Universitat Autònoma de Barcelona

DECIPHERING ISCHEMIC STROKE: MULTI-OMIC TECHNIQUES TO DISCOVER NEW BIOMARKERS AND THERAPEUTIC TARGETS

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ABSTRACT

Ischemic stroke is a leading cause of death and disability worldwide. A better comprehension of stroke pathophysiology is fundamental to improve its dramatic outcome. Beyond the approved thrombolytic therapies, there is no effective treatment to mitigate its progression. Besides, these reperfusion therapies have a short therapeutic window and severe side effects, reducing the number of patients that can benefit from them. Thus, there is an urgent need to find new complementary therapeutic strategies to attenuate or even reverse stroke progression. Moreover, identifying the patients at higher risk of presenting poor outcome (disability or death) after stroke is essential to facilitate decision-making processes, ultimately optimizing patients' management. In this regard, the identification of blood biomarkers that aid in the prediction of stroke prognosis is highly needed.

This Doctoral Thesis aims to contribute to the better understanding of stroke pathophysiology identifying new key molecules that can serve as therapeutic targets and/or as biomarkers of stroke prognosis.

First, we have described proteomic and transcriptomic changes in the human brain triggered after cerebral ischemia using –omic techniques and integrating this information through biostatistical tools to highlight key molecular factors in the stroke pathophysiology. In this regard, some of the proposed candidates show potential as stroke biomarkers and/or therapeutic targets.

Moreover, the influence of sex, age and comorbidities in stroke pathophysiology has been explored through integrative biostatistical tools by exploring similarities and differences in the brain proteome and transcriptome in a mice model of cerebral ischemia in (a) young males, (b) young females, (c) diabetic young males and (d) aged males. Interestingly, we found that the response to cerebral ischemia differed among the studied groups, highlighting the importance of

incorporating animals with different phenotypes in future stroke research pre-clinical studies.

Furthermore, three studies have been conducted to explore the role of selected candidates as blood biomarkers to predict stroke outcome. Interestingly, we found that Aquaporin-4 circulating level measured acutely after ischemic stroke predicts neurological improvement at 48 hours and at hospital discharge. In the same line, Alpha-1 antitrypsin (A1AT) circulating level measured in the hyperacute phase after stroke predicts poor functional outcome in-hospital and at mid-term. Of note, A1AT was a molecule found to be dysregulated in the brain after cerebral ischemia in the first study of this Doctoral Thesis. Moreover, we have identified that Endostatin, tumor necrosis factor receptor-1 and interleukin-6 are independent predictors of long-term mortality after stroke in a cohort of nearly 1000 patients. In all cases, the studied biomarkers added predictive value on top of clinical variables.

Finally, since all neuroprotective agents proposed to date have systematically failed to succeed in clinics, in this Thesis we have identified various two-by-two combinations of drugs with theoretical synergistic neuroprotective effects. To do so, we have used a systems biology-based approach to develop a mathematical model that simulates *in silico* ischemic stroke pathology. Remarkably, the efficacy of the drug combination formed by Ceruletide + A1AT has been demonstrated in a mouse model of transient cerebral ischemia and its potential interactions with recombinant tissue-plasminogen activator (rt-PA) have been discarded.

Overall, the results of this Doctoral Thesis contribute to advancing our knowledge of stroke pathophysiology, disclosing key molecules with plausible roles as therapeutic targets, prognostic biomarkers or both. In fact, we have described how A1AT, a serine proteinase inhibitor found to be upregulated in ischemic stroke, could be used both as a biomarker of in-hospital and mid-term poor functional outcome and as a therapeutic target when combined with Ceruletide.

RESUM

L'ictus isquèmic és una causa de mort i discapacitat majoritària al món. Una millor comprensió de la seva fisiopatologia és fonamental per millorar aquestes dades devastadores. Més enllà de les teràpies trombolítiques aprovades no hi ha cap tractament efectiu per mitigar-ne la progressió. A més, aquestes teràpies tenen una finestra terapèutica curta i efectes secundaris greus, reduint el nombre de pacients que se'n poden beneficiar. Per tant, hi ha una necessitat de trobar noves estratègies terapèutiques complementàries per atenuar la progressió de l'ictus. A més, identificar els pacients amb més risc de presentar mal pronòstic (discapacitat o mort) després d'un ictus és essencial per facilitar els processos de presa de decisions i optimitzar la gestió dels pacients. En aquest sentit, és necessària la identificació de biomarcadors sanguinis que ajudin a predir el pronòstic de l'ictus.

Aquesta Tesi Doctoral pretén contribuir a una millor comprensió de la fisiopatologia de l'ictus identificant noves molècules clau que poden servir com a dianes terapèutiques i/o com a biomarcadors pronòstic de l'ictus.

En primer lloc, hem descrit els canvis del proteoma i transcriptoma que es produeixen en el cervell humà després de la isquèmia cerebral mitjançant tècniques –òmiques. A més, hem integrat aquesta informació mitjançant eines bioestadístiques per destacar molècules clau en la fisiopatologia de l'ictus. Alguns dels candidats proposats mostren potencial com a biomarcadors i/o dianes terapèutiques per l'ictus isquèmic.

A més, s'ha explorat la influència del sexe, l'edat i les comorbiditats en la fisiopatologia de l'ictus mitjançant eines bioestadístiques d'integració explorant similituds i diferències en el proteoma i el transcriptoma cerebral en un model d'isquèmia cerebral en ratolins (a) mascles joves, (b) femelles joves , (c) mascles joves diabètics i (d) mascles vells. Curiosament, hem trobat que la resposta a la isquèmia cerebral és diferent entre els grups estudiats, posant de manifest la

importància d'incorporar animals amb fenotips diferents en futurs estudis pre-clínics.

A més, s'han realitzat tres estudis per explorar el paper de candidats seleccionats com a biomarcadors pronòstics de l'ictus. Curiosament, hem trobat que el nivell circulant d'Aquaporin-4 mesurat de manera aguda després d'un ictus isquèmic prediu la millora neurològica a les 48 hores i a l'alta hospitalària. En la mateixa línia, el nivell circulant d'alfa-1 antitripsina (A1AT) mesurat en la fase hiperaguda de l'ictus prediu un mal pronòstic funcional a l'hospital i a mig termini. Cal destacar que l'A1AT es va trobar sobre-expressada al cervell després de la isquèmia cerebral en el primer estudi d'aquesta Tesi. A més, hem identificat que l'endostatina, el receptor del factor de necrosi tumoral-1 i la interleucina-6 són predictors independents de la mortalitat a llarg termini després d'un ictus en una cohort de gairebé 1000 pacients. En tots els casos, els biomarcadors afegien valor predictiu a les variables clíniques.

Finalment, com que tots els agents neuroprotectors proposats fins ara han fracassat a les clínica, en aquesta Tesi hem identificat diverses combinacions de dos fàrmacs amb efectes neuroprotectors sinèrgics. Per fer-ho, hem utilitzat un enfocament basat en la biologia de sistemes per desenvolupar un model matemàtic que simula la patologia de l'ictus isquèmic *in silico*. Notablement, l'eficàcia de la combinació de fàrmacs formada per Ceruletide+A1AT s'ha demostrat en un model d'isquèmia cerebral en ratolí i s'han descartat les possibles interaccions amb l'activador tissular del plasminogen recombinant (rt-PA).

En conjunt, els resultats d'aquesta Tesi Doctoral contribueixen a aprofundir en el coneixement de la fisiopatologia de l'ictus, revelant molècules clau amb possibles funcions com a dianes terapèutiques, biomarcadors pronòstics o ambdues. De fet, hem descrit com l'A1AT es podria utilitzar tant com a biomarcador de mal pronòstic com a diana terapèutica quan es combina amb Ceruletide.

ABBREVIATIONS

A1AG1	Alpha-1-acid glycoprotein 1
A1AT	Alpha-1 antitrypsin
AMPA	A-amino3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANXA2	Annexin-2
ApoC-III	Apolipoprotein CIII
AQP4	Aquaporin-4
ATP	Adenosine triphosphate
AUC	Area under the curve
BBB	Blood-brain barrier
BI	Barthel Index
BNP	Brain natriuretic peptide
Ca^{2+}	Calcium
Cl^{-}	Chloride
CNS	Canadian Neurological Scale
CRP	C-reactive protein
CT	Computed Tomography
DAMPs	Damage-associated molecular patterns
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
DNM1L	Dynamin-1-like protein
DRP2	Dihydropyrimidinase-related protein 2
ELISA	Enzyme-linked immunosorbent assay
ESS	European Stroke Scale
FasL	Fas Ligand
FDA	Food and Drug Administration
GFAP	Glial fibrillary acidic protein
GOS	Glasgow Outcome Scale

GROA	Growth-related oncogene-α
Hsc70	Heat shock 70 kDa protein-8
IC	Infarcted core
ICAM-1	Intercellular adhesion molecule 1
ICH	Intracerebral hemorrhage
IDI	Integrative discriminative improvement
IGFBP-3	Insulin-like growth factor-binding protein-3
IL-6	Interleukin-6
K^+	Posatassium
LC-MS	Liquid chromatography coupled with mass spectrometry
Lp-PLA1	Lipoprotein-associated phospholipase A2
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MMPs	Metalloproteinases
MRI	Magnetic Resonance Imaging
mRS	Modified Rankin Scale
MS	Mass spectrometry
Na^+	Sodium
NCAM	Neuron cell adhesion molecule
NFAT	Nuclear factor of activated T cells
NIHSS	National Institutes of Health Stroke Scale
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NT-proBNP	N-terminal pro-B-type natriuretic peptide
OGD	Oxygen and glucose deprivation
PI	Peri-infarcted area
POC	Point-of-care
RNA	Ribonucleic acid

ROS	Reactive oxygen species
rt-PA	Recombinant tissue-plasminogen activator
RT-qPCR	Reverse transcriptase quantitative PCR
S100B	S100 calcium-binding protein
SAH	Subarachnoid hemorrhage
SSS	Scandinavian Stroke Scale
STAIR	Stroke Therapy Academic Industry Roundtable
TLS	Traffic Light System
TNF-R1	Tumor necrosis factor receptor-1
TOAST	Trial of Org 10172 in Acute Stroke Treatment
TTC	Triphenyltetrazolium chloride
VAP-1	Vascular adhesion protein-1
vWF	Von Willebrand factor
WB	Western blotting

INTRODUCTION

1.1. Stroke

Stroke is a neurologic disease caused by the disruption of blood flow into a brain region, causing oxygen and nutrients deprivation and ultimately leading to cell death and severe brain damage. Stroke can be caused either by the obstruction (ischemic stroke) or the rupture (hemorrhagic stroke) of a cerebral vessel. Blood flow interruption can be transitory, if it is restored spontaneously or through therapeutic strategies, or permanent [1]. Stroke symptoms vary depending on the cerebral region affected and stroke subtype, and normally comprise alterations in movement (hemiparesis or ataxia), in the language (aphasia or dysarthria), or in vision (diplopia or hemianopia) [2].

According to the World Health Organization (www.who.org) annually 15 million people worldwide suffer a stroke. Of these, 5 million are permanently disabled and another 5 million die, being stroke a major socioeconomic concern. Stroke is considered a multifactorial disease with various risk factors associated, which can be divided into modifiable and non-modifiable. Age, arterial hypertension, heart diseases, diabetes mellitus, dyslipidemia, smoking, alcoholism, obesity and physical inactivity are among the most common stroke risk factors [3]. Besides, genetic factors are also known to be non-modifiable risk factors, and they contribute to stroke risk through several potential mechanisms. For example, monogenic diseases in which stroke is a primary manifestation (Fabry disease or CADASIL (Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy)) or common variants of genetic polymorphisms have been associated with stroke risk [4].

1.1.2. Stroke subtypes

Depending on the cause of blood flow interruption stroke can be classified as ischemic stroke, which represents 87% of all strokes, and hemorrhagic stroke, which accounts for the remaining 13% of the cases (Figure 1) [5].

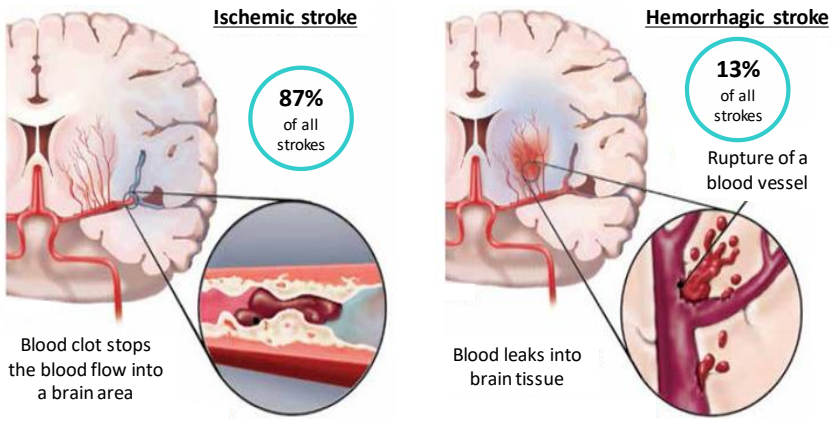


Figure 1. *Stroke subtypes (adapted from ®Heart and Stroke Foundation of Canada).*

Both types of stroke can be subclassified according to the brain region affected, the extension, the etiology or the duration of the event. Hemorrhagic strokes include intracerebral hemorrhage (ICH) if the bleeding occurs within the brain, and subarachnoid hemorrhage (SAH) if the bleeding occurs between the layers of tissue covering the brain within the subarachnoid space. ICH accounts for 10% of all strokes while SAH represents 3%. Regarding the location of ICH, the vast majority occurs in deep locations (36-69%), followed by lobar (15-32%), cerebellar (7-11%) and less frequently in the brain stem (4-9%). Although hemorrhagic strokes are less prevalent than ischemic strokes, they are more likely to be fatal. However, despite its severity, nowadays there is not an effective treatment available to treat hemorrhagic stroke patients [6].

Ischemic stroke can be further categorized depending on its etiology (the cause of the vascular obstruction) in 5 different subtypes (TOAST criteria, trial of Org 10172 in Acute Stroke Treatment). The most frequent subtypes of ischemic stroke are cardioembolic (20-30%), in which the obstruction is caused by an embolus of

cardiac source, and atherothrombotic (30-45%), caused by an atheromatous plaque that obstructs a brain vessel or by the rupture of a plaque that releases thrombotic material into the brain. Less frequent are small-vessel occlusions or lacunar strokes (10-23%), resulting from the occlusion of small arteries of the brain, and stroke of other determined etiology (2-11%), produced by rare causes such as nonatherosclerotic vasculopathies, hypercoagulable states, or hematologic disorders. Finally, there are some ischemic strokes in which after an exhaustive diagnosis study the exact cause of stroke cannot be determined and are classified as strokes of undetermined etiology (12-27%). This last kind of strokes can difficult patients' management by precluding the best secondary prevention strategy [7,8].

Given that the present Doctoral Thesis is focused on ischemic stroke, from now on stroke and ischemic stroke terms will be used indistinctly.

1.1.3. Ischemic stroke diagnosis and treatment

Stroke diagnosis is based on clinical parameters (medical history and neurological exploration), vascular exploration by Doppler echocardiography, cardiac exploration (electrocardiogram and transthoracic ultrasound) and neuroimaging (Computed Tomography (CT) or Magnetic Resonance Imaging (MRI)) [9,10]. In fact, neuroimaging is essential not only to allow a detailed evaluation of patients' neurologic state but also to differentiate ischemic stroke from stroke mimics, which are diseases with similar clinical presentations to stroke. This group of stroke mimics is very heterogeneous and includes, among others, seizures, brain tumors, sepsis, migraines and headaches. In fact, the overall frequency of mimics in the emergency rooms ranges between 15 and 25% of all suspected stroke presentations, being essential its discrimination from ischemic stroke [11,12].

Nowadays, the only approved pharmacologic therapy to treat acute ischemic stroke is thrombolytic therapy through the administration of intravenous

recombinant tissue-plasminogen activator (rt-PA). However, this treatment carries a significant risk of secondary bleeding in ischemic stroke and it is contraindicated in hemorrhagic strokes [13]. Moreover, rt-PA has a reduced time window of action, being only effective when administrated within 4.5 hours after symptoms onset [14]. Besides, this treatment cannot be administered in patients receiving anticoagulant therapies. All in all, due to its short therapeutic window, and because of the strict clinical eligibility criteria, the number of patients that can be treated with rt-PA is reduced to approximately 15% [15].

In addition, there are also non-pharmacologic approaches that have proven their efficacy in restoring vascular flow such as endovascular mechanical thrombectomy. In fact, this approach has brought a revolution in stroke patients' management, given the encouraging results obtained in comparison to rt-PA [16]. Interestingly, mechanical thrombectomy has enabled extending the therapeutic window to six hours and allowing to treat some selected patients up to 24h after symptoms onset [17,18]. What is more remarkable is that when endovascular mechanical thrombectomy is coupled with rt-PA, recanalization rates rise to more than 60% and patients' functional outcome improves substantially [19,20].

1.2. Ischemic stroke pathophysiology

The occlusion of a cerebral artery and the consequent blood flow interruption lead to a lack of oxygen and glucose supply into the brain. The absence of these vital fuels induces a series of biochemical and metabolic alterations, known as ischemic cascade, that finally provoke massive cell death [21]. After vessel occlusion, two major zones of injury can be differentiated: the infarcted core (IC) and the penumbra or peri-infarcted area (PI). The IC is the region with the most severe blood flow deficits (blood flow below 25%) resulting in rapid cell death progression [13]. Penumbra is the tissue surrounding the IC that suffers moderate to mild ischemia due to residual perfusion from collateral blood vessels. At the

early stages, PI region can represent from a third to half of the lesion volume. In this area, the tissue is functionally impaired but structurally intact and cells can remain viable for several hours. However, if blood supply is not re-established within a few hours, penumbra cells start dying and consequently, the structural lesion increases. For that reason, the PI area is the target for most pharmacologic interventions given that its rescue might reduce tissue death, resulting in neurological improvement and better outcomes [22].

1.2.1. Ischemic cascade

As a consequence of oxygen and glucose deficiency in the brain, the production of adenosine triphosphate (ATP) is impaired, ultimately leading to bioenergetics failure [23]. Therefore, energy-dependent ion transport pumps required to maintain ionic gradients are altered, causing neurons and glial cells depolarization. As a result, cell permeability is disturbed, causing an increase in intracellular sodium (Na^+) and calcium (Ca^{2+}) concentration as well as a massive extracellular release of potassium (K^+) [24].

Alterations in cell permeability trigger an immense release of neurotransmitters, mainly glutamate, into the extracellular space. The effect of an increase in synaptic glutamate concentration is the hyperactivation of N-methyl-D-aspartate (NMDA), a-amino3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic receptors, a process known as excitotoxicity. NMDA receptors are calcium-permeable, so the opening of these channels leads to further membrane depolarization and greater Ca^{2+} influx, exacerbating intracellular Ca^{2+} overload [24]. As a consequence of this ionic imbalance, Na^+ and chloride (Cl^-) influx and K^+ efflux is intensified, leading to a passive entrance of water into cells, resulting in cytotoxic edema [25]. In addition, Ca^{2+} influx also stimulates nitric oxide synthase (NOS) activity and increases the production of nitric oxide (NO), triggering the generation of reactive oxygen species (ROS). This ultimately leads to oxidative

stress, mitochondrial damage and disruption of the deoxyribonucleic acid (DNA) [26]. As a consequence of these biochemical and metabolic alterations, various signaling pathways are activated, resulting in an acute inflammatory response, which will be explained in detail in the next section.

In the end, brain cells that are compromised by excessive glutamate-receptor activation, Ca^{2+} overload, oxygen radicals or by mitochondrial and DNA damage can die by necrosis or apoptosis. Necrosis is the main mechanism that follows acute permanent vascular occlusion, so is the main mechanism occurring in the IC, whereas in the penumbra programmed cell death or apoptosis is the predominant one [25]. Via the release of cytochrome C from the outer mitochondrial membrane or through the activation of cell-surface death receptors, caspases and other pro-apoptotic molecules are activated. Finally, the activation of apoptotic molecules triggers DNA fragmentation, degradation of nuclear and cytoskeletal proteins, cross-linking of proteins, production of apoptotic bodies, expression of ligands for phagocytic cell receptors and ultimately uptake by phagocytic cells [27]. Figure 2 summarizes all these processes triggered by ischemia in the brain.

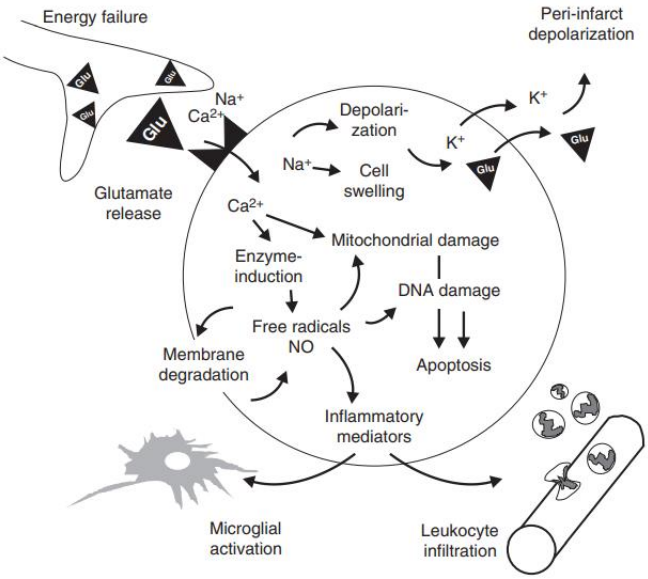


***Figure 2**. Pathophysiological mechanisms triggered after ischemic stroke: ischemic cascade (adapted from Dirnagl et al 1999)* [25,28].

1.2.2. Acute inflammatory response

After the ischemic event, the blockage of blood flow triggers an inflammatory response both locally, at a brain level, and also systemically. In the brain, the arterial occlusion leads to an ionic imbalance with the consequent activation of different signaling pathways that ultimately provoke the expression of pro-inflammatory genes, such as cytokines and chemokines, by injured brain cells [26]. As a consequence, the expression of cell adhesion molecules on the surface of endothelial cells is induced. Among others, E-selectin, P-selectin and intercellular adhesion molecule 1 (ICAM-1) are expressed, mediating the infiltration of leukocytes into the brain parenchyma, thus facilitating debris clearance in the infarcted region. In addition, endothelial cells express chemokines to direct the leukocytes to the diseased area [29]. Contrary to this beneficial role, pieces of evidence are pointing out that infiltrating immune cells also produce cytotoxic mediators that can extend the inflammatory response and increase brain damage, impairing the ischemic brain [21].

Additionally, damage-associated molecular patterns (DAMPs) released by dying brain cells are recognized by microglia, triggering its activation. Thus, both infiltrated and resident immune cells generate a burst of pro-inflammatory molecules in the infarcted region [30]. Besides, there is an increase in matrix metalloproteinases (MMPs) production, which mediate the destruction of the basal lamina, causing blood-brain barrier (BBB) leakiness, thus facilitating the entrance of more peripheral immune cells to the diseased area [31].

Accompanying the localized inflammatory response in the brain, an immune response at the systemic level is also triggered by stroke. After the ischemic event, there is a major release of pro-inflammatory mediators into the circulation, accentuated by the BBB disruption, that causes hyperactivation of peripheral immune cells. This overactivation leads to the exhaustion of mature leukocytes and the consequent recruitment of immature leukocytes, which are

not able to respond appropriately to brain damage [32]. The recruitment of this immature subpopulation ultimately causes lymphocytopenia, contributing to immunodepression and predisposing to post-stroke infections [33]. Moreover, the excessive concentration of pro-inflammatory mediators can activate both the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system with the consequent release of catecholamines and glucocorticoids. This results in the inhibition of pro-inflammatory pathways and the stimulation of anti-inflammatory mechanisms, via the release of growth factors and interleukins. This immunosuppression aids in the prevention of autoimmune reactions against antigens derived from the central nervous system that are present in the circulation due to BBB disruption [34].

At later stages, at a brain level, the immune system also works to abrogate the inflammatory response. The activated microglia and infiltrating macrophages phagocyte debris and dead cells. Moreover, they stimulate the production of anti-inflammatory molecules that suppress the immune response and inhibit the expression of adhesion molecules and the production of pro-inflammatory cytokines [35]. Furthermore, both microglial cells and macrophages release neuroprotective factors that promote neurogenesis and angiogenesis, playing a crucial role in the recovery of ischemic brain injury [36] (Figure 3).

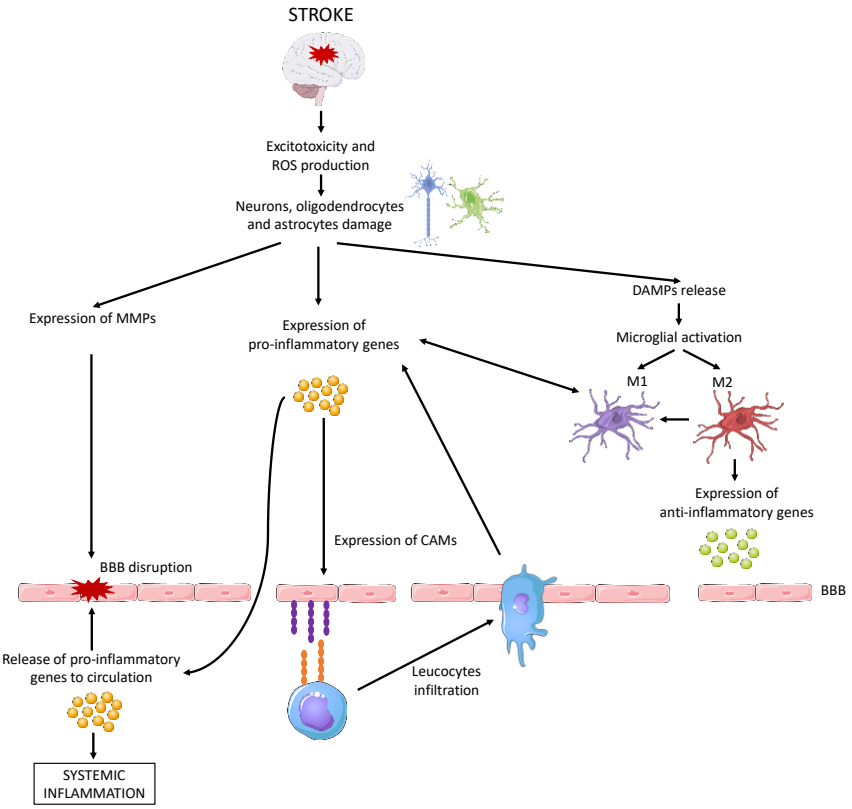


***Figure 3**. The acute inflammatory response following ischemic stroke. BBB, blood-brain barrier; CAM, Cellular adhesión molecule; DAMP, damage-associated molecular pattern; ROS, reactive oxygen species (adapted from Ramiro et al 2018)* [37].

1.3. Ischemic stroke prognosis

Stroke outcome can be evaluated at different levels including neurological, functional and vital prognosis. Various factors influence stroke prognosis. First of all, time of ischemia or time from symptoms onset is crucial and determines therapeutic strategies in the hyperacute phase, which ultimately will influence in patients' prognosis. In general, the longest the time of ischemia is, the worse will be the prognosis. In addition, other non-modifiable factors clearly influence stroke prognosis such as age and stroke severity [38]. More than 65% of all strokes occur in individuals older than 65 years [39], while only 10% of stroke

patients are under 50 years old [5,40]. Older patients are more likely to have a worse prognosis after stroke than younger ones [5]. In the same line, patients with more severe strokes are more likely to have worse outcomes. Moreover, the presence of comorbidities as well as stroke etiology can also determine stroke prognosis [38]. In addition, clinical complications that occur later during the course of the disease, including cerebral edema, hemorrhagic transformations, infections or secondary events, can also affect stroke morbidity [20,41–43].

In recent years, several attempts have been directed towards the generation of predictive models for stroke prognosis. These models have been evaluated and validated in terms of accuracy, with values around 80% in most of them. Many of these models include age and stroke severity as common predictor variables. However, none of them have been sufficiently well developed and validated to be useful in either clinical practice or research [44,45].

1.3.1. Neurological prognosis

Evaluating the neurological state of stroke patients is of special interest especially during the acute and sub-acute phase of the disease, given that it provides an objective measure of the cerebral damage caused and it allows monitoring the clinical evolution of the patient. The National Institutes of Health Stroke Scale (NIHSS) is the most extended scale to evaluate the neurological state of the patient. It is a 15-item scale with a total score ranging from 0 (without symptoms) to 42 (severe damage), that evaluates different aspects related to language, consciousness, motor strength, and sensory impulses, among others [46]. In addition, other scales allow evaluating the functional state of patients such as the Scandinavian Stroke Scale (SSS) [47], the Canadian Neurological Scale (CNS) [48] and the European Stroke Scale (ESS) [49], although they are not as extended in clinical practice as NIHSS.

Variations in the score of these scales over time allow assessing neurological evaluation of the patients. Regarding NIHSS, although there is not a fully established criterion, neurological improvement is usually considered as a 4-point or more decrease on NIHSS score, while neurological deterioration is defined as a 4-point or more increase on NIHSS score [50]. Those patients that have a variation lower than 4-points along time are considered to be stable. A neurological worsening within the first days after the ischemic event is defined as early neurological deterioration, and it occurs in approximately 15% of patients [51,52]. Early neurological deterioration has shown to be a good predictor of long-term poor outcome [52] and has been associated with stroke complications such as hemorrhagic transformation or cerebral edema [41,53].

1.3.2. Functional prognosis

Patients' functional state evaluation allows estimating the degree of disability or dependence to develop daily activities after an ischemic stroke. The functional state of patients can be evaluated through different scales such as the Barthel Index (BI) [54], the Glasgow Outcome Scale (GOS) [55] or the modified Rankin Scale (mRS) [56], being the last one the most popular and widely used. The mRS measures the global dependence of a patient to perform routine activities. Its total score ranges from 0 to 6, in which 0 points indicate the absence of dependency symptoms, 1 point define patients with a small disability despite presenting symptoms, 2 points for patients with minor disabilities, 3 to 5 points when disability is moderate to severe and 6 points correspond to patient's death. There is consensus in defining patients with mRS>2 as functionally disabled or dependent, while those patients with mRS≤2 are considered functionally independent [57]. This scale is commonly used to evaluate long-term patients' evolution normally at 3 or 6 months as well as years after the event. Moreover, this dichotomous categorization allows classifying patients as those having good outcome (functionally independent) and those having poor outcome

(functionally disabled) as an endpoint in many therapeutic and biomarkers clinical trials. Among stroke survivors, around 35-50% of patients present disability after the event [58,59].

1.3.3. Vital prognosis

Apart from being a major cause of disability, stroke also represents the fifth cause of death worldwide and is the first cause of death among women in our region. In fact, in 2016 around 10% of total deaths globally were due to stroke (5% due to ischemic stroke) which corresponded to more than 5.5 million deaths [60] (Figure 4). After ischemic stroke, the proportion of patients that die is influenced by different variables, such as stroke subtype or health resources of each country. According to literature, 40-60% of ischemic stroke patients would be dead 5 years after the event [61–63].

Although this devastating data, during the last decades, deaths due to stroke have significantly diminished. It is thought that this reduction can be attributed to a combination of improved interventions and programs based on scientific findings as well as reduced stroke risk factors, such as improved blood pressure control [64].

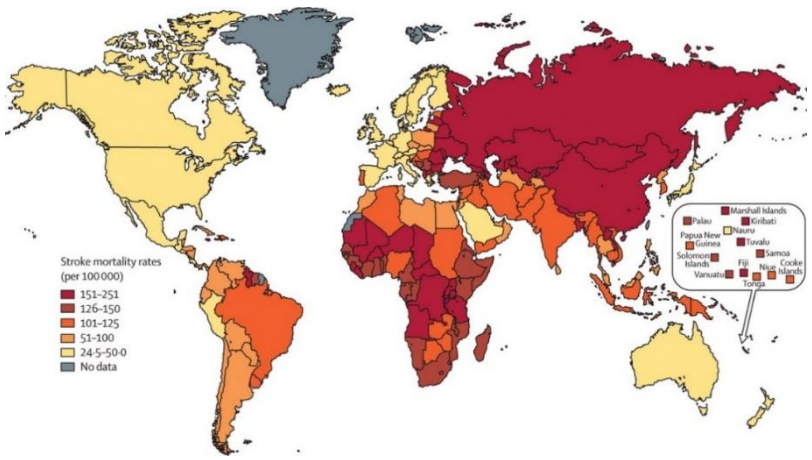


***Figure 4**. Age-adjusted and sex-adjusted stroke mortality rates. From Johnston et al. 2009* [65].

1.4. Animal models of cerebral ischemia

Due to the limited accessibility of the human brain, a large number of *in vitro* and in *vivo models* of cerebral ischemia have been described over the years. The *in vitro* models include cultured neurons with or without synaptic formation, glia and cultured brain slices [66]. However, *in vitro* models alone are not sufficient to evaluate stroke pathophysiology, given the importance of the brain vasculature to study the consequences of altered brain perfusion and given the involvement of other systems. For that reason, animal models of cerebral ischemia are essential to reproduce accurately specific aspects of human disease in order to represent the complex pathophysiology of stroke [67]. Besides, in these models variables such as size and location of the lesion can be taken under strict control, reducing variability and increasing reproducibility. Models of cerebral ischemia can be performed both in large and small animals. However, rodents are the most widely used due to economics, ethics and management issues [68].

Models of cerebral ischemia can be divided into models of focal and global ischemia. In global ischemia, the reduction of cerebral blood flow affects most of the brain or the entire brain, while focal ischemia is characterized by a reduction of cerebral blood flow in a specific brain region [69]. Models inducing focal ischemia are the ones representing more accurately human stroke pathology, and therefore, are the most widely used. Among them, middle cerebral artery occlusion (MCAO) is considered to be the closest to human ischemic stroke and has been used in more than 40% of neuroprotection experiments [70]. Focal cerebral ischemia can be induced via the mechanical occlusion of either the distal middle cerebral artery (MCA) or the proximal MCA. Also, by thrombotic occlusion either via injection of thrombin or blood clots into the MCA or by photo-thrombosis after intravenous injection of Rose Bengal. Distal occlusions only disturb cortical blood flow, while proximal occlusions result in brain infarcts that

involve both the striatum and cortex [69]. In addition, stroke models can also be classified in permanent or transient, if tissue reperfusion is allowed. The extension of brain damage mainly depends on the occlusion time, although other factors such as sex, strain and body temperature can also influence it [71].

Similarly to humans, the functional and neurological status of animals is a key parameter to be assessed after cerebral ischemia in order to determine the degree and extension of brain damage. Identifying behavioral deficits in animal models is essential for potential translational applications. Nowadays, there are several tests to determine the functional status of the animals and each one is sensitive to measure deficits associated with particular areas of the damaged brain. These tests can assess cognitive, motor and sensorimotor alterations. For example, the grip strength test or the hanging wire test mainly assess the strength of the animal, while the cylinder test discloses spatial and motor alterations. Composite scores such as Bederson Scale [72] and other neurological scoring scales evaluate postural, sensorial and motor deficits among others [73]. Besides revealing the status of the animals at a specific moment, these tests can also be used to monitor the animals over time in order to assess their improvement or worsening.

In addition, the evaluation of infarct volume is one of the main parameters measured. The size of the lesion can be assessed by different techniques, depending on the aim of the study, the sensitivity required and the availability of specialized devices. Some microscopic stainings such as haematoxylin-eosin or Nissl allow identifying necrotic and dead cells microscopically [74]. However, these techniques are not commonly used due to the development of macroscopic stainings that allow a faster visualization of the affected area without the usage of optic devices. These macroscopic staining techniques permit determining which cells are dead and unable to uptake the stain predicting which areas will become necrotic. The most commonly used are Triphenyltetrazolium chloride

(TTC) and cresyl violet staining [75]. The main limitation of these techniques is that the quantification of the lesion has to be performed post-mortem. The recent improvement of neuroimaging techniques such as MRI, has allowed using non-invasive techniques to quantify infarct volume techniques *in vivo*. However, it requires the availability of sophisticated devices that are not always accessible for all researchers and laboratories [76].

1.5. Development of therapeutic strategies for ischemic stroke

As previously exposed, the only approved drug to treat ischemic stroke is rt-PA which focuses on the recanalization of the blocked artery to reduce the extent of ischemic damage. There are at least two other primary categories of investigational therapies that are under development, which are neuroprotection and neurorepair approaches [77]. Neuroprotection strategies aim to modulate some initial processes of the ischemic cascade to block cell damage and consequent cell death, avoiding the progression of brain injury. On the other hand, neurorepair strategies aim to increase and sustain the intrinsic brain repair properties and to enhance the structural and functional reorganization of the ischemic brain. To do so, neurorepair therapies seek to enhance spontaneous angiogenesis and neurogenesis in order to restore the cerebral blood flow and promote neuroregeneration [78].

1.5.1. Neuroprotection

Neuroprotection in acute ischemic stroke consists of administering a therapy as soon as possible after symptoms onset to minimize the impaired brain damaged area while the ischemic brain is waiting for a reperfusion therapy to restore blood flow. It focuses on the modulation of key molecules from the ischemic cascade that are altered right after blood flow disruption [79]. These therapies are directed towards rescuing cells from the penumbra region, which are

metabolically impaired but still viable. Theoretically, the time window of these therapies extends up to 24 hours, depending on whether ischemia is reversible or not. However, the salvable tissue rapidly decreases within the first hours after stroke onset, being a large part of cells in penumbra already lost 6 hours after permanent ischemia [80]. For that reason, therapies should be initiated as soon as possible obeying the golden rule "time is brain", in order to rescue as many brain cells as possible [78]. Therefore, the ideal neuroprotectant would be characterized by an easy application in the pre-hospital setting so that patients would be able to receive the treatment before arriving at the hospital [81].

To date, more than 1000 neuroprotectants have shown promising results in preclinical models [82]. Due to the complexity of the ischemic cascade, various molecular targets have been modulated to achieve neuroprotection. They mainly include molecules related to excitotoxicity, calcium influx, ROS scavenging, nitric oxide production, inflammatory reactions and apoptosis [83]. Figure 5 summarizes the main sites of action of the most important neuroprotectants targeting the ischemic cascade tested to date.

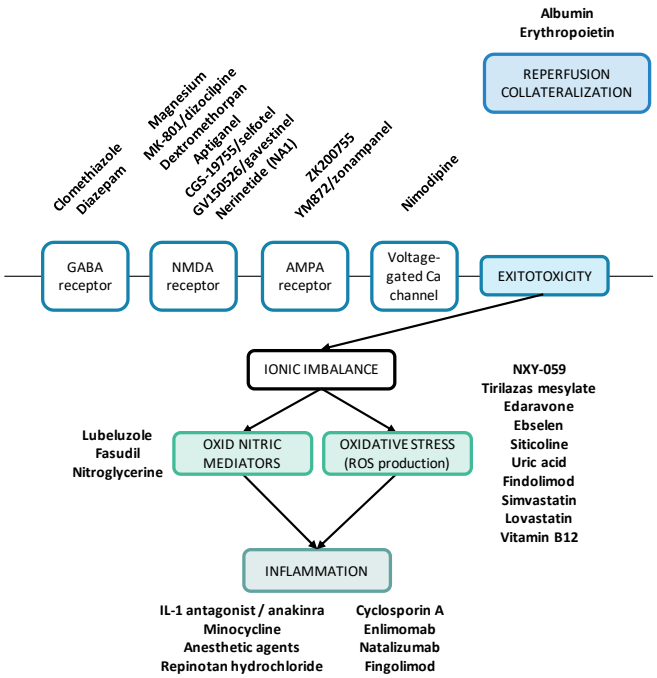


Figure 5. *Neuroprotective strategies targeting ischemic cascade. ROS, reactive oxygen species. (Adapted from Karsy et al. 2017)*[84].

Two hundred of these neuroprotectants have been tested in clinical trials (phase II or III) to date, but none of them has shown to be effective when translated into clinics [70]. Main possible reasons to explain this failure in clinical translation are summarized in table 1.

Table 1. *Potential reasons for translational failure of neuroprotective agents from pre-clinical to clinical studies*

Animal models	Human studies
Highly controlled, homogeneous population	Variable, heterogeneous population
Young animals	Old patients
Limited comorbidities	Numerous comorbidities
Induced onset of stroke	Spontaneous onset of stroke
Uniform etiology	Variable etiologies
Ischemic territory usually from MCA	Ischemic territory not restricted to MCA
Control over therapeutic time window (usually early treatment)	Less control over therapeutic time window (usually delayed treatment)
Controlled occlusion duration	Variable occlusion duration
Wide scope for dose optimization	Reduced scope for dose optimization
Multiple routes of administration	Limited routes of administration
Rapid availability of the drugs to the target area	Slow availability of the drugs to the target area
Infarct volume as the primary outcome	Function as the primary outcome
Sex: mostly male	Sex: male and female
Previously untreated with drugs	Previously treated with drugs

MCA: Middle cerebral artery. From Sutherland et al. 2012 [85].

In order to improve future study designs to make clinical implementation easier and feasible, the Stroke Therapy Academic Industry Roundtable (STAIR) published some recommendations to enhance the quality of preclinical research. Remarkably, these recommendations give special attention to the experimental model, species and strain selection. Firstly, they emphasize the necessity of considering both sexes when designing experiments. Secondly, they highly advocate including animals with comorbidities such as diabetes, obesity or hypertension as well as aged animals in pre-clinical studies, to better represent

the human population that most likely will suffer a stroke. Moreover, they also give importance to validating the results obtained in at least two independent laboratories, to increase its robustness and consequently increment the chance of success of clinical implementation [86–88].

1.5.2. New therapeutic strategies: neuroprotective agents' cocktail

In recent years, there has been growing interest in "drugs cocktail" strategy to treat complex diseases. This approach claims that targeting multiple key pathways or molecules with two or more therapeutic agents could be the optimum method to treat complex pathologies such as stroke. In other words, it is possible, that the individual modulation of single pathological mechanisms is not enough for attenuating the progression of a highly complex and multifactorial disease such as stroke [89]. In fact, this strategy has proven its efficacy in other complex conditions such as neurodegenerative [90,91] and neoplastic [92–94] diseases. Regarding stroke, the attractive results obtained in other fields made the cocktail therapy approach appealing to treat this disease. However, although modulating multiple mechanisms seems promising theoretically, the existent studies are too preliminary and further research is needed to corroborate its benefit. As an example, given the importance of inflammation and oxidative stress in the ischemic cascade, its modulation by Atorvastatin, an anti-inflammatory drug, and Probucol, an antioxidant drug, has been studied in a pre-clinical model of cerebral ischemia. Interestingly, the drug combination was able to reduce infarct volume and protect the ischemic brain but this effect was not observed when the drugs were given alone [95].

In the light of these results, simultaneously targeting several pathways of the ischemic cascade with a "neuroprotective agents' cocktail" could suppose the optimum therapeutic approach for ischemic stroke, a strategy that could change the present course of neuroprotection research.

1.6. Blood biomarkers in ischemic stroke

Biomarkers are defined as characteristics that can be objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic interventions [96]. Although biomarkers can be clinical or neuroimaging variables, they usually consist of biological molecules, such as proteins, metabolites or nucleic acids, among others, associated with a biological process of the disease [97]. An ideal biomarker for stroke should fulfill certain requirements if it has to be used in clinics. It should have high specificity and sensitivity for the indicated process and its measurement should be accurate, reproducible and easily interpretable by clinicians [37]. Moreover, it should add information on top of clinical variables to assist with diagnosis, determination of stroke subtype, prediction of outcome or response to therapy [98].

