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Reperfusion Injury after ischemic Stroke Study (RISKS): single-center, prospective observational study protocol

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Keywords:	Stroke < NEUROLOGY, Reperfusion Injury, Thrombolysis, Endovascular treatment, Blood Brain Barrier

Reperfusion Injury after ischemic Stroke Study (RISKS): single-center, prospective observational study protocol

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Strengths and limitations of this study

- Using a definite protocol, a prospective collection of data, and an adequate number of patients assuring statistically powered data, Reperfusion Injury after ischemic Stroke Study (RISKS) can integrate substantially scanty clinical information about biological factors involved in reperfusion injury after cerebral ischemia.
- RISKS combines advanced neuroimaging techniques for the study of blood brain barrier disruption with analyses of circulating biomarkers as potential predictors of reperfusion injury after acute phase interventions.
- A limitation of the study is the lack of a control group of patients with stroke not treated with revascularization therapies.
- Another limitation is that study will include patients with acute ischemic stroke treated with intravenous thrombolysis, endovascular treatment or both. This heterogeneity might influence levels of circulating biomarkers at 24 hours.
- A further limitation is the lack of standardization for the assessment of recanalization.

ABSTRACT

Introduction: treatments aiming at reperfusion of the acutely ischemic brain tissue may result futile or even detrimental because of the so-called "reperfusion injury". The processes contributing to reperfusion injury involve a number of factors, ranging from blood brain barrier (BBB) disruption to circulating biomarkers. Our aim is to evaluate the relative effect of imaging and circulating biomarkers in relation to reperfusion injury.

Methods and analysis: observational hospital-based study that will include 140 ischemic stroke patients, treated with systemic thrombolysis, endovascular treatment, or both. BBB disruption will be assessed with computed tomography perfusion (CTP) before acute treatment, and circulating levels of a large panel of biomarkers will be measured in blood samples before acute interventions and after 24 hours. Relevant outcomes will include: 1) reperfusion injury, defined as hemorrhagic transformation or cerebral edema at 24 hours; 2) clinical status three months after the index stroke. We will investigate the separate and combined effect of pre-treatment BBB disruption and circulating biomarkers on reperfusion injury and clinical status at three months. Study protocol is registered at http://www.clinicaltrials.gov (ClinicalTrials.gov ID: NCT03041753).

Ethics and dissemination

The study protocol has been approved by ethics committee of the Azienda Ospedaliero Universitaria Careggi (Università degli Studi di Firenze). Informed consent is obtained by each patient at time of enrollment or deferred when the participant lacks the capacity to provide consent during the acute phase. Researchers interested in testing hypotheses with the data are encouraged to contact the corresponding author. Results from the study will be disseminated at national and international conferences and in medical thesis.

Keywords: Stroke; Reperfusion Injury; Thrombolysis; Endovascular treatment; Blood Brain Barrier.

INTRODUCTION

Ischemic stroke is a major cause of death and disability. The target of treatments aiming at recanalization of the occluded vessel is the reperfusion of the ischemic brain tissue. However, reperfusion of the acutely ischemic tissue may result clinically futile, or even detrimental because of the so called "reperfusion injury".[1] Reperfusion injury is a pathophysiological term that describes complex biochemical mechanisms that may boost the damage of the ischemic tissue, even with successful recanalization of the occluded vessel.[2] Hemorrhagic transformation (HT) and cerebral edema (CE) may be considered as expression of the reperfusion injury. Preliminary evidence showed that the disruption of the blood brain barrier (BBB), which can be investigated by magnetic resonance (MR) techniques [3] or computed tomography (CT) using permeability maps,[4] is a key phenomenon of the reperfusion injury.[1, 2] Beside BBB disruption, experimental studies on cerebral ischemia have demonstrated that a number of biological factors, including inflammatory mediators, matrix metalloproteinases (MMPs), and endothelial function mediators may contribute to reperfusion injury.[5] Specifically, unbalance of MMPs levels and their natural inhibitors (TIMPs) seem to be associated with disruption of BBB and increased risk of HT.[6-8] However, scanty clinical data exist about relationships between circulating and imaging biomarkers of reperfusion injury.[9, 10] In this single-centre, prospective observational study of ischemic stroke patients treated with recanalization therapies, we plan to investigate the separate effect of pre-treatment BBB disruption and circulating biomarkers on reperfusion injury. Moreover, we will investigate the combined effect of BBB disruption and circulating biomarkers in relation to reperfusion injury. Results may help to identify imaging and circulating biomarkers useful for selection of patients in future clinical trials.

METHODS

Design

This is an observational prospective single center hospital-based study that will include 140 consecutive ischemic stroke patients. Circulating biomarkers sampling and CTP will be performed before acute interventions. Clinical, imaging and circulating biomarkers assessment will be repeated 24 hours after interventions. Clinical evaluation will be repeated three months after the index stroke (table 1). Study protocol is registered at http://www.clinicaltrials.gov (ClinicalTrials.gov ID: NCT03041753).

