, et al. Impact of Intracoronary Cell Therapy on Left Ventricular Function in the Setting of Acute Myocardial Infarction. A Collaborative Systematic Review and Meta-Analysis of Controlled Clinical Trials. *J Am Coll Cardiol* 2007; 50: 1761–1767.
189. Koyanagi M, Brandes RP, Haendeler J, et al. Cell-to-cell connection of endothelial progenitor cells with cardiac myocytes by nanotubes: A novel mechanism for cell fate changes? *Circ Res* 2005; 96: 1039–1041.
190. Hosoda T, Zheng H, Cabral-Da-Silva M, et al. Human cardiac stem cell differentiation is regulated by a mircrine mechanism. *Circulation* 2011; 123: 1287–1296.
191. Hur J, Yoon CH, Kim HS, et al. Characterization of Two Types of Endothelial Progenitor Cells and Their Different Contributions to Neovasculogenesis. *Arterioscler Thromb Vasc Biol* 2004; 24: 288–293.
192. Ingram DA, Mead LE, Tanaka H, et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* 2004; 104: 2752–2760.
193. Sieveking DP, Buckle A, Celermajer DS, et al. Strikingly Different Angiogenic Properties of Endothelial Progenitor Cell Subpopulations. Insights From a Novel Human Angiogenesis Assay. *J Am Coll Cardiol* 2008; 51: 660–668.
194. Meneveau N, Deschaseaux F, Séronde MF, et al. Presence of endothelial colony-forming cells is associated with reduced microvascular obstruction limiting infarct size and left ventricular remodelling in patients with acute myocardial infarction. *Basic Res Cardiol* 2011; 106: 1397–1410.
195. Wang C-H, Hsieh I-C, Su Pang J-H, et al. Factors associated with purity, biological function, and activation potential of endothelial colony-forming cells. *Am J Physiol Regul Integr Comp Physiol* 2011; 300: R586–R594.
196. Chu K, Jung K-H, Lee S-T, et al. Circulating endothelial progenitor cells as a new marker of endothelial dysfunction or repair in acute stroke. *Stroke* 2008; 39: 1441–1447.
197. Kawamoto A, Iwasaki H, Kusano K, et al. CD34-positive cells exhibit increased potency and safety for therapeutic neovascularization after myocardial infarction compared with total mononuclear cells. *Circulation* 2006; 114: 2163–2169.
198. Taguchi A, Soma T, Tanaka H, et al. Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. *J Clin Invest* 2004; 114: 330–338.
199. Ramos AL, Darabi R, Akbarloo N, et al. Clonal analysis reveals a common progenitor for endothelial, myeloid, and lymphoid precursors in umbilical cord blood. *Circ Res* 2010; 107: 1460–1469.

200. Madeddu P, Emanueli C, Pelosi E, et al. Transplantation of low dose CD34+KDR+ cells promotes vascular and muscular regeneration in ischemic limbs. *Faseb J* 2004; 18: 1737–1739.
201. Friedrich EB, Walenta K, Scharlau J, et al. CD34-/CD133+/VEGFR-2+ endothelial progenitor cell subpopulation with potent vasoregenerative capacities. *Circ Res*; 98. Epub ahead of print 2006. DOI: 10.1161/01.RES.0000205765.28940.93.
202. Schmidt-Lucke C, Fichtlscherer S, Aicher A, et al. Quantification of circulating endothelial progenitor cells using the modified ISHAGE Protocol. *PLoS One*; 5. Epub ahead of print 2010. DOI: 10.1371/journal.pone.0013790.
203. Seeger FH, Rasper T, Koyanagi M, et al. CXCR4 expression determines functional activity of bone marrow-derived mononuclear cells for therapeutic neovascularization in acute ischemia. *Arterioscler Thromb Vasc Biol* 2009; 29: 1802–1809.
204. Kim SW, Kim H, Cho HJ, et al. Human peripheral blood-derived CD31+ cells have robust angiogenic and vasculogenic properties and are effective for treating ischemic vascular disease. *J Am Coll Cardiol* 2010; 56: 593–607.
205. Saugstad JA. Non-coding RNAs in stroke and neuroprotection. *Frontiers in Neurology*; 6. Epub ahead of print 2015. DOI: 10.3389/fneur.2015.00050.
206. Jeyaseelan K, Lim KY, Armugam A. MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. *Stroke* 2008; 39: 959–966.
