SCUOLA DI DOTTORATO
UNIVERSITÀ DEGLI STUDI DI MILANO-BICOCCA

School of Medicine and Surgery

PhD program in NEUROSCIENCE Cycle XXXV

Curriculum in EXPERIMENTAL NEUROSCIENCE

Multicentre translational Trial of Remote Ischaemic Conditioning in acute ischaemic Stroke (TRICS)

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ACADEMIC YEAR 2021/2022

INDEX

LIST OF FIGURES 7
LIST OF TABLES 9
ABBREVIATIONS 10
ABSTRACT 15
1. INTRODUCTION 16
1.1 A brief history of stroke 16
1.2 Epidemiology 16
1.3 Stroke risk factors 18
Non-modifiable risks factors 18
Modifiable risks factors 19
1.4 Prevention 21
Primary prevention 21
Secondary prevention 22
1.5 Classification and aetiology of stroke 23
Ischaemic stroke 24
Haemorrhagic stroke 25
1.6 Pathophysiology of stroke 26
Collateral blood flow 27
Ischaemic cascade and reperfusion injury 27
1.7 Diagnosis of ischaemic stroke 31
1.8 Current therapies 32
1.9 Experimental models for ischaemic stroke 33
In vitro models 33
In vivo models 36
1.10 The gaps in stroke research 41
1.11 Quality and improvement of preclinical studies 42
General principles of preclinical studies design 42
Best prioritization practice in stroke research 44
Multicentric studies 46
1.12 Remote ischaemic conditioning 47
RIC in acute ischaemic stroke 47
Trials in AIS 49
Putative mechanism and effects of RIC 50

2. AIM OF THE STUDY 54
1) Harmonization phase 54
2) Interventional phase 54
3. MATERIALS AND METHODS 55
3.1 Structure of the TRICS network 55
3.2 Animals 56
3.3 Experimental design 57
3.4 Surgery protocol 57
Transient middle cerebral artery occlusion (tMCAo) 57
3.5 Inclusion and exclusion criteria 59
3.6 Interventions 61
3.7 Health monitoring 61
3.8 Behavioural testing 62
3.9 SPECIFIC METHODS IN THE HARMONIZATION PHASE 65
3.9.1 Training and evaluation of neurological deficits 65
3.9.2 Primary outcome 65
3.9.3 Experimental groups 66
3.9.4 Statistics 67
3.10 SPECIFIC METHODS IN THE INTERVENTIONAL PHASE 70
3.10.1 Outcomes 72
Primary outcome 72
Secondary outcome 72
3.10.2 Experimental groups 73
Sample size 73
3.10.3 Blinding and randomization 74
Sequence generation for randomization 75
Animal replacement 75
5. RESULTS 76
5.1 The interrater agreement on a total score range of the De Simoni neuroscore 80
5.2 The interrater agreement on the dichotomised score of De Simoni Neuroscore 81
5.3 Systematic errors during the execution of the De Simoni neuroscore in the first trial 83
5.4 The second trial of the harmonisation phase 85
5.5 The interrater agreement on the total score range of the De Simoni neuroscore in the second trial 87
5.6 The interrater agreement on the dichotomised score of De Simoni Neuroscore 88

5.7 Intra-rater score correlations between the two trials in mice and rats 91
6. DISCUSSION 94
7. CONCLUSIONS 99
8. BIBLIOGRAPHY 100
PUBLICATIONS AND SCIENTIFIC COMMUNICATIONS 119
ACKNOWLEDGEMENTS 122

LIST OF FIGURES

Figure 1.1 The Global distribution of ischaemic stroke incidence rates per 100000 people by stroke type and country, for both sexes, 2019

Figure 1.2. Proportion of DALYs attributable to risk factors by pathological type of stroke for both sexes combined

Figure 1.3 Illustration of stroke subtypes

Figure 1.4 Space-time progression of ischaemic brain damage

Figure 1.5 The complex series of mechanisms induced by cerebral ischaemia

Figure 1.6 Overview of models of cerebral ischaemia

Figure 1.7 Comparison of human stroke and experimental models of stroke in rodents

Figure 1.8 Proposed mediators involved in the two waves of protection following remote ischaemic conditioning (RIC)

Figure 3.1 TRICS network structure

Figure 3.2 Scheme of the vessel architecture in a mouse from a ventral view

Figure 3.3 Evaluation of intra-ischaemic deficits for the animals' inclusion

Figure 3.4 Health report adapted from the IMPROVE guidelines

Figure 3.5 General deficits of De Simoni neuroscore

Figure 3.6 Focal deficits of De Simoni neuroscore

Figure 3.7. Experimental plan of the interventional phase

Figure 3.8 Interface for data input by the surgeon

Figure 3.9 Interface for data input by the designed rater

Figure 5.1 Explanation of the general deficits of De Simoni neuroscore by tutorial video

Figure 5.2 Explanation of the focal deficits of De Simoni neuroscore by tutorial video

Figure 5.3 Experimental plan of the training phase

Figure 5.4 Experimental plan of the harmonization phase

Figure 5.5 Interrater agreement analysis on a total score range of De Simoni neuroscore in the first trial

Figure 5.6 The interrater reliability calculated on pairs of raters using the Cohen's κ coefficient. The correlation between scores from pairs of raters.

Figure 5.7 Typical animal handling errors during the De Simoni neuroscore first trial

Figure 5.8 Interrater agreement analysis on a total score range of De Simoni neuroscore in the second trial

Figure 5.9 Improved interrater agreement after the second trial

Figure 5.10 Intra-rater agreement

LIST OF TABLES

Table 1.1 The aetiology of stroke determines the strategy for the prevention of recurrent stroke

Table 1.2 Focus on differences and similarities between the experimental stroke models and humans

Table 3.1 Experimental groups of the harmonization phase

Table 3.2 Experimental groups of the TRICS preclinical trial

Table 5.1 Fleiss κ on the dichotomised score of De Simoni neuroscore

Table 5.2 Video randomization for mice and rats

Table 5.3 Video replaced in the second trial and randomization for both models

Table 5.4 Fleiss κ on the dichotomised score of De Simoni neuroscore

Table 5.5 Correlation of rater's trial 1 vs. trial 2 score on the same animals

Table 5.6 Update of the status of the TRICS BASIC project

ABBREVIATIONS

µBBB: microfluidic BBB

AF: atrial fibrillation

AHA-ASA: American Heart Association-American Stroke Association

AIS: acute ischaemic stroke

AP: alternative pathway

BBB: blood brain barrier

BCEC: brain capillary endothelial cells

BMI: body mass index

CCA: common carotid artery

CCS: Causative Classification of Stroke system

COX-2: cyclooxygenase

CP: classical pathway

CT: computed tomography

DALY: disability-adjusted life-years

DAMP: damage-associated molecular patterns

DAWN: DWI or CTP Assessment with Clinical Mismatch in the Triage of Wake-Up and Late Presenting Strokes Undergoing Neurointervention with Trevo

DEFUSE 3: Endovascular Therapy Following Imaging Evaluation for Ischemic Stroke 3

DIV-BBB: dynamic in vitro BBB

ECA: external carotid artery

ECASS: European Cooperative Acute Stroke Study

ECASS-ExTEND: European Cooperative Acute Stroke Study-4-Extending the time for thrombolysis in emergency neurological deficits

ECM: extracellular matrix

EDA: Experimental Design Assistant

EPO: erythropoietin

Erk/Akt: extracellular-signal regulated kinase/protein kinase B

ESO: european stroke organisation

ET: endothelin

EtOH: ethanol

EVT: endovascular treatment

EXTEND: Extending the Time for Thrombolysis in Emergency Neurological Deficits

EXTEND-IA-TNK: Tenecteplase versus Alteplase before Endovascular Therapy for Ischemic Stroke

FAo: femoral artery occlusion

FDA: food and drug administration

GOX/CAT: glucose oxidase and catalase system

HSP: heat shock protein

ICA: internal carotid artery

ICC: intraclass correlation coefficient

ICH: intracerebral haemorrhage

IL: interleukin

IP3: inositol trisphosphate

iPSCs: induced pluripotent stem cells

ISO: italian stroke organisation

ITT: intention-to-treat

IVT: intravenous thrombolysis

LP: lectin pathway

MBL: mannose-binding lectin

MCA: middle cerebral artery

mGluR: metabotropic glutamate receptors

MMPs: matrix metalloproteinases

mNIHSS: modified NIH Stroke Scale

MRI: magnetic resonance imaging

mRS: modified Rankin Scale

NC3Rs: national centre for the Replacement Refinement and Reduction of animals in research

NETs: neutrophil extracellular traps

NIHSS: National Institutes of Health Stroke Scale

NINDS: National Institute of Neurological Disorders and Stroke

NMDAR: anti-N-methyl-D-aspartate receptor

NO: Nitric oxide

NOS: Nitric oxide synthases

NVU: neurovascular unit

OFR: oxygen free radicals

OGD: oxygen-glucose deprivation

OR: odds ratio

PAMP: pathogen-associated molecular patterns

pRCTs: preclinical randomised controlled trial

PROTECT: PROphylaxis of Thromboembolic Events by Certoparin Trial

REGARDS: REasons for Geographic And Racial Differences in Stroke

REMOTE-CAT: REMOTE Ischemic Perconditioning Among Acute Ischemic Stroke Patients in CATalonia

REPOST: Remote Ischemic Conditioning in Patients With Acute Stroke

RESIST: REmote iSchemic conditioning In patients with acute Stroke

REVISE-2: A Proof-of-Concept Study Assessing the Safety and Efficacy of Remote Ischemic Conditioning for Acute Ischemic Stroke Patients Undergoing Endovascular Treatment

RIC: remote ischaemic conditioning

RICAMIS: Remote Ischemic Conditioning for Acute Moderate Ischemic Stroke (RICAMIS)

RICEPAC: Remote Ischaemic Conditioning in Endovascular Recanalization for Proximal Anterior Circulation Occlusion Study

ROS: Reactive Oxygen Species

RS: rankin scale

rtPA-RIC: Intravenous Rt-PA Thrombolysis Combined With Remote Ischemic Post-Conditioning for Acute Ischemic Stroke Patients

SAH: subarachnoid haemorrhage

SERIC AIS: Safety and Efficacy of Remote Ischemic Conditioning in Patients With Acute Ischemic Stroke

SPAN: Stroke Preclinical Assessment Network

STAIR: Stroke Therapy Academic Industry Roundtable

SWOP: second window of protection

TIA: transient ischemic attack

tMCAo: transient occlusion of MCA

TNF: Tumor Necrosis Factor

TOAST: Trial of Org 101072 in Acute Stroke Treatment

tPA: tissue plasminogen activator

WHO: World Health Organization

WT: wild-type

ABSTRACT

In view of fostering transferability of pre-clinical data on the efficacy of remote ischemic conditioning (RIC) in acute ischemic stroke, we designed two multi-centre translational trials in mice and rats of both sexes. We defined to model ischaemic stroke by the transient occlusion of the middle cerebral artery (tMCAo). The improvement of sensorimotor deficits at 48h after tMCAo in RIC-treated animals was defined as the primary outcome. This work presents the harmonization phase relative to the evaluation of sensorimotor deficits by De Simoni neuroscore. Each centre performed different tMCAo durations - 30, 45, 60 min - allowing sufficient variability in the outcome. Animals were monitored post-surgery according to the ARRIVE and IMPROVE guidelines and data was registered into an electronic case report form on RedCap. All animals were video recorded during the neuroscore and the videos (n=11 per species) were distributed and evaluated blindly by raters at all centres.

The study aimed at reaching an intraclass correlation coefficient (ICC)≥ 0.60 as satisfactory interrater agreement.

After a first remote training we obtained moderate agreement for mice (ICC=0.50 [0.22-0.77]) and rats (ICC=0.49 [0.21-0.77]). Errors were identified in animal handling and test execution. We thus performed a second training followed by a new blind evaluation replacing the videos with poor experimental execution. The interrater agreement improved for mice (ICC=0.64 [0.37-0.85]) and rats (ICC=0.69 [0.44-0.88]).

In conclusion, two dedicated training on the neuroscore allowed us to reach the agreement target for both species and thus next proceed with the interventional phase of the project.

1. INTRODUCTION

1.1 A brief history of stroke

The word 'stroke' was likely first introduced into medicine in 1689 by William Cole, coined from the need to classify non-traumatic cerebrovascular accidents. The term was related to the Greek word 'apoplexia' which implies being struck with a deadly blow, which likewise refers to a clinical concept characterized by rapid loss of consciousness, and various manifestations of brain dysfunction. The concept of apoplexy was mentioned first by Hippocrates (460BC-370BC). Hippocrates hypothesized that the pathogenesis of 'apoplexy' was linked to humoral theory. Then a Swiss physician, Wepfer, is credited with being the first to observe that apoplexy was associated with cerebral haemorrhage. Many changes occurred from the 17th century on with the advent of autopsies, and 'apoplexy' began to lose its unitary meaning [1]. In 1970, stroke is defined by the World Health Organization as '*rapidly developed clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than of vascular origin*' [2]. This definition is considered outdated by the American Heart Association-American Stroke Association (AHA-ASA) [3] due to significant advances in the '*nature, timing, clinical recognition of stroke and its mimics, and imaging findings that require an updated definition*'. More recently a new definition was proposed by the AHA-ASA. What separates this definition from historical precedent is the inclusion of the 'silent' brain, retinal and spinal infarcts, and silent cerebral haemorrhages [4].

1.2 Epidemiology

Stroke incidence and mortality are increasing along with modernization and advancing longevity. In the latest update of the Global Burden of Disease referred to the year 2019, stroke was the second leading cause of death (6.55 million people) and the third leading

cause of death and long-term disability combined (143.23 million, expressed by disability-adjusted life-years lost – DALYs) worldwide [5].

The main indicators used in stroke descriptive epidemiology are prevalence, incidence and mortality. Incidence is the number of new cases in a specific population in a fixed period. This is a dynamic measurement, which can change between different periods, places, and seasons. Prevalence is defined as the total number of people who have had a stroke at a specific moment within a population. Mortality represents the death rate in a defined population over a period. The actual incidence varies greatly among different geographical regions, populations and across time (**Figure 1.1**). From 1990 to 2019 the stroke burden, in terms of the absolute number of cases, increased substantially (70% increase in incident strokes, 43% deaths, 85% prevalent strokes and 32% DALYs) with a greater increase in low-income and middle-income countries than in high-income countries.

Stroke also has a large physical, psychological and financial impact on patients, families, the healthcare system and society.

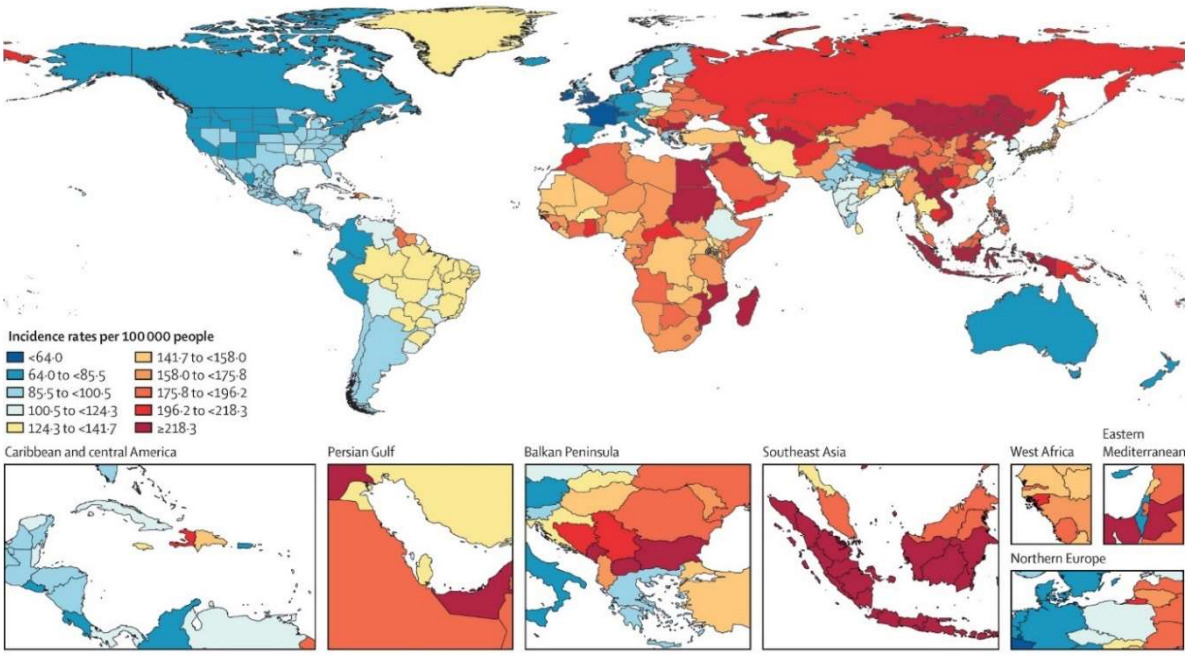


Figure 1.1 The Global distribution of ischaemic stroke incidence rates per 100000 people by stroke type and country, for both sexes, 2019. Data from the Global Burden of Disease Study [6].

1.3 Stroke risk factors

Stroke risk factors are an essential part of planning interventions to decrease the incidence. Primary prevention plays a fundamental role in decreasing the incidence of cardiovascular disorders and their related morbidity and mortality. Many factors increase the risk of stroke and these are usually divided into two categories: non-modifiable and modifiable risk factors.

Non-modifiable risks factors

Age, gender, ethnicity, family history of stroke and genetic mutations are non-modifiable risk factors for stroke. It has been shown that age has a cumulative effect on the risk of a first event; the incidence is highest between 45 and 85 years old. In general, stroke is a disease of age. The incidence of stroke increases with age and the relationship of sex

to stroke risk depends on age. Women have a higher risk than men at ages <30, lower in mid-life, and similar at ages ≥80 [7], due to hormonal factors such as fluctuations during and after pregnancy, as well as the use of oral contraceptive pills [8].

The incidence of stroke is higher among Blacks and Hispanic subjects than among Whites. As illustrated recently by the REGARDS study (REasons for Geographic And Racial Differences in Stroke)[9] one reason for the racial disparities could be the higher prevalence of stroke risk factors, such as hypertension, obesity, and diabetes mellitus, among blacks. However, these additional risk factors do not completely explain the increased risk seen in these ethnic groups.

The Framingham study showed the role of genetic factors in stroke risk, both paternal and maternal histories of stroke were related to a higher risk of stroke in the offspring [10]. Genetic variability may contribute to stroke risk through several potential mechanisms. Nowadays, genetics is placed in an overlapping location between non-modifiable and modifiable to represent the fact that genetic risk factors are increasingly recognized as potentially modifiable, either directly or through modification of gene-environment interactions [11].

Modifiable risks factors

Modifiable or potentially modifiable risk factors include several medical conditions and behavioural risk factors such as hypertension, smoking, diabetes, obesity, alcohol consumption, atrial fibrillation, elevated total cholesterol, lack of physical activity, poor diet, and oral estrogenic therapy [12]. In INTERSTROKE, a large case-control study [13], hypertension was by far the most important stroke risk factor. History of hypertension had the highest odds ratio (OR) for stroke at 2.64, followed by smoking at 2.09. Diabetes mellitus at 1.36 had an OR comparable to the high waist-to-hip ratio (1.65), high alcohol

intake (1.51), and depression (1.35), which in turn are risk factors for atrial fibrillation (AF).

Moreover, physical inactivity is associated with many poor health effects, including stroke. Diet influences the risk of stroke and the risk of other stroke risk factors, such as diabetes mellitus, hypertension, and dyslipidemia. In particular, salt intake is associated with an increased risk of hypertension and stroke, whereas increased potassium intake is associated with a decreased stroke risk [14].

Furthermore, levels of inflammatory biomarkers have been associated with an increased risk of stroke. Which way inflammation may contribute to stroke risk is through infection [15] or atherosclerosis, a condition recognized to have a highly inflammatory character [16].

In 2019, the five leading risk factors contributing to stroke death and disability combined were high systolic blood pressure, ambient particulate matter, high body mass index, high fasting plasma glucose concentrations and smoking (**Figure 1.2**).

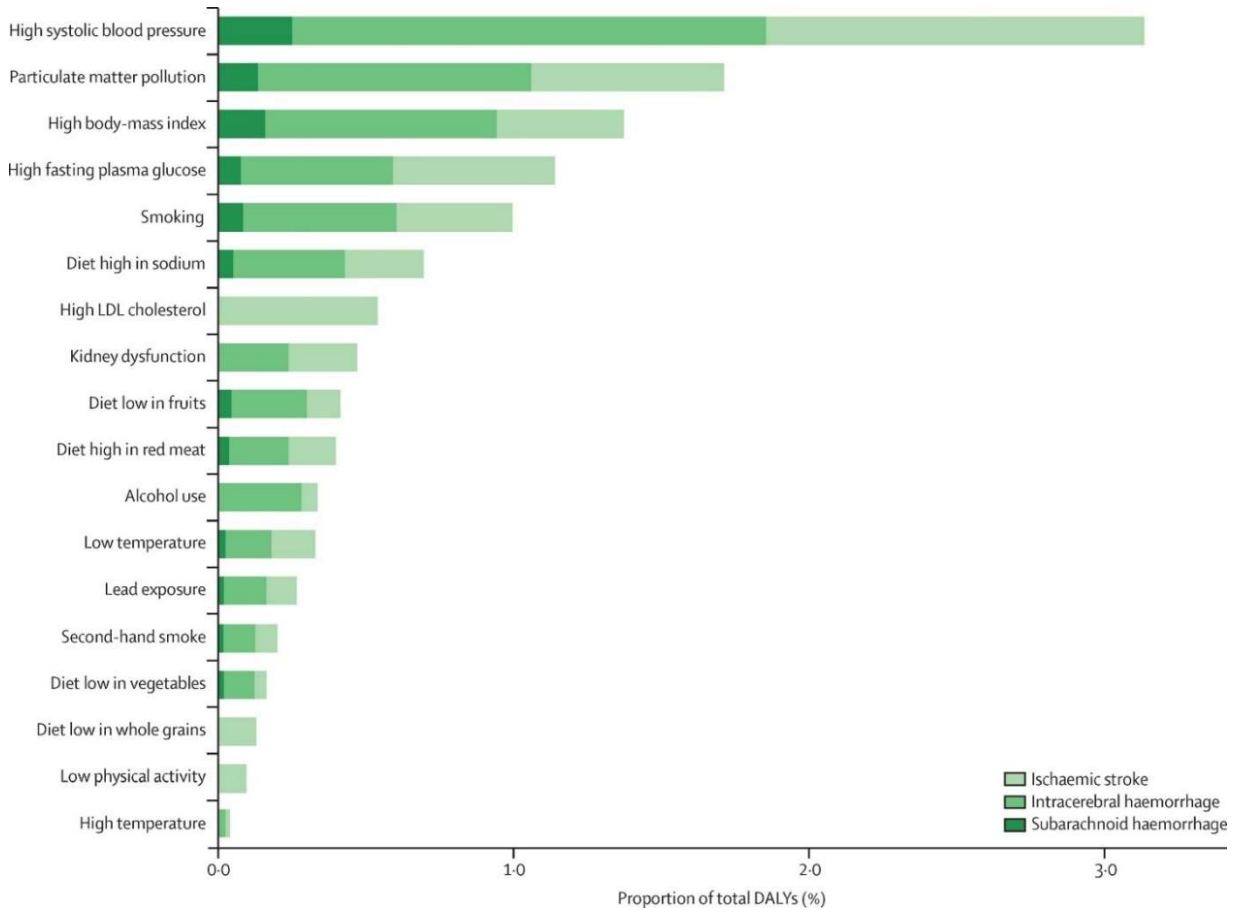


Figure 1.2 Proportion of DALYs attributable to risk factors by pathological type of stroke for both sexes combined. [6]

1.4 Prevention

Prevention requires knowledge of all the risk factors so that appropriate environmental, pharmacological or surgical treatment can be established [17]. The prevention of stroke can be grouped into primary and secondary prevention.