Research in stroke biomarkers mainly focuses on molecules that are present in peripheral blood, given that blood samples are easily accessible and their collection is minimally invasive. BBB disruption favors the release of brain antigens into the peripheral circulation, making these molecules promising stroke-associated biomarkers that could reflect in the circulatory system those pathological processes occurring in the brain after the ischemic event [37]. Thus, stroke biomarkers can be either brain-specific markers released from damaged tissue or other more systemic indicators such as those resulting from the inflammatory response at either a local or peripheral level [21]. However, other body fluids such as saliva, urine or cerebrospinal fluid as well as tissues such as brain necropsies, thrombus or atheroma plaques can also be used to discover stroke biomarkers.

In recent years, promising results in the field of ischemic stroke biomarkers have been obtained. Lipoprotein-associated phospholipase A2 (Lp-PLA2) and C-reactive protein (CRP) have been associated with the risk to suffer an ischemic

stroke as well as with stroke recurrence risk [99,100]. In fact, regarding Lp-PLA2, the use of a commercialized test to determine the risk to suffer a cardiovascular event has been approved (PLAC Activity Test by DiaDexus; https://www.placactivity.com). In reference to stroke diagnosis, the implementation in clinics of biomarkers or panels of biomarkers that allow discerning between mimics and ischemic stroke patients would ease patients' management shortening call-to-treatment time, making it possible to treat patients in a pre-hospital setting [101]. In this line, the simultaneous determination of D-dimer and caspase-3 or D-dimer, MMP-9, brain natriuretic peptide (BNP) and S100 calcium-binding protein (S100B) allows the discrimination between stroke and mimic patients [102,103]. In addition, other biomarkers have shown promising results in discerning between stroke subtypes, such as glial fibrillary acidic protein (GFAP) or S100B, which are substantially increased in circulation in hemorrhagic stroke patients when compared to ischemic stroke patients [104,105]. Similarly, N-terminal pro-B-type natriuretic peptide (NT-proBNP) has shown to be elevated in ischemic stroke patients, making it possible to discriminate them from hemorrhagic stroke patients [106].

Regarding stroke prognosis biomarkers, several candidates have been proposed. An accurate prediction of patients' evolution can facilitate decision-making processes, such as patients' inclusion in clinical trials or early admission to specialized stroke units, ultimately optimizing patients' management and reducing morbidity associated with stroke. Various studies have found an association between blood biomarkers and stroke prognosis, and distinct reviews and meta-analyses have been published [107–109]. However, few molecules have shown added predictive value in front of clinical variables. Remarkably, copeptin has shown additional value in predicting poor functional outcome and mortality three months after the event in front of a clinical model comprising age and basal NIHSS when measured within 24 hours after stroke [110].

1.6.1. The process to discover new biomarkers

The actual lack of stroke biomarkers used in clinical practice for any indication makes it necessary to do more studies focused on the discovery of new candidates. This process is complex and ideally consists of 4 phases: discovery, qualification, verification and validation. By following this process, the number of potential candidates is reduced in each phase while the number of samples needed increases (Figure 6).

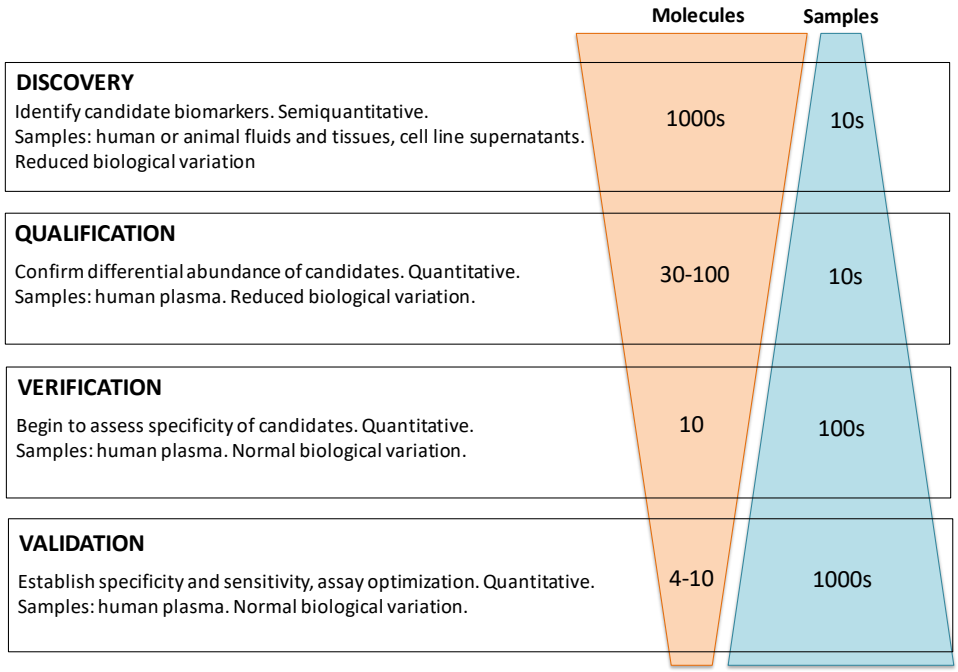


Figure 6. *Process flow for the development of novel biomarker candidates. 'Molecules' refers to the number of proteins expected to be evaluated as candidate biomarkers in each phase of development. 'Samples' refers to the sample requirements for each phase. (Adapted from Rifai et al. 2006)* [111].

The discovery phase comprises the unbiased and semiquantitative process by which the differential expression of specific molecules between different states is first defined. In this phase, both model systems and human biological samples can be used, and usually consists of a comparison between diseased and healthy tissues using high throughput massive screening techniques. This approach

avoids “contamination” by other diseases reducing confounding conditions. The result of this phase are extensive lists of molecules differentially expressed between the healthy and diseased states, and usually include false positives that need to be discarded during the following steps.

All the following phases consist of targeted quantitative approaches. The qualification phase allows confirming that the differential candidate expression observed in the discovery is maintained using alternative targeted methods, establishing a clear association between the candidate and the studied disease emphasizing marker sensitivity. Then, in the verification phase, the number of patients is substantially increased, incorporating the environmental, genetic and biological variation in the population to be tested. Thus, the biomarker candidate sensitivity is affirmed and specificity begins to be assessed. Finally, the validation phase is only performed in a reduced number of candidates that have successfully overcome previous phases. Here, candidates are tested through optimized techniques in thousands of patients that accurately reflect the full variation and heterogeneity of the targeted population. Then, validated biomarkers can be selected to be commercialized, and the immunoassay is refined to meet the rigorous standards required for clinical tests.

1.7. Tools to discover new biomarkers and/or therapeutic targets

Advances in technology are enabling the use of high throughput techniques, commonly known as –omic techniques, to deepen the knowledge of stroke pathophysiology. These techniques allow large unbiased screening processes of a large number of molecules altered due to cerebral ischemia that might be evaluated as biomarkers, therapeutic targets or both [37,112].

This large screening can be performed at any of the four basic molecular levels: at a gene level through genomics, at a ribonucleic acid (RNA) level via transcriptomics, at a protein level through proteomics and at metabolites level

via metabolomics. Although all these techniques are fundamental to understand stroke, the present Doctoral Thesis will focus on transcriptomics and proteomics.

1.7.1. Transcriptomics

Transcriptomics technologies are used to study an organism's transcriptome, the sum of all of its RNA transcripts, in a specific time. In this line, transcriptomic analysis allows the study of gene expression under different circumstances [113]. Studies exploring individual transcripts were being performed years before any transcriptomics approaches were available, through northern blotting, nylon membrane arrays, and later reverse transcriptase quantitative PCR (RT-qPCR) [114]. However, the rapid development of new technologies and the refinement of the existing ones have allowed to simultaneously detect tens to thousands of transcripts in the same sample [113].

Microarrays or chip methods have become one of the main techniques used to simultaneously assay a large number of transcripts. This technique consists of short nucleotide oligomers (probes) arrayed in a solid substrate. Transcript abundance is then determined by hybridization of fluorescently labeled transcripts to these probes [113]. This unbiased design allows the study of gene expression profiling at a greatly reduced cost, which has enabled to perform various transcriptomic studies in the field of stroke [115–117].

In addition to microarrays, RNA-sequencing methods have provided an even more complete characterization of RNA transcripts. This technique combines high-throughput sequencing methodology with computational methods to capture and quantify transcripts present in an RNA extract [118]. One of the main advantages of his approach is the possibility of identifying new transcripts that had never been described before, leading to the better characterization of the disease as well as to the improvement of hybridization techniques.

All these approaches have led to the identification of several transcript panels that are associated with stroke risk, stroke diagnosis and stroke etiology. As an example, different panels of gene transcripts expressed in blood cells enable to identify ischemic stroke patients with sensitivities and specificities of 80-90%, distinguishing these patients from controls and hemorrhagic stroke patients. Some of the gene transcripts included in these panels are those that encode for arginase, S100B, factor V, CD28 and nuclear factor of activated T cells (NFAT) [119–121].

1.7.2. Proteomics

Among all omics-based approaches, probably proteomics is the one that has experienced the biggest development. Proteomic techniques allow the study of the entire set of proteins in a specific moment, given that the composition of the proteome is in constant change over time and throughout the organism [122]. Technological advances have enabled to move from individual protein quantification to simultaneous identification of thousands of proteins.

Conventional techniques such as enzyme-linked immunosorbent assay (ELISA) and western blotting (WB) are used to analyze selective individual proteins. In addition, new antibody-based techniques have been developed in recent years greatly contributing to deciphering proteomic changes after ischemic stroke. Multiplexing these targeted methods allows the simultaneous identification of hundreds of proteins in a single sample. Examples of these techniques are aptamer microarrays (SomaLogic), microbead-based multiplexed immunoassays (Luminex) or the proximity extension assay (Olink) [123].

However, truly high-throughput identification of thousands of proteins per sample is only possible with mass spectrometry (MS) technology, being crucial to study the proteome. To identify and quantify proteins through MS, the proteins are extracted from the sample and digested into multiple peptides using

proteolytic enzymes. Further steps including enrichment and fractionation can be added to decrease the complexity of the sample. The obtained peptides are analyzed by liquid chromatography coupled with mass spectrometry (LC–MS), which allows separating even more the peptides depending on their chemical characteristics, such as size or electric charge. Finally, peptides are detected in the spectrometer depending on their mass-to-charge (m/z) ratio [124].

Regarding stroke, the brain proteome after ischemic stroke was first described ten years ago [125]. In addition, various proteins have been described to play a crucial role in stroke pathophysiology. As an example, thanks to a study where post-mortem brain samples of ischemic stroke patients' were analyzed by MS, Gelsolin, dihydropyrimidinase-related protein 2 (DRP2) and cystatin A were identified. Interestingly, these three proteins resulted to be independent predictors of poor outcome when measured in blood acutely after ischemic stroke [126].

1.7.3. Integromics

The rapid progress of all *–omics* technologies has triggered the development of tools to facilitate the analysis and interpretation of all the generated data. To date, various statistical methods are being used to independently analyze data from each individual *-omic* technique. However, such individual interpretations may ignore the existing crosstalk between different molecular units and may miss true biologically relevant information. For that reason, integrated analysis of the data obtained through different –omics techniques, what is known as integromics, is becoming critical to reveal interaction networks in complex biological systems (Figure 7) [127].

Besides, systems biology is emerging as a complementary and more refined integromics approach that aims to explore all information within the scope of disease-related signaling pathways. To that end, it combines experimental data

at multiple molecular levels with computational modeling. Systems biology visualizes the system as a whole and facilitates the identification of data with promising diagnostic, prognostic, or therapeutic value [128]. Moreover, it is also designed to track specific molecular interactions over time rather than a static map of molecular relationships within a specific disease.

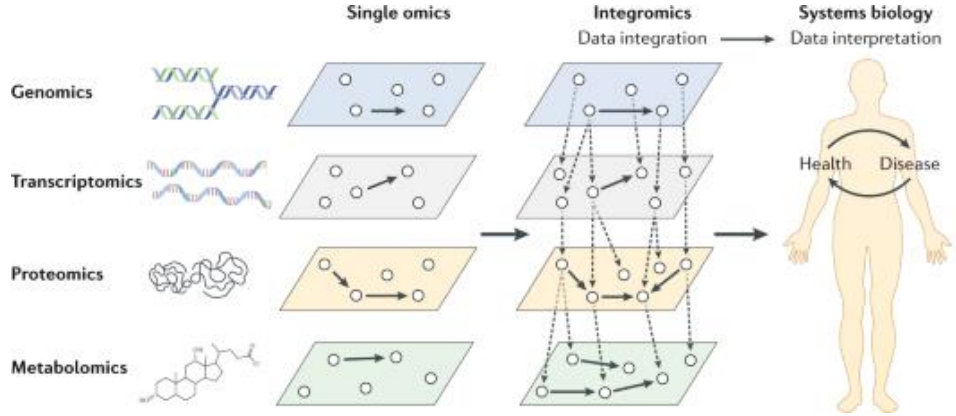


Figure 7. *Schematic representation of a multi-omics approach. Single omics data can be combined through integromics and systems biology. (From Montaner et al., 2020)* [112]

Regarding stroke, molecules identified through integromics for which changes are extremely large can be considered to be further studied as stroke biomarkers in clinical practice, given that it would be easier to establish a cut-off point to discriminate between conditions. In addition, candidate molecules for which changes are large or moderate could be considered as therapeutic targets [112].

In regard to stroke treatment, and in line with what has previously been discussed about neuroprotective agents' cocktail, integromics is expected to become an excellent source of multiple biologically interconnected features to be simultaneously modulated with combinational therapies for stroke.

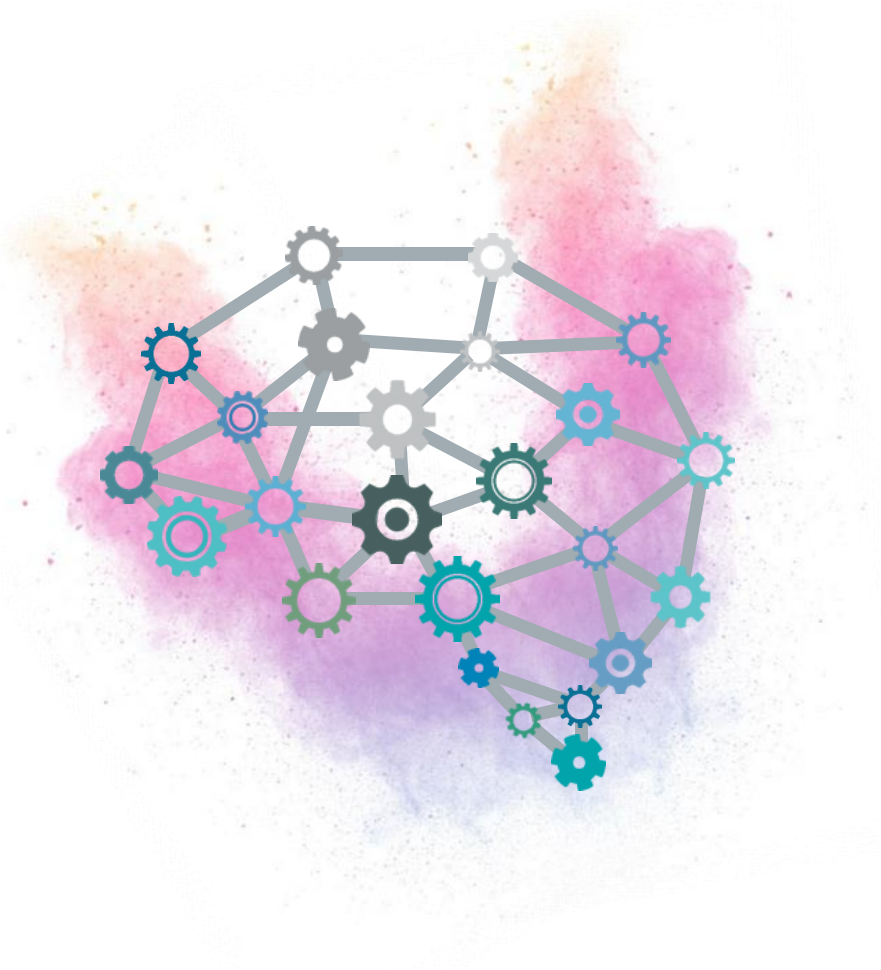
1.8. Unmet needs

As presented in previous sections, ischemic stroke patients' management is still challenging. It is well known that the clinical implementation of accurate blood

biomarkers would be crucial to improve and accelerate stroke diagnosis and prognosis. In this line, biomarkers that aid in predicting stroke patients' outcome might help to select those patients that should be admitted into specialized stroke units, rehabilitation programs, or into ongoing clinical trials to ensure optimal patients' care and ultimately improve their outcome [129]. In parallel, it is crucial to find new neuroprotective strategies to protect the ischemic brain beyond the current recanalization therapies, which might as well have a huge impact on stroke patients' outcome.

Both lines of research, however, have been studied for many years, despite no clinically useful findings have been achieved to date. In this regard, a better understanding of stroke pathophysiology is critical to detect molecules having key roles in the initial triggering of cerebral ischemia that, if detectable in blood, could be good biomarker candidates. Moreover, beyond stroke biomarkers, the therapeutic modulation of these key molecules might also become an interesting approach as a possible neuroprotective treatment to mitigate all subsequent pathological mechanisms implicated in the disease.

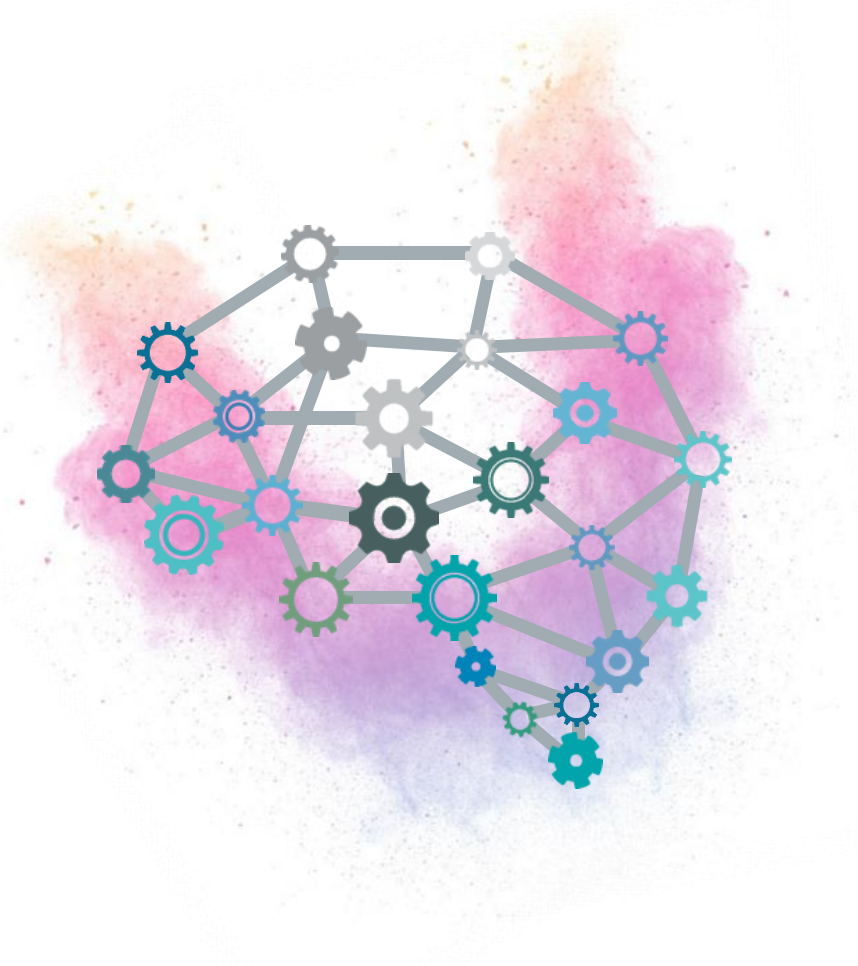
OBJECTIVES



The objectives of the present Doctoral Thesis are:

- To describe the human ischemic brain proteome and transcriptome for integrating these datasets through biostatistical tools to identify potential stroke biomarkers and/or therapeutic targets.
- To study the influence of sex, age and comorbidities in stroke pathophysiology by exploring similarities and differences in the brain proteome and transcriptome in a mice model of cerebral ischemia in (a) young males, (b) young females, (c) diabetic young males and (d) aged males.
- To describe the role of Alpha-1 antitrypsin and Aquaporin-4 as biomarkers of stroke outcome.
- To explore the role of inflammatory molecules as biomarkers of stroke mortality in the long term.
- To identify new drug combinations presenting synergistic neuroprotective effect through a drug repositioning approach and experimentally validate the neuroprotective effect of Alpha-1 antitrypsin + Ceruletide combination in a mouse model of cerebral ischemia.

METHODOLOGY



The present Doctoral Thesis can be divided into 3 main blocks. The first one aims to describe proteomic and transcriptomic changes at a brain level triggered after cerebral ischemia to better comprehend the pathophysiology of ischemic stroke and identify key molecules of the disease, and includes **Study 1** and **Study 2**. The second block aims to explore the role of different molecules as biomarkers for stroke prognosis, and comprises **Study 3**, **Study 4** and **Study 5**. Finally, the third block aims to explore the potential role of key molecules to be used as therapeutic targets to treat ischemic stroke, and includes **Study 6**.

Moreover, this Doctoral Thesis comprises studies performed using animal models and studies performed using samples from different cohorts of stroke patients. In this section, a brief description of the methodology and experimental design of each study is presented. Detailed information on materials and methods can be found in the corresponding *methods* section of the specific study. All human studies were approved by the Ethics Committee of Vall d'Hebron Hospital (PR[HG]85/04, PR[HG]89/03, PR[IR]87/10 and PR[AG]80/12) or the corresponding recruiting center. All animal studies were performed according to the ARRIVE guidelines and were approved either by the Ethics Committee of the Vall d'Hebron Institute of Research (protocol number 74/16, 03/19 and 73/20) or by the institutional review board (French ministry of Research and by the local ethical committee of Normandy (CENOMEXA).

3.1. Clinical studies in humans

For the development of **Study 1**, **Study 3**, **Study 4** and **Study 5**, samples from stroke patients have been used. In the following sections, the experimental design and characteristics of patients' cohorts are briefly described. Figure 8 summarizes the workflow of the clinical studies performed in humans included in this Doctoral Thesis.

3.1.1. Study 1: Integrative multi-omics analysis to characterize human brain ischemia

This study is divided into 3 main sections: a Discovery Phase developed in human brain samples employing RNA microarrays and MS, with an integrative bioinformatics analysis; a second Replication Phase for selected candidates performed in independent human brain samples using qRT-PCR and WB; and a third Qualification Phase developed in human blood samples using ELISA techniques.

For this study, two cohorts of patients were used, one to obtain brain samples and the other to obtain blood samples:

Brain samples: Eleven ischemic stroke patients that died during hospitalization in the Vall d'Hebron Hospital (Barcelona, Spain) within 360 hours of symptoms onset were included. Five patients received the standard thrombolytic treatment (intravenous 0.9 mg/kg rt-PA). Under the supervision of an experienced neuropathologist, brain samples from the IC and the corresponding healthy contralateral area were collected within the first hours after death.

Blood samples: Eleven patients with diagnosed acute ischemic stroke admitted to the emergency department of the Vall d'Hebron Hospital within the first 4.5 hours after symptoms onset were included. All patients received the standard thrombolytic treatment (intravenous 0.9 mg/kg rt-PA). Plasma samples were drawn at hospital admission (before any treatment was given), 24 hours and 1 week after symptoms onset. Additionally, plasma samples from 5 subjects free from brain lesions were included as a reference.

3.1.2. Study 3: Circulating Aquaporin-4 as a biomarker of early neurological improvement in stroke patients: A pilot study

This study aimed to investigate circulating Aquaporin-4 (AQP4) levels after ischemic stroke and their correlation with neurological outcome in-hospital. AQP4 determination was performed using ELISA and neurological outcome evaluation of patients was assessed using the NIHSS [46].

Forty-two patients with diagnosed acute ischemic stroke admitted to the emergency department of the Vall d'Hebron Hospital within the first 4.5 hours after symptoms onset were included. All patients received the standard thrombolytic treatment (intravenous 0.9 mg/kg rt-PA). Serum samples were drawn at hospital admission (before any treatment was given). In a subset of 12 patients, additional serial extractions were performed at 1 and 2 hours after rt-PA administration, and at 12 and 24 hours after stroke onset to obtain a temporal profile. Additionally, serum samples from 13 subjects free from brain lesions were included as a reference.

Stroke severity and neurological outcome were assessed using the NIHSS at hospital admission, at 12, 24 and 48 hours after symptoms onset and at hospital discharge. Neurological improvement was defined as a ≥4-point decrease and neurological deterioration as ≥4-point increase (or death) in NIHSS score at any time compared to the baseline score.

3.1.3. Study 4: Alpha-1 antitrypsin as a biomarker for stroke prognosis

This study aimed to determine if circulating levels of alpha-1 antitrypsin (A1AT) measured acutely after stroke were associated with the functional outcome either in-hospital or at mid-term. A1AT determination was conducted by ELISA and functional outcome evaluation of patients was assessed using the mRS [56].

Eighty patients with an acute ischemic stroke and 25 patients with an ICH admitted to the emergency department at Vall d'Hebron University Hospital (Barcelona, Spain) within the first 6 hours after symptoms onset were included. Forty-one ischemic stroke patients received rt-PA (intravenous 0.9 mg/kg), 3 were subjected to mechanical thrombectomy and 19 underwent both reperfusion strategies. Plasma samples were drawn at admission (before any treatment was given). Additionally, a control group of 8 healthy subjects free from inflammatory or infectious disease was included to set reference A1AT levels.

The clinical follow-up of each stroke patient was carried out until 3rd month after stroke. Functional outcome was evaluated 3 months after stroke using the mRS; patients with a mRS score lower or equal than 2 were classified as having 'good outcome' and patients with a mRS from 3 to 6 as having 'poor outcome'.

3.1.4. Study 5: Blood biomarkers to predict long-term mortality after ischemic stroke.

This multicenter study aimed to identify blood biomarkers that measured during the hyperacute phase of ischemic stroke could predict long-term mortality. A 14-biomarker panel including Apolipoprotein CIII (ApoC-III), D-dimer, Endostatin, Fas Ligand (FasL), Growth-related oncogene-α (GROA), Heat shock 70 kDa protein-8 (Hsc70), Insulin-like growth factor-binding protein-3 (IGFBP-3), Interleukin-6 (IL-6), Neuron cell adhesion molecule (NCAM), NT-proBNP, S100B, Tumor necrosis factor receptor-1 (TNF-R1), vascular adhesion protein-1 (VAP-1) and Von Willebrand factor (vWF) was analyzed by immunoassays.

Nine hundred forty-one ischemic stroke patients admitted at the emergency department of 6 Hospitals in Catalonia (Germans Trias i Pujol University Hospital, Vall d'Hebron University Hospital and Bellvitge University Hospital at Barcelona, Josep Trueta University Hospital in Girona, Joan XXIII University Hospital in

Tarragona, and Verge de la Cinta Hospital in Tortosa) within the first 6 hours after symptoms onset were included. Three hundred sixty-three patients received rt-PA (intravenous 0.9 mg/kg), 72 were subjected to mechanical thrombectomy and 389 underwent both reperfusion strategies. Plasma samples were drawn at hospital admission (before any treatment was given).

Patients were followed up for a median of 4.8 years and were contacted by telephone and interviewed using a structured questionnaire designed specifically for this study. Cause of death was recorded for each patient and mortality due to stroke was considered when the patient died due to the index stroke or a recurrent cerebrovascular event along follow-up.

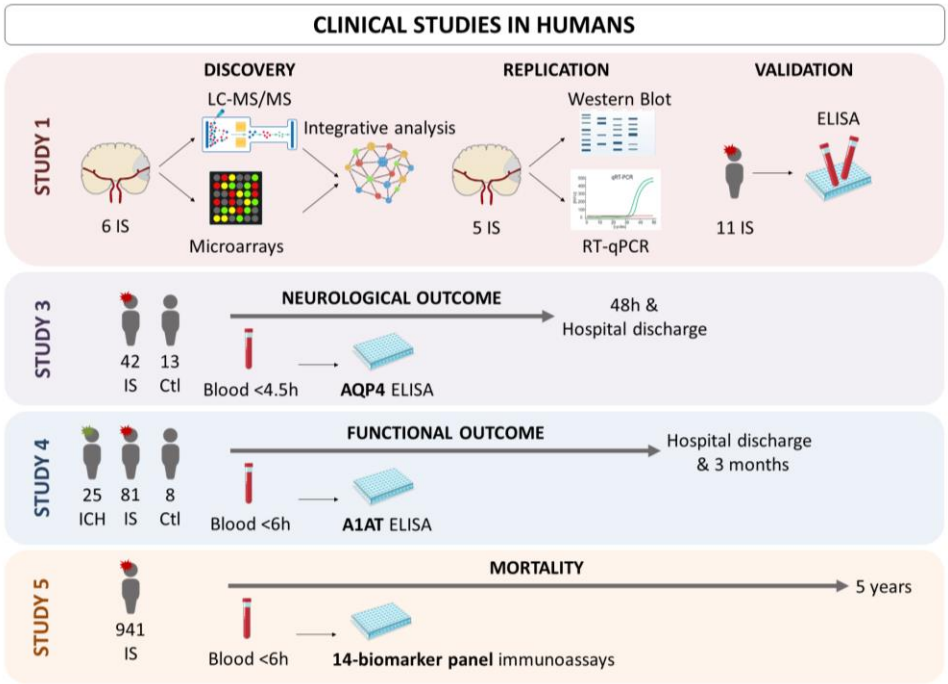


***Figure 8**. Workflow chart of clinical studies in humans included in this Doctoral Thesis*

3.2. Experimental studies in mice

For the development of **Study 2** and **Study 6** animal models of cerebral ischemia have been used. In the following sections, the experimental design and animal

models used are briefly described. All animal procedures were performed in compliance with the Spanish legislation and following the Directives of the European Union and were approved by the Ethics Committee of the Vall d'Hebron Research Institute. Figure 9 summarizes the workflow of the experimental studies in mice included in this Doctoral Thesis.

3.2.1. Study 2: Influence of sex, age and diabetes on brain transcriptome and proteome modifications following cerebral ischemia

This discovery study aimed to explore the role of age, sex and diabetes in the molecular changes produced by cerebral ischemia. Concretely, we aimed to explore proteomic and transcriptomic changes at a brain level triggered during the hyperacute phase of cerebral ischemia in mice of four different groups: (1) young male mice, (2) young female mice, (3) aged male mice and (4) diabetic young male mice. Transcriptomic changes were analyzed using RNA microarrays while proteomic changes with MS. Finally, an integrative analysis was performed to reveal key common and exclusive proteins and genes playing a crucial role in the first stages of the disease.

Transient ischemia in the territory of the MCA was induced by introducing an intraluminal filament through the external carotid artery to occlude the MCA. Animals were subjected to 90 minutes of MCAO and two hours after MCAO induction (30 minutes after reperfusion) animals were sacrificed to obtain the brain.

3.2.2. Study 6: Ceruletide and alpha-1 antitrypsin as a novel combination therapy for ischemic stroke.

This study is divided in 5 different phases. First, we used a systems biology-based approach based on artificial intelligence and pattern recognition tools to

integrate available biological, pharmacological and medical knowledge into mathematical models to simulate *in silico* the complexity of the stroke. Second, we screened these models to identify combinations of two drugs with synergic neuroprotective effects. The combination of ceruletide and A1AT was selected to be further studied. Third, the synergistic neuroprotective effect of this drug combination was validated *in vivo* in mice MCAO model. Fourth, incompatibilities with rt-PA were discarded both *in vitro* (using a clot lysis assay) and *in vivo* (using a mouse thromboembolic model). Finally, the synergistic mechanism of action of the drug combination was evaluated in vitro using WB, ELISA and Olink® proteomic arrays.

Two different mice models of cerebral ischemia were used in this study:

To evaluate the neuroprotective synergistic effect of the drug combination formed by ceruletide and A1AT in mice, transient ischemia in the territory of the MCA was induced by introducing an intraluminal filament through the external carotid artery to occlude the MCA. Animals were treated with drugs or vehicle intravenously ten minutes after MCAO and subjected to 90 minutes MCAO. The neurological state of animals was evaluated on a composite neurological scale 80 minutes and 24 hours after MCAO. Twenty-four hours after MCAO induction animals were sacrificed to obtain the brain and infarct volume was assessed using TTC staining.

To test incompatibilities of the drug combination with rt-PA in vivo we used a thromboembolic stroke model in mice with rt-PA-induced reperfusion. In brief, murine alpha-thrombin was injected into the MCA to induce the formation of a clot in situ. Ten minutes after, the drugs or vehicle were intravenously administered and ten minutes later (20 minutes after thrombin injection), rt-PA was injected intravenously to allow reperfusion. The neuromuscular function of mice was determined using the grip strength test before surgery (baseline), and

24, 48 and 72 hours after cerebral ischemia. Infarct volume was assessed by MRI 24 hours after stroke.

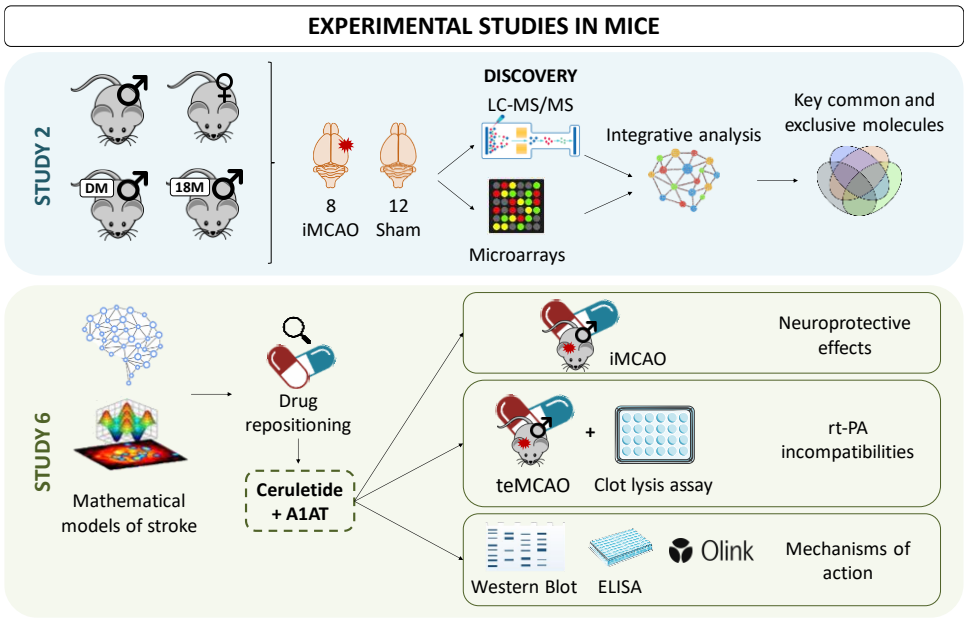


***Figure 9**. Workflow chart of experimental studies in mice included in this Doctoral Thesis*

RESULTS

4.1. Integrative multi-omics analysis to characterize human brain ischemia.

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4.2. Influence of sex, age and diabetes on brain transcriptome and proteome modifications following cerebral ischemia

Influence of sex, age and diabetes on brain transcriptome and proteome modifications following cerebral ischemia

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Abstract

Ischemic stroke is a major cause of death and disability worldwide. Translation into the clinical setting of neuroprotective agents showing promising results in pre-clinical studies has systematically failed. One of the reasons that could explain this failure is that the animal models used to test neuroprotectants do not represent properly the population affected by stroke, as the majority of pre-clinical studies are performed in healthy young male mice. In this regard, we aimed to determine if the response to cerebral ischemia differed depending on age, sex and the presence of comorbidities. Thus, we explored proteomic and transcriptomic changes triggered during the hyperacute phase of cerebral ischemia (by transient intraluminal middle cerebral artery occlusion) in the brain of: (1) young male mice, (2) young female mice, (3) aged male mice and (4) diabetic young male mice. Moreover, we compared the proteomic and transcriptomic changes of each group using an integrative enrichment pathways analysis to disclose key common and exclusive altered proteins, genes and pathways in the first stages of the disease. We found 61 differentially expressed genes (DEG) in male mice, 77 in females, 699 in diabetic and 24 in aged mice. Of these, only 14 were commonly dysregulated in all four groups. The enrichment pathways analysis revealed that the inflammatory response was the biological process with more DEG in all groups of animals, followed by hemopoiesis and lymphoid organ development. Our findings indicate that the response to cerebral ischemia regarding proteomic and transcriptomic changes differs depending on the sex, age and comorbidities, highlighting the importance of incorporating these groups of animals in future stroke research studies.

1. Introduction

Ischemic stroke is a leading cause of death and disability worldwide [1]. Nowadays, recombinant tissue-plasminogen activator (rt-PA) and tenecteplase (TNK) are the only approved drug to treat acute ischemic stroke, although cerebral blood flow can also be restored by removing the obstructive clot through mechanical thrombectomy in selective patients [2,3]. However, these treatments carry significant risks and secondary side effects, reducing the number of patients that can benefit from them [4,5]. Neuroprotective agents have been proposed as adjuvant therapies to be given during the hyperacute phase to minimize the impaired brain-damaged area while the ischemic brain is waiting for reperfusion therapy to restore the blood flow [6]. To date, more than 1,000 neuroprotective agents have shown promising results in preclinical models [7], but translation into the clinical setting has systematically failed [7–9].

The failure of the clinical trials with neuroprotective agents for the acute treatment of ischemic stroke makes the scientific community reconsider the most classical designs of the preclinical studies. One of the reasons that could explain this failure in translation from “bench to bedside” is that the animal models used to test neuroprotectants do not represent properly the demographic and health characteristics of stroke patients [10]. Normally, pre-clinical studies include young animals, while it is known that more than 65% of all strokes occur in subjects older than 65 years [11] and only 10% in individuals under 50 years old [12,13]. Regarding sex, most pre-clinical studies are performed on male animals, while stroke has a great impact on both sexes [14], being the first cause of death in women in some European countries [13,15]. Finally, the vast majority of pre-clinical studies are performed in healthy animals, although stroke patients usually present a high incidence of comorbid conditions such as hypertension, diabetes and/or obesity, which are known to be among the most common stroke risk factors [16]. In this regard, the Stroke Therapy Academic Industry Roundtable (STAIR) published some recommendations to improve the quality of preclinical research and to make clinical implementation more efficient. Of note, these recommendations give special attention to species, strain and experimental model selection. In brief, they highlight the necessity of using both sexes in pre-clinical studies and highly recommend including animals with comorbidities and aged animals in order to better represent the human population that most likely will suffer stroke [17–19].

Taking all together, it is plausible to think that the response to cerebral ischemia might be different depending on sex, age and comorbidities, and understanding these differences at molecular level in the brain is fundamental to better comprehend stroke pathophysiology and to improve the “bench to bedside” translation. With this background, we aimed to explore proteomic and transcriptomic changes at a brain level triggered during the hyperacute phase of cerebral ischemia in mice of four

different groups: (1) young male mice, (2) young female mice, (3) aged male mice and (4) diabetic young male mice. We aimed to compare proteomic and transcriptomic changes of each group using an integrative enrichment pathways analysis to reveal key common and exclusive dysregulated proteins, genes and pathways playing a crucial role in the first stages of the disease, with the hypothesis that those molecular changes could further determine the response to a given drug.

2. Materials and methods

2.1. Study design

The study presented here consists of a discovery study performed on RNA and protein extracts from mice ischemic brains of four different groups: (1) young male mice, (2) young female mice, (3) aged male mice and (4) diabetic young male mice, using mass spectrometry and RNA microarrays, with further integrative bioinformatics analysis. Each group included 8 ischemic animals, and 4 sham-control animals were used to discard genes and proteins differentially altered due to other phenomena rather than the ischemic event. Of note, analysis of young male mice was performed in a first set of experiments [20]. The other 3 groups of animals were performed in a second set of experiments simultaneously.

2.2. Animals

All animal procedures were performed in compliance with the Spanish legislation and in accordance with the Directives of the European Union and were approved by the Ethics Committee of the Vall d'Hebron Research Institute (protocol number 03/19 and 73/20). All experiments were conducted in a randomized manner and in adherence to the ARRIVE guidelines [21]. Group description: (1) C57BL/6J male mice 8/12-week-old (refered as *males*), (2) C57BL/6J female mice 8/12-week-old (refered as f*emales*), (3) C57BL/6J male mice 18-month-old (refered as *old*) and (4) BKS-Lepr$^{db/db}$/JOrlRj diabetic male mice 8/12-week-old (refered as *diabetics*) (Janvier Labs, France). Animals were kept in a climate-controlled environment on a 12-h light/12-h dark cycle. Food and water were available *ad libitum*. Analgesia (Buprenorfine, 0.05 mg/kg, s.c, Divasa Farma-Vic S.A, Spain) was administered to all animals to minimize pain and discomfort. Anesthesia (isoflurane, 4% for induction, 2% for maintenance in medicinal air, Abbot Laboratories, Spain) was given via facemask during all surgical procedures described below.

A total of 57 animals were used for the present study. Among them, 9 were excluded after applying the following criteria: incomplete occlusion or reperfusion after removal of the filament (n=2) and death during the experimental protocol (n=7), while the other 48 were included.

2.3. Transient middle cerebral artery occlusion (MCAO) model

Transient ischemia in the territory of the middle cerebral artery (MCA) was induced by introducing an intraluminal filament through the external carotid artery, as described before [22]. Briefly, animals were anesthetized and body temperature was maintained at 37ºC using a heating pad. The regional cerebral blood flow (CBF) was monitored close to the region irrigated by the MCA during the whole process by affixing a laser Doppler probe (Moor Instruments, UK) to the skull. Afterwards, animals were placed in the supine position and the right bifurcation of the external carotid artery and internal carotid artery were exposed. Then, a silicone-coated nylon monofilament (Doccol Corporation, USA; 602256PK10for *males* and *females*, 702256PK5 for *diabetics* and 602456PK10 for *old* animals) was introduced through the external carotid artery to occlude the MCA. MCA occlusion (MCAO) was confirmed by a reduction in the cortical CBF recorded by the laser Doppler probe. Next, the incision was closed with a silk suture and animals were allowed to recover from anesthesia. Ninety minutes later, mice were re-anesthetized and the filament was removed to induce reperfusion of the MCA. After reperfusion, mice were allowed to recover from anesthesia under supervision. Only animals that exhibited a reduction of 80% of CBF after filament introduction and a recovery of 80% after filament removal were included in the study. Sham animals underwent the same surgical procedures without the insertion of the nylon filament, and therefore, without MCAO.

2.4. Brain collection and extraction of protein and RNA

Mice were deeply anesthetized (5% isoflurane) 30 minutes after reperfusion and transcardially perfused with 20 mL of cold saline. Immediately after perfusion, mouse brains were quickly removed and sectioned into 6 slices of 1mm in cold conditions. The slice corresponding to the bregma anatomical point (representing the core of the infarct [23]) was carefully dissected to separate the right (ipsilateral, IP) and left (contralateral, CL) hemispheres separately. Each hemisphere was flash-frozen in liquid nitrogen and stored at -80ºC.

Flash-frozen tissues were pulverized into powder in liquid nitrogen, and total fractions of protein and RNA were then isolated using the MirVana™ Paris™ kit (Thermo Fisher Scientific Inc., USA) following manufacturers' instructions. RNA and protein fractions were stored at -80ºC until further use.

2.5. Transcriptomics study

Total RNA concentrations from brain samples were measured with a Nanodrop 1000 Spectrophotometer (ThermoFisher) and RNA integrity was assessed using the Agilent 2100 BioAnalyzer (Agilent Technologies, USA). Gene expression patterns were analysed using Genechip© Mouse Clariom S 24x arrays plates (Affymetrix, ThermoFisher). Starting material was 100 ng of total RNA of each sample. Briefly, sense ssDNA was generated from total RNA with the GeneChip WT Plus Reagent Kit (Affymetrix) following manufacturer's instructions. Then, sense ssDNA was fragmented, labelled and

hybridized to the arrays with the GeneChip WT Terminal Labeling and Hybridization Kit (Affymetrix). Arrays plates were scanned and processed with Affymetrix GeneChip Command Console to obtain expression array intensity .cel files.