207. Qu K, Wang Z, Lin X-L, et al. MicroRNAs: Key regulators of endothelial progenitor cell functions. *Clin Chim Acta* 2015; 448: 65–73.
208. Minami Y, Satoh M, Maesawa C, et al. Effect of atorvastatin on microRNA 221 / 222 expression in endothelial progenitor cells obtained from patients with coronary artery disease. *Eur J Clin Invest* 2009; 39: 359–67.
209. Zhu S, Deng S, Ma Q, et al. MicroRNA-10A* and MicroRNA-21 modulate endothelial progenitor cell senescence via suppressing high-mobility group A2. *Circ Res* 2013; 112: 152–164.
210. Van Solingen C, De Boer HC, Bijkerk R, et al. MicroRNA-126 modulates endothelial SDF-1 expression and mobilization of Sca-1+/Lin- progenitor cells in ischaemia. *Cardiovasc Res* 2011; 92: 449–455.
211. Meng S, Cao J, Wang L, et al. MicroRNA 107 partly inhibits endothelial progenitor cells differentiation via HIF-1beta. *PLoS One* 2012; 7: e40323.
212. Wang H-W, Huang T-S, Lo H-H, et al. Deficiency of the microRNA-31-microRNA-720 pathway in the plasma and endothelial progenitor cells from patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 2014; 34: 857–69.
213. Werner N, Kosiol S, Schiegl T, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 2005; 353: 999–1007.
214. Schmidt-Lucke C, Rössig L, Fichtlscherer S, et al. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: Proof of concept for the clinical importance of endogenous vascular repair. *Circulation* 2005; 111: 2981–2987.
215. Zhao Y-H, Yuan B, Chen J, et al. Endothelial Progenitor Cells: Therapeutic Perspective for Ischemic Stroke. *CNS Neurosci Ther* 2013; 19: 67–75.
216. Umemura T, Soga J, Hidaka T, et al. Aging and hypertension are independent risk factors

for reduced number of circulating endothelial progenitor cells. *Am J Hypertens* 2008; 21: 1203–1209.
217. Loomans CJ, de Koning EJ, Staal FJ, et al. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes* 2004; 53: 195–199.
218. Fadini GP, Miorin M, Facco M, et al. Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. *J Am Coll Cardiol*; 45. Epub ahead of print 2005. DOI: 10.1016/j.jacc.2004.11.067.
219. Ludwig A, Jochmann N, Kertesz A, et al. Smoking decreases the level of circulating CD34+ progenitor cells in young healthy women--a pilot study. *BMC Womens Health* 2010; 10: 20.
220. Sobrino T, Hurtado O, Moro MA, et al. The increase of circulating endothelial progenitor cells after acute ischemic stroke is associated with good outcome. *Stroke* 2007; 38: 2759–2764.
221. Yip H-K, Chang L-T, Chang W-N, et al. Level and value of circulating endothelial progenitor cells in patients after acute ischemic stroke. *Stroke* 2008; 39: 69–74.
222. Bogoslovsky T, Spatz M, Chaudhry A, et al. Stromal-Derived Factor-1 Correlates With Circulating Endothelial Progenitor Cells and With Acute Lesion Volume in Stroke Patients. *Stroke* 2011; 42: 618–625.
223. Zhou W, Zhu D, Yang G, et al. Circulating endothelial progenitor cells in Chinese patients with acute stroke. *Hypertens Res* 2009; 32: 306–310.
224. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature* 2005; 438: 932–936.
225. Risau W. Mechanisms of angiogenesis. *Nature* 1997; 386: 671–674.
226. Fischer C, Schneider M, Carmeliet P. Principles and therapeutic implications of angiogenesis, vasculogenesis and arteriogenesis. *Handb Exp Pharmacol* 2006; 176: 157–212.
227. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nature Reviews Molecular Cell Biology* 2007; 8: 464–478.
228. Potente M, Gerhardt H, Carmeliet P. Basic and therapeutic aspects of angiogenesis. *Cell* 2011; 146: 873–887.
229. Jain RK. Molecular regulation of vessel maturation. *Nature Medicine* 2003; 9: 685–693.
230. Cooke JP, Losordo DW. Modulating the Vascular Response to Limb Ischemia: Angiogenic and Cell Therapies. *Circ Res* 2015; 116: 1561–1578.
231. Ma F, Morancho A, Montaner J, et al. Endothelial progenitor cells and revascularization following stroke. *Brain Res* 2015; 1623: 150–159.