Table 1 Schedule of assessment

Clinical Data	0 (pre-treatment)	24-36h	7d/discharge	3 months
Demographic variables	x			
Functional status (mRs)	x			x
Vascular risk factors	x			
Medications	x	x	x	x
NIHSS	x	x	x	x
Blood pressure	x	x	x	x
Neuroimaging				
Plan CT	x	x		
CT Perfusion	x			
CT Angiography	x			
Recanalization assessment (CT angiography, MR angiography, transcranial doppler)		x		
Blood samples	x	x		

CT= computer tomography; MR= magnetic resonance; mRs= modified Rankin scale

Study population

The study will include patients with acute ischemic stroke in the anterior circulation with National Institutes of Health Stroke Scale (NIHSS) ≥7 [11] within 12 hours from last time seen well, treated with systemic thrombolysis, endovascular treatment or both. Post-stroke functional status will be measured with the modified Rankin Scale (mRS) administered at 3 months by visit or phone interview. We will rate HT grade using the European Cooperative Acute Stroke Study (ECASS II) criteria [12] and CE according to the Safe Implementation of Thrombolysis in Stroke-Monitoring Study (SITS-MOST) protocol [13]. Collection of clinical data will be blinded to the biomarkers' results. Key inclusion and exclusion criteria are summarized in table 2.

Table 2 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Acute stroke eligible for systemic thrombolysis or endovascular treatment or both	Ischemic stroke in posterior circulation
Age ≥ 18 years	Contraindication for iodine contrast medium
NIHSS ≥ 7	> 12 hours from last time seen well

Imaging protocol

Cerebral imaging will include baseline plain CT, CT angiography and CT perfusion at baseline. CT will be repeated at 24 h, and at any time when clinical deterioration will be observed. Collection of imaging data will be blinded to both clinical and laboratory data. Baseline and follow up CT scans will be assessed by three stroke physicians (FA, BP, VP) for presence of early ischemic signs (Alberta Stroke Programme Early CT score, hyperdensity of middle cerebral artery), presence and severity of small vessel disease markers. We will rate white matter changes with the Van Swieten Scale (VSS), then will combine the posterior (range 0-2) and anterior (range 0-2) scores into a five-point ordinal scale (0-4).[14] We will record the presence and number of lacunes, defined as round or ovoid shaped small CSF-attenuation areas <=2cm in diameter in subcortical white and deep grey matter.[15] We will define brain atrophy as deep or cortical, and will score with a three-point ordinal scale as none, moderate or severe against a reference CT brain template,[16] then will combine the single scores into global atrophy five-point ordinal scale. Presence and site of vessel occlusion will be assessed with CT angiography scans. Presence and grade of collaterals will be assessed with multi-phase CT angiography sequences. Collaterals will be graded into good, moderate and poor, according with the original publication. [17] We will assess presence and grading of HT on the 24 hours CT scan according to ECASS II criteria,[12] when visible at the follow-up CT scan. CTP studies will be performed with a two-phase acquisition protocol and permeability maps will be generated for each patient by Olea Sphere 3.0 (Olea Medical, LA Ciotat,

France) at baseline with a deconvolution-based delay-insensitive algorithm (figure 1). For permeability calculation, we will use Extended Tofts Model (Ktrans = ml/100g/min) [18].

In case of patients with no clinical improvement, we will assess recanalization at 24 hours with either CT angiography, MR angiography or transcranial Doppler. In patients treated with endovascular procedures with effective recanalization and clinical improvement, we will not reassess recanalization at 24 hours and we will assume the recanalization to be as it was at the end of the endovascular treatment.

Laboratory protocol

Blood will be collected before starting and 24 hours after acute interventions. Tubes will be centrifuged at room temperature at 1500 × g for 15 minutes, and the supernatants will be stored in aliquots at −80°C. The complete list of biomarker under study is depicted in table 3. Novel candidates that will emerge over the course of the study will be considered for analysis. Thrombus obtained after endovascular procedure will be placed into RNA stabilization reagent and stored at -80° C for global gene expression analysis.

Table 3 List of biomarkers

Biomarkers	Methods
Metalloproteinases and inhibitors	
MMP 1,2, 3, 8, 9,12, 13	Multiplex tecnology (BioPlex 200 System; BioRad, Italy).
TIMP 1, 2, 4	R&D assays (R&D System, Milan, Italy)
MMP 2, 9 Activity	Polyacrylamide gel (Precast Bio-rad Criterion 10% Zymogram Gelatin)
Endothelial dysfunction and inflammation	
vWf, D-Dimer, PAI-1	Immunoturbidimetric method ACL TOP (Instrumentation Laboratory) PAI-Ag ELISA (Technoclone)
IL-1, IL-1beta; IL-4, IL-10, IL-6, IL-8, IL-12, IL17, IFNγ, TNF-alpha, VEGF, MIP-1 alpha / Beta, IP-10, MCP-1, CRP, A2M, haptoglobin, P, E-selectine	Multiplex tecnology (BioPlex 200 System; BioRad, Italy)

