Universitat de Girona

**Final degree project**
**by Èlia Martínez Nogales**

# "Pilot study about the effect of rt-PA administration on the excitotoxicity mediated brain damage and its repercussion on the functional prognosis of patients with ischaemic stroke"

## Research Protocol

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Studies Degree in Medicine, 2013 – 2014

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Pag. 2

**TABLE OF CONTENTS**

**1. ABSTRACT**

Fibrinolytic therapy with Recombinant Tissue-Plasminogen Activator (rt-PA) is currently the only effective treatment for ischaemic stroke in its acute phase. Even though its use generally improves the prognosis of those patients likely to receive it, rt-PA administration is associated to several risks, such as haemorrhagic transformation of the ischaemic lesion and activation of excitotoxic mechanisms that may contribute to an increase in mortality or to a poor outcome in certain occasions, specially when arterial recanalization is not achieved or the rt-PA is lately administrated.

Since in the last few years the role of glutamate in the neurotoxicity associated to ischaemia has been widely studied and it is known that high plasma glutamate levels are predictors of ischaemic lesion growth and poor neurological outcome, it is necessary to find out which factors can contribute to glutamate release in the brain.

The aim of this study is to determine if rt-PA administration is related to an increase in plasma glutamate levels, as well as to define if higher plasma glutamate levels at admission are related to different evolution and prognosis of our patients, both in those in which recanalisation is achieved and not.

A series of cases of patients with hemispheric cerebral infarction admitted in our hospital during a year will be studied, and the data obtained from them will be compared to the data obtained from a control group, the samples of wich were taken years ago, before rt-PA was routinely used.

**2. INTRODUCTION**

   Background and current knowledge on the topic

Stroke is currently the second cause of death in the world [1], leading cause of dependency in adult patients and the world's second most common cause of dementia, making it one of the diseases with the highest social and economic costs, as strokes are events that result in severe disability among survivors.  In 2009 Spain's Statistic Institute ranked stroke as the second most frequent cause of death in Spanish population and its prevalence in Spain is estimated at 7% of the urban population older than 65. The incidence of stroke in Spain is 128 people per 100000 in the general population [2]. Research in this field and hunting for effective therapies are specially relevant in consequence.

When talking about the causes of stroke, about 80-85% of the cases are ischaemic strokes (cerebral infarction) whereas the other 15-20% have an haemorrhagic origin.

The ischaemic stroke is then a very prevalent disease and it consumes about the 3-4% of the health expense. Research in this field and hunting for effective therapies are specially relevant in consequence, not only for sanitary but also socio-economic reasons.

Several clinical trials on the use of drugs with different mechanisms of action have been done in the last years. From those, only thrombolytic therapy with recombinant tissue plasminogen activator (rt-PA) has demonstrated to be effective when administrated within the first 4.5 hours from the onset of the sypmtoms [4,5].

Nevertheless, rt-PA administration is not risk free. For instance, the use of this drug increases the risk of haemorrhagic transformation of the ischaemic lesion, which might entail the neurological early damage that some patients present, and the consequent worsening of the prognosis of those patients. This secondary effect and the limited therapeutical window for the use of this drug (only 4.5 hours from the onset of the symptoms) is itself an important limitation against the generalization of the thrombolytic therapy use. Actually, less than 5% of the patients with ischaemic stroke are receiving this treatment.

Several studies have been undertaken with the aim of demonstrating the efficacy of the rt-PA use beyond the currently accepted therapeutic window, selecting those patients who are more likely to benefit from that, using neuroimaging techniques. However, recent data seem to prove that rt-PA administration could be associated with an increasing in mortality, specially when it is lately administrated. Although the exact mechanisms through which the drug could be associated with mortality increasing are not exactly known yet, it is known that this drug has potential neurotoxic effects related to the activation of excitotoxic mechanisms. These excitotoxic mechanisms could be related to this complication.

Actually, it is well known that the main cause of neurological deterioration and mortality within the first hours of the evolution of the ischaemia is the ischaemic lesion growth, usually due to the "penumbra recruitment". Penumbra is the area of tissue where the blood stream is diminished but it is still likely to survive if the blood stream is recovered. It has been demonstrated that the main factor involved in both ischaemic area growth and neurological deterioration is the glutamate release that occurs at the begining of the patophysiology of the ischaemic process [6-10].