232. Martí-Fàbregas J, Crespo J, Delgado-Mederos R, et al. Endothelial progenitor cells in acute ischemic stroke. *Brain Behav* 2013; 3: 649–655.
233. Liu J, Wang Y, Akamatsu Y, et al. Vascular remodeling after ischemic stroke: Mechanisms and therapeutic potentials. *Progress in Neurobiology*, 2013. Epub ahead of print 2013. DOI: 10.1016/j.pneurobio.2013.11.004.
234. Moore MAS. Putting the neo into neoangiogenesis. *Journal of Clinical Investigation* 2002; 109: 313–315.
235. Favia A, Desideri M, Gambara G, et al. VEGF-induced neoangiogenesis is mediated by NAADP

and two-pore channel-2-dependent Ca2+ signaling. *Proc Natl Acad Sci* 2014; 111: E4706–E4715.

236. Tsopanoglou NE, Maragoudakis ME. The role of thrombin in angiogenesis. In: *Thrombin: Physiology and Disease*. 2009, pp. 93–113.
237. «Schrey-Petersen S, Stepan H. Anti-angiogenesis and Preeclampsia in 2016. *Current Hypertension Reports*; 19. Epub ahead of print 2017. DOI: 10.1007/s11906-017-0706-5.
238. Agarwal I, Karumanchi SA. Preeclampsia and the anti-angiogenic state. *Pregnancy Hypertension* 2011; 1: 17–21.
239. Biron KE, Dickstein DL, Gopaul R, et al. Amyloid triggers extensive cerebral angiogenesis causing blood brain barrier permeability and hypervascularity in alzheimer's disease. *PLoS One*; 6. Epub ahead of print 2011. DOI: 10.1371/journal.pone.0023789.
240. Kang D-H, Anderson S, Kim Y-G, et al. Impaired angiogenesis in the aging kidney: Vascular endothelial growth factor and Thrombospondin-1 in renal disease. *Am J Kidney Dis* 2001; 37: 601–611.
241. Martínez P, Esbrit P, Rodrigo a, et al. Age-related changes in parathyroid hormone-related protein and vascular endothelial growth factor in human osteoblastic cells. *Osteoporos Int* 2002; 13: 874–881.
242. Chang E, Yang J, Nagavarapu U, et al. Aging and survival of cutaneous microvasculature. *Journal of Investigative Dermatology* 2002; 118: 752–758.
243. Hull ML, Charnock-Jones DS, Chan CLK, et al. Antiangiogenic agents are effective inhibitors of endometriosis. *J Clin Endocrinol Metab* 2003; 88: 2889–2899.
244. Groothuis PG, Nap AW, Winterhager E, et al. Vascular development in endometriosis. *Angiogenesis* 2005; 8: 147–156.
245. Wang S, Park JK, Duh EJ. Novel targets against retinal angiogenesis in diabetic retinopathy. *Current Diabetes Reports* 2012; 12: 355–363.
246. Capitão M, Soares R. Angiogenesis and Inflammation Crosstalk in Diabetic Retinopathy. *J Cell Biochem* 2016; 2443–2453.
247. Pozarowska D, Pozarowski P. The era of anti-vascular endothelial growth factor (VEGF) drugs in ophthalmology, VEGF and anti-VEGF therapy. *Central European Journal of Immunology* 2016; 41: 311–316.
248. Tah V, Orlans HO, Hyer J, et al. Anti-VEGF therapy and the retina: An update. *Journal of Ophthalmology*; 2015. Epub ahead of print 2015. DOI: 10.1155/2015/627674.
249. De Palma M, Biziato D, Petrova T V. Microenvironmental regulation of tumour angiogenesis. *Nature Reviews Cancer* 2017; 17: 457–474.
250. Ramjiawan RR, Griffioen AW, Duda DG. Anti-angiogenesis for cancer revisited: Is there a role for combinations with immunotherapy? *Angiogenesis* 2017; 20: 185–204.
251. Yang W-H, Xu J, Mu J-B, et al. Revision of the concept of anti-angiogenesis and its applications in tumor treatment. *Chronic Dis Transl Med* 2017; 3: 33–40.
252. Koch AE. The role of angiogenesis in rheumatoid arthritis: recent developments. *Ann Rheum Dis* 2000; 59: i65–i71.
253. Konisti S, Kiriakidis S, Paleolog EM. Angiogenesis in rheumatoid arthritis. In: *Angiogenesis*

and Vascularisation: Cellular and Molecular Mechanisms in Health and Diseases. 2013, pp. 339–365.