2.6. Proteomics study

Due to equipment availability, proteomics study was performed following two different protocols depending on the group of mice. Young male mice proteomic study was previously published [20]. Briefly, after in-gel digestion of the proteins tryptic digests were analyzed using a LC–MS approach in a linear trap quadrupole (LTQ) Orbitrap Velos mass spectrometer (ThermoFisher).

For the remaining groups of animals, previous to LC-MS analysis, samples were digested with trypsin in-solution. Initial buffer exchange to 8M Urea 50mM ammonium bicarbonate (AB) was performed using 0.5mL 3KDa cut-off Amicon Ultra ultrafiltration devices (Merck-Millipore). Total protein content was quantified using RCDC kit (Bio-Rad), and 15 µg of each protein extract were taken for tryptic digestion. Samples were first reduced with DTT to a final concentration of 10mM, for 1h at room temperature (RT), and then alkylated with 20mM of iodoacetamide (IAA) for 30min at rt in the dark. Carbamidomethylation reaction was quenched by addition of N-acetyl-L-cysteine to final concentration of 35mM followed by incubation for 15min at RT in the dark. Samples were diluted with 50 mM AB to a final concentration of 1M Urea, and then modified porcine trypsin (Promega Gold) was added in a ratio of 1:20 (w/w), and the mixture was incubated overnight at 37 °C. The reaction was stopped with formic acid (FA) to a final concentration of 0.5%. The tryptic digests were then purified on SCX spin columns (PolyLC), evaporated to dryness and stored at -20ºC until analyzed.

For LC-MS/MS analysis tryptic digests were diluted in 3% ACN, 1% FA and 500 ng of the sample was loaded to a 300 µm × 5 mm Pep-Map C18 (Thermo Scientific) at a flow rate of 15 µl/min using a Thermo Scientific Dionex Ultimate 3000 chromatographic system (Thermo Scientific). Peptides were separated using a C18 analytical column (NanoEase MZ HSS T3 column, 75 µm × 250 mm, 1.8 µm, 100Å, Waters) with a 210 min run, comprising four consecutive steps, first 3 min of isocratic gradient at 3%B, from 3 to 35% B in 180 min, from 35 to 50% B in 5 min, from 50 to 85% B in 1 min, followed by isocratic elution at 85 % B in 5 min and stabilization to initial conditions (A= 0.1% FA in water, B= 0.1% FA in CH3CN). The column outlet was directly connected to an Advion TriVersa NanoMate (Advion) fitted on an Orbitrap Fusion Lumos™ Tribrid (Thermo Scientific). The mass spectrometer was operated in a data-dependent acquisition (DDA) mode. Survey MS scans were acquired in the orbitrap with the resolution (defined at 200 m/z) set to 120,000. The lock mass was user-defined at 445.12 m/z in each Orbitrap scan. The top speed (most intense) ions per scan were fragmented in the linear ion trap (CID) and detected in the Ion Trap. Quadrupole isolation was employed to selectively isolate peptides of 350-1700 m/z. The predictive automatic gain control (pAGC) target was set to 1.5e5. The maximum

injection time was set to 50 ms for MS1 and 35 ms for MS2 scan. Included charged states were 2 to 7. Target ions already selected for MS/MS were dynamically excluded for 30 s. The mass tolerance of this dynamic exclusion was set to ±2.5 ppm from the calculated monoisotopic mass. Spray voltage in the NanoMate source was set to 1.7 kV. RF Lens were tuned to 30%. Minimal signal required to trigger MS to MS/MS switch was set to 5000 and activation Q was 0.250. The spectrometer was working in positive polarity mode and singly charge state precursors were rejected for fragmentation.

Progenesis® QI for proteomics software v 3.0 (Nonlinear dynamics, UK) was used for MS data analysis using default settings. The results from LCMS runs were automatically aligned to a selected reference sample. Alignments were then manually supervised. Normalization of MS signals, based on the median of ratiometric distribution of the abundance measurements, was performed automatically by the Progenesis® QI for proteomics software. Only features within the 400 to 1,600 m/z range, from 50 to 180 min of retention time, and with positive charges between 2 to 4 were considered for identification and quantification. Peaklists (mgf files) were generated using Progenesis® QI for proteomics and searched from Proteome Discoverer 2.1 (Thermo Fisher Scientific Inc., USA) using the Mascot search engine (v5.1, Matrix Science, UK). Protein identification was carried out using the SwissProt-MusMusculus database (2020, Jul 01: 55,400 entries) setting precursor mass tolerance to 10 ppm and fragment mass tolerance to 0.5 Da. Oxidized methionine was considered as variable amino acid modification and carbamidomethylation of cysteines as fixed modification. Trypsin was selected as the enzyme allowing up to two missed cleavages. Significance threshold for the identifications was set to $p<0.05$, minimum Mascot ions score of 20. Only those proteins quantified and identified with at least 2 unique peptides were considered for further statistical analyses.

2.7. Bioinformatics and statistical analyses

Statistical and bioinformatics analyses of data were performed using custom scripts in R language (version 4.0.2) and R Studio (R Core Team, 2017, Vienna, Austria) with common Bioconductor packages.

As the male transcriptomic and proteomic studies were carried out in a first set of experiments separately from the other 3 groups, all groups have been analyzed independently.

2.7.1. Transcriptomics data

Transcriptomics data analyses has been performed separately for each group of animals. R olgo package was used to upload .cel files. A quality control of the data was performed using *arrayQualityMetrics* package [24], a principal component analysis (PCA) and intensity boxplots for each file. Afterwards, Robust Multiarray Average algorithm [25] was used for pre-processing microarray data in order to perform background adjustment, normalization and summarization of probe set

expression values. Next, genes whose standard deviation (SD) was below the 75 percentile of all SD were filtered out from the whole dataset.

Selection of differentially expressed genes was based on a linear model analysis with empirical Bayes modification for the variance estimates [26], using limma package. This analysis has been performed considering sample pairing. False Discovery Rate (FDR)-based corrections for multiple testing were also calculated [27]. Fold-change (FC) was calculated by dividing the IP to the CL expression value for each animal.

Sham animals' data were used to discard genes differentially expressed due to other phenomena rather than cerebral ischemia. With this purpose, genes differentially expressed when comparing IP and CL both in sham and MCAO animals (FDR<0.25) with a difference in logFC<0.5 were discarded.

2.7.2. Proteomics data

Protein abundance quantification was based on the sum of the peak areas within the isotope boundaries of peptide ion peaks. Proteins identified by identical peptide sets were grouped to satisfy the principles of parsimony. Only those proteins quantified and identified with at least 2 unique and non-conflicting peptides (it is, features assigned unambiguously to peptides belonging to the protein, as assessed by Progenesis software). Protein abundance values were log-10 transformed and column-wise standardized [28]. A quality control of the data was performed using a principal component analysis (PCA) and intensity boxplots for each file.

Selection of differentially expressed proteins was based on a linear model analysis with empirical Bayes modification for the variance estimates [26], using limma package. This analysis has been performed considering sample pairing. False Discovery Rate (FDR)-based corrections for multiple testing were also calculated [27]. Fold-change (FC) was calculated by dividing the IP to the CL expression value for each animal.

2.7.3. Integrative analyses

An integrative analysis of the transcriptomics data of MCAO *male*, *female*, *old* and *diabetic* mice has been carried out. Venn diagrams were used to represent common and exclusive differentially expressed genes of studied groups. The common genes were used independently as input for STRING protein-protein interaction (PPI) network analysis. The interactions were based on text mining, experiments, databases, co-expression, neighborhood, gene fusion and co-occurrence, considering a medium confidence (0.4) [29].

For the integrative analysis, *ActivePathways* and its corresponding R package were used. In brief, this tool merges p-values and performs ranked hypergeometric tests to determine enriched pathways and processes [30]. First, a matrix was created with all the analyzed genes and its corresponding p-values for each dataset. Then, the p-values were merged using the Brown's method [31], obtaining a ranked gene list. Finally, ranked hypergeometric tests were carried out to determine if a pathway is enriched

in the ranked gene list. The following functional gene sets were used: Gene Ontology (GO) Biological Processes and GO Molecular Functions [32,33].

In order to visualize the enriched pathways as a network, Cytoscape software [34] and the EnrichmentMap app [35] were used.

3. Results

3.1. Transcriptomics results

We determined changes in gene expression that occur in the brain within the hyperacute phase after ischemic stroke by analyzing mouse-brain samples obtained 2 hours after cerebral ischemia induction (30 minutes after reperfusion) or sham surgery. The changes in gene expression were elucidated by means of microarrays, comparing the IP and CL regions and correcting by sham animals for each studied group.

Quality control of samples was performed for each group of animals. Regarding *diabetic*, *female* and *old* mice all samples passed the quality control. However, after performing quality control in *male* mice, 2 animals (4 samples) were clearly separated from the rest in PCA with lower intensities than the other individuals, which remained after normalization. For that reason, these 2 animals were removed from the analysis. Afterward, all samples passed the quality control.

3.1.1. Differentially expressed genes

In *male* mice, we found 61 differentially expressed genes (DEG) after MCAO when comparing the IP and the CL brain regions (FDR<0.25), while in sham control animals there were no DEG. In *female* mice, there were 77 DEG between IP and CL brain regions in MCAO animals (FDR<0.25), while only 8 DEG were found in sham animals (FDR<0.25) and there were no common dysregulated genes between *female* MCAO and sham animals. In *diabetic* mice, 699 DEG were found when comparing the IP and the CL brain regions in MCAO animals (FDR<0.25), while in sham control animals there were no DEG. Finally, in *old* mice we found 24 DEG were found when comparing the IP and the CL brain regions in MCAO animals (FDR<0.25), while in sham control animals there were no DEG. Details of the top 15 DEG after cerebral ischemia of each studied group are shown in table 1.

Young male mice, *males*				Young female mice, *females*				Diabetic young male mice, *diabetic*				Aged male mice, *old*			
SYMBOL	**logFC**	**P.Value**	**FDR**	**SYMBOL**	**logFC**	**P.Value**	**FDR**	**SYMBOL**	**logFC**	**P.Value**	**FDR**	**SYMBOL**	**logFC**	**P.Value**	**FDR**
CCL3	2.4958	1.73E-15	8.80E-12	***ATF3***	1.4557	1.42E-06	0.00284	*HSPA1A*	2.4544	1.02E-09	5.17E-06	***NPAS4***	2.9274	1.53E-08	7.75E-05
FOSB	1.9774	7.77E-12	1.58E-08	***PTGS2***	1.5976	1.76E-06	0.00284	***FOS***	2.4266	9.29E-09	1.63E-05	***CCL3***	2.0499	1.08E-07	2.74E-04
CCN1	1.8493	1.10E-11	1.58E-08	*CCRL2*	1.1296	2.21E-06	0.00284	***CCN1***	1.9876	9.61E-09	1.63E-05	***FOS***	1.6891	8.48E-07	0.00143
FOS	2.1172	1.25E-11	1.58E-08	***FOS***	1.5602	2.24E-06	0.00284	***CCL3***	2.6185	1.55E-08	1.97E-05	*IL1A*	1.7778	2.60E-06	0.00329
NPAS4	2.6951	7.08E-11	7.18E-08	***CCL3***	2.1777	2.93E-06	0.00298	***FOSB***	2.1383	4.27E-08	4.33E-05	***PTGS2***	1.3054	5.90E-06	0.00599
ATF3	1.6062	1.09E-09	9.22E-07	***NPAS4***	2.2737	4.40E-06	0.00372	*GADD45G*	1.7414	5.81E-08	4.91E-05	***FOSB***	1.5304	9.64E-06	0.00815
JUNB	1.4066	2.36E-09	1.67E-06	*ADAMTS1*	1.0314	6.65E-06	0.00482	*IER2*	1.2020	9.91E-08	7.18E-05	***DUSP6***	0.9922	3.29E-05	0.02382
RGS2	1.4974	2.80E-09	1.67E-06	***CCN1***	1.3156	9.48E-06	0.00601	*THBS1*	1.8039	1.14E-07	7.22E-05	*HSPA1A*	1.6433	7.39E-05	0.04390
GADD45G	1.3902	2.96E-09	1.67E-06	***NR4A1***	1.0643	1.54E-05	0.00868	***NPAS4***	3.1196	3.98E-07	2.25E-04	***JUN***	0.8117	8.45E-05	0.04390
HSPA1A	1.5433	3.32E-09	1.68E-06	***FOSB***	1.5493	1.88E-05	0.00951	***DUSP6***	1.1689	5.27E-07	2.67E-04	*CCL4*	1.1057	8.66E-05	0.04390
EGR4	1.4966	8.59E-09	3.79E-06	*RND3*	0.8880	2.34E-05	0.01079	***NR4A1***	1.6955	7.58E-07	3.49E-04	*FKBP11*	-1.0158	1.84E-04	0.08205
NR4A1	1.4166	8.97E-09	3.79E-06	*RGS1*	1.1871	2.88E-05	0.01216	*EGR4*	1.6425	9.33E-07	3.94E-04	*GM9958*	-0.9378	2.12E-04	0.08205
PTGS2	1.3968	1.51E-08	5.88E-06	*GADD45G*	1.0496	3.97E-05	0.01547	***RGS2***	1.3091	1.09E-06	4.23E-04	***NR4A1***	1.1258	2.21E-04	0.08205
BTG2	1.2922	2.79E-08	1.01E-05	***DUSP6***	0.8763	4.39E-05	0.01547	***JUN***	1.1095	1.17E-06	4.23E-04	*CCRL2*	0.9681	2.27E-04	0.08205
EGR2	1.3976	4.45E-08	1.51E-05	*GALNT12*	-1.1040	4.58E-05	0.01547	*AKAP12*	0.9770	1.51E-06	5.10E-04	***CCN1***	1.2480	2.56E-04	0.08651

*Table 1**.** Top 15 differentially expressed genes (FDR<0.25) between the infarcted hemisphere and the contralateral healthy hemisphere 2 hours after cerebral ischemia induction. Genes that are differentially expressed in all the groups of animals are highlighted in bold. FDR: false discovery rate; logFC: Logarithmic fold change.*

3.1.2. Integrative analysis of transcriptomic results

To understand the mechanisms underlying ischemic stroke, we sought to identify common DEG after cerebral ischemia in all groups, as they might point at crucial therapeutic targets in the acute phase of the disease. We found 14 DEG in the ischemic brain that were common in all four groups of animals (IP vs CL, FDR<0.25). In addition, it is worth noting that 27 genes were dysregulated only in *female* mice, 640 in *diabetic* mice, 4 in *old* mice and 16 in *male* mice (Figure 1, Supplemental table S1).

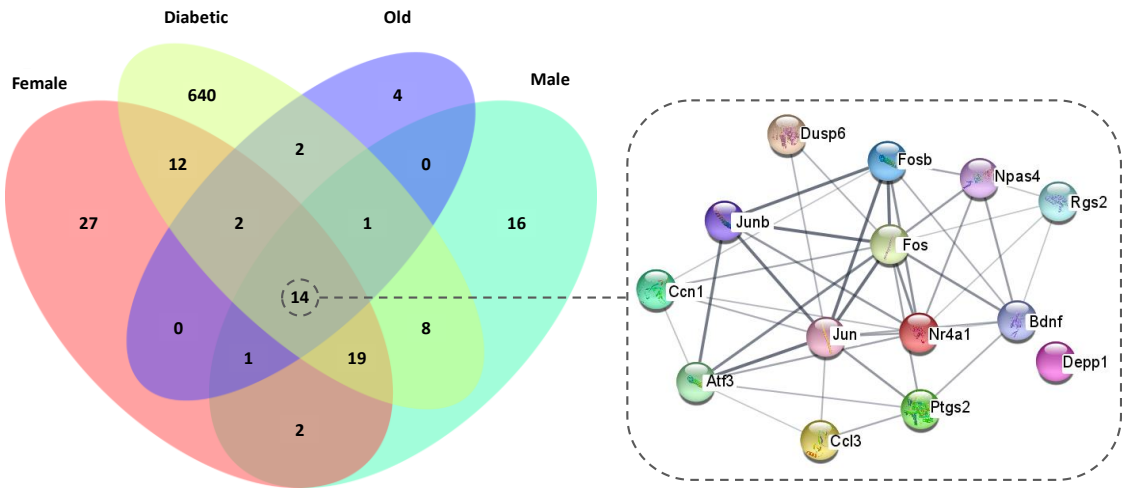


Figure 1. Venn diagram of differentially expressed genes in the ischemic brain (FDR<0.25) in all studied groups. The 14 common differentially expressed genes are shown through a STRING network revealing main interactions between molecules. The strength of the correlation between two genes (nodes) 20 is reflected by the thickness of the edge. Diabetic: diabetic young male mice; Female: young female mice; Male: young male mice; Old: aged male mice

The multi-omics pathway enrichment analysis was conducted on the integrated gene data to further understand transcriptomic alterations triggered by cerebral ischemia. Interestingly, this analysis pointed out enriched pathways on every single dataset but also enriched pathways that are only found through data integration that are not apparent in any single omics dataset independently [30]. This analysis identified several biological processes whose genes are significantly enriched within the hyperacute phase of cerebral ischemia. The top 20 enriched biological processes (sorted by p-value) and the number of DEG for each group are shown in Figure 2A. We created an enrichment map that shows pathways whose genes are significantly enriched in transcriptomics datasets. Remarkably, the acute inflammatory response was the biological process that encompassed more DEG, followed by hematopoietic or lymphoid organ development, hemopoiesis, MAPK cascade and leukocyte differentiation. Moreover, the

enrichment map of biological processes revealed 478 genes that were significantly enriched in 214 biological processes (Figure 2B). Of these 214 biological processes, 39 were common in all groups, 14 were only dysregulated in *females*, 22 in *diabetics*, 8 in *olds*, 5 in *males* and 48 were found only in the integrated gene list of all groups (Figure 2C). Furthermore, the main molecular functions of these DEG were related to phosphoprotein phosphatase activity, followed by chemokine receptor binding, receptor ligand activity and transcription regulator activity (Figure 2D).

3.2. Proteomics results

We also analyzed protein changes occurring early after cerebral ischemia in the brain using mass spectrometry. All samples in each group of animals passed the quality control.

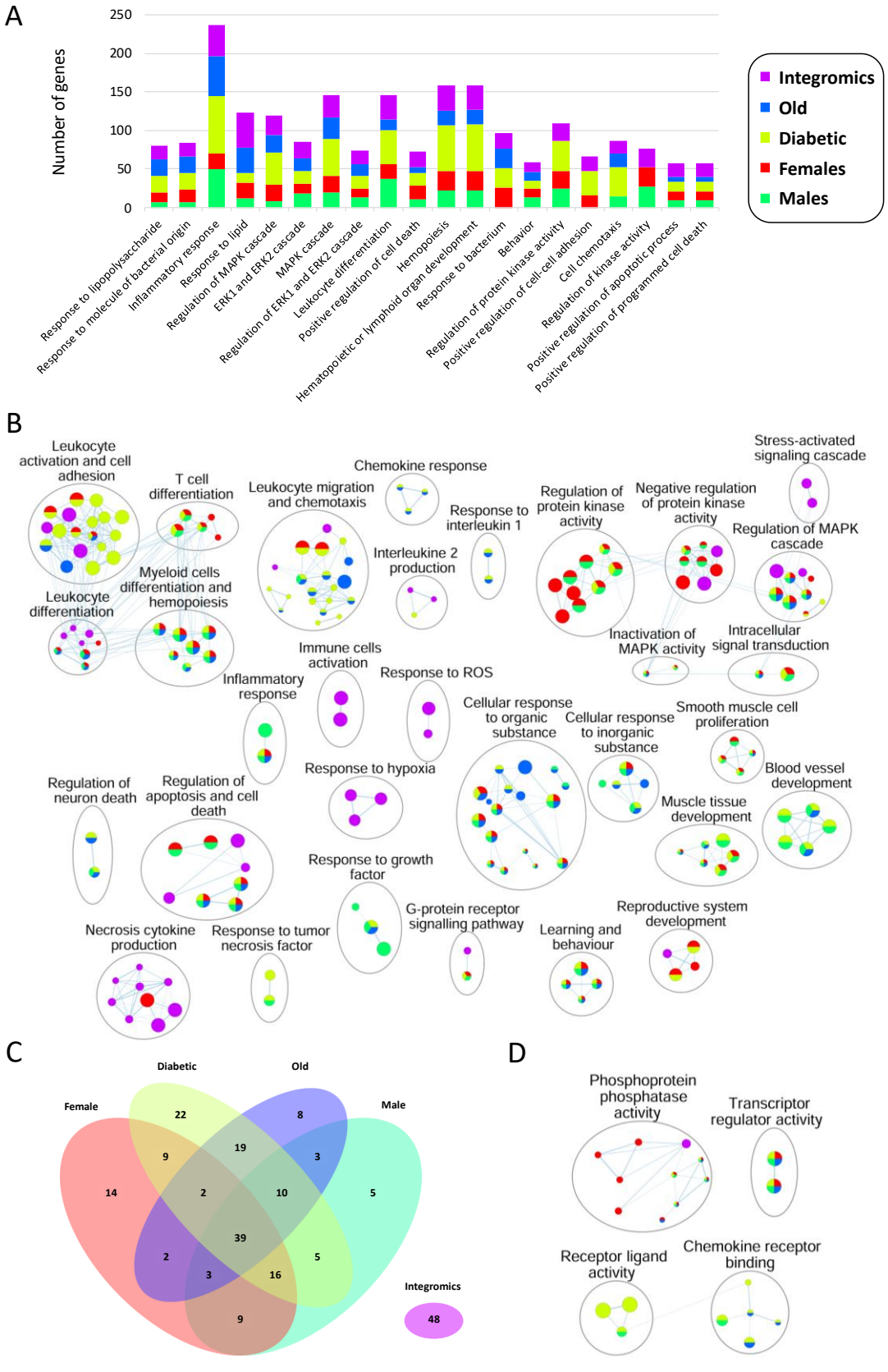
After MCAO induction, 4 proteins were found to be differentially expressed between the IP and the CL brain regions in male mice (FDR<0.25), 14 in *female* mice and 799 in *diabetic* mice. However, there were no differentially expressed (FDR<0.25) proteins in *old* mice when comparing both regions. Moreover, there were no proteins differentially expressed (FDR<0.25) between the IP and the CL in sham animals of any of the groups. The top 15 proteins differentially expressed after cerebral ischemia for each group are shown in table 2.

3.2.1. Integrative analysis of proteomic results

We aimed to identify common proteins dysregulated in the ischemic brain after stroke in all groups, as they will be playing a key role in stroke pathophysiology. However, we found no proteins differentially expressed shared between all the groups. Interestingly, females and diabetic mice were the only groups sharing dysregulated proteins, with 10 common proteins.

Figure 2. Pathway enrichment analysis revealing the main biological processes and molecular functions of differentially expressed genes in the brain 2 hours after cerebral ischemia. ***A*** *Top 20 enriched biological processes; bar plot showing the number of differentially expressed genes of each altered biological process, sorted by increasing p-value from left to right. Each color indicates the corresponding transcriptomic dataset, and purple (integromics) color corresponds to a subset of enriched pathways with combined evidence that were only detected through integrating the data of all groups.* ***B*** *Enrichment map of biological processes altered acutely after ischemic stroke. Nodes in the network represent biological processes, and similar biological processes with many common genes are connected. Nodes are colored according to the supporting omics datasets. Only pathways with 2 or more nodes were represented.* ***C*** *Venn diagram of the enriched biological processes in all studied groups.* ***D*** *Enrichment map of*

molecular functions altered after ischemic stroke. Nodes in the network represent molecular functions and similar molecular functions with many common genes are connected. Nodes are colored according to the supporting omics datasets. Diabetic: diabetic young male mice; Female: young female mice; Male: young male mice; Old: aged male mice



Young male mice, *males*				Young female mice, *females*				Diabetic young male mice, *diabetic*				Aged male mice, *old*			
UNIPROT	**logFC**	**P.Value**	**FDR**	**UNIPROT**	**logFC**	**P.Value**	**FDR**	**UNIPROT**	**logFC**	**P.Value**	**FDR**	**UNIPROT**	**logFC**	**P.Value**	**FDR**
Q8CI32 / Bag5	-0.3250	2.82E-08	7.01E-05	Q8BKX1 / Baiap2	0.4799	1.62E-05	0.0413	Q8VBY2 / Camkk1	-0.4021	1.55E-07	3.85E-04				
P11798 / Camk2a	-0.2119	9.70E-05	0.1205	Q8CGY8 / Ogt	-0.2743	1.29E-04	0.1068	O70433 / Fhl2	-0.6287	5.24E-07	6.51E-04				
Q6PHZ2 / Camk2d	-0.1114	3.09E-04	0.2427	Q920P5 / Ak5	-0.2659	1.43E-04	0.1068	Q62318 / Trim28	-0.2786	1.21E-06	0.0010				
O35927 / Ctnnd2	-0.0972	3.91E-04	0.2427	F6SEU4 / Syngap1	0.4880	1.93E-04	0.1068	Q8R570 / Snap47	-0.4342	6.25E-06	0.0037				
				P68404 / Prkcb	-0.2485	2.09E-04	0.1068	P27005 / S100a8	0.7479	7.42E-06	0.0037				
				P08414 / Camk4	-0.3514	4.61E-04	0.1680	P08414 / Camk4	-0.5602	9.31E-06	0.0039				
				Q9Z2Y3 / Homer1	0.2486	5.01E-04	0.1680	Q64337 / Sqtm1	-0.4070	1.33E-05	0.0041				
				E9Q0K9 / Elmod1	-0.3820	5.27E-04	0.1680	Q9WV34 / Mpp2	-0.2949	1.34E-05	0.0041				
				Q8BFT9 / Svop	0.2494	7.22E-04	0.2046	Q8BFR5 / Tufm	-0.3072	1.68E-05	0.0046				
				Q61329 / Zfhx3	0.1805	8.62E-04	0.2200	Q8K0E8 / Fgb	0.8275	1.99E-05	0.0049				
				Q8VEA4 / Chchd4	0.2649	1.01E-03	0.2201	Q3UHD6 / Snx27	-0.5525	2.30E-05	0.0049				
				E9QK62 / Ngef	-0.2693	1.04E-03	0.2201	Q68FF6 / Git1	-0.4539	2.38E-05	0.0049				
				P97822 / Anp32e	-0.1824	1.19E-03	0.2332	Q920P5 / Ak5	-0.5258	2.73E-05	0.0052				
				O88737 / Bsn	0.2131	1.28E-03	0.2332	P68404 / Prkcb	-0.2960	3.87E-05	0.0069				
								P04627 / Araf	-0.4653	4.54E-05	0.0070				

Table 2. Top 15 proteins differentially expressed (FDR<0.25) between the ipsilateral hemisphere and the contralateral hemisphere 2 hours after cerebral ischemia induction FDR: false discovery rate; logFC: Logarithmic fold change.

4. Discussion

The present study identifies key transcriptomic and proteomic changes triggered acutely after cerebral ischemia in brain samples of mice of different groups: (1) young male mice, (2) young female mice, (3) aged male mice and (4) diabetic young male mice, through mass spectrometry and microarrays. Moreover, the transcriptomics data generated has been integrated and analyzed together to reveal molecules and pathways with key roles in the hyperacute phase of stroke pathophysiology.

To date, various studies have devoted their efforts to describe proteomic and transcriptomic changes that occur in the brain after stroke using mice models of cerebral ischemia. These studies have provided valuable knowledge that has contributed to a better characterization of stroke pathology. This better comprehension of the disease has aided in selecting new candidates to be studied as neuroprotectants and/or biomarkers [36–38]. However, the vast majority of these studies are performed in young healthy male mice, with relatively fewer studies using females, and anecdotic studies using mice with comorbidities and aged mice [10]. However, it has been demonstrated that the response triggered after stroke is affected by sex, age and comorbidities [39,40]. Importantly, it has been suggested that this lack of precision in representing the population mostly affected by stroke in pre-clinical studies, might be one of the reasons explaining the systematic failure in translating the promising neuroprotection results obtained in pre-clinical studies into the clinical setting [10,41]. Taking all this into account, we aimed at evaluating similarities and differences in protein and gene changes triggered acutely after cerebral ischemia in mice of four different groups differing in sex, age and the presence of comorbidities to elucidate if differences in sex or comorbidities could explain the off-target molecules of choice when using standard young male models.

Regarding the transcriptomics results, it is worth noting that the number of DEG after cerebral ischemia is similar between *males* and *females* (61 and 77 respectively), while in *old* animals the number of DEG is reduced to 24 and in *diabetic* animals there is an important increase up to 699. In this line, our results suggest that the post-ischemic expression level of transcripts might be attenuated with age. In fact, an age-dependent decline in transcriptional homeostasis has been described before and is being extensively studied, although to date is poorly understood [42,43]. On the contrary, the expression level of transcripts after cerebral ischemia could be exacerbated by the presence of comorbidities such as diabetes, indicating that cerebral ischemia might be altering a higher number of molecular processes and pathways in that latter group of animals.

Considering the top 15 genes that are differentially expressed in each group (regarding their FDR), most of them are common in all four groups and mainly encode for transcription factors, chemokines and GTPases, which are known to be key players during the hyperacute phase of the disease [44–47]. From all the DEG, it is worth noting that 35 of them are shared between *males* and *females*, but 27 are exclusive for females and 16 for males, highlighting that divergences due to sex are an important factor to be taken into account. In fact, the existence of significant sexual dimorphism in stroke response has been described before. For example, genetic and epigenetic factors, differential activation of cell-death programs, cell-cell signaling pathways, and systemic immune responses have been proposed as contributors to sex differences in ischemic stroke [39]. Actually, various cell signaling pathways such as ischemia-induced cell death, mitochondrial metabolism and inflammation are different between males and females [48–50]. As an example, we have found that the acute inflammatory response is one of the biological processes more altered within the first hours after stroke, and our results show that *males* had a greater number of DEG related to this process than *females*. It has been shown that sex hormones can regulate the immune system, as the majority of immune cells have receptors for these molecules [51]. In the same line, progesterone has been associated with a reduction of pro-inflammatory cytokines, while depending on the context estrogen can either promote or inhibit the inflammatory response [52–54]. In this regard, there are various neuroprotective agents targeting inflammation that have shown promising results in pre-clinical studies but have failed in clinical translation, such as Anakinra, Enlimomab and Natalizumab [55]. Considering our results, it is plausible to think that the differences observed in the regulation of inflammatory response between *males* and *females*, might be one of the possible reasons influencing the failure of translating the preliminary results obtained into the whole population. In reference to *old* mice, our results show that there is a significant reduction in the number of DEG after the insult, being most of the DEG shared with the other groups and only 4 of them exclusively dysregulated in *old* mice. Interestingly, a previous study exploring the effect of age in the transcriptional response to ischemic stroke, reported that aged mice differentially regulated more genes than young mice 3 days after the event [56]. In the present study, we have found that within the hyperacute phase of the disease (2 hours after the occlusion) aged animals have less DEG than their young counterparts, which might suggest that the transcriptional response to cerebral ischemia might be a slower process in aged mice, although at later time points the response might be exacerbated. Regarding the biological processes and molecular functions altered after ischemic stroke, we found clear differences between aged mice and the other groups regarding cell adhesion processes and regulation of protein kinase activity, which seem to be unaltered in aged animals. Protein kinases are involved in the majority

of cellular pathways, especially cell signaling and signal transduction [57,58]. In addition, cell adhesion processes are triggered after cerebral ischemia to mediate the infiltration of leukocytes into the brain parenchyma [59]. The fact that we did not find significant DEG involved in these two processes in aged mice, highlights the importance of further studying their relationship with aging and stroke response. In this regard, considering that aged patients have poorer outcomes and higher rates of mortality after stroke, pharmacologically modulating molecules involved in these biological processes during the hyperacute phase of the disease in the aged population might be an interesting approach to be evaluated as neuroprotective strategies [60].

The presence of comorbidities such as obesity, diabetes and hypertension are known risk factors for ischemic stroke [16]. Moreover, patients with these comorbidities have poorer outcomes after cerebral ischemia [61–63]. We found that diabetic mice had a significant increase in the number of DEG after stroke, being 10 times higher than in the other groups, which might suggest that cerebral ischemia triggers the activation and modulation of an augmented number of biological processes in that group of animals. It has been reported before that after cerebral ischemia diabetic mice had pronounced systemic and vascular inflammation, augmented blood-brain barrier disruption, increased pro-inflammatory response, metabolic dysregulation, severe brain damage and worse neurological deficits [64–66]. Our results support this idea, given that the number of DEG in diabetic mice related to these biological processes is significantly higher than in the other groups. Interestingly, we found an increased number of DEG related to cell adhesion and leukocyte activation that were exclusively dysregulated in diabetic mice. In fact, it has been described that diabetic mice had an augmented number of infiltrating leukocytes in the brain after stroke, indicating that these cells might be responsible for the exacerbated ischemic brain injury in this group of animals [66].

The proteomics results are in agreement with the ones found in transcriptomics. Diabetic animals had an enormous number of proteins altered in the ischemic core in comparison to all other groups, while in *old* animals we did not find any dysregulated protein, maybe due to the early time-point in which samples have been obtained. Regarding *males* and *females*, we found a similar number of altered proteins in both groups, although it was higher in females, as it was observed in the transcriptomics results. In general, our results suggest that in the studied time-point genes had a higher contribution to the observed alterations triggered after cerebral ischemia than proteins. One possible reason for this might be the early time point in which we have evaluated the changes triggered after ischemia. In this regard, performing a similar study and analysis at later time points will give complementary and valuable information to better characterize divergences and similarities in cerebral ischemia response between the studied

groups. In addition to this, various biological reasons could be influencing the observed divergence between altered proteins and genes such as differential post-transcriptional mechanisms, the tight regulation of translation, and differences in the turnover of proteins and mRNAs [67–69]. Given the scarce amount of dysregulated proteins that we obtained in 3 of the studied groups, we were not able to perform an integrative analysis.

Altogether, we show robust data supporting differences in the molecular responses triggered by cerebral ischemia depending on the sex, age and the presence of comorbidities. These divergences might be one of the reasons explaining the choice of inappropriate therapeutic targets and the posterior failure in clinical translation of the main neuroprotective agents tested in pre-clinical studies up to the date. In order to improve future study designs to make clinical implementation more effective, the Stroke Therapy Academic Industry Roundtable (STAIR) published some recommendations to enhance the quality of preclinical research. Remarkably, these recommendations give special attention to the experimental model, species and strain selection and emphasizing the necessity of considering both sexes when designing experiments. In addition, they highly advocate including animals with comorbidities such as diabetes, obesity or hypertension as well as aged animals in pre-clinical studies, to better represent the human population that most likely will suffer a stroke [17–19]. The results obtained in the present study robustly support the necessity of implementing these recommendations when designing pre-clinical studies to test neuroprotective agents. In this regard, molecules to be targeted in pre-clinical studies should be selected considering the differences and similarities that exist between groups. Moreover, the presence of molecules exclusively dysregulated in one group of animals might open the door to future studies directed towards personalized medicine in subcohorts of stroke patients. For example, if the functional analysis of molecules that are dysregulated in diabetic, *males* and *females* but not in *old* mice showed neuroprotective effects, a pharmacologic activation of these molecules in aged ischemic brains might improve the functional outcome in elderly stroke patients. Conversely, if one of the many molecules dysregulated in diabetic mice shows negative neurodegenerative or even neurotoxic functions, a pharmacological inhibition in diabetic ischemic brains could bear neuroprotective effects. This approach will definitely provide a personalized management of stroke patients, by treating each patient considering its clinical characteristics.

This study stands for some limitations that had to be taken into account. First, regarding the experimental design, ideally, young male mice should have been performed simultaneously to the other groups, to reduce inter-assay variability. In this line, to minimize the variability, each group of animals was analyzed separately and corrected by its own control samples. Second, in the present study we have incorporated 4 different groups of animals differing in sex, age or the

presence of diabetes. However, it would have been also interesting to include additional groups such as aged females, diabetic females or mice with other comorbidities in order to enrich the obtained information. Third, time extrapolations between mice and humans are poorly understood and not well established. A better comprehension of this equivalence is needed to better comprehend the implications of the results presented here. Finally, infarct volume was not assessed in the present study due to the lack of sensitivity of TTC (triphenyltetrazolium chloride) staining to determine the lesion volume 2 hours after occlusion.

All in all, in the present study we have explored similarities and differences in protein and gene changes in the brain after cerebral ischemia depending on age, sex and the presence of diabetes. In this regard, we have revealed that the molecular response to stroke varies depending on the phenotype of the animals, reinforcing the necessity of following STAIR recommendations when designing future studies in order to improve "bench to bedside" translation

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Supplemental table S1. Top 30 differentially expressed genes (FDR<0.25) between the infarcted hemisphere and the contralateral healthy hemisphere 2 hours after cerebral ischemia induction exclusive from each group of animals. FDR: false discovery rate; logFC: Logarithmic fold change.

Young male mice				Young female mice				Diabetic young male mice				Aged male mice			
SYMBOL	**logFC**	**P.Value**	**FDR**	**SYMBOL**	**logFC**	**P.Value**	**FDR**	**SYMBOL**	**logFC**	**P.Value**	**FDR**	**SYMBOL**	**logFC**	**P.Value**	**FDR**
Cldn20	-0.7665	1.84E-04	0.0291	Galnt12	-1.1040	4.58E-05	0.0155	S100a9	1.4113	2.29E-06	6.44E-04	Fkbp11	-1.0158	1.84E-04	0.0820
Themis2	0.7839	1.97E-04	0.0302	Olfr690	-1.0280	4.99E-05	0.0158	S100a8	1.5050	5.19E-06	1.14E-03	Gm9958	-0.9378	2.12E-04	0.0820
Dgat2l6	-0.6872	3.30E-04	0.0468	Bcl2a1c	0.8490	1.09E-03	0.1478	Ccl7	1.1113	1.18E-05	2.22E-03	Dnajb13	0.6565	1.14E-03	0.2467
Gata2	-0.6329	9.20E-04	0.1166	Foxf1	0.7005	1.12E-03	0.1478	Pstk	-0.9176	1.58E-05	2.77E-03	Krtap12-1	-0.7606	1.17E-03	0.2467
Asb5	-0.6768	1.02E-03	0.1245	Tnfrsf10b	0.8084	1.14E-03	0.1478	Ccl2	0.9432	2.94E-05	4.25E-03				
Bmp15	0.6382	1.03E-03	0.1245	Mmp10	-0.8206	1.25E-03	0.1520	Creb5	0.7937	3.27E-05	4.61E-03				
Cited2	0.6148	1.20E-03	0.1385	Olfr492	0.9696	1.32E-03	0.1524	Hp	0.9923	3.90E-05	5.20E-03				
Ccl19	-0.6130	1.60E-03	0.1655	Slc25a25	0.5373	1.66E-03	0.1724	Zdbf2	0.8195	5.38E-05	6.82E-03				
Inmt	-0.6311	1.71E-03	0.1703	Gm13283	0.6909	1.67E-03	0.1724	C2cd4d	0.9371	1.04E-04	0.0120				
Pcsk1	0.5781	1.83E-03	0.1789	Clca1	0.5786	1.74E-03	0.1765	Gpr3	0.7008	1.18E-04	0.0130				
Otud1	0.5725	2.03E-03	0.1868	Olfr577	-0.6981	1.88E-03	0.1816	Paxbp1	0.7046	1.24E-04	0.0131				
Cd163	-0.5586	2.23E-03	0.1985	Tekt4	-0.6663	1.90E-03	0.1816	Retnlg	1.1193	1.45E-04	0.0145				
Zfp36l2	0.5582	2.23E-03	0.1985	Ptger4	0.5667	1.97E-03	0.1850	Nts	1.1122	1.46E-04	0.0145				
Cenpp	0.6483	2.38E-03	0.2042	Cd14	0.7055	2.25E-03	0.2035	Baz1a	0.8028	1.47E-04	0.0145				
Clk1	0.5498	2.64E-03	0.2232	Det1	-0.6143	2.35E-03	0.2035	Cdc42ep3	0.6720	1.48E-04	0.0145				
Serpina3j	-0.5403	2.87E-03	0.2382	Spata4	-0.6911	2.54E-03	0.2106	Med18	-0.7637	1.54E-04	0.0146				
				Fbxo33	0.4942	2.58E-03	0.2106	Nsun3	-0.8242	1.55E-04	0.0146				
				Trhr2	-0.5152	2.79E-03	0.2113	Lilrb4a	1.1014	1.59E-04	0.0147				
				Stil	0.8193	2.80E-03	0.2113	Plk4	-0.7627	1.74E-04	0.0155				
				Tas2r139	0.5987	2.80E-03	0.2113	Sinhcaf	0.8658	2.06E-04	0.0174				
				Olfr401	0.6691	2.83E-03	0.2113	Maff	0.9657	2.32E-04	0.0190				
				Erbb3	0.5773	2.83E-03	0.2113	Adamts9	0.7490	2.57E-04	0.0206				
				Arl5b	0.5858	3.04E-03	0.2113	Hmgb4	0.8023	2.76E-04	0.0216				
				Klf4	0.5976	3.04E-03	0.2113	Cxcl2	0.7927	2.89E-04	0.0222				
				Fkbp5	-0.4809	3.20E-03	0.2196	Gdpgp1	-0.8443	3.09E-04	0.0234				
				Zwilch	-0.5154	3.52E-03	0.2380	Zfp273	-0.6387	3.18E-04	0.0237				
				Fam78a	-0.6184	3.67E-03	0.2414	Mreg	-0.7207	3.30E-04	0.0243				
								Gm9930	0.8930	3.39E-04	0.0245				
								Ehd3	-0.6637	3.78E-04	0.0266				
								Rab2b	-0.6580	4.06E-04	0.0279				

4.3. Circulating Aquaporin-4 as a biomarker of early neurological improvement in stroke patients: A pilot study.

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4.4. Alpha-1 antitrypsin as a biomarker for stroke prognosis

(Manuscript in preparation)

Alpha-1 antitrypsin as a biomarker for stroke prognosis

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Abstract

Patients' outcome prediction after stroke is challenging. Alpha-1 antitrypsin (A1AT) is a serum proteinase inhibitor with anti-inflammatory and anti-apoptotic properties that increases in circulation within few hours after acute inflammatory responses. In the present study we aimed to explore the potential role of A1AT as a prognosis stroke biomarker.

Blood from 80 ischemic and 25 hemorrhagic stroke patients was drawn at hospital admission before any treatment was given. Stroke patients were followed after the event and functional outcome was evaluated at 3rd month according to the modified Rankin Scale (mRS; mRS≥3 = poor outcome).

Baseline A1AT levels were higher in those patients showing poor functional outcome at hospital discharge ($p<0.001$) and 3 months after the event ($p<0.001$). Cut-off values with the highest specificity and sensitivity for each time point were selected, and the resulting dichotomized variables were added to a logistic regression model including age, sex, baseline NIHSS and diabetes. A1AT baseline level was a strong independent predictor of poor functional outcome at both studied time points (Adjusted odds ratio (ORadj): 7.373 [1.804-30.143], $p=0.005$ at discharge; ORadj: 4.492 [1.210-16.668], $p=0.025$ at 3rd month). Moreover, the addition of A1AT to the clinical model increased its discriminatory ability by 5.29% at hospital discharge and by 4.02% at the 3rd month as measured by the integrated discrimination improvement (IDI) index. Overall, our data suggest that A1AT could be used as a biomarker for stroke outcome prediction stroke either in-hospital or at mid-term.

1. Introduction

Stroke is a leading cause of mortality and disability worldwide [1]. In fact, among stroke survivors, around 35-50% of patients present disability after the event [2,3]. Identifying those patients that are at higher risk of presenting poor outcome is crucial to guide decision-making processes to provide optimal stroke patients' management.

