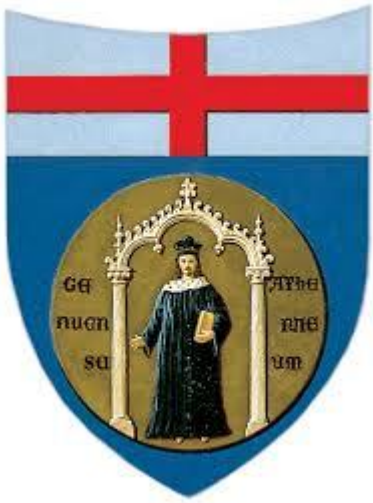

UNIVERSITÀ DEGLI STUDI DI GENOVA

SCUOLA DI SCIENZE MEDICHE E FARMACEUTICHE

CORSO DI LAUREA IN MEDICINA E CHIRURGIA

"RADIOLOGICAL CEREBRAL REPERFUSION AT 24 H AFTER ACUTE ISCHEMIC STROKE: PREDICTION OF GOOD CLINICAL AND FUNCTIONAL OUTCOMES AND POTENTIAL CORRELATION BETWEEN REPERFUSION AND SERUM INFLAMMATORY BIOMARKERS"

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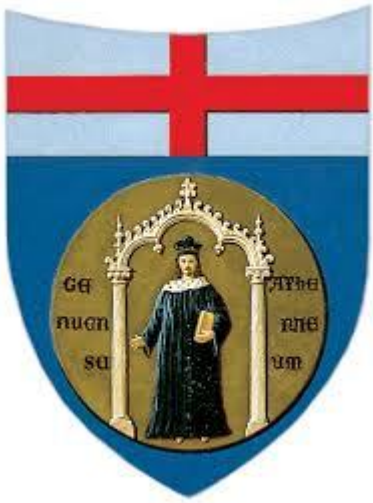

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1.Introduction

1.1. Epidemiology, classification, and etiology of ischemic stroke

Stroke is classically defined as a sudden, focal (or global) neurological deficit lasting more than 24 h or leading to death, with no apparent cause other than of vascular origin, including cerebral infarction, intracerebral hemorrhage, and subarachnoid hemorrhage *(1)*. Stroke is the second most common cause of mortality just behind ischemic heart disease and the second most common cause of disability worldwide. In 2016, around 14 million people worldwide are affected by stroke each year; of these, 5.5 million face death and another 5 million are permanently disabled *(2)*. Therefore represent the largest determinant to global neurological Disability-Adjusted Life Years (DALYs) *(3).* The overall rate of stroke-related mortality is decreasing in develop and developing countries, but the global burden of stroke-related disability and death is increasing. Thus, stroke implicates a serious public health problem and an important clinical and socio-economic cost for several health systems.

1.1.1. Stroke subtypes

The etiology of ischemic stroke affects prognosis, management, and ultimately outcome. Especially the identification of the underlying cause is critical being a leading determinant of treatment approach. Stroke classification should distinguish mainly between hemorrhagic stroke, subarachnoid hemorrhage and brain ischemia. Overall, about 87% of strokes are due to ischemia, while the amount of hemorrhagic stroke composed of both intracerebral and subarachnoid hemorrhage is of about 13% *(4)*. According with the TOAST classification, thrombotic strokes may be further categorized into five main causes: i) cardio-embolic when the occlusive thrombus comes from heart cavities, ii) large artery atherosclerotic if subsequent to a complicated atherosclerotic plaque causing significant occlusion or stenosis of a major brain artery (more commonly in internal carotids and vertebral artery) or intracranial cortical artery (belonging to Willis's circle and proximal branches); iii) lacunar, due to a pathology of the small cerebral vessels; iv) other determined etiology such as arterial dissection, vasculitis, or pro-thrombotic disorders; v) cryptogenic, or from an undetermined etiology *(5).* Stroke category may be usually identified by medical history and physical examination. However, brain imaging and

ancillary test are usually required to confirm a presumptive clinical diagnosis. The most common cause in acute ischemic stroke (AIS) is atherosclerosis. Combined mechanisms may also occur.

1.1.2. Prevalence and incidence

Currently, approximately 11% of the world's population people are at least 65 years old; by 2050, in according to the forecasts over 65 population is expected to increase to 22% and over 80 will account for 9,5% of the total population *(6)*. As populations are growing and aging, the prevalence of AIS considerably increases with age in both males and females. There were 80 million prevalent cases of stroke globally in 2016 and the American Heart Association forecasts a 20.5% increase in stroke prevalence by 2030 *(7)*. The prevalence of stroke-related symptoms was found to be fairly high in a general population free of a previous diagnosis of stroke or transient ischemic attack (TIA); thus, suggesting that a consistent amount of strokes may be underdiagnosed. Each year, almost 800.000 people experience a new or recurrent stroke. Around 600.000 of these are first attacks, and 200.000 are recurrent attacks. *(4)* In the past two decades in high-income countries, the stroke incidence rate is reduced but despite this promising trend, the aging population and accumulating risk factors lead to a rising long-life risk of stroke. Such risk of stroke for adult is approximately 25 % starting at age 25 and is estimated to have increased from 22% to 24% over the past decades, with the risk of ischemic stroke exceeding the risk of hemorrhagic stroke (8).

1.1.3. Age, ethnicity, and gender

Ischemic stroke shown important differences depending on age and sex distribution. Typically, stroke burden rapidly increased up with age and becomes the dominant cause of neurological morbidity between ages of 60 and 84 years. The individuals most at risk are the elderly, indeed being AIS a well-established age-related pathologies the rate doubles every decade after the age of 55 *(9);* although not surprisingly most stroke cases affect people aged 65 and over, approximately 10% of all strokes occur in individuals 18 to 50 years of age *(10).* Annual age-adjusted incidence for first-ever stroke was higher in black individuals than white. African Americans and Hispanics have a risk between 2 and 4 times higher than stroke. Substantially men have a higher incidence of ischemic stroke at younger and

middle-age groups than women, but at older ages these differences reverses; thus, in women over 85 years incidence rates higher than in males, substantially due to greater longevity of women and intrinsic factors *(4)*. As a non-adjustable risk factor, age plays a key role in determining sexual dimorphism in stroke epidemiology. Ischemic stroke-related mortality in women increases with age, probably due to loss of neuroprotective effect of sex hormones in postmenopausal women. Moreover, women have weaker outcomes and are more likely to die after an ischemic brain event. This is partly due to the fact that on average, women are older when suffering from stroke. In addition, women are under-represented in clinical trials and this may select possible gender bias. For these reasons the total amount of stroke-related deaths was around 575,000 stroke deaths in women, compared with 396,000 in men. This epidemiological differences across gender, especially in post-menopausal women, may affects outcome and in future move the magnifying glass towards a personalized management and treatment *(11)*.

1.1.4. Risk factor distribution

Non-modifiable risk factors include age, sex, ethnicity and genetic factors have already been mentioned above. The identification of modifiable risk factors is vital for implementing primary and secondary stroke prevention measures, through pharmacological interventions and lifestyle changes. High blood pressure represents the most important modifiable risk factor for stroke: the increase in the average blood pressure values corresponds to a linear increase in the risk of stroke (12). The reduction of systemic blood pressure, both by means of pharmacological agents and with lifestyle changes, has been shown to reduce the risk of stroke in hypertensive patients (13). Diabetes doubles the risk of acute cerebrovascular events, with particular effect in young patients (14). The association between diabetes mellitus and stroke risk differs between sexes, diabetic women hold a higher risk of ischemic stroke than men (15). Pharmacological and dietary correction of glycemic levels has been shown to reduce stroke risk in diabetic patients (16). Atrial fibrillation and atrial heart disease are important risk factors for the onset of ischemic stroke, increasing fivefold. According to the most classical theory, atrial fibrillation would cause blood stasis in the heart chambers, favoring the formation of thrombi responsible for cerebral cardio-embolism and therefore stroke. However, more recently it has been highlighted that the cause of cardio-embolism is not

properly atrial fibrillation but more likely atrial cardiomyopathy, However, in fibrillating patients primary and secondary prevention with anticoagulant therapy has proven effective in reducing the risk of ischemic stroke (17). The risk of ischemic stroke increases with high cholesterol levels and decreases with increasing HDL cholesterol (18). The cholesterol-lowering drug treatment reduces the risk of ischemic stroke. Physical inactivity correlates with several negative health effects, including an increased risk of stroke (19). Those who regularly practice physical activity have a reduced risk of developing cerebrovascular pathologies, probably also thanks to the reduction of blood pressure values and better control of diabetes. As regards the diet, it is known that an increased amount of salt increases the risk of stroke through the effect on blood pressure values. In addition, the Mediterranean diet, as well as a diet rich in fruit and vegetables have a protective effect (20). Cigarette smoking doubles the risk of stroke onset (21) with a linear relationship between the amount of cigarettes consumed per day and the frequency of acute cerebrovascular events (22). Discontinuation of smoking reduces the risk of stroke by eliminating the negative effect of tobacco after a period of 2-4 (23). In this context, promoting healthy lifestyles is a priority, given that epidemiological studies carried out in recent years have demonstrated the reversibility of risk, namely that by reducing modifiable risk factors, it is possible to delay or reduce the number of events that occur in the population (24).

In consideration of epidemiological data, there is an urgent need to implement the accuracy of stroke diagnosis in order to identify patients with better clinical and functional response, and characterize patients based on risk, with the aim of reducing mortality, disability and associated costs. Differentiating ischemic from hemorrhagic stroke is not possible through clinical findings and neuroimaging is needed.