254. Maruotti N, Cantatore FP, Ribatti D. Putative effects of potentially anti-angiogenic drugs in rheumatic diseases. *European Journal of Clinical Pharmacology* 2014; 70: 135–140.
255. Navaratna D, Guo S, Arai K, et al. Mechanisms and targets for angiogenic therapy after stroke. *Cell Adhesion and Migration* 2009; 3: 216–223.
256. Acevedo LM, Weis SM, Cheresh DA. Robo4 counteracts VEGF signaling. *Nature Medicine* 2008; 14: 372–373.
257. Takeshita S, Zheng LP, Brogi E, et al. Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest* 1994; 93: 662–670.
258. Wei L, Erinjeri JP, Rovainen CM, et al. Collateral growth and angiogenesis around cortical stroke. *Stroke* 2001; 32: 2179–2184.
259. Ding G, Jiang Q, Li L, et al. Angiogenesis detected after embolic stroke in rat brain using magnetic resonance T2*WI. *Stroke* 2008; 39: 1563–8.
260. Krupinski J, Kaluza J, Kumar P, et al. Some remarks on the growth-rate and angiogenesis of microvessels in ischemic stroke. Morphometric and immunocytochemical studies. *Patol Pol* 1993; 44: 203–209.
261. Manoonkitiwongsa PS, Jackson-Friedman C, McMillan PJ, et al. Angiogenesis after Stroke is Correlated with Increased Numbers of Macrophages: The Clean-up Hypothesis. *J Cereb Blood Flow Metab* 2001; 21: 1223–1231.
262. Yu SW, Friedman B, Cheng Q, et al. Stroke-evoked angiogenesis results in a transient population of microvessels. *J Cereb Blood Flow Metab* 2007; 27: 755–763.
263. Drews J. Paul Ehrlich: Magister Mundi. *Nat Rev Drug Discov* 2004; 3: 797–801.
264. Serlin Y, Shelef I, Knyazer B, et al. Anatomy and physiology of the blood-brain barrier. *Seminars in Cell and Developmental Biology* 2015; 38: 2–6.
265. Banks WA. From blood-brain barrier to blood-brain interface: New opportunities for CNS drug delivery. *Nature Reviews Drug Discovery* 2016; 15: 275–292.
266. Neuwelt E, Abbott NJ, Abrey L, et al. Strategies to advance translational research into brain barriers. *The Lancet Neurology* 2008; 7: 84–96.
267. Neuwelt EA, Bauer B, Fahlke C, et al. Engaging neuroscience to advance translational research in brain barrier biology. *Nature Reviews Neuroscience* 2011; 12: 169–182.
268. Campbell M, Humphries P. The blood-retina barrier tight junctions and barrier modulation. *Adv Exp Med Biol* 2013; 763: 70–84.
269. Chen Y, Liu L. Modern methods for delivery of drugs across the blood-brain barrier. *Advanced Drug Delivery Reviews* 2012; 64: 640–665.
270. Hawkins BT. The Blood-Brain Barrier/Neurovascular Unit in Health and Disease. *Pharmacol Rev* 2005; 57: 173–185.
271. Iadecola C. The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease. *Neuron* 2017; 96: 17–42.

272. Abbott NJ, Rönnbäck L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. *Nature Reviews Neuroscience* 2006; 7: 41–53.
273. Armulik A, Genové G, Mäe M, et al. Pericytes regulate the blood-brain barrier. *Nature* 2010; 468: 557–561.
274. Chen J, Luo Y, Hui H, et al. CD146 coordinates brain endothelial cell–pericyte communication for blood–brain barrier development. *Proc Natl Acad Sci* 2017; 114: E7622–E7631.
275. Hall CN, Reynell C, Gesslein B, et al. Capillary pericytes regulate cerebral blood flow in health and disease. *Nature* 2014; 508: 55–60.
276. Ransohoff RM, Engelhardt B. The anatomical and cellular basis of immune surveillance in the central nervous system. *Nature Reviews Immunology* 2012; 12: 623–635.
277. Belayev L, Busto R, Zhao W, et al. Quantitative evaluation of blood-brain barrier permeability following middle cerebral artery occlusion in rats. *Brain Res* 1996; 739: 88–96.
278. Rosenberg G a, Estrada EY, Dencoff JE. Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain. *Stroke* 1998; 29: 2189–2195.