From the neurologist's perspective, the prediction of stroke patients' prognosis is complex. Stroke outcome is known to be highly influenced by the baseline demographic and clinical characteristics of the patients and the etiology and stroke location among many other factors. However, clinical complications that occur later during the course of the disease, including hemorrhagic transformations, cerebral edema, infections or secondary events, might also be key factors that affect stroke morbidity [4–7]. In this scenario, the identification of blood biomarkers that aid in predicting stroke patients' outcome might help to select those subjects that should be admitted into specialized stroke units, rehabilitation programs, or into on-going clinical trials to ensure ideal patients' care and ultimately improve their outcome [8].

Alpha-1 antitrypsin (A1AT) is the most abundant serine proteinase inhibitor in human blood [9]. Its main function is the inhibition of neutrophil elastase, but it is also involved in proteostasis and has anti-inflammatory and anti-apoptotic properties [10]. In addition, it has been proposed that A1AT may play a role in the stability of arterial walls, given that several studies have reported an association between A1AT levels and intracranial and aortic aneurysms formation and rupture [11–13]. Severe deficiency of A1AT has been related with the development of emphysema, bronchiectasis and/or liver disease [14]. In contrast, A1AT has been shown to increase rapidly in circulation during acute systemic inflammatory responses, including stroke [15,16]. In this same regard, higher levels of A1AT in the bloodstream are also associated with an elevated risk of developing stroke [17]. However, the association between A1AT and stroke outcome and its plausible role as a biomarker for stroke prognosis remains unknown. To that end, this study aimed to determine whether A1AT circulating levels measured acutely after stroke could aid in predicting stroke patients' outcome in the subacute and mid-term phases of the disease.

2. Materials and methods

2.1. Study population

Patients with an acute ischemic stroke (IS) or an intracerebral hemorrhage (ICH) admitted to the emergency department at Vall d'Hebron University Hospital (Barcelona, Spain) within the first 6 hours after symptoms onset were prospectively studied. A total of 80 IS and 25 ICH patients were recruited for the present study. Inclusion criteria for this study included the following parameters: suspected stroke at the first medical assessment with persistent symptoms when arriving at the emergency room;

age > 18 years; <6 hours from symptom onset to blood sample collection; blood collection preceding thrombolytic treatment (if eligible); and signed informed consent. Forty-one ischemic stroke patients received tissue plasminogen activator (t-PA) in a standard 0.9mg/kg dose (10% bolus, 90% continuous infusion during 1 hour), 3 were subjected to mechanical thrombectomy and 19 underwent both reperfusion strategies.

Additionally, a control group of 8 healthy subjects free from inflammatory or infectious disease [18] was included to set reference A1AT levels.

2.2. Clinical protocol

The study was approved by the Ethics Committee (PR(AG)80/2012) [19] and conducted in accordance with the Declaration of Helsinki. All patients or relatives gave informed consent. A detailed history of vascular risk factors was obtained from each patient. Stroke severity at hospital admission was assessed using the National Institutes of Health Stroke Scale (NIHSS). The clinical follow-up of each stroke patient was carried out until 3rd month after stroke. Functional outcome was evaluated according to the modified Rankin Scale (mRS) [20]; patients with a mRS score lower or equal than 2 were classified as having 'good outcome' and patients with a mRS from 3 to 6 as having 'poor outcome'.

2.3. Blood sample collection and A1AT measurement

Peripheral blood samples were drawn in all patients at hospital admission (<6 hours after stroke onset) before any treatment was given. Blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes, centrifuged at 1500 g for 15 min at 4 ºC, and plasma aliquots were frozen at -80 ºC until biomarker analysis.

Commercial ELISA (Enzyme-Linked ImmunoSorbent Assay) kits were used and manufacturer's instructions were followed to quantify circulating levels of A1AT (Cat#. 108799, Abcam, UK). Optical density was measured using a SynergyTM Mx microplate reader (BioTek Instruments Inc, USA). Each sample was assayed twice and the mean value of both measurements was used. Samples with a coefficient of variation between duplicates higher than 20% were excluded from the analysis. All samples were within the detectable range of the assay.

2.4. Statistical analyses

SPSS statistical package 20.0 (IBM Corporation, Armonk, NY, USA) was used for statistical analyses, and graphs were generated using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Normality was assessed by Kolmogorov–Smirnov test. For normal distributed continuous variables t-test (mean ± SD) was used, whereas for variables with non-normal distribution Mann–Whitney test was used (median and interquartile range). Chi-squared test was performed to assess intergroup differences for categorical variables, expressed as frequencies. Correlations between continuous variables were calculated using Spearman's test. Receiver operating characteristics (ROC) curves were used to obtain the cut-off points of A1AT to predict outcome with optimal accuracy (both sensitivity and specificity).

Forward stepwise multivariate logistic regression analyses for stroke outcome were performed with all clinical variables associated with each studied end-point. Using the selected cut-off points, baseline A1AT level with the selected cut-off point was added to the clinical model at the last step to assess its independent association and to build a new predictive model. The R software (v3.4.4; R Development Core Team 2012, Vienna, Austria) was used to compare the areas under the ROC curve (AUC) from the predictive models, comparing the goodness of the fit of the two logistic regression models (Likelihood-ratio test, lmtest package). In all cases, a p-value <0.05 was considered statistically significant at a 95% confidence level. The integrated discrimination improvement (IDI) index determined the added value of A1AT to the clinical models for the studied end-points (Hmisc and PredictABEL R packages).

3. Results

There were no significant differences between ischemic and hemorrhagic stroke patients regarding demographic and clinical characteristics (table 1). Circulating levels of A1AT were detected in all subjects, and no differences were found among ischemic and hemorrhagic stroke patients (p=0.950).

Factors	Ischemic strokes (n=80)	Hemorrhagic strokes (n=25)	p-value
Age, years	75 (64-81)	75 (66-82)	0.647
Gender (Male), n (%)	41 (50.6%)	14 (56%)	0.638
Admission NIHSS score	10 (6.5-18)	9 (4-19)	0.559
Smokers, n (%)	11 (15.1%)	6 (27.3%)	0.212
Arterial hypertension, n (%)	55 (68.8%)	22 (88%)	0.057
Diabetes mellitus, n (%)	23 (28.4%)	6 (24%)	0.667
Dyslipidemia, n (%)	43 (53.1%)	11 (44%)	0.427
Atrial Fibrillation, n (%)	24 (29.6%)	5 (20%)	0.345
Previous stroke, n (%)	13 (16%)	5 (20%)	0.737
Cardiopathy, n (%)	15 (18.5%)	2 (8.3%)	0.348
TOAST			
Atherothrombotic	17 (21%)	-	
Cardioembolic	29 (35.8%)	-	
Undetermined	27 833.3%)	-	
Lacunar	6 (7.4%)	-	
Other causes	2 (2.5%)	-	
Admission A1AT levels (mg/dL)	123.87 (100.62-158.36)	123.94 (101.82-154.98)	0.950

Table 1. Demographic and clinical information from patients included in the study. A1AT: Alpha-1 antitrypsin; ICH: Hemorrhagic stroke patients; IS: ischemic stroke patient; NIHSS: National Institutes of Health stroke scale; TOAST: Trial of Org 10172 in Acute Stroke Treatment.

Next, we aimed to evaluate the practical use of A1AT as a biomarker of functional outcome after stroke. Of all stroke patients, 56 (53.3%) exhibited a poor functional outcome by the time of hospital discharge

(in-hospital outcome). The median time from stroke onset to hospital discharge was 5.5 (3-10) days. Those patients were older, had a higher baseline NIHSS score and a higher prevalence of atrial fibrillation than patients presenting good functional outcome at the studied time-point (Table 2). Notably, baseline circulating levels of A1AT were also higher in patients with poor functional outcome than those with good functional outcome [110.4 (92.5-134.6) mg/dL vs 148.1 (115.9-179.6) mg/dL, p<0.001] (Figure 1A).

In the same line, we also assessed patients' outcome 3 months after stroke (mid-term outcome). At that time, 53 patients (51.5%) exhibited poor functional outcome. Those patients were older, had a higher baseline NIHSS score and a higher prevalence of atrial fibrillation and diabetes than patients presenting good functional outcome (Table 2). Remarkably, baseline circulating levels of A1AT were also higher in patients with poor mid-term functional outcome than those with good mid-term functional outcome [110.2 (91.9-140.3) mg/dL vs. 147.7 (113.6-174.8) mg/dL, p<0.001] (Figure 1B).

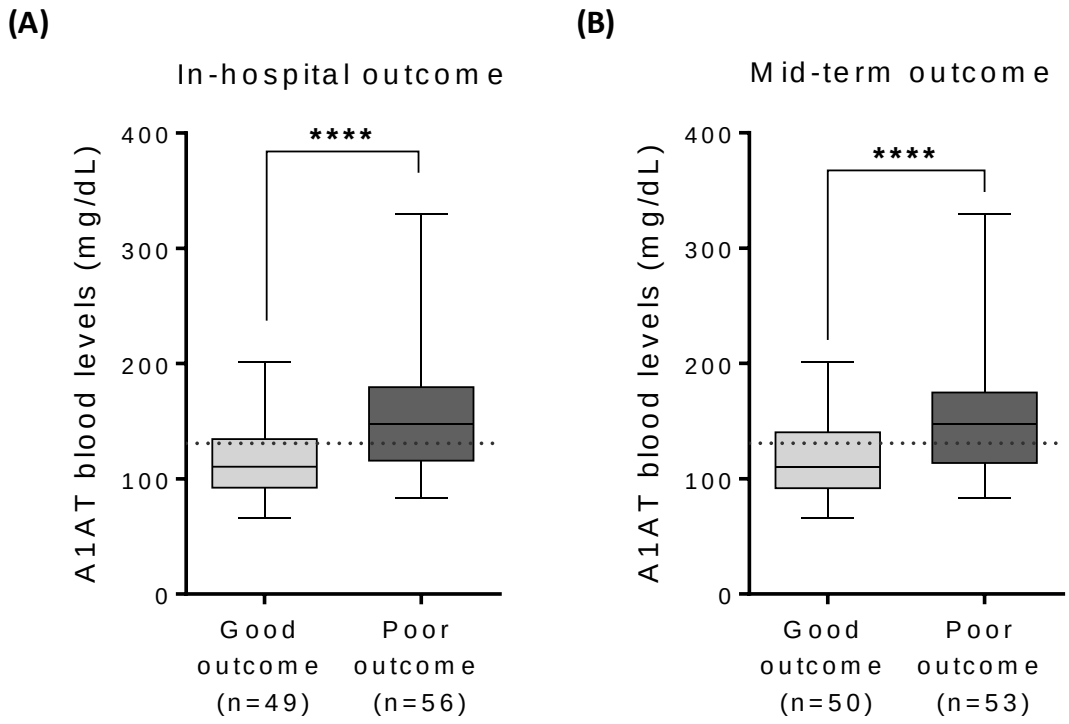

*Figure 1. Association between A1AT levels at admission and functional outcome (A) in-hospital (p<0.001) and (B) at mid-term (p<0.001). Box-plots represent the median and interquartile range (IQR). Error bars indicate the 95% confidence interval. **** indicates p<0.001. Dashed lines represent median values in control population (n=8) as reference. A1AT: Alpha-1 antitrypsin.*

We next sought to determine the best cut-off points of A1AT levels in terms of accuracy to predict outcome at both studied time-points. Through a ROC curve analysis, we determined a cut-off point of

156.96 mg/dL for A1AT significantly associated poor functional outcome at hospital discharge, with 41% sensitivity and 91.8% specificity (p<0.001) (Table 2). We created a predictive clinical model for stroke outcome including age and the NIHSS score at admission, which resulted to be independent predictors of in-hospital poor outcome. Notably, A1AT remained an independent factor associated with poor outcome at hospital discharge when a baseline level of A1AT>156.96 mg/dL was included in this clinical model (Table 3). Moreover, the addition of A1AT in the clinical model substantially improved the goodness-of-fit of the model, increasing the AUC by 3% [logarithmic likelihood ratio (logLR) clinical model =-46.42; logLR clinical model + A1AT = -41.82; p=0.002] (Figure 2A). Also, A1AT increased the discriminatory ability of the clinical model alone by 5.29%, as measured by IDI index (Table 3).

In the same line, we also determined a second cut-off point of 161.21 mg/dL for A1AT that was significantly associated with mid-term poor functional, with 35.8% sensitivity and 92% specificity (p=0.001) (Table 2). We created a predictive clinical model for mid-term stroke outcome including age, NIHSS score at admission and diabetes, which resulted to be independent predictors of this end-point. Of note, A1AT remained an independent predictor of mid-term poor outcome when a baseline level of A1AT>161.21 mg/dL was included in this clinical model (Table 3). The addition of A1AT as an independent variable also considerably improved its goodness-of-fit, increasing the AUC a 2% (logLR clinical model =-51.81; logLR clinical model + A1AT = -48.94; p=0.017) (Figure 2B). Moreover, A1AT further increased the discriminatory ability of the clinical model by 4.02%, measured by IDI index (Table 3).

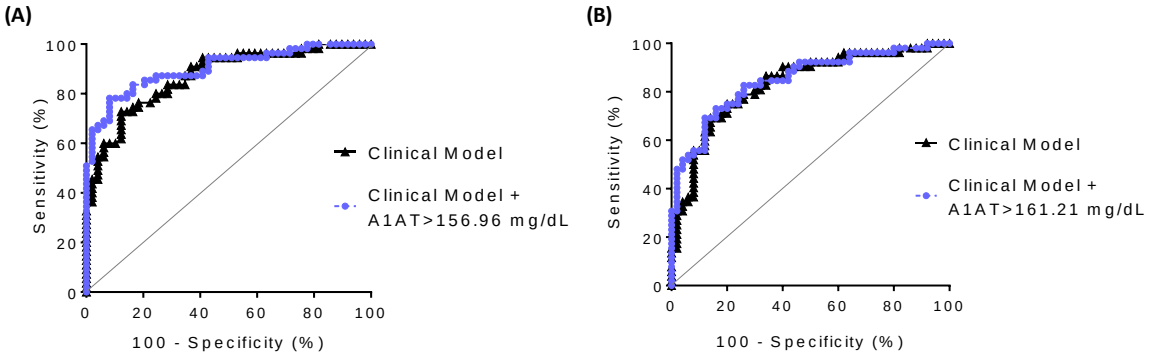

Figure 2. ROC curve of the multivariate regression models created to predict poor functional outcome in-hospital and at mid-term after stroke in our study population. Both clinical models were adjusted by basal NIHSS, age and sex, and diabetes was included in the case of mid-term poor functional outcome. **(A)** ROC curves to predict in-hospital poor functional outcome. **(B)** ROC curves to predict mid-term poor functional outcome. A1AT: Alpha-1 antitrypsin.

Factors	**In-hospital** functional outcome		p-value	**Mid-term** functional outcome		p-value
	Good outcome (n=49)	Poor outcome (n=56)		Good outcome (n=50)	Poor outcome (n=53)	
Age, years	71 (59-78)	79 (70-83)	**<0.001**	71.5 (59-77)	79 (70-83)	**<0.001**
Gender (Male), n (%)	26 (53.1%)	28 (50%)	0.754	26 (52%)	29 (54.7%)	0.782
Admission NIHSS score	6 (3-11)	17 (10-20.5)	**<0.001**	8 (3-13)	16 (9-20.5)	**<0.001**
Smokers, n (%)	10 (22.7%)	7 (14%)	0.273	8 (17.8%)	8 (17%)	0.924
Arterial hypertension, n (%)	32 (65.3%)	44 (80%)	0.092	32 (65.3%)	43 (81.1%)	0.070
Diabetes mellitus, n (%)	11 (22.5%)	18 (32.1%)	0.268	9 (18%)	19 (35.8%)	**0.042**
Dyslipidemia, n (%)	25 (51%)	28 (50%)	0.917	24 (48%)	29 (54.7%)	0.495
Atrial Fibrillation, n (%)	6 (12.2%)	23 (41.1%)	**0.001**	6 (12%)	23 (43.4%)	**<0.001**
Previous stroke, n (%)	7 (14.3%)	7 (12.7%)	0.836	6 (12%)	10 (18.9%)	0.336
Cardiopathy, n (%)	5 (10.4%)	12 (21.4%)	0.130	5 (10.2%)	12 (22.6%)	0.092
A1AT (mg/dL)	110.4 (92.5-134.6)	148.1 (115.9-179.6)	**<0.001**	110.2 (91.9-140.3)	147.7 (113.6-174.8)	**<0.001**

Table 2. Univariate analysis. Clinical characteristics associated with in-hospital and mid-term functional outcome. P<0.05 is marked in bold. A1AT: Alpha-1 antitrypsin; NIHSS: National Institutes of Health stroke scale.

	In-hospital poor functional outcome		**Mid-term** poor functional outcome	
	Only clinical model	**Clinical model + A1AT**	**Only clinical model**	**Clinical model + A1AT**
Logistic regression (OR_{adj})				
Basal NIHSS	1.245 (1.139-1.360), **p<0.001**	1.261 (1.143-1.392), **p<0.001**	1.168 (1.083-1.260), **p<0.001**	1.166 (1.077-1.262), **p<0.001**
Age	1.094 (1.037-1.153), **p=0.001**	1.086 (1.028-1.148), **p=0.004**	1.081 (1.028-1.138), **p=0.002**	1.076 (1.021-1.134), **p=0.006**
Diabetes	-	-	3.320 (1.111-9.921), **p=0.032**	2.951 (0.969-8.982), p=0.057
A1AT>156.96 mg/dL		7.373 (1.804-30.143), **p=0.005**	-	-
A1AT>161.21 mg/dL	-	-		4.492 (1.210-16.668), **p=0.025**
ROC curves				
AUC	87.1% (80.4-93.7)	90.2% (84.4-96.0)	83.4% (75.5-91.3)	85.0% (77.6-92.4)
Likelihood ratio	**p=0.002**		**p=0.017**	
IDI statistics				
IDI	Ref	5.29% (1.75-11.13), **p=0.032**	Ref	4.02% (0.04-8.05%), **p=0.05**
IDI events	-	2.49%	-	1.97%
IDI nonevents	-	2.80%	-	2.05%

Table 3. Predictive models for poor outcome at hospital discharge and at mid-term after stroke. For logistic regression models, Adjusted Odds ratio (ORadj) [95% confidential interval (CI)] and p-values are given (p<0.05 are marked in bold). A1AT was added to the clinical logistic regression model using the cut-off point of 156.96 mg/dL for in-hospital poor functional outcome and 161.21 mg/dL for mid-term poor functional outcome. AUC is given for each model with 95% CI. A1AT: Alpha-1 antitrypsin; AUC: area under the curve. IDI: integrated discrimination improvement index. Percentages of reclassification are given for events, non-events and for the sum of both (with 95% CI).

4. Discussion

Stroke outcome prediction is relevant to aid in decision-making processes and to ensure optimal patients' management after stroke. Nowadays, outcome prediction after cerebral ischemia is mainly based on patients' clinical information, such as age, comorbidities and stroke severity [21,22]. Various studies support the idea that the addition of blood biomarkers to these clinical models to predict stroke outcome might improve their predictive capacity [23–25]. In the present study, we have explored the association of A1AT levels measured within hyperacute phase of stroke with its sub-acute and mid-term prognosis. Here we have shown that circulating A1AT levels measured at patients' hospital admission are an independent predictor of poor functional outcome both at hospital discharge and 3 months after stroke.

Increased bloodstream levels of A1AT have previously been described to be associated with poorer outcomes in hepatocellular carcinoma and pancreatic cancer [26,27]. However, information regarding A1AT and its role in stroke pathophysiology is limited. Augmented levels of A1AT have been associated with a higher risk of developing stroke [17]. Moreover, increased levels of A1AT have been found in the infarct core of the brain when compared to the healthy contralateral area [28,29]. A pilot study measuring A1AT levels within the first 24 hours after ischemic stroke in 18 patients showed an association of these levels with calcification on major cervico-cranial arteries [30]. In addition, they reported that higher circulating A1AT levels were also associated with larger infarct volumes but not with the functional outcome at hospital discharge. Concerning neurological outcome, another study described that circulating levels of A1AT measured within the first 24 hours after atherotrombotic ischemic stroke were higher in those patients with poor neurological outcome 1 month after the event [31].

In the present study, we found no differences in A1AT levels in the bloodstream between stroke patients and controls in the hyperacute phase of the disease (<6 hours after symptoms onset). In fact, it has been previously described that A1AT progressively increases in circulation within the first 24 hours after stroke [29], reaching maximum levels 72-96 hours after the event [16]. Interestingly, we have observed that patients showing in-hospital and mild-term poor functional outcome had increased circulating levels of A1AT by the time of hospital admission. Therefore, bloodstream A1AT levels measured early after stroke, before any treatment is given, have demonstrated a good predictive capacity of stroke patients' outcome. Our results point out that A1AT baseline levels would predict more accurately in-hospital than mid-term stroke outcome. In this line, it would be interesting to explore the association of A1AT levels with post-stroke complications that occur during patients' hospitalization, which are known to highly influence patients' outcome. Of note, we have shown for the first time that this molecule is associated with poor outcome in both ischemic and hemorrhagic stroke patients, broadening the clinical applicability of this biomarker. It is also worth mentioning that

A1AT is routinely measured in the clinical laboratories of the vast majority of hospitals [32], which further paves the way for rapid clinical implementation of A1AT measurement to predict stroke outcome.

The pathophysiological reason for this increased A1AT circulating levels in patients presenting poorer outcomes is complex to determine. The administration of A1AT after cerebral ischemia in animal models have shown to exert neuroprotective effects by reducing the infarct size [33]. This beneficial effect has been attributed to the capacity of A1AT to modulate the activity of immune system cells [34]. Interestingly, it has been demonstrated that A1AT protects neurons from necrotic and apoptotic cell death after oxygen and glucose deprivation (OGD) through the reduction of pro-inflammatory cytokines levels and the increase of anti-inflammatory cytokines. In this line, A1AT is also able to protect astroglia and oligodendroglia from OGD-induced cell death, reinforcing its important role in protecting the ischemic brain [35]. Considering the results of the present study, we can hypothesize that the increased A1AT levels observed in patients presenting poor outcome may reflect a protective response mechanism that would take place as an attempt to mitigate the pathological processes triggered due to cerebral ischemia, although without success. However, it can not be discarded that this upregulation of A1AT was contributing to the expansion of the ischemic lesion and consequently to the worsening of stroke patients. In this line, further studies are needed to figure out the biological and functional implications of A1AT in the context of stroke.

The present study stands some limitations that need to be addressed in future studies. First, we are aware that our sample size is relatively small, therefore, our results should be validated in larger cohorts of patients. Second, it would be interesting to know if any subject presented A1AT deficiency, given that although it is quite improbable, it should be influencing the results presented here.

In conclusion, our results demonstrate that bloodstream A1AT levels measured acutely after stroke might potentially serve as a predictor of poor functional outcome after stroke at the sub-acute phase and at mid-term. Thus, A1AT could become a very useful variable to take into account when deciding the optimal clinical procedure for every patient after their admission to the stroke care unit. Ultimately, a good and rapid determination of patients' outcome would help to reduce sanitary costs and to anticipate the inclusion of patients in specialized units to receive intensive therapies, to finally improve their outcome and quality of life.

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6. Declaration of conflicting interests

A.S., L.R. and J.M. are co-inventors of a patent covering "Biomarkers for stroke prognosis" (Application nº EP19382599.9) owned by Vall d'Hebron Institute of Research (VHIR).

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4.5. Blood biomarkers to predict long-term mortality after ischemic stroke.

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Blood Biomarkers to Predict Long-Term Mortality after Ischemic Stroke

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Abstract: Stroke is a major cause of disability and death globally, and prediction of mortality represents a crucial challenge. We aimed to identify blood biomarkers measured during acute ischemic stroke that could predict long-term mortality. Nine hundred and forty-one ischemic stroke patients were prospectively recruited in the Stroke-Chip study. Post-stroke mortality was evaluated during a median 4.8-year follow-up. A 14-biomarker panel was analyzed by immunoassays in blood samples obtained at hospital admission. Biomarkers were normalized and standardized using *Z*-scores. Multiple Cox regression models were used to identify clinical variables and biomarkers independently associated with long-term mortality and mortality due to stroke. In the multivariate analysis, the independent predictors of long-term mortality were age, female sex, hypertension, glycemia, and baseline National Institutes of Health Stroke Scale (NIHSS) score. Independent blood biomarkers predictive of long-term mortality were endostatin > quartile 2, tumor necrosis factor receptor-1 (TNF-R1) > quartile 2, and interleukin (IL)-6 > quartile 2. The risk of mortality when these three biomarkers were combined increased up to 69%. The addition of the biomarkers to clinical predictors improved the discrimination (integrative discriminative improvement (IDI) 0.022 (0.007–0.048), $p < 0.001$). Moreover, endostatin > quartile 3 was an independent predictor of mortality due to stroke. Altogether, endostatin, TNF-R1, and IL-6 circulating levels may aid in long-term mortality prediction after stroke.

Keywords: ischemic stroke; biomarker; mortality; endostatin; IL-6; TNF-R1

1. Introduction

Stroke is a leading cause of death and long-term disability worldwide. In fact, in 2017, someone died due to stroke every 3.5 min, a total of 2.7 million individuals in the whole year, so it is a major health and socioeconomic concern [1]. An early prediction

of this fatal outcome after cerebral ischemia can facilitate decision-making processes, such as patients' inclusion to clinical trials or early admission to specialized stroke units, ultimately optimizing patients' management. However, predicting stroke outcome remains challenging. Various attempts to identify characteristics associated with poor outcomes have been made. Different predictive models for mortality in stroke patients have been developed, with an accuracy around 80% in most models [2–5], although none of them are routinely used in clinical practice yet. Many of these models include clinical variables, such as age and stroke severity, as predictors of poor outcome and mortality [6–8]. It has been suggested that the addition of blood biomarkers to a clinical model might improve its prediction capacity. However, the vast majority of studies have explored the role of blood biomarkers in predicting outcome and mortality early after stroke, being the outcome measured in most studies 3 months after the event [9–12]. Whether circulating proteins measured acutely after ischemic stroke can predict long-term mortality is still unclear.

For this reason, in the present study, we aimed to analyze whether blood biomarkers measured during acute ischemic stroke could predict its long-term mortality. To that end, the association between 14 molecules analyzed in the blood in the acute phase of stroke and five-year mortality was assessed in 941 ischemic stroke patients.

2. Materials and Methods

2.1. Study Design

A prospective longitudinal study was conducted to evaluate long-term mortality in 941 adult patients with ischemic stroke, previously recruited in the Stroke-Chip study (PR(AG)80/2012) [13]. In summary, inclusion criteria were suspected stroke at first medical assessment, with persistent symptoms when arriving at the emergency room; age > 18 years; <6 h from symptom onset to blood sample collection; blood collection preceding thrombolytic treatment; and signed informed consent. The only exclusion criteria was impossibility of collecting blood samples. Moreover, patients with an unclear diagnosis 1 month after the index event were excluded from the analysis. Stroke diagnosis was performed by trained neurologists at each center, according to the World Health Organization [14] definition, and confirmed by neuroimaging. Clinical and demographic data, as well as biomarker results obtained during the acute phase in the Stroke-Chip study, were analyzed with data obtained over a median follow-up of 4.8 years to explore predictors of long-term mortality.

The study was approved by the Ethics Committee of each recruiting center, and all patients or relatives gave written informed consent.

2.2. Blood Sample Collection and Biomarker Measurement

Blood samples were drawn at hospital admission, within 6 h after symptom onset, and before any treatment was given. Blood was collected into ethylenediaminetetraacetic acid (EDTA) tubes, centrifuged at 1500× *g* for 15 min at 4 °C, and plasma aliquots were frozen at −80 °C until biomarker analysis. A 14-biomarker panel was used in the original study to distinguish between strokes and stroke mimics, as well as ischemic stroke from intracranial hemorrhage, and these were analyzed in the present study. This panel included apolipoprotein CIII (ApoC-III), D-dimer, endostatin, Fas ligand (FasL), growth-related oncogene-α (GROA), heat shock 70 kDa protein-8 (Hsc70), insulin-like growth factor-binding protein-3 (IGFBP-3), interleukin-6 (IL-6), neuron cell adhesion molecule (NCAM), N-terminal pro-B-type natriuretic peptide (NT-proBNP), S100 calcium-binding protein (S100B), tumor necrosis factor receptor-1 (TNF-R1), vascular adhesion protein-1 (VAP-1), and Von Willebrand factor (vWF). Biomarker measurements were performed using immunoassays, according to the manufacturers' instructions and blinded to clinical diagnoses, as described before [13]. All samples were tested in duplicate, and the mean coefficient of variation (CV) was <20%. Inter-assay variation was checked by testing in duplicate a commercial internal control (human serum type AB, male, from clotted; Sigma-Aldrich, St. Louis, MO, United States, cat #H6914) in every plate. Biomarker values were log-transformed with a base of 10 and divided by the internal control value of each plate.

Due to the high intra-assay variability for some molecules, all values were standardized by plate by *Z*-scores (mean 2, standard deviation [SD] 1).

2.3. Clinical Assessment

All of the patients included in the present study were contacted by telephone and interviewed, using a structured questionnaire designed precisely for this study. When a patient was not available, a family relative or caregiver was interviewed, and if these attempts failed, data were compiled by chart review (PR(AG)397/2016). Cause of death was recorded for each patient. Mortality due to stroke was considered when the patient died due to the index stroke or a recurrent cerebrovascular event during follow-up.

Clinical and radiological variables related to stroke onset were collected for the original Stroke-Chip study [13]. Stroke-related variables comprise, among others, stroke severity, according to the National Institutes of Health Stroke Scale (NIHSS); symptomatic hemorrhagic transformation, based on the European Cooperative Acute Stroke Study III criteria (any hemorrhagic transformation with worsening of $\geq$4 NIHSS points); etiology (Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification) [15]; and affected cerebral artery territory, according to the Oxford Community Stroke Project (OCSP) classification [16].

2.4. Statistical Analysis

Statistical analyses were conducted with IBM SPSS Statistics, version 22.0 for Windows (SPSS Inc., Chicago, IL, United States) and R software (version 3.4.4). Mortality rates during follow-up were assessed with the Kaplan–Meier product limit survival method, using the log–rank test to check statistical significance between groups and conducting simple Cox proportional hazard models to determine differences in continuous variables. The "survivalROC" R package was utilized to execute time-dependent receiver-operating characteristic (ROC) curve analyses to calculate quartile cut-offs for biomarkers with *p*-values <0.1 in the simple Cox models with the best specificity and sensitivity to predict mortality; optimal quartile cut-offs were obtained using the Youden index maximum value (sensitivity + specificity − 1). All variables with a *p*-value <0.1 on univariate analysis were entered into multiple Cox regression models with the forward stepwise method, in order to detect factors independently associated with mortality during follow-up. Two models were performed, one with only clinical variables and another with both categorized biomarkers and clinical variables. The results are presented as hazard ratios (HRs) with 95% confidence interval (CIs). ROC curves were obtained to assess the performance of the models and determine their ability to predict mortality. In order to evaluate the additional value to the logistic regression models of the selected biomarker combinations with the clinical predictors, DeLong's method was used to compare areas under the curve (AUCs) of ROC curves. The integrative discriminative improvement (IDI) and the net reclassification improvement (NRI) were used to evaluate the incremental effect of adding significant biomarkers to the model built only with clinical data. The "survIDINRI" R package was utilized to obtain IDI and NRI indices in prediction models with censored survival data. A *p*-value lower than 0.05 was considered statistically significant in all tests.

3. Results

3.1. Demographics

Of the 941 ischemic stroke patients in the original Stroke-Chip study [13], four were lost to follow-up, leaving 937 patients that fulfilled the inclusion criteria for the present study.

Baseline demographic and clinical characteristics of stroke patients are shown in Table 1. Mean ± SD age was 72.8 ± 13.0 years, and 53.9% of patients were men. Arterial hypertension was the most common vascular risk factor in the sample (n = 686, 73.2%), followed by dyslipidemia (n = 461, 49.2%). The median baseline NIHSS score was 7 (interquartile range (IQR) = 3–15), and median follow-up was 4.8 years (IQR = 1.6–5.2) (Table 1).

Table 1. Demographic and clinical characteristics of patients.

		n = 937	Death during Follow-Up		*p*-Value	Death Due to Stroke		*p*-Value
			No (*n* = 574)	Yes (*n* = 363)		No (*n* = 804)	Yes (*n* = 133)	
Age (years, mean ± SD)		72.8 ± 13.0	68.4 ±12.9	79.7 ± 9.6	<0.001	71.5 ±13.1	80.4 ± 9.7	<0.001
Gender (male, *n*, %)		505 (53.9)	325 (56.6)	180 (49.6)	0.033	435 (54.1)	70 (52.6)	0.683
Smoking (*n*, %)		151 (16.1)	116 (20.2)	35 (9.6)	<0.001	133 (16.5)	18 (13.5)	0.317
Alcoholism (*n*, %)		64 (6.8)	48 (8.4)	16 (4.4)	0.021	55 (6.8)	9 (6.8)	0.828
Arterial Hypertension (*n*, %)		686 (73.2)	391 (68.1)	295 (81.3)	<0.001	573 (71.3)	113 (85.0)	0.001
Diabetes mellitus (*n*, %)		240 (25.6)	121 (21.1)	119 (32.8)	<0.001	199 (24.8)	41 (30.8)	0.107
Dyslipidemia (*n*, %)		461 (49.2)	285 (49.7)	176 (48.5)	0.719	392 (48.8)	69 (51.9)	0.508
Atrial fibrillation (*n*, %)		329 (35.1)	155 (27.0)	174 (47.9)	<0.001	263 (32.7)	66 (59.6)	<0.001
Coronary artery disease (*n*, %)		152 (16.2)	74 (12.9)	78 (21.5)	0.001	121 (15.0)	31 (23.3)	0.017
Previous Stroke (*n*, %)		162 (17.3)	81 (14.1)	81 (22.3)	0.002	132 (16.4)	30 (22.6)	0.086
Previous mRS (median, IQR)		0 (0–1)	0 (0–0)	1 (0–3)	<0.001	0 (0–0)	1 (0–3)	<0.001
Functionality (mRS > 2) (*n*, %)		141 (15.5)	30 (5.4)	111 (31.4)	<0.001	107 (13.7)	34 (26.6)	<0.001
Systolic blood pressure (mmHg)		156.2 ± 28.9	157.3 ± 28.5	154.4 ± 29.5	0.151	156.2 ± 28.9	156.3 ± 28.8	0.993
Diastolic blood pressure (mmHg)		83.0 ± 16.9	84.3 ± 16.8	80.8 ± 16.8	0.006	82.8 ± 16.6	84.0 ± 18.4	0.504
Glycemia (mg/dL)		133.5 ± 46.6	127.5 ± 42.3	142.9 ± 51.3	<0.001	131.6 ± 45.7	144.8 ± 50.1	0.001
Baseline NIHSS score (median [IQR])		7.0 (3–15)	5.0 (2–10)	12.5 (6–19)	<0.001	6.0 (2–12)	17.5 (11–21)	<0.001
Type of ischemic stroke	TIA (*n*, %)	102 (10.9)	80 (13.9)	22 (6.1)	<0.001	100 (12.5)	2 (1.5)	<0.001
	Ischemic stroke (*n*, %)	835 (89.1)	494 (86.1)	341 (93.9)		704 (87.6)	131 (98.5)	
OCSP classification	TACI (*n*, %)	306 (37.6)	123 (25.3)	183 (56.1)	<0.001	214 (31.1)	92 (73.6)	<0.001
	PACI (*n*, %)	310 (38.1)	210 (43.1)	100 (30.7)		288 (41.9)	22 (17.6)	
	LACI (*n*, %)	127 (15.6)	103 (21.1)	24 (7.4)		123 (17.9)	4 (3.2)	
	POCI (*n*, %)	70 (8.6)	51 (10.5)	19 (5.8)		63 (9.2)	7 (5.6)	
TOAST classificaiton	Cardioembolic (*n*, %)	387 (41.6)	210 (36.8)	177 (49.2)	<0.001	325 (40.7)	62 (47.0)	<0.001
	Atherothrombotic (*n*, %)	131 (14.1)	87 (15.2)	44 (12.2)		114 (14.3)	17 (12.9)	
	Lacunar (*n*, %)	120 (12.9)	100 (17.5)	20 (5.6)		118 (14.8)	2 (1.5)	
	Undetermined (*n*, %)	275 (29.5)	158 (27.7)	117 (32.5)		224 (28.0)	51 (38.6)	
	Other causes (*n*, %)	18 (1.9)	16 (2.8)	2 (0.6)		18 (2.3)	0 (0.0)	
Reperfusion therapy (*n*, %)		389 (41.6)	232 (40.5)	157 (43.4)	0.119	322 (40.1)	67 (50.4)	0.017
tPA (*n*, %)		363 (38.8)	213 (37.2)	150 (41.4)	0.037	298 (37.2)	65 (48.9)	0.005
Thrombectomy (*n*, %)		72 (7.7)	46 (8.0)	26 (7.2)	0.770	60 (7.5)	12 (9.0)	0.559
Symptomatic hemorrhagic transformation (*n*, %)		17 (1.8)	1 (0.2)	16 (4.4)	<0.001	4 (0.5)	13 (9.8)	<0.001

mRS: modified Rankin scale; NIHSS: National Institutes of Health Stroke Scale; IQR: interquartile range; TIA: transient ischemic attack; OCSP: Oxfordshire Community Stroke Project; TACI: total anterior circulation infarct; PACI: partial anterior circulation infarct; LACI: lacunar infarct; POCI: posterior circulation infarct; TOAST: Trial of ORG 10172 in Acute Stroke Treatment; tPA: tissue plasminogen activator.

3.2. Clinical Characteristics and Mortality

Three hundred and sixty-three patients (38.7%) died during the 4.8-year follow-up. Table 1 shows demographic and clinical differences between those patients who died and those who survived. Deceased patients were older, had higher prevalence of hypertension and atrial fibrillation, and a higher baseline NIHSS score. Patients' cause of death during follow-up is summarized in Figure 1, which shows that the index stroke was the most common cause of death among patients (*n* = 107).

In the multiple regression analysis, the independent predictors of mortality were age (hazard ratio (HR) = 1.063, 95% confidence interval (CI) = 1.049–1.078, $p < 0.001$), female sex (HR = 1.559, 95% CI = 1.243–1.956, $p < 0.001$), hypertension (HR = 1.359, 95% CI = 1.028–1.797, $p = 0.031$), previous modified Rankin scale (mRS) (HR = 1.247, 95% CI = 1.152–1.351, $p < 0.001$), glycemia (HR = 1.002, 95% CI = 1.000–1.004, $p = 0.043$) and baseline NIHSS (HR = 1.089, 95% CI = 1.089–1.073, $p < 0.001$).

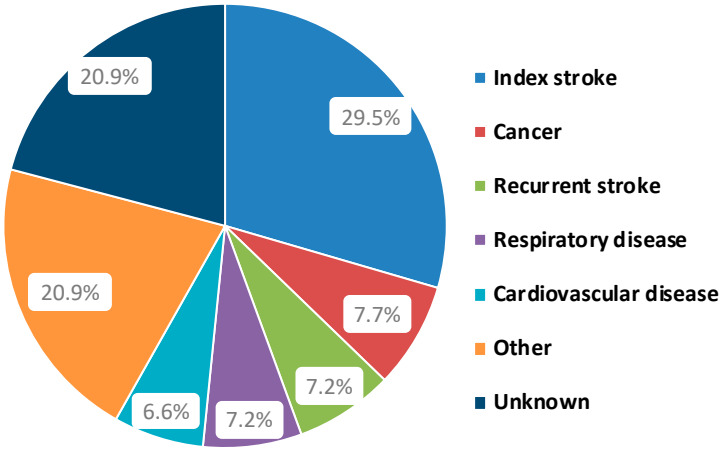

Figure 1. Patients' cause of death during follow-up.

3.3. Blood Biomarkers and Mortality

Patients who died during the follow-up time had higher baseline levels of D-dimer, endostatin, IL-6, NT-proBNP, VAP-1, vWF, and TNF-R1, and lower levels of apolipoprotein CIII (APOC-III) than patients who survived. For APOC-III, D-dimer, endostatin, IL-6, NT-proBNP, vWF, and TNF-R1, the optimal quartile cut-off point was quartile 2, whereas for VAP-1, quartile 3 was optimal (Table 2).

Table 2. Blood biomarker mean values and best quartile cut-off points to predict mortality during follow-up.

Biomarkers	Death during Follow-Up		*p*-Value	Cut-Off (Quartile)	Death during Follow-Up		*p*-Value
	No (*n* = 574)	Yes (*n* = 363)			No (*n* = 574)	Yes (*n* = 363)	
APOC-III	2.10 ± 0.92	1.88 ± 1.03	<0.001	>Q2	304 (53.4%)	162 (44.8%)	0.007
D-dimer	1.84 ± 0.93	2.51 ± 0.84	<0.001	>Q2	217 (38.1%)	247 (69.0%)	<0.001
Endostatin	1.89 ± 0.84	2.49 ± 0.97	<0.001	>Q2	222 (38.0%)	245 (68.2%)	<0.001
GroA	1.97 ± 0.97	2.02 ± 0.97	0.286	—	—	—	
IL-6	1.77 ± 0.93	2.35 ± 0.89	<0.001	>Q2	214 (39.4%)	232(67.1%)	<0.001
NT-proBNP	1.86 ± 0.88	2.59 ± 0.88	<0.001	>Q2	220 (38.6%)	247(68.2%)	<0.001
VAP-1	1.97 ± 0.96	2.21 ± 1.05	<0.001	>Q3	118 (20.7%)	115 (31.9%)	<0.001
vWF	1.87 ± 0.99	2.28 ± 0.89	<0.001	>Q2	246 (42.9%)	224 (61.7%)	<0.001
IGFBP-3	1.97 ± 0.91	2.05 ± 1.09	0.208	—	—	—	
FAS-L	1.99 ± 0.98	1.96 ± 1.00	0.677	—	—	—	
TNF-R1	1.82 ± 0.85	2.45 ± 1.03	<0.001	>Q2	205 (38.3%)	226 (69.5%)	<0.001
NCAM	1.97 ± 0.99	2.03 ± 0.97	0.416	—	—	—	
S100B	1.99 ± 0.99	2.07 ± 0.96	0.322	—	—	—	
Hsc70	2.04 ± 0.94	2.06 ± 0.97	0.757	—	—	—	

APOC-III: apolipoprotein CIII (µg/mL); GroA: growth-related oncogene α (pg/mL); IL-6: interleukin 6 (pg/mL); NT-proBNP: N-terminal pro-B-type natriuretic peptide (pg/mL); VAP-1: vascular adhesion protein-1 (pg/mL); vWF: von Willebrand factor (%); IGFBP-3: insulin-like growth factor binding protein-3 (pg/mL); FasL: Fas ligand (pg/mL); TNF-R1: tumor necrosis factor receptor-1 (pg/mL); NCAM: neural cell adhesion molecule (pg/mL); S100B: S100 calcium-binding protein B (pg/mL); Hsc70: heat shock 70 kDa protein-8 (ng/mL).

In the multiple regression model, after adjusting for those clinical variables associated with mortality in univariate analysis, endostatin > quartile 2 (HR = 1.373, 95% CI = 1.061–1.776, $p = 0.016$), TNF-R1 > quartile 2 (HR = 1.392, 95% CI = 1.071–1.808, $p = 0.013$) and IL-6 > quartile 2 (HR = 1.316, 95% CI = 1.025–1.690, $p = 0.032$) were independent predictors of long-term mortality, together with age (HR = 1.058, 95% CI = 1.042–1.073, $p < 0.001$), female sex (HR = 1.674, 95% CI = 1.308–2.142, $p < 0.001$), hypertension (HR = 1.338, 95% CI = 0.992–1.806, $p = 0.057$), previous mRS (HR = 1.232, 95% CI = 1.131–1.343, $p < 0.001$), glycemia (HR = 1.002, 95% CI = 1.000–1.004, $p = 0.071$) and baseline NIHSS (HR = 1.087, 95% CI = 1.069–1.105, $p < 0.001$).