1.2. Neuroimaging study of acute ischemic stroke

Imaging studies has a crucial role to exclude hemorrhage in the acute ischemic stroke, to assess the degree of brain injury, to rule out stroke mimics (such as tumor), to detect the vascular lesion responsible for the ischemic deficit and to evaluate the status of large cervical and intracranial arteries, to estimate the infarct core and penumbra volumes, and to guide acute interventions, including patient

selection for reperfusion therapies (25). In the hyperacute phase, a non-contrast computed tomography (NCCT) scan is commonly required to exclude or confirm hemorrhage; it is highly sensitive for this indication and widespread available. An NCCT scan should hence be acquired as soon as the patient is stable. The initial brain imaging study can be explored alternatively with a magnetic resonance imaging (MRI). Although MRI with diffusion-weighted imaging (DWI) is superior to NCCT for the very early recognition of acute ischemia, often is not quickly available in many centers and is more limited by patient contraindications or intolerance than CT. Thus, for the triage of patients MRI should be preferred rather than CT only if it does not delay treatment. Use of advanced imaging such as CT angiography (CTA) and CT perfusion imaging (CTP) are able to provide important information concerning early ischemia and perfusion injury in the assessment of acute stroke, allowing the distinction between the size of tissue that is irremediably damaged (called ischemic core) and that which is severely hypoperfused but potentially recoverable by reperfusion (called penumbra). Moreover, CTA is suitable for the detection of intracranial large vessel stenosis and occlusion. In this background, it can be helpful for the selection of patients for acute therapy, especially mechanical thrombectomy. CTA can be obtained concomitantly with head CT for patients with acute ischemic stroke. CTP is also useful for the triage of patients for endovascular interventions, in particular those in the late time window (>6 hours from stroke onset or from the time last known to be normal). Hence, this assessment allows an accurate selection of patients who are potential to benefit from therapy (26).

1.3. Approach to reperfusion therapy for acute ischemic stroke

The early pharmacological or mechanical restoration of the blood flow in the ischemic brain is recommended to treat acute ischemic stroke (27,28). The effectiveness of thrombolysis is mainly driven by the establishment of early recanalization and/or reperfusion (29). Noteworthy, the selection of suitable candidates for reperfusion therapy requires a neurological evaluation and a neuro-imaging study. Inclusion or exclusion criteria are listed in *Table 1.*

Table 1. Inclusion and Exclusion characteristics of patients with Ischemic Stroke who could be treated with intravenous rtPA within 3 hours from symptom onset

Inclusion criteria
Diagnosis of ischemic stroke causing measurable neurological deficit
Onset of symptoms <4,5 h before treatment begins
Age ≥18 y
Exclusion criteria
Significant head trauma or prior stroke in the previous 3 months
Symptoms suggest SAH
Arterial puncture at non compressible site in previous 7 days
History of previous intracranial hemorrhage
Intracranial neoplasm, AVM, or aneurysm
Recent intracranial or intraspinal surgery
Elevated blood pressure (systolic >185 mm Hg or diastolic >110 mm Hg)
Active internal bleeding
Acute bleeding diathesis, including but not limited to
Platelet count <100 000/mm^3
Heparin received within 48 h resulting in abnormally elevated aPTT above the upper limit of normal
Current use of anticoagulant with INR >1.7 or PT >15 s
Current use of direct thrombin inhibitors or direct factor Xa inhibitors with elevated sensitive laboratory tests (eg, aPTT, INR, platelet count, ECT, TT, or appropriate factor Xa activity assays)
Blood glucose concentration <50 mg/dL (2.7 mmol/L)
CT demonstrates multilobar infarction (hypodensity >1/3 cerebral hemisphere)
Relative exclusion criteria
Recent experience suggests that under some circumstances, with careful consideration and weighting of risk to benefit, patients may receive fibrinolytic therapy despite ≥1 relative contraindications. Consider risk to benefit of intravenous rtPA administration carefully if any of these relative contraindications is present:
Only minor or rapidly improving stroke symptoms (clearing spontaneously)
Pregnancy
Seizure at onset with postictal residual neurological impairments
Major surgery or serious trauma within previous 14 d
Recent gastrointestinal or urinary tract hemorrhage (within previous 21 d)
Recent acute myocardial infarction (within previous 3 mo)
Notes
The checklist includes some FDA-approved indications and contraindications for administration of intravenous rtPA for acute ischemic stroke. Recent guideline revisions have modified the original FDA-approved indications. A physician with expertise in acute stroke care may modify this list.

Onset time is defined as either the witnessed onset of symptoms or the time last known normal if symptom onset was not witnessed.

In patients without recent use of OACs or heparin, treatment with intravenous rtPA can be initiated before availability of coagulation test results but should be discontinued if INR is >1.7 or PT is abnormally elevated by local laboratory standards.

In patients without a history of thrombocytopenia, treatment with intravenous rtPA can be initiated before availability of platelet count but should be discontinued if platelet count is <100 000/mm^3

Adapted from Demaerschalck et al., 2016

All adult patients with suspected AIS should be quickly screened for treatment with intravenous thrombolytic therapy. The prompt goal of reperfusion therapy for AIS is to recover blood flow to the territory of brain that are ischemic but not yet infarcted. The long-term goal is to improve clinical and functional outcomes by reducing mortality and stroke-related disability. Intravenous recombinant tissue plasminogen activator (r-TPA) is the cornerstone of reperfusion therapy and the most important factor is the early treatment: the earlier the treatment, better the outcome. Intravenous thrombolytic therapy with alteplase improves functional outcome (defined by a modified Ranking scale score mRS; *Table 2*) at three to six months when given within 4.5 hours of acute ischemic stroke onset.

Table 2. The Modified Rankin Scale and Corresponding Sections of the Structured Interview

Modified Rankin Scale	Structured Interview for the Modified Rankin Scale
5=Severe disability: bedridden, incontinent, and requiring constant nursing care and attention.	5=Severe disability; someone needs to be available at all times; care may be provided by either a trained or an untrained caregiver. Question: Does the person require constant care?
4=Moderately severe disability: unable to walk without assistance, and unable to attend to own bodily needs without assistance.	4=Moderately severe disability; need for assistance with some basic ADL, but not requiring constant care. Question: Is assistance essential for eating, using the toilet, daily hygiene, or walking?
3=Moderate disability; requiring some help, but able to walk without assistance.	3=Moderate disability; need for assistance with some instrumental ADL but not basic ADL. Question: Is assistance essential for preparing a simple meal, doing household chores, looking after money, shopping, or traveling locally?
2=Slight disability; unable to carry out all previous activities but able to look after own affairs without assistance.	2=Slight disability; limitations in participation in usual social roles, but independent for ADL. Questions: Has there been a change in the person's ability to work or look after others if these were roles before stroke? Has there been a change in the person's ability to participate in previous social and leisure activities? Has the person had problems with relationships or become isolated?
1=No significant disability despite symptoms; able to carry out all usual duties and activities.	1=No significant disability; symptoms present but no other limitations. Question: Does the person have difficulty reading or writing, difficulty speaking or finding the right word, problems with balance or coordination, visual problems, numbness (face, arms, legs, hands, feet), loss of movement (face, arms, legs, hands, feet), difficulty with swallowing, or other symptom resulting from stroke?
0=No symptoms at all.	0=No symptoms at all; no limitations and no symptoms.

Adapted from Wilson et al. 2002

Even more beneficial effects for eligible patients are evident when administered within 3 hours, where the recommendation is highest. The benefit of intravenous thrombolysis for acute ischemic stroke decline considerably over time from symptom onset (30,31). In fact, it has been documented that for each 15-minute reduction in the time of administration of r-TPA treatment was related with an increased odds of being discharged at home rather than in an institution and a decreased hazard of death before discharge and symptomatic hemorrhagic transformation of ischemic stroke (32). The results showed that the benefits of early initiation of r-TPA are independent of the patient age or severity of stroke. Beyond 4.5 h, harm may outweigh benefit. Therefore, any usefulness over 4.5 hours of intravenous thrombolytic treatment has not yet been established. Some restricted studies suggest that the use of alteplase over the time threshold may be beneficial in some selected patients through imaging criteria indicative of recent cerebral infarction and/or significant salvageable brain tissue (33).

Simultaneously to thrombolytic treatment the evaluation for mechanical thrombectomy should be carried on. Patients with suspected AIS involving the anterior circulation should be evaluated as soon as possible with CTA or magnetic resonance angiography (MRA). Nevertheless, intravenous alteplase treatment should not be delayed by angiography or mechanical thrombectomy. If results of CTA or MRA confirm a proximal intracranial large artery occlusion, a mechanical thrombectomy within 6 hours is recommended (34). Unfortunately, the exact onset of stroke symptoms is often unknown, typically when stroke occurs before awakening from sleep. Whenever possible, assessment of brain perfusion status and clinical-core mismatch assessed with diffusion-weighted magnetic resonance imaging (DW-MRI) or CTP should be performed in those patients being of paramount relevance for therapeutic approach (35). Patients beyond 24 hours from ischemic stroke symptom onset are not suitable for any reperfusion treatment, focusing on supportive care.

Although recanalization and reperfusion are closely related, these pathophysiological conditions can be radiologically identified. Recently, the clinical benefit of recanalization achieved by endovascular treatment was demonstrated in various clinical trials, which also reported a prognostic relevance of reperfusion toward improved clinical and radiological outcomes (35–38). Full or partial recanalization up to 24 hours after onset of acute stroke after thrombolysis is

associated with a more positive outcome (39). Time to treatment is probably the most important determinant of good achievement of revascularization but a number of additional variables may have a role: clot features (including location, size, composition and origin) hyperglicemia, cerebral microbleeds and early ischemic change on neuroimaging (40). It is important to note that thrombolysis may fail to induce recanalization (41,42), but reperfusion may be maintained by collateral circulation. Significant reperfusion without recanalization can be observed in almost 30% of patients within the first 6 h (especially in ultra-early reperfusion) of symptoms onset. Reperfusion without recanalization was frequent and probably recruitment of leptomeningeal collaterals resulting in retrograde collateral flow may explain this phenomenon. However, a delayed increase in collateral flow may promote harmful reperfusion injury due to deterioration of collaterals. Thus, acute and delayed changes in collaterals might lead to different effects. Furthermore, several studies have proposed that collateral flow, when present and not related of canalization, is suitable to improve clinical and imaging outcomes (43).