Primary prevention

The aim of the primary prevention is to act on the modifiable risk factors. According to WHO [6] strokes prevention strategies include reducing the risk associated with hypertension (high systolic blood pressure), elevated lipids, diabetes (high fasting

plasma glucose), smoking, low physical activity, unhealthy diet, abdominal obesity (high BMI), atrial fibrillation and correcting heart valve disorders, carotid artery stenosis and vertebral stenosis.

Secondary prevention

Stroke survivors represent a high-risk population and are the focus of secondary prevention strategies. For secondary prevention - i.e., in a patient that has already suffered a stroke - guidelines differ depending on the stroke mechanism. The cause of ischaemic stroke is important as it can guide therapeutic strategies for the prevention of recurrent stroke. Secondary prevention consists of preventing relapses and recurrent stroke episodes. Most recurrences after both haemorrhagic and ischaemic strokes are ischaemic[18]. Secondary prevention is based on: the administration of anticoagulant treatment after TIA or ischaemic stroke[19]. The choice of treatment is dependent on the patient's characteristics (**Table 1.1**).

Aetiology	Investigation	Secondary stroke prevention strategy
Atherosclerosis (for example, artery-to-artery embolism and intracranial atherosclerosis)	• CT angiography • Magnetic resonance angiography • Carotid Doppler ultrasonography	• Antiplatelet therapy • Blood-pressure-lowering medication • High-potency statin • Carotid endarterectomy or stent for >50% symptomatic carotid stenosis
Cardioembolism (caused by, for example, atrial fibrillation, left ventricular akinetic segment, infective endocarditis, patent foramen ovale and cardiac tumours)	• Holter/loop recorder • Echocardiography	• Anticoagulation therapy • Left atrial appendage occlusion • Antibiotics • Percutaneous closure
Small vessel disease	• Brain MRI	• Antiplatelet therapy • Blood-pressure-lowering medication • High-potency statin
Arterial dissection	• CT angiography • Magnetic resonance angiography • T1 fat saturated neck MRI	• Antiplatelet therapy • Anticoagulation therapy
Cerebral vasculitis	• CT angiography • Magnetic resonance angiography • Catheter angiography • Cerebrospinal fluid examination • Brain and leptomeningeal biopsy	• High-dose steroids • Cyclophosphamide
Reversible cerebral vasoconstriction syndrome	• CT angiography • Magnetic resonance angiography • Catheter angiography	• Calcium channel antagonists • Avoidance of corticosteroids
Moyamoya disease	• CT angiography • Magnetic resonance angiography • Catheter angiography	• Conservative or revascularization (for example, superficial temporal to middle cerebral artery bypass)
Fabry disease	• MRI • Blood spot enzyme test	• Enzyme replacement
Antiphospholipid syndrome	• Lupus inhibitor assay • Anti-cardiolipin IgG assay • Anti β_2-glycoprotein antibody assay	• Anticoagulation therapy
Sickle cell anaemia	• Blood film • Haemoglobin electrophoresis	• Transfusion
Polycythaemia vera	• Haemoglobin measurement • Haematocrit measurement • *JAK2* mutation status	• Venesection • Aspirin • Cytoreduction therapy
Essential thrombocytosis	• Platelet count • *JAK2* mutation status	• Aspirin • Cytoreduction therapy

Table 1.1 The aetiology of stroke determines the strategy for the prevention of recurrent stroke. [20]

1.5 Classification and aetiology of stroke

Strokes can be broadly classified into ischaemic and haemorrhagic strokes (from the Greek *ískhaimos* = “stoppage of blood flow” and *haimorrhagía* = “a violent bleeding”, **Figure 1.3**).

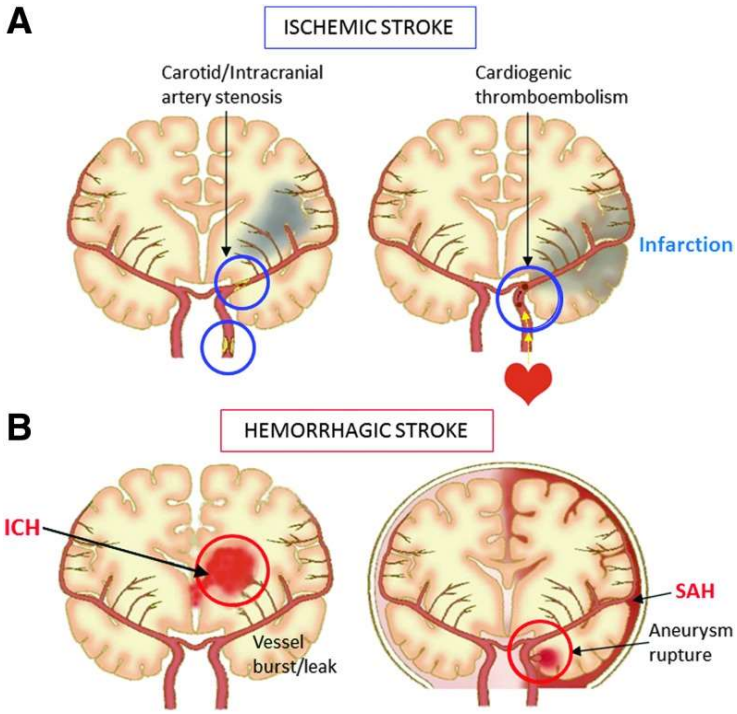


Figure 1.3 Illustration of stroke subtypes. (A) Ischaemic stroke occurs as a result of an obstruction within a blood vessel supplying blood to an area of the brain; usually by a clot from a small vessel lesion, intracranial/carotid stenosis or cardioembolic origin. **(B)** Haemorrhagic stroke occurs when an artery in the brain leaks or bursts (ICH), or an aneurysm ruptures (SAH). ICH, intracerebral haemorrhage; SAH, subarachnoid haemorrhage [21].

Ischaemic stroke

Ischaemic stroke is caused by blockage of an artery in the brain and is responsible for 87% of all cases. According to the TOAST classification[22], there are five main types of ischaemic strokes. These are large vessel atherosclerosis, small vessel diseases (lacunar infarcts), cardioembolic strokes, strokes of determined cause and strokes of undetermined cause. In 2003 a group of physicians interested in developing an evidence-based etiologic classification scheme for acute ischaemic stroke launched the CCS (Causative Classification of Stroke System) project [23]. The CCS was devised to overcome the limitations of the TOAST system and to achieve high reliability. In 2009 it

was proposed a new system to phenotype patients with ischaemic stroke, called ASCO (A for atherosclerosis, S for small-vessel disease, C for cardiac pathology, and O for other causes, [24]) to better describe the overlap between diseases underlying a cerebral ischaemic event in a stroke patient. Based on the experience over years, the same research group propose an updated version called ASCOD (A for atherosclerosis, S for small-vessel disease, C for cardiac pathology, O for other causes and D for dissection) phenotyping in 2013 [25]. Compared to other stroke-subtyping classifications ASCOD grades all diseases present in each patient, captures the overlap between the diseases, and weights the potentially causal relationship between every disease detected and the ischaemic stroke [26].

Most strokes are caused by occlusion of a major cerebral artery, usually the middle cerebral artery (MCA). Major causes of large arterial occlusion include thrombosis and embolism, most often caused by atrial fibrillation. Occlusion of small arteries and arterioles (small vessel disease) leads to small ischaemic lesions in the basal ganglia and subcortical white matter.

Haemorrhagic stroke

The other major type of stroke can be divided in:

- Intracerebral haemorrhage (ICH) - where bleeding occurs in the brain parenchyma;
- Subarachnoid haemorrhage (SAH) - extravasation of blood into the subarachnoid space between the pial and arachnoid membranes.

Haemorrhagic stroke on the whole accounts for 10% of all strokes. While less frequent than ischaemic stroke, haemorrhagic stroke is responsible for much of stroke's global

burden, especially in low- and middle-income countries, where mortality rates approach 80%.

In this thesis, I will only discuss about ischaemic stroke.

1.6 Pathophysiology of stroke

The pathophysiology of ischaemic stroke is extremely complex and involves the activation of a series of detrimental signalling cascades. The progression of ischaemic brain damage following impaired blood flow involves the initial development of a core of irreversibly injured necrotic tissue within the affected vascular bed, followed by late-phase injury development in the peri-infarct area, a potentially salvageable area surrounding the core (**Figure 1.4**).

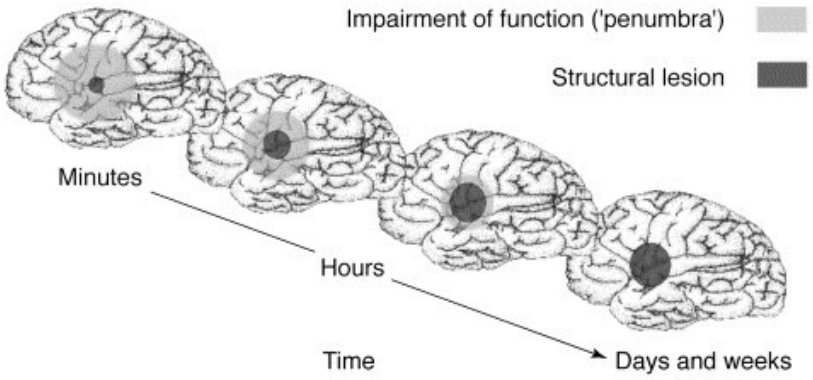


Figure 1.4 Space-time progression of ischaemic brain damage. Early in the course of a stroke, clinical symptoms mostly reflect an impairment of function (light grey) but not necessarily a structural lesion (dark grey). Over time, some areas either spontaneously, or because of therapy, recover function, which explains why symptoms in patients can regress while the structural lesion grows [27].

Deprivation of oxygen and glucose supply to the brain tissue leads to immediate failure of energy-dependent ion pumps and channels resulting in the release of potentially toxic concentrations of excitatory neurotransmitters and subsequent death of the vulnerable neurons.

Collateral blood flow

The blood flow to the brain is managed by two internal carotids anteriorly and two vertebral arteries posteriorly - the circle of Willis. Ischaemic stroke is caused by deficient blood and oxygen supply to the brain; haemorrhagic stroke is caused by bleeding or leaky blood vessels.

When an artery is occluded, alternative blood flow pathways can sustain viability in the penumbral brain region for a period of time. Brain collateral circulation is an essential compensatory mechanism in response to acute brain ischaemia. However, collaterals arteries represent a complex trait, highly influenced by genetic, age-related variability and sex. The circle of Willis includes the anterior communicating artery in humans, while this vessel is totally absent in rodents [28]. Rats and mice are commonly used to model stroke and exhibit similar variations. In particular, in mice has been reported that only 10% of C57BL/6 have a complete circle of Willis [29]. Poor collateral blood flow leads to rapid progression of infarction and limited response to reperfusion therapies [30]. Recent findings support the feasibility and efficacy of collateral therapeutics as a hyperacute therapy in experimental ischaemic stroke before reperfusion [31].

Ischaemic cascade and reperfusion injury

Ischaemic stroke leads to oxygen depletion in the brain, which has several cellular and molecular consequences that affect neuronal and glial function in addition to vascular

alterations and inflammation. The neuronal function relies on the continuous availability of ATP. ATP is required for the function of the Na^+/K^+-ATPase ion pump. This pump actively exchanges Na^+ for K^+, maintaining low levels of Na^+ and high levels of K^+ inside the cell, which are vital for the secondary transport of sugars, neurotransmitters, and amino acids across the membrane the cell requires to function [32]. Cerebral blood flow leads to failure of the ion pump within minutes, causing anoxic depolarization that forms the ischaemic core of the infarct [33]. Depolarizations initiated in the infarcted neurons spread to the surrounding penumbra, which is unable to compensate for the increased metabolic demands due to decompensation in the collateral circulation. Neuronal cell death causes the unregulated release of neurotransmitters from pre-synaptic neurons, with the excitatory amino acid glutamate being of particular importance. The binding of glutamate to postsynaptic receptors - mGluR and NMDAR - causes an influx of excess calcium inside the cell. The activation of ionotropic glutamate receptors results in the inflow of Ca^{2+} from the extracellular into the intracellular compartment, leading to mitochondrial Ca^{2+} overload and the activation of calcium-dependent catabolic enzymes. The activation of metabotropic glutamate receptors induces the IP_3-dependent signal transduction pathway, leading to the stress response of the endoplasmic reticulum. The increase of intracellular Ca^{2+} can also lead to the induction of neuronal nitric oxide synthase, with a subsequent increase in free radicals that can lead to DNA damage, intracellular structure breakdown, and induce processes leading to cell death in the form of apoptosis, autophagy, or necrosis [34,35].

Brain infarcts evoke a strong inflammatory response which contributes to the progression of ischaemic brain injury [36]. The ischaemic environment drives macrophage recruitment, and this results in the co-presence of infiltrating blood-borne macrophages and resident reactive microglia in the lesioned site[37]. Resident macrophages and invading

myelomonocytic cells release cytokines - such as TNFα and IL-1β - chemokines, reactive oxygen species (ROS), neutrophil extracellular traps (NETs), and MMP-9 which lead to further disruption of the BBB, brain edema and neuronal death [38]. This inflammatory response is thought to be the mechanism that contributes to reperfusion injury, where the opening of an occluded artery leads to further damage.

Over the past years, growing attention has been given to the activation of the complement system [39], a component of innate immunity which is activated on recognition of danger signals (DAMP), in addiction to activation following pathogen-associated molecular pattern (PAMP) recognition. Soon after cerebral ischaemia, complement components synthesized by local activated endothelial cells, neurons and glial cells, as well as complement derived from the liver, are implicated in the progression of the disease. Depending on the trigger, complement activation may proceed through three separate pathways, namely, the classical (CP), alternative (AP) and lectin pathways (LP). The LP depends on initiator molecules including mannose-binding lectin (MBL), ficolins (-1, -2 and -3) and collectin-11 that can recognise and bind carbohydrates exposed on the surface of altered or damaged cells in brain ischaemia [40]. In experimental studies the deleterious role of MBL in ischaemic injury has been documented, reporting that MBL genetic deletion or its pharmacological inhibition are highly protective in models of cerebral ischaemia [41]. Similar to MBL, ficolins can activate the LP on binding with their targets, thus promoting downstream complement activation in stroke-induced sterile inflammation [42]. Emerging clinical evidence showed that the ficolins are consumed within 6 hours after ischaemic stroke and identified ficolin-1 and -3 as sensitive prognostic markers indicating the unfavourable 3-month outcome of stroke patients [43,44]. A role for ficolin-2 has been also identified for prothrombotic conditions such as atherosclerosis[45], one of the main risk factors for ischaemic stroke. In particular atherosclerotic patients

with elevated levels of ficolin-2 were more likely to have an adverse cardiovascular event over 18 months after endarterectomy [45].

The immune system and post-ischaemic inflammation differ over the time course of the disease in a bivalent manner. While many aspects of inflammation are beneficial and aimed at restoring tissue homeostasis, collateral damage by the acute inflammatory response contributes to the ischaemic damage (**Figure 1.5**).

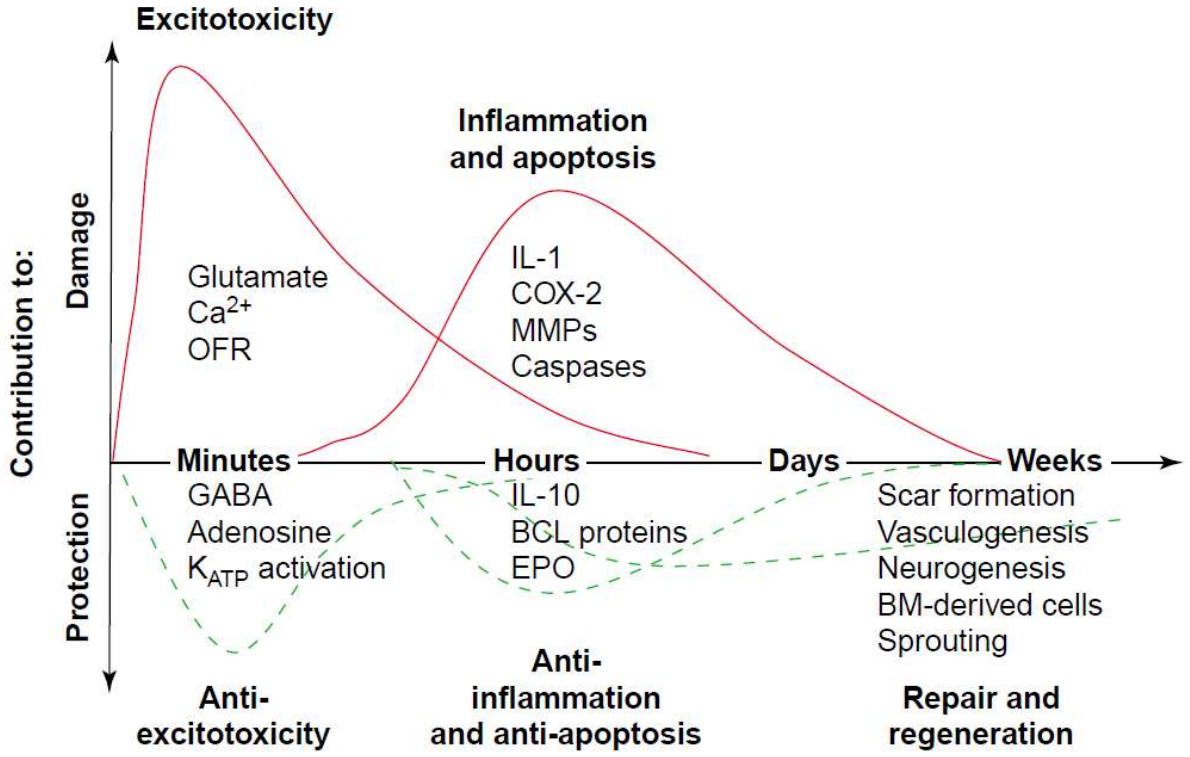


Figure 1.5 The complex series of mechanisms induced by cerebral ischaemia. The *x*-axis reflects the evolution of the cascades over time and the *y*-axis illustrates the impact of each element of the destructive (top) and protective (bottom) cascades on the final outcome. The red dotted lines envelop major pathophysiological entities of tissue destruction in stroke and the green broken lines envelop corresponding protective tissue responses. BM: bone marrow; COX-2: cyclooxygenase 2; EPO: erythropoietin; IL: interleukin; MMPs: matrix metalloproteinase; OFR: oxygen free radicals [46].

1.7 Diagnosis of ischaemic stroke

The American Stroke Association (2015) promoted the F.A.S.T. campaign (Face drooping, Arm weakness, Speech difficulty, Time to call) to improve awareness of stroke and to expedite the activation of emergency services for stroke victims. It is critical to establish the time of onset of symptoms because this determines whether a patient meets the 4.5-hour eligibility window for thrombolytic treatment. Non-contrast computed tomography scans immediately determine whether the stroke is ischaemic or haemorrhagic, which is critical because treatments are different. Stroke severity is determined by performing a complete neurologic exam and assessing the patient using the National Institutes of Health Stroke Scale (NIHSS). The NIHSS is a 15-item scale that can be performed in about 5 minutes (National Stroke Association, 2015). The scale incorporates an assessment of language, motor function, sensory loss, consciousness, visual fields, extraocular movements, coordination, neglect, and speech. A score of greater than 16 predicts a high probability of death or severe disability, whereas a score of less than 6 predicts a good recovery. It was later modified to the current 11 items (mNIHSS) for use in the National Institute of Neurological Disorders and Stroke recombinant tissue-type plasminogen activator (NINDS r-tPA) trial [47]. During the first years of use, the scale was found to be reliable for use by neurologists, nurses and non-neurologist physicians but only after a training in a research context [48].

In 1957, John Rankin introduced the original Rankin Scale [49]. The outcome was graded I-V and later modified for use in the United Kingdom Transient Ischaemic Attack (UK-TIA) trial in 1988, adding a grade of 0 for no symptoms [50]. An additional grade 6 was added later, denoting death, completing the 7-grade ordinal scale in use today [51]. Symptom severity scales used in the acute phase such as the NIHSS focus on limitations

in body functions and structure, while functional outcome scales such as the modified Rankin Scale (mRS) primarily concern limitations in activity and participation.

1.8 Current therapies

Treatment of acute ischaemic stroke consists in a multidisciplinary approach that requires the involvement of critical care specialists. The goal of treatment in the acute phase of ischaemic stroke is to salvage brain tissue by restoring blood flow as soon as possible. There are two main therapeutic avenues to achieve tissue reperfusion in acute ischaemic stroke: pharmacological treatment administered intravenously to lyse the thrombus (intravenous thrombolysis – IVT) using alteplase or tenecteplase, and endovascular treatment (EVT) to mechanically remove the thrombus.

Currently, tissue plasminogen activator (tPA), a thrombolytic drug that breaks down the clot, is the only FDA-approved therapeutic for the treatment of ischaemic stroke within 4.5 hours of symptoms onset. The European Cooperative Acute Stroke Study (ECASS III trial) showed that alteplase was safe and effective in a time window of 3 hours with strict inclusion criteria that included patients ≤80 years of age, without a history of both diabetes mellitus and prior stroke, with an NIHSS score ≤25, not taking any oral anticoagulation, and without imaging evidence of ischaemic injury involving more than one-third of the middle cerebral artery (MCA) territory [52]. In 2009 the AHA/ASA extended the IV-tPA window from 3 to 4.5 hours with additional exclusion criteria [53]. In 2019 AHA/ASA updated the guidelines for the early management of patients with acute ischaemic stroke, suggesting that the efficacy and safety of IV-tPA could be extended up to 9 hours and that revascularization could be extended up to 24 hours [54]. Based on results of recent trials - EXTEND and ECASS-4 ExTEND [55,56] - the European Stroke Organization (ESO) guidelines recommend IVT 4.5 to 9 hours from symptom onset but

only in patients where EVT is not planned and in centres with access to the imaging modalities used in the trials (perfusion-diffusion MRI or CT perfusion) showing potentially salvageable brain tissue [57]. Treatment with tPA outside the therapeutic time window can result in a haemorrhagic transformation, which can cause additional damage to the brain. Tenecteplase is another thrombolytic agent, with high fibrinogen specificity and a long half-life. It had promising results in recent clinical trials, EXTEND-IA-TNK [58] but at this time, it is not FDA-approved for IV thrombolysis in AIS patients.

