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Ana Isabel Lopes Luís

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Stem cells and stroke

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ABBREVIATIONS

ANOVA	One-way analysis of variance
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BrdU	5-bromo-29-deoxyuridine-59-monophosphate immunohistochemistry
cDNA	Complementary deoxyribonucleic acid
DA	Dopaminergic neurons
DCX	Doublecortin
DG	Dentate gyrus
ECASS III	European Cooperative Acute Stroke Study III
EGF	Epidermal growth factor
EPE	*Entidades Públicas Empresariais*
EPO	Erythropoietin
ESC	Embryonic stem cell
ESO	European Stroke Organization
ESS	European Stroke Scale
EU	European Union
FGF-2	Fibroblast growth factor-2
FISH	Fluorescent in situ hybridization
G-CSF	Granulocyte colony-stimulating factor
GDNF	Glia cell line-derived neurotrophic factor
GDNF-hMSCs	Glia cell line-derived neurotrophic factor gene modified human marrow stromal cells
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GZ	Granular zone
hATSCs	Human adipose tissue stromal cells
hMSCs	Human marrow stromal cells
IAT	Intra-arterial thrombolysis
ICH	Intracerebral hemorrhage
i.e.	*id est*
IGF-1	Insulin-like growth factor-1
INR	International normalized ratio
iPS	Induced pluripotent stem cells
IV	Intravenous
LACI	Lacunar infarct
LGE	Lateral ganglionic eminence
MCA	Middle cerebral arterial
MCAO	Middle cerebral artery occlusion
mRS	Modified Rankin scale
MSCs	Mesenchymal stem cells
NIHSS	National Institutes of Health Stroke Scale
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NINDS	National Institute of Neurological Disorders and Stroke
NO	Nitric oxide
NSCs	Neural stem cells
NT2N	human neural stem cell line NT2N (also called hNT cells) is derived from NTerra-2/D1 teratocarcinoma cell line

OB	Olfactory bulb
OCSP	Oxfordshire Community Stroke Project
OPCs	Oligodendrocyte precursor cells
PACI	Partial anterior circulation infarct
PIGF	Placental growth factor
PIGF-hMSCs	Human marrow stromal cells placental transfected with adenoviral vectors carrying placental growth factor gene
POCI	Posterior circulation infarct
PSA-NCAM	Poly-sialated neural cell adhesion molecule
RMS	Rostral migratory stream
rt-PA	Recombinant tissue plasminogen activator
SAM	*Sistema de Apoio ao Médico*/Medical Support System
SCF	Stem cell factor
SGZ	Subgranular zone
SITS	Safe Implementation of Treatments in Stroke
SVZ	Subventricular zone
TACI	Total anterior circulation infarct
TOAST	Trial of Org 10172 in Acute Stroke Treatment
US	United States
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

ABSTRACT

Stroke is a major cause of morbidity and mortality worldwide, heading the causes of disability and representing the third leading cause of death in the western world. Yet, apart from thrombolysis in the acute phase of ischemic stroke, no effective therapy is currently available to promote recovery and reduce the heavy burden stroke represents. In the background of this imperative clinical need, endogenous and exogenous stem cells have been studied as a therapeutic option following stroke. This arising strategy is briefly reviewed in this article, pointing out some of the knowledge acquired in basic research, pre-clinical studies and in the few developed clinical trials. In order to appraise the potential clinical necessity of alternative therapeutic strategies, such as stem cell therapy, in a close scenario, a retrospective and observational study was conducted in a stroke unit of a University Hospital, at Oporto, attempting to identify and characterize stroke patients admitted within a quadrimester, their baseline characteristics, type of treatment received and neurological and functional evolution at 2 to 4 months follow-up. A total of 136 patients were studied, 77.2% with ischemic and 22.8% with hemorrhagic stroke. Among 51 patients admitted within the therapeutic window (approved at the time), 34 were submitted to thrombolysis. The sooner thrombolysis was given to ischemic stroke patients the greater seemed to be the benefit.

Keywords: stem cells; stroke; cell therapy; neurogenesis

INTRODUCTION

Stroke overview, the clinical challenge and rational for cell therapy

The generic term stroke refers to the sudden impairment of brain function caused by a variety of pathologic changes involving one (focal) or several (multifocal) cervicocerebral blood vessels [135]. The majority of strokes are ischemic (87%), with the remaining being result of intracerebral (10%) and subarachnoid hemorrhage (3%) [75].

Stroke is a major cause of morbidity and mortality worldwide, heading the causes of disability and representing the third cause of death, in the western world, behind heart disease and cancer [11,23,36,41,66,74-76]. Of all stroke survivors, 30%–50% do not regain functional independence and 15%–30% are permanently disabled (i.e., not able to walk, talk clearly, or feed themselves with a favored hand), a devastating reality to both patients and carers [36,66,93]. It is as well the second most common cause of dementia, the most frequent cause of epilepsy in the elderly, and a frequent cause of depression [104].

Stroke also represents a substantial economical burden, with stroke care estimated to cost more than 5% of many countries' healthcare budgets [101]. For instance, in 2006, in the European Union (EU), the overall cost of stroke was around €38.1 billion [103], and the expected direct and indirect cost of stroke for 2010, in the United States (US), is $73.7 billion [75].

In the US, each year around 795 000 people experience a new or recurrent stroke. About 610 000 of these are first attacks, and 185 000 are recurrent attacks. Approximately 1 in 4 people dies within 1 year after having an initial stroke and mortality data from 2006 revealed that stroke accounted for approximately 1 of every 18 deaths in the US. This means that on average, every 40 seconds, someone in the US has a stroke, and every 4 minutes someone dies of it [75].

Annually, also in the US, around 55 000 more women than men have a stroke. As a matter of fact, the stroke incidence rate is higher for men compared with women at younger ages, but the scenario changes as they grow older. The male-to-female incidence ratio was 1.25 in those 55 to 64 years of age, 1.50 in those 65 to 74 years of age, 1.07 in those 75 to 84 years of age, and 0.76 in those ≥85 years of age [75].

There are also noticeable racial differences in stroke incidence. In fact, blacks have a risk of first-ever stroke that is almost twice that of whites. The age-adjusted stroke incidence rates in people 45 to 84 years of age are 6.6 per 1000 population in black men, 3.6 in white men, 4.9 in black women, and 2.3 in white women [75].

In Europe, each year, stroke accounts for 1.24 million deaths (among 900 million total estimated population): over 1 in 6 women (approximately 17%) and over 1 in 10 men (around 11%) dying from the disease [103]. Large differences in incidence, prevalence and mortality have been reported between Eastern and Western European countries. This has been attributed to difference in risk factors, with higher levels of hypertension and other risk factors resulting in more severe stroke in Eastern Europe. But even within Western Europe notable regional variations have also been found. Yet, stroke in Europe is also the most important cause of morbidity and serious, long-term disability, and demographic changes will result in an increase in both incidence and prevalence [104].

In truth, the population in Europe is aging rapidly. In 2004, 13.7% of the European population was aged 65 years or older which was twice the world level (with the higher percentages in Italy, 18.9%, Germany, 18.0%, Greece, 18.0%, and Sweden, 17.2%) (82). Besides, the percentage of people aged 65 or older is expected to double to about 30% in 2050 [112]. This trend, also observed in the US, at a shorter term [23], seems to reflect the longer life expectancy in Western countries [112]. In 2006, the European average life expectancy at birth was 75.1 years, and 78.6 for the 25 Members States of the European Union. The US life expectancy in 2007 was 77.9 years (80.4 years to women and 75.3 years to men) [86], continuing a long-term increasing trend [46]. Life expectancy at age 65 was 18.6 years in 2007, an increase of 6 percent since 2000 [86].

With age, the risk of stroke increases steeply. It more than doubles each decade of life over the age 55. So, as the elderly segment of the population continues to rapidly grow, substantial advances in the prevention and treatment of stroke are of paramount relevance [23].

Despite more than four decades of intense investigations, no therapy that significantly prevents stroke-induced brain damage and neurological dysfunction has emerged [74].

Recombinant tissue plasminogen activator (rt-PA), a thrombolytic agent, is the only currently available intervention to reduce the size of the cerebral infarct. Classically, the thrombolytic therapy with rt-PA has been approved for use only if administered within 3 hours of the onset of ischemia, being this limit recommended as a standard in all international stroke treatment guidelines until 2008 [23,126]. However, since January 2009, according to the European Stroke Organization (ESO), intravenous rt-PA is recommended within 4.5 hours of onset of ischemic stroke [104], on the basis of the finding of the European Cooperative Acute Stroke Study III (ECASS III) showing a modest, but significant benefit of intravenous alteplase given 3 to 4.5 hours after the onset of symptoms in ischemic stroke patients [44]; and on the basis of the data from Safe Implementation of Treatments in Stroke (SITS) centers revealing that alteplase remains safe when given at that period (3-4.5 hours after the onset of symptoms), offering an opportunity for patients who cannot be treated within the standard 3 hours frametime [126]. These studies lend support to those suggesting a potentially longer timeframe for intravenous thrombolysis [93]. Nonetheless, treatment benefit is time-dependent, with the best results observed if given within 90 minutes [23,44]. So, to maximize the benefit, patients should be treated with alteplase as early as possible (“time is brain”) [44].

Unfortunately, due to the narrow time window as well as a number of contraindications, rt-PA therapy has been restricted to a small proportion of patients evaluated in the emergency room (about 5% of stroke victims). Of those who receive it, rt-PA may be expected to yield around a 30% increase in the number of patients avoiding long-term neurologic deficits [23].

Aspirin has a wide utility but modest efficacy and anticoagulation has proven ineffective not being recommended for the treatment of patients with acute ischemic stroke [36,104].

Advances in endovascular stroke therapy have come from different directions, including pharmacologic and mechanical [93,115] (table 1), and they may improve reperfusion sufficiently to improve survival, but these have yet to be substantially confirmed by randomized clinical trials [19,23,69,94]. Nevertheless, physiologic time to reperfusion will remain critical no matter which tools prove most effective and safest [19].

Table 1. **Current and investigational reperfusion strategies in Acute Ischemic Stroke** [93]

Reperfusion approaches
Recanalization or antegrade
Intravenous (IV) and/or Intra-arterial thrombolysis (IAT)
Endovascular thrombectomy
Endovascular thromboaspiration
Mechanical thrombus disruption
Transcranial or endovascular augmented fibrinolysis
Endovascular thrombus entrapment
Temporary endovascular bypass
Alternative
Global reperfusion (flow augmentation or transarterial retrograde reperfusion)
Transvenous retrograde reperfusion (flow reversal)

Adapted from Nogueira, *et al*, 2009

Unfortunately, although a plethora of neuroprotective compounds have shown promise in animal models, no neuroprotection intervention has shown improved outcome. Various neuroprotection approaches remain under investigation [23,36].

Regardless of increasing focus on evidence-based primary and secondary prevention, strokes still occur. And, even with optimal stroke unit care (including thrombolysis) fewer than one in three patients recover fully from stroke [104]. Thus, rehabilitation is necessary to optimize functional recovery in the remainder [135]. Although there is lack of robust evidence for several of the common interventions employed in post-stroke rehabilitation, there is expert consensus in favor of it, as a way to minimize stroke impairment and to reach and maintain optimal physical, intellectual, psychological and/or social function [101,104]. Sadly, once recovery has reached a plateau and the neurological deficits are fixed, there is no known effective treatment [135].

Intracerebral hemorrhage (ICH), for which no effective treatment strategy is currently available, constitutes one of the most devastating forms of stroke [8-9,81]. The major risk factor for ICH is arterial hypertension, accounting and contributing for about 60% of cases. Hemorrhagic stroke prognosis is poor, with an overall mortality rate of about 40% at 1 month and most of the survivors are left with persistent, severe neurological deficits. Indeed, it is estimated that 90% of surviving patients are dependent on a caregiver at 1 month, and 80% at 6 months. The available therapy is mainly supportive, including maintenance of homeostasis and treatment of brain edema [8-9]. Hematoma growth is frequent and it represents an independent determinant of death and disability [81]. Given the prognostic significance of hematoma expansion and continuous bleeding, therapies aiming to prevent it, through the administration of haemostatic agents, seem an attractive approach [8]. However, it was observed that the promising haemostatic therapy with recombinant activated factor VII (*FAST study*) reduces growth of the hematoma, but does not improve survival or functional outcome after ICH [81].

In selected patients with space-occupying hematomas, surgery may relive the mass effect, but the indication to decompressive surgery remain a subject of ongoing debate [8]. Damage to the brain parenchyma is inevitable

once the bleeding has occurred, and no effective treatment for improving the outcome, other than neurological rehabilitation, is currently available [8].

Given the incapability to efficiently mitigate the devastating effects of stroke, it is vital that novel therapeutic strategies be developed to both minimize the initial neural trauma, as well as repair the damage brain once the pathological cascade of stroke has run its course [16].

In the background of this imperative clinical need, numerous studies have been published investigating the potential application of endogenous and exogenous stem cell therapies for treatment of stroke. In addition to their potential for generating a variety of new functional cell types, stem cells have the ability to respond actively to their environment, migrate to areas of injury, and secrete neuroprotective compounds. These properties may provide them therapeutical potential both in the acute phase and at later time points after conventional medical approaches would no longer be effective [23].

Objectives:

This work major aim is to briefly review the potential application of endogenous and exogenous stem cell therapies for treatment of stroke. This work will also include, as a second part, but a related one, a retrospective and observational study set to identify and characterize the patients admitted in the Stroke Unit of S. João Hospital EPE, Porto, Portugal, since September 2008 until December 2008, namely the remaining disability and its possible relationship with treatment approach. Based on this studied scenario, it is intended to appraise the clinical need of alternative therapeutic strategies and thus support the discussion of stem cell therapy in stroke.

Review Methods

In relation to search strategy and selection criteria used on this review, five data bases were consulted: Pubmed; Isi Web of Knowledge; Science Direct; Scopus and Scirus, using the following query: (stroke OR CVA OR "Intracranial Embolism and Thrombosis" OR "cerebral infarction" OR "brain infarction" OR "Intracranial Hemorrhages" OR "cerebrovascular disorders") AND (stem cell OR "Progenitor Cells") and no specified limits were added. The first approach was article selection by title and/or abstract, including the potential relevant ones. Preference was given to the most recent publications, commonly referenced and highly regarded older publications. It was also searched the reference list of articles included and often further searches were made to find additional information on a selected field.

STEM CELLS: SUBSTRATE FOR REGENERATION

Definitions and types of stem cells

The definition of a stem cell continues to evolve as more knowledge is achieved on the subject [36]. Stem cells have two main defining features: multipotency and self-renewal [3,18,23,34,36,42,137-138]. Multipotency is the capability to differentiate into several morphological and functional cell types [4,23,53]. As a result, stem cells should be able to functionally reconstitute appropriate tissues *in vivo* [4]. Self-renewal refers to the ability of stem cells to make identical copies of themselves [4,23,53].

A self-renewing cell division must give rise to at least one daughter cell with the same differentiation and self-renewal potential as the parent cell. Stem cells display extensive proliferation capacity and are often supposed to be able to divide indefinitely, yielding a virtually unrestricted resource of cells [23].

A multiplicity of stem cells can be identified differing on their potency or on the variety of cell types they can give rise to [23]. The best example of potency is the fertilized human egg. In fact, the zygote is a totipotent cell that generates embryonic and extraembryonic tissues. Nevertheless, being a transitory cell, that does not self-renew, it is not usually regarded as a stem cell. Embryonic stem cells (ESCs), isolated from the inner cell mass of the blastocyst, have the widest potential of any true stem cell. These cells are pluripotent, which implies that they are capable of giving rise to all cell types within the developing embryo [3,23,48].

Outstandingly, there are also a large number of stem cells in the adult mammal. However, this adult stem cells are tissue-specific, meaning that they are capable of generating certain local cell types, but not those from unrelated tissues [23]. Often they are referred as multipotent cells [3]. The hematopoietic stem cell, which can give rise to all blood cell types, is the best well-known tissue-specific stem cell. But tissue-specific stem cells have been identified in several organs (heart, muscle, skin, gut, liver, pancreas and brain) [23]. These resident stem cells provide a continual source for a physiological organ-specific cell replacement during normal cell turnover, as well as they may contribute to regeneration in disease/injury conditions [42].

Remarkably, recently it was demonstrated that is possible the induction of pluripotent stem (iPS) cells from terminally differentiated somatic cells via nuclear reprogramming with retroviral vectors containing complementary deoxyribonucleic acid (cDNA) encoding genes known to be associated with the ESC state [23].

Stem cell behavior

True self-renewing stem cells usually divide uncommonly when *in vivo*, possibly as a way to protect the integrity of their genetic material, since continuous proliferation increases the mutation risk and subsequently the risk of tumorgenesis [23].

To generate enough cells for *in vivo* regeneration while minimize the stem cell proliferation, adult stem cells go through asymmetric self-renewing divisions, giving rise to one stem cell and one committed progenitor cell, which can proliferate rapidly and thus generate a large number of more differentiated progeny [23].

Frequently, progenitor cells are unipotent, differentiating into only one differentiated cell type, though keeping the property of self-renewal [3,23].

For a long time, it has been conceived that stem and progenitor cells can either self-renew or generate more restricted cell daughters, but cannot move backward along this development sequence or "dedifferentiate". However, it has been suggested that, under certain conditions, some adult stem cells have the ability to give rise to cell types from unrelated organs, a phenomenon labeled transdifferentiation. It remains uncertain whether those rare cells with greater potency may actually exist *in vivo*, or whether this event represents an artifact of *in vitro* cultures conditions. Besides, some of the first cases initially reported to be *in vivo* transdifferentiation, where latter confronted with the demonstration that some cell types may fuse with mature cells from unrelated organs. Others have posited that specified stem cells may have the capacity to dedifferentiate [23].

Stem cell applications

Cell replacement therapy, i.e., the replacement of cell types lost as a result of disease or injury is the most commonly discussed role for stem cells. This replacement may be accomplished either by *in vitro* predifferentiation of stem cells into the desired cell type followed by transplantation to the affected area, or by direct transplantation of stem cells followed by spontaneous *in vivo* differentiation of stem cells into the needed cell types [23].

In conditions, such as Parkinson's disease or diabetes mellitus type 1, where restricted populations are selectively lost (dopaminergic cells in the brain and beta cells in the pancreas) and function may be restored by replacement of cells with similar properties, the transplantation of predifferentiated cells may be particularly useful. In contrast, in stroke, where multiple cell types are lost and thus different cell types are needed to restore function to a broadly damaged area, the use of undifferentiated cells may be of better value [23,47]. As a matter of fact, usually strokes affect not only different neuronal phenotypes, but also astrocytes, oligodendrocytes, endothelial cells [47,49,110]. Thus reconstitution of the complex and widespread neuronal-glial-endothelial interrelationships may require access to a broader array of lineage species than more committed phenotypes. Hence, cells for transplant may need to initially remain immature and phenotypically plastic [110]. Another distinctive characteristic of stroke is that the injury process is acute and limited in time and there is no ongoing degenerative process or major immunological attack. Therefore the brain may be more hospitable to transplantation [47].

Another strategy to promote cell replacement involves the mobilization of resident tissue-specific stem cells to provide the substrate for cell replacement [23].

Although spontaneous neurogenesis may occur in certain brain regions in response to brain injury it is not sufficient to allow functional recovery. So, different approaches have been chased in order to magnify this endogenous regenerative response [23].

