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ALBA SIMATS ORIOL

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Aquesta Tesi Doctoral s'ha dut a terme al laboratori d'Investigació Neurovascular de l'Institut de Recerca de Vall d'Hebron, amb el suport d'una beca predoctoral de l'Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR), de la Generalitat de Catalunya (2015 FI_B00952).

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Optimism is the faith that leads to achievement.
Nothing can be done without hope and confidence.

Helen Keller

L'optimisme és la fe que guia a l'assoliment.
Res es pot fer sense esperança i confiança

Hellen Keller

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RESUM

L'ictus isquèmic és una de les principals causes de mort i discapacitat arreu. Actualment, l'únic tractament efectiu és la teràpia de reperfusió, ja sigui a través del tractament intravenós amb l'activador tissular del plasminogen (rt-PA) o la retirada del clot mitjançant trombectomia mecànica intra-arterial. No obstant, només un percentatge reduït dels pacients poden beneficiar-se d'aquestes teràpies, doncs presenten una finestra terapèutica molt estreta, així com nombroses contraindicacions mèdiques i efectes secundaris adversos. És per això que hi ha una necessitat urgent de trobar noves estratègies terapèutiques complementàries capaces de bloquejar la progressió de la lesió i neuroprotegir el cervell isquèmic en els estadis inicials de la malaltia.

L'ús de biomarcadors sanguinis per al diagnòstic i el pronòstic de l'ictus isquèmic acceleraria la presa de decisions mèdiques i l'administració de tractaments als pacients afectats. Malgrat nombroses biomolècules hagin estat proposades per a tals finalitats, actualment cap d'elles ha aconseguit mostrar una millora significativa respecte les proves de neuroimatge i els paràmetres clínics que s'utilitzen avui en dia, de manera que descobrir nous biomarcadors sanguinis d'ictus també és una necessitat vigent.

En aquesta Tesi Doctoral s'explora el paper de nous biomarcadors d'ictus en els estadis inicials de la malaltia, de manera que a banda de bons indicadors, pensem que la seva modulació terapèutica pot esdevenir també interessant com a possible tractament neuroprotector. Així, els treballs que la integren pretenen contribuir a la identificació de noves biomolècules que puguin servir com a biomarcadors, dianes terapèutiques o ambdues aplicacions que avui en dia encara manquen de resolució.

D'una banda, hem aprofundit en l'estudi del paper dual de certs mediadors inflamatoris com a biomarcadors i dianes terapèutiques alhora. Amb aquest propòsit, hem demostrat que la inhibició d'un biomarcador de transformacions hemorràgiques, la VAP-1/SSAO, és una estratègia prometedora per tal d'evitar l'aparició d'aquestes complicacions associades a l'administració de l'rt-PA. També hem identificat per primer cop la quimiocina CCL23 com un bon biomarcador de pronòstic de l'ictus isquèmic, l'administració exògena de la qual ha mostrat indicis de millorar l'estat neurològic dels animals afectats per la isquèmia.

Amb l'objectiu de descobrir nous candidats, s'han dut a terme dos estudis emprant diferents tècniques *–òmiques* en models experimentals d'isquèmia cerebral. S'han estudiat els canvis al proteoma del líquid cefaloraquidi de rata, així com les alteracions a nivell del transcriptoma i del proteoma del cervell isquèmic de ratolí, ambdós durant la fase aguda de la malaltia. Ambdós estudis han permès identificar i explorar en detall una sèrie de gens i proteïnes rellevants en la fisiopatologia de la isquèmia cerebral, alguns dels quals han estat avaluats pel seu possible ús com a biomarcadors de diagnòstic i pronòstic de l'ictus isquèmic. Els resultats obtinguts en ambdós estudis fan atractiva una futura exploració de les molècules destacades com a nous biomarcadors o dianes terapèutiques per l'ictus.

Finalment, donada la manca d'èxit dels agents neuroprotectors proposats fins al moment, en aquesta Tesi hem optat per una aproximació innovadora basada en el reposicionament de fàrmacs per identificar, a través d'una simulació matemàtica de l'ictus isquèmic, combinacions de fàrmacs amb efecte terapèutic sinèrgic. Dues de les combinacions de fàrmacs testades *in vivo* han demostrat una capacitat significativa per reduir l'extensió de la lesió cerebral d'animals isquèmics, obrint la porta a nous tractaments neuroprotectors per l'ictus.

En conjunt, els resultats d'aquesta tesi doctoral contribueixen a millorar el coneixement de la fisiopatologia de la isquèmia cerebral, així com al descobriment de futurs biomarcadors i dianes terapèutiques de l'ictus, sent ambdues necessitats urgents per reduir l'impacte devastador d'aquesta malaltia arreu del món.

ABSTRACT

Ischemic stroke is one of the leading causes of death and disability worldwide. Currently, the only effective treatments are reperfusion therapies, via the intravenous administration of recombinant tissue plasminogen activator (rt-PA) or the mechanical removal of the thrombus with stent-retriever or aspiration devices. However, only a small percentage of patients can benefit from these therapies due to their short therapeutic window and their severe side effects. Thus, there is an urgent need to find complementary therapeutic strategies to mitigate the stroke progression and rescue the brain tissue from ischemic injury.

The use of blood biomarkers for the diagnosis and prognosis of ischemic stroke would accelerate decision-making processes and treatments of the affected patients. Although numerous molecules have been proposed for these purposes, none of them have shown a significant improvement over the neuroimaging and clinical parameters that are currently used, so new blood biomarkers for stroke are also sought.

This Doctoral Thesis explores the role of new stroke biomarkers in the initial stages of the disease, since it is hypothesized that beyond its role as indicators of stroke, their therapeutic modulation can also be interesting as a neuroprotective strategy to treat ischemic stroke. Thus, the studies comprised in this Thesis aim to contribute to the identification of new molecules that can serve as blood biomarkers, therapeutic targets or both applications at once.

On the one hand, we studied the dual role of certain inflammatory mediators as blood biomarkers and therapeutic targets. We have shown that the inhibition of VAP-1/SSAO, a biomarker of rt-PA-associated hemorrhagic transformation, is a promising strategy to avoid the appearance of these complications. Moreover, we have also identified for the first time the CCL23 chemokine as an interesting biomarker of ischemic stroke prognosis, whose exogenous administration has also shown potential to improve the neurological status of animals after cerebral ischemia.

Furthermore, two *–omics* studies have been conducted to discover new candidates in experimental models of stroke. Specifically, the proteome changes in the rat cerebrospinal fluid and the transcriptome and proteome alterations in the mouse brain have been deeply evaluated during the acute phase of cerebral ischemia. Both studies highlighted key molecular factors in the stroke pathophysiology, some of which have been tested for their role as biomarkers for stroke diagnosis and prognosis. Moreover, the results from both studies envision a future exploration of these outstanding molecules as new biomarkers or therapeutic targets for ischemic stroke.

Finally, since all neuroprotective agents proposed so far have systematically failed to succeed, in this Thesis we aimed for an innovative drug repositioning approach based on a mathematical simulation of ischemic stroke disease. This *in silico* model has been used to identify combinations of drugs with a synergistic therapeutic effect on neuroprotection. Two of the resulting drug combinations have demonstrated *in vivo* promising neuroprotective actions by reducing the extension of the brain lesion, which suggests their efficacy as new treatments for ischemic stroke.

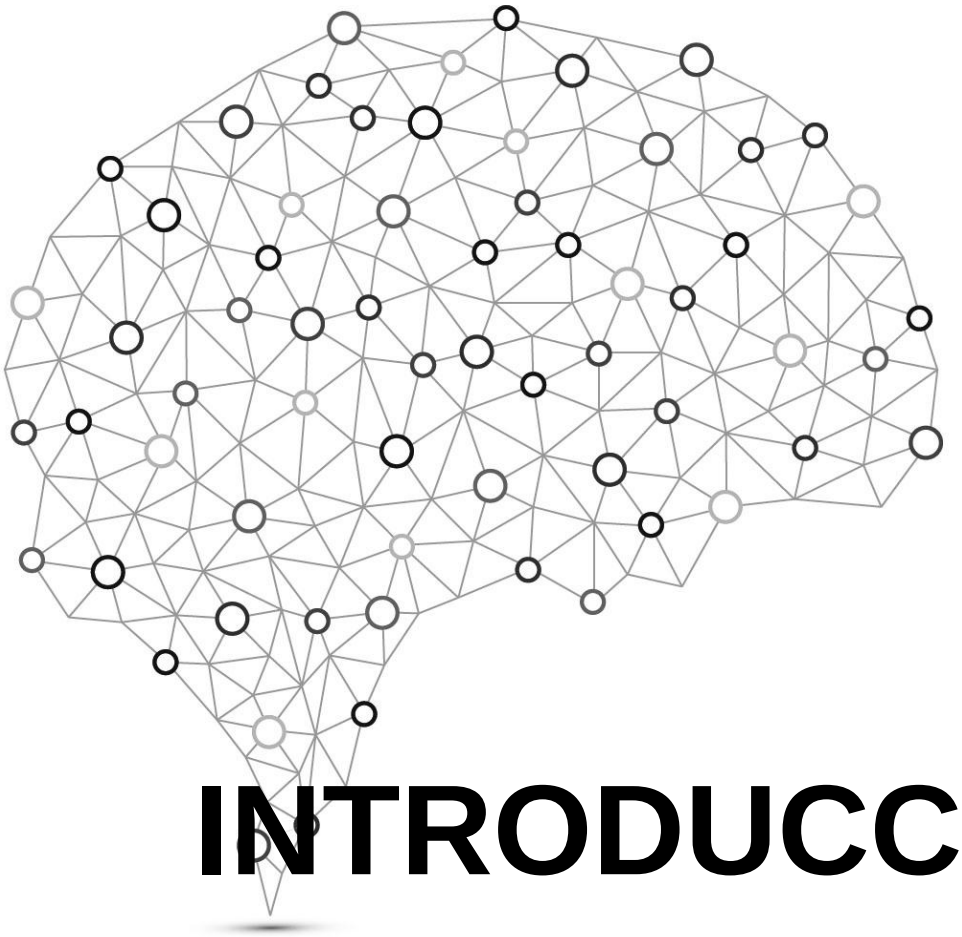
Overall, the results of this Doctoral Thesis contribute to our understanding of the stroke pathophysiology, as well as to the discovery of future blood biomarkers and therapeutic targets for stroke, being two urgent clinical needs to reduce the devastating impact of this disease worldwide.

ABREVIATURES

AIT	atac isquèmic transitori
AMPA	de l'anglès amino-3-hydroxy-5-methyl-4-isoxazolepropionic àcid
AREG	de l'anglès amphiregulin
AUC	de l'anglès area under the curve
ATF3	de l'anglès activating transcription factor 3
ATP	de l'anglès adenosine triphosphate
BBB	de l'anglès brain-blood barrier
BI	de l'anglès barthel index
BNP	de l'anglès brain natriuretic peptide
BPI	de l'anglès bactericidal/permeability-increasing protein
Ca^{2+}	ió de calci
CADASIL	de l'anglès cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CAM	de l'anglès cellular adhesion molecule
CAMK2	de l'anglès calcium/calmdoulin protein kinase 2
CCA	de l'anglès canonical correlation analysis
CCL	de l'anglès chemokine (C-C motif) ligand
CKB	de l'anglès creatine kinase B-type
Cl^-	ió clorur
Cldn20	de l'anglès claudin 20
CMPK	de l'anglès uridine monophosphate - cytidine monophosphate kinase
CNS	de l'anglès Canadian neurological scale
CoA	de l'anglès co-intertia analysis
CRP	de l'anglès C-reactive protein
CSF	de l'anglès cerebrospinal fluid
Cstad	de l'anglès CSA-conditional, T cell activation-dependent protein
CTNND2	de l'anglès catenin delta 2
CysA	de l'anglès cystatin a
DNA	de l'anglès deoxyribonucleic acid
Drp2	de l'angles dihydropyrimidinase-related protein 2
ECASS	de l'anglès European cooperative acute stroke study
EPO	eritropoetina
ELISA	de l'anglès enzyme-linked immunosorbent assay
ESS	de l'anglès European stroke scale
FABP	de l'anglès fatty acid-binding protein
FosB	de l'anglès FBJ murine osteosarcoma viral oncogene homolog B
Gadd45g	de l'anglès growth arrest and DNA-damage-inducible protein gamma

G-CSF	de l'anglès granulocyte colony-stimulating factor
Gels	de l'anglès gelsolin
GFAP	de l'anglès glial fibrillary acidic protein
GO	de l'anglès gene ontology
GOS	de l'anglès glasgow outcome scale
GRPCs	de l'anglès G-protein coupled receptors
HDAC	de l'anglès histone deacetylase
HI	infart hemorràgic
ICAM-1	de l'anglès intracellular adhesion molecule 1
ICAT	de l'anglès isotope coded affinity tag
IgG	Immunoglobulina G
IL-	interleucina
IL-1Ra	de l'anglès interleukin 1 receptor anatagonist
IMPROVE	de l'anglès ischemia models procedural refinaments of *in vivo* experiments
IFN-ɣ	interferó gamma
ITRAQ	de l'anglès isobaric tag for relative and absolute quantification
JAM	de l'anglès junctional adhesion molecule
K^+	ió potassi
kDa	de l'anglès, kilodaltons
KEGG	de l'anglès kyoto encyclopedia of genes and genomes
LC	de l'anglès líquid chromatography
MAPK	de l'anglès mitogen-activated protein kinase
MCA	de l'anglès middle cerebral artery
MCAO	de l'anglès middle cerebral artery occlusion
MCEMP1	de l'anglès mast cell-expressed membrane protein 1
MMP	de l'anglès matrix metalloproteinases
MMP9	de l'anglès matrix metalloproteinase 9
MPO	de l'anglès myeloperoxidase
MR16-1	anticòs anti-receptor de la interleucina 6
mRS	de l'anglès modified rankin scale
MS	de l'anglès mass spectometry
MULTIPART	de l'anglès multicentre preclinical animal research team
Na^+	ió sodi
NIHSS	de l'anglès national institutes of health stroke scale
NMDA	de l'anglès n-methyl-d-aspartatic acid
nNOS	de l'anglès neuronal nítric oxide synthase
NO	de l'anglès nitric oxide
NOS	de l'anglès nitric oxide synthase
PCA	de l'anglès principal component analysis

PCR	de l’anglès polymerase chain reaction
PDXP	de l’anglès pyridoxal phosphatase
PEA	de l’anglès proximity extension assay
PH	de l’angles parenquimatous hemorrhage
POC	de l’anglès point of care
PRM	de l’anglès parallel reaction monitoring
PSD-95	de l’anglès postsynaptic density protein 95
PSGL-1	de l’anglès P-selectin glycoprotein ligand 1
Rgs2	de l’anglès regulator of G-protein signaling 2
RNA	de l’anglès ribonucleic acid
RNS	de l’anglès reactive nitrogen species
ROS	de l’anglès reactive oxygen species
rt-PA	de l’anglès recombinant tissue plasminogen activator
S100B	de l’anglès S100 calcium binding protein B
SELEX	de l’anglès systematic evolution of ligands by exponential enrichment
sICAM-1	de l’anglès soluble intracellular adhesion molecule 1
SILAC	de l’anglès stable isotope labeling with aminoacids in cell culture
SPAN	de l’anglès stroke prognostication using age and NHISS index
SSS	de l’anglès scandinavian stroke scale
SSAO	de l’anglès semicarbazide-sensible amine oxidase activity
STAIR	de l’anglès stroke treatment academic industry roundtable
TGF-β	de l’anglès transformin growth factor beta
TH	transformacions hemorràgiques
TMT	de l’anglès tandem mass tags
TNF-α	de l’anglès, tumor necrosis factor alpha
TOAST	de l’anglès trial of Org. 10172 in acute stroke treatment
TTC	de l’anglès triphenyltetrazolium chloride
VAP-1	de l’anglès vascular adhesion protein 1
VLA-4	de l’anglès very late antigen 4
VCAM-1	de l’anglès vascular adhesion molecule 1



INTRODUCCIÓ

1.1 L'ictus

1.1.1 Epidemiologia i aspectes clínics

L'ictus o infart cerebral és un trastorn neurològic produït per l'alteració del flux sanguini en una determinada zona del cervell. Aquest trastorn vascular es produeix com a conseqüència del trencament (ictus hemorràgic) o obstrucció (ictus isquèmic) d'un vas o artèria cerebral i pot pertorbar la funcionalitat de la regió del cervell afectada de manera transitòria (si es restableix el flux espontàniament o mitjançant accions terapèutiques) o bé permanent. La manca sobtada d'oxigen i nutrients que es produeix al cervell com a conseqüència d'aquesta interrupció del flux sanguini pot donar lloc a tota una sèrie de símptomes neurològics, que depenen del tipus d'ictus causant de la lesió i de la zona de l'encèfal afectada. Els més comuns, però, són l'alteració de la parla (afàsia), l'afectació de la capacitat de moviment d'un costat del cos (hemiparèsia), la pèrdua de visió (hemianòpsia, diplopia) i el vertigen, entre d'altres [1].

Dades recents sobre la incidència de l'ictus demostren que aquesta malaltia continua sent un dels trastorns neurològics més devastadors arreu. Segons les dades obtingudes de la World Health Organitzation (www.who.org), l'ictus causa més de 5,5 milions de morts anuals a nivell mundial, i és responsable de les discapacitats neurològiques a llarg termini de més de 5 milions de supervivents. Des del punt de vista socioeconòmic, l'ictus suposa doncs una important càrrega per al sistema sanitari, no només a nivell econòmic (doncs suposa d'un 3% a un 4% dels costos sanitaris globals), sinó també per la pèrdua de qualitat i d'anys de vida degut a la discapacitat [2, 3].

L'ictus es tracta d'una patologia multifactorial. És per això que el control dels principals factors de risc associats a l'ictus és una de les eines més importants per a la seva prevenció. Aquests factors es classifiquen en modificables i no modificables [4]. Els factors de risc modificables, com la hipertensió, la diabetis, les dislipèmies, la obesitat o el tabaquisme, són molt comuns entre el conjunt de la població i un mal control d'aquests suposen un gran increment del risc de patir un ictus [5]. Altres factors de risc com la fibril·lació auricular o els atacs isquèmics transitoris són menys freqüents però més específics que els factors de risc comuns i també suposen un gran augment de la predisposició a patir aquesta malaltia. Per contra, es consideren factors de risc no modificables l'edat, el sexe, l'ètnia o algunes variants genètiques, ja siguin monogèniques (com la malaltia de Fabry o CADASIL [Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy]) o poligèniques [6].

1.1.2 Subtipus d'ictus

En funció de quina sigui la causa de l'alteració del flux sanguini, l'ictus es classifica en ictus hemorràgic o ictus isquèmic (Figura 1).

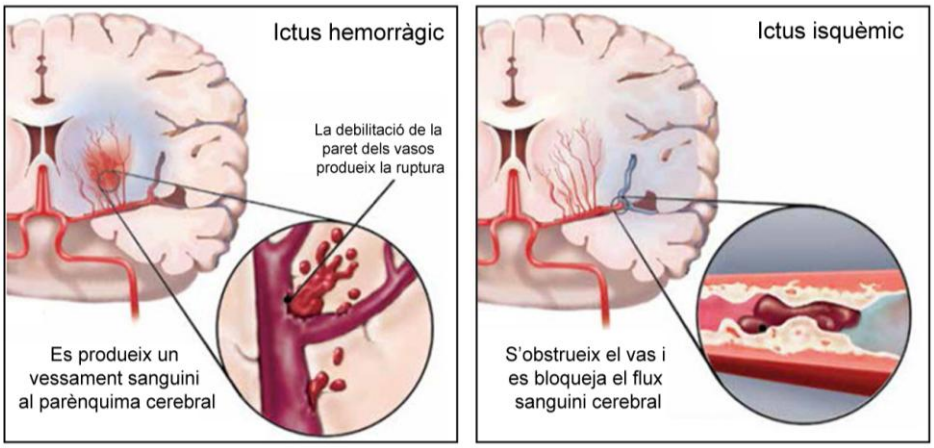


Figura 1. Subtipus d'ictus (Adaptat de ®Heart and Stroke foundation of Canada).

L'ictus hemorràgic es produeix com a conseqüència de la ruptura d'un vas sanguini i representa entre un 10 i un 20% del total dels ictus [7]. Els ictus hemorràgics es classifiquen en hematomes intracerebrals, quan l'extravasació de sang es produeix cap al parènquima, o hemorràgies subaracnoïdals, quan el vessament de sang es produeix directament a l'espai subaracnoïdal [8]. Els pacients que pateixen un ictus hemorràgic presenten una afectació severa i el seu pronòstic és força més dramàtic que el d'altres tipus d'ictus [9]. Tot i aquesta gravetat, actualment encara no es disposa de cap tractament efectiu per aquest tipus de trastorn cerebrovascular [10]. A més, segueix sent el subtipus d'ictus menys estudiat fins ara i encara es desconeixen en gran mesura els mecanismes moleculars que intervenen en la seva fisiopatologia.

L'ictus isquèmic, en canvi, representa entre un 80 i 90% de tots els ictus [11]. Ja que l'objectiu principal de la present Tesi Doctoral és l'ictus isquèmic, d'aquí en endavant s'aprofundirà extensivament en aquest subtipus d'ictus i s'utilitzaran els termes ictus i ictus isquèmic indiferentment. L'ictus isquèmic es caracteritza per la interrupció del flux sanguini en una determinada zona del cervell degut a una oclusió vascular. En funció de quina sigui la causa de l'obstrucció vascular (etiologia), els ictus isquèmics es classifiquen en 5 subtipus diferents (criteris TOAST, trial of Org 10172 in Acute Stroke Treatment) [12]. Els dos subtipus majoritaris d'ictus isquèmic són el cardioembòlic, en el que l'obstrucció es produeix per un coàgul o èmbol sanguini procedent del cor, i l'aterotrombòtic, causat per una placa d'ateroma que obstrueix la llum del vas o per la ruptura d'aquesta placa

d'ateroma que circula i arriba en forma de trombe al cervell. Menys freqüents serien els ictus lacunars, patologies de vas petit d'origen no aterotrombòtic en els que el flux sanguini de petites artèries o arterioles queda interromput per un trombe o l'estrenyiment del vas, i els ictus de causa inhabitual, produïts per causes poc freqüents com les malalties sistèmiques, infeccions, neoplàsies, alteracions metabòliques o de la coagulació, o per aneurismes, migranyes, malformacions arteriovenoses, entre d'altres [1]. Finalment, aquells ictus isquèmics en els quals després de la realització d'un estudi diagnòstic exhaustiu no s'ha pogut identificar una única causa específica que expliqui la patologia es consideren ictus isquèmics d'origen indeterminat [13].

Tant l'ictus hemorràgic com l'ictus isquèmic es poden classificar a diferents nivells, en funció de la zona cerebral afectada, la seva duració i extensió, o la causa de la lesió cerebral (Figura 2).

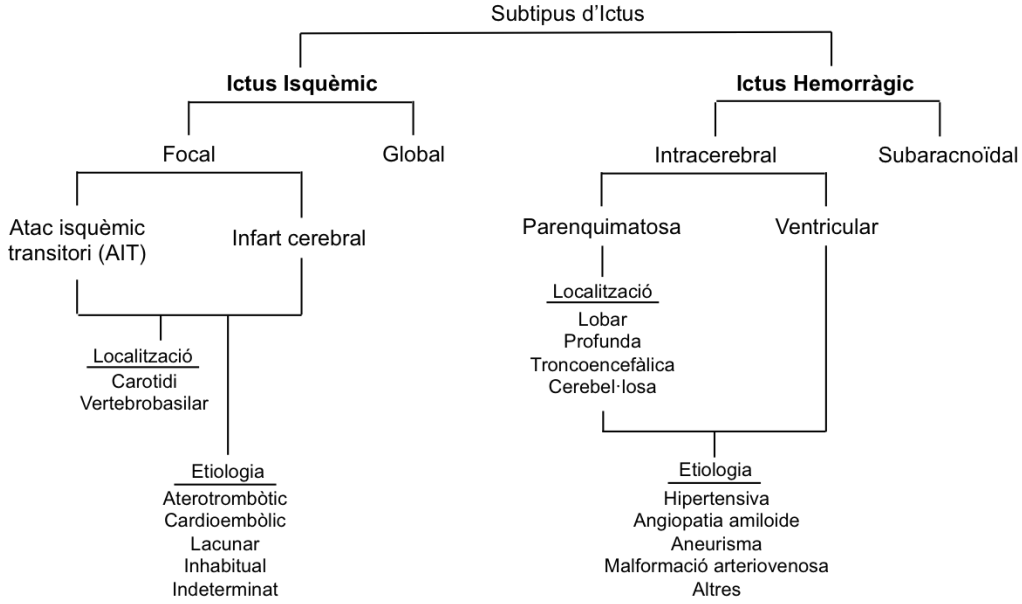


Figura 2. Classificació dels subtipus d'ictus (Adaptada de Díez-Tejedor et al., 2001) [1].

1.1.3 Diagnòstic de l'ictus isquèmic i estratègies de recanalització

Actualment el diagnòstic de l'ictus isquèmic realitzat únicament per mitjà de variables clíniques no és possible. És per això que es basa en l'avaluació conjunta de paràmetres clínics (com l'anamnesis i l'exploració neurològica), una exploració vascular (mitjançant una ecografia doppler-dúplex), un estudi cardíac (a través d'un electrocardiograma i ecografia transtoràcica) i proves de neuroimatge (bé per tomografia computeritzada o per ressonància magnètica cranial) [14]. Aquestes últimes han esdevingut imprescindibles per a tal finalitat; ambdues estratègies d'imatge permeten una avaluació detallada de l'estat neurològic del pacient, facilitant el diagnòstic de l'ictus isquèmic durant la fase aguda i

alhora diferenciant-lo d'aquelles altres patologies que presenten símptomes clínics similars als de l'ictus (anomenades *mímics*). Aquest grup de *mímics* és molt heterogeni i engloba patologies de diferent grau d'afectació neurològica, ja siguin aquelles que comporten algun altre tipus de lesió estructural del teixit cerebral, com els tumors o els traumatismes cranioencefàlics, o bé aquelles que sense causar danys cerebrals morfològics també donen lloc a símptomes clínics similars als de l'ictus, com poden ser les migranyes, el vertigen, les malalties toxico-metabòliques o les neuropaties perifèriques. Els *mímics* representen aproximadament entre un 20 i un 25% dels pacients que inicialment presenten un quadre clínic compatible amb l'ictus i és de vital importància la seva correcta i ràpida discriminació front la patologia isquèmica [15].

Diagnosticat l'ictus isquèmic, l'únic tractament farmacològic eficaç actualment en la fase aguda és el tractament trombolític per mitjà de l'administració intravenosa de l'activador tissular del plasminogen recombinant (rt-PA, *recombinant tissue plasminogen activator*) [16]. Molecularment, aquest fàrmac catalitza la conversió del plasminogen a plasmina, que alhora catalitza la degradació de la fibrina i el fibrinogen acumulats en el coàgul sanguini. Aquesta degradació dóna lloc a la dissolució d'aquest trombe i la recanalització del vas obstaculitzat, així com la reperfusió del teixit isquèmic afectat. És important destacar que malgrat sigui l'únic tractament farmacològic autoritzat, només d'un 10 a un 15% dels ictus isquèmics poden beneficiar-se d'aquesta estratègia terapèutica [17]. Els estrictes criteris d'inclusió que presenta aquest fàrmac fan que només puguin tractar-se aquells pacients menors de 80 anys que, sense superar les 4,5 hores des de l'aparició dels símptomes clínics de l'ictus, no estiguin medicant-se amb anticoagulants i no presentin evidència de possibles complicacions hemorràgiques. Aquesta estreta finestra terapèutica impedeix, així, que gran part dels pacients puguin beneficiar-se d'aquest tractament, normalment degut al temps requerit per a transportar el pacient a l'hospital i per a realitzar-li un diagnòstic complet. Desgraciadament, l'administració de l'rt-PA en els pacients aptes pot presentar també certs efectes adversos i complicacions, tals com la no recanalització de l'artèria obstruïda o la possible aparició de transformacions hemorràgiques, un dels efectes secundaris més greus derivats de la trombòlisi [18]. De les característiques d'aquests tipus de complicacions, però, en parlarem més endavant (apartat 1.1.4.1).

A banda de l'rt-PA, hi ha altres aproximacions no farmacològiques que també han demostrat eficàcia per a recanalitzar el vas obstruït, com la trombectomia endovascular mecànica. Aquesta aproximació ha revolucionat el tractament de l'ictus en els últims anys, doncs presenta resultats més prometedors que l'actual tractament farmacològic [19]. Tot i estar lluny de l'ideal, la recanalització mecànica ha permès ampliar lleugerament la finestra terapèutica, de manera que, en alguns casos, els pacients es poden beneficiar d'aquesta estratègia fins a 24h després de l'inici dels símptomes de l'ictus [20, 21]. A més, s'ha vist

que s'assoleix una major taxa de recanalització i un millor pronòstic dels pacients quan aquests són sotmesos a la recanalització mecànica després de l'administració de rt-PA [22].

1.1.4 Pronòstic de l'ictus isquèmic

El pronòstic de l'ictus pot avaluar-se des d'un punt de vista vital i/o funcional. A nivell vital, l'ictus isquèmic causa un 7% del total de les defuncions a nivell nacional i fins a un 25% de les morts per causes cardiovasculars [23]. Malgrat aquestes xifres són encara avui en dia devastadores, segons l'últim Informe Nacional de Salut (2016) les morts com a conseqüència de l'ictus han disminuït fins a un 50% des de l'inici del segle XXI fins enguany [23]. Aquesta millora en el pronòstic vital dels pacients es creu que pot estar força relacionada amb una millora de les cures hospitalàries, sobretot durant la fase aguda de la malaltia [24].

De manera paral·lela a aquesta disminució de la mortalitat, s'ha observat però un augment de la morbiditat després d'un ictus. Així, des d'un punt de vista funcional, l'ictus suposa també una de les principals causes de discapacitat arreu. Dades del Ministeri de Sanitat i Política Social reporten que un 43,8% dels pacients que sobreviuen a l'ictus presenten discapacitat i dependència funcional 6 mesos després de patir la lesió cerebrovascular (dades obtingudes del Sistema Nacional de Salut), mentre que a nivell mundial, aquesta xifra assoleix les primeres posicions en el rànquing de malalties que causen dependència funcional [11].

L'estat funcional dels pacients s'avalua a través de diferents escales i sistemes de puntuacions, tals com l'índex de Barthel (BI: Barthel Index) [25], la GOS (Glasgow Outcome Scale) [26] i l'escala modificada de Rankin (mRS: modified Rankin Scale) [27], aquesta última essent la més utilitzada avui en dia. La mRS mesura el grau d'independència global dels pacients per a la realització d'activitats bàsiques de la vida diària a través d'un sistema de puntuacions del 0 al 6, on obtenen 0 punts aquells pacients sense símptomes de dependència funcional, 1 punt aquells pacients que presenten algun símptoma però en absència de discapacitat, 2 punts quan presenten una discapacitat lleu, de 3 a 5 punts quan la discapacitat és moderada o severa i 6 punts quan els pacients moren. Per definició de l'escala en qüestió, es considera que una puntuació de mRS>2 reflexa una dependència funcional dels pacients, mentre que es consideren independents els pacients amb valors inferiors a 2 [28]. Aquesta classificació dicotòmica s'utilitza comunament, doncs, per determinar l'estat i explorar el pronòstic dels pacients a curt i llarg termini després d'un esdeveniment isquèmic (a l'alta hospitalària o al cap d'1, 3, 6 mesos o 1 any després de l'ictus).

Predir quina serà aquesta evolució (pronòstic vital i funcional) durant la fase aguda de l'ictus, quan resulta ser potencialment valuosa per a una correcta actuació clínica, segueix sent però un gran repte pels neuròlegs, ja que són nombrosos els factors que poden condicionar i influir el pronòstic dels pacients afectats per la malaltia.

En primer lloc, el **temps d'evolució** des del inici dels símptomes, o temps d'isquèmia, condiciona altament les estratègies terapèutiques o tractaments a seguir en la fase aguda de la patologia isquèmica, així com també influeix en la recuperació clínica del pacient i la resposta favorable al tractament. És evident que com major sigui el temps d'isquèmia, menor probabilitat hi ha d'un pronòstic prometedor [29]. Una de les estratègies que ha permès reduir el temps d'isquèmia i alhora, doncs, millorar el pronòstic de l'ictus i evitar les seves complicacions és la implantació del *Codi Ictus* i la creació d'Unitats d'Ictus especialitzades [30]. Ambdues eines permeten l'activació extrahospitalària d'una sèrie de procediments que permeten reconèixer la urgència, avisar l'equip mèdic i organitzar el transport del pacient al centre especialitzat corresponent de la manera més ràpida i efectiva possible. No obstant, aquestes Unitats d'Ictus especialitzades no es troben a l'abast de tothom i és per això que complementàriament s'han desenvolupat també programes mèdics d'assistència remota (tele-assistència o Teleictus) [31]. Aquests faciliten el contacte directe entre els hospitals comarcals i els de referència, aquests últims dotats de neuròlegs de guàrdia que avaluen mitjançant videoconferències les proves de neuroimatge i la situació del pacient, i ordenen si s'escau l'aplicació del tractament trombolític en els mateixos hospitals regionals.

L'**estat neurològic** dels pacients és un altre dels factors més determinants del pronòstic de l'ictus. Aquest s'avalua mitjançant diferents escales que permeten contemplar de manera quantitativa la gravetat del dany cerebral ocasionat. L'escala que més s'utilitza avui en dia és la NIHSS (National Institutes of Health Stroke Scale) [32]. Aquesta contempla 15 ítems i una puntuació global que oscil·la entre el 0 (sense símptomes) i el 42 (gravetat severa), i avalua diferents aspectes relacionats amb la consciència, la capacitat visual i sensorial, la mobilitat de les extremitats i el llenguatge del pacient, entre d'altres. A banda d'aquesta escala, es coneixen, però, altres sistemes que també permeten determinar l'estat neurològic dels pacients, tals com l'escala canadenca (CNS: Canadian Neurological Scale) [33], la escandinava (SSS: Scandinavian Stroke Scale) [34] i l'europea (ESS:European Stroke Scale) [35], tot i que totes elles són menys utilitzades que la NIHSS.

Les variacions en la puntuació d'aquestes escales al llarg del temps també ajuden a predir l'evolució de l'estat neurològic dels pacients. En el cas de la NIHSS, malgrat no existeixi un criteri plenament establert, es considera que el pacient millora o empitjora neurològicament quan es produeix una disminució o augment de la puntuació de l'NIHSS de 4 o més punts, respectivament [36]. En canvi, un pacient es qualifica com a estable quan la diferència de

la NIHSS en el temps és menor de 4 punts. Transcorregudes les primeres 24h des de l'inici de l'ictus, entre un 8 i un 14% dels pacients pateix deteriorament neurològic [37, 38]. Aquest empitjorament durant la fase subaguda ha resultat ser, a més, un bon predictor de mal pronòstic a llarg termini i s'ha associat al desenvolupament de complicacions subjacents tals com l'edema cerebral o la transformació hemorràgica [39].

A banda del temps d'isquèmia i l'estat neurològic dels pacients, hi ha una sèrie de variables clíniques no modificables que també han demostrat ser potencialment condicionants de l'ictus isquèmic i la seva evolució. Entre elles, destaquen l**'edat** i el **sexe** [40]**.** Pel que fa a la incidència de l'ictus, tan sols un 10% del total es donen en pacients joves (menors de 50 anys) [11, 41] i més d'un 65% succeeixen en individus majors de 65, dels quals aproximadament un 35% inclús superen els 74 anys de vida [42]. El fet que gran part dels pacients que pateixen un ictus siguin d'edat avançada, doncs, repercuteix en el pronòstic i evolució d'aquests, de manera que les taxes de mortalitat i morbiditat associades a l'ictus segueixen sent avui en dia destacadament superior en individus majors de 75 anys [11]. No obstant, malgrat els pacients més grans semblen ser més susceptibles d'un pitjor pronòstic, sovint és difícil predir amb certesa l'evolució d'aquest conjunt de pacients, especialment pel que fa la resposta al tractament endovenós. Això és degut a que actualment no s'inclouen pacients d'edat avançada en la gran majoria d'assajos clínics, fet que dificulta l'aplicació dels resultats obtinguts als pacients d'edat superior al rang d'inclusió d'aquests estudis [16]. L'edat pot computar-se en combinació amb la gravetat de la lesió (mesurada a través de la NIHSS) mitjançant l'índex SPAN-100 (Stroke Prognostication using Age and NIHSS index), una escala de classificació dicotòmica (SPAN≥100 o positiu vs. SPAN<100 o negatiu) resultant de la suma de l'edat en anys i la puntuació de la NIHSS en un temps determinat [43]. De manera similar a l'escala de gravetat, es coneix que els pacients SPAN-100 positius presenten una menor incidència de bon pronòstic i una major taxa de complicacions subjacents a l'ictus isquèmic.

El sexe, amb interacció directa amb l'edat, també ha demostrat ser un gran influent de la incidència i pronòstic de l'ictus isquèmic [44, 45]. Les taxes d'incidència de l'ictus són substancialment inferiors en dones que en homes de mitjana edat, mentre que aquestes diferències es redueixen o fins i tot es reverteixen en edats avançades, on la incidència en dones és aproximadament igual o fins i tot superior a la dels homes [11]. Aquestes variacions s'atribueixen en part al paper neuroprotector i antiinflamatori dels estròg ens, que predomina en l'edat adulta però s'anul·la amb la menopausa [46]. A més, les dones que pateixen un ictus són de mitjana quatre anys més grans que els homes que també desencadenen tal afectació cerebrovascular (75 vs. 71 anys, respectivament) [11]. Així doncs, que aquesta major proporció d'afectats sigui envellida i de sexe femení pot explicar

també que la taxa de mortalitat associada a l'ictus esdevingui lleugerament major en dones que en homes.

Finalment, altres factors no modificables, com la situació funcional prèvia, la presència de comorbiditats del pacient o l'etiologia de l'ictus, també tenen importància en el pronòstic d'aquesta patologia i poden tenir alhora un gran impacte sobre l'evolució clínica del pacient [40].

1.1.4.1 Complicacions de l'ictus isquèmic

L'ictus isquèmic pot donar lloc a una sèrie de complicacions que poden modificar el curs de la malaltia i condicionar també el pronòstic del pacient afectat. Aquestes complicacions varien en les diferents etapes de l'ictus, fet que ens permet agrupar-les en complicacions de fase aguda, subaguda i crònica.

Durant la **fase aguda**, les principals complicacions associades a l'ictus isquèmic són la manca de recanalització o la reoclusió de l'artèria afectada i l'aparició de sagnats cerebrals, ambdós processos altament relacionats amb les estratègies de trombòlisi. L'rt-PA només assoleix la recanalització del vas obstruït en un 50% dels casos aproximadament, mentre que la trombectomia mecànica ha mostrat ser efectiva fins en un 80% dels casos [47–49]. La localització exacta de l'oclusió, la mida i composició del coàgul, les comorbiditats del pacient (principalment la pressió arterial i la glucèmia) o la presència de flux sanguini residual són alguns dels factors que poden condicionar l'eficàcia del fàrmac trombolític. No obstant, fins i tot quan es produeix la recanalització de l'artèria, aquesta pot tornar-se a obstruir (reoclusió). Es calcula que aquest fenomen es dóna en un 34% dels pacients que assoleixen una recanalització inicial per acció de l'rt-PA [50], sent els ictus de major gravetat i els que presenten estenosis de la caròtida ipsilateral els més susceptibles a desenvolupar aquesta complicació [51]. Les transformacions hemorràgiques són les complicacions de fase aguda més severes i perilloses actualment. Donada la seva rellevància en un dels capítols d'aquesta Tesi Doctoral, hem cregut convenient destinar un apartat específic sencer per parlar de la seva incidència, classificació, tractament i pronòstic (Apartat 1.1.4.1.1).

Més tard en el temps, durant la **fase subaguda** de l'ictus, es desencadenen també una sèrie de mecanismes moleculars que poden donar lloc a altres complicacions secundàries. Per exemple, l'augment substancial de la permeabilitat de la barrera hematoencefàlica (BBB: brain-blood barrier) permet l'entrada massiva de fluids i components de la circulació cap a la zona cerebral afectada, el que pot donar lloc a l'edema cerebral, entre d'altres [52]. Aquest edema pot inclús culminar en el que coneixem com a infart maligne si la progressió

d'aquest és tal que finalment produeix una hipertensió intracranial severa i desplaça estructures cerebrals més enllà de la línia mitja [53]. Una altra complicació freqüent de la fase subaguda, malgrat també pot aparèixer en qualsevol altre moment, és el desenvolupament de crisis epilèptiques [54]. Aquestes, juntament amb altres complicacions associades a les pròpies comorbiditats del pacient (com les complicacions cardiològiques) o bé derivades directament de l'ictus (com la disfàgia, la immobilitat o la necessitat de dispositius mèdics vitals), causen un augment de la taxa de discapacitat i deteriorament dels pacients, allarguen les seves estàncies hospitalàries i suposen un gran increment dels costos sanitaris associats a aquesta patologia.

Unes altres complicacions força temudes durant la fase subaguda de l'ictus isquèmic són les infeccions. S'ha descrit que un 30% dels pacients que han patit un ictus es veuen afectats també per una infecció, les més comuns essent les pneumònies i les infeccions del tracte urinari [55]. A nivell molecular, tal i com veurem més endavant (apartat 1.2.2), la resposta inflamatòria associada a l'ictus isquèmic acaba donant lloc a un estat d'immunosupressió [56]. Aquest estat es tradueix bàsicament en una baixada de les defenses del pacient i una impossibilitat de fer front als diversos agents patògens externs que trobem el dia a dia, fet que facilita la infecció. A més, cal tenir present que les estades hospitalàries o en centres especialitzats de manera prolongada poden augmentar el risc de contraure aquestes infeccions, doncs la majoria de centres sanitaris presenten una elevada presència de microorganismes patògens fàcilment adquiribles pels pacients ingressats (infeccions nosocomials). La prevenció, detecció i tractament de les infeccions associades a l'ictus és un tema important d'estudi actualment [57–59], doncs les infeccions suposen un pitjor pronòstic pels pacients que les contrauen [60]. Nombrosos esforços s'estan destinant per intentar disminuir la incidència d'aquestes complicacions severes, així com millorar el tractament i pronòstic dels pacients que les pateixen després de l'ictus.

Finalment, durant la **fase crònica** de l'ictus les complicacions que en deriven estan majoritàriament relacionades amb les seqüeles que romanen en l'individu a nivell motor i sensorial, i que alteren sovint la capacitat per a realitzar les activitats bàsiques del dia a dia. Alguns exemples són la osteoporosi, la incontinència urinària i els dolors, entre d'altres [61]. També es poden donar alteracions psicològiques, com la depressió, que afecta aproximadament fins a un terç dels supervivents de l'ictus [62] i cognitives, com la demència, que malgrat no ser considerada una conseqüència directa de l'ictus, el seu desencadenament està altament associat a aquesta malaltia [63].

Els pacients que han patit un ictus presenten, a més, un risc elevat de patir un nou esdeveniment vascular, ja sigui un ictus recurrent o un altre esdeveniment isquèmic. El risc cumulatiu de recurrència assoleix fins a un 10% durant el primer any i un 25% en els cinc anys següents [64]. Per tal de frenar l'aparició d'aquests nous esdeveniments és important

mantenir un control estricte dels principals factors de risc, així com portar un estil de vida més saludable. A més, l'ús de tractaments específics que evitin l'aparició de nous episodis també està altament recomanat, però varien en funció de l'etiologia del primer esdeveniment isquèmic.

1.1.4.1.1 Transformacions hemorràgiques en l'ictus isquèmic

Les transformacions hemorràgiques (TH) són sagnats localitzats habitualment a l'àrea infartada que s'ocasionen majoritàriament en el procés de la restauració del flux sanguini de la vasculatura potencialment debilitada per la pròpia isquèmia. Malgrat l'aparició de TH pot produir-se de manera independent al tractament, l'administració de l'agent trombolític rt-PA juga un paper clau en l'aparició d'aquestes complicacions severes i es coneix que augmenta la incidència d'aquests sagnats fins a 10 vegades més que en els pacients no tractats [65]. A banda de promoure la degradació del coàgul obstructiu, l'rt-PA pot provocar efectes tòxics si arriba al parènquima cerebral, doncs estimula la degradació de la matriu extracel·lular, promou la infiltració de leucòcits, l'activació de la micròglia i l'alliberació de radicals lliures en la zona de la lesió isquèmica [66].

Les TH poden oscil·lar des de petites hemorràgies petequials asimptomàtiques fins a grans hematomes intraparenquimatosos. La classificació més coneguda es basa en criteris radiològics, i categoritza les TH en quatre grups (Taula 1) (Figura 3) [67].

Tipus d'hemorràgies		**Característiques radiològiques**
HI-1	Infart hemorràgic tipus 1	Petites petèquies a la perifèria de l'infart
HI-2	Infart hemorràgic tipus 2	Petèquies confluents a l'àrea de l'infart però sense efecte massa
PH-1	Hemorràgia parenquimatosa tipus 1	Sang homogènia en <30% de l'àrea infartada. Pot tenir lleuger efecte massa
PH-2	Hemorràgia parenquimatosa tipus 2	Sang homogènia en >30% de l'àrea infartada amb efecte massa evident.

Taula 1. Classificació de les TH segons criteris radiològics, establerta per l'ECASS (European Cooperative Acute Stroke Study) [65].

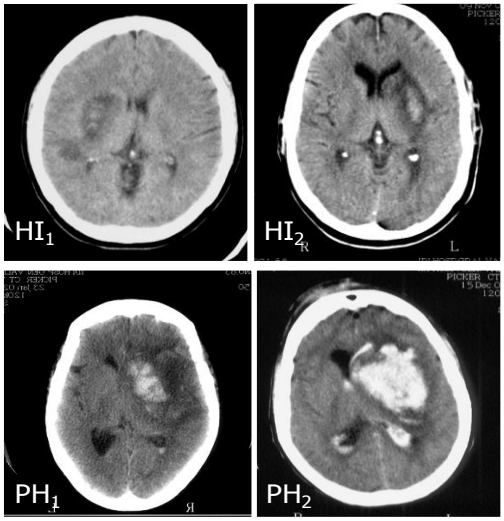


Figura 3. Classificació dels tipus de TH (Adaptat de Montaner et al. 2003) [68] per tomografia computaritzada segons la classificació ECASS [65].

Les TH poden agrupar-se també en simptomàtiques o asimptomàtiques. La definició més utilitzada per classificar la simptomatologia de les TH té origen en l'assaig clínic ECASS (European Cooperative Acute Stroke Study), on es descriu la transformació simptomàtica com qualsevol signe radiològic d'hemorràgia intracraneal que succeeix durant les primeres 36 hores i que s'associa directament a un deteriorament neurològic, és a dir, a un augment de 4 o més punts en la NIHSS [69, 70]. Les TH simptomàtiques presenten una incidència de fins un 7% dels pacients que es tracten amb rt-PA, mentre que en absència de teràpies trombolítiques, aquestes complicacions només s'han reportat en un 1,5% dels casos [71].

L'hematoma parenquimatós remot es coneix com qualsevol sagnat intracraneal localitzat fora de la zona infartada. Aquest tipus de sagnat afecta entre un 1,3% i un 3,7% dels pacients que pateixen un ictus i també pot aparèixer després de l'administració de rt-PA [72]. No obstant, s'ha vist associat majoritàriament a altres alteracions tals com les coagulopaties, les estructures vasculars anòmales o les angiopaties [18].

A banda de l'rt-PA, les TH s'han associat també a un gran nombre de factors clínics, tals com la gravetat i la mida de la lesió isquèmica, l'edat avançada, la hipertensió o els nivells de glucosa en sang, entre d'altres [73]. Malgrat la seva preocupant incidència, encara avui en dia no es disposa de cap estratègia terapèutica efectiva per prevenir o tractar les TH. El tractament dels pacients amb TH depèn bàsicament de l'abast del sagnat i els símptomes i efectes que aquest produeixi en el pacient, de manera que només aquells grans hematomes que causin un deteriorament neurològic sever seran extrets per mitjà d'una craniotomia [74].

1.2 Fisiopatologia de l'ictus isquèmic

La conseqüència immediata de l'oclusió d'una artèria cerebral, sigui degut a un coàgul, una trombosi o una hipoperfusió sistèmica, és una dràstica interrupció del flux sanguini cerebral. El resultat és la manca del subministrament d'oxigen i glucosa en una zona determinada del cervell, fet que desencadena una sèrie de mecanismes, tant a nivell cel·lular com tissular, que acaben ocasionant la mort neuronal del teixit afectat, ja sigui per necrosis (mort com a conseqüència de la lesió cel·lular massiva) o per apoptosis (mort cel·lular programada a través de mecanismes dependents d'energia).

Aquesta restricció del reg sanguini es produeix de manera heterogènia en el conjunt del territori afectat. Així, s'anomena nucli o core a la zona central que normalment està directament irrigada per l'arteria obstruïda i on el flux sanguini està gairebé completament restringit. En canvi, es coneix com a penombra isquèmica a la zona circumdant a la regió central que manté un flux sanguini residual capaç de mantenir la viabilitat cel·lular durant un cert temps determinat. La penombra isquèmica és de gran interès a nivell terapèutic ja que malgrat està metabòlicament compromesa per la isquèmia, manté la integritat morfològica i és susceptible de recuperació si s'aconsegueix restaurar el subministrament de sang a la zona afectada [75]. El destí d'aquesta zona, però, depèn de la presència de circulació colateral, el grau d'isquèmia de l'artèria afectada i, sobretot, del temps transcorregut des de l'inici de l'ictus isquèmic fins a la recuperació del flux sanguini a la zona danyada.

1.2.1 Cascada isquèmica

Immediatament després d'iniciar-se la isquèmia es produeix una reducció sobtada del contingut d'ATP cel·lular, conseqüència de l'esgotament del reservori energètic de les cèl·lules i la fallida metabòlica (Figura 4). El resultat és l'alteració de tots els mecanismes cel·lulars dependents d'ATP, principalment les bombes iòniques, que desencadenen la despolarització o pèrdua d'activitat elèctrica de les membranes cel·lulars de neurones i glia i una subseqüent pertorbació de la permeabilitat d'aquestes [76]. Aquest fet dificulta el manteniment de l'homeòstasi iònica de les cèl·lules i causa un augment massiu de la concentració intracel·lular de sodi (Na+) i calci (Ca^{2+}), així com un alliberament extracel·lular desmesurat de potassi (K^{+}) [77].

Com a conseqüència d'aquesta alteració de la permeabilitat cel·lular es produeix una alliberació massiva de neurotransmissors a l'espai extracel·lular, principalment glutamat. El glutamat causa l'activació dels receptors de N-metil-D-aspartat (NMDA), d'àcid amino-3-hidroxi-5-metil-4-isoxazol (AMPA) i dels receptors metabotròpics de la neurona post-

sinàptica [76]. Aquest procés, conegut com a **excitotoxicitat**, provoca una segona entrada massiva de Ca^{2+} a l'interior de la cèl·lula i incrementa encara més el contingut intracel·lular d'aquest ió. A més, com a conseqüència d'aquestes variacions iòniques s'intensifica també l'influx de Na^{+} i clorur (Cl^{-}) i l'eflux de K^{+}, donant lloc a l'entrada passiva d'aigua a l'interior cel·lular i provocant edema per trencament de l'equilibri osmòtic de les cèl·lules. L'entrada intracel·lular de Ca^{2+} també estimula la síntesi d'òxid nítric (NO) i l'activitat de l'òxid nítric sintasa (NOS), que provoquen la formació de radicals lliures i **estrès oxidatiu**. A més, s'activen també diferents enzims lítics (proteases, lipases i endonucleases) i es desacobla la fosforilació oxidativa, tot comprometent encara més la disponibilitat energètica cel·lular [78].

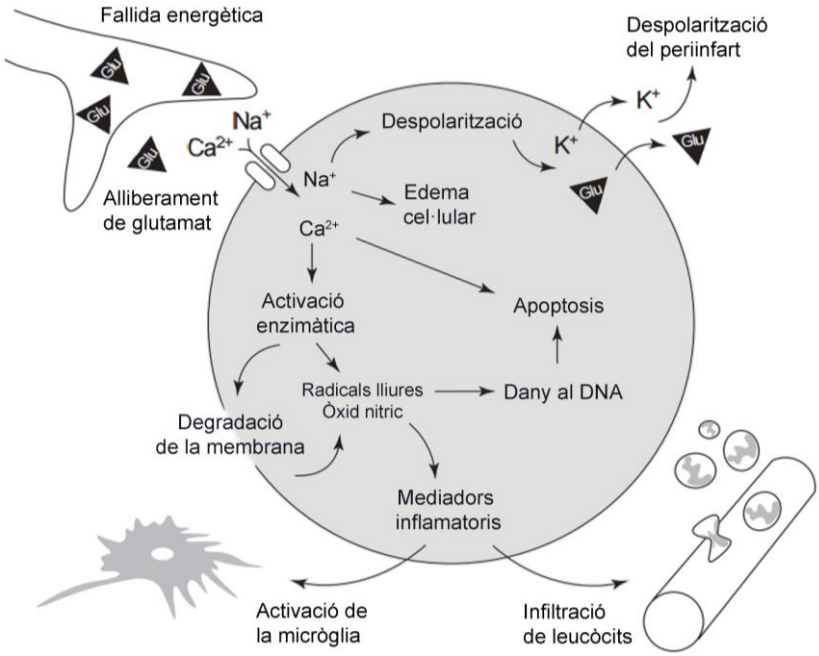


Figura 4. Principals processos fisiopatològics de l'ictus isquèmic (Adaptat de Dirnagl et al., 1999) [79].

Paral·lelament, la sobrecàrrega intracel·lular de Ca^{2+} activa alhora un seguit de vies de senyalització que desencadenen una **resposta inflamatòria** aguda i massiva, tal i com s'explica en detall en el següent apartat [80].

Finalment, tots aquests canvis i alteracions cel·lulars provoquen l'activació de gens i proteïnes implicades en la mort cel·lular programada, també coneguda com **apoptosis**. Per mitjà de la via intrínseca de l'apoptosi, l'augment massiu de Ca^{2+} provoca l'alliberació de citocrom C dels mitocondris i l'activació directa de la caspasa-3 [81]. A través de la via extrínseca, en canvi, s'activen els receptors de mort cel·lular de la superfície de la cèl·lula, els quals causen l'activació directa de la caspasa-8, seguit d'un gran ventall d'altres molècules pro-apoptòtiques. Ambdues vies de senyalització, però, acaben provocant danys irreparables al material genètic, així com la degradació de proteïnes nuclears i del

citoesquelet, la formació de cossos apoptòtics, l'expressió i translocació a la superfície cel·lular de lligands per a les cèl·lules fagocítiques i finalment, la seva pròpia mort i fagocitosi [82].

1.2.2 Resposta inflamatòria de fase aguda

Immediatament després de produir-se l'oclusió d'un vas sanguini cerebral es desencadena una resposta inflamatòria que condiciona de manera significativa el progrés de la lesió isquèmica. El desenvolupament d'aquesta resposta inflamatòria té lloc simultàniament a nivell local, tant sigui per l'acció de les cèl·lules residents del cervell o les que hi infiltren des de la sang, i a nivell perifèric (resposta inflamatòria sistèmica), degut a l'augment considerat d'espècies inflamatòries en el sistema circulatori.

A nivell local, la reducció del flux sanguini cerebral i el subseqüent augment desmesurat de Ca^{2+} intracel·lular dóna lloc a l'activació d'un seguit de vies de senyalització que culminen en l'expressió de diversos gens pro-inflamatoris. El resultat final és l'alliberació de citocines i quimiocines per part dels diferents tipus cel·lulars metabòlicament compromesos per la isquèmia amb el fi d'activar i mobilitzar altres cèl·lules inflamatòries cap a la zona afectada per donar resposta a l'estímul isquèmic [83] (Figura 5).

Les cèl·lules de la micròglia, derivades del sistema hematopoiètic, constitueixen les principals cèl·lules fagocítiques i d'immunitat residents al sistema nerviós central. La seva funció principal és detectar canvis en l'homeòstasi cerebral i contribuir, en tal cas, al desencadenament d'una resposta inflamatòria local i aguda front un estímul determinat. Immediatament després de la interrupció del flux sanguini cerebral, la micròglia s'activa al reconèixer una sèrie de senyals d'alarma (anomenades *alarmins*) procedents de les cèl·lules metabòlicament compromeses per la isquèmia [84] i participa en primera instància en l'eliminació del debrís i les restes de les cèl·lules isquèmiques moribundes (fagocitosis).

Conjuntament amb els astròcits, la micròglia estimula l'alliberació massiva de senyals pro-inflamatòries que propaguen la lesió isquèmia i promouen la infiltració d'altres cèl·lules immunitàries a la zona cerebral afectada [85]. La secreció d'espècies reactives de l'oxigen (ROS: reactive oxygen species) i del nitrogen (RNS: reactive nitrogen species) estimula la producció de més radicals lliures per part de les neurones isquèmiques i causa danys directes als components cel·lulars essencials que garanteixen la integritat de les cèl·lules [86]. Paral·lelament a aquest estrès oxidatiu, tant la micròglia com els astròcits provoquen una explosió de citocines i quimiocines pro-inflamatòries. D'entre totes, el factor necròtic tumoral alfa (TNF-α) i la interleucina 1 beta (IL-1β) són les primeres que inicien la resposta inflamatòria. Aquestes estimulen l'expressió d'altres citocines i molècules d'adhesió

cel·lular (CAM: cellular adhesion molecules) a nivell de l'endoteli, entre les que destaquen la molècula d'adhesió intercel·lular de tipus 1 (ICAM-1) i la molècula d'adhesió vascular de tipus 1 (VCAM-1), ambdues amb un paper clau com a lligands de les integrines presents a la superfície de la membrana de les cèl·lules circulants [87, 88]. Aquestes molècules d'adhesió promouen el *rolling* de leucòcits, l'activació i adhesió d'aquests a la paret vascular i la seva infiltració cap a la zona cerebral danyada (Figura 6).

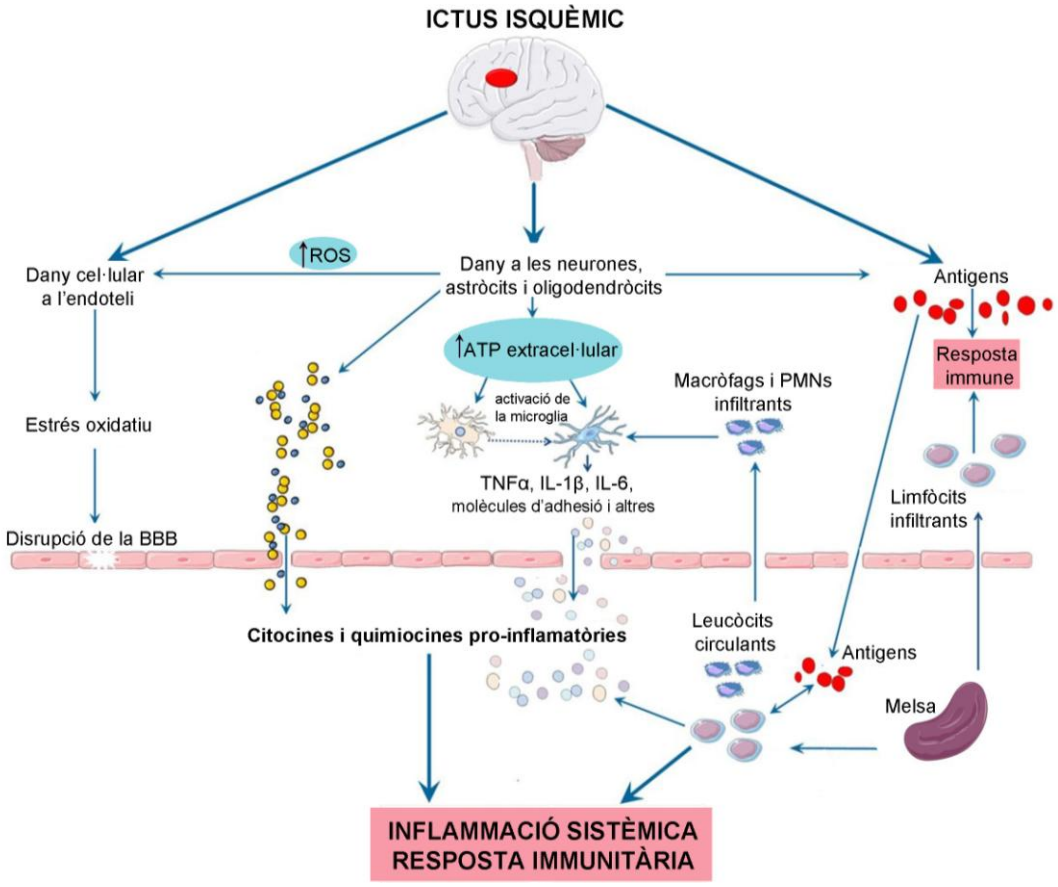


Figura 5. Paper de la resposta immunitària i inflamatòria després de l'ictus isquèmic (Adaptat de Chen et al., 2017) [89]. Abreviacions: ATP: Adenosina trifosfat; BBB (Blood-Brain-Barrier) barrera hematoencefàlica; IL-1β: interleucina 1 beta; IL-6: interleucina 6; ROS: espècies reactives de l'oxigen; PMNs: cèl·lules polimorfonuclears; TNFα: factor necròtic tumoral alfa.

Aquest procés es dóna de manera progressiva i pautada, essent les primeres cèl·lules en ser reclutades els neutròfils, seguits dels monòcits, macròfags i finalment els limfòcits [90]. Malgrat l'ideal seria que la infiltració d'aquestes cèl·lules circulants ajudés a combatre l'insult, hi ha evidències clares que demostren que aquestes produeixen un efecte perjudicial en el parènquima cerebral durant la fase aguda de la lesió [91]. Els leucòcits infiltrats també augmenten la producció de ROS i altres mediadors pro-inflamatoris, que actuen inespecíficament sobre tot tipus cel·lular present a la zona afectada, exacerbant així el dany tissular i contribuint a la propagació de la lesió cerebral.

Les senyals pro-inflamatòries, majoritàriament alliberades per les cèl·lules infiltrants, estimulen també la producció de metalloproteinases de matriu (MMP), encarregades de degradar les proteïnes de la matriu extracel·lular, tals com el col·lagen, els proteoglicans, la laminina o la fibronectina [92, 93]. Les MMPs, en conjunt amb altres mediadors pro-inflamatoris i les ROS, debiliten la paret dels vasos sanguinis i comprometen la integritat de la BBB [94, 95]. Aquest procés culmina amb un increment substancial de la permeabilitat de la BBB, que alhora fa possible una major migració de cèl·lules sanguínies i mediadors inflamatoris cap a la zona afectada i viceversa, i prolonga la resposta inflamatòria a la isquèmia.

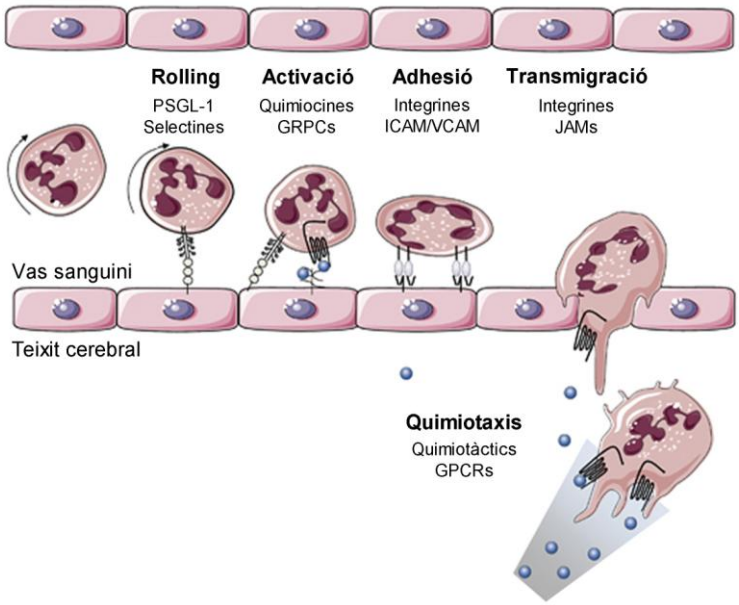


Figura 6. Fases del reclutament de leucòcits cap a la zona infartada del cervell (Adaptat de Bestebroer et al., 2010) [96]. GRPCs: receptors acoblats a proteïna G; ICAM: molècula d'adhesió intracel·lular; JAM: molècula d'adhesió d'unions *junction,* PSGL-1: lligand de selectina P; VCAM: molècula d'adhesió vascular.

A nivell perifèric, l'augment de la permeabilitat de la BBB es tradueix en un increment massiu d'antígens i components inflamatoris al sistema circulatori. Aquests alhora estimulen l'alliberació de més senyals inflamatòries per part de les cèl·lules circulants, així com la producció de ROS, l'activació del complement de plaquetes i la coagulació [97–99]. L'arribada d'aquests mediadors inflamatoris a la melsa també provoca una massiva producció de limfòcits, així com l'alliberació de més mediadors inflamatoris [100]. Tot això culmina en un estat d'híper-inflamació en el sistema circulatori, que es coneix com a inflamació sistèmica i pot inclús derivar al síndrome de resposta d'inflamació sistèmica (SIRS: systemic inflammatory response syndrome) [101].

Aquest excés de mediadors inflamatoris circulants pot donar lloc, també, a l'estimulació del sistema nerviós simpàtic, parasimpàtic i l'eix hipotalàmic-pituïtari-adrenal. L'activació d'aquests sistemes estimula l'alliberació de glucocorticoides i catecolamines, l'expressió d'IL-4, IL-10 i el factor de creixement transformant beta (TGF-β), i l'activació de diverses vies de senyalització antiinflamatòries que inhibeixen l'expressió dels actuals mediadors inflamatoris [56]. La situació resultant és, doncs, un estat global de immunosupressió, en el que el sistema immune deixa de ser capaç de fer front a estímuls patògens externs [102]. Aquesta ineficiència és responsable de la susceptibilitat que presenten els pacients isquèmics a les infeccions, tal i com s'ha explicat anteriorment.