However, up to 69% of stroke patients are ineligible to receive tPA due to delayed hospital presentation [59]. Over the last years, the time window for AIS treatment has expanded thanks to another option for recanalization treatment: EVT [60]. In this procedure, the blood clot causing the ischaemia is extracted by threading a catheter with a wire-caged device called a stent retriever into the blocked arterial vessel in the brain. The procedure should be done within 6 hours of acute stroke symptoms. Recent studies show success with thrombectomy up to 8 hours after the onset of stroke symptoms [61]. The latest breakthrough came in 2018 when results from two RCTs - DEFUSE 3 and DAWN - showed that EVT can be effective in selected patients with an occlusion in the anterior circulation up to 24 hours after symptom onset [62,63].

Preventative treatments, such as anticoagulants and blood pressure-lowering and cholesterol-lowering drugs, may be administered as well, as there is an increased risk of having a second stroke immediately after the initial stroke.

1.9 Experimental models for ischaemic stroke

In vitro models

The complexity of ischaemic stroke can not be modelled in an *in vitro* system with single cells or pieces of brain tissue with the absence of intact blood vessels and blood flow as

well as the lack of infiltration of leukocytes. Nevertheless, *in vitro* models allow the investigation of specific basic biochemical and molecular mechanisms under conditions of energy deficiency similar to ischaemia. There are two principal ways to induce ischaemia-like events outside a living organism: the deprivation of oxygen and glucose (OGD, [64]) or the chemical or enzymatic blockade of cellular metabolism [65]. The OGD model is based on exposing cells to N_2/CO_2 equilibrated medium without glucose and maintaining cells in a hypoxic/anoxic chamber. The chemical method relies on inhibition of the mitochondria electron transport chain; less common is the enzymatic induction of hypoxia, which relies on manipulating the glucose oxidase and catalase (GOX/CAT) system [66–68] and 2-deoxyglucose [69].

Promising drug candidates have failed to translate into clinically drug compounds partly due to the blood-brain barrier (BBB) properties of the brain capillary endothelial cells. *In vitro* models of the ischaemic BBB have been developed to gain a better understanding of changes in barrier properties during ischaemia. The vasculature of the brain is surrounded by capillary endothelial cells endowed with barrier properties, and adjacent perivascular cells, including astrocytes and pericytes that wrap the abluminal capillary surface supporting and maintaining healthy BBB functioning [70]. BBB models can be divided into the study of isolated components of the BBB, such as BCEC (brain capillary endothelial cells) monolayers, and those seeking to replicate the more complex composition and communication between cells, such as co-cultures in Transwells (2D systems) and 3D systems [71]. Most of the knowledge derived from *in vitro* stroke studies is based on cells grown as monolayers. However primary cell isolation and purification are time-consuming, and cells can lose phenotypic identity with increased passage number, limiting their application. In addition, possible contamination with other cell types can affect reproducibility [72,73]. This simplified culture system led to the optimisation and

standardization of applications for 2D cultures. 2D cultures are utilized to understand the role of a specific factor rather than the interaction of cell types that work together to coordinate blood flow or permeability. As such 2D cultures are unable to mimic the complicated microenvironment cells experience in tissue. For these reasons, the 3D systems are considered more realistic models of the human brain. The 3D systems are broadly categorized into three groups: dynamic in vitro BBB model (DIV-BBB), microfluidic BBB (μBBB) and BBB-on-a-chip. The 3D models are potential tools for performing drug uptake, dosing assay and drug discovery. In particular, models using patient-derived induced pluripotent stem cells (iPSCs) and the inclusion of the blood flow on a chip are promising tools for understanding disease mechanisms [74,75]. The advent of iPSC technology has obviated the scarcity of human-derived brain slices and has boosted the development of 3D models of the brain, such as brain spheroids or organoids [76]. The scaffold-free techniques can be generated by growing cells in 3D self-assembled spherical clusters (sometimes referred to as cell aggregates or spheroids), which do not contain added biomaterials and the ECM present is produced only by cells themselves. Instead, scaffold-based techniques can be obtained by seeding/dispersing cells into 3D solid or liquid matrices made from either natural or synthetic materials and using the material to provide cell-matrix interaction and guide cell behaviour [77,78]. Spheroid and organoid models presented some limitations - i.e. the lack of vascularisation, causing the development of a hypoxic, necrotic core and further hampering the growth and maturation of neural organoids and spheroids; the lack of microglia which has important roles in immune defence and maintenance of central nervous system homeostasis [79]; and the heterogeneity in terms of shape, size and composition that limits controlled experiments and future potential screening approaches [80] - which researchers are currently trying to overcome [81–84].

In vitro studies must be combined with *in vivo* experiments to come closer to the reality of human stroke, obtaining a full picture of ischaemia.

In vivo models

An *in vivo* model is a construct of a real physical component or property observed in nature. Although stroke has been studied in different species (e.g., dogs, rabbits, monkeys), rodents are the most widely used [85]. Rodents present some advantages including low costs for transportation, storage, and feeding, and the availability of unique strains that can be genetically engineered to over or under-express selected target genes. Furthermore, small animals are less controversial from an ethical point of view than primates and higher mammals. There are two models of cerebral ischaemia: global and focal. Global ischaemia develops after transient circulatory arrest with resuscitation or after near drowning. Focal ischaemia occurs after transient or permanent blood flow reduction in the territory of a cerebral artery and its branches. Typically, flow reduction is caused by the embolic or thrombotic occlusion of a cerebral artery, most frequently the middle cerebral artery (MCA).

Experimental focal ischaemia is most commonly studied using models of permanent or transient occlusion of an MCA as this is highly relevant to the clinical situation where 87% of strokes are caused by the occlusion of the MCA. The models of focal cerebral ischaemia can be categorized into two groups: models requiring craniectomy and models retaining skull integrity. Within the two groups, the models can be divided into models leading to permanent and models leading to transient ischaemia (**Figure 1.6**).

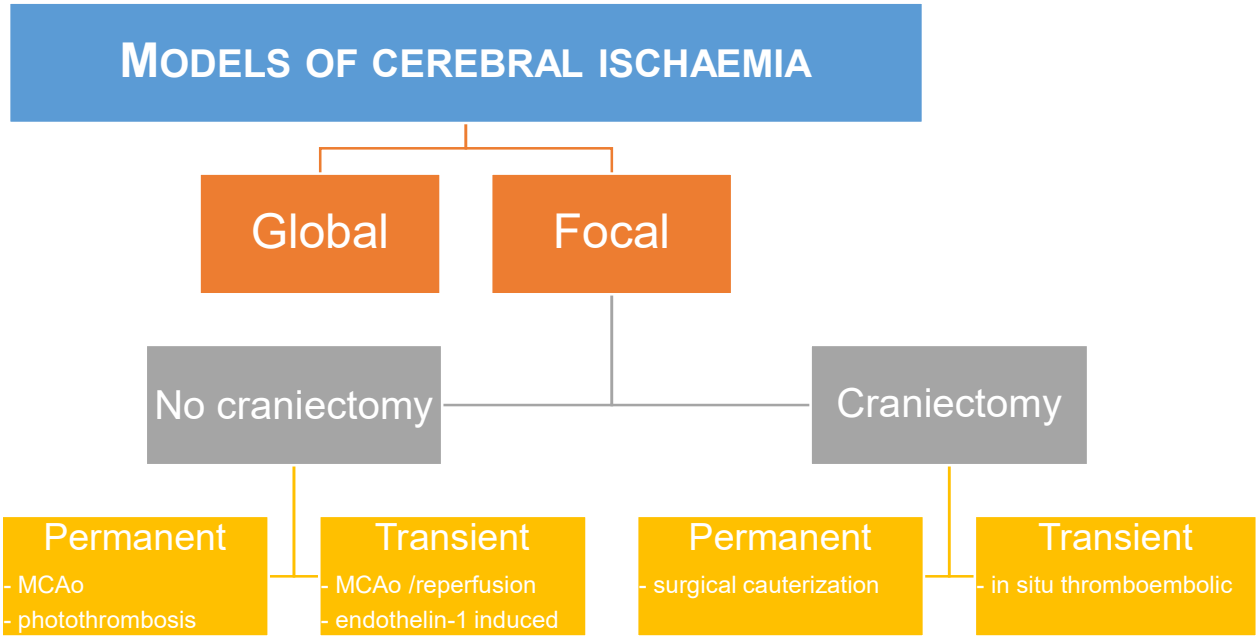


Figure 1.6 Overview of models of cerebral ischaemia.

Each model does not contemplate the full complexity of human pathology but helps focus on specific pathogenic aspects. The different stroke models present advantages and disadvantages in replicating the human disease (**Table 1.2**). Models resulting in permanent ischaemia mimic clinical stroke without reperfusion, whereas transient models reflect human stroke with therapy-induced or spontaneous reperfusion, which accounts for the majority of all stroke cases. In some ways, the reperfused brain imitates the restoration of blood flow in humans after spontaneous lysis of a thromboembolic clot. During reperfusion, NO generation and free radical production are particularly pronounced and contribute to 'reperfusion injury' [86,87].

The so-called photothrombotic model produces a cortical infarction, while skull integrity is preserved. The relevance of this model for stroke research is debatable for the basic differences in early lesion development [88].

Endothelin-1, a potent vasoconstrictor, can be used to induce ischaemia by direct application on the exposed MCA or cortex, requiring craniectomy or can be injected stereotactically next to a cerebral artery [89]. The disadvantages concern the limited control of the intensity and duration of ischaemia since the vasoconstrictive effect of endothelin-1 is dose-dependent.

Thromboembolic animal models are closer to human stroke, non-invasive, and suitable for studying the thrombolytic agents, the effects of thrombolysis and combined strategies and reperfusion injury. The model of in situ thromboembolic stroke uses microinjection of murine thrombin to trigger *in situ* clot formation. This experimental model shows two important similarities to human pathology: the fibrin-rich clots may be spontaneously lysed as occurs in non-treated patients; the early administration of tPA, the only pharmacological therapy approved in humans, is beneficial and reduces the lesion size [90,91]. However, in contrast to other embolic models, the model requires craniectomy.

The most frequently used experimental model of ischemic stroke in rodents is the intraluminal filament with occlusion of the MCA. The procedure consists of inserting a filament (mono-nylon suture) into the point of origin of the MCA through the internal carotid artery (ICA). The filament model does not require craniotomy and can be used to either model permanent ischemia or transient ischemia [92,93] by the withdrawal of the filament, allowing reperfusion at different time points, from 30 min to 120 min [94]. The prompt reperfusion of the MCA after the removal of the filament is far from the pathophysiology of spontaneous human stroke, in which it occurs by gradual reperfusion. The abrupt removal more closely mimics the therapeutic situation of mechanical thrombectomy which is expected to be increasingly applied to stroke patients [95].

Experimental model	Close to reality	Far from reality/distorting reality
In vitro models	Principal mechanisms and molecular pathways of cell death under ischemia-like conditions	Absence of intact blood vessels and blood flow Lack of infiltration of leukocytes
Endovascular suture model	Localization of the infarct (mostly MCAO), penumbra, blood-brain barrier injury, inflammatory processes and cell death pathways (Permanent and transient ischemia) No craniectomy	Large infarcts, mimics rather malignant infarction [16] Involvement of the hypothalamus with consecutive hyperthermia (rat) [79] Prompt reperfusion by withdrawal of the filament [55] Exception: mimics closely endovascular mechanical thrombectomy Thromboembolism/thrombolysis not modeled Anesthesia
Craniectomy models with direct vessel occlusion	Penumbra, blood-brain barrier injury, inflammatory processes and cell death pathways (Permanent and transient ischemia)	Prompt reperfusion by reversal of the mechanical occlusion [55] Exception: mimics closely endovascular mechanical thrombectomy Thrombembolism/thrombolysis not modeled Craniectomy Anesthesia
Photothrombotic stroke	Small cortical infarcts and small subcortical infarcts (Permanent ischemia only) Recovery and plasticity mechanisms in chronic stroke Modifications with stroke induction in freely moving rats and mice allow real-time analysis of a number of parameters in acute stroke without distortion through anesthesia [81, 136]	Simultaneous development of cytotoxic and vascular edema with rapid breakdown of the blood-brain barrier No penumbra (whether the "ring" model accurately models penumbra under discussion) [16] Anesthesia
Endothelin-1 model	Infarcts of variable sizes in nearly any brain region Subcortical stroke Recovery and plasticity mechanisms in chronic stroke (Transient ischemia only)	Minimal edema [112] Endothelin-1 and endothelin-1 receptors present also on neurons and astrocytes [94, 95]—may interfere with post-stroke recovery mechanisms [16]
Thromboembolic clot models	Thromboembolic infarcts Transient ischemia with unpredictable time point of lysis of the embolus Pathophysiology of embolic stroke including primarily cytotoxic edema superimposed later on by vasogenic edema with breakdown of the blood-brain barrier, presence of a penumbra, development of spreading depressions as well as an inflammatory response Possibility to test thrombolytic therapies	(Animal model)
Microsphere models of embolic stroke	Thromboembolic infarcts (Permanent ischemia) (Mini-)penumbras, pathophysiology of ischemic cell death, inflammation	Permanent ischemia without possibility of reperfusion Multiple vessels occluded Capillaries and arterioles are blocked resulting in redistribution of the blood flow and immediate disruption of the blood-brain barrier and vasogenic edema [132]
Macrosphere models of embolic stroke	Thromboembolic infarcts (Permanent ischemia) Pathophysiology including penumbra, ischemic cell death, inflammation Occlusion can be postponed allowing to induce ischemia while the rat lies in an MRI or PET scanner	Permanent ischemia without possibility of reperfusion
Spontaneous stroke models: spSHR rat	Subcortical infarcts (Small) vessel pathology	(Animal model)

Table 1.2 Focus on differences and similarities between the experimental stroke models and humans [96].

Stroke is a disease characterized by heterogeneities on several levels, each of which influences disease processes. For a correct interpretation of the experimental outcome, however, differences in brain anatomy [97], infarct localization and functional differences [27] between animal models and humans need to be taken into account (**Figure 1.7**).

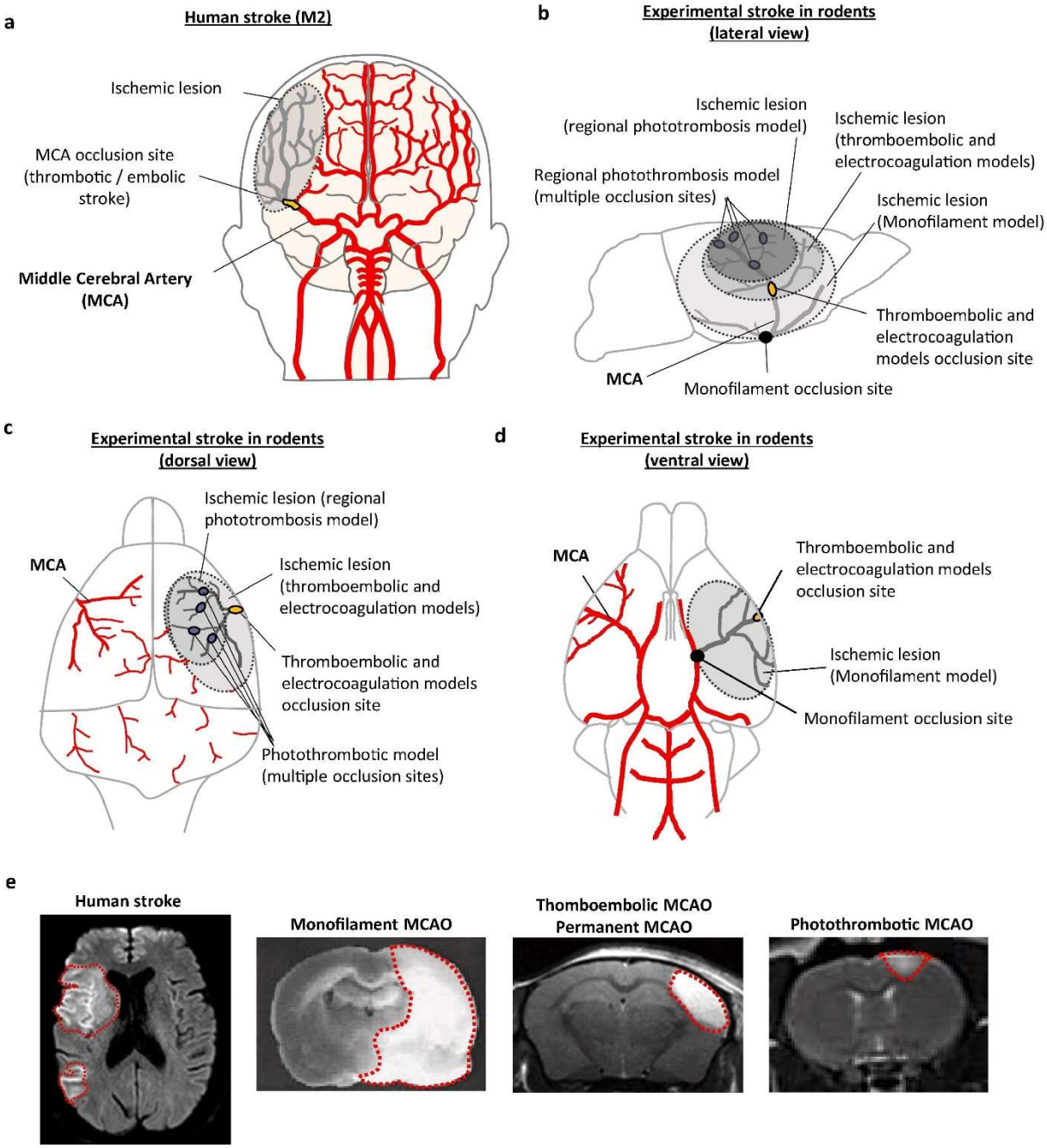


Figure 1.7 Comparison of human stroke and experimental models of stroke in rodents.

a) Schematic view of the human brain vasculature with thrombotic/embolic occlusion of the M2 segment of the MCA. **b)** Schematic view of the rodent brain vasculature (lateral view). In thromboembolic and electrocoagulation experimental stroke models, there is only one site of MCA occlusion, whereas in regional photothrombotic stroke there are multiple occlusion sites within the area illuminated by the laser. In both cases, lesions are limited to the brain cortex. The

occlusion site in the monofilament experimental model is located at the origin of the MCA, leading to a bigger ischemic volume including both cortical and subcortical brain regions. **c)** Schematic view of the rodent brain vasculature (dorsal view). **d)** Schematic view of the rodent brain vasculature (ventral view). **e)** Comparison of ischemic lesions (delimitated area) visualized by MRI in human stroke (M1-M2 occlusion) and different experimental models of stroke [98].

1.10 The gaps in stroke research

Understanding and limiting the variability in preclinical models will allow better testing of potential therapeutic strategies and help expand our understanding of ischaemic stroke pathogenesis.

Three aspects to consider in preclinical models are the heterogeneous nature of the disease; the presence of comorbidities and appropriate outcome measures. Each MCAo model has strengths and limitations when trying to model the complex heterogeneous nature of stroke in humans. The different in brain anatomy and functional organization is relevant concerning infarct localization. *In vivo* stroke models use animals with a lissencephalic brain, while gyrencephaly is a mammalian-specific trait present in humans [99]. Moreover, male animals are usually used instead of females to avoid hormonal imbalances. Possible gender differences are widely disregarded. Inbred strains are used that fail to replicate the human genetic variety. Usually very young and healthy animals are used for the modelling of stroke. They are kept under standard laboratory conditions with little exercise and no variation in diet.

There are several modifiable comorbidities related to stroke that should also be considered in animal models. For instance, hypertension and diabetes, and external factors related to daily life e.g. obesity, tobacco and alcohol consumption [100].

Another factor that contributes to contradictory results is the timing of outcome assessment between mouse and human studies. Most of the preclinical studies focus on neurological outcomes on the first days after stroke, while in most clinical studies this is measured in patients up to several months after the stroke (usually 3 months). Moreover many experimental stroke studies only report one outcome measure - decreased infarct sizes or short-term behavioural analysis [101]. Inclusion of these aspects in preclinical research and following the STAIR (Stroke Therapy Academic Industry Roundtable) recommendations [102] would increase the value, robustness and translational potential of the results.

1.11 Quality and improvement of preclinical studies

Reproducibility is an essential characteristic in any field of experimental sciences. For years, the scientific community has been challenged by the reproducibility of published data. Cumulative errors are the primary causes of irreproducibility, i.e. study design, biological reagents and reference materials, laboratory protocols and data analysis and reporting. These flaws contributed to the 'translational crisis' of stroke pre-clinical research. Several studies have indeed identified and tested different therapeutic targets with promising results in preclinical trial. However none of the compounds identified in preclinical research has translated effectively in clinical trials. Over the past decade, evidence has accumulated indicating that scientific validity and reproducibility are alarmingly poor throughout biomedical research [103–106].

General principles of preclinical studies design

To minimize the effects of biological heterogeneity when designing a study to test a new product or therapy, investigators should adopt or develop standardized specimens, materials and protocols, and use multiple disease-relevant *in vitro* models and animal

models. There are different measures to improve the design, conduct, and reporting of animal studies to maximize both their internal and their external validity. These include, but are not limited to, randomization, allocation concealment, blinded assessment of outcome, sample size calculation, and measures to avoid publication bias. Tools that protect investigators from biases and statistically poor results can act as effective safeguards against reproducibility failures in preclinical studies.

-Internal validity

Internal validity refers to the scientific robustness of a study's design, conduct, analysis and reporting. Several studies in preclinical and clinical research evidenced poor study design, the presence of bias, the lack of blinding, randomization and *a priori* size calculation are contributors to low internal validity [107–109].

To improve internal validity randomization, blinding, inclusion and exclusion criteria need to be specified before data are collected [110]. It is recommended a pre-registration of detailed laboratory protocols at a platform, such as http://www.pre-clinicaltrials.eu or http://www.animalstudyregistry.org.

Studies that lack internal validity will always lack external validity (http://www.consort-statement.org/checklists/view/32–consort-2010/120-generalisability).

-External validity

For the assessment of external validity, research findings from one setting need to be generalized to other settings. Poor external validity may thus contribute to both poor reproducibility of a research finding (e.g., when the same study replicated in a different laboratory by a different experimenter produces different results) and translational failure (e.g., when a treatment shown to be efficacious in an animal model is not efficacious in a clinical trial in humans) [103]. External validity consists of potentially modifiable features (e.g. representativeness of animal samples, the clinical relevance of animal models) and

unmodifiable features (animal-human species differences). Importantly, some of the strategies employed to increase internal validity may at the same time decrease external validity [111–113]. For example, common strategies of standardizing experiments by using homogenous study populations to maximize test sensitivity compromise the external validity of the research findings, resulting in the lack of generalization of the findings. For these reasons, a compromise has to be reached. A greater pressure must be placed on researchers, reviewers, and journal editors to avoid negligence in experimental design, conduct, and publication.

Best prioritization practice in stroke research

High-quality research produces results that can be confidently used as the basis for generating new knowledge or for application purposes. A stroke occurs because of a variety of vascular pathologies and injury mechanisms, some of which are difficult to model in preclinical models. Preclinical research endpoints do not generally reflect clinical outcomes. Thus high-quality research, successful collaborative research and training enable several partners in a consortium to replicate each other results and then share the obtained knowledge. Large and inclusive consortia can have a more comprehensive view of what is already available or underway in the specialty [114,115]. This awareness might improve efficiency in the use of resources and provide more informative results.