279. Huang ZG, Xue D, Preston E, et al. Biphasic opening of the blood-brain barrier following transient focal ischemia: effects of hypothermia. *Can J Neurol Sci* 1999; 26: 298–304.
280. Durukan A, Marinkovic I, Strbian D, et al. Post-ischemic blood-brain barrier leakage in rats: One-week follow-up by MRI. *Brain Res* 2009; 1280: 158–165.
281. Yang Y, Rosenberg GA. Blood-brain barrier breakdown in acute and chronic cerebrovascular disease. *Stroke* 2011; 42: 3323–8.
282. Kassner A, Merali Z. Assessment of Blood-Brain Barrier Disruption in Stroke. *Stroke* 2015; 46: 3310–3315.
283. Heye AK, Culling RD, Vald??s Hern??ndez MDC, et al. Assessment of blood-brain barrier disruption using dynamic contrast-enhanced MRI. A systematic review. *NeuroImage: Clinical* 2014; 6: 262–274.
284. Khatri R, McKinney AM, Swenson B, et al. Blood-brain barrier, reperfusion injury, and hemorrhagic transformation in acute ischemic stroke. *Neurology*; 79. Epub ahead of print 2012. DOI: 10.1212/WNL.0b013e3182697e70.
285. Ozkul-Wermester O, Guegan-Massardier E, Triquenot A, et al. Increased blood-brain barrier permeability on perfusion computed tomography predicts hemorrhagic transformation in acute ischemic stroke. *Eur Neurol* 2014; 72: 45–53.
286. Jickling GC, Liu D, Stamova B, et al. Hemorrhagic transformation after ischemic stroke in animals and humans. *J Cereb Blood Flow Metab* 2014; 34: 185–99.
287. Yaghi S, Willey JZ, Cucchiara B, et al. Treatment and Outcome of Hemorrhagic Transformation After Intravenous Alteplase in Acute Ischemic Stroke: A Scientific Statement for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke* 2017; STR.0000000000000152.
288. Liu J, Jin X, Liu KJ, et al. Matrix Metalloproteinase-2-Mediated Occludin Degradation and Caveolin-1-Mediated Claudin-5 Redistribution Contribute to Blood-Brain Barrier Damage in Early Ischemic Stroke Stage. *J Neurosci* 2012; 32: 3044–3057.

289. Kazmierski R, Michalak S, Wencel-Warot A, et al. Serum tight-junction proteins predict hemorrhagic transformation in ischemic stroke patients. *Neurology* 2012; 79: 1677–1685.
290. Rempe RG, Hartz AM, Bauer B. Matrix metalloproteinases in the brain and blood–brain barrier: Versatile breakers and makers. *J Cereb Blood Flow Metab* 2016; 36: 1481–1507.
291. Sandoval KE, Witt KA. Blood-brain barrier tight junction permeability and ischemic stroke. *Neurobiology of Disease* 2008; 32: 200–219.
292. Yaghi S, Eisenberger A, Willey JZ. Symptomatic Intracerebral Hemorrhage in Acute Ischemic Stroke After Thrombolysis With Intravenous Recombinant Tissue Plasminogen Activator. *JAMA Neurol* 2014; 71: 1181.
293. Vandelli L, Marietta M, Gambini M, et al. Fibrinogen decrease after intravenous thrombolysis in ischemic stroke patients is a risk factor for intracerebral hemorrhage. *J Stroke Cerebrovasc Dis* 2015; 24: 394–400.
294. Niego B, Medcalf RL. Plasmin-dependent modulation of the blood-brain barrier: A major consideration during tPA-induced thrombolysis? *Journal of Cerebral Blood Flow and Metabolism* 2014; 34: 1283–1296.
295. Montaner J, Molina CA, Monasterio J, et al. Matrix metalloproteinase-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis in human stroke. *Circulation* 2003; 107: 598–603.
296. Weiser RE, Sheth KN. Clinical predictors and management of hemorrhagic transformation. *Curr Treat Options Neurol* 2013; 15: 125–149.
297. Group TNI of ND and S rt-PSS. Tissue Plasminogen Activator for Acute Ischemic Stroke. *N Engl J Med* 1995; 333: 1581–1588.
298. Jin R, Yang G, Li G. Molecular insights and therapeutic targets for blood-brain barrier disruption in ischemic stroke: Critical role of matrix metalloproteinases and tissue-type plasminogen activator. *Neurobiology of Disease* 2010; 38: 376–385.