Una mica més tard en el temps, aquests mediadors antiinflamatoris acaben vencent el balanç de components pro- versus antiinflamatoris, i donen lloc a la resolució de la inflamació aguda. L'alliberació d'aquests senyals antiinflamatoris provoca un canvi de rol en la micròglia resident i els macròfags infiltrants, que adopten una paper antiinflamatori per eliminar els components cel·lulars que provenen de les cèl·lules moribundes i alliberar factors protectors que permetin recuperar en certa mesura la zona potencialment danyada per la isquèmia [103].

1.3 Models animals d'isquèmia cerebral

Els estudis preclínics o experimentals en models animals d'isquèmia cerebral han aportat gran part dels coneixements actuals sobre l'ictus isquèmic. Degut a la limitada accessibilitat del cervell humà i el caràcter agut de la malaltia en qüestió, els models preclínics han esdevingut imprescindibles per a l'estudi en detall dels mecanismes fisiopatològics que contribueixen al desenvolupament i progressió d'aquesta patologia, així com pel descobriment de noves estratègies terapèutiques o de diagnòstic de l'ictus isquèmic.

L'objectiu principal d'aquests models és reproduir en animals d'experimentació una situació d'isquèmia cerebral el més similar possible a l'observada en la pràctica clínica. A banda de mimetitzar les condicions humanes, els models experimentals permeten estudiar la patologia isquèmica des d'un punt de vista reduccionista, és a dir, permeten el control d'aquelles variables clíniques que formen la gran complexitat de la malaltia humana i faciliten l'estudi de cadascun d'aquests factors a nivell individual. A més, els models *in vivo* presenten una alta reproductibilitat i escassa variabilitat en la mida i localització de la lesió cerebral induïda, tant entre animals com entre experimentadors, donant lloc a una valuosa simulació el més estrictament possible dels fenòmens biològics implicats en la malaltia [104]. Per altra banda, els models experimentals d'isquèmia cerebral s'han implementat en

una gran varietat d'espècies animals, com porcs, conills o primats, el que ha permès aprofundir més en l'estudi de la patologia. No obstant, els rosegadors són de llarg els més emprats, tant per qüestions econòmiques, de comoditat en el maneig d'aquests animals o d'acceptació ètica.

Els models animals d'isquèmia cerebral es classifiquen en models d'isquèmia global o focal, en funció del territori cerebral afectat. La isquèmia cerebral global afecta tot el cervell, mentre que la isquèmia cerebral focal afecta a un sol territori vascular.

Els models d'isquèmia cerebral global es basen en l'oclusió de les grans artèries que irriguen l'encèfal (Taula 2). Es coneixen diferents models que aconsegueixen aquest tipus d'isquèmia (que afecta els dos hemisferis cerebrals), tot i que els més usats són el model dels quatre vasos, on s'oclouen les artèries vertebrals i les caròtides comuns d'ambdós costats [105] o el model dels dos vasos, en que solament s'oclouen les dues artèries caròtides comuns [106]. Malgrat aquests models són quirúrgicament senzills, avui en dia són poc utilitzats com a models d'ictus isquèmic per la disparitat vers la realitat de la malaltia.

ISQUÈMIA GLOBAL	Isquèmia completa	Aturada cardíaca
		Oclusió aòrtica
		Decapitació
		Torniquet al coll
	Isquèmia incompleta	Hemorràgia o hipotensió
		Isquèmia hipòxica intracranial
		Oclusió de dos vasos i hipotensió
		Oclusió de quatre vasos
		Oclusió unilateral de l'artèria caròtida comú (CCA)
ISQUÈMIA FOCAL	Isquèmia focal	Oclusió de l'artèria cerebral mitja (MCAo), distal o proximal
		MCAo i oclusió ipsilateral de la CCA
		MCAo i oclusió bilateral de la CCA
		Infart cerebral espontani
	Isquèmia multifocal	Embolització amb coàguls sanguinis
		Embolització amb microesferes
		Foto-trombosi

Taula 2. Principals models d'isquèmia cerebral en rosegadors (Adaptat de Liu i McCullough, 2011) [107].

Els models experimentals que reprodueixen més estrictament la patologia cerebrovascular humana, doncs, són els focals. Aquest es basen en la interrupció del flux sanguini de l'artèria cerebral mitja (MCA) en tan sols un dels dos hemisferis cerebrals per mitjà de diferents estratègies i aproximacions (Taula 2). L'oclusió de la MCA pot realitzar-se a nivell proximal (oclusió de gran vas) o a nivell distal (oclusió de petit vas) a través de mecanismes mecànics o trombòtics. Mecànicament, l'oclusió de la MCA pot realitzar-se a través de la introducció d'un filament, per lligament o electrocoagulació de l'artèria en qüestió, mentre que l'oclusió trombòtica té lloc mitjançant la introducció d'un coàgul sanguini o per injecció de trombina o endotelina-1 directament a la MCA [108]. Els models focals afecten principalment al territori vascular de la MCA; les oclusions proximals comprometen inicialment la zona de l'estriat i avancen cap al còrtex amb el pas del temps, mentre que les oclusions distals únicament afecten la regió vascular de la MCA del còrtex. Els models d'isquèmia cerebral focal, a més, presenten certa complexitat quirúrgica i l'aparició de complicacions durant o després de la cirurgia pot augmentar la taxa de mortalitat.

A banda de l'estratègia utilitzada per a l'oclusió del vas, els models es poden classificar en transitoris o permanents, en funció de la duració de la isquèmia i de si permeten o no la reperfusió del teixit isquèmic. L'extensió de la lesió isquèmica dels models transitoris depèn en major part del temps d'isquèmia, tot i que altres factors com la localització de l'oclusió, el sexe, l'espècie i la soca de l'animal, la temperatura corporal o la pressió arterial també hi poden influir [109].

L'avaluació de la mida de la lesió és un dels principals paràmetres d'estudi de bona part dels models experimentals d'isquèmia cerebral. Per a tal finalitat es coneixen múltiples estratègies que varien en funció de l'objectiu i la finalitat de l'estudi, la sensibilitat requerida, el temps d'isquèmia i la disponibilitat d'equipaments especialitzats. En els seus inicis, la visualització de la zona isquèmica es limitava a l'avaluació histològica de les característiques morfològiques del teixit cerebral. Més endavant, però, es van desenvolupar tincions que permeten, de manera eficaç i senzilla, un marcatge microscòpic de la lesió afectada per la isquèmia, com les tincions d'hematoxilina i eosina o les variacions múltiples de la tinció de Nissl [110]. El posterior desenvolupament de tincions macroscòpiques, que faciliten un marcatge encara més ràpid i sense necessitat d'aparells òptics per a la seva correcta visualització, va suposar també un gran avenç per a la delimitació de les lesions isquèmiques. La tinció amb clorur de tetrazole (TTC) és probablement avui en dia la més utilitzada arreu, doncs es considerada un mètode fàcil de realitzar i que permet obtenir resultats de manera ràpida i fiable [111]. Recentment, la millora substancial de les tècniques de neuroimatge ha permès utilitzar estratègies no invasives per a la visualització dels infarts cerebrals en els models experimentals. A

diferència de les tincions post-mortem, les tècniques no invasives permeten realitzar mesures repetides al llarg del temps en un mateix animal, sense suposar cap malestar ni contraindicació física. L'eina més utilitzada arreu per a la detecció *in vivo* de la lesió cerebral en animals és destacadament la ressonància magnètica [112, 113]. No obstant, però, la utilització d'aquestes tècniques està subjecta a la disponibilitat d'aparells especialitzats, sovint força costosos i no accessibles en molts centres d'investigació.

Donat que un dels principals objectius del maneig de l'ictus és la restauració de la funció conductual dels pacients, identificar els dèficits neurològics en els models animals d'ictus és també essencial per a l'aplicació translacional de possibles tractaments terapèutics. Així doncs, complementàriament a la mida de la lesió, també s'utilitzen tests de comportament que permeten avaluar l'estat neurològic i funcional dels animals, així com realitzar un seguiment de la seva millora o deteriorament al llarg del temps (valor pronòstic). Avui en dia, es coneixen múltiples tests per avaluar aquest comportament [114]. L'elecció del més adient és un factor clau a tenir en compte en el disseny experimental inicial, doncs pot condicionar la qualitat i sensibilitat dels resultats que se n'obtinguin. Per a tal elecció cal tenir en compte una sèrie de factors condicionants: en primer lloc, els dèficits que mostren els animals isquèmics difereixen en funció de la zona i l'extensió de la lesió cerebral, de la mateixa manera que els diversos tests difereixen en la selectivitat i sensibilitat pels diferents dèficits, ja siguin motors, sensorials, posturals, reflexius, d'equilibri, etc. Per exemple, el test del filferro penjant (de l'anglès *Hanging Wire*) o la prova de força d'adherència (*Grip Strength test*) contemplen principalment la força motriu, el test del cilindre (*Cylinder test*) avalua el comportament motor i espacial, i el test de la cantonada (*Corner Test)* o les escales de puntuació combinada (com el test de Bederson) contemplen una mescla de valors posturals, sensorials, d'equilibri i funció motora, entre molts d'altres [115–117]. En segon lloc, en funció dels dèficits que es vulguin avaluar, cal tenir també present que els tests de comportament són sensibles al moment de l'assaig. Per exemple, una prova de comportament específica pot mostrar diferències significatives entre grups al cap d'una setmana de la isquèmia cerebral, però no ha de ser necessàriament efectiva en temps més aguts. És per això que la tria del temps òptim d'assaig per a cada prova és també un factor crític per a obtenir resultats fiables [115]. Finalment, la soca i l'espècie animal, així com l'edat i la presència de comorbiditats, poden també influir en la susceptibilitat dels animals a la isquèmia i condicionar el comportament dels mateixos en els tests escollits per a l'estudi [118–121].

Malgrat els models experimentals han esdevingut una eina imprescindible tant per l'estudi de la fisiopatologia de l'ictus com pel desenvolupament d'estratègies terapèutiques, aquests models presenten certes limitacions que cal prendre en consideració a l'hora de realitzar estudis preclínics. De manera destacable, tot i que el control i reducció de certes

variables fisiològiques (principalment sexe, edat i comorbiditats tals com la hipertensió, la diabetis o l'obesitat) pot semblar un clar avantatge per a l'experimentació animal respecte els estudis realitzats amb mostres humanes, aquest fet suposa un emmascarament de la realitat i complexitat clínica i pot causar l'obtenció de resultats no translacionals fruit d'aquest reduccionisme. És per això que s'aconsella cada vegada més la validació dels resultats obtinguts en rosegadors de diferent sexe i edat i, si és possible, que presentin alguna o vàries d'aquestes comorbiditats observades en la pràctica clínica (veure criteris STAIR, apartat 1.4.1).

Així doncs, a l'hora d'escollir un model animal experimental cal tenir en compte els avantatges i desavantatges que cadascun d'aquests presenta en funció del tipus d'estudi que es vulgui realitzar. Cal tenir present el procés fisiopatològic que es pretén estudiar, ja que el model d'isquèmia dependrà de si s'està interessat en el procés isquèmic o el de reperfusió, el tractament o la neuroprotecció a fase aguda, els mecanismes posteriors de neuroreparació o alguna de les complicacions associades a la isquèmia, entre d'altres. També cal tenir present l'àrea cerebral afectada en cada model experimental, així com la recuperació de l'animal i la mortalitat que s'experimenta, si el que es pretén és fer estudis a llarg termini. La restauració del flux sanguini cerebral també serà un paràmetre a considerar quan l'objectiu sigui l'administració de substàncies que arribin a l'àrea infartada [109]. Finalment, l'elecció de la soca, el sexe i la presència o no d'altres malalties concomitants també podran condicionar els resultats de l'estudi i cal tenir-les en consideració abans d'iniciar la fase experimental.

1.4 Noves estratègies terapèutiques de l'ictus isquèmic

A banda de millorar les estratègies de reperfusió actuals, grans esforços estan sent destinats a la cerca i estudi de teràpies protectores i reparadores de la zona cerebral afectada (Figura 7). Les estratègies de **neuroprotecció** tenen com a finalitat principal interferir en algun dels processos inicials de la cascada isquèmica per tal de bloquejar o frenar els mecanismes de dany tissular i mort cerebral, prevenint així un major desencadenament i progressió de la lesió. Les estratègies reparadores o de **neuroreparació**, en canvi, es centren en potenciar, accelerar i estimular la recuperació funcional espontània dels pacients que han patit un ictus. Aquestes estratègies terapèutiques intenten, doncs, regenerar el teixit danyat i restablir els circuits neuronals afectats per tal de recuperar les funcions sensorials i motores perdudes per motiu de la isquèmia [122].

Ja que la present Tesi Doctoral té com objectiu contribuir en la cerca de noves estratègies terapèutiques que permetin frenar el dany i la mort cerebral durant la fase aguda i subaguda de l'ictus, hem cregut convenient destinar un apartat específic a la neuroprotecció i les estratègies neuroprotectores objecte d'estudi des de les últimes dècades.

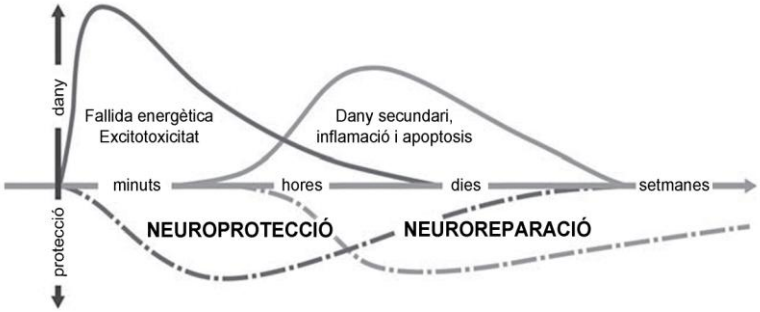


Figura 7. Característiques fisiopatològiques de la isquèmia cerebral al llarg del temps (Adaptat de Janowski et al., 2015) [123].

1.4.1 La neuroprotecció

La neuroprotecció, coneguda tradicionalment com la "protecció de les neurones", es centra en l'atenuació i bloqueig de mediadors claus de la cascada isquèmica. L'eficàcia dels agents neuroprotectors està condicionada per l'existència de teixit cerebral isquèmic que, tot i estar metabòlicament compromès, encara és potencialment viable, és a dir, no presenta danys irreparables. Malgrat l'àrea d'aquest teixit viable disminueix dràsticament durant les primeres hores després de l'episodi isquèmic (Figura 8), el període de temps en que es creu que encara hi ha possibilitat de protegir la zona salvable s'estén fins a les primeres 72 hores [124]. Així, un dels factors claus que condicionaran l'eficàcia dels agents neuroprotectors és el temps, doncs tal i com resumeix molt bé la frase "*time is brain*", una major rapidesa a l'hora d'iniciar aquests tractaments comportarà idealment una major protecció de la zona circumdant a la lesió isquèmica, evitant el seu creixement [122].

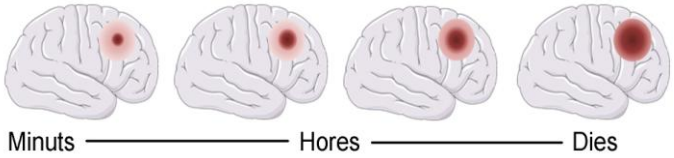


Figura 8. Creixement de la zona isquèmica amb el temps. S'indica el creixement del nucli de la zona isquèmica (vermell fort) i la disminució proporcional de la zona de penombra (rosa).

Un gran ventall d'estratègies neuroprotectores han estat proposades i dissenyades per bloquejar la progressió de la lesió cerebral després de l'oclusió d'un vas sanguini (Taula 3).

Classificació	Tractaments	Mecanismes d'acció
Anti-excitotòxics	Sulfat de magnesi	Disminueix l'alliberació de glutamat; bloqueja canals iònics; indueix hipotèrmia
	Tat-NR2B9c (NA-1)	Inhibeix la formació del complex del receptor NMDA/PSD-95/nNOS; inhibeix la producció d'NO
	Donepezil	Inhibeix l'alliberació de glutamat i glicina; inhibeix els ions de calci i sodi
Antioxidants	Edavarone, Ebselen	Recaptadors de ROS
	Nitrones (NXY-059)	Recaptadores de ROS
	Àcid úric	Recaptador de ROS
	Vitamina B2	Recaptadora de ROS
	Inhibidors de NOS	Inhibidor de les diferents isoformes de NOS
	DP-b99	Quelant de ions metàl·lics divalents
Factors de creixement hematopoètics	G-CSF i EPO	Redueixen l'excitotoxicitat del glutamat; tenen propietats antiinflamatòries i anti-apoptòtiques; estimulen la neurogènesi i l'angiogènesi
Antiinflamatoris	IL-1ra	Inhibeix la infiltració de neutròfils i prevé la disrupció de la BBB
	Natalizumab	Atenua la infiltració de leucòcits
	Fingolimod	Anàleg del receptor d'esfingosina 1-fosfat; inhibeix la sortida de limfòcits dels òrgans limfoides
Immunosupressors	Ciclosporina A	Propietats antiinflamatòries i anti-excitotòxiques
Estatines	Lovastatina, Simvastatina, Atorvastatina	Inhibidors de l'hidroximetil-glutaril-coenzim-A reductasa
Antibiòtics	Minociclina	Té propietats antiinflamatòries i antioxidants; redueix l'activació de la micròglia i la MMP9; anti-apoptòtic
	Dapsona	Té propietats antioxidants, anti-apoptòtiques i antiinflamatòries
Altres	Albúmina	Produeix hemodilució i resposta microvascular; millora la circulació col·lateral i del CSF; redueix l'edema cerebral
Estratègies no farmacològiques	Hipotèrmia	Redueix la demanda energètica; preserva les reserves d'energia; disminueix el lactat, el glutamat i ROS; prevé l'apoptosis; redueix la disrupció de la BBB i l'edema cerebral

Taula 3. Estratègies neuroprotectores: principals tractaments que han estat objecte d'investigació en models experimentals d'isquèmia durant les últimes dècades (Adaptada de Minnerup et al., 2012, Moretti et al., 2015 i Bonaventura et al., 2016) [83, 125, 126]. BBB: barrera hematoencefàlica; CSF: líquid cefaloraquidi; EPO: eritropoetina; G-CSF: factor estimulant de colònies de granulòcits; IL-1ra: antagonista del receptor d'interleucina 1; NO: òxid nítric; NOS: sintasa d'òxid nítric; nNOS: sintasa d'òxid nítric de tipus neuronal; NMDA: N-metil D-aspartat; MMP9: metal·loproteïnasa de matriu 9; PSD-95: proteïna de densitat postsinàptica 95; ROS: espècies reactives de l'oxigen.

La majoria d'aquestes teràpies provenen d'estudis bàsics en animals d'experimentació, on han mostrat eficàcia com a neuroprotectors en el cervell isquèmic. Destacadament, la majoria d'aquests fàrmacs han estat dirigits a frenar el desencadenament dels processos excitotòxics, modular l'estrès oxidatiu i atenuar la resposta inflamatòria, entre d'altres.

No obstant, dels milers de compostos que han estat testats en models animals d'isquèmia cerebral, només uns 200 han arribat a estudiar-se en assajos clínics (fase II o III), i encara cap d'ells ha aconseguit mostrar resultats prometedors per a la seva implantació en la pràctica clínica [127]. Aquest fracàs translacional pot explicar-se, en part, per les grans diferències metodològiques entre els estudis experimentals i els assajos clínics (Taula 4).

Models animals	**Estudis en humans**
Població homogènia i controlada	Població heterogènia i variable
Animals joves	Pacients d'edat avançada
Comorbiditats limitades	Nombroses comorbiditats
Inici induït de l'ictus	Inici espontani de l'ictus
Etiologia uniforme	Etiologies variades
Territori isquèmic subjacent a la MCA	Territori isquèmic no restringit a la MCA
Finestra terapèutica variable	Finestra terapèutica estricta
Control exacte de la duració de l'oclusió	Duració de l'oclusió variable
Ampli abast per optimitzar la dosis	Reduït abast d'optimització de la dosis
Múltiples vies d'administració	Vies d'administració limitades
Volum d'infart com a objectiu primari	Discapacitat funcional com a objectiu primari
Estudis *in vivo* i *in vitro*	Estudis *in vivo*
Ús majoritari del sexe masculí	Sexe masculí i femení
Estudis de paràmetres subcel·lulars	Pocs estudis subcel·lulars amb mostres post-exitus
Sense tractaments previs amb altres fàrmacs	Tractaments previs amb altres fàrmacs

Taula 4. Principals diferències entre els estudis preclínics i clínics que han portat al fracàs translacional de moltes estratègies neuroprotectores (Adaptat de Moretti et al., 2015) [126]. MCA: arteria cerebral mitja.

Amb l'objectiu de posar remei a aquest fracàs translacional, la creació del grup de treball STAIR (Stroke Treatment Academic Industry Roundtable) va permetre documentar una sèrie de criteris i recomanacions per a l'estudi de l'ictus en experimentació animal (STAIR 1999). Des de llavors, el grup STAIR ha continuat reportant actualitzacions i millores d'aquestes recomanacions, totes elles basades, entre d'altres, en el refinament dels criteris experimentals per al descobriment de noves teràpies neuroprotectores [128–132].

De manera destacable, aquests criteris posen especial èmfasis en l'elecció del model experimental, l'espècie i la soca animal. Recomanen considerar les diferències existents entre sexes i, sobretot, incloure animals amb diferents comorbiditats (diabetis, obesitat,

hipertensió), tal i com succeeix en els humans. Destaquen també la necessitat de realitzar estudis transparents, aleatoritzats i duts a terme per investigadors cecs a les condicions experimentals, així com reportar els càlculs de la mida mostral, la potència dels resultats i els criteris d'inclusió i exclusió dels animals, amb la idea de millorar la qualitat dels estudis preclínics. Finalment, aquestes recomanacions remarquen amb insistència la necessitat de verificar els efectes neuroprotectors d'un determinat fàrmac en un mínim de dos laboratoris diferents, per garantir la fiabilitat dels resultats assumint la variabilitat metodològica que pot existir entre centres. En aquest mateix sentit, el consorci MULTIPART (Multicentre Preclinical Animal Research Team) va néixer el 2013 amb l'objectiu d'establir i implementar una plataforma internacional per realitzar estudis a nivell preclínic i multicèntric. Simultàniament, les directrius IMPROVE, sorgides d'aquest consorci i publicades recentment, pretenen donar suport als investigadors i personal tècnic per refinar les pràctiques dels models experimentals d'isquèmia cerebral [133], amb l'objectiu final d'homogeneïtzar els estudis preclínics i aconseguir, així, una eficaç translació a la clínica d'aquells tractaments neuroprotectors candidats que finalment mostrin un alt valor potencial.

La manca d'èxit de les estratègies testades fins ara, però, fa que la cerca de nous agents neuroprotectors continuï essent una de les principals prioritats dins el món de la investigació de l'ictus. Malgrat els estudis preclínics no hagin pogut proporcionar fàrmacs eficaços pels humans, aquests han esdevingut una gran font d'informació sobre els esdeveniments moleculars que succeeixen en el desencadenament d'aquesta malaltia. Les continues millores en el coneixement dels mecanismes fisiopatològics poden, alhora, esdevenir un molt bon punt de partida per a la futura identificació de noves dianes terapèutiques potencialment més valuoses que les identificades fins al moment. No obstant, encara queda camí per recórrer i la necessitat d'entendre al complet els processos patològics que tenen lloc immediatament després de l'oclusió d'un vas sanguini cerebral segueix vigent.

1.5 Biomarcadors de l'ictus isquèmic

Un biomarcador es defineix com aquella característica mesurable de manera objectiva que permet ser utilitzada com a indicador d'un procés biològic normal, patogènic o de resposta a una determinada intervenció terapèutica [134]. Malgrat el concepte de biomarcador pot fer referència a dades clíniques o de neuroimatge, cada cop més freqüentment s'utilitza per designar aquelles molècules biològiques de naturalesa complexa, com les proteïnes, pèptids, lípids, metabòlits, químics o fins i tot àcids nucleics, que es troben associades a determinats processos biològics subjacents a la malaltia estudiada en qüestió.

En el marc de l'ictus isquèmic, s'espera que els biomarcadors siguin mesurables de manera ràpida i fiable i que permetin cobrir algun dels aspectes de la patologia, ja sigui el seu diagnòstic, la identificació de l'etiologia, l'avaluació del risc i resposta al tractament o el pronòstic dels pacients que han patit la malaltia. A més, per tal que un bon biomarcador tingui una capacitat predictiva potencialment bona per a alguna d'aquestes indicacions ha de presentar una alta sensibilitat, és a dir, una elevada capacitat per detectar com a tal aquells individus que realment han desenvolupat la indicació en qüestió (verdaders positius), i una alta especificitat, referint-se a la capacitat de detectar correctament aquelles persones que no han patit aquella indicació determinada (verdaders negatius). Sovint, aquestes característiques no són fàcilment assumibles per a un sol indicador biològic. És per això que l'agrupació de dos o més biomarcadors en panells ha permès millorar lleugerament aquests aspectes tècnics i assolir en molts casos millors resultats que un biomarcador individual [135, 136].

Tot i que l'estudi dels biomarcadors en el context de l'ictus és relativament recent, els estudis orientats al descobriment d'aquests han experimentat un augment substancial durant els últims anys. No obstant, l'interès i els recursos emprats per a tal finalitat encara no han estat suficients per fer arribar a la pràctica clínica diària cap dels biomarcadors d'ictus proposats fins avui en dia. Aquesta manca d'èxit s'atribueix, en part, a la complexitat biològica del cervell i a la seva heterogeneïtat cel·lular, que fa que la resposta a la isquèmia de cadascuna de les poblacions cerebrals existents presenti un patró molecular específic que incrementa la variabilitat del conjunt de molècules associades a la isquèmia en global. A més, la presència de la BBB, tot i estar estructuralment alterada, pot dificultar la sortida massiva del cervell d'aquestes possibles molècules indicadores d'isquèmia, probablement tot comprometent també així la detecció d'aquestes i el seu ús com a bons biomarcadors d'ictus.

Tot i aquestes dificultats, es coneixen certs biomarcadors que durant aquests últims anys han presentat resultats força prometedors. Pel que fa al diagnòstic de l'ictus, el descobriment de biomarcadors o panells de biomarcadors potencialment valuosos per diagnosticar l'ictus i la seva subseqüent implementació a la clínica permetria identificar d'una manera immediata aquesta patologia i discriminar-la d'altres que presenten quadres clínics similars (*mímics*). A més, reduiria els trasllats urgents innecessaris als hospitals i alhora acceleraria el tractament d'aquells pacients realment diagnosticats d'ictus isquèmic. La determinació simultània del D-dímer i la caspasa 3, o el D-dímer, la MMP9, el pèptid natriurètic cerebral (BNP, Brain Natriuretic Peptide) i la S100B, per exemple, han mostrat una correcta diferenciació dels ictus, tant isquèmics com hemorràgics, front el grup dels *mímics* [136, 137]. A banda, per a la discriminació del subtipus concret d'ictus també s'han proposat biomarcadors com la proteïna gliofibrilar àcida (GFAP, glial fibrillary acidic

protein) o la S100B, ambdues proteïnes alliberades per necrosis astroglial. Aquestes presenten perfils cinètics diferents entre els pacients isquèmics i hemorràgics, de manera que es troben substancialment més elevades en l'ictus hemorràgic durant les primeres hores després de l'inici dels símptomes [138, 139]. De manera similar, nivells elevats del fragment N-terminal del pèptid precursor del BNP (NT-proBNP) han demostrat una bona capacitat discriminatòria dels pacients isquèmics front els hemorràgics [140].

A banda del diagnòstic, una correcta discriminació de l'etiologia de l'ictus permet proporcionar un tractament secundari específic per evitar, en la major mesura possible, la recurrència de futurs esdeveniments vasculars. Per exemple, quan es diagnostica un ictus d'etiologia aterotrombòtica es recomana l'ús d'agents anti-agregants de plaquetes (àcid acetilsalicílic, clopidogrel, dipiridamol), doncs disminueixen el risc de recurrència fins un 25%, mentre que aquells que pateixen un ictus d'origen cardioembòlic se'ls recepta l'ús d'anti-coagulants, que eviten la recurrència d'aquest tipus d'esdeveniments aproximadament un 50% dels casos [141]. Desafortunadament, encara avui en dia més d'un 30% dels ictus isquèmics es classifiquen com a criptogènics, principalment perquè no es detecta cap mecanisme etiològic potencial [142]. De fet, fins i tot en molts casos no s'aconsegueix identificar l'origen després de realitzar totes les proves de diagnòstic disponibles, fet que suposa un gran contratemps per al correcte tractament de prevenció secundària. En aquest context, s'espera que en funció de la naturalesa de l'ictus, el patró d'expressió proteic en circulació variï i permeti identificar biomarcadors específics capaços de discriminar l'origen de l'ictus en un percentatge elevat de casos. Fins al moment, però, només s'ha trobat que els biomarcadors sanguinis BNP i D-dímer presenten una rellevància potencialment destacada per la identificació dels ictus d'etiologia cardioembòlica [143, 144].

Pel que fa a la predicció del pronòstic i complicacions de l'ictus, també s'han plantejat diversos biomarcadors candidats al llarg de les últimes dècades. Una predicció acurada de l'evolució del pacient ajudaria a prendre decisions anticipades en el tractament i maneig dels pacients, i permetria reduir la taxa de morbiditat associada a la malaltia. D'entre totes les molècules avaluades, però, poques han demostrat la seva utilitat clínica al no aportar valor predictiu addicional important sobre les variables clíniques que s'utilitzen habitualment per a tal finalitat. Estudis recents han proposat la copeptina, una molècula derivada del pèptid precursor de la vasopressina que s'expressa en condicions d'estrès, i el BNP com a predictors en fase aguda de discapacitat o mort a llarg termini [145, 146]. Nivells elevats de MMP9 o proteïna d'adhesió vascular 1 (VAP-1; vascular adhesion protein 1) han demostrat també ser bons indicadors de transformacions hemorràgiques [68, 147], mentre que la proteïna C reactiva (CRP: C-reactive protein) i la IL-6 s'han presentat com a possibles candidats per identificar aquells pacients amb alt risc de patir infeccions

després de la isquèmia cerebral [148]. No obstant, la falta de confirmació o millora d'aquests resultats en cohorts de pacients més grans i heterogènies impedeix a hores d'ara la incorporació d'aquests biomarcadors a la pràctica clínica, fet que demostra que cal mantenir de manera intensa la cerca de candidats que cobreixin aquestes mancances actuals pel diagnòstic i pronòstic de l'ictus isquèmic.

1.5.1 Mostres biològiques

Des dels seus inicis, la recerca de biomarcadors d'ictus s'ha centrat en la identificació de marcadors biològics en **sang,** majoritàriament en forma de sèrum o plasma. La fàcil accessibilitat d'aquestes mostres i el fet que la seva obtenció sigui poc invasiva pel pacient han fet que aquest fluid biològic esdevingui l'ideal per a tal finalitat. La sang esdevé en molts moments un reflex força immediat del que succeeix en qualsevol altra part del cos, i permet identificar fàcilment alteracions moleculars que estan tenint lloc en òrgans subjacents. En el cas de l'ictus, però, aquesta capacitat de reflectir els canvis biològics que es donen a nivell local en una determinada zona del cervell a vegades està condicionada per l'existència de la BBB. Malgrat els avantatges immunològics d'aquesta barrera protectora són clars, aquesta alhora pot impedir la sortida massiva d'indicadors de processos patogènics cerebrals, fet que podria limitar o enrederir la seva detecció en sang, així com condicionar l'excel·lent ús d'aquest fluid com a eina biològica per a detectar bons biomarcadors d'ictus en fase híper-aguda. Cal tenir present, però, que molts pacients amb un ictus isquèmic pateixen un augment substancial de la permeabilitat d'aquesta BBB, o fins i tot la disrupció total d'aquesta, fet que augmenta l'intercanvi de molècules entre els dos compartiments i facilita la detecció dels mediadors cerebrals d'isquèmia en el sistema circulatori [149].
A banda de les limitacions biològiques, la sang també presenta una gran complexitat i heterogeneïtat i, juntament amb el fet que la concentració dels seus components presenta un rang dinàmic molt extens, de fins a 12 ordres de magnitud, fa que l'ús de la sang per a la identificació de nous indicadors patològics presenti sovint complicacions tècniques al ser analitzada [150].

Un altre fluid biològic utilitzat per a la identificació de biomarcadors en l'ictus és el **líquid cefaloraquidi** (CSF, cerebrospinal fluid), doncs permet identificar i estudiar molt directament aquells canvis i alteracions moleculars que es produeixen al cervell durant la isquèmia. Això es deu al contacte directe existent entre el CSF i l'espai extracel·lular del parènquima cerebral, el qual permet que gran part dels productes de degradació o rebuig que s'originen a les cèl·lules s'eliminin a través del CSF [151]. Aquesta estreta relació fa que els canvis en la concentració de determinats biomarcadors procedents de cervell

siguin generalment més pronunciats en el CSF que en la sang, donant avantatge clar a aquest fluid biològic per a ser una millor i més imminent font de biomarcadors patològics. De fet, això ja succeeix en altres malalties neurològiques, on el CSF s'utilitza per confirmar el diagnòstic clínic previ de, per exemple, la meningitis, l'encefalitis, la malaltia de Guillain-Barré, les paraneoplàsies o l'esclerosis múltiple, entre d'altres [152]. En el cas de l'ictus, el CSF també hauria d'esdevenir idealment el fluid biològic capaç de detectar amb més sensibilitat i rapidesa aquelles molècules indicadores d'una alteració cerebrovascular. No obstant, els estudis de descobriment de biomarcadors en mostres de CSF han sigut limitats, doncs l'obtenció d'aquest requereix d'una tècnica força invasiva i contraindicada per aquells pacients amb un risc elevat de sagnat [153, 154]. De fet, l'obtenció de mostres de CSF a l'arribada del pacient a l'hospital suposaria una incompatibilitat per a l'administració de rt-PA, i provocaria l'exclusió d'aquell pacient a ser candidat a rebre teràpia trombolítica.

L'**orina** també es considera una bona font de marcadors biològics en diferents patologies, inclòs l'ictus [155]. Malgrat es tracta d'un fluid molt abundant, de relativa fàcil obtenció i amb una composició molt més minimalista que la sang, la gran variabilitat que pot experimentar en funció de la dieta, els medicaments o les afectacions renals dels pacients, fa que aquest manqui d'atractiu per a ser una bona mostra on trobar biomarcadors d'ictus.

La saliva, el líquid bronco-alveolar, el líquid sinovial o les llàgrimes són exemples d'altres fluids biològics que han estat utilitzats per estudis d'identificació de marcadors en diverses malalties [156, 157]. No obstant, en el context de l'ictus aquests fluids no tenen gran interès, doncs la seva utilitat i aplicabilitat a la pràctica clínica és limitada.

A banda dels fluids biològics, les mostres de **teixit cerebral** també han esdevingut una gran font d'informació biològica. És obvi, però, que en el cas de l'ictus l'obtenció de teixit procedent del cervell *in vivo* és inviable. No obstant, l'estudi del teixit cerebral humà *post-mortem* ha suposat un bon punt de partida i ha fet més accessible l'exploració de les alteracions biològiques a nivell molecular derivades de la isquèmia. Aquest tipus de teixit, però, presenta certes limitacions que cal tenir en compte en funció de quin sigui l'objectiu de l'estudi. Com a destacable, el teixit cerebral *post-mortem* reflexa les alteracions moleculars del cervell en el moment en que el pacient traspassa, que poden allunyar-se en gran mesura dels canvis moleculars que succeeixen a fases inicials d'aquesta malaltia, quan és de major interès pel seu diagnòstic i tractament. També, cal destacar que la obtenció d'aquest tipus de mostra és sovint costosa i requereix una preservació específica i acurada per tal d'assegurar que els diferents components moleculars (majoritàriament material genètic, proteïnes i lípids) no s'alterin durant el període de conservació i

mantinguin les característiques del moment de l'èxitus durant un llarg període de temps [158]. Per a tal finalitat, doncs, és important limitar al màxim el temps transcorregut des de la mort del pacient fins a la preservació del teixit cerebral i controlar estrictament les condicions de conservació d'aquest.

Una de les alternatives a l'ús de teixit cerebral humà *post-mortem* és la utilització de teixit cerebral animal. Els models d'isquèmia en animals d'experimentació permeten reproduir la fisiopatologia humana, i alhora faciliten l'obtenció de mostres biològiques en el moment precís que es vulgui estudiar al llarg de les diferents fases de la malaltia. Malgrat aquesta estratègia també presenta limitacions (bàsicament fruit de les diferències existents entre espècies [159, 160]) cada cop són més els estudis que inicien els seus experiments amb mostres animals per després confirmar, verificar i validar els descobriments assolits en mostres humanes, ja siguin de teixit cerebral o altres fluids biològics [161–163].

1.5.2 Procés de descobriment de nous biomarcadors

La actual manca clara de biomarcadors d'ictus isquèmic, sigui per una o altra indicació, crea la necessitat de realitzar estudis orientats a la identificació de nous candidats per a tal finalitat. No obstant, el procés de descobriment de nous biomarcadors és complex i costós, i com a conseqüència sovint acaba fracassant abans d'arribar a assolir l'objectiu final. Aquest procés consta idealment de quatre fases ben caracteritzades (descobriment, qualificació, verificació i validació) que permeten reduir paulatinament el nombre de candidats potencialment vàlids com a biomarcadors i alhora augmentar substancialment la mida de les cohorts de pacients amb les que comprovar l'eficàcia d'aquests (Figura 9) [164].

La fase de descobriment sovint utilitza sistemes model (ja siguin *in vivo* o *in vitro*) o materials biològics humans per a realitzar una comparació binària entre mostres amb i sense presència del procés fisiopatològic estudiat, en molts casos a través de tècniques de cribratge massiu. Aquesta simplificació evita la contaminació dels resultats per influència d'altres processos patològics i disminueix la presència de possibles variables de confusió. Els resultats d'aquesta fase comprenen grans quantitats de components diferencialment expressats entre les dues condicions estudiades i sovint inclouen abundants falsos positius que requereixen ser filtrats i descartats en les fases posteriors del procés.

La fase de qualificació permet corroborar els resultats observats durant la fase de descobriment per mitjà de l'ús de tècniques analítiques diferents a les anteriors, en molts casos dirigides específicament als candidats en estudi. Aquesta fase, juntament amb la de descobriment, centren la seva atenció en establir una associació clara entre el candidat a

biomarcador i la malaltia estudiada, emfatitzant la sensibilitat de la molècula per sobre la seva especificitat.

La fase de verificació inclou un augment substancial de la mida de la cohort (centenars de pacients), començant a plasmar la variabilitat ambiental, genètica, biològica i estocàstica del conjunt de la població humana. En aquesta fase té lloc la confirmació de la sensibilitat del candidat a biomarcador i es comença a contemplar la seva especificitat.

Finalment, la fase de validació només es desenvolupa en un nombre reduït de candidats que han superat amb èxit la fase anterior. En aquesta fase s'avaluen els candidats per mitjà de tècniques optimitzades en grans cohorts (varis milers de pacients) que reflecteixin a la perfecció la variabilitat i heterogeneïtat del conjunt de la població. Els candidats que finalment siguin validats positivament podran ser seleccionats per a la seva comercialització, fet que implica el refinament i perfeccionament del mètode de detecció per assolir els requeriments estàndards dels tests clínics actuals.

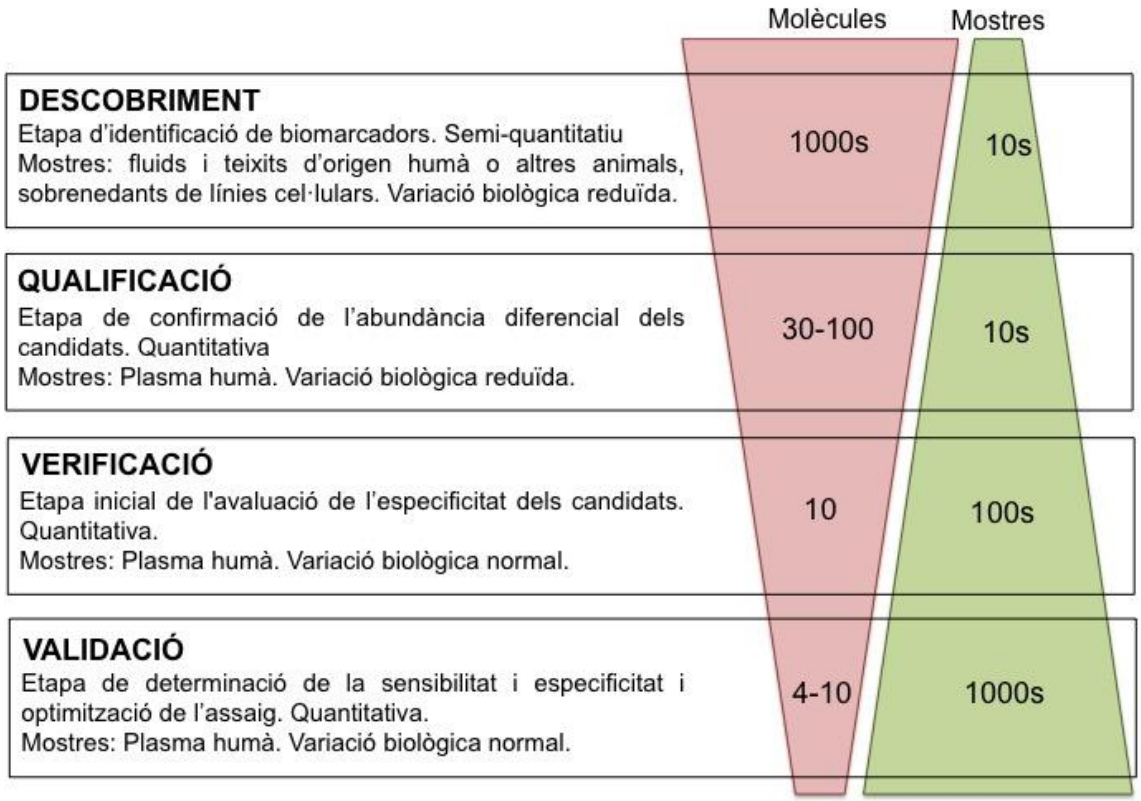


Figura 9. Procés de descobriment de nous biomarcadors (Adaptat de Rifai N, 2006) [164]. Molècules fa referència al número de candidats a ser avaluats en cada fase; Mostres fa referència al número de mostres en que s'avaluen aquests candidats.

1.6 Cerca de nous biomarcadors i/o dianes terapèutiques

L'aparició i millora de les tecnologies d'alt rendiment, conegudes generalment com a tècniques *–òmiques*, està permetent incrementar de manera massiva el coneixement de processos patològics que succeeixen en l'ictus isquèmic. Aquestes tècniques *-òmiques* faciliten específicament un cribratge simultani de nombroses molècules diferencialment alterades durant la isquèmia cerebral, tot millorant el coneixement dels mecanismes moleculars associats a aquesta patologia. Alhora, aquesta major comprensió provoca una millora substancial en el descobriment de candidats que opten a esdevenir bons biomarcadors d'ictus isquèmic, possibles dianes terapèutiques o fins i tot ambdós alhora (Figura 10).

Aquest cribratge massiu pot realitzar-se a qualsevol dels quatre nivells moleculars bàsics, a través de l'anàlisi de gens (genòmica), RNA (transcriptòmica), proteïnes (proteòmica) i metabòlits (metabolòmica). Malgrat els quatre blocs són indispensables per a la comprensió global d'un determinat procés patològic, la present Tesi Doctoral es centrarà d'ara en endavant en més detall en les estratègies de transcriptòmica i proteòmica.

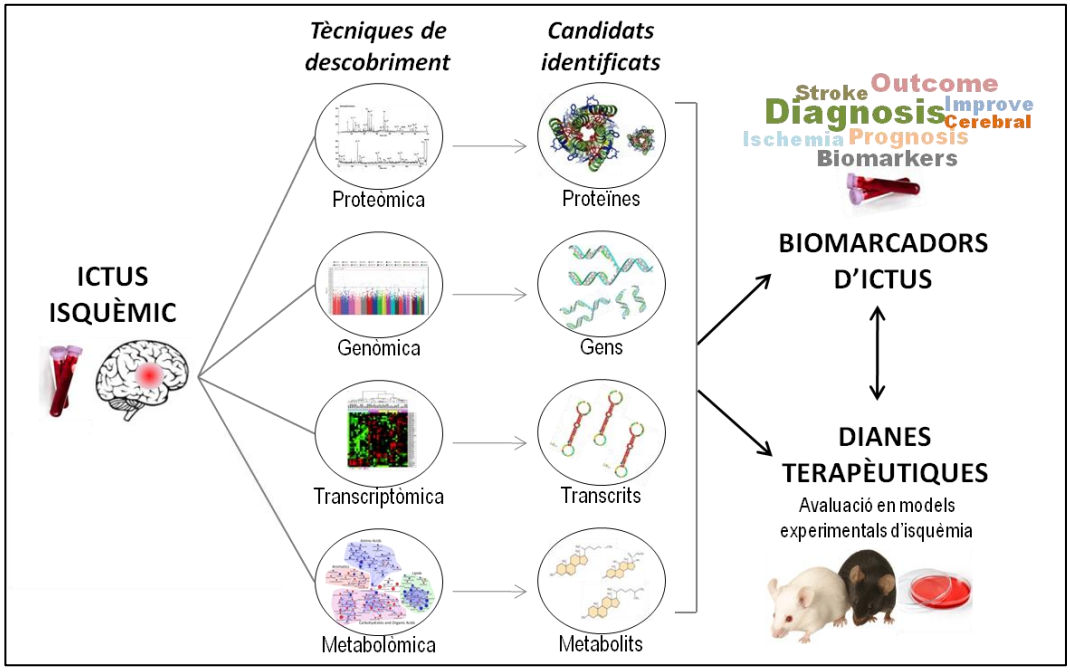


Figura 10. Procés de descobriment de biomarcadors i dianes terapèutiques de l'ictus isquèmic. Adaptat de Simats et al., 2016 [165].

1.6.1 Transcriptòmica

La transcriptòmica estudia el perfil d'expressió dels gens en un determinat moment i sota unes determinades condicions fisiològiques. Aquesta tecnologia està oferint la possibilitat

d'aconseguir informació de l'estructura i funció de bona part dels gens que conformen el genoma, contribuint alhora a la caracterització dels mecanismes involucrats en processos biològics específics.

Originàriament, els estudis d'expressió gènica estaven limitats a la detecció individual de transcrits predeterminats per mitjà de tècniques com el Northern blot o la PCR quantitativa. La millora de les tecnologies existents i el descobriment de noves tècniques, però, ha permès assolir avui en dia la detecció simultània de des de desenes fins a milers d'àcids nucleics en una mateixa mostra.

Els **microarrays** o tecnologies xip han esdevingut una de les eines principals que han permès aquesta detecció simultània i no esbiaixada de gran diversitat de transcrits [166]. Els microxips destaquen per la utilització de sondes específiques dissenyades per a detectar, hibridar i permetre la quantificació de bona part dels transcrits d'una mostra prèviament amplificada sobre una única plataforma de mida de l'ordre dels micròmetres. Els avenços en aquesta tecnologia d'hibridació l'han feta fàcil, ràpida i econòmicament accessible, i han donat peu a una gran quantitat d'estudis de transcriptòmica, també en el camp de l'ictus [167, 168].

A banda dels estudis de microarrays, les estratègies de **seqüenciació d'RNA**, (NGS: next generation sequencing), han estat les altres grans responsables de la massiva expansió que ha experimentat el camp de la transcriptòmica en els darrers anys [169]. Un dels avantatges clars d'aquestes noves tècniques és la possibilitat d'identificar nous transcrits que no hagin estat prèviament descrits, fet que contribueix altament en la caracterització del procés biològic que s'estudia i, indirectament, contribueix a millorar i optimitzar les estratègies que es basen en hibridacions amb sondes prèviament descrites.

No obstant, nous mètodes estan sorgint per complementar i refinar aquestes estratègies de transcriptòmica més massives. En primer lloc, la PCR quantitativa s'ha aconseguit multiplexar, de manera que per mitjà de marcatges específics per a cadascuna de les sondes que s'utilitzen, sovint a través de la fluorescència, és factible la detecció i quantificació dels nivells d'expressió de varis gens simultàniament en una mateixa mostra [170]. També, de manera destacable, algunes d'aquestes tecnologies emergents es basen en la detecció directa de RNA per mitjà de microesferes magnètiques (com per exemple els assajos de QuantiGene™ basats en la tècnica Luminex®, de la casa ThermoFisher) o codis de colors (com la tècnica del Nanostring®, www.nanostring.com) [171]. A grans trets, aquestes tècniques faciliten la quantificació específica de cada molècula de transcrit de RNA de manera directa, ja que no utilitzen enzims de retrotranscripció. A més de facilitar el procés i proporcionar una quantificació més real, aquestes tècniques són aplicables a l'anàlisi de mostres difícils de processar o amb mètodes de conservació menys eficients,

com per exemple, mostres tissulars fixades amb formalina i submergides en parafina (principal mètode de conservació a nivell dels biobancs cerebrals) o fins i tot mostres que resten emmagatzemades des de fa molts anys [166]. Cal tenir present, però, que la identificació dels transcrits mitjançant aquestes tècniques emergents està condicionada a l'existència de sondes específiques conegudes. En aquest mateix sentit, es limita també la identificació dels candidats a la qualitat (afinitat i especificitat) de les sondes disponibles en cada cas. Es consideren, doncs, bones estratègies per validar l'estat de múltiples transcrits candidats que han sigut prèviament caracteritzats per altres tècniques de transcriptòmica.

En el camp dels biomarcadors d'ictus les estratègies de transcriptòmica ja han aportat alguns candidats plausibles. Un dels estudis pioners en aquest camp va proposar un panell complex de diversos gens l'expressió circulant dels quals va demostrar tenir capacitat discriminatòria entre els pacients que havien patit o no un ictus isquèmic [172, 173]. L'aplicació d'aquest panell, però, quedava força lluny del seu ús com a eina de diagnòstic en la pràctica clínica, doncs utilitzava dades d'expressions combinades generades a partir de tres extraccions de sang obtingudes durant les primeres 24h d'hospitalització. No obstant, l'estudi va resultar ser una demostració conceptual de que els canvis transcripcionals induïts per l'ictus poden servir per a la identificació d'aquesta patologia amb relativa precisió, i va ser el desencadenant de molts estudis posteriors en aquest àmbit. Recentment, a través de microarrays d'expressió gènica també en mostres de plasma s'ha descobert, per exemple, que l'expressió individual del gen *MCEMP1* (*Mast cell–expressed membrane protein 1*) en circulació presenta un cert potencial per a diagnosticar els ictus, tant isquèmics com hemorràgics, així com per a pronosticar l'evolució dels pacients que n'han patit un [174]. També, partint de mostres de cervell *post-mortem* de pacients amb ictus hemorràgic, s'ha identificat un increment de la interleucina 8 (IL-8), tant a nivell de transcrit com proteic en la zona cerebral circumdant a l'hematoma, i s'ha provat el rol com a biomarcador diagnòstic d'ictus hemorràgic de la IL-8 a nivell circulatori [175].

1.6.2 Proteòmica

De totes les tècniques *–òmiques*, la proteòmica és probablement la que ha experimentat un major desenvolupament durant aquests últims anys, fins al punt de plantejar-se assolir la caracterització completa del proteoma humà amb el projecte internacional *Human Proteome Project* (http://www.thehpp.org), anàleg del *Human Genome Project*. Aquest té com a finalitat principal assolir el mapa proteic complet de l'ésser humà amb l'objectiu de

millorar el coneixement i comprensió de la biologia humana a nivell cel·lular i assentar les bases moleculars per al desenvolupament de noves aplicacions mèdiques de diagnòstic, pronòstic, terapèutiques i preventives.

El desenvolupament de les noves tècniques d'identificació i detecció de proteïnes ha permès passar de la quantificació individual de proteïnes a la quantificació exacta i precisa de centenars o milers d'aquestes de manera simultània, gràcies a la optimització i millora de les tècniques proteòmiques ja existents i al desenvolupament de noves eines tecnològiques per a dur a terme tal finalitat.

Les matrius d'afinitat basades en l'ús d'**anticossos** han contribuït àmpliament al coneixement del proteoma. La optimització del procés de producció i l'afinitat i especificitat que aquests presenten, són dues característiques que han fet dels anticossos unes eines força utilitzades en la ciència en general. D'entre les diferents opcions possibles, destaquen els panells o matrius d'anticossos, doncs permeten detectar fins a centenars de proteïnes simultàniament en una mateixa mostra. Alguns exemples d'aquests panells són la antiga matriu de múltiplex SearchLight®, o les matrius basades en la tecnologia Luminex® o Quantibody® (de RayBiotech), totes derivades de la tècnica d'assaig per immuno-adsorció lligat a enzims (ELISA: Enzyme-Linked ImmunoSorbent Assay) [176]. També s'han desenvolupat noves tècniques mixtes que utilitzen anticossos units a sondes de DNA que, sota determinades condicions i per mitjà de l'amplificació d'aquestes seqüències aconsegueixen una quantificació molt més acurada i sensible que els anticossos simples (per exemple, l'assaig d'extensió per proximitat (PEA: proximity extension assay), d'Olink proteomics, www.olink.com).

Més recentment, però, l'aparició d'alternatives a aquests anticossos ha suposat un reforç considerable per a l'estudi del proteoma. En concret, cal destacar la tecnologia basada en **aptàmers**, petites cadenes senzilles d'àcid nucleic que per mitjà de la seva estructura tridimensional reconeixen i detecten proteïnes amb una alta afinitat i especificitat [177]. Aquesta tecnologia ha permès millorar certes limitacions dels anticossos com són els costs i processos de producció, l'estabilitat, i la possibilitat de detectar molècules que per mitjà d'anticossos resulta complexa, doncs presenten certa toxicitat o no són immunogèniques (Taula 5).

Aquestes dues tècniques estan condicionades a l'existència de lligands específics (anticossos o àcids nucleics) per cada proteïna en concret, fet que restringeix també el descobriment de noves molècules i limita la identificació dels candidats a la qualitat (afinitat i especificitat) dels lligands disponibles en cada cas [178].

A banda dels anticossos i aptàmers, la continua millora de **l'espectrometria de masses** (MS) i els seus instruments ha permès consolidar-la com una de les tècniques fonamentals per la proteòmica. La identificació i quantificació de proteïnes per MS requereix una digestió enzimàtica prèvia per tal de fragmentar les proteïnes de la mostra en els seus respectius pèptids. En determinats casos, aquesta digestió pot inclús intensificar-se per mitjà d'estratègies de fraccionament anteriors a aquest procés enzimàtic. Un cop digerida la mostra, els pèptids resultants són injectats paulatinament a l'espectròmetre de masses a través d'un cromatògraf líquid acoblat (LC-MS), que permet separar encara més els pèptids en funció de les seves característiques químiques (ja sigui la mida dels pèptids, la seva càrrega elèctrica o l'afinitat a la matriu del cromatògraf, entre d'altres) [179]. Finalment, els pèptids es detecten a l'espectròmetre en funció de la seva relació massa-càrrega (m/z) [179].

	Anticossos	**Aptàmers**
Temps de desenvolupament	~4-6 mesos	~1-3 mesos
Procés de desenvolupament	La producció inicial requereix una resposta immunitària en un model animal	Enriquiment d'una llibreria d'oligonucleòtids a través del procés SELEX
Mida	150-170 kDa (IgG)	12-30 kDa (30-80 nucleòtids)
Concentració òptima de treball	Depèn de l'aplicació	De 5 a 10 vegades inferior que els anticossos en determinades aplicacions
Mida mínima de la diana	600 Daltons	60 Daltons
Procés de fabricació	Producció *in vivo* o *in vitro*	Síntesis química
Modificació	Els anticossos poden conjugar-se amb un sol tipus de molècula de senyalització o unió	Els aptàmers poden conjugar-se a l'extrem 3' i al 5'
Estabilitat	Els anticossos són susceptibles a les altes temperatures i canvis de pH. Quan es desnaturalitzen no poden reparar-se	Els aptàmers són estables a temperatura ambient i si es desnaturalitzen poden reparar-se
Disponibilitat a llarg termini	Necessitat de mantenir la població cel·lular congelada per a la producció d'anticossos	Només es necessita conèixer la seqüencia de nucleòtids per a la producció d'aptàmers

Taula 5. Diferències principals entre anticossos i aptàmers (Adaptat de BasePair Biotechnologies Inc., www.basepairbio.com). Abreviacions: SELEX: evolució sistemàtica dels lligands per enriquiment exponencial; IgG: immunoglobulines; kDa: quilo daltons.

La MS ofereix la possibilitat de quantificar proteïnes per mitjà de diferents estratègies de marcatge, ja sigui de forma biosintètica (com la tècnica SILAC [Stable Isotope Labeling with Aminoacids in Cell culture]) [180] o a través de la derivatització química sobre l'extracte de proteïnes (mitjançant les tècniques iCAT [Isotope Coded Affinity Tag], iTRAQ

[Isobaric Tag for Relative and Absolute Quantification] o TMT [Tandem Mass Tags]) [181–183]. Avui en dia, però, l'evolució dels instruments de MS cap a una major sensibilitat i resolució ha permès també la consolidació de la tècnica de MS sense la utilització d'aquests marcatges (anomenada també MS *label-free*), que sovint poden aportar errors derivats del processament i marcatge previ de les mostres [184].

En el cas de les tècniques de *label-free*, l'LC-MS permet la quantificació final dels pèptids per mitjà de la mesura de les intensitats dels senyals dels pèptids precursors que es detecten a l'espectre o segons el comptatge total d'espectres de cada pèptid [185]. La primera opció permet quantificar els pèptids a través de la mesura de l'àrea sota el pic (AUC: area under the curve), que és directament proporcional a la quantitat de pèptid present a la mostra analitzada [186]. La segona estratègia, en canvi, es basa en el fet que com més abundant és un pèptid, major nombre d'espectres presenta, que alhora també és proporcional a l'abundància de la proteïna total [187]. Ambdues estratègies són comunament acceptades i l'elecció d'una o altra es basa majoritàriament en el tipus i característiques de l'experiment que es pretén dur a terme. També cal tenir en compte que ambdues aproximacions poden presentar certes limitacions; la quantificació basada en l'AUC pot resultar complexa quan dos pèptids co-elueixen conjuntament a la cromatografia líquida i presenten una mateixa relació m/z, mentre que la quantificació per comptatge total d'espectres es complica si pèptids de la mateixa proteïna presenten propietats fisicoquímiques diferents, doncs introdueixen variabilitat i biaix en els mesuraments per LC-MS [186].

Malgrat l'LC-MS sense marcatge es tracta d'una estratègia revolucionària que presenta una gran precisió i sensibilitat a l'hora de detectar pèptids, segueix sent una aproximació semi-quantitativa, i requereix una validació addicional dels resultats obtinguts per mitjà d'altres tècniques. Per a tal confirmació, les tècniques més utilitzades segueixen sent aquelles enfocades a la immunodetecció dels candidats, majoritàriament per Western blot o ELISA. No obstant, cada cop s'estan imposant amb més força aquelles estratègies també basades en MS que permeten una quantificació més exhaustiva de les proteïnes prèviament seleccionades en una fase inicial. Una d'aquestes estratègies és la proteòmica dirigida, anomenada de monitoratge d'una reacció paral·lela (PRM: Parallel Reaction Monitoring). Aquesta tècnica permet la identificació i quantificació de proteïnes per MS d'una manera més personalitzada, focalitzada, i eficaç [188]. A grans trets, l'SRM es basa en la quantificació precisa de pèptids determinats a través de la seva quantificació relativa respecte un estàndard intern de concentració coneguda prèviament afegit a la mostra [189]. Aquesta estratègia aporta més sensibilitat que les tradicionals tècniques d'MS i permet una quantificació independent de l'ordre de magnitud dels pèptids en un nombre elevat de mostres biològiques. A més, permet multiplexar fins a vàries desenes de pèptids

conjuntament, de manera que facilita un anàlisi més detallat de les mostres i redueix substancialment el temps d'adquisició dels resultats. És per tot això que es creu que l'PRM pot suposar una millora a l'actual procés de validació dels nous descobriments i esdevenir un pas intermedi entre la identificació inicial de noves molècules, que es duu a terme en un nombre reduït de mostres, i la seva validació en grans cohorts, que en el millor dels casos assoleix el miler de pacients [189].

Les estratègies de proteòmica també han permès ja el descobriment de varis candidats que opten a esdevenir bons biomarcadors en l'àmbit de l'ictus. En mostres de cervell humà *post-mortem* analitzades per espectrometria de masses (MS) també s'ha pogut identificar un panell de tres proteïnes (gelsolina (Gels), cistatina A (CysA) i proteïna 2 relacionada amb la dihidropirimidinasa (Drp2)) que, mesurades de manera aguda en mostres sanguínies, presenten cert potencial per a predir el pronòstic funcional a llarg termini dels pacients que han patit un ictus isquèmic [190]. Per altra banda, el descobriment de la proteïna d'unió als àcids grassos (FABP; fatty acid-binidng protein) en mostres *post-mortem* de CSF mitjançant MS, va permetre assajar-la com a potencial biomarcador diagnòstic d'ictus en circulació durant la fase aguda de la malaltia [191]. Altres biomarcadors han sigut identificats directament en mostres de plasma, bé per MS (on la combinació de les Apolipoproteïnes C III i I permetent discriminar els ictus isquèmics dels hemorràgics [192, 193]) o amb tècniques d'immunoassaig (amb la identificació de MMP9 com a predictor de TH relacionada amb l'administració de rt-PA [194]).

1.6.3 Integròmica

Degut als avenços substancials de les tècniques *–òmiques*, les quals permeten generar ràpida i eficaçment grans quantitats de dades, el processament i interpretació biològica dels resultats s'ha convertit en el factor limitant en la majoria d'estudis i investigacions. La informació que s'obté de cadascuna de les tècniques *-òmiques* de manera individual es considera merament una manera de catalogar els elements moleculars que participen en una determinada acció o procés. La comprensió global del sistema que aquests conformen, però, requereix la integració de múltiples conjunts de dades, que alhora permeten descriure més amplia i profundament les relacions entre els diferents components integrants del sistema en estudi [195] (Figura 11).

La interpretació global d'aquestes connexions es coneix com a **biologia de sistemes**, disciplina que es pot definir comunament com a l'estudi d'un organisme o sistema vist com una xarxa integrada i interactiva dels gens, proteïnes, metabòlits i reaccions bioquímiques

que li confereixen la vida [196]. Així, la biologia de sistemes intenta modelitzar aquests organismes a través d'eines matemàtiques i racionals que posen de manifest les interaccions entre els diferents elements que els formen, ja que en última instància aquestes esdevenen les responsables de la complexitat morfològica i funcional de tots els sistemes biològics complexos.

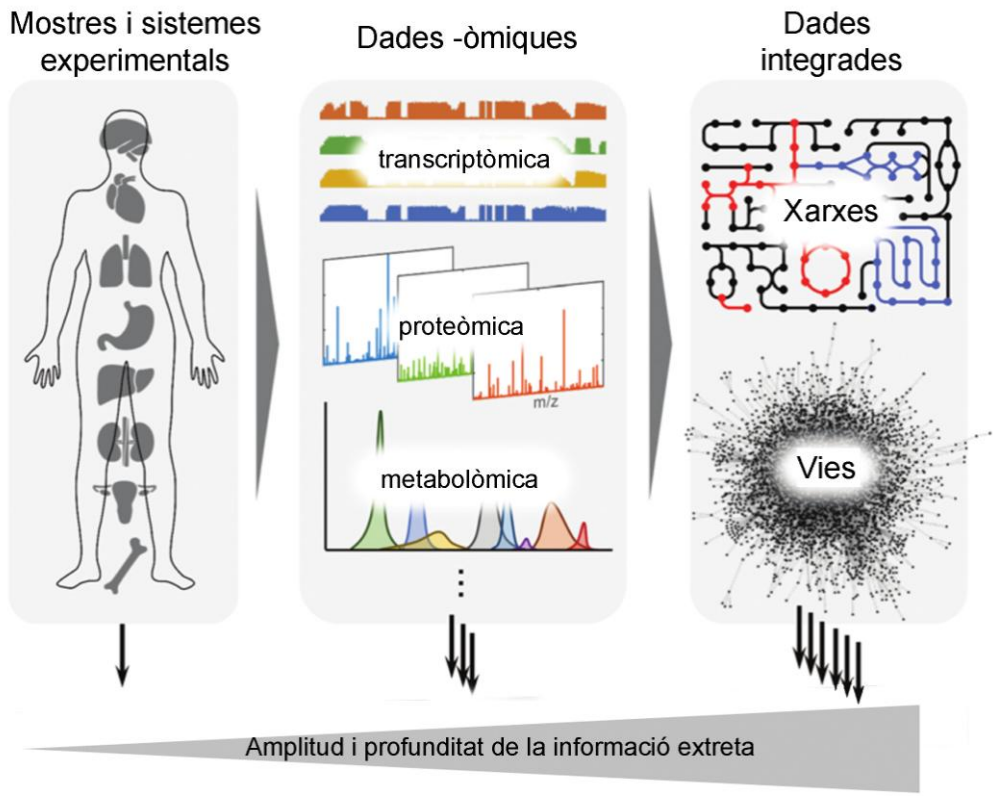


Figura 11. Integració de dades *-òmiques*. D'esquerra a dreta els panells representen l'increment de l'amplitud i la profunditat de la informació que pot ser extreta de cada conjunt de dades (Adaptat de Robinson et al., 2016) [197].