Given that the fibrinolytic therapy with rt-PA is currently the only effective treatment of ischaemic stroke, and that several studies on its usage beyond the currently accepted therapeutic window are being performed, it is crucial to determine if the drug administration does really entail an increase in mortality, and also the mechanisms possibly involved in this complication. If the relationship is proven, it would mean the need of research on new therapeutic targets to avoid the complication.

### Excitotoxicity and ischaemic lesion growth

Cerebral ischaemia happens as a consequence of an occlusion of a cerebral artery, originating a blood flow reduction in the area supplied by that artery. Blood flow decrease is not uniform in the whole area supplied by that artery, so that only in certain areas, where the blood flow is smaller than 10mL/100g/min, a rapid neuronal death occurs. The areas supplied with blood flows between 10-22mL/100g/min are hypoperfused but still likely to survive, and are called *penumbra* area.

Penumbra is likely to be saved if arterial recanalization and blood stream restoration are achieved. The ischaemic lesion growth is due to the recruitment of the penumbra area and depends on several factors, among which excitotoxicity, and more specifically glutamate release happening in the first phases of the ischaemic process, plays an important role [11]. In fact, the oxygen and glucose deprivation secondary to the ischaemia induces the release of glutamate by presinaptic neurons. Glutamate causes a long and intense activation of the glutamate specific receptors, which results in: 1) a massive entrance of $Ca^{2+}$ into the neurons and its desintegration (necrosis) because of the activation of enzymes such as lipases, proteases, kinases, phosphatases and endonucleases; 2) the entrance of sodium into the neurons, contributing to the neuronal lesion by the intracellular edema [12].

It has been demonstrated that patients with ischaemic stroke of less than 24 hours of evolution and neurological deterioration have higher glutamate levels both in plasma and cerebrospinal fluid (CSF) [6,13] than those patients who keep stable [7].
In that same study it was shown that plasma glutamate levels >200mmol/L and >8.2mmol/L in CSF are predictive of neurological deterioration (OR=26.1, 95% CI 6.9 – 98.6; and OR=40.9, 95% CI 7.6 – 220 respectively; p<0.0001) even after adjunsting for the final infarct volume in the analysis [7].

Besides, glutamate levels remain elevated for at least 24 hours after the stroke in patients with progressive stroke, whereas they decrease to normal values in less than 6 hours from the onset of the stroke in those patients with a non progressive infarction [8]. The correlation between glutamate in plasma and CSF is excellent, which means that plasma glutamate levels are reflecting the process ongoing in the brain. Moreover, it has been recently proven that glutamate levels are the most powerful predictive factor of lesion growth in patients with ischaemic stroke, regardless of other clinical, radiologic and molecular factors that could also be involved in the ischaemic lesion expansion [10].

This association between glutamate levels and ischaemic lesion growth confirms ~~the~~ previously published experimental data, which have shown the efficacy of the administration of antiglutamaterigic drugs in the reduction of the final infarction volume [14]. Even though the efficacy of administrating antiglutamatergic drugs has not been proven in the clinical practise yet [15], it has been proven that the activation of glutamate metabolism in peripheral blood, which is due to the activation of oxalacetate trasaminase enzyme and glutamate piruvate transaminase, results in a decrease in the final infarct volume in both experimental [16] and clinical [17,18] settings. This effect was also demonstrated to be associated with a decrease in the percentage of patients with neurological deterioration [17,18].

rT-PA and excitotoxicity

Several published data based on investigations on cell cultures and experimental models of cerebral ischaemia have demonstrated that the administration of rt-PA might have neurotoxic effects. These data reveal a major neuronal resistance towards ischaemia if the cells belong to genetically modified animals in whom rt-PA has no effect (rt-PA knockout) [19, 20] as well as a reduction in the final volume of infarction in the same group of animals [21, 22].

These same studies have demonstrated that there are different mechanisms through which rt-PA can mediate neurotoxic effects. Glutamate may increase the release of proteolytic enzymes, such as matrix metalloprotease-9, which damages the extracellular matrix and favours the development of haemorrhagic trasformation, as well as an increase of excitotoxic damage, an effect that seems to be related to the stimulation of glutamate receptors (mostly NMDA) by the drug [23].

Experimental data show that rt-PA is able to bound to the subunits NR1 [24] and NR2 [25] of the NMDA receptor and it induces an increased release of intracelular $Ca^{2+}$, amplifying the neuronal damage mediated for excitotoxicity. In fact, the isolated administration of rt-PA in the striatum is not toxic, whereas the combined administration of rt-PA and NMDA (N-Methyl-D-Aspartic acid, an aminoacid derivative that acts as a specific agonist of the NMDA receptor mimicking the action of glutamate) increases the cerebral excitotoxic damage in the 50% of the cases. This effect is related to the cleavage of the NR1 subunit of the NMDA receptor [25]. The administration of antagonists of the subunit NR2 of the NMDA receptor such as MK-801 also diminishes the excitotoxic cerebral damage mediated by rtPA [26]. This effect seems to be related with a decrease in the entrance of $Ca^{2+}$ into the cell.

### Limitations of the previous research

Although several publications in the last few years have demonstrated the participation of the excitotoxic mechanisms in the brain damage secondary to ischaemia, data demonstrating the excitotoxic effect of rt-PA administration are scarce and limited to the experimental field. So far, there are no clinical data to clarify whether rt-PA administration could exacerbate the ischaemic brain damage through an increased excitotoxicity, or not. In this respect, it would be important to know if rt-PA administration may result in an increase in the release of glutamate or, if the increased excitotoxic brain damage related to rt-PA could be related to higher levels of glutamate at the moment of admission; if proven, rt-PA adminsitration could have a deleterious effect in some cases, specially in those cases in which the arterial recanalization is not achieved.

Given that rt-PA is the only approved treatment for acute ischaemic stroke and that this disease is itself associated with a high mortality and disability, it is very important to determine whether this treatment can aggravate the brain damage related to ischaemia and also to determine in which circumstances this possibility is more likely to happen.

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## 4. STUDY OBJECTIVES

Primary aims:

1.- To determine if rt-PA administration in patients with ischaemic stroke is associated with an increase in the plasma levels of glutamate.

2.- To determine the effect of the pre rt-PA administration glutamate levels in the clinical evolution of patients with ischaemic stroke treated with this drug, both in patients with and without arteral recanalization after the administration of the treatment.

Secondary aim:

1.- To determine if the increase in the levels of plasma glutamate associated to rt-PA administration is associated to an increased likelihood of neurological deterioration and /or mortality in patients treated with this drug.

## 5. HYPOTHESIS

The hypothesis that generate the main goals in this study are:

1.- rt-PA administration in patients with ischaemic stroke results in an increase in plasma levels of glutamate.

2-. The evolution and prognosis of patients treated with rt-PA is different depending on the glutamate levels previous to the rt-PA administration and on the effect of rt-PA administration on arterial recanalization.

The hypothesis that generates the secondary goal is:

1.- The increase in the plasma levels of glutamate associated to rt-PA administration is associated with a higher probability of neurological deterioration and/or mortality in patients treated with this drug.

## 6. METHODS

6.1.- Study Design

Descriptive longitudinal study of a series of cases with diagnosis of hemispheric cerebral infarction treated with rt-PA.

6. 2.- Subject selection and withdrawal

The study will include 100 consecutive patients aged >17 admitted at the Hospital Universitari Dr. Josep Trueta (Girona) with an hemispheric cerebral infarction treated with rt-PA if they accomplish the administration criteria of this drug and having signed the informed consent.

Patients with exclusion criteria for rt-PA administration as well as patients in coma or in a state suggestive of imminent death, patients with lacunar syndrome, patients with rapid recovery of the neurological symptoms, patients with hepatic, renal, haematological or immune diseases, thyroid disfunction, infections in the days before the stroke and solid tumours (due to a possibility of interference with the analytic determinations), and those patients unable to complete the follow-up of the study will not be included.

In our Hospital, thrombolytic therapy has been administrated since 1999 and in the last few years a mean of 70 patients per year were treated with rt-PA. Considering a 20% of withdrawals due to clinical criteria or to the impossibility of sample obteining (mostly because of the transfer to another hospital for a neurovascular procedure), we estimate a total of 50 patients being included in the study within the timing of this.

Since  the obtention of blood samples at the same times established in this protocol in patients not treated with rt-PA would not be posible for ethical reasons, glutamate levels from a control group of patients admitted in the Stroke Unit of our hospital in the past, when rt-PA was not routinely administered, and who signed the informed consent for the analysis of this biomarker during ther admission, will also be analysed retrospectively.

6.3.- Main Variables for the analysis of goals

     6.3.1 Clinical Variables

Neurological deterioration: Increase of >3 points in the NIHSS score Stroke Scale between admission and 72h of evolution.

     6.3.2 Biochemical Variables

Plasma glutamate levels: Serial samples will be obtained before rt-PA administration (pre-rt-PA) and at 2, 6, 12, 24 and 48 hours after rt-PA administration.

Blood samples from the control group will be analysed at the same times.

### 6.3.3 Neuroradiological variables

- Arterial recanalization: will be evaluated by comparing the angio-CT performed before the rt-PA administration and the one performed 1-5h after the drug administration.

- Haemorrhagic transformation of the ischaemic lesion: will be evaluated in the non contrast Cranial CT scan performed 24±12h post-rt-PA administration, as it is stablished in the rt-PA administration protocol.

- Final infarct volume: evaluated in the cranial CT scan performed at 30±2 days of evolution.

## 6.4. Data collection and analysis

### 6.4.1 Inclusion of patients

At the moment of admission in the emergency room, a first anamnesis will be done (specially focused on the neurological sympthoms and the specific time of their onset) and the inclusion/exclusion criteria for the administration of the thrombolytic therapy, as well as the inclusion/exclusion criteria for our study will be considered. The informed consent will be requested to the patient and/or their relatives in case the patient is not able to consent by himself.

### 6.4.2 Biochemical evaluation

Blood samples will be taken from our patients for the analysis of glutamate levels before and 2, 6, 12, 24 and 48 hours after the rt-PA administration. Blood samples will be centrifuged at 3000rpm for 10 minutes; then 3 aliquots of serum and 3 aliquots of citrated plasma will be separated (2cc per aliquot aprox.) and stored at -80º C until the moment of analysing.

Glutamate levels will be analysed by HPLC-fluorescence (High Performance Liquid Chromatography) using a modificaton of the AccQTAG method by Waters.

### 6.4.3 Radiological evaluation

According to the existing stroke protocol in our Hospital, the following examinations will be performed in our patients:

- Angio-CT immediately after taking the first blood sample and before rt-PA administration. A second angio-CT will be performed 1-5h later for the assessment of arterial recanalization. The level of arterial occlusion and/or the recanalization will be assessed according to the TIMI criteria.

- Non contrast Cranial CT scan 24±12 hours post-rt-PA for the assessment of haemorrhagic transformation, which will be classified according to the ECASS II criteria.

- Non contrast Cranial CT scan 30±2 days after the stroke for measuring final infarct volume.

The CT scan obtained images will be evaluated by a neurologist belonging to the Hospital, who will not have information on the clinical evaluation of the patient nor the laboratory data.


### 6.4.4 Clinical evaluation of the patients

The patients will be admited at the Stroke Unit under semi-intensive cardiovascular and neurological monitoring, according to the protocol of the Unit. The severity of the neurological deficit will be quantified by using the NIHSS score at the moment of admission and at 2, 24, 48, 72 later, as well as at days 7±1 (or at the moment of discharge in case it is before) 30±2 and 90±7 of evolution.

The NIHSS score will be evaluated by a neurologist.

Neurological deterioration associated to haemorrhagic transformation will be considered positive if there is an increase of >3 points in the NIHSS score within the first 72 hours of evolution associated with the presence of haemorrhagic transformation in the Cranial CT scan.

The functional situation of the patients will be evaluated at day 90±7 by using both the modified Rankin scale and the Barthel scale, according to the usual protocol in our hospital.


## 7. STATISTICAL ANALYSIS

- Quantitative continous variables will be expressed as mean and standard deviation if their distribution is normal; if it is not, they will be expressed as median and interquartile range.

- Qualitative variables will be described as percentages.