Besides cell replacement therapy, stem cells appear to serve supportive roles. For instance, some stem cells show evidence of robust tropism for injury. Although in an injured area most resident cells may die, other stem cells seems to be attracted to that area of injury and may secrete molecules that promote survival and regeneration. Stem cells may as well be recruited to tumors, where they are related with decrease neoplasms growth via secretion of antiangiogenic and proinflammatory compounds [23].

Stem cells' tropism to injured areas has interesting some groups that aim to use stem cells genetically engineered to deliver therapeutic compounds specifically to the area of injury. This approach could play an important role for ischemic stroke, in which compromised blood flow may make delivery of drugs to the affected region more challenging after systemic administration [23].

ENDOGENOUS STEM CELLS AND STROKE

Neural stem cells

After decades of dogma stating that "the brain does not regenerate" and no new neurons are born in the adult mammalian brain it is now largely confirmed that neural stem cells (NSCs) are actually present throughout life and that spontaneous adult neurogenesis occurs primarily in three brain regions: the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG), the subventricular zone (SVZ) of the lateral ventricles and the posterior periventricular area (Fig.1) [23,36,74,132]. Although these three are the most well established neurogenic regions of adult mammalian brain, some studies indicated the presence of minute amounts of NSCs in other brain areas, as striatum, spinal cord [133] and neocortex [76,97,132].

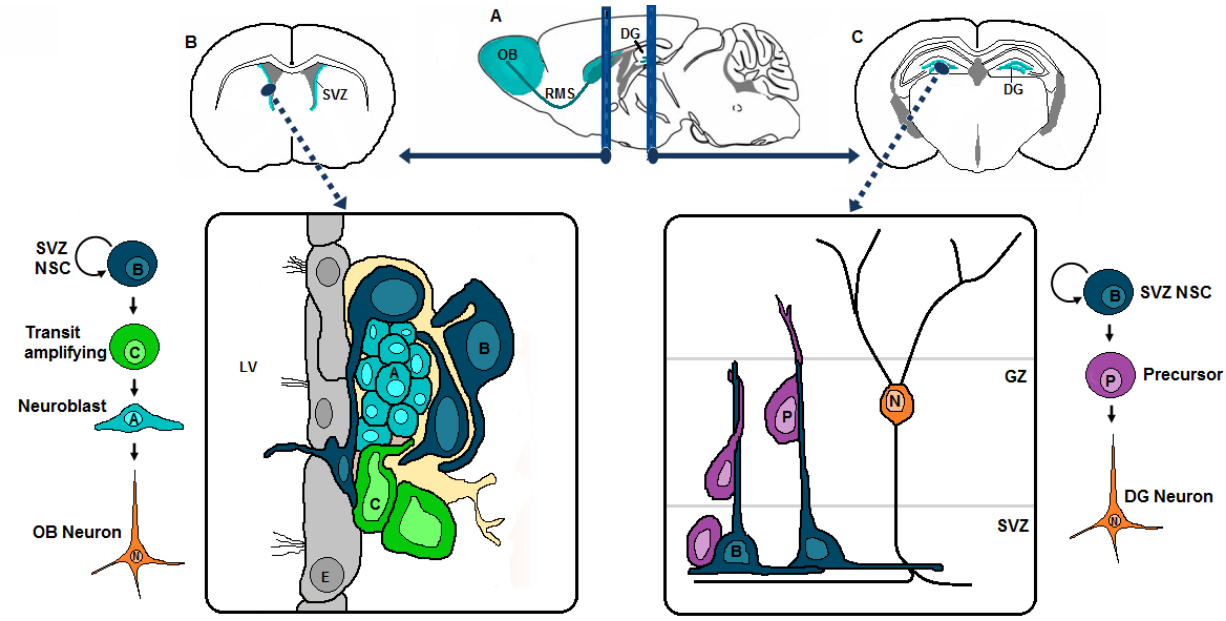


Figure 1 - Neurogenesis persists in adult mammals throughout life
a: the parasagittal section of a mouse brain demonstrates the brain regions involved in rodent adult neurogenesis (red). b,c: coronal sections at the approximate regions of the dashed lines. b: the SVZ, where neural stem cells persist throughout life. Slowly dividing type B cells (expressing glia fibrillary acidic protein – GFAP) give rise to rapidly dividing type C cells (that serve to increase the number of progeny), which in turn give rise to migratory neuroblasts (type A cells). These reach the olfactory bulb (OB) via the rostral migratory stream (RMS), and there preferentially differentiate into granule interneurons (that functionally integrate into local circuitry) and into dopamine periglomerular neurons. c: the dentate gyrus, where new neurons are also born throughout life. Type B cells in the subgranular zone (SGZ) give rise to precursor cells, which migrate up the radial projection of type B cells to become granule neurons in the granular zone (GZ). Adapted from Burns, et al, 2009[31]

NSCs can be segregated from the adult brain and then cultured *in vitro,* in the presence of basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). *In vitro,* NSCs form cellular clusters named neurosphere [23]. These stem cells are able to generate neurons as well as astrocytes and oligodendrocytes. *In*

vivo research revealed that NSCs have characteristics of astrocytes and that they are at the end of a development continuum that starts with neuroepithelial cells in the neural tube, followed by radial glia that function both as parent and radial migratory guide for newly born neurons in embryonic brain. Toward the end of development, radial glia disappears in most parts of the brain, but some persist as multipotent astrocytes confined mainly to the adult brain regions mentioned above, where they are known thereafter as NSCs (Fig.2) [23,132].

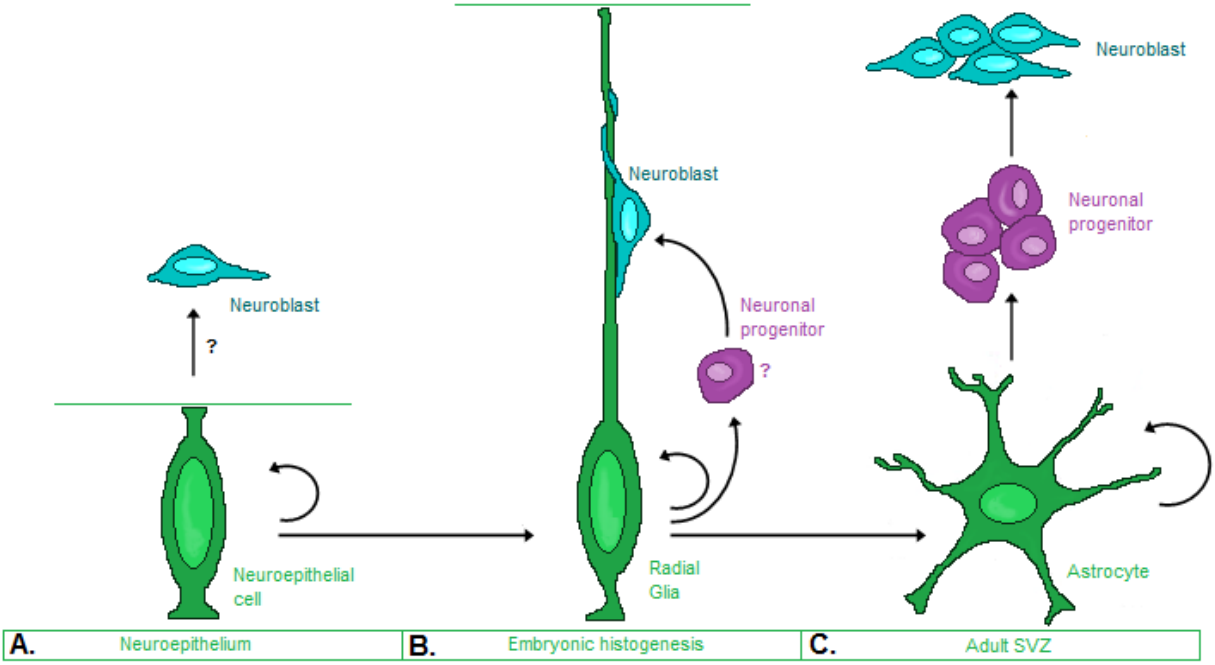


Figure 2 - **Hypothesis for neural stem-cell development**
A. Neural stem cells (green) in the early neuroepithelium expand from the ventricle to the pia. B. Radial glia (green) is thought to be a neural stem cell, possibly an elongated form of the neuroepithelial cell. These cells are known to divide symmetrically or asymmetrically to produce neurons (blue) that migrate into the cortex along the fibre of their progenitor. Radial glia may produce neurons directly or indirectly through transit amplifying cell types (violet). C. Later in the development, radial glia transform into cortical astrocytes. Cells derived from radial glia may come to reside in the adult subventricular zone (SVZ) (blue), these astrocyte-like cells behave as stem cells, since they self-renew and produce neurons, probably through intermediate cell types (violet). Adapted from Alvarez-Buylla *et al* 2001[6].

In the SVZ of the adult brain, NSCs are found nearby to a layer of ependymal cells that line the lateral ventricles. This area has four different types of cells: type A, B, C and E. Of these, the most numerous are the glial fibrillary acidic protein (GFAP)-positive type B cells, which are considered as the primary adult neural stem cells. Type B cells are the astrocytes of the SVZ and form chains of young migratory neuroblasts, designated as A cells, that migrate to the olfactory bulb (OB) through the rostral migratory stream (RMS), where they differentiate into olfactory interneurons. The highly proliferative type C cells are at the base of the migratory chain of A cells and are believed to be immature precursors, the intermediaries between B and A cells. Type E cells correspond to ependymal cells [74,132]. It is interesting to realize that the neuroblast migration occurs even in the absence of olfactory bulb, suggesting that the neural cell migration is not target mediated, but rather an inherent property [132].

In the dentate gyrus and the posterior periventricular area NSCs have quite limited self-renewing capacity when compared to the NSCs of the SVZ. Besides, while the SVZ have four different cell types, DG is thought to contain only one type of progenitor cells [74]. In DG, neural progenitors are situated nearby the hilus in the SGZ, where they continuously proliferate, and then migrate to the granule cell layer, differentiate into mature neurons and extend axonal projections into the Ammon's horn of the CA3 area [74,132].

The subependymal layer of posterior periventricular area surrounding the hippocampus is thought to contain the true stem cells that replenish the hippocampal neurons. Indeed, it was demonstrated that the newly proliferated progenitors migrate and repopulate the hippocampal CA1 after ischemic cell death [132].

Knowing that the adult brain homes stem cells with neurogenic potential brings up the thrilling possibility of exploiting them as a way to restore neurons and glia both lost following stroke [23,99]. However, in a normal adult brain, neurogenesis, defined as the generation of new neurons, occurs in a limited capacity. Around 9000 new neuronal cells (0.1% of the granule cell population) are generated in the DG of mice per day [74].

Even though increased proliferation of NSCs frequently leads to augmented neurogenesis, it is not a direct measure of neurogenesis, as differentiation of the newly born cells to a neural lineage is also a requisite. In the same way, increased neurogenesis does not necessarily means that there will be an augment in the net number of functional neurons. For that to happen newly born neurons must first survive, which usually is associated with a successful synaptic integration into brain circuitry [23].

It appears that the major function of neurogenesis in the adult brain is to replace the neurons lost regularly in certain brain areas. But then again, the resident neural progenitors can also be the emergency supply that could be induced to replace neurons lost due to acute insults. It is fascinating to notice that a lot of conditions may be capable of affecting neurogenesis in the adult brain, upregulating it (stroke, positive life experiences, for instance, exercise, learning, enriched environment, caloric restriction) or downregulating it (negative life experiences, such as stress, drug abuse, irradiation, hight-fat diet, diabetes, inflammation…) [91,132].

Ischemia-induced neurogenesis

Models of brain ischemia

Quite a few rodent models of brain ischemia were created. To generate "global ischemia" it can be used transient bilateral carotid artery stenosis, which induces selective cell types destruction, such as CA1 pyramidal cells in the hippocampus [23]. Nevertheless, most often the ischemic models are focal, generally involving occlusion of the middle cerebral artery (MCAO). Differently from global ischemia, during focal ischemia blood perfusion is only blocked to specific regions of brain. The MCAO model results in damage mostly to striatum and, in a minor part, to the overlying cortex [23,76,132]. In order to create selective cortical lesions it can be used occlusion distal to the striatal branches [23].

When aiming a permanent occlusion it can be used ligation, cauterization or laser-induced photothrombosis. If a transient occlusion is intended to model an ischemia-reperfusion injury, it can be used an intraluminal filament through the carotid artery into the proximal middle cerebral artery (MCA) [23]. When this transient occlusion is shorter than half an hour the damage preferentially affects the striatal tissue, but if the occlusion lasts for 90-120 minutes both striatum and cortex will be affected [10].

Increased neurogenesis in ischemic neurogenic regions

In 1998, a pioneering study by Liu *et al* [73], reported, for the first time, increased neurogenesis in the DG after global ischemia. In order to determine whether ischemia affects neurogenesis, newly divided cells in the DG were examined after transient global ischemia in adult gerbils. A 12-fold increase in DG cell birth was observed 1-2 weeks after 10 minutes of global ischemia (with bilateral common carotid artery occlusions), using 5-bromo-29-deoxyuridine-59-monophosphate (BrdU) immunohistochemistry. It was also demonstrated that newborn cells differentiate into a neural phenotype by 26 days after the onset of ischemia and are primarily located in the granule cell layer at 7 months post-ischemia. As ischemic preconditioning (which protects CA1 neurons from subsequent ischemic damage) did not alter the level of increased neurogenesis, neurogenesis in this case was not attributable to actual neural loss. Thus, the level of progenitor proliferation in SGZ, although affected by the duration of global ischemia, is not influenced by the intensity of CA1 cell death [73]. Afterwards, several studies confirmed that global cerebral ischemia stimulates neurogenesis in the DG of adult rodents [58], monkeys and other species [74,122]. Strikingly, stroke induced neurogenesis has recently been observed in the adult human brain [57].

Increased neurogenesis has also been reported in the SVZ and posterior periventricular areas following global ischemia [23,52,74,76,90,132]. The newly generated progenitors in the SVZ, after global ischemia, express many immature neural markers including highly poly-sialated neural cell adhesion molecule (PSA-NCAM), doublecortin (DCX), nestin and β-III tubulin, and migrate along the RMS to the olfactory bulb [52,122].

Using models of focal ischemia it was also reported a significantly enhanced progenitor proliferation in the SVZ as well as in the DG [58,98,136]. In fact, different groups observed that, after transient focal unilateral ischemia enhanced proliferation begins bilaterally in both SVZ and SGZ as early as 2 days, reaches the peak at 1-2 weeks and declines to the basal levels by the 3-4 week of reperfusion [33,58,132,136].

The degree of post-ischemic progenitor proliferation was shown to be influenced by the duration of MCAO, with a 120 minutes occlusion inducing more proliferation than a 30 min MCAO [10].

Neurogenesis in ischemic non-neurogenic brain regions

Differently from the DG where there is no known evidence of migration of NSCs from the subgranular zone out of the DG to restore other brain areas [56], in 2002, for the first time, it was observed, in a rat model of transient MACO [98], that neuroblasts from an expanded ipsilateral SVZ are activated after ischemia, deviate from their normal route, toward the olfactory bulb (via RMS), and migrate in chains toward the ischemic penumbra of striatum and cerebral cortex. It was also observed that some of these neuroblasts persisted in damaged neostriatum and with time differentiate into cells with neuronal morphologies, that expressed markers of matures striatal medium spiny neurons. However, the functional integration of newly generated neurons in the neostriatum was not shown [98].

Some previous studies had reported post-ischemic neurogenesis in the cerebral cortex following focal ischemia [39,54]. However, the origin of the neurons present in the cortex is being debated [132], with some stating that they could correspond to apoptotic neurons, instead of new born neurons [23]. A subsequent study showed robust migration of DCX positive neuroblasts from the rostral SVZ (but not from the DG), via RMS and lateral

cortical stream, into the penumbra of ischemic cortex. But survival and maturation of these cells at later time points was not investigated [56].

Currently it is accepted that changes in the SVZ leading to striatal neurogenesis following ischemic injury persist long term, and new neurons continue to be added to the striatum for at least several months [70,121] or even for a year [63] after stroke.

Nakatomi *et al*. [90] showed that the newly proliferated progenitors migrate from the posterior periventricular area and repopulate the hippocampal CA1 following ischemic cell death. And later it was demonstrated that this phenomena leads to recovery of spatial learning and memory function in adult rats. In fact, so far this study was the most convincingly showing functional integration of the new neurons in the adult brain following transient global ischemia. The great level of neuroregeneration shown in this study permitted prospective labeling of periventricular NSCs with a green fluorescent protein (GFP) transfered by lentivirus, allowing direct demonstration of the functionality of the new neurons via electrophysiological studies [23,132].

Regulation and augmentation of ischemia-induced neurogenesis

Presently it is widely accepted that neurogenesis occurs in response to ischemic lesions. However, it also seems clear, that this limited response needs to be potentiated in order to achieve a significant level of regeneration. In fact, differently from what happens with amphibians and reptiles that present a robust regeneration of central nervous system after injury, achieving a post-injury brain that is structurally and functionally comparable to the pre-lesion state, it remains uncertain whether mammalian baseline levels of stroke-induced neuroregeneration is of any functional value at all. Some claim that this scarce neuroregenerative capacity is the price paid for increased complexity of mammalian brain. More and more it has been shown that some physiological stimuli, endogenous molecules and exogenous agents are capable of regulate adult neurogenesis [23]. The significance of some of these factors will be briefly indicated, since no exhaustive review on this topic is intended.

Factors that modulate post-ischemic neurogenesis

As previously mentioned, increased neurogenesis is reported not only in the ipsilateral DG and SVZ, but also contralaterally, what suggests that diffusible factors may play a role in promoting post-ischemic proliferation. Several diffusible mitogens including growth factors, cytokines and cell division modulators are known to be upregulated during brain ischemia [132]. Some biologic characteristics of these diffusible factors will be briefly reviewed.

Epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2) are factors that expand the stem cell pool, and thus appear to be useful to provide an adequate cellular substrate for neuroregenerative response. Both growth factors are known to play a significant role in neurogenesis *in vivo* and regarded as essential to preserve the pluripotence of neural stem cells cultures *in vitro* [132]. After cerebral ischemia the FGF-2 expression suffers a notable increase and it was shown that the FGF-2 knockout mice present an attenuation of the neurogenesis induced by focal ischemia [132]. Leker *et al* [70] showed that adenovirus programmed to deliver FGF-2 over longer time periods enhanced neurogenesis up to 90 days after ischemia, which was

accompanied by continuous improvement in behavior, suggesting FGF-2's role in long-term neurological repair. EGF may have an analogous effect [132]. Infusion of a combination of EGF and FGF-2 was shown to increase neuroproliferative potential in the posterior periventricular area following global ischemia [90] and in the DG and SVZ after focal ischemia [124]. The amplified progenitor proliferation in rats infused with both FGF-2 and EGF was also shown to be associated with improved postischemic memory formation and retention [90].

Brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) and the stem cell factor (SCF) are endogenous substances which synthesis is induced by transient focal ischemia [132]. Also, it was demonstrated that BDNF promotes neurogenesis, leading to increased numbers of neurons in the olfactory bulb and promoting the appearance of new neurons in the striatum [23]. The infusion of GDNF into lateral ventricles after MCAO in the adult rats revealed a considerably enhance in the neurogenesis both in SVZ and DG [33]. SCF is known to be a major player in the embryonic neurogenesis and it was demonstrated to be upregulated in hypoxia-treated cultures of embryonic cells from mouse cerebral cortex [132]. Jin *et al.* showed that SCF significantly stimulated BrdU uptake into cultured stem cells and when cultures were exposed to hypoxia conditioned medium containing SCF antibodies, BrdU labeling was reduced. Moreover, SCF infusion enhanced the BrdU incorporation in SVZ and SGZ and cells in which SCF stimulates BrdU incorporation were of neuronal lineage [55].