D'eines matemàtiques per a tal finalitat se'n coneixen una gran varietat, que oscil·len des d'aproximacions estadístiques que minimitzen la complexitat dels sistemes i en faciliten la seva comprensió, fins a models matemàtics molt més elaborats i costosos que emulen més precisament el comportament fisiològic de l'organisme o sistema.

D'aproximacions estadístiques també se'n coneixen diverses. Matemàticament, aquestes es basen en (I) l'agrupació de les dades en subconjunts o *clusters* en funció de les característiques comunes que aquestes presenten o en (II) la descomposició de les dades en un nombre reduït de variables, anomenades components, per tal d'obtenir una representació bidimensional o tridimensional d'aquestes que permet explicar les diferències observades d'una manera reduccionista [198, 199]. Malgrat ambdues

estratègies són vàlides, el mètode reduccionista té actualment més cabuda en el món de la integració de dades –*òmiques* ja que simplifica la complexitat de les dades i dóna una visió global simplificada del seu conjunt. Alguns exemples de mètodes que permeten aquesta projecció de la informació resultant en un nombre reduït de dimensions són l'anàlisi de components principals (PCA: principal component analysis), l'anàlisi de correlacions canòniques (CCA, canonical correlation analysis) o l'anàlisi de co-inèrcia (CoA, co-intertia analysis), ambdós últims essent útils quan es pretenen combinar dos o més conjunts de dades simultàniament, entre molts d'altres [198].

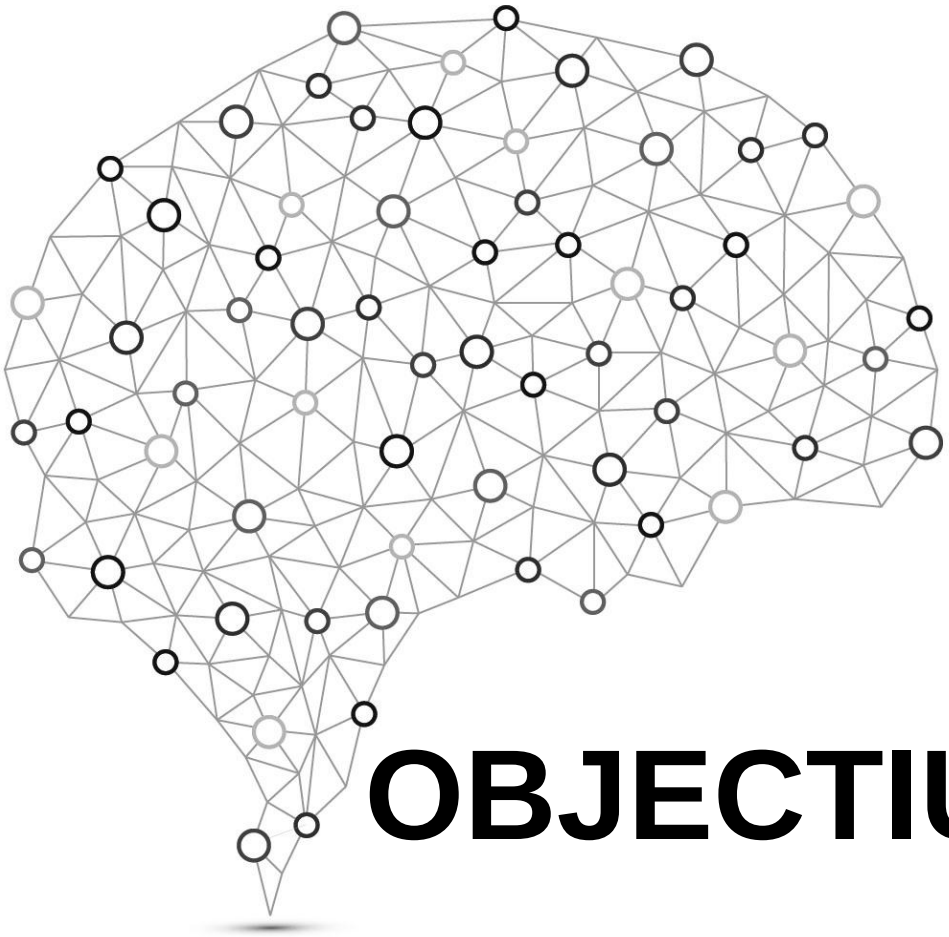
Paral·lelament a aquestes aproximacions estadístiques, també es coneixen els anàlisis basats en la funcionalitat de les molècules [200]. Aquests anàlisis tenen com a finalitat principal identificar associacions potencials del conjunt de molècules en estudi (majoritàriament gens i proteïnes) amb vies, processos biològics i funcions moleculars prèviament descrites a la literatura. Moltes d'aquestes eines obtenen la informació de grans bases de dades públiques, com la del consorci de la *Gene Ontology* (GO database), la de l'enciclopèdia de gens i genomes de Kyoto (KEGG: Kyoto Encyclopedia of Genes and Genomes) o l'anomenada REACTOME (REACTOME pathway database), entre d'altres.

Totes aquestes eines estadístiques i bioinformàtiques, doncs, representen un bon punt de partida per la comprensió de les relacions entre proteïnes, gens i metabòlits i la seva funcionalitat sota una determinada condició, fet que ja ha permès una millor caracterització de molts processos patològics desconeguts [201]. El seu ús dins el món dels estudis basats en tècniques –*òmiques* està creixent exponencialment, i es creu que podran millorar la comprensió conjunta de molts processos que fins ara només es contemplaven des d'un sol nivell molecular.

Més enllà d'aquestes aproximacions, es coneixen altres mètodes que permeten la creació de models matemàtics més complexos que simulen *in silico* el comportament global de la fisiologia humana. La majoria d'aquests models no només es formen a partir de dades procedents d'experiments amb tecnologies –*òmiques* sinó que també s'alimenten en gran mesura del coneixement bibliogràfic previ sobre la malaltia o procés en qüestió. A més, aquests models adquireixen aquesta major complexitat a través d'estratègies d'intel·ligència artificial, també conegudes com aprenentatge automàtic (de l'anglès, *machine learning*) [202]. L'aplicació d'aquestes eines complexes és una estratègia recent, innovadora i amb gran potencial per desenvolupar-se dins el camp de la biologia de sistemes aplicada. Aquests models matemàtics faciliten la identificació dels components o xarxes de components claus per al desenvolupament de la malaltia en qüestió, així com determinar quines vies poden esdevenir importants per a modular la progressió de la patologia i aturar-ne el seu desencadenament [203]. A banda d'aquesta millor

caracterització molecular, un dels usos més coneguts d'aquestes eines d'intel·ligència artifical és el reposicionament de fàrmacs (de l'anglès, *drug repositioning*) [204]. Aquesta estratègia ja ha aportat avui en dia nous candidats potencialment valuosos per a tractar patologies neuronals, com per exemple l'esclerosis lateral amiotròfica o el dany de les terminacions nervioses perifèriques [205, 206]. També destaca l'ús d'aquests models matemàtics intel·ligents per a facilitar la identificació de nous biomarcadors, l'activitat o expressió diferencial dels quals està associada a un estat patològic segons el model matemàtic prèviament configurat [207].

Malgrat tot aquest ventall de models bioestadístics i bioinformàtics, tant els més bàsics com els que presenten gran complexitat matemàtica, hagin esdevingut de gran utilitat per a millorar la comprensió dels sistemes biològics en conjunt, encara hi ha camí per recórrer i és de vital importància seguir desenvolupant noves estratègies que facilitin en un futur proper una total comprensió de la patologia humana de l'ictus en el seu conjunt, que alhora permeti millorar la seva identificació i les estratègies terapèutiques per combatre-la.



OBJECTIUS

Objectius de la Tesi Doctoral

- Explorar en detall el paper de la inflamació com a procés biològic clau en el desenvolupament de l'ictus isquèmic, des de la seva aplicació per al diagnòstic i pronòstic de l'ictus fins al seu possible rol com a diana terapèutica.

- Estudiar el rol terapèutic d'un inhibidor de la proteïna d'adhesió vascular 1 [VAP-1/SSAO], un conegut biomarcador del pronòstic de l'ictus, en un model d'isquèmia cerebral en rata.

- Estudiar el paper dual com a biomarcador i diana terapèutica de la quimiocina CCL23 com a molècula candidata de la via de la inflamació.

- Descriure el proteoma del líquid cefaloraquidi en la fase aguda de l'ictus en un model experimental d'isquèmia cerebral en rata per tal d'identificar nous biomarcadors de diagnòstic i/o dianes terapèutiques de l'ictus isquèmic.

- Descriure les alteracions del proteoma i transcriptoma del cervell isquèmic integrant les dades a través d'eines bioestadístiques amb el fi d'identificar possibles candidats a nous biomarcadors i/o dianes terapèutiques de l'ictus isquèmic.

- Identificar noves combinacions de tractaments que presentin efecte neuroprotector sinèrgic per mitjà d'estratègies integratives de reposicionament de fàrmacs, i avaluar experimentalment el seu efecte protector en un model d'isquèmia cerebral en ratolí.

RESULTATS

Neuroinflammatory biomarkers: From stroke diagnosis and prognosis to therapy

Neuroinflammatory biomarkers: From stroke diagnosis and prognosis to therapy

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Abstract

Stroke is the third leading cause of death in industrialized countries and one of the largest causes of permanent disability worldwide. Therapeutic options to fight stroke are still limited and the only approved drug is tissue-plasminogen activator (tPA) and/or mechanical thrombectomy. Post-stroke inflammation is well known to contribute to the expansion of the ischemic lesion, whereas its resolution stimulates tissue repair and neuroregeneration processes. As inflammation highly influences susceptibility of stroke patients to overcome the disease, there is an increasing need to develop new diagnostic, prognostic and therapeutic strategies for post-stroke inflammation. This review provides a brief overview of the contribution of the inflammatory mechanisms to the pathophysiology of stroke. It specially focuses on the role of inflammatory biomarkers to help predicting stroke patients' outcome since some of those biomarkers might turn out to be targets to be therapeutically altered overcoming the urgent need for the identification of potent drugs to modulate stroke-associated inflammation.

Review article

Keywords: Stroke, cerebral ischemia, inflammation, biomarkers, therapeutic targets

1. Introduction

Stroke produces more than 9% of all deaths and is one of the leading causes of permanent disability worldwide. The most common cause of stroke is the occlusion of blood circulation by a thrombus (or embolism) although it can also be produced by the rupture of a vessel and subsequent bleeding in a certain region of the brain. Strokes can, therefore, be classified as ischemic or hemorrhagic. Ischemic strokes represent between 80% and 85% of all stroke cases, while hemorrhagic strokes are around 15%.

Despite many years of intensive research, therapeutic options for stroke patients remain very limited. Thrombolytic treatment with tissue Plasminogen Activator (tPA) is still the only Food and Drug Administration (FDA)-approved therapy for treating hyperacute ischemic strokes, together with mechanical thrombectomy, whereas no treatment is routinely used to overcome hemorrhagic strokes. tPA improves functional outcome and reduces neurological deficits in stroke patients. However, due to risks of hemorrhagic transformation, tPA can only be administrated within the first 4.5h of ischemic stroke onset, reducing by up to 10% the number of patients that qualify for this treatment [1]. This therapeutic window has been slightly extended with the new endovascular approaches supported by several positive trials published this year (MR CLEAN, EXTEND-IA, ESCAPE, SWIFT PRIME, REVASCAT, etc.). However, most stroke

patients only receive supportive care, which underscores the need of new therapeutic agents to fight stroke.

Although stroke is a highly complex disease, inflammation is known to be a major contributor to stroke pathophysiology [2]. During a stroke, cerebral brain injury evokes a massive upregulation of the inflammatory response. However, whether this response has beneficial or detrimental effects has long been the subject of controversy, with agreement still remaining elusive. Hence, the immune system is considered a promising tool to modulate brain damage during and after stroke. This review provides a brief overview of the pathophysiology of ischemic stroke, focusing attention on the underlying inflammatory process and its association with the discovery of stroke biomarkers and the possibility that these biomarkers might not only become diagnostic tools but may even turn out to be targets to be therapeutically blocked or stimulated in order to modulate the inflammatory response after the ischemic challenge.

2. Neuroinflammation in stroke: a dual role

During stroke, the lack of cerebral blood flow in the ischemic core causes a complete reduction of oxygen and glucose supply to cerebral neurons and other supporting cells. The absence of these vital fuels induces a series of biochemical and metabolic alterations that finally lead to massive cell death [3]. The area surrounding the dying core is known as the ischemic penumbra, a region with still viable cerebral tissue. Cells within this zone are functionally impaired but structurally intact, which makes that region potentially salvageable and suitable for therapeutic interventions. However, if oxygen and glucose supplies are not restored in this surrounding area, neuronal apoptosis processes are initiated, which results in a reduction of the salvageable tissue and an increase in the ischemic lesion.

Dying cells from the ischemic area release damage signals, commonly known as brain-released alarmins, which are recognized by the microglia [4]. Under immunological stimuli, these resident immune cells become activated and act as relevant players in the immune system's response together with peripheral leukocytes, which infiltrate the brain from circulation within a short period of time [5]. Both local and peripheral immune cells consequently produce an explosion of pro-inflammatory mediators surrounding the ischemic region, which further increase the permeability of the blood-brain barrier (BBB) and facilitate the infiltration of beneficial leukocytes to clear away the large amount of debris caused by cell death. To that end, pro-inflammatory cytokines act on endothelial cells, upregulating the expression of leukocyte adhesion molecules, and stimulating the synthesis of chemokines, which guide leukocytes to the site of injury. Here also the complement system has added importance as a player in inflammation, specifically in leukotaxis [6]. Several components derived from the cascade-like activation of the complement system act as opsonins (C3b, C4b, C5b), enhancing the inflammatory phagocytic response, while other components (C6, C7, C8, C9, C5b) form a membrane attack complex involved in cellular lysis. This cellular damage is mediated by the increased expression of complement cascade activators (C1q, C3) and receptors by dying neurons and glial cells during the acute phase of cerebral ischemia [7].

Nonetheless, there is a growing body of evidence reporting that infiltrating immune cells also impair the ischemic brain. Infiltrated leukocytes produce inflammatory cytotoxic mediators that prolong the inflammatory response, increase brain damage and contribute to edema formation and hemorrhagic transformation, secondary complications that commonly influence stroke outcome [2]. The presence of these inflammatory mediators during the acute phase of stroke can, thus, be a threat to neuronal cell survival and repair.
Because of this inflammatory collateral damage, many attempts have been made to improve stroke outcome by using strategies that suppress the immunologic response after stroke.

Regulatory T cells (T_{reg}) and B cells (B_{reg}) are two subpopulations of lymphocytes that act as important neuroprotective modulators of the immune response under many pathological conditions such as cerebral ischemia [8]. T_{reg} and B_{reg} have been characterized as stroke-limiting protective cells that preserve the immune homeostasis by counteracting the production of pro-inflammatory mediators and modulating the activation of effector lymphocytes and microglia in the ischemic region. However, despite the advances in the understanding of the function of T_{reg} and B_{reg}, further research should be conducted in order to assess whether their modulation might have application as a novel therapeutic approach for this devastating disease.

Furthermore, it is widely acknowledged that in severe strokes the extension of brain lesion highly correlates with the strength of the neuroinflammatory reaction. In extreme cases, the massive burst of circulating pro-inflammatory mediators is frequently excessive, which causes the ineffectiveness of the immune system to respond to secondary pathological stimuli elsewhere [9]. The disproportionate over-activation of the peripheral immune cells culminates in the exhaustion of mature leukocytes, a fact that causes the need to recruit immature leukocytes instead. This subpopulation is, however, unable to respond appropriately to brain injury, which leads to the deregulation of the immunological signaling pathways. This is the case for monocytes, where the recruitment and expansion of their immature subpopulation causes lymphocytopenia, a condition that significantly contributes to post-stroke immunosuppression [4].

In addition, the excessive concentration of pro-inflammatory mediators can promote the release of glucocorticoids and catecholamines by the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. This results in the stimulation of anti-inflammatory pathways, through the release of interleukin (IL)-4, IL-10 and transforming growth factor (TGF)-β, and the inhibition of pro-inflammatory mechanisms, which include the decreased expression of IL-1β, tumor necrosis factor (TNF)-α, several chemokines and several proteins involved in antigen-presentation processes [10]. The rapid and inappropriate activation of these mechanisms also contributes to stroke-related immunosuppression, which enhances the risk of infection after cerebral ischemia. In line with this, 23% to 65% of stroke patients develop infections, predominantly respiratory, urinary and catheter-related infections, after stroke onset [11]. Moreover, patients that develop post-stroke infections are thought to suffer worse outcomes with an increased mortality risk.

Beyond these detrimental consequences and side effects, the immune system also works to resolve this post-ischemic inflammation at later stages. The production of anti-inflammatory mediators and the removal of the remaining inflammatory molecules are major points in the mechanisms of inflammation suppression, which takes place after the initial burst of inflammation. During this period, the infiltrating pro-inflammatory macrophages (M1 macrophages) turn into anti-inflammatory (M2 macrophages) when stimulated by IL-4, IL-10 and TGF-β, among others [12]. These M2 macrophages antagonize the inflammatory response through the clearance of residual necrotic debris and the release of neuroprotective factors, including insulin-like growth factor and fibroblast growth factor, both participating in the recovery of ischemic brain injury by promoting neuroregeneration [13]. However, the mechanisms through which pro-inflammatory macrophages shift to an anti-inflammatory state during the recovery phase of stroke are not fully understood.

Due to this dual function of the immune system (Figure 1), the inflammatory response after ischemia requires close control during stroke progression and resolution: permitting leukocyte infiltration to clear cell debris in the acute phase, avoiding harmful effects produced by either the accumulation of these circulating cells into the brain and their suppression at the peripheral level, and promoting their regenerative benefits some days later. Adjustments at this level seem promising as new therapies for stroke.

3. Brain-blood relationship

As the brain is an extremely high-demand system and neurons need tight regulation of the extracellular microenvironment, the integrity of the BBB is essential. Under physiological conditions, the BBB permits the maintenance of ion concentrations within very narrow ranges, allows the entry of nutritional supplies to brain cells and avoids the inflow of neurotoxic and harmful molecules [14]. Moreover, the BBB regulates and restricts the access of immune cells and immune mediators to the brain compartment, which provides privileged brain protection against peripheral insults. Under physiological conditions, leukocyte recruitment across the BBB is responsible for the maintenance of the immunologic privilege of the central nervous system (CNS). However, under pathological conditions, there is a reduction of the BBB tightness and an increase in the barrier leakiness (process referred as *BBB disruption*), which increases the BBB permeability, alters the natural immunological isolation of the brain and allows massive immune cell infiltration to aid the clearing of debris and repair of damage. In addition, this disruption of the BBB also contributes to a massive and uncontrolled exchange of molecules between the brain and the peripheral circulatory system and vice versa. The release of brain specific molecules into the peripheral circulation alerts the immune system to the presence of a damaged brain area and contributes to increasing the inflammatory response to the ischemic challenge.

The extent of this BBB rupture is determined by the severity and duration of stroke: moderate BBB leakage can be partly reversible, while a severe breakdown of the BBB leads to a massive entry of circulating immune cells that culminate in irrevocable deregulation of BBB permeability.

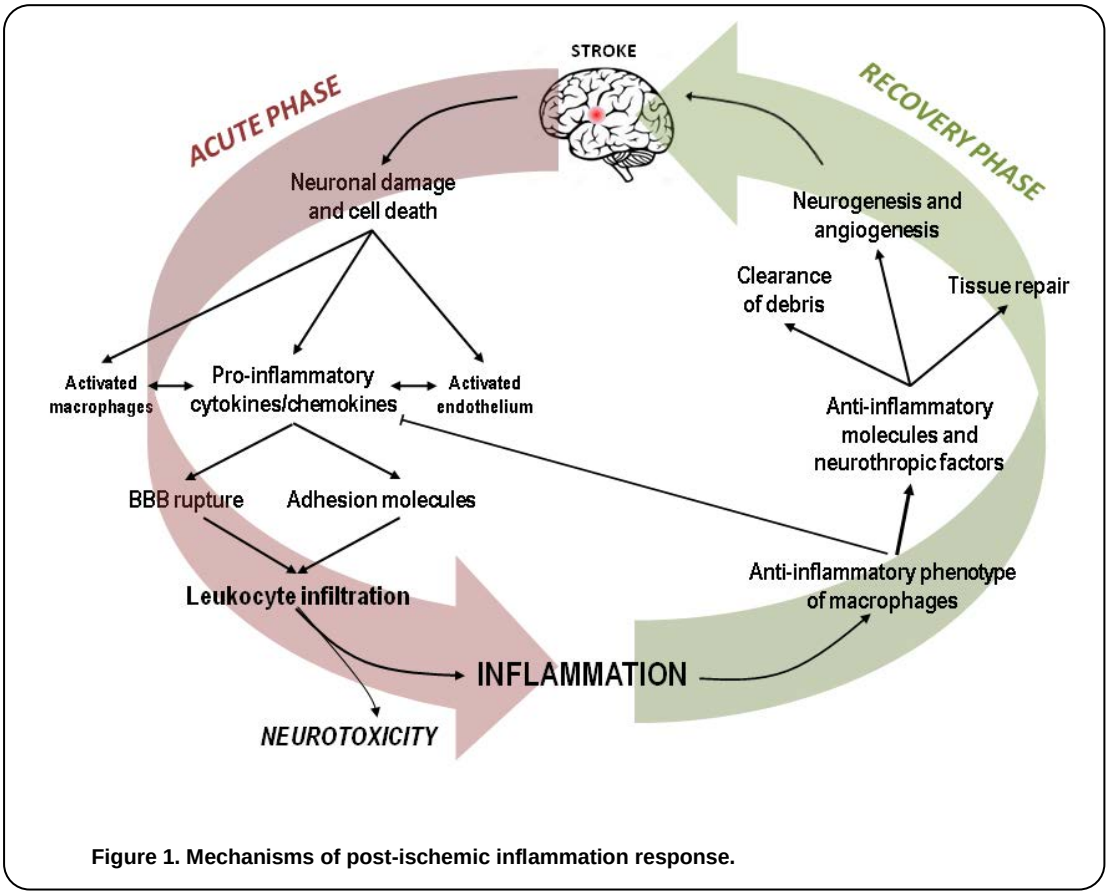

Figure 1. Mechanisms of post-ischemic inflammation response.

Although the BBB disruption mechanisms are not perfectly understood, endothelial barrier failure is suggested to be an immediate consequence of the disruption of the junctional complexes in the interendothelial space of the cerebral microvasculature [14]. Numerous

studies demonstrate that the molecular structure and function of junctional adhesion proteins is altered under acute inflammation, resulting in a variation in their physiological localization within endothelial plasma membranes [15]. This redistribution correlates with an increased BBB permeability, suggesting junctional complexes as major mediators of BBB leakage.

Moreover, matrix metalloproteinases (MMPs) also play an important role in BBB deregulation. Recognized findings indicate that the expression of several MMPs, such as MMP-2, MMP-3, and MMP-9, increases after ischemia [16]. Different cell types, including resident cells (neurons, microglia, astrocytes and endothelial cells) and infiltrated inflammatory cells synthesize and secrete MMPs, which are proteolytically cleaved and activated in the extracellular space. Active MMPs disrupt the BBB by degrading basal lamina constituents, such as collagen, fibronectin, laminin, proteoglycans and others, resulting in vessel wall weakness and BBB leakage. Consequently, higher leukocyte infiltration into brain parenchyma is permitted, which sometimes culminates in cerebral hemorrhages within the ischemic zone of the brain. In addition, it is well known that some of these leukocytes, specifically neutrophils, contain vesicles loaded with several types of MMPs that may be released after tPA interacts with those leukocytes located at the basal lamina, which might promote tPA related bleedings [17].
However, despite their detrimental effects, MMPs are also known to be key mediators of tissue remodeling during stroke recovery. Evidence suggests that MMPs also participate in two major processes in the later phases: angiogenesis and neurogenesis. MMPs are known to increase the availability of growth factors (e.g. vascular endothelial growth factor) involved in tissue remodeling and vascularization by processing their precursors [18]. Moreover, MMPs also modulate the extracellular matrix to allow the mobilization of both progenitor vascular and neuronal cells and process several guidance molecules (epidermal growth factor and neuregulins) that stimulate the formation and growth of new neurons in contiguous regions [19].
Therefore, MMPs play a dual role within stroke: while they contribute to increase brain damage during the acute phase of the disease, MMPs participate in remodeling processes during the recovery phase of stroke. This dual role of MMPs is comparable with the duality of inflammation, which starts being particularly deleterious during the acute phase of stroke but finally contributes to tissue repair in the recovery phase of the disease. Thus, MMP inhibition has been suggested as a possible therapeutic intervention to palliate and even prevent BBB disruption during the early phase of the ischemic challenge [20].
It should be noted, however, that the disruption of the BBB integrity can also facilitate the entrance of therapeutic drugs to the ischemic brain, which would favor the early arrival of future treatments to overcome stroke-related neuronal damage. Moreover, the rupture of the endothelial barrier also favors the release of brain antigens from lysed cells into the peripheral circulatory system. These brain-derived antigens are considered possible biomarkers of ischemia and/or of stroke-associated inflammation that reflect in the circulatory system the pathological processes that are going on in the brain after stroke.

4. Biomarkers in stroke

Biomarkers are defined as *characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to therapeutic interventions* (Biomarkers Definitions Working Group 2001). Clinical biomarkers can be detectable molecules from blood, urine or other biological fluids (but also imaging tests, etc.) that refer to measurable indicators used to predict physiological states of a disease. Under ideal conditions, biomarkers should fulfill certain requirements if they are to be used in clinics; their measurements should be accurate, precise and reproducible. Moreover, biomarkers should also present high sensitivity and specificity, be relatively easy to interpret by clinicians and add information on top of clinical variables. In the case of stroke, molecular biomarkers should reflect the associated ongoing processes; 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. Therefore, all molecular processes described in the sections above should be taken into consideration for the detection of these candidate biomarkers.

Stroke biomarkers are considered valuable tools for different clinical scenarios, for all the steps between diagnosis and stroke outcome (http://stroke-biomarkers.com). The critical aspect of all these steps is that the selected biomarkers should be useful when making clinical decisions (i.e. whether to apply a treatment or to indicate another diagnostic test based on the results of that test) (Table 1).

The **diagnosis** of ischemic stroke depends on its differentiation from hemorrhagic stroke and other mimicking entities and still relies on clinical assessment and neurological imaging, delaying or even limiting the acute treatment of stroke. Hence early screening to allow rapid ischemic stroke identification would speed stroke patient management and treatment, thus increasing the percentage of patients who receive reperfusion therapies after the event. Although many attempts have been made to find biomarkers for stroke diagnostic, a robust, sensitive and rapid blood test is still lacking.

Table 1
Stroke biomarkers as valuable tools in clinics. BNP, b-type natriuretic peptide; CRP, C-reactive protein; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6; MMP-2, matrix metalloproteinase-2; MMP-13, matrix metalloproteinase-13; NURR1, nuclear receptor related 1 protein; TNF-α, tumor necrosis factor-alpha; tPA, tissue-plasminogen activator.

Clinical scenario	Biomarker use	Examples
Diagnosis	Facilitate the rapid identification of ischemic stroke	CRP D-dimer, BNP, MMP2, S100β D-dimer, caspase-3
	Speed stroke patient management and treatment	
Prognosis	Influence on medical decisions (discharge, rehabilitation, palliative care…)	CRP IL-6 TNF-α ICAM-1 MMP-13
	Prognosticate stroke-associated infections	$CD14^{high}CD16^{-}$ Th1/Th2 profile Cortisol and noradrenalin Copeptin Procalcitonin
Treatment response	Identify patients with unsuccessful tPA-induced recanalization	Soluble endothelial protein C Soluble thrombomodulin
	Predict the appearance of secondary intracranial hemorrhage	Fibronectin NURR1 MMP-9

Several candidate biomarkers for the diagnosis of stroke are involved in inflammatory processes. For instance, C-reactive protein (CRP), an acute-phase protein involved in the activation of several inflammatory mechanisms such as the complement system, is reported to be very useful for rapid diagnosis and prediction of ischemic lesion volume in the acute phase of stroke [21]. In addition, it has been demonstrated that CRP levels increase by 6% the discriminative value of initial neurological severity for the diagnosis of ischemic stroke [22],

which suggests its potential but still marginal function in the early evaluation of patients with suspected stroke.
Therefore, the combination of different biomarkers in a panel has grown in importance during the last decade. Panels permit the measurement of several molecular alterations simultaneously, improving the specificity or the sensitivity required for such a clinical scenario. A kit combining D-dimer, B-natriuretic peptide, MMP-9 and S100β protein was evaluated as one of the first panels used to diagnose stroke. Although the combination of these four molecules provided information that complements clinical triage, the sensitivity and specificity rates (93% and 24% respectively) that were reported make the clinical application of this kit non-viable [23]. Similarly, the combination of six different biomarkers (caspase-3, D-dimer, sRAGE, chimerin II, secretagogin and MMP-9) suggested D-dimer and caspase-3 levels as the most accurate combination for stroke diagnosis (Se 87%; Sp 55%) [24]. However, biomarker panels need further development in order to gain accuracy and reach, ideally, 100% sensitivity and specificity. For the use of biomarkers for stroke diagnosis, these high percentages of exactitude are totally necessary due to the severe complications that can arise from incorrect therapeutic treatments resulting from erroneous diagnoses.

Several studies have also focused on biomarkers to predict stroke outcome and treatment response, which can be completely different between patients. Stroke **prognosis** might also influence medical decisions about sending stroke patients to scarce specialized stroke units, palliative care, rehabilitation programs or deciding on the best moment for discharge. Although the prognostic value of several clinical variables has been explored [25], non-modifiable factors such as age and initial neurological severity are the main predictors that might be improved when combined with inflammatory biomarkers. Despite the fact that most of these inflammatory biomarkers are not specific to ischemic stroke, the levels of several inflammatory mediators correlate with stroke severity and outcome. Inflammatory biomarkers such as CRP or several pro-inflammatory cytokines, specially IL-6, among other molecules have been widely associated with poor functional outcome after cerebral ischemic events [26]. In addition, some inflammatory markers have also been related to infarct volume, including TNF-α, intercellular cell adhesion molecule 1 (ICAM-1) and MMP-13 [27], [28].
Interestingly, some groups are focusing their efforts on dissecting the specific factors that influence stroke outcome. For instance, several studies have focused on biomarkers of **stroke-associated infections**: IL-6 has been suggested as a key biomarker to predict infections after stroke, as well as functional disability and mortality [29]. Another biomarker that has recently gained importance for the detection of infectious complications is $CD14^{high}CD16^{-}$ circulating monocytes, which are involved in the inflammatory response after stroke and are known to be associated with poor outcome and mortality [30]. Furthermore, Salat *et al.* reported that the evaluation of the Th1/Th2 profile (conducted through the evaluation of a set of different cytokines from both pro-inflammatory (Th1) and anti-inflammatory characteristics (Th2)) increases the predictive accuracy of the clinical model from 85.5% to 93.4%, suggesting that the inflammatory state can contribute to the prediction of stroke-associated infections [31]. Additionally, levels of neuroendocrine molecules (such as cortisol and noradrenalin) have been found to be of particular interest for the identification of stroke patients who subsequently develop infections within a short period of time, suggesting their role as predictors of post-stroke infections [32]. Equally, copeptin, a peptide derived from the pre-pro-hormone that gives rise to arginine vasopressin, has demonstrated its added value as a predictor of poor outcome, mortality and infectious complications [33]. De Marchis *et al.* reported that the addition of copeptin to a validated model encompassing a patient's NIHSS and age improved the prediction of functional outcome by 11.8% and the prediction of mortality by 37.2%. [34]. In addition, large amounts of evidence report copeptin as a good prognostic measure in predicting acute infectious illnesses, including sepsis, pneumonia and lower respiratory tract infections, which are the commonest infections that patients get after stroke [35]. Furthermore, the high accuracy of procalcitonin, a precursor peptide of the hormone calcitonin, for the diagnosis of bacterial

infections in hospitalized patients is also well documented, although its role in predicting stroke associated infections is not clear. Circulating levels of procalcitonin during the acute phase of stroke are also reported to be elevated in patients with poor functional outcome and non-survivors 1 year after stroke. In this line, Wang *et al.* reported that a model combining NIHSS score and procalcitonin levels showed an Area Under the ROC Curve (AUC) of 0.85 for outcome and 0.94 for mortality, which improved the predictive capacity by 11% and 17% respectively [36]. However, the accuracy, sensitivity and specificity of all these biomarkers is still not enough to be used in clinics, and further research should be carried out to improve these parameters.

Recent reviews have compiled detailed information about all the neuroendocrine and inflammatory markers related to stroke prognosis [37], [38]. Nevertheless further studies will be required to identify more robust biomarkers to allow the detection of the individual risk of stroke-associated infections, which would help clinicians to anticipate this complication and guide preventive antibiotic treatment, since it is evident that providing antibiotics for all patients is a futile strategy [39]. In this regard, it has to be highlighted that there is an ongoing clinical trial (STRAWINSKI) based on the procalcitonin-based guidance of antibiotic therapy, which supports the idea of the use of suitable biomarkers to guide physicians in an early antibiotic treatment to prevent post-stroke infections [40].

In terms of **treatment response**, it is well known that stroke patients respond differently to thrombolysis and mechanical reperfusion therapies. The most feared side effect of a therapeutic intervention with tPA is hemorrhagic transformation. In addition, success in recanalization after the administration of tPA is not always achieved and depends on different factors, including timing and the degree of blood flow reperfusion. The rate of complete recanalization of tPA-treated patients within the first hour after treatment is known to be around 20%, which increases from 30% to 60% within the following 6 to 24 hours after tPA administration [41]. Hence, specific biomarkers that selectively evaluate the response to tPA, predict the appearance of secondary intracranial hemorrhages and identify patients with unsuccessful tPA-induced recanalization have gained in importance during the last few decades. In terms of hemorrhages, fibronectin is considered a potential biomarker in the diagnosis of hemorrhagic transformation. Plasmatic levels of fibronectin are highly correlated with vascular damage, as endothelial cells, together with hepatocytes, are major producers of this protein [42]. Castellanos *et al.* documented that circulating levels of fibronectin above 3.6 mg/mL were able to predict tPA-associated parenchymal hemorrhages with 100% sensitivity and 60% specificity [43]. Although further research should support the robustness of this biomarker and improve its specificity by determining better cutoff points, fibronectin is currently considered the best indicator of hemorrhagic transformation after stroke. Furthermore, serum levels of the transcription factor nuclear receptor related-1 (NURR1) before tPA administration have been widely associated with the occurrence of symptomatic hemorrhagic transformation [44]. Equally, the semicarbazide-sensitive amine oxidase activity of vascular adhesion protein-1 (VAP-1/SSAO) measured before thrombolytic treatment has also been considered a good indicator of parenchymal hemorrhage transformation after tPA administration [45]. Additionally, a large amount of evidence demonstrates that elevated MMP-9 levels are also predictors of hemorrhagic transformation and thrombolysis failure; MMP-9 levels measured before thrombolytic treatment (baseline levels) are reported to be very good predictors of tPA-associated hemorrhages [46]. Furthermore, Castellanos *et al*. reported high sensitivity and specificity for the detection of parenchymal hematomas by MMP-9 levels above 140 ng/mL (Se 92%; Sp 74%) [43]. On the other hand, MMP-9 levels measured after the administration of tPA are well known to correlate with non-reperfusion of the ischemic region (thrombolysis failure) [47].

In addition, the identification of patients resistant to tPA thrombolysis would be of great interest in deciding whether stroke patients may benefit from alternative therapies. Very recently REVASCAT, a multicenter phase 3 study, demonstrated that mechanical thrombectomy with a stent retriever, carried out within the first 8 hours and in combination with medical therapy, reduced the severity of post-stroke disability and improved functional outcome after stroke [48].

Thus, due to the efficacy of this mechanical method, specific biomarkers to anticipate unsuccessful recanalization after tPA administration are very promising. Soluble endothelial protein C receptor and soluble thrombomodulin, two endothelial receptors that participate in coagulation and inflammation by mediating the protein C pathway, have been documented to be inversely correlated with recanalization rates after tPA treatment, which suggests their role as possible biomarkers to predict recanalization failure [49]. However, more research on this clinical indication for biomarkers is inevitable.

Despite the progress made over the last few decades, **biomarker limitations** are still a current issue that precludes their clinical application. Firstly, several factors might be major source of confounding variables: such as age, gender and metabolic factors, and a variety of comorbid conditions. It is known that other inflammatory pathologies, such as hyperglycemia, cancer or autoimmune diseases, can lead to anomalous levels of inflammatory markers in stroke patients. For instance, asymptomatic atherosclerosis, which is also commonly associated with ischemic stroke, is known to influence the levels of two specific molecular markers, CRP and homocysteine [50]. Secondly, as blood is employed as the most suitable biological sample, the identification of clinically significant biomarkers becomes difficult due to the presence of tens of thousands of circulating proteins. Additionally, differences in protein abundance, which can be up to eleven orders of magnitude, can also hinder the discovery of small specific indicators of disease. Finally, the different "statistical power" needed to ensure that the clinical use of the biomarkers is powerful enough to influence decision-making processes has not yet been agreed upon within the medical community.
In addition, an extra limiting factor is the extended period of time required for the validation and commercialization of a discovered biomarker and its translation to the clinics. For instance, troponin, described as a myocardial infarction biomarker, reached the clinics 35 years after its discovery (1960-1995), and prostate-specific antigen (PSA), a biomarker for prostate cancer, was available in hospitals 17 years after its discovery (1979-1996).
Thus, due to all these limitations, the search for new stroke biomarkers should be further intensified and perfected.

5. Biomarker discovery techniques

Biomarker discovery efforts are no longer solely based on the biological function of candidate biomarkers; they are now more widespread due to the urgent need for specific and sensitive clinical indicators of disease. The emergence of *-omics* technology is enhancing the opportunities for discovering new biomarkers and therapeutic targets for stroke (Figure 2). Therefore, discovery through these new massive and high-quality techniques is increasing knowledge of stroke pathophysiology at different molecular levels.

Although this review is mainly focused on proteins as potential biomarkers in stroke, different studies have focused their attention to other methodologies. For instance, **genomics** (the study of the sequence, structure and function of genes) has led to the identification of stroke-related genes and their variants. Genome wide association studies (GWAs) are commonly performed in genomics to detect single nucleotide polymorphisms (SNPs), where one nucleotide has been replaced by another. Some molecular genetic variations are reported to be related to stroke, such as modifications in the *PITX2 and ZFHX3* gene regions*,* which are linked with cardioembolic stroke, or variations in *HDAC9,* which are related to large vessel disease [51]. However, little is known about genetic modifications of the genes involved in the inflammatory process, although further research needs to be carried out. Beyond genomics is **transcriptomics** (the study of the expression profile of genes), which facilitates the determination of the complete set of RNA transcripts that are produced after stroke onset. The

study of RNA expression in blood from stroke patients, which mainly comes from leukocytes, is becoming a major source of information about changes in immune cells related to brain ischemia. Inflammatory response genes of circulating mononuclear leukocytes from stroke patients are known to differ significantly from those in healthy controls [52]. These differences might reflect the state of the inflammatory response after stroke and may contribute to a better characterization of the pathophysiology of the disease. Changes in the expression profiles of blood are also valuable tools in assessing the differences between strokes from different etiologies. For instance, the expression profiles in blood from cardioembolic stroke patients differs by more than seventy genes from those in blood from large-vessel atherosclerotic stroke patients (fold change>1.5, $p<0.05$) [53] and surprisingly many inflammatory genes are part of the cardioembolic signature. Of these genes, a minimum of 23 genes differentiated the two etiologies with a sensitivity of more than 90% , which suggests that the characterization of gene expression profiles is a promising method in predicting stroke etiology and, consequently, they add diagnostic information to aid decision-making regarding patient management. However, although numerous gene expression studies are providing insights into the stroke pathophysiology, further research is needed in order to evaluate the functionality of expression profiles and ensure their applicability as an additional tool for clinicians.

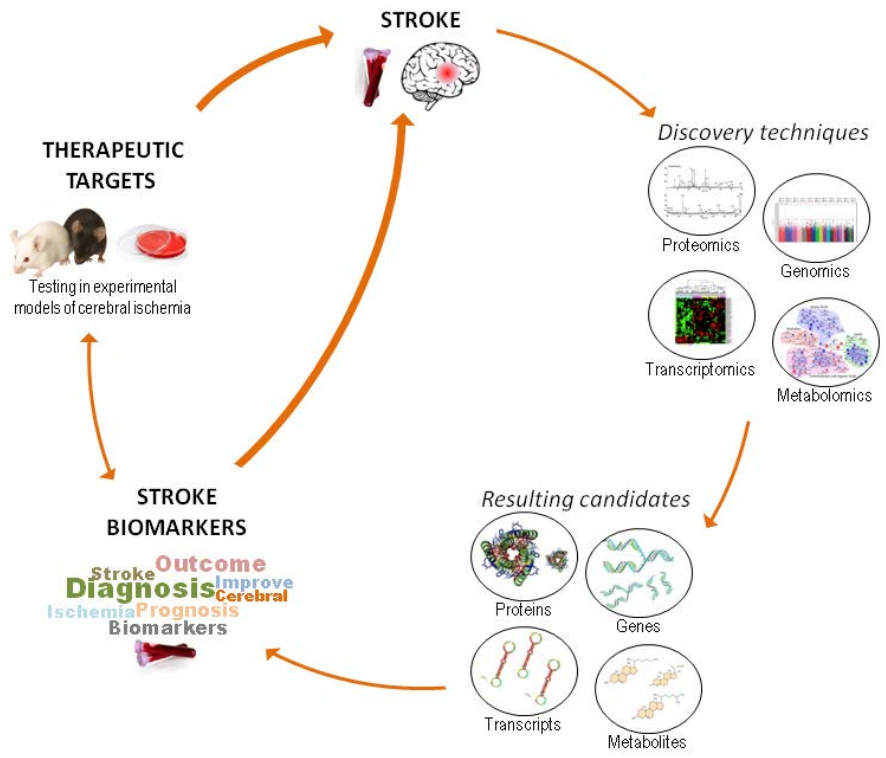

Figure 2. Process of discovery of stroke biomarkers and therapeutic targets.

In conjunction with transcriptomics, **microRNAs** or miRNAs (small nucleotides sequences that regulate gene expression) and their expression patterns have recently been associated with the diagnosis and prognosis of vascular diseases, including ischemic stroke [54]. Emerging studies documented circulating miRNA-233, which is highly involved in the regulation of the cell cycle and the immune system, as a possible biomarker candidate in diagnosing stroke, as its expression has been found to be upregulated after cerebral ischemia in mice [55]. Similarly, miRNA-145 levels are increased in ischemic stroke patients, suggesting both miRNA-233 and -

145 as diagnosis indicators but also as possible therapeutic targets for stroke patients [56]. Besides, **metabolomics** (the study of chemicals from the metabolism) is one of the most novel approaches to finding new biomarkers for stroke. The profiles of metabolites might help to identify patients that suffered an ischemic stroke, but might also provide information about the ischemic cascade and help better understand the complexities of stroke pathophysiology. For instance, spatiotemporal dynamics of metabolites after middle cerebral artery occlusion (MCAO) in mice revealed an enhancement of amino acid- and tricarboxylic acid-related metabolism during pathological progression [57]. However, as it is a very recent approach, no data related to inflammatory processes have been published yet. Nonetheless, metabolomics still has its limitations: there are a large number of unknown molecule profiles that have not been identified yet, which complicates the understanding of stroke pathology at this level.

Although these above-mentioned new *-omics* techniques are progressively gaining in importance, **proteomic** studies (the study of the entire set of proteins expressed by a genome under a particular situation) are still the most promising tools for discovering new biomarkers, as proteins seem easily detectable in quick point-of-care devices. However, although many advances have recently been made regarding proteomics, the methodology is still under development and needs further improvement to easily analyze the entire disease-affected proteome.

Many protein candidates related to the inflammatory response after stroke have been proposed using proteomics. After performing a systemic search of all inflammatory candidates identified in proteomic studies, we summarized them in Table 2. According to these findings, stroke induces the upregulation of many inflammatory proteins involved in the activation of the immune system, but also in cell adhesion and inflammatory signaling pathways. In addition, most inflammatory proteins identified in proteomic studies have been found with increased levels in stroke subjects compared with their controls, which also supports the massively increased degree of inflammation and its potential function during the acute phase of the ischemic challenge.

6. Diagnostic and therapeutic pathways of interest

Despite the fact that many proteins related to inflammation have been found to be altered during stroke (Table 2), only some of them have been studied as potential key proteins involved in both the detection and the modulation of the inflammatory processes, making them useful as both biomarkers and therapeutic targets. This is interesting since theoretically when they are used as targets for stroke treatment it might also be possible to measure them in the blood stream as surrogate markers of the efficacy of the drugs of interest. Thus, from the proteins in Table 2, some of the most relevant candidates are explained in detail in the following sections.

Other proteins than those identified in the systemic search are also gaining in importance in stroke pathophysiology due to their role in relevant canonical pathways, although they are not reviewed in detail here. This is the case for chemokines, an important family of inflammatory proteins that act as both deleterious and restorative molecules depending on the phase of the disease. Although a better understanding of the specific role of chemokines in stroke pathophysiology is still needed, the most important chemokine families related to stroke (CC and CXC families) have already been reviewed elsewhere [58]. As many chemokines are upregulated after stroke, it has been suggested they may be promising candidates for stroke diagnosis, prognosis and therapy, but further research should be conducted to corroborate this hypothesis. In addition, another relevant group of proteins is the family of “A disintegrin And Metalloproteinase with ThromboSpondin motifs” (ADAMTS). Although their pathological role in stroke is not fully understood, ADAMTS are believed to be involved in the disease, since several studies in experimental models reported the upregulation of some of its members after cerebral ischemia [59]. Future studies might determine their specific role in the disease and evaluate their potential as stroke biomarkers and/or therapeutic targets.

Table 2
Stroke-associated inflammatory proteins identified in proteomic studies. References are provided as PubMed reference numbers (PMID). OGD, oxygen-glucose deprivation; tMCAO, transient middle cerebral artery occlusion.

Protein name	Abbreviation	Biological function	Regulation after ischemic stimulus	Study type	PMID
14–3–3 protein beta	14–3–3β	Inflammatory signaling and cell adhesion	↑	Rat cerebellar granule neurons exposed to glutamate excitotoxicity	17530875
14–3–3 protein epsilon	14–3–3ε	Inflammatory signaling	↑	Rat brain endothelial cells exposed to OGD	17203967
			↑	Rat brain after global cerebral ischemia	17536849
14–3–3 protein zeta	14–3–3θ	Inflammatory signaling	↑	Rat brain after hypoxic–ischemic insult	25197337
			↓	Rat brain endothelial cells exposed to OGD	17203967
Annexin 1	ANXA1	Anti-inflammatory	↓	Rat brain endothelial cells exposed to OGD	17203967
			↑	Human brain after ischemic stroke	24007662
			↑	Rat neuroblastoma cell line exposed to OGD	19916522
Annexin 2	ANXA2	Pro-inflammatory	↓	Human neuroblastoma cell line exposed to OGD	23321784
			↓	Rat brain after hypoxic–ischemic insult	25197337
			↑	Rat neuroblastoma cell line exposed to OGD	19916522
			↑	Human brain after ischemic stroke	24007662
Annexin 3	ANXA3	Anti-inflammatory	↑	Rat brain after hypoxic–ischemic insult	25197337
			↑	Rat brain after reversible cerebral ischemia	17823964
Annexin 5	ANXA5	Anti-inflammatory	↓	Rat neuroblastoma cell line exposed to OGD	19916522
			↓	Rat brain after hypoxic–ischemic insult	25197337
Apolipoprotein L1	APOL1	Immune system	↑	Human plasma from patients following lacunar infarction	24752076
CD5 precursor	Pre-CD5	Immune system	↑	Human brain after ischemic stroke	20667078
CD14	CD14	Immune system	↑	Human brain after ischemic stroke	20667078
CD59	CD59	Immune system	↑	Human brain after ischemic stroke	20667078
CD115	CD115	Immune system	↑	Human brain after ischemic stroke	20667078
Chemokine CXC motif ligand 1	CXCL1	Immune system	↑	Human brain endothelial cells exposed to oxidative stress	21164131
Complement 1 binding protein	C1BP	Immune system	↑	Rat cerebellar granule neurons exposed to glutamate excitotoxicity	17530875
Complement 3	C3	Immune system	↑	Human plasma from patients following lacunar infarction	24752076
			↑	Rat plasma after transient focal cerebral ischemia	22103620
			↑	Rat brain after transient focal cerebral ischemia	21950801
			↑	Rat brain after intracerebral hemorrhage	24583080
			↑	Human brain microdialysates after ischemic stroke	14675487
			↑	Human brain after ischemic stroke	20940630
Complement 4	C4	Immune system	↑	Human brain microdialysates after ischemic stroke	21142207
Complement 4 binding protein alpha	C4BPA	Immune system	↑	Human plasma from patients following lacunar infarction	24752076
Complement 5	C5	Immune system	↑	Human plasma from patients following lacunar infarction	24752076
Complement 8 alpha chain	C8A	Immune system	↓	Human brain after ischemic stroke	20667078
Complement 9	C9	Immune system	↑	Rat brain after hypoxic–ischemic insult	25197337
Complement factor H-related 5	CFHR5	Immune system	↓	Human brain after ischemic stroke	20667078
Connective tissue growth factor	CTGF	Pro-inflammatory	↑	Rat brain endothelial cells exposed to OGD	17203967
Cyclooxigenase 1	COX1	Immune system	↑	Rat neuroblastoma cell line exposed to OGD	19916522
E-selectin ligand 1	ESL1	Immune system	↑	Rat brain endothelial cells exposed to ischemic insult	17203967
Ficolin-3 isoform 1	FCN3	Immune system	↑	Human brain after ischemic stroke	20667078
Haptoglobin	Hp	Immune system	↑	Rat plasma after transient focal cerebral ischemia	22103620
Immunoglobulin gamma-2B	IgG-2B	Immune system	↑	Rat brain after hypoxic–ischemic insult	25197337
Immunoglobulin heavy chain	IgH	Immune system	↑	Human brain after ischemic stroke	20940630
			↑	Human brain microdialysates after ischemic stroke	14675487
Immunoglobulin kappa, C region	IGK-C	Immune system	↓	Rat brain after hypoxic–ischemic insult	25197337
Immunoglobulin light chain	–	Immune system	↑	Human brain microdialysates after ischemic stroke	14675487
		Immune system	↑	Human brain after ischemic stroke	20940630
Integrin alpha-2B	ITGA2B	Cell adhesion and signaling	↑	Human plasma from patients following lacunar infarction	24752076
Integrin alpha-D	ITGAD	Cell adhesion and signaling	↑	Rat brain after hypoxic–ischemic insult	25197337
Integrin-linked kinase	ILK	Cell adhesion and signaling	↑	Rat brain after hypoxic–ischemic insult	25197337
Interleukin-8	IL8	Immune system	↑	Human brain endothelial cells exposed to oxidative stress	21164131
Leukocyte immunoglobulin-like receptor subfamily A member 3	LILRA3	Immune system	↑	Human brain after ischemic stroke	20667078
Macrophage migration inhibitory factor	MMIF	Immune system	↑	Rat brain after hypoxic–ischemic insult	25197337
			↑	Rat brain endothelial cells exposed to OGD	17203967
Macroglobulin-α precursor	Pre-AXM	Immune system	↑	Rat brain endothelial cells exposed to OGD	17203967
Macroglobulin-α1	A1M	Immune system	↑	Rat brain after intracerebral hemorrhage	24583080
Macroglobulin-α2	A2M	Immune system	↑	Rat plasma after transient focal cerebral ischemia	22103620
Microglobulin-β2	B2m	Immune system	↑	Human urine samples after ischemic stroke	22615742
			↑	Human brain microdialysates after ischemic stroke	21142207 14675487
			↑	Human brain samples after ischemic stroke	20667078
Murinoglobulin-1	MUG1	Immune system	↑	Rat brain after intracerebral hemorrhage	24583080
		Immune system	↑	Rat brain after hypoxic–ischemic insult	25197337

Table 2 (*continued*)

Protein name	Abbreviation	Biological function	Regulation after ischemic stimulus	Study type	PMID
Murinoglobulin-1 precursor	Pre-MUG1	Immune system	↑	Rat brain after transient focal cerebral ischemia	21950801
Murinoglobulin-2	MUG2	Immune system	↑	Rat brain after hypoxic–ischemic insult	25197337
Neutrophil defensin 1 precursor	Pre-DEFA1	Immune system	↑	Human brain microdialysates after ischemic stroke	21142207
Neutrophil gelatinase-associated lipocalin 2	NCL2	Immune system	↑	Human brain samples after ischemic stroke	20667078
Polymeric-immunoglobulin receptor	PIGR	Immune system	↑	Human urine samples after ischemic stroke	22615742
Selectin	SEL	Cell adhesion and signaling	↑	Rat brain endothelial cells exposed to OGD	17203967
Serine/cysteine proteinase inhibitor clade G member 1 splice variant 2	SERPING1	Immune system	↑	Human plasma from patients following lacunar infarction	24752076
Talin-1	TLN1	Cell adhesion and signaling	↑	Human plasma from patients following lacunar infarction	24752076
Tachykinin peptides	TAC	Pro-inflammatory	↑	Human brain endothelial cells exposed to oxidative stress	21164131
Thrombospondin 1	THBS-1	Cell adhesion and signaling	↑	Human plasma from patients following lacunar infarction	24752076
			↑	Human brain endothelial cells exposed to oxidative stress	21164131
Transforming growth factor alpha	TGF-α	Pro-inflammatory	↑	Rat brain after preconditioned-induced ischemic insult	15030391
Transforming growth factor beta	TGF-β	Pro-inflammatory	↑	Rat brain after preconditioned-induced ischemic insult	15030391
			↑	Rat brain endothelial cells exposed to OGD	17203967
Vinculin	VCL	Cell adhesion and signaling	↑	Rat brain after transient focal cerebral ischemia	21950801

6.1. Cell adhesion molecules

The major role of inflammatory leukocyte-endothelial cell adhesion molecules in the pathogenesis of stroke is well characterized [60]. Their increased expression after stroke onset facilitates the infiltration of leukocytes into the brain parenchyma, which contributes to the emerging inflammatory response at the site of injury.

One of the best-known molecules involved in leukocyte adhesion is **ICAM-1**. After ischemia, proinflammatory cytokines such as TNF-α, IL-1 and interferon (IFN)-γ up-regulate ICAM-1 on both cerebral endothelial cells and neurons, which appears to be an important feature in driving leukocyte infiltration from blood to brain parenchyma [61]. Soluble ICAM-1 is increased in the blood and cerebrospinal fluid of acute ischemic stroke patients and is associated with neurological deterioration and early death after stroke [62]. On the other hand, the absence of differences in soluble ICAM-1 levels between ischemic strokes and healthy controls has also been documented [60]. This paradox, however, can be partially explained by differences in the methodology used to detect ICAM-1 and the influence that some medications can have over the expression of adhesion molecules, such as non-steroid anti-inflammatory drugs [63]. Therefore, the use of circulating ICAM-1 levels as a biomarker of stroke diagnosis and inflammatory progression after ischemic stroke is not fully corroborated. Further research needs to be conducted to determine the ability of plasma ICAM-1 levels to discriminate ischemic stroke from other neurological and inflammatory diseases.

Beyond its role as a biomarker, the inhibition of ICAM-1 has been shown to be neuroprotective by reducing neuronal damage in ischemic rats [64]. Studies of ICAM-1 knock-out mice also reported an improvement in blood flow and a reduction in infarct volume after an ischemic challenge [65], suggesting the impairment of leukocyte migration as a possible therapeutic strategy in improving stroke outcome. Furthermore, at the clinical level the Enlimomab Acute Stroke Trial (EAST) tested the efficacy and safety of inhibiting ICAM-1 through the administration of a murine anti-ICAM-1 antibody. Unfortunately, the Enlimomab-treated group of patients reported a significantly higher fatality rate than the placebo group [66], demonstrating the inefficacy of this therapeutic drug in stroke patients. Nevertheless, there is still some controversy in regards to the experimental design of EAST. The choice of the anti-ICAM-1

treatment regimen and the decision to perform a consecutive 5-day administration are suggested not to be optimally extrapolated parameters compared with the settings used in their respective studies performed in animal models of ischemia [67]. For this reason, ICAM-1 is still suggested to be a possible therapeutic target for improving ischemic stroke outcome, as further studies might corroborate its benefits.

Similar studies have been conducted regarding **lymphocyte function-associated antigen-1** (LFA-1), the major ligand of ICAM-1, which also participates in leukocyte infiltration during ischemic stroke [68]. LFA-1 is a β_2-integrin constitutively expressed in leukocytes that mediates the process of adhesion of circulating blood cells to endothelial cells. Although no studies reported LFA-1 levels as a possible biomarker of stroke-associated inflammation, studies with blocking antibodies against LFA-1 have shown beneficial effects in animal models of cerebral ischemia, where a reduction in infarct size was partially attributed to a lower rate of neutrophil infiltration into brain parenchyma and less accumulation of blood cells in cerebral venules after ischemia, following the same results as the inhibitors of ICAM-1 [68].

Vascular cell adhesion molecule 1 (VCAM-1) is also involved in stroke pathophysiology. VCAM-1, also known as CD106, is a cell surface protein expressed in the endothelium that mediates cell-cell recognition and leukocyte adhesion. VCAM-1 also participates in the downstream signal transduction originated after endothelium activation, directing the immune response to ischemia. High levels of soluble VCAM-1 have been detected in circulation after stroke [69]. Many other inflammatory diseases such as cardiopathies and cancer also present increased levels of soluble VCAM-1 in plasma, suggesting VCAM-1 as an indicator of an inflammatory state [70].
The upregulation of VCAM-1 after stroke is well documented in neurons and endothelial cells after ischemia, which is presumably caused by the elevated levels of cytokines after the ischemic event [63]. Ligand binding to VCAM-1 induces several metabolic and structural changes in endothelial cells that facilitate migration of leukocytes into the brain, which include the production of ROS and the subsequent activation of several MMPs [71]. Recently, VCAM-1 has also been found to participate in neuronal apoptosis after intracerebral hemorrhage, due to the pronounced increase of this adhesion molecule around the hematoma [72]. Thus, all these data attest to the involvement of VCAM-1 in the pathological processes following stroke.

The major ligand of VCAM-1 is **very late antigen-4** (VLA-4). VLA-4 is an integrin that is constitutively expressed in leukocyte plasma membranes. Upon leukocyte activation, VLA-4 undergoes conformational changes to bind VCAM-1, which contributes to leukocyte penetration of the brain tissue. Thus, VCAM-1/VLA-4 interaction has particular relevance in the immune response and leukocyte infiltration into areas of inflammation. Circulating VLA-4 levels have not been reported in stroke patients up to now, although further studies should check whether these proteins can be found in circulation (soluble form) and be used as inflammatory biomarkers.
Several experiments in animal models of stroke have been conducted in order to study the effects of VLA-4 blockage. Of these, some studies reported reduced infarct volumes in ischemic rats treated with VLA-4 inhibitors after 24 and 48 hours of the challenge [73]. This therapeutic effect seems mainly due to the depletion of T-cells and the impairment of the rolling and adhesion of neutrophils [74]. On the other hand, other studies failed to confirm the beneficial effect of blocking VCAM-1/VLA-4 axis in animals suffering from acute ischemic stroke [75]. Although differences in species and drugs can partially explain the discrepancies, the importance of VLA-4 for immune cell trafficking has also been thrown into question by several researchers as the biological effect of VCAM-1/VLA-4 interaction in an ischemic brain and its contribution to infarct growth or tissue regeneration is not fully understood [76].
In spite of the controversy, VLA-4 blockage therapy is available in clinics. Currently, there is an ongoing phase 2 clinical trial that evaluates the effect of Natalizumab on infarct volume in acute ischemic stroke (*https://www.clinicaltrials.gov/ct2/show/NCT01955707).* Natalizumab, a

recombinant IgG4 monoclonal antibody that binds to alpha subunits of VLA-4 integrins to prevent leukocyte transmigration through the endothelial cell barrier, has already been described for the treatment of autoimmune diseases, such as multiple sclerosis (MS). Results from this trial are still not available, although they are eagerly awaited.

All these data suggest that anti-leukocyte strategies may be useful as possible neuroprotective therapies. The inhibition of leukocyte infiltration also suppresses many injury processes associated with this phenomenon, including apoptosis, edema, and injury caused by free radicals and oxidative stress.
Similarly, many other adhesion molecules that participate in cell-cell interaction and leukocyte infiltration into the ischemic brain have been found to be upregulated after stroke (Table 2).
Thus, further research to improve therapeutic strategies against leukocyte migration might result in effective treatments to reduce the excess of inflammation, slow down brain injury, and ameliorate stroke outcome during the acute phase of stroke.

6.2. Matrix metalloproteinases

MMPs comprise a family of zinc-dependent endopeptidases involved in many biological processes, including the activation of inflammatory mediators (chemokines and cytokines), the cleavage of cell receptors, and the release of death signals. MMPs also participate in cell proliferation, neuronal regeneration, angiogenesis and apoptosis [16]. During the acute phase of ischemic stroke, MMPs are known to degrade basal lamina, weaken brain blood vessels and force BBB disruption and leakage. **MMP-9** is a 92-kDa type-IV collagenase secreted in a latent form and activated in the extracellular space after proteolytic processing. MMP-9 is upregulated under several central nervous system disorders, such as Alzheimer's disease, brain tumors, multiple sclerosis, neuropsychiatric diseases and ischemic stroke. Recent studies report that baseline MMP-9 levels are increased in patients who undergo an hemorrhagic transformation following tPA administration, suggesting MMP-9 as a good predictor of this secondary complication in ischemic stroke patients treated with tPA [46]. Thus MMP-9 inclusion in future panels of biomarkers to assess stroke outcome and treatment response should be considered.
The inhibition of MMP-9 has been widely studied as a possible therapeutic strategy to diminish the severity of ischemic stroke and avoid possible complications of the disease. The administration of neutralizing monoclonal antibodies directed against MMP-9 has been reported to significantly reduce brain injury after stroke [77]. However, other tested strategies do not directly inhibit MMP-9 activity, but modulate different pathological processes that indirectly attenuate MMP-9 expression and activity, among other cellular injury pathways. For instance, the administration of *Rutin*, a flavonoid glycoside that scavenges ROS and consequently reduces MMP9 activity, before the induction of ischemia or right after blood flow occlusion in animal models resulted in the attenuation of BBB rupture and showed a reduction of neuronal apoptosis [78]. Similarly, the administration of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a member of the immunoglobulin superfamily that suppresses immunological signal transduction, resulted in the inhibition of MMP-9 secretion by neutrophils, which decreases BBB damage caused by this type of cells during post-ischemic inflammation [79].
Thus, although MMP9 inhibitors have not been evaluated yet in stroke patients, all these studies in experimental models of cerebral ischemia suggest them as therapeutic options during the acute phase of stroke, when they are suggested to improve the outcome of patients suffering from stroke.

6.3. Signaling proteins

14-3-3 proteins are an emerging family of multifunctional molecules that regulate different signaling pathways within the cells. This group of seven different proteins has recently gained relevance due to their participation in various biological processes, including cell growth, proliferation, differentiation, survival, apoptosis, adhesion and other signaling pathways.

Moreover, evidence also shows the involvement of this family of proteins in inflammation [80]. 14-3-3 members are widely known to modulate toll-like receptor (TLR) signaling pathways during inflammatory diseases [80]. 14-3-3 proteins, specifically isoforms 14-3-3σ and 14-3-3ε, are downregulated during the early phase of inflammation, when the production of pro-inflammatory molecules is enhanced. However, both proteins appear to be upregulated during the chronic phase of inflammatory processes, contributing to the clearance of the content of pro-inflammatory cytokines and promoting the balance through the stimulation of anti-inflammatory effectors. In addition, 14-3-3ε proteins are also able to inhibit apoptosis through the sequestration of several apoptotic mediators, such as the pro-apoptotic protein Bad (p-Bad) [81]. Thus, again a dual role in inflammation is raised in relation to 14-3-3 proteins.

In terms of stroke, the expression of 14-3-3 proteins has been found to be upregulated in ischemic brains (Table 2). 14-3-3 protein levels are increased in both the cytoplasm and nuclei of human neurons within the ischemic penumbra [82] and are also induced in astrocytes from ischemic lesions, contributing to an abnormal increase of this type of cell (a process known as astrocytosis) after an episode of neuronal destruction [83].

While high levels of 14-3-3 proteins in ischemic brains after stroke are well documented, studies reporting the presence of these molecules in circulation have been published for non-neuronal inflammatory diseases such as cancer [84] but not in the field of neuroinflammatory diseases. These data suggest that further studies need to be performed to evaluate the abundance of these proteins in stroke patients' circulation and their role as clinical biomarkers. The hypothetical finding of higher 14-3-3 protein levels in stroke cases would indicate severe inflammation in the brains of these patients, which, in combination with other biomarkers for stroke, would help to prognosticate outcome and therapy efficacy in stroke patients.

Although 14-3-3 proteins are naturally upregulated after stroke, therapeutic strategies to further increase their expression are promising tools for modulating ischemic injury. Several pharmacological compounds from the thiazolidinedione group of drugs have been shown to protect against cerebral ischemia in animal stroke models by modulating 14-3-3 proteins. Rosiglitazone, troglitazone and pioglitazone, which are commonly used as antidiabetic drugs, activate peroxisome proliferator-activated receptors (PPARs), a group of transcription factors that stimulate the expression of several 14-3-3 proteins [85]. Specifically, rosiglitazone administration after reperfusion in a rat model of cerebral ischemia has shown neuroprotective effects, which are attributed to the rapid activation of PPAR-γ that, by up-regulating 14-3-3ε proteins, prevents neuronal apoptosis by sequestering pro-apoptotic factors (e.g. p-Bad) in the cytoplasm. However, these aforementioned drugs are not specific for 14-3-3 proteins, which means that the expression of other proteins can also be enhanced with these pharmacological compounds. Therefore, future studies are needed to robustly confirm the beneficial effects of the enhancement of 14-3-3 proteins exclusively.