ICAM-1, VCAM-1	Multiplex tecnology (BioPlex 200 System; BioRad, Italy)

MMP=metalloproteinase; TIMP=Tissue inhibitor of metalloproteinase; PAI-1=Plasminogen activator inhibitor-1; IL= interleukin; IFN=interferon; TNF= tumor necrosis facror; VEGF= vascular endothelial growth factor; IP-10=IFN-gamma-inducible protein 10; MIP= macrophage inflammatory protein; CRP= C reactive-protein; A2M= alpha2-macroglobulin; ICAM-1: Intercellular Adhesion Molecule 1; VCAM-1=Vascular cell adhesion protein 1.

Treatment or intervention

No adjunctive selective study-related interventions are planned. Patients are treated following current guidelines.

Primary endpoint

We will evaluate the following composite endpoint:

- relevant HT (defined as hemorrhagic infarction type 2 or any type of parenchymal hemorrhage [PH1 and PH2]) at 24hours CT

  or

- relevant CE (defined as brain swelling comprising 1/3 of the hemisphere or causing midline shift) at 24 hours CT.

Secondary endpoints

- symptomatic HT (defined as any deterioration in NIHSS score or death combined with intracerebral hemorrhage of any type) at 24 hours CT
- categorical shift in mRS score 3 months after the index stroke

Statistical analysis

We will describe general characteristics of the study population, and test differences using Pearson χ^2 for categorical variables, analysis of variance (ANOVA) and Mann Whitney-U test for numeric variables, as appropriate. BBB disruption and differences in biomarkers levels between baseline and

24 hours will be evaluated according to their distribution. In case of skewed distribution of biomarker values, we will consider the possibility of log-transformation of data. For imaging data, as a main explanatory variable we will use BBB disruption values. For biomarkers data, as a main explanatory variable we will use single patient's relative pre- and post-thrombolysis variation (Δ median value) of biomarker levels, calculated according to the formula: (24-hour post-tPA biomarker levels–pre-tPA biomarker levels)/pre-tPA biomarker levels.[6] Differences in these Δ values will be analyzed in relation to demographic and clinical features and across subgroups of patients with different outcomes. The net effect of each biomarker (baseline, 24 hours, and Δ values) on outcomes will be also estimated by both logistic regression and ordinal models, including potentially confounding covariates, such as age, sex, baseline stroke severity, onset-to-treatment time.

Data Monitoring Body

Data are currently registered in the web-based registry available at http://www.stroketeam.it/risks/. Quality controls are done on a weekly basis. Imaging data are instantly checked for protocol conformity. Biosamples are processed using standard and harmonized operating procedures.

Sample size

We plan to include 140 patients. The sample size was calculated using observational data from a pilot study conducted in 32 similar stroke patients by our group.[19] Permeability values above the threshold within the ischemic core were found in 38% of stroke patients. Considering a statistical power of 0.80 (alpha error of 0.05), we estimated a sample size of 140 patients, to demonstrate an effect of significant BBB disruption on relevant HT in at least 22% of patients.

Enrollment started on October 2015 and is ongoing. Using a definite protocol, a prospective collection of data, and an adequate number of patients, this study will integrate clinical information about biological factors involved in reperfusion injury after cerebral ischemia.

DISCUSSION

Circulating and neuroimaging biomarkers of reperfusion injury measured in the acute ischemic stroke setting represent potentially useful predictors of undesirable complications after recanalization procedure. Three small studies focusing just on the role of CTP, reported quite high sensitivity and specificity to predict HT or CE after acute ischemic stroke.[20-22] A relationship between MMP9 and BBB disruption assessed by CTP has not be proven yet and scanty clinical data exist on MRI. In 41 acute ischemic stroke patients, baseline MMP-9 proved a significant predictor of BBB disruption, as assessed using gadolinium enhancement of cerebrospinal fluid on fluid-attenuated inversion recovery (FLAIR) MRI at 24 hours from symptoms onset.[9] In a larger cohort of 180 acute stroke patients, T2 FLAIR hyperintensities, possible expression of vasogenic edema turned out to be associated with both baseline MMP-9 level and risk of hemorrhage.[10]

The RISK Study combines advanced neuroimaging techniques for the study of BBB disruption with analyses of circulating biomarkers as potential predictors of reperfusion injury after acute phase interventions. The following considerations motivate this study: (i) in the human setting, the biological mechanisms of reperfusion injury remain incompletely described and understood; (ii) a better knowledge of the underlying mechanisms may help discriminating patients at risk for complications from those who may definitely benefit from targeted interventions; (iii) the validation of surrogate diagnostic markers of reperfusion damage such as BBB disruption may help designing and conducting clinical trials testing effectiveness of reperfusion injury antagonists.