In turn, successful recanalization does not consistently lead to reperfusion in case of distal thrombus embolization (44) or no-reflow (NR) phenomenon. The NR phenomenon is defined as a reduction of blood flow despite vessels patency. This phenomenon is commonly observed in coronary circulation and seems to be due to extensive damage to the microvascular circulation. During a ST-segment elevation myocardial infarction (STEMI) vessels patency can be obtain by percutaneous coronary intervention (PCI) within 120 minutes. Incidence of NR is variable and depend also upon the method used for detection, but in some studies it reaches up to 60% of STEMI. (45,46) If no reflow phenomenon occurs, there is a significant attenuation in beneficial effects of perfusion therapy. The exact pathophysiology is unknown but multiple factors probably contribute to reperfusion failure at microvascular level. They includes activation of inflammatory pathways, reperfusion injury, vasoconstriction and others. Insufficient restoring of microcirculatory despite complete recanalization significantly impairs the effectiveness of thrombolysis in stroke. The understanding of underlying mechanisms and strategies to restoring microcirculatory function has then became critical, being penumbra survival largely dependent of blood supply (47).

Despite these substantial differences, the terms “recanalization” and “reperfusion” have long-time been used interchangeably (48). Nowadays, advances in imaging methods are applied to discriminate such entities. Indeed, it is now possible to routinely visualize and discriminate the ischemic core, the penumbral tissue and the state of collateral blood supply. Therefore, advanced imaging-based procedure are increasingly applied to patient screening and extend the time window for thrombolytic strategies.

1.4. Ischemia-related inflammation

Post-stroke inflammation deserves a growing attention in the pathophysiology of stroke reperfusion. To date, only a limited amount of patients benefit from reperfusion treatment, due to late presentation to emergency department (30). Late thrombolytic treatment via recombinant r-TPA or mechanical thrombectomy increased the risk of hemorrhagic transformation. Revascularization indeed leads to a sudden restoration of oxygenated blood, with generation of free oxygen radicals, which may paradoxically exacerbate ischemic brain injury. This process is known as ischemia-reperfusion (I/R) injury (49). Post-stroke inflammation is a major determinant of I/R. Reactive oxygen species (ROS) associated with damage-associated molecular patterns (DAMPs), released by ischemic brain cells, determine a post-stroke immune burst. DAMPs may stimulate aberrant activation of microglial cells, which act as resident immune cells. They then activate several inflammatory pathways leading to cytokines and chemokines release, which increased the permeability of brain vascular endothelium and cause blood brain barrier (BBB) disruption, brain edema, hemorrhagic transformation and increase of lesion size. Inflammatory signaling also polarizes resident immune cells to produce effector molecules such as matrix metalloproteinases-9 (MMP-9), ROS and nitric oxide. Simultaneously, the peripheral leukocyte infiltration further enhances post-stroke I/R. Especially circulating monocytes recruited from the bloodstream become macrophages within the ischemic tissue and sustain inflammatory response through the release of various pro inflammatory molecules, such as TNF-α, INF-β, IL-6, IL1-β among the others (50). ROS generation is mainly sustained by impaired mitochondrial function and polymorphonuclear neutrophils (PMNs) degranulation. The activation of such ischemia-induced inflammatory burst establishes a vicious

circle further sustains deterioration of cerebral ischemic injury (51). Post-stroke inflammation is also involved in micro-vascular damage and no reflow phenomenon. Several observational studies over the years have studied these pathways but pre-clinical results have not yet been fully translated into clinical practice and the most of clinical trials have failed to improve I/R and post stroke outcome.

1.5. Inflammatory biomarkers: focus of serum osteopontin

A relevant role for serum osteopontin (OPN) has been increasingly described. OPN is an extracellular matrix glycoprotein, mainly known for the activity in bone metabolism and turnover. However, a growing body of evidence indicate that OPN is expressed by a wide variety of cells and tissues and is involved in both physiological processes — such as bone mineralization, tissue remodeling (52) and immune regulation (53,54), — and pathological ones: chronic inflammatory diseases (55), autoimmune diseases (56,57), neoplasms (58,59), metabolic diseases (60) and cardiovascular diseases (61,62). OPN secretion is largely sustained by macrophages and acts as strong chemo-attractant factor (63). Higher levels of serum OPN levels in injured arteries or in atherosclerotic plaques have previously been demonstrated reach a peak at day seven after ischemic lesions, and are positively correlated with worse ischemic lesion volumes and poorer neurological score (64). A similar progression was confirmed even in acute myocardial infarction after successful reperfusion (65). In a cross-sectional study, higher serum levels of OPN have been found in stroke patients compared with controls, confirm the findings of previous works (66). Furthermore, OPN mRNA and protein have been visualized within the ischemic core by immunohistochemistry through electron microscope early after AIS (three days). Later, at day 7 after AIS, a prominent increase of OPN-expressing macrophage was observed within the infarcted brain. Such pattern of expression remained unaltered for two weeks after AIS and then progressively reduces after four weeks (67). Is then conceivable that OPN may have a role in post-stroke I/R.

In light of that, the present study has been designed to compare the prognostic value of reperfusion vs. recanalization after an AIS. Serial examination with computerized tomography (CT) were performed and long-term (day 90) functional outcome was

set as primary endpoint. We have further planned to investigate potential correlations between recanalization/reperfusion and serum inflammatory biomarkers, namely OPN.

2. Methods

2.1. Patients selection and clinical assessment

We retrospectively analyzed clinical and radiological data from n=55 consecutively AIS patients admitted to the Neuroscience Department of Ferrara University Hospital during the period from April 2016 to December 2017. All patients were treated according with the current guidelines (68,69).

Inclusion criteria were: i) presentation at the hospital within 8 hours from symptom onset; ii) baseline and follow-up confirmation of a large vessel anterior AIS based on CT findings represented by the absence of intracranial hemorrhage on Non-contrast CT (NCCT) at admission, the presence of a territorial hypoperfused area on CT perfusion (CTP) at onset and the presence of a territorial hypoattenuated area on NCCT at 24 hours; iii) baseline and follow-up CT imaging carried out at established time-points after symptom onset (NCCT, CT angiography [CTA] and CTP at admission, NCCT, CTA and CTP at days 1 and NCCT at day 90).

Exclusion criteria included: the detection of brain stem infarct or intracerebral hemorrhage at admission NCCT, the inability to complete multimodal CT protocol at baseline and follow-up, history of strokes with residual deficit; contraindications to iodinated contrast agent, pregnancy, age <18 years, clinical instability and/or poor quality of CT acquisition due to motion artifacts. Lacunar AIS were also excluded, due to the low sensitivity of CTP in detecting them (70,71). To select a more homogenous population, we further excluded minor strokes (defined by a NIHSS <4) as well as patients receiving intra-arterial thrombolysis and mechanical thrombectomy. As previously reported (43), we further excluded patients with lacunar/undetermined or posterior circulation stroke, NIHSS <4 at enrollment and those receiving intra-arterial thrombolytic therapy as well.

Type of reperfusion therapy (if any) was recorded. The Trial of ORG 10172 in Acute Stroke Treatment (TOAST) criteria were used to categorize AIS subtypes (5), whereas the National Institutes of Health Stroke Scale (NIHSS) (*Table 3)* was used to score the disease severity at onset, day 1, and day 90 after AIS (72).

The Local Ethics Committee approved this study, in accordance with the guidelines of the Declaration of Helsinki. The All patients gave informed consent before entering in the study.

Table 3. The National Institutes of Health Stroke Scale (NIHSS)

0= No stroke symptoms; 1-4 Minor stroke; 5-15 Moderate stroke; 16-20 Moderate to severe stroke; 21-42 Severe stroke

Current NIHSS		
Level of consciousness	Alert	0
	Not alert, arousable	1
	Not alert, obtunded	2
	Unresponsive	3
LOC questions	Answers both correctly	0
	Answers one correctly	1
	Incorrect	2
LOC commands	Obeys both correctly	0
	Obeys one correctly	1
	Incorrect	2
Gaze	Normal	0
	Partial gaze palsy	1
	Forced deviation	2
Visual fields	No visual loss	0
	Partial hemianopsia	1
	Complete hemianopsia	2
	Bilateral hemianopsia	3
Facial palsy	Normal	0
	Minor paralysis	1
	Partial paralysis	2
	Complete paralysis	3
Motor arm(a) Left(b) Right	No drift	0
	Drift before 10 s	1
	Falls before 10 s	2
	No effort against gravity	3
	No movement	4
Motor leg(a) Left(b) Right	No drift	0
	Drift before 10 s	1
	Falls before 10 s	2
	No effort against gravity	3
	No movement	4
Ataxia	Absent	0
	One limb	1
	Two limbs	2

Current NIHSS		
Sensory	Normal	0
	Mild loss	1
	Severe loss	2
Language	Normal	0
	Mild aphasia	1
	Severe aphasia	2
	Mute or global aphasia	3
Dysarthria	Norma	0
	Mild	1
	Severe	2
Extinction/inattention	Normal	0
	Mild	1
	Severe	2
Total score 0-42		

Adapted from Lyden 2017

2.2. Study endpoint and power estimation

The primary endpoint was to determine whether the occurrence of radiological reperfusion might predict long term (day 90) good functional outcome, defined as a modified Rankin scale (mRS) at day 90 <2 (43,73,74). The sample size was computed based on an expected prevalence of complete reperfusion of 45%, taking into account a prevalence of good functional outcome of 65% and a minimum of six-fold increased incidence in reperfused patients (43,74). According with our power calculation for two proportion comparison, the minimum sample size requested to detect a six-fold increase in the incidence of good functional outcome with a power of 80% and with a two-sided alpha error of 5% was of 12 patients. As secondary endpoint, we investigated the ability of reperfusion to predict early favorable clinical response, defined as a reduction of ≥8 points on the NIHSS (43) during the first day after AIS, and the occurrence of hemorrhagic transformation during the first 7 days after AIS. Finally, potential correlations between reperfusion and serum biomarkers of inflammation has been explored. Two blinded independent investigators adjudicated the study endpoints.