In summary, all these findings suggest the relevance of the overexpression of 14-3-3 proteins in protecting against cell death after cerebral ischemia. 14-3-3 proteins are, therefore, considered to be promising targets for modulating brain injury and neuronal damage.

6.4. Annexins

Annexins are membrane-bound proteins involved in exocytosis and endocytosis, vesicle trafficking, membrane reorganization, and ion-transport across membranes, among others. Little is known about annexin molecules regarding ischemic stroke, but they have been found with increased levels after the occurrence of the disease (Table 2). **Annexin A1** (ANXA1), previously known as lipocortin 1, regulates several cellular functions in a variety of cell types, including differentiation and proliferation, but also plasma membrane repair and cellular apoptosis. Additionally, ANXA1 is of increasing importance in inflammation due to its potent anti-inflammatory properties: ANXA1 prevents the massive stimulation of the oxidative metabolism resulting from the release of arachidonic acid [86], impedes neutrophil adhesion to the endothelium, promotes macrophage phagocytosis of leukocyte debris and inhibits microglia activation, among other activities [87]. However, although the anti-inflammatory functions of

ANXA1 have been well documented, the detailed mechanisms of action of some of these functions remain elusive. Increased circulating ANXA1 levels have been found in inflammatory disorders such as sepsis [88], but no data is available regarding ANXA1 plasmatic levels in stroke patients. Further research should, thus, assess the expression profile of this protein during the stroke time-course.
Studies in animal models of cerebral ischemia report that the inhibition of ANXA1 by antibodies against the protein increase infarct volume and enhance the appearance of edema [89]. Similarly, the administration of ANXA1 to ischemic rats causes the opposite, a marked inhibition of infarct volume and decreased cerebral edema formation, suggesting ANXA1 as a possible neuroprotective target [73].

Annexin A2 (ANXA2) is a cell-surface protein that works as a receptor for plasminogen and its activator, tPA. The aggregation of ANXA2, plasminogen and tPA all together cause an increase in tPA efficacy in converting plasminogen to plasmin, suggesting ANXA2 as a key modulator of tPA response. Because of this, the evaluation of ANXA2 as a possible candidate to determine treatment response after thrombolytic therapy would be of great use. Furthermore, ANXA2, in combination with other circulating molecules (myosin heavy chain 7, desmin and insulin-like growth factor 7), has been proposed as a good biomarker to detect heart failure [90]. Hence, the evaluation of circulating ANXA2 levels in blood from stroke patients remains of interest and might possibly be carried out in the near future.
In animal models of ischemia, the administration of ANXA2 recombinant protein in combination with tPA has been shown to improve long-term neurological outcome after stroke. Additionally, the combination of both drugs permitted the reduction of tPA dose, which also diminishes the side effects of these thrombolytic treatments [91]. Thus, therapeutic interventions with ANXA2 might also be beneficial when combined with other effective treatments.

Annexin A5 (ANXA5) is another intracellular membrane-bound protein of the group of annexins. Although its functions are not perfectly understood, it is known that a major role of ANXA5 is the inhibition of prothrombin activation, which suggests its potent activity as an anticoagulant agent. In addition, ANXA5 is also involved in protection from excessive inflammatory activities, as it sequesters negatively charged phospholipids when exposed to cell surfaces, which consequently prevents the stimulation of phospholipid-induced pro-inflammatory processes [92]. Circulating levels of ANXA5 have been found to be increased in patients suffering from Alzheimer's disease and dementia with Lewy bodies [93]. On the other hand, ANXA5 serum levels are demonstrated to be lower in patients with type 1 diabetes [94]. Although no data can be found regarding ANXA5 levels in stroke patients' circulation, there is evidence reporting that antibodies against this protein are detectable in plasma samples of patients suffering from stroke. Lee *et al*. reported that endogenous levels of circulating anti-ANXA5 antibodies were found to be very high in ischemic stroke patients after the challenge [95], which perfectly matches the decreased levels of ANXA5 found in ischemic brain tissues (Table 2). Due to the anticoagulant function of ANXA5, endogenous antibodies against ANXA5 have been related to thrombotic events, as they inhibit the anticoagulant activity of ANXA5. Hence, the decrease in the activity of ANXA5, by stimulating the production of endogenous antibodies against ANXA5 or through the administration of ANXA5 inhibitors, might be a possible therapeutic strategy to decrease the occurrence of hemorrhagic transformation after stroke. In addition, ANXA5 co-localizes with phosphatidylserine groups, which are expressed on cell surfaces when they are dying [96]. This could suggest the role of ANXA5 in apoptotic processes, which may be another interesting target to modulate in future studies after further investigation.

6.4. Complement system

Activation of the complement system has been well documented in several neuroinflammatory diseases, including neurodegenerative processes and stroke. Specifically, the complement system has been associated with ischemic insults since the 1970s when Hill and Ward described the presence of C3 fragments in rat myocardial tissue [6]. Components of the complement system, including effectors and receptors, are mainly produced by neurons, astrocytes and microglia during brain damage [7]. Pro-inflammatory mediators, ROS and apoptotic cells triggered by stroke mainly drive the expression of this group of proteins. The biosynthesis of complement factors in the central nervous system is now well established and their upregulation has been well documented in plasma, serum and brain tissue after ischemic stroke. The levels of C3 and C5 complement factors are reported to peak at day 1 and between day 7 and 14 after cerebral ischemia respectively [97]. Studies in animal models of cerebral ischemia also revealed that the expression of complement receptors is upregulated in neurons and microglia after ischemic injury [98]. The activation of the complement system is, thus, increasing inflammatory and immune activities, which intensify the deleterious consequences of inflammation in ischemic brains during the first days after stroke.

Circulating levels of many complement factors are elevated in stroke with different etiologies, such as cardioembolic stroke and small vessel disease [99]. It is well documented that complement C3 levels positively correlate with poor outcome in cardioembolic stroke patients [100]. Similarly, C3 factor has been reported to be a predictor of poor outcome after 3 months of stroke, although further research has to confirm this hypothesis firmly [101]. These findings suggest the role of several complement proteins as possible biomarkers to discriminate between ischemic stroke subtypes and predict stroke outcome. However, these markers are again focused on inflammation, which indicates that they will not be specific for stroke, but for inflammatory diseases. For that reason, the combination of these biomarkers with other more specific indicators of ischemia will form a complex panel to precisely diagnose stroke cases.

In an attempt to determine the efficacy of complement inhibition in ischemic brains, several studies in animal models have evaluated different neuroprotective strategies as inhibitors of the complement system. However, none of the strategies tested so far directly modulate complement components, instead they modulate activators of this signaling system. For instance, the inhibition of oxidative stress with the administration of N-ter-butyl-α-phenylnitrone (PBN) antioxidant or the use of copper/zinc-superoxide dismutase transgenic mice results in the suppression of complement factor C3 expression. The decrease in C3 protein accumulation in neurons and microglia reduces infarct volume and improves the neurological status after cerebral ischemia in mice [102]. These data, thus, demonstrate the contribution of ROS in the activation of the complement system and the role of these processes in brain injury after stroke.

Although several approaches in animal models have succeeded in the suppression of complement activation after stroke, these strategies are still far from reaching the clinics. This can be explained by the fact that nearly all these methods have been based on the indirect modulation of the complement system (e.g. modulation of signaling pathways that culminate in the inhibition of complement molecules, among others), rather than the specific inhibition of its components. For instance, the beneficial effects of the suppression of oxidative stress are usually difficult to attribute to the inhibition of the complement system, instead they are due to the decrease of many other deleterious effects of ROS. In future studies, therefore, specific inhibitors of the complement system should be evaluated to better characterize the effects of the complement system and its inhibition.

As shown in Figure 3, several inflammatory molecules described in this section are interlinked. It is well documented that 14-3-3 proteins are related to annexins, which are also linked to complement components. It is suggested that a better understanding of these inflammatory pathways and their upstream connections might provide new therapeutic candidates to be modulated. In other words, the targeting of a specific protein that connects several inflammatory pathways together might be interesting as a new therapeutic strategy, as it would alter the activity of various pathological proteins implicated in the pathophysiology of stroke.

7. Conclusions

It is clear that inflammation is highly implicated in the pathophysiology of stroke. Accumulating evidence gives support to the dual role of inflammation during ischemic stroke, which displays beneficial but also adverse effects according to the phase of the disease.

Anti-inflammatory strategies are providing promising tools for regulating the inflammatory processes that take place during the acute phase of stroke, which predominantly produce deleterious effects. While several therapeutic strategies have been successful in both experimental models of ischemia and clinical trials, the translation from bench-to-bedside is still missing. Perhaps a better understanding of the inflammatory mechanisms involved during ischemic stroke would increase the knowledge of possible therapeutic targets to be modulated to fight against the disease and its complications. In addition, a better characterization of stroke pathophysiology would also provide new therapeutic options to rapidly stimulate neuroregeneration processes after the ischemic challenge.

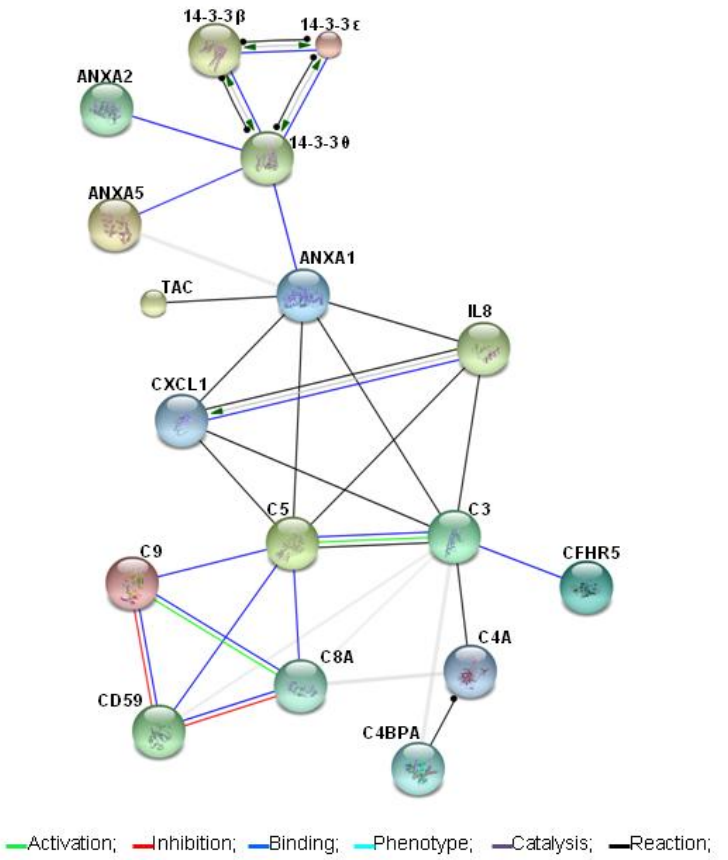

Figure 3. STRING action network related to several inflammatory proteins involved in stroke pathophysiology. STRING database was queried with a subset of inflammatory proteins from Table 2. Protein-protein interactions were provided and non-connected proteins were excluded from the figure. Functional associations and types of interaction were represented in colored lines between proteins as indicated in the legend. Abbreviations: ANXA1, annexin-1; ANXA2, annexin-2; ANXA5, annexin-5; C3, complement-3; C4A, complement-4A; C4BPA, complement-4 binding protein A; C5, complement-5; C8A, complement-8A; C9, complement-9; CFHR5, complement factor H-related protein 5; CXCL1, chemokine CXC motif ligand-1; IL8, intereukin-8.

In addition, the lack of sensitive and rapid blood tests to diagnose and aid stroke prognostication is still a major cause of concern worldwide. Because of the numerous

advantages that would result from the elaboration of robust biomarkers for stroke, we consider that research in the years ahead might focus attention on their discovery, which not only provides potential methods for stroke diagnosis and prognosis, but also contributes to the understanding of the pathophysiology of this devastating disease. Hence, the combination of inflammatory biomarkers with other indicators of ischemic injury would improve the potential of stroke biomarkers and facilitate a better clinical assessment of patients during the acute phase of the disease, which is of vital importance in reducing the prevalence of stroke-related disability and mortality.

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Protective effect of R-Tech Ueno VAP-1-inhibitor molecule (U-V296) on an “in vivo” stroke model

Effect of VAP-1/SSAO blockade on an in vivo ischemic stroke model of hemorrhagic transformations

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Leukocyte infiltration into the brain is known to exacerbate tissue injury after ischemic stroke. Vascular adhesion protein (VAP-1), also named as semicarbazide-sensitive amine oxidase (SSAO or VAP-1/SSAO), plays an important role in adhesion and transmigration of leukocytes, which together with the SSAO-derived toxic metabolites, compromise the integrity of the brain-blood barrier (BBB) following an ischemic event. The aim of this study was to examine the effectiveness of VAP-1/SSAO blockade in reducing the extension of the ischemic lesion and preventing the occurrence of Tissue Plasminogen Activator (tPA)-related hemorrhagic transformations (HT), all associated to the BBB weakness. To that end, male Wistar rats were subjected to cerebral ischemia through the Middle Cerebral Arterial Occlusion (MCAO) intraluminal model and intravenously treated with a VAP-1/SSAO inhibitor (named UV296, Sucampo Pharma, LLC, Japan), tPA or both. The neuroprotective effect of UV296 was evaluated in rats by assessing their neurological state, infarct volume and the occurrence of tPA-related HT 48h after the ischemic event. Results indicated that UV296 treatment did not reduce the extension of the ischemic lesion and neither improved the neurological status of treated animals. UV296 administration prior to tPA, however, prevented the appearance of tPA-related HT, which supports previous published findings reporting that anti-VAP-1/SSAO drugs may succeed in the prevention of secondary complications in ischemic stroke patients treated with tPA.

Stroke is one of the leading causes of death and disability worldwide. Despite research efforts, effective therapies to prevent ischemic-related brain damage and neurological deterioration are still limited. Amongst the clot-busting strategies to restore brain perfusion[1,2], which are the only available treatments at present, intravenous administration of tissue plasminogen activator (tPA) remains still today the gold standard pharmacologic therapy for ischemic stroke[3]. Despite its high effectiveness on stroke patients has been widely demonstrated[4], tPA treatment increases 10-fold the risk of developing thrombolysis-related hemorrhagic transformations (HT), which still present nowadays significant alarming rates of mortality and morbidity[5]. To attenuate the incidence of HT following tPA treatment, therapeutic vascular and brain protective approaches that aimed to modulate key mediators of the underlying mechanisms of bleedings have been a matter of investigation over the last few decades[6].

Vascular adhesion protein-1 (VAP-1) is a circulating and membrane-bound ectoenzyme involved in leukocyte recruitment. Upon inflammation, VAP-1 strongly up-regulates on the endothelial cell surface to mediate leukocyte adhesion and transmigration into the inflamed tissue through its semicarbazide-sensitive amine oxidase (SSAO) activity[7]. This enzymatic activity of VAP-1/SSAO also metabolizes primary amines that ultimately enhance the expression of additional pro-inflammatory mediators, overall promoting cellular damage within the ischemic brain.

Both, leukocyte infiltration and the endogenous overproduction of pro-inflammatory mediators, enhance brain cellular damage and compromise the integrity of the blood-brain barrier (BBB)[8]. Hence, it has been postulated that the inhibition of VAP-1/SSAO activity might be a promising therapeutic strategy to attenuate the entrance of leukocytes and preserve the BBB integrity, which might eventually reduce the incidence

of fatal hemorrhagic complications after tPA treatment of stroke patients.

The aim of this study was to explore the efficacy of the combined treatment of tPA and a VAP-1/SSAO inhibitor, named UV296 (Sucampo Pharma, LLC, Japan). Specifically we were interested in studying whether this therapeutic agent could reduce the extension of the ischemic lesion and influence on the fatal incidence of intracranial hemorrhage after the tPA treatment. To that end, male rats were subjected to mechanical transient cerebral ischemia and subsequently treated with UV296 and tPA to evaluate the incidence of neurological deficits, infarct volumes and HT 48h after the ischemic event.

MATERIALS AND METHODS

Ethics statement

All procedures were approved by the Animal Ethics Committee of the Vall d'Hebron Research Institute (27/15 CEEA) and were conducted in compliance with the Spanish legislation, in accordance with the Directives of the European Union. Experiments were performed in male rats (Wistar, Charles River) weighting 250-300g. Rats were kept in a climate-controlled environment on a 12-h light/12-h dark cycle. Food and water were available *ad libitum*. Analgesia (Buprenorfin, 0.05 mg/Kg, s.c. Divasa Farma-Vic S.A., Barcelona, Spain) was given to all rats to reduce their pain and discomfort.

Experimental design

Animals were randomly allocated to 6 experimental groups: (A) Sham animals treated with vehicle; (B) Middle cerebral artery occlusion (MCAO) animals treated with vehicle (MCAO controls); (C) MCAO animals treated with vehicle and tPA (tPA controls); (D) MCAO animals treated with high dose of UV296 (1 mg/kg); (E) MCAO animals treated with low dose of UV296 (0.1 mg/kg) and tPA; (F) MCAO animals treated with high dose of UV296 and tPA.

A total of 117 animals were needed to complete the study. Among them, 60 animals were included in the study and 58 were excluded after applying the following criteria: incomplete occlusion after the filament insertion (n=7), incomplete reperfusion after the filament removal (n=11), incorrect drug administration (n=3), death before experimental protocol finished (n=9), death during the first 24 h (n=23) and death between 24 and 48 h after the occlusion (n=5).

Intraluminal tMCAO model

Rats were submitted to a transient MCAO model using an intraluminal filament as described previously[9]. Briefly, rats were anesthetized under spontaneous respiration with 2% isoflurane (Abbot Laboratories, Kent, UK) in oxygen during surgery and body temperature was maintained at 37ºC. A neck incision was done on the right side in order to exposure the right bifurcation of the external carotid artery (ECA) and the internal carotid artery (ICA) and a silicone-coated nylon monofilament (Doccol Corporation, reference number: 403723PK10) was introduced to occlude the middle cerebral artery (MCA). After occlusion, animals were allowed to recover from anesthesia for 90 minutes until reperfusion. Sham-operated animals were submitted to the same processes but filament was removed immediately after its introduction.

In order to monitor regional cerebral blood flow (CBF) and ensure the correct occlusion of the MCA, the day before surgery a cranial trepanation was performed to attach a laser-Doppler probe (Moor Instruments, Devon, UK). Only animals that exhibited a reduction >75% in regional CBF after filament placement and a recovery of >75% after filament removal were included in the study.

UV296 and tPA administration

VAP-1 inhibitor (UV296) was provided by Sucampo Pharma, LLC (Japan). Powder UV296 was dissolved in saline at 0.02 mg/ml (low dose UV296, 0.1 mg/kg) or 0.2 mg/ml (high dose UV296, 1mg/kg), mixed for 5 minutes and kept at 4 ºC until use. Fifteen minutes after MCAO occlusion, 1.5 milliliter of UV296, either at low or high dose, or vehicle (saline) was intravenously administered.

Initial tPA solution was prepared by dissolving 50 mg of tPA powder in 25 ml of solvent (2mg/ml) (Actilyse, Boehringer, Ingelhem, Germany) and was kept frozen until use. At

the time of use, tPA solution was thawed and activated prior to administration. In brief, a blood clot formed in an 18-mm PE-50 catheter was rinsed intensely in saline to remove temperature and supernatant (activated tPA) was collected[10]. Immediately after reperfusion, animals treated with this activated tPA underwent a 20-minute intravenously administration of the activated antithrombotic solution using an automatic injector in a 10 mg/kg dose (75 ul/min).

Neurological deficit

Rats were assessed using a 9-point neurological deficit scale. Four consecutive tests were conducted: (I) spontaneous activity (moving and exploring=0, moving without exploring=1, no moving or moving only when pulled by the tail=2); (II) left drifting during displacement (none=0, drifting only when elevated by the tail and pushed or pulled=1, spontaneous drifting=2, circling without displacement or spinning=3); (III) resistance to left forepaw stretching (stretching not allowed=0, stretching allowed after some attempts=1, no resistance=2) (IV) parachute reflex (symmetrical=0, asymmetrical=1, contralateral forelimb retracted=2). Neurological score was assessed in a blinded manner at 80 minutes, 24 hours and 48 hours after occlusion of the MCA.

Infarct volume and HT evaluation

After the neurological test at 48h, all animals were euthanized. Blood samples were drawn through transcardiac puncture and cold heparin solution and cold saline solution were transcardially injected one after another for 10 minutes each using an infusion pump.

Immediately after perfusion, brain was removed and brain tissue was cut into 6 serial 2-mm coronal sections to further proceed with the quantification of the infarct volume and hemorrhage transformations.

surrounding red blood cells and cut into pieces of 1.5 mm length each. Twelve pieces were incubated with 2 ml of tPA solution (2mg/ml) for 20 minutes at room Infarct volume was assessed using 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining as described[11]. TTC images were captured using a Cano Scan 4200F and infarct volume was measured in a blinded manner using Image J software by integration of infracted areas. Infarct volume data was expressed as percentage of the ipsilateral hemisphere.

Three different methods were used to evaluate HT severity: (I) classification of HT by visual score as previously described[12] (no hemorrhage transformation=0, hemorrhagic infarction type 1 (HI-1)=1, hemorrhagic infarction type 2 (HI-2)=2, parenchymal hematomas type 1 (PH-1)=3, parenchymal hematomas type 2 (PH-2)=4); (2) determination of the area of parenchymal hemorrhage by integration of hemorrhagic areas (expressed in mm^2) using the Image J software; and (3) classification of HT by counting the total number of hemorrhagic events. HTs were all evaluated in a blinded manner.

Statistical Analysis

Experimental data were analyzed using SPSS 20.0 (IBM, New York, NY, USA). Graphs were performed using GraphPad Prism 6. Normality for continuous variables was assessed by Kolmogorov-Smirnov test. Those normally distributed were analyzed by Student's t-test and mean ± SEM are given. Mann-Whitney U test was done for non-parametric ones and median (IQR) were given. Pairwise analysis was performed using paired t-test for normal distributed variables and Wilcoxon for non-parametric ones.

Experimental group	% Occlusion	% Reperfusion
MCAO-Vehicle	8.78 ± 3.189	91.44 ± 26.02
MCAO-Vehicle-tPA	11.77 ± 8.454	91.48 ± 25.48
MCAO-0.1mg/kg UV296	11.32 ± 6.758	106.3 ± 25.80
MCAO-1.0mg/kg UV296-tPA	7.856 ± 2.972	118.4 ± 65.83
MCAO-1.0mg/kg UV296-tPA	10.22 ± 3.873	124.1 ± 42.16

Table 1. Mean percentages of occlusion and reperfusion of the MCA during the MCAO surgery.

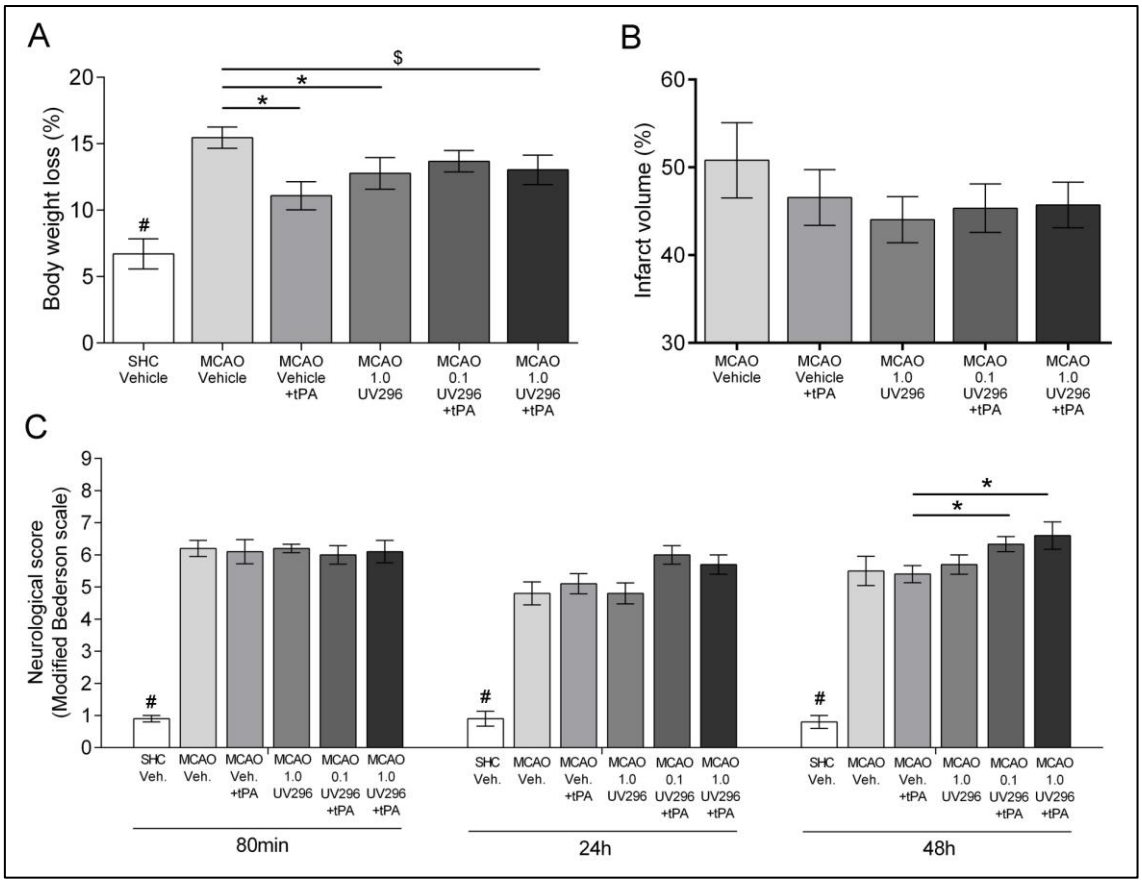

Figure 1. Effect of tPA and UV296 on infarct volume and neurological outcome after cerebral ischemia. (A) Percentage of body weight loss after MCAO. **(B)** Infarct volume 48h after MCAO, expressed as percentage of the ipsilateral hemisphere. **(C)** Neurological deficit evaluation at 80min, 24h and 48h following MCAO. Abbreviations: MCAO: middle cerebral artery occlusion. In all graphs, mean ± SEM are shown, *indicates $p<0.05$, $^{\$}$indicates $p<0.1$ and $^{\#}$indicates differences between sham and all other MCAO groups. N=10 animals/ group.

RESULTS

All ischemic animals included in the study showed similar occlusion and reperfusion percentages of the MCA (Table 1), and ischemia-related mortality rates were not statistically different between groups (data not shown). Ischemic animals receiving tPA or high dose of UV296, either alone or in combination, lost less body weight than those which did not receive tPA (p=0.011 for MCAO + tPA; p=0.035 for MCAO + 1.0mg/kg UV296 and p=0.09 for MCAO + 1.0mg/kg UV296 + tPA) (Figure 1A).

No significant differences were either detected on infarct volume percentages among experimental groups (Figure 1B). Neurological outcome showed no differences between groups at 80 minutes and 24 hours. However, 48h after ischemia, animals treated with tPA in combination with UV296, either at low or high dose, presented worse neurological outcome compared to animals treated with tPA alone (p=0.028 for MCAO + 0.1mg/kg UV296 + tPA and p=0.029 for MCAO + 1.0mg/kg UV296 + tPA) (Figure 1C).

Hemorrhages were observed in most of the animals treated with tPA, although some cases were also found in animals that did not received the thrombolytic drug. The most frequent hemorrhage identified was HI type I, followed by HI type II, whereas no PH type hemorrhages were observed (Figure 2A).

Significant differences were seen regarding HT rates between ischemic animals treated and non-treated with tPA; rats receiving tPA presented higher visual hemorrhagic scores (p=0.007, Figure 2B), increased number of hemorrhagic events (p=0.029, Figure 2C) and larger hemorrhagic areas (p=0.029, Figure 2D) compared with animals that did not

receive the thrombolytic therapy. Interestingly, the administration of high dose UV296 in combination with tPA reversed the appearance of these tPA-related intracranial hemorrhages, since those animals did not show differences in any of the three used methods of HT evaluation when compared with animals that did not undergo thrombolysis (p=0.509 for visual score, p=0.525 for number of hemorrhagic events and p=0.396 for hemorrhagic area). Moreover, animals treated with high dose of UV296 and tPA also presented a lower visual score and a decreased number of hemorrhagic events compared with animals treated with tPA alone, despite statistical significance was not fully reached in this case (p=0.075 and p=0.089, respectively). **DISCUSSION**

Hemorrhagic complications derived from the thrombolytic treatment with tPA cause high mortality and morbidity rates on stroke patients[5]. Since it is still the only pharmacological drug available to treat ischemic strokes, contributions aimed at preventing the incidence of these side effects are urgently needed. In this work, we studied the efficacy of UV296, a VAP-1/SSAO inhibitor, to reduce the extension of the ischemic lesion, ameliorate the neurological outcome and specifically tackle the devastating incidence of tPA-related HT in an experimental model of cerebral ischemia in rats.

Following an ischemic event, VAP-1/SSAO is well-known to mediate adhesion and transmigration of circulating immune cells by binding and catalyzing the removal of an amino group present on the surface of leukocytes, which temporary connect these two cell types to facilitate their migration through the inflamed vasculature[7,13].

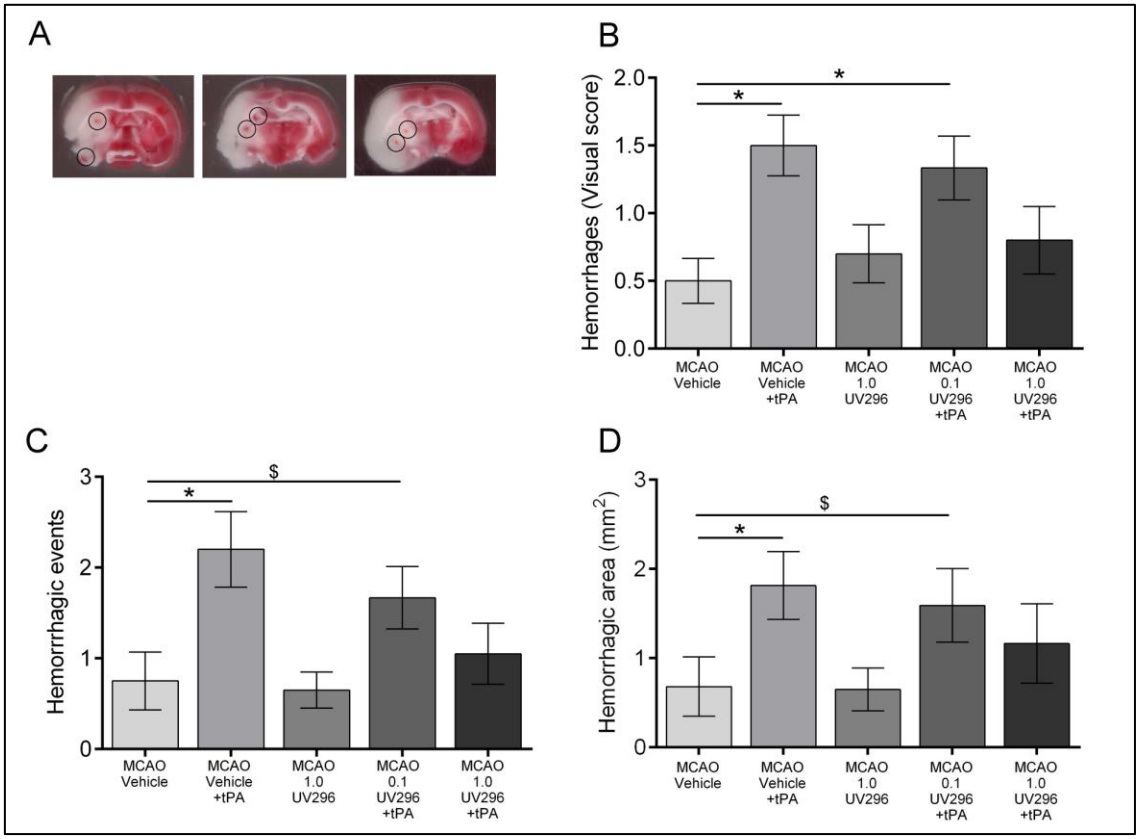

Figure 2. Evaluation of hemorrhagic transformations (HT) after tPA and UV296 treatment following cerebral ischemia. (A) Representative image showing the presence of HT (circles) in an ischemic brain of rats treated with tPA. HT severity was assessed through 3 different methods: **(B)** Visual hemorrhage score; **(C)** Number of different hemorrhagic events; and **(D)** determination of the mean area of parenchymal hemorrhages, expressed in mm^2. In all graphs, mean ± SEM are shown, *indicates $p<0.05$ and $^{\$}$indicates $p<0.1$ in comparison to the reference group (MCAO vehicle). N=10 animals/ group.

Since leukocyte infiltration participate in stroke disease progression by further releasing pro-inflammatory mediators to the site of brain injury[14], the attenuation of the entrance of these blood cells is suggested as a therapeutic approach to palliate the severity of the ischemic brain lesion[15]. In this respect, VAP-1/SSAO activity has been specifically proposed as a feasible target for stroke treatment[16], inasmuch as its mitigation has provided valuable neuroprotective effects in different experimental models of cerebral ischemia and intracerebral hemorrhage[17–20]. Unfortunately, the inhibition of VAP-1/SSAO by the administration of UV296 has not shown any improvement in the extension of the ischemic lesion in our hands. These observations differ from previous published studies and cast doubt on the capacity of this specific VAP-1/SSAO inhibitor, UV296, to neuroprotect the ischemic brain[17–20]. Despite future assessments might confirm UV296 optimal inhibitory properties, future contributions might also aim at fully determine whether the blockade of only VAP-1/SSAO molecule is sufficient to palliate leukocyte entrance and disease progression, or other crucial mediators of these pathological mechanisms might be co-targeted simultaneously to obtain synergistic protective effects through the inhibition of different and complementary mechanisms involved in leukocyte infiltration[6].

Curiously, the combination of tPA and UV296, either at low or high dose, slightly worsens the neurological outcome of ischemic animals at 48h. This increase in the neurological deficit, however, was not associated to a more severe body weight loss, neither correlated with the extension of the ischemic lesion nor with the appearance of HT, which insinuates that it might not be directly linked to the ischemic pathophysiology. Moreover, since animals treated with UV296 alone did not show this neurological worsening, it is probably the combination of tPA and UV296, rather than UV296 itself, what leads to the observed neurological deterioration at latter stages after stroke onset. However, the exact underlying mechanisms are a matter of much speculation for the time being and need further assessment.

Infiltrated leukocytes, in particular neutrophils, exert harmful effects due to their endothelial damaging properties[15,21]. Since tPA administration exacerbates the devastating effects of these infiltrating neutrophils by potentiating the release of their granules and further stimulating apoptosis in endothelial cells[6], it has been proposed that preventing neutrophil migration into the ischemic tissue might be a therapeutic option to maintain the integrity of the BBB and avoid tPA-related BBB leakage and bleedings on tPA-treated ischemic stroke patients.

The occurrence and severity of tPA-associated HT is not only caused by tPA itself, but it is known to also increase by the presence of tPA-related thrombolyisis products, such as plasmin, derived from the binding of tPA to thrombi[22]. These thrombolytic products have been also reported to play an important role in the disruption of the BBB, although the underlying mechanisms still remain speculative. In order to ensure the appearance of hemorrhagic transformations in our intraluminal model of cerebral ischemia, tPA was pre-incubated with blood clots to facilitate the release of products derived from the thrombolysis. This approximation enables us to ensure that tPA-treated animals developed significantly more HT than non-treated ischemic rats, as commonly reported before[23]. Interestingly, our study showed that the administration of high dose UV296 before tPA prevented the appearance of these tPA-related HT. These results are in line with previous studies reporting that VAP-1 may be an aggravator of the ischemia-induced vascular damage, which is directly linked to the appearance of hemorrhagic events[24]. Molecularly, these fatal adverse effects are well attributed to the up-regulation of VAP-1/SSAO-derived toxic metabolites and the promotion of leukocyte extravasation[17,24]. This is why the inhibition of VAP-1/SSAO has been proposed effective in preventing vascular injury following ischemic stroke[24], which from a clinical point of view it might be seen as an approach to reduce the risk of tPA-related HT and improve the outcome of ischemic stroke patients. Future experiments, however, might deeper confirm these preliminary potential effects of UV296 on the prevention of HT, and might also

consider their verification in other more physiological animal models of HT, such as the embolic model[25]. Furthermore, additional contributions aimed at determining whether the inhibition of VAP-1/SSAO is also capable preventing the risk of bleedings on the vast majority of ischemic stroke patients that undergo thrombolysis, regardless of the recanalization strategy[26].

In summary, tPA-related HT is a fatal complication of ischemic stroke patients that urgently needs to be prevented. The inhibition of VAP-1/SSAO by its specific inhibitor UV296 has been here proposed as a potent candidate to avoid tPA-related bleedings, despite further studies might be conducted to fully corroborate its potential effectiveness.

of preventing HT after mechanical thrombectomy, the main complementary clot-busting approach to the tPA treatment, are also required. Its effectiveness in this alternative scenario might further contribute to

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CCL23: a new chemokine involved in human brain damage

Unraveling the therapeutic relevance of CCL23 in an experimental stroke model in rats – A pilot study

CCL23: A new CC chemokine involved in human brain damage

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Background: CCL23 role in the inflammatory response after acute brain injuries remains elusive. Here, we evaluated whether CCL23 blood levels associate with acquired cerebral lesions and determined CCL23 predictive capacity for assessing stroke prognosis. We used pre-clinical models to study the CCL23 homologous chemokines in rodents, CCL9 and CCL6.

Methods: Baseline CCL23 blood levels were determined on 245 individuals, including ischemic strokes (IS), stroke-mimics and controls. Temporal profile of circulating CCL23 was explored from baseline to 24h in 20 of the IS. In an independent cohort of 120 IS with a three-month follow-up, CCL23 blood levels were included in logistic regression models to predict IS outcome. CCL9/CCL6 cerebral expression was evaluated in rodent models of brain damage. Both chemokines were also profiled in circulation and histologically located on brain following ischemia.

Results: Baseline CCL23 blood levels did not discriminate IS, but permitted an accurate discrimination of patients presenting acute brain lesions (p=0.003). IS exhibited a continuous increase from baseline to 24h in circulating CCL23 (p<0.001). Baseline CCL23 blood levels resulted an independent predictor of IS outcome at hospital discharge (ORadj: 19.702 [1.815-213.918], p=0.014) and mortality after 3 months (ORadj: 21.47 [3.434-134.221], p=0.001).
In preclinics, expression of rodent chemokines in neurons following cerebral lesions was elevated. CCL9 circulating levels decreased early after ischemia (p<0.001), whereas CCL6 did not alter within the first 24h after ischemia.

Conclusions: Despite pre-clinical models do not seem suitable to characterize CCL23, it might be a novel promising biomarker for the early diagnosis of cerebral lesions and might facilitate the prediction of stroke patient outcome.

Keywords: CCL23, brain injury, stroke, biomarker, inflammation

CC chemokine ligand 23 (CCL23; also known as chemokine β8-1 (Ckβ8-1), myeloid progenitor inhibitory factor 1 (MPIF-1) and macrophage inflammatory protein 3 (MIP-3)) is a new member of the small CC chemokine family that has recently been associated with the pathophysiology of various inflammatory conditions. CCL23 was originally identified as a potent suppressor of hematopoiesis due to its capacity to inhibit colony formation of myeloid progenitors[1], [2]. However, secreted CCL23 also functionally contributes to

modulation of the immune response via promoting leukocyte trafficking as well as directing the migration of monocytes, macrophages and activated T lymphocytes to local sites of injury[3]. Circulating CCL23 interacts with CC chemokine receptor 1 (CCR1), subsequent up-regulating several adhesion molecules that promote the migration of circulating immune cells to the inflamed microenvironment[4]. Activation of the CCL23-CCR1 axis also stimulates the production of other pro-inflammatory cytokines, including macrophage inflammatory protein (MIP)-1α, interleukin (IL)-1β and tumor necrosis factor (TNF)-α[3], and boosts angiogenesis via promotion of endothelial cell migration through up-regulation of several matrix metalloproteinases in the endothelium[5], [6]. CCL23 plays a pathological role in the development or progression of several inflammatory diseases, such as rheumatoid arthritis, chronic rhinosinusitis, chronic kidney dysfunction and systemic sclerosis[7]–[11]. Notably, circulating CCL23 levels have also been correlated with markers of atheroprogression, such as aortic wall thickness and plaque burden[4].

Despite substantial evidence of the role of CCL23 in inflammation, its relevance to immune-mediated inflammatory responses in the injured brain is not known. Acquired brain injuries, such trauma, stroke, and tumors, damage brain structure and function and exhibit known immune- and inflammatory-mediated mechanisms. Despite major advances in the treatment of these pathologies, there is a persistent need for reliable methods to rapidly diagnose them and improve the acute management of affected patients, which may influence patient outcome and overall survival. The need for a fast interventional procedure is especially important in cases of ischemic stroke (IS) because life-saving pharmacological and mechanical thrombolytic therapies are available for only the hyperacute phase of the disease[12], [13]. This narrow treatment window substantially limits the number of patients who benefit from these therapies.

Implementation of peripheral blood biomarkers has emerged as a promising strategy to accelerate the accurate diagnosis and prognosis of acquired brain diseases. Biomarkers are expected to participate in the mechanisms of the pathophysiology of brain injury, including metabolic changes, oxidative stress and the exhaustive inflammatory response initiated early after lesion onset[14]. Brain damage-related inflammatory markers are expected to play an important role in the identification of these cerebral diseases and reflect lesion severity or predict patient outcome, thus potentially facilitating clinical decisions and providing optimal support to these patients.

We hypothesized that CCL23 might serve as a blood biomarker of cerebral injury and a predictor of the outcome of IS patients. Therefore, the present study examined whether blood CCL23 levels are associated with acquired cerebral lesions and assessed the importance of this chemokine in the prediction of stroke patient outcome. We used preclinical models of brain injury (cerebral ischemia and traumatic brain injury (TBI)) and performed a molecular study of two previously proposed CCL23 homologous chemokines in rodents, CC chemokine ligand 9 (CCL9) and CC chemokine ligand 6 (CCL6), to further describe and characterize these chemokines in the immune-mediated inflammatory response to acquired brain damage.

MATERIALS AND METHODS

Clinical study

Individuals and sample collection

All patients were admitted to the Emergency Department of Vall d'Hebron University Hospital (Barcelona, Spain). The local ethical committee approved all studies detailed below (PR[HG]89/2003, PR[HG]85/2004, PR[AG]96/2009, PR[AG]157/2011) and all individuals or relatives gave informed consent in agreement with the Declaration of Helsinki.

1. **Blood samples**

Cohort 1: Patients were recruited from March 2003 to January 2008 and underwent a complete diagnostic protocol (including neuroimaging) that allowed clinicians to

correctly discriminate between stroke and stroke mimics. A detailed history of demographic and vascular risk factors was obtained for each individual. Neurological severity was assessed by the National Institutes of Health Stroke Scale (NIHSS) and stroke etiology was classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST). All IS patients received standard thrombolytic treatment (rt-PA) in a standard dose of 0.9 mg/kg (10% bolus, 90% one-hour continuous infusion) within the first 4.5 h after symptoms onset. Patients with a known inflammatory or malignant disease were excluded from the study. Peripheral venous blood samples were drawn from each patient at hospital arrival (baseline), before treatment. In a subset of 20 IS patients, blood samples were obtained at serial time points; at baseline and at 2 h, 12 h and 24 h after thrombolytic treatment. Blood samples were centrifuged at 1,500 g at 4°C for 15 min and serum were stored at -80°C until further use. From this sample collection, patients were randomly selected in order to include approximately 30% rate of stroke mimics[15], with a total of 150 IS and 85 stroke-mimics. Additionally, 10 healthy volunteers were included as controls (Table SI).

Cohort 2: Patients were recruited from August 2012 to November 2013 within the first 6 hours after symptoms onset and underwent similar clinical and neuroradiological protocol than patients in Cohort 1, as well as baseline blood serum sample collection and storage. A total of 120 IS patients were included in this second cohort; when eligible, they received rt-PA and/or mechanical thrombectomy to remove the clot. The clinical follow-up of each patient was done until 3rd month after stroke. Functional outcome was evaluated according to the modified Rankin Scale (mRS); patients with a mRS score below 2 were classified as “good outcome” and patients with a mRS from 2 to 6 as “poor outcome”.

2. Brain tissue samples

Postmortem tissue samples from the infarct core of four IS patients were collected within the first hours after death by an experienced neuropathologist (Table SII). An additional brain tissue sample from a patient who died from a non-neurological disease was used as a control. Cortical brain areas were fixed into 10% buffered formalin for 3 weeks and embedded in paraffin blocks.

CCL23 blood immunoassays

Quantitative determinations of CCL23 levels in circulation were performed as described in the Data Supplement.

Statistical analyses

SPSS statistical package 22.0 (IBM, USA) was used for statistical analyses and graphs were generated using GraphPad Prism 6.0 (GraphPad Software, Inc.).

Normality was assessed by Kolmogorov-Smirnov test. For normal distributed continuous variables ($p>0.05$), analyses were performed using ANOVA or t-test followed by Bonferroni post hoc (mean ± SD). For variables with non-normal distribution, Mann-Whitney U or Kruskal-Wallis test followed by Dunn’s Multiple Comparison test were used (median and interquartile range). CCL23 repeated measurements for the temporal profile were analyzed with Friedman test and Wilcoxon test. Chi-squared test was used 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 CCL23 circulating levels with optimal accuracy (both sensitivity and specificity) for discriminating IS prognosis.

Forward stepwise multivariate logistic regression analyses for poor functional outcome and mortality were performed with all clinical variables associated with each endpoint at $p<0.1$. Odds ratio (OR) and 95% confidence interval (CI) were adjusted by sex. Using the selected cut-off points, baseline CCL23 levels were added to the clinical model to assess its independent association and to build new predictive models.

The R software (v3.3.2, R development core team 2012, Austria) was used to compare the areas under the ROC curve (AUC) from the predictive models, using DeLong’s method

(pROC package). The integrated discrimination improvement (IDI) and net reclassification improvement (NRI) indexes determined the added value of CCL23 to the clinical models for the studied endpoints (Hmisc and PredictABEL packages). For the NRI test, pre-specified clinically relevant thresholds of predicted risk (<10% and >90% risk) were used to calculate the patients' reclassification into risk outcome groups when CCL23 was added to the model[16]. To assess whether there was any discrimination improvement when adding CCL23 as a predictive variable in the model, we performed the pre- and post-test probability analysis with 95% CI (http://araw.mede.uic.edu/cgi-bin/testcalc.pl). Pre-test probability was predetermined as 50% and post-test probabilities were calculated using clinical models and models that include CCL23 as a variable.

Kaplan-Meier curves were depicted for survival till 3 months after stroke and significant differences were assessed using Log-Rank Test. Cox proportional Hazards regression analysis was performed to examine the relationship of the survival distribution to CCL23 levels. Hazard ratios and 95% CI are given.

In all cases, a two-sided p-value <0.05 was considered significant at a 95% confidence level.

CCL23 histological examination

CCL23 was evaluated by means of standard immunohistochemistry in ischemic and non-ischemic human brain tissues (Data Supplement).

Experimental study

Ethics statements

The experimental study was conducted in compliance with the Spanish, German and NIH Guide for the Care and Use of Laboratory Animals legislations. All experiments were conducted in a randomized manner and in adherence to the ARRIVE guidelines.

All procedures were approved by the Ethics Committee for Animal Experimentation of the Vall d'Hebron Institute of Research (Barcelona, Spain), the German governmental committees (Regierung von Oberbayern, Munich, Germany) and the Massachusetts General Hospital Institutional Animal Care and Use Committee (Massachusetts, USA).

Male Wistar rats (250-300g) and male wild-type C57BL/6J mice (8-12 weeks-old) were obtained from Charles River Laboratories. All animals were kept in a climate-controlled environment on a 12-hour light/12-hour dark cycle. Food and water were available ad libitum. Non-antiinflammatory analgesics were given to all before starting any surgical procedure to minimize their pain and discomfort. Analgesia and sedation protocols were followed as approved by the local governmental committee.

Animal models and experimental design

Transient intraluminal MCAO model (tMCAO)

Transient cerebral infarction was induced in rats by mechanical occlusion of the MCA, as previously described [17], [18]. Further details are provided in the Data Supplement.

Permanent distal MCAO model (pdMCAO)

Focal cerebral ischemia was induced by permanent occlusion of the MCA distal of the lenticulostriate arteries, as previously described[19]. Further details are provided in the Data Supplement.

Traumatic Brain Injury (TBI) model

TBI was induced through a controlled cortical impact (CCI), as previously described [20]. Further details are provided in the Data Supplement.

Sample collection

Blood and brain samples were collected at their respective times as described in the Data Supplement.

CCL9 and CCL6 mRNA quantification, histological examination and blood immunoassay

CCL6 and *CCL9* mRNA expression was evaluated in the rodent brain by standard quantitative real-time PCR (Data Supplement). Histological examination was performed by means of standard immunohistochemistry / immunofluorescence and quantitative determinations of CCL9 and

CCL6 levels in circulation were performed using an ELISA kit (Data Supplement).

RESULTS

Clinical study

Fig. 1A shows a schematic representation of the different cohorts of patients included in this study.

CCL23 as a blood biomarker for the early diagnosis of cerebral damage

CCL23 levels were assessed in blood samples after patient admission to the hospital within the first 6 h from symptom onset for the diagnosis of IS. The CCL23 plasma levels of IS patients (61.22%) were compared with those of controls (4.1%) and patients who presented stroke-mimicking conditions (stroke mimics) (34.7%). The stroke mimic group included patients who presented brain-damaging pathologies, such as tumors (18.82%) and severe TBI (2.36%), or mimicking disorders that do not acutely alter brain structure, including cryptogenic epileptic seizures (24.70%), migraine auras (10.58%), peripheral neuropathies (15.29%) and other disorders (vertigo, intoxications, global transient amnesia or delirium) (17.65%).

A detailed descriptive analysis of the demographic and risk factors of all the studied groups of patients is provided in Table SI. Briefly, compared with the control and stroke mimic groups, the IS group presented a higher prevalence of arterial hypertension, atrial fibrillation and coronary artery disease; moreover, the IS group tended to smoke more than the other groups. By contrast, more controls exhibited dyslipidemia than did IS patients or stroke mimic patients. Regardless of the brain condition, patient age slightly influenced differences in blood CCL23 levels (R=0.163, p=0.010).

Notably, blood CCL23 levels were higher in the IS and stroke mimic groups than in the control group (p<0.001 for both comparisons) (Fig. 1B). However, baseline blood CCL23 levels did not differentiate IS patients from stroke mimic patients (p=0.468). Further analysis revealed that CCL23 discriminated patients with acute brain-damaging diseases (stroke, brain tumors and TBI) from patients with other non-tissue-damaging brain diseases (p=0.003) (Fig. 1C).

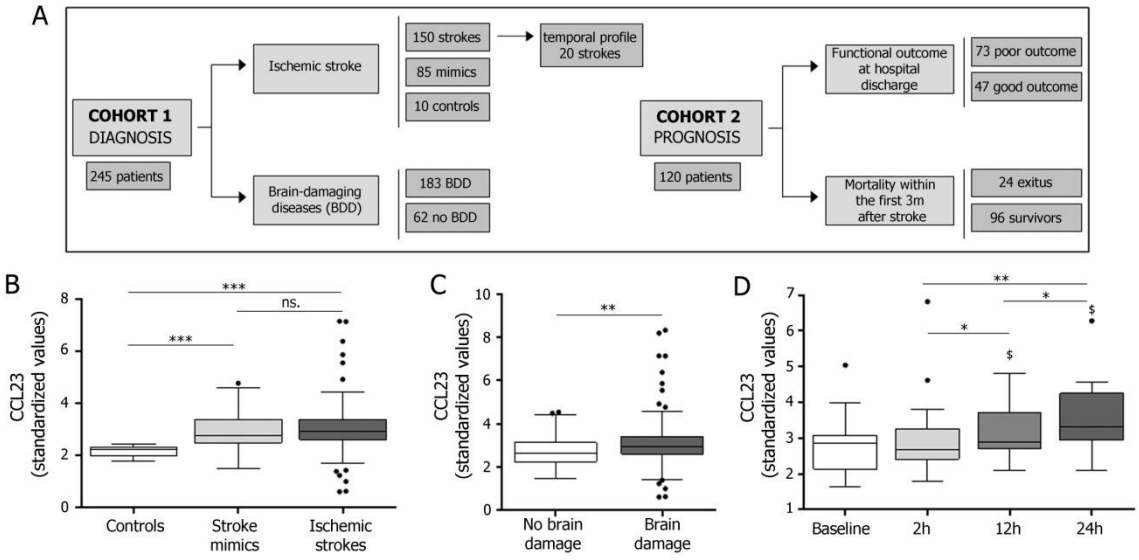

Figure 1. CCL23 blood levels discriminate brain damaging diseases. A. Schematic representation of the cohorts of patients. **B.** Blood levels of CCL23 in controls, stroke mimics and IS patients. **C**. Blood levels of CCL23 in patients suffering from diseases that do and do not entail brain damage. **D.** Temporal profile of CCL23 blood levels in IS patients (n=20). $^{\$}$Indicates significant differences (p<0.05) compared with baseline levels. In all graphs, *p<0.05, **p<0.01, ***p<0.001, and median and interquartile range are indicated.

CCL23 may be a stroke prognosis blood biomarker

The temporal profile of CCL23 after stroke exhibited a continuous increase from baseline (before any therapy was administered) to 24 h after thrombolytic treatment ($p<0.001$) (Fig. 1D). We assessed CCL23 in an independent cohort of 120 IS patients in a three-month follow-up period to investigate whether CCL23 levels were associated with IS patient outcome.

CCL23 blood levels predict patient outcome at hospital discharge

Seventy-three of the 120 patients (60.8%) exhibited poor functional outcome at the time of hospital discharge and presented higher baseline CCL23 levels than did patients who exhibited good functional outcome, as measured by using the modified Rankin Scale (mRS) (523.3 (366.1-802.3) pg/mL vs. 451.1 (302.1-540.8) pg/mL, respectively ($p=0.026$)). Moreover, CCL23 blood levels on admission positively correlates with the NIHSS score at the time of hospital discharge ($p=0.033$; $r=0.119$).

Compared with good functional outcome patients, poor functional outcome patients were older, had more severe strokes (using the NIHSS score) and had a lower prevalence of smoking (Table 1). Through receiver operating characteristic (ROC) curve analysis, we determined a cut-off point for CCL23 (906.51 pg/mL) that was associated with poor functional outcome at hospital discharge, with 19.2% sensitivity and 97.9% specificity ($p=0.006$) (Fig. 2A). Multivariate logistic regression analysis, with adjustment by sex, confirmed that age and the NIHSS score at admission were independent predictors of poor outcome at the end of a hospital stay (Table 2). CCL23 remained an independent predictor of poor outcome when a baseline level of CCL23>906.51 pg/mL was included in this clinical model. The area under the curve (AUC) did not improve significantly, but CCL23 increased the discriminatory ability of the clinical model by 6.17% and provided correct reclassification of 15% of patients into the appropriate predictive risk category (Table 2). Clinical variables alone reached a 60% probability of correctly predicting poor outcome, whereas post-test analysis with the addition of baseline CCL23 levels to the clinical model increased the detection of poor outcome to 78% and decreased the false negative rate to 28%.

CCL23 blood levels predict mortality within the first three months after stroke

Twenty-four of the 120 patients (20%) died within the first three months after stroke. The patients who died were older, had higher NIHSS scores and had a higher prevalence of previous IS (Table 1). Baseline CCL23 levels were elevated in patients who died (642.7 (412.0-1044.7) pg/mL vs. 464.3 (314.2-638.2) pg/mL ($p=0.013$)). We determined a cut-off point of 1027.3 pg/mL for CCL23 (29.1% sensitivity and 96.9% specificity) that was significantly associated with mortality ($p<0.001$). The multivariate logistic regression model, adjusted by sex, confirmed that age and NIHSS score at admission were independent predictors of death within the first 3 months after an ischemic event. CCL23 remained an independent predictor of mortality after the addition of a baseline level of CCL23>1027.3 pg/mL to this clinical model, but it did not significantly improve the AUC. Notably, compared with the clinical model alone, CCL23 enhanced the discrimination of the model by 13.37% (Table 2). The addition of CCL23 to the clinical variables also improved the reclassification rate of patients into the appropriate predictive risk category to 26.72%. Importantly, the new clinical model that included CCL23 significantly increased the accurate prediction of death during the follow-up period from 20% to 67%.

Similar results were obtained by using Kaplan-Meier curves, which confirmed that mortality was significantly higher in patients with baseline CCL23 levels above 1027.3 pg/ml and revealed that death occurred principally within the first month after stroke (Fig. 2B). The highest significant separation between survival curves occurred from day 26 to 45 after an ischemic event ($p<0.001$).

COX regression analysis, adjusted by sex and including age and admission NIHSS score, demonstrated that patients with CCL23 levels above the cut-off value were seven times

Table 1 *Univariate analysis. Clinical characteristics associated with in-hospital outcome and third-month mortality*

		Hospital discharge outcome			Three-month mortality		
Factors	Ischaemic strokes (n = 120)	Good outcome (n = 47)	Poor outcome (n = 73)	P-value	Survivor (n = 96)	Exitus (n = 24)	P-value
Age, years	81 (69–85)	76 (65.5–83)	82 (78–86)	**0.003**	79 (67.5–85)	86 (82–92)	**0.003**
Sex (male)	63 (52.5%)	26 (55.3%)	32 (54.2%)	0.328	49 (51%)	14 (58.3%)	0.552
Admission NIHSS score	10 (5–17)	5 (2.5–10)	14 (7–20)	**<0.001**	9 (4–15.5)	16.5 (14–19)	**<0.001**
Smokers	14 (11.7%)	9 (19.1%)	3 (5.1%)	**0.023**	12 (12.5%)	2 (8.3%)	0.570
Arterial hypertension	91 (75.8%)	37 (78.7%)	44 (74.6%)	0.617	73 (76%)	18 (75%)	0.915
SBP, mmHg	160 (139–181)	150 (130–179)	160 (144–186.5)	0.439	159 (140–180.5)	166 (139–187)	0.850
DBP, mmHg	83.5 (72–91)	79.5 (70–90)	83 (74–90)	0.375	80 (70.5–90)	84.5 (77–105)	0.547
Glycemia, mg dL^{-1}	121.5 (105–152)	118 (100–145.5)	124 (105.5–165.5)	0.085	120 (104–151.5)	125 (112–185)	0.555
Diabetes mellitus	28 (23.3%)	8 (17%)	16 (27.1%)	0.217	22 (22.9%)	6 (25%)	0.829
Dyslipidaemia	60 (50%)	24 (51.1%)	28 (47.5%)	0.712	48 (50%)	12 (50%)	1.000
Atrial fibrillation	48 (40%)	14 (29.8%)	27 (45.8%)	0.093	37 (38.5%)	11 (45.8%)	0.514
Ischaemic cardiopathy	21 (17.5%)	7 (14.9%)	10 (16.9%)	0.774	14 (14.6%)	7 (29.2%)	0.093
Previous stroke	31 (25.8%)	9 (19.1%)	16 (27.1%)	0.337	21 (21.9%)	10 (41.7%)	**0.048**
TOAST classification				0.154			0.293
Atherothrombotic	17 (14.2%)	4 (8.5%)	11 (18.5%)		12 (12.5%)	5 (20.8%)	
Cardioembolic	51 (42.5%)	16 (34%)	28 (47.5%)		39 (40.5%)	12 (50%)	
Lacunar	32 (26.7%)	8 (17%)	4 (6.8%)		12 (12.5%)	0 (0%)	
Undetermined	12 (10%)	14 (29.8%)	13 (22%)		25 (26%)	7 (29.2%)	
CCL23 > 906.51 pg mL^{-1}		1 (2.1%)	11 (18.6%)	**0.008**	–	–	–
CCL23 > 1027.3 pg mL^{-1}		–	–	–	3 (3.1%)	7 (29.2%)	**<0.001**

Statistical significant differences between groups are expressed as bold P-value. DBP, diastolic blood pressure; NIHSS, National Institutes of Health Stroke Scale; SBP, systolic blood pressure; TOAST, aetiology stroke subtype classification.

more likely to die early after an ischemic event (HR adj=6.989 (2.53-19.307), p<0.001).

CCL23 is located in circulating and infiltrating neutrophils in ischemic brains

Brain tissue sections from IS patients who died between 13 h and 5 days after stroke onset were used to identify CCL23 in infarcted tissue (Table SII). CCL23 staining was limited to MPO-expressing neutrophil granulocytes inside blood vessels and the brain parenchyma (Fig. 2C-2D). No CCL23 staining was observed in resident brain cells in any of the studied cases. Sections from non-injured brain regions were negative for CCL23 staining.

Experimental study

We further investigated CCL23 in different experimental models of acute brain injury. However, as rodents lack the *CCL23* gene, we examined two homologous CC chemokines in mice and rats, CCL9 (also known as macrophage inflammatory protein 1 γ (MIP-1γ), macrophage inflammatory protein-related protein 2 (MRP-2), and small inducible cytokine subfamily A member 9/10 (SCYA9/10)) and CCL6 (also known as C10 and small inducible cytokine subfamily A member 6 (SCYA6)). Both CCL9 and CCL6 exhibited a moderate degree of sequence identity (41% and 40%, respectively) and a high degree of 3D structural similarity with human CCL23 (Fig. SI).

CCL9 and CCL6 expression is up-regulated in the brain shortly after cerebral injury

Despite no CCL23 has been detected in the human brain, previous studies reported CCL6 brain expression in rodents [21]. We therefore assessed CCL9 and CCL6 brain expression after cerebral injury to examine the regulation of these chemokines after brain damage.

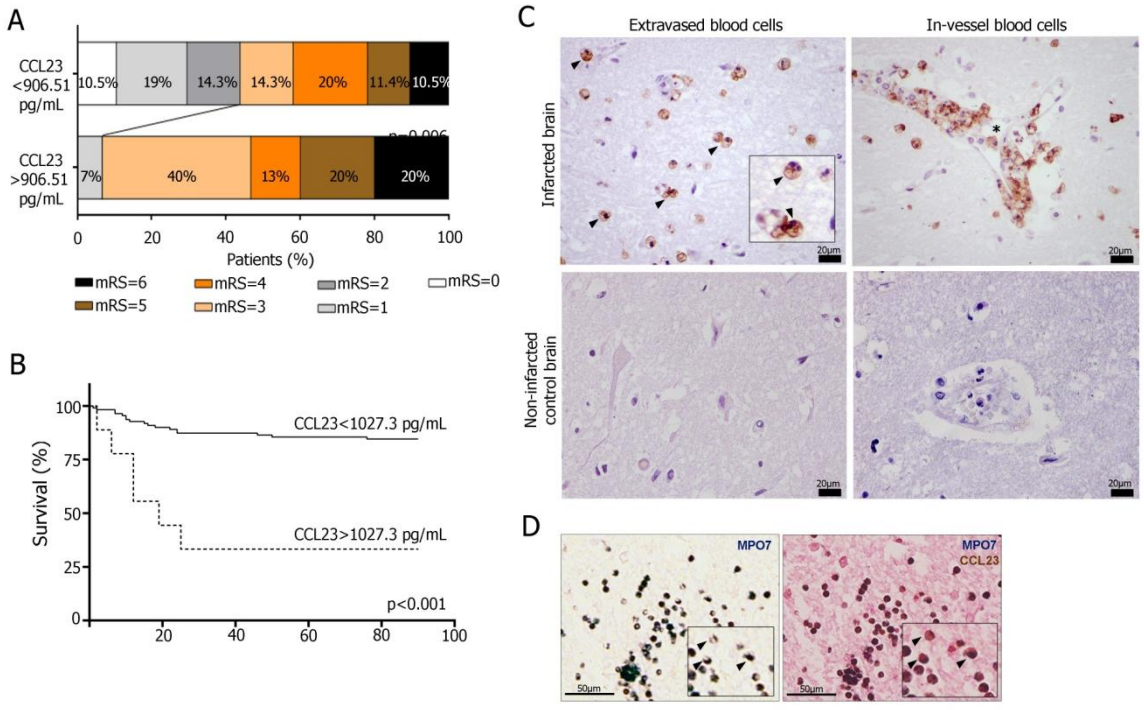

Figure 2. CCL23 blood levels in ischemic stroke patients. A. Stacked bar graph of patients' outcome at hospital discharge according to CCL23 blood levels. Outcome is measured by modified Rankin scale (mRS). Percentages of patients in each category are given. **B.** Kaplan-Meier survival analysis at third month after ischemic event of patients presenting high CCL23 blood levels compared with patients presenting low CCL23 blood levels (n=120). **C.** Representative immunohistochemical stating of CCL23 in infarcted and non-infarcted human brain tissues. Blood cells infiltrated in brain parenchyma are indicated with arrows. Asterisk indicates blood cells within blood vessels. Scale bar = 20 µm. **D**. Consecutive immunological staining of CCL23 and MPO7. Arrows indicate representative co-staining of CCL23 and MPO7. Scale bar = 50 µm. In all graphs, *p<0.05, **p<0.01, ***p<0.001, and median and interquartile range are indicated.