Researchers should design new randomized trials considering realistic sample size calculations, methods for data collection, and a statistical analysis plan. For this purpose, the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) has launched a useful free online tool, Experimental Design Assistant

(EDA). It is useful for the design of experiments that use the minimum number of animals, the methods to reduce subjective bias, and for appropriate statistical analysis. Resources are also available to support the design and conduct of multicentre animal studies (www.multi-part.org). Waste in research can also be reduced through initiatives aimed at simplifying, centralizing, and harmonizing protocols and guidelines. External validation and replication of findings by independent teams are essential to avoid bias. Before the experiment starts, researchers should also establish clear inclusion/exclusion criteria. They also should establish rules about which animals will be excluded from the analysis, without knowledge of treatment group allocation. During the interventional phase, animals should be allocated to an experimental group by blinded randomization. The assessment of the outcome should be carried out by someone who is not aware of treatment group allocation. All experiments should be set out according to the principles outlined in the international consensus statement on Good Laboratory Practice in the modelling of cerebral ischaemia [116] and the ARRIVE guidelines [117]. Preclinical stroke research should be conducted according to other available guidelines, specifically designed for stroke studies: the IMPROVE guidelines. These guidelines provide a number of recommendations to minimise the level of severity in the most common rodent models of middle cerebral artery occlusion, while sustaining or improving the scientific outcomes. The recommendations cover basic requirements pre-surgery, selecting the most appropriate anaesthetic and analgesic regimen, as well as intraoperative and post-operative care [118].

All efforts and scientific quality should be maintained in reporting data and publication. Additionally, public availability of raw data and complete scripts of statistical analyses could be required by journals and funding agencies sponsoring new research.

Multicentric studies

In recent years, studies have identified and tested a large number of therapeutic targets with promising results in preclinical models. Despite our increasing knowledge, a countless number of therapeutic studies failed to support efficacy in clinical trials [119], suggesting the existence of a missing link in the transition from 'bench to bedside' that is either not understood or easily solved. Preclinical randomized controlled trials (pRCTs) have been proposed as a suitable tool for 'bridging the gap' between experimental research and clinical trials. pRCTs are a valuable tool to increase the reliability of experimental stroke research. The first rigorous pRCT on natalizumab in experimental ischaemic stroke has been successfully performed in Europe - including six research centres - using two different stroke models in mice [120]. It showed remarkable similarity with the results of a concomitant multicentre clinical trial on the same drug in human ischaemic stroke [121]. Despite its successes, this first pRCT also had several difficulties. Researchers identified and summarized two fundamental requirements. First, the experimental protocols must be harmonized among the participating study centres, using as much detail as possible; moreover, the study protocol and analysis strategy should be established *a priori* and made publicly available using platforms for study pre-registration. Second, a pRCT should adopt all of the key elements of clinical efficacy trials, including *a priori* sample size calculation, randomization of the treatment groups, blinding of all investigators concerning treatment allocation, cross-validation between several independent study centres, centralized study organization, and analysis by an independent research centre. Using this study design, results obtained from separate research centres can be pooled as if they were acquired in a single experiment [122]. In 2022, Lyden and colleagues [123] published promising results from the Stroke Preclinical Assessment Network (SPAN), a randomized, placebo-controlled, blinded,

multilaboratory trial which uses a multi-arm multistage protocol to select one or more putative stroke treatments.

Translational stroke research needs an overhaul to render more predictive results. It is therefore important to create the conditions to foster extensive collaborations between different laboratories engaged in preclinical research on ischaemic stroke. A nationwide network of preclinical stroke research laboratories, the Italian Stroke Organization (ISO) Basic Science, was created to perform multicentre translational research studies on highly promising therapeutic strategies in experimental ischaemic stroke, to overcome the barrier between the bench and bedside.

This thesis represents a work carried out within the ISO Basic Science with the aim of clarifying the therapeutic efficacy of remote ischaemic post-conditioning (RIC).

1.12 Remote ischaemic conditioning

The extent of brain damage following an ischaemic stroke can be limited by rapidly rescuing the 'ischaemic penumbra' that is at-risk but not yet infarcted tissue. Among new protective approaches is ischemic conditioning, defined to as intermittent ischaemia and reperfusion cycles, previously shown as a promising potential cardio-protective technique by Murry and colleagues in 1986 [124]. After extensive evaluation in the field of cardiac ischaemia [125–127], the paradigm of remote (i.e. far from the site of injury) ischaemic conditioning (RIC) has been translated to ischaemic stroke [127].

RIC in acute ischaemic stroke

In ischaemic conditioning, transient, intermittent ischaemia is induced either in the organ undergoing spontaneous ischaemia (i.e. conventional conditioning) or at a distance from the affected organ (i.e. indirect or remote ischaemic conditioning). RIC consists of

repetitive inflation and deflation of a cuff around the limb to pressures above systolic blood pressure, intending to protect distant organs such as the heart, kidney or brain. According to the timing of the intervention, RIC can be divided into three types: pre-, per- and post-conditioning. Pre-conditioning (pre-RIC) is a process of endogenous protection in which small, sublethal doses of a harmful agent, such as ischaemia, induce a tolerant phenotype that protects the organism against a later lethal dose of the same agent. Some research showed that ischaemic pre-RIC treatment reduces cerebral damage improving infarct size [128] and neurological outcomes [129–132]. It might be applied as protective conditioning in the event of a future stroke in at-risk individuals. However, its use in the clinical setting has been limited due to the unpredictable nature of cerebral infarctions. Per-conditioning (per-RIC) refers to RIC applied during the early acute stroke phase before reperfusion [133]. Post-conditioning (post-RIC) is a process in which transient insult is applied to a non-vital remote organ following reperfusion of a previously ischaemic organ. Rapid post-RIC might provide neuroprotection when applied immediately after ischaemic stroke and initial reperfusion, and delayed post-RIC could be equally applied days after a stroke [134]. Per- and post-RIC have promise for translation, as they are non-invasive and can be administered pre-hospital (i.e. in an ambulance while transferring stroke victims to the emergency centre) or following recanalization therapy.

RIC has been shown to be effective in pre-clinical models of acute brain ischaemia [135], both alone and in combination with revascularization therapies [136,137]. The first evidence for the protective effects of RIC in cerebral ischaemia comes from Ren and colleagues [138]. They found that induction of a pre-RIC stimulus to the femoral artery reduced infarct size after focal cerebral ischaemia in rats. Then, the focus of researchers shifted toward the application of ischaemic conditioning during and after acute ischaemic stroke (AIS) in animal models, showing the reduction in infarct size and improvement in neurological

scores [139]. Post-RIC showed a reduction in infarct size of 63% when RIC was applied immediately after reperfusion, while a 43% of reduction in infarct size was present when RIC was applied 3 hours post-stroke induction. Additional investigations advanced the theory of "two-time windows for protection" [140–142]. The early phase of protection occurs immediately, within minutes after the conditioning application, and lasts for about 3 hrs. The late phase starts 18-24 hrs after post-conditioning and lasts for 4 days (**Figure 1.8**). In addition to the short-lasting benefits of acute RIC, long-term benefits induced with repeated daily conditioning were investigated by Hess *et al*. [143,144]. Despite the effects of RIC in animal studies, only few clinical trials explored the effect of RIC in patients with stroke [145–150]. These studies show that RIC is well tolerated and has no severe adverse effects in AIS patients [151]. According to a recent review [152,153] up to now, 19 studies applied RIC. Among them, 6 studies applying per-RIC to acute ischaemic stroke patients have been completed and 13 are ongoing.

Trials in AIS

Trials of RIC in cerebrovascular disease are underway and small trials have been completed [154]. Interpreting results will, however, be challenging since they are full of heterogeneity in terms of RIC protocols and stroke subtype assessed. Preclinical data have demonstrated that RIC during acute ischaemia is effective when applied both alone and in combination with revascularization therapies [137]. In clinical trials, findings on clinical endpoints of RIC for acute ischaemic stroke are even more limited. Neurological outcomes did not differ significantly between patients undergoing pre-hospital RIC and controls. Only four ongoing studies have clinical endpoints as primary outcomes: REMOTE-CAT, SERIC AIS, RESIST, and RICAMIS. Other trials that are investigating the effect of RIC on radiological biomarkers, such as brain infarction volume - rtPA-RIC

(NCT02886390); PROTECT I (NCT03915782); REVISE-2 (NCT03045055); RICEPAC (NCT03152799); REPOST - are planned or ongoing. On the other hand, a meta-analysis by Zhao et al. found that post-RIC may not only reduce the risk of recurrent stroke but also the mRS and the NIHSS [155]. For that, there are still proposals to study the effect of RIC in other subgroups of patients. Diamanti et al. [156] designed the multicentre phase II study TRICS-9 to assess the efficacy of RIC in patients with AIS within 9 hrs of onset who are not candidates for recanalization therapies.

The lack of conclusive evidence of RIC efficacy in AIS may be due to:

- incompleteness in methodological reporting, paucity of studies using female rodents, use of invasive RIC, different exposure to anaesthetic conditions, missing of infarct volume measurements at multiple times including very delayed time points (in preclinical studies);
- lack of heterogeneous protocols and patient stratification, different RIC applications and timing, patient's comorbidities and comedications (in clinical trials).

Putative mechanism and effects of RIC

RIC can target different cellular and molecular processes occurring during ischaemia and reperfusion. The exact mechanisms of this remote organ protection from ischaemia are unknown and could differ between pre-, per-, and post-conditioning. Until now, three pathways are thought to play a role in RIC protection: humoral signalling, neural pathway or modulation of the systemic immune system [157]. The underlying mechanisms of RIC include neurovascular protection, induced anti-inflammatory action and neuronal protection against excitotoxicity; paired together with mitochondrial protection, circulating

inflammasome activation and/or transcriptional regulation of the neuroprotective pathway[158].

Although the three protective signal pathways are distinct, both neuronal and immunological pathways are linked to the humoral pathway. The humoral hypothesis postulates that the IR cycles in a distant site, like a limb for ischaemic stroke, may have humoral (blood-borne) nature. After every brief ischaemic cycle, followed by a brief reperfusion cycle, the factors secreted during the ischaemic conditioning flow in the bloodstream toward the target organ. The secretion and the humoral release of some factors may lead to the activation of sensory afferent nerves. For example, the release of autocoids - adenosine, bradykinin, catecholamines, opioids, and prostaglandins - at the site of remote ischaemia may initiate neuronal and humoral signal transduction, contributing to the protective effects of RIC [159]. Some autacoids can stimulate the afferent neural pathways, while others, such as nitric oxide (NO) and endothelin (ET), are mainly characterized by vasoactive effects on the blood vessels [144]. RIC has been shown to have a systemic anti-inflammatory influence. RIC can inhibit not only the activation of the brain resident immune cells - microglia and astrocytes - following an acute ischaemic stroke but also the recruitment of circulating peripheral immune cells - neutrophils, monocytes, and T cells - into the ischaemic brain [160,161].

After RIC, there are two proposed waves of protection (**Figure 1.8**).

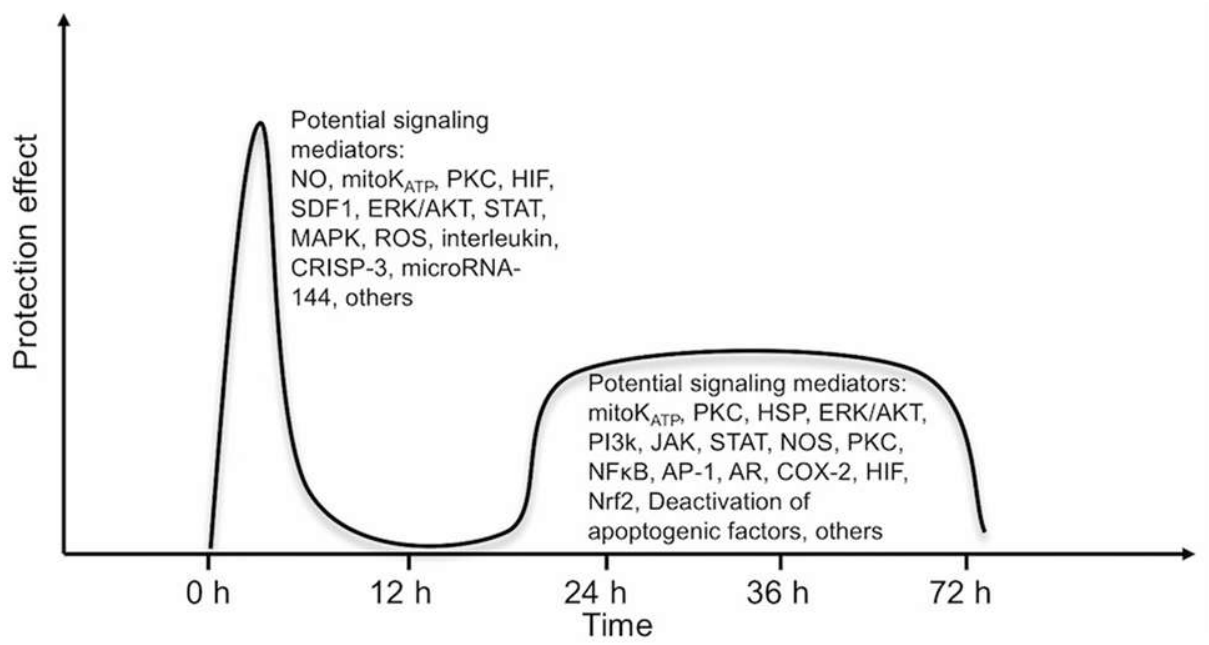


Figure 1.8 Proposed mediators involved in the two waves of protection following remote ischaemic conditioning (RIC). [162]

The first wave is immediate and short, beginning at 0 until 2 hours. During this time, there are changes in ion permeability, protein phosphorylation, and release of several protective mediators - NO, microRNAs, Erk/Akt, and hypoxia-inducible factor (HIF) [138,163]. The second wave, referred to as the second window of protection (SWOP), follows 12–24 hours later and lasts 48–72 hours [164]. SWOP may change inflammation response, improve endothelial function and activate protein expression - heat shock proteins (HSP), nitric oxide synthase (NOS), Erk/Akt) [165–168].

Remote ischaemic conditioning is a promising therapy for AIS. The current preclinical evidence contains many gaps that presently may limit successful clinical translation. Future well-designed randomized controlled studies should include a more comprehensive description of methods, inclusion criteria, the optimal time windows for

RIC application and the population that could most benefit from this non-invasive, safe and cheap strategy.

2. AIM OF THE STUDY

There are two main topics investigated in this thesis: 1) the harmonization of surgical procedures and behavioural protocols across the seven centres involved; 2) the assessment of RIC efficacy in the rodent model of endovascular MCA occlusion followed by reperfusion.

1) Harmonization phase

A model of transient ischaemia (tMCAo) was applied in mice and rats of both sexes (n=11 for each species). This phase was carried out considering different aspects:

I) to increase the variability in the outcome, a tMCAo model of 30, 45 or 60 minutes was applied in mice and of 50, 75 or 100 minutes in rats;

II) to limit bias associated with the De Simoni neuroscore evaluations, I prepared tutorial videos on sham and ischemic animals;

III) to determine the reliability of the behavioural score assigned by each rater, all animals were video recorded;

IV) to analyse the data, all videos were sent to coordinating centres and thus randomized. Subsequently, the assessments by each centre were forwarded to our Institute for statistical analysis of the interrater agreement. All analyses were performed blindly.

2) Interventional phase

I used a model of tMCAo (60 minutes of occlusion) in mice (n=24) of both sexes. The tMCAo model (100 minutes of occlusion) was applied in rats of both sexes. RIC was applied by a single transient occlusion of the right femoral artery by surgical clipping performed after reperfusion of the proximal MCA. The main aim of this phase was to test the RIC efficacy in two well characterize models of ischaemia.

3. MATERIALS AND METHODS

3.1 Structure of the TRICS network

The TRICS network is centrally managed by the coordinating centre, the University of Milano-Bicocca, and consists of 7 research laboratories (see Experimental design). The relationship among all TRICS participants is illustrated in **Figure 3.1**. The coordinating centre is charged with collecting all data, performing quality control on all data, conducting image acquisition and coordinating all manuscripts. The TRICS research laboratories are charged with reaching a consensus on the best practices for all protocols, conducting stroke surgeries and behavioural testing in mice and/or rats.

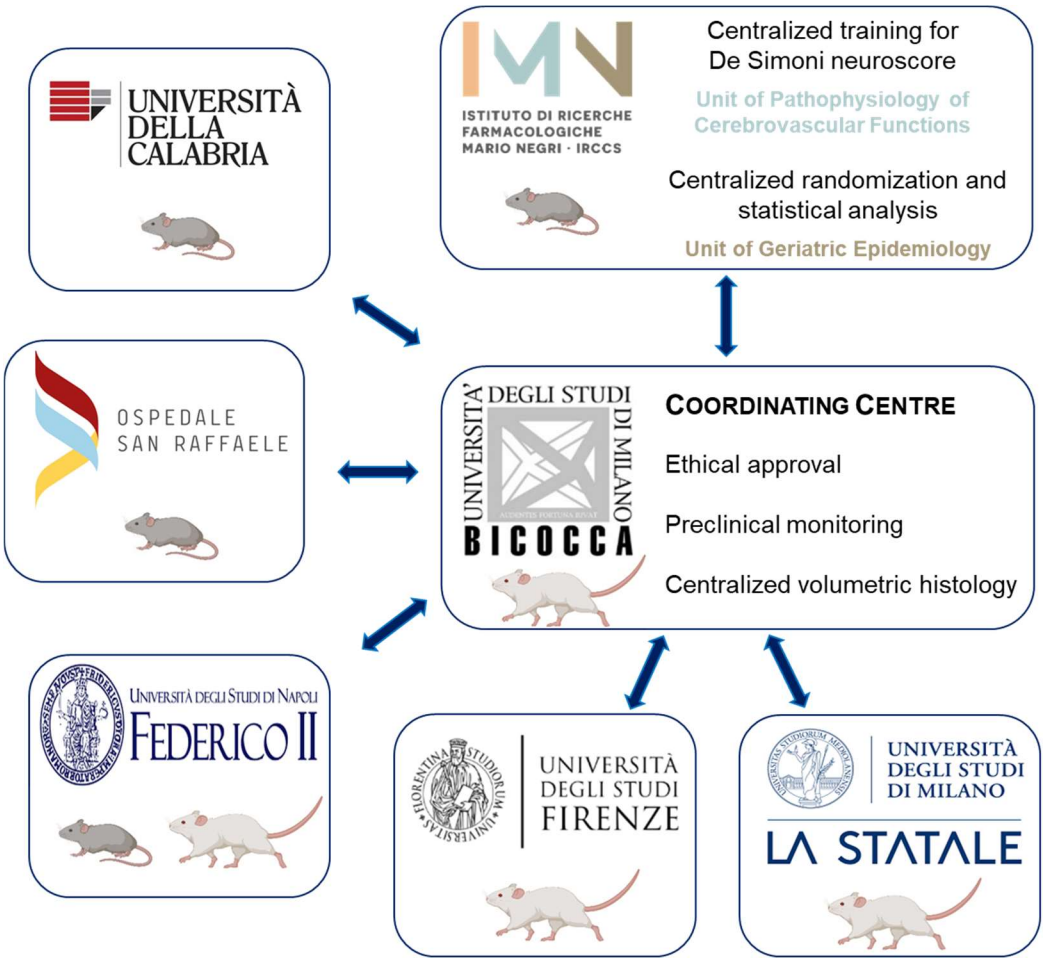


Figure 3.1 TRICS network structure.

3.2 Animals

Procedures involving animals and their care were conducted in conformity with institutional guidelines that comply with national (Decree-Law No. 26/2014; authorization No. 1056/2020-PR, protocol FB7CC.43, by the Ministry of Health) and international (EEC Council Directive 2010/63/UE; Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council 2011) laws and policies (Quality Management System Certificate-UNI EN ISO9001: 2008 - Reg. N° 6121). All experiments on animals were approved by the Ethics Committee of the University of Milano-Bicocca, the Coordinating Centre of the TRICS project. The experimental protocol of which the study is part was registered with the following number: PCTE0000177 on https://preclinicaltrials.eu.

At 'Mario Negri' Institute, animals were housed in a Specific Pathogen Free (SPF) *vivarium*, 5 per cage and kept at constant temperature (21 ± 1 °C) and relative humidity (60%) with a regular light/dark schedule (12/12 hours). Food and water were available *ad libitum*. Before beginning any procedure, mice were housed for at least 1 week in their home cages under the conditions described above. Before and after surgery, the animals were housed in a single cage.

The strains used in the project were:

- C57BL/6J wild-type (WT) mice (body weight of 24g ± 10%; Charles Rivers Laboratories, Italy) male and female 1:1 (regardless of oestrous cycle);
- Sprague-Dawley rats (body weight of 250g ± 5%), male and female 1:1 (regardless of oestrous cycle).

3.3 Experimental design

Animals were assigned to surgery and experimental group following a pre-published protocol [169]. To minimize the variability, all surgeries were performed by the same investigator in each centre. All subsequent evaluations were done by blinded investigators. All experiments were carried out in animal facilities of the seven Italian academic and research institutions:

- Istituto di Ricerche Farmacologiche Mario Negri (IRFMN), the University of Calabria (UniCal) and San Raffaele Hospital (HSR) that use mice as animal model;
- The University of Firenze (UniFi), the University of Milano-Bicocca (UniMiB) and the University of Milano Statale (UniMi) that use rats as animal model;
- The University of Napoli (UniNa) uses both species as animal models.

Experiments were conducted according to the plan detailed in **Figure 3.7, 5.5**.

3.4 Surgery protocol

Transient middle cerebral artery occlusion (tMCAo)

Anaesthesia was induced by 3% isoflurane inhalation in an N_2O/O_2 (70/30%) mixture and maintained with 1.5% isoflurane inhalation in the same mixture. During the surgery, the animal was placed supine on a thermostatic bed (LSI-Letica, Spain) equipped with a rectal probe to monitor and maintain the temperature at 37 ± 0.5°C. The surgical site was disinfected with clorexyderm 4% solution and a 1 cm incision was made in the midline of the neck. Using a dissecting microscope (Seto, Japan), the right common carotid artery (CCA) was carefully dissected free from the surrounding nerves (without harming the vagal nerve), was isolated and a ligature was made upstream of the bifurcation between the internal (ICA) and external (ECA) carotid artery using 7–0 string. An arteriotomy was performed downstream of the ligation to allow insertion of a silicone rubber-coated

monofilament (for mice: size 7–0, diameter 0.06–0.09 mm, length 10±1 mm; diameter with coating 0.23 mm; coating length 6 mm; for rats: size 5–0, diameter with coating 0.33mm; coating length 5–6 mm; Doccol Corporation, Redlands, California, USA) into the ICA, which was pushed cranially to occlude the origin of the middle cerebral artery (MCA). Since the diameter of the MCA is smaller than the anterior cerebral artery, only the first was blocked by the filament (**Figure 3.2**).