The risk of death increased up to 69% when these three biomarkers were combined (Figure 2). The area under the ROC curve (AUC) of the predictive model was significantly higher when combining biomarkers with clinical variables (85.5%; 95% CI = 81.7–87.5%) than when clinical variables were used alone (84.4%; 95% CI = 82.9–88.1%) (De Long's test $p = 0.017$) (Figure 3).

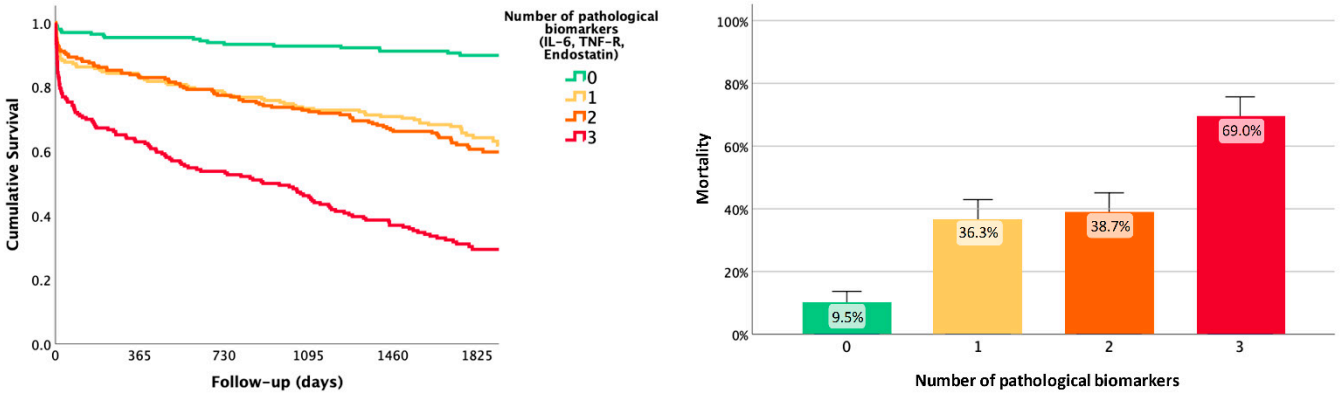

Figure 2. Combination of predictive blood biomarkers (endostatin > quartile 2, TNF-R1 > quartile 2, and IL-6 > quartile 2). Risk of death increased up to 36.3% when adding any of the biomarkers, up to 38.7% when combining two of three biomarkers, and up to 69% when combining all three biomarkers. IL-6: interleukin-6; TNF-R: tumor necrosis factor receptor-1.

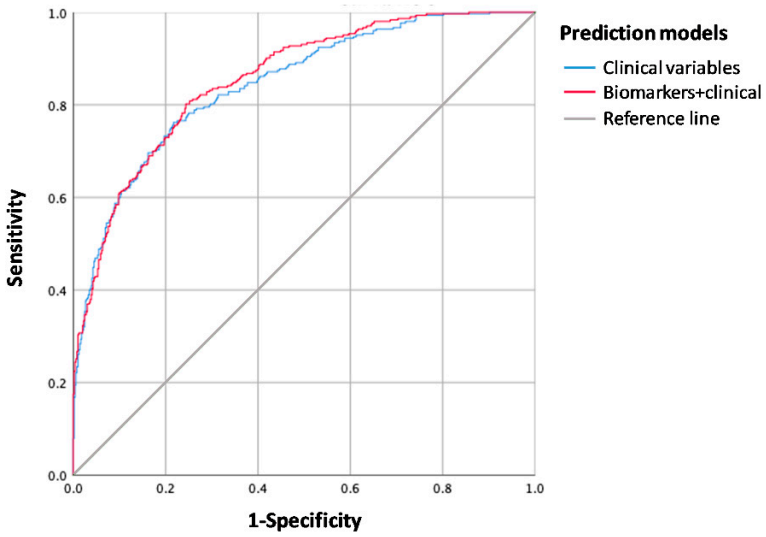

Figure 3. Receiver-operating characteristic (ROC) curve of the predictive model for long-term mortality. The predictive capacity of the model was slightly higher when combining clinical variables and blood biomarkers (endostatin, IL-6, and TNF-R1) than when using clinical variables alone.

The IDI value was 0.022 (95% CI = 0.007–0.048, $p < 0.001$) (IDI events = 0.011 and IDI non-events = −0.011), and the NRI was 0.218 (95% CI = 0.157–0.349, $p < 0.001$) (NRI events = 0.600 and NRI non-events = 0.382), so the inclusion of the biomarkers significantly improved the model when compared to the model built only with clinical variables.

3.4. Mortality Due to Stroke

One hundred and thirty-three patients (14.2%) died due to stroke during follow-up. Of those, 107 (29.5%) died due to the index event, and 26 (7.2%) due to a recurrent stroke. Table 1 shows demographic and clinical differences between those patients who died

because of stroke and those who survived. Patients who died due to stroke were older, had higher prevalence of hypertension, atrial fibrillation, and coronary artery disease, and had a higher baseline NIHSS score.

In the multivariate analysis, the independent predictors of mortality due to stroke were age (HR = 1.058, 95% CI = 1.037–1.079, $p < 0.001$), hypertension (HR = 1.852, 95% CI = 1.131–3.030, $p = 0.014$) and basal NIHSS (HR = 1.138, 95% CI = 1.111–1.167, $p < 0.001$).

Patients who died because of stroke during the follow-up time had higher levels of D-dimer, endostatin, IL-6, NT-proBNP, VAP-1, vWF, and TNF-R1 than patients who did not die. Each biomarker was divided into quartiles and optimal quartile cut-off points to predict long-term mortality due to stroke. For D-dimer, IL-6, NT-proBNP, vWF, and TNF-R1, the optimal quartile cut-off point was quartile 2, whereas for endostatin and VAP-1 quartile 3 was the optimal (Table 3).

Table 3. Blood biomarker mean values and best quartile cut-off points to predict mortality due to stroke during follow-up.

Biomarkers	Death Due to Stroke		*p*-Value	Cut-Off (Quartile)	Death Due to Stroke		*p*-Value
	No (*n* = 804)	Yes (*n* = 133)			No (*n* = 804)	Yes (*n* = 133)	
APOC-III	2.03 ± 0.98	1.95 ± 0.93	0.329	—	—	—	
D-dimer	2.02 ± 0.95	2.58 ± 0.84	<0.001	>Q2	371 (46.4%)	93 (72.1%)	<0.001
Endostatin	2.06 ± 0.91	2.58 ± 1.05	<0.001	>Q3	173 (21.7%)	61 (46.2%)	<0.001
GroA	1.97 ± 0.97	2.11 ± 1.01	0.103	—	—	—	
IL-6	1.94 ± 0.95	2.40 ± 0.89	<0.001	>Q2	361 (47.3%)	85 (68.0%)	<0.001
NT-proBNP	2.07 ± 0.94	2.59 ± 0.92	<0.001	>Q2	382 (47.8%)	85 (63.9%)	<0.001
VAP-1	2.03 ± 0.99	2.24 ± 1.05	0.021	>Q3	190 (23.8%)	43 (32.6%)	0.027
vWF	1.98 ± 0.97	2.35 ± 0.89	<0.001	>Q2	386 (48.0%)	84 (63.2%)	<0.001
IGFBP-3	2.01 ± 0.96	1.97 ± 1.14	0.768	—	—	—	
FAS-L	1.98 ± 0.99	1.98 ± 0.98	0.945	—	—	—	
TNF-R1	1.99 ± 0.94	2.51 ± 1.09	<0.001	>Q2	351 (47.1%)	80 (69.6%)	<0.001
NCAM	1.97 ± 0.99	2.11 ± 0.93	0.177	—	—	—	
S100B	2.01 ± 0.99	2.09 ± 0.90	0.434	—	—	—	
Hsc70	2.04 ± 0.96	2.11 ± 0.88	0.421	—	—	—	

ApoCIII: apolipoprotein CIII (μg/mL); GroA: growth-related oncogene α (pg/mL); IL-6: interleukin 6 (pg/mL); NT-proBNP: N-terminal pro-B-type natriuretic peptide (pg/mL); VAP-1: vascular adhesion protein-1 (pg/mL); vWF: von Willebrand factor (%); IGFBP-3: insulin-like growth factor binding protein-3 (pg/mL); FasL: Fas ligand (pg/mL); TNF-R1: tumor necrosis factor receptor-1 (pg/mL); NCAM: neural cell adhesion molecule (pg/mL); S100B: S100 calcium-binding protein B (pg/mL); Hsc70: heat shock 70 kDa protein-8 (ng/mL).

In the multiple regression model, after adjusting for significant clinical variables, endostatin > quartile 3 was an independent predictor of mortality due to stroke (HR = 1.835, 95% CI = 1.196–2.815, $p = 0.005$), together with age (HR = 1.054, 95% CI = 1.029–1.079, $p < 0.001$), hypertension (HR = 1.626, 95% CI = 0.953–2.772, $p = 0.074$), and basal NIHSS (HR = 1.144, 95% CI = 1.110–1.178, $p < 0.001$). The AUC of the predictive model was moderately higher when combining biomarkers with clinical variables (83.6%; 95% CI = 80.2–86.9%) than when clinical variables were used alone (82.5%; 95% CI = 79.1–86.0%), although this difference was not significant (De Long's tests $p = 0.066$) (Figure 4). The inclusion of the biomarkers improved the model when compared to the model built only with clinical variables, given that the IDI value was 0.020 (95% CI = 0.002–0.050, $p = 0.02$) (IDI events = 0.016 and IDI non-events = −0.004) and the NRI was 0.296 (95% CI = 0.043–0.390, $p = 0.04$) (NRI events = 0.472 and NRI non-events = 0.176).

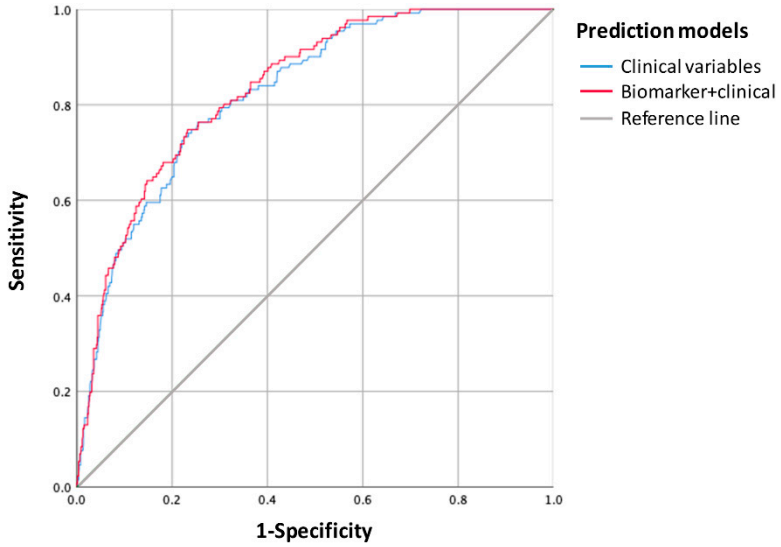

Figure 4. Receiver-operating characteristic (ROC) curve of the predictive model for long-term mortality due to stroke. The predictive capacity of the model was slightly higher when combining clinical variables with a blood biomarker (endostatin) than when using clinical variables alone.

4. Discussion

The present study explored the association of 14 blood biomarkers measured in the acute phase with long-term mortality after ischemic stroke. We followed up with a cohort of 941 ischemic stroke patients over a median time of almost 5 years after the event, in order to determine whether some blood biomarkers can predict mortality in this population. The proportion of patients that die after ischemic stroke is influenced by different variables, such as subtype of stroke or health resources of each country. According to the literature, 40–60% of ischemic stroke patients die within 5 years after the event [17–19]. In the present study, we found that 38.7% of ischemic stroke patients died during the median 4.8-year follow-up period, and those who died were older, had higher prevalence of some vascular risk factors, such as hypertension and atrial fibrillation, and had a higher baseline NIHSS score.

The independent predictors of mortality were age, sex, hypertension, previous mRS, and basal NIHSS, which are among the most frequent clinical and demographical variables associated with stroke mortality, as reported in the literature [8,20]. In addition, from the proposed biomarkers, we found that after adjusting for clinical variables, increased levels of endostatin, IL-6, and TNF-R1 were independent predictors of long-term mortality following stroke. In fact, when these three biomarkers were combined, the risk of death increased up to 69%. The best biomarkers identified in the present study are known key players in pathophysiological pathways implicated in stroke, such as angiogenesis (endostatin) and inflammatory response (IL-6 and TNF-R1).

It is widely reported that cerebral ischemia triggers an inflammatory response both in the brain and peripheral circulation, which leads to up-regulation of inflammatory cytokines such as IL-6 [21]. Increased circulating levels of IL-6 have been regularly associated with poor-outcome after stroke [22]. The value of IL-6 to predict mortality after cerebral ischemia has also been studied before. Various studies support that increased baseline levels of IL-6 are associated with risk of death 3 months after the event [23–26]. Regarding long-term mortality, it has been revealed that IL-6 could also predict mortality both 1 year [27] and 2 years [28] after stroke. In the present study, we propose that IL-6 could also predict mortality even at later time-points, being associated with increased risk of mortality 5 years after cerebral ischemia.

TNF-R1 is a transmembrane death receptor that is able to detect the presence of extracellular death signals and ultimately trigger cell apoptosis [29]. TNF-R1 can be

activated by both membrane-bound and soluble forms of TNF-α [30]. It is well known that within the first hours after cerebral ischemia, cells of the ischemic core and penumbra suffer irreversible damage that ultimately leads to cell death by apoptosis [21]. Increased levels of TNF-α have previously been associated with poor outcome after ischemic stroke [31,32]. However, the association of TNF-R1 in stroke outcome and long-term mortality has never been described before. Here we found that those patients with increased risk of long-term death after stroke showed higher baseline levels of TNF-R1, reinforcing the crucial role of the TNF signaling pathway in stroke prognosis.

Endostatin is derived from type XVIII collagen. It inhibits both the proliferation and migration of endothelial cells, ultimately leading to inhibition of angiogenesis [33]. Regarding outcome, it has been previously reported that tissue plasminogen activator (tPA)-treated stroke patients showing higher endostatin level at hospital admission had an impaired functional outcome three months after the event [34]. In relation to mortality, one study including 3463 acute ischemic stroke patients revealed that increased baseline endostatin levels were associated with increased risk of mortality and severe disability at 3 months [35]. However, to date, the association of endostatin and long-term mortality after stroke has not been explored. Interestingly, in the present study, we found that those patients who did not survive during follow-up had higher baseline levels of endostatin at hospital admission. This is in line with what has previously been reported, and therefore endostatin may be a useful biomarker to predict both early and long-term mortality. In addition to this, we have reported for the first time that baseline endostatin levels are also associated with long-term mortality due to stroke, making endostatin even more interesting as a promising stroke biomarker.

Remarkably, the risk of long-term death when these three biomarkers were combined increased up to 69%, while the risk was below 10% when none of these biomarkers were pathological. The combination of blood biomarkers and clinical variables showed a slight but significant additional predictive value over clinical data in AUC results, although the clinical model included stroke severity. Moreover, these findings are supported by positive results in NRI and IDI procedures. In addition, endostatin was also able to predict long-term mortality due to stroke, given that the combination of endostatin and clinical variables moderately increased the predictive value when compared to clinical data. All in all, Il-6 TNF-R1 and endostatin seem to be interesting candidates for further exploration as long-term mortality biomarkers after stroke. In this regard, the modulation of these molecules and pathways through angiogenic or anti-inflammatory drugs might be an attractive approach to improve patients' outcome and reduce mortality. In fact, some pre-clinical studies already point in that direction [21].

The main strengths of the present study are the large sample size and the standardized follow-up after a long time of almost five years. However, our study has some limitations. First, in some cases, data were collected by chart review, which might lead to some missing data. Second, the specific cause of death of 20% of patients is unknown, so some deaths due to stroke may not have been diagnosed. Third, the need to standardize the results could have led to an underestimation of the biomarker effect. Finally, these patients were recruited in 2012–2013, so the actual high rates of thrombectomies that are being performed in hospitals, which may contribute to the reduction in mortality, might not be fully represented by the present cohort. For that reason, our results should be replicated in an independent cohort before solid conclusions can be drawn.

In conclusion, we have identified that the acute upregulation of endostatin, IL-6, and TNF-R1 after ischemic stroke can predict long-term mortality. Along this line, a panel comprising these three biomarkers could be used to help identify high-risk patients, so that more aggressive therapeutic strategies can be targeted to those most likely to benefit. Additional studies are needed to validate these findings, in order to elucidate the real impact on stroke patients' management.

Author Contributions: L.R., L.A., E.S., A.B., and J.M conceived and designed the study; J.Z., M.H.-P., X.U., B.L., M.T., and A.B. collected data and blood samples from ischemic stroke patients; L.R. and M.Q. analyzed the data; L.R. and P.G.-R. wrote the paper; J.Á.-S. and J.M. obtained funding for study development. All authors have read and agreed to the published version of the manuscript.

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4.6. Ceruletide and alpha-1 antitrypsin as a novel combination therapy for ischemic stroke.

(Manuscript under revision)

Ceruletide and alpha-1 antitrypsin as a novel combination therapy for ischemic stroke

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Abstract

Ischemic stroke is a primary cause of morbidity and mortality worldwide. Beyond the approved thrombolytic therapies, there is no effective treatment to mitigate its progression. Drug repositioning combinational therapies are becoming promising approaches to identify new uses of existing drugs to synergically target multiple disease-response mechanisms underlying complex pathologies. Here, we used a systems biology-based approach based on artificial intelligence and pattern recognition tools to generate in silico mathematical models mimicking the ischemic stroke pathology. Combinational treatments were acquired by screening these models with more than 5 million two-by-two combinations of drugs. A drug combination (CA) formed by ceruletide and alpha-1 antitrypsin showing a predicted value of neuroprotection of 92% was evaluated for their synergic neuroprotective effects in a mouse pre-clinical stroke model. The administration of both drugs in combination was safe and effective in reducing by 39.42% the infarct volume 24h after cerebral ischemia. This neuroprotection was not observed when drugs were given individually. Importantly, potential incompatibilities of the drug combination with tPA thrombolysis were discarded in vitro and in vivo by using a mouse thromboembolic stroke model with t-PA-induced reperfusion, revealing an improvement in the forepaw strength 72h after stroke in CA treated mice. Finally, we identified the predicted mechanisms of action of ceruletide and alpha-1 antitrypsin and we demonstrated that CA modulates EGFR and ANGPT-1 levels in circulation within the acute phase after stroke. In conclusion, we have identified a promising combinational treatment with neuroprotective effects for the treatment of ischemic stroke.

Keywords: Ischemic stroke, Neuroprotection, Ceruletide, Alpha-1 antitrypsin, Combinational therapy.

INTRODUCTION

Ischemic stroke is among the leading causes of morbidity and mortality worldwide [1]. The only current therapy for ischemic stroke aims at restoring cerebral blood flow by removing the obstructive clot, via the intravenous administration of tissue plasminogen activator (t-PA) or tenecteplase (TNK) or through mechanical thrombectomy using stent-retriever or aspiration devices [2,3]. Despite the effectiveness of reperfusion strategies, their short therapeutic window and some potential side effects preclude offering those therapies to all stroke patients. Therefore, there is still an important need to find alternative stroke therapies to rescue brain tissue from the ischemic injury that could be implemented to the largest number of patients possible. In this regard, an increasing number of pre-clinical and clinical studies have now moved their focus of attention to the pre-hosiptal phase or the early post-arrival Emergency Department [4]. In such scenario, patients could be treated even before the stroke diagnosis is confirmed by neuroimaging techniques in the medical facility and under the hypothesis that treatments might be most effective when initiated as soon as possible after stroke onset.

Since ischemic stroke is well-known to be a multifactorial and heterogeneous disease, there is growing awareness that targeting a single pathological activated pathway might be insufficient to impact on the progression of the disease [5], a fact that could explain the systematic failure of translation into clinics of neuroprotectants tested so far [6–8]. In this sense, a global understanding of the different pathways that are disturbed in this multifactorial disorder is becoming critical to successfully find new therapeutic agents to reverse stroke progression [9]. Systems biology has emerged as a new discipline to precisely decipher this complexity. To that end, it considers organisms as sophisticated network-based maps of highly interrelated molecules and connected biological pathways. Systems biology provides insights into those relevant elements whose differential activity is associated with the disease, and simulates the behavioral response of the pathology to the modulation of any disease-related mediators [10].

Approaches based on systems biology are also particularly suited for drug repositioning strategies [11]. The integration of current biological and clinical knowledge with data on therapeutic responses facilitates the identification of new uses for existing drugs as promising healing agents outside their original medical indication. In fact, there are already examples of drug repositioning approaches in the field of ischemic stroke applying the concept of systems biology [12,13]. More importantly, systems biology-based drug repositioning strategies are also making significant contributions to the identification of combinational treatment approaches, in which two or more therapeutic agents are combined to synergically target multiple disease-response mechanisms to improve the management of complex pathologies, including stroke. On top of that, the simultaneous combination of drugs is also

expected to overcome toxicity and dose-associated side effects by lowering the effective dose of each individual compound [14,15].

In this study, we aimed to use a systems biology-based approach based on artificial intelligence and pattern recognition tools to integrate available biological, pharmacological and medical knowledge into mathematical models to simulate *in silico* the complexity of the stroke [16]. By screening these stroke-mimicking models, we have identified a drug combination formed by ceruletide and alpha-1 antitrypsin (CA), two already Food and Drug Administration (FDA)-approved drugs, with theoretical synergic neuroprotective effects. Following our idea of maximizing the number of patients that could benefit from this therapeutic strategy, we have experimentally validated in a mouse stroke model the efficacy of this drug combination in protecting the ischemic brain when administered acutely after stroke induction. Moreover, by using both *in vivo* and *in vitro* approaches, we have proved that this drug combination can be safely administered together with t-PA, which supports the future translation of these findings into clinical trials. Last, we also provided evidence that the neuroprotective effects of ceruletide and alpha-1 antitrypsin are partly driven by the modulation of peripheral mediators in circulation.

MATERIAL AND METHODS

1. In silico stroke modeling, repositioning and identification of CA MoA

Therapeutic Performance Mapping System (TPMS) technology, a top-down systems biology approach (Anaxomics S.L., Spain) was applied, as previously described [16–18], and validated [19–21] (Supplementary methods). This technology starts off from bibliography and database-based characterization of drugs and conditions [18,22], and contextualizes them in the human protein network by the use of publicly available protein-protein functional interaction information. The network is then converted to a dynamic model through its training with known pathophysiological relationships (i.e. drug-indication and drug-adverse reactions relationships), and two types of models are built (Fig. 1A): Artificial Neuronal Networks (ANN) [17], with predictive capabilities, and sampling methods [18], with descriptive capabilities. In the current study, we defined 13 relevant processes for ischemic stroke through literature revision, 387 functionally-related proteins were assigned to each of them and this molecular definition was embedded on the human protein network (Fig. 1B). TPMS-based models were constructed from that network, and a combination of both modelling strategies were applied for drug repositioning over DrugBank database [23], using as a benchmark the value obtained for drugs proved unsuccessful to treat stroke so far (Fig. 1C) [24,25]. This strategy led to CA combination identification, and sampling methods-based models were used for *in silico* identification

of the molecular mechanisms modulated by the combination for further experimental validation (Supplementary methods, Supplementary table S1).

2. Drugs

Ceruletide (caerulein, Sigma-Aldrich, Germany) and alpha-1 antitrypsin (Prolastin-C, Grifols S.A, Spain) were used in the experiments. The initial dose of each drug was chosen according to an extensive literature-based research [26–39]. Ceruletide was given at a dose of 0.1mg/kg dissolved in sterile double-distilled water (ddH_2O), and alpha-1 antitrypsin at a dose of 60mg/kg or 480mg/kg, resuspended in its commercial solution for injection (solvent) and prepared according to manufacturer's instructions. Sterile ddH_2O and Prolastin-C solvent were used as vehicles. Drugs and vehicles were intravenously administered through the retro-orbital sinus (safety study and transient MCAO stroke model) or tail vein (thromboembolic stroke model). Drugs were always administered sequentially.

3. Animals

All animal procedures were conducted either in compliance with the Spanish or French legislation and following the European Communities Council guidelines (2010/63/EU). Moreover, they were approved either by the Ethics Committee of the Vall d'Hebron Institute of Research (protocol number 74/16) or by the institutional review board (French ministry of Research and by the local ethical committee of Normandy (CENOMEXA). All experiments were conducted in a randomized manner and in adherence to the ARRIVE guidelines [40]. Sample sizes were not predetermined since preliminary data was not available. C57BL/6J male mice were used in the experiments (8–12-week-old; Janvier Labs, France), kept in a climate-controlled environment on a 12-h light/12-h dark cycle with food and water available *ad libitum*. All efforts were made to minimize the possible suffering, pain or discomfort of the animals. Anesthesia (isoflurane, 5% for induction; 2% for maintenance in medical air or 70%/30% mixture of NO2/O2) was given to mice via facemask during all surgical procedures described below. Rectal temperature was controlled at 37+/-0.5°C throughout all the surgical procedures using a feedback-regulated heating system.

A total of 135 animals were used for the study. Of these, 24 were excluded after applying the following criteria: inappropriate occlusion or reperfusion of the middle cerebral artery (n=11); massive surgical bleedings (n=5); death during the surgical procedure (n=5) or premature death within 24h after ischemia (n=3).

4. Transient middle cerebral artery occlusion (MCAO) model

Transient ischemia in the territory of the right middle cerebral artery (MCA) was induced by introducing an intraluminal filament through the external carotid artery, as described elsewhere [41].

In brief, the regional cerebral blood flow (CBF) was monitored close to the region irrigated by the MCA during the entire surgical procedure by affixing a laser Doppler probe (Moor Instruments, UK) to the skull. After surgical exposure of the right bifurcation of the external carotid artery and internal carotid artery, a silicone-coated nylon monofilament (Doccol Corporation, USA; reference number: 602256PK10Re) was introduced to occlude the MCA. The incision was closed with a silk suture and the animal was allowed to recover from anesthesia. The filament was left in place for 90 minutes. Afterward, mice were re-anesthetized and the filament was gently pulled out to induce reperfusion of the MCA. Only animals that exhibited a reduction of 80% of CBF after filament introduction and a recovery of 80% after filament removal were included in the study. Ten minutes after MCA occlusion, the drugs or vehicle were administered intravenously via the retro-orbital sinus in a blinded manner.

4.1. Neuroscore

An investigator blinded to the treatments evaluated all mice and scored them on a composite neurological scale, adapted from previous studies [42,43]. In brief, neurological score ranges from 0 (healthy) to 39 and represents the sum of the general deficits (0-13): hair [0-2], ears [0-2], eyes [0-3], posture [0-3] spontaneous activity [0-3]; and focal deficits (0-26): body symmetry [0-2], gait [0-4], climbing on a surface held at 45º [0-3], circling behavior [0-3], front limb symmetry [0-4], compulsory circling [0-3], whiskers response to a light touch [0-4] and gripping of the forepaws [0-3]. All animals were evaluated 80 minutes after MCAO induction (post-occlusion) and 24h after ischemia.

4.2. Sample collection and infarct size measurement

Under deep terminal anesthesia (5% isoflurane), blood samples were drawn through cardiac puncture and collected in EDTA tubes. Plasma was promptly obtained by centrifugation (3000g, 10 min, 4ºC) and kept at -80ºC until further use. Then, mice were transcardially perfused with 20 ml of heparinized cold saline, the brain was immediately collected and cut into 6 serial 1mm coronal sections to assess infarct volume using 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) staining, as previously described [44]. TTC images were captured using a CanoScan 4200F (Canon, Japan) and infarct volume was measured using Image J software by an investigator blinded to the treatments. Infarct volumes were calculated by integration of the lesion areas as previously described [45]. Results were finally adjusted for edema by dividing the measured value by the scaling factor I/C (I: area of the ipsilateral hemisphere; C: area of the contralateral hemisphere), and expressed in cubic millimeters (mm^3). After scanning, brains were immediately frozen at -80ºC until further use.

5. Thromboembolic model

The distal occlusion of the right MCA by thrombin injection and t-PA induced reperfusion was conducted as previously described [46]. In brief, before the surgical procedure, a pipette was made

with hematologic micropipettes (calibrated at 15 mm/µL; Assistent ref. 555/5; Hoecht, Sondheim-Rhoen, Germany) by using an electrophysiology puller (PC-10; Narishige) and pneumatically filled with purified murine alpha-thrombin (0.05 mg; Stago BNL). Mice were placed in a stereotaxic device and the right MCA was exposed after a small craniotomy and the excision of the dura mater. The pipette was introduced into the lumen of the MCA and 1 µL of purified murine alpha- thrombin (1UI) was injected to induce the formation of a clot *in situ*. The efficacy of the occlusion was assessed using laser doppler flowmetry, with the optic fiber placed above the MCA territory before and up to 10 min after MCA occlusion (MCAo).

Ten minutes after thrombin injection, a catheter was inserted into the tail vein to allow the intravenous administration of the drugs or vehicle in a blinded manner. Ten minutes later (20 after thrombin injection), 200 µL of human recombinant t-PA (10 mg/kg, Actilyse®, Boeringher Ingelheim) was injected (10% bolus, 90% perfusion for 40 minutes). The control group received the same volume of saline under identical conditions.

5.1. Grip strength

The grip strength test (BIOSEB, France) was used to assess neuromuscular functions in mice by determining the strength displayed by the animal relative to the forepaw-grasping reflex. Five tests per mouse were performed to reduce variabilities, with 1 minute resting time in between to obtain the mean strength. Measurements were registered in grams the day before MCAO surgery (baseline acquisition) and at 24 hours, 48 hours and 72 hours of ischemia and expressed as % of baseline values to normalize individual with its baseline values (=100%).

5.2. Infarct size measurement

Neuroimages were acquired by magnetic resonance imaging 24 hours after ischemia. Experiments were carried out on a Pharmascan 7 T/12 cm system using surface coils (Bruker, Germany). T2-weighted images were acquired using a multi-slice multi-echo sequence (TE/TR 33/2500 ms with 70 × 70 × 500 μm^3 spatial resolution) and used for lesion size as quantification using the ImageJ software. T2*-weighted sequences were used to monitor if animals underwent hemorrhagic events (TE/TR 7.7/500 ms with 70 x 70 x 500 μm^3 spatial resolution). Two-dimensional time-of-flight angiographies (TE/TR 12/7 ms) were acquired to analyze the MCA angiogram and identify undesired spontaneous recanalization (data not shown).

6. Blood biochemical parameters

Routine clinical biochemistry parameters including urea, creatinine, creatine kinase (CK), aspartate transaminase (AST) and alanine transaminase (ALT), were analyzed and quantitatively measured in the clinical laboratories from Hospital Vall d'Hebron (Barcelona, Spain).

7. Western blot

Frozen TTC-stained ischemic and contralateral hemispheres from each animal were homogenized with freshly prepared ice-cold lysis buffer (1% phosphatase inhibitor cocktail 3 (Sigma-Aldrich), 0.1M PMSF (Sigma-Aldrich) 0.5% Aprotinin (Sigma-Aldrich) diluted in ready-to-use RIPA buffer (Sigma-Aldrich)). One gram of tissue per sample was homogenized in 1 mL of lysis buffer with a homogenizer drill (5mm-diameter bit). Then the tissue was centrifuged (15.294 g, 12 min, 4ºC) and supernatants were stored at -80ºC until further use. The total protein concentration of the homogenates was measured by the BCA method (PierceTM BCA Protein Assay, ThermoFisher, USA). Equal protein amounts (30-40ug) were resolved in a 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 10% non-fat milk and incubated with the following primary antibodies: ANGPT1 (Proteintech, USA; 1/500), EGFR (Biorbyt, UK; 1/1000), β-actin (Sigma-Adrich, 1/5000) or β-tubulin (Abcam, 1/2000). Membranes were then incubated with the secondary antibody linked to horseradish peroxidase (HRP) (Sigma-Aldrich) (anti-rabbit HRP for ANGPT1 and EGFR, 1/2000; or anti-mouse HRP for β-actin and β-tubulin, 1/2000). The substrate reaction was developed with chemiluminescent reagent Luminol (ThermoFisher) and analyzed with Odyssey Li-Cor. Western blots were quantified using Image-J free software. Positive signals were corrected by β-actin or β-tubulin signal, used as a loading control. Afterward, each ipsilateral hemisphere (IP) band-signal was normalized by the respective contralateral hemisphere (CL) band-signal to obtain signal ratios.

8. Olink® proteomic array

The *Target 96 Mouse Exploratory panel* was performed by Olink® Proteomics (Uppsala, Sweden) to simultaneously measure 92 proteins that encompass a broad range of biological functions and pathways (Supplementary table S2). Thirty microliters of frozen brain lysates from the IP and CL hemispheres from both experimental groups (n=6/group) were analyzed with the Olink® Proteomics technology in accordance with the stablished protocols and policy. Data is provided as normalized protein expression (NPX) values, on log2 scale Olink Proteomics' arbitrary units. The quality of each sample was assessed by evaluating the deviation from the median value of the controls (pooled plasma samples) for each individual sample, which was less than 0.3 NPX from the median. Intra-assay coefficient of variance was of 4% (reference intra CV<15%).

9. ELISA immunoassay

A sandwich ELISA array was assayed to determine the levels of EGFR and ANGPT1 in mouse plasma, using commercially available ELISA kits (EGFR: Cat. #ab201275, Abcam, UK and ANGPT1: Cat.#EK1296, Boster, USA). Optical densities (OD) were measured by duplicate in a SynergyTM Mx microplate reader

(BioTek Instruments Inc, USA). Samples displaying a CV higher than 20% were excluded from the analysis.

10. *In vitro* clot formation and lysis assay

A pool of plasma was obtained from 10 healthy volunteers from the ISSYS cohort [47] (5 men and 5 women, age 78 [75.25-82.25], with no medical records of coagulation disorders). In brief, blood samples were collected in citrate tubes and centrifuged at 1500g, 15 min at 4ºC. All 10 plasma samples were mixed together for 2h at 4ºC and then kept at -80ºC until further use.

The assay was conducted in a microtiter 96-well plate with twenty-five ul of citrate plasma added to 75 ul of assay buffer (0.05M Tris-HCl (Sigma-Aldrich), 1M NaCl (Sigma-Aldrich), 1.245ng (83 ng/ml) of t-PA (Actylise®) at pH=7.4). A final volume of 50ul of activation mix (7.5mM CaCl2 and 0.03U/mL thrombin) was also added to start the clot reaction. OD was immediately read at 405nm and every 40 sec for 40 min total assay time at 37ºC using a BIO-TEK Elx-808 microplate reader and Gen5 software. Internal controls without t-PA were also run in each independent experiment. Three independent experiments were performed for each experimental condition and all samples were run per triplicate. Only those with a CV < 20% were used for the final analysis.

Ceruletide and alpha-1 antitrypsin were loaded at two different doses: low dose (CA, 83ng/mL and 498ng/mL, respectively) and high dose, 10-times higher than the low dose (CA 10x, 830 ng/mL and 4980 ng/mL, respectively). To determine the *in vitro* low doses, we sought to accurately reproduce the *in vivo* physiological ratio between the circulating concentration of t-PA and drugs in the *in vitro* environment. For that purpose, we first extrapolated the concentration of ceruletide and alpha-1 antitrypsin in circulation immediately after its administration, calculated based on a standard mouse of 25g (8-12 weeks) and 80 mg/kg of blood content [48]. We then calculated the *in vitro* dose of ceruletide and alpha-1 antitrypsin based on the ratio between the *in vivo* (10mg/kg, 125ug/mL) and *in vitro* (0.083 ug/mL) t-PA concentration [49], following the formula: [drug *in vitro*] = [drug *in vivo*] x [tPA *in vitro*] / [tPA *in vivo*].

Drugs were also tested at two different time-points: before starting clot formation (t0) and once the clot was already formed (t1, 14.78 $\pm$ 4.5 min from starting point), when reactions reached their maximum OD.

11. Statistical analysis

GraphPad Prism 6.0 was used for analyzing data and creating graphs. Results are given as mean ± SD (normally distributed variables) or median ± interquartile range (non-normal distributed variables). Data Gaussian distribution was checked using the Shapiro-Wilk and Kolmogorov-Smirnov tests.

Comparisons among independent experimental groups were performed through unpaired Student's t-tests or one-way ANOVA followed by a Tukey's multiple comparison test for normal-distributed variables, and Mann-Whitney test or a Kruskall-Wallis test, followed by a Dunn's multiple comparison test for non-normal distributed variables. Data comparison over time within each experimental group was performed by paired Student's t-test (normal variables) or Wilcoxon test (non-normal variables). A p-value< 0.05 was considered to be statistically significant in all cases.

The analysis of the proteomic data was performed with R-studio software (R-studio, Boston, USA). Differentially expressed proteins were selected based on a linear model analysis for paired samples, implemented in the Bioconductor limma package [50]. Statistically significant proteins were considered when False Discovery Rate (FDR)- adjusted p-value <0.10. Enrichment analysis on differentially-expressed proteins between experimental groups was performed using the EnrichR web-based tool through the Fisher Exact test [51]. Logarithmic base 10 transformation of the p-value was computed to obtain p-value ranking.

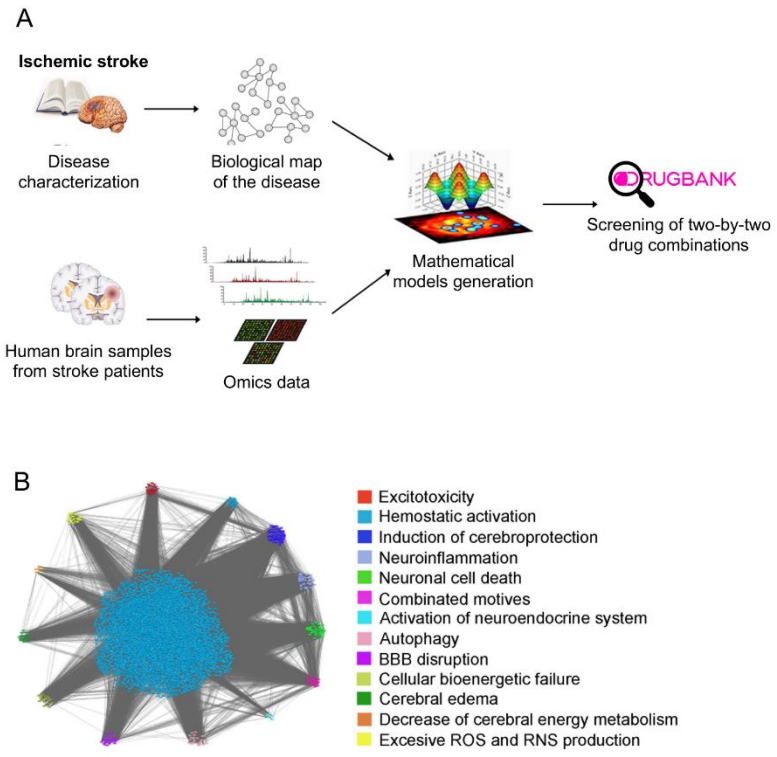
RESULTS

1. The combination of ceruletide and alpha-1 antitrypsin presents synergic neuroprotective potential *in silico.*

To discover potential neuroprotective drug combinations to successfully treat ischemic stroke, we initially applied a machine-learning approach to simulate and computationally reproduce the stroke pathophysiology (Fig. 1A). In brief, we generated systems biology-based artificial maps for stroke modeling that covered 13 well-known pathological pathways of stroke extracted from literature review, which encompassed a total of 6640 proteins (Fig. 1B). These maps were then computationally-converted into mathematical stroke models and fed with biological knowledge available related to stroke (See Supplementary methods). To further enrich these bibliography-based stroke models, we also incorporated valuable datasets from 4 different proteomics and transcriptomics studies previously conducted in our lab on human brain samples from patients who died due to ischemic stroke [52–54].

Once the mathematical simulation of stroke was created, disease-orientated drug repositioning neuroprotective solutions were then acquired by perturbing these virtual stroke models with already approved drugs from the DrugBank Database (v4.3) [23]. First, to validate and demonstrate the accuracy of the *in silico* generated models of ischemic stroke, we screened 25 drugs that have been previously tested as neuroprotectants in clinical stroke trials (Fig. 1C). Since none of them has been approved for the therapeutic use during clinical stroke management [24,25], used the ANN predictive

values obtained in our mathematical models to establish a threshold for the optimal identification of the new candidates in this study.



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Drug name	ANN prediction
Maxipost (BMS-204352)	79.39%
Nalmefene (Cervene)	79.15%
Fosphenytoin	74.85%
Trafermin	49.52%
Enlimomab	46.81%
Ancrod	46.35%
Magnesium	45.39%
UK-279,276	43.62%
ONO-2506	42.58%
Dipyridamole	38.08%
Repinotan	36.22%
Lubeluzole	34.83%
Buspirone	34.83%
Nimodipine	34.79%
Zonampanel (YM872)	33.84%
Aptiganel (CNS-1102, Cerestat)	32.65%
Diazepam	32.53%
Clomethiazole	32.03%
Natalizumab	32.01%
Ebselen	31.26%
Flunarizine	30.84%
Pentoxifylline	29.28%
Abciximab	28.82%
Pethidine	28.5%
Dextromethorphan	28.36%

Figure 1. In silico mathematical models of ischemic stroke. (A) Schematic representation of the experimental design. (B) Snapshot of the full protein network modelled for ischemic stroke, visualized through the Cytoscape software platform. (C) Artificial Neural Network (ANN) predictive value of treatments already tested in clinical studies of ischemic stroke that have failed to show neuroprotective effect in patients. Gradient of colors (from red to green) indicates increasing % of ANN predictive value.

We next screened the virtual stroke models with more than 5 million two-by-two combinations of drugs to identify potential neuroprotective drug combinations that surpassed that threshold. Furthermore, we only considered combinations with synergistic effect (ANN predictive value of the combination >20% of the individual drug values). Additionally, and in order to further identify those with high translational potential, we also applied several specific filter criteria to the screening (see Supplementary methods), including: approval status for the individual drugs; individual drugs could not have previous knowledge of association to hypotension or hemorrhages; and all individual drugs could not present incompatibilities for intravenous administration (aiming to widen the therapeutic management to more patients within the hyper-acute phase of stroke).

From all drug combinations that fulfilled the established criteria, we selected the combination formed by ceruletide and alpha-1 antitrypsin to be further studied in the *in vivo* stroke models. For the

selection of CA among all candidate combinations, different criteria were taken into consideration. Twenty-five drug combinations with the highest ANN predictive value (ranging from 88% to 96%) that fulfilled all established criteria were pre-selected. Of those, 16 drug combinations containing monoclonal antibodies were initially discarded due to higher difficulties in their administration and translation into clinics. Six out of the 9 remaining combinations presented the highest ANN predictive value (92%), including CA. Final selection among these candidates was performed based on literature information, their market status and the ANN predictive value of each individual drug compared to that of the combination. Regarding CA, the ANN of A1AT alone was 40%, of Ceruletide 51%, whereas the ANN value of the combnation rised up to 92%, showing as well a high value of the predicted synergic effect (81%). Moreover, pieces of evidence in the literature gave us information about the possible protective role of both proteins, since A1AT showed an anti-inflammatory [32,55,56] and an anti-apoptotic role [39], while ceruletide may modulate neurotoxicity [57,58], and also some studies supported the neuroprotective potential of the individual drugs [26,59].