Table 2 *Predictive models comparative for poor outcome at discharge and long-term mortality.*

	Poor outcome at discharge		Long-term mortality	
	Only clinical	Clinical + CCL23	Only clinical	Clinical + CCL23
Logistic regression (OR_{adj})				
Admission NIHSS score	1.219 (1.116–1.332), **<0.001**	1.248 (1.131–1.378), **<0.001**	1.143 (1.052–1.242), **0.002**	1.141 (1.041–1.250), **0.005**
Age	1.041 (1.002–1.080), **0.037**	1.042 (1.002–1.083), **0.038**	1.075 (1.013–1.141), **0.002**	1.095 (1.016–1.181), **0.018**
Sex	0.992 (0.388–2.536), 0.987	0.803 (0.295–2.191), 0.669	0.549 (0.193–1.562), 0.261	0.348 (0.105–1.155), 0.085
CCL23 > 906.51 pg mL^{-1}	–	19.702 (1.815–213.918), **0.014**	–	–
CCL23 > 1027.3 pg mL^{-1}	–	–	–	21.47 (3.434–134.221), **0.001**
ROC curves				
Area under curve (AUC)	0.813 (0.732–0.893)	0.843 (0.771–0.915), 0.153	0.812 (0.728–0.897)	0.857 (0.774–0.941), 0.167
Categorical NRI				
NRI	Ref.	15% (4.95–25.05), **0.003**	Ref.	26.72% (11.05–42.4), **<0.001**
NRI events	–	8.62%	–	13.04%
NRI nonevents	–	6.38%	–	13.68%
IDI statistics				
IDI	Ref.	6.17% (1.56–10.78), **0.008**	Ref.	13.37% (3.23–23.51), **0.009**
IDI events	–	2.77%	–	10.77%
IDI nonevents	–	3.41%	–	2.61%

For logistic regression models, OR_{adj} (95% CI) and *P*-values are given. Biomarkers were added to clinical logistic regression model using the cut-off point of 906.51 ng mL^{-1} for outcome at discharge and 1027.3 ng mL^{-1} for long-term mortality. Clinical model is always used as a reference model (Ref.). AUC: area under the curve; area is given for each model with 95% CI. NRI: net reclassification improvement index (risk categories used: ≤10%. 10–90%, >90%). Percentages of reclassification are given for events, nonevents and for the sum of both (with 95% CI). IDI: integrated discrimination improvement index; index is given for events, nonevents and for the sum of both (with 95% CI). Statistical significance is highlighted in bold.

CCL9 but not *CCL6* mRNA expression was significantly higher in the infarcted brain hemisphere of ischemic rats than in the brain of sham-operated rats 30 min after 90-min transient middle cerebral artery occlusion (tMCAO) (Fig. 3A and 3B).

mRNA up-regulation of both chemokines was acutely pronounced 24 h after ischemic onset and exhibited greater than a 4-fold expression increase in the infarcted hemisphere of ischemic rats ($p<0.001$ for both chemokines). The increased expression of CCL9 and CCL6 mRNA at 24 h was also observed in the infarcted hemisphere of mice subjected to a different approach of cerebral ischemia, a permanent distal occlusion of the middle cerebral artery (pMCAO) (Fig. 3C).

Furthermore, in a mouse TBI model (controlled cortical impact), transcription of *CCL9* and *CCL6* was significantly up-regulated 2 h after an ischemic event ($p=0.006$ for *CCL9*; $p=0.039$ for *CCL6*). This finding demonstrated the rapid up-regulation of *CCL9* and *CCL6* during the hyper-acute phase of not only ischemic stroke, but also of other acute brain injuries (Fig. 3D).

CCL9 and CCL6 localize to neurons after cerebral ischemia in rats

We determined the cellular source of brain CCL9 and CCL6 after acute brain injury. Rat brains obtained 2 and 24 h after cerebral ischemia were immunostained for CCL9 and CCL6. Positively immunostained cells overlapped with cortical neurons from the infarcted and contralateral sides of the brain at both studied time points (Fig. 4A-4B). The staining pattern of both chemokines was inconsistent throughout all cortical areas, but certain isolated groups of cells exhibited the characteristic features of pyramidal neurons. CCL9- and CCL6-positive staining was more pronounced in the ischemic region than in the contralateral region, suggesting a potential role of these chemokines in the brain after IS.

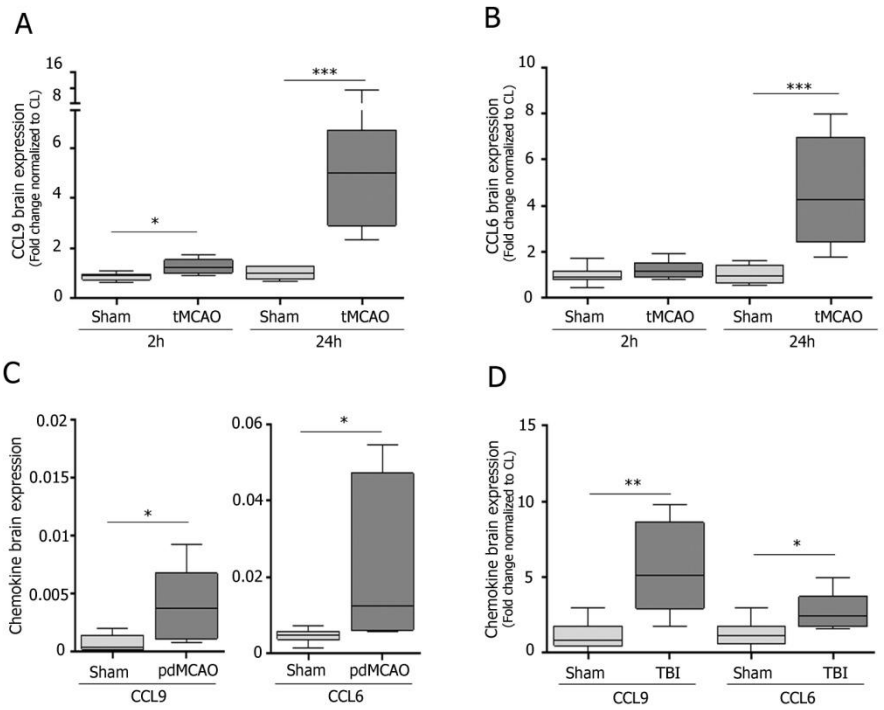

Figure 3. CCL9 and CCL6 cerebral expression after brain damage in rodents. *CCL9* (**A**) and *CCL6* (**B**) brain mRNA expression in sham-control and ischemic rats 2h and 24h after tMCAO (n=5/7 for sham-control animals; n=10 for tMCAO animals). Fold change expression are reported for each animal. **C**. *CCL9* and *CCL6* brain mRNA expression in sham-control and ischemic mice 24h after pdMCAO (n=6/group). Brain expressions of the infarcted area of ischemic animals are expressed in relation to the brain expression of sham-control animals. **D**. *CCL9* and *CCL6* brain mRNA expression in sham-control and TBI mice 2h after the induction of the challenge (n=6/group). Fold change expression are reported for each animal. In all graphs, $^{\#}p<0.1$, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ and median and interquartile range are indicated.

CCL6-positive staining was also observed 24 h after the ischemic challenge in blood cells infiltrating the parenchyma and endothelium (Fig. 4C). Notably, based on their morphology, these CCL6-positive blood cells exhibited the characteristic segmented nuclei of polymorphonuclear granulocytes, as reported for CCL23.

CCL9 and CCL6 present a different profile in circulation after cerebral ischemia in rats

Blood CCL9 and CCL6 levels were measured at different time points (2 h, 24 h and 48 h) after tMCAO and sham-control surgery in rats to investigate the circulating profile after cerebral ischemia.

The circulating levels of CCL9 after cerebral ischemia were lower in the tMCAO group than in the naïve group at all studied time points (p=0.001 at 2 h, p=0.021 at 24 h and p=0.016 at 48 h) (Fig. 4D). By contrast, blood CCL9 levels in sham-operated animals were not lower than those in naïve animals until 48 h (p=0.003 at 48 h) and remained higher than those in ischemic animals during the first hours after cerebral ischemia (p<0.001 at 2 h). Blood CCL6 levels were undetectable in samples obtained from healthy naïve rats and from rats 2 h after the induction of cerebral ischemia or sham-control surgery (Fig. 4E). Circulating CCL6 levels exhibited a similar gradual increase at later time points in ischemic and sham-control animals (p=0.030 and p=0.104 for naïve versus sham-operated or tMCAO animals, respectively, at 48 h).

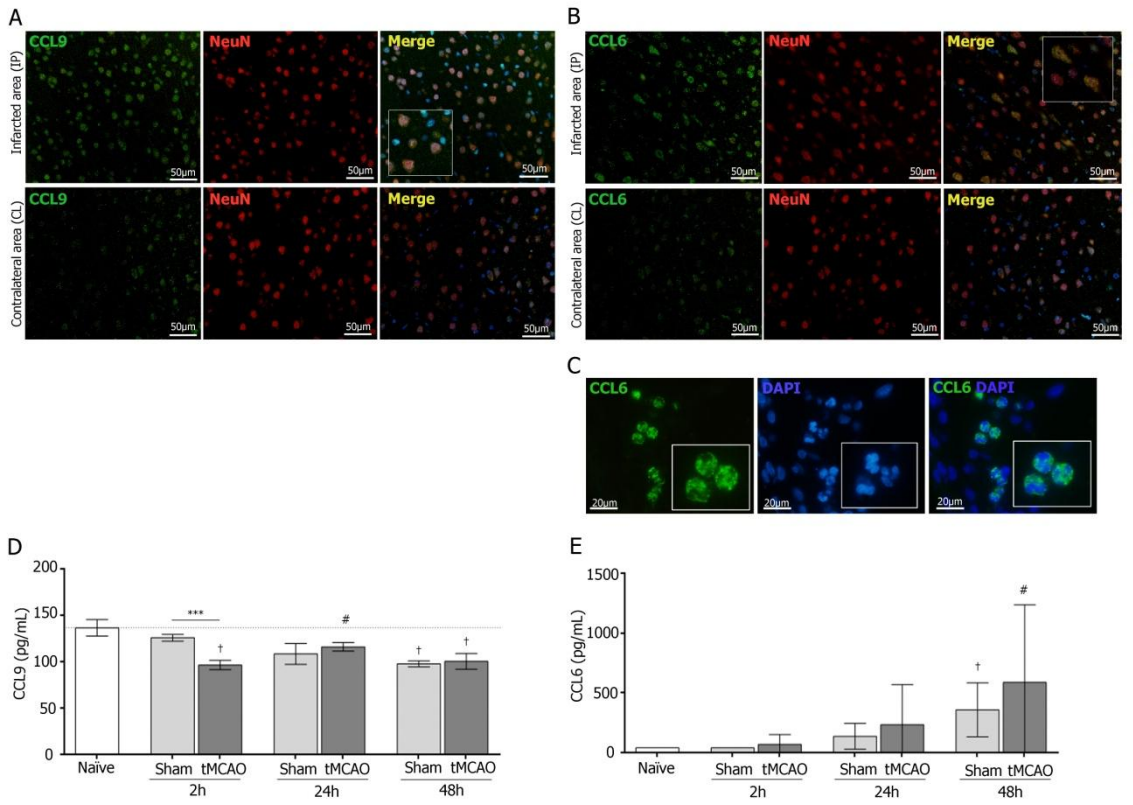

Figure 4. CCL9 and CCL6 brain and blood profiles after brain damage in rodents. Representative immunofluorescence staining of CCL9 **(A)** and CCL6 **(B)** in infarcted and contralateral neurons from ischemic rats 2h after tMCAO. Scale bar = 50 µm. **C**. Representative immunohistochemistry and immunofluorescence staining of CCL6 in polymorphonuclear granulocytes within the brain at 24h after tMCAO in rats. Scale bar = 20 µm. Blood CCL9 (**D**) and CCL6 (**E**) levels in naïve, sham-control and ischemic rats 2h, 24h and 48h after tMCAO (n=6 for naïve and sham-control animals; n=8/12 for 2h and 24h post-tMCAO animals; n=7 for 48h post-tMCAO animals). In all graphs, $^{\#}$p<0.1, ***p<0.001 and † indicates p<0.05 for the comparison with the reference group (naïve animals). Mean and SD are indicated.

DISCUSSION

Information regarding the CCL23 chemokine is limited. To the best of our knowledge, no previous study has described a strong relationship between CCL23 and acquired brain diseases. This investigation provides new evidence that patients with acute cerebral lesions present higher baseline levels of circulating CCL23 than do patients with conditions mimicking these brain injuries or healthy controls. Moreover, the CCL23 levels in circulation at baseline were associated with poor IS outcome and may have greater value than typical clinical markers for the prediction of stroke-related functional outcome and mortality.

The hyperacute inflammatory reaction triggered by an acquired cerebral lesion involves immediate activation of the chemotactic transduction pathways, which promote the recruitment of mature immune cells to the injured brain. We demonstrated that circulating CCL23 increased rapidly after an acquired cerebral lesion. In agreement with its previously published chemotactic functions, circulating CCL23 may rapidly move toward the damaged brain and favor infiltration of peripheral immune cells to the site of injury[10]. The present study further revealed that circulating and brain parenchyma-infiltrating neutrophils expressed CCL23 after IS, which supports the previous described ability of human neutrophils to produce this chemokine in an inflamed environment[22]. Notably, these cells even sustained CCL23 expression once they were inside the brain, probably to further enhance the on-going inflammatory response. Additionally, cerebral expression of CCL23 was maintained up to the subacute phase of IS, suggesting that cerebral CCL23 may exert functions other than the promotion of inflammation at later times after stroke. One possible hypothesis is that released CCL23 in the brain might participate in processes related to neuronal repair, including endothelial cell migration and angiogenesis, as suggested by the described functions of this chemokine in vitro[6]. However, further studies are needed to verify this hypothesis.

The present study demonstrated that the circulating CCL23 levels may potentially serve as a biomarker to diagnose acute inflammatory reactions in the brain after cerebral damage. Other chemotactic molecules have been previously proposed as potential biomarkers of acquired brain lesions, especially IS. Particular emphasis has been placed on CXC chemokine ligand 12 (CXCL12) (also known as stromal cell-derived factor 1 (SDF-1)), which participates in leukocyte migration and contributes to vascular remodeling and neovascularization of ischemic tissue[23], [24]. Comparable to CCL23, CXCL12 is also significantly elevated in the circulation of IS patients, and its serum levels are positively correlated with infarct volume[25], [26]. However, the potential of CXCL12 for stroke diagnosis has been questioned. Studies have reported differences in circulating CXCL12 levels between IS patients and healthy controls, but no variations between IS patients and non-ischemic patients have been reported[27]. These previous results are similar to our findings on CCL23; we were unable to discriminate between IS patients and stroke mimics based on CCL23 levels. These results cast doubt on the relevance of these chemokines for the specific diagnosis of acute cerebral conditions, such as IS, but CCL23 may play a prominent role in the identification of acute inflammatory responses shortly after a brain-damaging event. Future clinical implementations of these brain damage-related inflammatory markers, including CCL23, may improve clinical treatment by permitting early identification of acute brain lesions regardless of the cause and may also be useful for ruling out brain-damaging diseases in patients who present nonspecific symptoms of altered brain functions (such as vertigo or confusion).

Identification of robust biomarkers to predict patient outcome after any cerebral lesion also remains challenging. In this study, the early measurement of circulating CCL23 was an independent prognostic factor for functional disability and mortality within the first three months after IS. Specifically, CCL23 provided substantial prognostic information for clinical data, which may facilitate the decision-making processes of clinicians for optimal patient care, including admission to specialized

stroke-recovery units where patients could potentially receive complementary therapies and benefit from stroke rehabilitation programs that improve functional outcomes and quality of life[28], [29]. Moreover, in our cohort, CCL23 exhibited high specificity for the prediction of poor outcome after stroke and may assist physicians in decision-making processes involving aggressive therapies, such as hemicraniectomy in malignant middle cerebral artery (MCA) infarcts or guide withdrawal of care orders.

Similar to CCL23, the abovementioned CXCL12 and CC chemokine ligand 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP-1), increase in circulation after stroke, and higher blood levels indicate poor long-term outcome and stroke recurrence[30]–[32]. These results support the hypothesis that specific pro-inflammatory chemokines may potentially reflect the stroke-related inflammatory response, which is a key factor predicting patient outcome, and high circulating CCL23 levels during the first days after stroke may influence the disease course, as well as patient prognosis and survival. Nevertheless, we cannot conclude from these findings whether this pronounced up-regulation of CCL23 contributes to the extension of the ischemic lesion or merely take place as a protective response mechanism for an attempt to mitigate the on-going pathological process. Further studies might determine the functional significance of this chemokine after stroke.

We complemented our clinical findings with descriptive studies in experimental models of cerebral damage to further characterize the role of CCL23 after brain injury. These studies were chiefly focused on ischemic animals subjected to the intraluminal tMCAO model. However, a different stroke model (pdMCAO) and a model of TBI were also used to support these novel findings and assess whether those results were common features to acute brain-damaging diseases. We primarily investigated two previously described rodent chemokines as potential CCL23 homologues in mice and rats, CCL9 and CCL6[33]–[35]. Both CCL9 and CCL6 participate in the suppression of hematopoiesis, stimulate cell activation and recruitment and contribute to immune cell trafficking in a similar manner to CCL23 via the CCL9/6-CCR1 pro-inflammatory axis[36]–[39]. Even though the human and rodent counterparts exhibited similar functions under inflammatory conditions, our findings revealed slight discrepancies in the performance of these homologous chemokines, not only between species but also between rodent CCL9 and CCL6.

The present study reported CCL9 expression in rodent brains. Notably, our findings indicated that *CCL9* mRNA expression markedly increased early after IS or TBI, supporting the involvement of this chemokine in the molecular mechanisms underlying cerebral damage. However, we cannot exclude the specific functions of CCL9 in neurons, and future studies should investigate the mechanisms and pathways of CCL9 in cerebral lesions. We also demonstrated high levels of CCL9 in the circulation of naïve rats. Other authors have reported similar results in mice and have postulated that CCL9 may have unrecognized homeostatic functions for normal body maintenance[39]–[41]. In our study, the induction of focal cerebral ischemia in rats produced a sudden decrease in blood CCL9 levels early in the acute phase of this pathological process. Certain circulating immune cells alter their homeostatic chemokine expression patterns during acute inflammation and release potent inflammatory mediators that contribute to the rapid amplification of the inflammatory response[40]. This scenario may explain the observed decrease in circulating CCL9. However, unknown CCL9 functions may also result in a decrease in its physiological levels in circulation after stroke.

In contrast to CCL9 expression, CCL6 expression in rodent brain tissue has been previously reported by Kanno and colleagues[21], who demonstrated that microglia express CCL6 under physiological and inflammatory conditions and that CCL6 participates in the inflammatory-related recruitment of outlying resident brain cells. We did not find CCL6 expression in microglia within infarcted brains; however, this chemokine was expressed in neuronal cells, and its up-regulation was highly pronounced in the injured region of the brain within the first

24 h after cerebral damage. No circulating CCL6 was detected acutely after cerebral ischemia or in naïve animals. A slight increase in CCL6 in the blood was observed at later time points, but this increase was not specific to ischemic animals since it was also observed in sham-control animals. Many studies have demonstrated an early increase in circulating CCL6 under pro-inflammatory conditions[36], [42], [43]. Coelho and colleagues demonstrated that CCL6 neutralization enhances sepsis-related mortality in mice, and superacute administration of recombinant CCL6 after the induction of a severe systemic inflammatory response significantly improved the survival of these mice[36]. However, other models of inflammation have demonstrated that CCL6 is strongly induced at later stages and has beneficial effects that favor the resolution of inflammation[42], [43]. Notably, we have also reported CCL6-positive staining in blood cells infiltrating the brain parenchyma and endothelium 24 h after an ischemic challenge, thus reinforcing the hypothesis that CCL6 production by blood immune cells may gradually increase after an ischemic event. Whether the role of CCL6 is crucial in the early stages of cerebral lesions is currently unknown.

Overall, these results question homology and cast doubt on the similarities among CCL23, CCL9 and CCL6, since they seem not to share all functional features following brain damage, especially in brain. Thus, our findings on CCL9 and CCL6 complicate the use of preclinical models for further study of the detailed mechanisms of brain damage or repair related to human CCL23 in the brain.

Our study has several limitations that should be overcome in future studies. We observed a substantial increase in circulating CCL23 levels in patients presenting different types of acquired brain injuries, but we focused our study on CCL23 in IS patients. Therefore, further studies are needed to investigate CCL23 levels in brains and blood from patients with other cerebral diseases, including TBI and brain tumors. Moreover, the sample size of healthy control patients in the first cohort of this study was relatively small; future studies should use larger cohorts of patients with cerebral lesions and healthy subjects. Finally, despite increased CCL9 and CCL6 brain expression was observed after different approaches of acute cerebral lesions (cerebral ischemia and TBI) further determinations of CCL9 and CCL6 brain localization and circulating abundances were only assessed in the ischemic rat. It would be interesting, however, to further corroborate and expand these findings in other experimental models of acute brain injury, as well as to continue investigating their beneficial or deleterious functional effects following these brain injuries.

In conclusion, this study provided the first reported evidence of the outstanding potential of CCL23 as a promising biomarker for the diagnosis of cerebral lesions and reported that circulating CCL23 levels may serve as a biomarker of stroke patient outcome and subsequently affect decision-making regarding the management and treatment of these patients. We also demonstrated that two previously described CCL23 homologous proteins (CCL9 and CCL6) in rodents are involved in brain-damaging diseases. However, these rodent chemokines do not share all features with human CCL23 under brain damage-related inflammatory conditions.

CONFLICT OF INTEREST STATEMENT

None.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article

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CCL23: A new CC chemokine involved in human brain damage

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SUPPLEMENTARY METHODS

CCL23 blood immunoassays

A sandwich ELISA array including myeloid progenitor inhibitory factor 1 (MPIF-1/CCL23) was assayed to assess the levels of this chemokine in circulation (SearchLight®, Aushon BioSystems, USA). The detection of the chemiluminescent signal was performed with a cooled CCD camera (Pierce, USA) and data was analyzed with ArrayVision 8.0 software (Imaging Research, USA). The sensitivity for CCL23 measurement in this array was 1.17 pg/mL.

Human MPIF-1 ELISA Kit (RayBiotech Inc., USA) was used to assess temporal circulating profiles of CCL23 in a subset of ischemic stroke patients and baseline CCL23 levels in a subgroup of stroke mimics (sensitivity: 7 pg/ml), and human CCL23/MPIF-1 ELISA kit (R&D Systems Inc., USA) was used to test serum samples in Cohort 2 (sensitivity: 15.6 pg/mL). In these ELISA kits, optical density at 450 nm was measured in a Synergy™ Mx microplate reader (BioTek Instruments Inc., USA).

All CCL23 immunoassays were performed following manufacturer's instructions and blinded to clinical data. Each serum sample was assayed in duplicate and the mean value of both measurements was reported in pg/mL. When CCL23 values were under the detectable range of the assay, the limit of detection value was assigned to them. Replicates with a coefficient of variation (CV) >20% were discarded for statistical analyses. In those cases in which the inter-assay CV was higher than 20%, values were standardized prior to statistical analysis by calculating the Z-score value by substracting the mean and dividing by the standard deviation (SD) of each plate and adding three units to avoid results below zero in any sample.

To ensure an optimal interpretation of the CCL23 measurements, we performed an additional analysis to determine whether CCL23 determinations under the different CCL23 ELISA kits were comparable. Robust correlations were obtained between SearchLight® ELISA kit and RayBiotech ELISA kit (n=20, R=0.636, p=0.003) and between RayBiotech ELISA kit and R&D ELISA kit (n=20, R=0.881, p<0.001).

CCL23 and MPO7 brain immunostainings

Human CCL23 was evaluated by means of immunohistochemistry in human brain tissues. In brief, 3µm sections were mounted onto glass slides and kept at 4ºC until use. Sections were heated at 65ºC for 1 h and were subjected to a standard deparaffinization procedure with a final wash in 0.1% Tween-Tris buffered saline (TBST). Brain sections were submitted to 15 min of

antigen retrieval using citrate buffer (pH 6, 95º) and were blocked with 10% goat serum (Millipore, USA) in TBST. Slides were hereafter incubated overnight at 4ºC with a rabbit antibody against Ct-CCL23 (anti-macrophage inflammatory protein 3, 1/50, Abcam, UK) diluted in blocking solution. Slides were incubated with biotinylated anti-rabbit IgG (1/250) (Vector Laboratories Inc., USA) followed by horseradish peroxidase-conjugated streptavidin (1/500) (Vector Laboratories Inc.). Finally, brain sections were incubated with Liquid DAB+ (diaminobenzidine) (Brown, Dako, USA), stained with Harris hematoxylin (Sigma Aldrich Inc., USA) and mounted on coverslips using DPX mounting medium (Sigma Aldrich Inc.). Negative controls were performed without applying the primary antibody. Brain sections were analyzed using an Olympus BX61 microscope (Olympus, Japan) and the same intensities and parameters were set for all slides. Images were processed with the Olympus CellSens Imaging software (Olympus).

MPO7 was also evaluated by means of immunohistochemistry using the same protocol. In this case, a mouse primary antibody against MPO7 (mieloperoxidase-7, 1/25, Abcam), a biotinylated anti-mouse IgG (1/250, Vector Laboratories Inc.) and a horseradish peroxidase-conjugated streptavidin (1/500) (Vector Laboratories Inc.) were used. Sections were finally incubated with Vector SG Peroxidase (HRP) substrate kit (Blue, Vector Laboratories Inc.).

To perform consecutive histochemical tests, mounted sections stained with MPO7 were incubated 15 min in xylene to remove the coverslips, and the above-described protocol was restarted again to stain the same brain section with CCL23.

Animal models of brain damage

Transient intraluminal MCAO model (tMCAO)

Transient infarction in the territory of the MCA was induced in rats by introducing an intraluminal filament, as previously described [1,2]. In summary, animals were anesthetized under spontaneous respiration with volatile anesthesia (2% isoflurane) in oxygen and body temperature was maintained at 37ºC during the whole procedure. After surgical exposure of the bifurcation of the external carotid artery (ECA) and internal carotid artery (ICA) on the right side, a silicone-coated nylon monofilament (Doccol Corporation, USA, reference number: 403723PK10) was introduced to occlude the MCA. After occlusion, animals were allowed to recover from anesthesia. Ninety minutes later, animals were re-anesthetized and filament was removed to induce reperfusion of the cerebral blood flow (CBF). Regional CBF was monitored during the surgery with a laser Doppler probe (Moor Instruments, UK), and only animals that exhibited a reduction of >75% of CBF after filament introduction and a recovery of >75% after filament removal were included in the study.

Three independent groups were studied with this model of cerebral ischemia: 1) 90-min tMCAO followed by 30-min reperfusion (2 h post-tMCAO), 2) followed by 24 h reperfusion (24 h post-MCAO) and 3) followed by 48 h reperfusion (48 h post-tMCAO).

1) 2 h post-tMCAO group

A total of 34 rats were used. Twelve animals were subjected to sham surgery and 22 were submitted to tMCAO. One sham-operated animal died during the surgery and 9 of the ischemic animals were excluded after applying the following criteria: inappropriate occlusion or reperfusion of the MCA (n=6), surgical bleedings (n=1), death during surgery (n=2).

2) 24 h post-tMCAO group

Thirty-five rats were used. Six rats were sham-operated animals and 29 were submitted to tMCAO. Fourteen of the ischemic animals were excluded after applying the following criteria: inappropriate occlusion or reperfusion of the MCA (n=4), death during surgery (n=1), death within the first 24 hours after MCAO (n=5) or incomplete cardiac perfusion during euthanasia (n=4).

3) 48 h post-tMCAO group

A total of 20 animals were needed. Seven were sham-control animals and 13 were submitted to tMCAO. Six out of those 13 were excluded due to the following criteria: inappropriate occlusion

or reperfusion of the MCA (n=1), death within the first 24 h after MCAO (n=3) and death between 24 h and 48 h after tMCAO (n=2).
Eight naïve rats were included as healthy controls for the tMCAO experiments.

Permanent distal MCAO model (pdMCAO)

Focal cerebral ischemia was induced by permanent occlusion of the MCA distal of the lenticulostriate arteries as previously described [3]. Briefly, mice were anesthetized with volatile anesthesia (isoflurane) and placed in lateral position. After skull exposure, the MCA was permanently occluded using bipolar electrocoagulation forceps. Permanent occlusion of the MCA was visually verified before suturing the wound. During the surgery, body temperature was maintained using a feedback-controlled heating pad.
A total of 12 animals were used to perform the pdMCAO sub-study. Six were submitted to the pdMCAO and six were used as sham-operated control mice.

Traumatic Brain Injury (TBI) model

TBI was induced through a controlled cortical impact (CCI), as previously described [4]. In brief, mice were anesthetized under isoflurane effects and positioned in a stereotactic frame. A 5-mm craniotomy was performed over the left parietotemporal cortex, and the bone flap was removed without disrupting the dura. CCI was performed with a 3-mm flat-tip impounder, 5 m/second velocity, 0.6 mm depth and 150 milliseconds impact duration. All mice were allowed to recover under a heat lamp and were conscious and able to move after approximately 5 min.
Fifteen mice were used. From those, seven were sham-operated animals and eight were submitted to a CCI. One mouse from the sham group and two mice from CCI group were excluded due to tissue cutting injury-associated visible bleedings on the brain surface during craniotomy procedure or right after CCI. Finally, n=6 mice per group in both sham and CCI groups were included.

Sample Collection

All animals were deeply anesthetized at their respective times. Blood samples were collected from rats through cardiac puncture, contained in EDTA collection tubes and centrifuged at 1,500 g at 4°C for 10 min. Plasma samples were stored at -80°C until further use.
All animals were transcardially perfused with cold saline and ipsilateral and contralateral hemispheres of the brain were carefully isolated and separately frozen at -80ºC. Four additional rats were also transcardially perfused with cold saline, and their brains were removed and formalin fixed, and embedded with paraffin. Sections of 3 µm were cut, mounted onto glass slides and kept at 4ºC.

Quantitative real-time PCR (qPCR)

Total RNA was isolated from brain tissue samples using the MirVana™ Paris™ (Thermo Fisher Scientific) or the Arcturus PicoPure RNA Isolation kit (Applied Biosystems), following the manufacturer's instructions. RNA concentrations were measured with a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc.). Reverse transcription was performed with the High Capacity complementary DNA Archive Kit (Applied Biosystems, USA) or the RT2 PreAMP cDNA synthesis Kit (Qiagen). Real-time polymerase chain reaction was completed with TaqMan® Gene Expression Assays (Applied Biosystems), using the following probes: CCL6 (Rn01456400_m1), CCL9 (Rn01471276_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Rn99999916_s1) and beta-2 microglobulin (B2M) (Rn00560865_m1). The amplification procedure was conducted on an ABI7900HT real-time polymerase chain reaction system (Applied Biosystems) and data was analyzed with the Applied Biosystems SDS 7900 system software. The amplification of each sample was run in triplicate. Relative quantification (RQ) values were calculated according to the $2^{-\Delta\Delta CT}$ method, with reference to the expression of

these housekeeping genes (GAPDH and B2M). For mouse samples after pMCAO, CCL6 and CCL9 expression was determined by obtaining results from a RT2 Profiler PCR Array for chemokines and chemokine receptors (PAMM-022Z, Qiagen). This array was run on a Roche LightCycler 480 following the manufacturer's instructions. Data were analyzed with RT2 Profiler PCR Array Data analysis software (version3.5) from SABiosciences and results for other chemokines than CCL6 and CCL9 of were previously reported [5]. Values are expressed as fold change of the ipsilateral compared to the contralateral hemisphere, for the tMCAO and TBI groups, and as relative quantifications of the ipsilateral hemisphere alone for the pMCAO group.

CCL9 and CCL6 brain immunostaining

CCL9 and CCL6 were evaluated by means of immunohistochemistry and/or immunofluorescence in rat brain tissues obtained 2 h and 24 h after the ischemic challenge. Paraffin-embedded samples were used. Three micrometers sections were mounted onto glass slides and kept at 4ºC until use. The immunohistochemical procedure that was followed was the same as that used for the CCL23 staining in human brain tissues. In this case, antigen retrieval procedure was carried out using EDTA/Tris buffer (pH 9, 95ºC) and the primary antibodies were Macrophage Inflammatory Protein 1 gamma/CCL9 Antibody (1/100) (Bioss Antibodies Inc., USA) and anti-CCL6 antibody (1/500) (Abcam).

For the immunofluorescence staining, 3-micrometer sections of paraffin-embedded samples were heated at 65ºC for 1 h and were subjected to a standard deparaffinization procedure with a final wash in 0.1% Tween-Tris buffered saline (TBST). Brain sections were submitted to 15 min of antigen retrieval using EDTA/Tris buffer (pH 9, 95º) and were blocked with 10% goat serum (Millipore, USA) in TBST. Slides were hereafter incubated overnight at 4ºC with rabbit anti-CCL6 or anti-CCL9 (1/500) (Abcam) and mouse anti-NeuN antibodies (1/200) (Millipore). The following day, slides were incubated with AlexaFluor® 488 anti-rabbit IgG and AlexaFluor® 568 anti-mouse IgG (Life Technologies, USA). A final incubation with Sudan Black B was performed to reduce brain tissue autofluorescence[6] and sections were mounted on coverslips using Vectashield with 46-diamidino-2-phenyl indole (DAPI; Vector laboratories). Negative controls were performed without applying the primary antibody. Brain sections were analyzed using an Olympus BX61 microscope (Olympus, Japan) and the same intensities and parameters were set for all slides. Images were processed with the Olympus CellSens Imaging software (Olympus).

CCL9 and CCL6 blood immunoassays

Quantitative determinations of rat CCL9 and CCL6 were conducted in plasma were performed using Rat MIP-1 gamma ELISA kit (MyBioSource, Inc. USA) and Rat C-C Motif Chemokine 6 (CCL6) ELISA Kit (Cusabio Biotech Co., USA), respectively. All procedures were conducted as per manufacturers' instructions. Optical densities were assessed with a microplate reader (Synergy Mx). The minimum detection value of these kits was 2.0 pg/mL and 39 pg/mL for CCL9 and CCL6, respectively. Each sample was assayed twice and the mean value of both measurements was used. Replicates with a CV >20% were discarded for statistical analyses. When CCL6 values were under the detectable range of the assay, the limit of detection value was assigned to them. The mean inter-assay CV was lower than 20% for both proteins.

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SUPPLEMENTARY TABLES

Factors	Ischemic strokes (n=150)	Stroke mimics (n=85)	Healthy controls (n=10)	p-value
Age, years	71.91 ± 13.23	67.08 ±16.519	68.5 ±11.63	0.150
Sex (Male), n (%)	77 (51.3%)	49 (57.6%)	4 (40%)	0.453
Admission NIHSS score	15.47 ±5.34	-	-	-
Smokers, n (%)	28 (20.4%)	3 (6%)	1 (10%)	0.052
Arterial hypertension, n (%)	85 (58.6%)	10 (20%)	3 (30%)	**<0.001**
Diabetes mellitus, n (%)	30 (20.5%)	6 (12%)	0 (0%)	0.128
Dyslipidemia, n (%)	43 (29.9%)	5 (10%)	5 (50%)	**0.005**
Atrial Fibrillation, n (%)	53 (36.3%)	2 (4%)	-	**<0.001**
Ischemic cardiopathy, n (%)	25 (17.1%)	2 (4%)	0 (0%)	**0.027**
Previous stroke, n (%)	25 (17.1%)	11 (22%)	0 (0%)	0.442
TOAST classification				
Atherothrombotic, n (%)	37 (25.7%)	-	-	-
Cardioembolic, n (%)	69 (47.9%)	-	-	-
Undetermined, n (%)	33 (22.9%)	-	-	-

Table SI. Demographic and clinical factors from ischemic strokes, stroke mimics and controls. Age and NIHSS score at admission are expressed as mean ± SD, and all other clinical variables are expressed as frequency of patients that present the specified condition. Statistically significant p-values are highlighted in bold. Abbreviations: NIHSS: National Institutes of Health stroke scale; TOAST: etiology stroke subtype classification.

Case	Age (years)	Sex	Admission NIHSS score	TOAST etiological classification	Exitus (hours after stroke onset)	Exitus to necropsy timelapse (hours)
1	72	Male	19	Atherothrombotic	13	3
2	75	Male	22	Undetermined	19	5
3	80	Male	20	Cardioembolic	101	4.5
4	50	Female	19	Lacunar	123	9

Table SII. Demographic and clinical data of the ischemic stroke patients included in the brain study.

SUPPLEMENTARY FIGURE

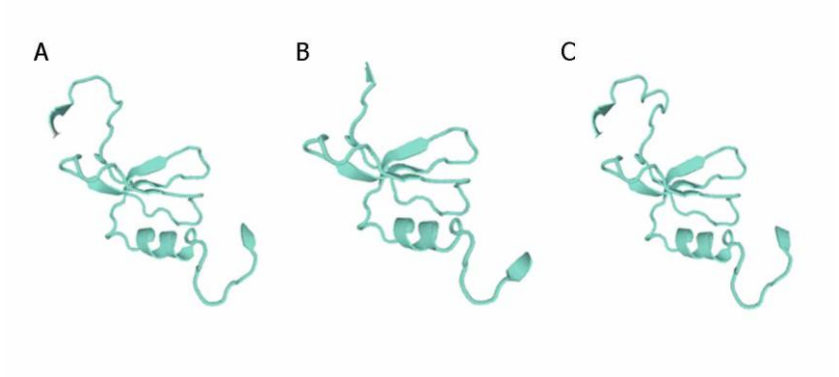

Figure SI. Tridimensional structure of the human chemokine CCL23 (**A**) and rodent CCL6 (**B**) and CCL9 (**C**). Images obtained from the SWISS-MODEL repository, Swiss Institute of Bioinformatics, Switzerland.

(Annex to "CCL23: A new CC chemokine involved in human brain damage")

Unraveling the therapeutic relevance of CCL23 in an experimental stroke model in rats – A pilot study

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Introduction and aims

CCL23 is a functionally-unknown human chemokine that has been recently reported to be a promising biomarker for the diagnosis of cerebral lesions and the prognosis of stroke patients' outcome [1]. Beyond this biomarker capacity, we aimed to explore whether CCL23 is also clinically relevant as a therapeutic target for the treatment of ischemic stroke. To that end, we performed a pilot study to evaluate CCL23 capacity to modify functional outcome and infarct volume after cerebral ischemia in rats by intravenously administrating human recombinant CCL23 (hrCCL23) early after stroke onset.

Materials and methods

All experimental procedures were conducted in compliance with the Spanish legislation and in accordance with the Directives of the European Union, and performed as thoroughly explained in [1], unless otherwise stated here. In brief, rats were randomly assigned to one of the 2 subsequent treatments: hrCCL23 (R&D Systems, cat#.131-M1-025/CF) or Vehicle (PBS). All rats were subjected to 90-min middle cerebral artery occlusion (MCAO) followed by 48h of reperfusion. Treatments (hrCCL23 or Vehicle) were intravenously administered via the retro-orbital sinus immediately after MCAO and 24h after reperfusion (n=7/group) (Figure 1A). The dose of hrCCL23 (3.7 µg/kg) was derived from a combination of dose equivalence calculations from the mean human CCL23 circulating levels in the acute phase of ischemic stroke [1] and literature-based doses from other studies regarding CC chemokines [2], [3]. Neurological deficit scores were evaluated immediately before reperfusion and at 24h and 48h after MCAO using a 9-point neurological deficit score (Modified Bederson test) [4]. Infarct volumes were also evaluated at 48h using 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining as described [5]. All neurological scores and infarct volumes were assessed in a blind manner.

Results

Treatment with hrCCL23 did not cause any effect on the extension of the ischemic lesion 48h after MCAO (p=0.603) (Figure 1B). No differences in the mortality rate were either observed between hrCCL23-treated and non-treated animals 48h after ischemia (Figure 1C). Interestingly, a slight improvement in the neurological state of animals treated with hrCCL23 was sensed 24h and 48h after ischemia when compared to the vehicle group (p=0.097 for both time-points) (Figure 1D).

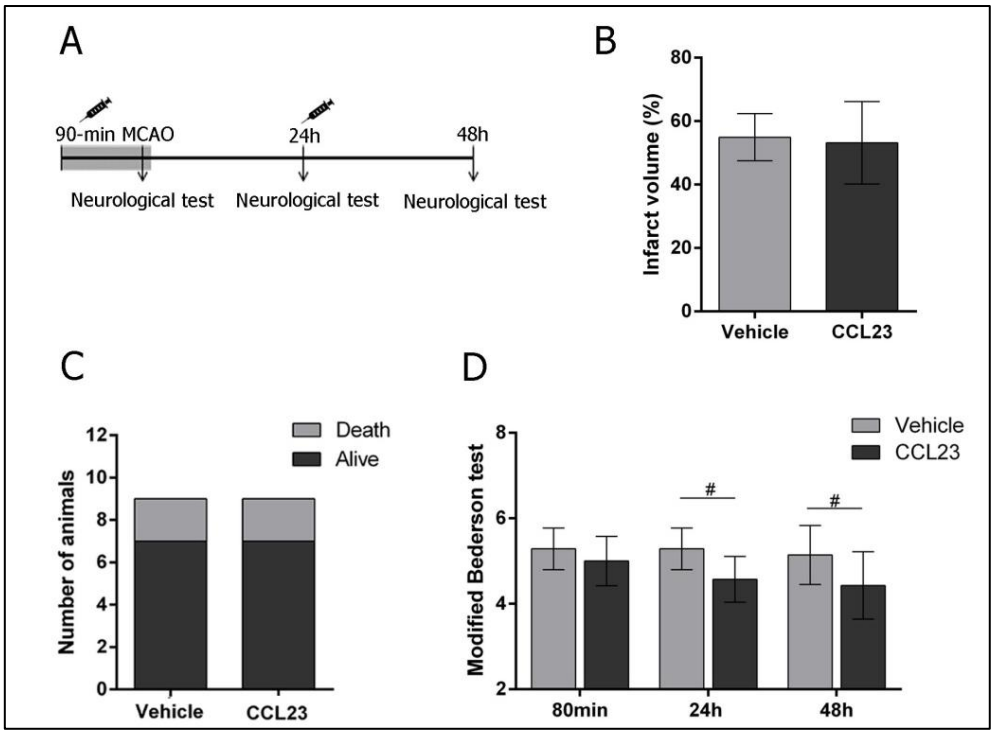

Figure 1. **Effect of hrCCL23 treatment after cerebral ischemia.** (**A**) Experimental design of the study. (**B**) Effect of hrCCL23 treatment on infarct volume, expressed as percentage of the contralateral hemisphere (n=7/group). (**C**) Graph showing mortality rates. Bars represent the number of animals that survived or died 48h after MCAO. (**D**) Effect of hrCCL23 treatment on the neurological deficit at 3 time-points after MCAO. Statistical comparisons were performed through a (B) Mann-Whitney test or a (D) two-way ANOVA followed by Sidak's multiple comparison tests and columns indicate mean ± standard deviation. # $p<0.1$.

Conclusions

In this pilot study we showed that hyper-acute hrCCL23 treatment was not able to reduce brain lesions in rats subjected to transient cerebral ischemia. Conversely, we observed that hrCCL23 tended to improve the neurological state of treated animals, which suggest a tentative –but still quite hypothetic- neuroprotective effect for this functionally-unknown chemokine on the outcome of ischemic animals.

At this preliminary point, we are aware that we cannot certainly assume any reliable beneficial involvement of CCL23 in the protective mechanisms that fight against ischemia. However, we consider that these initial findings are a promising starting point from which future contributions might arise. In this same regard, we strongly believe that new studies with larger cohort of animals, adjusting doses and administration times, performed on different stroke models or using other species and strains are needed to fully determine whether hrCCL23 has a therapeutic relevance in ischemic stroke.

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Characterization of the rat cerebrospinal fluid proteome following acute cerebral ischemia using an aptarmer-based proteomic technology

Characterization of the rat cerebrospinal fluid proteome following acute cerebral ischemia using an aptamer-based proteomic technology

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The limited accessibility to the brain has turned the cerebrospinal fluid (CSF) into a valuable source that may contribute to the complete understanding of the stroke pathophysiology. Here we have described the CSF proteome in the hyper-acute phase of cerebral ischemia by performing an aptamer-based proteomic assay (SOMAscan) in CSF samples collected before and 30min after male Wistar rats had undergone a 90min Middle Cerebral Artery Occlusion (MCAO) or sham-surgery. Proteomic results indicated that cerebral ischemia acutely increased the CSF levels of 716 proteins, mostly overrepresented in leukocyte chemotaxis and neuronal death processes. Seven promising candidates were further evaluated in rat plasma and brain (CKB, CaMK2A, CaMK2B, CaMK2D, PDXP, AREG, CMPK). The 3 CaMK2 family-members and CMPK early decreased in the infarcted brain area and, together with AREG, co-localized with neurons. Conversely, CKB levels remained consistent after the insult and specifically matched with astrocytes. Further exploration of these candidates in human plasma revealed the potential of CKB and CMPK to diagnose stroke, while CaMK2B and CMPK resulted feasible biomarkers of functional stroke outcome. Our findings provided insights into the CSF proteome following cerebral ischemia and identified new outstanding proteins that might be further considered as potential biomarkers of stroke.

Stroke is one of the most frequent causes of mortality worldwide and is still considered the leading cause of permanent adult disability in developed countries.[1] Despite major improvements have been achieved in the management of stroke patients in regards to acute therapies[2,3], there is still a need for improving the diagnosis, prognosis and the treatment of patients that had suffered from this devastating disease. To that end, a complete understanding of the key molecular changes that happen as a consequence of the reduction of glucose and oxygen supply to the brain is necessarily required, and has been a matter of study during the last decade for many experts on this field.[4]

Due to limited accessibility to the brain, the study of these cellular and molecular alterations remains laborious. It is not surprising, then, that remarkable efforts have been directed to the systemic identification and evaluation of molecular mediators of ischemic injury that accurately reflect the stroke pathology and its severity. Remarkably, due to the exchange of molecules between brain and blood, circulating biomarkers have been the first line of research thanks to the simplicity and the minimally-invasive blood collection procedure. Blood evaluation has permitted the identification of potential mediators of the stroke pathophysiology in terms of diagnosis and prognosis, despite none of them has reached its clinical implementation yet.[5,6] However, because the brain is strictly protected from circulation by the brain-blood barrier (BBB) and considering that there is still a lack of complete understanding of how different brain molecules reach the peripheral circulatory system, blood biomarkers research has not

yet provided enough information to fully understand all the alterations that occur in the brain following stroke.

To overcome these issues, the exhaustive study of other biological samples, such as the cerebrospinal fluid (CSF), which is in much more close contact with the brain than the peripheral circulation, has emerged as a complementary approach for the characterization of stroke pathophysiology. As known for other neurological diseases, this brain protective and supportive body-fluid provides an excellent opportunity to evaluate the very early signs of neuronal degeneration.[7,8] In the case of ischemic stroke, however, the clinical usefulness of CSF for an early diagnosis of stroke is challenging, since collection methods require an invasive procedure that might still be difficult to be perform in clinical situations of emergency and supposes an absolute contraindication for intravenous thrombolytic therapies. Nevertheless, CSF evaluation in the field of ischemic stroke might provide additional insights about the pathogenic alterations underlying cerebral ischemia, and may contribute to the complete interpretation of stroke pathology and the identification of potential therapeutic targets for its pharmacological modulation.[9] In this context, the use of pre-clinical models has proven to be notably relevant to examine those biological fluids that are difficult to obtain from human, as for the case of CSF.

The emerging proteomic technologies provide new high-throughput approaches that allow the simultaneous identification of a huge quantity of proteins in biological samples, including fluids, especially serum or plasma, and, although to a lesser extent, post-mortem brain tissue of ischemic stroke subjects.[10] Due to the wide range of protein abundance in the circulating proteome, however, the identification of low abundant brain-specific molecules in body fluids is frequently a limitation.[11] To overcome this challenge, continuous advances in these proteomics' approaches are being made. Concretely, the SOMAscan proteomic assay, which is based on the usage of modified nucleic acid aptamers (named SOMAmers: Slow Off-rate Modified Aptamers), facilitates the simultaneous identification of an extensive set of proteins across a wide range of concentrations and abundances by replacing direct protein measurement into a straightforward DNA quantification, independently of high abundant protein confounders.[12]

In this study, our aim was to describe the CSF proteomic profile at a very early stage after cerebral ischemia in rats by using the SOMAscan proteomic technology. Moreover, we further explored our results to deeply describe seven selected candidates with a potential involvement in the stroke pathophysiology. To that end, these molecules were examined in blood and brain tissue of ischemic and sham-control animals and were tested in stroke patients for their ability as diagnosis and prognosis biomarkers.

RESULTS

Proteomic profiling of CSF after cerebral ischemia. To characterize the CSF proteomic profile in the hyperacute phase of cerebral ischemia, we harvested CSF from MCAO and sham-control animals at two different time-points: before (pre) and after (post) their respective surgeries (Figure 1B). No tangible differences in relative protein abundances were detected in CSF samples from sham-control animals, with only 47 proteins (4.16%) differentially regulated in CSF between the pre and post sham-control surgical procedure. Interestingly, a large amount of proteins (738; 65.4% of total) increased in rat CSF after the induction of cerebral ischemia compared to their pre samples (Figure 1C and Supplementary table S1).

CSF differentially-expressed proteins after MCAO and sham-control surgery were further characterized through bioinformatics analysis. The disease- and function-related analysis (Figure 2A) revealed that the inflammatory response that early appears in the brain after an ischemic event was the most overrepresented biological process in the CSF proteome 2h after cerebral ischemia. Specifically, the activation and accumulation of leukocytes, and several inflammatory

processes related to the infiltration of immune cells into the brain, including immune cells movement and leukocytes chemotaxis, were largely represented in the CSF from MCAO animals. Furthermore, particular interest was also directed towards the specific biological processes encompassed in the category of neurological disease, since molecular mechanisms of cell death of brain cells, specifically of cortical neurons, were the foremost annotations reported in the CSF at this early time-point after ischemia (Figure 2A).

Notably, the analysis of the main canonical pathways revealed that the neuroregulin and the platelet-derived growth factor (PDGF) molecular pathways were also highlighted processes in the acute phase of ischemia, as reflected by their highlighted relevance in the CSF proteome exclusively from ischemic animals (-log(B-H p-value)>7). Moreover, additional strength was given to inflammation in the early phase of ischemia, since chemokine signaling pathways were also highly represented in the CSF proteome profile after cerebral ischemia (Figure 2B).

Selection of proteins with significantly altered expression in CSF after cerebral ischemia. Twenty-two out of the 738 differentially-expressed proteins after MCAO (2.98%) were also up-regulated after sham-control surgery, namely common in both dataset comparisons. Since our attention was focused on the key proteomic alterations after cerebral ischemia, and in order to minimize the influence of the non-ischemic processes associated to the surgery procedure,

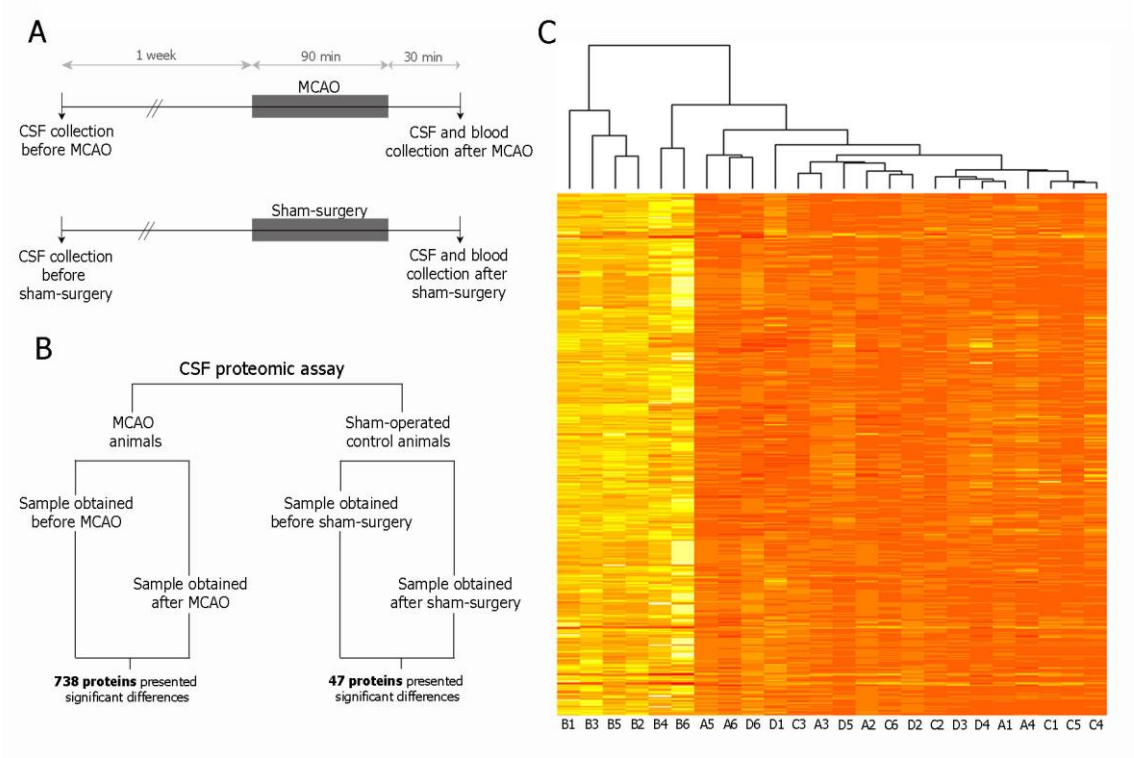

Figure 1. A. Schematic representation of the design of the experimental study. **B.** Schematic representation of data analyzed by the SOMAscan proteomic assay. **C.** Color map of relative fluorescence units (RFU) of the 738 proteins that were significantly altered after the ischemia. Squares are color coded; bright yellow color indicates high RFU and pale orange color indicate low RFU. The brighter the color is, the higher RFUs are. A1-A6 indicate samples obtained before MCAO; B1-B6 indicate samples obtained 2h after MCAO; C1-C6 indicate samples obtained before sham surgery; D1-D6 indicate samples obtained after sham surgery. Abbreviations: CSF: cerebrospinal fluid; MCAO: middle cerebral artery occlusion.

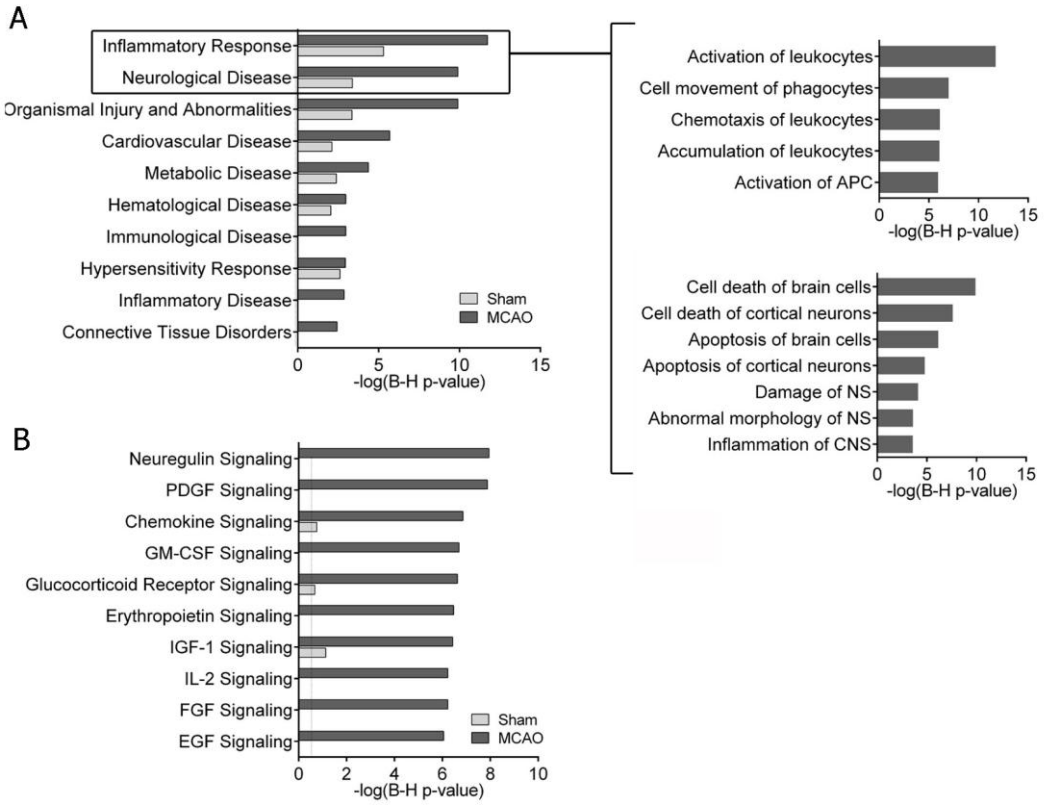

Figure 2. IPA analysis of differentially expressed proteins in CSF after cerebral ischemia in rats. Analyses were classified by disease and disorders (**A**) and canonical pathways (**B**) and compared with the CSF from the control group of sham-operated animals. Magnification of the top 2 canonical pathways from A is also provided. Top 10 categories ranked by MCAO corrected p-value are listed in all graphs.

these 22 common proteins were discarded for further analysis. Therefore, we finally identified 716 proteins that were exclusively up-regulated due to brain ischemia.

Of those stroke-associated proteins, 46 (6.42%) presented more than a 4-fold up-regulation in the CSF after MCAO compared to pre MCAO (the top 20 proteins are shown in Table 1).

For further studies, the top five proteins from this list (CKB, CaMK2B, CaMK2D, PDXP and AREG) were chosen as promising candidates to be examined in detail. Calcium/Calmodulin-dependent protein kinase II subunit alpha (CaMK2A) was additionally chosen for further exploration, given the outstanding importance of their analogues (CaMK2B and CaMK2D), and Uridine monophosphate/cytidine monophosphate (UMP/CMP) kinase (CMPK) protein was also included as a candidate due to its relevance in a previous study from our group (FDR p=0.008, logFC=1.127)[13] (Figure 3A). None of the selected candidates showed an association with the acute neurological deficits of ischemic animals (data not shown).

Analysis of the selected proteins in the rat brain and blood. The proteomic profile of plasma samples, which were also obtained 2h post MCAO or sham-control surgery from the same set of animals, was run in parallel. Of all selected candidates, only CKB and the 3 members of the CaMK2 protein family showed an increase in plasma of MCAO animals compared to sham controls (Figure 3B). Additionally, these higher circulating levels were positively correlated with the CSF levels for the CaMK2 members (R=0.736, p=0.006 for CaMK2B; R=0.589, p=0.044 for CaMK2D; and R=0.626, p=0.029 for CaMK2A), whereas no correlation between plasma and CSF levels post MCAO was seen for CKB (R=0.161, p=0.617). Conversely, neither PDXP nor AREG or CMPK showed differences in their circulating plasma levels

between MCAO and sham-control animals at the studied time-point, and neither of them showed correlation between plasma and CSF levels post MCAO.

Protein candidates were further characterized in brain homogenates from the same set of MCAO and sham-control animals. No appreciable differences were detected for PDXP, AREG and CKB levels between the affected and healthy brain hemispheres of MCAO animals and sham controls, as reported by the ratio between ipsilateral (IP) and contralateral (CL) protein levels (p=0.956, p=0.190 and p=0.736, respectively).

Table 1. Top 20 proteins increased in the CSF after MCAO. Proteins selected for further examination are highlighted in bold.

Protein name	Acronym	Pre vs. Post MCAO	
		FDR p-value	Log(FC)
Creatin kinase B-type	**CKB**	**0.0393**	**3.4370**
Calcium/Calmodulin dependent protein kinase II subunit beta	**CAMK2B**	**0.0393**	**2.7742**
Pyridoxal phosphate phosphatase	**PDXP**	**0.0393**	**2.7283**
Amphiregulin	**AREG**	**0.0393**	**2.6482**
Calcium/Calmodulin dependent protein kinase II subunit delta	**CAMK2D**	**0.0393**	**2.6411**
Junctional adhesion molecule C	JAM3	0.0393	2.5815
Cytokine receptor common subunit gamma	IL2RG	0.0393	2.5596
40S ribosomal protein S7	RPS7	0.0102	2.5538
Fibroblast growth factor 16	FGF16	0.0393	2.5361
Ubiquitin-conjugating enzyme E2 L3	UBE2L3	0.0393	2.5222
Interleukin-23 receptor	IL23R	0.0393	2.5061
Vacuolar protein sorting-associated protein VTA1	VTA1	0.0183	2.5013
Calcium/Calmodulin dependent protein kinase II subunit alpha	**CAMK2A**	**0.0393**	**2.5007**
Seizure 6-like protein 2	SEZ6L2	0.0393	2.4662
Glycoprotein hormones alpha chain / Lutropin subunit beta	CGALHB	0.0393	2.4515
Protein kinase C iota type	PRKCI	0.0393	2.4042
NKG2D ligand 3	ULBP3	0.0393	2.3961
Desert hedgehog protein	DHH	0.0393	2.3926
Fibroblast growth factor 7	FGF7	0.0393	2.3710
Apoptosis regulator Bcl-2	BCL2	0.0393	2.3698

However, CMPK and the three members of the CaMK2 family (CaMK2B, CaMK2D and CaMK2A) did show a decrease in their levels in the ischemic hemisphere compared to the healthy side of the brain and sham controls (p=0.032, p=0.075, p=0.037 and p=0.011, respectively) (Figure 3C-3D). Interestingly, the simultaneous evaluation of the total levels of CaMK2 (phosphorylated and non-phosphorylated forms) resulted in no differences between MCAO and sham-control animals (p=0.817), whereas the detection of the phosphorylated form alone did show a slight increase in the infarcted hemisphere compared to the contralateral in ischemic animals (p=0.090) (Supplemental Figure S2).

The specific brain localization in ischemic rats was conducted for each selected protein with commercially-available antibodies for immunohistochemistry. Relative abundances between IP and CL hemispheres were consistent with the aforementioned results for all candidates. CKB immunostaining was consistent through all brain regions and co-localized with the astrocytic marker GFAP (Glial Fibrillary Acidic Protein) (Figure 4A), but not with the microglial marker Iba-1 (data not shown).

CaMK2B positive staining was observed in neurons from the pyramidal layers of the cerebral cortex and in striatal neurons. Moderate staining of CaMK2B was also shown in hypothalamic nuclei from the periventricular region and in the hippocampus (Figure 4B). Staining of AREG was detected in both, pyramidal and granular neuronal layers. Moreover, AREG-positive signal was also seen in the third coronal brain depth and was localized just below the ending lateral ventricles, close to the reticular nucleus of the thalamus (Figure 4C).

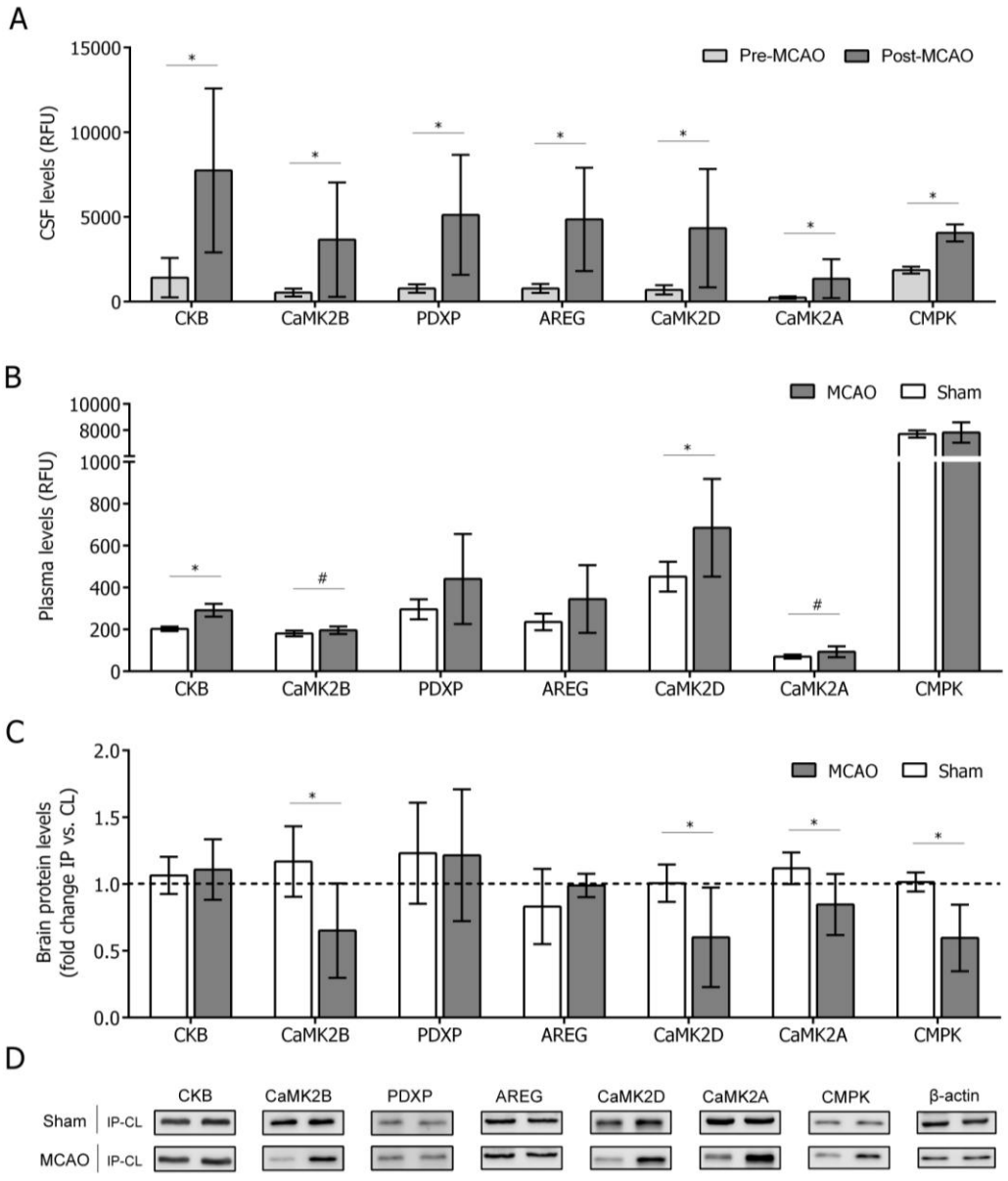

Figure 3. Exploration of protein levels in CSF, plasma and brain of ischemic and sham-control animals. Plots of protein abundance data (RFU: relative fluorescent units) in (**A**) CSF samples obtained pre- and post-MCAO and (**B**) plasma samples obtained 2h after MCAO or sham-control surgery (n=6 samples per experimental group for all cases). (**C**) Plot of protein abundance (relative quantification, ratio IP vs. CL hemisphere of each animal) in brain homogenates samples obtained 2h after MCAO or sham-control surgery (n=4 samples for sham; n=8 samples for MCAO). In all cases, IP and CL samples from each animal are run in the same gel and individually corrected by their respective β-actin loading controls. (**D**) Representative Western Blots signals used for quantification of proteins in brain. Bars indicate mean ± SD. For all graphs, *p<0.05 and $^{\#}$p<0.1. Abbreviations: IP: ipsilateral hemisphere; CL: contralateral hemisphere.