Results of a pilot study conducted by our group [19] and the successful enrollment of our first 70 patients, may corroborate applicability and feasibility of our study protocol.

ETHICS AND DISSEMINATION

The study protocol has been approved by the Azienda Ospedaliero Universitaria Careggi (Università degli Studi di Firenze) Ethic Committee (Prot. 2015/0015162 Rif. 138/12).

Informed consent is obtained by each patient before enrollment**.** A full explanation of the study, a written “Patient Information Sheet” (detailing rationale, design and personal implications of trial

entry) and informed consent form is provided. The consent process is deferred when the participant lacks the capacity to provide consent. Researchers interested in testing hypotheses with the data are encouraged to contact the corresponding author. Results from the study will be disseminated at national and international conferences and in MD and PhD medical theses.

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Authors' contributions: DI, NM, PV, PN, FA, BP conceived the study and its design, managed its coordination, drafted the manuscript, developed the methodology, conducted collection, extraction and analysis of the data. BG, AMG, DG, MM, EF, SM, SN, FG, GP, AF, PP, SV, SG, CS, ML, AP, FP, AS, LP participated in the design of the study and all authors critically revised the manuscript and approved the final version of the manuscript.

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Competing interests None declared.

Ethics Approval Azienda Ospedaliero Universitaria Careggi (Università degli Studi di Firenze) Ethic Committee (Prot. 2015/0015162 Rif. 138/12).

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Figure 1 Computed tomographic perfusion. Permeability map of middle cerebral artery territory on the affected side compared with the contralateral hemisphere.

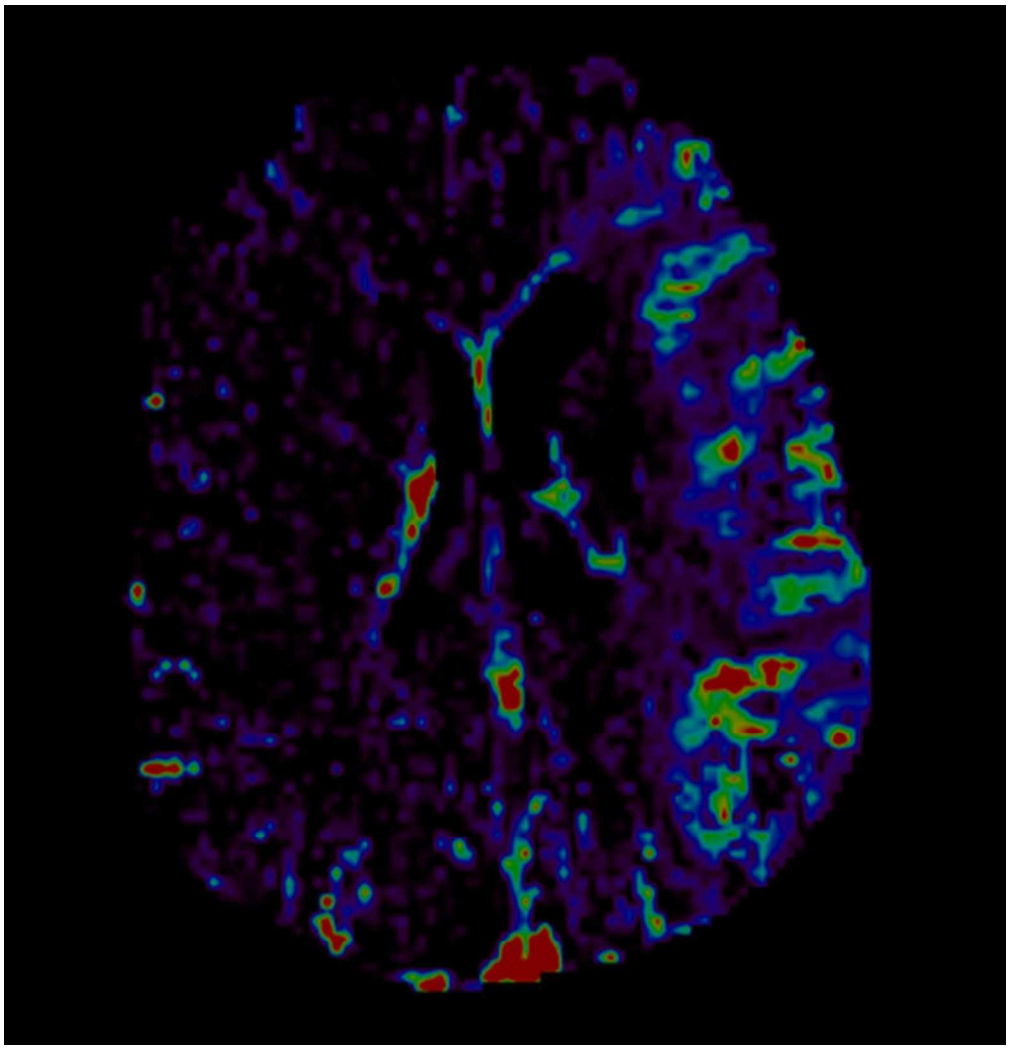

Figure 1

103x107mm (300 x 300 DPI)