2. Ceruletide and alpha-1 antitrypsin in combination showed neuroprotection in a mouse model of stroke

To further explore the predicted synergic effect of ceruletide and alpha-1 antitrypsin, we aimed to experimentally test the neuroprotective effect of the drug combination in a mouse model of cerebral ischemia. To prevent any toxic effect derived from the pre-selected doses and the co-administration of the two drugs at the same time, we first investigated whether the administration of both drugs in combination was safe. To that end, we initially evaluated safety outcomes on naïve animals receiving three alternate-day doses of the drug combination (CA) or the respective drug vehicles (Fig. 2A). Clinical blood biochemical parameters measured eight days after the first drug dose showed similar levels between treatment groups (Supplemental table S2). Moreover, animals receiving either CA or vehicle treatment did not present significant body weight loss (Fig. 2B), overall suggesting no apparent side effects from these drugs when given in combination.

After considering the administration of the drug combination safe, we next intended to validate its theoretical neuroprotective effects *in vivo* (Fig. 2C). To that end, both drugs from the combination were given sequentially to mice immediately after MCAO. Our results show that the administration of CA exhibited neuroprotection 24h after stroke by reducing 39.42% the infarct volume compared to the vehicle group ($p=0.022$, Fig. 2D). In addition, only CA-treated mice improved their neurological scale scoring over time, when comparing post-occlusion and 24h deficits ($p=0.012$, Fig. 2E).

We also demonstrated the synergic effects of CA in front of single drug treatment (Fig. 2F), since none of the drugs reduced the infarct volume when given alone compared to the vehicle group ($p=0.956$ for

ceruletide and p=0.888 for alpha-1 antitrypsin). However, both individual treatments showed an improvement in the neurological outcome 24h after ischemia compared to post-occlusion deficits (p=0.005 for ceruletide and p=0.003 for alpha-1 antitrypsin, Fig. 2G).

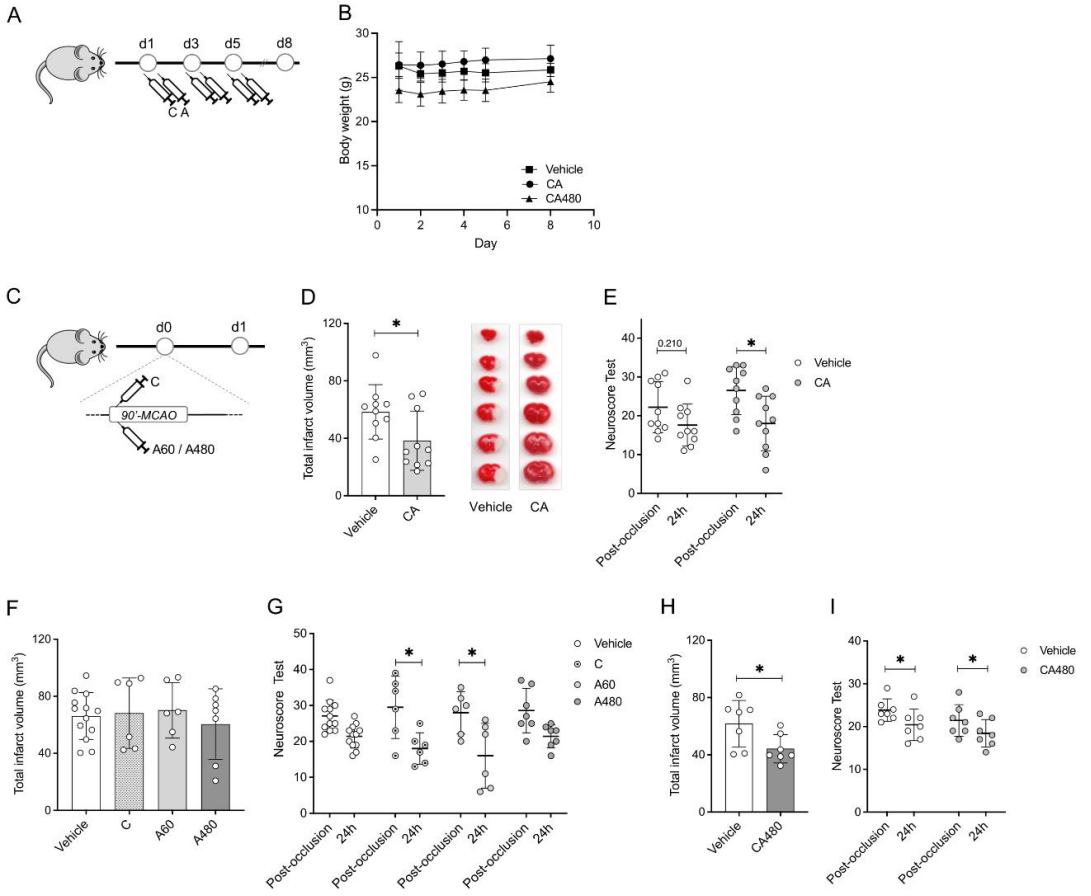

*Figure 2. Administration of CA showed neuroprotection after stroke. (A) Experimental design of the safety study. (B) Body weight monitoring of animals treated with vehicle or ceruletide 0.1 mg/kg and alpha-1 antitrypsin at a dose of 60 mg/kg (CA60) or 480 mg/kg (CA480). (C) Experimental design of the efficacy study. (D) Infarct volumes (mm3) of animals treated with vehicle (n=10) or ceruletide (0.1 mg/kg) + alpha-1 antitrypsin (60 mg/kg) (CA, n=10) 24h after cerebral ischemia. (E) Neurological deficits of animals evaluated post-occlusion (80 min after MCAO induction) and 24h after the ischemic event, expressed in % based on each respective baseline score. (F) Infarct volumes (mm3) of animals treated with vehicle (n=10) or single drugs (n=6 for Ceruletide (C), n=6 for alpha-1 antitrypsin at a dose of 60mg/kg (A60) and n=7 for alpha 1 antitrypsin at 480mg/kg (A480). (G) Neurological deficits of animals evaluated post-occlusion and 24h after the ischemic event, expressed in % based on each respective baseline score. (H) Infarct volumes (mm3) of animals treated with vehicle (n=10) or ceruletide (0.1 mg/kg) + alpha-1 antitrypsin (480 mg/kg) (CA480, n=10) 24h after cerebral ischemia. (I) Neurological deficits of animals evaluated post-occlusion and 24h after the ischemic event, expressed in % based on each respective baseline score. In all cases, mean ± SD is shown. * indicates p<0.05 and **p<0.01.*

3. The synergic neuroprotective effects of CA persist regardless of the alpha-1 antitrypsin dose

To evaluate whether the observed therapeutic effects were dose-dependent, we tested the neuroprotective effect of the drug combination after increasing the dose of alpha-1 antitrypsin up to 480 mg/kg, while maintaining the dose of ceruletide at 0.1mg/kg. This criterion was established based on previous literature supporting the use of higher doses of alpha-1 antitrypsin in humans, but not of ceruletide [60,61].

In terms of safety, and consistent with the lower dose results, animals receiving the higher dose of alpha-1 antitrypsin in combination with ceruletide (CA480) did not suffer any body weight loss. Also, all biochemical blood parameters showed similar levels between vehicle- and CA480-treated animals (Fig. 2B, Supplementary Table S3).

Efficacy results were also similar to those reported for the lower dose of alpha-1 antitrypsin: mice treated with the CA480 treatment showed a significant infarct volume reduction compared to the vehicle treatment ($p=0.031$, Fig. 2H), whereas the single treatment with alpha-1 antitrypsin at a high dose did not show infarct reduction ($p=0.913$, Fig. 2F). The neurological outcome improved over time for both experimental groups similarly ($p=0.011$ for CA480, $p=0.010$ for vehicle, Fig. 2I), whereas this improvement was not observed in animals treated only with alpha-1 antitrypsin at a high dose ($p=0.162$, Fig. 2G). Based on these results, further experiments to study the beneficial effects of the CA treatment were conducted with ceruletide (0.1 mg/kg) and alpha-1 antitrypsin at the lower dose (60 mg/kg).

4. CA does not impair the thrombolytic activity of t-PA

To test the translational potential of our CA treatment in the context of stroke thrombolysis, we made use of a thromboembolic stroke model in mice with t-PA-induced reperfusion (Fig. 3A). Infarct volume was here evaluated through magnetic resonance imaging 24h after stroke onset and functional deficits were recorded by means of the grip strength test daily within 3 days after stroke. Animals receiving t-PA alone or together with CA showed a substantial reduction of the ischemic lesion when compared to the non-thrombolyzed vehicle group ($p=0.025$ for t-PA; $p= 0.033$ for t-PA + CA; Fig. 3B). However, no differences were observed between t-PA-treated animals, either alone or together with CA ($p=0.896$). None of the animals receiving t-PA alone or together with CA presented possible signs of hemorrhagic transformation, an event that was only observable in one animal from the non-thrombolyzed vehicle group. Interestingly, the administration of CA before thrombolysis also showed a trend towards an improvement in the forepaw strength 72h after stroke compared to the vehicle-treated group ($p=0.054$), which was unexpectedly not observed in t-PA- and vehicle-treated mice.

These results suggest a possible effect of the drug combination on improving the functional outcome after stroke beyond the hyper-acute phase of the disease (Fig. 3C).

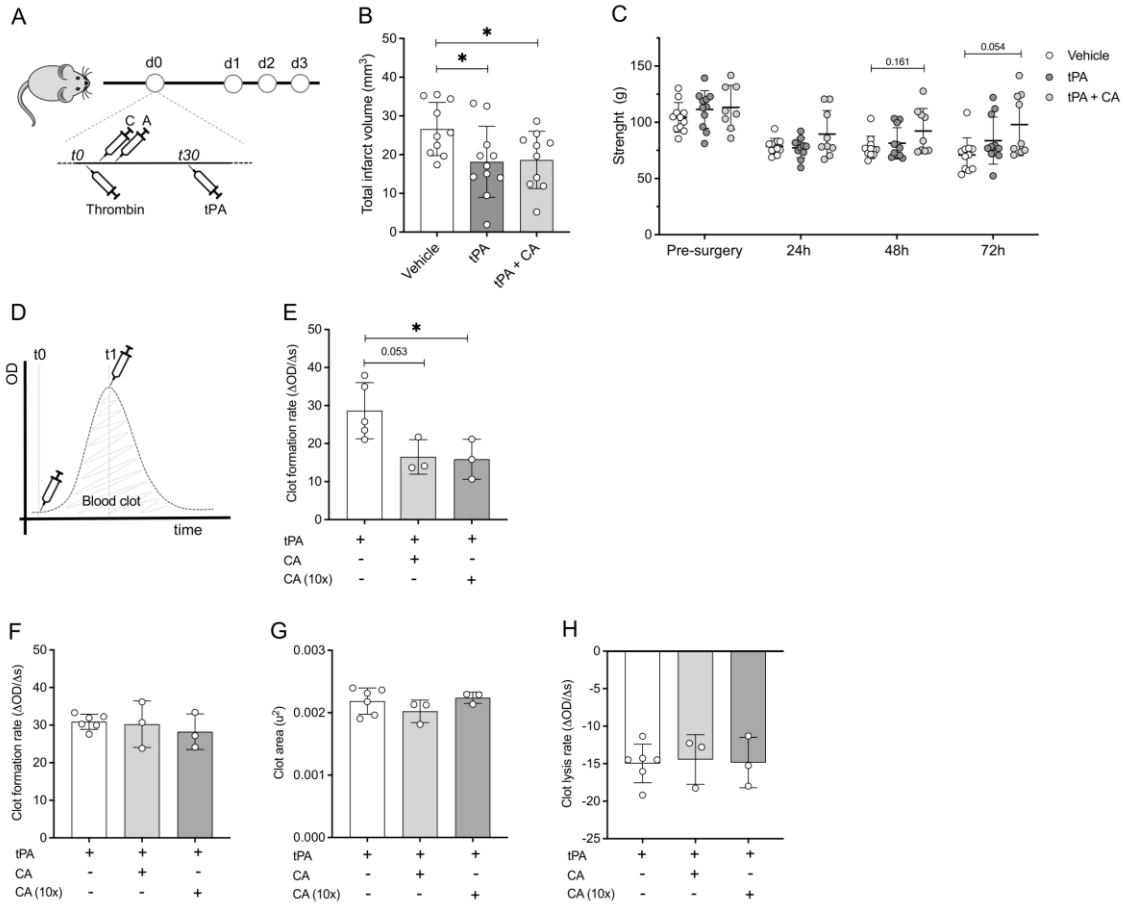

*Figure 3. CA does not impair the thrombolytic activity of t-PA. (A) Experimental design. Drugs were given intravenously at a dose of 0.1 mg/kg for ceruletide and 60 mg/kg for alpha-1 antitrypsin. (B) Infarct volumes (mm3) of animals treated with vehicle (vehicle, n=10), t-PA (n=11) and t-PA together with ceruletide + alpha-1 antitrypsin (tPA + CA, n=9) 24h after cerebral ischemia. (C) Grip strength test measurements performed before MCAO and 24, 48 and 72h after surgery. Histograms represent strength of both forepaws. Data was assessed in grams and converted in % normalized for each mouse with its corresponding baseline values. (D) Representation of the clot formation and lysis experiment showing the two time-points of drug administration: t0 and t1. (E) Clot formation rate when drugs were added at t0. (F) Clot formation rate when drugs were added at t1. (G) Clot area and (H) clot lysis rate parameters of the experiments performed when drugs were added at t1. In all cases, mean ± SD is shown. * indicates p<0.05 and #p<0.1.*

To further explore whether the drug combination can be safely administered together with t-PA in humans, we made use of an *in vitro* clot/lysis assay approach, which simulated the formation of a blood clot and its lysis by means of the protease activity of t-PA [49]. When CA was added to the clot/lysis

assay at baseline (t0, Fig. 3D), we observed a significant reduction in the rate of clot formation at both, the CA therapeutic dose and overdosed (10 times higher), compared to the vehicle-treated group (Fig. 3E). This result pointed at a possible effect of CA in the prevention of blood clotting. To specifically evaluate the effect of the neuroprotective therapy on the t-PA-related clot lysis, CA was added to the assay after the clot was formed (t1, Fig. 3D). This way, equal clot formation rates were ensured before any treatment was given (Fig. 3F). However, no differences were observed in the total area under the clot formation and lysis curve (Fig. 3G), neither in the clot lysis rate (Fig. 3H), indicating no alterations of CA on the human t-PA clot-lysis profile *in vitro*.

5. Evaluation of the synergic mechanism of action of the CA

To elucidate a mechanistic background for our neuroprotective findings, we initially performed proteomics on the ipsilateral and contralateral tissue of CA- and vehicle-treated mice (Fig. 2A). Out of the 92 explored proteins, 39 of them were differentially expressed (DE) between the IP and the CL brain hemispheres of vehicle-treated mice, but only 21 between brain hemispheres from animals treated with CA (Fig. 4A-4B, Supplementary table S4). Interestingly, 19 of these proteins were DE in both experimental groups, suggesting a minor effect of the treatment on these concrete molecules. In support of this hypothesis, we also found that the fold changes of these 19 DE proteins did not statistically differ between experimental groups (Fig. 4B).

To further unravel the effect of the drug combination in the ischemic brain, we next focused our attention on the proteins that had been either up- or down-regulated by the CA treatment, compared to the vehicle-treated group. By analyzing these proteins for overrepresentation of biological functions, we found that our treatment exerted effects by modulating cytokine-mediated pro-inflammatory signaling pathways, necrotic and apoptotic processes, as well as by promoting endothelial cell proliferation and angiogenesis (Fig. 4C).

To further gain insight into the mechanism underlying the synergic neuroprotective effects of ceruletide and alpha-1 antitrypsin, we next made use once more of the *in silico* mathematical simulation of ischemic stroke. This time, by applying sampling methods strategies, models draw up the predicted mechanisms of action that could explain the beneficial effects of CA (Fig. 4D). Interestingly, modulated mechanisms involved the Endothelial Grown Factor Receptor (EGFR) and angiopoietin 1 (ANGPT1), among other more ubiquitous proteins, such as the Ras GTPase, PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha) and the hypoxia-inducible factor 1-alpha (HIF-1a) (Fig. 4D). Since it is already known that ANGPT1 has a crucial role in stroke pathophysiology [62,63] and due to the proposed implication of EGFR in promoting cell proliferation and survival [64], we further investigate these two proteins in our CA- or vehicle-treated stroke mice.

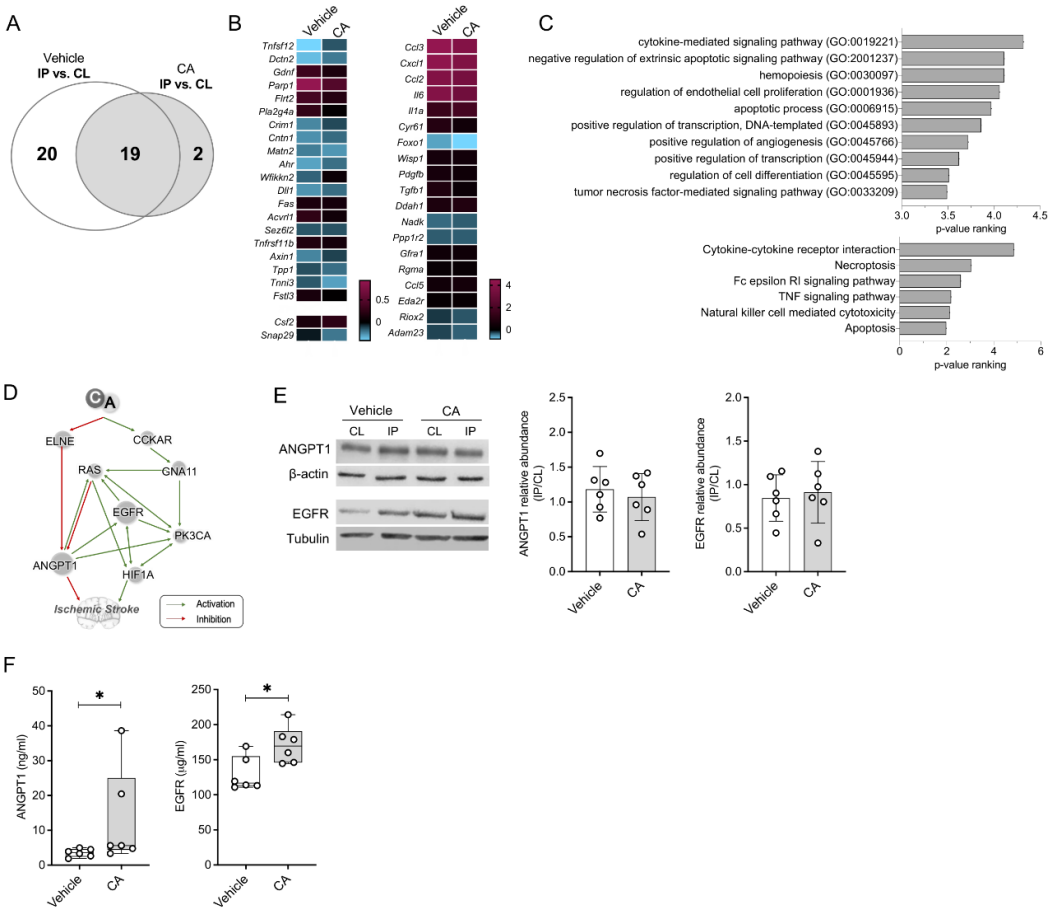

*Figure 4. Identification and validation of the synergic mechanisms of action of CA. (A) Venn's diagram of the differentially expressed proteins between the IP and CL brain hemispheres of vehicle- and CA-treated mice evaluated in the Olink proteomic array. (B) Heat maps of the differentially expressed proteins between the IP and CL brain hemispheres of mice treated with vehicle (left) or CA (right). (C) Biological significance analysis of the proteins modulated by CA. (D) Predicted molecular mechanism of action of CA on ischemic stroke obtained from the in silico-generated mathematical models of stroke. (E) Representative Western Blot images and quantification of brain levels of ANGPT1 and EGFR in vehicle- and CA-treated animals (n=5/group). The ratio between IP and CL levels within each animal is depicted. (F) Quantification of ANGPT1 and EGFR levels in mouse plasma samples (n=7/group). In all cases mean ± SD is shown. * indicates p<0.05 and **p<0.01. Abbreviations: ANGPT1, Angiopoetin-1; CCKAR, Cholecystokinin receptor type A; CL, Healthy contralateral brain hemisphere; EGFR, epidermal growth factor receptor; ELNE, Neutrophil elastase; GNA11, G Protein Subunit Alpha 11; HIF1A; Hypoxia-inducible factor 1-alpha; IP, Ipsilateral brain hemisphere; PK3CA, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha.*

Experimental testing of ANGPT1 and EGFR did not show differences in brain protein expression when comparing treated and non-treated animals (Fig. 4E). On the contrary, circulating plasma EGFR and ANGPT1 levels were found elevated in animals treated with CA, compared to the vehicle group (Fig.

4F). However, no differences were observed in animals treated with either agent alone (Supplementary Figure S1), reinforcing the synergistic effect of the drug combination. Altogether, these results support the notion that the proposed CA treatment might lead to a synergic neuroprotective effect by modulating EGFR and ANGPT1 systemically.

DISCUSSION

During the past decades, a large number of studies have devoted their efforts to identify potential neuroprotective strategies to treat ischemic stroke, most of which were directed at modulating single biological motives involved in the pathophysiology of the disease. However, the failure of many of these drugs in experimental and clinical studies has become a conceptual proof that perhaps the individual modulation of single pathological mechanisms might not be sufficient for attenuating the progression of a highly complex and multifactorial disease such as stroke [5]. Thus, the simultaneous targeting of several pathways of the ischemic cascade with a combination of neuroprotective agents could offer a better therapeutic approach for stroke. In fact, this strategy has already proven its high efficacy in other complex pathologies, including in neurodegenerative [65,66] and neoplastic [67–69] diseases: to treat amyotrophic lateral sclerosis, a drug cocktail consisting of an inhibitor of microglial activation (Minocycline), a glutamate antagonist (Riluzone), and a calcium-channel antagonist (Nimodipine) has already shown neuroprotective effects in vivo compared to the minimal effect described for the single treatments [66]. Similarly, to treat early-stage breast cancer, a drug cocktail formed by a DNA-intercalating agent (Anthracycline), an immunosuppressor (Cyclophosphamide) and an antimetastatic drug (Taxane) is being already used in clinical practice [67]. These attractive results reinforces the idea that the synergic action of drugs targeting different pathways might be required to successfully treat complex and multifactorial diseases such as stroke, but no combinational stroke treatment has been yet studied in clinical trials so far [70].

With this concept in mind, we here aimed at discovering potential combinational treatments for stroke by means of a systems biology and drug repositioning approach. Our objective was focused on finding a therapy that could benefit the largest number of patients, and therefore it should be given as soon as possible after stroke onset, even before the stroke diagnosis is confirmed in the medical center, to maximize its effectiveness [4]. Systems biology has shown to be in our hands a powerful method to accurately reproduce the whole process of stroke. Indeed, all known biological processes of the pathophysiology of stroke were precisely depicted in our stroke models, including among others neuronal cell death, brain-blood barrier disruption, ROS and RNS production, and neuroinflammation. Also, our *in silico* simulation of stroke incorporated valuable data from proteomics and transcriptomic studies conducted on brain necropsies from ischemic stroke patients. These datasets provided substantial unbiased raw information of the main molecules that change in the brain following an

ischemic stroke at both the protein and the gene levels, which overall improved the performance of the mathematical models used in the present investigation.

To guarantee the therapeutic success of the outcoming drug combinations, we initially tested in our newly-generated mathematical models of stroke the performance of 25 drugs that have previously failed their translation into clinics. These include, among others, agents targeting the glutamate-induced excitotoxicity, such as Aptiganel [71] or Lubeluzole [72]; the acute inflammatory response and the infiltration of immune cells into the damaged brain, including UK-279,276 [73], Natalizumab [74] and Enlimomab [75] or the overload of cellular calcium, for instance through Nimodipine [76] and Flunarizine [77]. The screening of these drugs allowed us to set a threshold that all the newly-identified combination of drugs had to overcome. This criterion made more likely the success when experimentally testing in pre-clinical models and even in future clinical trials any combination of drugs resulting from this screening approach.

Alpha-1 antitrypsin is a well-known serine protease inhibitor with described anti-inflammatory and anti-apoptotic properties [78]. Less is known about the therapeutic qualities of ceruletide, which was initially described as a neuromodulator of the dopaminergic system [79] and has been also involved in the reduction of glutamate-induced neuronal death [57,80]. Our findings suggest that the neuroprotective effect of the CA treatment could be attributed to an enhanced synergic interaction of both drugs, rather than an additive action, since we could not demonstrate drugs' individual therapeutic efficacy. Also, our data do support that the neuroprotective effect of CA is not dependent on alpha-1 antitrypsin dose, since we observed a similar reduction of the infarct volume when mice were treated with either the low or the high dose of alpha-1 antitrypsin in combination with ceruletide. Nevertheless, treatment with alpha-1 antitrypsin alone has previously shown protection against cerebral ischemic injury in other preclinical studies [26]. Differences in species, the modeling of stroke and the drug doses and routes of administration, among others, might be some of the reasons behind the discrepancy.

Our results also suggested that the synergic neuroprotective effects of the drug combination might be triggered through systemic actions. We have demonstrated that the combinational treatment but not the single agents increased ANGPT-1 and EGFR levels in circulation, well-known growth factors that promote angiogenesis [81] and cell proliferation [64], respectively. Of note, the upregulation and activation of these molecules have been already described to induce neuroprotection by reducing the infarct size and the brain-blood barrier leakiness in stroke preclinical models [81–84], which could also explain the therapeutic findings of our combinational treatment. Concretely, ANGPT1 helps in the maintenance of the vascular quiescence by inhibiting the increased vascular permeability against inflammatory mediators [85] protecting cell-to-cell contacts and tight junctions of the endothelial cells

[86]. Moreover, decreased circulating levels of ANGPT1 within the hyperacute phase in ischemic stroke patients have been associated with poorer outcomes [63]. Thus, it has been suggested that therapeutic interventions upregulating ANGPT1 could potentially improve stroke outcomes [87], which is in line with our findings. However, no data is available regarding the possible protective effects that increased circulating EGFR levels may exert in the brain, thus future studies are needed to elucidate its neuroprotective mechanism. Besides these alterations in circulation, we could only identify minor molecular changes in the brain due to the treatment. Enrichment analysis revealed that ceruletide and alpha-1 antitrypsin might be modulating inflammation and cell-death processes in the brain, both well-known key mechanisms defining stroke outcome [88,89]. Besides, processes related to endothelial cell proliferation and angiogenesis are also stimulated by the treatment, perhaps favoring an increased microvessel density in the infarcted region which has been associated with improved stroke outcomes [90]. Altogether, further studies are needed to confirm these initial findings and explore in more detail the molecular mechanisms of the observed therapeutic effect of ceruletide and alpha-1 antitrypsin in combination.

It should be also highlighted that no significant toxic or side effects were found in any of our experiments. In this same regard, we have demonstrated that the drug combination could be safely administered together with t-PA, overall paving the way for a future translation of this encouraging therapeutic strategy to clinical trials in stroke patients. Interestingly, this treatment would particularly benefit most those patients who cannot receive t-PA treatment due to any of the well-known medical contraindications, but it will neither cause any deleterious effect in those who are treated with t-PA at their arrival to the hospital.

The present study also stands for some limitations that should be overcome in future studies. Firstly, the experimental validation of the neuroprotective effects has only been performed in young male mice. Performing studies in different models of cerebral ischemia, including permanent strokes that resemble clinical presentations of large vessel strokes, in animals of different sexes, ages and comorbidities, as proposed in the STAIR recommendations [91] would accelerate the translation into clinics. In addition, these future studies should also include complementary behavioral and functional assessment approaches to fully evaluate the potential of this treatment for improving stroke outcome and recovery, thus confirming these initial but encouraging findings. Secondly, drug administration time-points here used correlate with a clinical scenario in which therapeutic interventions are performed within the pre-hospital phase of care, such as in the ambulance, or early post-arrival emergency department time period. Delayed administration of the drug combination also needs to be tested to fully corroborate its acute neuroprotective capacity after stroke. Similarly, the long-term impact of such acute treatment, within the sub-acute and chronic phases of the disease, deserves

further attention in future preclinical and clinical trials. Third, further studies are needed to fully elucidate the specific mechanisms of action of the drug combination. In this regard, exploring transcriptomic and proteomic changes in the brain with new and high-throughput technologies would probably improve the identification of the underlying effects of the combinational treatment in the ischemic brain. In the same line, it would be also interesting to deepen into the systemic alterations triggered by the drug combination, to confirm whether the beneficial action of the combinational treatment is mainly driven by the modulation of stroke-triggered peripheral mediators, beyond a local action in the ischemic brain.

In conclusion, we have here used a new systems biology approach to generate mathematical models that simulate the pathophysiology of ischemic stroke. This *in silico* representation allowed us to identify a drug combination formed by ceruletide and alpha-1 antitrypsin which showed synergic neuroprotective effects after ischemia *in vivo*. Overall, our findings shed light on a new powerful strategy for developing future therapies for ischemic stroke.

DECLARATIONS

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Conflicts of interest

A.S., L.R. and J.M. are co-inventors of a patent covering "Methods and compositions for treating ischaemia in a subject" (Application nº EP19382600.5), co-owned by VHIR and Anaxomics Biotech. Other authors declare no conflicts of interest.

Authors' contributions

A.S and J.M conceived and designed the experiments. R.V, L.A and T.S generated the in silico mathematical model and performed the drug repositioning experiment. A.S, L.R and C.O performed all animal experiments. H.R, P.G-R and A.S conducted the *in vitro* experimental studies. A.R and J.M supervised the experiments. A.S and L.R drafted the manuscript. All authors have critically reviewed the article content and approved it in its final version.

Ethical approval

All animal procedures were conducted in adherence to the ARRIVE guidelines [40], either in compliance with the Spanish or French legislation and following the European Communities Council guidelines (2010/63/EU). They were approved either by the Ethics Committee of the Vall d'Hebron Institute of Research (protocol number 74/16) or by the institutional review board (French ministry of Research and by the local ethical committee of Normandy (CENOMEXA).

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Supplementary materials

A. Supplementary methods

1. In silico stroke modeling

In brief, TMPS approach included the following steps (Figure 1A):

I. **Generation of molecular maps for ischemic stroke disease**. A manually-curated list of known molecular mediators that characterize the pathology of ischemic stroke was created by carefully reviewing full-length published articles (387 proteins; Supplementary table S1). The generation and extension of the subsequent network map was conducted by incorporating all known relationships of the molecular mediators from this list, based on the following sources: KEGG [1], REACTOME [2], BIOGRID [3], INTACT [4], HDPR [5], MATRIXDB [6], MIPS [7], DIP [8] and MINT [9]. The final network included 6640 proteins (data not shown).

II. **Generation of the mathematical models.** Static network maps were transformed into mathematical models through the pattern recognition techniques based on the optimization of genetic algorithms, as previously published elsewhere [10–12]. The neural network model used was a multilayer perceptron (MLP) neural network classifier [13,14]. The machine learning methodology consisted of a model constructed by stratified clusters of neural networks, which was trained with a gradient of algorithms to approximate the values of a given truth table [11] (Supplementary table S2). The truth table incorporated a set of functional values and restrictions based on the available biological knowledge about the molecular mediators of the constructed network. Two types of models were constructed: Artificial Neuronal Networks (ANN) [12], with predictive capabilities, and sampling methods [15], with descriptive capabilities.

III. **Creating experimental data-driven mathematical models and identifying reversion points.** Proteomics and transcriptomics data from human brain samples from patients who died due to stroke were also used to further characterize the truth table. In brief, own data from 4 different studies were included into the mathematical models: 2 different strategies of mass spectrometry (MS)-based proteomics with brain homogenates [16,17], MS analysis of microdissected neurons and brain blood vessels [18] and microarray-based transcriptomics with brain homogenates [17]. Repeats and contradictions among MS-based studies were disregarded and gene information was transformed to protein data before inclusion into the model. In all cases, infarct, peri-infarcted and healthy contralateral regions were compared and 1876 differentially expressed proteins were finally included in the models (data not shown), as previously described. Through this strategy, 3 different sampling methods-based mathematical models were finally generated by selectively integrating into them all high-throughput information: (I) ‘healthy brain model’ (i.e., without

data), (II) 'infarct zone model' (i.e. with infarct data) and (III) 'peri-infarct zone model' (i.e. with peri-infarct data); each of them simulating the differently affected brain regions. The proteins in each of these models were queried in order to assess whether their reversion [19] respect their original state in the model promoted the conversion of the 'peri-infarct zone model' to a 'healthy brain model' and without promoting, or avoiding, the conversion of the 'peri-infarct zone model' to the 'infarct zone model' (ischemia model reversion proteins).

IV. **Drug repositioning strategy.** Once the mathematical models were generated, drug repositioning solutions were acquired by evaluatng multiple sets of stimuli applying ANN models to identify potential functional relationships between these stimuli and ischemia or ischemia model reversion proteins [12]. The stimuli corresponded to two-by-two combinations of approved drugs from the DrugBank Database (v4.3) [20]. Approximations to the best treatment solution for ischemic stroke were obtained based on two complementary approaches, which identified the best drug combinations according to the criteria: (1) treating ischemic stroke both in the 'healthy brain model' setting and in the ''peri-infarct zone model' or (2) targeting the ischemia model reversion proteins. To set an ANN prediction value threshold, we screened in our mathematical models a set of previously unsuccessful treatments studied in clinical trials for ischemic stroke [21,22] (Fig. 1C), and we considered 80% as minimum threshold of the ANN predictive value all identified drug combinations had to fulfill for either criteria (1) or (2). ANN predictive values show the probability of the resulted relationship to be a true positive (evaluating its performance against the truth table). Besides the 80% ANN predictive value filter, accepted combinations needed to show synergistic behavior respect the individual drugs applying the highest single agent criteria [23], i.e. at least 20% increase in ANN predictive value compared to the individual drug with highest predictive value. To further restrict the discovery of potential drug combinations, the following specific filters, based on available marketing and clinical data on the drugs, were applied: (1) an approved-administration for all individual drugs; (2) no association to hypotension or hemorrhages according to public databases; and (3) no incompatibilities for intravenous administration. The following associated DrugBank categories were not considered in the study: affinity labels, artificial tears, buffers, dietary supplements, food additives, food preservatives, imaging agents, pesticides, photoaffinity labels, pigmenting agents and ultrasound contrast agents.

V. **Mechanisms of action (MoA) elucidation through sampling methods models analysis.** The TPMS technology [15] also permitted the identification of the predicted molecular mechanisms involved in the beneficial effects of the selected drug combinations. By using sampling methods, a huge number of possible solutions that comply with all biological restrictions within the truth table were identified. To track back the observed biological effects on concrete molecules, the stroke-

mimicking mathematical model is challenged with the stimulus (drug combinations) to trace the most probable solutions within the whole population and thereby identify the most probable mechanism of action that achieves a physiological response when the system is stimulated. An easy-to-read representation of the most probable MoA for each drug combination is drawn. Only the most mathematically relevant molecules from a mathematical point of view are represented.

Supplementary table S1. Summary of the data (number of entries in the database for each data type) used as for model construction (network and truth table).

Data type	Number of entries
In-house databases information	
Considered Interactions	304480
Considered Proteins	14273
Characterized Drugs	4686
Drug Targets	2096
Characterized Clinical Conditions	209
Clinical Conditions Key Proteins Characterized	3500
Training set information	
Curated drug-indications restrictions	107447 (1205 positive)
Drug-ADRs restrictions	19350 (573 positive)
Drug-indications/ADRs protein correlations	2655

ADR: Adverse Drug Reactions

Supplementary table S2. Proteins measured with the *Target 96 Mouse Exploratory panel* by Olink® Proteomics.

Assay	Uniprot ID	OlinkID	Assay	Uniprot ID	OlinkID	Assay	Uniprot ID	OlinkID
Clmp	Q8R373	OID05027	Ppp1r2	Q9DCL8	OID05060	Cxcl9	P18340	OID05093
Matn2	O08746	OID05028	Adam23	Q9R1V7	OID05061	Map2k6	P70236	OID05094
Cpe	Q00493	OID05029	Itgb6	Q9Z0T9	OID05062	Itgb1bp2	Q9R000	OID05095
Gcg	P55095	OID05031	Cyr61	P18406	OID05063	Il17f	Q7TNI7	OID05096
Gdnf	P48540	OID05032	Dlk1	Q09163	OID05064	Il1b	P10749	OID05097
Yes1	Q04736	OID05033	Ahr	P30561	OID05065	Casp3	P70677	OID05098
Il17a	Q62386	OID05034	Ccl2	P10148	OID05066	Apbb1ip	Q8R5A3	OID05099
Foxo1	Q9R1E0	OID05035	Eno2	P17183	OID05067	Wisp1	O54775	OID05100
Tnfrsf11b	O08712	OID05036	Plin1	Q8CGN5	OID05068	Cdh6	P97326	OID05101
Tgfb1	P04202	OID05037	Wfikkn2	Q7TQN3	OID05069	Pdgfb	P31240	OID05102
Pla2g4a	P47713	OID05038	Flrt2	Q8BLU0	OID05070	Igsf3	Q6ZQA6	OID05103
Il6	P08505	OID05039	Qdpr	Q8BVI4	OID05071	Tgfbr3	O88393	OID05104
Prdx5	P99029	OID05040	Fas	P25446	OID05074	Cxcl1	P12850	OID05105
Tgfa	P48030	OID05041	Erbb4	Q61527	OID05075	Pak4	Q8BTW9	OID05106
Ccl5	P30882	OID05042	Riox2	Q8CD15	OID05076	Cntn4	Q69Z26	OID05107
Epo	P07321	OID05043	Plxna4	Q80UG2	OID05077	Ghrl	Q9EQX0	OID05108
Axin1	O35625	OID05044	Epcam	Q99JW5	OID05078	Lpl	P11152	OID05109
Fst	P47931	OID05046	Ccl3	P10855	OID05079	Fstl3	Q9EQC7	OID05110
Rgma	Q6PCX7	OID05047	Crim1	Q9JLL0	OID05080	Dctn2	Q99KJ8	OID05111
Nadk	P58058	OID05048	Vsig2	Q9Z109	OID05081	Il5	P04401	OID05112
Tnni3	P48787	OID05049	Hgf	Q08048	OID05082	Eda2r	Q8BX35	OID05113
Notch3	Q61982	OID05050	Sez6l2	Q4V9Z5	OID05083	Ntf3	P20181	OID05114
Snap29	Q9ERB0	OID05051	Il1a	P01582	OID05084	Tnfsf12	O54907	OID05115
Cntn1	P12960	OID05052	Il23r	Q8K4B4	OID05085	Ccl20	O89093	OID05116
Clstn2	Q9ER65	OID05053	Dll1	Q61483	OID05086	Fli1	P26323	OID05117
S100a4	P07091	OID05054	Ddah1	Q9CWS0	OID05087	Tpp1	O89023	OID05118
Ca13	Q9D6N1	OID05055	Il10	P18893	OID05088	Tnr	Q8BYI9	OID05119
Mia	Q61865	OID05056	Tnfrsf12a	Q9CR75	OID05089	Vegfd	P97946	OID05120
Cant1	Q8VCF1	OID05057	Acvrl1	Q61288	OID05090	Parp1	P11103	OID05121
Kitlg	P20826	OID05058	Lgmn	O89017	OID05091	Tnf	P06804	OID05122
Gfra1	P97785	OID05059	Csf2	P01587	OID05092			

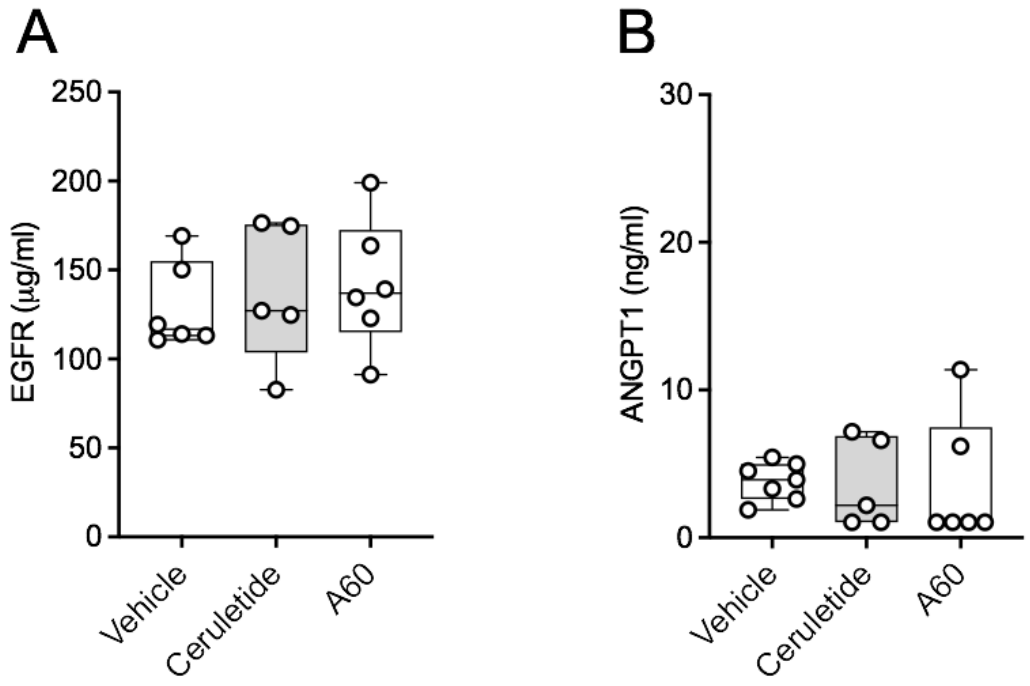
Supplementary table S3. Blood biochemical parameters

	Vehicle	CA		CA480	
	n=7	*n=5*	*p-value*	*N=5*	*p-value*
Urea, mg/dL	46.0 ±4.69	49.75 ± 5.68	0.163	50.2 ± 5.38	0.113
Creatine kinase, UI/L	407.0 ± 242.6	314.5 ± 147.1	0.927	638.3 ± 415.0	0.442
AST, UI/L	103.6 ± 50.83	89.0 ± 31.58	0.775	133.5 ± 77.45	0.933
ALT, UI/L	32.33 ± 15.37	22.80 ± 6.68	0.392	29.20 ± 9.65	0.696
Creatinine, mg/L	0.161 ± 0.01	0.167 ± 0.01	0.724	0.164 ± 0.01	0.840

Supplementary table S4. Differentially expressed proteins between IP and CL brain hemispheres of vehicle and CA-treated mice. In bold, common differentially expressed proteins between both experimental groups.