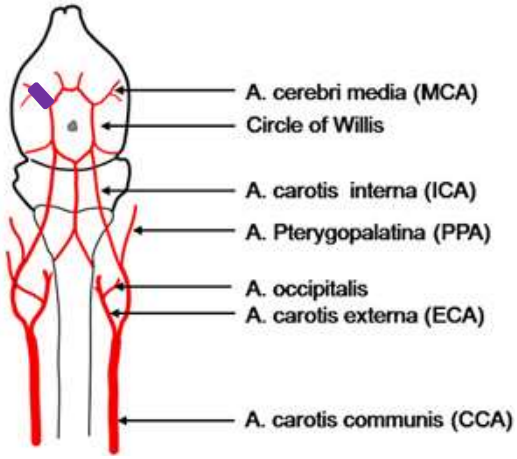


Figure 3.2 Scheme of the vessel architecture in a mouse from a ventral view. The violet line represents the occlusion of the middle cerebral artery (MCA) (adapted from Engel *et al.* [170]).

To ensure better variability in the outcome, during the harmonization phase of the project three different occlusion times were performed: 30, 45 or 60 minutes for mice and 50, 75 or 100 minutes for rats. In the interventional phase, mice were subjected to tMCAo for 60 minutes and rats for 100 minutes, both with a reperfusion period of 48 hours. For pain relief, Emla creme (Aspen Pharma) was topically applied to the wound. Animals stayed for 60 or 100 minutes of occlusion in a heated cage. Afterwards, second anaesthesia was performed and blood flow was restored by carefully removing the Doccol filament.

Sham-operated animals (MCA-) received a midline neck incision and the subsequent exposure of the carotid sheath. The external carotid artery and its branches were isolated without being ligated.

After surgery, animals were housed in single cages

3.5 Inclusion and exclusion criteria

Rats and mice have been included in the study if cerebral ischaemia was successfully induced, that was, filament correctly positioned in the MCA origin. After MCA occlusion, the following intra-ischaemic clinical assessment score was applied. Animals were judged ischaemic, and included in the trial if presenting ≥3 of the following deficits (**Figure 3.3**) during the intra-ischaemic period:

1. The palpebral fissure had an ellipsoidal shape (not the normal circular one);
2. One or both ears extended laterally;
3. Asymmetric body bending on the ischaemic side;
4. Limbs extended laterally and do not align with the body.

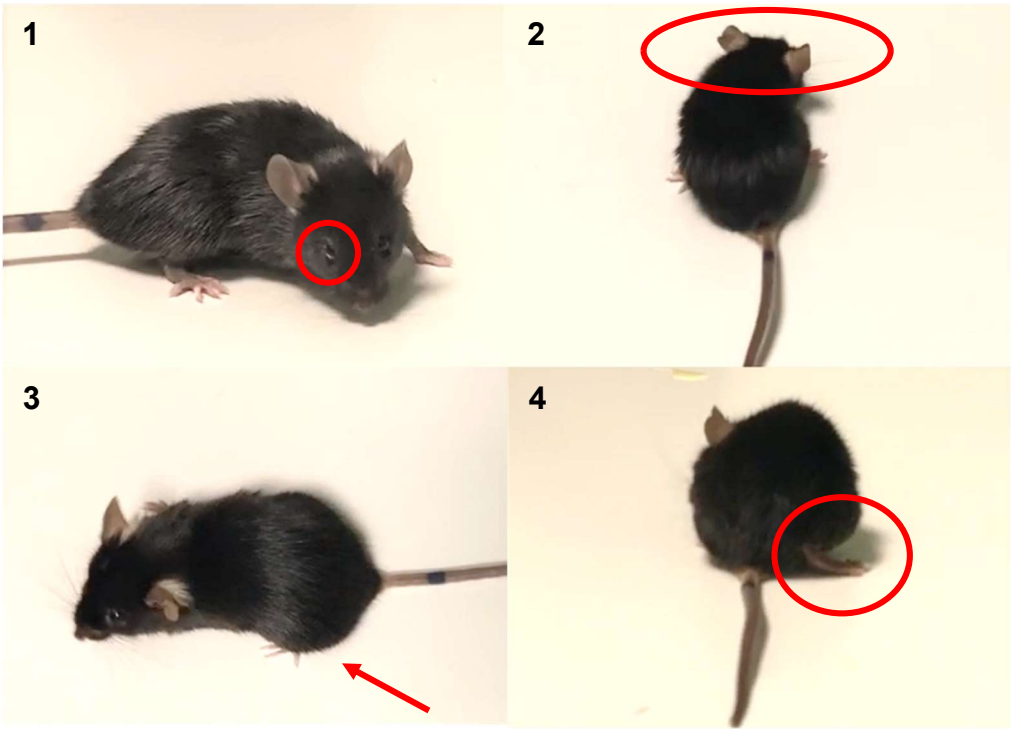


Figure 3.3 Evaluation of intra-ischaemic deficits for the animals' inclusion. (1) Ellipsoidal shape of palpebral fissure. **(2)** Ears extended laterally. **(3)** Asymmetric body bending on the ischaemic side. **(4)** Extended limb not align with the body.

Animals were excluded in case of:

1. Death during MCAo surgery;
2. Major experimental protocol violations: errors or surgical complications (eg, major arterial or venous haemorrhage, section of the vagus nerve, carotid artery dissection, filament entrapment or displacement) during MCA occlusion procedure; errors in ischaemia time.

According to these criteria, we considered that approximately 30% of animals would be excluded.

3.6 Interventions

Remote Ischaemic post-Conditioning (RIC) was induced by surgical transient occlusion of the femoral artery (FAo) [167]. Briefly, the femoral artery was identified, isolated and occluded with microserrefines clips (18055-05 for rats, and 18055-04 for mice; Fine Science Tools) to stop the blood flow for 20 min in rats and 10 min in mice (**Figure 3.7**). The achievement of femoral artery blockage was verified by visual inspection of the distal femoral artery territory.

Sham-operated animals (RIC-) were subjected to the same surgical procedure except for the occlusion of the femoral artery which did not take place.

3.7 Health monitoring

Animals were observed twice at 24 and 48 hours after surgery, before the behavioural testing. A predefined Middle Cerebral Artery occlusion (MCAo) health report (available at https://figshare.com, DOI: 10.6084/m9.figshare.13031861), prepared based on the Ischaemia Models: Procedural Refinements Of in Vivo Experiments (IMPROVE) guidelines, were filled at baseline, at 24 hours and 48 hours with information on animal welfare. Animals showing signs of moderate distress (orange box in **Figure 3.4**), according to the MCAo health report, received subcutaneous buprenorphine 0.05–0.1 mg/kg every 12 hours (this dose is used for both rats and mice). Animals showing signs of severe distress (red box in **Figure 3.4**), according to the MCAo health report, were sacrificed before the end of the experiment. These animals, if sacrificed after RIC/sham application, were retained in the intention-to-treat (ITT) analysis, and given the highest (i.e., worst) score in the behavioural testing.

ID CODE				
		YES	NO	comment
Low distress	Reduced food and water intake			
	Abnormal behaviour upon handling (increased or decreased reaction to being handled)			
	Lethargy and reduced motility			
	Piloerection / staring coat			
	Discharge from the eyes and nose			
Moderate distress	Animal not drinking			
	Animal not eating (including wet mash)			
	Surgical wound complication			
	Absence of faeces			
	Audible respiratory noises (rasping, wheezing), intermittent, without respiratory effort			
	Weight loss exceeding 10%.			
High distress	Presence of barrel rolling			
	Presence of tonic clonic seizures			
	Continuous laboured respiration with increased respiratory effort			
	Animal not moving, unresponsive to stimulation, or in a lateral recumbent position			
	Weight loss exceeding 20%.			
Death	Dead animal			
ANALGESIC DRUG AND DOSE				
EUTHANASIA PERFORMED				
COMMENTS				

Figure 3.4 Health report adapted from the IMPROVE guidelines [118].

3.8 Behavioural testing

At 48 hours after the induction of the ischaemia, each centre performed the De Simoni neuroscore [171]. An evaluator for each centre was designated, selecting a different person than that who carried out the surgeries. Each animal was assigned a score ranging from 0 (absence of deficits) to 56 (worst neurological result). The general deficits describe the general well-being of the mouse with a score between 0 and 28. This score includes information on the physical appearance of the mouse, i.e.: fur (0-2), ears (0-2), eyes (0-4), posture (0-4), spontaneous activity (0-4) and presence of epileptic seizures (0-12) (**Figure 3.5**). Focal deficits describe neurological damage with a score between 0 and 28 and were evaluated through observations on body symmetry (0-4), gait (0-4), ability to climb a 45° inclined plane (0-4), circling behaviour (0-4), forelimb symmetry (0-4),

compulsory circling (0-4) and whisker response (0-4) (**Figure 3.6**). All evaluations were entered on the RedCap platform and sent to the coordinator for statistical analysis.

GENERAL DEFICITS	
Hair	○ Hair neat and clean. ○ Localized piloerection and dirty hair in 2 body parts (typically nose and eyes). ○ Piloerection and dirty hair in more than 2 body parts.
Ears Animal on OBT. Observation at the beginning with no interference, then stimulating by snapping fingers.	○ Normal. Ears are stretched laterally and behind. They react to noise. ○ Stretched laterally but not behind (one or both). They react to noise. ○ Stretched laterally but not behind (one or both). They do not react to noise.
Eyes Animal on OBT. Observation with no interference or stimulation.	○ Open, clean and quickly follow the surrounding environment. ○ Open and characterized by aqueous mucus. Slowly follow the surrounding environment. ○ Open and characterized by dark mucus. ○ Ellipsoidal shaped and characterized by dark mucus. ○ Closed.
Posture Place the animal on the palm and swing gently.	○ The animal stands in the upright position with the back parallel to the palm (during the swing, it stands rapidly). ○ The animal stands humpbacked. During the swing, it flattens the body to gain stability. ○ The head or part of the trunk lies on the palm. ○ The animal lies on one side, barely able to recover the upright position. ○ The animal lies in a prone position, not able to recover the upright position.
Spontaneous activity Animal on OBT. Observation with no interference or stimulation.	○ The animal is alert and explores actively. ○ The animal seems alert, but it is calm and sluggish. ○ The animal explores intermittently and sluggishly. ○ The animal is somnolent and numb, few movements on-the-spot. ○ No spontaneous movements.
Epileptic behavior Animal on OBT. The worse epileptic behavior detected during the whole observational period should be recorded and reported according to the following score.	○ None. ○ The animal is reluctant to handling, shows hyperactivity. ○ The animal is aggressive, stressed and stares. ○ The animal shows hyperexcitability, chaotic movements and presence of convulsion following handling. ○ Generalized seizures associated with wheezing and unconsciousness.

Figure 3.5 General deficits of De Simoni neuroscore.

FOCAL DEFICITS	
Body symmetry Animal on OBT, observation of undisturbed resting behavior and description of the virtual nosetail line.	○ Normal. BODY: normal posture, trunk elevated from the bench, with fore and hindlimbs leaning beneath the body. TAIL: straight. ○ Slight asymmetry.BODY: leans on one side with fore and hindlimbs leaning beneath the body. TAIL: slightly bent. ○ Moderate asymmetry. BODY: leans on one side with fore and hindlimbs stretched out. TAIL: slightly bent. ○ Prominent asymmetry. BODY: bent, on one side lies on the OBT. TAIL: bent. ○ Extreme asymmetry. BODY: highly bent, on one side constantly lies on the OBT. TAIL: highly bent.
Gait Animal on OBT. Observation of undisturbed movements.	○ Normal. Gait is flexible, symmetric and quick. ○ Stiff, inflexible. The animal walks humpbacked, slower than normal mice. ○ Limping with asymmetric movements. ○ Trembling, drifting, falling. ○ Does not walk spontaneously. When stimulated, the animal walks no longer than three steps
Climbing Animal on a gripping surface 45° to OBT. Place the animal in the centre of the gripping surface.	○ Normal. The animal climbs quickly. ○ Climbs with strain, limb weakness present. ○ Holds onto slope, does not slip or climb. ○ Slides down slope, unsuccessful effort to prevent fail. ○ Slides immediately, no effort to prevent fail.
Circling behavior Animal on OBT. Observation of the animal walking undisturbed on the OBT.	○ Absent. The animal equally turns left or right. ○ Predominantly one-sided turns. ○ Circles to one side, although not constantly. ○ Circles constantly to one side. ○ Pivoting, swaying, or no movement.
Forelimb symmetry Animal suspended by the tail. Movements and position of forelimbs are observed.	○ Normal. Both forelimbs are extended towards the bench and move actively. ○ Light asymmetry. Contralateral forelimb does not extend entirely. ○ Marked asymmetry. Contralateral forelimb bends towards the trunk. The body slightly bends on the ipsilateral side. ○ Prominent asymmetry. Contralateral forelimb adheres to the trunk. ○ Slight asymmetry, no body/limb movement.
Compulsory circling Forelimbs on bench, hindlimbs suspended by the tail. This position reveals the presence of the contralateral limb palsy.	○ Absent. Normal extension of both forelimbs. ○ Tendency to turn to one side. The animal extends both forelimbs but starts to turn preferably to one side. ○ Circles to one side. The animal turns towards one side with a slower movement compared to healthy mice. ○ Pivots to one side sluggishly. The animal turns towards one side failing to perform a complete circle. ○ Does not advance. The front part of the trunk lies on the bench. Slow and brief movements.
Whisker response Animal on the bench. Using a pen, touch gently the whiskers and the tip of the ears from behind, first one the lesioned and then on the contralateral side.	○ Normal symmetrical response. The animal turns the head towards the stimulated side and withdraws from the stimulus. ○ Light asymmetry. a. The animal withdraws slowly when stimulated on the ischemic side. b. Normal response on the contralateral side. ○ Prominent asymmetry. a. No response when stimulated on the ischemic side. b. Normal response on the contralateral side. ○ Absent response ipsilaterally, slow response when stimulated on the contralateral side. ○ Absent response bilaterally.

Figure 3.6 Focal deficits of De Simoni neuroscore.

3.9 SPECIFIC METHODS IN THE HARMONIZATION PHASE

3.9.1 Training and evaluation of neurological deficits

A centralised training for the correct administration of the De Simoni neuroscore was performed by the Unit of Pathophysiology of Cerebrovascular Functions in 'Mario Negri' Institute in multiple meetings before starting randomization for the harmonization phase. In this part of the study in order to limit variability in the interpretation of the De Simoni neuroscore, I prepared two tutorial videos, one on a sham mouse and one on an ischaemic mouse, showing in a detailed way the execution of the assessment.

During the harmonization phase, each centre recorded on video the behavioural testing. The total amount of recorded videos was n=11 for mice and n=11 for rats among all the centres. Each evaluator analyzed each of the 11 videos by the De Simoni neuroscore, blinded to the experimental conditions. The assigned scores were analysed centrally to provide feedback to limit subjective evaluations by the evaluators.

3.9.2 Primary outcome

Assessment of the interrater reliability of the De Simoni neuroscore measured at 48 hours using intraclass correlation coefficient (ICC), setting a target of ICC≥ 0.60 as a requirement to start the interventional phase.

3.9.3 Experimental groups

In protocol paper [169], the use of 120 total animals per species was anticipated. In the harmonization phase, we performed tMCAo in a fixed number of mice and rats per centre (**Table 3.1**):

Experimental groups	**MCAo+ 30 min**	**MCAo+ 45 min**	**MCAo+ 60 min**
Total mice number (first trial)	4	3	4
Total mice number (second trial)	2	2	7
Experimental groups	**MCAo+ 50 min**	**MCAo+ 75 min**	**MCAo+ 100 min**
Total rats number (first trial)	4	3	4
Total rats number (second trial)	3	2	6

Table 3.1 Experimental groups of the harmonization phase

3.9.3.1. Sample size

The sample size for the interrater agreement was calculated by a statistician, starting from the knowledge that 4 raters were available for each species. The expected intraclass correlation coefficient was estimated to be approximately 0.80. When the sample size is 11, a two-sided 95% confidence interval computed using the large sample normal approximation for an intraclass correlation was calculated to extend about 0.17 from the observed intraclass correlation.

3.9.4 Statistics

3.9.4.1 Intraclass correlation coefficient

The intraclass correlation (ICC) assesses the reliability of ratings by comparing the variability of different ratings of the same subject to the total variation across all ratings and all subjects. To limit the use of animals, the power analysis performed indicated that n=11 animals for species were necessary. The sample size was calculated starting from the knowledge that 4 raters were available for each species. The expected intraclass correlation coefficient (ICC) was estimated to be approximately 0.80. The ICC ranges from -1 (perfect disagreement) to 0 (absence of agreement) to +1 (perfect agreement). When the sample size is 11, a two-sided 95% confidence interval computed using the large sample normal approximation for an intraclass correlation was calculated to extend about 0.17 from the observed intraclass correlation. In our study, we set the minimum ICC value to be reached at 0.60 in order to consider the evaluations between the centres sufficiently in agreement.

Fleiss's Kappa

Fleiss's Kappa is a tool to test the agreement between raters. The κ coefficient ranges from 0 (no agreement or agreement that one would expect to find by chance) to 1 (perfect match).

$$\kappa = \frac{\bar{P} - \bar{P}_e}{1 - \bar{P}_e}$$

in which ($1-P_e$) gives the degree of agreement that is reachable above the case and ($P-P_e$) gives the degree of the agreement reached above the case.

The first step involves the calculation of the values of P_j and P_i, obtained by applying the following formulas:

$$p_j = \frac{1}{Nn}\sum_{i=1}^{N} n_{ij}, \qquad 1 = \sum_{j=1}^{k} p_j$$

$$P_i = \frac{1}{n(n-1)}\sum_{j=1}^{k} n_{ij}(n_{ij}-1)$$

$$= \frac{1}{n(n-1)}\sum_{j=1}^{k}(n_{ij}^2 - n_{ij})$$

$$= \frac{1}{n(n-1)}\left[\left(\sum_{j=1}^{k} n_{ij}^2\right) - (n)\right]$$

where

n is the number of evaluators per subject and therefore the number of evaluators (*n*=4 for mice and *n*=4 for rats); *N* is the number of subjects (in the harmonization phase of the project: *N*=11 mice and *N*=11 rats); *k* is the number of categories based on which the evaluations are carried out (in our case *k*=2, “good” and “bad”, in the dichotomized evaluation of De Simoni neuroscore).

The subjects are indexed by *i= 1, ... N* and the categories are indexed by *j= 1, ... k*; n_{ij} is the number of evaluators who have assigned the same category to the same subject. P_j represents the proportion of all evaluations of the same category. The following formulas are used to calculate P and Pe:

$$\bar{P} = \frac{1}{N}\sum_{i=1}^{N} P_i$$

$$= \frac{1}{Nn(n-1)}\left(\sum_{i=1}^{N}\sum_{j=1}^{k} n_{ij}^2 - Nn\right)$$

$$\bar{P}_e = \sum_{j=1}^{k} p_j^2$$

Cohen's Kappa

The evaluations that each of the 7 centres obtained from carrying out the De Simoni neuroscore on the 11 videos of the mice and 11 of the rats were then compared in pairs. The interobserver agreement of De Simoni neuroscore comparing pairs of raters was described using Cohen's κ, ranging from κ=0 (equivalent to chance) to κ=1 (perfect agreement).

$$\kappa = \frac{p_o - p_e}{1 - p_e} = 1 - \frac{1 - p_o}{1 - p_e},$$

3.10 SPECIFIC METHODS IN THE INTERVENTIONAL PHASE

In the interventional phase of the project, the study was conducted according to the plan depicted in **Figure 3.7**. Wild-type C57BL6J male and female mice were subjected to 60 min tMCAo. RIC was induced at 10 min after reperfusion for a duration of 10 min. Sprague-Dawley male and female rats were subjected to 100 min tMCAo. RIC was induced at 20 min after reperfusion for 20 min. Animals were monitored at 24 and 48 hours post-surgery according to the IMPROVE guidelines to identify those needing the administration of a painkiller (i.e. Buprenorphine) or sacrifice according to the humane endpoint. At 48 hours after tMCAo animals underwent the De Simoni neuroscore.

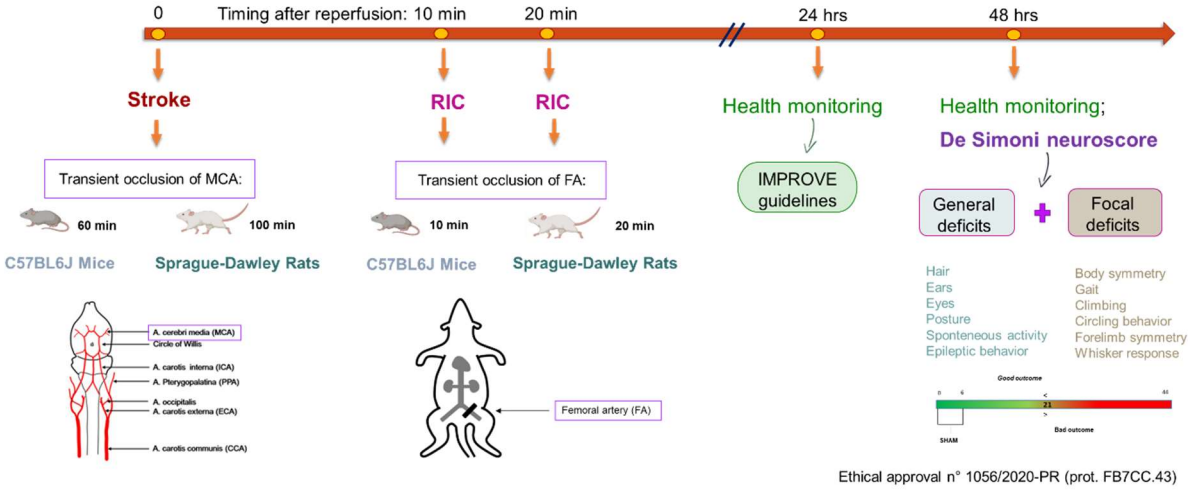


Figure 3.7 Experimental plan of the interventional phase.

Data were collected and managed using REDCap electronic data capture tools [172] hosted at the Istituto di Ricerche Farmacologiche "Mario Negri" IRCCS. In each centre, the surgeon (**Figure 3.8**) and the person designed for the health monitoring and De Simoni neuroscore assessment (**Figure 3.9**) were provided with a personal username and a password that were used to input data on the platform.