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Strengths and limitations of this study

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ABSTRACT

Introduction: treatments aiming at reperfusion of the acutely ischemic brain tissue may result futile or even detrimental because of the so-called "reperfusion injury". The processes contributing to reperfusion injury involve a number of factors, ranging from blood brain barrier (BBB) disruption to circulating biomarkers. Our aim is to evaluate the relative effect of imaging and circulating biomarkers in relation to reperfusion injury.

Methods and analysis: observational hospital-based study that will include 140 ischemic stroke patients, treated with systemic thrombolysis, endovascular treatment, or both. BBB disruption will be assessed with computed tomography perfusion (CTP) before-treatment, and-levels of a large panel of biomarkers will be measured before-intervention and after 24 hours. Relevant outcomes will include: 1) reperfusion injury, defined as radiologically relevant hemorrhagic transformation at 24 hours; 2) clinical status three months after the index stroke. We will investigate the separate and combined effect of pre-treatment BBB disruption and circulating biomarkers on reperfusion injury and clinical status at three months. Study protocol is registered at http://www.clinicaltrials.gov (ClinicalTrials.gov ID: NCT03041753).

Ethics and dissemination

The study protocol has been approved by ethics committee of the Azienda Ospedaliero Universitaria Careggi (Università degli Studi di Firenze). Informed consent is obtained by each patient at time of enrollment or deferred when the participant lacks the capacity to provide consent during the acute phase. Researchers interested in testing hypotheses with the data are encouraged to contact the corresponding author. Results from the study will be disseminated at national and international conferences and in medical thesis.

Keywords: Stroke; Reperfusion Injury; Thrombolysis; Endovascular treatment; Blood Brain Barrier.

INTRODUCTION

Ischemic stroke is a major cause of death and disability. The target of treatments aiming at recanalization of the occluded vessel is the reperfusion of the ischemic brain tissue. However, reperfusion of the acutely ischemic tissue may result clinically futile, or even detrimental because of the so called "reperfusion injury".[1] Reperfusion injury is a pathophysiological term that describes complex biochemical mechanisms that may boost the damage of the ischemic tissue, even with successful recanalization of the occluded vessel.[2] Hemorrhagic transformation (HT) may be considered as expression of the reperfusion injury. Preliminary evidence showed that the disruption of the blood brain barrier (BBB), which can be investigated by magnetic resonance (MR) techniques [3] or computed tomography (CT) using permeability maps,[4] is a key phenomenon of the reperfusion injury.[1, 2] Experimental studies on cerebral ischemia have demonstrated that a number of biological factors, including inflammatory mediators, matrix metalloproteinases (MMPs), and endothelial function mediators may contribute to reperfusion injury.[5] Specifically, unbalance of MMPs levels and their natural inhibitors (TIMPs) seem to be associated with disruption of BBB and increased risk of HT.[6-8] However, scanty clinical data exist about relationships between circulating and imaging biomarkers of reperfusion injury.[9, 10] In this single-centre, prospective observational study of ischemic stroke patients treated with recanalization therapies, we plan to investigate the separate effect of pre-treatment BBB disruption and circulating biomarkers on reperfusion injury. Moreover, we will investigate the combined effect of BBB disruption and circulating biomarkers in relation to reperfusion injury. Results may help to identify imaging and circulating biomarkers useful for selection of patients in future clinical trials.

METHODS

Design

This is an observational prospective single center hospital-based study that will include 140 consecutive ischemic stroke patients. Circulating biomarkers sampling and CTP will be performed

before acute interventions. Clinical, imaging and circulating biomarkers assessment will be repeated 24 hours after interventions. Clinical evaluation will be repeated three months after the index stroke (table 1). Study protocol is registered at http://www.clinicaltrials.gov (ClinicalTrials.gov ID: NCT03041753).

Table 1 Schedule of assessment

Clinical Data	0 (pre-treatment)	24-36h	7d/discharge	3 months
Demographic variables	x			
Functional status (mRs)	x			x
Vascular risk factors	x			
Medications	x	x	x	x
NIHSS	x	x	x	x
Blood pressure	x	x	x	x
Neuroimaging				
Plan CT	x	x		
CT Perfusion	x			
CT Angiography	x			
Recanalization assessment (CT angiography, MR angiography, transcranial doppler)		x		
Blood samples	x	x		

CT= computer tomography; MR= magnetic resonance; mRs= modified Rankin scale

Study population

The study will include patients with acute ischemic stroke in the anterior circulation with National Institutes of Health Stroke Scale (NIHSS) ≥7 [11] within 12 hours from last time seen well, treated with systemic thrombolysis, endovascular treatment or both. Post-stroke functional status will be measured with the modified Rankin Scale (mRS) administered at 3 months by visit or phone interview. We will rate HT grade using the European Cooperative Acute Stroke Study (ECASS II) criteria [12]. Collection of clinical data will be blinded to the biomarkers' results. Key inclusion and exclusion criteria are summarized in table 2.