Vehicle-treated mice			CA-treated mice		
Protein	**logFC**	**adj.P.Val**	**Protein**	**logFC**	**adj.P.Val**
Ccl3	4.519	1.78E-08	**Ccl2**	3.455	3.10E-06
Cxcl1	4.325	2.44E-08	**Cxcl1**	3.795	3.10E-06
Ccl2	3.861	3.69E-08	**Il6**	3.236	3.10E-06
Il6	3.915	4.13E-07	**Ccl3**	3.898	8.78E-06
Il1a	1.491	1.45E-05	**Foxo1**	-0.822	6.80E-05
Cyr61	0.946	1.47E-04	**Il1a**	1.851	1.29E-03
Foxo1	-0.595	1.65E-04	**Cyr61**	0.474	1.48E-03
Wisp1	0.584	1.80E-04	**Nadk**	-0.302	4.27E-03
Pdgfb	0.605	2.66E-04	**Wisp1**	0.507	5.99E-03
Tgfb1	0.818	9.34E-04	**Tgfb1**	0.296	9.84E-03
Ddah1	0.761	1.24E-03	**Ccl5**	0.548	1.08E-02
Nadk	-0.353	2.12E-03	**Pdgfb**	0.393	1.23E-02
Ppp1r2	-0.298	2.78E-03	**Ddah1**	0.878	2.22E-02
Tnfsf12	-0.413	4.07E-03	**Adam23**	-0.318	3.92E-02
Dctn2	-0.356	4.37E-03	**Gfra1**	0.403	6.95E-02
Gdnf	0.329	6.46E-03	Csf2	0.238	7.10E-02
Parp1	0.931	6.75E-03	**Ppp1r2**	-0.305	8.08E-02
Gfra1	0.399	9.31E-03	**Rgma**	0.216	8.08E-02
Flrt2	0.390	1.42E-02	**Eda2r**	0.214	9.32E-02
Rgma	0.237	1.56E-02	Snap29	-0.203	9.32E-02
Pla2g4a	0.276	2.34E-02	**Riox2**	-0.276	9.77E-02
Crim1	-0.235	2.41E-02			
Ccl5	0.494	4.21E-02			
Cntn1	-0.256	4.21E-02			
Matn2	-0.217	4.21E-02			
Ahr	-0.296	4.43E-02			
Wfikkn2	-0.166	4.43E-02			
Dll1	-0.263	4.85E-02			
Fas	0.151	4.85E-02			
Acvrl1	0.248	5.46E-02			
Eda2r	0.176	5.82E-02			
Sez6l2	-0.140	5.82E-02			
Tnfrsf11b	0.186	6.31E-02			
Riox2	-0.173	7.68E-02			
Axin1	-0.231	8.25E-02			
Tpp1	-0.126	8.25E-02			
Tnni3	-0.144	8.77E-02			
Adam23	-0.214	9.12E-02			
Fstl3	0.137	9.40E-02			

Supplementary Figure S1. Quantification of ANGPT1 and EGFR levels in mouse plasma samples treated with individual agents or vehicle (n=7 in vehicle, n=5 in ceruletide, n=6 in alpha-1 antitrypsin). In all cases mean ± SD is shown**.** Abbreviations: ANGPT1, Angiopoetin-1, EGFR, epidermal growth factor receptor**.**



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DISCUSSION

After years of in-depth research and despite advances in the understanding of stroke pathophysiology, the therapeutic options to treat acute ischemic stroke remain scarce. In the same line, although many efforts have been directed towards the identification of stroke biomarkers to predict its prognosis, no biomarker with sufficient predictive power has been found to date. For that reason, the research included in the present Doctoral Thesis pretends to contribute to the identification of molecules that could be used in the future either as therapeutic targets or as prognostic biomarkers, or even for both indications.

5.1. Omic techniques to identify key molecules in cerebral ischemia

With the idea of better understanding stroke pathophysiology and identifying molecules with a key role in ischemic stroke, two different discovery studies (**Study 1** and **Study 2**) have been performed with a distinct experimental design but with a common feature: the usage of –omic techniques. These –omic techniques have emerged as high-throughput approaches that enable the accurate identification of molecular pathways altered due to ischemic stroke.

One of the main differences between these two studies is the study population: **Study 1** is performed on human ischemic stroke patients' brain samples while **Study 2** is performed in brain samples obtained from mice subjected to cerebral ischemia. Considering that we aimed to identify new molecules that could become both prognostic biomarkers and therapeutic targets for stroke, each study allowed us to obtain complementary information. **Study 2** permitted us to explore molecular changes triggered by cerebral ischemia in the hyperacute phase of the disease, specifically 2 hours after cerebral ischemia induction. Improving the characterization of stroke pathophysiology during this stage is crucial, given that it might help identifying those molecules with a key role in the initial stages of the disease. Once identified, the therapeutic modulation of these

molecules can attenuate or even reverse the progression of the disease. On the other hand, the fact that **Study 1** is performed on human brain samples from patients that died due to ischemic stroke, avoids the molecular differences found between species that might difficult the translation of pre-clinical results to clinical practice.

Thereupon, the first study of this Doctoral Thesis (**Study 1**) consists of the exploration of proteomic and transcriptomic changes that occur at a brain level after cerebral ischemia in deceased stroke patients. Interestingly, the data of both –omics datasets were analyzed together through an integrative approach to interpret the results in their biological context. A total of 128 proteins and 2716 genes were found to be significantly dysregulated after stroke when comparing the infarcted area with the contralateral brain region. Using this integrative approach, we found a 60% coincidence between proteomics and transcriptomics datasets, revealing an important crosstalk between proteins and genes after stroke. Interestingly, the other 40% of divergence discloses that proteins and genes might bring distinct and complementary information, reinforcing the importance of integrating the information obtained in each dataset. This integrative analysis revealed the main canonical pathways altered after stroke, including an increase in platelet activation and aggregation or downregulation of neurotransmitter release and neuronal system activity. These molecules related to main altered canonical pathways could become candidates to be further studied as therapeutic targets to treat ischemic stroke.

In addition, 28 genes and 9 proteins were selected to be validated in independent human brain samples employing orthogonal techniques, and finally, the results of 6 genes (ANXA1, ANXA2, IL8, NCDN, RAB3C and ST4A1) and 8 proteins (A1AG1, A1AT, DNM1L, JAM3, NCDN, RAB3C, ST4A1 and VTDB) were verified. Of note, some of the validated molecules resulted to be among the most significant of the overrepresented canonical pathways in the ischemic brain such as annexins

(ANXA1 and ANXA2) that play a role in the matrisome regulation, or JAM3 and SRGN which participate in hemostasis processes. Interestingly, those validated molecules together with others presented in **Study 1** are interesting molecules to be further studied as therapeutic targets or biomarkers for stroke, which will be discussed in the following section.

Following the main objective of the present Doctoral Thesis of identifying stroke biomarkers and with the idea of a future translation into clinics of our findings, we explored if the verified proteins were detectable in peripheral blood of ischemic stroke patients. If detectable, these molecules could be promising molecules to be further studied as potential stroke biomarkers. In this regard, we found that circulating levels of A1AT and A1AG1 increased within the first 24 hours and returned to baseline 1 week after the event. Therefore, if these results are confirmed in larger cohorts of patients, these two molecules could be promising candidates to serve as biomarkers for stroke diagnosis, provided that patients with stroke mimicking conditions have similar levels to healthy subjects included in **Study 1**. Moreover, to the best of our knowledge, it is the first time that NCDN, RAB3C and ST4A1, molecules almost exclusively expressed in the brain, are detected in peripheral blood. This could open the door to explore the potential role of these molecules as biomarkers, not only for ischemic stroke but for other neurological diseases in which they could be playing an important role.

Altogether, in this study we have integrated for the first time data from proteomics and transcriptomics techniques through a biostatistical approach to identify new key molecules in human stroke pathophysiology. Thus, we have provided novel information about protein and gene expression changes triggered in the human brain after cerebral ischemia. In this regard, the dysregulated molecules that we have found might be promising candidates to become blood biomarkers and/or therapeutic targets in the future, as it is discussed in the following section.

5.1.1. New candidate biomarkers and/or therapeutic targets for ischemic stroke

Thanks to the **Study 1** we have disclosed a list of molecules with crucial roles in stroke that could be potential candidates to be explored as biomarkers or therapeutic targets.

As examples of possible therapeutic targets, we found that DNM1L (Dynamin-1-like protein) was downregulated in the infarct core. This protein is a GTPase implicated in mitochondrial division and distribution, vesicle endocytosis and mitochondria apoptosis and necrosis [130]. Moreover, alterations in this protein can cause energy production disruption ultimately leading to cell death [131]. Remarkably, it has been reported that DNM1L inhibition produces neuroprotection both *in vitro* and *in vivo* [132,133]. In the same line, we found that ANXA2 (Annexin-2), a protein involved in cell cycle regulation, proliferation, cell survival, cell division and angiogenesis [134,135], was up-regulated in the infarcted brain region. It has been demonstrated that the administration of ANXA2 in combination with rt-PA after experimental cerebral ischemia has neuroprotective properties by reducing the hemorrhagic transformation risk and the infarct volume and by improving functional recovery [136–139].

In addition, some molecules of **Study 1** have also been studied before as possible stroke biomarkers. A1AG1 (Alpha-1-acid glycoprotein 1), an acute-phase response protein that modulates the immune response and angiogenesis [140], has been proposed before as a potential biomarker for stroke. A pilot study including 30 ischemic stroke patients, reported that plasma levels of A1AG1 were significantly higher in patients at 3 and 7 days after ischemic stroke than in healthy subjects [141]. Moreover, it has been suggested that A1AG1 could also work as a biomarker for stroke prognosis, given that ischemic stroke patients with poorer neurological outcomes one month after the event had higher baseline circulating levels of A1AG1 than those with good outcome [142].

Considering that the potential role as biomarkers and/or therapeutic targets of some of the molecules described in **Study 1** has been reported before, further exploration and/or modulation of the dysregulated molecules presented seems an interesting approach to take into consideration. In this regard, we selected A1AT, a protein found to be upregulated in the ischemic brain in **Study 1**, to study its potential role as a biomarker for stroke prognosis in **Study 4** and as a therapeutic target in **Study 6**.

5.1.2. Influence of sex, age and comorbidities on cerebral ischemia pathways

With the aim of better understanding ischemic stroke pathophysiology and deciphering the reason of translation into clinics of pre-clinical results failure, we designed **Study 2**. In this study, we explored proteomic and transcriptomic changes in the brain triggered in the first stages of cerebral ischemia in mice of four different groups: (1) young male mice, (2) young female mice, (3) aged male mice and (4) diabetic young male mice. Moreover, we compared these proteomic and transcriptomic changes of each group using an integrative enrichment pathways analysis to reveal key common and exclusive dysregulated proteins, genes and pathways playing a crucial role in the first stages of the disease.

Broadly speaking, we found notorious differences in protein and gene expression between groups. Regarding transcriptomic alterations, we found that the number of differentially expressed genes (DEG) after cerebral ischemia was similar between males and females, while in aged mice there was an important reduction in the number of DEG and in diabetic mice a very significant increase. These results suggest that the post-ischemic expression level of transcripts might be attenuated with age as described before [143,144] and exacerbated by the presence of comorbidities such as diabetes. Interestingly, we found 27 genes exclusively dysregulated in females and 16 in males, highlighting that divergences

between sexes exist and are an important factor to be considered. Moreover, we found differences between sexes regarding biological processes altered after cerebral ischemia. Concerning the inflammatory response, the biological process more altered after stroke according to our findings, we found that males had a greater number of DEG involved in this process than females. Interestingly, different neuroprotectants that are targeting the inflammation such as Anakinra, Enlimomab and Natalizumab have shown promising results in pre-clinical studies but have failed in translation into clinics [84]. Thanks to the study presented here, we have shown that sex differences in inflammation triggered after cerebral ischemia exist, a fact that could be one of the possible reasons explaining this failure.

In reference to the effect of age, we found that in aged mice there is a significant reduction of DEG after stroke. Moreover, we found notorious differences between aged mice and the other groups of animals regarding cell adhesion processes and regulation of protein kinase activity, which seem to be unaltered in aged animals. Considering that we did not find DEG involved in these two processes in aged mice, it would be interesting to further explore their relationship with aging and stroke response. Once the biological significance of this was fully understood, the pharmacological modulation of molecules participating in these processes in the aged population might be an interesting approach to be evaluated, considering that the aged population has poorer outcomes after stroke [145].

Our results also pointed out that the effect of comorbidities is an important factor to be considered when designing experiments. We found that diabetic mice had 10 times more DEG than the other groups, highlighting that cerebral ischemia triggers the alteration of an increased number of biological processes in that group of animals. Interestingly, we found an increased number of DEG involved in cell adhesion and leukocyte activation that were exclusively dysregulated in diabetic mice. In this line, it has been reported that diabetic mice had an

augmented number of infiltrating leukocytes in the brain after stroke, which is in line with the results presented here, indicating that these cells might be responsible for the exacerbated ischemic brain injury in this group of animals [146].

Regarding the proteomics results, they go in the same direction as the transcriptomic ones. However, our results point out that genes had a higher contribution to the observed alterations triggered after stroke than proteins. One possible explanation might be the early time point in which these changes have been studied. Therefore, performing a similar study and analysis at later time points will give complementary and valuable information to better characterize divergences and similarities in cerebral ischemia response between the studied groups.

What we can undoubtedly conclude from **Study 2** is that differences in the response to cerebral ischemia depending on age, sex and the presence of comorbidities exist, and this should be taken into consideration when designing new experiments. Of note, these divergences might be one of the possible reasons explaining the failure from bench to bedside of the main neuroprotectants tested so far. Hence, the results obtained in the present study reinforce the necessity of implementing STAIR recommendations [86–88] in future pre-clinical studies testing neuroprotectants.

5.2. Biomarkers to predict stroke prognosis

Considering that ischemic stroke is among the first causes of death and long-term disability worldwide [147], identifying those patients at higher risk of presenting poor outcome after stroke is essential to facilitate decision-making processes, ultimately optimizing patients' management. Stroke outcome prediction remains challenging, and it is known to be highly influenced by the baseline demographic and clinical characteristics of the patients and the etiology and stroke location

among many other factors. Nowadays, stroke outcome prediction is exclusively based on clinical scores that include variables such as stroke severity, comorbidities and age. In this regard, the identification of blood biomarkers that add value to this prediction might help to ensure ideal patients' care and ultimately improve their outcome [129].

Taking this into account, in the present Doctoral Thesis three studies have been directed towards the identification of blood biomarkers that could aid in predicting stroke outcome. Interestingly, each study evaluated the association of the selected molecules with stroke outcome at different levels and time points: **Study 3** explores neurological outcome in the acute-subacute phase of ischemic stroke; **Study 4** assesses functional outcome after stroke both in-hospital and at mid-term; and finally, **Study 5** analyses vital prognosis at long-term. Thus, with this approach we intended to cover different stages of the disease as well as different applications of prognostic biomarkers.

In **Study 3** we aimed to explore the association of AQP4 circulating levels and neurological outcome in ischemic stroke patients. AQP4 is the most abundant water channel in the brain and it is essential to maintain cerebral water balance, [148,149], being implicated in edema formation, an aggravating factor among ischemic stroke patients related to poor neurological and functional outcome [150–152]. To date, various studies have reported increased brain expression of AQP4 after ischemic stroke, both in humans [153,154] and in animal models [155–157]. However, the presence of this protein in circulation after stroke and its plausible role as a biomarker has never been studied before. We found no differences in AQP4 circulating levels between ischemic stroke patients and controls at hospital admission, but we observed that stroke patients presented a peak of AQP4 levels in the blood within the very early phase after the event. This increase might suggest that the changes in AQP4 levels previously described in the brain might be reflected in circulation some hours later. Interestingly, we

found that AQP4 levels measured at hospital admission might serve as a predictor of early neurological improvement (measured by NIHSS) during the acute and sub-acute phase of ischemic stroke. In fact, patients showing early neurological improvement, either 48 hours after stroke or at hospital discharge, had higher circulating AQP4 levels at baseline, suggesting a protective role of this protein. In this line, previous studies also pointed out this beneficial role using AQP4 knockout mice [158–160]. We determined a cut-off point for AQP4 of 2.52 ng/ml and of 1.72 ng/ml associated with patients' neurological improvement 48 hours after stroke and at hospital discharge, respectively. In both cases, the addition of AQP4 to the clinical model improved significantly its predictive capacity and its discriminatory ability. Interestingly, early neurological improvement has been proposed to be a straightforward surrogate indicator of a good outcome at later time points [161,162]. Taking all this into consideration, the usage of AQP4 as a biomarker for early stroke prognosis may aid in the decision-making and optimal patients' management, ultimately improving their outcome and quality of life after stroke.

With the idea to continue seeking biomarkers for stroke prognosis, we took advantage of the data generated in **Study 1** to further explore the role of A1AT in stroke pathophysiology. In that first study, we found that A1AT was upregulated in the infarcted area of the brain, and increased in circulation within 24 hours after stroke onset. In this regard, in **Study 4** we aimed to determine whether A1AT circulating levels measured acutely after stroke could aid in predicting stroke patients' functional outcome in the subacute and mid-term phases of the disease. We measured A1AT circulating levels at hospital admission (<6h after symptoms onset) in ischemic and hemorrhagic stroke patients and in control subjects, and we found no differences in A1AT levels between groups at this time point. This is in line with what we found in **Study 1** and with what other studies reported, that A1AT progressively increases in circulation within the first 24 hours after the event and reaches maximum levels 72-96 hours after stroke [163]. Of note, we

found that stroke patients showing poor functional outcome in-hospital and 3 months after the event had higher circulating levels of A1AT at hospital admission. We determined a cut-off point for A1AT of 156.96 mg/dL and of 161.21 mg/dL associated with poor functional outcome at hospital discharge and 3 months after the event respectively. In both cases, the addition of A1At to the clinical model substantially improved the goodness-of-fit of the model and its discriminatory ability. Thereupon, measuring A1AT levels in the hyperacute phase of stroke could aid in predicting the functional outcome of patients. Our results suggest that A1AT measurement would predict more accurately in-hospital than mid-term functional outcome, although it could improve the predictive capacity of clinical models at both times. It is worth highlighting that the clinical implementation of A1AT measurement to predict stroke outcome seems feasible and easy to implement, given that A1AT is routinely measured in the clinical laboratories of the majority of hospitals [164]. Moreover, A1AT is associated with poor functional outcome in both ischemic and hemorrhagic stroke patients, broadening the clinical applicability of this biomarker. In this regard, A1AT determination could become a useful tool to speed decision-making processes for every patient after their admission to the stroke care unit, ultimately improving their outcome and quality of life.

Finally, considering that ischemic stroke represents the fifth cause of death worldwide [60], in **Study 5** we aimed to determine whether the measurement of a panel containing 14 biomarkers acutely after stroke could predict long-term mortality. Remarkably, a large number of studies have explored the role of blood biomarkers in predicting mortality early after stroke, normally 3-months or 1 year after the event, missing what happens at later time points [107–109,165]. In this study, nearly a thousand ischemic stroke patients were followed for a median time of almost 5 years after the event to determine whether some of these 14 biomarkers could predict long-term mortality.

We found that 38.7% of ischemic stroke patients died during follow-up, which is in line with what other studies have reported [61–63]. In the studied population, we found that endostatin>quartile 2, IL-6>quartile 2 and TNF-R1>quartile 2 were independent predictors of long-term mortality after stroke. In fact, these three molecules are known to have a key role in pathophysiological pathways implicated in stroke such as inflammatory response (IL-6 and TNF-R1) and angiogenesis (endostatin). Both endostatin and IL-6 have been associated before with poor outcome and mortality after stroke [166–172] but at earlier time points. On the contrary, the association of TNF-R1 with stroke mortality has never been studied before. Interestingly, when combining these three biomarkers, the risk of death increased up to 69% while the risk was below 10% when none of these biomarkers were pathological. Moreover, the addition of these biomarkers to the clinical model increased its predictive capacity, supporting that they are interesting candidates to be further explored as long-term mortality biomarkers after stroke. In addition, endostatin>quartile 3 was also able to predict long-term mortality due to stroke (death due to the index stroke or a recurrent cerebrovascular event), given that the combination of endostatin and clinical variables slightly increased the predictive value when compared to clinical data alone. All in all, IL-6, TNF-R1 and endostatin are interesting candidates to be further studied as biomarkers to predict long-term mortality. Moreover, the modulation of these molecules and/or pathways through angiogenic or anti-inflammatory drugs might be an attractive approach to improve patients' outcome and reduce their mortality, as some pre-clinical studies are pointing out [37].

5.2.1. The future of biomarkers for stroke prognosis

Predicting patients' outcome after stroke can aid in decision-making processes, such as selecting those patients that should be admitted into specialized stroke units or rehabilitation programs. Moreover, patients at higher risk of poorer

outcomes might be selected to receive more aggressive therapeutic strategies. Ultimately, this would help to reduce sanitary costs and to ensure ideal patients' care, consequently improving their outcome and quality of life [129]. Moreover, some treatments that have been tested so far in clinical trials and have failed might be beneficial for a subgroup of patients, for example for those having poorer outcomes, so biomarkers could also give valuable information in this field. Ideally, those biomarkers that show independent association with any of the prognostic variables (functional outcome, neurological outcome and/or mortality) and also provide predictive value, could be used in clinical practice incorporating them in clinical models. In the present Doctoral Thesis, different biomarkers have been identified with increased predictive capacity over clinical models to predict stroke outcome (Table 2).

***Table 2**. Summary of identified molecules with prognostic value*

Protein	Assessment time	Prognostic variable	AUC increase	IDI
AQP4	<4.5h	Neurological improvement at 48h	13%	8.93%
		Neurological improvement at hospital discharge	7.0%	11.39%
A1AT	<6h	Poor functional outcome at hospital discharge	3.1%	5.29%
		Poor functional outcome at 3rd month	1.6%	4.02%
Endostatin IL-6 TNF-R1	<6h	Mortality at 5 years	1.1%	2.2%
Endostatin	<6h	Mortality due to stroke at 5 years	1.1%	2.0%

AUC: Area under the curve; IDI: Integrative discriminative improvement; AQP4: Aquaporin4; A1AT: Alpha-1 antitrypsin, IL-6: Interleukin-6, TNF-R1: Tumor necrosis factor receptor-1.

To implement these or other biomarkers into clinics, the results need to be validated in independent larger cohorts and they need to be cost-effective. Moreover, for those molecules used to predict the outcome in short-term, as the

ones to predict early neurological improvement, a point-of-care device (POC) should be available to rapidly measure their circulating levels. Huge advances are being performed in the design of these devices and their capacity to measure distinct molecules such as proteins, DNA and RNA in different kinds of samples (saliva, urine, serum, whole blood…) [173].

In this regard, how can we improve the discovery of prognostic biomarkers to facilitate their clinical implementation? First of all, multicenter prospective studies should be designed in order to reduce the number of false positive biomarkers. Although this kind of studies have a considerable cost, they are essential to ensure reliable results. Moreover, it is important to have in mind that the lesions produced in the brain due to cerebral blood flow interruption are not static and are in constant change. Therefore, molecular changes that occur during the hyperacute phases can vary from the ones that take place in later time points. In this regard, in biomarker studies, it is important to consider the time when the molecule is being measured and the time at which outcome is being predicted. Nowadays, the vast majority of studies predicting outcomes at the 3rd month or years after the event are being performed with biomarkers measured during the first hours of symptoms onset. In this line, better comprehending the role of the molecules altered during the hyperacute phase in stroke outcome is crucial. It would be interesting to know if these molecules are associated with stroke outcome because they remain altered during large periods of time or because they initiate other processes and pathways in the initial stages of the disease that influence stroke outcome at later time points.

In addition, stroke outcome is known to be highly influenced by clinical complications that occur later during the course of the disease, including hemorrhagic transformations, cerebral edema, infections or secondary events [20,41–43]. These events occurring during and after stroke generate a huge heterogeneity in the evolution of patients. In this line, identifying biomarkers that

could predict these complications may aid in preventing their occurrence. As an example, recognizing those patients at higher risk of developing post-stroke infections and treating them with prophylactic drugs may have an impact on their outcome.

5.3. Combination of therapeutic agents to treat ischemic stroke

During the last years, big efforts have been directed towards the identification of potential neuroprotective agents to mitigate or even reverse ischemic stroke progression. The vast majority of strategies were directed at modulating single biological motives or mechanisms altered after stroke, such as oxidative stress, excitotoxicity, inflammation or apoptosis among others [70,83,174].

Although more than 1000 neuroprotective agents have shown optimistic results in preclinical models [70], the translation of these results into the clinics has systematically failed. As has been discussed in the previous sections, stroke is a very complex disease with various molecular processes and pathological mechanisms altered. In this regard and considering the previous failure in clinical translation, we hypothesize that the single modulation of individual pathological mechanisms might not sufficient to attenuate or reverse the progression of a multifactorial disease such as stroke. Hence, combining two or more drugs that modulate different pathological pathways might be an effective therapeutic strategy to obtain synergic effects to effectively treat ischemic stroke [175,176]. However, it has been suggested that the administration of more than one drug could increase undesirable side effects. On the contrary, numerous pieces of evidence support that the “drug cocktail” approach would allow reducing the doses of each drug, consequently reducing the side effects while maintaining the neuroprotective effect [177].

With this idea in mind, in **Study 6** we have taken advantage of an innovative approach to identify combinations of two drugs with synergic neuroprotective

effects. Using a systems biology-based approach we have developed a mathematical model that simulates *in silico* ischemic stroke pathology. Of note, we guaranteed the accuracy of our mathematical model by screening more than 30 drugs previously tested in clinical trials of stroke that have failed to show enough neuroprotective effects to be translated into clinics. Thus, we verified that our model was able to predict a low degree of efficacy for these neuroprotectants, allowing us to establish a threshold above the limit of protection of those unsuccessful tested drugs to select our new candidates. We think that establishing this threshold was crucial to limit the candidate drug combinations to those with a high probability of success. After screening more than 5 million two-by-two combinations of drugs, the combination formed by A1AT and ceruletide was selected to be further studied.

We found that animals subjected to 90 minutes cerebral ischemia and treated with the drug combination showed reduced infarct volume and improved neurological status. Interestingly, our findings support the idea that the neuroprotective effect exerted by this drug combination could be attributed to a synergistic interaction of both drugs instead of to an additive effect since individual drugs showed no neuroprotective effects. It is worth noting that no significant toxic or side effects were found in any of our experiments. Despite the safety of these drugs in humans was an indispensable criterion for their inclusion in the initial screening of drug combinations by the mathematical model, the administration of both treatments together has also been proven to be safe in mice. Also, we have demonstrated that the drug combination could be safely administered together with rt-PA, overall paving the way for a future translation of this encouraging therapeutic strategy to clinical trials in stroke patients.

Taking all this information together, we think that the combination of these drugs might be effective due to their complementary effects in modulating various pathological mechanisms altered after stroke. However, despite these

encouraging results, further studies need to be performed to corroborate our findings. As it has been shown in **Study 2**, sex, age and comorbidities have a great influence on the response in front of stroke. In this regard, the neuroprotective synergistic effect of this drug combination should be confirmed in females, in animals of different ages and in animals with comorbidities, as well as on other species, as proposed in the STAIR recommendations [88]. It would also be of interest, to perform dose-response studies and to establish the therapeutic window, to optimize the therapeutic effect of the drug combination.

One of the strengths of this study is that drug repositioning allows identifying new uses of existing Food and Drug Administration (FDA) approved drugs. Therefore, the drugs of the proposed combination have previously been approved for clinical uses, although they are used for other purposes than neuroprotection. This fact allows accelerating the future translation of this strategy into clinics, given that safety and toxicity studies in humans have been performed previously. Moreover, identifying and validating new medical applications of commercialized drugs allows reducing time and expenses in time-to-market.

5.4. Key molecules might become both biomarkers and therapeutic targets for stroke management

The better characterization of molecular changes that occur after cerebral ischemia, allows identifying molecules with key roles in stroke pathophysiology. Interestingly, these molecules might have a dual role: might serve as stroke biomarkers but at the same time might become interesting therapeutic targets. In the present Doctoral Thesis, and thanks to **Study 1** and **Study 2**, we have identified key molecules altered in the brain after ischemic stroke. The lists of molecules derived from these studies provide novel candidates to be evaluated as stroke biomarkers and/or therapeutic targets.

As it has been discussed through the sections of this Doctoral Thesis, stroke is a complex disease with different mechanisms involved in its pathophysiology. As we have found in **Study 2**, inflammation has a crucial role in the triggering and the progression of stroke pathophysiology. In fact, the plausible dual role of inflammatory molecules as stroke biomarkers and therapeutic targets has been extensively reviewed (Annex I). The results summarized in this review pointed out a plausible use of inflammation-related proteins as blood biomarkers, therapeutic targets and even surrogate biomarkers of the efficacy of therapeutic strategies directed against the same target molecule, a fact that will provide more personalized management of stroke patients.

Following this idea, here we have described how A1AT, a molecule found to be upregulated in the ischemic brain in **Study 1**, could be used both as a biomarker of in-hospital and mid-term poor functional outcome (**Study 4**) and as a therapeutic target when combined with Ceruletide (**Study 6**). The beneficial and neuroprotective effects of A1AT have been attributed to its capacity of modulating the activity of immune system cells. It has been shown that A1AT protects neurons, astroglia and oligodendroglia from apoptotic and necrotic cell death after oxygen and glucose deprivation (OGD) by increasing pro-inflammatory cytokines and reducing anti-inflammatory cytokines [178]. In **Study 4**, we have observed increased A1AT circulating levels in patients presenting poorer outcomes. We can hypothesize that these augmented levels may reflect a protective response mechanism that would take place as an attempt to mitigate the pathological processes triggered due to cerebral ischemia, although without success. However, we can not discard that this upregulation in A1AT levels was contributing to the expansion of the ischemic lesion and consequently to the worsening of stroke patients. In this line, further studies are needed to figure out the biological and functional implications of A1AT in the context of stroke.

As another example of a molecule presenting this dual role, in **Study 3** we found that AQP4 could be used as a biomarker of early neurological improvement. It has been reported that the inhibition of AQP4 in animal models of cerebral ischemia could have neuroprotective roles by reducing brain edema and improving the outcome [179,180].

In the current context of stroke in which the only approved treatments are reperfusion therapies, is essential to explore new biomarkers and therapeutic targets such as the ones presented in this Doctoral Thesis. In an ideal scenario, some of these molecules could be measured in the future in POCs to rapidly diagnose ischemic stroke and increase the number of patients that can benefit from the actual reperfusion therapies. Moreover, by measuring these molecules we could also select those patients at higher risk of presenting poorer outcomes to receive more aggressive therapeutic strategies. In addition, since many stroke patients do not respond properly to these reperfusion therapies, the pharmacological modulation of the candidate molecules presented as a complementary therapeutic tool could be an interesting approach to improve stroke patients' management and consequently their outcome. In this line, in the following section, we will propose 3 different possible future therapeutic approaches.

5.4.1. Future directions and possible therapeutic approaches

Compiling all the information and data generated in the present Doctoral Thesis, we propose three different possible therapeutic approaches that could be interesting to be further studied. In a first scenario, we propose to use the Traffic Light System (TLS) approach. In this case, when an ischemic stroke patient arrives at the Emergency Department, a blood sample would be obtained to measure a panel of biomarkers. This panel should include molecules known to be altered after cerebral ischemia with key roles in stroke pathophysiology. Moreover, it

should have been demonstrated before in pre-clinical studies that the pharmacological modulation of these molecules exerts neuroprotective effects. In this regard, once the panel of biomarkers is measured, the patient would receive a drug cocktail of pharmacologic agents to modulate only those molecules that are altered (a cut-off point for each biomarker should be determined before).

In a second scenario, we propose to take advantage of prognostic biomarkers. First of all, we would need a good biomarker to predict the outcome of patients, and we should establish a cut-off point to determine which patients are the ones at higher risk to present poor outcome (with enough accuracy). Thus, when an ischemic stroke patient arrives at the Emergency Department, the biomarker should be measured and those patients at higher risk of poorer outcomes could be selected to receive more aggressive therapeutic strategies. Moreover, as we have demonstrated in the present Doctoral Thesis, it would be possible that the molecule predicting patients' outcome is the same that could be pharmacologically modulated.

In a last scenario, the therapeutic agents that each patient should receive would be determined according to its phenotype and clinical characteristics. As we have discussed, the response to cerebral ischemia differs depending on clinical variables such as sex, age and the presence of comorbidities. However, there are also common molecules and pathways altered after an ischemic stroke independently of patients' clinical characteristics. In this line, we consider that an interesting strategy would be having a common drug cocktail for all types of patients modulating shared altered molecules, but also specific drugs to modulate molecules dysregulated only in some clinical phenotypes. As an example, recent studies support that combined treatment with Uric Acid and rt-PA after ischemic stroke only exerts neuroprotective effects in women but not in men [181]. In this regard and following the therapeutic strategy proposed here,

only women would be treated with this therapeutic agent, together with other "common drugs" that would receive all types of ischemic stroke patients.

In all cases, all therapeutic strategies proposed here should be accompanied by either rt-PA or thrombectomy if the patient passes the eligibility criteria.

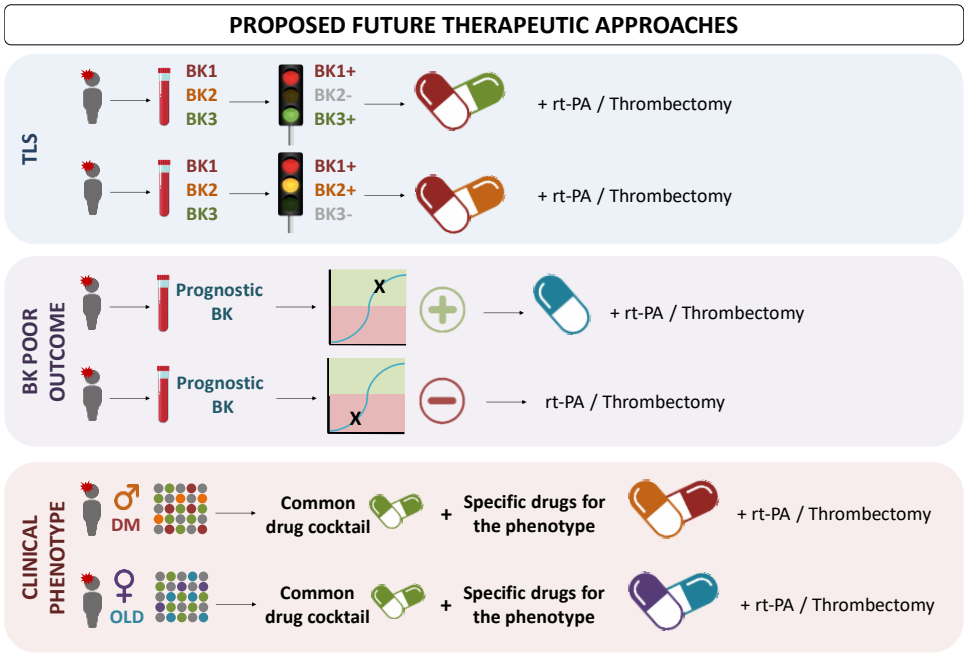
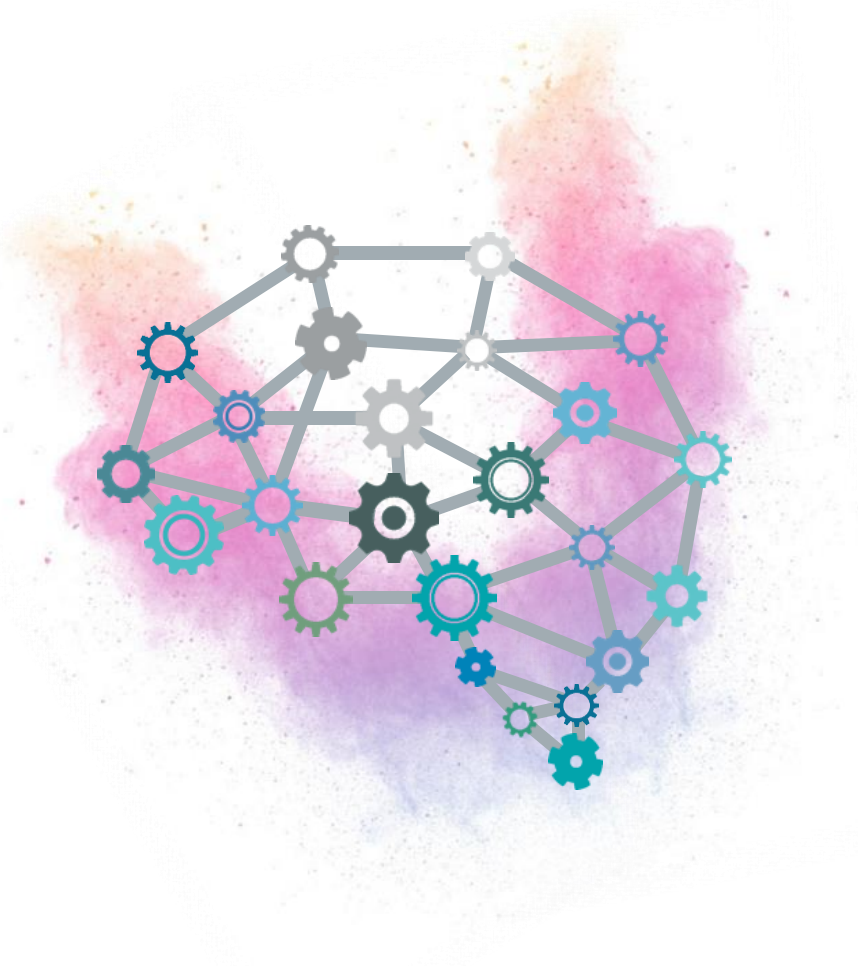


Figure 10. *Proposed future therapeutic approaches derived from the present Doctoral Thesis. BK: Biomarker; DM: Diabetes Mellitus; rt-PA: Recombinant tissue-plasminogen activator; TLS: Traffic light system.*

CONCLUSIONS



Conclusions

- Through the study of the proteome and the transcriptome of the human ischemic brain during the acute phase of ischemia and their integrative biostatistical analysis, we have identified 128 proteins, 2716 genes and several canonical pathways significantly dysregulated after stroke. Some of the proposed candidates show potential as stroke biomarkers and/or therapeutic targets.

- The integrative biostatistical study of proteomic and transcriptomic changes associated with the influence of sex, age and comorbidities in stroke pathophysiology shows that the response to cerebral ischemia differs among those studied groups, highlighting the importance of incorporating animals with different phenotypes in future neuroprotection related studies

- Aquaporin-4 circulating level measured acutely after ischemic stroke predicts neurological improvement at 48 hours and at hospital discharge. Alpha-1 antitrypsin circulating level measured in the hyperacute phase after stroke predicts poor functional outcome in-hospital and at mid-term. Both molecules add predictive value on top of clinical variables.

- Endostatin, tumor necrosis factor receptor-1 and interleukin-6 are independent predictors of long-term mortality. When data of these 3 biomarkers are combined the risk of mortality increases up to 69%, adding predictive value over clinical data.

- Thanks to a systems-biology-based drug repositioning approach various two-by-two combinations of drugs with theoretical synergistic neuroprotective effects have been identified. The efficacy of the drug combination formed by Ceruletide + Alpha-1 antitrypsin has been demonstrated in a mouse model of transient cerebral ischemia and its potential interactions with rt-PA have been discarded.

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ANNEX

8.1. Inflammatory molecules might become both biomarkers and therapeutic targets for stroke management

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Therapeutic Advances in Neurological Disorders

Review

Inflammatory molecules might become both biomarkers and therapeutic targets for stroke management

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Abstract: Stroke is the fifth leading cause of death and the most frequent cause of disability worldwide. Currently, stroke diagnosis is based on neuroimaging; therefore, the lack of a rapid tool to diagnose stroke is still a major concern. In addition, therapeutic approaches to combat ischemic stroke are still scarce, since the only approved therapies are directed toward restoring blood flow to the affected brain area. However, due to the reduced time window during which these therapies are effective, few patients benefit from them; therefore, alternative treatments are urgently needed to reduce stroke brain damage in order to improve patients' outcome. The inflammatory response triggered after the ischemic event plays an important role in the progression of stroke; consequently, the study of inflammatory molecules in the acute phase of stroke has attracted increasing interest in recent decades. Here, we provide an overview of the inflammatory processes occurring during ischemic stroke, as well as the potential for these inflammatory molecules to become stroke biomarkers and the possibility that these candidates will become interesting neuroprotective therapeutic targets to be blocked or stimulated in order to modulate inflammation after stroke.

Keywords: biomarkers, cerebrovascular disease, inflammation, neuroprotection, stroke, therapeutic targets

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Introduction

Stroke is caused by the disruption of the blood flow into a brain region and the consequent oxygen and nutrient deprivation, resulting in cell death and severe brain damage. It is the fifth most common cause of death and the most frequent cause of permanent disability in adults worldwide. Ischemic stroke, which is caused by the obstruction of a blood vessel by a thrombus, represents 87% of all strokes.[1]

After years of in-depth research and despite advances in the understanding of stroke pathophysiology, the therapeutic options for acute stroke remain very scarce. Currently, the only approved therapy for treating acute ischemic stroke is the administration of intravenous recombinant tissue–plasminogen activator (rt-PA) together with endovascular mechanical thrombectomy to remove the thrombus. However, rt-PA treatment carries a significant risk of secondary bleeding.[2] Furthermore, it has a reduced time window of action, given that it is effective only when administered within 4.5 h after the onset of symptoms.[3] This limitation reduces the number of stroke patients who can be treated to approximately 15%.[4] Although mechanical thrombectomy has recently demonstrated its efficacy even 24 h after the onset of the cerebrovascular event,[5] neuroprotective therapies are sought in order to rescue the compromised tissue in the peri-infarct zone of the brain. Although different neuroprotective therapies have shown good results in experimental models, none of them has achieved good results at the clinical level. For that reason, alternative therapies are urgently needed to combat

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stroke. In addition, stroke diagnosis is currently based on medical history, neurological exploration and neuroimaging; therefore, there are no rapid diagnostic tools, such as a blood test, available in clinics. Such a test would help accelerate stroke treatment and optimize patients' management to ameliorate stroke outcomes.

Given the complexity of stroke, different mechanisms are thought to be involved in its pathophysiology. In fact, increasing evidence shows that inflammation plays an important role in the progression of stroke.[6] For that reason, therapeutic targeting of postischemic inflammation in acute stroke has gained interest as a potential neuroprotective strategy. Here, we provide an overview of the inflammatory processes occurring during ischemic stroke, the potential for these inflammatory molecules as stroke biomarkers and the possibility that these biomarkers will become important therapeutic targets to be blocked or stimulated in order to modulate inflammation after stroke. To that end, studies in which pharmacological agents are specifically directed to modulate inflammatory molecules in the context of stroke will be reviewed.

In our attempt to focus on translational evidence as much as possible, studies including knockout animals for inflammation-related genes are considered beyond the scope of this review. In addition, studies indirectly modulating inflammatory molecules will not be considered either, given that we want to describe the specific role of each individual protein in the pathophysiology of ischemic stroke.

Postischemic stroke inflammation

After the ischemic event, the blockage of blood flow produces a reduction of glucose and oxygen availability, which leads to an impaired adenosine triphosphate production and ultimately causes a bioenergetics failure.[7] In addition, an ionic imbalance occurs within minutes after the arterial occlusion, generating a reduced reuptake of glutamate and subsequently leading to excitotoxicity and apoptosis. At the same time, catabolic enzymes are activated, producing an increase in the generation of reactive oxygen species. This fact triggers the expression of pro-inflammatory genes, such as cytokines and chemokines, by the injured brain cells.[8] Consequently, the expression of cell adhesion molecules on the endothelial cell surface is induced, including P-selectin, E-selectin and intercellular adhesion molecule-1 (ICAM-1). These molecules mediate the infiltration of leukocytes into the brain parenchyma and facilitate the clearance of debris in the infarct area. Moreover, endothelial cells increase the expression of chemokines in order to guide leukocytes to the site of injury.[9] However, there is evidence showing that beside their beneficial role, infiltrating immune cells also impair the ischemic brain by producing cytotoxic mediators that can extend the inflammatory response and increase brain damage.[10]

In addition, dying brain cells secrete damage-associated molecular patterns (DAMPs) that are recognized by microglia and cause their activation. Thus, both resident and infiltrated immune cells produce a burst of pro-inflammatory molecules within the infarct area.[11] This fact couples with an increase in matrix metalloproteinase (MMP) production, which mediates the destruction of the basal lamina, increasing blood–brain barrier (BBB) permeability and facilitating the entrance of additional peripheral immune cells into the affected brain area.[12]

Furthermore, the complement cascade, a major constituent of innate immunity, has been shown to play an important role in stroke. The activation of the complement system is mediated by an increased expression of complement cascade receptors and activators (C1q and C3) by damaged cells in the site of injury. Its activation generates opsins (C3b, C4b and C5b), which enhance the phagocytic response, as well as inflammatory mediators and a membrane attack complex (C5b, C6, C7, C8 and C9) that has cytolytic activity.[13] In addition, complement system activation has been implicated in adhesion molecule upregulation, chemotaxis and leukocyte activation.[14]

Acute stroke not only causes a local inflammatory response in the ischemic brain but also triggers a systemic immune response. After the ischemic event, there is a major release of pro-inflammatory mediators into the systemic circulation, causing an overactivation of peripheral immune cells. This excessive activation leads to an exhaustion of mature leukocytes, provoking the recruitment of immature leukocytes that are unable to respond properly to brain damage.[15] In fact, the recruitment and expansion of this immature subpopulation ultimately lead to lymphocytopenia, contributing to a significant immunodepression that predisposes

patients to poststroke infections and influence stroke outcome.[16] Furthermore, the disproportionate concentration of pro-inflammatory mediators can stimulate the activation of the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system, with the consequent release of catecholamines and glucocorticoids. All this leads to the inhibition of pro-inflammatory pathways and the stimulation of anti-inflammatory mechanisms through the release of interleukins and growth factors. In addition, this immunosuppression helps prevent autoimmune reactions against the central nervous system (CNS) antigens that are present in the bloodstream due to BBB leakage.[17]

Finally, in the brain, the immune system also works to terminate the inflammatory response at later stages. To that end, infiltrating macrophages and activated microglia are known to phagocytose dead cells and debris. Moreover, they promote the production of anti-inflammatory molecules that contribute to the suppression of the immune response, and at the same time, inhibit the expression of adhesion molecules and the production of pro-inflammatory cytokines.[6] In addition, both macrophages and microglial cells release neuroprotective factors required for the recovery of the ischemic brain injury that promote neurogenesis and angiogenesis, among other processes.[18] Figure 1 summarizes the postischemic inflammatory cascade, highlighting key proteins that play a relevant role in the process.