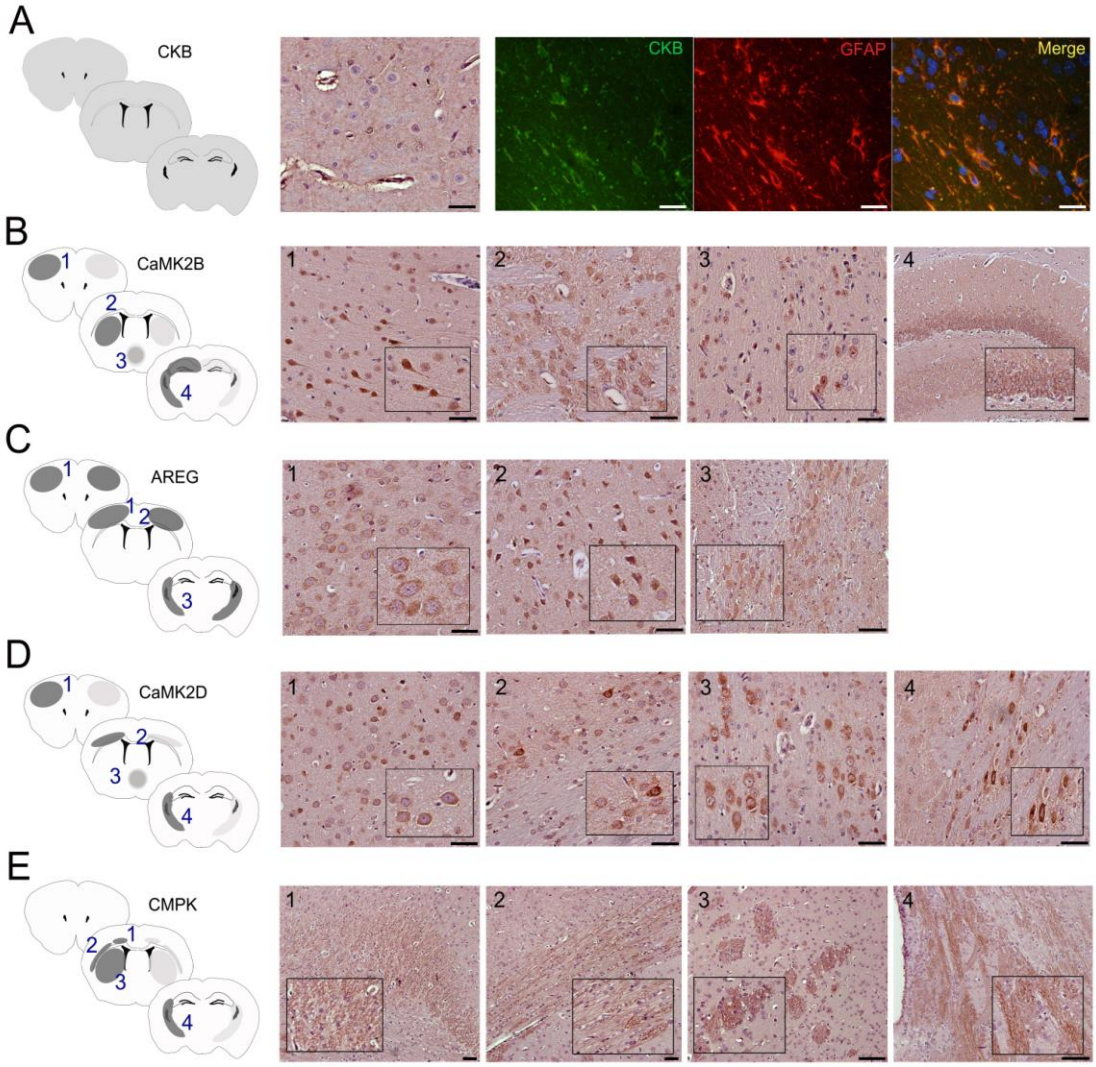

Figure 4. Histological localization of protein candidates in the ischemic brain. (A) CKB, **(B)** CaMK2B, **(C)** AREG, **(D)** CaMK2D and **(E)** CMPK histological examination in three established cortical depths of the ischemic brain (see Supplemental figure S1). In the schematic representation of brain, right hemisphere corresponds to IP and left hemisphere corresponds to CL. Dark grey regions indicate higher abundance of positive staining than pale gray zones. CKB (green) was co-localized with GFAP marker in the red channel and nuclear detection was conducted with DAPI in the blue channel. Scale bar =50 µm.

CaMK2D positive staining was shown in cortical neurons from the granular layer IV and the multiform layer VI, it was also identified in the periventricular region of the hypothalamus, similar to its analogue CaMK2B, and presented robust positive staining in neurons from the internal capsule of the corticospinal tract (Figure 4D). Finally, CMPK staining was observed in areas close to the *cingulum*, external and internal capsule, and was notably pronounced in neuronal bundles from the striatum (Figure 4E).

Translation to potential biomarkers in stroke patients. We further aimed to assess the potential role of CKB, CaMK2B, CaMK2D, CaMK2A and CMPK as biomarkers of stroke. With that purpose, we evaluated circulating levels of these proteins in human blood samples from ischemic stroke (<6h from symptoms onset) and control subjects. Demographical and clinical characteristics of all patients are described in Supplemental table S2.

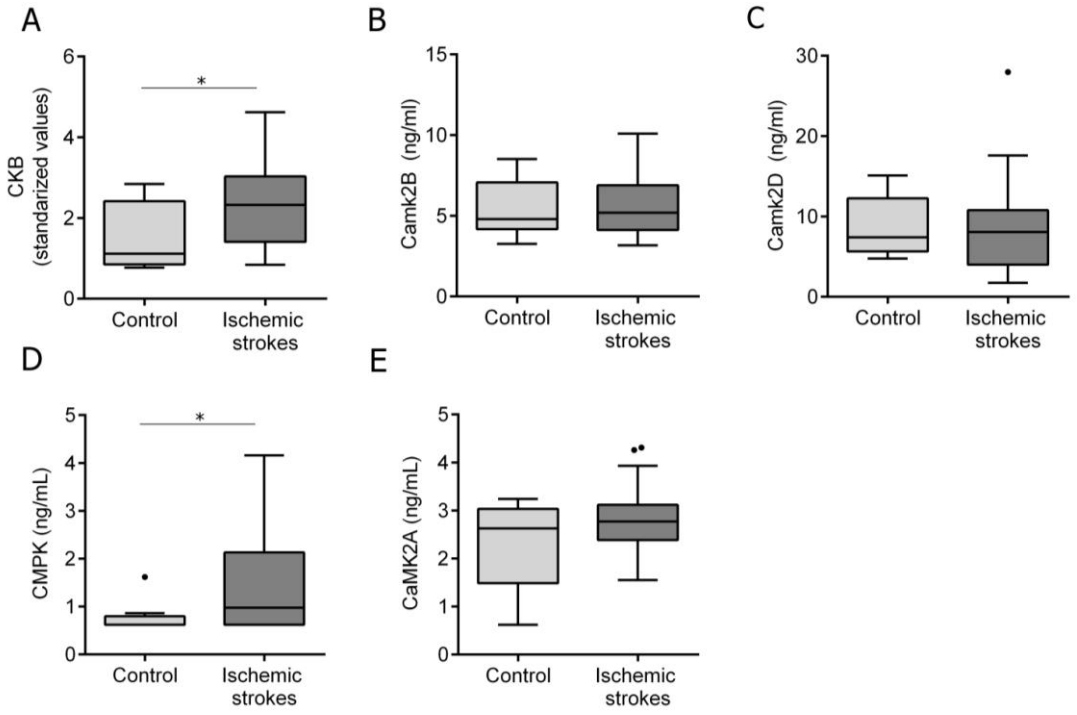

Figure 5. Plasma levels of CKB, CaMK2-family members and CMPK. Circulating levels in ischemic stroke patients (n=38) and controls (n=8) of all selected candidates. Blood samples were obtained on patient admission to the hospital (<6h from symptoms onset). Median and Tukey confidence intervals are represented in each graph. Standardized values for CKB were calculated using Z-score (see Supplemental materials and methods data). For all graphs, *p<0.05.

In brief, ischemic stroke subjects were older and presented higher prevalence of atrial fibrillation and ischemic cardiopathy than control individuals. However, differences in the circulating levels of the selected candidates could not be explained by any of these demographical and clinical variations (data not shown).

Notably, circulating levels of CKB and CMPK were higher in ischemic strokes than controls (p=0.050 and p=0.047, respectively) (Figure 5A, 5D).

However, neither CaMK2B nor CaMK2D or CaMK2A showed differences in their circulating levels between both studied groups (p=0.719, p=0.904 and p=0.687, respectively) (Figure 5B, 5C, 5E).

Next, we aimed to evaluate the practical use of these proteins as biomarkers of long-term functional outcome after stroke. Seventeen out of 38 ischemic stroke patients (44.7%) exhibited a poor functional outcome by the third month after stroke. Notably, baseline circulating levels of CaMK2B and CMPK were higher in those patients with poor functional outcome than patients with good functional outcome (6.23 ± 1.96 ng/mL vs. 5.02 ± 1.41 ng/mL, p=0.046 for CaMK2B; 1.43 [0.9-2.39] ng/mL vs. 0.62 [0.62-1.09] ng/mL, p=0.019 for CMPK), but did not associate with stroke severity, as measured by the NIHSS at admission (p=0.734 and p=0.773 respectively).

We found a cut-off point of 5.016 ng/mL for CaMK2B (70% sensibility, 69% specificity) and 0.759 ng/mL for CMPK (76% sensibility, 71% specificity) that were significantly associated with poor functional outcome at the studied time-point (p=0.020 and p=0.014, respectively). The predictive clinical model including the variables significantly associated with poor outcome in the univariate analysis (Supplemental table S3) was further adjusted by age, sex and NIHSS score at admission, which are well-known factors that highly influence stroke prognosis.[14] The addition of each biomarker individually to this predictive model showed that both of them remained independent predictors of poor outcome (OR=9.82 [1.13-85.88], p=0.039 for CaMK2B; OR=9.40 [1.53-57.67], p=0.015 for CMPK). The combination of both biomarkers in the

same predictive model was also an independent predictor of poor outcome (OR=6.38 [1.33-30.69], p=0.020).

DISCUSSION

The study being presented here aimed to map the rat cerebrospinal fluid proteome in the hyperacute phase of cerebral ischemia by using an aptamer-based proteomic technology as a research tool to identify potential stroke biomarkers. The SOMAscan approach facilitated the screening of hundreds of proteins in CSF, and permitted the evaluation and characterization of its protein composition following an event of cerebral ischemia.

The cerebrospinal fluid is the physiological medium that surrounds and mechanically supports the central nervous system (CNS). The CSF circulates unidirectionally from the brain ventricles to the subarachnoid space in the cortex and serves as a transporter of biochemical messages from one brain region to another. Approximately an 80% of the CSF originates from the filtration of arterial blood in the choroid plexus, whereas the rest is thought to principally derive of the drainage of brain interstitial fluid (ISF).[15,16] The connection between CSF and ISF is strictly necessary for an optimal maintenance of the neuronal microenvironment in the brain parenchyma. Under cytotoxic conditions, ISF conducts metabolic waste to clear away cellular degradation products, leading to an accumulation of catabolites diffusing out from brain to the CSF.[17] Following an ischemic event, unique signatures of brain-specific molecules from damaged parenchyma come together in the CSF. The early accumulation of these brain-derived factors in the CSF might accurately reflect the current status of the brain, and its collection might in turn facilitate a rapid diagnosis of the on-going CNS pathological processes and accelerate therapeutic interventions as required.

Bioinformatics' analysis of the CSF composition after cerebral ischemia reinforced the well-known importance of the inflammatory response in the ischemic brain.[18] After only 2 hours from ischemia onset, the signaling cascade of inflammation was well established and molecularly reflected in the CSF. Concretely, mediators of the mobilization of activated leukocytes to the ischemic site of injury, including proteins involved in cellular movement and chemotactic factors, were of considerable relevance in the ischemic CSF proteome. The diffusion into the CSF of proteins that participate in pathways of neuronal cell death was also found to be of great magnitude at the studied time-point. As it is well-known, the breakdown of neuronal cell integrity causes a devastating loss of neurons in the core of the ischemic lesion within a short period of time from disease onset. The identification and functional characterization of mediators of (or induced by) this rapid stroke-related massive injury might serve as valuable therapeutic targets for the reduction of the brain lesion. This is the case of the neuroregulin and PDGF pathways, since both signaling pathways have been related to neuroprotection: neuroregulin has shown potential therapeutic value by preventing macrophage/monocytes infiltration and astrocyte activation[19], whereas PDGF has been proposed to be angiogenic in stroke.[20]

The top seven outstanding proteins found at high levels in the CSF following cerebral ischemia in the present study have been extensively described in the context of the stroke pathophysiology. CK-BB, composed by two identical CKB subunits, is the most abundant creatine kinase (CK) isoenzyme in the brain. CK isoforms play a key role in energy transduction and homeostasis, especially in high-demanding tissues, by catalyzing the reversible exchange between creatine and creatine-phosphate, thus facilitating the conversion of ADP to ATP and vice versa to cover cell energy requirements and storage.[21] CK in the isoforms of CK-MM and CK-MB have been widely studied for the diagnosis of myocardial infarction[22], but less is known about CK-BB disease relevance. In concordance with our results, CK-BB has been well reported to immediately increase in blood circulation and CSF following acute cerebral injuries, including cerebrovascular traumatisms, meningitis and strokes[23–25], and its role as a plausible diagnostic biomarker for

ischemic stroke has been previously postulated.[26,27] CK-BB provenance has been slightly controversial; some studies have proven that astrocytes and neurons are the main CK-BB source, whereas others, including us, could only proof that astrocytes express CK-BB in brain.[28] Our findings indicate that there is a strong CK-BB accumulation in the CSF and blood following ischemia in rats; despite we were not able to detect differences in CK-BB levels in brain parenchyma between the infarcted and healthy hemispheres of the brain.

AREG (amphiregulin) was another outstanding protein found to have increased levels in CSF after cerebral ischemia. AREG is a member of the epidermal growth factor (EGF) family of proteins. This EGF-like molecule induces cell differentiation and proliferation and contributes to wound healing and tissue repair following infection or injury.[29] In brain, AREG remarkably increases in response to ischemic conditions in cortical, striatal and hippocampal neurons, and attenuates neuronal damage by inhibiting the pro-inflammatory-related endoplasmic reticulum stress.[30] In the present study, AREG markedly accumulate in the CSF of ischemic animals but their plasma and in-brain levels did not change after the insult. This controversy might be explained in part by a direct balance between a higher intracellular release caused by the on-going massive cell disruption and death in the ischemic core, and a simultaneously higher expression of this protein in an attempt to slow-down and retrain stroke-related neuronal damage. Besides, AREG has been also linked to the propensity to develop hemorrhages in thrombolytic-treated ischemic stroke patients.[31] Concretely, AREG increases the expression of MMP-9 and VEGF, both molecules strongly associated with hemorrhagic complications after stroke.[32,33] Thus, although the time-point studied here precludes the study of hemorrhagic complications and their association with AREG levels, further experiments should be conducted to explore it as a therapeutic target.

PDXP, also named as chronophin, regulates the synthesis of pyroxidal 5'-phosphate (PLP), the coenzymatically active form of vitamin B6. It removes the phosphate group from PLP and degrades it to 4-pyridoxic acid.[34] PDXP participates in the biosynthesis of various neurotransmitters and organic molecules, and is found at high levels in brain and testis.[35,36] PDXP is also an important regulator of cofilin or actin-depolymerization factor (ADF).[37] ADF/cofilin maintains a regular intracellular reservoir of ATP-G-actin monomers by the depolymerization of older ADP-F-actin, thus regulating actin dynamics and contributing to the construction and remodeling of a great variety of polarized structures within cells.[38] Activated ADF/cofilin (non-phosphorylated form) has been shown to mediate apoptotic processes, and its inhibition (phosphorylated form) has shown promising neuroprotective results by increasing neuronal viability and survival.[39] PDXP is known to activate ADF/Cofilin, thus further contributing to mechanisms of neuronal damage and death.[40] PDXP itself is activated in response to different intracellular and extracellular signals of stress, such as oxidative stress or the drop of intracellular ATP that occurs after cerebral blood flow impairment.[34,37] PDXP levels increase in brain following cerebral ischemia, and decrease after the administration of neuroprotective free radical scavengers, such as ferulic acid.[34,41] In our study, we reported high levels of PDXP in CSF early after ischemia, but we were not able to detect changes in brain levels neither in plasma at the studied time-point. It remains to be tested whether PDXP increases in the ischemic brain at later stages after ischemia, which would potentially serve as a therapeutic target to mediate the mechanisms of stroke-related tissue injury.

CMPK reversibly catalyzes the addition of a phosphate group to UMP and CMP to consequently form UDP and CDP, which are further converted to their triphosphorylated form to be used as substrates of the DNA and RNA polymerases.[42] In brain, these nucleotides are strictly required for the synthesis of neuronal membrane phospholipids, and thus influence various membrane-dependent processes such as neurotransmitter release and neurite outgrowth.[43] A decrease expression of CMPK has been related to aging[44], whereas an

increased expression has been seen in brain cortex from patients with temporal lobe epilepsy and following sciatic nerve injury in rodents.[45,46] In terms of ischemia, decreased levels of both, nucleotides and CMPK, has been observed in the infarcted region of the brain after transient cerebral ischemia[13,47], which we further corroborated by also reporting decreased levels of CMPK in the early ischemic rat brain. Besides, at the same time-point following ischemia, we observed an increased accumulation of CMPK in the CSF, which suggest that this lower content of CMPK in the ischemic core could be a direct consequence of a massive release of components from disrupted cells within the injured area, rather than a brain injury-related decrease in CMPK expression. On the other hand, despite we could not observe any increase in CMPK levels in circulation 2h after ischemia in rats, we reported higher CMPK blood levels in ischemic patients within the first 6h from symptoms compared to controls. Moreover, we could explore the role of CMPK as marker of stroke patient poor prognosis. As reported in the field of cancer[48], we observed that circulating levels of CMPK at admission were associated with poor long-term outcome, independently of the patient clinical factors. Taking into consideration the involvement of CMPK in mechanisms to combat cell disruption and neuronal cell death[49], higher CMPK levels following ischemia could be associated with severe ischemic lesions and a final patient poor outcome. Nevertheless, further studies are needed to verify both, the observed diagnostic and prognostic value of CMPK, and evaluate whether CMPK can also serve as a valuable target for therapeutic strategies.

Finally, CaMK2 members (CaMK2A, CaMK2B and CaMK2D) belong to a group of Ser/Thr protein kinases that has been previously involved in glutamate excitotoxicity-induced neuronal cell death.[50] CaMK2 members are primary activated under ischemic conditions by binding of Ca^{2+}/Calmodulin and achieve full activity after autophosphorylation of all former subunits by their neighbors.[50] Phosphorylated CaMK2 remains active and maintains its autonomous activity after dissociation of Ca^{2+}/Calmodulin, which facilitates and prolongs CaMK2 functional activity.[51] Glutamate citotoxicity causes translocation of the activated CaMK2 members to the post-synaptic sites and extra-synaptic clusters[52,53], where are involved in synaptic plasticity and serve as a reservoir of CaMK2 to avoid their massive phosphorilation within cells, respectively. Active CaMK2 members have a controversial role in mediating both apoptotic cell death and cell viability.[50] Some of the CaMK2-downstream targets, including different Ca^{2+} channels, promote neuronal apoptosis by enhancing death-inducing overload of cellular Ca^{2+} [50], while the modulation of other factors, such as the inhibition of caspase 2 and Bad or the enhanced expression of Bcl-xL, might also attenuate those apoptotic processes.[54,55] In accordance, both cerebroprotective but also neurotoxic properties of CaMK2 inhibition have been described previously.[56–58] However, more promising results have been reported in terms of neuroprotection by suppressing CaMK2 activity before, during or after the ischemic insult, both in vitro and in vivo.[58–60] In our study, we described decreased levels of the non-phosphorylated inactive CaMK2 and increased levels of the phosphorylated active CaMK2 in the ischemic brain hemisphere compared to the healthy side of the brain and sham controls, as others have also stated.[61] This early increase in the active forms of CaMK2 might be promoted by their involvement in ischemia-related neural plasticity processes and might also participate in the early on-going apoptotic mechanisms within the ischemic lesion. Moreover, CaMK2 levels accumulated in the CSF early after ischemia, and this increase was also reflected in the peripheral circulatory system of ischemic rats. However, these results did not match with those from the pilot study, where circulating levels of CaMK2 were not increased in patients that had suffered an ischemic stroke, and hampers the use of these proteins as potential blood biomarkers of stroke diagnosis. Notably, CaMK2B, but not CaMK2A neither CaMK2D, appeared as a valuable indicator of stroke patient functional outcome. CaMK2B association to disease prognosis has been previously reported in cancer[62] but, as far as we know no previous study has explored CaMK2B prognosis-value

by measuring its levels in the circulation. Thus, in this preliminary study we are newly describing that circulating levels of CaMK2B showed a potential to predict stroke patients outcome, though larger studies are required to corroborate these findings and to assess whether this predictive capacity is unique to CaMK2B, which is the most brain-specific protein of the CaMK2 members[35], and whether it turns out to also be a potential target to therapeutically modulate the progression of the ischemic lesion.

Importantly, we could not identify a consistent pattern among brain, CSF and blood levels relation between the studied candidates. The lack of a reliable correlation between these three compartments hampers the fully comprehension of their interrelationship in stroke pathology and leads to presume that there might be several unknown mechanisms that selectively and distinctively regulate the exchange of molecules between one and another compartment following an ischemic event. We propose that further efforts should be made to understand this brain-CSF-blood connection, which would offer a huge breakthrough for the complete understanding of stroke disease.

Our work presents some limitations that should be taken into consideration for further studies. First, although the animal model chosen in this study is highly suitable to evaluate the biological alterations of acute ischemic stroke, some additional injury due to reperfusion cannot be avoided, which can lead to the identification of surrounding processes of ischemic stroke itself that are not found in all clinical scenarios.[63] Second, due to technical incompatibilities, we were not able to determine the lesion volumes in the ischemic animals, which would have also been an interesting parameter to analyze. Besides, the SOMAscan platform used here for candidate discovery is initially designed to detect serum human proteins. Despite a great majority of those human-based aptamers show high reactivity based on homology to their rodent counterparts, we cannot discard the presence of potential confounding proteins identified in our datasets. However, we present further analysis with other techniques and tissues confirming the relevance of some of the selected proteins. Finally, despite our clinical pilot study supports the findings on experimental data, we are aware of its limitation due to the reduced number of patients included. Larger and independent cohorts should be studied to validate these results in a near future.

This work also has some strengths that should be highlighted. This study has originated an extended source of important contributors of stroke pathology, which has been validated through the achieved congruence between the discovery datasets and the further exploration of the selected candidates in brain and blood. In this same line, previously known candidate biomarkers for stroke diagnosis and prognosis, including interleukin 6[64,65], C-reactive protein[66,67], CC-chemokine ligand 23[68] or D-dimer[69,70], have also been corroborated here, since they were also highly increased in CSF 2h after stroke onset (data not shown). Hence, this additional information further validates and gives potential effect to our study, which we expect it to serve as a potential tool to elucidate future mediators of stroke pathology, provide other feasible biomarkers of stroke and support further potential discovery studies in the field of cerebral ischemia.

In conclusion, we have used here for the first time a high multiplexed, sensitive and quantitative proteomic tool for mapping the rat cerebrospinal fluid proteome in the hyperacute phase of cerebral ischemia. The SOMAscan proteomic approach has facilitated an unbiased screening of hundred of proteins in CSF and plasma samples from ischemic animals, and has originated an extended source of important contributors of stroke pathology that might be supporting future studies in this field. Through this strategy we have been able to firstly identify and primary evaluate the role of three promising proteins as stroke blood biomarkers, which provides added value on the design and development of the present study.

METHODS

Animals. All experimental procedures were conducted 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 Institute of Research (protocol number 58/14). All experiments were conducted in a randomized manner and in adherence to the ARRIVE guidelines. Male Wistar rats were used in the experiments (7-12 weeks; Charles River Laboratories Inc., Wilmington, MA, USA). Animals were kept in a climate-controlled environment on a 12h light/12h dark cycle. Food and water were provided *ad libitum*. Analgesia (Buprenorfine, 0.05 mg/kg, s.c, Divasa Farma-Vic S.A, Barcelona, Spain) was administered before surgeries to all animals to minimize pain and discomfort. All animals were anesthetized with isoflurane (4% for induction; 2% for maintenance in air, Abbot Laboratories, Spain) and body temperature was maintained at 37ºC during all surgical procedures.

A total of 41 rats were needed to complete the study: 14 animals were subjected to sham-control surgery and 26 to transient middle cerebral artery occlusion (MCAO). One sham-control animal died during surgery and 10 ischemic animals were excluded after applying the following criteria: inappropriate occlusion or reperfusion of the middle cerebral artery as described below (n=6), massive surgical bleedings (n=1) and death during the surgery procedure (n=3).

Model of transient MCAO. Cerebral ischemia was induced by mechanical occlusion of territory of the MCA, as previously described.[71,72] In brief, the day before surgery, cranial trepanation was performed to attach a laser-Doppler probe (Moor Instruments, Devon, UK) and monitor regional cerebral blood flow (CBF). The following day, a silicone-coated nylon filament (Doccol Corporation, reference number: 403723PK10) was introduced through the external carotid artery and pushed to the internal carotid artery to occlude the MCA. Animals were allowed to recovery during the MCA occlusion period. Ninety minutes later, animals were re-anesthetized to induce a 30-min reperfusion by removal of the filament. Successful occlusion and reperfusion of the MCA was guaranteed by the reduction or increase in the CBF recorded by the laser Doppler probe. Only animals that exhibited a CSF reduction >75% after filament placement and a recovery of >75% after filament removal were included in the study. Sham-control surgery was performed by the same surgical procedures without insertion of the nylon-coated filament.

Acute neurological deficit. Rats were assessed using the modified Bederson scale, as described elsewhere[73]. Neurological deficits were evaluated in a blinded manner at 80 minutes of MCAO, before reperfusion.

CSF collection. CSF was collected from rats one week before (pre) and 2 hours after MCAO or sham-control surgery (post) (Figure 1A). CSF was collected by using a minimally-invasive method of CSF sampling, previously described by Mahat and colleagues.[74] In brief, animals under anesthesia effects were placed in a stereotactic apparatus and properly fixed to create an angle of approximately 110º between the base of the stereotaxic frame and the animal snout. After localizing the rhomboid depressed region, corresponding to the cisterna magna, a 25G-needle connected to a collection apparatus was vertically inserted and slowly advanced until CSF started flowing into the collection tube. After obtaining the sample, the collection tube was clipped to avoid blood contamination and the needle was withdrawn from the cisterna magna.

If blood contamination occurs at any point during CSF collection, the collection tube was cut off from the point of blood contamination. Only clear CSF without visual sings of blood contamination was collected and frozen at -80ºC until further use.

Rat brain and blood samples. Immediately after CSF collection blood samples were drawn through transcardiac puncture in EDTA tubes and immediately centrifuged at 3,000g for 10 minutes at 4ºC to obtain plasma, which was frozen at -80ºC until further use. Animals were then euthanized and transcardially perfused with cold saline. Brains were

carefully removed and IP and CL hemispheres were separated. Each hemisphere was individually frozen at -80ºC. Frozen hemispheres were cut into 1mm coronal slices over dry ice. Slice corresponding to the bregma point, where the infarction territory is located, were selected for all animals, thawed and homogenized using mirVana™ PARIS™ RNA and Native Protein Purification kit (ThermoFisher Scientific Inc., Waltham, MA, USA). Protein homogenates were centrifuged at 12,000g for 10 min at 4ºC and the cleared protein extract was quantified using bicinchoninic acid (BCA) assay (ThermoFisher Scientific Inc.) and frozen at -80º until further use.

Brains from MCAO animal were also used for immunohistological purposes. Those rats were euthanized and transcardially perfused with cold saline and their brains carefully removed and fixed with formalin. Fixed brains were carefully cut into segments at three precisely controlled depths: (1) 2.1±0.2 mm anterior to bregma, (2) bregma and (3) 1.5±0.2 mm posterior to bregma (Supplementary Figure S1). The formed tissue sections were mounted on blocks and embedded with paraffin. Tissue slices of 3 µm were cut, mounted onto glass slides and kept at 4ºC for the IHC experiments.

SOMAscan proteomic assay. The SOMAscan assay was performed by SomaLogic Inc. (Boulder, CO, USA). SOMAScan is a relative quantitative proteomics assay based on the usage of SOMAmers, which are modified nucleic acid aptamers that permit the identification and quantification of a library of human proteins, most of which show reactivity based on homology to their rodent counterparts.[12] Concretely, 1,129 (1.1k assay) and 1,310 (1.3k assay) proteins were measured in CSF and plasma samples, respectively, from 6 MCAO and 6 sham-control animals. Animals were selected according to the total volume of CSF collected (minimum CSF volume required = 100µl), and all samples were analyzed individually. Three different dilutions were performed to achieve all dynamic logs provided by the SOMAscan technology[75]. Normalization and calibration steps were then performed following SOMAscan technical instructions in order to remove systemic biases in the raw assay data. All samples passed SomaLogic quality control. Moreover, relative fluorescent unit (RFU) output from the array was subjected to background subtraction by establishing an accurate limit of detection threshold. To that end, a no-protein control sample was included in the assay run, which enabled the qualitative evaluation of baseline signal for each SOMAmer. Concretely, background subtraction discarded 216 (19.1%) and 29 (1.4%) proteins from the CSF and plasma datasets, respectively.

Comparisons between pre and post CSF samples were performed using paired t-test, corrected by the False Discovery Rate (FDR adjusted p-value), within each experimental group (R software 3.3.2, R development core team 2012, Austria, multtest R package) (Figure 1B). Proteins presenting an FDR adjusted p-value<0.05 were considered statistically significant. Base 2 logarithmic fold-changes (LogFC) were calculated for each protein by substracting abundance logarithmic values of the pre to the post CSF samples (LogFC = log [Post-CSF value / Pre-CSF value]). Comparisons on plasma samples were performed by using t-test (R software 3.3.2). Proteins presenting a p-value<0.05 were considered statistically significant. Correlations between CSF and plasma levels were calculated using Pearson's test.

Bioinformatics analysis. All data on rat CSF were analyzed by Ingenuity Pathway Analysis (IPA, QIAGEN, USA). All proteins screened in the SOMAscan proteomic approach were introduced in the software. For each experimental group, a filter of FDR adjusted p-value<0.05 for the comparison of proteins between pre and post CSF samples was applied. For the MCAO group, an additional cut-off of logFC >|1.5| was also used.

Data sets from MCAO and sham experimental groups were then characterized with the Ingenuity Pathways Knowledge Base, focusing the attention on the central nervous system and neuronal, immune and microvasculature cells types, and using all data sources (human, mouse and rat). A list of the main enriched biological processes, canonical pathways and molecular functions

for each experimental data set were obtained according to the overlap p-values, calculated using right-tailed Fisher's exact test corrected by the Benjamini-Hochberg multiple test (significant B-H p-value ≤0.05). Furthermore, both IPA analysis (MCAO and sham) were then compared side-by-side.

Western Blot and Immunohistochemistry/ Immunofluorescence. Seven protein candidates from the proteomics list (AREG, CaMK2A, CaMK2B, CaMK2D, CKB, CMPK and PDXP) were evaluated in rat brain protein homogenates and tissue sections by means of western blot (n=4/8 shams and ischemic animals, respectively) and immunohistochemistry or immunofluorescence (n=2 ischemic animals). Detailed protocols and procedures can be found in Supplemental materials and methods data.

Human blood samples. All human studies were approved by the Ethics Committee of Vall d'Hebron Hospital (ischemic stroke - PR[AG]157/2011- and control - PR[IR]87/2010 - individuals) and written informed consent was obtained from all subjects or relatives in accordance with the Declaration of Helsinki. Ischemic stroke patients admitted to the emergency department of the Vall d'Hebron University Hospital (Barcelona, Spain) from December 2013 to November 2014 within the first 6h after neurological symptoms onset. On admission, patients underwent a standardized protocol of brain imaging to differentially diagnose ischemic stroke. Trained neurologists assessed stroke severity using the National Institutes of Health Stroke Scale (NIHSS) and obtained demographic and clinical data from all patients. When eligible, ischemic stroke patients received the standard thrombolytic treatment (intravenous 0.9 mg/Kg recombinant tissue-plasminogen activator, rt-PA) and/or mechanical thrombectomy to remove the arterial clot. The clinical follow-up of each stroke patient was conducted at the 3rd month after stroke. At that time-point, functional outcome was evaluated according to the modified Rankin Scale (mRS); patients with a mRS score from 0 to 2 were classified as "good outcome" group and patients with a mRS from 3 to 6 as "poor outcome" group.
Blood samples from all patients were drawn on admission (<6h from symptoms onset) and before administration of any treatment in EDTA tubes. Samples were then centrifuged at 1,500g at 4°C for 15 minutes and plasma was stored at -80°C until further use. From this sample collection, a total of 38 ischemic stroke patients were selected for this study. Additionally, a total of 16 volunteers were included as controls.

Human ELISA. Five protein candidates (CaMK2A, CaMK2B, CaMK2D, CKB and CMPK) were further evaluated in blood samples from the aforementioned ischemic stroke patients and controls using commercially available ELISA kits. Detailed methods are available in Supplemental materials and methods data.

Statistical analyses. SPSS statistical package 22.0 was used for statistical analyses and GraphPad Prism 6.0 for creating graphs. Rat brain and human plasma protein data distribution was assessed by Shapiro-Wilk and Kolmogorov-Smirnov test, respectively. To determine differences in protein levels between experimental groups, Student t-test (normally distributed variables) or Mann-Whitney (non-normally distributed variables) were used. To analyze differences on clinical variables among patients, continuous factors were analyzed by Student's test (normally distributed, mean and SD values) or Mann-Whitney test (non-normally distributed, median and interquartile range (IQR)), while categorical variables were assessed by Pearson chi-squared test (frequencies). Correlations between protein levels and clinical continuous variables were analyzed using Pearson's test (normally distributed variables) or Spearman's test (non-normally distributed variables).
Receiver operating characteristics (ROC) curves were used to obtain the cut-off points of circulating protein levels with optimal accuracy (both sensitivity and specificity) for discriminating ischemic stroke prognosis. Forward stepwise multivariate logistic regression analysis for poor functional outcome was performed with all clinical

variables associated with this endpoint at p<0.1. Odds ratio (OR) and 95% confidence interval (CI) were adjusted by sex, age and NIHSS score at admission. Using the previous identified cut-off points, baseline levels of each associated prognostic biomarker were added to the clinical model, singly or in combination, to assess their independent association and to build new predictive models.

P-value <0.05 was considered significant at a 95% confidence level in all cases.

Data Availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTION STATEMENT

A.S, T.G-B and J.M conceived and designed the experiments. A.S performed all animal experiments. A.S, L.R and N.G did the western blot and immunohistochemical replication. A.P and A.B. helps in recruiting and selecting the cohort of patients and A.S performed the ELISAs with human blood samples. A.S and D.G performed the statistical analysis. A.R, T.G-B and J.M supervised the experiments and A.S drafted the manuscript. All authors have critically reviewed the article content and approved it in its final version.

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COMPETING FINANCIAL INTEREST

There is NO competing interest.

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Characterization of the rat cerebrospinal fluid proteome following acute cerebral ischemia using an aptamer-based proteomic technology

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS

Western Blot

Top protein candidates from the proteomics list were chosen to be evaluated in rat brain protein homogenates from MCAO and sham-control animals (n=8/4 per group, respectively). Equal protein amounts of 25 µg were resolved in 12% sodium dodecyl sulfate-polyacrylamide gels under reducing conditions and transferred onto nitrocellulose membranes (Amersham Bioscience, Amersham, UK). Membranes were blocked with 10% non-fat milk and the following primary antibodies were incubated: Rabbit anti-amphiregulin (AREG) (1/1,000) (Bioss Antibodies, USA), mouse anti-calcium/calmodulin-dependent protein kinase II subunit alpha (CaMK2A) (1/10,000) (ThermoFisher Scientific Inc.), mouse anti-calcium/calmodulin-dependent protein kinase II subunit beta (CaMK2B) (1/5,000) (ThermoFisher Scientific Inc.), rabbit anti-calcium/calmodulin-dependent protein kinase II subunit delta (CaMK2D) (1/5,000) (Proteintech Group Inc., Rosemont, USA), rabbit anti-calcium/calmodulin-dependent protein kinase II (CaMK2, total) (1/1,000) (Abcam, Cambridge, UK), rabbit anti-T-286 phosphorylated calcium/calmodulin-dependent protein kinase II (CaMK2, phospho-T286) (1/10,000) (Abcam), mouse anti-creatine kinase B-type (CKB) (1/5,000) (Abcam), rabbit anti-uridine monophosphate/cytidine monophosphate (UMP/CMP) kinase (CMPK) (1/1,000) (Proteintech Group Inc.), goat anti-pyridoxal phosphate phosphatase (PDXP) (0.1µg/ml) (Novus Biologicals, Littleton, USA) and anti-β-actin (1/500) (Sigma Aldrich Inc., MO, USA). Membranes were then incubated with secondary antibodies linked to horseradish peroxidase (HRP) (ThermoFisher Scientific Inc.) (anti-rabbit HRP (1/1,000), anti-mouse HRP (1/1,000) or anti-goat HRP (1/5,000)) with gentle agitation. Finally, substrate reaction was developed with chemiluminescent reagent Luminol (Amersham Biosciences) and analyzed with Odissey® Fc Imaging System (Li-Cor, USA). Western blots were then quantified using Image-J free software. Positive signals were corrected by β-actin signal, used as loading control. Afterwards, IP quantified bands were normalized by their respective CL bands and statistical comparison was performed between the IP/CL ratios of the experimental groups.

Immunohistochemistry and immunofluorescence

Selected candidates were evaluated by means of immunohistochemistry (IHC) or immunofluorescence (IF) in paraffin-embedded rat brain slides (n=2, MCAO animals). In brief, brain sections were heated at 65ºC and were subjected to a standard deparaffinization procedure with a final wash in 0.1% Tween-Tris buffered saline (TBST). Subsequently, all brain sections were submitted to antigen retrieval using citrate buffer (pH 6, 95º), allowed to temper and blocked with 10% goat serum (Millipore Corporation, MA, USA) in TBST for 1h.

For IHC purposes, slides were then incubated with the aforementioned primary anti-rat antibodies in the following conditions: AREG (1/500), CaMK2B (1/250), CaMK2D (1/250), CMPK (1/100) and CKB (1/100). Then, slides were incubated with the corresponding biotinylated secondary antibody against IgG (1/250) (Vector Laboratories Inc., Burlingame, CA, USA), and consecutively with HRP-streptavidin (1/100) (Vector Laboratories Inc.). Finally, brain sections were submerged to Liquid DAB+ (diaminobenzidine) (Dako, Carpinteria, CA, USA) and stained with Harris hematoxylin (Sigma Aldrich Inc.) and mounted on coverslips using DPX mounting medium (Sigma Aldrich Inc.).
For IF, slides were incubated with the primary anti-rat antibodies against CKB (1/100), Glial Fibrillary acidic protein (GFAP) (1/200) (ThermoFisher Scientific Inc.) and Ionized calcium binding adaptor molecule 1 (Iba-1) (1/750) (Abcam) followed by AlexaFluor® 488 anti-rabbit IgG and AlexaFluor® 568 anti-mouse IgG (Life Technologies, USA) respectively, and with 0.3% Sudan Black B (Sigma Aldrich Inc.) to reduce brain tissue autofluorescence [3]. Sections were mounted on coverslips using Vectashield with 46-diamidino-2-phenyl indole (DAPI; Vector laboratories).
In all cases, negative controls were performed without applying the primary antibody. All brain sections were analyzed using an Olympus BX61 microscope (Olympus, Japan) and the same intensities and parameters were set for all slides. Images were processed with the Olympus CellSens Imaging software (Olympus) and overlaying fluorescent images were created with Image J free software.

Human ELISA

Outstanding candidates from the animal study were evaluated in a pilot study with blood samples from ischemic stroke patients and controls using commercially available ELISA kits. Circulating levels of CaMK2A (Cat.# DL-CAMK2a- Hu, DLdevelop, China), CaMK2B (Cat.#E01C1335, BlueGene BioTech Co., China), CaMK2D (Cat.#E01C1336, BlueGene BioTech Co.), CKB (Cat.#E-EL-H2433, Elabscience Biotechnology Co., China) and CMPK (Cat.#E14030h, Wuhan EIAab Science Co., Ltd, China) were assessed following manufacturer's instructions. Each candidate was evaluated in all 38 samples from ischemic stroke patients, and in 8 randomly-selected samples from the 16 selected volunteers (controls). Each sample was assayed per duplicate and the mean value was used. Optical densities (OD) were measured in a Synergy TM Mx microplate reader (BioTek Instruments Inc, USA). Samples with a coefficient of variation (CV) higher than 20% were excluded for the analysis. Standard curves from each plate were used as inter-assay controls for each commercial kit. When values were under the detectable range of the assay, the limit of detection of the assay value was assigned to them. When inter-assay CV was higher than 20%, values were standardized prior to statistical analysis by calculating the Z-score value by dividing the mean of each 96-well plate kit by the standard deviation and adding two units to avoid results below zero in any sample.

SUPPLEMENTARY TABLES

Table S1 is accessible via the following link*:*

https://drive.google.com/open?id=1_RFq2t2iYRc6zL7DSfhQNc2t46WNdfeY

Factors	Ischemic strokes (n=38)	Healthy controls (n=16)	p-value
Age (years)	76.55 ±10.02	68.47 ±5.18	**0.016**
Sex(Male)	17 (44.7%)	8 (50%)	0.723
Admission NIHSS score	10.34 ± 6.33	-	-
Smokers	4 (12.1%)	2 (25%)	0.578
Alcohol consumption	3 (7.9%)	2 (25%)	0.268
Arterial hypertension	29 (76.3%)	10 (62.5%)	0.333
Diabetes mellitus	10 (26.3%)	2 (12.5%)	0.229
Dyslipidemia	15 (39.5%)	4 (25%)	0.309
Atrial Fibrillation	20 (52.6%)	0 (0%)	**<0.001**
Ischemic cardiopathy	17 (44.7%)	2 (12.5%)	**0.024**
Coronary artery disease	4 (10.3%)	1 (6.3%)	0.923
Previous stroke	6 (15.8%)	0 (0%)	0.163

Table S2. Demographic and clinical factors of ischemic stroke patients and healthy controls. Age and NIHSS score at admission are expressed as mean ± SD and all other clinical variables are expressed as frequency of patients that present the specified condition. Statistically significant p-values are highlighted in bold. Abbreviations: NIHSS: National Institutes of Health stroke scale.

Table S3. Univariate analysis. Clinical characteristics and factors associated with outcome at third month after stroke. Statistical significant differences between groups are expressed as bold p-value. Abbreviations: NIHSS: National Institutes of Health stroke scale; SBP: systolic blood pressure; DBP: diastolic blood pressure; TOAST: etiology stroke subtype classification.

Factors	3-month outcome		
	Good outcome (n=17)	Poor outcome (n=21)	p-value
Age, years	79 (74-82)	81 (69-83)	0.45
Gender (Male)	8 (47.1%)	9 (42.9%)	0.796
Admission NIHSS score	10 (4-11)	11 (5-17)	0.186
Smokers	3 (21.4%)	1 (5.3%)	0.288
Arterial hypertension	13 (76.5%)	16 (76.2%)	1
SBP, mm Hg	148 (140-155)	142 (115.5-167.5)	0.304
DBP, mm Hg	80 (74.5-82)	74 (62.5-85)	0.685
Glycemia, mg/dL	110 (102.5-133)	142 (129-176.5)	**0.02**
Diabetes mellitus	4 (23.5%)	6 (28.6%)	1
Dyslipidemia	8 (47.1%)	7 (33.3%)	0.389
Atrial Fibrillation	7 (41.2%)	13 (61.9%)	0.203
Ischemic cardiopathy	6 (35.3%)	11 (52.4%)	0.292
Coronary artery disease	0 (0%)	4 (19%)	0.113
Previous stroke	4 (23.5%)	2 (9.5%)	0.378
TOAST			0.766
- Atherothrombotic	5 (29.4%)	3 (14.3%)	
- Cardioembolic	7 (41.2%)	14 (66.7%)	
- Lacunar	1 (5.9%)	1 (4.8%)	
- Undetermined	4 (23.5%)	3 (14.3%)	

SUPPLEMENTARY FIGURES

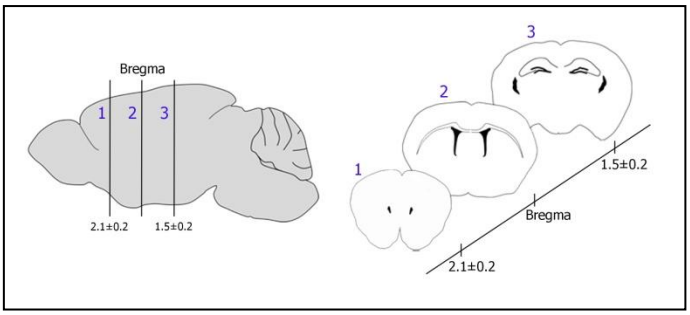

Figure S1. Representative illustration of the three cortical depths in which brains were cut for immunostaining purposes: ***1*** indicates 2.1 ± 0.2 mm anterior to bregma, ***2*** indicates the bregma point and ***3*** indicates 1.5 ± 0.2 posterior to bregma.

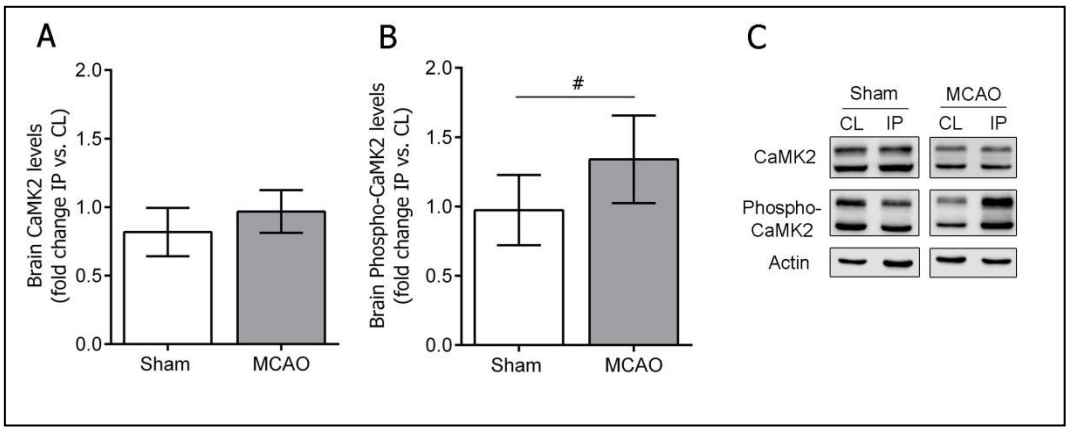

Figure S2. CaMK2 and phosphorylated CaMK2 protein levels in the brains of ischemic and sham-control animals. Plot of (**A**) total CaMK2 and (**B**) phosphorylated CaMK2 protein abundances (relative quantification, ratio IP vs. CL hemisphere of each animal, both run in the same blot) in brain homogenates samples obtained 2h after MCAO or sham-control surgery (n=4 samples for sham; n=8 samples for MCAO). (**C**) Representative Western Blots signals of the two detected bands for each used antibody. $^{\#}$ indicates $p<0.1$. Abbreviations: IP: ipsilateral hemisphere; CL: contralateral hemisphere.

Describing the mouse brain proteome and transcriptome after acute cerebral ischemia through a multiomics-integrative approach

Describing the mouse brain proteome and transcriptome after acute cerebral ischemia through a multiomics-integrative approach

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Stroke remains a major leading cause of death and disability worldwide. Despite continuous advances, a better characterization of the stroke pathophysiology is still required to elucidate key mediators that facilitate a prompt stroke diagnosis and the identification of effective therapeutic treatments to combat stroke consequences. High-throughput *-omics* technologies are enabling large-scale studies on stroke pathology at different molecular levels. Data integration resulting from these *-omics* approaches is becoming crucial to unravel the interactions among all different molecular elements and to highly contribute to interpret all findings in a complex biological context. Here, we have used advanced data integration methods for multi-level joint analysis of transcriptomics and proteomics datasets depicted from the mouse brain 2h after cerebral ischemia. By modeling net-like correlation structures, we identified a set of differentially expressed genes and proteins with a relevant association in stroke pathology. Of those, *Ccl3, Atf3, Fosb, Gadd45g, Rgs2, 4933427D14Rik, Cldn20 and Cstad* genes and CTNND2 and CAMK2A proteins were further replicated in a new cohort of ischemic mice and changes in their expression pattern were also assessed later in time. In summary, we have used for the first time an integrative approach that enabled us to elucidate, by means of biostatistical tools, key elements of the stroke pathophysiology. This innovative strategy is expected, therefore, to complement or even go beyond the traditional analysis for single-omics datasets and emphasize complex unnoticed associations among different molecular elements on stroke pathology.

Stroke remains a major leading cause of death and disability worldwide [1], representing a major negative social health problem and contributing to the rising costs of healthcare in many developed countries [2]. Effective therapies based on reperfusion mechanisms are currently in use [3], [4]. However, only a reduced percentage of ischemic stroke patients can benefit from them, since there is a very narrow therapeutic time window for their implementation and absolute contraindications for those who present risk of bleedings [5], [6]. Therefore, contributions identifying new therapeutic strategies and targets to neuroprotect the ischemic brain are still urgently needed.

A prompter and accurate diagnosis of ischemic stroke might also increase the number of patients that benefit from all these therapies, inasmuch as at this moment diagnosis is currently based on neurological exploration and costly imaging techniques [7]. However, neuroimaging devices are not always accessible, especially in primary health centers and in underdeveloped countries, which delay the stroke diagnosis in some scenarios. Blood biomarkers are expected to become potential substitutes for these neuroimaging approaches [8]–[10] since they might facilitate stroke discrimination versus other neurological disorders that present similar clinical symptoms, accelerating the treatment, helping in patients'

monitoring for adverse effects or supporting patients prognosis at later stages of the disease [11], [12].

For this, a better characterization of the stroke pathophysiology is strictly required to both facilitate the identification of molecular indicators of ischemic stroke and provide novel insights into potential therapeutic targets to restrain or even reverse the progression of ischemia damage. In this context, the *-omics* technologies have emerged as new sophisticated large-scale analytical tools that enable an accurate identification of the biological changes in the brain at different molecular levels [13]. On top of that, joint analyses of multiple datasets from different *–omics* technologies are becoming crucial to unravel the relationships among the different molecular components and globally interpret all findings in a complex biological context [14]. The combination of these *multi-omics* through integrative analyses, thus, is expected to further enhance the comprehension of the molecular dynamics underlying ischemic stroke and might provide a framework where the complexity of the interactive molecular networks prevails over the individual alterations of each component separately.

In the present study we aimed at identifying the main transcriptomic and proteomic changes that occur in the mouse brain early after cerebral ischemia. Specifically, we were interested in joining data from both *–omics* techniques by means of integrative analyses to reveal potential networks of genes and proteins with a substantial involvement in the pathogenesis of ischemic stroke. We further explored the results by selecting a relevant network from these integrative analyses to replicate our findings for several of their components and further explore them in the ischemic brain over time.

MATERIALS AND METHODS

1. Experimental design

All animal procedures were conducted 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 60/16). All experiments were conducted in a randomized manner and in adherence to the ARRIVE guidelines [15]. C57BL/6J male mice were used in the experiments (8-12 week-old; 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 air, Abbot Laboratories, Spain) was given to mice via facemask during all surgical procedures described below.

A total of 37 animals were used to complete the whole study. In the discovery phase, 15 animals were needed. Among them, 3 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=1). In the replication phase, 18 animals were used. From those, 6 were excluded due to incomplete occlusion or reperfusion after removal of the filament (n=2), death during the experimental protocol (n=3) or poor brain perfusion after euthanasia (n=1).

2. Transient cerebral ischemia model (tMCAO)

Transient infarction in the territory of the middle cerebral artery (MCA) was induced by introducing an intraluminal filament, as described elsewhere [16]. In summary, 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 by affixing a laser Doppler probe (Moor Insturments, UK) to the skull. Then, animals were placed in the supine position and 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. Occlusion of the MCA was confirmed by a reduction in the CBF recorded by the laser Doppler probe.

After occlusion, animals were allowed to recover from anesthesia. Ninety minutes later, mice were re-anesthetized and filament was removed to induce reperfusion of the CBF. Only animals that exhibited a reduction of CBF of 80% after filament introduction and a recovery of 75% after filament removal were included in the study. After reperfusion, animals were allowed to recover for 30 minutes or 4.5 hours (corresponding with 2h or 6h from the beginning of the MCA occlusion), according to their experimental group. Sham surgery was performed by the same surgical procedures without insertion of the nylon-coated filament.

3. Brain tissue collection and extraction of protein and RNA

Animals were euthanized and transcardially perfused with cold saline to remove blood from brain vessels. Immediately after euthanasia, mouse brains were quickly removed and sectioned into 1mm slices over ice. Slice corresponding to the bregma point, where the infarction territory is located, was carefully dissected to isolate the right (ipsilateral, IP) and left (contralateral, CL) hemispheres separately (Supplementary Figure S1). Each hemisphere was flash frozen in liquid nitrogen and stored at -80ºC.

Flash frozen tissues were pulverized into a powder in liquid nitrogen over dry ice, and total fractions of protein and RNA were then isolated using the MirVana™ Paris™ (Thermo Fisher Scientific Inc., USA). RNA and protein fractions were kept at -80ºC until further use.

4. Discovery phase study

The discovery phase was performed on RNA and protein extracts from brain samples from 8 ischemic animals euthanatized 2h after the MCAO onset. Four sham-control animals were also included in this phase to discard the selection of gene and protein candidates differentially altered due to other reasons rather than cerebral ischemia itself.

4.1 Transcriptomics study

4.1.1 Mouse brain microarrays

Total RNA concentrations from mouse brain samples were measured with a Nanodrop 1000 Spectrophotometer (ThermoFisher) and RNA integrity was assessed using the Agilent 2100 BioAnalyzer (Agilent Technologies, USA). All samples showed similar RNA integrity numbers (between 5 and 7).

The Genechip© Mouse Clariom S 24x arrays plate (Affymetrix, ThermoFisher) was used to analyze gene expression patterns on a whole-genome scale on a single array. 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) according to the 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 plate was scanned and processed with Affymetrix GeneChip Command Console to obtain expression array intensity .cel files.

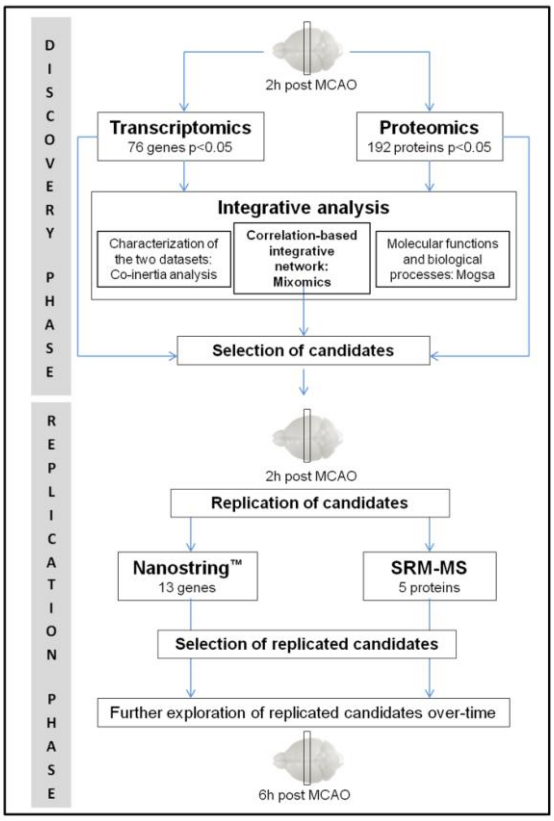


Figure 1. Workflow chart of the different stages of our study.

4.1.2 Microarray statistical analyses

Statistical and bioinformatics analyses were performed using custom scripts in R language

version 3.4.2 (R Core Team, 2017) with common Bioconductor packages. In brief, after following a standard quality control [17], Robust Multi-array Average algorithm (RMA, [18]) was used for pre-processing microarray data in order to perform background adjustment, normalization and summarization of probe set expression values. Then, genes whose standard deviation (SD) was below the 50 percentile of all SD, without a known Entrez Gene database identifier and without a valid annotation to the Gene Ontology database were filtered out from the whole dataset and the final amount of 9324 genes was considered for the statistical analysis. Selection of differentially expressed elements was based on a linear model analysis with empirical Bayes modification for the variance estimates [19], considering statistically significant those genes with a p-value<0.05 when IP and CL brain samples were compared. Logarithmic fold-changes (LogFC) were calculated by applying a base 2 logarithm to each gene expression in each sample and then subtracting the CL to the IC expression value for each animal. Subtracted values were finally averaged within each experimental group.

4.2 Proteomics study

4.2.1 In-gel fractionation and digestion

Protein extracts from mouse brain samples were quantified using the BCA Protein Assay (ThermoFisher). Twenty µg of each sample were resolved in 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels under reducing conditions and stained with colloidal coomasie G250 (Bio-Rad, USA). Each lane was divided in five segments that were individually processed to perform in-gel digestion of the proteins. Briefly, stained gel fragments were cut into small pieces, washed with 50 mM ammonium bicarbonate / 50% ethanol and dehydrated with ethanol. Reduction was performed by incubating samples with 10 mM dithiothreitol (DTT) for 1h at 56°C, followed by alkylation with 55 mM iodoacetamide for 30 min at dark. After washing and dehydration with acetonitrile, gel pieces were covered with 2.7 ng/µl trypsin (Promega, USA) in 25 mM ammonium bicarbonate and digestion was run overnight at 37°C. Peptide extraction was carried out by incubation at 37ºC with acetonitrile and further incubation with 0.2% trifluoracetic acid (TFA). The eluted peptides were dried in a Savant™ SpeedVac™ High Capacity Concentrator (ThermoFisher) and stored at -20°C until further use.

4.2.2 Liquid chromatography-Mass spectrometry analysis (LC-MS)

Tryptic digests from excised bands were analyzed using a LC-MS approach in a linear ion trap (LTQ) Orbitrap Velos mass spectrometer (ThermoFisher). Peptide mixtures were fractionated by on-line nanoflow liquid chromatography using an EASY-nLC system (Proxeon Biosystems, ThermoFisher) with a two-linear-column system: digests were loaded at 4µL/min onto a trapping guard column (EASY-column, 2 cm long, ID 100 µm, packed with Reprosil C18, 5 µm particle size) and then eluted from the analytical column (EASY-column, 10 cm long, ID 75 µm, packed with Reprosil C18, 3 µm particle size). Separation was achieved by using a mobile phase of 0.1% formic acid in water (Buffer A) and acetonitrile with 0.1% formic acid (Buffer B) and applying a linear gradient from 0 to 35% of buffer B for 120 min at a flow rate of 300 nL/min. Ions were generated applying a voltage of 1.9 kV to a stainless steel nano-bore emitter.

The mass spectrometer was operated in a data-dependent mode. A scan cycle was initiated with a full-scan MS spectrum (from m/z 300 to 1600) acquired in the Orbitrap with a resolution of 30,000. The 20 most abundant ions were selected for collision-induced dissociation fragmentation in the LTQ when their intensity exceeded a minimum threshold of 1000 counts, excluding singly charged ions. Automatic gain control (AGC) target values were set to 1×10^6 ions for survey MS and 5000 ions for MS/MS experiments. The maximum ion accumulation time was 500 and 200 ms in the MS and MS/MS modes, respectively. The normalized collision energy was set to 35%, and one microscan was acquired per spectrum. Ions subjected to MS/MS with a relative mass window of 10 ppm were excluded from further sequencing for 20 s. For all precursor masses a window of 20 ppm and isolation width of 2

Da was defined. Orbitrap measurements were performed enabling the lock mass option (m/z 445.120024) for survey scans to improve mass accuracy.

4.2.3 Protein identification and quantitative differential analysis

Progenesis® QI for proteomics software v3.0 (Nonlinear dynamics, UK) was used for MS data analysis using default settings. The results from each of the five gel fractions were independently analyzed and all MS runs were automatically aligned to a selected reference sample. Alignments were then manually supervised and automatically normalized to all features. Only features within the 400 to 1,600 m/z range, oscillating from 5 to 115 min of retention time according to the gel fragment, and with positive charges between 2 to 4 were considered for identification and quantification. Peak lists were analyzed using the Mascot search engine (v5.1, Matrix Science, UK). Protein identification was carried out using the SwissProt-MusMusculus database (2017_10: 16.942 entries), setting precursor mass tolerance to 10 ppm and fragment mass tolerance to 0.8 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 cleavage. Significant threshold for protein identification was set to $p<0.05$ for the probability-based Mascot score and at least 2 spectra per peptide. Finally, the five fractions were combined into one single Progenesis experiment. Label-free protein abundance quantification was based on the sum of the peak areas within the isotope boundaries of peptide ion peaks. To allow comparison across different sample runs, the abundance of each protein was normalized to all proteins. Only those proteins quantified and identified with at least 2 unique and non-conflicting peptides were considered for the statistical analysis (44.8%, 2485 proteins). For data normalization, protein abundance values were log-10 transformed and column-wise standardized [20]. Selection of differentially expressed proteins was based on a linear model analysis implemented in the Bioconductor limma package [21], considering statistically significant those proteins with a p-value<0.05 when IP and CL brain samples were compared. Logarithmic fold-change (LogFC) was calculated by applying a base 2 logarithm to each protein expression in each sample and then subtracting the CL to the IC expression value for each animal. Subtracted values were finally averaged within each experimental group.

4.3 Integrative analyses of ischemic-altered gene and protein datasets

For the integrative analyses only samples from ischemic animals were considered. Individual pre-processing of ischemia-related differentially expressed gene and protein datasets through Principal Component Analysis (PCA) resulted in the identification of two samples (corresponding to the IP and CL hemispheres from one single animal) that behaved as outliers, which were beforehand excluded for the integrative analyses (Supplemental Figure S2). Thus, the integrative analyses of significant differentially expressed genes and proteins were performed with samples corresponding to the IP and CL of 7 different MCAO animals. Besides, the protein TXN2 was momentarily precluded from these integrative analyses, since its relatively high fold change expression in the ischemic hemisphere masked all other candidates with a similar abundance profile when data was integrated.

Three different integrative tools were used, all of them implementing omics-focused versions of already known dimension reduction techniques. First, Multiple Co-Inertia Analysis (MCIA, [22]) included in made4 R package (v1.52.0, [23]) was performed to maximize the covariance between gene and protein data sets for each group of samples. Second, mixOmics R package (v6.3.1, http://mixomics.org) was used to perform a Regularized Canonical Correlation Analysis (rCCA, [24]) between gene and protein data sets. In brief, data was properly transposed and scaled and the tuning of the rCCA parameters were iterated until achieving a cross validation (CV) score of 0.8 (lambda 1=0.1318 and lambda 2=0.001). Resulting relevance network was plotted for a

correlation cut-off of R≥0.75. Third, the mogsa R package (v1.12.2, [25]) was employed to annotate and weight genes and proteins data sets against gene sets from the Gene Ontology (GO). This analysis was based on the application of the Multiple Factor Analysis (MFA, [26]) and was fed with the gene sets corresponding to GO annotations in Mus musculus for Hallmark (v5.2, [27]) and Broad Institue's C2 Cannonical Pathways molecular signature databases (v5.2, http://software.broadinstitute.org/gsea/login.jsp). Default parameters were used, but applying weighting of the individual data sets. The mogsa R package generated gene set scores (GSS) by computing all gene and protein contributions to each gene set found to be enriched [28]. GSS were further decomposed with respect to each dataset (genes and proteins).

5. Replication phase study

The replication phase was conducted on brain samples from 6 ischemic animals obtained 2h after ischemia. Replicated candidates were further evaluated on brain samples from 6 ischemic animals 6h after ischemic onset.