299. Kim EY, Na DG, Kim SS, et al. Prediction of hemorrhagic transformation in acute ischemic stroke: Role of diffusion-weighted imaging and early parenchymal enhancement. *Am J Neuroradiol* 2005; 26: 1050–1055.
300. Leigh R, Christensen S, Campbell BC V, et al. Pretreatment blood-brain barrier disruption and post-endovascular intracranial hemorrhage. *Neurology* 2016; 87: 263–269.
301. Leigh R, Jen SS, Hillis AE, et al. Pretreatment blood-brain barrier damage and post-treatment intracranial hemorrhage in patients receiving intravenous tissue-type plasminogen activator. *Stroke* 2014; 45: 2030–2035.
302. Martins AI, Sargento-Freitas J, Silva F, et al. Recanalization Modulates Association between Blood Pressure and Functional Outcome in Acute Ischemic Stroke. *Stroke* 2016; 47: 1571–1576.
303. Jickling GC, Liu D, Ander BP, et al. Targeting neutrophils in ischemic stroke: translational insights from experimental studies. *J Cereb Blood Flow Metab* 2015; 35: 888–901.
304. Merali Z, Huang K, Mikulis D, et al. Evolution of blood-brain-barrier permeability after acute ischemic stroke. *PLoS One*; 12. Epub ahead of print 2017. DOI: 10.1371/journal.pone.0171558.
305. Lin CY, Chang C, Cheung WM, et al. Dynamic changes in vascular permeability, cerebral blood

volume, vascular density, and size after transient focal cerebral ischemia in rats: evaluation with contrast-enhanced magnetic resonance imaging. *J Cereb Blood Flow Metab* 2008; 28: 1491–1501.

306. Schmidt-Kastner R, Meller D, Bellander BM, et al. A one-step immunohistochemical method for detection of blood-brain barrier disturbances for immunoglobulins in lesioned rat brain with special reference to false-positive labelling in immunohistochemistry. *J Neurosci Methods* 1993; 46: 121–132.
307. Manaenko A, Chen H, Kammer J, et al. Comparison Evans Blue injection routes: Intravenous versus intraperitoneal, for measurement of blood-brain barrier in a mice hemorrhage model. *J Neurosci Methods* 2011; 195: 206–210.
308. Jaffer H, Adjei IM, Labhasetwar V. Optical imaging to map blood-brain barrier leakage. *Sci Rep*; 3. Epub ahead of print 2013. DOI: 10.1038/srep03117.
309. Reiber H, Peter JB. Cerebrospinal fluid analysis: Disease-related data patterns and evaluation programs. *Journal of the Neurological Sciences* 2001; 184: 101–122.
310. Marchi N, Fazio V, Cucullo L, et al. Serum transthyretin monomer as a possible marker of blood-to-CSF barrier disruption. *J Neurosci* 2003; 23: 1949–55.
311. Kapural M, Krizanac-Bengez L, Barnett G, et al. Serum S-100beta as a possible marker of blood-brain barrier disruption. *Brain Res* 2002; 940: 102–104.
312. Saha GB, MacIntyre WJ, Go RT. Radiopharmaceuticals for brain imaging. *Semin Nucl Med* 1994; 24: 324–349.
313. Schoknecht K, Shalev H. Blood-brain barrier dysfunction in brain diseases: Clinical experience. *Epilepsia* 2012; 53: 7–13.
314. Wunder A, Schoknecht K, Stanimirovic DB, et al. Imaging blood-brain barrier dysfunction in animal disease models. *Epilepsia* 2012; 53: 14–21.
315. Sourbron SP, Buckley DL. On the scope and interpretation of the Tofts models for DCE-MRI. *Magn Reson Med* 2011; 66: 735–745.
316. Armitage PA, Farrall AJ, Carpenter TK, et al. Use of dynamic contrast-enhanced MRI to measure subtle blood-brain barrier abnormalities. *Magn Reson Imaging* 2011; 29: 305–314.
317. Tofts PS, Brix G, Buckley DL, et al. Estimating kinetic parameters from dynamic contrast-enhanced T1- weighted MRI of a diffusable tracer: Standardized quantities and symbols. *Journal of Magnetic Resonance Imaging* 1999; 10: 223–232.