2.3. Imaging acquisition protocol

All images were conducted on 64-slice scanners and included i) NCCT carried out from the skull base to the vertex; ii) CTA performed from the carotid bifurcation to vertex; iii) CTP that covered a total of 4 cm from the basal ganglia to the lateral ventricles. CTP studies were obtained with a dynamic first-pass bolus-tracking methodology according to a one-phase imaging protocol consisting of an acquisition of 50-seconds continuous (cine) scans, which started 5 seconds after the automatic injection of 40 ml of non-ionic contrast agent at the rate of 4 ml/sec.

2.4. Imaging processing and analysis

According to EXTEND-IA trial (75), the severity of arterial occlusion was judged on CTA at onset using a modified version of the Thrombolysis in Myocardial Infarction (TIMI) grading system: complete occlusion (TIMI score=0-1); partial occlusion (TIMI score=2); no occlusion (TIMI score=3). Patients with TIMI score ranging between 0 and 1 were categorized as occluded, whereas a TIMI score of 2 and 3 defined the not occluded ones. The site of occlusion was assessed as previously indicated (76). All CTP scans were assessed using a commercially available delay-sensitive

deconvolution software (CT Perfusion 3, GE Healthcare, Waukesha, WI). For each CTP scan, time-density curves for the arterial input function and venous output functions were obtained from the anterior cerebral artery and superior sagittal sinus, respectively. The AIF was corrected for partial volume averaging using the VOF-TDC. Cerebral blood flow (CBF), cerebral blood volume (CBV) and mean-transit-time (MTT) CTP maps were generated for each patient by deconvolution of tissue TDCs and the AIF. CBF, CBV and MTT values were expressed in ml/min/100g, ml/100g and seconds, respectively. Large blood vessels were automatically excluded from calculation by the software. Color coded functional CTP map scales were set at 0-100 ml/min/100g for CBF, 0-8 ml/100g for CBV and 0-20 seconds for MTT. After identification by visual inspection on MTT and CBV maps, three different regions of interest (ROIs) were drawn freehand by two neuro-radiologists (A.B. and E.F., with 5- and 20-year experience, respectively) on every section in which they were visible according to the classical CTP mismatch model (77): i) MTT lesion indicating total hypoperfusion; ii) CBV lesion referring to infarct core; iii) MTT-CBV lesion representing ischemic penumbra (Figure 1).

Figure 1

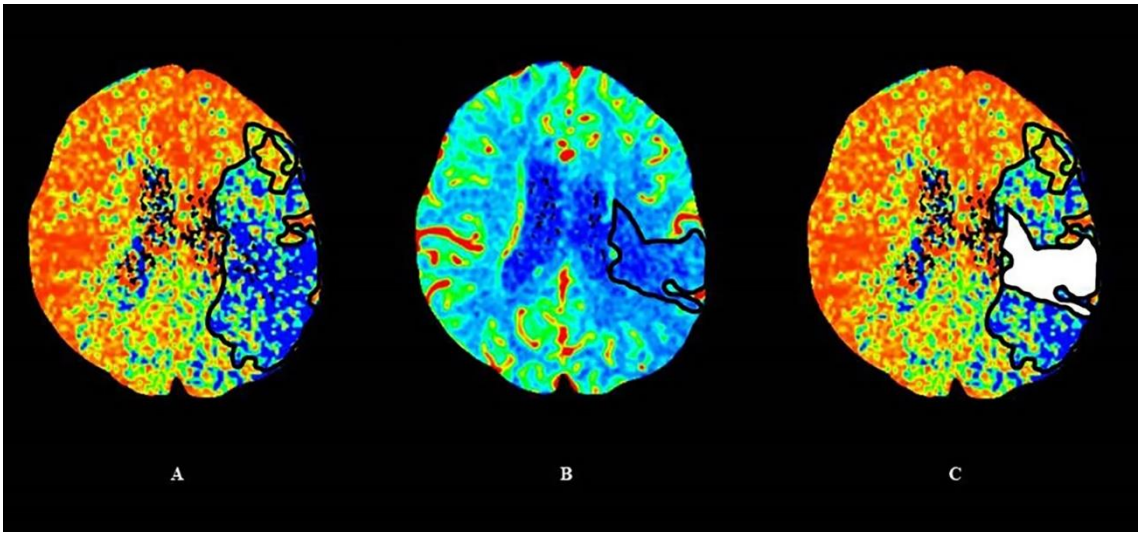

Figure 1 Color coded functional CT perfusion map. After identification by visual inspection on mean-transit-time (MTT) and cerebral blood volume (CBV) maps, 3 different regions of interest were drawn freehand on every section according to the classical CTP mismatch model: **A** MTT lesion indicating total hypoperfusion; **B** CBV lesion referring to infarct core; **C** MTT-CBV lesion representing ischemic penumbra.

This last ROI was outlined on MMT maps where the ROI corresponding to CBV defect were automatically superimposed. Discrepancies between readers were resolved by consensus adjudication. The sum of these lesion areas was then multiplied by slice thickness to obtain core and penumbra (CTP MTT-CBV mismatch) volumes, respectively. Final infarct volume was measured on follow-up NCCT at 3 months after symptom onset with a multi-slice planimetric method by summation of the hypodense areas, manually traced on each slice in which they were detectable, multiplied by slice thickness (78). The type of HT was recorded by NCCT at 24 hours and at 7 days post ictus. In agreement with EXTEND-IA trial (75), recanalization was scored on CTA at 24 hours with an adaptation of the TIMI grading system: persistent occlusion (TIMI score=0-1); partial recanalization (TIMI score=2); full recanalization (TIMI score=3). Patients with TIMI score ranging from 2 to 3 were considered as reanalyzed, whereas patients with TIMI score of 0 and 1 were classified as not reanalyzed. All patients not occluded on CTA at admission had a TIMI score of 3 at 24 hour-CTA and therefore, were considered as reanalyzed. Radiological reperfusion was evaluated by reperfusion index that measures the percentage reduction of baseline MTT lesion at 24 hours. For this purpose, visually identified MTT defect volume was obtained by a manual multi-slice planimetric method at admission and at 24 hours (79). Patients with a reperfusion index >75% were considered as reperfused. Reduction of the ischemic volume was expressed as the difference between total hypoperfusion volumes at admission (baseline) and at 24 hours.

2.5. Blood collection and quantification

Blood samples were collected at different time points using a butterfly to reduce membrane shear stress and then drawn in tubes to obtain serum. Samples were collected at baseline and at day 1 after AIS. Hematology parameters and blood chemistry, including plasma glucose, triglycerides, total cholesterol, high-density lipoprotein and low-density lipoprotein cholesterol (to assess dyslipidemia) were measured by routine auto-analyzer.

2.6. Measurement of a serum biomarker associated with reperfusion

Serum osteopontin (OPN), a biomarker associated with cerebral reperfusion (80,81), were measured by colorimetric enzyme-linked immunosorbent assay (ELISA), following manufacturer’s instructions (R&D Systems, Minneapolis, MN).

The limits of detection for OPN was 62.5 pg/ml. Mean intra- and inter-assay coefficients of variation (CV) were below 8% for all markers (82). ELISA is useful method in detecting and quantifying biomarker proteins in serum and biological fluids. ELISA is a test that employs antibodies to detect a substance, usually antigen or antibodies. The use of a solid support (usually a microtitration plate) is essential. There are several variants of the ELISA test, which differ according to the component to be detected, but the two main methodologies of ELISA are divided into:

- Direct test, in which the presence of an antigen is determined. the direct test can be further classified into simple and sandwich ELISA. In the simple method the antigen is absorbed on the plaque and detected with an enzyme-labeled antibody. In the sandwich method, the antibody (capture Ab) that is used to capture the antigen is absorbed on the plate, the absorbed antibodies are then exposed to the sample that could contain the antigen and a second, labeled antibody (detection Ab) is added to detect that the antigen has been captured.
- Indirect test, in which principle consists in detecting the presence of a specific antibody in the sample. Indirect test is widely used to identify the presence of antibodies against a specific antigen in the patient's blood plasma to ascertain whether there has been exposure to a specific pathogen. This is done more commonly in the HIV test. The test includes four main stages: first of all, there is an antigen fixation: the antigen, specific to the desired antibody, is incubated on a microtitration plate. Then there is the fixation of the antibodies to be dosed: the sample to be dosed is incubated (serum containing the antibodies), as well as standards (solutions containing known concentrations of antibodies). Specific antibodies attach to antigens. Third stage is the fixation of detection antibodies: secondary antibodies conjugated to one are incubated peroxidase. Finally, the specific substrate to the enzyme is incubated. In case the reaction is positive (presence of the desired antibodies) the substrate will be transformed and induce a color change. The intensity of the coloring is proportional to the amount of reaction present. At the end of each step, the plate is washed with a detergent solution to remove the components of the sample that have unsecured bonds.

Figure A

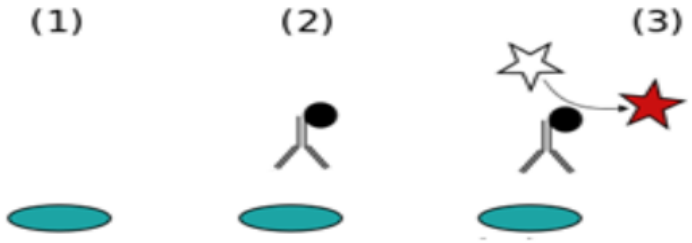

Figure A. Direct ELISA.

The antigen which we are going to test is added in a well of the microtitration plate (**A1**). A solution of non-reacting protein is added to cover the area uncoated by the antigen. The primary antibody conjugated with an enzyme is added (**A2**). The substrate for the enzyme is added. Generally, this substrate changes the color of the solution, reacting with the enzyme (**A3**). If there are high concentration of antigen, the color change will be stronger. A spectrometer is used to quantify the color intensity.