Erythropoietin (EPO) and vascular endothelial growth factor (VEGF) promote the differentiation of bone-marrow stem cells into circulating mature red cells [76]. In adition, EPO is an important mitogen that is upregulated in the SVZ following ischemia and promotes post-ischemic neurogenesis [23,132]. And besides increasing the number of proliferating cells in the SVZ, EPO was shown to augment the levels of other growth factors, such as VEGF and BDNF. EPO is known to promote differentiation of type B stem cells into type C transient amplifying cells, leading to a decrease in the true stem cell population and increasing the number of lineage protected neuronal precursors [114]. VEGF is an angiogenetic growth factor also upregulated by cerebral ischemia that showed to promote neurogenesis both *in vitro* and *in vivo* and to increase the survival and proliferation of transplanted human NSCs [76,132].

Insulin-like growth factor-1 (IGF-1) has well-established angiogenic, anti-inflammatory and anti-apoptotic properties. This factor is also upregulated in response to ischemia, and infusion of antibodies to IGF-1 attenuates ischemia-induced proliferation in the DG [33] and SVZ [134]. In fact, IGF-1 treatment has neuroprotective effects in mice with ischemic stroke associated with improved long-term clinical outcome [76].

Nitric oxide (NO) is known to be formed in excess after cerebral ischemia and it is acknowledge as a modulator of stroke-induced neurogenesis [132]. NO is formed by 3 different isoforms of NO synthase (NOS): neuronal NOS, inducible NOS and endothelial NOS. In relation to modulation of post-ischemic neurogenesis, the role of these NOS isoforms seems to be different. In fact, studies indicate that NO produced by neuronal NOS is inhibitory, while NO formed by inducible NOS is beneficial for post-ischemic neurogenesis [74].

Granulocyte colony-stimulating factor (G-CSF) is in charge of bone-marrow-derived stem cell differentiation in circulating neutrophilic granulocytes. Yet, in experimental models of focal ischemia, the administration of G-CSF after reperfusion was associated with neuroprotection mediated by diverse

mechanisms including activation of anti-apoptotic pathways, reduction of focal inflammatory response, neurogenesis and angiogenesis potentiation, enhancement of cell proliferation of the SGZ of the DG and promotion of stem cell mobilization and homing to brain [76]. Furthermore, the concomitant administration of G-CSF and SCF in an animal model of brain ischemia was associated with more than fifty percent infarct volume reduction and significantly increased angiogenesis [123].

The significance of neurotransmitters in modulating post-ischemic neurogenesis stills needs to be better understood. Disturbed glutamatergic neurotransmission, especially modifications on receptor expression and function, is recognized to play a significant role in post-ischemic neuronal death. Other neurotransmitters such as serotonin or dopamine are also thought to modulate neurogenesis in the adult brain. Thus serotonin reuptake inhibitors, which are commonly used as antidepressants, have been shown to potentiate neurogenesis presenting an association between increased serotonin and neurogenesis. Dopamine, a neurotransmitter related with mood and motivation, has been suggested to play a significant role in neurogenesis in adult brain [132].

Are there neural stem cells in non-neurogenic regions?

Several were the articles that showed compelling evidence of neuroblast migration from the SVZ or rostral migratory stream (RMS) to areas of injury. Yet, in a number of them, one cannot rule out the possibility that some newly generated neuronal cells may be born locally. Despite scarce evidence of normal neurogenesis in the majority of brain regions, in truth, cells isolated from different areas in the brain (such as cerebellum, cortex, white matter and spinal cord) show neural stem cells' properties *in vitro*, since they can differentiate into neurons, astrocytes and oligodendrocytes. What exact features do these cells have? Their precise identity remains uncertain. They could correspond to quiescent NSCs or even to glial cells that display neural potential within certain circumstances. Actually, oligodendrocytes precursor cells have been shown to generate neurons *in vitro*, and endogenous NSCs now seem to belong to the astroglial lineage [23]. Furthermore, studies have suggested that genetic manipulation of parenchymal progenitors, such as oligodendrocyte precursor cells (OPCs), may perhaps represent a way to promote neuroregeneration after stroke [23,95].

Effect of age on neuroregeneration

Although it is well known that aging is a risk factor for stroke and that human stroke frequently occurs in the aged brain environment, the majority of rodent studies of stroke has been performed on young animals, and thus may not fully replicate the effects of ischemia on neural tissue in aged subjects [22-23]. Several lines of evidence have indicated that neurogenesis decreases abruptly in intact aged brain, both in the SVZ and the DG. So, one may conjecture that in old age it may be observed less neurogenic response to stroke. However, data indicates that decreased neurogenesis with age may be a reflection of brain microenvironment changes, rather than modification in the number or properties of NSCs themselves [14,23]. This interpretation is supported by the possibility of enhancing neurogenesis in the aged brain by suitable stimulation. While neurogenic regions of aged brain provide poor support for grafted neural stem cells, improving the environment enhances survival of implanted cells [14].

Furthermore, with age, basal levels of corticosteroids increase, decreasing the NSC proliferation [22-23]. Correction of this augment by adrenalectomy translates into a considerable neurogenesis increase in the DG. In addition, the levels of several cytokines decline with age, namely fibroblast growth factor 2 (FGF2), IGF-1, and VEGF, as well as the level of EGF signaling, and infusion of such cytokines in aged brains reverses age-related declines [23]. Moreover, isolation and *in* vitro culture of NSCs from young and old rodent brains results in similar numbers of neurospheres (i.e. proliferative clusters of cells) [22-23] and it was shown that *in vivo* magnitude of striatal neurogenesis after stroke is similar in young and old rats [31].

Besides, it has been observed stroke-induced neurogenesis in the human brain of aged patients [57,78,85].

Thus, it seems that the aged brain may present a similar neurogenic potential for response to injury [23].

Is there a role for endogenous bone marrow in neuroregeneration after stoke?

The role of endogenous bone marrow in stroke has been a topic of some controversy [23].

Different bone marrow derived cells have interesting relationships with the brain. Microglia derive from the hematopoietic lineage and assume roles of immune surveillance (comparable to macrophages in other organs), rapidly responding to brain injury and demonstrating both pro and anti-inflammatory properties, which are of paramount importance to minimize injury following stroke [23]. Perivascular cells, inside the basal lamina of vessels, play an active role in vascular regulation and remodeling and therefore are thought to have an important function in stroke [23,64]. The number of circulating endothelial progenitor cells was shown to increase in acute ischemic stroke patients, and seems to be associated with a good prognosis, consistently with the increasingly understood importance of early angiogenesis after stroke [23,116].

Recently, several reports have advocated that bone marrow-derived cells may contribute to endogenous neurogenesis, claiming that the cerebral ischemia promotes recruitment and mobilization of stem cells from the bone marrow to brain [23,74]. However, while some groups, relying specially on fluorescent *in situ* hybridization (FISH), presented evidence suggesting bone marrow plasticity, others categorically report a nonexistence of bone marrow-derived neurogenesis, using transgenic labels that showed absence, following stroke, of derived neurons and glia in the cerebrum after bone marrow transplantation or by demonstrating that bone marrow-derived cells fuse with various somatic cells. Thus, although the idea of “turning blood into brain” is intriguing, further investigation is needed to clarify it [23].

Putting it briefly…

Numerous endogenous molecular mechanisms synergistically promote the postischemic progenitor cell proliferation, survival, migration to the areas of injury and eventually functional integration. Several exogenous agents including a variety of growth factors promote the post-ischemic neurogenesis [132]. However definitively assessing the role of neurogenesis in observed functional recovery remains an ongoing challenge [23]. There are quite a few obstacles needing to be defeated, such as inducing sufficient proliferation to repair the devasting neuronal damage following ischemia, promoting the survival of the newly formed cells in the hostile environment, and more importantly to induce proper connectivity of the newly formed cells with the existing circuitry [132].

EXOGENOUS STEM CELLS

Introduction to cell transplantation

So far, the majority of studies have shown relatively limited cell replacement from endogenous NSCs. In addition, the technology for endogenous NSCs mobilization is quite recent, whereas replacement of lost neural cells by transplantation has been studied for decades. This approach could have the advantages of greater control over cell fate, ability to deliver the desired amount of cells, and reduce the risks associated with mitogen infusion [23].

A number of cell sources for stem cell transplantation have been considered with different aims that vary from replacement of the lost circuitry to delivery of neuroprotective or immunomodulatory compounds, as it will be discussed further below [23].

However stroke poses special conditions that impact the potential success of transplantation to enhance neurological recovery [110].

Transplantation variables

In order to maximize the number of surviving functional cells present at the appropriate site of injury it is important to account for diverse parameters that may differ according to the cell type used or to the predominant goal: neuroprotection or cell replacement [23].

Anatomy

As it was previously mentioned, since stroke affects multiple cell types (including neurons, glia and endothelial cells), cells used to transplantation, ideally, need to maintain initially an immature state and differentiate into several specific cell types after engraftment [76,110]. Furthermore, stroke may also affect simultaneously white and grey matter and disrupt various neuroanatomical pathways including motor, sensory, cerebellar and visual tracts as wells as networks for attention, language, and praxis. Therefore, patients may present a large spectrum of clinical features, differently from what happens in many neurodegenerative conditions. So at least one question arises: which stroke lesions are amenable to cell transplantation? The majority of preclinical ischemia studies are conducted using a middle cerebral artery stroke model which represents damage mainly to striatum and also, in a smaller proportion, to cortex [76,110]. The striatum and the rest of the basal ganglia are anatomically well defined and stereotactically accessible by following a trajectory under the Sylvian fissure. Cortical lesions also may be accessible to transplantation [110], but only a few authors have investigated cell therapy for cortex infarcts and still there are not any conclusive results on the possibility on restoring cortical damage [76].

On other words, it is necessary to make available therapeutic strategies depending on the zone affected by the stroke.

Another aspect to account for is the size and extent of the infarct. Ideally, a small number of cells would reasonable cover the affected area. Yet, in extensive cerebral damage, the number of cells need to restore function may be discouraging [40].

Timing

The optimal time for implant following the cerebral infarct is not yet well defined [40,76,110]. Dynamic changes of the ischemic lesion's environment occur over time. In the acute phase, the main threat to the new tissue introduced into the peri-infarct region is the release of exocitotoxic neurotransmitters, free radicals, as well as proinflammatory mediators [41,76,110]. Besides cells may be undergoing apoptosis in the penumbra over several weeks following stroke [40,110]. The activated inflammatory response leading to microglial activation may inhibit the growth and survival of both transplanted and endogenous cells. But on the other hand, it may be better to take advantage of local repair processes that happen during the stroke acute phase, such as the release of cytokines and neurotrophic factors, which could potentially favor implant growth, survival, differentiation, and/or integration [76,110]. Further, the ischemic environment also promotes the generation of new neurons in periventricular regions and in the cerebral cortex [110]. And also postponing transplantation for weeks, poses the disadvantage of allowing the formation of scar tissue, which may adversely affect implanted cells [76,110]. The way transplantation will affect on-going neurogenesis is unknown. The timing choice should also take into consideration the natural course of recovery from stroke. Stroke impairments, depending on the type and severity, have wide variety courses of improvement. And in addition, individual brains are very differently wired. For instance, while some patients who become aphasic after an infarct of the left caudate region regain some language function by using the intact cerebral hemisphere, others don't [110]. Thus, for a lot of neurologists it is rational to delay transplantation until neurologic deficits have *plateaued* [76,110].

Vascular supply

Vascular supply is vital to support graft survival [76,110]. Even before the challenge of neuron differentiation and survival of neurons, the ischemic environment defies functional restoration. A large infarct with massive necrosis may yield an infarct core that is inhospitable to newly delivered cells due to lack of blood supply and absence of an appropriate extracellular support. One approach has involved the transplantation of NSC-embedded scaffolding into the infarct cavity to promote the formation of reciprocal connections between graft and host [23].

But on the other hand, the angiogenesis in the ischemic border, result of the vascular sprouting from mature endothelial cells of pre-existing blood vessels, creates a hospitable microenvironment for neural plasticity [76]. In fact, it has been described that endothelial cells release factors that stimulate the self-renewal of both embryonic and adult neural stem cells, inhibit their differentiation, and promote their production of neurons. Besides affecting neurogenesis, angiogenesis influences also the migration of SVZ neuroblasts toward the ischemic boundary where it takes place [120].

Route and site of stem cell delivery

Another crucial point in stem cell transplantation is the route of cell administration [23,40-41,76]. They may be delivered systemically into the vasculature or locally into the brain [23]. Several studies indicate that stem cells can home to sites of injury in the CNS and induce functional recovery after stereotactic intraparenchymal [61], intracerebroventricular [88], intravenous [27-29,119] and intra-arterial [113] transplantation [15,41].

A comparative study [59] revealed that all routes resulted in cells targeting the lesion and in similar functional recovery. However more cells were found at the lesion site when using the intracerebral route, followed by intracerebroventricular and intravenous delivery [59]. Nevertheless, researchers in this study assessed exclusively the absolute number of cells nearby the lesion. And some argue that they took no account of whether these cells were therapeutically distributed to all injured areas of brain parenchyma on a microscopic level. As a matter of fact, many believe that intravascular delivery of stem cells may lead to a wider distribution of cells around the lesioned area as compared with focal perilesional transplants, thereby leading to superior stem cell–injured tissue interactions. Cells travel in the bloodstream and follow a chemoattractant gradient generated by inflammation in the injured brain [41]. Unfortunately, stem cells delivered intravenously pass through the systemic and pulmonary circulation systems and home to other organs as well (kidneys, lungs, spleen), which significantly reduces cell homing to the injured brain [15,17,41]. An alternative route to intravascular delivery is intra-arterial, which would circumvent body circulation, since the first pass of stem cells injected into the carotid artery would be the brain [41].

Nevertheless, interestingly, entry of intravenously injected cells into the central nervous system is not required for therapeutic effects, indicating that peripheral mechanisms play a role as well [17,49]. In fact, the majority of the studies involving intravenous cell delivery either failed to demonstrate, or at best showed only a very small proportion of, the injected cells entering the injured brain. But, despite this poor transendothelial migration, intravenous cell delivery still leads to enhanced functional recovery [17,41].

Stem cell direct delivery into the infarct core generally yields poor cell survival, while injection into the penumbra routinely yields both surviving cells and neuroprotection [23]. On the other hand, transplantation might be performed distant from the infracted area, for instance at the contralateral side, characterized by healthy and well vascularized surroundings. Veizovic *et al* [125] have shown long-term recovery, from deficits induced occlusion of the middle cerebral artery, after grafts of the transgenic murine stem cell line MHP36 were placed in the intact hemisphere contralateral to the stroke lesion to avoid exposure to the inflammatory environment of the developing lesion. Transplanted cells did not stay at the site of implantation, but extensively migrated through the striatum and somatosensory cortex in both the intact and lesioned hemispheres, with approximately a third of cells crossing from the intact to the lesioned side. The presence of cells on both sides of the brain suggested that functional recovery may involve both limited repair of pathways on the damaged side and interactions with processes of reorganization in the intact side [88,125].

While in one study [131], Willling *et al,* found out intravenous delivery of human umbilical cord stem cells at 24 hours was more beneficial than intracerebral delivery, Borlongan *et al* [16], observed the opposite when using bone marrow cells.

The route of stem cells delivery may also influence the type of recovery. For instance, Modo *et al* [88], showed that intraparenchymal grafts of the transgenic murine stem cell line MHP36 only enhanced sensoriomotor function, whereas intracerebroventricular grafts only affected learning and memory.

Therefore, the optimum route of delivery still needs to be established considering the specific cell type or the mechanism of action underlying the beneficial effect [15].

Immunosuppression

Immunosuppression role and importance remain heavily debated. NSCs may be minimally immunogenic [87], whereas marrow stromal cells may account for a robust inflammatory response resulting in rapid acute rejection [23]. Immunosuppressive drugs, including cyclosporine A, may also promote sprouting of host neural cells, potentially leading to functional enhancement independent of the grafted cells. However given the serious side effects of immunosuppression its use remains a ongoing debate topic [23].

Predifferentiation

In preparation for stem cells transplantation the capacity of extensive proliferation capacity is of utmost importance in order to generate adequate numbers of cells. Still, if cells fail to exit the cell cycle at some appropriate point after transplantation, the result may ultimately be lethal. Similarly, although pluripotent or multipotent stem cells may be required to guarantee capacity to generate the cell types desired after transplantation, uncontrollable inappropriate differentiation may give way to undesired cell types in the brain. On the other hand, entirely differentiated neuronal cells may have long projections and be too fragile to transplant. Hence, cells committed to the neural lineage are commonly used when the goal is to produce functioning neural cells in vivo. Thus, when a specific phenotype is desired, such as dopaminergic neurons for Parkinson's disease, early postmitotic cells are normally used. Whereas in stroke, where multiple cell types are needed, as it was already said, multipotent cells may be appropriate. These variables, however, remain under investigation [23].

Pretreatment

It is important to notice that the most common fate of transplanted cells is cell death. Apart from the concern about potentially limited stem cells supply, having large numbers of dead cells in the graft may exacerbate stroke-related deficits [23,89]. Differentiation toward a neural lineage itself is also directly associated with cell death, especially in non-neurogenic regions, where neurotrophic signals are scarce. Consequently a range of methods have been developed both to block apoptotic programs and to prevent their initiation via provision of various neurotrophic compounds [23].

Genetic stability

For decades, fetal cells have been used in animal models of Parkinson's disease, ischemic brain injury, and several other pathologies. And quite a few human clinical trials have showed that in the case of dopamine cell transplants, grafts may survive and function for long periods of up to 14 years after transplantation [23,84]. Still, limited accessibility to fetal tissue, has raised the investigation for other sources of cells, including stem cells [23].

Classically stem cells are cultured for long periods *in vitro*, which results on many more cell divisions than it would be experienced by any normal cell in the body. Thus there is a greater likelihood of mistakes during DNA replication and cell division. Pluripotent cells, such as germ cells and ESCs, express high levels of telomerase that in theory allow unlimited proliferation. *In vivo*, the genetic payload of germ cells is maintained in pristine condition from generation to generation despite countless cell divisions accumulated over the millennia.

However, this is not true for ESCs which may congregate considerable karyotypical abnormalities after extended passaging *in vitro*. In adult stem cells, that express some telomerase, but not enough to prevent telomere shortening, the situation may even be of bigger concern. Thus, to facilitate self-renewing *in vitro*, while minimizing karyotypic modifications, some authors advise the use of cells that have been passaged a minimal number of times, using specific passaging techniques, or cells that have been immortalized via overexpression of proto-oncogenes such as Ras, or even direct overexpression of telomerase. Immortalized cell lines may present some advantageous growth and differentiation characteristics. For instance, certain immortalized NSC lines are capable of generating dopaminergic neurons (DA) neurons, whereas primary NSCs generally do not. However, the use of immortalized cells raises discussion. While some authors suggest that immortalized cell lines offer the best way to guarantee the use of well-characterized, stable cells, others believe that immortalization renders an unacceptably augmented risk for following malignant transformation. Attending to this concern, some investigators groups have generated conditionally immortalized cell lines that are immortal only in the presence of specific agents [23].

Cell types and transplantation

Clearly, the most important issue in cell transplantation would be the availability of an appropriate cell type with the potential to proliferate *in vivo*, and structurally and functionally integrate into the brain. As it has been repeated, it seems rational to use cellular elements immature and phenotypically plastic so as to differentiate into different cell types [96]. Up to now, several cell types have been studied as potential candidates for neural repair in ischemic stroke, some of which are discussed below.

Embryonic stem cells

These cells are considered to be the most plastic of all stem cell sources, combining the self-renewal capacity with the hypothetical ability to generate a whole-body variety of different cell types [23,74,76]. Thus this stem cell source has been postulated as ideal for cell replacement after stroke [23,76]. In fact, neuro-glial differentiation and electrophysiological evidence of synaptic integration has been obtained in vivo after human and mouse derived embryonic stem cells transplantation [23,76]. The setback of this potency is the formation of teratomas after ESCs transplantation in vivo. Thus one of the major challenges when using ESCs is the need of directing them towards a particular cell differentiation before transplantation, avoiding the presence of free undifferentiated cells within the graft [23,139]. Yet few methods are associated with the acquisition of homogeneous population destitute of undifferentiated cells with potential to develop teratoma [23].

Neural stem/progenitor cells

NSCs cells may be isolated from different parts of the central nervous system and, when in culture, they organize in clusters, named neuropheres, which are not homogeneous collections of cells, but instead are heterogeneous collection of true stem cells, committed progenitors and differentiated progeny. This diverges from the homogeouns aggregates of ESCs. Another distinctive aspect of NSCs, from ESCs, is the smaller variety of neurons they can generate. However, although they tend to adopt a forebrain profile, NSCs can be successfully differentiated into cortical projection neurons, interneurons, hippocampal pyramidal neurons and

also into astrocytes, oligodendroytes and perhaps even into endothelium. Thus these cells should be capable of replacing most cell types affected by stroke. Synaptic integration and action potential generation from these neurons have been shown *in vivo* [23].

Porcine fetal cells

In animal models of neurodegenerative diseases, transplanted fetal neurons have showed to survive, integrate and ameliorate functional deficits. However, although fetal human transplants have showed benefit to patients with Parkinson's disease, the limited availability of human fetal tissue and the ethical concerns around this subject, turned investigators attention to xenotransplantation of this kind of cells. Fetal porcine neurons are considered to be relatively safe [96,111]. In fact, transplantation of fetal cells from porcine lateral ganglionic eminence (LGE) (primordial striatum) has shown to improve deficits in animal models of Huntington's disease as well as in rat ischemic models [111]. On ischemic models, grafts differentiated into glia and neurons, there was evidence of synaptogenesis (both within the graft and within the host) and the transplanted animals showed functional improvement [111]. Nevertheless, when considering the use of this cells in humans, the risk of rejection of this foreign cells emerges as a concerning aspect [36,96,111]. Other worry is the potential transmission of porcine viruses, such as the porcine endogenous retrovirus. However this stills a matter of ongoing debate [36,111].

Immortalized human stem cell lines - Teratocarcinoma cells

Because of the ethical dilemmas concerning embryonic stem cell research and the limitations of xenotransplantation some groups investigated alternative cell sources, such as transformed cell lines [111]. The immortalized human neural stem cell line NT2N (also called hNT cells) is derived from NTerra-2/D1 teratocarcinoma cell line [74]. And it was originally (more than 20 years ago) derived from a human testicular tumor [41,111]. Differently from other teratocarcinoma cell lines, the NT2 cells show an exclusive commitment to a neural lineage upon exposure to retinoid acid, giving rise to a neuronal cell population virtually indistinguishable from terminally differentiated post-mitotic neurons [23,41]. These are the only cells that have been tested in human clinical trials for the treatment of stroke [23]. The potential for de-differentiation of these cells back to a malignant state, following transplantation is a major concern [41]. And thus, regarding their tumorigenic nature, teratocarcinoma cells will likely continue to raise questions of safety [23]. Besides, if on one hand predifferentiation of teratocarcinoma cells prior to transplantation gives rise to homogenously differentiated post-mitotic neuronal cells, on the other, mature transplanted neurons have a higher risk of cell death [23].

Bone marrow stromal cells

A wide diversity of cells can be obtained from the bone marrow, many of which have been suggested to have beneficial effects after transplantation into the ischemic brain [23]. In fact, when exposed to EGF or BDNF, both *in vitro* and *in vivo*, or when cultures with neural cells, human marrow stromal cells (hMSCs) differentiate into cells, that express neural precursor cells markers. These cell lines seem to be associated with several advantages: it is easy to obtain bone marrow cells as it is expanding them *in culture*; use of patient's own MSCs

would theoretically eliminate the risk of rejection. However, differentiation mechanism for these cells is poorly understood and whether these cells produce neuronal synaptic networks with plasticity or trophic factors alone is questionable [96]. The truth is substantial functional benefits have been observed after both intracranial and intravascular delivery [23].

Porcine fetal cells

In animal models of neurodegenerative diseases, transplanted fetal neurons have showed to survive, integrate and ameliorate functional deficits. However, although fetal human transplants have showed benefit to patients with Parkinson's disease, the limited availability of human fetal tissue and the ethical concerns around this subject, turned investigators attention to xenotransplantation of this kind of cells. Fetal porcine neurons are considered to be relatively safe [96,111]. In fact, transplantation of fetal cells from porcine lateral ganglionic eminence (LGE) (primordial striatum) has shown to improve deficits in animal models of Huntington's disease as well as in rat ischemic models [111]. On ischemic models, grafts differentiated into glia and neurons, there was evidence of synaptogenesis (both within the graft and within the host) and the transplanted animals showed functional improvement [111]. Nevertheless, when considering the use of this cells in humans, the risk of rejection of this foreign cells emerges as a concerning aspect [36,96,111]. Other worry is the potential transmission of porcine viruses, such as the porcine endogenous retrovirus. However this stills a matter of ongoing debate [36,111].

Other sources of stem cells suitable for cell therapy in stroke / Adipocite stem cells

Intracerebral implantation of stem cells derived from adipose tissue also showed significant recovery of the functional deficit [40]. It has been reported that both that human adipose tissue stromal cells (hATSCs) and hMSCs share many of the same adhesion and receptor molecules and have similar expression profiles of protein and mRNA. And some postulate that hATSCs may have similar potentials to hMSCs in tissue engineering and regenerative medicine [60]. Kang *et al*, isolated hATSCs from human liposuction tissues and induced neural differentiation with azacytidine. After induction, hATSCs revealed immunocytochemical characteristics of neural lineages. Labeled hATSCs were injected into the lateral ventricle of the rat brain, migrated to various parts of the brain, specially to the injured cortex, and showed to improve functional deficits in a MCAO ischemic brain model. Some of the engrafted cells expressed neural markers. Still these findings do not clearly support transdifferentiation of hATSCs into neurons *in vivo*, since the electrophysiological properties of the engrafted cells were not determined and one cannot exclude cell fusion, as it was demonstrated to happen *in vitro* between embryonic stem cells and adult stem cells, such as neural stem cells and MSCs. Yet, the cell fusion for itself is unlikely to not be enough to explain the percentage of neural marker expressing cells among engrafted cells observed in this experiment (about 10%) [60].

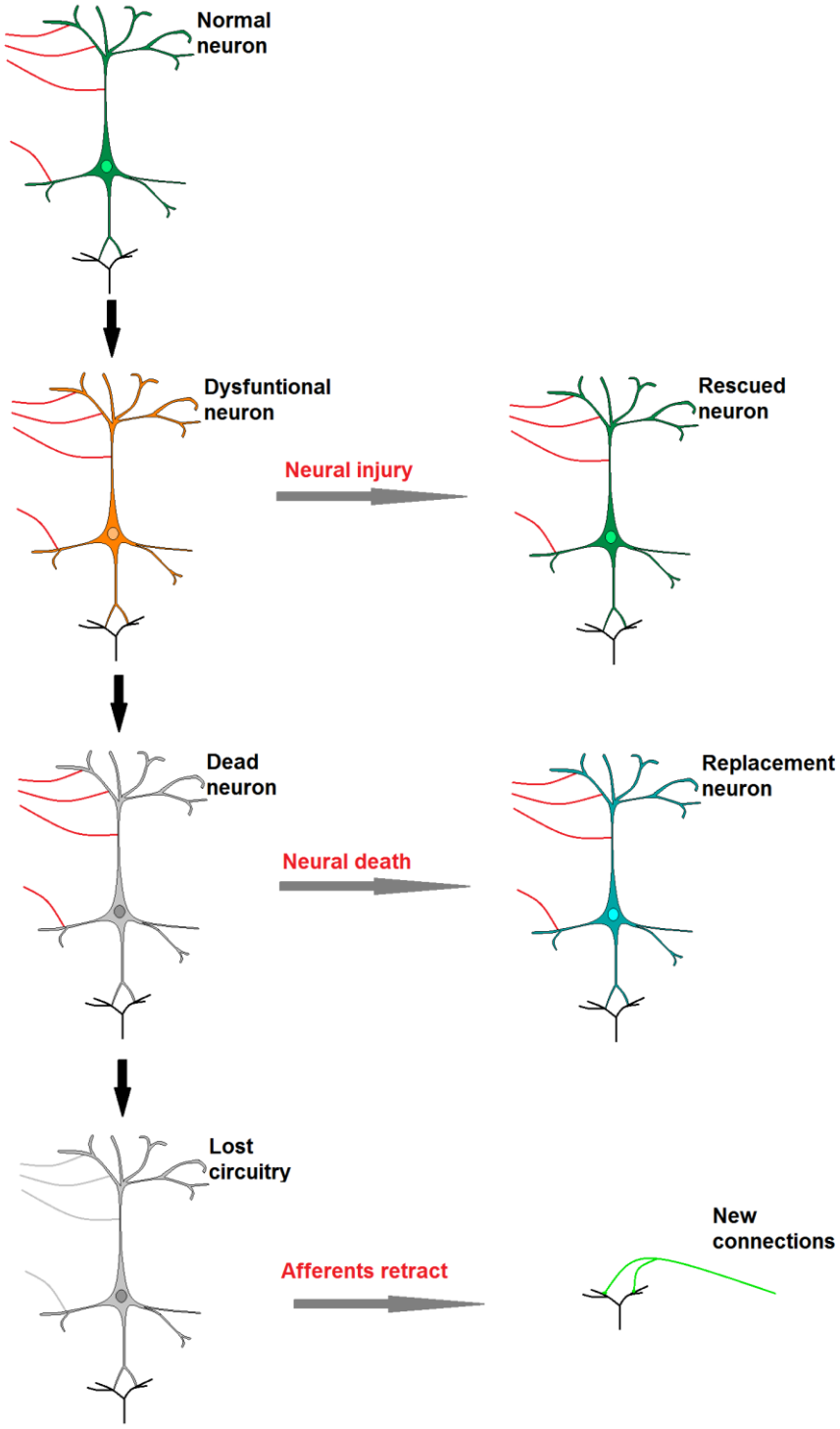


Figure 3 - Postulated mechanisms of exogenous stem cell action.

After brain injury, exogenous stem cells may use several means to restore function. Assuming neuroprotective roles, through modulating inflammation or secretion of neuroprotective compounds, exogenous cells may rescue dysfunctional neurons, preserving existing neural circuitry. If the host neuron dies, stem cells can, conceptually, be used to replace this lost neuron. Nevertheless, for that to effectively happen, it would be necessary the preservation of surrounding cytoarchitecture. Considering original circuits cannot be maintained due to retraction of afferent projections after the neuron dies, or due to cavitation of the injury site, stem cells may contribute in the generation of new circuits – synaptic plasticity, (directly via synaptic incorporation into the new circuitry, or indirectly by promoting the formation of new synaptic connection). Adapted from Burns, *et al*, 2009[31]

CELL TRANSPLANTATION FOR DELIVERY OF TROPHIC MOLECULES

Given the migratory potential of certain cell types towards areas of injury, stem cells may work as suitable vehicles for delivery of specific molecules that may be complicated to carry into the ischemic area via systemic administration at adequate concentration. Anti-inflammatory, proangiogenic and "prosurvival" molecules are some of the possible candidates for this *ex vivo* gene therapy. Molecules that promote endogenous neurogenesis may, as well, be of use. In truth, taking into account the small proportion of adult-born neurons that survive, the delivery of proangiogenic and prosurvival drugs may be a way of promoting the survival of migrating neuroblasts that find themselves at the infarct site. Many of the trophic effects demonstrated by neural and bone marrow-derived stem cells are poorly understood; thus the delivery of such stem cells in conjunction with specific gene delivery may offer additional benefit. Indeed, several studies seemed to borne out this supplementary benefit [23]. For instance, Chen *et al*, showed that the transplantation of MSCs with brain derived neurotrophic factor (BDNF) into the ischemic boundary zone of rat brain, facilitates MSCs survival and differentiation, and improves functional recovery after middle cerebral artery occlusion, emphasizing that bone marrow cells may be an useful vehicles for both cells and gene therapy for neurotransplantation [26]. Liu *et al*, conducted a study to test the hypothesis that placental growth factor (PIGF), a substance proven to be angiogenic to impaired non-neural tissue, contributes to the therapeutic benefits of hMSCs in cerebral ischemia. The efficacy of systemic delivery of hMSCs and hMSCs transfected with a fibre-mutante F/RGD adenoviral vectors carrying a human PlGF gene (PIGF-hMSCs) was compared in a permanent MCAO model. The results showed that both hMSCs and PIGF-hMSCs reduced lesion volume, induced angiogenesis and elicited functional improvement compared with the control sham group, with the effect being superior in the PIGF-hMSC group. Enzyme-linked immunosorbent assay of the infarcted hemisphere revealed an increase in PIGF in both hMSC groups, but a greater increase in the PIGF-hMSC group. Therefore, these data support the hypothesis that PIGF contributes to neuroprotection and angiogenesis in cerebral ischaemia, and cellular delivery of PIGF to the brain can be achieved by intravenous delivery of hMSCs [72]. On another similar study, hMSCs were transfected with the glia cell line-derived neurotrophic factor (GDNF) gene, once more using a fiber-mutant F/RGD adenovirus vector. The aim was to investigate whether GDNF gene-modified hMSCs (GDNF-hMSCs) could contribute to functional recovery in a rat permanent MCAO. MRI and behavioral analyses revealed that rats receiving GDNF-hMSCs or hMSCs intravenously presented increased recovery from ischemia compared with the control group, and, yet again the effect was greater in the GDNF-hMSC group [50]. It was, as well, demonstrated that intraventricular delivery of BDNF adenovirus-infected hATSCs results in adipose tissue-derived BDNF-expressing cells within the brain parenchyma and that hATSCs transduced with BDNF adenovirus increase the ability to improve the behavioral recovery. This results suggest that hATSCs similarly to MSCs might be useful vehicles for both cell and gene therapy [60].

CLINICAL TRIALS OF STEM CELLS TRANSPLANTATION FOR STROKE PATIENTS

Stem cell therapy in stroke patients is giving the first steps. So far, only a few clinical trials [13,65,67,102,109] evaluated the safety and the efficiency of different stem cells on stroke (see table 2).[1]

Table 2 - Synopsis clinical studies involving stem-cells after stroke

Author, year	Type of study	Patients (active / controls)	Type of stem cells	Stroke type	Time since stroke onset	Mode of delivery	Immuno Ssupression	Comments
Kondziolka *et al*, 2000[67]	Phase I trial	12/0	NT2N cells	Ischemic involving basal ganglia (8 cases) or basal ganglia and cerebral cortex (4 cases)	6 months to 6 years	Stereotactic transplantation into region of the stroke	Methylprednisolone during surgery, Cyclosporine 1 week prior to surgery and continued for 8 weeks	No effect on functional outcome, PET showed increased metabolic activity. No cell-related adverse effects 5 years after cell transplantation
Kondziolka *et al*, 2005[65]	Phase II randomized trial	14/4	NT2N cells	Ischemic (9 cases) or hemorrhagic (9 cases), envolving basal ganglia but no motor cortex	1 to 6 years	Stereotactic transplantation into region of the stroke	Methylprednisolone during surgery, Cyclosporine 1 week prior to surgery and continued for 6 months	No effect on functional outcome. No evidence of a significant benefit in motor function, but it indicate the safety and the feasibility of neuron transplantation
Savitz *et al*, 2005[109]	Phase I trial	5 (of 12 planned)/ 0	Fetal porcine cells (LGE)	Ischemic involving the striatum (middle cerebral artery infact)	1.5 to 10 years	Stereotactic transplantation into region of the stroke	None (cells pre-treated with anti-MHC antibody)	Study stopped early after 2 serious adverse events
Bang *et al*, 2005[13]	Phase I and II randomized trial	5/25	MSC+	Ischemic in middle cerebral artery territory	4-5 weeks and 7-9 weeks	Intravenous injection	None	Questionable study quality, i.e. 10 patients lost follow-up No cell-related adverse effects 1 year after cell transplantation
Rabinovich *et al*, 2005[102]	Case series	10	Human fetal cells	Ischemic (7 cases) or hemorrhagic (3 cases) involving the middle cerebral artery territory	14–24 moths	Subarachnoidal injection	_	Some patients developed fever and meningism 48 hours after transplantation

LGE – lateral ganglionic eminence; MHC – major histocompatibility complex; MSC – mesenchymal stem cells; NT2N – neuroteratocarcinoma neurons; PET – positron emission tomography

[1] Before presenting the following trials a disclosure has to be made. For Kondziolka *et al*, 2000, Kondziolka *et al*, 2005 and Savitz *et al*, 2005 no fulltext was available, despite several attempts to obtain them. Thus, the citation in the text refers to the data retrieved from the abstract of these articles, complemented with the articles where these studies are cited.

Neuroteratocarcinoma cell trials

Phase I clinical trial

In 2000, Kondziolka *et al* [67] (cited by England *et al,* 2009[36]; Kondziolka *et al*, 2008 [66]; Kondziolka *et al*, 2002 [68]; Locatelli *et al*, 2009 [76]; Padma, 2009 [96]), published a small phase I open-label trial approved two years before by the US Food and Drug Administration (FDA) that intended to study the safety and feasibility of human neuronal cellular transplantation.

Twelve patients, aged 44 to 75 years, that had experienced basal ganglia stroke and fixed motor deficits, 6 months to 6 years before (stable for at least 2 months), received one of two doses of neuroteratocarcinoma cells (NT2N), via CT-guided stereotactic-targeting of cell implants. Based on preclinical safety data, doses of 2 and 6 million cells were considered appropriated. Immunosuppression with methylprednisolone and cyclosporine was covered for 8 weeks. Serial evaluations (12 to 18 months) showed no adverse cell-related serologic or imaging-defined effects. Five years after the surgery no adverse events were reported related to the implant. Six of the eleven PET scans performed at 6 months, showed high uptake of fluorodeoxyglucose at the site of transplantation, suggesting either graft survival or inflammatory response. Even though this trial was not designed to evaluate efficacy (small size of the study and the absence of a control group), the total European Stroke Scale (ESS) score improved in six patients (3 to 10 points), with a mean improvement of 2.9 points in all patients (p=0.046) and in some patients it correlated with increased PET metabolic activity [36,67-68,76,96]. The ESS score increase tended to be larger in the group of four patients receiving six million cells, both in the total scores and in the composite motor subscale scores [68].

One patient died 27 months after the cell implantation of an unrelated cause (myocardial infarction), and an autopsy was performed. The histopathological study of the brain revealed a population of immunoreactive grafted cells with no signs of inflammation or neoplasia, suggesting the prolonged survival of the grafts, even in the absence of a continuous immunosuppressant regimen. This patient had experience no clinical improvement after transplantation [36,41,68,76,96].