Biomarkers in stroke

Biomarkers are defined as characteristics that can be objectively measured and evaluated as indicators of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic interventions.[19] Clinical biomarkers can be detectable molecules from different biological fluids such as blood, urine, or saliva. Ideally, biomarkers should have high specificity and sensitivity for the indicated process, and their measurements should be accurate, reproducible, and easily interpretable by clinicians. In terms of clinical application, stroke biomarkers are considered relevant tools for different phases, including both the diagnosis and the prognosis of stroke. These biomarkers should be representative of the brain injury processes triggered by stroke. As previously described, BBB disruption favors the release of brain antigens into the peripheral circulation, making these molecules promising stroke-associated biomarkers that could reflect in the circulatory system those pathological processes occurring in brain after the ischemic event. In addition, both local and peripheral inflammatory response can produce systemic indicators that might serve as stroke biomarkers.

Different inflammation-related proteins have been found altered after ischemic stroke and have shown a potential role in the detection and modulation of inflammation, making them potential biomarkers or therapeutic targets for stroke management. Those being studied for both roles will be reviewed in the following sections. Table 1 summarizes the main studies performed for each of the proteins described in this review.

Main inflammatory players in stroke

Cytokines

Cytokines are small proteins that through extracellular signaling regulate different biological functions such as innate and acquired immunity, inflammation, proliferation and repair. Cytokines have both pro- and anti-inflammatory properties and play an important role in the progression of the stroke-associated inflammation.[152] After the ischemic event, cytokines are upregulated in the brain, expressed by both cells of the immune system and resident brain cells such as neurons and microglia.[153]

Pro-inflammatory cytokines. Interleukin-1 (IL-1) is produced in the CNS by microglia, astrocytes, endothelium and neurons. It has two different forms, one intracellular (IL-1α) and one secreted (IL-1β). Both forms act through the IL-1 type I receptor (IL-1RI) which is expressed by immune and endothelial cells. This receptor is competitively blocked by the naturally occurring IL-1 receptor antagonist (IL-1Ra), also secreted by endothelial and immune cells and binds to the receptor without inducing any effect.[154] In reference to the plausible role of the IL-1 family as stroke biomarkers, there is some controversy. Distinct studies revealed that ischemic stroke patients have higher circulating levels of IL-1β within 24 h after the ischemic event when compared with controls, as well as when compared with patients with other neurological conditions such as Alzheimer disease and Parkinson disease.[20–24] However, two other studies have reported that IL-1β levels in serum or plasma are not higher in stroke patients when compared

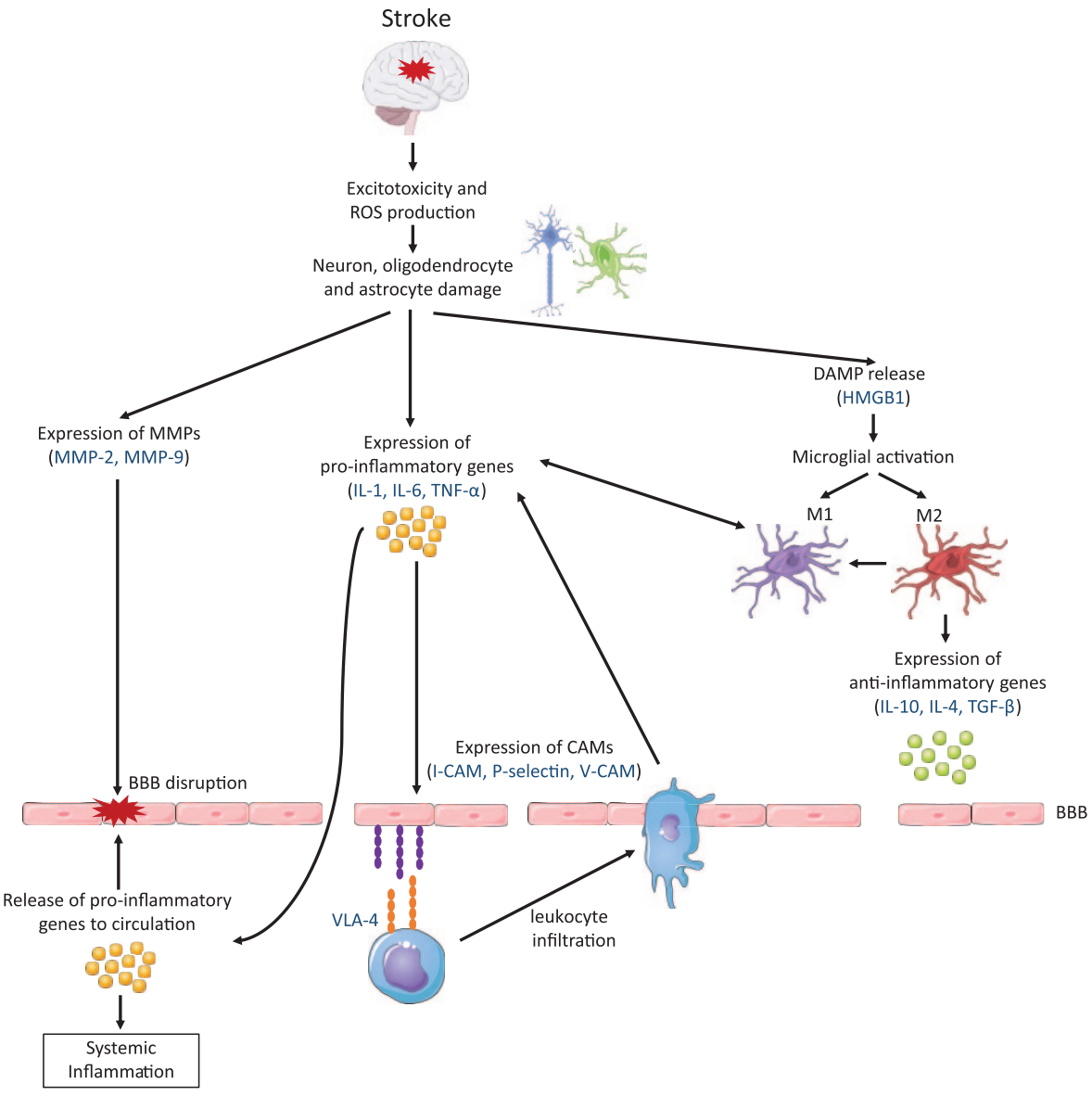

Figure 1. The inflammatory cascade following a stroke.
BBB, blood–brain barrier; CAM, cellular adhesion molecule; DAMP, damage-associated molecular pattern; HMGB1, high-mobility group box 1; I-CAM, intercellular adhesion molecule; IL, interleukin; MMP, matrix metalloproteinase; ROS, reactive oxygen species; TGF, transforming growth factor; TNF, tumor necrosis factor; V-CAM, vascular cell adhesion molecule; VLA, very late antigen.

with healthy controls, at 12 h[25] and 72 h after the onset of symptoms.[26] These contradictory results observed among studies might be explained, in part, by the differences in the time of evaluation of the biomarker or the sample size of the stroke patient cohorts (up to 120 for those studies reporting higher levels in stroke patients *versus* less than 50 patients in those reporting no differences). In addition, it has been shown that plasma levels of IL-1Ra are higher in patients than in controls within the first 24 h and even at 72 h after the ischemic event.[25–27] However, these elevated levels of IL-1Ra may not be sufficiently high to prevent IL-1 activation of receptors in target cells.[27] Several studies have investigated the therapeutic effect of the modulation of this pathway. Various studies have shown that IL-1β administration increases the infarct size in mice after transient middle cerebral artery occlusion (tMCAO)[28] and rats after permanent (pMCAO) or transient ischemia.[33,34]

Table 1. Main studies performed on inflammatory-related proteins having an important role in stroke pathophysiology.

Protein	Role as biomarker		Experimental studies			Human clinical trials	
	Type	Blood levels	Intervention	Rodent model	Infarct volume changes	Design	Results
IL-1β/IL-1Ra	Diagnosis	Higher levels of IL-1β in IS than in controls within 24 h after the event.[20–24] No differences in IL-1β levels between IS and controls at 12 or 72 h after the event.[25,26] Higher levels of IL-1Ra in IS than in controls[25–27]	IL-1Ra/IL-1β administration	tMCAO mouse	IL-1Ra ↓[28,29] IL-1β ↑[28]	34 IS: 17 received intravenous IL-1Ra over 3 days and 17 placebo.[30,31]	Drug is safe and well tolerated; it produced a reduction of the inflammatory response (white cell counts and pro-inflammatory proteins) and reversed peripheral immunosuppression; improved 3-month outcome
				tMCAO rat	IL-1Ra ↓[32] IL-1β ↑[33]	80 IS: 39 receiving subcutaneous IL-1Ra for 3 days and 41 receiving placebo[39]	Drug reduces plasma inflammatory markers; no improvement in 3-month outcome
				pMCAO rat	IL-1Ra ↓[34,35–38] IL-1β ↑[34]		
VCAM-1/VLA-4	Diagnosis/prognosis	Higher levels of VCAM-1 in IS than controls;[23,24,40–43] association between higher VCAM-1 levels and worse outcome[44,45]	Anti-VLA-4 administration	tMCAO mouse	No change[46,47] ↓[46,48,49]	161 IS: 79 received intravenous natalizumab and 82 placebo[50]	Natalizumab did not reduce infarct volume; natalizumab-treated group had better functional outcome than placebo group
				pMCAO mouse	No change[48] ↓[47]	270 participants receiving Natalizumab in a low or a high dose, or placebo	Ongoing [ClinicalTrials.gov identifier: NCT02730455]

(Continued)

Table 1. (Continued)

Protein	Role as biomarker		Experimental studies			Human clinical trials	
	Type	Blood levels	Intervention	Rodent model	Infarct volume changes	Design	Results
				tMCAo rat	↓[51,52]		
ICAM-1	Diagnosis/ prognosis	Higher levels of ICAM-1 in IS than controls within 24 h after the ev ent,[21,23,24,53,54] although another study found no difference;[40] one small study showed lower levels of ICAM-1 in IS than in controls;[55] association between higher ICAM-1 levels and larger infarct size and worse long-term outcome,[21] although no association also found[40,56]	Anti-ICAM-1 administration	tMCAO rat	↓[57–59]	32 IS: several dose ranges of enlimomab within first 24 hours + four daily doses[60]	160 mg on day 1 + 40 mg/ day for 4 days produce the desired blood levels (⩾10 μg/ml) without increasing adverse effects
				pMCAO rat	No change[58]	625 IS: 317 received enlimomab and 308 placebo[61]	Enlimomab-treated group had worsening of neurological functions, increased mortality and adverse drug reactions compared with placebo group
TNF-α	Diagnosis/ prognosis	Higher levels of TNF-α in IS than in controls;[21,23,24,62–65] smaller studies showed no differences in TNF-α between IS and controls;[25,26,66,67] associations between higher TNF-α levels and poor outcome[20,62] and larger lesion size[65,68] are not clear enough[26,69,70]	anti-TNF/ TNF-bp administration	tMCAO mouse	Anti-TNF ↓[71]		

Table 1. (Continued)

Protein	Role as biomarker		Experimental studies			Human clinical trials	
	Type	Blood levels	Intervention	Rodent model	Infarct volume changes	Design	Results
				pMCAO mouse	TNF-bp ↓[72,73]		
				tMCAO rat	Anti-TNF ↓[74,75]		
				pMCAO rat	Anti-TNF ↓[76] TNF-bp ↓[77]		
TGF-β	Diagnosis/ prognosis	Controversial results: lower,[78,79] equal[80,81] or higher[82] levels of TGF-β in IS than controls; association between high late TGF-β levels and large infarct volume and stroke severity[81]	TGF-β/TGF-β antagonist administration	tMCAO mouse	TGF-β ↓[83]		
				pMCAO mouse	TGF-β ↓[84]		
				tMCAO rat	TGF-β antagonist ↑[85]		
MMP-9/ MMP-2	Diagnosis/ prognosis	Higher levels of MMP-9 in IS than controls[86–93] and stroke mimics;[94–96] no differences between stroke patients (ischemic and hemorrhagic) and controls;[97] association between higher MMP-9 levels and worse outcome and larger infarct volumes;[21,68,86,89,93,98–106] association between higher MMP-9 levels and hemorrhagic transformation following rt-PA administration;[92,107–110] lower levels of MMP-2 in IS than controls;[93,102] smaller study showed no differences[92]	Anti-MMP-9 + anti-MMP-2 administration (SB-3CT)	tMCAO mouse	↓[111]		

(Continued)

Table 1. (Continued)

Protein	Role as biomarker		Experimental studies			Human clinical trials	
	Type	Blood levels	Intervention	Rodent model	Infarct volume changes	Design	Results
				pMCAO mouse	↓[112]		
IL-6	Diagnosis/ prognosis	Higher levels of IL-6 in IS patients compared with controls[20,21,23,24,26,62,66,67,113–120] and stroke mimics;[94] association between higher IL-6 levels and poor functional outcome, infections[44,121] and larger infarct volumes[122,123]	IL-6 administration	pMCAO mouse	No change[124]		
				pMCAO rat	↓[125]		
IL-10	Diagnosis/ prognosis	Lower[78,115,117,126,127]or equal[24,128]IL-10 levels when comparing IS and controls; no association between IL-10 and infarct volume[70,129] or stroke outcome;[70, 126,130,131] association between higher levels of IL-10 and infections[132]	IL-10 administration	pMCAO rat	↓[133–135]		
IL-4	Diagnosis/ prognosis	Higher levels of IL-4 in IS than controls;[116] no association between IL-4 and neurological worsening[69]	IL-4 administration	tMCAO mouse	↓[136] No change[137]		
P-selectin	Diagnosis	Higher levels of P-selectin in IS than in controls[23,24,138–140]	Anti-P-selectin administration	tMCAO mouse	↓[141]		
				tMCAO rat	No change[142]		

Table 1. (Continued)

Protein	Role as biomarker		Experimental studies			Human clinical trials	
	Type	Blood levels	Intervention	Rodent model	Infarct volume changes	Design	Results
				pMCAO rat	↓[143,144]		
HMGB1	Diagnosis/ prognosis	Higher levels of HMGB1 in IS than in controls;[86,145–147] association between higher HMGB1 levels and poor outcome,[86,148] although another study found no association[147]	HMGB/ anti-HMGB1 administration	tMCAO rat	HMGB1 ↑;[149] anti-HMGB1 ↓[149]		
ANXA1	Diagnosis	No differences in ANXA1 levels between IS and controls or stroke mimics.[150]	ANXA1/anti-ANXA1	pMCAO rat	ANXA1 ↓;[151] anti-ANXA1 ↑[151]		

Inflammatory-related proteins are sorted from the most to the least studied.
ANXA1, annexin A1; HMGB1, high-mobility group box 1; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; IL-1β, interleukin-1 beta; IS, ischemic stroke patients; MMP, matrix metalloproteinase; pMCAO, permanent middle artery occlusion; rt-PA, recombinant tissue–plasminogen activator; SB-3CT, methylthiirane; TGF-β, transforming growth factor beta; tMCAO, transient middle cerebral artery occlusion; TNF-α, tumor necrosis factor alpha; TNF-bp, tumor-necrosis-factor-binding protein; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4.

On the other hand, the administration of recombinant human IL-1Ra greatly reduces (up to 60%) the infarct volume after MCAO in both mice[28,29] and rats. [32,34–38] In line with this, there are studies showing an improved functional outcome after the administration of IL-1Ra.[155,156] The results obtained in these studies indicate a deleterious role of IL-1 and a plausible mechanism of neuroprotective action through the blockage of this pathway. This has led to the development of clinical trials testing the therapeutic potential of recombinant human IL-1Ra (anakinra) in stroke. In a phase II randomized clinical trial, 34 ischemic stroke patients within 6 h of the onset of symptoms received anakinra (100 mg loading dose over 60 s, followed by a 2 mg/kg/h infusion over 72 h) or placebo, intravenously. This study showed that the drug was safe and well tolerated and that patients receiving anakinra showed a reduced inflammatory response, with lower levels of neutrophils and total white cell counts as well as C-reactive protein (CRP) and IL-6 blood levels. Moreover, although no differences were observed in infarct volumes, 3-month clinical outcome was better in the treated group than in the placebo group.[30] This could be related to the reversal of the peripheral immunosuppression, given that the production of cytokines such as tumor necrosis factor alpha (TNF-α), IL-6 and IL-10 was reduced in the anakinra-treated group.[31] Very recently, a new phase II trial has explored the subcutaneous administration of IL-1Ra, which increased the half-life of the drug and facilitated its administration. In this study, 39 ischemic stroke patients received a total of six doses of anakinra (100 mg administered twice a day for 3 days) and 49 patients received placebo. The results confirmed that IL-1Ra administration significantly reduced plasma inflammatory markers associated with worse outcome, such as IL-6. However, patients receiving IL-1Ra subcutaneously did not show an improvement on the 3-month outcome when compared with the placebo group.[39] Therefore, although the overall results seem promising, further studies need to be done before its translation to practice. Moreover, it would be interesting to evaluate the efficacy of anakinra on reducing the infarct volume to better understand the role of IL-1 in the stroke pathophysiology.

Interleukin-6 (IL-6) is a pro-inflammatory cytokine that acts as a messenger molecule. It is secreted by different cells including microglia, astrocytes, leukocytes and endothelial cells in response to cerebral injury.[157] Several studies show that there is an increase in IL-6 circulating levels in stroke patients early after the ischemic event in comparison with controls[20,21,23,24,26,57,62,66,113–120] and even in front of stroke-mimicking conditions such as syncope, seizures, and primary headache disorders.[94] Moreover, high IL-6 levels in blood 24 h after the ischemic event are associated with poor long-term functional outcome[44,121] and with large infarct volumes.[122,123] In addition, it has been shown that an acute increase of IL-6 levels in ischemic stroke patients is associated with poststroke infections within the first week after the event.[121] However, while higher circulating levels of IL-6 are related to morbidity, there is also evidence that IL-6 can have neuroprotective functions after ischemic stroke.[158] For example, it has been described that IL-6 produced by injured brain cells promotes poststroke angiogenesis.[159] For that reason, some studies have explored the neuroprotective role of IL-6 in experimental stroke. One study performed using a pMCAO mouse model showed that IL-6 administration right after reperfusion did not reduce infarct volume but improved functional outcome.[124] Similarly, another independent study performed in a transient tMCAO mouse model showed an improved functional outcome after IL-6 administration.[160] Loddick and colleagues showed that the intracerebroventricular injection of recombinant human IL-6 protein reduced infarct size in rats.[125] In addition, IL-6 prevented learning disability and hippocampal neuron loss when administered in gerbils.[161] All these findings could be indicating different effects of IL-6 between central and systemic inflammation, as well as at different times poststroke. Thus, IL-6 seems to be a good biomarker for stroke diagnosis and prognosis but further studies need to be done to better evaluate its therapeutic effect. Moreover, a blockade of this pathway might also be of interest in order to further evaluate the dual role of this protein.

TNF-α is another important mediator implicated in the pathophysiology of stroke. TNF-α has two forms: a transmembrane form that regulates local inflammation by cell-to-cell interactions, and a soluble form generated by TNF-α-converting enzyme (TACE).[162] The soluble form acts at the systemic level amplifying the phagocytic and cytotoxic action of macrophages and enhancing the expression of other cytokines, such as IL-6 and IL-1. In reference to TNF-α blood levels after ischemic stroke, there are contrasting reports. Some studies have reported that circulating

TNF-α levels do not change after the ischemic event within the first 24 h,[25,26,66,67] whereas others reported an increase in the TNF-α blood levels after stroke in comparison with controls.[21,23,24,62–64,163] However, the lack of differences reported in some studies could be, partly due to a reduced sample size. Specifically, studies reporting no differences include fewer patients (from 19 to 34 ischemic stroke patients) than the ones showing increased TNF-α levels (from 23 to 131 ischemic stroke patients, including 4 studies with more than 100 patients). Moreover, the role of TNF-α as a biomarker for stroke prognosis has also been studied, and contradictory results have also been obtained. Some studies have found an association between higher TNF-α levels and poor outcome[20,62] or larger lesion size.[65,68] However, some other studies have not found these associations.[26,69,70] Thus, further studies need to be done before any conclusions can be drawn about the use of TNF-α as a stroke biomarker. In reference to the experimental models, TNF-α is probably one of the most extensively studied cytokines in the field of stroke research. In a tMCAO mouse model, Yang and colleagues showed that intracerebroventricular injection of an antibody against TNF-α after the brain artery occlusion significantly decreased the infarct volume.[71] Moreover, three independent studies showed that intraperitoneal, intravenous and intracerebroventricular administration of anti-TNF-α in rats decreased the infarct volume using tMCAO[74,75] and pMCAO models.[76] In line with this, other studies showed that either intracranial, intraperitoneal, intravenous or even topic administration of TNF-binding protein (TNF-bp, which binds to and inhibits TNF-α) decreased the infarct volume following pMCAO in mice[72,73] and when administered intravenously in rats.[77] Finally, Wang and colleagues proved that the administration of a TACE inhibitor reduced the infarct volume in rats.[164] Thus, all these studies seem to indicate that TNF-α has a detrimental role in stroke, since its inhibition seems to be a promising tool for stroke treatment. To corroborate the findings reported in experimental models, it might be interesting to perform a clinical trial in ischemic stroke patients to evaluate the therapeutic potential of inhibiting TNF-α.

Anti-inflammatory cytokines. Interleukin-4 (IL-4) is a cytokine produced mainly by leukocytes. Its signaling contributes to a potent anti-inflammatory response through the inhibition of pro-inflammatory cytokines and chemokines, among other functions.[165] Although it is poorly studied as a stroke biomarker, Kim and colleagues found that acute ischemic stroke patients had higher levels of IL-4 in serum than controls,[116] while similar IL-4 levels were found in ischemic stroke patients with or without neurological worsening.[130] In reference to animal models, functional and cognitive improvement was shown following continuous (starting 6 h after ischemia and lasting for one week) IL-4 administration in a tMCAO mouse model, but without differences in infarct volume when compared with the control group.[137] In contrast, others have found that IL-4 administration decreased infarct volume and improved the behavioral performance and neurological recovery 14 days after stroke in a tMCAO mouse model.[136] Finally, IL-4 administration in rat tMCAO did not reduce the number of degenerating neurons and produced an increase in the number of macrophages/microglia in the infarct zone.[166]

Interleukin-10 (IL-10) is an anti-inflammatory cytokine expressed mainly by monocytes in response to brain injury. It has different immunomodulatory functions during the inflammatory response, being particularly important during the resolution phase. In fact, IL-10 reduces the activation of T cells, macrophages and monocytes, as well as attenuating the synthesis of pro-inflammatory cytokines. Moreover, it also reduces leukocyte adhesion and extravasation through the endothelium.[167] Various studies have measured the circulating levels of this protein after ischemic stroke at different time points. Two studies have reported that patients have lower levels of IL-10 than controls within 12 h after the onset of symptoms.[115,117] However, an independent study reported no differences between patients and controls at this same time point.[24] Moreover, it has also been reported that circulating levels of this protein remain decreased at 48 h,[126] 72 h,[127] and even more than 28 days after the ischemic event.[78] However, a lack of differences in IL-10 circulating levels between patients and controls 72 h after the ischemic event has also been reported.[128] Therefore, the majority of studies seem to indicate a plausible role of IL-10 as a biomarker for stroke diagnosis, but more studies need to be performed. In addition to this, two independent studies revealed that circulating levels of IL-10 were not associated with the initial infarct volume.[70,129] Moreover, some studies have explored the potential of IL-10 as an outcome predictor, although with some controversy as

well. One study found that lower IL-10 levels 24 h after the ischemic event were associated with neurological worsening at 48 h.[130] However, another found that high circulating levels of IL-10 at 48 h after the event were independently associated with severe neurological deficits and predicted 3-month adverse clinical outcome.[126] Another study revealed that those patients who survived after the ischemic event, had decreased levels of IL-10 at 24 h but increased levels of IL-10 at 72 h and at 6 days compared with those patients who died.[131] However, Sahan and colleagues showed no significant association between prognosis and IL-10 levels.[70] In addition to this, some studies showed that patients who developed infections within the first week after the ischemic event had higher IL-10 levels within the first 24 h.[132] Regarding these results, further studies need to be carried before any practical conclusion can be drawn. In reference to experimental studies, two independent studies demonstrated that intracerebroventricular and intravenous administration of IL-10 reduces the infarct volume after pMCAO in rats.[133,134] Moreover, Ooboshi and colleagues reported that postischemic gene transfer of IL-10 into the lateral ventricle reduced the infarct size in a pMCAO rat model.[135] Hence, all experimental studies seem to indicate that the administration of IL-10 has a neuroprotective effect, but its application as a new stroke therapy needs to be further studied before its translation to the clinical setting.

Transforming growth factor beta (TGF-β) is produced by microglia, macrophages and astrocytes after ischemia and it regulates a variety of functions, such as cell growth and differentiation, immune function, and apoptosis.[168] In reference to the differences observed in the circulating levels of this cytokine between ischemic stroke patients and controls, there are contradictory results. Two independent studies reported decreased levels of TGF-β in patients after acute ischemic stroke in comparison with controls,[78,79] while two other studies found no differences.[80,81] Moreover, another study reported that TGF-β levels were significantly elevated 24 h after the ischemic event.[82] In addition, Stanzani and colleagues reported that higher TGF-β levels on the fourth day were associated with larger infarct volumes and an increased severity of ischemic stroke.[81] However, all studies carried out so far have had limited sample sizes, therefore, larger studies need to be undertaken. In addition, various studies have explored the modulation of TGF-β as a potential treatment in experimental stroke. Ma and colleagues reported that the intranasal administration of TGF-β produced a significant reduction of the infarct volume in a tMCAO mouse model.[83] Moreover, another study showed that the intracerebroventricular administration of TGF-β decreased the lesion size after pMCAO in mice.[84] A different approach was attempted by Ruocco and colleagues, who showed that the administration of a TGF-β antagonist increased the infarct volume in a rat tMCAO model,[85] thus demonstrating the beneficial role of TGF-β in the ischemic brain.

Cell adhesion molecules

Cell adhesion molecules (CAMs) play a key role in the trafficking and recruitment of leukocytes to activated endothelia in acute ischemic stroke. In fact, after the ischemic event, there is an increase in CAM expression on the cerebral endothelium. During the progression of inflammation, soluble isoforms of CAMs are shed from the cell surface and released into the bloodstream. CAMs are divided into three groups: the immunoglobulin gene superfamily (ICAM-1 and 2, VCAM-1, PECAM-1 and MAdCAM-1), the selectins (P-, E- and L-selectin), and the integrins (CD11, CD18, CD29 and CD49).[9] Of these, only those CAMs described in the following sections have been studied as both biomarkers and therapeutic targets for stroke.

ICAM-1 is one of the most studied CAMs in the context of ischemic stroke. In reference to changes in the ICAM-1 plasma levels that occur after ischemic stroke, there are contradictory results. Various independent studies reported higher levels of this protein in ischemic stroke patients compared with controls within 24 h after the ischemic event.[21,23,24,53,54] However, another study reported no difference in ICAM-1 levels when comparing patients and controls at the same time point.[40] In addition, reduced levels of this protein at 72 h after the onset of ischemic stroke symptoms were also reported; however, this study included only 14 stroke patients, therefore, these results might not be as robust as those of the other studies, which all included more than 100 ischemic stroke patients.[55] Controversies also exist in reference to ICAM-1 levels and stroke outcome. While two studies reported no association between ICAM-1 levels and stroke severity within the first week after

the event and neither with functional outcome at 10 days or 3 months,[40,56] Sotgiu and colleagues found a significant association with higher admission ICAM-1 levels and worse 3-month outcome, as well as with larger infarct size.[21] In addition, various studies have evaluated the therapeutic potential of anti-ICAM-1 antibody in experimental stroke. Three independent studies reported a reduction of the infarct size after intravenous or intraperitoneal administration of anti-ICAM-1 in a tMCAO rat model.[57–59] However, this treatment seems not to be able to reduce the infarct size after a pMCAO in rats.[58] In addition to this, the inhibition of ICAM-1 as a potential stroke therapy has also been studied at the clinical level. Enlimomab is a murine monoclonal antibody that binds to ICAM-1, inhibiting neutrophil adhesion and migration through the brain endothelium. It was tested for safety, pharmacokinetics and biological activity in 32 stroke patients. The aim of this trial was to identify the dosing regimen that would achieve therapeutic serum levels (above 10 μg/ml), which were based on an *in vitro* study. To that end, a loading dose of enlimomab (ranging between 60 and 160 mg of antibody) was administered within 24 h after the onset of symptoms followed by four daily maintenance doses (ranged between 20 and 80 mg/day). The results obtained revealed that a regimen of initial 160 mg followed by a maintenance dose of 40 mg/day produced the desired blood levels of enlimomab and was well tolerated given that it did not increase the risk of adverse events in stroke patients.[60] Once the safe dosage of the treatment was established, the Enlimomab Acute Stroke Trial (EAST) tested the efficacy of this compound in a total of 625 patients with ischemic stroke (317 received enlimomab and 308 placebo). Unfortunately, the enlimomab-treated group of patients had worsening of neurological functions, an increased mortality and adverse drug reactions (infections and fever) compared with the placebo group, showing the inefficacy of this therapeutic drug in stroke patients.[61] The negative results obtained in this trial have been attributed to a detrimental immunoactivation due to the administration of a mouse antibody. In fact, Furuya and colleagues reproduced this design in an experimental study in which murine antibodies to ICAM-1 were serially administered to rats. They found that serial administration of the antibody-sensitized rats to produce antimouse antibodies, covered up any potential benefit from the inhibition of leukocyte infiltration to the site of injury.[169]

Another important CAM in the stroke pathophysiology is vascular cell adhesion molecule-1 (VCAM-1), also known as CD106. VCAM-1 is a protein that is expressed on the surface of endothelial cells and mediates cell-to-cell recognition and adhesion, as well as the activation and subsequent passage of leukocytes into the inflamed region.[65] The major ligand of VCAM-1 is very late antigen-4 (VLA-4), which is an integrin constitutively expressed on the membrane of leukocytes. In reference to the plasma levels of these proteins, the circulating levels of VLA-4 in ischemic stroke patients have not been studied for the moment. In addition, several distinct studies have found that soluble VCAM-1 levels in circulation are higher after stroke than in stroke-free controls.[23,24,41–43] Nonetheless, it seems that these increased VCAM-1 levels are found only in thromboembolic stroke patients and not in those with lacunar strokes.[40] In addition, one study showed that higher levels of VCAM-1 at admission were associated with the risk of recurrence or death within 1 year after the ischemic event.[45] Similarly, an association was found between higher VCAM-1 levels 2–3 weeks after the event and a worse 3-month outcome in ischemic stroke patients.[44] The interaction between VLA-4 and VCAM-1 is crucial for the transmigration of leukocytes to the site of injury; for that reason, the inhibition of this interaction by specific antibodies seems to be a promising strategy to combat stroke.[48] In fact, several experimental studies have analyzed the effect of VCAM-1/VLA-4 axis blockade in stroke animal models. Although not all studies obtained the same results, the vast majority have reported beneficial effects of this blockade. In 2001, two independent studies reported for the first time that VLA-4 blockade by antibodies reduced the infarct size and improved the neurological function after a transient occlusion in rats.[51,52] However, the results obtained in mouse models are not as consistent. VLA-4 blockade produced a reduced infarct size and improved neurological function only when the antibody was administered after 30–45 min of occlusion in a tMCAO mouse model,[46,48,49] and not after 60 min of tMCAO[46] or after pMCAO.[48] The discrepancies obtained between the studies are probably due to important differences in the design and methodology. To find more robust results, a group of researchers from six different centers performed a multicenter and randomized preclinical trial to explore the efficacy of VLA-4 blockade in two distinct mouse models of stroke. The

results of this study revealed that the treatment with anti-VLA-4 significantly reduced infarct volume after distal pMCAO, which produced small cortical infarctions. However, after 60 min of tMCAO, the procedure that produces larger lesions, the blockade of VLA-4 did not reduce infarct size, suggesting that the efficacy of this treatment depends on infarct severity and localization.[47] Even with this information in mind, the efficacy of VLA-4 blockade has also been studied in stroke patients. Natalizumab is a recombinant immunoglobulin G4 monoclonal antibody that binds to the alpha subunit of the VLA-4 integrin to block the leukocyte transmigration. Last year, the ACTION trial, a double-blind phase II study exploring the safety and efficacy of natalizumab in reducing the infarct volume in acute ischemic stroke was concluded. A total of 161 stroke patients were randomly assigned to 300 mg intravenous natalizumab ($n = 79$) or placebo ($n = 82$). It revealed that a single dose of natalizumab administered within the first 9 h after the onset of symptoms was not able to reduce infarct volume. However, functional outcome at 30 and 90 days was improved in patients receiving natalizumab.[50] An ongoing ACTION 2 trial [ClinicalTrials.gov identifier: NCT02730455] with 270 participants is currently testing the efficacy of this treatment using a high (600 mg) or a low dose (300 mg), given that the ACTION trial revealed that patients with higher serum natalizumab concentrations showed better clinical outcomes. The results from the ACTION 2 trial are still not available, although a press release on February 2018 from the company behind the study points to the failure of the trial since the primary and secondary efficacy endpoints were not met.

P-selectin is an adhesion molecule expressed on the surface of activated endothelial cells and platelets that mediates the rolling of leukocytes.[138] In reference to changes in the circulating levels of this protein after ischemic stroke, various studies have reported that ischemic stroke patients have higher levels of P-selectin than controls.[23,24,138–140] Therapies targeting P-selectin have also been shown as effective in various experimental studies. Connolly and colleagues demonstrated for the first time that the blockade of P-selectin after tMCAO in mice reduced the infarct volume and improved the functional outcome.[141] Similarly, a reduction of the infarct volume was also reported after anti-P-selectin administration following pMCAO in rats.[143,144] However, Goussev and colleagues found that the administration of the anti-P-selectin antibody upon reperfusion after tMCAO in rat did not reduce the infarct size.[142] Thus, further studies need to be performed in translational experimental models in order to confirm the potential role of this protein as a therapeutic target, but the results obtained for the moment do seem promising.

Damage-associated molecular patterns

DAMPs are endogenous molecules released from injured cells that can initiate or maintain the inflammatory response. A well-known DAMP implicated in ischemic brain injury is a protein named high-mobility group box 1 (HMGB1), a nuclear protein that functions as a nucleosome stabilizer and transcription factor. In addition to this, HMGB1 is able to activate endothelial cells, increasing VCAM-1, ICAM-1 and E-selectin expression, and consequently, allowing leukocyte extravasation. Moreover, HMGB1 can act itself as a pro-inflammatory cytokine because it is secreted by activated immune cells and can mediate the systemic inflammatory response.[15] In reference to the circulating levels of this protein in ischemic stroke patients, distinct studies have reported an elevation of HMGB1 after stroke in comparison with controls.[86,145–147] Moreover, increased plasma levels of HMGB1 have been described as associated with a poor functional outcome within 1 month or 1 year after stroke.[86,148] However, Schulze and colleagues reported no association between HMGB1 levels and outcome or infarct size.[147] Experimental studies on a tMCAO rat model have reported that the intracerebroventricular injection of HMGB1 increased the severity of infarction, whereas the administration of anti-HMGB1 reduced the infarct size, improved the neurological function[149] and decreased the edematous area.[170] Although the results obtained seem promising, more experimental studies using different animal models are still needed to confirm the findings reported.

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are zinc-binding proteolytic enzymes that remodel the extracellular matrix. In addition to this, MMPs are also involved in different biological processes such as the cleavage of cell receptors, the release of death signals, the activation of inflammatory mediators, cell proliferation and apoptosis.[12]

MMPs are secreted by neurons, glial and endothelial cells, as well as by infiltrating leukocytes as inactive enzymes; thus, they must be cleaved by other proteases to become active.[171] The most studied MMPs in the field of ischemic stroke are MMP-9 and MMP-2, which are gelatinases that specifically degrade type IV collagen, laminin, and fibronectin, the major components of the basal lamina around cerebral blood vessels.[172] In fact, a huge amount of studies have reported increased circulating levels of MMP-9 in ischemic stroke patients within 24 h after the event in comparison with controls[86–93] and even stroke mimics.[94–96] However, these differences in MMP-9 plasma levels were not observed in one study comparing controls with stroke patients (including both the ischemic and hemorrhagic stroke subtypes).[97] In reference to MMP-2, two independent studies have reported decreased levels of this protein in ischemic stroke patients when compared with controls.[93,102] However, the lack of differences between patients and controls has also been reported.[92] In addition to this, higher MMP-9 levels have also been associated with larger infarct volumes and worse 3-month outcomes, including mortality.[21,68, 86,89,93,98,99,102–106] Furthermore, distinct studies have reported that increased levels of MMP-9 at admission (before rt-PA administration) are associated with hemorrhagic transformation following the thrombolytic treatment.[92,107–110] For that reason, MMP-9 levels at admission could work as a good biomarker to predict this secondary complication. In reference to experimental models of ischemic stroke targeting MMPs, a highly selective inhibitor that targets both MMP-2 and MMP-9 [(4-phenoxyphenylsulfonyl) methylthiirane (SB-3CT)] has been tested. Gu and colleagues explored whether the administration of SB-3CT after transient ischemia in mice could be neuroprotective. They found that the blockade of MMP-2 and MMP-9 produces a decrease in the infarct volume in comparison with vehicle-treated controls and ameliorates the functional outcome.[111] Moreover, the same results were obtained when administering SB-3CT after pMCAO in mice.[112] Thus, all experimental studies performed so far seem promising, although further studies need to be undertaken before any conclusions can be drawn. Moreover, no one has deeply studied the effect of specifically blocking MMP-9 or MMP-2, which may be interesting in terms of revealing the specific role of each protein in stroke pathophysiology.

Annexins

Annexins are a family of proteins involved in vesicle transport, membrane scaffolding, apoptosis, proliferation, differentiation and inflammation. Annexin A1 (ANXA1), previously known as lipocortin 1, is the only annexin that has been studied in the context of stroke as a possible biomarker and therapeutic target. There is an increasing evidence of the role of ANXA1 in different anti-inflammatory processes, such as the regulation of macrophage phagocytosis and neutrophil migration through the inhibition of neutrophil adhesion to the endothelium.[173] One study exploring a small cohort of patients has reported no differences in the ANXA1 plasma levels between ischemic stroke patients, controls and patients with stroke-mimicking conditions.[150] In addition, studies in animal models of cerebral ischemia found that the intracerebral administration of ANXA1 reduced the infarct size after pMCAO in rats, whereas the inhibition of ANXA1 by antibodies produced larger lesions in the same model.[151] Hence, only one study has explored the effect of modulating this protein in experimental models; thus, it is necessary to perform more studies in order to confirm these promising results. Moreover, it would be interesting to explore the potential role of this protein as a stroke biomarker in large cohorts of patients.

Limitations and future steps

As described earlier, the understanding and modulation of the postischemic inflammatory response is gaining interest among scientists in the field of stroke. In recent years, several studies have explored the potential of inflammatory molecules to become stroke biomarkers or therapeutic targets. Although the majority of the results obtained seem promising, there also exist contradictory results and lack of robustness in certain cases. However, it is important to bear in mind that ischemic stroke is a complex disease in which several biological processes are altered, and different molecules may interact. Moreover, the inflammatory response triggered after the ischemic event shows dynamic behavior; thus, the implicated molecules may have different roles depending on the moment they act. For that reason, studies exploring the role of some inflammatory proteins as stroke biomarkers may not be as robust as expected, mainly due to the differences in the time of measurement of the proteins among exploratory studies, and the limited sample size of several

studies. In addition to this, almost all studies assaying the potential usage of inflammatory proteins as biomarkers for stroke diagnosis are comparing circulating levels of these proteins between ischemic stroke patients and healthy controls; this experimental design is far from reality and does not ensure the efficacy of these biomarkers in stroke diagnosis. Studies exploring acutely the differences in circulating concentrations of these proteins between strokes and stroke-mimicking conditions such as seizures, headaches or brain tumors, seem thus necessary, as the inflammatory response is also altered in some of these mimics, who are the real confounders in stroke diagnosis.

In addition, the pharmacological modulation of the inflammatory response seems to be a promising strategy for stroke treatment as reported in several experimental studies. However, the existence of different ischemic stroke models and differences in experimental designs, including different administration times and protocols, may generate opposite results among studies that are also influenced by the dynamic nature of the inflammatory processes following stroke. To make feasible the translation of these treatments to the clinical setting, it seems necessary to adjust the experimental designs to a situation closer to the pathophysiology and timing occurring in humans. Moreover, the application of the current guidelines for preclinical studies in stroke would add great value to the search for new therapies.[174,175]

The results summarized in this review underlined a plausible use of inflammation-related proteins as blood biomarkers, therapeutic targets and even surrogate biomarkers of the efficacy of therapeutic strategies directed against the same target molecule. This will definitely provide a more personalized management of stroke patients. For that reason, a better characterization and understanding of the stroke pathophysiological processes such as inflammation is necessary to overcome the current limitations, as well as a deeper understanding of the importance of precise times in the diagnosis and treatment response in stroke.

The current context of stroke, in which reperfusion therapies are the only approved treatments, seems ideal for the exploration of new biomarkers and therapeutic targets, such as those described here. In an ideal scenario, some of these inflammatory-related molecules might be measured by future point-of-care devices in order to rapidly diagnose ischemic stroke. This might help increasing the number of patients who can benefit from the actual reperfusion therapies. Moreover, since many stroke patients do not respond properly to these therapies, the pharmacological modulation of inflammatory-related molecules as a complementary therapeutic tool could be an interesting approach to improve stroke patients' management and consequently, their outcome.

Conclusion

Despite improvements in ischemic stroke management, appropriate diagnostic tools and therapies beyond vascular reperfusion are still missing. There is an urgent need for pharmacological agents to reduce the ischemic damage. Inflammation is a key process involved in the pathophysiology of stroke and its modulation seems a promising strategy for neuroprotection. Various studies have demonstrated that inflammatory molecules could work as biomarkers for stroke diagnosis or prognosis. Moreover, numerous animal model studies have shown that pharmacological modulation of these inflammatory molecules can reduce infarct size and improve functional outcome. However, the translation from bench to bedside has not been yet accomplished. To that end, a better understanding of the postischemic inflammatory response would increase the chances to find new diagnostic and therapeutic strategies.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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