Figure B

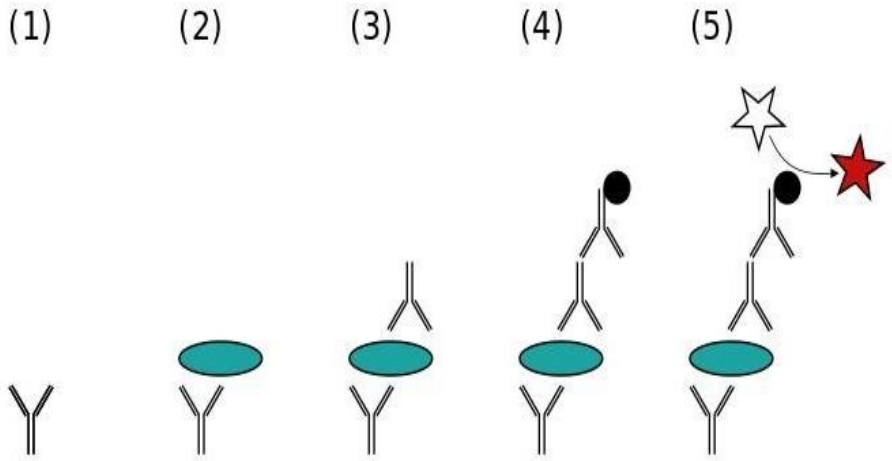

Figure B. Indirect ELISA.

The well of the microtitration plate is prepared with the capture antibody **(B1)**. The sample containing the antigen is added to the plate **(B2)**. The detection antibody is added, which forms a complex with the antigen **(B3)**. A secondary antibody, labeled with an enzyme, is added which binds the detection antibody **(B4)**. The enzyme substrate is added; the bond generally causes a change in the color of the solution **(B5)**. At the end of each step, the plate is washed with a detergent solution to eliminate the components of the sample that have non-specific bonds.

In our study we used the "sandwich" procedure. General ELISA protocol in summary include:

- Addiction of a solution of the primary antibody (capture Ab), specific for the antigen to be searched, to each wells of a special polystyrene test plate. Seal the plate and incubate overnight. The bottom of the well is saturated with the antibody that adheres and the excess is washed off using a wash buffer.
- Block plates by adding reagent diluent to each well and incubate for a minimum of 1 hour. Repeat wash using an autowasher.
- Addition of the sample or standards in reagent diluent, per well. Incubate for 2 hours. The antigen wanted, if present, binds specifically with the antibody and the excess is washed off.
- Addition of secondary antibody (detection Ab) diluted in reagent diluent, to each well. Incubate for 2 hours. This antibody is conjugated with an enzyme, typically peroxidase or alkaline phosphatase (in our case it was used horseradish-peroxidase HRP), and washing with a buffer solution; The secondary antibody binds selectively to the antigen, if present, and the excess is washed off.
- A streptavidin–biotin complex was used as a bridge between the enzyme and the antibody. Incubate for 20 minutes. A well wash is necessary. Biotin is a water-soluble vitamin that has affinity for Streptoavidin. Streptoavidin-HRP (60Kd protein isolated from the bacterium Streptomices avidinii, is linked to the enzyme HRP) has 4 active sites with high affinity for biotin.
- If the enzyme used is a peroxidase, H_2O_2 plus tetramethylbenzidine (TMB) is added as a substrate. Incubate for 20 minutes. This substance causes a reaction with the streptavidin-HRP, conjugated to the secondary antibody producing a blue coloration visible to the naked eye. If the antigen characteristic of the pathogenic organism is absent in the well, there will also be no enzyme conjugated to the secondary antibody and therefore the reaction cannot take place.
- Addition of sulfuric acid to stop the reaction between the streptavidin-HRP and substrate. This producing a yellow coloration of wells where antigen is present.
- Reading of the result, expression of the reaction which produces a chromogenic or fluorogenic reaction on the substrate, subsequently

measurable through a spectrophotometer (450 λ). The concentration of antigen present in the sample is then calculated based on the intensity of the coloration.

Figure C

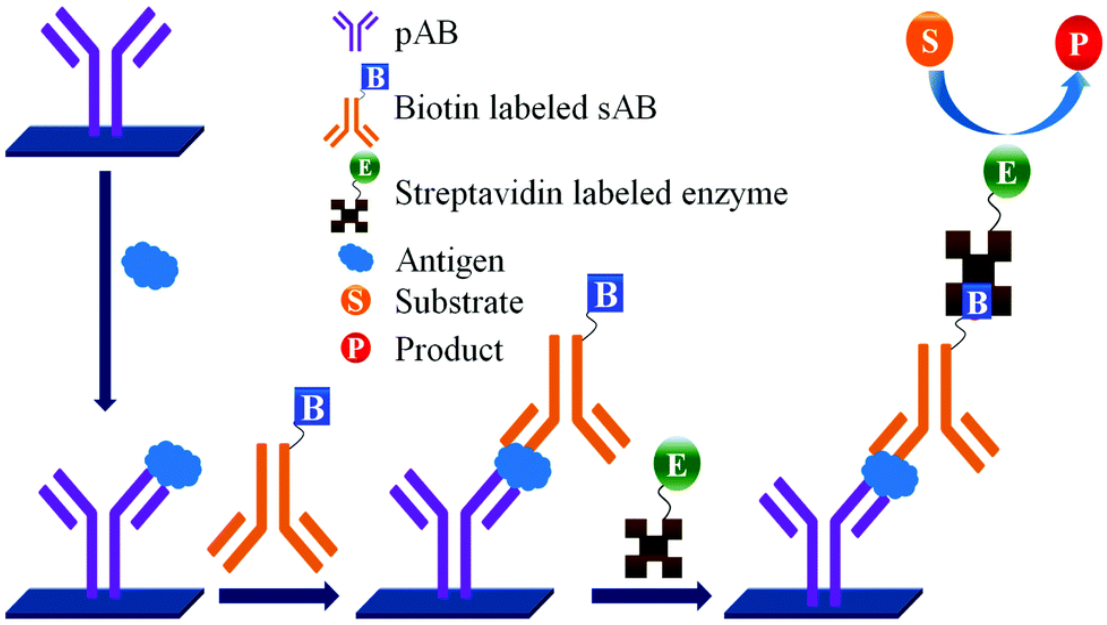

Figure C: Addiction of capture Ab. The capture antibody binds to the researched antigen. The detection antibody that binds Biotin is added. Streptoavidin-HRP is added. Therefore, the biotin-streptoavidin complex serves to indirectly bind the enzyme to the secondary antibody. The enzyme is the Horseradish peroxidase (HRP). Add the substrate of the enzyme, which is hydrogen peroxide and TMB. The enzyme catalyzes the cleavage reaction of hydrogen peroxide (H_2O_2). Then, in a second and consequent reaction, the oxygen that is formed oxidizes a chromogen TMB, whose oxidized product appears blue. Addiction of sulfuric acid and further oxidation the color turns yellow. The final colored complex can be read with a spectrophotometer at 450 nm.

Adapted from Nimse et al., 2016

2.7. Statistical analysis

Analyses were performed with IBM SPSS Statistics for Windows, Version 23.0 (IBM CO., Armonk, NY). Categorical data are presented as relative and absolute frequencies. Continuous variables were expressed as median and interquartile range (IQR) as the normality assumption was not demonstrated. Difference between two time-points were presented as delta (Δ). Intergroup comparisons were drawn by Fisher's exact test and Mann-Whitney *U*-test, as appropriate. Conversely, the dichotomized classification of reperfusion and recanalization was compared by McNemar test Instead comparison between paired sample was drawn by Wilcoxon rank sum test Ranked. Multiple linear regressions were performed to model the lesion growth and final infarct volumes with the recanalization and reperfusion. Multivariate logistic regression was used to evaluate the predictive role of recanalization and reperfusion toward good clinical response and good functional outcome. Finally, the prognostic ability was assessed in a post-hoc manner based upon receiver operator characteristic (ROC) curve (MedCalc 12.5, MedCalc Software, Ostend, Belgium). The area under the curve (AUC) was given with 95% confidence interval (CI). For all statistical analyses a 2-sided p-value <0.05 was considered as statistically significant.

3. Results

3.1. Patients' characteristics

Baseline clinical characteristics of the whole cohort (n=55) are listed in Table 4.

Table 4. Clinical characteristics of study population (n=55) at admission.

Demographic	
Age, years (IQR)	69 (55-76)
Male, no (%)	29 (52.7)
Hypertension, no (%)	32 (58.2)
Atrial fibrillation, no (%)	23 (43.4)
Active smokers, no (%)	18 (33.3)
Previous smokers, no (%)	5 (9.1)
Diabetes, no (%)	4 (7.3)
Dyslipidemia, no (%)	13(23.6)
Biochemical	
Total WBC#, no. x 10^9 (IQR)	7.91 (6.70-9.54)
Neutrophil count, no. x 10^9 (IQR)	4.83 (3.71-6.74)
Lymphocyte count, no. x 10^9 (IQR)	2.1 (1.53-2.58)
Serum glycaemia, mg/dL (IQR)	112 (97-143)
INR*, no. (IQR)	1.09 (1.02-1.19)
Clinical/ radiological	
Time window to CT†	
0-3 hours, no. (%)	45 (81.8)
3-6 hours, no. (%)	9 (16.4)
6-8 hours, no. (%)	1 (1.8)
TOAST‡ classification	
Atherothrombotic, no. (%)	32 (58.2)
Cardio-embolic, no. (%)	23 (43.4)
Intravenous r-TPA§, no (%)	39 (70.9)
NIHSS‖	10 (7-14)
Total hypoperfused volume, ml (IQR)	88.03 (36.48-129.18)
Ischemic core volume, ml (IQR)	5.74 (0.51-17.18)
Ischemic penumbra, ml (IQR)	59.63 (27.04-102.32)

Data are expressed as median (interquartile range [IQR]) or number [no.] (percentages [%]).
WBC: white blood cells
* INR: international normalized ratio
† CT: computerized tomography
‡ TOAST: Trial of Org 10172 in Acute Stroke Treatment
§ r-r-TPA: recombinant tissue plasminogen activation
|| NIHSS: National Institutes of Health Stroke Scale

Patients' median age was 69 years (55-76), with a slight prevalence of hypertension (58.2%). In most cases the time window between symptom onset and CT study performed was less than 3 hours (81.8%), thus accounting for a high rate of reperfusion therapy with r-TPA (70.9%). More precisely, in our study population, 49/55 (89.1%) patients were treated (n=39 with intravenous thrombolysis with r-TPA and n=10 with mechanical thrombectomy), whereas 6/55 (10.9%) did not receive any therapy due to the presence of hypodensity >1/3 cerebral hemisphere, as indicated by ASPECTS≤7, on admission NCCT (n=3), and the current use of anticoagulant with INR>1.7 (n=3). At day 1 after AIS, reperfusion was present in 24 patients (43.6%). (Table 5). In 15 patients (27.3%) there was a pattern characterized by recanalization without reperfusion (Table 5).