Table 2 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria

Acute stroke eligible for systemic thrombolysis or endovascular treatment or both	Ischemic stroke in posterior circulation
Age ≥ 18 years	Contraindication for iodine contrast medium
NIHSS ≥ 7	> 12 hours from last time seen well

Imaging protocol

Cerebral imaging will include baseline plain CT, CT angiography and CT perfusion. CT will be repeated at 24 h, and at any time if clinical deterioration occurs. Collection of imaging data will be blinded to both clinical and laboratory data. Baseline and follow up CT scans will be assessed by three stroke physicians (FA, BP, VP) for presence of early ischemic signs (Alberta Stroke Programme Early CT score, hyperdensity of middle cerebral artery), presence and severity of small vessel disease markers. Cerebral Edema will be assessed according to the Safe Implementation of Thrombolysis in Stroke-Monitoring Study (SITS-MOST) protocol [13]. We will rate white matter changes with the Van Swieten Scale (VSS), then will combine the posterior (range 0-2) and anterior (range 0-2) scores into a five-point ordinal scale (0-4).[14] We will record the presence and number of lacunes, defined as round or ovoid shaped small CSF-attenuation areas <=2cm in diameter in subcortical white and deep grey matter.[15] We will define brain atrophy as deep or cortical, and will score with a three-point ordinal scale as none, moderate or severe against a reference CT brain template,[16] then will combine the single scores into global atrophy five-point ordinal scale. Presence and site of vessel occlusion will be assessed with CT angiography scans. Presence and grade of collaterals will be assessed with multi-phase CT angiography sequences. Collaterals will be graded into good, moderate and poor, according to the original publication. [17] We will assess presence and grading of HT on 24 hours CT scan according to ECASS II criteria,[12] when visible at the follow-up CT scan. CTP studies will be performed with a two-phase acquisition protocol and permeability maps will be generated for each patient by Olea Sphere 3.0 (Olea Medical, LA Ciotat, France) at baseline with a deconvolution-based delay-insensitive algorithm (figure 1). For permeability calculation, we will use Extended Tofts Model (Ktrans = ml/100g/min) [18].

In case of patients with no clinical improvement, we will assess recanalization at 24 hours with either CT angiography, MR angiography or transcranial Doppler. In patients treated with endovascular procedures with effective recanalization and clinical improvement, we will not reassess recanalization at 24 hours and we will assume the recanalization to be as it was at the end of the endovascular treatment.

Laboratory protocol

Blood will be collected before and 24 hours after acute interventions. Tubes will be centrifuged at room temperature at 1500 × g for 15 minutes, and the supernatants will be stored in aliquots at −80°C. The complete list of biomarker under study is depicted in table 3. Novel candidates that will emerge over the course of the study will be considered for analysis. Thrombus obtained after endovascular procedure will be placed into RNA stabilization reagent and stored at -80° C for global gene expression analysis.

Table 3 List of biomarkers

Metalloproteinases and inhibitors		
Biomarkers	**Methods**	**Rationale**
MMP 1,2, 3, 7, 8, 9, 10, 12, 13, EMMPRIN	Multiplex tecnology (R&D System, Milan, Italy)	Numerous studies have documented increases in matrix metalloproteinases (MMPs), specifically MMP-9 levels following stroke with disruption of the blood brain barrier (BBB) [19]
TIMP 1, 2, 3, 4	R&D assays (R&D System, Milan, Italy)	
MMP 2, 9 Activity	Polyacrylamide gel (Precast Bio-rad Criterion 10% Zymogram Gelatin)	
Endothelial dysfunction and inflammation		
Biomarkers	**Methods**	**Rationale**
vWf, D-Dimer, PAI-1	Immunoturbidimetric method ACL TOP (Instrumentation Laboratory) PAI-Ag ELISA (Technoclone)	In experimental models systemic inflammation seems to alter the kinetics of cerebrovascular tight junction determining BBB disruption [20]
IL-1, IL-1beta; IL-4, IL-10, IL-6, IL-8, IL-12, IL17, IFNγ, TNF-alpha, VEGF, MIP-1	Multiplex tecnology (BioPlex 200 System; BioRad, Italy)	

alpha / Beta, IP-10, MCP-1, CRP, A2M, haptoglobin, P-selectin, E-selectin		
ICAM-1, VCAM-1	Multiplex tecnology (BioPlex 200 System; BioRad, Italy)	

MMP=metalloproteinase; TIMP=Tissue inhibitor of metalloproteinase; PAI-1=Plasminogen activator inhibitor-1; IL= interleukin; IFN=interferon; TNF= tumor necrosis factor; VEGF= vascular endothelial growth factor; IP-10=IFN-gamma-inducible protein 10; MIP= macrophage inflammatory protein; CRP= C reactive-protein; A2M= alpha2-macroglobulin; ICAM-1: Intercellular Adhesion Molecule 1; VCAM-1=Vascular cell adhesion protein 1.