318. Woolf DK, Taylor NJ, Makris A, et al. Arterial input functions in dynamic contrast-enhanced magnetic resonance imaging: Which model performs best when assessing breast cancer response? *Br J Radiol*; 89. Epub ahead of print 2016. DOI: 10.1259/bjr.20150961.
319. Mehrabian H, Chandrana C, Pang I, et al. Arterial input function calculation in dynamic contrast-enhanced MRI: An in vivo validation study using co-registered contrast-enhanced ultrasound imaging. *Eur Radiol* 2012; 22: 1735–1747.
320. Gordon Y, Partovi S, Müller-Eschner M, et al. Dynamic contrast-enhanced magnetic resonance imaging: fundamentals and application to the evaluation of the peripheral perfusion. *Cardiovasc Diagn Ther* 2014; 4: 147–64.

321. Silva EA, Kim E-S, Kong HJ, et al. Material-based deployment enhances efficacy of endothelial progenitor cells. *Proc Natl Acad Sci U S A* 2008; 105: 14347–52.
322. Vasa M, Fichtlscherer S, Aicher a, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001; 89: E1–E7.
323. Kim YS, Meyer JS, Garami Z, et al. Flow diversion in transcranial Doppler ultrasound is associated with better improvement in patients with acute middle cerebral artery occlusion. *Cerebrovasc Dis* 2006; 21: 74–78.
324. Schoning M, Walter J, Scheel P. Estimation of cerebral blood flow through color duplex sonography of the carotid and vertebral arteries in healthy adults. *Stroke* 1994; 25: 17–22.
325. Jiang Q, Zhang RL, Zhang ZG, et al. Magnetic resonance imaging characterization of hemorrhagic transformation of embolic stroke in the rat. *J Cereb Blood Flow Metab* 2002; 22: 559–68.
326. Yang Y, Rosenberg GA. Blood-brain barrier breakdown in acute and chronic cerebrovascular disease. *Stroke* 2011; 42: 3323–3328.
327. Chopp M, Zhang ZG, Jiang Q. Neurogenesis, angiogenesis, and MRI indices of functional recovery from stroke. In: *Stroke*. 2007, pp. 827–831.
328. Vlachos IS, Zagganas K, Paraskevopoulou MD, et al. DIANA-miRPath v3.0: Deciphering microRNA function with experimental support. *Nucleic Acids Res* 2015; 43: W460–W466.
329. Eltzschig HK, Eckle T. Ischemia and reperfusion--from mechanism to translation. *Nat Med* 2011; 17: 1391–1401.
330. Yoon CH, Hur J, Park KW, et al. Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: The role of angiogenic cytokines and matrix metalloproteinases. *Circulation* 2005; 112: 1618–1627.
331. Bhatwadekar AD, Yan Y, Stepps V, et al. MiR-92a corrects CD34+ cell dysfunction in diabetes by modulating core circadian genes involved in progenitor differentiation. *Diabetes* 2015; 64: 4226–4237.
332. Tan KS, Armugam A, Sepramaniam S, et al. Expression profile of microRNAs in young stroke patients. *PLoS One*; 4. Epub ahead of print 2009. DOI: 10.1371/journal.pone.0007689.
333. Zheng Y, Xu Z. MicroRNA-22 Induces Endothelial Progenitor Cell Senescence by Targeting AKT3. *Cell Physiol Biochem* 2014; 34: 1547–1555.
334. Zhao X, Huang Y, Wang Y, et al. MicroRNA profile comparison of the corneal endothelia of young and old mice: implications for senescence of the corneal endothelium. *Mol Vis* 2013; 19: 1815–25.
335. Li Q, He Q, Baral S, et al. MicroRNA-493 regulates angiogenesis in a rat model of ischemic stroke by targeting MIF. *FEBS J* 2016; 283: 1720–1733.
336. Kuehbacher A, Urbich C, Zeiher AM, et al. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. *Circ Res* 2007; 101: 59–68.
337. Templin C, Volkmann J, Emmert MY, et al. Increased Proangiogenic Activity of Mobilized CD34+ Progenitor Cells of Patients With Acute ST-Segment-Elevation Myocardial Infarction: Role of Differential MicroRNA-378 Expression. *Arterioscler Thromb Vasc Biol* 2017; 37: 341–349.
338. Yang S-F, Lee W-J, Tan P, et al. Upregulation of miR-328 and inhibition of CREB-DNA-binding

activity are critical for resveratrol-mediated suppression of matrix metalloproteinase-2 and subsequent metastatic ability in human osteosarcomas. *Oncotarget* 2015; 6: 2736–2753.