Table 5. Radiological characteristic at day 1.

		p-value
Reperfusion, n (%)	24 (43.6)	
Recanalization, n (%)	39 (70.9)	
Reperfusion *vs.* recanalization		<0.001
No-reperfusion, no-recanalization	16 (29.09)	
Recanalization without reperfusion ("no-reflow"), n (%)	15 (27.3)	
Reperfusion without recanalization, n (%)	0 (0.0)	
Reperfusion and recanalization, n (%)	24 (43.6)	

Data are expressed as median (interquartile range [IQR]) or number [no.] (percentages [%]). Comparison was drawn by Fisher's exact test.

Conversely, no patients had reperfusion without recanalization, whereas in 24 patients (43.6%) both reperfusion and recanalization were observed. A McNemar test on the dichotomized classification of reperfusion and recanalization confirmed a significant discrepancy between these two parameters ($p<0.001$) (Table 5).

3.2. Reperfusion and recanalization weakly influences inflammatory biomarkers and radiological features of cerebral injury

Ischemic core and penumbra volumes did not differ in patients categorized as recanalized vs. no recanalized and reperfused vs. no reperfused (Figure 2A-C).

Figure 2

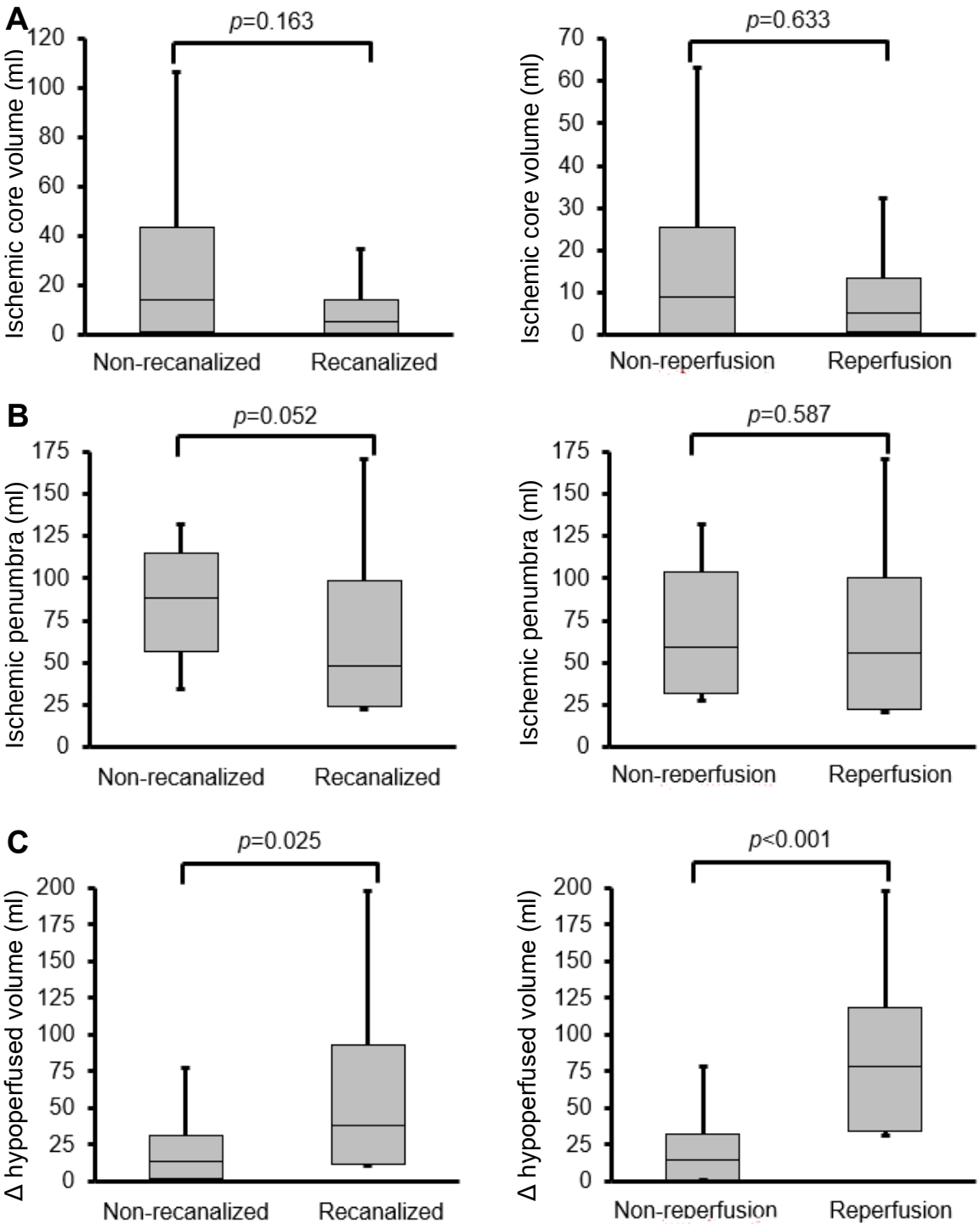

Figure 2. Box-whisker representative plots of the impact of recanalization/reperfusion of radiological findings. The values of ischemic core volume **(A)**, ischemic penumbra **(B)** and difference (Δ) in hypoperfused volume between onset at 24 hours **(C)** are represented in non-recanalized/recanalized and non-reperfused/reperfused patients.

Conversely, reperfusion determined a great reduction of the ischemic volume during the first day after AIS (Δ baseline-day 1: 78.59 *vs.* 14.59 ml; $p<0.001$). A lesser but significant benefit was also observed in the group of recanalized patients (Δ baseline-day 1: 37.95 vs. 13.30 ml; p=0.025). OPN was tested as a known circulating mediators increased after cerebral reperfusion (80,81). Noteworthy, when non-reperfused and reperfused patients were analyzed separately, no difference was shown in demographic, biochemical and clinical/radiological parameters (Table 6).

Table 6. Clinical differences among no-reperfused and reperfused patients at baseline.

	No-reperfused (n=31)	Reperfused (n=24)	*p*-value
Demographic			
Age, years (IQR)	67 (58-76)	71 (46-78)	0.939
Male, no (%)	17 (54.8)	12 (50.0)	0.789
Hypertension, no (%)	19 (61.3)	13 (54.2)	0.783
Atrial fibrillation, no (%)	12 (38.7)	11 (45.8)	0.783
Active smokers, no (%)	13 (41.9)	5 (21.7)	0.151
Previous smokers, no (%)	3 (9.7)	2 (8.3)	1.000
Diabetes, no (%)	3 (9.7)	1 (4.2)	0.624
Dyslipidemia, no (%)	10 (32.3)	3 (12.59	0.116
Biochemical			
Total WBC#, no. x 10^9 (IQR)	7.91 (6.99-10.01)	7.8 (6.26-9.01)	0.396
Neutrophil count, no. x 10^9 (IQR)	5.11 (3.69-7.36)	4.45 (3.74-6.42)	0.665
Lymphocyte count, no. x 10^9 (IQR)	2.1 (1.65-2.74)	2.13 (1.18-2.58)	0.773
Serum glycaemia, mg/dL (IQR)	116 (103-166)	107 (91-130)	0.096
INR*, no. (IQR)	1.08 (1.01-1.13)	1.13 (1.02-1.28)	0.098
Clinical/ radiological			
Time window to CT†			0.214
0-3 hours, no. (%)	24 (77.4)	21 (87.5)	
3-6 hours, no. (%)	7 (16.422.6)	2 (8.3)	
6-8 hours, no. (%)	0 (0.0)	1 (4.2)	
TOAST‡ classification			
Atherothrombotic, no. (%)	19 (61.3)	13 (54.2)	
Cardio-embolic, no. (%)	12 (38.7)	11 (45.8)	0.783
Intravenous r-TPA§, no (%)	21 (67.7)	18 (75.0)	0.765
NIHSS‖	11 (7-20)	10 (7-14)	0.208

Comparison were drawn by Mann-Whitney U test, Fisher's exact test or Kruskal-Wallis test, as appropriate. Data are expressed as median (interquartile range [IQR]) or number [no.] (percentages [%]).

WBC: white blood cells * INR: international normalized ratio

† CT: computerized tomography

‡ TOAST: Trial of Org 10172 in Acute Stroke Treatment

§ r-TPA: recombinant tissue plasminogen activation

|| NIHSS: National Institutes of Health Stroke Scale

MTT: mean-transit-time

** CBV: cerebral blood volume

Whereas no difference was observed at baseline, a significant increase was observed the day after AIS ($p=0.002$), thus determining a significant difference among the two study groups ($p=0.004$) (Figure 3).