5.1 Digital multiplexed gene expression assay

The Nanostring's nCounter® XT Assay (NanoString Technologies®, USA) was performed at the Genomics Core Facility, Center Esther Koplowitz, Barcelona (Spain). The assay was conducted according to manufacturer's instructions. Briefly, Nanostring's nCounter Elements™ XT probes were created for candidate genes from the discovery phase, along with 3 housekeeping genes (beta-2-microglobulin (*B2m*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and peptidyl-prolyl cis-trans isomerase A (*Ppia*)) (Supplemental Table S3). One hundred ng of the total RNA from mouse brain samples was hybridized with the complete Master Mix containing the pool of probes. Then, samples were loaded onto the NanoString PrepStation, placed into the nCounter cartridge and transferred to the nCounter digital analyzer for image capture and data acquisition of fluorescent reporters. Measurements were taken at high sensitivity with 280 fields of view (FOVs). Data analysis and normalization with standard procedures was performed with the NanoString-supplied software (v3.0). Briefly, following background subtraction, raw intensity values (RNA counts) were normalized to the geometric mean of the positive control spike-in RNAs, followed by a second normalization to the geometric mean of the housekeeping genes. Values were finally expressed as fold-change of the IP compared to the CL hemispheres for each animal.

5.2 Targeted mass spectrometry: Parallel Reaction Monitoring (PRM)

5.2.1 In-solution digestion

Protein extracts from mouse brain samples were subjected to buffer exchange to 8M Urea in 50mM ammonium bicarbonate using 3KDa cut-off Amicon Ultra ultrafiltration devices (Merck-Millipore, USA). Afterwards, total protein content was quantified using the RCDC kit (Bio-Rad), and 4 µg of each protein extract were processed. Samples were reduced with 10 mM DTT for 1h, and then alkylated with 20mM iodoacetamide for 30min at dark. Carbamidomethylation reaction was quenched by addition of 35 mM N-acetyl-L-cysteine for 15min at dark. Samples were diluted with 50 mM ammonium bicarbonate to a final concentration of 1M Urea before being digested with trypsin in a ratio of 1:20 (w/w) overnight at 37 °C. The reaction was stopped with 0.5% formic acid, and the tryptic digest was kept at -20ºC until further analysis.

5.2.2 Targeted LC-MS analysis

Proteotypic peptides from the selected protein candidates, along with 3 housekeeping proteins (B2M, GAPDH and PPIA), were selected from the LC-MS experimental results on the discovery phase or from MS data repositories (Peptide Atlas, SRM Atlas) (Supplemental table S3). Isotopically labeled versions of target peptide sequences were purchased from ThermoFisher. Analysis of the heavy labeled peptides spiked into a pool of the samples yielded concentration response curves with high correlation coefficients (typically >0.9) and low coefficients of

variation (≤20%) over a 10-fold concentration range.

Prior to LC-MS analysis, known amounts of each labeled peptide (ranging from 1-18000 fmol per µg of digest) were mixed with sample digests, from which 500 ng were analyzed using LTQ Orbitrap Velos MS. In brief, peptide mixtures were fractionated in the EASY-nLC 1000 system: digests were loaded at 4µL/min onto a trapping guard column (Acclaim PepMap 100 nanoviper, 2 cm long, ID 75 µm and packed with C18, 3 µm particle size; ThermoFisher) and then eluted from the analytical column (25 cm long, ID 75 µm, packed with Reprosil Pur C18-AQ, 3 µm particle size (Dr. Maisch GmbH). Separation was finally achieved by the same procedure than in untargeted LC-MS.
The LTQ Orbitrap Velos MS was operated in parallel reaction monitoring (PRM) mode. PRM was used to acquire full MS/MS spectra from target peptides, from which a precursor ion mass list was generated based on their sequence into Skyline (v4.1.0. MacCross Lab Software, USA). AGC target value was set for 5000 ions in MS/MS. The maximum ion accumulation time was 50 ms. Normalized collision energy was set to 38% and one microscan was acquired per spectrum. For all precursor masses an isolation width of 2 Da was defined.

5.2.3 Quantitative differential analysis

Raw data was imported to Skyline software to analyze the results. Chromatographic ion extractions of the 3 to 5 transitions that gave the highest intensities were used to quantify each peptide. For peptides giving significant amounts of two different charge state ions, signals were acquired for both. Ratios between the unlabeled endogenous peptide and the labeled internal standard (L/H) were then used to calculate endogenous peptide levels within each sample. In brief, amounts of each endogenous peptide were extrapolated from their respective concentration-response curves and normalized to the geometric abundance average of the housekeeping proteins. For those proteins with more than one representative peptide, the geometric mean of all peptide values was also computed for each sample. Final values are expressed as fold-change of the IP compared to the CL hemisphere.

5.3 Mouse brain ELISA

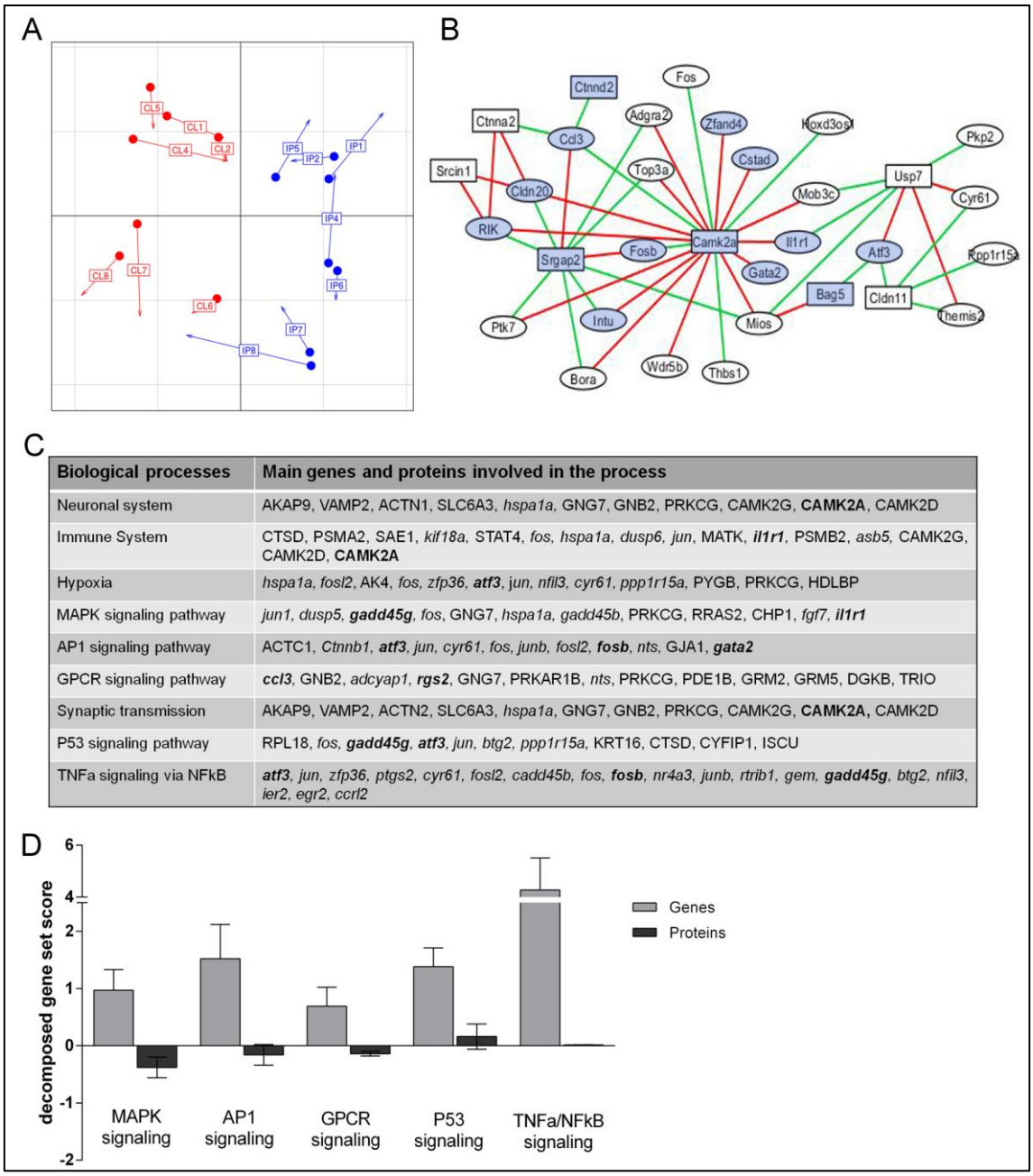
Two outstanding gene candidates were evaluated at protein level in mouse brain by means of commercial ELISA immunoassays, following manufacturer's instructions (Regulator of G-protein signaling 2, Cat.# MBS9321001, MyBiosource, USA; Gadd45g, Cat.#OKEH01666, Aviva Systems Biology, USA). Samples of both IP and CL hemispheres from 10 MCAO animals, 5 from each experimental group (2h and 6h after ischemia), were assayed per duplicate and the mean value was used. Optical densities were measured in a Synergy TM Mx microplate reader (BioTek Instruments Inc, USA). Samples with a coefficient of variation (CV) higher than 20% were excluded for the analysis. All samples were successfully detected within the concentration range of each ELISA assay.

5.4 Statistical analyses

SPSS statistical package v22.0 was used for statistical analyses and GraphPad Prism v6.0 for creating graphs. Mouse protein and gene data distribution was assessed by Shapiro-Wilk ($p>0.05$ indicates normal distribution). Differences in protein or gene levels between IP and CL hemispheres were assessed by paired t-test (normally distributed variables) or Wilcoxon signed-rank test (non-normally distributed variables). All other comparisons between experimental groups were evaluated through the Student t-test (normally distributed variables) or the Mann-Whitney test (non-normally distributed variables).

RESULTS

A summarized workflow chart of the whole study is shown in Figure 1.



Biological processes	Main genes and proteins involved in the process
Neuronal system	AKAP9, VAMP2, ACTN1, SLC6A3, *hspa1a*, GNG7, GNB2, PRKCG, CAMK2G, **CAMK2A**, CAMK2D
Immune System	CTSD, PSMA2, SAE1, *kif18a*, STAT4, *fos*, *hspa1a*, *dusp6*, *jun*, MATK, ***il1r1***, PSMB2, *asb5*, CAMK2G, CAMK2D, **CAMK2A**
Hypoxia	*hspa1a*, *fosl2*, AK4, *fos*, *zfp36*, ***atf3***, *jun*, *nfil3*, *cyr61*, *ppp1r15a*, PYGB, PRKCG, HDLBP
MAPK signaling pathway	*jun1*, *dusp5*, ***gadd45g***, *fos*, GNG7, *hspa1a*, *gadd45b*, PRKCG, RRAS2, CHP1, *fgf7*, ***il1r1***
AP1 signaling pathway	ACTC1, *Ctnnb1*, ***atf3***, *jun*, *cyr61*, *fos*, *junb*, *fosl2*, ***fosb***, *nts*, GJA1, ***gata2***
GPCR signaling pathway	***ccl3***, GNB2, *adcyap1*, ***rgs2***, GNG7, PRKAR1B, *nts*, PRKCG, PDE1B, GRM2, GRM5, DGKB, TRIO
Synaptic transmission	AKAP9, VAMP2, ACTN2, SLC6A3, *hspa1a*, GNG7, GNB2, PRKCG, CAMK2G, **CAMK2A**, CAMK2D
P53 signaling pathway	RPL18, *fos*, ***gadd45g***, ***atf3***, *jun*, *btg2*, *ppp1r15a*, KRT16, CTSD, CYFIP1, ISCU
TNFa signaling via NFkB	***atf3***, *jun*, *zfp36*, *ptgs2*, *cyr61*, *fosl2*, *cadd45b*, *fos*, ***fosb***, *nr4a3*, *junb*, *rtrib1*, *gem*, ***gadd45g***, *btg2*, *nfil3*, *ier2*, *egr2*, *ccrl2*

Figure 2. Integrative analysis of transcriptomic and proteomic data in mouse cerebral ischemia. (**A**) Distribution of samples based on their transcriptomic (dot) and proteomic (arrow) information for contralateral (CL, red) and ipsilateral (IP, blue) samples. Circle and arrows from each sample are joined by a line, the length of which is proportional to the divergence between those samples in the two datasets. (**B**) Relevance network of top correlations between genes (circles) and proteins (rectangles). Red-colored edges indicate positive correlations; green-colored edges indicate negative correlations. Selected candidates for replication are marked in blue. (**C**) Altered biological processes and signaling pathways in which the selected candidates (in bold) are involved. Genes are indicated in italics, proteins are shown in regular type. (**D**) Decomposed gene set score (GSS) of the five outstanding intracellular signaling pathways. The higher decomposed GSS, the larger contribution of the dataset to the specific process or pathway.

Differentially expressed proteins and genes early after cerebral ischemia

To elucidate the specific gene and protein changes that early occur in brain following cerebral ischemia, we evaluated mouse brain samples obtained 2h after ischemic or sham-control surgery by means of a transcriptomics and a proteomics approach. After cerebral ischemia, 76 genes and 192 proteins were found differentially regulated between IP and

CL brain regions (Supplementary tables S1 and S2). In contrast, only 2 genes and 60 proteins were altered between IP and CL hemispheres after sham-control surgery.
Gene and protein datasets from sham-control animals were used to discard the selection of any relevant component disturbed due to other reasons rather than cerebral ischemia itself. In this regard, none of the genes altered after ischemia were deregulated after sham-control surgery, and only 8 stroke-related differentially expressed proteins were also altered in brains from sham-control animals (marked with an asterisk in Supplementary tables S1 and S2). Thus, these proteins were dismissed from any future selection, so all highlighted candidates hereafter were assumed to be specifically deregulated due to cerebral ischemia.

Integrative analysis of differentially abundant proteins and genes after ischemia

To give a deep understanding of the mechanisms underlying cerebral ischemia, we conducted a comprehensive integration analysis of both *-omics* datasets from MCAO animals. Overall, the final amount of 76 genes and 191 proteins (after excluding TXN2) deregulated after ischemia were used to joint data in these integrative approaches (Figure 2).

By means of the multiple co-inertia analysis (MCIA), we initially examined how our experimental samples behaved when projecting gene and protein components within the same bi-dimensional space (Figure 2A). We clearly differentiated two separated co-structures, corresponding to each brain hemisphere, which confirmed that integrated data from both datasets had the ability to plainly discriminate phenotypical changes due to the ischemia.

Besides, by modeling net-like correlation structures (rCCA) between our protein and gene datasets, we identified an integrated network in which high correlated elements were represented (Figure 2B). Positive and negative correlations were found among 24 genes and 7 proteins. Interestingly, two clusters were visually highlighted around CAMK2A and SRGAP2, with 9 and 19 directly-connected genes respectively, suggesting an important role of these candidates in the pathophysiology of stroke.

From this correlation-based integrated network, 15 representative candidates were selected to replicate their performance in the hyper-acute phase of cerebral ischemia (in blue in Figure 2B). Besides, 3 other candidates were also included due to their leading position in the analysis of the gene individual dataset (sorted by p-value; *Gadd45g* and *Rgs2*) and in the protein dataset (sorted by logFC; TXN2). Overall, 18 candidates (13 genes and 5 proteins) were chosen to be further replicated in a new cohort of ischemic animals (Table 1).

Gene candidates	P-value	Log FC
Atf3	$3.59 \cdot 10^{-5}$	1.438
Ccl3	$2.90 \cdot 10^{-6}$	2.207
Cenpp	$2.22 \cdot 10^{-2}$	0.525
Cldn20	$7.36 \cdot 10^{-3}$	-0.666
Cstad	$3.39 \cdot 10^{-2}$	-0.453
Fosb	$4.25 \cdot 10^{-4}$	1.660
Gadd45g	$4.47 \cdot 10^{-3}$	1.302
Gata2	$1.73 \cdot 10^{-2}$	-0.496
Il1r1	$2.85 \cdot 10^{-2}$	-0.524
Intu	$3.87 \cdot 10^{-2}$	-0.416
Rgs2	$8.24 \cdot 10^{-3}$	1.637
Zfand4	$2.65 \cdot 10^{-2}$	-0.511
4933427D14Rik	$4.53 \cdot 10^{-2}$	-0.467

Protein candidates	P-value	Log FC
BAG5	$1.68 \cdot 10^{-5}$	-0.394
CAMK2A	$4.72 \cdot 10^{-4}$	-0.274
CTNND2	$6.41 \cdot 10^{-4}$	-0.119
SRGAP2	$7.22 \cdot 10^{-3}$	0.123
TXN2	$1.07 \cdot 10^{-2}$	1.511

Table 1. Selected candidates on the discovery phase of the study. Statistical significance was evaluated between ipsilateral (IP) and contralateral (CL) samples of ischemic animals. Logarithmic fold-change (LogFC) was calculated by applying a base 2 logarithm to each gene or protein expression in each sample and then subtracting the CL to the IC value for each animal.

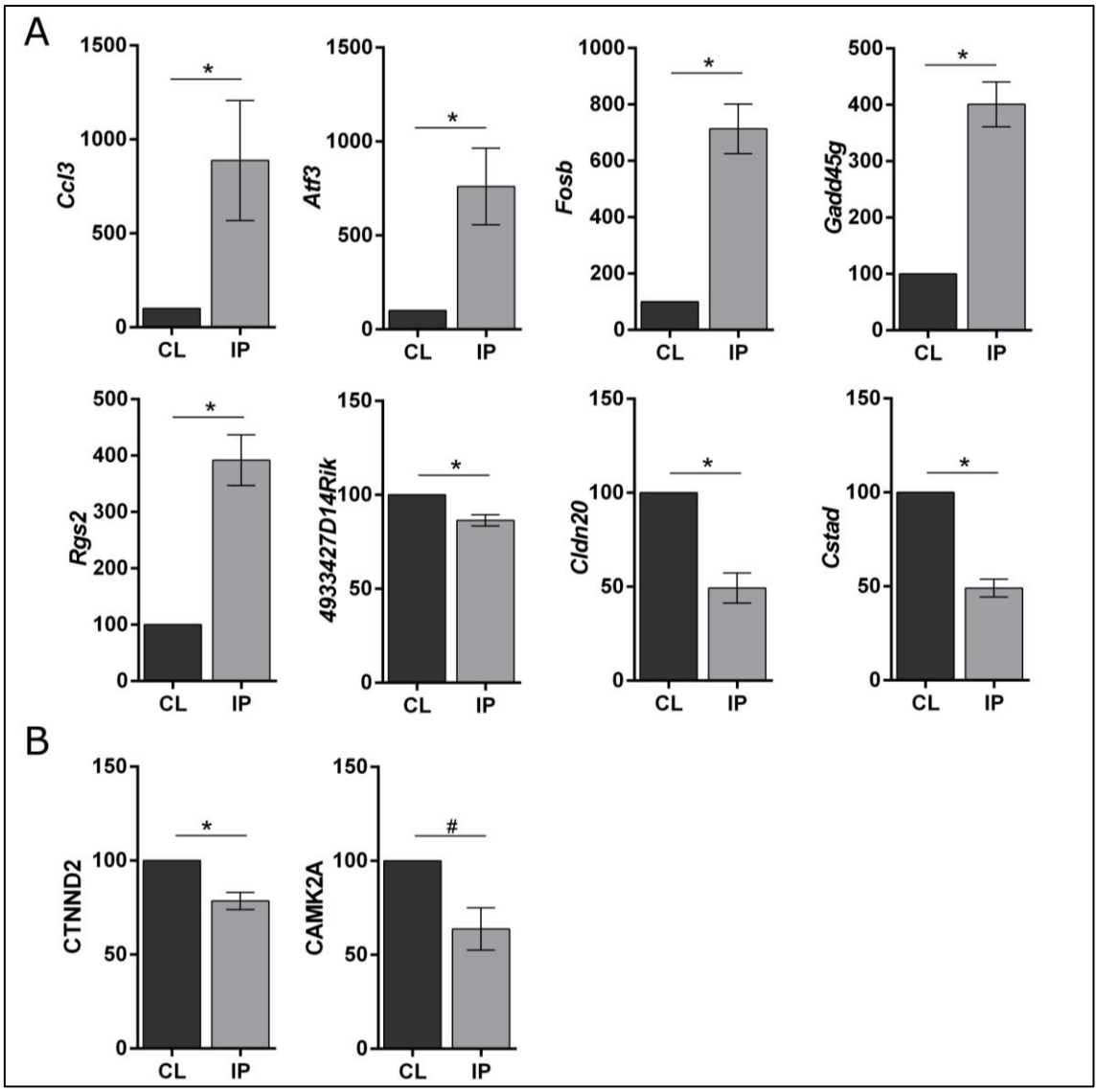


Figure 3. Replication of the selected candidates for genes (A) and proteins (B) in the mouse brain 2h after cerebral ischemia. Raw values from all samples were normalized by the expression of their housekeeping genes and then normalized by the signal of its respective contralateral hemisphere (Y axis). Graphs indicate mean ± SEM. In all cases, * indicates $p<0.05$, ** indicates $p<0.01$ and $^{\#}$indicates $p\leq0.1$. IP: ipsilateral brain hemisphere; CL: contralateral brain hemisphere.

Deeper information about the main biological processes and molecular functions in which these selected candidates were involved was also obtained (mogsa). In this regard, we were able to confirm that the neuronal system was the most distinguished biological process differentially altered in our ischemic brain samples (Figure 2C). Concretely, molecular datasets indicated there was an altered transmission across synapses and unbalanced excitotoxicity mechanisms, which are all well known altered functions in acute cerebral ischemia. Moreover, changes in the immune system were already appreciable at this early studied time-point as well, reinforcing its involvement in the acute pathophysiology of stroke. Mediators and contributors of hypoxic signaling pathways were also over-represented in our datasets, together with several intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway, the activator protein-1 (AP-1) transcription pathway, the G protein-coupled receptors (GPCR) network, the p53 pathway and the tumor necrosis factor alpha (TNF-α) signaling pathway. Interestingly, a deeper exploration of these altered pathways identified that genes were

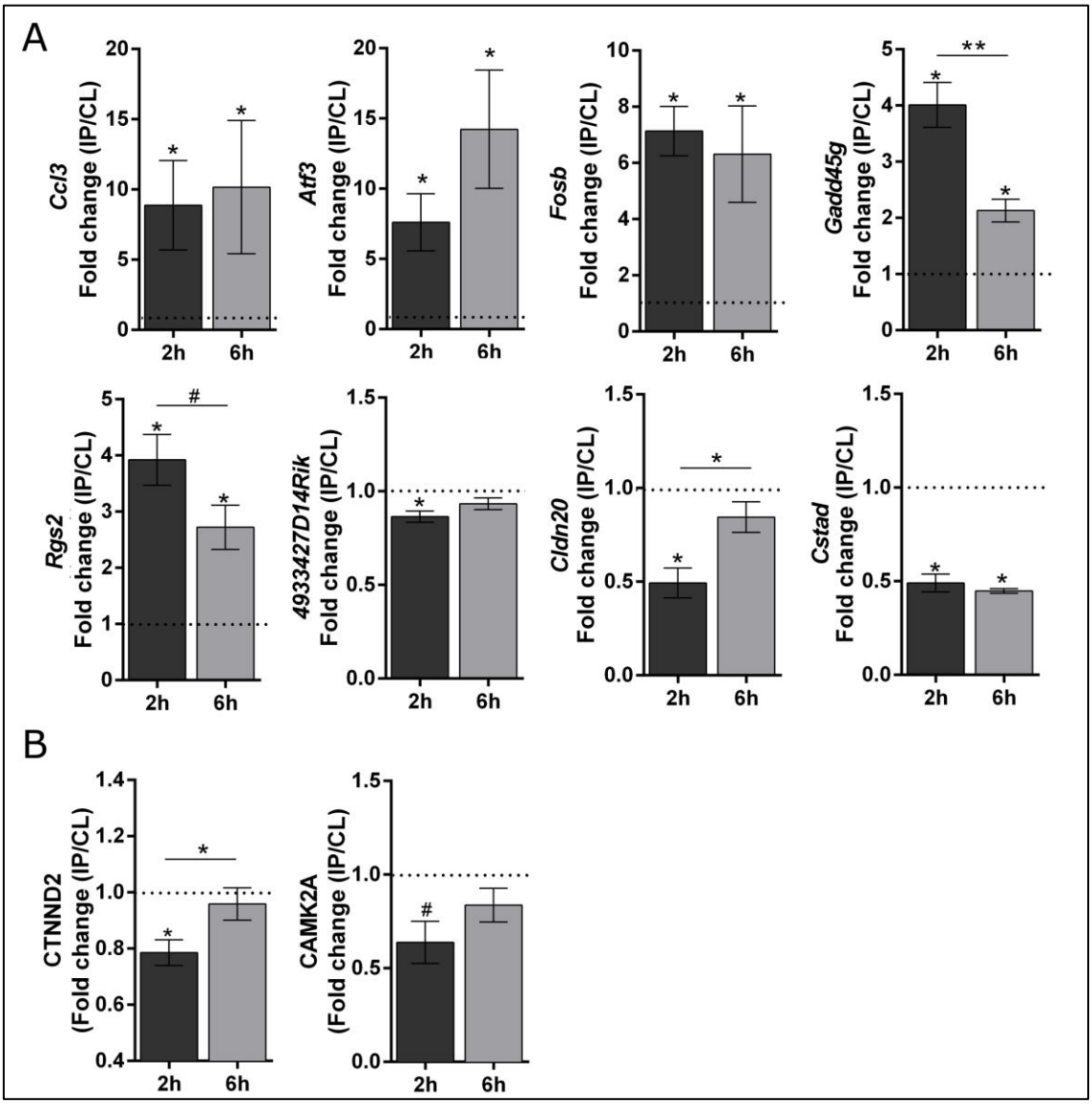


Figure 4. Exploration of the replicated genes (A) and proteins (B) over time after mouse brain ischemia. Fold change expression was calculated as ratios between IP and CL hemispheres for each individual animal and time-point. Graphs indicate mean ± SEM. Statistical differences between IP and CL hemispheres at each studied time-point are indicated with symbols on top of each bar. Significantly relevant comparisons of fold change between 2h and 6h are indicated with horizontal bars. In all cases, * indicates $p<0.05$ and # indicates $p<0.1$. IP: ipsilateral brain hemisphere; CL: contralateral brain hemisphere. Dashed lines indicate a 1-fold change in expression (equal values for IP and CL samples).

contributing substantially more than proteins to the alteration of the specific signaling cascades at the studied time-point (Figure 2D).

Replication of the selected candidates

Gene and protein replication was performed in a new cohort of MCAO animals, in which brain samples were also collected 2h after the ischemia onset (Figure 3). Eight out of the 13 genes (61.5%) were correctly replicated in this new cohort of ischemic animals (Figure 3A): *Ccl3*, *Atf3*, *Fosb*, *Gadd45g* and *Rgs2* significantly increased in the IP compared to the CL hemisphere, while *4933427D14Rik*, *Cldn20* and *Cstad* decreased in the IP versus the CL brain region. Besides, from the 5 selected proteins, 2 were not detectable through PRM strategy and could not be evaluated in this phase of the study. For the

other 3 candidates, 2 showed a pattern of abundance similar to the discovery phase: both CAMK2A and CTNND2 protein levels decreased in the IP hemisphere compared to their CL (Figure 3B).

Brain abundance of selected genes and proteins over time after ischemia

To further characterize our replicated candidates, we evaluated them at a later time-point after cerebral ischemia (6h after MCAO). Expression of *Ccl3*, *Atf3* and *Fosb* remained at high levels 6h after the ischemic event, since no differences in the IP/CL ratios were detected between the 2 studied time-points (Figure 4A). Conversely, *Gadd45g* and *Rgs2* genes showed a decrease by half in their fold-change expression 6h after MCAO compared to that at 2h, but still remained high in the IP compared to the CL hemisphere. With regards to the early down-regulated genes, *4933427D14Rik* did not significantly reverse the initial ischemia-related decrease in fold-change expression, despite changes between IP and CL hemispheres were not further maintained 6h after ischemia. At this later time-point *Cldn20* expression returned to normal levels, whereas *Cstad* maintained decreased over time.

CTNND2 protein levels also reverse their initial down-regulation 6h after ischemia (Figure 4B). CAMK2A, however, did not statistically show this recovery, but differences between IP and CL hemispheres at 6h were no longer consistent either.

Exploration of RGS2 and GADD45G protein levels in brain

We further focused our attention to *Rgs2* and *Gadd45g* candidates, since both showed a similar gene expression profile, with an acute up-regulation within the first hours after ischemia. Thus, we aimed at determining the protein profile of these two candidates at the same explored time-points for gene expression. Two hours after ischemia, neither RGS2 nor GADD45G protein levels differed between IP and CL hemispheres, since the IP/CL ratios resulted to be close to one (Figure 5A, 5B). However, 6h after ischemia both proteins significantly changed their levels between the two studied regions of the brain: RGS2 protein levels decreased in the IP hemisphere compared to the CL, whereas GADD45G levels showed a significant increase in the ischemic region compared to the healthy hemisphere.

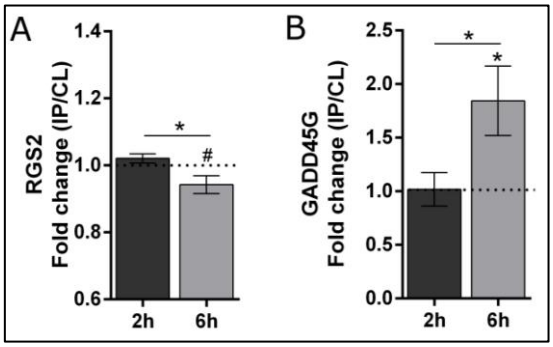


Figure 5. Brain protein levels of RGS2 (A) and GADD45G (B) at 2h and 6h after ischemia. Raw values from all samples were normalized by the signal of its respective contralateral hemisphere. Graphs indicate mean ± SEM. Statistical differences between IP and CL hemispheres at each studied time-point are indicated with symbols on top of each bar. Significantly relevant comparisons of fold change between 2h and 6h are indicated with horizontal bars. In all cases, * indicates $p<0.05$. Dashed lines indicate a 1-fold change in expression (equal values for IP and CL samples).

DISCUSSION

The study being presented here identifies key molecular signatures of cerebral ischemia by means of an integrative analysis of transcriptomic and proteomic data compiled from the mouse ischemic brain during the hyper-acute phase of cerebral ischemia, when is of primary interest for a real translational research, both in the diagnosis and therapeutic context.

During the past decades numerous studies aimed at unraveling the main molecular changes that occur in brain following cerebral ischemia. The vast majority of these contributions have been based on the single characterization of protein or gene alterations separately, which have actively constructed knowledge about the stroke pathophysiology to the point of achieving a reliable -but still

uncompleted- understanding of the main mechanisms of cerebral ischemia at different molecular levels. Beyond the exploration of individual contributions of genes, proteins and metabolites to stroke pathology, a global comprehension of all their complex interactions, associations and connections is expected to further improve the identification of biologically relevant disease-associated mediators of stroke. On this basis, we applied recently developed multivariate projection-based approaches to transcriptomics and proteomics datasets in an effort to explore these relationships between genes and proteins. MCIA pinpointed the separation between the infarcted and healthy regions of the brain, which was clearly marked for both genes and proteins. Then, we use the mixOmics biostatistical tool to help us comprehend the underlying correlation-based connections between the differentially altered genes and proteins. It is worth mentioning that these correlations do not imply a direct biological connection among components, but they have been here used as a novel and complementary theory-based statistical strategy to highlight relevant associations among constituents of the stroke pathology. Deep details about the underlying biological interpretation of all these connections will need to be elucidated in the future. A first approximation, however, has been here obtained through the exploration of the main biological processes and molecular pathways in which our candidates were involved, and has been deepened by the existing literature hereunder.

Representative candidates from this correlation-based relevance network were selected to be replicated and further explored over time. Three of them (*Gadd45g, Rgs2 and Ccl3*) belonged to intracellular mechanisms of acute cell response to the ischemic insult, mostly the MAPK or the GPCR signaling pathways. GADD45 proteins are involved in cellular stress response mechanisms, including cell cycle arrest, DNA repair and apoptosis [29], which are mediated by the p38 MAPK, either by direct interaction between GADD45 and MAPK or by the GADD45-induced activation of upstream kinases for MAPK [30]. At present, the exact implications of GADD45 in brain are still unclear. Nonetheless, by extrapolating experimental data from other cell types, accumulating evidence suggests that ischemia might lead to increased GADD45 levels, which would in once be involved in hypoxia-induced cell death mechanisms [31]. In the present study, we detected a pronounced increase in brain *Gadd45g* gene expression very early after ischemia, which was already reduced by half 6h after the ischemic event, and a subsequent increase in its protein levels later in time. These results are in line with previous publications that reported abrupt changes in *Gadd45* gene expression and protein levels following transient global ischemia in rats [32], despite its concrete functional relevance still needs to be elucidated.

A similar temporal profile of gene expression was observed for *Rgs2* in our experiments. RGS family members interact with alpha subunits of G proteins and accelerate the hydrolysis of Gα-bound guanine nucleotide triphosphate (GTP), thereby reducing the amplitude and duration of the ligand-induced GPCR effects. RGS are therefore known to act as negative regulators of a wide variety of GPCR, including adrenergic, glutamatergic and GABAergic receptors, among others [33]. In response to short-term activation of the GPCR signaling pathway, *Rgs2* gene expression transiently up-regulates in different cell types, such as in cardiomyocytes [34], fibroblasts [35] and astrocytes [36]. In this latest cell type, the ischemia-related increase in *Rgs2* expression has been further associated to enhanced protein kinase C (PKC) and MAPK-mediated apoptosis. In fact, RGS modulation has become a new line of investigation as potential drug targets in a wide variety of pathologies, including brain and cardiovascular diseases and diabetes [37]. Since the loss of RGS-mediated regulation of G proteins has been reported to be protective against cardiac ischemic injury [38], it would be interesting to elucidate whether this therapeutic effect is also observed in the ischemic brain. In this regard, future efforts might be also directed towards determining whether the observed stroke-induced decrease in RGS2 protein levels is subjected to any endogenous compensatory

mechanism related to this hypothesized RGS-related neuroprotective effect.

In contrast to these previous candidates, CCL3 has been studied a lot in the context of stroke. Similar to what we have found here, this chemokine (also named as macrophage inflammatory protein 1 alpha, MIP1α) is well known to early increase in the ischemic brain in a wide variety of experimental stroke models [39]. CCL3 in brain plays an important role in activating and attracting inflammatory cells to the site of injury [40]. CCL3 intracellular effects are mediated by the stimulation of G protein-coupled cell surface receptors CCR1 and CCR5, which results in calcium release and increased expression of pro-inflammatory mediators, among others [41]. The exogenous administration of CCL3 in rodents has resulted in increased infarct volumes [42], which suggest a plausible role for this chemokine in the pathophysiology of stroke. These findings are supported also in stroke patients, in which CCL3 levels showed an early and sustained increase in circulation and correlated with post-stroke-related functional disability [43].

Immediately after stroke, there is also a fast and abrupt over-activation of gene expression as an intracellular response mechanism to tackle the ischemic insult. Since we are focused on very early time-points after ischemia, it is not surprising that two other selected candidates, *FosB* and *Atf3*, have a role in the regulation of gene transcription processes. The FOS family comprises four different members that dimerize with proteins of the Jun family, thereby forming the transcription factor complex AP-1. Concretely, *FosB* gene encodes for FOSB protein, which can also derive after chronic stimulation to a truncated splice variant ΔFOSB. After an acute ischemic insult, there is a substantial increase in FOSB, which is thought to be involved in the regulation of programmed cell death mechanisms [44]. Latter in time, there is a gradual accumulation of the truncated form ΔFOSB, which lacks this potent transcriptional activity, but triggers proliferation of neuronal progenitor-like cells, overall promoting the maturation of neurons [45]. Hence, although it is already known that the splicing of *FosB* pre-mRNA is endogenously regulated by the amount of the unspliced transcript [46], future contributions might aim at exploring whether these splicing machinery can be chemically modified for therapeutic purposes at earlier time-points after ischemia.

The other transcription factor, ATF3, is a stress-induced member of the cAMP-responsive element-binding protein (CREB) family of transcription factors highly involved in the repression of inflammatory gene expression in multiple cell types and diseases [47]–[49]. In experimental models of stroke, exogenous ATF3 up-regulation is known to confer protection against ischemia-related glutamate cytotoxicity both *in vitro* [50] and *in vivo* [51], and resulted in decreased neuronal apoptosis [52]. In contrast, ATF3 suppression in mice resulted in larger infarct volumes, worse neurological outcome and a dramatically increase of inflammatory gene expression and cell recruitment [53]. In this study, we have noticed an immediate increase in *Atf3* gene expression following cerebral ischemia, which even sustained high at 6h after the ischemic event. These results are in accordance with previous publications in the context of stroke [54], which report that *Atf3* up-regulation lasts throughout the first 48h, peaking at approximately 24h [50], [55]. All in all, these findings provide support for the proposal that the up-regulation of ATF3 after ischemia seem to be directed to the mitigation of the stroke-induced neurotoxicity, despite endogenous levels might not be sufficient to achieve that end. Future studies might elucidate whether therapies directed towards this increase in ATF3 levels might provide the desired neuroprotective efficacy.

The correlation-based relevance network of connected genes and proteins also depicted 4933427D14Rik, *Cstad* and *Cldn20,* 3 genes with a decrease expression in the ischemic region. *4933427D14Rik* has been recently described by the Encyclopedia of DNA Elements (ENCODE) Consortium [56]. Despite it is known to have a broad expression in the central nervous system, no biological information is still available in the literature about this gene. We have here found that *4933427D14Rik* gene expression

acutely down-regulates in the affected brain after cerebral ischemia, despite these changes seem not to maintain too much over time. Based on this observed expression profile, future contributions aimed at characterizing the main biological and functional implications of this gene are highly encouraged.

On the other hand, Mascarell and colleagues described for the first time in 2004 the *Cstad* gene, which encodes for two still unknown proteins located in mitochondria. To the best of our knowledge, *Cstad* gene expression has not been previously explored in the context of stroke. In fact, *Cstad* overexpression has only been described after the activation of T cells in the presence of CSA, also named as cyclosporin A [57]. It is noteworthy that CSA has been proposed as a neuroprotectant for stroke and traumatic brain injuries [58]. Since we here found an abrupt and maintained decrease in *Cstad* gene expression following cerebral ischemia, one might hypothesized that the CSA-induced overexpression of *Cstad* could be contributing to the previously observed CSA-related neuroprotective effects after stroke, thus studies focused on *Cstad* in the context of stroke pathology might be initiated.

Besides, Claudins are a large family of proteins with a pivotal role in tight junctions of all epithelial and endothelial tissues, including the brain-blood barrier (BBB) [59]. Whilst little is known about CLDN20 specific function in brain, other Claudins, such as CLDN1, CLDN3 and CLDN5, have been well characterized in the central nervous system [60]. Similar to what we have found here for *Cldn20*, *Cldn5* gene expression levels decrease in brain following cerebral ischemia in rats [61]. Interestingly, we also observed a recovery of *Cldn20* expression 6h after ischemia, which has not been described for *Cldn5*. Since the specific molecular functions of CLDN20 in brain are completely unknown, future contributions aimed at describing whether this isoform plays a key role in brain endothelia after cerebral ischemia and how its modulation could serve as a therapeutic approach are still required.

In regards to protein candidates, we identified CTNND2 (or neural plakophilin-related Armadillo-repeat protein (NPRAP)) as an early-phase down-regulated protein after ischemia. CTNND2 is an adhesion junction-associated protein highly express in the brain, and specifically enriched in the postsynaptic and dendirtic compartments [62], where plays a pivotal role as a signaling sensor and integrator [63]. Concretely, CTNND2 interacts and stabilizes brain cadherins by linking them to the actin cytoskeleton and to a wide variety of post-synaptic scaffold molecules, including post-synaptic density protein 95 (PSD-95) and excitatory neurotransmitter receptors, such as the ionotropic N-methyl-D-aspartic acid receptor 2A (NR2A) and the metabotropic glutamate receptor 1K (mGluR1K) [64]. In line with our results, Jones and colleagues also described that treatment of cortical neurons with glutamate resulted in a prompt down-regulation of CTNND2. In fact, they reported that glutamate-induced toxicity also promoted CTNND2 dissociation from NR2A and mGluR1K, being all these occurrences observed at very early time-points, when apoptosis was not even noticed. The exact consequences of this observed sudden and transient down-regulation of CTNND2 levels following ischemia, however, still need to be elucidated.

Similar to CTNND2, CAMK2A protein levels also showed a sudden decrease in brain due to the ischemic challenge, which tends to recover after 6h from the ischemic onset. This multifunctional Serine/Threonine protein kinase family has been well characterized in the context of ischemic stroke. CAMK2 have been described to stimulate calcium channels, thus promote neuronal apoptosis by enhance death-inducing overload of calcium [65]. On the other hand, CAMK2 have been also reported to inhibit apoptotic-related mediators, such as caspase 2 or Bad, and increase expression of Bcl-xL, which attenuates those apoptotic neuronal processes [66], [67]. All in all, there is still controversial thoughts about CAMK2 role, despite preclinical studies in stroke models seem to be tipping the scale towards the inhibition of CAMK2 as a plausible neuroprotective strategy [68]–[70]. In accordance with what we found here, this group and others have previously reported

decreased levels of several CAMK2 members, including CAMK2A, following cerebral ischemia in rodents (unpublished data, [71]). However, these decreased levels of total CAMK2 are known to be associated with an increase in their phosphorylated form, which in fact resulted to be the active state for the kinases [71]. Thus, protective strategies aimed at decreasing the active and phosphorylated form of CAMK2 might be further studied in detail.

Altogether, although this study aimed to merely explore the transcriptome and proteome of the ischemic mouse brain, we have here taken advantage of a novel and complementary biostatistical strategy to joint and integrate data from these two *-omics* techniques to eventually identify relevant elements within the stroke pathology. Interestingly, certain identified candidates have already been well characterized in the context of stroke pathology, such as *Atf3*, *Ccl3* or CAMK2A, which overall provides high reliability to this novel integrative approach. On this basis, we believe further efforts might be directed towards the exploration of all other pinpointed stroke-related elements as potential therapeutic targets to modulate or even reverse stroke pathology, as well as their role as biomarkers to diagnose stroke or even predict stroke patients' outcome. Overall, this innovative perspective of data analysis is expected to further depict relevant associations between genes and proteins that might not have been determined from separate analysis of each *–omics.* Thus, we undoubtedly believe that these emerging integrative approaches will continue to grow in scale and in popularity to complement or even go beyond the traditional biostatistical tools used to analyze single-*omics* datasets.

In conclusion, this breakthrough integrative approach enabled us to identify, replicate and explore over time 10 stroke-associated elements in the mouse ischemic brain. Despite future contributions are still required to deeply characterize these candidates, we believe the methodology used here is a novel and effective approach to contribute to increasing knowledge on the molecular mechanisms of ischemic stroke pathology to ultimately provide biomarkers and therapeutic target candidates for stroke patients management and treatment.

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AUTHORS' CONTRIBUTIONS

A.Simats, T.García-Berrocoso and J.Montaner conceived and designed the experiments. A.Simats performed all animal experiments, supported by L.Ramiro. F.Canals helped in the design of the study and supplied the Progenesis LC-MS® software, and L.Martín, A.Sabé, N.Colomé conducted the proteomics experiments. F.Briansó, R.Gonzalo and A.Sánchez analyzed transcriptomics and proteomics data from the discovery phase, F.Briansó performed the integrative analysis, and A.Simats analyzed data from the replication phase. A.Rosell, T.García-Berrocoso and J.Montaner supervised the experiments and A.Simats drafted the manuscript.

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SUPPLEMENTARY MATERIAL

Describing the mouse brain proteome and transcriptome after acute cerebral ischemia through a multiomics-integrative approach

Alba Simats[1], Teresa García-Berrocoso[1], Ferran Brianso[2,4], Laura Ramiro[1], Ricardo Gonzalo[2], Luna Martín[3], Anna Sabé[3], Nuria Colomé[3], Alex Sánchez[2,4], Francesc Canals[3], Anna Rosell[1], Joan Montaner[1]

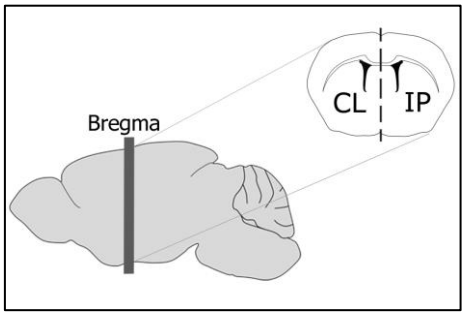
SUPPLEMENTAL TABLES

Supplemental table S1 and **Supplemental table S2** are accessible via the following link*: https://drive.google.com/open?id=13ppFht1avpHW2mid4EajDBa2fHH3YOZc*

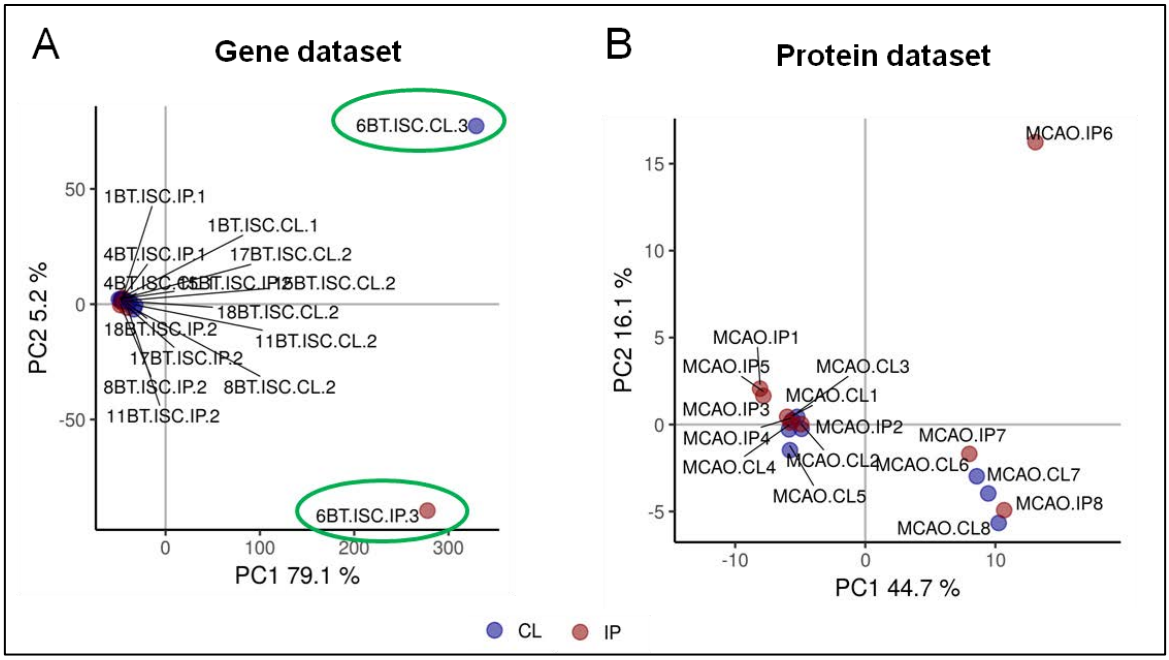
Supplemental table S3. Replication phase of the study. Nanostring™ probes references and PRM-MS peptides sequences are indicated. Statistical significance was evaluated between ipsilateral (IP) and contralateral (CL) samples of ischemic animals. Significant p-values are marked in bold. Fold-change (FC) expression indicates IP/CL ratio.

Digital multiplexed gene expression assay			
Gene names	**NCBI References for Nanostring™ probes**	**P-value**	**FC**
Atf3	NM_007498.3	**0.031**	7.603
Ccl3	NM_011337.1	**0.031**	8.875
Cenpp	NM_025495.3	>0.999	1.086
Cldn20	NM_001101560.1	**0.031**	0.494
Cstad	NM_030137.2	**0.031**	0.491
Fosb	NM_008036.2	**0.031**	7.134
Gadd45g	NM_011817.1	**0.031**	4.008
Gata2	NM_008090.4	0.156	0.756
Il1r1	NM_001123382.1	0.156	0.872
Intu	NM_175515.5	0.218	0.919
Rgs2	NM_009061.2	**0.031**	3.919
Zfand4	NM_001081317.1	0.218	0.850
4933427D14Rik	NM_028963.2	**0.031**	0.863
Ppia	NM_008907.1	-	-
Gapdh	NM_001001303.1	-	-
B2m	NM_009735.3	-	-
Targeted mass spectrometry: Parallel Reaction Monitoring (PRM)			
Protein names	**Peptide sequences for PRM-MS**	**P-value**	**FC**
BAG5	IVPFYSGGNCVTDEFEEGIQDIILR	*Not detected*	
	ELEQNANHPHR		
CAMK2A	FTEEYQLFEELGK	**0.062**	0.638
	ITQYLDAGGIPR		
CTNND2	ASYAAGPASNYADPYR	**0.031**	0.785
	TSTAPSSPGVDSVPLQR		
SRGAP2	HGLQHEGIFR	*Not detected*	
	GLEHPLFPK		
TXN2	TTFNVQDGPDFQDR	0.218	1.105
PPIA	FEDENFILK	-	-
	VSFELFADK	-	-
GAPDH	IVSNASCTTNCLAPLAK	-	-
	GAAQNIIPASTGAAK	-	-
B2M	TPQIQVYSR	-	-

SUPPLEMENTAL FIGURES



Supplemental Figure S1. Schematic representation of brain collection and isolation. IP: ipsilateral hemisphere; CL: contralateral hemisphere



Supplemental Figure S2. Principal Component Analysis of gene (**A**) and protein (**B**) individual datasets. Contralateral hemisphere samples are shown in blue, whereas ipsilateral hemisphere samples are marked in red. Green circles indicate outlier samples excluded for the integrative analyses.

Identification of novel neuroprotective drug combinations for the treatment of ischemic stroke through a systems biology-based drug repositioning approach

Identification of novel neuroprotective drug combinations for the treatment of ischemic stroke through a systems biology-based drug repositioning approach

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Ischemic stroke is a primary cause of morbidity and mortality worldwide. Beyond the standard thrombolytic therapies, there is still no effective treatment to mitigate or even reverse the progression of stroke disease. Since potential candidates from pre-clinical studies have failed to translate into clinical success, effective neuroprotective drug discovery is still urgently needed. Drug repositioning has become a promising alternative approach to identify new uses of existing drugs in any indication outside its original medical scope. Besides, combinational treatment approaches based on this drug-repositioning technique are also emerging as powerful strategies to synergistically and simultaneously target more than one disease-response mechanism underlying complex pathologies. In this study, drug-repositioning solutions were acquired through the screening of an *in silico* generated mathematical model mimicking ischemic stroke pathology with multiple two-by-two combinations of FDA-approved drugs. Through this strategy, drug combination A (CA) and B (CB) were selected and evaluated for their synergic neuroprotective effects in a pre-clinical stroke model. To that end, male mice were subjected to transient cerebral ischemia by 90-min Middle Cerebral Arterial Occlusion (MCAO) and intravenously treated with drug combinations during occlusion, individual drug treatments (D1-4) or their respective vehicles. Both drug combinations resulted effective in attenuating infarct volume and neurological deficits 24h after ischemia. Individual drugs, however, did not reduce the ischemic lesion, despite D1 and D2 also improved the neurological function of ischemic animals. In conclusion we have here identified, by means of a systems-biology and drug-repositioning approach, two different drug combinations with potential neuroprotective actions in ischemic stroke disease. These encouraging results support future pre-clinical studies and investigations to explore their mechanisms of action.

<u>Note:</u> Drug names are not disclosed in this manuscript due to confidentiality issues related with intellectual property filing process.

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 recombinant tissue plasminogen activator (rt-PA) or through mechanical thrombectomy using stent-retriever or aspiration devices [2], [3]. Despite the effectiveness of reperfusion strategies, their short therapeutic window and the some of their severe side effects precludes offering those therapies to all stroke patients. Therefore, there is an important need to find alternative stroke therapies to rescue brain tissue from ischemic injury. A large number of pre-clinical studies have been conducted in rodent models of cerebral ischemia to evaluate the efficacy of thousands of drugs aiming to attenuate or even to

reverse the progression of the ischemic disease. Unfortunately, the translation of effective neuroprotectants from pre-clinical studies into the clinics has systematically failed so far [4], [5].

Those failures are in part due to the fact of targeting only one altered pathway with a specific drug, that seems too simple since ischemic stroke is a highly complex and heterogeneous disease. Therefore, a global understanding of all different pathways that are disturbed in this multifactorial disorder is becoming critical to find new therapeutic agents that could successfully reverse or mitigate stroke progression [6]. To that end, systems biology has recently emerged as a new discipline that aims at deciphering this complexity by considering humans as sophisticated network-based maps of interrelated molecules and connected biological pathways. Systems biology provides insights into those relevant elements whose differential activity is globally associated with a particular state of disease, and simulates the behavioral response of the human pathology to the single or multiple modulation of disease-related mediators [7]. Approaches based on systems biology are therefore particularly suited for drug repositioning strategies [8], in which 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 to treat any pathology outside its original medical scope. More importantly, systems biology-based drug repositioning strategies are also making significant contributions to the identification of combinational treatment approaches. In this regard, there is now a growing interest in the mixture of two or more therapeutic agents to synergistically target multiple disease-response mechanisms, which might overall permit an increased control and treatment of complex pathologies. 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 or by countering biological compensatory mechanisms, which are known to mask the protective effects of many pharmacological agents [9], [10].

Our aim is to use a systems biology-based technique based on artificial intelligence and pattern recognition models to integrate available biological, pharmacological and medical knowledge into mathematical models that simulate *in silico* the complex behavior of human stroke disease [11]. We have screened these stroke-mimicking models to identify potential neuroprotective combinations of FDA-approved drugs and, importantly, we have experimentally validated the therapeutic effects of two promising drug combinations in a mouse model of transient cerebral ischemia.

MATERIAL AND METHODS

Therapeutic Performance Mapping System (TMPS) technology

We have used the TPMS technology, a top-down systems biology approach (Anaxomics S.L., Spain), for drug repositioning [11]. In brief, TMPS approach included the following steps (Figure 1A):

(1) 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 (287 proteins; data not shown[1]). 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 [12], REACTOME [13], BIOGRID [14], INTACT [15], HDPR [16], MATRIXDB [17], MIPS [18], DIP [19] and MINT [20]. The final network included 6640 proteins (data not shown[1]).

(2) Generation of the mathematical models. Static network maps were transformed into mathematical models (topological maps) through the use of Artificial Neuronal Networks

[1] *Protein names are not disclosed in this manuscript due to confidentiality issues related with intellectual property filing process.*

(ANN) and pattern recognition techniques based on the optimization of generic algorithms, as previously published elsewhere [21]. The multilayer perceptron (MLP) neural network classifier was the algorithm used for ANN [22].

(3) Feeding of the mathematical models with experimental data. 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. 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. 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 ([23], unpublished data), MS analysis of microdissected neurons and brain blood vessels [24] and microarray-based transcriptomics with brain homogenates (unpublished data). 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 model (data not shown[2]). Through this strategy, 4 different mathematical models were finally generated by selectively integrating into them all high-throughput information. These models simulate (I) healthy and (II) disease conditions (ischemia), and (III) infarct and (IV) peri-infarct brain regions.

(4) Solving the mathematical models: drug repositioning strategy. Once the mathematical models were generated, drug repositioning solutions were acquired by perturbing them with multiple sets of stimuli, which corresponded to two-by-two combinations of drugs from the DrugBank Database (v4.3) [25] (Table 1).

Category	No. of drugs
Screened combinations	~5.000.000
Evaluated combinations with Artificial Neural Network that fulfill the restrictive filters	1.133
Drug combinations with potential mechanism of action against ischemic stroke according to the scientific literature	142
Pre-selected combination candidates	25

Table 1. Drug combination selection process

Approximations to the best treatment solution for ischemic stroke were obtained based on three complementary approaches, which identified the best drug combinations for (1) treating ischemic stroke in general (disease model), (2) recovering the peri-infarct area and (3) promoting the conversion of the peri-infarct zone model to a healthy brain model and avoiding the conversion of the peri-infarct zone model to the infarct zone model. All identified drug combinations had to fulfill a minimum threshold of ANN predictive value of 80%. This cut-off point corresponded to the maximum ANN predictive value obtained from screening in our mathematical models a set of previous unsuccessful treatments studied in clinical trials for ischemic stroke [26] (Table 2). To further restrict the discovery of potential drug combinations, the following specific filters were applied: (1) an approved-administration for all individual drugs; (2) a theoretical synergic effect of drugs when combined (higher than 20%); (3) no association to hypotension or hemorrhages according to public databases; (4) incompatibilities for intravenous administration; (5) identified in the two proposed ANN strategies (disease and peri-infart area models) or in model reversion

[2] *Gene and protein names are not disclosed in this manuscript due to confidentiality issues related with intellectual property filing process.*

strategies. The following associated DrugBank categories have not been 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.

Drug Name	ANN prediction
Maxipost (BMS-204352)	79.39%
Nalmefene (Cervene)	79.15%
Fosphenytoin	74.85%
Enoxaparin	54.46%
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%
Simvastatin	35.00%
Lubeluzole	34.83%
Buspirone	34.83%
Nimodipine	34.79%
Heparin	34.31%
Zonampanel (YM872)	33.84%
Aptiganel (CNS-1102, Cerestat)	32.65%
Diazepam	32.53%
Clomethiazole	32.03%
Natalizumab	32.00%
Ebselen	31.26%
Flunarizine	30.84%
Acetylsalicylic acid	29.91%
Pentoxifylline	29.28%
Abciximab	28.82%
Pethidine	28.5%
Dextromethorphan	28.36%

Table 2. Artificial Neural Network (ANN) predictive value of treatments already tested in ischemic stroke. None of the evaluated treatments showed neuroprotective effect for ischemic stroke in clinical studies [26]. Gradient of colors (from red to green) indicates increasing % of ANN predictive value.

Animals

All animal procedures were conducted 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 Institute of Research (protocol number 74/16). All experiments were conducted in a randomized manner and in adherence to the ARRIVE guidelines [27]. C57BL/6J male mice were used in the experiments (8-12 week-old; 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, subcutaneously; 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 air; Abbot Laboratories, Spain) was given to mice via facemask during all surgical procedures described below.

To evaluate the safety of the selected doses and combinations of drugs, a total of 38 animals were used. Of those, 18 animals were used for initial re-adjustments of drug doses, whereas 20 were used for the final safety evaluation of drug combinations. From these latest, 4 animals were discarded for the blood tests due to insufficient volume of sample. To evaluate efficacy of drug combinations, 99 animals were needed. Of those, 23 were excluded after applying the following criteria: inappropriate occlusion or reperfusion of the middle cerebral artery (n=16); massive surgical bleedings (n=2); death during the surgical procedure (n=4) and death within 24h after ischemia (n=1).

Administration of selected drugs

Two drug combinations were finally selected: combination A (CA) formed by drug D1 and drug D2, and combination B (CB) formed by drug D3 and drug D4 (data not shown[3]). The dose of each drug was chosen according to an extensive literature-based research on published studies, and all initially selected doses had been evaluated in rodents before (20mg/kg for D1; 2mg/kg for D2; 0.1mg/kg for D3 and 60mg/kg for D4). However, as detailed in the results section, animals receiving the initial doses for D1 and D2 showed severe side-effects and final doses were both lowered by half (10mg/kg for D1 and 1mg/kg for D2). D2 was finally used in its injectable in-solution form. All drugs were prepared and kept according to manufacturer's instructions until their use. Drug and vehicle administrations were performed via the retro-orbital sinus [28]. For drug combinations, each individual drug was injected separately, one in each retro-orbital sinus. Administration volumes vary among drugs (range between 25 µl and 50 µl), and for vehicles, same volumes of their respective drugs were administered to animals.

Analysis of biochemical parameters in blood

On day 8, after three alternate-day doses of each drug combination or their respective vehicles, all animals were anesthetized and blood samples were drawn through cardiac puncture, collected in EDTA tubes and centrifuged for 10 min at 3000g at 4ºC. Plasmas were kept at -80ºC until further use. Routine clinical biochemistry parameters (including urea, creatinine, bilirubin, alkaline phosphatase, creatine kinase, aspartate transaminase (AST) and alanine transaminase (ALT)) were analyzed in plasma samples in the Clinical laboratories from Hospital Vall d'Hebron (Barcelona, Spain).

Animal model of cerebral ischemia

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 elsewhere [29]. In summary, 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 by affixing a laser Doppler probe (Moor Instruments, UK) to the skull. Then, animals were placed in the supine position and 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. MCA occlusion (MCAO) was confirmed by a reduction in the cortical CBF recorded by the laser Doppler probe and the incision was closed with a silk suture. Within the first 10 minutes of occlusion, animals were treated with individual drugs, drug combinations or vehicles as described above, according to their experimental group, and were allowed to

[3]*Drug and protein names are not disclosed in this manuscript due to confidentiality issues related with intellectual property filing process.*

recover from anesthesia. Ninety minutes later, mice were re-anesthetized and the filament was removed to induce reperfusion of the MCA. Only animals that exhibited a reduction of 80% of CBF after filament introduction and a recovery of 75% after filament removal were included in the study.

Evaluation of neurological deficits and infarct volume

An investigator blinded to the treatments evaluated neurological deficits and infarct volumes. Eighty minutes and 24h after MCAO each mouse was scored on a neurological function scale, adapted from previous studies [30], [31]. 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].

Following the neurological test at 24h all animals were euthanatized. Blood samples were also drawn through cardiac puncture and cold saline solution was then transcardially injected to remove blood from brain vessels. Immediately after saline perfusion, brain was removed and cut into 6 serial 1mm coronal sections. Infarct volume was assessed on these coronal sections using 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, USA) staining, as previously described [32]. TTC images were captured using a CanoScan 4200F (Canon, Japan) and infarct volume was measured using Image J software. Infarct volumes were calculated by integration of the lesion areas, corrected for edema and expressed in cubic millimeters (mm^3), as previously described [33].

Statistical analyses

GraphPad Prism 6.0 was used for analyzing data and creating graphs. Results are given as mean ± SEM. 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.

RESULTS

Identification of neuroprotective drug combinations

To identify potential neuroprotective drug combinations to treat ischemic stroke, we applied machine-learning tools as depicted in Figure 1A. To generate our systems biology-based artificial maps for stroke modeling 13 main pathological pathways were covered, which encompassed a total of 6640 proteins (Figure 1B) (data not shown[4]). In brief, these maps were created and computationally converted into mathematical stroke models incorporating all biological knowledge available, including our own proteomics and transcriptomics datasets from human brains of ischemic stroke patients. Disease-orientated drug repositioning neuroprotective solutions were then acquired by perturbing these virtual stroke models with multiple two-by-two combinations of drugs. From all drug combinations that fulfilled the established criteria (Table

[4] *Drug and protein names are not disclosed in this manuscript due to confidentiality issues related with intellectual property filing process.*

1), 25 were initially identified with an outstanding synergic neuroprotective effect, which include a total of 32 individual drugs.

From those, 2 combinations were highlighted as potential candidates to neuroprotect the ischemic brain: combination A (CA), formed by drug D1 and drug D2, and combination B (CB), which included drug D3 and drug D4. Selection was based on those combinations with higher ANN predictive value of neuroprotection against ischemic stroke injury, higher value of the predicted synergic effect and with experimental evidences reported in the scientific literature supporting the neuroprotective potential of the individual drugs.