Treatment or intervention

No adjunctive selective study-related interventions are planned. Patients are treated following current guidelines.

Primary endpoint

- relevant HT (defined as hemorrhagic infarction type 2 or any type of parenchymal hemorrhage [PH1 and PH2]) at 24hours CT

Secondary endpoints

- symptomatic HT (defined as any deterioration in NIHSS score or death combined with intracerebral hemorrhage of any type) at 24 hours CT
- categorical shift in mRS score 3 months after the index stroke

Statistical analysis

We will describe general characteristics of the study population, and test differences using Pearson χ^2 for categorical variables, analysis of variance (ANOVA) and Mann Whitney-U test for numeric variables, as appropriate. BBB disruption and differences in biomarkers levels between baseline and 24 hours will be evaluated according to their distribution. In case of skewed distribution of biomarker values, we will consider the possibility of log-transformation of data. Considering a

possible effect of systemic thrombolysis on biomarker levels, the patient cohort will be divided in two groups on the basis of treatment received at baseline (systemic thrombolysis vs mechanical thrombectomy alone). For imaging data, as a main explanatory variable we will use BBB disruption values. For biomarkers data, as a main explanatory variable we will use single patient's relative pre- and post-thrombolysis variation (Δ median value) of biomarker levels, calculated according to the formula: (24-hour post-tPA biomarker levels–pre-tPA biomarker levels)/pre-tPA biomarker levels.[6] Differences in these Δ values will be analyzed in relation to demographic and clinical features and across subgroups of patients with different outcomes. The net effect of each biomarker (baseline, 24 hours, and Δ values) on outcomes will be also estimated by both logistic regression and ordinal models, including potentially confounding covariates, such as age, sex, baseline stroke severity, onset-to-treatment time.

Data Monitoring Body

Data are currently registered in the web-based registry available at http://www.stroketeam.it/risks/. Quality controls are done on a weekly basis. Imaging data are instantly checked for protocol conformity. Biosamples are processed using standard and harmonized operating procedures.

Sample size

We plan to include 140 patients. The sample size was calculated using observational data from a pilot study conducted in 32 similar stroke patients by our group.[21] Permeability values above the threshold within the ischemic core were found in 38% of stroke patients. Considering a statistical power of 0.80 (alpha error of 0.05), we estimated a sample size of 140 patients, to demonstrate an effect of significant BBB disruption on relevant HT in at least 22% of patients.

Enrollment started on October 2015 and is ongoing. Using a definite protocol, a prospective collection of data, and an adequate number of patients, this study will integrate clinical information about biological factors involved in reperfusion injury after cerebral ischemia.

Patient and Public Involvement

Patient and Public Involvement in the development of the research question, in outcome measures and in the design of this study could not be planned. Results will be disseminated through patient's association.

DISCUSSION

Circulating and neuroimaging biomarkers of reperfusion injury measured in the acute ischemic stroke setting represent potentially useful predictors of undesirable complications after recanalization procedure. On the basis of the results of three small studies, CTP seems to have high sensitivity and specificity to predict HT and CE after acute ischemic stroke. [22-24] MRI data from 41 patients evaluated for acute stroke have shown an association between BBB disruption at 24 hours (defined as gadolinium enhancement of cerebrospinal fluid on fluid-attenuated inversion recovery-FLAIR MRI) and baseline levels of MMP9. [9] In a larger cohort of 180 acute stroke patients, T2 FLAIR hyperintensities, possible expression of vasogenic edema in the setting of BBB disruption, turned out to be associated with both baseline MMP-9 level and risk of hemorrhage.[10] The RISK Study combines advanced neuroimaging techniques for the study of BBB disruption with analyses of circulating biomarkers as potential predictors of reperfusion injury after acute phase interventions. The following considerations motivate this study: (i) in the human setting, the biological mechanisms of reperfusion injury remain incompletely described and understood; (ii) a better knowledge of the underlying mechanisms may help discriminating patients at risk for complications from those who may definitely benefit from targeted interventions; (iii) the validation of surrogate diagnostic markers of reperfusion damage such as BBB disruption may help designing and conducting clinical trials testing effectiveness of reperfusion injury antagonists.

Results of a pilot study conducted by our group [21] and the successful enrollment of our first 100 patients, may corroborate applicability and feasibility of our study protocol.

ETHICS AND DISSEMINATION

The study protocol has been approved by the Azienda Ospedaliero Universitaria Careggi (Università degli Studi di Firenze) Ethic Committee (Prot. 2015/0015162 Rif. 138/12).