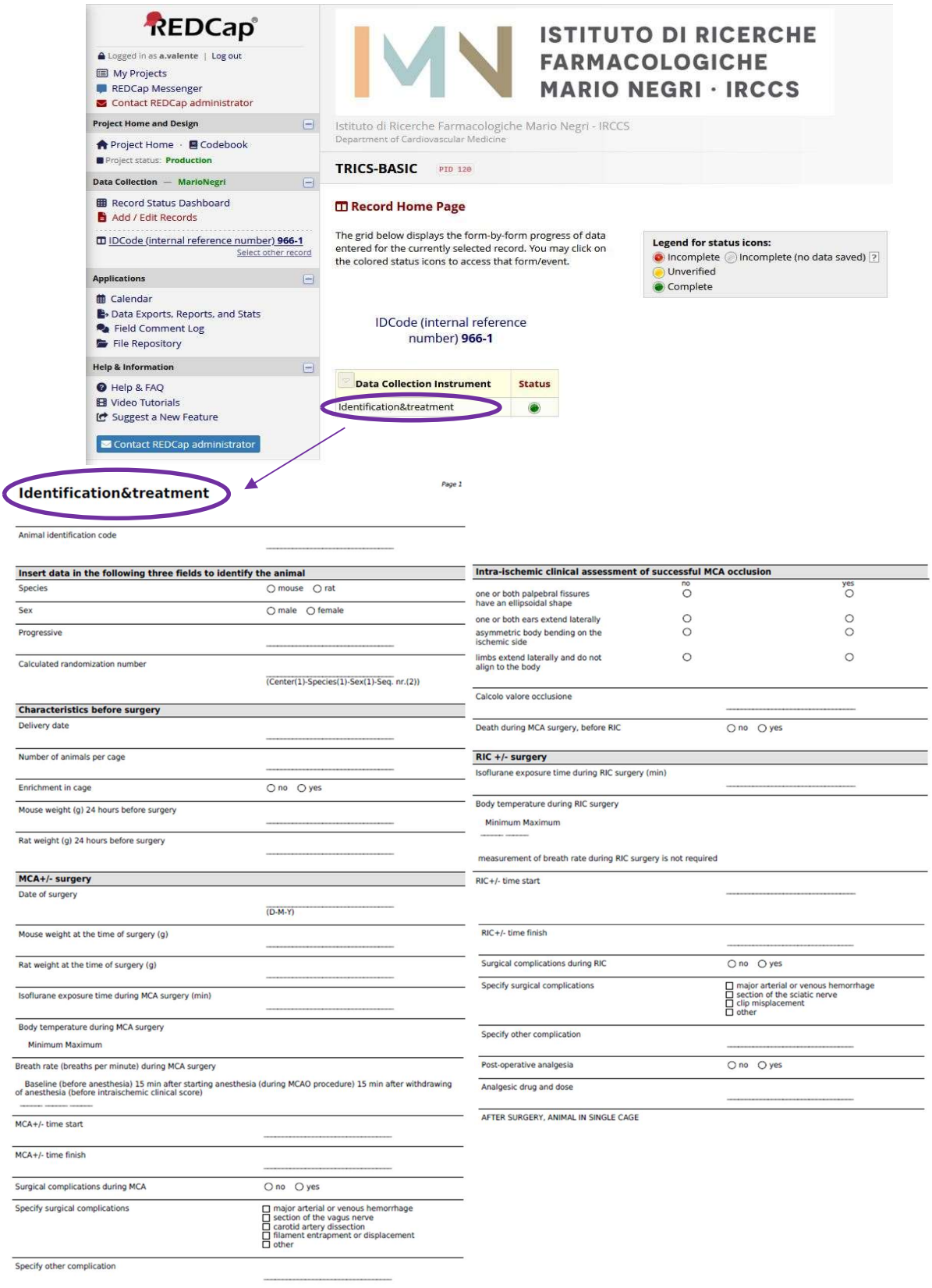


Figure 3.8 Interface for data input by the surgeon.

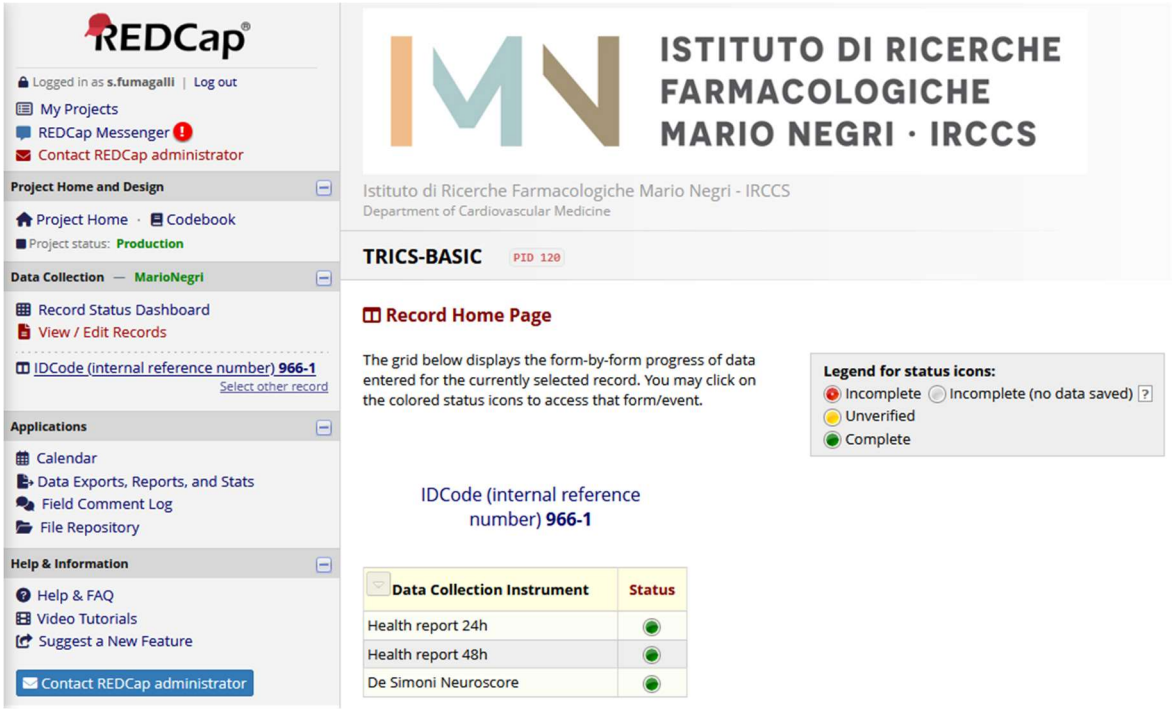


Figure 3.9 Interface for data input by the designed rater.

3.10.1 Outcomes

Primary outcome

The difference in the proportion of rats or mice with a good functional outcome was measured by the De Simoni composite neuroscore (13 items, range 0-56 points) at 48 hours after MCA occlusion, that is, the proportion of animals scoring 20 or less. A dichotomised functional outcome (0-20, good outcome; 21-56 bad outcome) was chosen according to the translational approach of this study.

Secondary outcome

- Infarct volume (mm^3) measured by volumetric histology at 48 hours after MCA occlusion.
- The difference in the composite neuroscore, as a continuous variable, at 48 hours after MCA occlusion.

3.10.2 Experimental groups

In the interventional phase, we had four experimental groups:

1. MCA+/RIC+ (MCA occlusion, treated with RIC; treatment group);
2. MCA+/RIC- (MCA occlusion, sham femoral artery surgery; control group);
3. MCA-/RIC+ (sham carotid artery surgery, treated with RIC; single sham group);
4. MCA-/RIC- (sham carotid artery surgery, sham femoral artery surgery; double sham group).

We performed surgery on a fixed number of animals per centre (**Table 3.2**):

Experimental groups	MCAo+/RIC+	MCAo+/RIC-	MCAo-/RIC+	MCAo-/RIC-
Total mice number (male/female ratio 1:1)	40	40	8	8
Mice per centre (four laboratories)	10	10	2	2
Total rats number (male/female ratio 1:1)	40	40	8	8
Rats per centre (four laboratories)	10	10	2	2

Table 3.2 Experimental groups of the TRICS preclinical trial

Sample size

The number of animals to be used in the experiments was defined separately by MCA arms. The statistician used Power and Precision, V.4.1 (Biostat, Englewood, New Jersey, USA) for power calculations. The primary objective of the proposed study is to test the null hypothesis that the proportion of good functional outcome (according to dichotomised De Simoni composite neuroscore) is identical in the two arms (MCA+ arm and MCA- arm). We know that good functional outcome is present in approximately 20% of the RIC- animal. We posed that the minimum difference in proportions should be equal

to or higher than 30 percentage points to be of substantive translational significance, that is, RIC+ animals should have a good functional outcome of 50% against the standard 20% in RIC- animals.

Each of the two trials had therefore a total of 80 animals randomised to the MCA+ arm. In line with the ethical requirement of reducing the number of animals used for preclinical research, we included four animals per centre per MCA- arm, since they served as internal controls for neurobehavioural assessment.

3.10.3 Blinding and randomization

To facilitate blinding, each centre designed a surgeon. No person assisting the surgeon in any way assessed functional outcomes. The designed surgeon received 30 sealed, non-transparent, non-resealable envelopes per treated species. Envelopes were marked with a code from Male01 to Male15 and from Female01 to Female15. Envelopes were preferred to online randomisation due to logistic constraints: unavailability of online access in some animal care areas. The external envelope with the pre-treatment (MCA+/MCA-) contained another envelope: this internal envelope contained the treatment option (RIC+/RIC-) and was opened only after a successful MCA surgery to guarantee allocation concealment to surgeons. All envelopes and randomization lists were prepared by personnel not involved in the implementation of the procedures with animals.

Post-operative animal care, behavioural assessment and image acquisition were all completed by investigators unaware of the assigned treatment.

Sequence generation for randomization

Two randomised lists were produced, separately by species and stratified by centre and sex. The lists were produced using a pseudo-random number generator, using permuted blocks of random size: a procedure implemented using JMP Pro was used to generate the list. The allocation ratio (MCA+/RIC+: MCA+/RIC-: MCA-/RIC+: MCA-/RIC-) was 10:10:2:2. Since the two sexes were equally represented, for each sex every centre treated 5+5+1+1 animals, with three available replacements.

Animal replacement

Animals sacrificed before the RIC application were replaced, up to a total number of three per centre, per species and per sex. The new animal received the same MCA treatment as the replaced animal. This missing randomisation (MCA+/MCA-) did not imply the primary analysis since this analysis was done only on the difference between RIC+ and RIC-, and this last randomisation took place independently and after MCA intervention.

5. RESULTS

In the training phase of the project, I prepared video tutorials on sham and ischaemic mice explaining the evaluation of sensorimotor deficits using the De Simoni neuroscore (**Figure 5.1, 5.2**). Video tutorials on sham and ischaemic rats were prepared by UniNa following our scoring system. Tutorials were administered to each rater of the participating centres before starting the evaluation phase in order to standardize the procedure for behavioural testing.

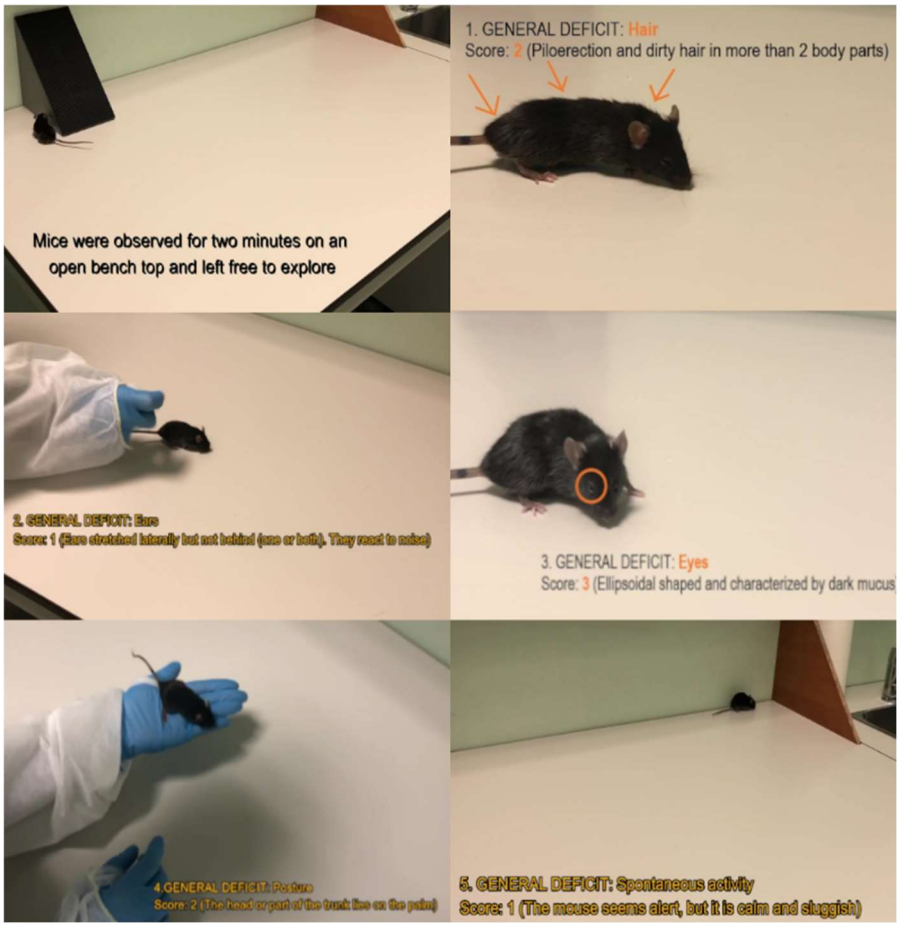
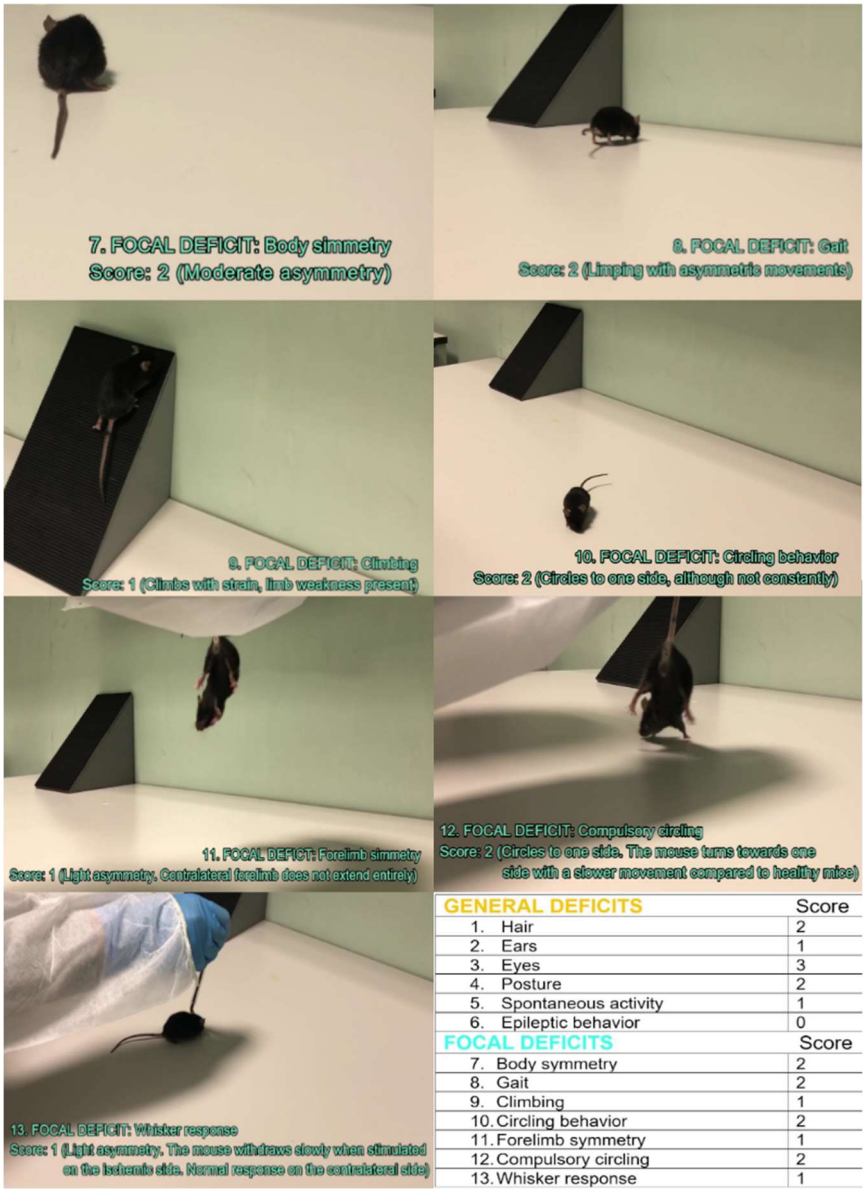


Figure 5.1 Explanation of the general deficits of De Simoni neuroscore by tutorial video.



GENERAL DEFICITS	Score
1. Hair	2
2. Ears	1
3. Eyes	3
4. Posture	2
5. Spontaneous activity	1
6. Epileptic behavior	0
FOCAL DEFICITS	**Score**
7. Body symmetry	2
8. Gait	2
9. Climbing	1
10. Circling behavior	2
11. Forelimb symmetry	1
12. Compulsory circling	2
13. Whisker response	1

Figure 5.2 Explanation of the focal deficits of De Simoni neuroscore by tutorial video.

The study was then conducted according to the plan depicted in **Figure 5.3**. Wild-type C57BL6J mice and Sprague-Dawley rats were subjected to tMCAo with different duration, namely 30, 45 or 60 minutes for mice and 50, 75 or 100 for rats. Animals were monitored at 24 and 48 hours post-surgery according to the IMPROVE guidelines to identify those needing the administration of a painkiller (i.e. Buprenorphine) or sacrifice

according to the humane endpoint (**Figure 3.4**). None of the operated mice or rats was sacrificed before the evaluation of the sensorimotor deficits.

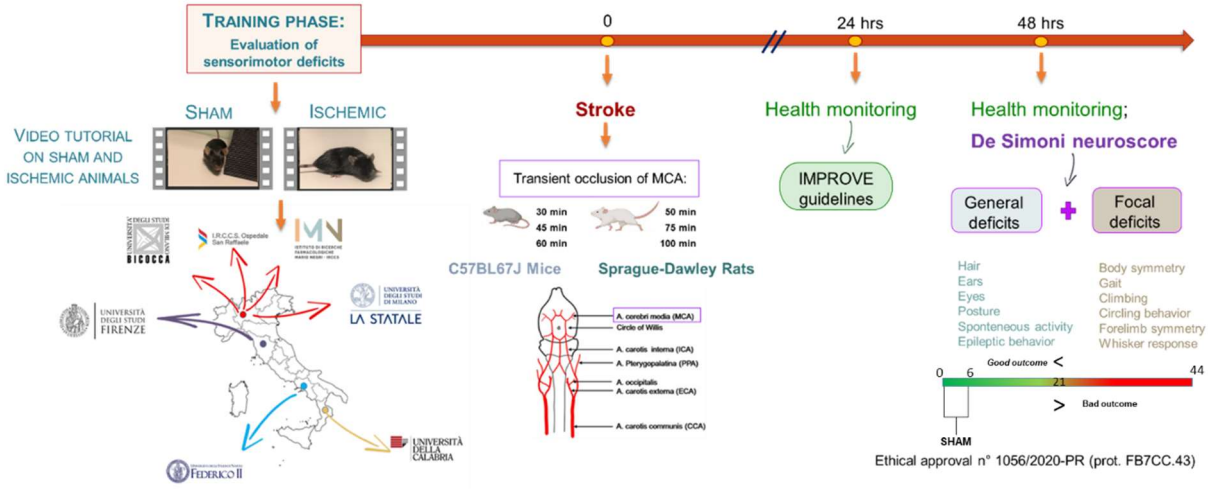


Figure 5.3 Experimental plan of the training phase.

At 48 hours after tMCAo animals underwent the De Simoni neuroscore while being video recorded. The videos were collected by the coordinating centre. Blinding was done by a figure outside the study who changed the number that identifies the name of the animal's video. Videos were numbered from T01 to T11 for mice and R01 to R11 for rats (see **Table 5.2** in section **5**). The videos were uploaded to an online platform with free access to all the centres (Google Drive, shared folder TRICS Basic project, sub-folder Inter-Rater Agreement). Thus, an evaluator for each centre, different from the surgeon, analysed and assigned a score to the 11 videos. All evaluations were entered on the RedCap online platform and retrieved by the coordinator for statistical analysis (**Figure 5.4**).

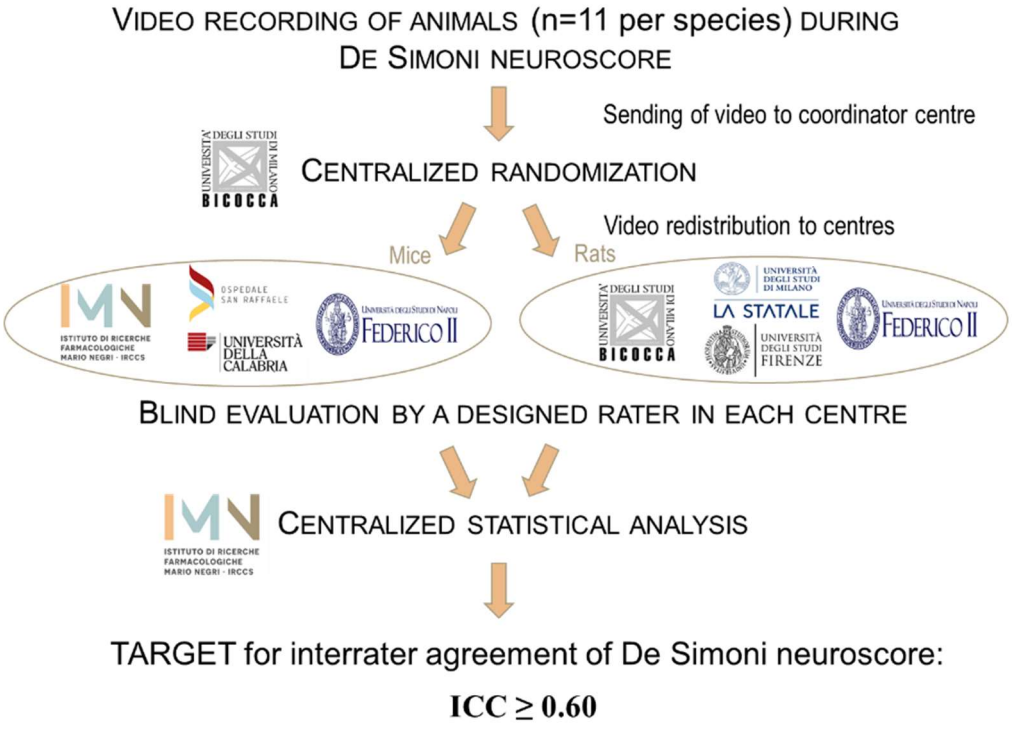


Figure 5.4 Experimental plan of the harmonization phase.

5.1 The interrater agreement on a total score range of the De Simoni neuroscore

The interrater agreement on the total score range of the De Simoni neuroscore (0-56) was described using the Intraclass Correlation Coefficient, ranging between ICC=0 (equivalent to chance) and ICC=1 (perfect agreement), setting a target of ICC≥ 0.60 as a satisfactory agreement. The analysis showed a moderate agreement for mice ICC=0.50 [0.22-0.77] (**Figure 5.5 A**) and for rats ICC=0.49 [0.21-0.77] (**Figure 5.5 B**), failing to reach the target set at ICC 0.60.