Phase II clinical trial

Subsequently, in 2005 [65], the same researchers (cited by England *et al,* 2009 [36] and Locatelli *et al*, 2009 [76]) presented a phase II randomized open-label trial, using the same cell line. Eighteen patients with stable deficit (stable for at least 2 months), one to six years after ischemic or hemorrhagic basal ganglia stroke, were randomized to receive either 5 or 10 million implanted cells (7 patients per group) or to serve as non-surgical control group (4 patients), having only rehabilitation. All patients underwent stroke rehabilitation. One patient had a single seizure the day after surgery and another had a subdural hematoma evacuated 1 month after transplantation. However, no cell-associated adverse effects were observed. Transplanted patients showed a trend towards improvement in functional outcomes on several scales compared to baseline measurements before transplantation, but there were no statistically significant differences compared to the four controls [36,65,76].

Porcine cell trial

Phase I clinical trial

Savitz *et al*, in 2005 [109], as cited by (cited by England *et al,* 2009[36]; Locatelli *et al*, 2009 [76]; Padma, 2009 [96] and Wechsler, 2009 [127]) studied the safety and feasibility of fetal porcine stereotactic cell transplantation in 5 patients who had experienced basal ganglia infarct between 1.5 and 10 years before and had stable neurological deficits. In order to prevent rejection, cells were pretreated with an anti-major histocompatibility complex class I antibody, thus obviating the need for immunosuppression after transplantation. The study was prematurely terminated by FDA after two patients developed serious adverse effects. One patient had temporary worsening of motor deficits 3 weeks after transplantation, due to cortical vein occlusion, that resolved after 10 days. The other, one week after transplantation, developed seizures in the setting of hyperglycemia and a ring-enhancing lesion on MRI remote from the transplant site. The connection between these 2 complications and the transplants is uncertain. Two patients showed functional improvement (in speech, language, and/or motor impairments) over several months that persisted at 4 years [36,76,96,109,127].

Autologous mesenchymal stem cells

Phase I/II clinical trials

In 2005, Bang *et al* [13], presented a randomized controlled phase I/II trial of autologous mesenchymal stem cells (MSCs) transplantation in patients with cerebral infarcts within the middle cerebral arterial (MCA) territory and with severe neurological deficits. Thirty patients, within 7 days of stroke, were randomly allocated into one of two treatment groups: the MSC group (n=5), that received intravenous infusion of $1x10^8$ autologous MSCs and the control group (n=25) that did not receive any additional interventions. MSCs were acquired from bone marrow aspirates and then cultured *ex vivo* to achieve satisfactory quantities before re-injection into the patient 4-5 and then 7-9 weeks after symptom onset ($5x10^7$ cells each time). Neurological deficits and improvements in function were compared between the groups for a year after symptom onset. The Barthel index and the modified Rankin score identified a non-significant trend toward improved scores in patients treated with MSCs and NIHSS score changes were not substantial. With this article it was showed that it is feasible and perhaps safe to administer ex vivo–cultured autologous MSCs intravenously in patients with ischemic stroke [13,36,76,96]. The assumptions made by the authors in relation to the results have been a matter of critics [12,32,96].

Human fetal cells

Case series

Rabinovich *et al* (2005) transplanted human fetal cells (consisting of cells from immature nervous and hemotopoietic tissues) into the subarachnoidal space of 10 patients, 4 to 24 months after their ischemic or

hemorrhagic stroke of MCA territory. The control group was studied retrospectively and consisted of 11 patients comparable with the experimental group by the severity of cerebral involvement, the previous standard rehabilitative therapy received and duration of observation. Six months after transplantation, functional status of the patients was evaluated using Karnovskii score. According to the authors, the treatment significantly improved quality of life compared to the control group, but outcome measures were not clearly described. Some patients developed fever (up to 38.5ºC) and meningism during 48 h post-transplantation No serious complications of cell therapy were described [76,102].

Regarding for the small sample size and the lack of double-blinded controls of these initial clinical trials, the significance of the results is uncertain. These clinical trials are hardly comparable as a result of the differences in the target population, type of stem cells used, timing of injection and mode of delivery. However they point that stem cell therapy may be technically feasible in stroke patients. Nevertheless, safety should still be a matter of concern.

Currently, there are several planned/ongoing clinical trials (table 3) that will certainly expand the actual knowledge about the safety and feasibility of the different exogenous stem cell therapy and enlighten on the potential clinical and functional effects on stroke patients.

Table 3 - Planned clinical trials using stem cells in stroke patients

Upcoming studies	Clinical trial phase	Study design	Sample proposed	Primary Outcome measures	Secondary Outcome measures	Arms	Investigators	Study start date	Estimated study completion date
Safety of IV Autologous Mononuclear Cells and Marrow Stromal Cells After Stroke NCT00908856	II	Treatment, Randomized, Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor), Parallel Assignment, Safety Study	33	-death	-myocardial infarction -pulmonary embolism -ischemic stroke -deep venous thrombosis -other arterial or venous thrombosis -Infection requiring IV antibiotics	1. Placebo 2. Autologous mononuclear cells: a single intravenous autologous bone marrow mononuclear cell transfusion (4days after stroke onset) 3. Autologous marrow stromal cells: a single intravenous autologous marrow stromal cell transfusion (23days after stroke onset)	Steven C. Cramer, MD, MMSc University of California, Irvine	January 2010	December 2012
Cell Therapy by Intravenous Injection of Mesenchymal Stem Cells After Stroke NCT00875654	II	Treatment, Randomized, Open Label, Parallel Assignment, Safety Study	30	-Feasibility and tolerance of the iv injection of autologous MSCs in patients with carotid ischemic stroke	-Clinical and functional effects of the iv injection of MSCs in patients with carotid ischemic stroke -Determination of the most effective dose of stem cells -To define the best criteria for a future trial (phase III) -To define the best target population for a future study	1. No Intervention = Control group without intervention nor placebo 2. Experimental = First dose of stem cells 3. Experimental = Second dose of stem cells	Olivier Detante, MD University Hospital, Grenoble	January 2010	December 2013 (primary outcome 2010)
Phase II Study of Autologous Peripheral Blood CD34 Stem Cell Implantation in Chronic Stroke Patients NCT00950521	II	Treatment, Randomized, Open Label, Active Control, Parallel Assignment, Efficacy Study	30	-NIHSS	-ESS -EMS -Barthel index and MMSE -MRI and CT scans	1. PBSC Treatment: brain implant of autologous peripheral blood stem cell(CD34+) - 2-8 millions - plus convention stroke treatment that include rehabilitation and antiplatelet medication	Shinn-Zong Lin, MD, DMSci China Medical University Hospital	June 2009	December 2010

						2. Control: Active Comparator Control group receive conventional stroke treatment that include rehabilitation and antiplatelet medication			
Autologous Bone Marrow Stem Cells in Middle Cerebral Artery Acute Stroke Treatment NCT00761982	I/II	Treatment, Non-Randomized, Single Blind (Outcomes Assessor), Active Control, Parallel Assignment, Safety/Efficacy Study	20	-Absence of new neurological deficits and adverse effects during the timeframe.	-Improvement in clinical function as assessed by the MRS, BS and NIHSS stroke scale.	1. Intraarterial infusion of autologous bone marrow stem cells into middle cerebral artery	Jesús Hernández, MD Hospital Universitario Central de Asturias Hospitales Universitarios Virgen del Rocío	September 2008	March 2010 September 2009 (Final data collection date for primary outcome measure)
A Phase I/II Safety and Tolerability Study Following the Autologous Infusion of Immuno-Selected CD34+ Subset Bone Marrow Stem Cells Into Patients With Acute Total Anterior Circulation Ischaemic Stroke NCT00535197	I/II	Basic Science, Non-Randomized, Open Label, Single Group Assignment, Safety Study	10	-Safety will be evaluated in terms of adverse events graded according to CTC toxicity criteria and laboratory test results	-Improvement in clinical function as assessed by the MRS, and NIHSS.	1. Infusion of autologous CD34+ stem cells into middle cerebral artery	Nagy Habib, Professor Imperial College London U.K.	September 2007	June 2010 May 2010 (Final data collection date for primary outcome measure)
Safety/Feasibility of Autologous Mononuclear Bone Marrow Cells in Stroke Patients NCT00859014	I	Treatment, Open Label, Uncontrolled, Single Group Assignment, Safety/Efficacy Study	10	-Safety and feasibility of bone marrow mononuclear cell autologous (stem cell) transplantation in patients with acute stroke	-Functional outcome	1. Peripheral IV infusion of autologous stem cell	Sean I. Savitz, M.D University of Texas Heath Science Center-Houston	January 2009	January 2014
Phase I Study of Autologous Bone Marrow Cell Transplantation in Patient With	I	Treatment, Non-Randomized, Open Label, Historical Control, Single Group Assignment, Safety	15	-Absence of new neurological deficits during the procedure	- Improvement of neurological deficits - Improvement in the neuroimaging exams	1. Up to 10 patients receive up to $500x10^6$ autologous BMCs injected intra-arterially into the middle cerebral artery through	Charles André, Gabriel Freitas, Rosalia Mendez-Otero, Lea Mirian	December 2005	December 2010 July 2010 (Final data collection

Ischemic Stroke NCT00473057		Study		and/or in the 4 months follow-up.		percutaneous approach. Procedure is monitored by TCD and EEG. 2. Five patients receive up to 500x10^6 autologous BMCs injected intravenously	Fonseca, Federal University of Rio de Janeiro		date for primary outcome measure
Phase 1/2A Study of Intravenous Autologous Bone Marrow Mononuclear Cell Transplantation for Patients After Cerebral Embolism NCT01028794	I/II	Treatment, Non-Randomized, Open Label, Historical Control, Parallel Assignment, Safety/Efficacy Study	12	-Improvement of NIHSS -Frequency of change for the worse in NIHSS	-Mean level of MRS -Frequency of death	1. IV administration of autologous bone marrow derived mononuclear cells obtained from 25ml of bone marrow on day 7-10 after stroke (only once in that period) 2. IV administration of autologous bone marrow derived mononuclear cells obtained from 50ml of bone marrow on day 7-10 after stroke (only once in that period)	Akihiko Taguchi, MD.PhD Department of Cerebrovascular Disease, National Cardiovascular Center	May 2008	March 2012

IV – intravenous; MSC – mesenchymal stem cells; BMC – bone marrow cells; TCD – transcranial Doppler; EEG – electroencephalogram; NIHSS - National Institute of Health Stroke Scale; ESS – European stroke scale; EMS – European stroke motor subscale; MRS – modified Rankin Score; BS – Barthel Scale; MMSE - Mini-Mental State Examination; MRI - Magnetic resonance imaging; TC - computed tomography

Source: www.clinicaltrials.gov last visited on 14th February 2010

CASE STUDY

BACKGROUND

According to the World Health Organization (WHO), in 2008, Portugal had an estimated population of 10.7 million inhabitants, 17.1% of whom were older than 65 years old [128] (which represents a higher percentage, than the European Union average, 14.0%) [129]. The life expectancy at birth, in 2004, was 78.3 years (74.9 years for males and 81.6 years for females) continuing a long-term increasing trend, and the disability-adjusted life expectancy was 69.2 years (data from 2002, World Health Report) [128].

The major cause of death in Portugal is stroke [100], being responsible for almost twice more deaths then ischemic heart disease in 2004 [128]. Quoting Sá, 2009 [106]: *one can say that on a world scale stroke mortality rate ranges between 20 and 250 per 100 000 inhabitants per year, with Portugal closer to the superior limit*. From overall deaths in 2008 (104.280), 32.3% were caused by diseases of the circulatory system and 22.9% were related with cancer, these being the two leading mortality causes in Portugal [51].

In 2004 (last WHO available data), in Portugal, cerebrovascular diseases standardized death rate (SDR) per 100 000 inhabitants was 101.38 for all ages, 812.4 for people older than 65 years old and 13.5 among people younger than 65 years old. Therefore, Portuguese stroke mortality rate represents twice as much as that of neighboring Spain and three times that of France [128].

Data from 2008, provided by the Portuguese Health Ministry (*citied by* Sá, 2009)[106], shows a decrease on stroke related mortality among individuals younger than 65 years old (9.9 per 100 000). However, this value remains high when compared to other Western Europe countries, where stroke mortality rate before 65 years old is around 5 to 6 deaths per 100 000 inhabitants [106]. In truth, despite a decreasing tendency in stroke mortality in Portugal, it remains one of the highest in the world, only surpassed by China, Russia and some East European countries [37].

In several internal medicine departments, stroke is the most frequent cause of hospitalization and accounts for a high prevalence of irreversible patients' sequels (representing the cause of death or prolonged disability in 50% of those affected). Thus stroke embodies an enormous personal, social and economical burden, with stroke patients consuming a large proportion of the state health care budget (with around 27 billion *escudos* in 1997 in direct costs alone) [37].

Not many epidemiology studies were conducted in Portugal, and data concerning stroke resulting disability in Portugal is scarce.

In 2004, Correia *et al* [30], published a prospective study conducted to determine stroke incidence and case fatality in rural and urban population in Northern Portugal, during a two years period. The studied revealed a high stroke incidence in rural (3.05 per 1000) and urban (2.69 per 1000) Northern Portugal when compared to that reported in other Western Europe regions, substantiating that the high official stroke mortality in this country is associated with a high incidence [30]. High incidence of stroke (2.40 per 1000) was also reported in western central Portugal by Rodrigues [105].

Is currently accepted that treatment by multidisciplinary teams specialized in stroke improves patients outcome, with substantial decrease in acute phase mortality and neurological impairments, when compared to conventional treatment in the departments of internal medicine and/or neurology. Though no unifying definition of stroke unit is available, this term is usually applied to a designated area with specialized care, operation protocols, specifically assigned staff, educational programs, continuous training, research and permanent improvement in quality care [37]. So far, it seems that patient's management of patients within a stroke unit reduces mortality by about 20% and improves functional outcome by about the same amount [35].

In Portugal by July 2002, five stroke units were already operating, and by 2008 the number had rise to 28 stroke units. The number of patients admitted to stroke units in Portugal evolved from 3410, in 2007, to 6911, in 2008, 1159 through *Via Verde de AVC* - acute cerebrovascular accident green way - (in contrast to the 502 patients admitted through this system the year before). Stroke Unit of a University Hospital (S. João Hospital, EPE), one of the most representative stroke units in Northern Portugal, admitted 438 stroke patients in 2008, according to the Portuguese Health Ministry [5].

Given the impact of stroke and because stroke mortality is probably decreasing more rapidly than its incidence, the proportion of stroke survivors is likely to increase, so it is imperative to devise strategies to minimize the overall stroke burden.

Objective: This study was set to: analyze and compare the degree of disability of stroke patients admitted, during a quadrimester, to a stroke unit (generally considered to be the most effective acute stroke service), at two time points, the admission and 2 to 4 months within the stroke onset; seek for potential outcome differences between thrombolytic versus conservative therapy in ischemic stroke patients; and evaluate the degree of disability associated to hemorrhagic stroke and its evolution after the available intervention strategies. Based on this scenario background, it was also intended to appraise the clinical need of alternative therapeutic strategies, namely stem cell therapy.

METHODS

Study population and design

A retrospective, observational, single center study was performed using data from all patients admitted to the Stroke Unit of an University Hospital (S. João Hospital, EPE) located at Oporto, Portugal (estimated population of Oporto and metropolitan area: 1.7 million inhabitants [77]; Wikipedia), with a primary diagnosis of acute ischemic stroke or hemorrhagic stroke, from 1st September 2008 to 31st December 2008. For tracing this population it was used a Neurology Service database where all these diagnosed cases were identified. Then, data related with each patient was obtained from the Hospital database systems (SAM – that stands for "Sistema de Apoio ao Médico", that means Medical Support System – and ALERT®) where the information concerning the patients, namely the records from the admission at the Hospital [namely from the "Via Verde AVC" at the emergency room – acute cerebrovascular accident green way, an instituted protocol to deal with all patients with a suspected stroke), from the Stroke Unit and from the follow up at the outpatient clinic, is registered.

Main outcome measures

It was collected information on patient demographic characteristics (age and gender), known cardiovascular risk factors (such as diabetes mellitus, high blood pressure, smoke habits, dyslipidemia, atrial fibrillation), as well as, previous history of stroke.

The time at the onset of stroke symptoms, hospital arrive time, and, when applicable, door-to-needle time were retrieved.

The majority of ischemic stroke episodes were classified, at the admission, by the Oxfordshire Community Stroke Project (OCSP) classification [7,83] as: total anterior circulation infarct (TACI), partial anterior circulation infarct (PACI), lacunar infarct (LACI) or posterior circulation infarct (POCI). Ischemic stroke pathophysiology was accessed using TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification [1], which is based on clinical symptoms as well as results of further investigations; on this basis, a stroke was classified as being due to 1. thrombosis or embolism due to atherosclerosis of a large artery, 2. embolism of cardiac origin, 3. occlusion of a small blood vessel, 4. other determined cause, 5. undetermined cause (two possible causes, no cause identified, or incomplete investigation).

Hemorrhagic stroke was classified as subarachnoid hemorrhage or intracerebral hemorrhage [35].

In order to rate baseline stroke severity, as well as the subsequent evolution, the information based in two scales was collected (or, if possible inferred) from the admission registries and from the follow up records at 2 to 4 months after the stroke event. These scales were: the modified Ranking Scale (mRS), which measures functional dependence on a scale of 0 (no symptoms at all) to 6 (death) [44], and the National Institutes of Health Stroke Scale (NIHSS) [2], that quantifies the neurologic impairments, and ranges from 0 to 42, with higher values reflecting more severe cerebral infarcts (<5 mild impairment; ≥25, very severe neurologic impairment) [44].

The study sample was organized in two major groups: ischemic stroke patients and hemorrhagic stroke patients. The ischemic stroke patients group was subdivided in 4 groups, according to the hospital arrive time and type of treatment received, thrombolysis or conservative therapy: group 1 – patients admitted within the first 3 hours after stroke onset that were submitted to thrombolytic therapy; group 2 – patients admitted within the first 3 hours after stroke onset that were NOT submitted to thrombolytic therapy; group 3 – patients admitted within 3 to 4.5 hours after stroke onset that were NOT submitted to thrombolytic therapy; and group 4 – patients admitted after 4.5 hours after stroke onset that were NOT submitted to thrombolytic therapy.

Statistic analysis

The software used for statistic analysis was GraphPad Prism 4.0, 2003. Data was reported in frequency tables using standard descriptive statistics. Chi-square and Fisher's exact test were used to compare categorical variables; Student's *t* test was used to perform comparisons between pairs of continuous variables, multigroup continuous variables comparisons were performed using one-way analysis of variance (ANOVA) with Neuman-Keuls correction, and for multiple group nonparametric categorical variables comparison were used Kruskal-Wallis test and Dunn's post-test. Ninety-five percent confidence intervals were calculated for odds ratios and for relative risk. All variables included in the model were statistically significant at $p<0.05$.

Ethical considerations

The data collection protocol of the current study was submitted and approved by the University Hospital Ethics Committee and it was obtained consent from the Stroke Unit and the Neurology Service Directors, as well as from the Clinical Hospital Director and the Hospital Administrative Council. Data confidentiality and privacy was respected.

RESULTS

During the selected four-month period 136 stroke patients were admitted to the stroke unit. Of these, 105 (77.2%) have had an ischemic stroke and 31 (22.8%) a hemorrhagic stroke.

Fifty one ischemic stroke patients (48.6%) were admitted at the emergency department within 3 hours after stroke onset, meaning that they were admitted within the therapeutic window, up to that time approved for thrombolytic therapy (according to guidelines approved back then; 2008). Among them, only 34 (32.4% of the overall ischemic group, which represents 66.7% of those admitted within the therapeutical window) received treatment with intravenous alteplase, while a conservative approach was adopted for the remaining patients. Since the reason not to perform thrombolysis in this group of patients, admitted within the rt-PA therapeutic window, was not specified in their medical records, all the following criteria of exclusion for this treatment, showed in table 4, were searched on each patient's registered medical data. Table 5 shows the inferred reasons why these patients were not submitted to alteplase.

Table 4 - **Exclusion criteria for the treatment of acute ischemic stroke with recombinant tissue plasminogen activator (alteplase) within 3 hours of stroke onset [2]**

EXCLUSION CRITERIA
Historical
Stroke or head trauma in the previous 3 months
Any history of intracranial hemorrhage
Intracranial neoplasm
Major surgery in the previous 14 days
Gastrointestinal or urinary tract bleeding in the previous 21 days
Myocardial infarction in the previous 3 months
Arterial puncture at a noncompressible site in the previous 7 days
Clinical
Spontaneously clearing stroke symptoms or only minor and isolated neurologic signs
Seizure at the onset of stroke is an exclusion if the residual impairments are due to postictal phenomenon; seizure is not an exclusion if the clinician is convinced that residual impairments are due to stroke and not to post ictal phenomenon.
Symptoms of stroke suggestive of subarachnoid hemorrhage
Persistent blood pressure elevation (systolic ≥185 mmHg, diastolic ≥110 mmHg)
Active bleeding or acute trauma (fracture) on examination
Laboratorial
Platelets <100,000/mm3*
Serum glucose <50 mg/dL (<2.8 mmol/L)
International normalized ratio (INR) >1.7 if on oral anticoagulant*
Elevated partial thromboplastin time (aPTT) if on heparin*
Head CT scan
Evidence of hemorrhage
Evidence of a multilobar infarction with hypodensity involving >33 percent of the cerebral hemisphere

* Although it is desirable to know the results of these tests, thrombolytic therapy should not be delayed while results are pending unless (1) there is clinical suspicion of a bleeding abnormality or thrombocytopenia, (2) the patient has received heparin or warfarin, or (3) use of anticoagulants is not known.

Adapted from Adams *et al* 2007

Table 5 - Potential reasons not to perform thrombolysis on group 2 patients

Most likely thrombolysis exclusive criteria – group 2 (n=17)	
Minor or rapid improving neurological deficits	7
High blood pressure (refractory)	1
INR increased	1
Recent stroke (another one)	1
Unknown reason	7

More than half of the stroke ischemic patients (51.4%) arrived at the hospital over 180 minutes after stroke, thus late for a pharmacological therapy with rt-PA. Five of those patients (4.8%) were admitted 3 to 4.5 hours and the other 49 (46.7%) over 4.5 hours after stroke onset.

Hence patients were grouped according to the type of stroke and those with ischemic stroke according to hospital arrive time and treatment received (Fig.4).

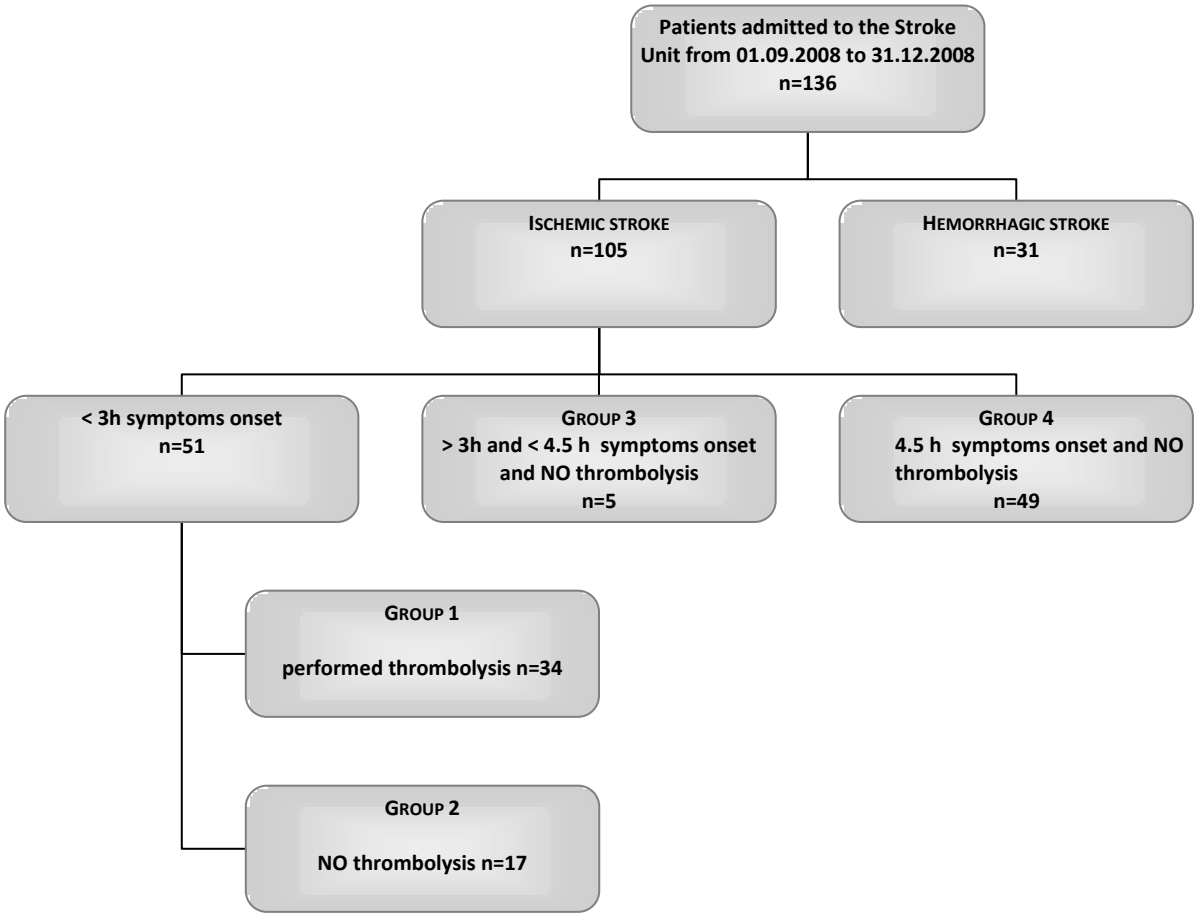


Figure 4 - Amount of patients admitted to the stroke unit between 1st September and 31st December 2008

Number of hemorrhagic and ischemic strokes and its distribution according to hospital time arrive. Group 1 – patients admitted within the first 3 hours after stroke onset that were submitted to thrombolytic therapy; group 2 – patients admitted within the first 3 hours after stroke onset that were NOT submitted to thrombolytic therapy; group 3 – patients admitted within 3 to 4.5 hours after stroke onset that were NOT submitted to thrombolytic therapy; and group 4 – patients admitted after 4.5 hours after stroke onset that were NOT submitted to thrombolytic therapy.

Demographic and clinical characteristics of ischemic and hemorrhagic stroke patients are shown in tables 3 and 4, respectively. Data concerning those characteristics was available for all patients.

Table 6 - Demographic and clinical characteristics of Ischemic Stroke Patients

Characteristic		Study group				P value
		Thrombolysis	NO thrombolysis			
		Group 1 < 3h symptoms onset and performed thrombolysis (n=34)	Group 2 < 3h symptoms onset and NO thrombolysis (n=17)	Group 3 3h and < 4.5 h symptoms onset and NO thrombolysis (n=5)	Group 4 4.5 h symptoms onset and NO thrombolysis (n=49)	
Demographic						
Age (years old)	**Mean ± SD**	70.4 ± 13.6	66.7 ± 16.1	69.8 ± 14.6	66.4 ± 11.9	0.57† (1)
	Minimum	38	39	45	38	
	Maximum	91	83	83	85	
Age by gender	**Male (n, mean ± SD)**	n=14 64.4 ± 14.0	n=9 63.7 ± 17.3	n=2 58.0 ± 18.4	n=28 64.5 ± 12.3	0.93† (1)
	Female (n, mean ± SD)	n=20 74.6 ± 11.8	n=8 70.0 ± 15.2	n=3 77.7 ± 4.7	n=21 69.0 ± 11.2	0.34† (1)
Gender	**Male n (%)**	14 (41.2)	9 (52.9)	2 (40.0)	28 (57.1)	
	Female n (%)	20 (58.8)	8 (47.1)	3 (60.0)	21 (42.9)	0.51‡
Medical history						
Previous history of stroke		4	3	3	6	1.00§
Atrial fibrillation		11	2	1	24	0.18§
Cardiovascular risk factor						
	Diabetes *mellitus*	5	7	0	14	0.10‡
	High blood pressure	23	10	4	31	0.81‡
	Tobacco	9	6	2	19	0.70‡
	Dyslipidemia	14	10	1	29	0.17‡
Number of cardiovascular risk factor per patient						
	0	3	1	0	3	_
	1	14	4	2	11	0.27‡
	2	12	8	1	23	0.51‡
	3	3	3	0	9	_
	4	1	1	0	3	_

† ANOVA with the Newman-keuls correction
‡ Chi-square test
§ *p* value refers to the comparison of group 1 and 4.

Table 7 - Demographic and clinical characteristics of Hemorrhagic Stroke Patients

Characteristic		Study group
		Hemorrhagic stroke (n=31)
Demographic		
Age (years old)	**Mean ± SD**	63.8±10.7
	Minimum	45
	Maximum	83
Age by gender	**Male (n, mean ± SD)**	n=14 64.4 ± 14.0
	Female (n, mean ± SD)	n=17 68.2 ± 10.6
Gender	**Male n (%)**	14 (45.2)
	Female n (%)	17 (54.8)
Medical history		
Previous history of stroke		7
Cardiovascular risk factor		
	Diabetes *mellitus* n (%)	7 (22.6)
	High blood pressure n (%)	24 (77.4)
	Tobacco n(%)	11 (35.5)
	Dyslipidemia n(%)	16 (51.6)
Number of cardiovascular risk factor per patient		
	0	1
	1	9
	2	15
	3	6
	4	0
Previous use of aspirin or antiplatelet drugs (n/%)		9 (29.0)
Previous use of anticoagulation drugs (n/%)		3 (9.7)

The average age of the sample was 66.98±12.79 (mean±SD) years and the gender distribution was homogeneous between groups (p=0.63; Chi-square test) (with women slightly outnumbering men, 50.7% and 49.3%, respectively). There were no significant differences, concerning ages, between patients of group 1, 2, 3 and 4 of ischemic stroke and those of hemorrhagic study group (ANOVA with the correction of Newman-Keuls, p=0.35). There were also no significant differences between ages (66.98±12.79, n=105 vs 63.84±10.74, n=31; Student's t test; p=0,22), proportions of female and male (Fisher's exact test; p=0.68) between ischemic and hemorrhagic stroke patients. The most prevalent cardiovascular risk factor among both ischemic and hemorrhagic stroke patients was hypertension, with analogous distribution on both groups (p=0.27, Fisher's exact test).

Most of pretreatment characteristics of the four ischemic stroke groups were similar (Table 6 and 8). The age of ischemic stroke onset was 67.9±13.2 (mean±SD) years with no significant differences between groups (ANOVA with the correction of Newman-Keuls, p=0.57). The groups were homogeneous with respect to gender distribution (p=0.51, Chi-square test), cardiovascular risk factors, previous history of stroke and presence of atrial fibrillation.

NIHSS score at admission was significantly different among ischemic stroke groups (p=0.0006, Kruskal-Wallis test with Dunn's correction). Therefore NIHSS score at the admission was significantly higher on group 1, when compared to that in groups 2 (p<0.01) and 4 (p<0.001).

As a matter of fact, overall stroke severity at presentation was moderate, with a median NIHSS score of 7 (table3). Furthermore, ischemic and hemorrhagic stroke severity at admission was quite similar, with a NIHSS median score of 7 for both stroke types.

The majority of patients (93.3%) had an estimated mRS score of 0 to 2, which indicates functional independence. The pre-existing disability distribution (defined as mRS score greater than 2 before stroke) was quite alike in all groups with no significant differences observed (table 8).

Table 8 - Baseline Characteristics of the patients

Characteristic		Study group						P value
		Ischemic Stroke				Hemorrhagic stroke (n=31)	All strokes (n=136)	
		Ischemic stroke: thrombolysis		Ischemic stroke: NO thrombolysis				
		Group 1 (n=34)	Group 2 (n=17)	Group 3 (n=5)	Group 4 (n=49)			
Independence before stroke (mRS 0–2)	N	33	15	4	47	28	127	
	%	84.8	88.2	80	95.9	90.3	93.3	1.00*
NIHSS score at admission	Mean±SD ±SEM	12.44±6.09 ±1.04	6.35±7.31 ±1.77	7.60±5.86 ±2.62	6.80±6.13 ±0.88	8.70±7.15† ±1.31	8.61±6.84† ±0.59	
	Median (IQR)	11.5 (11)	3 (12)	8 (16)	5 (9)	7 (10)†	7 (10)†	0.0006‡

* Chi-square test.
† NIHSS at the admission not available for one patient.
‡ Kruskal-Wallis test with Dunn's correction.

The scores on the modified Rankin scale indicate the following: 0, no symptoms at all; 1, no significant disability despite symptoms (able to carry out all usual duties and activities); 2, slight disability (unable to carry out all previous activities but able to look after own affairs without assistance); 3, moderate disability (requiring some help but able to walk without assistance); 4, moderately severe disability (unable to walk without assistance and unable to attend to own bodily needs without assistance); 5, severe disability (bedridden, incontinent, and requiring constant nursing care and attention); 6, death.

Table 9 - Classification of subtypes of acute Ischemic Strokes

Ischemic stroke subtypes	Study group				P value
	Group 1 (n=34)	Group 2[a] (n=17)	Group 3[b] (n=5)	Group 4[c] (n=49)	
Oxfordshire Community Stroke Project Classification (OCSP)[25] n(%)					
TACI – total anterior circulation infarcts	18 (52.94)	4 (26.67)	1 (25.00)	6 (13.04)	0.0007*†
LACI – partial anterior circulation infarcts	4 (11.76)	4 (26.67)	2 (50.00)	18 (39.13)	0.07†
PACI – lacunar circulation infarcts	10 (29.41)	3 (20.00)	1 (25.00)	8 (17.39)	0.53†
POCI – posterior circulation infarcts	2 (5.88)	4 (26.67)	0 (0)	14 (30.43)	_
TOAST *criteria (n/%)					
1. Large-artery atherosclerosis (embolus or thrombosis)	8 (23.53)	2 (11.76)	1 (20.00)	12 (24.49)	0.73†
2. Cardioembolism (high-risk or medium risk)	12 (35.29)	6 (35.29)	2 (40.00)	6 (12.24)	0.05†
3. Small-vessel occlusion (lacune)	2 (5.88)	2 (11.76)	1 (20.00)	9 (18.37)	_
4. Stroke of other determined cause	0 (0)	0 (0)	0 (0)	2 (4.08)[d]	_
5. Stroke of undetermined cause (two or more causes identified; negative evaluation; incomplete evaluation)	12 (35.29)	7 (41.18)	1 (20)	20 (40.82)	0.79†

a. absence of data of OCSP for two patients; b. absence of data of CCSP for one patient; c. absence of data of OCSP for three patients; d. One patient had a left carotid artery dissection (female, 41 years old) and the other had a left vertebral artery dissection (female, 70 years old).
* *p* statistically significant
† Chi-square test.
‡ TOAST – Trial of Org 10172 in acute stroke treatment

The ischemic stroke groups were different in stroke subtype according to OCSP classification, with a significant higher frequency of TACI related strokes on group 1 (p=0.0007, Chi-square test). A balanced distribution of all stroke subtypes on the remaining groups was observed. The pathophysiology of ischemic stroke accessed by TOAST classification showed a homogeneous group distribution with no significant differences between groups. In all four groups, there was a high proportion of strokes of undetermined cause. The great majority of patients included in this category had a negative evaluation (which means that it was impossible to discriminate the ethiopathogenesis despite the workup) and only one patient had two potential causes identified.

The large majority of hemorrhagic strokes were intracerebral haemorrhages (91.32%), with a small proportion of primary subarachnoid hemorrhages. In relation to hemorrhagic stroke etiology, as it was mentioned before, hypertension was the cause most often named responsible and there was a large proportion of cases where a cause could not be pointed out (table 10). All cases of cerebral aneurysms required an invasive approach: 3 of them underwent a craniotomy with aneurysm clipping (two of them of the left middle cerebral artery and one of the left anterior cerebral artery) and 1 was submitted to endovascular coiling of the cerebral aneurysm of one of the posterior inferior cerebellar arteries), the rest of hemorrhagic strokes had a conservative approach.

Table 10 - Hemorrhagic stroke subtypes and etiology

Hemorrhagic stroke n=31			
Cerebral hemorrhagy		**Subarachnoid hemorrhage** n(%)	4 (12.90)
		Intracerebral hemorrhage n(%)	27 (87.10)
Probable cause	**Subarachnoid hemorrhage**	**Cerebral aneurysm** n(%))	4 (100.00)
	Intracerebral hemorrhage	**Cerebral amyloidosis** n(%)	2 (7.41)
		Hypertension n(%)	13 (48.15)
		Undetermined n(%)	12 (44.44)

As shown in table 11, the median time from ischemic stroke onset to thrombolysis was 145.0 (IQR 65) minutes and the median door-to-treatment time was 61 (IQR 37) minutes. The patients' mean delay to hospital, among those admitted within 3 hours, was 92.38±44.85 minutes. (This data concerns only the patients who were access for both scores at follow up, n=48). However time elapsed between presumably stroke onset and hospital admission was significantly shorter for patients submitted to thrombolytic therapy (p=0.007, Student's t test).

Table 11 - Time since stroke onset until thrombolysis initiation and door-needle time

		Patients admitted within 3 hours after stroke onset		**P value**
		Group 1 (n=33)	**Group 2 (n=15)**	
Time elapsed between stroke onset and hospital admission	**Mean±SD ±SEM**	80.94±40.29 ±7.01	117.53±45.34 ±11.71	0.007*
	Median (IQR)	76 (42)	114 (69)	
Time since stroke onset until thrombolysis initiation	**Mean±SD ±SEM**	148.1±39.7 ±6.91	Not applicable	Not applicable
	Median (IQR)	145.0 (65)		
Door-needle time	**Mean±SD ±SEM**	67.15±34.31 ±5.97	Not applicable	Not applicable
	Median (IQR)	61 (37)		

* Student's t test.

Outcome measures (mRS and NIHSS scales) at 2 to 4 months were complete or possible to infer (from the medical records that described the functional independence of patients and the neurological impairments) to 68.6% (n=33) of stroke ischemic patients. Using the descriptive medical notes at the follow-up evaluation, NIHSS scale at 2-4 months was estimated, employing the same rationale of that proposed by Williams *et al* algorithm for retrospective NIHSS scoring [130]. For the 6 in-hospital case fatalities the last NIHSS registered (< 2 months) was used (except for one of the patients, which had no available NIHSS assessment apart from the admission). The other 25.7% patients did not return to the hospital for the three-month follow-up visit. For those, missing data were imputed using the principle of carrying the last score forward, so the last assessed mSR and NIHSS scores (prior to 2 months) were used, but for three of them it was only possible to retrieve information of one of the scales score.