Informed consent is obtained by each patient before enrollment**.** A full explanation of the study, a written "Patient Information Sheet" (detailing rationale, design and personal implications of trial entry) and informed consent form is provided. The consent process is deferred when the participant lacks the capacity to provide consent. Researchers interested in testing hypotheses with the data are encouraged to contact the corresponding author. Results from the study will be disseminated at national and international conferences and in MD and PhD medical theses.

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Authors' contributions: DI, NM, PV, PN, FA, BP conceived the study and its design, managed its coordination, drafted the manuscript, developed the methodology, conducted collection, extraction and analysis of the data. BG, AMG, DG, MM, EF, SM, SN, FG, GP, AF, PP, SV, SG, CS, ML, AP, FP, AS, LP participated in the design of the study and all authors critically revised the manuscript and approved the final version of the manuscript.

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Competing interests None declared.

Ethics Approval Azienda Ospedaliero Universitaria Careggi (Università degli Studi di Firenze) Ethic Committee (Prot. 2015/0015162 Rif. 138/12).

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Figure 1 Computed tomographic perfusion. Permeability map of middle cerebral artery territory on the affected side compared with the contralateral hemisphere.

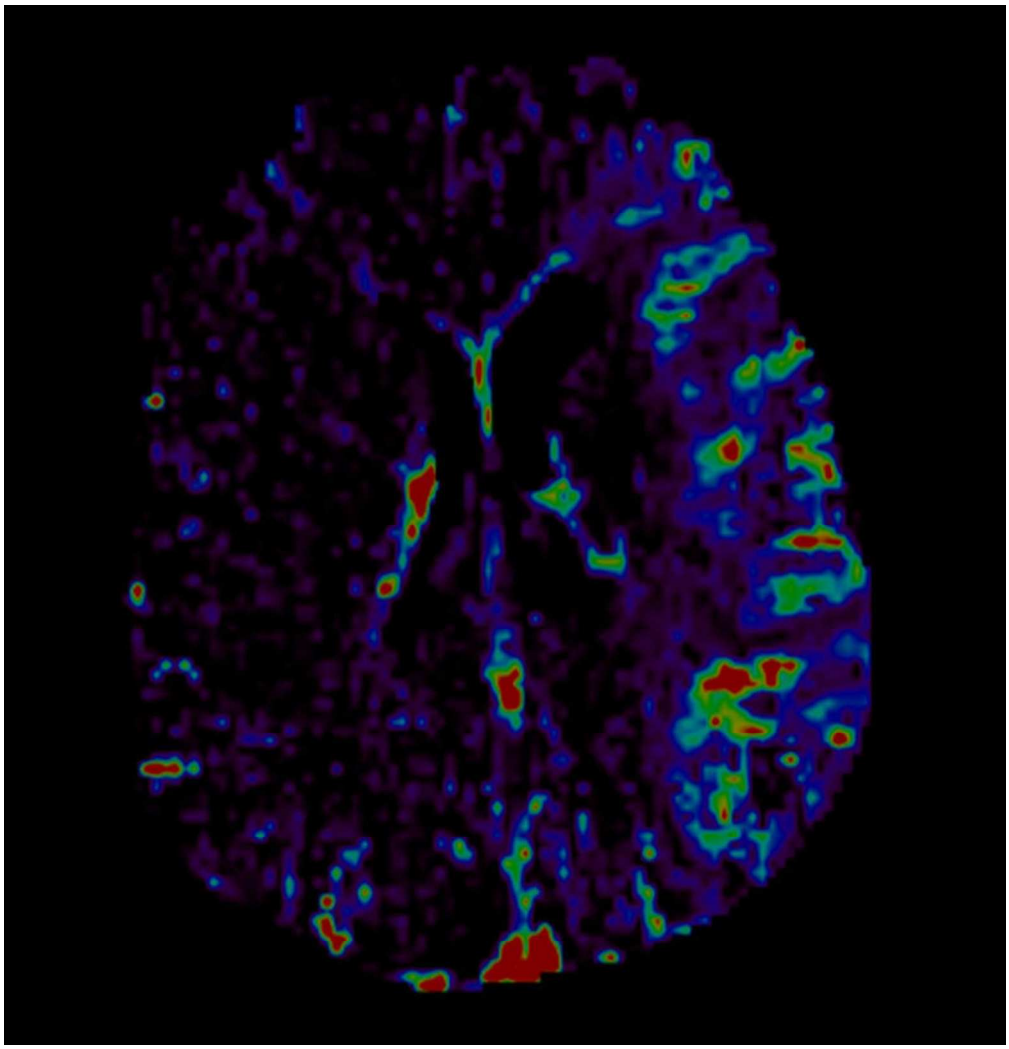

Figure 1

103x107mm (300 x 300 DPI)