Figure 3

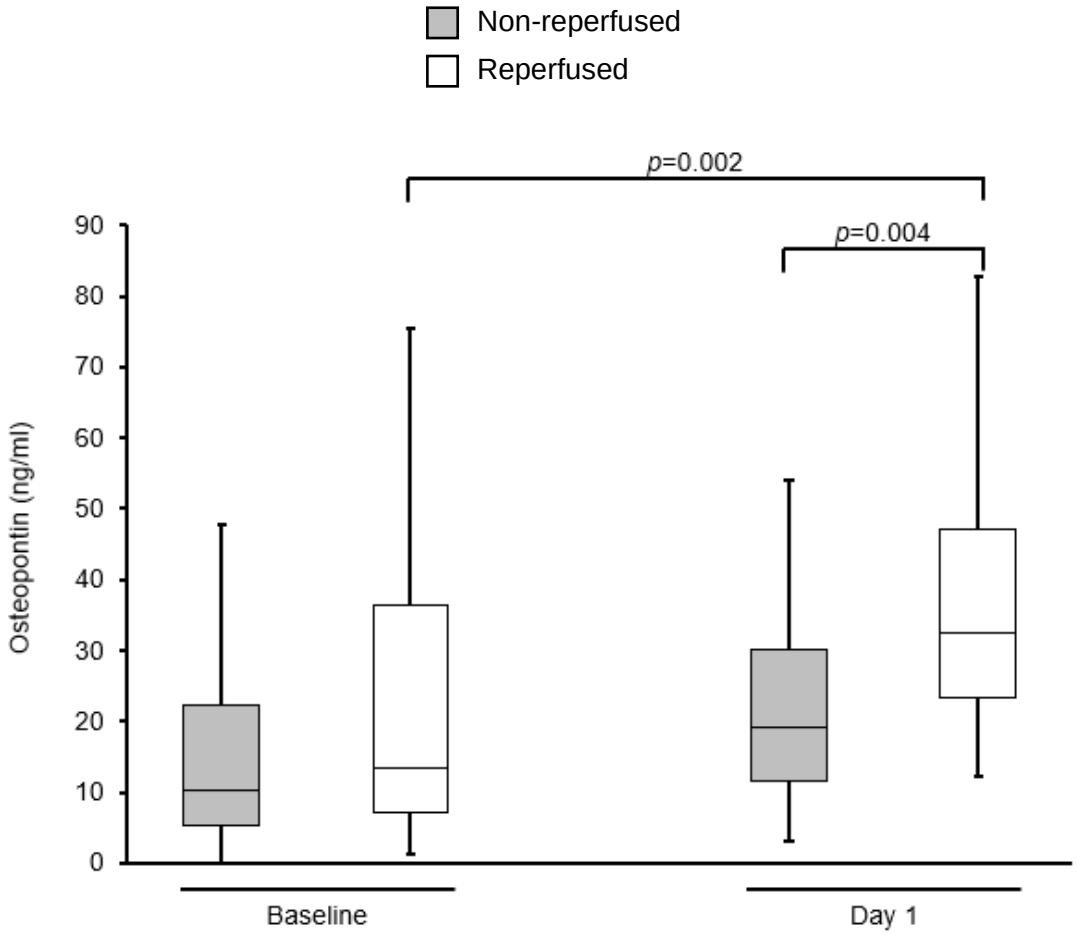

Figure 3. Box-whisker representative plots of the modification in circulation osteopontin (OPN). Circulating OPN levels represented in non-reperfused/reperfused patients were quantified at baseline and after 1 day from stroke onset. Intergroup comparisons and overtime change were assayed.

Multilinear regression analysis further confirmed an independent association of volume lesion reduction at day 1 with both recanalization (29.425 [6.271-52.580]; β=0.265; *p*=0.014) and reperfusion (B 46.803 [95% CI 29.138-64.469]; β=0.461; *p*<0.001) (Table 7). However, only the reperfusion was independently associated with final infarct volume at day 90 after AIS (B -54.236 [95% CI 93.203- -15.270]; β= -0.333; *p*=0.007) (Table 7).

Table 7. Multiple linear regression for predicting the reduction of ischemic lesion volume at day 1 and the final infarct at day 90.

	B (95% CI)	B	*p*-value
Volume lesion reduction			
Age	-0.069 (-0.640-0.502)	-0.019	0.809
Gender, male	7.361 (-8.481-23.202)	0.073	0.354
Glycemia	-0.105 (-0.324-0.115)	0.109	0.341
Time window to CT#	-4.948 (-23.525-13.628)	-0.044	0.594
Intravenous r-TPA*	-2.095 (-21.594-17.404)	-0.019	0.830
NIHSS† at onset	-0.139 (-1.753-1.475)	-0.016	0.863
Ischemic core volume	0.554 (0.290-0.819)	0.334	**<0.001**
Ischemic penumbra	0.663 (0.496-0.830)	0.587	**<0.001**
Reperfusion at day 1	46.803 (29.138-64.469)	0.461	**<0.001**
Recanalization at day 1	29.425 (6.271-52.580)	0.265	**0.014**
Final infarct volume			
Age	-0.851 (-41.309-191.681)	-0.142	0.180
Gender, male	-22.466 (-57.409-12.477)	-0.139	0.202
Glycemia	-0.058 (-0.542-0.426)	-0.027	0.809
Time window to CT	42.518 (1.540-893.945)	0.233	**0.042**
Intravenous r-TPA	22.079 (-20.931-65.090)	0.124	0.307
NIHSS at onset	2.289 (-1.271-5.849)	0.163	0.202
Ischemic core volume	1.270 (0.687-1.854)	0.476	**<0.001**
Ischemic penumbra	-0.060 -(0.428-0.308)	0.033	0.745
Reperfusion at day 1	-54.236 (-93.203- -15.270)	-0.333	**0.007**
Recanalization at day 1	-1.606 (-52.681-49.469)	-0.009	0.950

Volume of lesion growth is defined as the Δ baseline-day 1.
CT: computerized tomography WBC: white blood cells
* r-TPA: recombinant tissue plasminogen activator
† NIHSS: National Institutes of Health Stroke Scale

3.3. Reperfusion at 24 hours is a strong predictor of good outcome after an AIS

Reperfusion, but not recanalization, was able to predict good clinical response at 24 hours also after adjustments for age, gender, glycaemia, time window to CT, intravenous thrombolysis, NIHSS, ischemic core and penumbra volumes at onset (OR 16.054 [1.423-181.158]; *p*=0.025) (Table 8). Though non-significant, reperfusion shown a trend towards the prediction of hemorrhagic transformation during the first 7 days after AIS (adjusted OR 0.153 [0.022-1.047]; *p*=0.056) (Table 8). However, the most relevant result was the predictive ability of reperfusion towards 90-day good functional outcome, assessed by mRS (adjusted OR 25.801 [1.483-448.840]; *p*=0.026) (Table 8).

Table 8. Multiple logistic regression for predicting good clinical response at day 1 (reduction of ≥8 points on the NIHSS or a NIHSS score ≤1), occurrence of hemorrhagic transformation and good functional outcome (modified Rankin Scale, 0-1) at day 90.

	OR (95% CI)	*p*-value
Good clinical response (day 1)		
Age	1.090 (1.002-1.185)	**0.044**
Gender, male	3.316 (0.475-23.150)	0.227
Glycemia	0.928 (0.870-0.990)	**0.024**
Time window to CT#	1.751 (0.108-28.333)	0.693
Intravenous r-TPA*	0.746 (0.056-9.940)	0.825
NIHSS† at onset	1.235 (0.978-1.559)	**0.076**
Ischemic core volume	0.946- (0.898-0.996)	**0.034**
Ischemic penumbra	1.002 (0.982-1.023)	0.811
Recanalization at day 1	7.721 (0.296-201.188)	0.219
Reperfusion at day 1 ≥75%	16.054 (1.423-181.158)	**0.025**
Hemorrhagic transformation (onset-day 7)		
Age	1.014 (0.941-1.092)	0.720
Gender, male	0.476 (0.076-2.976)	0.427
Glycemia	1.020 (0.998-1.042)	0.076
Time window to CT	0.156 (0.007-3.573)	0.245
Intravenous r-TPA	1.197 (0.119-12.010)	0.879
NIHSS at onset	1.052 (0.862-1.284)	0.616
Ischemic core volume	1.074 (1.007-1.147)	**0.030**
Ischemic penumbra	1.000 (0.981-1.019)	0.984
Recanalization at day 1	0.744 (0.065-8.575)	0.812
Reperfusion at day 1 ≥75%	0.153 (0.022-1.047)	0.056
Good functional outcome (day 90)		
Age	0.950 (0.854-1.058)	0.351
Gender, male	0.348 (0.035-3.471)	0.370
Glycemia	0.968 (0.939-0.998)	**0.034**
Time window to CT	0.071 (0.004-1.347)	0.078
Intravenous r-TPA	0.050 (0.002-1.054)	0.054
NIHSS at onset	0.734 (0.569-0.949)	**0.018**
Ischemic core volume	0.964 (0.922-1.007)	0.095
Ischemic penumbra	1.008 (0.985-1.032)	0.507
Recanalization at day 1	1.618 (0.178-14.700)	0.669
Reperfusion at day 1 ≥75%	25.801 (1.483-448.840)	**0.026**

CT: computerized tomography WBC: white blood cells
* r-TPA: recombinant tissue plasminogen activator
† NIHSS: National Institutes of Health Stroke Scale

ROC curve analysis, further characterized the predictive ability of reperfusion by showing an AUC of 0.777 (sensitivity 88.89%, specificity 64.86%; $p<0.001$) and 0.792 (sensitivity 88.89%, specificity 75.68%; $p<0.001$) for the good clinical response and good long-term clinical outcome, respectively (Figure 4).