The assessed overall mortality at 2 to 4 months was 4.41%. In fact, six patients who had experienced an ischemic stroke died while in the hospital, and hemorrhagic transformation was observed in 4 patients (8.82% in the group that received treatment with rt-PA and 2.04% in group 4; $p>0.05$, Chi-square test) (Table 12). Only one patient with a hemorrhagic transformation recovered to a level of functional independence, one of them became moderately disable (mRS score 3), another severely disabled (mRS score 5) and one last one died (mRS score 6).

Table 12 - Ischemic stroke adverse events

Serious adverse events	**Study group**			
	Group 1 (n=34)	**Group 2[a] (n=17)**	**Group 3[b] (n=5)**	**Group 4[c] (n=49)**
Hemorrhagic transformation n(%)	3 (8.82)	0 (0)	0 (0)	1 (2.04)
In-hospital case fatality n(%)	2(5.88)	2 (11.76)	0 (0)	2 (4.08)

At 2-4 months, a significant improvement of more than 8 points in the NIHSS scale was observed on patients that received thrombolysis (group 1), when compared to those admitted 4.5 hours after stroke onset (group 4; $p=0.002$, Fisher's exact test) (table 13). No significant decrease of more than 8 points in NHISS score was observed in patients submitted to thrombolytic therapy, as compared with those who did not receive alteplase within 3 and 3-4.5 hours after stroke onset.

However, when the analysis was restricted to more than 4-point decrease of NIHSS score, it was observed a significant neurologic improvement in patients submitted to alteplase, as compared with patients who received a conservative approach ($p=0.002$, chi-square test).

In truth, a more than 4-point improvement in NIHSS score at follow-up was significantly more frequent in patients that received thrombolytic therapy (group 1) when compared to those admitted within the same frame time (group 2; $p=0.01$, Fisher's exact test) and to those admitted after 4.5 hours (group 4; $p=0.002$, Fisher's exact test).

Furthermore there were no significant differences between the ischemic stroke groups in rates of functional independence (mRS score 0-2), or rates of excellent recovery/excellent functional outcome (defined as mRS score of 0 or 1) ($p>0.05$, Chi-square test) (table 13). Distribution of scores on the mRS scale are shown in table 13 and graphic 1.

Table 13 - NIHSS and mRS scores after 2-4 months of follow-up among the 4 groups of ischemic stroke patients

At follow-up	Study group				P value*
	Group 1 n=33[a] n(%)	Group 2 n=15[b] n(%)	Group 3 n=5 n(%)	Group 4 n=48[c] n(%)	
mRS score of 0 or 1[d]	10 (30.3%)	7 (46.7%)	2 (40.0%)	15 (31.3%)	0.68
mRS score of 0 – 2[e]	13 (39.4%)	10 (62.5%)	3 (60%)	25 (52.1%)	0.33
NIHSS score 0 or 1 or > 8 points improvement from baseline	12 (36.4%)	7 (46.7%)	3 (60.0%)	24 (50.0%)	0.59
NIHSS score > 4 points improvement from baseline	18 (54.5%)	2 (12.5%)	2 (40.0%)	9 (18.8%)	0.002
NIHSS score > 8 points improvement from baseline	6 (18.2%)	1 (6.7%)	1 (20.0%)	1 (1.3%)	_

a One patient was excluded because there was no NIHSS score record; b Two patients were excluded because there was no NIHSS score record for one of the patients and there was no mRS score record to another; c One patient excluded because there was no mRS score record; d Excellent recovery.; e Functional independence.

* Chi-square test

Table 9 - NIHSS and mRS scores after 2-4 months of follow-up among group 1 and 2 of ischemic stroke patients

	Study groups		Odds ratio (95% CI)	P value*
	Group 1 Thrombolysis n=33[a] n (%)	Group 2 No thrombolysis n=15[b] n (%)		
mRS score of 0 or 1	10 (30.3%)	7 (46.7%)	0.5 (0.1-1.7)	0.34
mRS score of 0 – 2	13 (39.4%)	10 (62.5%)	0.3 (0.1-1.1)	0.12
NIHSS score 0 or 1 or > 8 points improvement from baseline	12 (36.4%)	7 (46.7%)	0.7 (1.2-2.3)	0.54
NIHSS score > 4 points improvement from baseline	18 (54.5%)	2 (12.5%)	7.8 (1.5-40.2)	0.01
NIHSS score > 8 points improvement from baseline	6 (18.2%)	1 (6.7%)	3.1 (0.3- 28.4)	0.41

* Fisher's exact test

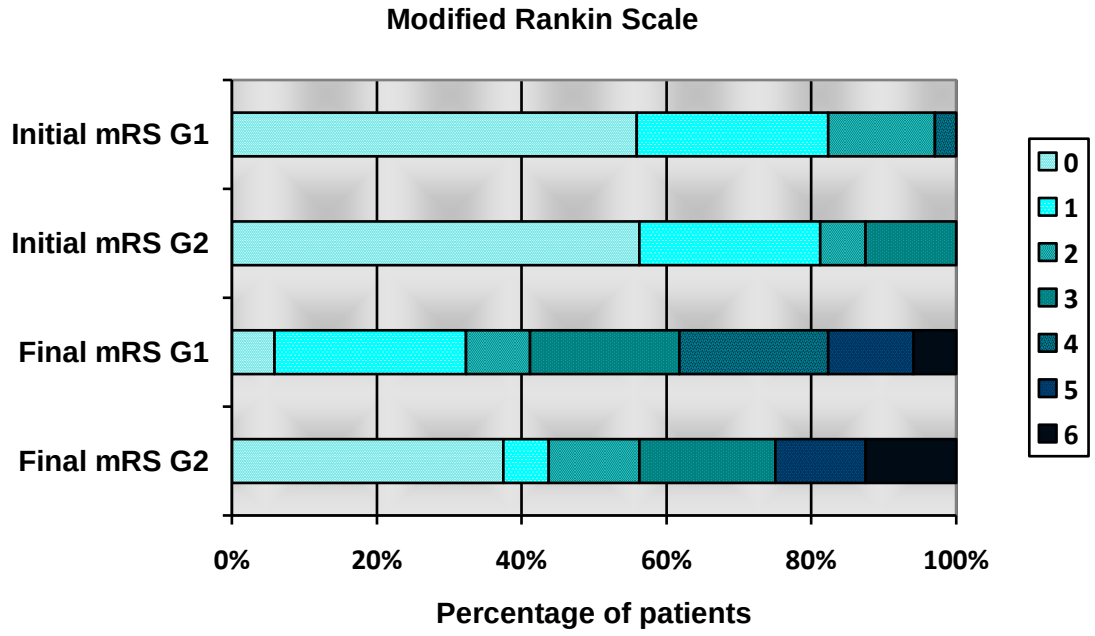
a One patient was excluded because there was no NIHSS score record.

b Two patients were excluded because there was no NIHSS score record for one of the patients and there was no mRS score record to another.

Table 14 - Distribution of scores on the Modified Rankin Scale.

Modified Rankin Scale		0	1	2	3	4	5	6
Group 1 (n=34)	**Initial G1** (n/%)	19(55.88)	9(26.47)	5(14.71)	0(0)	1(2.94)	0(0)	0(0)
	Final G1 (n/%)	2(5.88)	9(26.47)	3(8.82)	7(20.59)	7(20.59)	4(11.76)	2(5.88)
Group 2 (n=16)[a]	**Initial G2** (n/%)	9(56.25)	4(25.00)	1(6.25)	2(12.50)	0(0)	0(0)	0(0)
	Final G2 (n/%)	6(37.50)	1(6.25)	2(12.50)	3(18.75)	0(0)	2(12.50)	2(12.50)

a Missing data from one patient.



Graphic 1 - Distribution of scores on the Modified Rankin Scale.
The distribution of scores is shown for group 1 (patients admitted to the hospital within 3 hours after stroke onset that were submitted to thrombolysis) at admission (initial mRS G1) and 2 to 4 months after stroke onset (final mRS G1) and also for group 2 (patients admitted to the hospital within 3 hours after stroke onset that were submitted to a conservative treatment) at admission (initial mRS G1) and 2 to 4 months after stroke onset (final mRS G2). The scores on the modified Rankin scale indicate the following: 0, no symptoms at all; 1, no significant disability despite symptoms (able to carry out all usual duties and activities); 2, slight disability (unable to carry out all previous activities but able to look after own affairs without assistance); 3, moderate disability (requiring some help but able to walk without assistance); 4, moderately severe disability (unable to walk without assistance and unable to attend to own bodily needs without assistance); 5, severe disability (bedridden, incontinent, and requiring constant nursing care and attention); 6, death.

With respect to proportion of patients whose neurological status accessed by NIHSS scale improved (NIHSS score reduction), there were no significant differences between patients admitted to the hospital within 3 hours that received thrombolysis (group 1) and the ones who did not (group 2), as it may be seen in table 15. The NIHSS mean score reduction was 6.96±0.87 (mean±SEM) for group 1 and 3.73±1.60 (mean±SEM) for group 2, as graphically represented (graph 2).

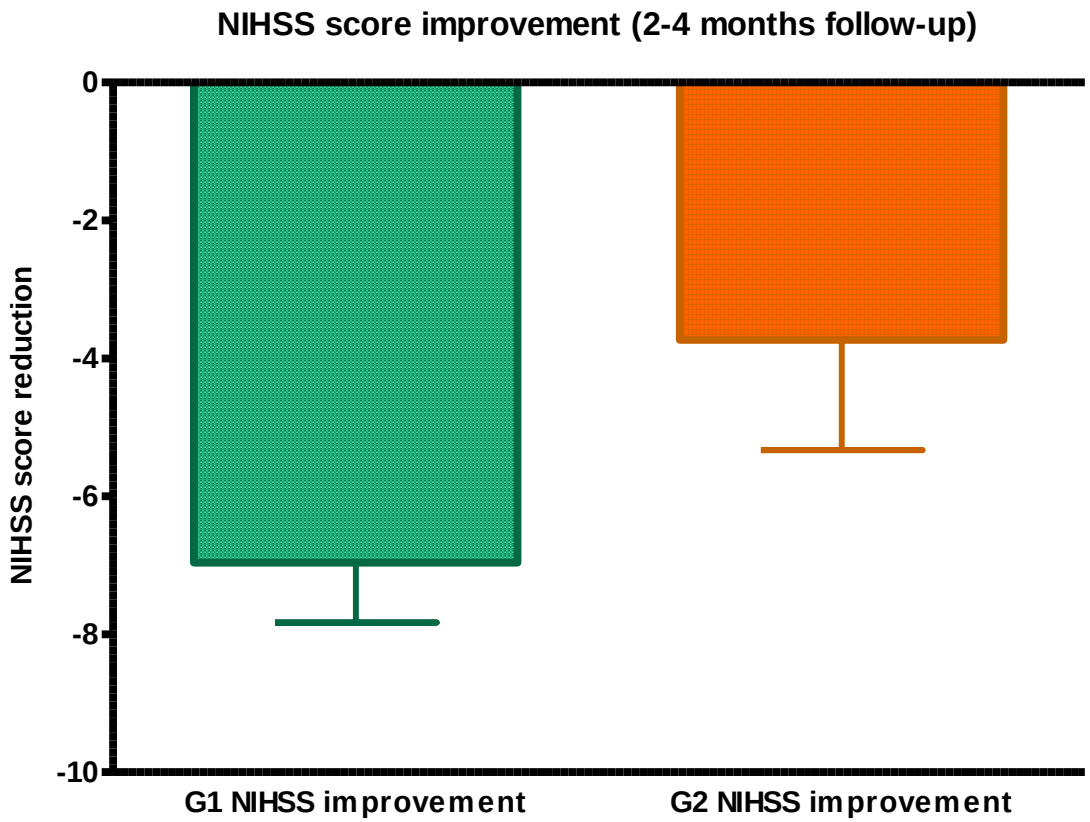
Table 15 - NIHSS variation at 2-4 months follow-up.

NIHSS score at 2-4 months follow-up	Group 1 (n=33)[a]	Group 2 (n=15)[b]	P value*
Reduction (Improved neurologic status)	27	11	0.70
Increase (Decline in neurologic status)	5	2	1.00
Same score as in admission	1	2	0.23

a. Unavailable data for one patient.

b. Unavailable data for two patients.

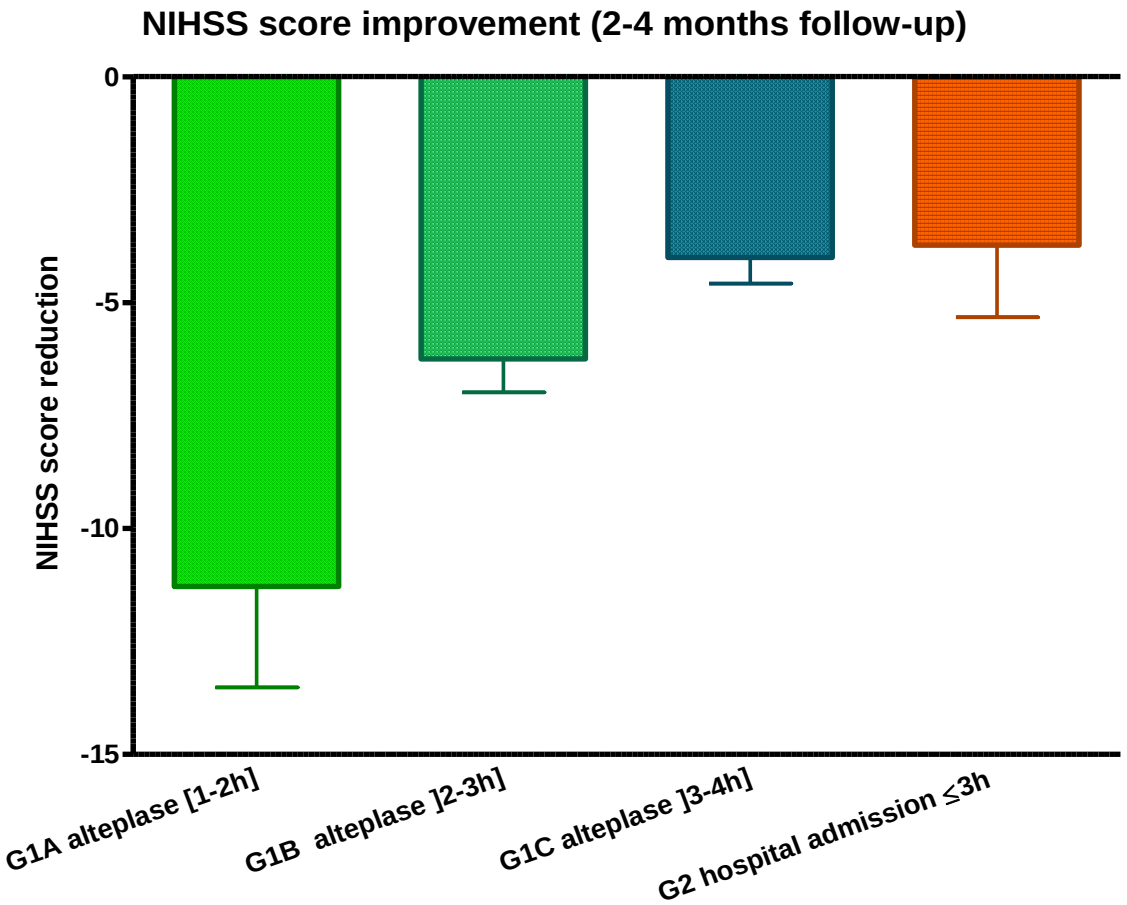
* Fisher's exact test.



Graphic 2 - Mean±SEM NIHSS reduction/improvement at 2-4 months follow-up among patients who were admitted within 3 hours after stroke onset at the hospital.

In order to access the potential advantage of thrombolysis in the study sample a sub-analysis was done. Although all patients who received thrombolysis (group 1) were admitted to the hospital within the first 3 hours after stroke onset, not all of them received rt-PA on this time frame. In fact, for six patients the administration of alteplase started beyond 180 minutes, ranging from 185 to 223 minutes.

So group 1 was divided in 3 subgroups: group 1A – submitted to rt-PA 1-2 hours after stroke onset; group 1B – submitted to rt-PA between 2-3 hours after stroke onset; and group 1C – submitted to rt-PA 3-4 hours after stroke onset, and NIHSS score improvement at 2-4 months follow-up compared with group 2 patients (graphic 3). Using Kruskal-Wallis test with Dunn's correction it was possible to observe that the overall medians of NIHSS score improvement varied significantly (p<0.05). Thus, thrombolysis performed 1 to 2 hours (group 1A), or 2 to 3 hours (group 1B) after stroke onset significantly improved the patients neurological outcome accessed by NHISS scale when compared with patients not submitted to this treatment (group 2) (p<0.05). Besides the administration of alteplase between 185 and 223 minutes (group 1C) showed no significant difference in NIHSS score improvement in relation to the patients with a conservative approach (group 2) or with those treated with alteplase on the first 3 hours.



Graphic 3 - Mean±SEM NIHSS improvement at 2-4 months follow-up among patients who were admitted within 3 hours after stroke onset at the hospital and had a reduction in NIHSS score at the follow-up.

DISCUSSION

First of all, it is important to notice that the sample of this study only include patients admitted to the stroke unit, therefore may not be representative of the stroke patient population admitted at the whole Hospital.

The mean age of stroke onset (around 67 years) among the patients admitted at the stroke unit was similar to that reported in other studies (namely clinical trials [21,43] and stroke unit reports [24]).

Risk factors for stroke can be roughly classified as modifiable or fixed. The modifiable ones, for instance: hypertension, diabetes and smoking, are common and influence patients health in several ways. Atrial fibrillation and transient ischemic attack are less prevalent and more specific risk factors [35]. The frequency of atrial fibrillation among this study ischemic stroke patients was 36.2%.

Differently from ischemic heart disease that in more than 90% is explained by identifiable risk factors, in stroke, risk factors that have been identified explain only about the 60% of the attributable risk. Thus investigation seems to be needed in order to find the risk factors (namely genetic) that may account for the 40% gap [35].

In this stroke unit-based study, hypertension stood out as the most prevalent risk factor for both ischemic (64.8%) and hemorrhagic strokes (77.4%), with no significant differences among these stroke subtypes, but with a slight trend to a bigger proportion in the last group. However it is important to highlight that no information was retrieved on whether the blood pressure was or not controlled or even treated. Several studies among the years have been corroborating the association between stroke and hypertension [8] and some have stated that this gradient of association is steeper between blood pressure and hemorrhagic stroke [117]. A population-based case-control study, that aimed to estimate the proportion of strokes occurring among treated hypertensive patients that could be attributed to uncontrolled blood pressure, after adjustment for potential confounders, found that uncontrolled blood pressure was moderately associated with ischemic stroke (n=460); risk ratio=1.5 [95% CI, 1.2 to 1.9] and strongly related to hemorrhagic stroke (n=95); risk ratio=3.0 [95% CI, 1.7 to 5.4] and estimated that 27% of the ischemic strokes and 57% of the hemorrhagic strokes among treated patients were attributable to uncontrolled blood pressure [62].

In truth, arterial hypertension is a highly common problem in Portugal affecting around 40% of adults (2004) [79]. According to recent data from Portuguese Health Ministry, around 2 million Portuguese are hypertensive, but only half are aware of this condition, around ¼ is medicated and in no more than 16% the blood pressure is controlled [45]. Thus hypertension is insufficiently diagnosed and treated and so Portugal is still the European Union country with the highest mortality rate due to stroke [38].

The proportion of hemorrhagic strokes in this selected population was higher than is usually reported. According to the literature, of all strokes, around 87% of strokes are ischemic, 10% are intracerebral hemorrhage and 3% are subarachnoid hemorrhage strokes. In this study, however, there were 10% less ischemic strokes (77.2%), at the expense of a greater percentage of hemorrhage strokes (22.8%). Thus, although the proportion of subarachnoid hemorrhage in this study sample, 2.9%, overlaps the usually reported (3%), an unexpected elevated percentage of intracerebral hemorrhage of 19.9% was observed.

It is described that intracerebral hemorrhage is more than twice as common as subarachnoid hemorrhage [20]. Yet, in this study it is almost 7 folds (6.8) more frequent, which represents a substantial difference.

Besides, it was possible to retrieve the cause of all subarachnoid hemorrhages in this study, once they were all due to cerebral aneurysms. This occurrence is not surprising considering the small size of the sample and that the rupture of saccular aneurysms is the most common cause of this stroke subtype [35].

In contrast, the etiology of intracerebral hemorrhage was impossible to determine in an important percentage of patients (44.4%). Advancing age and hypertension are the most important risk factors. In fact, the pathophysiological change in small arteries and arterioles due to sustained hypertension is generally regarded as the most important risk factor for intracerebral hemorrhage. Cerebral amyloid angiopathy is also increasingly recognized as a cause of lobar intracerebral hemorrhage in the eldery [20]. These two mentioned causes were the only ones identified, with a high proportion of cases considered as resulting of hypertension (48.2%) and a small percentage as being result of cerebral amyloidosis (7.41%). Other causes include vascular malformations, ruptured aneurysms, coagulation disorders, use of anticoagulants and thrombolytic agents, hemorrhage into a cerebral infarct, bleeding into brain tumors, and drug abuse [20].

In this study the known hemorrhagic transformations were considered as ischemic strokes that had a complication. Thus, unless the stroke only gave symptoms after the hemorrhagic transformation, this was not a direct cause of an increased number of intracerebral hemorrhage. In relation to the use of anticoagulants and thrombolytic agents, this feature was only assessed to the patients with a hemorrhagic stroke, therefore no comparison group exists. However among the patients with an undetermined cause of hemorrhagic stroke, two had INR above the normal limit (INR 2.63 and INR 1.81), which might have contributed. No other potential correlated causes were found on patients records.

Taken as whole, considering the high prevalence of hypertension in Portugal and the tremendous influence attributed to this risk factor in the etiopathology of it in intracerebral hemorrhage, it seems plausible to think that hypertension may be the disguised cause behind this higher proportion of intracerebral hemorrhage cases.

The widespread sentence “time is brain” draws the attention to the rapid and irretrievable loss of human nervous tissue as stroke progresses and emphasizes the emergency of acute therapeutic intervention. The quantitative estimation of the pace of neural circuitry loss in human ischemic stroke is astonishing. According to Saver (2006) [108], in a patient suffering a “typical” large vessel acute ischemic stroke, each hour without treatment represents lost of something like 120 million neurons, 830 billion synapses, and 714km of myelinated fibres, which means that each minute of treatment delay represents the destruction of 1.9 million neurons, 14 billion synapses and 12 km of myelinated fibres. This is the equivalent of saying that, compared with the normal rate of neuron loss in brain aging, the ischemic brain ages 3.6 years each hour without treatment [108].

Less than half of the ischemic stroke patients 48.6%, in this study, were admitted at the hospital within 3 hours. Among them, the mean pre-hospital delay was around 90 minutes (n=51). Therefore the majority of ischemic patients arrived beyond the therapeutical rt-PA window recommended at the time. Thus, five patients (around 5%) were admitted between 3 and 4.5 hours, and for this small group the pre-hospital mean-delay was 222 minutes and for the other 46.7% that arrived past 4.5 hours it was impossible to gauge the delay.

Curiously, patients admitted within 3 hours that received thrombolysis, when compared with the ones admitted at the same frame time that did not, had both a significantly higher NIHSS score at admission and a significantly shorter symptom-to-door time. So, apparently the most severe patients (rated by NIHSS scale) had a shorter pre-hospital delay. But this finding finds support in the literature. In fact, studies have reported a correlation between increasing stroke severity and shorter pre-hospital delay [43,82]. Besides it has been observed an association between stroke severity (and number of stroke symptoms) and reduced time from symptom onset to first call for medical help and transport by the emergency management of stroke [82]. This brings up the importance of educating patients to recognize stroke symptoms and to seek immediate medical attention whatever their initial clinical severity, because of the significant potential risk of early and marked progression of mild/moderate stroke symptoms [82].

In relation to door-to-needle time the NINDS recommendations published in 1996 [92] advocate 60 minutes from the door to drug administration and here, the median door-to-treatment time was 61 (IQR 37) minutes, thus close the goal proposed.

Another interesting finding in this study results was that group 1 patients (hospital arrival within 3h of stroke onset) besides being the group with the most severe disability at admission classified by the NIHSS scale, was also the group with a higher frequency of TACI, which is consistent with the literature. Baseline clinical stroke syndrome classification (OCSP classification), by which stroke can be classified into four clinical syndromes, is usually associated with distinct natural histories. TACI is typically associated with greatest severity and worse outcome, PACI with the highest risk of recurrence, whilst LACI syndrome has the mildest severity and POCI the most favourable outcome [118]. Accordingly, it is not surprising that the group with higher mean NIHSS score at admission is also the one with higher prevalence of TACI.

Usually TACI is associated with cardioembolism and also with large-artery atherosclerosis stroke. Here, if we exclude the undetermined cause, it can be seen that the cardioembolism and large-artery atherosclerosis are the most prevalent causes pointed out in group 1 [80].

In this population there were a high percentage of strokes of undetermined cause, as classified by TOAST. Overall around 38% (n=40) of ischemic stroke patients were classified under “undetermined cause”. This percentage is similar to that obtained on a study based on National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) Stroke Data Bank where among 1273 ischemic strokes, 40% (n=508) where labelled as infarcts of undetermined cause [107].

In relation to the functional independence at follow-up, defined as 0-2 score in the mRS, there were no significant differences among groups (thrombolysis *vs* no thrombolysis).

When neurological improvement at follow-up was considered as a reduction of more than 8 points at NIHSS scale a significant difference was only observed between group 1 (hospital admission within 3 hours and thrombolytic therapy) and 4 (hospital arrive time larger than 4.5 hours) (p=0.02, Fisher’s exact test), and no differences where found between group 1 and 2 (patients admitted within 3 hours that did not receive thrombolysis).

When considering neurological improvement as a four-point reduction of NIHSS score, significant differences where noticed between patients submitted to thrombolysis and the ones who weren't. Thus there a significant reduction in group 1 as compared to group 2 (p=0.01, Fisher's exact test) and 4 (p=0.002, Fisher's exact test). No statistically significant difference was observed between group 1 and 3, which is probably related with the small number of patients in this last group.

Comparing the potential effect of thrombolysis at different timings it seems clearly the early therapy maximizes benefit. In fact, alteplase administered between 60-120 and 120-180 minutes significantly reduces de NIHSS score as compared as patients admitted within 3 hours after stroke onset that received a conservative approach.

Patients submitted to rt-PA between 3 and 4 hours did not seem to benefit or have any perceptible detriment. However the number of patients (n=4) in this circumstances represents a small sample for us to infer any conclusions. However, latest publications in this field have been showed a modest but significant improvement in the clinical outcomes in patients with acute ischemic stroke of intravenous alteplase administered between 3 and 4.5 hours [44,126].

One can speculate that repercussion of thrombolytic therapy might have been compromised by the higher stroke severity among the group who received it.

It was observed a trend to a slightly increased hemorrhagic transformation in thrombolytic group, but it was not statistically significant (groups 1 vs 4 p=0.3, Fisher's exact test), what might be related with the small size of the sample. Clinical trials have been showing an association between thrombolytic treatment in patients with acute ischemic stroke and increased risk of intracranial hemorrhage [44].

Other study limitations

First of all the present results must be interpreted within the context of a retrospective study with its inherent limitations.

Indeed, several limitations have been pointed out while discussing the results and some other will be highlighted here. One has to do with the necessity to estimate NIHSS score at 2-4 months of follow-up based on the medical record information. As a matter of fact, it has been described validity of retrospectively scored NIHSS [130], showing that NIHSS scored from patients' history and physical examination records is almost identical to the NIHSS score obtained prospectively by a stroke team physician [71]. However, some of the follow-up records were pauci-descriptive. If, in general, the admission and discharge records at the stroke unit were quite homogeneous and well structured, alluding to a large broad of critical information, the content of the medical records at the follow-up was quite divergent among physicians. Thus the same limitation applies to the estimation of mRS score at follow-up.

Another critique worth mentioning is the considerable follow-up loss at 2-4 months, and the subsequent need of using the last assessed mRS and NIHSS, with potential bias, since the outcome of these patients refers to an earlier stage.

In relation to stroke related mortality it was only possible to assess in-hospital case fatality incidence, being impossible to retrieve information on patients that missed the follow-up visit (that were assumed to maintain the same neurological and functional condition).

The motives why patients were not eligible for treatment with intravenous rt-PA had to be presumed, because exclusion criteria were not available for the majority of patients admitted within the therapeutic window approved at the time.

Another aspect worth evaluate, that could have interfere with the patients outcome at follow-up, would have been the amount and type of physiotherapy (if any), speech, occupational therapy or other rehabilitation interventions.

CONCLUSION

Stroke represents a devastating disease leaving behind a trail of death and marked long-term disability in adults.

Portugal arises in this scenario as an aging country, with high rate of hypertension and where the leading cause of death is stroke. This burden insinuates to become heavier as the elderly segment of the population steeply grows and the proportion of stroke survivors increases. So it seems of paramount importance to devise strategies that might minimize stroke consequences.

First, given the enormous apparent impact of stroke in Portugal, it seems extremely relevant to invest on retrieving epidemiological information precious to a better insight of potential areas of improvement, from stroke primary prevention, to treatment and rehabilitation.

This stroke unit's retrospective study showed some interesting results. Thus, judging from this case study, although there is a clear prevalence of ischemic strokes, the intracerebral hemorrhagic stroke assumes higher prevalence than usually, as compared to the international reports, which might be a reflex of the high prevalence of untreated and uncontrolled hypertension, among the Portuguese population.

According to this study results, thrombolytic therapy within 3 hours significantly improves the neurological impairments measured by NIHSS scale. However it does not seem to have repercussion on patient's functional independence.

Therefore, apart from optimizing the current therapeutical tools, new therapeutic strategies for stroke should be considered.

Stem cells with their potential for generating a wide spectrum of new functional cell types associated with the capacity to actively respond to their environment, travel to injured areas and secrete neuroprotective compounds, represent a potential therapy in acute stroke and at latter time points when the there is no longer effective treatment to offer to the patient.

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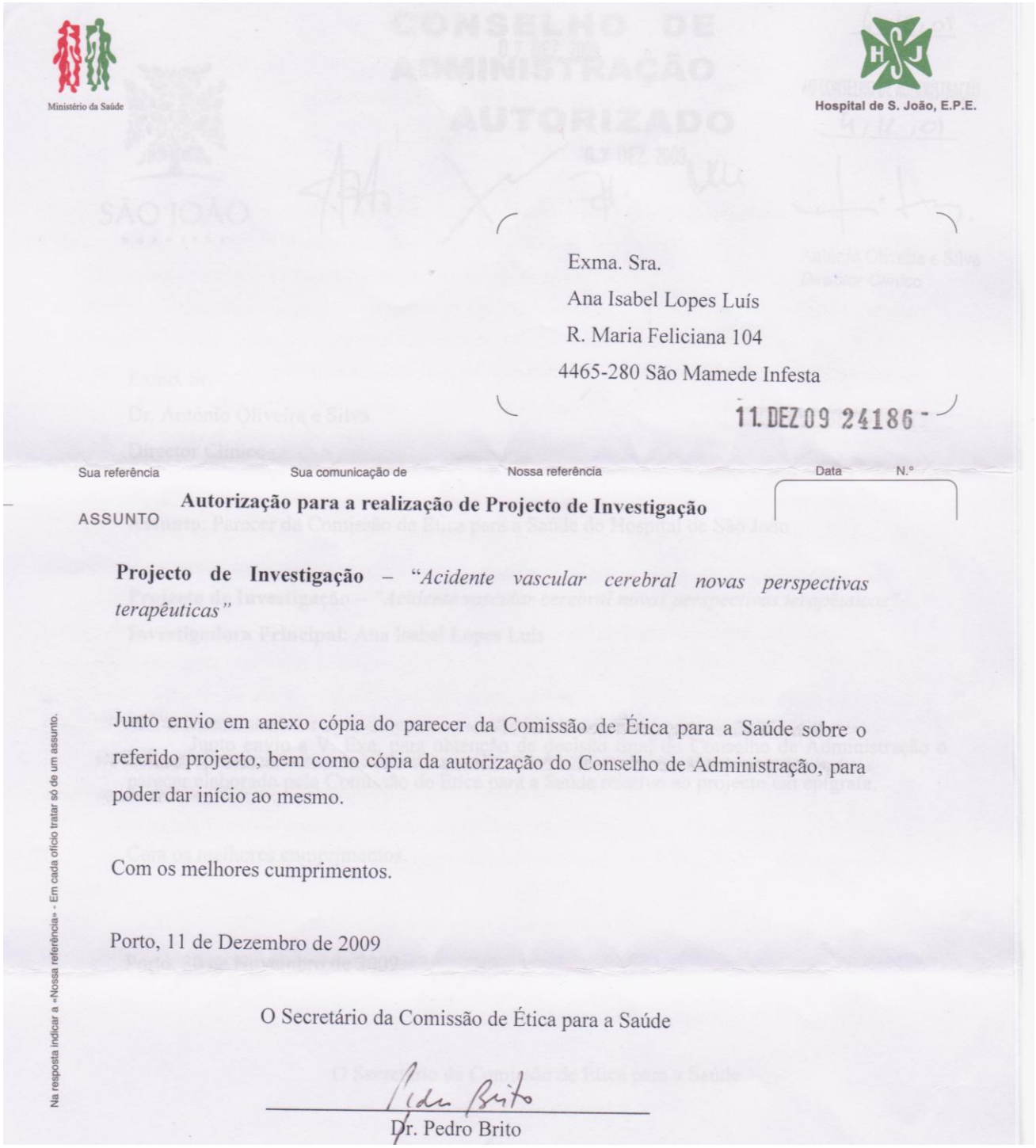
ANNEX - CONSENT BY THE UNIVERSITY HOSPITAL ETHICS COMMITTEE



Ministério da Saúde

Hospital de S. João, E.P.E.

Exma. Sra.
Ana Isabel Lopes Luís
R. Maria Feliciana 104
4465-280 São Mamede Infesta

11 DEZ 09 24186

Sua referência	Sua comunicação de	Nossa referência	Data	N.º

ASSUNTO **Autorização para a realização de Projecto de Investigação**

Projecto de Investigação – "*Acidente vascular cerebral novas perspectivas terapêuticas*"

Junto envio em anexo cópia do parecer da Comissão de Ética para a Saúde sobre o referido projecto, bem como cópia da autorização do Conselho de Administração, para poder dar início ao mesmo.

Com os melhores cumprimentos.

Porto, 11 de Dezembro de 2009

O Secretário da Comissão de Ética para a Saúde

Dr. Pedro Brito

Na resposta indicar a «Nossa referência» - Em cada ofício tratar só de um assunto.

À Direcção Clínica
4/12/09

CONSELHO DE ADMINISTRAÇÃO
07 DEZ. 2009
AUTORIZADO
07 DEZ. 2009

AO CONSELHO DE ADMINISTRAÇÃO
4/12/09

SÃO JOÃO
HOSPITAL

António Oliveira e Silva
Director Clínico

Exmo. Sr.
Dr. António Oliveira e Silva
Director Clínico

Assunto: Parecer da Comissão de Ética para a Saúde do Hospital de São João

Projecto de Investigação – *"Acidente vascular cerebral novas perspectivas terapêuticas"*
Investigadora Principal: Ana Isabel Lopes Luís

Junto envio a V. Exa. para obtenção de decisão final do Conselho de Administração o parecer elaborado pela Comissão de Ética para a Saúde relativo ao projecto em epígrafe.

Com os melhores cumprimentos.

Porto, 30 de Novembro de 2009

O Secretário da Comissão de Ética para a Saúde

Dr. Pedro Brito

Hospital São João
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Cont. nº 507645367
Capital Estatutário 112.000.000,00 Euros

Comissão de Ética para a Saúde do HSJ

Parecer

Projecto de investigação intitulado "Acidente Vascular Cerebral – novas perspectivas terapêuticas. Uma revisão a propósito de casos admitidos na unidade de Acidente Vascular Cerebral do Hospital de S. João".

Estudo que se propõe vir ser desenvolvido na Unidade de Acidente Vascular Cerebral do Hospital de S. João EPE pela estudante finalista do curso de Medicina da FMUP, Ana Isabel Lopes Luís, no âmbito da realização do Mestrado Integrado de Medicina da FMUP, sob a orientação do Professor Manuel Joaquim Vaz da Silva.

Do ponto de vista científico trata-se de um estudo retrospectivo e observacional em que a investigadora se propõe recolher os dados relativos aos doentes admitidos na Unidade de Acidente Vascular Cerebral no primeiro semestre de 2009, com o objectivo de analisar o nível de incapacidade na admissão, e compará-lo com o que se verificar 2 a 4 meses depois; comparar as alterações laboratoriais e imagiológicas nos dois períodos e correlacioná-las com os níveis de incapacidade, entre grupos que receberam e não receberam trombólise; avaliar o grau de incapacidade associado ao AVC hemorrágico e a sua evolução e avaliar a necessidade de terapêuticas alternativas no AVC designadamente terapias celulares.

Está previsto o acesso à informação contida nos processos clínicos dos doentes, através do elo de ligação, que será o Professor Manuel Joaquim Vaz da Silva. Está igualmente assegurada a utilização da ficha clínica avaliável.

Não está prevista a realização de questionários.

Dada a natureza do estudo, não estão previstos riscos, incómodos ou benefícios imediatos para os doentes incluídos. Não está prevista a obtenção de consentimento informado e não existe promotor do estudo.

A investigadora dispõe da competência científica para a realização do estudo que está autorizado pela Drª Goreti Moreira, responsável pela Unidade de AVC do Hospital de S. João EPE.

Os resultados obtidos serão apenas utilizados para fins académicos.

Não está prevista qualquer forma de retribuição a participantes e a natureza do estudo não impõe a necessidade de seguro.

Em face da análise do protocolo, proponho a sua aprovação pela CES do HSJ.

Porto, 26 de Novembro de 2009

O relator

Prof. Manuel Pestana

CES

COMISSÃO DE ÉTICA PARA A SAÚDE

8. TERMO DE RESPONSABILIDADE

Eu, abaixo-assinado, Ana Isabel Lopes Luís, na qualidade de Investigador Principal, declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (com as emendas de Tóquio 1975, Veneza 1983, Hong-Kong 1989, Somerset West 1996 e Edimburgo 2000) e da Organização Mundial da Saúde, no que se refere à experimentação que envolve seres humanos.

Porto, 15 / Novembro / 2009

Ana Isabel Lopes Luís

O Investigador Principal

PARECER DA COMISSÃO DE ÉTICA PARA A SAÚDE DO HOSPITAL DE S. JOÃO

emitido na reunião plenária da CES de ___/___/___

A Comissão de Ética para a Saúde APROVA por unanimidade o parecer do Relator, pelo que nada tem a opor à realização deste projecto de investigação.

Prof. Doutor Filipe A...
Presidente da Comissão de Ética