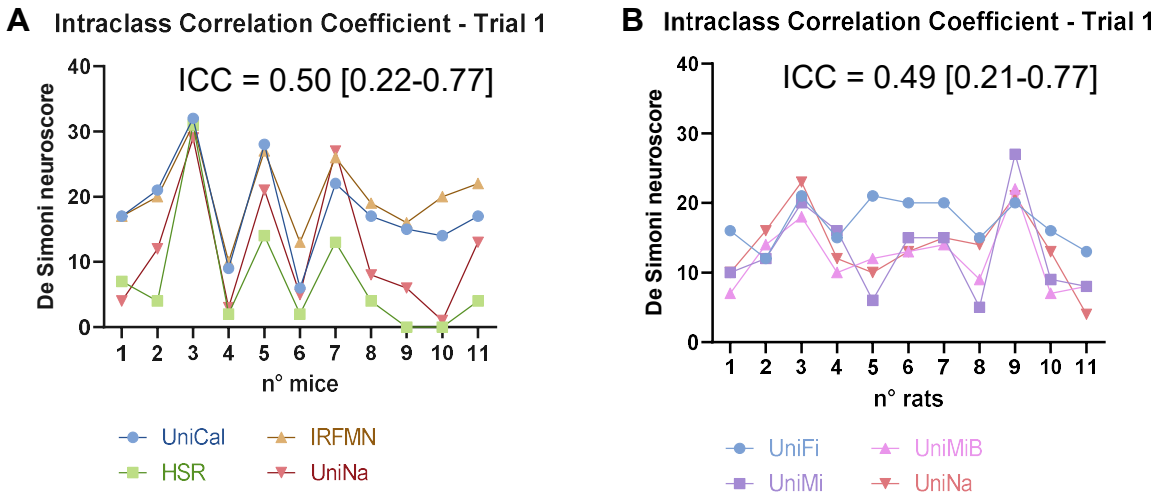


Figure 5.5 Interrater agreement analysis on a total score range of De Simoni neuroscore in the first trial. The interrater reliability is presented as ICC values with a 95% interval of confidence.

5.2 The interrater agreement on the dichotomised score of De Simoni Neuroscore

I repeated the analysis after score dichotomisation, i.e. "good" outcome if the total score was <21 and "bad" outcome if the total score was ≥21. This score cut-off was defined based on a previous work using the De Simoni neuroscore [171]. The Fleiss κ on the dichotomised score was κ=0.54 for mice and κ=0.36 for rats (**Table 5.1**).

Mice – Trial 1				**Rats – Trial 1**			
n_{ij}	**good**	**bad**	**P_i**	**n_{ij}**	**good**	**bad**	**P_i**
1	4	0	1.000	**1**	4	0	1.000
2	3	1	0.500	**2**	4	0	1.000
3	0	4	1.000	**3**	2	2	0.333
4	4	0	1.000	**4**	4	0	1.000
5	1	3	0.500	**5**	3	1	0.500
6	4	0	1.000	**6**	4	0	1.000
7	1	3	0.500	**7**	4	0	1.000
8	4	0	1.000	**8**	4	0	1.000
9	4	0	1.000	**9**	1	3	0.500
10	4	0	1.000	**10**	4	0	1.000
11	3	1	0.500	**11**	4	0	1.000
total	32	12		**Total**	38	6	
P_j	0.73	0.27		**P_j**	0.86	0.14	
P	0.818			**P**	0.848		
P_e	0.603			**P_e**	0.764		
Fleiss κ	**0.542**			**Fleiss κ**	**0.357**		

Table 5.1 Fleiss κ on the dichotomised score of De Simoni neuroscore.

n_{ij}: the number of evaluators who have assigned the same category to the same subject. **P_i**: the proportion of all evaluations of the same category; compute how many rater-rater pairs are in agreement, relative to the number of all possible rater-rater pairs. **P**: the mean of the P_i's. **P_e**: the hypothetical probability of chance agreement.

In order to identify possible 'outlier centres', the interrater reliability was calculated on pairs of raters using Cohen's κ coefficient, also ranging from κ=0 (equivalent to chance) to κ=1 (perfect agreement). In mice, considering the dichotomised score, I observed: fair

agreement between HSR and UniCal (κ=0.30), HSR and IRFMN (κ=0.30); moderate agreement between HSR and UniNa (κ=0.42); substantial agreement between UniCal and IRFMN (κ=0.61), UniCal and UniNa (κ=0.79), UniNa and IRFMN (κ=0.79) (**Figure 5.6 A**). In rats, I obtained: poor agreement between UniFi and UniMi (κ=0), UniFi and UniMiB (κ=0); fair agreement between UniFi and UniNa (κ=0.39); substantial agreement between UniMi and UniNa (κ=0.62), UniMiB and UniNa (κ=0.62); perfect agreement between UniMi and UniMiB (κ=1) (**Figure 5.6 B**). Thus HSR for mice and UniFi for rats provided slightly different scores than other centres.

I then correlated the total score given by each rater using the Pearson or Spearman correlation coefficient depending on data distribution. In mice, I found Spearman r=0.88 (UniCal-HSR, p=0.0006), Pearson r=0.93 (UniCal-IRFMN, p<0.0001), Pearson r=0.84 (UniCal-UniNa, p=0.0011), Spearman r=0.74 (HSR-IRFMN, p=0.0119), Spearman r=0.78 (HSR-UniNa, p=0.0059), Pearson r=0.88 (IRFMN-UniNa, p=0.0004) (**Figure 5.6 C**). In rats, I found Pearson r=0.44 (UniFi-UniMi, p=0.1711), Pearson r=0.57 (UniFi-UniMiB, p=0.0695), Pearson r=0.47 (UniFi-UniNa, p=0.1484), Pearson r=0.84 (UniMi-UniMiB, p=0.0012), Pearson r=0.73 (UniMi-UniNa, p=0.0115), Pearson r=0.80 (UniMiB-UniNa, p=0.0028) (**Figure 5.6 D**).

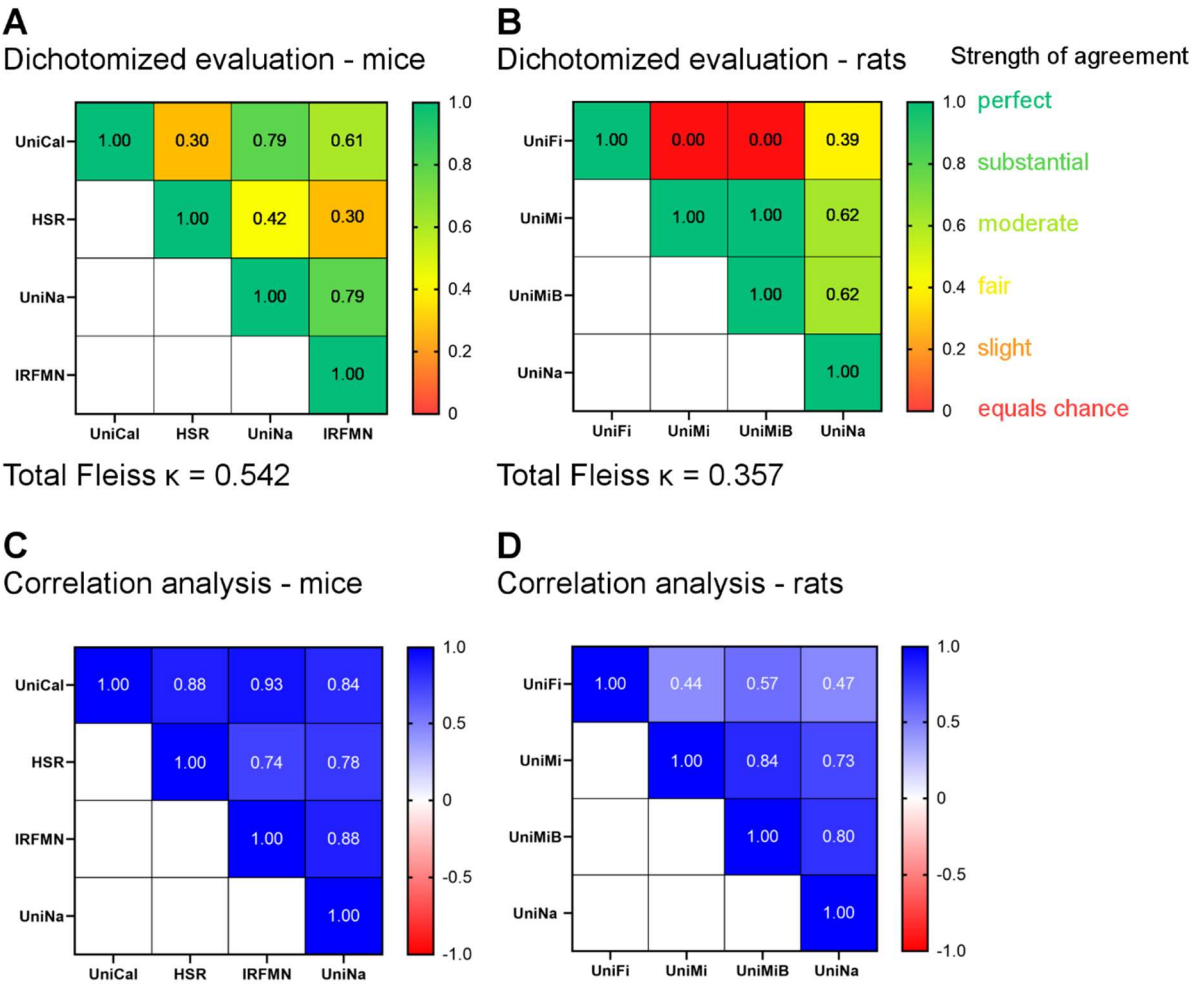


Figure 5.6 The interrater reliability calculated on pairs of raters using Cohen's κ coefficient. Red tones indicate poor while green tones strong agreement (A, B). **The correlation between scores from pairs of raters.** Red tones indicate poor correlation while blue tones strong correlation (C, D).

5.3 Systematic errors during the execution of the De Simoni neuroscore in the first trial

In order to identify the reasons for the poor agreement in the first trial - i.e. lower than our target of ICC≥ 0.6 - I critically revised all videos to identify any experimental issues. I noticed errors during the evaluation of general and focal deficits, as reported in **Figure**

5.7. In particular, during the observation of eyes (**Figure 5.7 A, E**) some raters stimulated animals instead of leaving them freely moving on the bench. I also observed the improper use of wool gloves (**Figure 5.7 B**) and plastic sheets (**Figure 5.7 F**) to assess animals' balance, thus altering the grip of the paws. The animals had to be placed on the palm covered with nitrile gloves without holding them by the tail. Some raters used an incorrect surface to assess climbing (**Figure 5.7 C, G**) instead of the gripping surface tilted to 45 degrees to the bench. I observed errors in animal handling for evaluation of whisker response on the lesioned and contralateral side (**Figure 5.7 D, H**), i.e. the use of pointed tweezers, the wrong position of the observer visible from the animals.

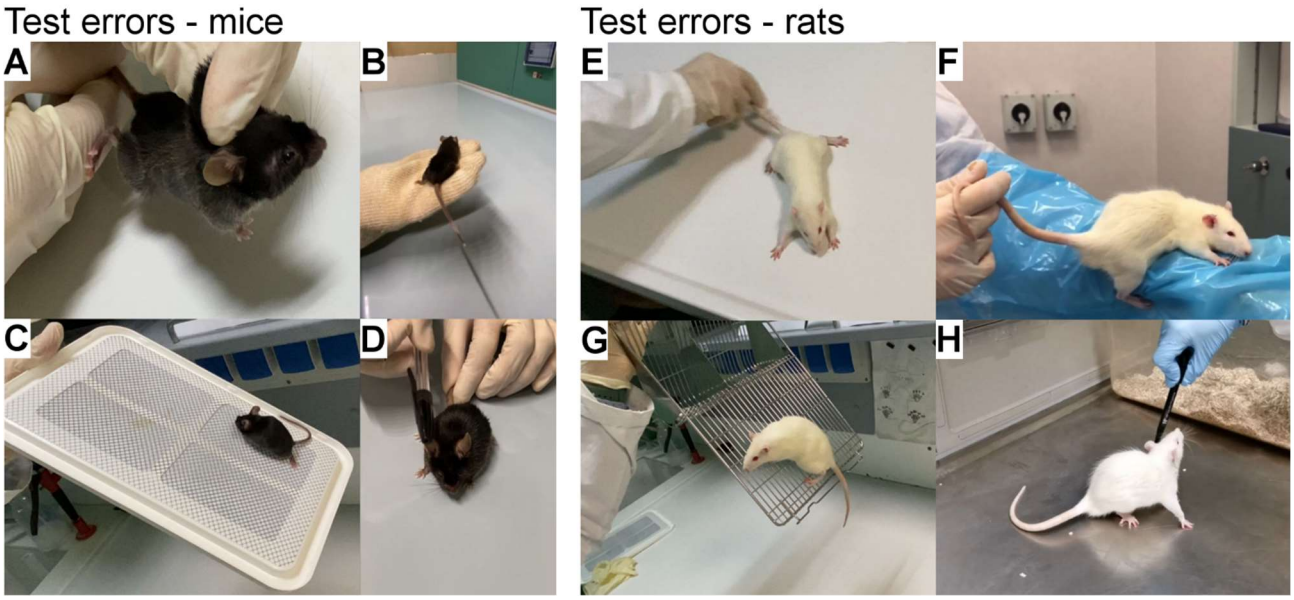


Figure 5.7 Typical animal handling errors during the De Simoni neuroscore first trial. In particular: **(A, E)** interference when observing the eyes; **(B)** improper use of wool gloves and **(F)** plastic sheet to assess animals' balance; **(C, G)** incorrect surface to assess climbing; **(D, H)** wrong handling during the evaluation of whisker response.

5.4 The second trial of the harmonisation phase

Our group, the coordinating unit for the De Simoni neuroscore, replaced the videos with poor experimental execution with new correct ones. All videos were blinded randomised again (**Table 5.2**) and redistributed in each centre for evaluation.

Species	ID CENTRE CODE	tMCAo (min)	TRIAL1	TRIAL 2
Mouse 1	IRFMN	30	T5	T3
Mouse 2	UniCal	30	T9	T1
Mouse 3	UniNa	30	T2	Replaced
Mouse 4	HSR	30	T4	Replaced
Mouse 5	IRFMN	45	T10	T5
Mouse 6	UniCal	45	T11	T7
Mouse 7	HSR	45	T1	Replaced
Mouse 8	IRFMN	60	T3	T4
Mouse 9	UniCal	60	T7	T6
Mouse 10	UniNa	60	T8	Replaced
Mouse 11	HSR	60	T6	T2
Rat 1	UniFi	50	R4	Replaced
Rat 2	UniNa	50	R7	R1
Rat 3	UniMi	50	R1	R4
Rat 4	UniMiB	50	R11	R9
Rat 5	UniMiB	75	R9	R10
Rat 6	UniFi	75	R3	Replaced
Rat 7	UniMi	75	R10	R3
Rat 8	UniFi	100	R5	Replaced
Rat 9	UniMi	100	R2	R8
Rat 10	UniNa	100	R6	R2
Rat 11	UniMiB	100	R8	R5

Table 5.2 Video randomization for mice and rats.

In particular, in the second trial we replaced four videos for the mouse model and three for the rat model (**Table 5.3**):

Species	ID CENTRE CODE	tMCAo (min)	TRIAL 2
Mouse 1	IRFMN	60	T10
Mouse 2	IRFMN	60	T11
Mouse 3	IRFMN	60	T9
Mouse 4	IRFMN	60	T8
Rat 1	UniMiB	100	R6
Rat 2	UniMiB	100	R7
Rat 3	UniMiB	100	R11

Table 5.3 Video replaced in the second trial and randomization for both models.

The videos were performed on new animals. We decided to apply the same duration of occlusion for all mice (tMCAo= 60 min) and rats (tMCAo= 100 min), as reported in **Table 5.3**. Before the evaluation of the videos, I held meetings in the consortium where I could discuss the critical issues and how to tackle them. I made a detailed presentation to explain how to improve animals' observation in the first two minutes of the behavioural test (spontaneous activity, gait, and circling behaviour) before the animals get weary. Moreover, I pointed out the importance of a correct observation without interference for some deficits (i.e. hair, eyes, body symmetry, and whisker response) and a silent environment while performing the tests. Other problems regarded the evaluation of focal deficits done by the improper use of wool gloves and the surface used for the assessment of climbing which was not regular and plain. After noticing issues concerning the evaluation of the mice, I focused on the revision of the rats' video recordings. Consulting groups with more experience with rat models, we agreed that rats need more time to begin exploring than mice do. We also noticed further problems caused by the larger size of the animals which impacted the correct evaluation of some deficits (general deficit: posture). We thus agreed to adjust the dimensions of the gripping surface.

Overall I noticed that some raters had several difficulties due to inexperience with this type of behavioural test.

5.5 The interrater agreement on the total score range of the De Simoni neuroscore in the second trial

In the second trial the analysis showed a substantial agreement for mice, having an ICC=0.64 [0.37-0.85] (**Figure 5.8 A**) and for rats, ICC=0.69 [0.44-0.88] (**Figure 5.8 B**), both satisfactory according to our *pre-hoc* target (ICC≥0.60).

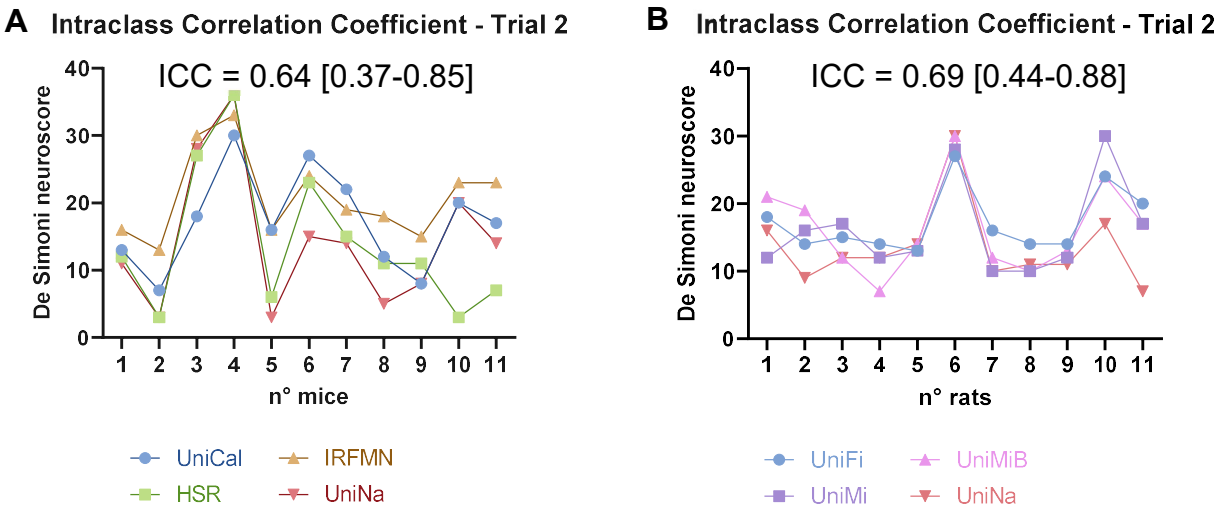


Figure 5.8 Interrater agreement analysis on a total score range of De Simoni neuroscore in the second trial. The interrater reliability is presented as ICC values with a 95% interval of confidence.

5.6 The interrater agreement on the dichotomised score of De Simoni Neuroscore

I repeated the analysis after score dichotomisation. The total Fleiss κ on the dichotomised score was κ=0.45 for mice and κ=0.69 for rats (**Table 5.4**).

Mice – Trial 2				**Rats – Trial 2**			
n_{ij}	**good**	**bad**	P_i	n_{ij}	**good**	**bad**	P_i
1	4	0	1.000	**1**	3	1	0.500
2	4	0	1.000	**2**	4	0	1.000
3	1	3	0.500	**3**	4	0	1.000
4	0	4	1.000	**4**	4	0	1.000
5	4	0	1.000	**5**	4	0	1.000
6	1	3	0.500	**6**	0	4	1.000
7	3	1	0.500	**7**	4	0	1.000
8	4	0	1.000	**8**	4	0	1.000
9	4	0	1.000	**9**	4	0	1.000
10	3	1	0.500	**10**	1	3	0.500
11	3	1	0.500	**11**	4	0	1.000
total	31	13		**total**	36	8	
P_j	0.70	0.30		P_j	0.82	0.18	
P	0.773			**P**	0.909		
P_e	0.584			P_e	0.702		
Fleiss κ	**0.454**			**Fleiss κ**	**0.694**		

Table 5.4 Fleiss κ on the dichotomised score of De Simoni neuroscore.

n_{ij}: the number of evaluators who have assigned the same category to the same subject. P_i: the proportion of all evaluations of the same category; compute how many rater-rater pairs are in agreement, relative to the number of all possible rater-rater pairs. **P**: the mean of the P_i's. P_e: the hypothetical probability of chance agreement.

The interrater reliability on pairs of raters was calculated using Cohen's κ coefficient. The interrater reliability, considering the dichotomised score, was in mice: slight agreement between UniCal and UniNa (κ=0.23), UniCal and IRFMN (κ=0.24); moderate agreement between UniNa and IRFMN (κ=0.42); substantial agreement between UniCal and HSR (κ=0.54), HSR and IRFMN (κ=0.62), HSR and UniNa (κ=0.74) (**Figure 5.9 A**). In rats, I observed moderate agreement between UniMiB and UniNa (κ=0.42); substantial agreement between UniFi and UniNa (κ=0.62), UniMi and UniNa (κ=0.62), UniFi and UniMiB (κ=0.74), UniMi and UniMiB (κ=0.74); perfect agreement between UniFi and UniMi (κ=1) (**Figure 5.9 B**). Thus in the second trial UniCal for mice and UniNa for mice and rats seemed to provide different scores than other centres, resulting in a different agreement than in the first trial.

With correlation analysis, I found in mice: Pearson r=0.69 (UniCal-HSR, p=0.0184), Pearson r=0.79 (UniCal-IRFMN, p=0.0037), Pearson r=0.74 (UniCal-UniNa, p=0.0092), Pearson r=0.80 (HSR-IRFMN, p=0.0033), Pearson r=0.79 (HSR-UniNa, p=0.0037), Pearson r=0.95 (IRFMN-UniNa, p<0.0001) (**Figure 5.9 C**). In rats I found Spearman r=0.54 (UniFi-UniMi, p=0.0855), Spearman r=0.63 (UniFi-UniMiB, p=0.0414), Spearman r=0.33 (UniFi-UniNa, p=0.3185), Spearman r=0.68 (UniMi-UniMiB, p=0.0250), Spearman r=0.38 (UniMi-UniNa, p=0.2470), Spearman r=0.44 (UniMiB-UniNa, p=0.1735) (**Figure 5.9 D**).

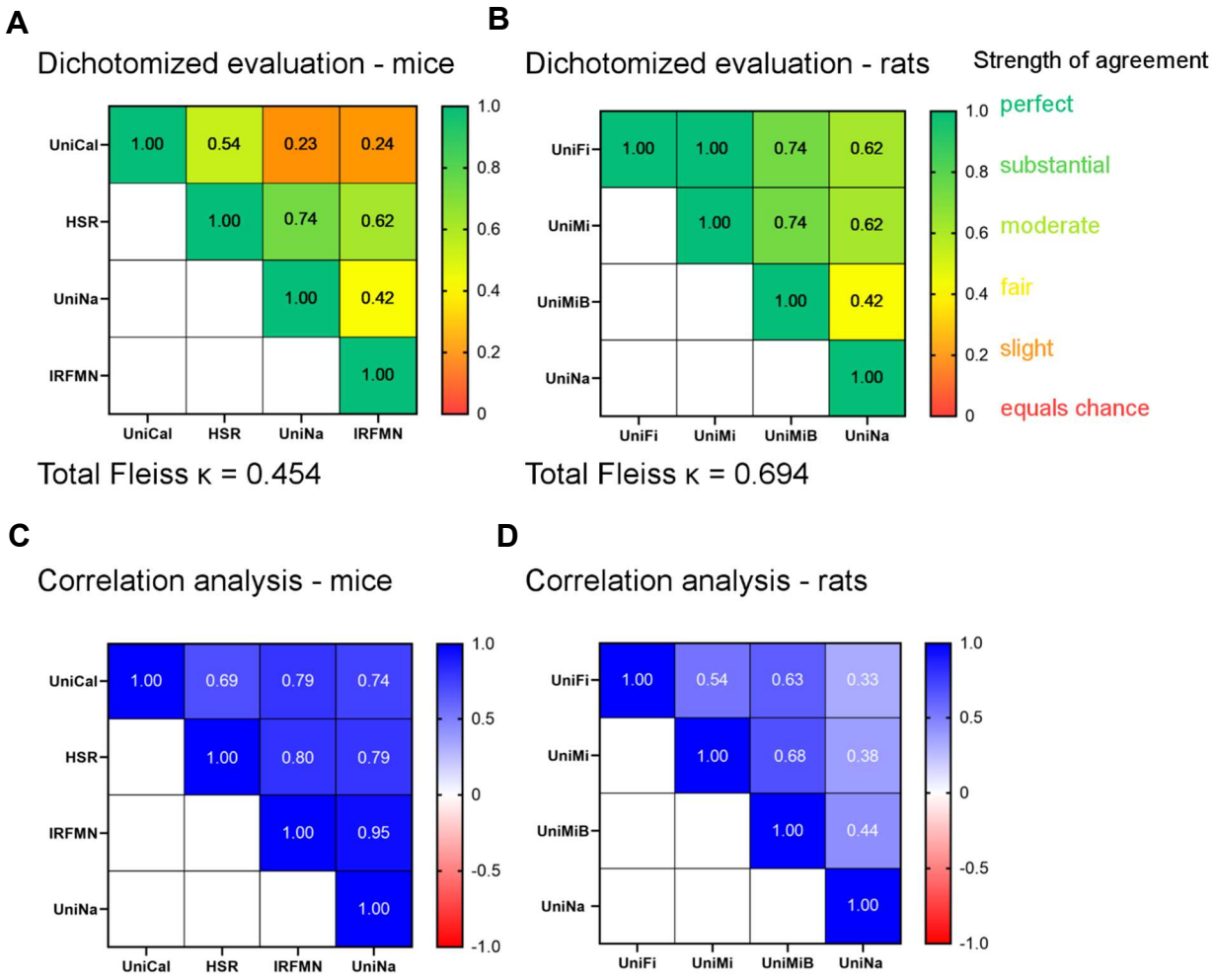


Figure 5.9 Improved interrater agreement after the second trial. (A, B) The interrater reliability was calculated on pairs of raters using Cohen's κ coefficient. Red tones indicate poor while green tones strong agreement. **(C, D)** The correlation between scores from pairs of raters. Red tones indicate poor while blue tones strong correlation.

5.7 Intra-rater score correlations between the two trials in mice and rats

Exploiting the videos that were evaluated in both trials (7 for mice and 8 for rats) after randomization, we could calculate the intra-rater agreement, i.e. how the two blind evaluations on the same animal correlated for each rater (**Figure 5.10**). The intra-rater score correlation revealed a good consistency between the two trials in mice, but not in rat evaluation.

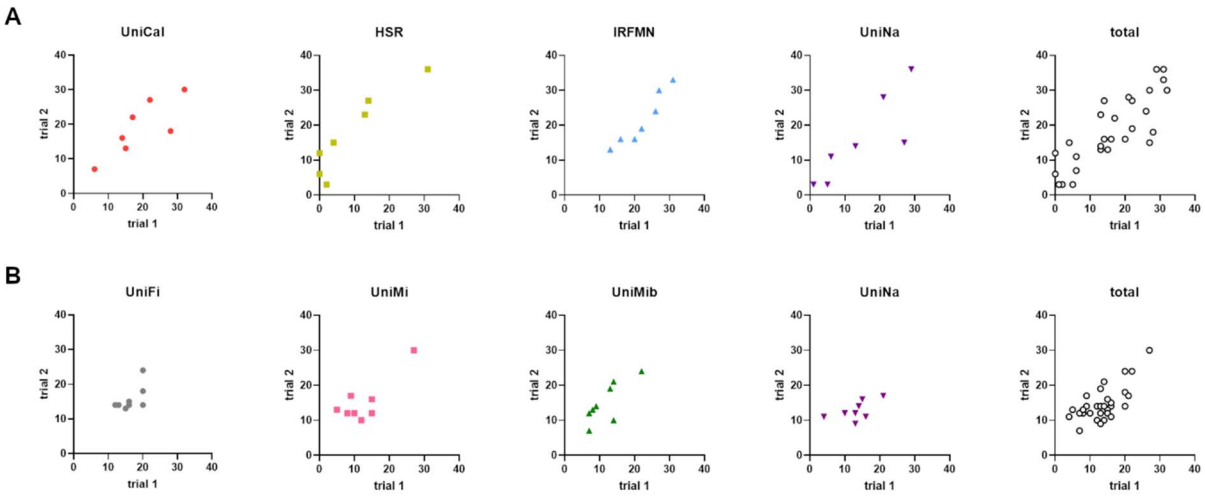


Figure 5.10 Intra-rater agreement. The correlation of trial 1 vs. trial 2 scores by the same rater on the same mouse **(A)** or rat **(B)**.

As reported in **Table 5.5**, raters evaluating mice were more consistent in the two trials compared to those evaluating rats, with an overall r of 0.83 ([0.66-0.92] CI 95%), p<0.0001, compared to 0.69 [0.45-0.84], p>0.0001. In the second trial, the total score on the same animals evaluated by each rater increased by +2.2 for mice and +1.2 for rats, indicating a better ability of raters to identify the deficits associated with the ischaemic models.

Species	Centre	Pearson r	95% CI	*p-value*
Mouse	UniCal	0.81	0.16-0.97	0.0257 (*)
	HSR	0.93	0.57-0.99	0.0025 (**)
	IRFMN	0.95	0.68-0.99	0.0011 (**)
	UniNa	0.84	0.25-0.98	0.0170 (*)
	Total	**0.83**	0.66-0.92	<0.0001 (***)
Rat	UniFi	0.61	-0.17-0.92	0.1088
	UniMi	0.81	0.24-0.96	0.0150 (*)
	UniMib	0.78	0.16-0.96	0.0229 (*)
	UniNa	0.59	-0.21-0.91	0.1262
	Total	**0.69**	0.45-0.84	<0.0001 (***)

Table 5.5 Correlation of rater's trial 1 vs. trial 2 score on the same animals. Correlations are presented as Pearson correlation coefficient (r) and 95% of confidence interval (CI).

The **Table 5.6** presents an update on the present status of interventional phase of the project, with all completed animals at each centre.

Update to: 26/04/2022

CENTRE	Species	n° randomised animals	% target	n° histologies performed	% target
IRFMN - Mario Negri	Mice	**24/24**	**100**	12/24	50
UniCal - Calabria	Mice	**24/24**	**100**	**24/24**	**100**
HSR - San Raffaele	Mice	0/24	0	0/24	0
UniNa - Napoli	Mice	**24/24**	**100**	**24/24**	**100**
UniNa - Napoli	Rats	**24/24**	**100**	**24/24**	**100**
UniMiB - Milano-Bicocca	Rats	20/24	83	18/24	75
UniFi - Firenze	Rats	18/24	75	12/24	50
UniMi - Milano-Statale	Rats	10/24	41	4/24	16
TOTAL		**144/192**	**75.00%**	**118/192**	**61.40%**

End of procedures: 30/07/2022

Table 5.6 Update of the status of the TRICS BASIC project

Animals were subjected to tMCAo surgery as described in **Figure 3.7**. Animals were randomised to receive the treatment: the remote ischemic post conditioning. A total of 144 animals were used in the interventional phase (update to 26/04/2022) as reported in **Table 5.6**. The coordinating centre performed histological analysis on 61.40% of the samples. The latter results for functional outcome assessed by De Simoni neuroscore and infarct volume assessed by the histological analysis were not analysed in this thesis.

6. DISCUSSION

In this thesis, we harmonized the behavioural procedures for the evaluation of sensorimotor deficits across the seven centres involved in the TRICS BASIC project. This work originally presents an effective workflow to standardize the assessment of the pre-defined primary outcome in a multicentre preclinical study.

Preclinical randomized controlled trials (pRCTs) have been proposed as a valuable tool for overcoming the existing limitations between preclinical *in vivo* studies and clinical trials. The first multicentre stroke study has been published in 2015 [120]. The design of the study was modelled on phase III clinical trials and involved six research independent centres in Europe, anticipating the results of a clinical trial on Natalizumab efficacy [121], thus proving pRCTs as reliable predictive tools. Despite its successes, this first pRCT also had several difficulties due to inexperience with this type of study. In particular, some parameters could not be fully harmonized between the participating centres - e.g. body temperature maintenance, requirements in post-surgical care, the use of anaesthesia and post-surgery analgesia, and the approval of the animal experiments by single ethics committees. Overall the study provided a critical view of used methods in experimental stroke research and defined the harmonization procedures as a necessary step in a pRCT. Based on the evidence provided by pRCTs, the Multi-PART (Multicentre Preclinical Animal Research Team) consortium, a European Union-funded international network of preclinical stroke researchers, set up a platform to conduct high-quality multicentre preclinical studies. However, pRCTs are still uncommon and have shown some weaknesses. Specifically, not all the good practices for solid clinical trials have been implemented in pRCTs, including trial pre-registration and protocol standardization.

In the TRICS project, the achievement of harmonization and standardization of protocols was predefined by a protocol paper published in 2020 [169]. At variance with previously published pRCT, TRICS included the presence of a 'preclinical monitor' who supervised centres' compliance to the experimental plan. Then TRICS detailed protocol was pre-registered (see https://preclinicaltrials.eu, ID: PCTE0000177) and presented a thorough implementation of the ARRIVE and IMPROVE guidelines [118,173]. The above-mentioned features of TRICS represent an effort to make the trial meeting the usual standards for clinical trials.

Our approach improved the reporting of research using animals, maximizing the information published and minimizing variations in the monitoring of animals in the different stages of experimentation. Several parameters were established to avoid confounders in the outcome evaluation and analysis. In greater detail, in this project, we used the most common experimental model of ischaemic stroke, including both male and female animals. The experimental group and the sample size were predefined by the designed statistician. We established common surgery and behavioural protocols across the involved centres. In addition, we identified the inclusion and exclusion criteria prior to randomisation. The animals were allocated to the surgery and the treatment using two randomization lists, separately by species and stratified by centres and sex. During the experiment and after surgery animals were monitored following a common checklist to ensure a satisfactory state of health. Animals were placed in an enriched environment, stimulating explorative and sensorimotor behaviour. In addition, to overcome the reduction of food and water intake after surgery, we placed wet food close to the animals. Treatment, surgery, and analysis of neuroscore results and infarct volume were performed by different researchers, or raters, who were blinded concerning the treatment

groups. Unblinding was performed after the statistical analyses were completed. Data were stored on the online platform RedCap which was accessible to study raters.

Stroke in animal models and humans is characterized by common functional deficiencies. This includes among others loss of limb function, sickness behaviour and sensorimotor deficiencies. Behavioural tests are helpful to monitor the progression and recovery of therapies in animal models. Nevertheless, almost all experimental studies provide infarct volumes as the main endpoints, whereas most clinical studies report neurological outcomes only [174]. Unlike in stroke clinical trials that use well-characterized behaviour rating scales (e.g. the modified Rankin Scale), in preclinical assessment there is no universally preferred behavioural scale or task. Recently the SPAN pRCT was launched and its published stage 1 results confirmed that a large, multilaboratory, preclinical assessment effort to reduce known sources of bias is feasible and practical [123]. In this trial, the researchers selected the corner test as the primary outcome measure. This test is used to evaluate persistent asymmetries in turning preference after focal ischemia. It requires preoperative testing to compare pre-stroke versus post-stroke values, as well as to identify a baseline. A major disadvantage of the corner test is that animals can be too sick and unmotivated to perform at early time points. They also rapidly lose motivation to perform when tested continuously. As the animals are introduced to the angle apparatus repeatedly, it is more difficult to observe a ‘proper' spontaneous turn since the animals lose their exploratory interest and become overly anxious by continuous handling. The results from SPAN network differed across six centres but there were no statistically significant difference among evaluation from different timepoints.

The TRICS group, after discussion in the consortium, instead selected the De Simoni composite neuroscore to determine stroke deficits in rodents as the primary outcome measure. The test is simple and it can be adapted to rats. Previous evidence from our

group showed that the ischaemic lesion is fully developed histopathologically at 48 hours and the De Simoni neuroscore highly correlated with the histological assessment of infarct volume. The test is performed only once at 48 hours and it takes into account the physical appearance of the rodent - hair, ears, eyes - certain sensory responses, seizure activity, basic gait, and limb and body symmetry assessments. It does not require any expensive equipments making it ideally used in all labs. However the execution of the test and a correct neuroscore assessment requires a trained investigator.

Key to standardization and quality check was to harmonize the evaluation of sensorimotor deficits by De Simoni neuroscore, which was successfully obtained in the present project. As a multicentre trial, the agreement among the individuals collecting data - here referred to as interrater agreement - can be immediately observed due to the fluctuation among the raters. Interrater agreement can vary on the individuals' different expertise with the specific assessments [175,176]. This is the reason why we decided to implement a training phase trial for data collectors, or raters, before the start of the trial, in order to reduce the variability in the way raters assess and interpret the neurobehavioural data. For this reason, in the first phase of the project, I prepared tutorials to illustrate to the centres involved in the project how to perform the De Simoni neuroscore in sham and ischaemic animals. Although the perfect agreement is difficult to achieve, a substantial agreement was deemed to be required before starting animal randomization, considering the translational aim of multicentre preclinical trials.

The interrater agreement on the total score range of the De Simoni neuroscore (0-56) was described using the intraclass correlation coefficient, setting a target of ICC≥ 0.60 as a substantial agreement, as per protocol paper [169]. It was noticeable that operators that used this test for the first time encountered more complications in the evaluation of ischaemic animals and made mistakes that regarded animal handling or the use of

unsuitable devices. Moreover the De Simoni neuroscore was applied to the ischaemic rats for the first time in this pRCT, and required some protocol adjustments, especially in animal handling and the observation timing on the bench. For these reasons, we held meeting in the consortium where I could discuss the critical issues and how to tackle them. We revised the assigned score by each rater on the single deficits of neuroscore. These meetings allowed us to discuss the difficulties encountered by the raters from each centre thus improving the evaluation. Furthermore based on the level of agreement lower than our target, as some videos showed experimental errors that I described above, we replaced them with new correct ones. Proceeding with the evaluation of new randomized videos, we thus started a second trial.

We improved the ICC on the total score range of De Simoni neuroscore from 0.50 in the first trial of the harmonization phase to 0.64 in the second trial for the evaluation of mice, and from 0.49 to 0.69 for that of rats. The fact that we could not obtain a substantial interrater agreement at the first trial largely depended on operators' mistakes, then corrected before the second trial. Also the raters using for the first time this neuroscore tended to give low deficit scores, thus failing to identify deficits when not overtly present. In line with this, considering the seven randomized videos analysed blindly in both trials, the overall increase of neuroscore compared to the first trial was +2.2 for mice and +1.2 for rats. When we analyzed the intra-rater agreement by correlating same rater's trial 1 vs. trial 2 score on the same animals, we obtained higher correlations for mice than rats.

Our study is the first specifically designed to increase reliability of neurobehavioural scoring as a primary outcome in multicentre preclinical trials. A multi-step, online harmonization phase proved to be feasible, easy to implement and highly effective to improve the agreement between the raters of different centres and with different skills.

This study provides that the De Simoni neuroscore can be used reliably in a community setting, and that trained researchers can perform an expert examination.

7. CONCLUSIONS

To conclude, our findings strongly indicate that the harmonization phase reduces bias in the neurobehavioural assessment used as a primary outcome in multicentre preclinical stroke trials and could be considered as a basic requirement before starting animal randomization. Our work proved the feasibility of the De Simoni neuroscore to both models of ischaemic stroke, a key finding in view of the interventional phase of the TRICS project. The approach used by the TRICS group may serve as a model for multilaboratory preclinical development to improve stroke research and hopefully in other disease areas.

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PUBLICATIONS AND SCIENTIFIC COMMUNICATIONS

Publications

Tettamanti M, Beretta S, Pignataro G, Fumagalli S, Perego C, Sironi L, Pedata F, Amantea D, Bacigaluppi M, Vinciguerra A, Valente A, Diamanti S, Mariani J, Viganò M, Santangelo F, Zoia C P, Rogriguez-Menendez V, Castiglioni L, Rzemieniec J, Dettori I, Bulli I, Coppi E, Gullotta G S, Bagetta G, Martino G, Ferrarese C, De Simoni M-G

Multicentre translational Trial of Remote Ischaemic Conditioning in Acute Ischaemic Stroke (TRICS): protocol of multicentre, parallel group, randomised, preclinical trial in female and male rat and mouse from the Italian Stroke Organization (ISO) Basic Science network

BMJ Open Science 2020; DOI:10.1136/bmjos-2020-100063

Mercurio D., Piotti A., Valente A., Oggioni M., Ponstein Y., Van Amersfoort E., et al.

Plasma-derived and recombinant C1 esterase inhibitor: Binding profiles and neuroprotective properties in brain ischemia/reperfusion injury

Brain, Behavior and Immunity 2021; DOI: 10.1016/j.bbi.2021.01.002

Valente A*, Mariani J*, Seminara S, Tettamanti M, Pignataro G, Perego C, Sironi L, Pedata F, Amantea D, Bacigaluppi M, Vinciguerra A, Diamanti S, Viganò M, Santangelo F, Zoia C P, Rogriguez-Menendez V, Castiglioni L, Rzemieniec J, Dettori I, Bulli I, Coppi E, Di Santo C, Ornella Cuomo, Gullotta G S, Butti E, Bagetta G, Martino G, De Simoni M-G, Ferrarese C, Fumagalli S**, Beretta S**, for the TRICS study group

Harmonization of sensorimotor deficit assessment in a registered multicenter pre-clinical randomized controlled trial using two models of ischaemic stroke

Submitted to *Journal of Cerebral Blood Flow & Metabolism* 2022

Abstracts

THE QUEST FOR IMPROVING EXPERIMENTAL STROKE RESEARCH: HARMONIZATION OF BEHAVIOURAL TEST IN A PRECLINICAL RANDOMIZED CONTROLLED TRIAL
Valente A., Fumagalli S., Mariani J., Seminara S., Beretta S., Ferrarese C. and TRICS groups
Poster at NeuroMi 2022 – November 2022, Milan, Italy

IS ALPHA-SYNUCLEIN (α-SYNUCLEIN) A FOE FOR BRAIN RECOVERY AFTER ISCHEMIC STROKE?
Bogale T. A., Mercurio D., **Valente A.**, Seminara S., Faustini G., Gussago C., Porrini V., Benarese M., Mota M., Rhein S., Mitola S., Schwaninger M., Fumagalli S., Bellucci A., Pizzi M.
Poster at National meeting of PhD students in Neuroscience – June 2022, Brescia, Italy

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Poster at PhD students meeting – June 2022, Istituto di Ricerche Farmacologiche "Mario Negri" IRCCS, Milan, Italy

ALPHA-SYNUCLEIN MODULATES VASCULAR RESPONSES FOLLOWING CEREBRAL ISCHEMIA-REPERFUSION INJURY
Bogale T. A., Mercurio D., **Valente A.**, Seminara S., Mitola S., Fumagalli S., Bellucci A., Pizzi M.
Poster at Brain & Brain pet 2022 – May/June 2022, Glasgow, UK

PRECLINICAL RANDOMIZED CONTROLLED TRIAL OF REMOTE ISCHEMIC CONDITIONING IN ACUTE ISCHEMIC STROKE: HARMONIZATION PHASE
Valente A., Fumagalli S., Mariani J., Seminara S., Beretta S., Ferrarese C. and TRICS groups
Poster at Brain & Brain pet 2022 – May/June 2022, Glasgow, UK

PRECLINICAL RANDOMIZED CONTROLLED TRIAL OF REMOTE ISCHEMIC CONDITIONING IN ACUTE ISCHEMIC STROKE: HARMONIZATION OF BEHAVIORAL TESTING ACROSS CENTRES
Valente A., Fumagalli S., Mariani J., Seminara S., Beretta S., Ferrarese C. and TRICS groups
Poster at Stroke Immunology Meeting – March 2022, Munich, Germany

MULTICENTRE TRANSLATIONAL TRIAL OF REMOTE ISCHEMIC CONDITIONING IN ACUTE ISCHEMIC STROKE (TRICS)
Oral presentation at PhD Days – January 2022, Milan, Italy

PRECLINICAL RANDOMIZED CONTROLLED TRIAL OF REMOTE ISCHEMIC CONDITIONING IN ACUTE ISCHEMIC STROKE: HARMONIZATION OF BEHAVIORAL TESTING ACROSS CENTRES

Valente A., Fumagalli S., Mariani J., Seminara S., Beretta S., Ferrarese C. and TRICS groups
Poster at NeuroMI – November 2021, Milan, Italy

THE PREDICTIVE ROLE OF FICOLIN-2 IN ATHEROSCLEROTIC PATIENTS: A VALID BIOMARKER FOR RUPTURE-PRONE PLAQUES ASSOCIATED WITH THE RISK OF ISCHEMIC STROKE
Valente A., Lo Presti F., Cotta Ramusino M., Perini G., Bonalumi G., Capelli M., De Simoni M-G., Costa A., Fumagalli S.
Poster at SINS National Congress – Italian society for neuroscience, September 2021

ACKNOWLEDGEMENTS

The work reported in this thesis has been performed at Istituto di Ricerche Farmacologiche 'Mario Negri', Milan. I have been supervised by Dr Stefano Fumagalli, head of the Unit of Pathophysiology of Cerebrovascular Functions at the Istituto 'Mario Negri' and by Prof Carlo Ferrarese, full professor at the University of Milano-Bicocca (School of Medicine and Surgery). This doctoral thesis was made possible through the help and support of supervisors, colleagues, friends and family. My heartfelt thanks go out to all of you. In particular, I would like to mention:

Dr Stefano Fumagalli, my supervisor at 'Mario Negri' Institute. Thank you for your wisdom and guidance. You are an inspiration as a researcher and it has been a pleasure to be your PhD student.

Prof Carlo Ferrarese, my tutor at the University of Milano-Bicocca. I am grateful for the academic support.

Thank you to the TRICS Basic group and all my co-authors, in particular Dr Maria Grazia De Simoni, Dr Simone Beretta, Dr Carlo Perego and Jacopo Mariani. It has been a pleasure working with you.

Thank you to all my colleagues working at the Department of Neuroscience: Domenico, Serena, Marco, Gizem, Laura, Joe, Chantal, Tizibt.

Thank you to my friends for keeping my spirits up during these three years, especially to Daniela.

Special thanks go to my family. In particular to my parents, Carmela and Luca, for your encouragement, guidance and financial aid, and to my sister Tiziana and Gianmichele, for your patience and continuous moral support.

Last but not least, thank you to my life partner, Dante. I would be lost without your love and support.