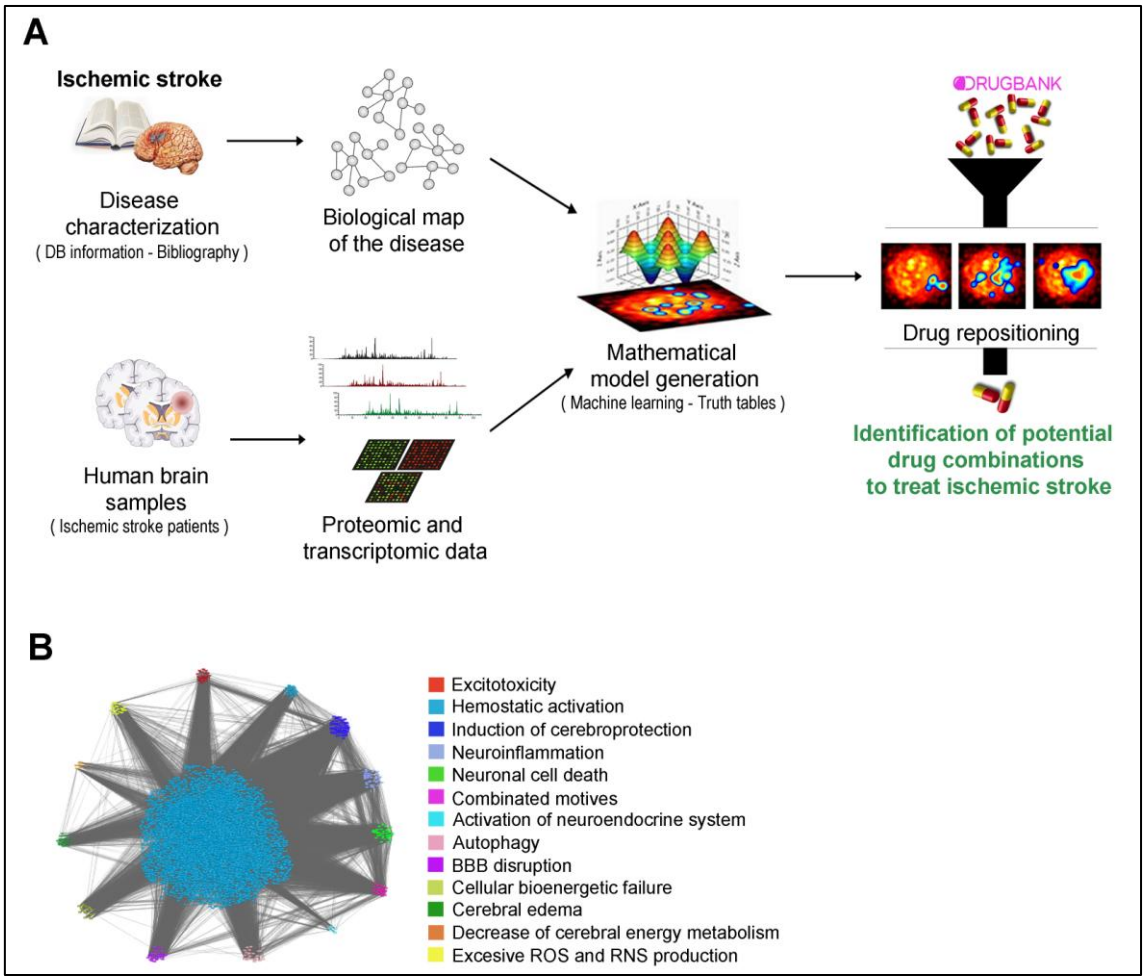


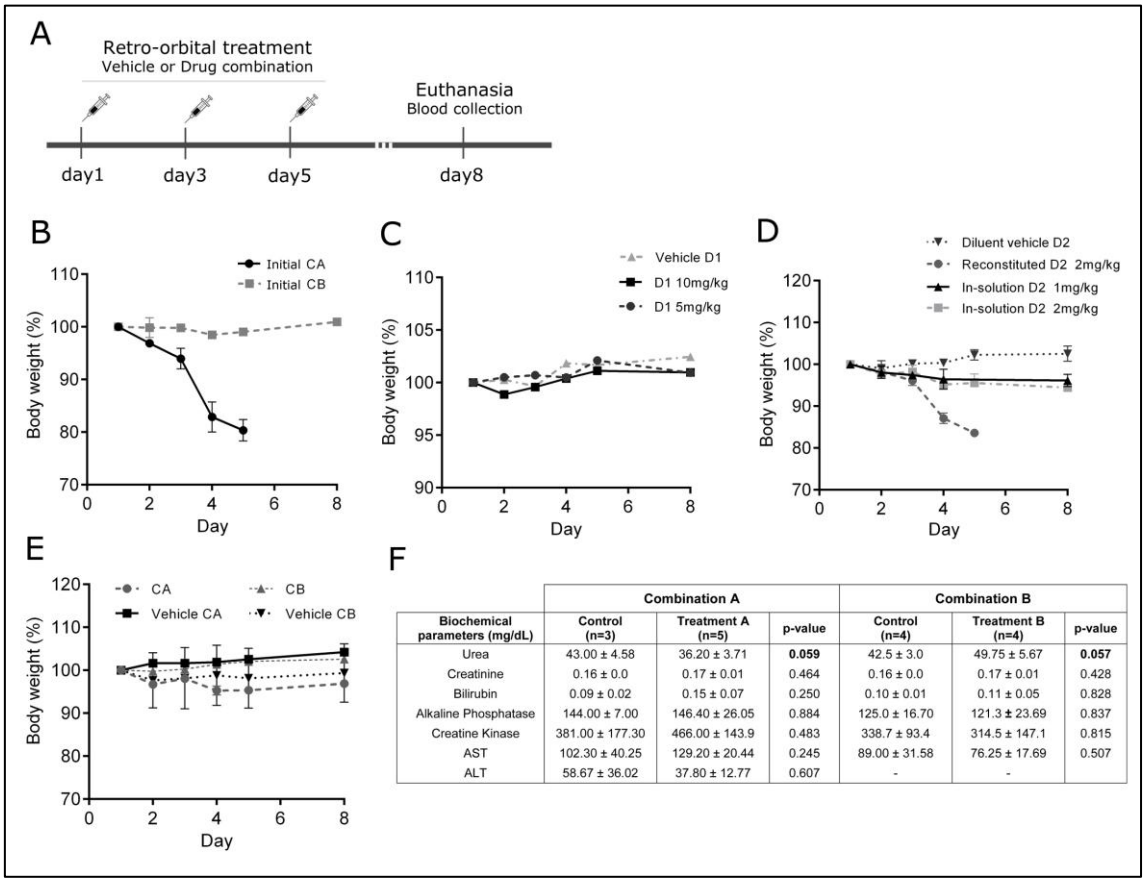
Figure 1. Schematic representation of the experimental design. (A) The starting material was a manually curated list of key proteins in stroke from the bibliography, from which a biological map of the disease was constructed. Using TPMS, network static maps were converted into topological maps associated with mathematical equations. Proteomics and transcriptomics data from human brain from ischemic stroke patients were used to enrich the mathematical model and to build a set of restrictions collated into a truth table, which all generated models had to fulfill. Drug repositioning strategy was used to search for combinations of FDA-approved drugs from the DrugBank. **(B)** Snapshot of the full protein network modelled for ischemic stroke disease, visualized through the Cytoscape software platform.

In vivo safety evaluation of the drug combinations' administration

To discard any toxic effect derived from the pre-selected drug doses and the co-administration of the two drugs at the same time, we initially evaluated safety outcomes on naïve animals receiving three alternate-day doses of each drug combination, or the respective vehicles (Figure 2A). Animals receiving initial CA drug combination doses (20mg/kg for D1 and 2mg/kg for D2) presented tremors and ambulation difficulties within the first 15 minutes after CA administration,

despite normal performance was observed during posterior follow-up. Moreover, CA-treated animals also showed a severe body weight loss (greater than 20% at day 5), so they had to be euthanatized according to our approved ethics' protocol for animal experimentation (Figure 2B). Evaluation of single administration of D1 and D2 revealed that D1 was responsible of the tremors and walking abnormalities, whether body weight loss was attributed to the single administration of D2 (Figure 2C-D). D1-derived side effects were prevented when the dose was reduced by one half and one fourth. D2 toxicity was attributed to an inadequate reconstitution of the lyophilized drug, since neither the diluent nor the injectable D2 in-solution showed these side effects (Figure 2D). Additionally the final dose of D2 was reduced by half.

Once doses were readjusted, a second phase of safety evaluation was conducted for both combinations and their vehicles in naïve mice, in which animals receiving either drugs in combination or vehicles did not report any body weight loss (Figure 2D). Moreover, 3 days after the last treatment administration, clinical biochemical parameters were analyzed in blood. Most biochemical parameters showed similar levels between vehicle- and drug-treated animals, except for urea (Figure 2E). Blood levels of urea tended to decrease in CA-treated animals compared to vehicles. However, they showed a slightly increase in CB-treated animals compared to their respective controls, though not statistically significant.



Biochemical parameters (mg/dL)	Combination A Control (n=3)	Combination A Treatment A (n=5)	Combination A p-value	Combination B Control (n=4)	Combination B Treatment B (n=4)	Combination B p-value
Urea	43.00 ± 4.58	36.20 ± 3.71	**0.059**	42.5 ± 3.0	49.75 ± 5.67	**0.057**
Creatinine	0.16 ± 0.0	0.17 ± 0.01	0.464	0.16 ± 0.0	0.17 ± 0.01	0.428
Bilirubin	0.09 ± 0.02	0.15 ± 0.07	0.250	0.10 ± 0.01	0.11 ± 0.05	0.828
Alkaline Phosphatase	144.00 ± 7.00	146.40 ± 26.05	0.884	125.0 ± 16.70	121.3 ± 23.69	0.837
Creatine Kinase	381.00 ± 177.30	466.00 ± 143.9	0.483	338.7 ± 93.4	314.5 ± 147.1	0.815
AST	102.30 ± 40.25	129.20 ± 20.44	0.245	89.00 ± 31.58	76.25 ± 17.69	0.507
ALT	58.67 ± 36.02	37.80 ± 12.77	0.607	-	-	

Figure 2. Safety evaluation of the selected drug combinations. (A) Schematic time-line of the experimental procedure. **(B)** Recorded body weight of animals treated with initial doses of CA or CB (n=2/group). (**C**) Recorded body weight of animals treated with D1 at 10mg/kg and 5mg/kg and their vehicle (n=2/group). (**D**) Recorded body weight of animals treated with reconstituted D2 (2mg/kg), diluent vehicle of D2 or in-solution D2 (1mg/kg or 2mg/kg) (n=2/group) (**E**) Recorded body weight of vehicle and final drug combination-treated animals (n=5/group). (**F**) Biochemical parameters analyzed in blood samples. The number of animals in each group is indicated in the panel. Abbreviations: ALT: alanine transaminase; AST: aspartate transaminase.

In vivo determination of the synergic neuroprotective effect of drug combinations

We validated the theoretical neuroprotective effect of the selected drug combinations (CA and CB) and the synergic effect in front of single drug treatment (D1 and D2 for CA; D3 and D4 for CB) in a mouse model of transient cerebral ischemia (Figure 3A). Both combinations of drugs (CA and CB) showed efficacy by protecting mouse brains from cerebral ischemia, since they showed a reduction of 39.54% and 39.42% in the infarct volume compared to their vehicles, respectively (p=0.018 and p=0.022, Figure 3B-C). However, none of the drugs when given alone was capable of reducing the infarct volume when compared to vehicle group (p=0.767 for D1, p=0.963 for D2, p=0.956 for D3 and p=0.888 for D4). Moreover, all animals improved their neurological outcome 24h after the ischemic challenge when compared to their respective baseline deficits (i.e. to neurological score at 80 min after MCAO), regardless of treatment group (p=0.002 for vehicle A; p=0.0039 for CA; p=0.031 for D1; p=0.062 for D2 and p=0.035 for vehicle B; 0.027 for CB; 0.062 for D3; p=0.062 for D4; Figure 3D, 3E). Interestingly, animals treated with CA or individual D1 even showed a further improvement compared to vehicle group at 24h after ischemia (p=0.006 for CA and p=0.008 for D1; Figure 3D), whereas no differences were observed in this same regards in CB-, D3- or D4-treated animals (Figure 3E).

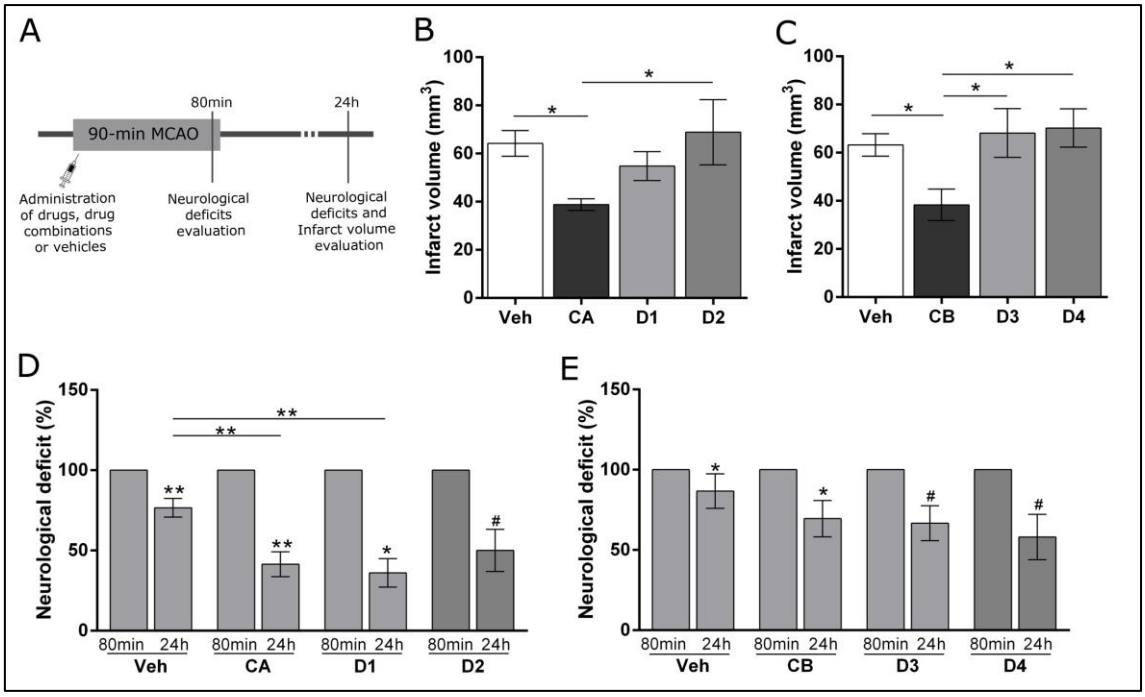


Figure 3. Efficacy evaluation of the selected drug combinations. (A) Schematic time-line of the experimental procedure. **(B)** Infarct volumes (mm^3) of animals treated with vehicle (n=16), CA (n=10), D1 (n=6) and D2 (n=6) 24h after cerebral ischemia. **(C)** Infarct volumes (mm^3) of animals treated with vehicle (n=16), CB (n=10), D3 (n=6) and D4 (n=6) 24h after cerebral ischemia. **(D)**, **(E)** Neurological deficits of animals 80 min (baseline) and 24h after the ischemic event, expressed in % based on each respective baseline score. For each experimental group, statistical differences between the two studied time-points are indicated with symbols on top of each 24h-bar. Other significantly relevant comparisons are indicated with horizontal bars between the two respective groups. In all graphs, mean ± SEM is shown. * indicates p<0.05, ** p<0.01 and # p<0.1.

DISCUSSION

During past decades, a large number of studies have devoted their efforts in identifying potential neuroprotective strategies to treat ischemic stroke, most of which were aiming 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. In this sense, we have used here systems biology and drug repositioning strategies to identify potential pairs of drugs that protect the ischemic brain by synergistically combining their therapeutic effects. To that end, we have created a network-based mathematical simulation of the stroke disease. We took advantage of our novel brain proteomics and transcriptomics data from deceased ischemic stroke patients to feed and curate the emergent mathematical models, which increased further their complexity, reliability and similarity to the human stroke pathology.

Moreover, we guaranteed the accuracy of our *in silico* generated models of ischemic stroke by screening more than 30 drugs previously tested in clinical trials of stroke. These include agents targeting the glutamate-induced excitotoxicity, such as Aptiganel [34] or Lubeluzole [35]; the acute inflammatory response and the infiltration of immune cells into the damaged brain, including UK-279,276 [36], Natalizumab [37] and Enlimomab [38] or the overload of cellular calcium, for instance through Nimodipine [39] and Flunarizine [40], among many others. However, at present none of these drugs have shown enough neuroprotective effects to be singly applied in the clinical management of stroke [41]. Thus, we verified that our models were able to properly predict a low degree of efficacy of those neuroprotectants, which allowed us to establish a threshold above the limit of protection of those unsuccessful tested drugs to select our new candidates.

Moreover, in this study we have experimentally validated the predicted neuroprotective effect of two selected combinations of drugs in a preclinical model of cerebral ischemia in mice. The neuroprotective effects shown by the administration of CA during cerebral ischemia could be attributed to the synergic and complementary effects of both individual drugs in combination. D1 selectively acts on the gamma aminobutyric acid (GABA) system. Despite D1 was originally developed as an anticonvulsant agent, several studies have proven its neuroprotective effects on experimental models of cerebral ischemia. Particularly, D1 has been involved in the mechanisms that enhance the GABA-mediated inhibitory system, which cause the hyperpolarization of neuronal membranes and results in a subsequent counterbalance of the glutamate-induced excitotoxic effects after ischemia. Similar to D1, a wide variety of modulators of GABAergic inhibitory system have been well studied in the past years [42]. In fact, some of those drugs have been later evaluated in clinical trials of ischemic stroke, but none of them could prove neuroprotection. On the other hand, D2 is well known to contribute to systemic and peripheral tissue calcium homeostasis. In brain, D2 has been related to the attenuation of age-related dementia and cognitive function decline. Besides, D2 neuroprotective effects following ischemic stroke are also being increasingly recognized. First of all, D2 is reported to inhibit NMDA receptors, which is expected to attenuate the ischemic-induced excessive calcium cell-entry and the subsequent neuronal excitotoxicity. Second, protective effects of D2 against ischemic-induced brain-blood barrier (BBB) dysfunction in cerebral endothelial cells have also been characterized. Specifically, D2 showed protection to the endothelium by activating membrane receptors provoking a reduction of intracellular reactive oxygen species and decreasing the ischemia-induced up-regulation of BBB-disruption mediators, such as the matrix metalloproteinase 9 (MMP-9). Besides, it has been also reported that D2 promotes the expression of neurothropic factors, which control the differentiation of neuronal stem cells in order to compensate and recover the neural functions that are lost because of the ischemic condition. On the top of that, there is substantial evidence that D2 further plays a role as a potent modulator of the immune system. In neurodegenerative diseases, D2 has been described to protect against glial-mediated inflammation by attenuating microglial cell activation and inducible nitride oxide synthase, as well as by preventing pro-inflammatory cytokines expression and anti-inflammatory cytokines up-regulation (including interleukin 10 (IL-10), IL-4 or the transforming growth factor-beta (TGF-β)). D2 has been also evaluated in clinical trials: its administration resulted in a successful improving of the functional outcome of patients that received D2 along with conventional treatments and physiotherapy for stroke recovery. These results are in line with our findings, in which we could demonstrate that D2 ameliorated the

neurological state of ischemic animals, despite infarct lesion was not reduced. Altogether, D2 seems to modulate a wide spectrum of pathways and motives following ischemic stroke, whereas D1 exclusively focus on the attenuation of neuronal synaptic transmissions. Thus, since neither D1 nor D2 single administration mitigate the ischemic lesion in our study, we hypothesize that both drugs might be synergistically reaching therapeutic effects through the joint modulation of the multiple pathways involved in the pathophysiology of stroke.

On the other side, treatment with CB, but not single D3 or D4 administrations, was also capable of reducing infarct lesions at 24h after ischemia and therefore combined actions of both drugs were also responsible for the observed neuroprotective effects in this case. Despite initial discovery of D3 was not related to brain pathologies, it has been found that an endogenous form of this molecule is expressed in neurons. D3 neuronal functions are not fully understood, but it is thought that it can play a role as a neuromodulator of the dopaminergic system. In this sense, sporadic studies over past 30 years have demonstrated a plausible role for D3 as a treatment for neuropsychiatric disorders, in which dopaminergic dysfunction exists. In regards to stroke less is known. D3 has been involved in the reduction of glutamate-induced death of cultured neurons and body temperature-lowering effects have also been attributed to D3. Although the exact mechanisms are still unclear, this effect is suggested to have a plausible neuroprotective impact on ischemic stroke patients' outcome [43]. Finally, D4 is a serum proteinase inhibitor with already known anti-inflammatory, anti-apoptotic and cytoprotective properties. The endogenous form of D4 increases in circulation within few hours after infections or acute inflammatory responses. The primary role of circulating D4 is to inhibit a wide range of proteases derived from the degranulating neutrophils, but can also act on the attenuation of other circulating proteases, most of which are produced by mast cells and lymphocytes or as a result of coagulation or digestion processes. Similarly, D4 also regulates non-circulating proteases, such as matrix metalloproteinases. Contrary to our results, therapeutic treatment with D4 has shown protection against cerebral ischemic injury in preclinical studies, in which other anti-inflammatory capabilities that extend beyond these anti-protease functions have been attributed to D4. Those include the inhibition of pro-inflammatory cytokines expression (including IL-1α, IL-6, and tumor necrosis factor alpha (TNFα)), the enhancement of anti-inflammatory cytokine IL-10 production and the attenuation of the lipopolysaccharide-related activation and migration of human monocytes and neutrophils. Overall, the combination of D3 and D4 in CB seems to be also strengthening the insufficient protective effects of each individual agent, despite the exact mechanisms through which they are acting are not completely understood yet. Interestingly, despite CB resulted effective in reducing infarct volumes, its neuroprotective effects were not depicted in the neurological outcome of ischemic animals, since no further neurological improvement was observed in CB-treated animals compared to the vehicle group. For this reason, our data suggests that further research might be conducted to fully evaluate whether CB synergic therapeutic effects are also reflected in functional and neurological outcomes following ischemic stroke. Adjustments on doses and administration times might also provide useful insights into this issue and demonstrate whether these therapeutic effects are maintained when drugs are given at a more advanced stage of the disease, when is clinically relevant to treat stroke patients.

All in all, simultaneous attenuation of different motives and pathways involved in the pathophysiology of stroke appeared here to be a good strategy to reverse the progression of the disease, and showed better results than the modulation of single components in an individual manner.

In conclusion, we have here used a new systems biology-approach to generate mathematical models that simulate the pathophysiology of the ischemic stroke disease. This *in silico* representation allowed us to identify 2 promising drug combinations that showed synergic therapeutic effects in an experimental model of cerebral ischemia in mice.

Future perspectives and next steps

Despite these encouraging results, further investigations are required. To begin with, we will use the generated stroke virtual model to identify the molecular downstream pathways that are modulated by our combinations of drugs and we will experimentally corroborate their mechanisms of action in our mice model of cerebral ischemia, as previously conducted elsewhere [21]. This would allow us to confirm whether they can produce more-than-additive effects by bringing out independent actions that converge at specific altered pathways following ischemic stroke. Besides, we will also conduct additional efficacy studies in which drug combinations will be given at more clinically relevant time-points (from minutes to hours after reperfusion) to delimitate the therapeutic window in which our drug combinations that better exert their neuroprotective effects. We will also evaluate drug compatibilities between our combinations and the actual thrombolytic therapies for ischemic stroke, and in case of success, we plan to validate our therapeutic effects in different models of cerebral ischemia, including animals of different sexes and ages and comorbidities, as proposed in the STAIR recommendations [44]. Hopefully, if this strategy works, translation to clinical trials in stroke patients is guaranteed, with the great advantage that these drugs have already proven to be safe in humans.

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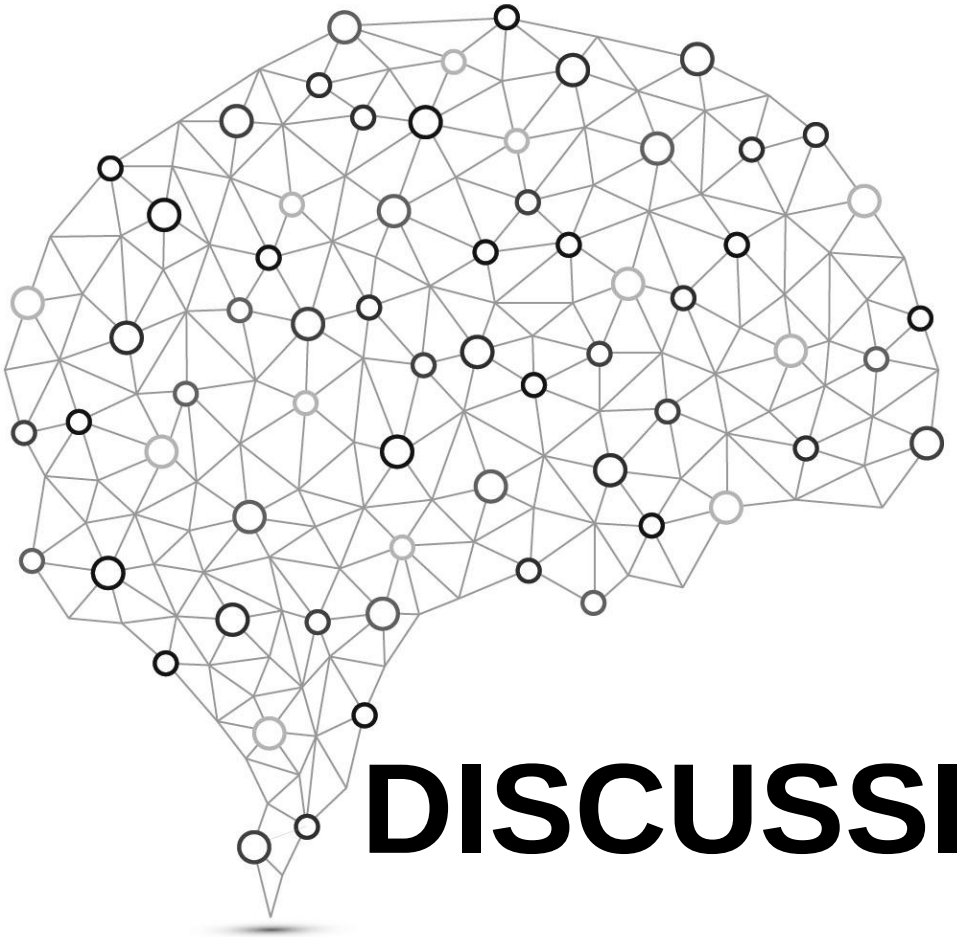


DISCUSSIÓ

Malgrat s'estan destinant nombrosos recursos i esforços a la identificació de marcadors sanguinis eficaços per al diagnòstic i pronòstic de l'ictus isquèmic durant la fase aguda de la malaltia, les troballes fins al moment no han resultat ser prou efectives. El mateix succeeix amb les teràpies neuroprotectores, doncs després de molts anys d'investigacions encara avui en dia no hi ha tractament eficaç per protegir el cervell isquèmic més enllà de les teràpies de reperfusió. És per això que la investigació continguda en aquesta Tesi Doctoral pretén contribuir principalment a la identificació de noves biomolècules que puguin servir en un futur per una, altra o ambdues d'aquestes finalitats que avui en dia encara manquen de resolució.

4.1 De biomarcadors a dianes terapèutiques: paper dual

Diagnosticar els pacients amb un ictus isquèmics i la seva etiologia per mitjà d'un test sanguini permetria agilitzar exponencialment la intervenció terapèutica dels pacients afectats per la malaltia. De manera similar, pronosticar de manera anticipada l'evolució d'aquests pacients i l'aparició de possibles complicacions associades a l'ictus ajudaria també a prendre les decisions mèdiques necessàries per tal de prevenir-les. A priori, ambdós escenaris causarien una disminució de la taxa de mortalitat associada a la malaltia i una millora substancial de l'estat funcional dels supervivents. Malauradament, però, encara no es coneix cap biomarcador sanguini que sigui potencialment més eficaç que les actuals eines clíniques que s'utilitzen per a tals finalitats. Així doncs, segueix vigent la gran necessitat de descobrir nous indicadors moleculars plausibles que permetin un diagnòstic i un pronòstic acurat i ràpid, sota qualsevol localització geogràfica i independentment de la situació socioeconòmica de la regió.

Un bon biomarcador d'ictus hauria de ser una molècula fàcilment i ràpidament detectable, idealment en sang, que la seva expressió diferencial sigui quasi immediata després de l'ictus isquèmic i/o s'associï a la progressió d'aquest. En base a aquestes característiques, és plausible pensar que aquests biomarcadors puguin tenir un paper clau en el desencadenament inicial de la malaltia [149, 208], de manera que a banda de bons indicadors, la seva modulació terapèutica també pugui esdevenir interessant com a possible tractament neuroprotector. Això permetria identificar i tractar els danys cerebrals associats a l'ictus des d'una fase molt primerenca, tot contribuint a frenar la progressió de la lesió isquèmica el més ràpid possible amb teràpies neuroprotectores, afavorint així una millor recuperació neurològica i funcional dels pacients.

En aquest mateix context, també és interessant estudiar l'ús d'aquestes molècules com a biomarcadors subrogats de resposta a tractaments dirigits contra les molècules en qüestió, complementant així els indicadors clínics actuals que s'utilitzen per avaluar el grau d'efectivitat de l'estratègia terapèutica. Aquest concepte ja s'utilitza a la pràctica clínica; en la hipercolesterolèmia, per exemple, els nivells de colesterol han esdevingut biomarcadors de diagnòstic de la patologia i alhora biomarcadors subrogats per avaluar l'eficàcia del tractament amb estatines, els principals agents hipolipemiants. Així, en el context de l'ictus, pensar en un possible escenari clínic on (1) s'utilitzi la mesura ràpida i precisa d'un biomarcador que permeti identificar la isquèmia cerebral, (2) s'administri un tractament neuroprotector dirigit contra aquesta mateixa molècula per tal de frenar la progressió de la malaltia, i (3) s'utilitzin a posteriori altre cop els nivells del biomarcador en qüestió per avaluar el bon funcionament de la teràpia, aproximaria l'ús de la medicina personalitzada en l'àmbit de la malaltia neurovascular (Figura 12).

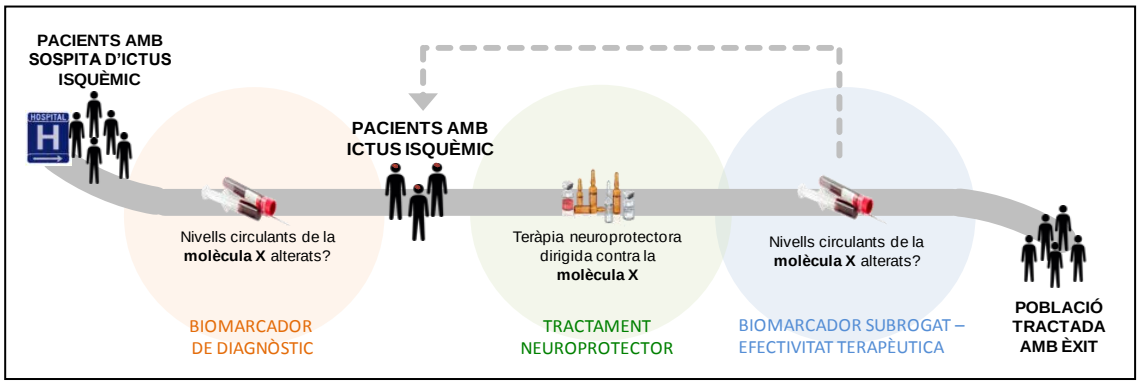


Figura 12. Esquema del paper dual de certes molècules inflamatòries com a biomarcadors i dianes terapèutiques de l'ictus.

Aquest concepte de paper dual d'una molècula com a biomarcador i diana terapèutica, de fet, ja està força establert dins la comunitat científica i s'ha proposat en vàries patologies cerebrals, com l'Alzheimer, els neuroblastomes i fins i tot també en l'ictus, tal i com s'explica més endavant [209–212]. La majoria d'estudis, però, confirmen experimentalment una de les dues funcions, ja sigui com a biomarcador o com a diana per neuroprotegir el cervell isquèmic, mentre que només hipotetitzen el possible rol del candidat per a l'altra finalitat. Són necessaris, doncs, futurs estudis orientats a caracteritzar exhaustivament i corroborar l'efectivitat de noves molècules per ambdues aplicacions proposades.

4.2 La neuroinflamació associada a l'ictus com a font de biomarcadors i dianes terapèutiques

L'ictus isquèmic desencadena una sèrie d'alteracions moleculars que provoquen una resposta inflamatòria aguda, tant a nivell local com perifèric, considerada un dels mecanismes més rellevants durant l'establiment i progressió de la malaltia [213]. Malgrat la neuroinflamació associada a l'ictus isquèmic ha estat objecte d'estudi durant molts anys, encara avui en dia no està del tot clar si la seva activació pot ser beneficiosa en certes fases de la malaltia [214, 215]. Sembla lògic pensar que l'activació d'una resposta inflamatòria com a conseqüència de la reducció sobtada del flux sanguini cerebral contribueixi a combatre el dany, promoure la supervivència del teixit i eliminar les restes cel·lulars alliberades per la mort de les cèl·lules afectades per la isquèmia. No obstant, hi ha nombroses evidències que demostren que la resposta inflamatòria desencadenada durant la fase aguda de la malaltia, tant a nivell local com perifèric, potencia el dany cerebral i propaga la lesió isquèmica a través de l'alliberació d'espècies citotòxiques i més mediadors inflamatoris que acaben donant lloc a un estat d'inflamació massiu [100, 103, 216]. Aquesta activació aguda de la resposta inflamatòria agreuja el dany cel·lular i alhora facilita la formació d'edema, l'aparició de transformacions hemorràgiques i fins i tot augmenta la probabilitat de contraure infeccions, principals complicacions associades al mal pronòstic de l'ictus isquèmic [217–219].

Donat que la resposta inflamatòria s'origina immediatament després de produir-se l'ictus, s'ha especulat que molècules clau mediadores d'aquest procés inflamatori podrien facilitar la identificació i predicció de la malaltia en les fases més inicials d'aquesta, així com esdevenir dianes interessants a modular per revertir el més aviat possible el desenvolupament de la lesió cerebral isquèmica. En aquest sentit, a l'**estudi 1** d'aquesta Tesi Doctoral s'ha detallat tota una sèrie de molècules inflamatòries que han mostrat tenir aquesta capacitat dual i han estat avaluades com a biomarcadors clau per a la detecció, la predicció i fins i tot la modulació de la progressió de l'ictus. De manera destacada, grans esforços han estat destinats a l'estudi de les **molècules d'adhesió cel·lular** que faciliten la invasió dels leucòcits circulants cap a la zona cerebral isquèmica, doncs es tracta d'un procés clau implicat en el dany neuronal secundari associat a l'ictus [88, 213, 220]. Per exemple, la ICAM-1 i la VCAM-1 han estat molt estudiades al llarg de les últimes dècades. Malgrat s'ha avaluat el seu possible rol com a biomarcadors de diagnosis, cap de les dues han tingut èxit: tot i que els nivells circulants de la ICAM-1 soluble (sICAM-1) i la VCAM-1 es troben elevats en els pacients que han patit un ictus [221, 222], s'ha vist que aquest augment no és específic, sinó que també s'observa en presència d'altres malalties també associades a una resposta inflamatòria, com les cardiopaties o el càncer [223]. Per contra, sembla estar més ben demostrat que nivells elevats de la sICAM-1 correlacionen amb el

deteriorament neurològic i la mort primerenca associada a l'ictus [221], fet que suggereix la sICAM-1 com un candidat a esdevenir un biomarcador de pronòstic de l'ictus isquèmic. A nivell terapèutic, s'ha demostrat reiteradament que la inhibició d'ICAM-1, ja sigui farmacològicament com mitjançant l'ús d'animals transgènics, té la capacitat de disminuir el dany neuronal associat a la isquèmia cerebral [224, 225]. No està tan clar, en canvi, l'efecte terapèutic d'inhibir l'acció del principal lligand de la VCAM-1, la VLA-4 (de l'anglès, *very late antigen-4*), doncs els resultats de diferents estudis experimentals han conclòs que la seva eficàcia pot estar condicionada per la mida i la localització de la lesió cerebral [226–230]. L'eficàcia neuroprotectora d'inhibir aquestes molècules també ha estat avaluada en assajos clínics (l'EAST [231], l'ACTION [232] i l'ACTION-2 [NCT02730455]) a través de l'administració dels anticossos monoclonals Enlimomab i Natalizumab, inhibidors de la ICAM-1 i VCAM-1 respectivament. En el cas de Enlimomab, l'elevada taxa de mortalitat mostrada pels pacients que rebien el tractament va aturar d'immediat els estudis relacionats amb aquest fàrmac. La comunitat científica, però, es manté molt crítica amb el disseny experimental utilitzat en aquest assaig clínic i qüestiona l'estratègia translacional seleccionada a partir dels prometedors resultats experimentals [233]. Concretament, s'ha suggerit per exemple que els antígens murins presents en la preparació de l'Enlimomab o fins i tot els mateixos anticossos contra l'ICAM-1 podrien haver estat els responsables del desencadenament d'una resposta inflamatòria massiva que suprimís qualsevol efecte beneficiós de la inhibició de l'ICAM-1 [75, 234]. En el cas del Natalizumab, malgrat els resultats de l'ACTION-2 encara no són públics, una nota de premsa de l'empresa que ha liderat l'estudi ha corroborat que els dos estudis han fracassat, doncs en cap d'ells s'ha observat una disminució de l'extensió de la lesió cerebral ni s'ha pogut demostrar una millora significativa del pronòstic funcional dels pacients amb ictus isquèmic.

Per contribuir en l'estudi de biomarcadors moleculars relacionats amb el procés inflamatori i la seva aplicació com a dianes terapèutiques per l'ictus isquèmic, l'**estudi 2** d'aquesta Tesi ha profunditzat en l'avaluació de la proteïna d'adhesió vascular (VAP-1). Aquesta proteïna pro-inflamatòria facilita el reclutament leucocitari a través de la seva activitat amina oxidasa sensible a semicarbazida (VAP-1/SSAO) i s'altera en un gran ventall de malalties, tals com la diabetis [235], l'aterosclerosi [236], la insuficiència cardíaca congestiva [237], la hipertensió [238], l'Alzheimer [239, 240] i l'ictus, tant isquèmic com hemorràgic [147, 241]. En el context de l'ictus isquèmic, a més, els nivells d'activitat plasmàtica de la VAP-1/SSAO s'han vist associats a l'aparició d'hemorràgies parenquimatoses després del tractament dels pacients isquèmics amb rt-PA [147]. Així, l'activitat de la VAP-1/SSAO s'ha proposat com un marcador novell de TH i s'ha suggerit que la seva monitorització en sang podria ajudar a la presa de decisions mèdiques per millorar la seguretat de l'administració dels agents trombolítics actuals, així com accelerar

la teràpia per trombectomia mecànica en aquells casos més susceptibles de desenvolupar TH associades a l'rt-PA.

Degut a la rellevància funcional d'aquesta molècula, nombrosos estudis han intentat modular l'efecte de la VAP-1/SSAO i, en conseqüència, la invasió leucocitària. S'han publicat un seguit d'estudis experimentals en els quals la inhibició de la VAP-1/SSAO ha mostrat capacitat per reduir la lesió cerebral després de la isquèmia, així com per millorar el pronòstic neurològic dels animals afectats [147, 242–244]. Molecularment, aquest bloqueig ha demostrat reduir la resposta inflamatòria associada a l'ictus, atenuar l'expressió d'altres molècules d'adhesió i disminuir la producció d'espècies reactives derivades de l'activitat SSAO. Tot i aquests estudis previs, en l'**estudi 2** d'aquesta Tesi no s'han reproduït aquestes troballes. En un model d'isquèmia cerebral transitòria en rates, l'administració d'un nou inhibidor de la VAP-1/SSAO, anomenat UV296, no ha mostrat capacitat per reduir l'extensió de la lesió isquèmica, ni en presència ni en absència de l'rt-PA. No obstant, equiparar aquests resultats amb els dels altres estudis positius prèviament publicats és difícil, doncs tots han fet servir inhibidors diferents que poden presentar distintes propietats fisicoquímiques vers l'acció sobre la VAP-1/SSAO. Cal destacar, però, que en tots aquests estudis previs s'ha administrat l'inhibidor de la VAP-1/SSAO lleugerament més tard en el temps, entre 1 i 6 hores després de la inducció de la lesió [147, 242], mentre que a l'**estudi 2** s'ha administrat l'UV296 durant l'oclusió arterial. També, altres contribucions han demostrat l'efecte beneficiós d'un inhibidor de la VAP-1/SSAO quan aquest s'administrava de manera continuada durant les primeres 60h després de la lesió [243]. Tot sembla apuntar, doncs, al fet que amb un nou disseny experimental modificant la dosi del UV296, la freqüència i el temps d'administració es podrien aconseguir els efectes beneficiosos prèviament observats per d'altres inhibidors de la VAP-1/SSAO.

Sabem que l'administració d'rt-PA exacerba els efectes citotòxics que els leucòcits infiltrants produeixen sobre la BBB [87, 88]. Per tant, la inhibició de la infiltració leucocitària associada a l'ictus no només s'ha proposat com un mecanisme de neuroprotecció del cervell isquèmic, sinó que també s'ha especulat sobre la seva possible capacitat per mantenir la integritat de la BBB i frenar l'aparició de TH associades a l'rt-PA. En aquest sentit, l'**estudi 2** també ha avaluat l'efecte de l'inhibidor UV296 sobre l'aparició d'aquestes hemorràgies. Els resultats han mostrat que la presència de l'agent trombolític augmenta substancialment l'aparició de TH en el model emprat, mentre que l'administració de l'agent UV296 abans de la infusió amb rt-PA ha mostrat capacitat per revertir aquest efecte deleteri. Malgrat els resultats són encara molt preliminars, hem pogut observar un possible paper neuroprotector de la inhibició de la VAP-1/SSAO enfocat a protegir la BBB front l'acció secundària de l'rt-PA. No obstant, futures contribucions destinades a estudiar més

en detall aquests efectes protectors són encara necessàries. En aquest sentit, per corroborar aquestes troballes inicials, l'ús d'un model embòlic d'isquèmia on s'aconsegueix la reperfusió de l'artèria per acció de l'rt-PA tot reproduint molt més fisiològicament la condició patològica humana, seria probablement el més òptim [245]. També caldria estudiar diferents temps d'administració de l'inhibidor de VAP-1/SSAO, així com la possibilitat d'administrar múltiples dosis d'aquest fàrmac al llarg de la fase aguda i subaguda de la malaltia [246], per observar si la inhibició de la VAP-1/SSAO pot resultar efectiva per ambdues finalitats proposades: la prevenció de les TH i la neuroprotecció del dany per la isquèmia [246].

En conjunt, aquests resultats obren la porta a futurs tractaments que combinin les teràpies trombolítiques actuals amb nous agents terapèutics com aquest, per tal de millorar la seguretat i eficàcia de l'rt-PA, poder ser administrats a un major nombre de pacients i millorar l'eficàcia del tractament actual. Aquestes noves eines també poden comprendre's com una aproximació a la teràpia personalitzada, on només els pacients amb ictus isquèmic que presentin nivells elevats de VAP-1/SSAO circulants, és a dir, siguin susceptibles a desenvolupar una TH durant el tractament trombolític, puguin ser tractats amb l'inhibidor VAP-1/SSAO per tal d'evitar de minimitzar l'aparició d'aquests sagnats associats a l'rt-PA (Figura 13).

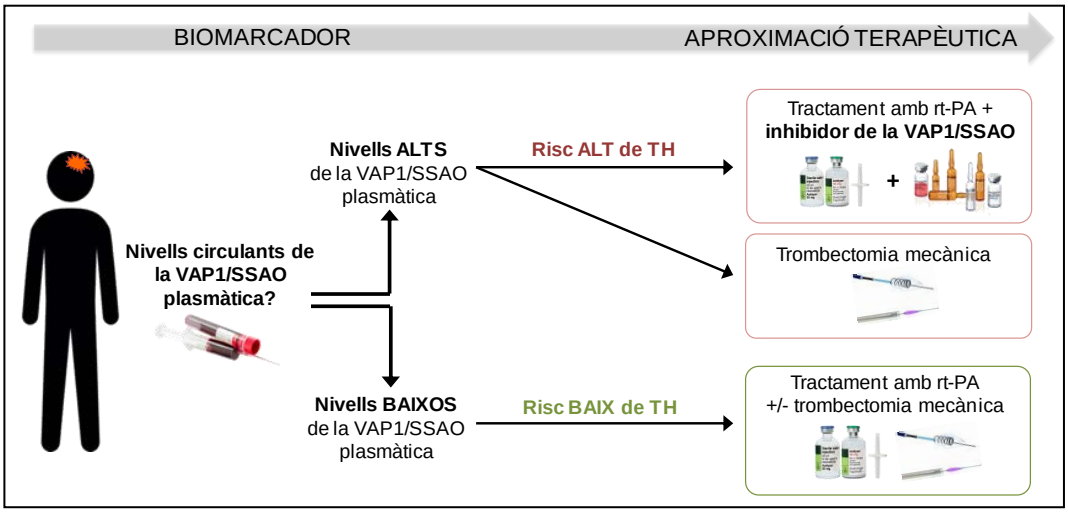


Figura 13. Esquema d'una possible aplicació clínica de la VAP-1/SSAO com a biomarcador de TH associades a l'rt-PA i la seva inhibició com a teràpia per prevenir aquests sagnats.

A banda d'aquestes molècules d'adhesió, la resposta inflamatòria aguda subjacent a l'episodi isquèmic provoca l'alliberació massiva de **citocines i quimiocines** que exacerben l'estat inflamatori global, moltes de les quals també han presentat aquesta dualitat funcional per esdevenir bons biomarcadors alhora que efectives dianes terapèutiques [212, 247]. La capacitat de diagnòstic d'aquestes molècules inflamatòries, però, és limitada. És

inevitable pensar que l'augment d'aquests mediadors inflamatoris en circulació pugui estar influït per una sèrie de comorbiditats, sense relació específica amb l'ictus, que poden esbiaixar la fiabilitat dels resultats, complicant la seva futura aplicació clínica. Per exemple, nivells d'IL-6 o CRP s'eleven substancialment després d'un esdeveniment isquèmic [212, 248], però també ho fan en presència de factors de risc cardiovascular com la hipertensió, la diabetis, l'obesitat, l'estrès o el consum de tabac, entre d'altres [249–251], dificultant així el seu ús individual com a biomarcador diagnòstic.

A l'**estudi 3** d'aquesta Tesi Doctoral s'ha descrit per primer cop el rol de la CCL23 com a possible biomarcador de diagnòstic de dany cerebral. Malgrat no ser específica de l'ictus, aquesta quimiocina ha mostrat capacitat per discriminar aquelles patologies que comporten dany cerebral agut, ja siguin ictus, tumors o traumatismes cranioencefàlics. A més, els nivells basals de CCL23 circulant no han mostrat cap associació amb els principals factors de risc vascular, suggerint la possibilitat que tingui un elevat grau d'especificitat comparat amb els altres candidats inflamatoris prèviament proposats. La seva utilitat clínica podria anar enfocada a l'ús en panells de múltiples biomarcadors, on candidats de diferents vies alterades en la patologia de l'ictus podrien combinar-se amb un alt grau de precisió per a detectar la malaltia, tal i com ja ha estat proposat en altres estudis [137, 252].

A diferència del diagnòstic, hi ha evidències clares que demostren que la resposta inflamatòria aguda si que està estretament associada al pronòstic dels pacients amb ictus isquèmic a mig i llarg termini [253]. En el transcurs de les últimes dècades s'han proposat diverses molècules inflamatòries circulants de fase aguda com a bons indicadors de pronòstic de l'ictus isquèmic; per exemple, nivells sanguinis alts de CRP, IL-6, TNF-α o IL-1β s'han associat a un pitjor pronòstic funcional i neurològic després d'un ictus isquèmic [254–257]. En aquest mateix sentit, a l'**estudi 3** d'aquesta Tesi Doctoral també s'ha estudiat el paper de la CCL23 com a candidat a ser un bon biomarcador de pronòstic. Els nivells circulants de la CCL23, mesurats en el moment d'admissió dels pacients a l'hospital, han mostrat una bona capacitat predictiva de la discapacitat funcional d'aquests a l'alta hospitalària, així com de la mortalitat associada a l'ictus a llarg termini. Així, en aquest estudi es proposa que l'avaluació dels nivells basals de CCL23 podria millorar un 15% la predicció del pronòstic funcional dels pacients i fins un 26,72% la capacitat de predir de manera anticipada la mortalitat associada a l'ictus, enfront de les variables clíniques actualment associades al mal pronòstic dels pacients (bàsicament edat, sexe i estat neurològic inicial). En conjunt, aquests resultats suggereixen la CCL23 com a bon biomarcador de pronòstic durant les primeres hores d'evolució, el qual podria jugar un paper clau per a la presa de decisions relacionades amb la inclusió dels pacients en

unitats d'ictus especialitzades o altres cures rellevants per a millorar el seu estat i evitar-ne el seu empitjorament a mig i llarg termini.

Moltes d'aquestes molècules inflamatòries també han estat avaluades com a dianes terapèutiques. Per exemple, la inhibició de l'IL-6 a través de l'administració d'un anticòs contra el seu receptor (MR16-1), la neutralització dels nivells endògens de TNF-α amb anticossos o el bloqueig farmacològic dels efectes de la IL-1 per mitjà d'un antagonista del seu receptor (IL-1Ra) ja han demostrat capacitat per reduir la lesió isquèmica i millorar el pronòstic neurològic i funcional en models preclínics de la malaltia [258–260]. L'efectivitat de l'IL-1Ra inclús s'ha testat en assajos clínics. Tot i que un primer assaig mostrava que l'administració intravenosa d'IL-1Ra tendia a millorar el pronòstic funcional dels pacients isquèmics [261], l'efecte de l'IL-1Ra s'ha seguit estudiant a través de la seva administració per via subcutània, més econòmica i més fàcilment administrable, la qual també ha mostrat capacitat per reduir els nivells dels principals mediadors inflamatoris circulants [262].

En aquesta Tesi Doctoral també s'ha plantejat el rol dual de la CCL23, identificada aquí com a biomarcador de pronòstic de l'ictus isquèmic, i s'ha dut a terme un estudi per avaluar la seva capacitat terapèutica (**Annex estudi 3**). Tot i no haver estat capaços de demostrar fermament els efectes beneficiosos de l'administració de la CCL23, els resultats obren un llarg camí a futurs estudis que tractin d'avaluar en detall aquests possibles beneficis terapèutics, reajustant la dosi efectiva i testant altres temps d'administració una mica més rellevants clínicament, és a dir, una mica més tard després de l'inici de la isquèmia cerebral. Estudis de pronòstic a mig i llarg termini (per exemple, des de 48h fins a 1-2 setmanes després de la isquèmia) també aportarien informació sobre la rellevància terapèutica d'aquesta estratègia en el pronòstic de l'ictus.

No obstant, a banda de tots aquests estudis d'eficàcia, també calen més estudis moleculars que descriguin com es comporta i com actua aquesta quimiocina. Amb aquesta finalitat exploratòria i donada la manca d'estudis previs en aquest àmbit, l'**estudi 3** incorpora també l'exploració i la caracterització d'aquesta molècula en models experimentals d'isquèmia. Malauradament, com que els rosegadors no tenen el gen de la CCL23 l'estudi està centrat en l'avaluació de la CCL9 i la CCL6, proposats com a homòlegs per la seva semblança estructural tot i que les seves similituds funcionals encara no estan del tot descrites [263–265]. Ambdues quimocines augmenten substancialment la seva expressió neuronal després de la isquèmia cerebral. No obstant, aquest increment sembla no ser específic de l'ictus, sinó que també s'ha observat en animals sotmesos a un traumatisme cranioencefàlic. D'altra banda, l'expressió de CCL6 també s'ha detectat en cèl·lules sanguínies polimorfonuclears, de manera similar a la CCL23 en humans. Tot i això, però, els nivells circulants de la CCL9 i la CCL6 no semblen comportar-se com els de la CCL23: mentre que la CCL9 disminueix dràsticament els seus nivells després de la

isquèmia, la CCL6 tendeix a incrementar-los un cop superada la fase aguda de la malaltia. Aquests resultats qüestionen les semblances funcionals entre totes aquestes quimiocines i dificulten enormement l'estudi de la CCL23 en models animals.

Cal destacar que aquest no és un cas aïllat, sinó que després d'anys d'estudi s'ha arribat a conèixer que hi ha altres molècules potencialment implicades en la resposta inflamatòria que s'alteren diferencialment en ratolí i humà, o fins i tot, només són presents en una de les dues espècies. Per exemple, els receptors de tipus *Toll*, peces clau en el sistema immunitari, han mostrat divergència entre ambdues espècies a nivell d'expressió i especificitat, i l'interferó gamma (INF-γ), mediador de la resposta inflamatòria tardana, estimula l'expressió de 18 GTPases en ratolí, mentre que en humans no és capaç d'activar-ne cap [266]. En un cas semblant al de la CCL23, la IL-8 va ser identificada com un dels majors estímuls pel reclutament de neutròfils en humans, mentre que el seu equivalent en ratolins encara està per descobrir [264]. A banda d'aquests exemples, hi ha altres discrepàncies entre les respostes immunitàries dels rosegadors i la dels humans que dificulten la translació dels descobriments en models animals en aquest context. Per exemple, es coneix que els rosegadors presenten un balanç dels subtipus de leucòcits que difereix en gran mesura del que presentem els humans. En condicions fisiològiques la població cel·lular circulant que predomina en els rosegadors són els limfòcits (75-90%), mentre que en els humans la posició predominant l'ocupen els neutròfils (50-70%) [267]. A més, la composició dels grànuls dels neutròfils també difereix entre especies: mentre que els grànuls dels humans presenten un alt contingut de defensines, mieloperoxidases (MPO), proteïnes bactericides (BPI) i altres petites proteïnes catiòniques riques en cisteïna, els grànuls dels ratolins no expressen defensines i el seu contingut de MPO i BPI és molt més escàs que en els humans [266]. Així mateix, cada cop són més les molècules (majoritàriament citocines, quimiocines i receptors) i els mecanismes que acaben caracteritzant-se diferencialment entre humans i rosegadors [267]. Tot i això, encara avui en dia és difícil establir quines són les conseqüències funcionals d'aquestes divergències, però val la pena pensar que la resposta inflamatòria inicial pot diferir substancialment i tenir-ho present a l'hora d'utilitzar models animals per estudiar processos com la neuroinflamació associada a l'ictus.

En conjunt, doncs, tot sembla indicar que certs mediadors inflamatoris presenten un prometedor paper dual com a biomarcadors i dianes terapèutiques en l'ictus.

4.3 Les tècniques *-òmiques* com a eines per a millorar la identificació de nous biomarcadors i dianes terapèutiques de l'ictus isquèmic

Amb l'objectiu d'aprofundir en la caracterització de molècules rellevants per a la fisiopatologia de l'ictus isquèmic, en aquesta Tesi Doctoral s'han realitzat dos estudis de descobriment de biomarcadors candidats, ambdós amb un disseny experimental diferent però amb un mateix nexe comú: les tècniques *-òmiques*. Donat que tenen l'objectiu d'identificar noves molècules que puguin esdevenir en un futur bons biomarcadors però també bones dianes terapèutiques per l'ictus, ambdós estudis es focalitzen en l'exploració de la fase aguda de la malaltia en model preclínic, concretament en les primeres dues hores des de l'inici de la isquèmia cerebral.

Millorar la caracterització de la patologia en aquestes fases tan primerenques és essencial, ja que pot afavorir substancialment la identificació d'aquelles molècules responsables del desencadenament inicial de la malaltia, la detecció i modulació terapèutica de les quals permetrà aturar la progressió d'aquesta el més aviat possible. L'estudi de la patologia isquèmica en aquests primers moments, tan valuosos clínicament, és possible gràcies a la utilització dels models experimentals. Aquest és un clar avantatge respecte els estudis realitzats en mostres post-mortem de cervell humà [247, 268–270], on les mostres procedeixen de necròpsies de pacients decessos que sovint presenten temps d'evolució de l'ictus molt diferents entre ells i lluny de la fase aguda de la malaltia, on és clínicament més interessant per identificar possibles biomarcadors i/o tractaments neuroprotectors.

Una altra avantatge de l'experimentació animal és poder reduir la variabilitat associada a la obtenció i conservació de les mostres, que sovint és difícil de controlar quan es realitzen les necròpsies humanes. No obstant, com ja s'ha comentat en l'apartat anterior, un clar inconveninent dels estudis preclínics és l'existència de diferències moleculars entre espècies, que poden dificultar la translació dels resultats en rosegadors cap a la pràctica clínica.

Semblant al que succeeix amb el teixit cerebral humà, l'estudi del CSF en el context de l'ictus isquèmic també és clínicament limitat. Obtenir aquest tipus de mostres en pacients isquèmics és una contraindicació per a l'ús de les teràpies trombolítiques actuals [153, 154], fet que descarta la seva utilitat clínica tant per al descobriment com per l'assaig de biomarcadors d'ictus isquèmic. A nivell experimental, però, l'estudi dels canvis moleculars que experimenta aquest fluid biològic pot esdevenir molt interessant com a punt de partida per a desxifrar el que està succeint al cervell isquèmic. També, l'estudi de les molècules alliberades del cervell cap al CSF després de la isquèmia pot considerar-se una molt bona oportunitat per identificar possibles biomarcadors que acabin inclús detectant-se en sang una mica més tard en el temps.

Així doncs, el primer dels estudis que es presenta (**estudi 4**) inclou l'avaluació de mostres de CSF de rata obtingudes durant la fase aguda de l'ictus a través d'una tècnica de proteòmica basada en aptàmers. S'han identificat un total de 716 proteïnes augmentades específicament al CSF com a conseqüència de la isquèmia cerebral, de les quals 7 (CKB, CAMK2B, PDXP, AREG, CAMK2D, CAMK2A i CMPK) han estat seleccionades i explorades més en detall en el cervell dels mateixos animals isquèmics, tant a nivell d'expressió diferencial com en termes de la seva localització cerebral (discussió detallada dels candidats a la secció 4.3.1). També, amb l'objectiu d'estudiar el seu plausible rol com a biomarcadors d'isquèmia cerebral, aquestes proteïnes han estat explorades en l'estudi en la circulació d'aquests mateixos animals, així com en mostres humanes de pacients isquèmics i controls.

Curiosament, cal destacar que no hem estat capaços d'identificar cap relació clara entre els nivells de les proteïnes estudiades i els diferents compartiments tissulars analitzats (cervell, CSF i sang). Sembla lògic pensar que l'augment massiu dels nivells de certes proteïnes al cervell pugui veure's reflectit també al CSF [271], malgrat aquesta hipòtesi no s'ha pogut confirmar per a tots els nostres candidats. Les divergències observades, però, no s'han pogut associar al pes molecular de les proteïnes estudiades, doncs hem de descartar la possible sortida als fluids de només aquelles molècules petites capaces de travessar les barreres fisiològiques afectades per la isquèmia. Tot sembla indicar, doncs, que la relació entre l'expressió d'aquestes molècules en cervell i els seus nivells en sang o CSF no es deu només a l'extravasació a través de la BBB disgregada, sinó que altres mecanismes també hi estan jugant un paper clau. Donat que la identificació d'un patró clar entre els nivells de proteïnes al cervell, al CSF i al sistema circulatori seria interessant per entendre en detall el paper d'aquestes proteïnes com a biomarcadors sanguinis, futurs estudis destinats a millorar la comprensió de les vies i mecanismes implicats en aquest procés esdevindrien, doncs, una molt bona font d'informació per identificar nous biomarcadors sanguinis procedents del cervell afectat per la isquèmia.

D'altra banda, val la pena destacar que la tecnologia basada en aptàmers utilitzada en aquest estudi està competint directament amb els tradicionals panells de múltiples anticossos, els quals encara avui en dia presenten moltes limitacions (Taula 5, *Introducció*). Els aptàmers han resultat inclús ser molt atractius pel seu rol plausible a la pràctica clínica, ja que la seva incorporació en aparells de detecció immediata (en anglès, *point-of-care (POC)*) està resultant prometedora [272]. També, aquestes petites cadenes senzilles d'àcids nucleics s'estan estudiant avui en dia com a possibles tractaments terapèutics [273, 274], de manera similar al que ja succeeix amb alguns anticossos [275].

L'**estudi 5** d'aquesta Tesi Doctoral centra els seus objectius en estudiar els principals canvis moleculars que ocorren al cervell després de la isquèmia cerebral a través d'estratègies de transcriptòmica i proteòmica, basades en les tècniques de microarrays i LC-MS, respectivament. En aquest estudi s'utilitza el teixit cerebral, tant de la zona infartada com de l'hemisferi no afectat, i s'avaluen els canvis moleculars també 2h després d'iniciar la isquèmia, durant la fase aguda de la malaltia. Com a fet remarcable, cal mencionar que el material biològic de partida és exactament el mateix per a l'estudi de transcriptòmica que per al de proteòmica, fet que ens ha permès comparar i integrar els resultats conjuntament en un mateix anàlisi. Individualment, s'han identificat 76 gens i 192 proteïnes diferencialment alterades després de la isquèmia cerebral. Malgrat a priori sembli un nombre reduït de candidats significativament alterats, cal tenir en compte que l'estudi d'una fase tan aguda d'aquesta patologia, quan probablement el desencadenament de la lesió isquèmica encara està en una fase molt inicial, fa que la variabilitat entre animals pugui accentuar-se. En aquest sentit, malgrat assumir certa variabilitat experimental, hem cregut convenient estudiar l'expressió dels gens i proteïnes de manera individual per a cada mostra. Aquest fet, però, no és tan freqüent quan els estudis de descobriment es realitzen en mostres humanes, on és força comú agrupar-les (de l'anglès, *sample pooling)* per reduir la variabilitat entre condicions experimentals. D'aquesta manera, aconsegueixen també millors resultats estadístics al comparar les condicions estudiades sense necessitar un nombre gaire elevat de mostres, que normalment són difícils d'obtenir [276–278].

A partir d'aquests elements diferencialment alterats en la isquèmia, amb la finalitat de seleccionar candidats interessants en el desencadenament de la fisiopatologia isquèmica, hem optat per una estratègia bioestadística d'integració de dades procedents de les dues tècniques *–òmiques*. Aquesta aproximació difereix en gran mesura de les estratègies de selecció de candidats utilitzades en estudis previs, la majoria de les quals s'han basat en la funcionalitat dels candidats més rellevants estadísticament a nivell individual [247, 268–270]. En aquest estudi, però, aquesta aproximació integradora pot ajudar-nos a destacar altres candidats significativament alterats per la isquèmia que, sense tenir una funció clara individualment, poden estar contribuint amb força a la progressió de la malaltia en el seu conjunt. Així, l'anàlisi integratiu que hem aplicat ens ha permès destacar una xarxa concreta de gens i proteïnes altament relacionats entre ells, a partir de la qual s'han seleccionat 18 candidats interessants (13 gens i 5 proteïnes) per replicar en una nova cohort d'animals isquèmics. Cal destacar, però, que en aquesta xarxa no es reflecteixen connexions biològiques directes, sinó que es basa merament en associacions bioestadístiques entre els diferents components. Caldrà, doncs, concretar en un futur l'existència de relacions funcionals, tant directes com indirectes, entre els elements destacats a la xarxa, així com estudiar si realment es destaquen en ella les molècules més

rellevants del conjunt de processos fisiopatològics que es donen en el desencadenament de la malaltia.

De tots els elements pre-seleccionats, 8 gens (*Ccl3, Atf3, Fosb, Gadd45g, Rgs2, 4933427D14Rik, Cldn20 i Cstad)* i 2 proteïnes (CTNND2 i CAMK2A) han estat replicats amb èxit en aquesta segona fase de l'estudi, i la seva expressió diferencial en cervell s'ha avaluat també a les 6h després de l'esdeveniment isquèmic (descripció detallada dels candidats a la secció 4.3.1). La replicació d'aquests candidats i la seva exploració més tard en el temps també s'ha realitzat utilitzant tècniques *–òmiques*. Per a replicar els resultats dels gens hem utilitzat una tècnica basada en el comptatge digital directe d'RNA (Nanostring®), mentre que per corroborar els resultats de proteïna s'ha utilitzat una estratègia de proteòmica dirigida, la PRM, en ambdós casos amb aproximacions en format múltiplex. En aquest sentit, cal remarcar que tradicionalment molts estudis de cribratges massius han estat dissenyats per saltar directament de la identificació de candidats a través de l'anàlisi d'un conjunt reduït de mostres per mitjà de tècniques *–òmiques* a la replicació d'aquests en grans conjunts de mostres per tècniques d'immunodetecció. Aquest canvi dràstic de tècnica i mida mostral ha donat lloc a molts fracassos experimentals, probablement fruit de la manca de passos intermedis que permetin replicar i corroborar els resultats del descobriment a petita escala i per tècniques basades en metodologies semblants a les inicials.

De manera destacable, la replicació dels resultats a través de la tècnica de la PRM pot presentar inclús un avantatge addicional per a una futura aplicació clínica, ja que aquesta tècnica ha demostrat poder assolir properament el seu ús als hospitals [279]. En tal situació, la PRM permetria identificar proteïnes específiques d'una manera independent a les tècniques d'immunodetecció, que sovint presenten limitacions a l'hora de diagnosticar específicament determinats biomarcadors [280].

De manera similar, també cal remarcar els grans esforços que s'estan destinant per traslladar la pràctica clínica les tècniques de quantificació d'RNA. Semblant al que succeeix actualment amb la tècnica de la PCR en el camps de la microbiologia i virologia, on s'utilitza per confirmar un diagnòstic patològic, les tècniques de comptatge digital de RNA podrien utilitzar-se també a nivell hospitalari en un futur proper [281]. En aquest sentit, és important recordar que aquestes tècniques presenten clars avantatges respecte a l'actual PCR quantitativa, ja sigui pel fet de no haver de pre-processar les mostres o per la capacitat de poder-se multiplexar per detectar centenars de transcrits simultàniament.

D'altra banda, donat que un dels objectius principals de l'**estudi 5** és la identificació de nous biomarcadors plasmàtics, sembla força interessant continuar aquest estudi amb l'avaluació a nivell del sistema circulatori de les molècules seleccionades, tal i com ja s'ha

fet a l'estudi 4 per alguns candidats. Seria bo començar per la seva detecció en la circulació dels mateixos ratolins utilitzats pel descobriment i la replicació inicial, implementant així un pas intermedi a la qualificació d'aquestes en grans cohorts de mostres sanguínies humanes. En cas d'assolir resultats prometedors, aquesta segona fase inclouria la quantificació dels nivells plasmàtics dels candidats en mostres de sang de pacients que hagin patit un ictus isquèmic agut i es compararien amb els de pacients controls o que presentin condicions mimetitzants, per avaluar el seu valor real com a biomarcadors de diagnòstic. Paral·lelament, el fet d'haver identificat molècules diferencialment expressades en el cervell isquèmic en una fase tan primerenca de la malaltia fa pensar en el seu possible rol com a desencadenants d'algun dels processos patològics que succeeixen en l'ictus. En aquest sentit, doncs, estudis orientats a la seva modulació podrien també ser plantejats.

Finalment, cal remarcar que a banda dels candidats proposats i avaluats més en detall, les grans llistes de gens i proteïnes que s'han obtingut en ambdós estudis, tant en cervell com en CSF, són fonts d'informació molt valuoses d'elements que podrien ser claus en la patologia de l'ictus. Així, podrien servir per corroborar troballes d'altres investigacions i donar consistència a futurs estudis complementaris als que es presenten aquí.

També, serà necessari estudiar com es comporten els candidats descoberts en aquests estudis experimentals en els humans, un pas crític que delimitarà l'èxit d'aquests candidats per a esdevenir en un futur bons biomarcadors o dianes terapèutiques de l'ictus isquèmic.

4.3.1 Nous candidats a biomarcadors i/o dianes terapèutiques

Al llarg dels estudis que formen aquesta Tesi Doctoral s'han anat identificant diferents elements moleculars que han presentat una rellevància destacada dins la fisiopatologia de l'ictus isquèmic. Alguns d'ells, com les proteïnes CAMK2, CKB i CMPK, o els gens *Ccl3, Atf3* i *Fosb*, tots prèviament descrits i estudiats en detall a la literatura, han permès corroborar els dissenys experimentals i les estratègies metodològiques que hem utilitzat en els diferents estudis de descobriment de candidats (estudis 4 i 5). D'altres, com els gens *Rgs2, