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THE ROLE OF ALPHA-1 ANTITRYPSIN IN THE PROGNOSIS OF ISCHAEMIC STROKE

DEGREE FINAL PROJECT

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ABBREVIATIONS

AAT	Alpha-1 antitrypsin.
ASPECTS	Alberta Stroke Program Early CT score.
BBB	Blood-brain barrier.
BP	Blood pressure.
CEIC	Clinical Research Ethics Committee.
CT	Computed tomography.
ECM	Extracellular matrix.
EIC	Early ischemic changes.
eNOS	Endothelial nitric oxide synthase
HI	Haemorrhagic infarction.
HJT	Hospital Dr. Josep Trueta.
HT	Haemorrhagic transformation.
IL	Interleukin**.**
INR	International normalized ratio.
IR	Ischaemia/reperfusion
LDL	Low density lipoprotein.
MAP2	Microtubule-associated protein 2.
MMP-9	Matrix metalloproteinase-9.
MRI	Magnetic resonance imaging.
mRS	Modified Rankin scale.
NIHSS	National Institute of Health Stroke Scale.
OGD	Oxygen and glucose deprivation.
PH	Parenchymal hematoma.
PMN	Polymorphonuclear.
ROS	Reactive oxygen species.
rt-PA	Recombinant tissue plasminogen activator.
SERPIN*A1*	Serine Protease Inhibitor, group A, member 1.
SICH	Symptomatic intracranial haemorrhage.
SOD	Superoxide dismutase.
SU	Stroke unit.
TNF-α	Tumor necrosis factor alpha.
ZO-1	Zonula occludens-1.

ABSTRACT

BACKGROUND: Ischaemic stroke is the second cause of death and the first cause of disability in Europe. Nowadays, the only available pharmacological treatment for acute ischaemic stroke is recombinant tissue plasminogen activator (rt-PA). However, it only achieves the reperfusion of the occluded vessel in 50% of the patients. Moreover, only 5-10% of the patients can receive this treatment due to its numerous contraindications. One of the possible complications of the treatment with rt-PA is the haemorrhagic transformation of the stroke. Although it only occurs in 2-7% of the patients, it worsens the recovery and prognosis of the patient. Another fact that might negatively influence in the outcome of the patient is alpha-1 antitrypsin (AAT) deficiency. AAT is the main antiprotease in bloodstream and its activity is crucial to maintain the balance between proteases and antiproteases in order to limit the proteolytic effect of proteases in tissues, for instance in blood-brain barrier (BBB). Thus, AAT deficiency might be related to BBB disruption, neuronal cell death and an increase and exacerbation of the inflammatory response after an ischaemic stroke, worsening the outcome of the patient.

OBJECTIVE: To analyse AAT levels in blood samples of patients with acute ischaemic stroke treated with rt-PA, at admission, 2, 6, 24 and 72 hours, and correlate these levels with the functional outcome of the patient at 3 months (modified RANKIN scale).

DESIGN AND PARTICIPANTS: The study will consist in a prospective cohort study including consecutive patients suffering from acute ischaemic stroke (independently of the aetiology of the stroke) treated with rt-PA in the Neurology Department of Hospital Dr. Josep Trueta, between 1 June 2020 and 31 November 2022.

METHODS: 5 blood samples will be extracted in the acute phase of stroke (at admission, 2, 6, 24 and 72 hours). Patients will be divided into two groups according to AAT levels at admission: normal levels of AAT (≥1-2 g/L) versus low levels of AAT (<1 g/L). 196 patients will be required in each group. 3 months after the stroke, the prognosis of the patient will be evaluated using the modified RANKIN scale. AAT levels in the acute phase of the stroke will be compared with levels at 3 months. Infarct volume and the presence of haemorrhagic transformation will be assessed with computed tomography (CT) scan at 24 hours of the stroke, and these variables will be correlated with AAT levels in the acute phase.

KEYWORDS: ischaemic stroke, rt-PA, haemorrhagic transformation, BBB, AAT.

1. INTRODUCTION

1.1 ISCHAEMIC STROKE

Definitions

Stroke is a cerebrovascular disease defined as a sudden alteration of cerebral bloodstream producing a focal neurological deficit. There are two types of stroke (Figure 1). Ischaemic stroke, which represents the 80% of all strokes, is caused by a lack of blood supply in a certain brain location and is clinically defined as more than one hour of evolution of focal neurological deficit added to a visible infarct zone in neuroimage. On the other hand, haemorrhagic stroke (20%) is produced by a blood vessel rupture and can be divided into subarachnoid haemorrhage (the stroke with the greatest morbimortality), and intraparenchymal or intracerebral haemorrhage (1).

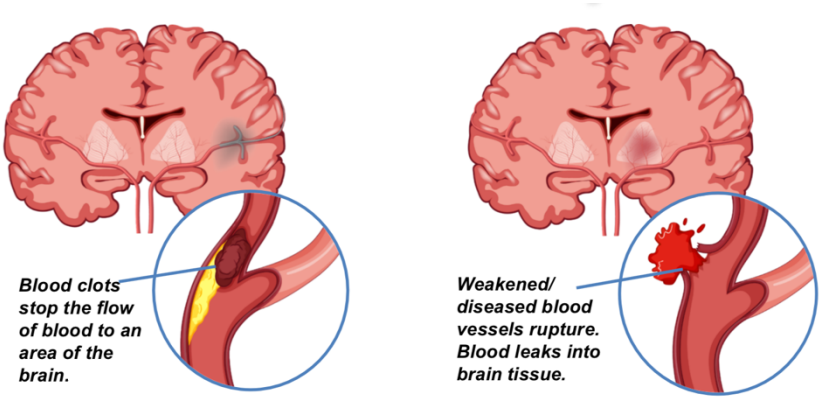


Figure 1. Main pathogenic mechanisms in stroke, ischaemic (left) and haemorrhagic (right) (2).

Epidemiology

Ischaemic stroke constitutes a great public health problem and a socioeconomical burden in developed countries. It is the second leading cause of death in Europe and the first one in women older than 65 years old and men older than 75. It affects 17 million people worldwide and it is the first cause of disability (3) among adults and the second cause of dementia. The incidence in Catalonia is 150-200/100.000 inhabitants/year (4).

Despite the incidence is increasing, mortality rate has been decreasing in the last two decades. The combination of these two factors has led to an increasing number of people living with the consequences of stroke and requiring primary and specialized medical assistance. The reduction in the mortality rate can be explained by the increasing awareness about primary prevention in patients with risk factors, and also by the improvement of medical assistance in the acute stroke phase thanks to the development of stroke units (SUs) (4).

Risk factors

Stroke risk factors can be classified as nonmodifiable and modifiable (Table 1). The main risk factor for ischaemic stroke is arterial hypertension. In fact, it is the only factor consistently associated with all types of stroke. Studies suggest that 90% of all strokes can be explained by modifiable risk factors, and that 80% of recurrent strokes can be prevented with the optimal control and/or the elimination of these factors (4).

The nonmodifiable risk factors include age (the incidence of stroke rises with the age (5)), sex (at younger ages, stroke is slightly more common in women; at older ages it is slightly higher in men), race and genetic factors (family history of stroke increases stroke risk by 30% (6)).

Table 1. The most frequent modifiable and nonmodifiable risk factors of ischaemic stroke. Adapted from (1).

Nonmodifiable	Modifiable
- Age - Sex - Race - Genetics	- Hypertension - Diabetes mellitus - Hyperlipidaemia - Embolic cardiopathies - Smoking - Obesity

Etiological classification (TOAST)

Ischaemic stroke can be classified as the following subtypes (Figure 2) according to its aetiology:

- **Large-artery atherothrombotic stroke**

It requires the presence of clinically generalized atherosclerosis (coexistence of ischaemic cardiopathy and/or peripheral vascular disease) or the demonstration of occlusion or stenosis of cerebral arteries equal to or greater than 50%, related to the symptoms of the patient.

- **Cardioembolic stroke**

It is produced in the context of an embolic cardiopathy (atrial fibrillation, valvulopathy, recent myocardial infarction, endocarditis, congestive heart failure, etc).

- **Small vessel disease (lacunar stroke)**

It is a small size stroke (<2cm of diameter by magnetic resonance imaging (MRI) or 1.5cm by CT) located in a cerebral perforating artery caused by microatheromatosis or lipohialinosis of the perforating arteries smaller than 200 μm.

- **Stroke of infrequent cause**

It is a stroke which its origin is not atherothrombotic, cardioembolic or lacunar. They are mainly caused by diseases that produce an arteriopathy different from atherosclerotic or by systemic disorders.

- **Unknown origin stroke (cryptogenic stroke)**

It is defined as a brain stroke not attributable to cardioembolism, large artery atherosclerosis, or small artery disease despite a standard vascular, cardiac, and serologic evaluation. It also includes patients with two or more equally plausible identified causes of stroke (1).

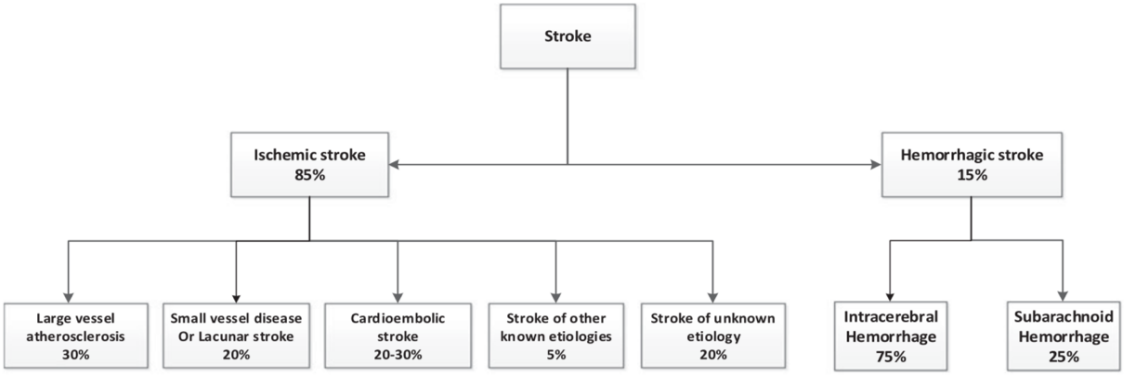


Figure 2. Stroke subtypes (7).

Clinical manifestations

The semiology caused by cerebral ischaemia depends basically on the size of the lesion, the cerebral parenchymal topography it affects, the vascular topography and the aetiology. Stroke is characterized by a sudden focal neurological deficit. Specifically, ischaemic stroke produces a sudden loss of the ability to talk and/or to understand, a loss of strength and sensitivity from one side of the body (face, arm and/or leg) and a loss of vision. Regarding sensitivity, ischaemic stroke produces a sudden sensitivity disorder noticed as a tingling feeling from one side of the body. Finally, another alarm sign is a very intense and sudden headache (1,5,8).

Diagnosis

Due to the suspicion of stroke the diagnostic process should meet the following objectives:

1. Confirm the diagnosis of stroke with neuroimaging (CT or MRI) and rule out other clinical entities.
2. Establish the type of stroke (ischaemic or haemorrhagic).
3. Determine the topography and extension of brain injury.
4. Know the situation of vascular system.
5. Know the aetiology and pathogenesis.

All these steps must be completed in the shortest possible time. This diagnostic process includes clinical history and general, neurological and neuroimaging examination (simple CT, multimodal CT, MRI, multiparametric MRI, supra-aortic trunk and/or transcranial doppler ultrasound) (1,8).

Prognosis

Approximately half of stroke survivors remain disabled (7) and 8%-20% die during the first month. Mortality at 5 years is 40%-60%. During the first week, mortality is usually attributable to neurological causes, while late mortality is mainly caused by medical disorders (5).

The prognosis of ischaemic stroke improves if treatment starts early, especially if it is carried out in a SU (8). SUs are dedicated areas in hospitals open round the clock, where stroke patients are admitted and cared for by a multidisciplinary team including medical, nursing and therapy staff. They are the most effective measure in reducing mortality and morbidity (9).

Long-term functional status of the patient with stroke is perhaps the most interesting way of establishing its prognosis. The functional situation is usually measured using the modified Rankin scale (mRS), whose scores range from 0 to 6 points (**Annex 1**). It evaluates the abilities of the patient to carry out the basic activities of daily life. Dichotomization of the scale in ≤2 or >2 points is commonly used to report good or bad functional outcome, since this cut point marks the difference between functional autonomy and dependence (10).

1.2 FIBRINOLYSIS WITH RECOMBINANT TISSUE PLASMINOGEN ACTIVATOR (rt-PA)

The available treatments for acute ischaemic stroke are reperfusion therapies, which include intravenous fibrinolysis with rt-PA and mechanical thrombectomy. Their main objective is the restoration of cerebral blood flow distal to the occluded artery that causes the symptoms to rescue the ischaemic tissue that has not yet experienced irreversible damage (ischaemic penumbra) (5,8).

rt-PA or *alteplase* is the only pharmacological treatment approved by the Food and Drug Administration for the treatment of acute ischaemic stroke. It is a fibrino-selective thrombolytic agent, chemically identical to endogenous t-PA, that breaks fibrin through the activation of plasminogen to plasmin (Figure 3). It achieves the dissolution of the thrombus, allows recanalization of the occluded artery, and finally, the reperfusion of the tissue (11).

Once simple CT scan confirms the ischaemic nature of stroke and rejects the presence of haemorrhage or other causes that might explain the symptoms, rt-PA is indicated in ischaemic stroke of less than 4.5 hours of evolution (8) (or until 9 hours under some specific conditions). The recommended dose of rt-PA is 0.9 mg/Kg, with a maximum dose of 90 mg. 10% of the total dose is administered in bolus, and the remaining dose is administered in continuous infusion during 60 minutes (8).

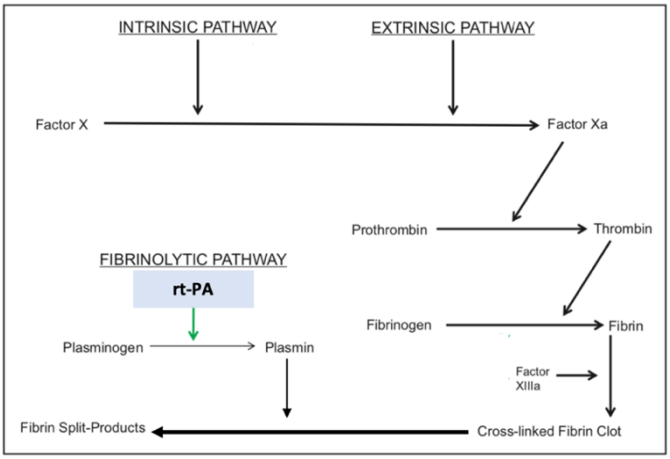


Figure 3. Mechanism of action of alteplase. Adapted from (12).

rt-PA improves survival and functional prognosis when administered early after the onset of stroke symptoms. However, the narrow therapeutic window (<4.5 hours) and its multiple contraindications (1) (Table 2) limit its use. In fact, less than 5-10% of the patients benefit from this treatment in the acute phase. On the other hand, arterial recanalization is only achieved in 50% of patients and the risk of haemorrhagic complications is not negligible (12).

Table 2. Contraindications for rt-PA.

Contraindications for rt-PA
• Sustained blood pressure (BP) >185/110 mmHg. • Platelet count <100,000 uL. • Acute haemorrhagic diathesis. • On an anticoagulant, with an international normalized ratio (INR) >1.7 (warfarin) or elevated partial thromboplastin time (heparin). • Treatment with direct thrombin inhibitors or direct factor Xa inhibitors. • Stroke or serious head trauma in the last 3 months. • Early established infarct involving more than 1/3 of the middle cerebral artery territory in CT • Suspected subarachnoid haemorrhage or known history of intracranial haemorrhage. • Major surgery in the last 14 days. • Myocardial infarction in the last 3 months. • Evidence of active bleeding or acute trauma (fracture) on examination. • Gastrointestinal or urinary tract haemorrhage in the last 21 days. • Serum glucose <50 mg/dL or >400 mg/dL.

1.3 HAEMORRHAGIC TRANSFORMATION OF ISCHAEMIC STROKE

One of the complications of the treatment with rt-PA is the appearance of haemorrhagic transformation (HT), which refers to an ischaemia-related brain haemorrhage, especially produced after thrombolytic therapy (13). Although any HT can occur up to ≥7 days, the clear majority occur within 24 hours (12). The incidence of symptomatic intracranial haemorrhage (SICH) after rt-PA at the standard dose of 0.9 mg/kg varies from 2% to 7% in clinical trials and prospective stroke registries (12), and the rate increases with the dose of rt-PA (14).

With regard to the type of haemorrhage, HT can be divided into haemorrhagic infarction (HI) and parenchymal hematoma (PH) (Figure 4). HI is an heterogeneous hyperdensity occupying a portion of an ischaemic infarct zone on CT images, whereas PH refers to a more homogeneous, dense hematoma with mass effect (12). Each of them has two subtypes. On radiographic images, HI1 is characterized by small hyperdense petechiae, whereas HI2 refers to more confluent hyperdensity throughout the infarct zone. Any of them causes mass effect. PH1 refers to an homogeneous hyperdensity occupying less than 30% of the infarct zone, with some mass effect, and PH2 occupies over 30% of the infarct zone, with significant mass effect (12).

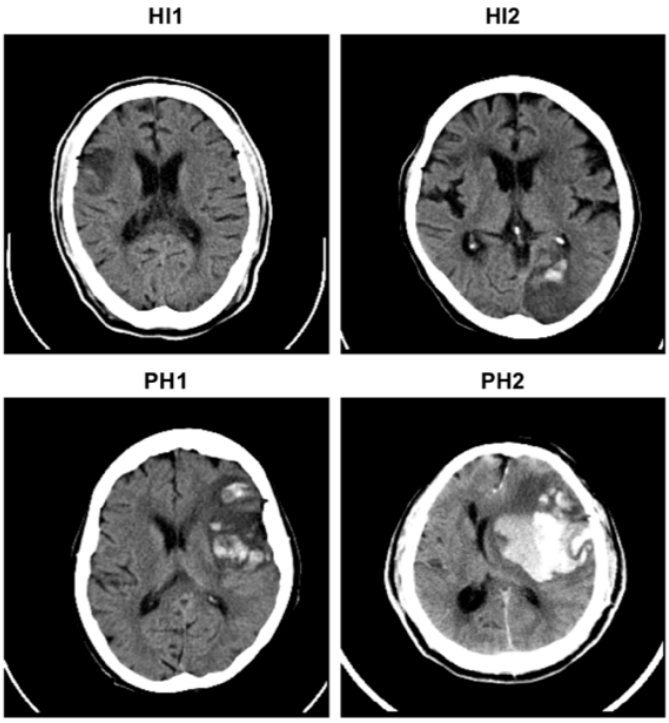


Figure 4. Subtypes of haemorrhagic transformation (14).

Signs of haemorrhage transformation should be monitored, such as a decrease in the level of consciousness, an increase in motor deficit, an increase in systolic BP, headaches and vomiting. HI usually occurs without clinical translation, while PH causes expansive effect, cerebral herniation and death (5). The diagnosis requires brain imaging (CT or MRI) 24 hours after the administration of rt-PA, showing acute haemorrhage. The factors increasing the risk of HT are: size of the lesion on CT (the presence of oedema is an excellent marker); age (advanced age is associated with an increased risk of HT); severity of stroke (assessed by NIHSS score); hypertension, diabetes mellitus, ischaemic heart failure or atrial fibrillation (5).

SICH following thrombolytic therapy is associated with very poor clinical outcomes. The fatality rate is between 50 and 80% and the rate of severe morbidity or mortality exceeds 90%. Thus, it would be of interest to encounter biomarkers likely to be independent predictors of HT following thrombolytic therapy (15).

HT following rt-PA is thought to be the result of reperfusion of cerebral vessels whose integrity has been disrupted by severe ischaemia (15). Thus, the disruption of the BBB predisposes to blood extravasation when the ischaemic tissue is reperfused (13). Recent data suggest that the signalling activities of t-PA in the neurovascular unit are responsible for some potentially neurotoxic side effects. Besides its intended role in clot lysis, t-PA is also an extracellular protease. It increases the activity of matrix metalloproteinase (MMP), which degrade the extracellular matrix (ECM) integrity, increasing the risks of neurovascular cell death, BBB disruption, oedema, and haemorrhage (13) (Figure 5).

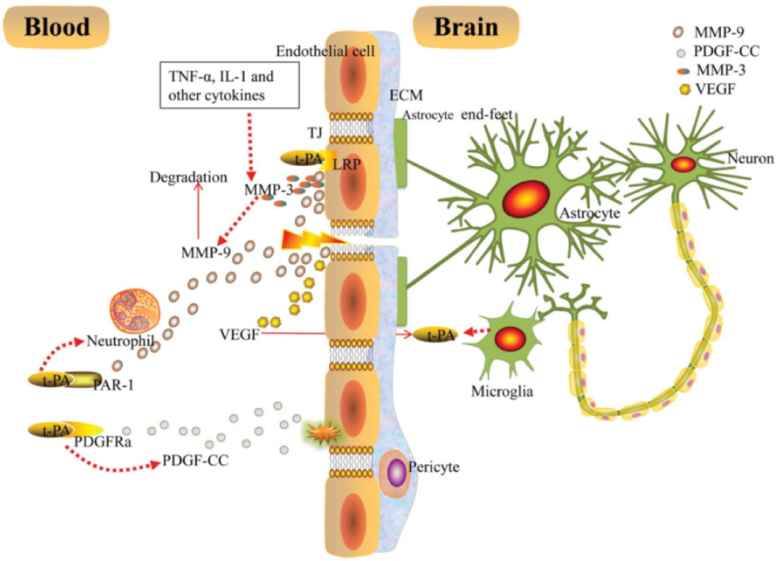


Figure 5. The blood brain barrier (BBB). Adapted from (16) .

1.4. BLOOD-BRAIN BARRIER (BBB)

A relevant structure damaged by the consequences of ischaemic stroke is the BBB. It consists of endothelial cells of the cerebral capillaries, and it is surrounded by pericytes, astrocytes end-feet and neurons, resulting in the neurovascular unit (Figure 6). BBB form a low permeability physical barrier between the blood and the brain due to the presence of tight junctions between adjacent endothelial cells. Thus, the BBB serves the important role of restricting the entry of molecules and immune cells from the systemic circulation into the central nervous system (17).

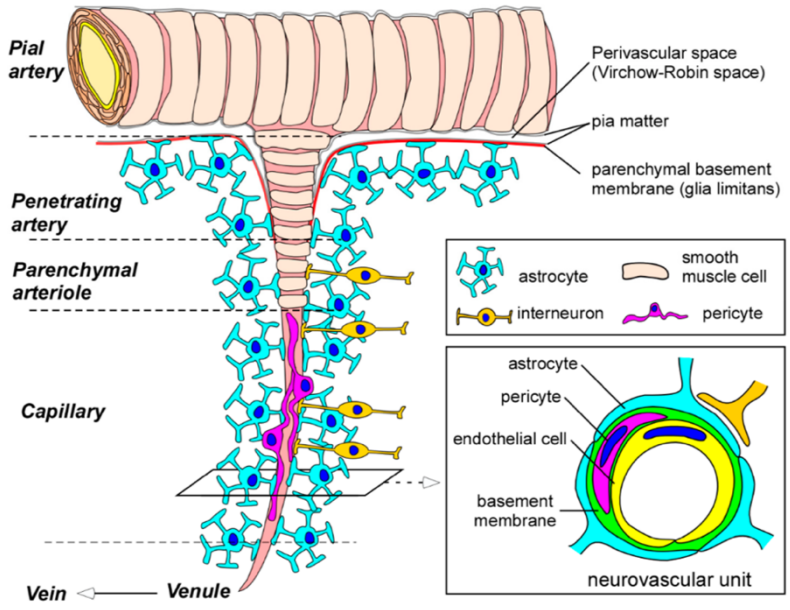


Figure 6. Blood-brain barrier (BBB) and the neurovascular unit (18).

The BBB has three main functions: it strictly limits the passive diffusion of macromolecules and pathogens from the blood to the brain; it mediates the transport of nutrients to the brain parenchyma as well as the efflux from the brain of toxic metabolites (Figure 7); and it regulates the migration of circulating immune cells (7).

The barrier function of the BBB can be disrupted by a number of different stimulus or pathologies, including hypoxia stress and stroke (17). Moreover, whereas the BBB may recover from ischaemia if reperfusion occurs early, delayed reperfusion often results in exacerbated BBB disruption. A key feature of BBB disruption is an increase in paracellular permeability of endothelial cells mainly caused by the degradation of tight-junction proteins, and the subsequent disassembly of junctions. Importantly, BBB disruption can contribute to brain injury after ischaemic stroke (19).

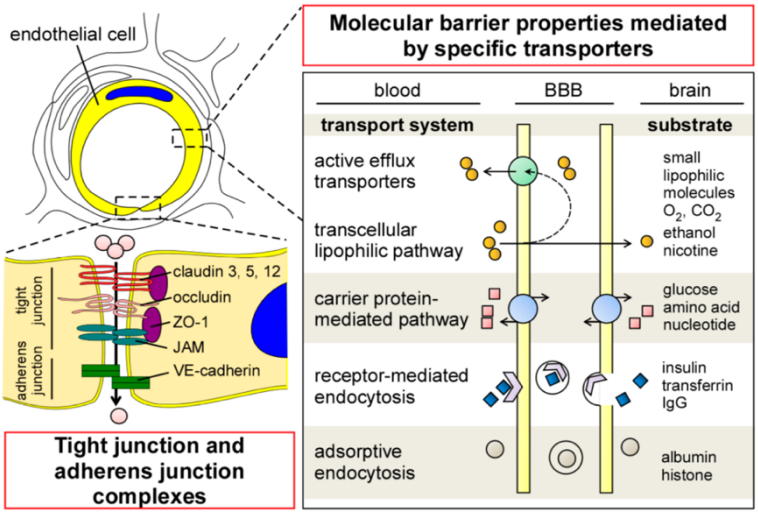


Figure 7. Physical and molecular properties of endothelial cells contributing to BBB integrity and function (18).

The study of the BBB has largely fallen into two major categories: *in vivo* perfusion models in animals, and *in vitro* cultures of endothelial cells from cerebral microvessels or other endothelial cell sources. However, the investigation of specific molecular mechanisms controlling BBB permeability and response to stimulus can best be approached using *in vitro* models of the BBB (17).

1.5. BLOOD BIOMARKERS IN ISCHAEMIC STROKE

Prediction of stroke prognosis represents a challenge for neurologists and researchers. An accurate and early estimation of which patients will improve or worsen in the following hours, which are at a higher risk of dying as a result of the stroke, or which will be disabled months after the event could be used to guide decision making in clinical practice (10).

The World Health Organization defines a biomarker as a substance, structure or process that can be measured in the body or its products, and it is able to influence or predict the incidence or outcome of a disease.

Until now, the majority of biomarkers studied are related with an important pathophysiological pathway in stroke and associated with diagnosis, aetiology, response to thrombolytic treatment or prognosis. However, its used has not yet been generalized (7). Biomarkers studied are related to processes such as inflammation, tissue remodelling, coagulation or fibrinolysis (10).

1.6. ALPHA-1 ANTITRYPSIN (AAT)

AAT is the most abundant serine protease inhibitor in serum and an acute phase glycoprotein, also called SERPIN*A1* (Serine Protease Inhibitor, group A, member 1) (Figure 8). It is a circulating glycoprotein with a molecular weight of 52-kDa and a half-life blood of about 5 days (20). AAT is synthesized and released essentially by hepatocytes (≈80%), and in additional amounts by monocytes, macrophages, alpha and delta pancreatic cells and alveolar epithelial cells type II (20).

At baseline, the organism produces 34 mg/kg/day of AAT, which is translated to plasma concentrations of about 1-2 g/L. Since it is an acute phase reactant, its plasma levels increase rapidly, 2-4 times, in response to inflammatory or infectious stimulus. Thus, plasma levels of AAT rise in response to ischaemic injury produced, for instance, by an ischaemic stroke (20).

The local concentration of AAT in inflammatory tissues can be increased up to 11 times, due to an increased in the synthesis by resident cells and by the contribution of migrated leukocytes. The local production of AAT is important to limit the degradation of the extracellular matrix by the proteases released by activated cells (20).

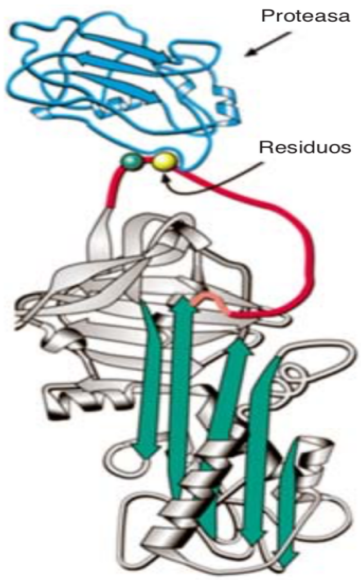


Figure 8. Formation of protease-antiprotease complex (22).

The infarcted area resulting from cerebral ischaemia is characterized by the presence of a primary necrotic core and a secondary apoptotic area. This neuronal damage is promoted by the presence of oxidative stress, excitotoxicity and BBB disruption. Afterwards, pro-inflammatory cytokines activate infiltrated neutrophils that release neutrophil elastase contributing to exacerbate the neuronal damage. Therefore, molecules as AAT with capacity to avoid the activity of neutrophils and to inhibit the activation of pro-inflammatory cytokines and proteases could help to prevent cerebral ischaemia mediated-cell death (21).

Effects of AAT on ischaemia damage

- PERMEABILITY

Tight junction associated proteins (occludin, claudin-5, zonula occludens-1 (ZO-1)), are important in maintaining the structure and function of tight junctions. *In vivo*, tight junctions become disrupted in the first hours after ischaemia resulting in increased BBB permeability (19). By contrast, AAT upregulates the reduced expression of ZO-1 and occludin caused by ischaemia/reperfusion (IR) injury, reducing cellular permeability (23).

Polymorphonuclear cells (PMNs) are rapidly activated during acute ischaemia. Similarly, activation of cerebral endothelial cells leads to an increased expression of adhesion molecules leading to transmigration of PMNs through BBB. PMNs have a key role in acute ischaemic cerebral injury and BBB disruption. Oxygen and glucose deprivation (OGD) may stimulate PMN activation and the release of its proteases, such as elastase (Figure 9), leading to endothelial damage and BBB disruption (24). The antiprotease effect of AAT might limit the action of elastase, reducing cellular permeability.

MMP-9 is a collagenase involved in the breakdown of ECM. It might be released in response to ischaemic insult from neurons, oligodenroglia, reactive astrocytes and activated microglia. Furthermore, tPA and other molecules have the capacity to activate MMP-9. This increase in MMP-9 is further associated with excitotoxicity, neuronal damage, apoptosis, oxidative stress, and most importantly BBB disruption leading to cerebral oedema and haemorrhagic transformation after ischaemia. MMP-9 is another target protease of AAT, limiting its effect to the ECM (25).

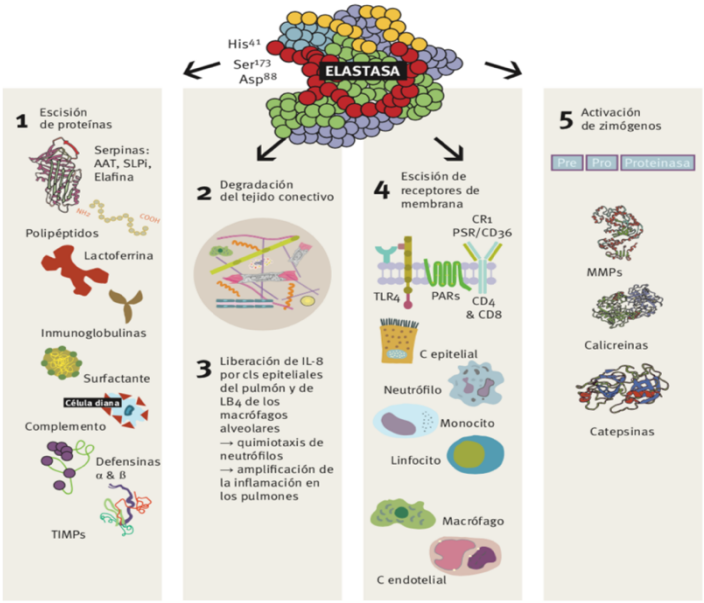


Figure 9. Derived effects from an excess of non-neutralized elastase (20).

- CELL APOPTOSIS AND PROLIFERATION

Induction of OGD increases caspase-3 levels in cortical, hippocampal and striatal cultures. AAT inhibits IR-induced cell death by inhibiting caspase-1 and -3 (21). Moreover, IR injury increases expression of apoptotic cascades (Rac1, phosphorylation of PAK and p38), whereas AAT significantly limits its expression, reducing cell apoptosis and increasing proliferation (23).

- OXIDATIVE STRESS

It is known that reactive oxygen species (ROS) play a critical role in IR injury, and superoxide and hydrogen peroxide are the main free radicals that contribute to it. During reperfusion, an excess of free radicals cannot be neutralized by endogenous antioxidant enzymes, which results in oxidative stress. Superoxide dismutase (SOD) is an antioxidant enzyme highly specific for superoxide elimination, thus reducing lesions caused by IR. AAT plays an antioxidative role by increasing the activity of SOD and the expression of endothelial nitric oxide synthase (eNOS) (23).

- PROTEASE-ANTIPROTEASE BALANCE

Under physiological conditions, serine proteases must only be activated in the presence of inflammatory processes. Therefore, there must exist a strict balance between proteases and antiproteases, which is maintained by AAT. The imbalance leads to an excessive proteolytic effect, causing pathologies affecting the structure of connective tissue basically in the lung (emphysema) and in the liver (hepatopathy) (22) (Figure 10).

Although the main function of AAT is the inhibition of the excessive elastase released by neutrophils, several studies have shown that it can also neutralize other serine proteases, like proteinase-3, cathepsin G, tryptase, trypsin, chymotrypsin or myeloperoxidase (20,26–28).

- INFLAMATION

Inflammation following ischaemic stroke is considered an unavoidable pathological process involved in post-ischaemic brain injury. Activated immune cells cross the disrupted BBB and accumulate at the site of injury, which aggravate cerebral infarction and contribute to the destruction of cerebral tissue (29).

Cerebral damage by OGD is associated with the activation of pro-inflammatory cytokines (interleukin (IL)-1α, IL- 1β, , IL-6, IL-8 and tumor necrosis factor alpha (TNF-α)), and the inhibition of anti-inflammatory interleukins (IL-10). Considerable evidence show that pro-inflammatory

cytokines modulate tissue and endothelial injury, and facilitate leukocyte migration into the ischaemic area (30). Addition of AAT after OGD promotes a significant reduction of pro-inflammatory cytokines, and an increase in IL-10 levels (21).

- CEREBAL TISSUE

Administration of AAT to mouse models submitted to brain ischaemic injury protects the cells, thereby reducing the infarct size (21). The physiopathologic mechanism might be explained by both a systemic and a local effect of AAT. On the one hand, circulating AAT crosses the disrupted BBB, reaching the impaired area of the brain and displaying local protective functions. On the other hand, AAT may indirectly reduce brain tissue damage by inhibiting the infiltration and migration of lymphocytes to the penumbra of the ischaemic region (26).

Cerebral ischaemia promotes an important damage to astrocytes and degeneration of white matter. It has been evaluated the capacity of AAT to protect astrocytes and oligodendrocytes from OGD-mediated degeneration. The results show a concentration-dependent protective effect suggesting a broad therapeutic range of AAT with the ability to protect neurons and neuroglia, and both grey and white matter (21).

- NEURONAL STRUCTURE

Cerebral ischaemia not only induces neuronal death, but it also compromises and reduces the dendritic network within the damaged area. In OGD conditions, a study observed a reduction of microtubule-associated protein 2 (MAP2) levels in the neuronal cultures studied. When AAT was added to cultures exposed to OGD, they detected a preservation of MAP2 levels near to control values. Reduction of MAP2 has been associated with a loss of synapses, whereas elevation of MAP2 levels promoted the formation and maturation of synaptic connections and neurogenesis. In this way, this data support the ability of AAT to enter cells and maintain the structure of neurons, allowing neuroplasticity to take place after OGD (21).

The effects of AAT on ischaemia are summarized in Table 3. Finally, Figure 10 summarizes the different topics explained before, from the moment of ischaemia to the appearance of haemorrhagic transformation.

Table 3. Effects of AAT on ischaemia damage.

Ischaemia damage	AAT effect
⇑ **PERMEABILITY** ⇓ tight junction expression	⇓ **PERMEABILITY** ⇑ tight junction expression ⇓ MMP-9 expression
⇑ **CELL APOPTOSIS +** ⇓ **CELL PROLIFERATION** ⇑ Rac1/Pak/p38 activation ⇑ caspase 1 and 3 expression	⇓ **CELL APOPTOSIS +** ⇑ **CELL PROLIFERATION** ⇓ Rac1/Pak/p38 activation Inhibits caspase 1 and 3
OXIDATIVE STRESS ⇓ SOD activity	**ANTIOXIDANT** ⇑ SOD activity, ⇑ eNOS expression
⇑ **PROTEASE ACTIVITY** ⇑ PMN activation	**ANTIPROTEASE** Serine protease inhibitor. Maintains protease-antiprotease balance
INFLAMMATION ⇑ pro-inflammatory cytokines expression	**ANTI-INFLAMMATORY** ⇓ pro-inflammatory cytokines expression. ⇑ anti-inflammatory cytokines expression
BRAIN TISSUE DAMAGE Damages astrocytes. White matter degeneration	**CYTOPROTECTIVE** Protects neurons, neuroglia, white and grey matter.
DAMAGES NEURONAL CYTOARCHITECTURE ⇓ MAP-2 levels → Loss of synapses	**MAINTAINS NEURONAL CYTOARCHITECTURE** Preserves MAP-2 levels → synapses formation and maturation → neuroplasticity.

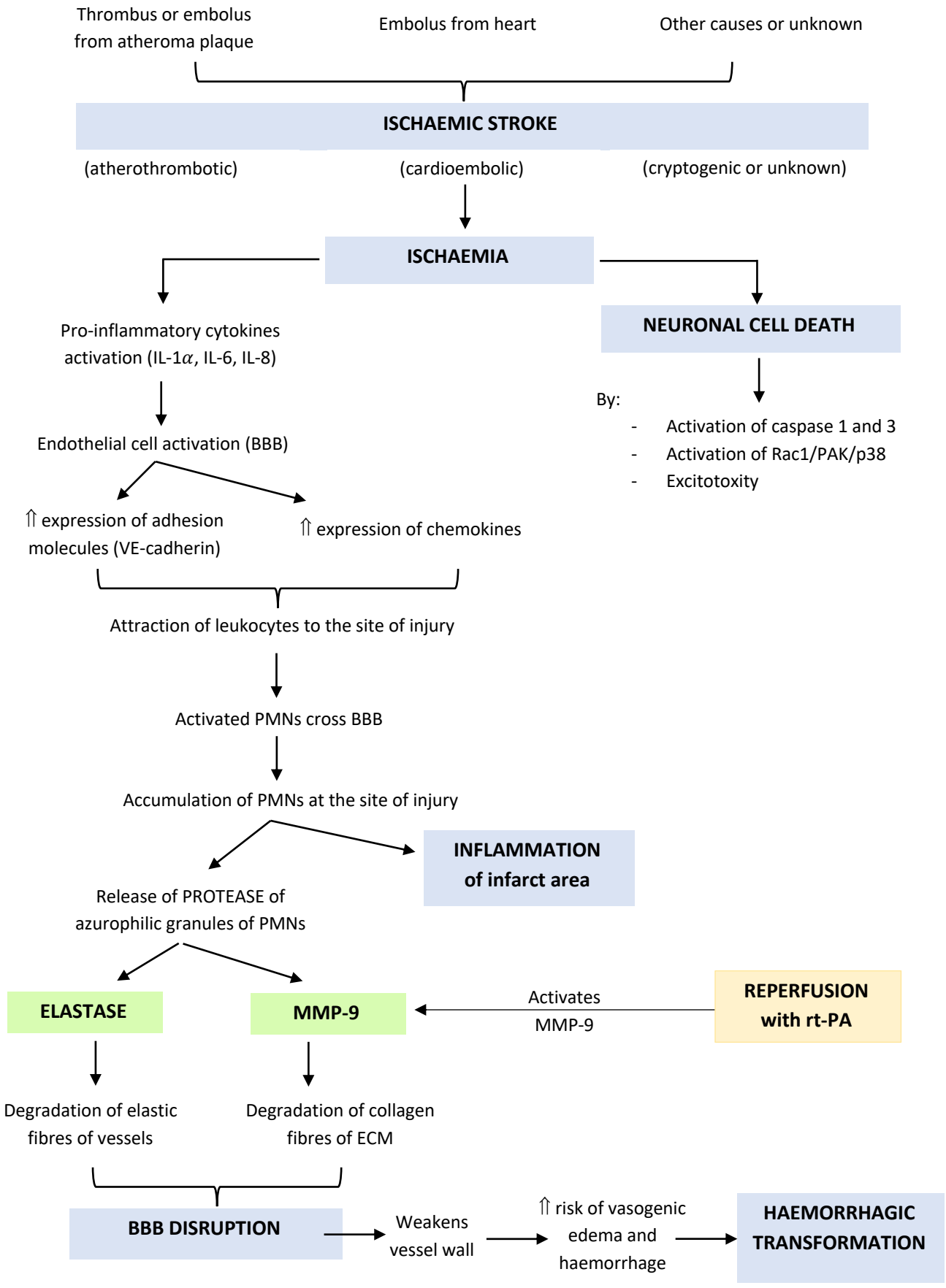


Figure 10. From ischaemic stroke to haemorrhagic transformation.

2. JUSTIFICATION

Ischaemic stroke is the second cause of death and the first cause of disability in Europe with a huge economic impact due to the residual deficiencies that leaves in most of the patients. Nowadays, the only available pharmacological treatment for acute ischaemic stroke is rt-PA. However, its fibrinolytic effect only achieves the reperfusion of the occluded vessel in 50% of the patients. Moreover, only 5-10% of the patients can receive this treatment due to its numerous contraindications. One of the possible complications of the treatment with rt-PA is the haemorrhagic transformation of the stroke. Although it only occurs in 2-7% of the patients, the fatality rate is between 50 and 80%. Despite the thrombolytic effect of rt-PA, reperfusion of the occluded vessel added to the fact that rt-PA damages BBB, rises the risk of haemorrhagic transformation, worsening the recovery and prognosis of the patient. Another fact that might negatively influence in the long-term outcome of the patient is AAT deficiency. AAT is the main antiprotease in bloodstream. It has anti-inflammatory, antiapoptotic and cytoprotective properties. Its activity is crucial to maintain the balance between proteases and antiproteases in the organism in order to limit the proteolytic effect of proteases in tissues, for instance in BBB. Thus, AAT deficiency might be related to BBB disruption, neuronal cell death and an increase and exacerbation of the inflammatory response.

Cabezas et al. found that the administration of human AAT to mouse neuronal cortical, hippocampal, striatal and glial cultures 30 minutes after OGD protects them from cell death (21). *Huong et al.* induced middle cerebral artery occlusion in rats (26). Administration of human AAT significantly improved stroke outcome. *Vila et al*. found that patients who suffered an ischaemic stroke due to arterial dissection had significantly lower levels of AAT than controls (31).

Despite some studies have documented the potentially beneficial effects of AAT on ischaemia damage, AAT has not yet been studied as an acute biomarker for ischaemic stroke. Identifying blood biomarkers of stroke is an active area of research, not only for the diagnosis, but for the long-term prognosis of the patient. Although many biomarkers have been identified, any of them has proved enough sensitivity and specificity in order to be used in clinical practice. Biomarkers representing endothelial dysfunction, especially in combination with neuroimaging markers, may exhibit a better correlation with clinical outcomes in ischaemic stroke.

For these reasons, this protocol expects to study if there is an association between low levels of AAT and poor functional outcome in ischaemic stroke patients treated with rt-PA. If low levels of AAT in the acute phase of stroke correlate with poor clinical outcome at 3 months and more incidence of haemorrhagic transformation, AAT could be used as a biomarker in the acute phase of stroke to predict the prognosis of patients treated with rt-PA.

The prevalence of low levels of AAT within all the bibliography searched only concerns the genetic AAT deficiency (S allele: 2-3%; Z allele: 1-3%). However, like some acute phase reactants, the AAT activity of a person without a genetic deficiency might be also lower than another patient when suffering an ischaemic stroke. Consequently, in this study we do not focus on the hereditary disorder of AAT. The aim is to know which baseline levels of AAT has the patient when suffers an ischaemic stroke, and if they are related with his prognosis.

3. HYPOTHESIS AND OBJECTIVES

3.1. HYPOTHESIS

Low blood levels of AAT will correlate with poor functional outcome (clinical scale and infarct volume) and more incidence of haemorrhagic transformation in patients with acute ischaemic stroke treated with rt-PA.

3.2. OBJECTIVES

Main objective

To analyse AAT levels in blood samples of patients with acute ischaemic stroke treated with rt-PA, at admission, 2, 6, 24 and 72h, and correlate these levels with the functional outcome of the patient at 3 months (modified RANKIN scale).

Secondary objectives

- To analyse AAT levels at 3 months and correlate them with levels in acute phase of the stroke.
- To correlate AAT levels in the acute phase of the stroke with final infarct volume.
- To correlate AAT levels in the acute phase of the stroke with the appearance of haemorrhagic transformation.

4. METHODS

4.1. STUDY DESIGN

The study will consist in a prospective cohort study including patients diagnosed, treated and followed in University Hospital Dr. Josep Trueta (HJT).

4.2. STUDY POPULATION

Participants

The study population will include consecutive patients suffering from ischaemic stroke (independently of the aetiology of the stroke) treated with rt-PA in the Neurology Department of HJT, between 1 June 2020 and 31 November 2022.

Inclusion criteria

- Patients older than 18 years old.
- Patients with a diagnosis of ischaemic stroke confirmed with CT or MRI image.
- Patients treated with rt-PA.
- Patients diagnosed, treated and followed in HJT.
- Patients who sign informed consent.

Exclusion criteria

- Contraindication of rt-PA (see **table 2**).
- Acute or chronic infection and/or inflammation.
- Serious, fatal or disabling illness that makes impossible the recovery or the follow-up.
- Patients who don't sign informed consent.

4.3. SAMPLING

Sample size

In a bilateral contrast with a significance level (α) of 5%, a statistical power of 80% and foreseeing a moderate effect, we will need 196 patients in each group (normal and low AAT levels). Computations were carried out with Prof. Dr. Marc Sáez's software based on the library pwr of the free statistical environment R (version 3.6.2).

Patient recruitment

A consecutive sampling will be used in order to recruit every patient arriving at the Neurology Department and meeting the inclusion criteria, in the period of the study.

4.4. VARIABLES AND MEASURMENTS

Dependent variables

Functional outcome: dichotomous nominal qualitative variable. It will be measured with the modified RANKIN scale (**Annex 1**), considering 0 – 2 score as functional independence, and 3-6 score as functional dependence/death.

Final infarct volume: continuous quantitative variable. A CT image will be performed 24 hours post ischaemic stroke. Infarct volume will be measured using the formula *AxBxC/2*, where A and B = larger perpendicular diameters of the zone of hypodensity and C = number of sections in which the infarct can be seen. It will be expressed in cm^3.

Haemorrhagic transformation: dichotomous nominal qualitative variable. A CT image will be performed 24 hours post ischaemic stroke. Haemorrhagic transformation will be expressed by yes or no, and classified according to the radiological classification mentioned in **1.3 Haemorrhagic transformation of ischemic stroke**.

Independent variable

AAT blood levels: dichotomous nominal qualitative variable. Patients will be divided into two groups according to AAT levels at admission: patients with normal levels of AAT (≥1-2 g/L), and patients with low levels of AAT (<1 g/L).

Co-variables

STROKE RISK FACTORS

Age: continuous quantitative variable. It will be measured in years.

Sex: dichotomous nominal qualitative variable. Male or Female.

Smoking habit: ordinal qualitative variable. Patients will be divided into three categories: smoker (currently smoking), non-smoker (never smoked) and ex-smoker (not currently smoking but did it in the past).

Arterial hypertension: dichotomous nominal qualitative variable. It will be expressed by yes or no, considering yes: systolic BP ≥140 mmHg and/or diastolic BP ≥90 mmHg.

Dyslipidaemia: dichotomous nominal qualitative variable. It will be expressed by yes or no, considering yes: total cholesterol >200 mg/dL, low density lipoprotein (LDL) >110 mg/dL and triglycerides >150 mg/dL.

Atrial fibrillation: dichotomous nominal qualitative variable. It will be expressed by yes or no. It is defined as a supraventricular arrhythmia, with a narrow QRS (<120 ms), absence of P waves and, usually, presence of f waves. It will be considered as present whether if it is paroxysmal (<7 days) or persistent (>7 days).

Diabetes mellitus: dichotomous nominal qualitative variable. It will be expressed by yes or no, considering yes: HbA1c > 6,5%, fasting serum glucose >126 mg/dL, serum glucose >200 mg/dL after 2 hours of an oral glucose tolerance test, glucose >200 mg/dL in patients with classical symptoms of hyperglycemia or hyperglycemia crisis.

Obesity: ordinal qualitative variable. It will be expressed by Body Mass Index: normal weight (18,5–24,9Kg/m2), overweight (25- 29,9Kg/m2) or obesity (≥30Kg/m2).

Socioeconomic level: expressed by education level and occupation, according to social class classification proposed by *Domingo et al.* (32).

VARIABLES RELATED TO STROKE PROGNOSIS

Time from stroke onset to rt-PA administration: continuous quantitative variable. It will be expressed in minutes.

- Clinical data and vital signs at admission

National Institute of Health Stroke Scale (NIHSS): ordinal qualitative variable. 11 items will be evaluated to assess stroke severity (**Annex 2**). 0: no stroke symptoms; 1-4: minor stroke; 5-15: moderate stroke; 16-20: moderate to severe stroke; 21-42: sever stroke.

Systolic and diastolic BP: continuous quantitative variables. They will be expressed in mmHg.

Basal glycemia: continuous quantitative variable. It will be expressed in mg/dL.

Leucocytes: continuous quantitative variable. It will be expressed in cells/mm^3.

Stroke subtype (TOAST): nominal qualitative variable. According to the TOAST classification (**1.1 Ischaemic stroke**), we will consider 5 subtypes of stroke: atherothrombotic, cardioembolic, lacunar, infrequent cause and cryptogenic.

- Neuroimaging data

Early ischaemic changes (ASPECTS) at admission: dichotomous nominal qualitative variable. It will be assessed by non-contrast CT scan at admission. The Alberta Stroke Program Early CT score (ASPECTS) is a topographic scoring system for the assessment of early ischemic changes (EICs). One point is substracted from a total score of 10 points, if an EIC is present in any of the ASPECTS regions. EICs include: hypoattenuation in the basal ganglia, hypoattenuation of the vascular territory of the middle cerebral artery, obscuration of the lentiform nucleus, loss of gray and white matter differentiation in the basal ganglia, cortical sulcal effacement, loss of insular ribbon, obscuration of Sylvian fissure sign or the “dense artery sign”.

Extensive early signs of ischaemia: dichotomous nominal qualitative variable. It will be expressed by yes or no, considering yes the presence in non-contrast CT scan at admission of early signs of brain infarction >1/3 of the middle cerebral artery territory or >1/2 of the anterior or posterior cerebral artery territory or an ASPECT score $\leq$ 6.

Final infarct volume: mentioned in **4.4. Dependent variables.**

4.5. DATA COLLECTION

Sociodemographic and clinical data

The neurologist will collect sociodemographic and clinical data at the moment of the stroke and 3 months after in the routine revision. Afterwards, data will be reported to the database of the study. The information that will be collected includes: identification of the patient and personal data, toxic habits (smoking, alcohol or drug consumption), presence/absence of comorbidities (arterial hypertension, cardioembolic disease, obesity, dyslipidaemia, diabetes mellitus, neoplasia, infection or other diseases), functional status before the stroke, treatments receiving at the moment, treatment received for the stroke, NIHSS at admission and mRS score at 3 months.

Laboratory and neuroimaging data

Blood pressure and basal glycemia will be determined at the admission of the patient at Emergency Department. Neuroimaging will be performed and informed by Radiology Department of HJT. CT image will be carried out at the moment of admission in order to reject the presence of haemorrhage and confirm ischaemic stroke. CT image will be repeated 24 hours after the stroke to assess the infarct volume and the appearance of haemorrhagic transformation.

Regarding AAT levels, a dedicated nurse will perform a total of six venous extractions in each patient within the acute phase and 3 months after the stroke in the exactly time mentioned below:

- 1st blood sample: at the moment of admission in Emergency Department.
- 2nd blood sample: 2 hours after rt-PA administration.
- 3rd blood sample: 6 hours after rt-PA administration.
- 4th blood sample: 24 hours after rt-PA administration.
- 5th blood sample: 72 hours after rt-PA administration.
- 6th blood sample: at 3 months (+/- 7 days) after the stroke.

After extraction, blood samples will be centrifuged at 1500g for 10min and stored at -80 ºC until their analysis in the IDIBGI Biobank. Serum AAT levels will be assayed in IDIBGI laboratory using a specific commercial high-sensitivity enzyme-linked immunosorbent assay (ELISA) (ELH-SerpinA1; RayBiotech).

Blood parameters mentioned in **4.4 Variables and measurements** (lipid profile, glycemia, leucocytes) will be analysed in the blood sample extracted at admission in the Clinical analysis Department of HJT.

5. STATISTICAL ANALYSIS

5.1. DESCRIPTIVE ANALYSIS

With the exception of infarct volume (continuous variable), the other two dependent variables (dichotomous qualitative variables) will be summarize with proportions in a cross tabs stratifying by the independent variable (normal and low). By regards of infarct volume, it will be summarized using the mean and standard deviation, or the median and interquartile range (IQR), depending on whether the distribution of the variable is symmetric or asymmetric, respectively, stratifying again by the independent variable (normal and low). These analysis will be repeated stratifying for co-variables. When the co-variables are quantitative, these will be categorized in quartiles.

5.2. BIVARIATE INFERENCE

The association between the qualitative dependent variables (functional outcome and haemorrhagic transformation) and the independent variable will be assessed by means of the chi-squared test, and the Fisher's exact test if the expected frequencies are lower than 5. Relation between the quantitative dependent variable (infarct volume) and the independent variable will be assessed by means of t-student, if the distribution of the frequencies is symmetrical, and the Mann-Whitney's U test, if the distribution is asymmetrical. These analysis will be repeated stratifying for co-variables. When the co-variables are quantitative, these will be categorized in quartiles.

5.3. MULTIVARIATE ANALYSIS

The association between qualitative dependent variables (functional outcome and haemorrhagic transformation) and the independent variable will be adjusted in the logistic regression controlling for all the co-variables. Relationship between the quantitative dependent variable (infarct volume) and the independent variable will be adjusted using linear regression, controlling for all the co-variables. The possible interaction between the AAT blood levels groups and the smoking status, will be evaluated in both cases.

6. WORK PLAN AND CHRONOGRAM

6.1. WORK PLAN

PERSONNEL OF THE RESEARCH TEAM

The research team will be composed by two neurologists, two laboratory researchers, a nurse and a statistical specialist.

The duration of the study is estimated in three years, and it is divided into six stages described below.

STUDY STAGES

- **Stage 1: team meeting (1 month)**

The research team will meet in order to stablish the steps to take and define the role of each of the members of the team.

- **Stage 2: bibliographical research, study design and elaboration of the protocol (4 months)**

The members of the team will accurately search for bibliography. The hypotheses and the main and secondary objectives of the study will be defined according to the medical experience of the members, the clinical needs and the current bibliography. Afterwards, they will discuss the best design to achieve the objectives. Finally, the elaboration of the protocol will be set by all the members of the team.

- **Stage 3: Clinical Research Ethics Committee (CEIC) approbation (2 months)**

Once the protocol is written, it will be presented to the CEIC in order to achieve its approval. Any objection given by the CEIC will be considered, revised and modified in the protocol.

- **Stage 4: recruitment of patients (30 months)**

After the approval of the protocol by CEIC, we will start the recruitment of the patients. Neurologist will recruit participants meeting the inclusion criteria. Considering a ratio 1:1 between normal and low levels of AAT, and the fact that approximately 180 patients with an ischaemic stroke are annually treated with rt-PA in HJT, we estimate 2.5 years to complete the recruitment. However, if the ratio is not 1:1 and the proportion of patients with low levels of AAT is minor, the time of recruitment will increase.

- **Stage 5: data collection (33 months)**

This period will start simultaneously with the recruitment of patients but will end 3 months after the last participant is included in the study in order to process the last data.

Database will be created in order to register personal and medical information about the patients. Blood samples will be extracted from the patients at the hospital in the acute phase (from admission to 72h) and will be included in the ICT collection of IDIBGI Biobank once the patient has signed the informed consent, and kept in suitable conditions in the Biobank, as explained in **4.5. Data collection**. CT image will be performed 24 hours after the stroke.

3 months after the stroke, patients will come to the routine revision with the Neurologist, who will evaluate functional status of the patient with the mRS scale. Likewise, a nurse will take another blood sample in order to analyse AAT levels at 3 months.

Blood samples will be analysed in IDIBGI every 2 months during the recruitment of the patients in order to classify them according to its AAT levels.

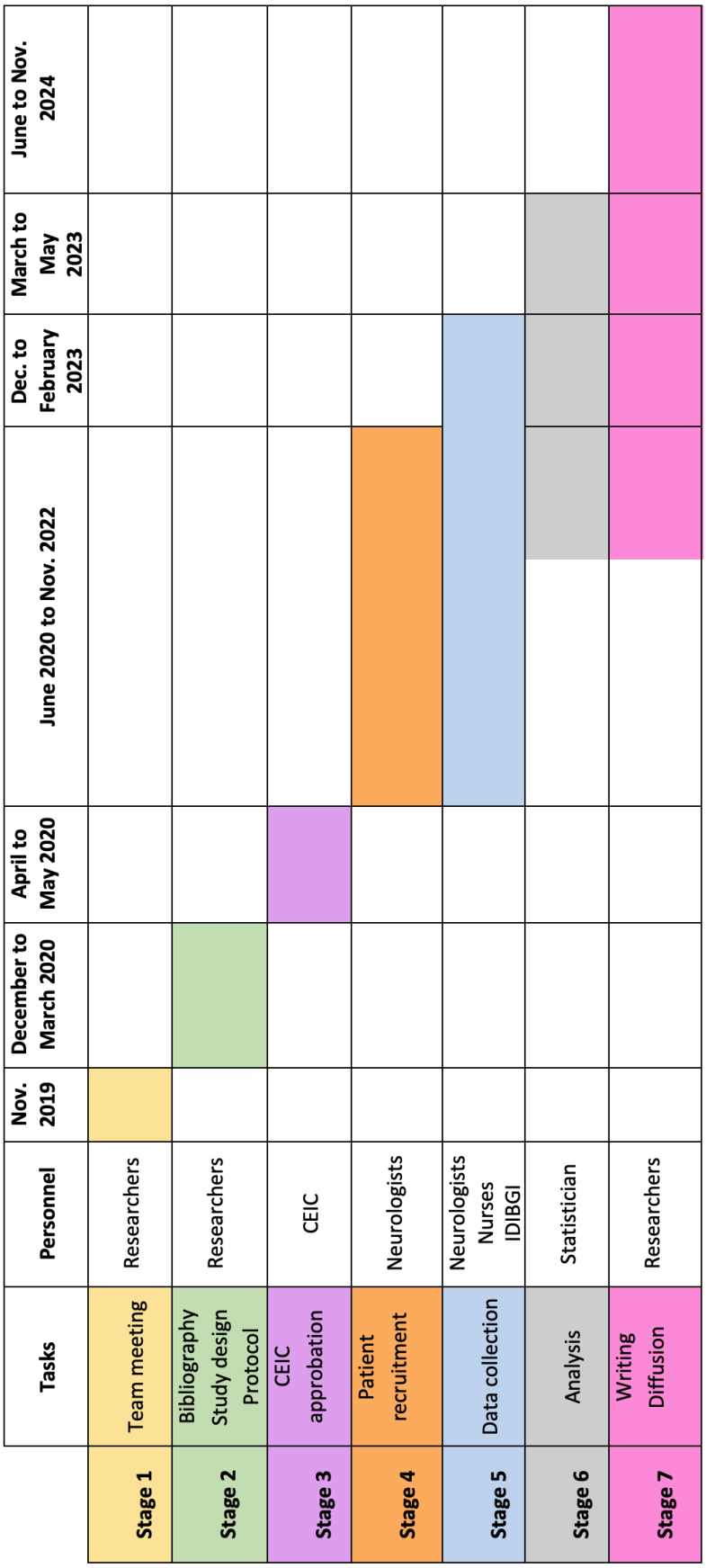
- **Stage 6: analysis of the results and statistical analysis (3 months)**

The results will be analysed as explained in **5. Statistical analysis**. A first analysis will be performed in the second year of the study in order to have the preliminary results, and at the end of the process of patient recruitment and data collection to analysis the final results.

- **Stage 7: writing, publication and diffusion of the results (6 months)**

This final stage will start in the second year of the study with de preliminary results. An article including the results, discussion and conclusions of the study will be published and promulgated in medical journals and national and international congresses.

6.2. CHRONOGRAM



7. ETHICAL CONSIDERATIONS

This project will be presented, evaluated and approved by the Clinical Research Ethics Committee (CEIC) of HJT. Any objection in the protocol will be considered and modified.

The study will be conducted according to the requirements expressed in the *Declaration of Helsinki of Ethical Principles for Medical Research Involving Human Subjects* defined by the World Medical Association (WMA) and reviewed in 2013, in order to ensure the human rights and ethical principales.

The autonomy of the patient will be respected according to *Ley 41/2002, de 14 de noviembre, básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica*. Therefore, all participants will be appropriately informed and will be given an information sheet (**Annex 3**) about the study before being included. Subjects will have to voluntarily sign the informed consent (**Annex 4**).

To guarantee and protect the confidentiality of all participants, personal and medical data needed for the study will be performed in accordance to *Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales*. Personal and clinical information of participants will be confidential and only used for the purpose of the research. Moreover, all data will be analysed anonymously.

Analysis of biological samples required in this study will respect biomedical regulation according to *Ley 14/2007, de 3 de julio, de Investigación Biomédica* for invasive procedures.

Participants will have the right to access, modify, oppose or remove their personal data, as well as to refuse to participate once the study has started.

8. LIMITATIONS AND STRENGTHS

Limitations

Related to the sampling

- Since the study will use a consecutive sampling, a great emphasis must be put within Emergency and Neurology Department to avoid forgetting to enter any patient in the study. Moreover, it is a non-probabilistic sampling. Thus, it may affect the external validity of the study.

Related to the design

- The prevalence of low levels of AAT within all the bibliography searched, only concerns the genetic AAT deficiency (S allele: 2-3%; Z allele: 1-3%). However, like other acute phase reactants, the AAT activity of a person without a genetic deficiency might be also lower than another patient, and this prevalence remains unknown. If the ratio between normal and low levels of AAT is not 1:1, the time of recruitment of the patients with low levels could be higher than expected, which would lengthen the duration of the study. Nevertheless, as AAT levels at admission will be analysed every 2 months in IDIGBGI to classify the patients, we will progressively know the prevalence of AAT deficiency.

Related to data collection

- AAT analysis with ELISA is a research-dependent technique. Even if analysis will be performed by the same researcher, it exists the risk of information bias. In order to avoid it, the researcher will accurate the technique to always perform it equal.

Other limitations

- AAT is an acute phase protein. Thus, its concentration can increase due to an infectious or inflammatory process. In order to control it, patients with a current infection or inflammation will be excluded of the study, and leukocyte analysis will be included among the variables of the study.

- As the first blood sample will be extracted at admission, we will not know previous basal AAT levels before ischaemic stroke. In order to solve it, AAT levels will be also analysed at three months, and we will assume that these are the baseline levels of the patient. However, as explained in **Justification**, the aim of the study is to assess the role of AAT as a poor outcome biomarker, not as a risk factor of ischaemic stroke.

- AAT levels measured in the systemic circulation might not reflect its levels and production in the brain.

- Other aetiologies of brain dysfunction may produce similar BBB dysfunction and release of AAT. Thus, AAT might not be a specific glycoprotein for cerebral ischaemia. However, the etiological study of stroke patients would rule out coexisting pathologies that could interfere with the study and if detected, would constitute a criterion of patient exclusion

Strengths

Related to data collection

- As the functional outcome of the patient will be assessed in the routine revision with the Neurologist 3 months after stroke, we do not expect losses between this period of time.

- Venous blood samples for the analysis of AAT during the acute phase of the stroke will be extracted from the peripheral venous access that is placed routinely in every patient while its hospitalization in the SU. Thus, they will not suppose a bothering for the patient.

- The variables that need to be assessed in CT scan (infarct volume and haemorrhagic transformation) will be measured in the CT scan which is performed routinely 24 hours after the administration of rt-PA. Thus, the study will not require extra CT scans, avoiding extra irradiation of the patients that will participate in the study.

9. BUDGET

Each patient will receive 1 information sheet (3 sides) and 1 informed consent (1 side). We will order 20 extra copies of both documents.

ITEM	COST PER UNIT	NUMBER OF UNITS	SUBTOTAL COSTS
Document printing	0.05 €/side	4 sides	x 392 patients +20
			83 €

We estimate that the study will require the hire of an statistician mainly during the period from the end of the second year to the conclusion of the study (12 months).

ITEM	COST PER UNIT	NUMBER OF UNITS	SUBTOTAL COSTS
Data management and statistical analysis	50 €/hour	60 hours	3,000 €

Blood extractions will be carried from HJT to IDIBGI, where they will be properly stored in Biobank IDIBGI until its analysis.

ITEM	COST PER UNIT	NUMBER OF UNITS	SUBTOTAL COSTS
Biobank IDIBGI	2,000 €	1	2,000 €

Serum AAT levels will be analysed using ELISA assay RayBio® Human Serpin A1 ELISA Kit (ELH-SERPINA1) from RayBiotech. Considering that the sample size includes 392 patients, and AAT will be analysed in 6 blood samples of each patient (at admission, 2 hours, 6 hours, 24 hours, 72 hours and 3 months), AAT will be analysed in a total of 2.352 blood samples during the study. Since each ELISA kit allows the analysis of 40 samples, we will need 59 kits.

ITEM	COST PER UNIT	NUMBER OF UNITS	SUBTOTAL COSTS
Serum AAT analysis (ELISA)	3,038 € 1,367 €	11 kits 5x96 well 2 kits 2x96 well	33,418 € 2,734 €
			36,152 €

The study will also require a nurse to extract and prepare blood samples at the established times, and to help with the introduction of database, phone calls and clinical controls. Considering that the study will require a total of 2,352 samples and that we estimate a dedication of approximately 30 minutes/sample, it means 1,176 hours of work.

ITEM	COST PER UNIT	NUMBER OF UNITS	SUBTOTAL COSTS
Personnel (nurse)	15 €/hour	1	17, 640 €

Once the article is written in English, the writing will be accurately revised by an expert of the language. Considering a maximum extension of 10 written pages, and an approximately number of 500 words/page, we estimate the following costs:

ITEM	COST PER UNIT	NUMBER OF UNITS	SUBTOTAL COSTS
Language revision	0.05 €/word	1	250 €

The written articles will be published in Open Access journals of high impact in cerebrovascular pathology area.

ITEM	COST PER UNIT	NUMBER OF UNITS	SUBTOTAL COSTS
Article publication	2,000 €	1	2,000 €

The diffusion of the results will be carried out by two members of the research team in a national stroke congress organized by *Sociedad Española de Neurología (SEN)* and in an international congress of *European Stroke Organisation Conference (ESOC)*. Travel expenses will include the inscription to the congress, transport, accommodation and diet allowances for each member.

ITEM	COST PER UNIT	NUMBER OF UNITS	SUBTOTAL COSTS
Conferences	2,000 €	2 members x2 congresses	8,000 €

BUDGET OVERVIEW

ITEM	**COST PER UNIT**	**NUMBER OF UNITS**	**SUBTOTAL COSTS**
MATERIAL AND SERVICES			
Document printing	0.05 €/side	4 sides	x 392 patients +20
			83 €
Data management and statistical analysis	50 €/hour	60 hours	3,000 €
Personnel (nurse)	15 €/hour	1	17, 640 €
			20,640 €
Biobank IDIBGI	2,000 €	1	2.000 €
Serum AAT analysis (ELISA)	3,038 € 1,367 €	11 kits 5x96 well 2 kits 2x96 well	33,418 € 2,734 €
			38,152 €
Language revision	0.05 €/word	1	250 €
Article publication	2,000	1	2,000 €
			2,250 €
TRAVEL			
Conferences	2,000	4	**8,000 €**
		TOTAL COST	**69,125 €**

10. IMPACT ON THE NATIONAL HEALTH SYSTEM

Ischaemic stroke is one of the most important causes of disability and mortality worldwide and it turns into enormous direct and indirect costs to healthcare services. Fast and accurate diagnosis of stroke is crucial for the immediate application of the right treatment to patients. rt-PA is a widely used thrombolytic therapy for the treatment of acute ischaemic stroke. However, despite its beneficial effect on the recanalization of the occluded artery, it may lead to the appearance of haemorrhagic transformation of the stroke, worsening the recovery and the outcome of the patient.

Thus, when regarding the prediction of the prognosis of an acute ischaemic stroke, it would be very useful to have biomarkers, like AAT, that give us objective and reliable information about whether the patient will improve or get worse in a few hours, will die in short or long term or will be in a functional situation of autonomy or disability a few months after the stroke. Furthermore, the prediction of potentially preventable or modifiable complications, such as haemorrhagic transformation, that may alter the final outcome of the patient, would have even a biggest impact in clinical practice, due to the possibility to establish direct actions towards its prevention or early treatment, including decision making regarding whether or not to administer thrombolytic therapy with alteplase e.v. in patients identified as high risk of complications.

This is the reason why identifying blood biomarkers of stroke is an active area of research since their potential use is not limited to diagnosis, but could be applied to prognosis and monitoring of the effectiveness of the treatment with rt-PA and/or the diagnosis of possible complications, such as haemorrhagic transformation after the thrombolytic therapy.

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12. ANNEXES

ANNEX 1 – Modified Rankin Scale (mRS)

0	**NO SYMPTOMS.**
1	**NO SIGNIFICANT DISABILITY.** Able to carry out all usual activities and work, despite some symptoms. Questions: - Does the patient have difficulty in reading or writing, speaking or finding the right word, problems with stability or coordination, visual discomfort, numbness (face, arms, legs, hands, feet), loss of mobility (face, arms , legs, hands, feet), difficulty in swallowing saliva or other symptoms after suffering the stroke?
2	**SLIGHT DISABILITY**. Able to look after own affairs without assistance, but unable to carry out all previous activities. Presents limitations in its usual activities and previous work, but is independent for the basic activities of daily living (ABVD). Questions: - Has there been any change in the patient's ability for their usual activities, work or care, compared to their pre-stroke situation? - Has there been any change in the patient's ability to participate in social or leisure activities? - Does the patient have problems with his personal relationships with others or has he been socially isolated?
3	**MODERATE DISABILITY.** Needs help for some instrumental activities but not for the ABVD, walks without the help from another person, and needs a caregiver at least twice a week. Questions: - Do you need help preparing food, home care, money management, shopping or using public transportation?
4	**MODERATELY-SEVER DISABILITY**. Unable to attend to own bodily needs without assistance, and unable to walk unassisted. Need a caregiver at least once a day, but not continuously, and can stay at home alone for a few hours.

	Questions: - Do you need help for eating, using the bathroom, daily hygiene or walking? Could you be alone for a few hours a day?
5	**SEVER DISABILITY**. Requires constant nursing care and attention, bedridden, incontinent.
6	**DEAD.**
	The scale runs from 0-6, running from perfect health without symptoms to death.

ANNEX 2 – National Institute of Health Stroke Scale (NIHSS)

NIVEL DE CONCIENCIA	Alerta Estuporoso Coma	0 1 2	
PREGUNTAS LOC	Responde ambas correctamente Responde una correctamente Incorrecto	0 1 2	
ÓRDENES LOC	Realiza ambas correctamente Realiza una correctamente Incorrecto	0 1 2	
MIRADA	Normal Parálisis parcial de la mirada Desviación oculocefálica	0 1 2	
CAMPOS VISUALES	Sin déficit campimétrico Hemianopsia parcial Hemianopsia completa Hemianopsia bilateral	0 1 2 3	
PARÁLISIS FACIAL	Movimientos normales y simétricos Paresia ligera Parálisis parcial Parálisis completa	0 1 2 3	
BRAZO IZQUIERDO	No claudica (BM 5) Claudica (BM 4) Algún esfuerzo contra gravedad (BM 3) Sin esfuerzo contra gravedad (BM 1-2) Ningún movimiento (BM 0)	0 1 2 3 4	
BRAZO DERECHO	No claudica (BM 5) Claudica (BM 4) Algún esfuerzo contra gravedad (BM 3) Sin esfuerzo contra gravedad (BM 1-2) Ningún movimiento (BM 0)	0 1 2 3 4	
PIERNA IZQUIERDA	No claudica (BM 5) Claudica (BM 4) Algún esfuerzo contra gravedad (BM 3) Sin esfuerzo contra gravedad (BM 1-2) Ningún movimiento (BM 0)	0 1 2 3 4	
PIERNA DERECHA	No claudica (BM 5) Claudica (BM 4) Algún esfuerzo contra gravedad (BM 3) Sin esfuerzo contra gravedad (BM 1-2) Ningún movimiento (BM 0)	0 1 2 3 4	
ATAXIA DE MIEMBROS	Ausente Presente en una extremidad Presente en dos extremidades	0 1 2	
SENSIBILIDAD	Normal Hipoestesia ligera a moderada Hipoestesia severa o anestesia	0 1 2	
LENGUAJE	Normal, sin afasia Afasia ligera a moderada Afasia severa. Broca, Wernicke Afasia global o mutismo	0 1 2 3	
DISARTRIA	Articulación normal Ligera a moderada Severa o anartria	0 1 2	
EXTINCIÓN	Sin anormalidad Parcial (una modalidad afecta) Completa (más de una modalidad)	0 1 2	
TOTAL			

ANNEX 3 – Information sheet

FULL D'INFORMACIÓ AL PARTICIPANT DE L'ESTUDI

DADES DE L'ESTUDI

Títol de l'estudi: THE ROLE OF ALPHA-1 ANTITRYPSIN IN THE PROGNOSIS OF ACUTE ISCHAEMIC STROKE. (El paper de l'alfa-1 antitripsina en el pronòstic de l'ictus isquèmic agut).
Investigador principal: Dr. Joaquín Serena Leal, Unitat d'Ictus del Servei de Neurologia, Hospital Universitari Dr. Josep Trueta de Girona.
Centre: Hospital Universitari Dr. Josep Trueta de Girona i Institut d'Investigació Biomèdica de Girona (IDIBGI)

Ens dirigim a vostè per informar-lo sobre un projecte d'investigació en el qual se'l convida a participar. Aquest projecte ha estat aprovat pel Comitè Ètic d'Investigació Clínica del nostre centre. L'objectiu d'aquest full d'informació és que rebi la informació correcta i suficient perquè pugui decidir si vol o no participar en aquest projecte.