Figure 4

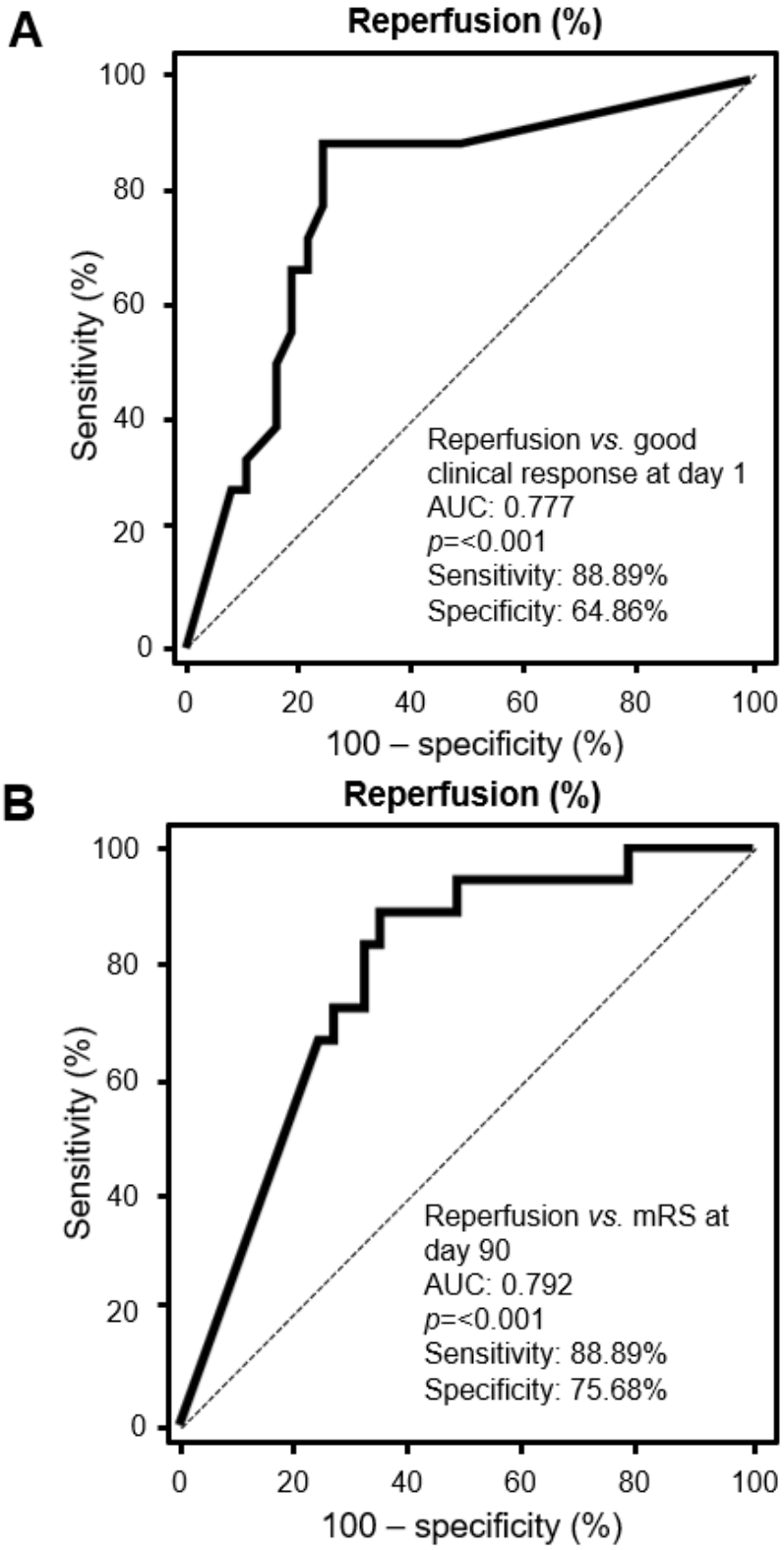

Figure 4. Receiver operator characteristic (ROC) curve analysis.
The predictive value of reperfusion toward good clinical response at 24 hours **(A)** and long-term functional outcome assessed by modified Rankin scale (mRS) at day 90 **(B).**

4. Discussion

In this study, reperfusion at 24 hours after AIS consistently overcame recanalization in predicting radiological evolution of ischemic lesion and clinical outcomes. Indeed, patients with MTT reperfusion index >75% experienced a greater reduction of ischemic lesion volume during the first day after AIS and a smaller infarct volume at 90 days. According with radiological findings, reperfusion was also associated to more favorable prognosis in terms of good clinical response at 24 hours and good functional outcome assessed 90 days after AIS. Noteworthy, we observed some discrepancies between recanalization and reperfusion rates. As compared to previous studies (43,79), we did not observe reperfusion without recanalization, whereas the recanalization occurred without reperfusion in fifteen patients. This implies that, in some cases, reopening of the occluded artery is not associated with the restoration of microcirculatory blood flow (41). On the other hand, it is important to emphasize that reperfusion can be non-nutritional inducing a luxury perfusion, exceeding metabolic demand, in non-viable tissue evolving into infarct (83) or a deterioration of ischemic but salvageable brain tissue due to an impairment of neurovascular unit mediated by molecular and cellular mechanisms (84). Nevertheless, the present study further emphasizes a conceptual difference between reperfusion and recanalization. The discrepancy between angiographically successful recanalization and non-favorable clinical outcome might be explained by a non-visible alteration in microcirculation, as occurs in case of distal thrombus embolization (44) or no-reflow phenomenon. In the latter case, swollen astrocyte, pericyte and endothelial cells determine the narrowing of microvascular lumen and failure of microcirculatory reperfusion despite clot removal (85,86). The capillary narrowing usually starts one hour after AIS and contributes to brain injury by promoting oxidative stress (85). Not surprisingly, more recent experimental studies (87) and clinical trials (88,89) pointed out the role of early (or even ultra-early) reperfusion as key determinant of stroke outcome (42,90,91). Our results are then consistent with previous studies based on both CT (79) and magnetic resonance (92–94), in supporting a role of reperfusion as surrogate marker of clinical outcome independently of recanalization. More specifically, we emphasized the role of earlier repeated follow-up imaging in improving the prognostic value of reperfusion (95).

Another critical matter of discussion is the role of inflammation in ischemic/reperfusion (I/R) injury (96). Various studies in the last decade emphasized the detrimental role of inflammatory response in AIS. We previously observed that delayed rise in serum OPN was associated with poor long-term clinical outcome after an AIS (64). Similarly, time-dependent changes in serum OPN were also correlated with left-ventricular volume and function in patients with myocardial infarction undergoing successful reperfusion (65). Yet, numerous clinical studies failed to demonstrate any positive outcomes of anti-inflammatory strategies in stroke patients (50). Rather, the timing of inflammatory response is increasingly emerging as main determinant of I/R injury outcome. Whereas persistent inflammation exerts negative effects on tissue repair, early inflammatory response seems to drive tissue healing (97). With this aim we focused on the early change in circulating OPN, reporting a significant rise in reperfused patients. Nevertheless, long-term kinetic of OPN after AIS and potential relationship with radiological findings still remains unknown and any potential explanation is highly speculative.

4.1. Study limitations

Firstly, the study cohort may not be considered representative of a general stroke population as whole and future population-based studies are warranted to validate our results. Whereas we were able to demonstrate the prognostic role of reperfusion independently of intravenous thrombolysis, our sample size was not powerful enough to discriminate the effect of other therapeutic approaches (i.e. intra-arterial thrombolysis and mechanical thrombectomy). Similarly, larger studies are required to investigate the setting of minor stroke, where the expected differences may be smaller. Secondly, recanalization/reperfusion imaging was obtained relatively late, and recanalization/reperfusion observed at 24 hours after AIS may not have the ability to salvage viable ischemic tissue at risk. However, the time from admission to recanalization/reperfusion imaging and to discharge imaging was similar for all patients, thus not introducing a bias in the analysis. Thirdly, the evaluation of reperfusion by CTP is incomplete because this technique is not able to obtain metabolic information which are crucial, in combination with hemodynamic parameters, to establish whether the restoration of microvascular circulation corresponds to an actual tissue recovery or leads to reperfusion injury (98). In this way, only positron emission tomography can provide both hemodynamic and

metabolic data useful to better understand tissue fate (99). Fourthly, the estimation of final infarct volume with 24-hour NCCT could be affected by a distortion of residual cavity due to retraction effects related to gliosis. However, it is currently accepted that final infarct volume is more accurately delineated in images obtained 30 or 90 days after stroke (100). Fifthly, it is well-known that threshold-based fully-automated software are superior to manually tracing technique we used in this study for quantitative volumetric analysis of CBV and MTT alterations (101). Nevertheless, the calculation of CBV and MTT lesion volume by a manual multi-slice planimetric method is still considered reliable in defining core and penumbra (102) and reperfusion on CTP (103). Finally, different studies have described a bi-modal delayed peak of OPN (a serum biomarker associated with reperfusion) after AIS (67). Therefore, future prospective cohorts should include earlier and later time points in order to able to correlate radiological evolution (in terms of recanalization/reperfusion) with overtime change in circulating OPN.

5. Conclusion

In conclusion, we were able to show that reperfusion occurring during the first day after AIS in predicting better radiological evolution of ischemic lesion and clinical outcome. Radiological reperfusion, but not recanalization, was significantly associated with early positive clinical response (defined as reduction on the NIHSS ≥8 at day 1 or a NIHSS ≤ 1 at 24 h) and good functional long-term outcome (defined as an mRS <2 at day 90) in multiple logistic regressions. Consequently, reperfusion at 24 hours may be the stronger predictor of, and consequently a reliable surrogate for, clinical outcome. Also, reperfusion, but not recanalization, was associated with an increase in serum levels of OPN, an inflammatory molecule potentially related with post-ischemic cerebral pathophysiology. Whereas no differences were quantified at baseline, a significant increase of circulating OPN levels was observed after day 1 from the stroke onset, therefore resulting a significant difference among reperfused and non-reperfused patients. In view of different studies that have described delayed peaks of OPN and that has been observed that there is a biphasic inflammation response, a critical step for optimal healing, and given that our study has focused on early increase in circulating OPN after reperfusion, assessed only at baseline and day 1, future studies are needed to evaluate the trend over several times. Nowadays, recent advances in acute reperfusion therapy and the possibility of a better comprehension of the fine-tuned mechanisms underlying cerebral ischemia/reperfusion salvage, should push towards the possibility of acting on the underlying inflammation pathways in such a way to identify effective protective mechanisms and investigate the predictive role of related serum inflammatory biomarkers in functional and clinical outcomes. Thus, larger clinical trials correlating radiological reperfusion and potential biomarkers are required to validate their predictive power on AIS clinical endpoints.

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