The multivariant statistical analysis will be performed according to the main objectives:

- For the first main objective, the presence of increase in serial post-rt-PA glutamate levels will be compared by using a longitudinal GEE model (Generalised Estimating Equations) for repeated measurements, adjusted for the pre-rt-PA glutamate levels and the use of rt-PA. It allows us to quantify the correlation of rt-PA administration and plasma glutamate levels at admission with the increase of plasma glutamate levels in the first hours of evolution.

- For both, the second main objective and the secondary objective a longitudinal GEE model will be used too. It allows us to quantify the correlation of the pre-rt-PA and serial post-rt-PA glutamate plasmatic measurements with the evolution (arterial recanalization, neurological deterioration, mortality) of our patients. Both of them will be adjusted for their possible confounding variables (age, hypertension, glucose levels…).

The statistical analysis of variables will be performed using the statistical SPSS program.


## 8. WORK PLAN

8.1. Inclusion and clinical evaluation of patients (September 2013 – September 2014).

The clinical evaluation of the patients will be done by Dr. Castellanos, Dr. Serena, Dr. Van Eendenburg and Ms. Elia Martínez.


8.2. Radiological evaluation (September 2013 – November 2014)

The evaluation of the images will  done by Dr. Puig and Dr. Pedraza (both of them, Neuroradiologists of the Institut de Diagnòstic per la Imatge de l'Hospital Universitari Dr. Josep Trueta) and will be performed according to this timing:

- Image obtaining: September 2013 – September 2014.
- Image analysis for data inclusion in the database: December 2013 – November 2014.

The evaluation of the images will be done without knowledge on the laboratory data and clinical evolution of the patients.


8.3. Blood samples obtention and storage: September 2013 – September 2014.

Samples obtained in the emergency room and the Stroke Unit will be stored by Martha Kazimierczak, from the Biomedical Research Institute of Girona.


8.4. Biochemical analysis of the samples: February 2014 – November 2014

The analysis of the samples will be performed monthly in the laboratory of Analytical Chemistry of the Departament of Chemist of the University of Girona by Dr. Juan Manuel Sánchez Navarro, Dr. Gemma Huguet and Dr. Elisabet Kádar.


The laboratory data will be evaluated without knowledge on the neuroimaging data and the clinical evolution of the patients.

8.5. Analysis of results: March 2014 – December 2014

A first analysis of the obtained data will be done by April 2014, and the main analysis of whole data will be accomplished by December 2014. The research team will be in touch for the analysis of preliminary data and the drafting of the obtained results.

## 9. ETHICAL ASPECTS

As it was said in section 6.2 (Subject selection and withdrawal), currently is not possible to have a control group for a further and prospective determination of their glutamate levels, since the patients in the control group could not be treated with rt-PA. However, in same conditions and times from stroke onset, also those patients should be likely to receive fibrinolytic therapy with rt-PA. For this reason, serial plasma glutamate levels from samples taken from patients in the Stroke Unit before this treatment was routinely administrated, will be analysed retrospectively; having had signed informed consent for future research.

## 10. BUDGET

Some of the procedures included in this study are already done as a routine in the common protocol for diagnosis, treatment and follow up of stroke.

The procedures and evaluations out of the common protocol are included in the budget below:

- Second Angio-CT scan, done 1-5 hours after rt-PA administration

- Second non-contrast Cranial CT scan, done after 30±2 days of evolution

- Serial blood sample obtention after rt-PA administration (2, 6, 12, 24 and 48 hours later).

Costs of execution: 78.710 €

| Neuroimaging | | 29.600€ |
|---|---|---|
| 100 angio-CT scans | 25.000 € | |
| 100 non-contrast Cranial CT scans | 4.600 € | |
| Laboratory | | 14.910€ |
| Hiring of laboratory services | 14.000 € | |
| 2 HPLC kits (100 tests/kit) | 2 x 455 = 910€ | |
| Nursery | | 34.200€ |
| Hiring of nursery services | 34.200€ | |
| | TOTAL | 78.710€ |

# ANNEX

**1. Information sheet for patients**

**2. Informed consent for patients**

**3. Informed consent for patients' legal representors**

**4. National Institutes of Health Stroke Scale**