ANTECEDENTS

L'ictus isquèmic és la segona causa de mort i la primera causa de discapacitat a Europa amb un enorme impacte econòmic a causa de les deficiències residuals que deixa en la majoria dels pacients. Avui en dia, l'únic tractament farmacològic disponible per l'ictus isquèmic agut és l'activador recombinant tissular del plasminogen (rt-PA). No obstant, el seu efecte fibrinolític només aconsegueix la reperfusió del vas tapat en un 50% dels pacients. A més, només el 5-10% dels pacients poden rebre aquest tractament donades les seves nombroses contraindicacions.

Una de les possibles complicacions del tractament amb rt-PA és la transformació hemorràgica de l'ictus. Tot i que només es produeix en un 2-7% dels pacients, la taxa de mortalitat està entre el 50 i el 80%.

La identificació de biomarcadors sanguinis per l'ictus és una àrea activa de recerca, no només per al diagnòstic, sinó per al pronòstic a llarg termini del pacient. Tot i que s'han identificat molts biomarcadors, cap d'ells ha demostrat prou sensibilitat i especificitat per ser utilitzat en la pràctica clínica.

D'altra banda, l'alfa-1 antitripsina (AAT) és la principal antiproteasa de la sang. Té propietats antiinflamatòries, antiapoptòtiques i citoprotectores. La deficiència d'AAT podria estar associada a un augment del risc d'ictus isquèmic i de transformació hemorràgica i, per tant, a un mal pronòstic a llarg termini. Així, si els nivells baixos d'AAT en la fase aguda de l'ictus es correlacionen amb un mal resultat clínic als 3 mesos i una incidència més gran de transformació hemorràgica, l'AAT es podria utilitzar com a biomarcador en la fase aguda de l'ictus per predir el pronòstic dels pacients tractats amb rt-PA.

Per aquestes raons, aquest protocol espera estudiar si hi ha una associació entre nivells baixos d'AAT i un mal pronòstic dels pacients que han patit un ictus isquèmic i van ser tractats amb rt-PA.

PARTICIPACIÓ

Se'l convida a participar en un estudi observacional en el qual es pretén estudiar un possible marcador en mostres de sang que s'associï amb una millor o pitjor evolució després del tractament habitual de l'ictus. La participació a l'estudi implica els següents procediments:

1. L'obtenció de 5 mostres de sang durant el seu ingrés a Unitat d'Ictus, que es guardaran al Biobanc de l'IDIBGI per tal de determinar els nivells d'AAT, així com altres determinacions analítiques vinculades a l'estudi. Aquestes extraccions no suposaran en cap cas una molèstia addicional ja que es recolliran de la via venosa que de rutina ja portarà a la Unitat d'Ictus.
2. La valoració del seu estat funcional als 3 mesos durant la visita rutinària amb el Neuròleg.
3. L'obtenció d'una última mostra de sang als 3 mesos per comparar els nivells d'AAT amb els de la fase aguda de l'ictus.

US DE LES SEVES DADES

Les seva informació personal i les mostres biològiques recollides com a part del projecte seran utilitzades únicament per a la realització del mateix. La seva privacitat estarà protegida i regulada per la "Ley Orgánica 3/2018, de 5 de diciembre, de Protección de Datos Personales y garantía de los derechos digitales". L'emmagatzematge de totes les mostres es realitzarà a les instal·lacions del Biobanc IDIBGI. El seu tractament i ús es realitzarà seguint el que especifica la "Llei de Recerca Biomèdica (14/2007)", i el "Reial Decret 1716/2011".

INFORMACIÓ DELS RESULTATS D'INVESTIGACIÓ

En el cas que aquestes investigacions proporcionin dades que puguin ser clínica o genèticament rellevants per a vostè i interessar la seva salut o la de la seva família, li seran comunicats si així ho estima oportú. Si vostè no desitja rebre aquesta informació, tingui en compte que la llei estableix que, quan la informació obtinguda sigui necessària per evitar un greu perjudici per a la salut dels seus familiars biològics, un comitè d'experts estudiarà el cas i haurà de decidir si és convenient informar als afectats o als seus representants legals.

Si per alguna raó vostè volgués conèixer els resultats de les investigacions que s'hagin produït com a conseqüència de la seva col·laboració, podrà posar-se en contacte amb els responsables del projecte, que l'informaran degudament.

PARTICIPACIÓ VOLUNTÀRIA

La seva participació en aquesta investigació és voluntària i l'alternativa és no participar. Si decideix no participar, aquesta decisió no afectarà la seva assistència mèdica. Així mateix, vostè pot retirar-se de l'estudi en qualsevol moment, sense donar explicacions.

BENEFICIS

Encara que aquest projecte no li prometi cap avantatge directe, vostè contribuirà a una major comprensió de la patologia estudiada. Els resultats de la investigació amb la seva mostra biològica poden ajudar als nous casos d'ictus isquèmic que s'esdevindran, i a la població en general.

PRIVACITAT I CONFIDENCIALITAT

Per garantir i protegir la confidencialitat de tots els participants, les dades personals i mèdiques necessàries per a l'estudi es realitzaran d'acord amb la "Ley Orgánica 3/2018, de 5 de desembre, de Protecció de dades personals i garantia dels drets digitals".

Per protegir la seva privacitat, les seves mostres de sang i dades personals seran identificades mitjançant un codi alfanumèric. Només els investigadors responsables del projecte podran connectar-los amb el seu nom. En cap moment el seu nom, adreça, o qualsevol altra informació que l'identifiqui serà utilitzada per als propòsits de la investigació.

L'accés a la seva informació personal quedarà restringit als investigadors del projecte, les autoritats sanitàries i al Comitè Ètic d'Investigació Clínica, quan ho necessitin per comprovar les dades i procediments de l'estudi, però sempre mantenint la confidencialitat dels mateixos d'acord amb la legislació vigent.

En cas de qualsevol dubte podrà contactar amb l'investigador principal, Dr. Joaquín Serena Leal, mitjançant el telèfon 619 677 404 (08:00 – 15:00 hores, de dilluns a divendres) o mitjançant el correu electrònic jserena.girona.ics@gencat.cat.

ANNEX 4 – Informed consent

FORMULARI DE CONSENTIMENT INFORMAT AL PARTICIPANT DE L'ESTUDI

Títol de l'estudi: THE ROLE OF ALPHA-1 ANTITRYPSIN IN THE PROGNOSIS OF ACUTE ISCHAEMIC STROKE (El paper de l'alfa-1 antitripsina en el pronòstic de l'ictus isquèmic agut).

Jo, _____, amb DNI _____:

- He llegit el full d'informació sobre l'estudi que m'han lliurat.
- He parlat amb l'investigador principal, o algun dels seus col·laboradors en l'estudi, el Dr._____
- He rebut suficient informació sobre l'estudi.
- He pogut fer preguntes sobre l'estudi i s'han respost satisfactòriament.
- Comprenc que la meva participació és voluntària.
- Comprenc que puc retirar-me de l'estudi en qualsevol moment, sense donar explicacions i sense que aquest fet comporti repercussions en la meva assistència mèdica.

Per tant, dono el meu consentiment a participar en aquest estudi així com al tractament de les meves dades personals i de salut que l'estudi requereixi.

Firma del participant

Firma del metge/investigador:

Data: __ /__ /__

Data: __ /__ /__

Hospital Universitari Dr. Josep Trueta, Girona.

**Tant el Full d'informació al pacient com el Consentiment informat estaran disponibles en una versió catalana i castellana, adaptant-se així a les preferències del pacient.

ANNEX 5 – Practices at the research laboratory

During the development of this final degree project, I have had the opportunity to carry out the practices both in Stroke Unit of the Neurology Department of Hospital Dr. Josep Trueta, and in the Cerebrovascular Pathology Research group of *Institut d'Investigació Biomèdica de Girona* (IDIBGI).

For the duration of my stay in IDIBGI, I have participated in the testing of the optimal AAT dose for an *in vitro* BBB model. The cell line used is bEnd.3, which is an immortalized mouse brain endothelial cell line (Figure 12). These cells form monolayers in culture and have key characteristics of the BBB, such as the expression of tight junction proteins. Studies have used bEnd.3 cells exposed to OGD conditions to study BBB disruption after ischaemia (19).

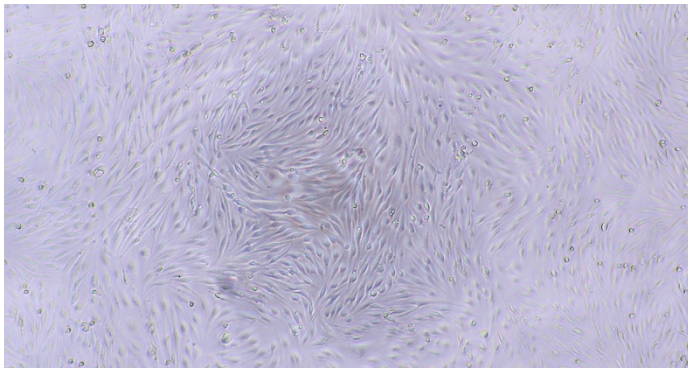

Figure 12. bEnd.3 endothelial cell line.

My participation with the cerebrovascular pathology group has allowed me the acquirement of some laboratory abilities and the realization of the following procedures:

- Cell culture in T75 culture flasks
- Cell maintenance every 3-4 days.
- Cell passage and seeding into 96-well culture plates.
- OGD performance and cell reoxygenation.
- Cytotoxicity evaluation through analysis of lactate dehydrogenase (LDH).
- Cell viability through analysis of metabolic activity with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.
- Observation of cells with an inverted microscope and familiarization with its morphology.

The study of the optimal AAT dose could be used in future studies with the *in vitro* BBB models to assess the possible protector role of AAT on the BBB.