339. Lechler T. Adherens junctions and stem cells. *Subcell Biochem* 2012; 60: 359–377.
340. Wallez Y, Huber P. Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. *Biochimica et Biophysica Acta - Biomembranes* 2008; 1778: 794–809.
341. Urbich C, Kaluza D, Frömel T, et al. MicroRNA-27a/b controls endothelial cell repulsion and angiogenesis by targeting semaphorin 6A. *Blood* 2012; 119: 1607–1618.
342. Rosenblum S, Wang N, Smith TN, et al. Timing of intra-arterial neural stem cell transplantation after hypoxia-ischemia influences cell engraftment, survival, and differentiation. *Stroke* 2012; 43: 1624–1631.
343. Sargento-freitas J, Aday S, Nunes C, et al. Endothelial progenitor cells enhance blood – brain barrier permeability in subacute stroke. *Neurology* 2018; 90: e127–e134.
344. Han JH, Ho SSY, Lam WWM, et al. Total cerebral blood flow estimated by color velocity imaging quantification ultrasound: a predictor for recurrent stroke? *J Cereb Blood Flow Metab* 2007; 27: 850–856.
345. Demchuk AM, Burgin WS, Christou I, et al. Thrombolysis in Brain Ischemia (TIBI) Transcranial Doppler Flow Grades Predict Clinical Severity, Early Recovery, and Mortality in Patients Treated With Intravenous Tissue Plasminogen Activator. *Stroke* 2001; 32: 89–93.
346. Ringleb P a, Bousser M, Ford G, et al. Guidelines for Management of Ischaemic Stroke and Transient Ischaemic Attack 2008 The European Stroke Organization (ESO) Committee and the ESO Writing Committee Executive. *Stroke* 2008; 25: 6855–6859.
347. Fiorelli M, Bastianello S, Von Kummer R, et al. Hemorrhagic Transformation Within 36 Hours of a Cerebral Infarct Relationships With Early Clinical Deterioration and 3-Month Outcome in the European Cooperative Acute Stroke Study I (ECASS I) Cohort. *Stroke* 1999; 30: 2280–2284.
348. Li Y, Lu Z, Keogh CL, et al. Erythropoietin-induced neurovascular protection, angiogenesis, and cerebral blood flow restoration after focal ischemia in mice. *J Cereb Blood Flow Metab* 2007; 27: 1043–54.
349. Zechariah A, ElAli A, Doeppner TR, et al. Vascular endothelial growth factor promotes pericyte coverage of brain capillaries, improves cerebral blood flow during subsequent focal cerebral ischemia, and preserves the metabolic penumbra. *Stroke* 2013; 44: 1690–1697.
350. Helisch A, Wagner S, Khan N, et al. Impact of mouse strain differences in innate hindlimb collateral vasculature. *Arterioscler Thromb Vasc Biol* 2006; 26: 520–526.
351. Ichijo M, Iwasawa E, Numasawa Y, et al. Significance of development and reversion of collaterals on mri in early neurologic improvement and long-term functional outcome after intravenous thrombolysis for ischemic stroke. *Am J Neuroradiol* 2015; 36: 1839–1845.
352. Kanazawa M, Igarashi H, Kawamura K, et al. Inhibition of VEGF signaling pathway attenuates hemorrhage after tPA treatment. *J Cereb Blood Flow Metab* 2011; 31: 1461–1474.
353. Alessio AM, Beltrame MP, Flores Nascimento MC, et al. Circulating progenitor and mature endothelial cells in deep vein thrombosis. *Int J Med Sci* 2013; 10: 1746–1754.
354. Mizuma A, Yamashita T, Kono S, et al. Phase II Trial of Intravenous Low-Dose Granulocyte Colony-Stimulating Factor in Acute Ischemic Stroke. *J Stroke Cerebrovasc Dis* 2016; 25: 1451–7.

355. Zaidat OO, Yoo AJ, Khatri P, et al. Recommendations on angiographic revascularization grading standards for acute ischemic stroke: A consensus statement. *Stroke* 2013; 44: 2650–2663.
356. Zhang ZG, Zhang L, Jiang Q, et al. VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. *J Clin Invest* 2000; 106: 829–838.
357. Yin K-J, Hamblin M, Chen YE. Angiogenesis-regulating microRNAs and Ischemic Stroke. *Curr Vasc Pharmacol* 2015; 13: 352–65.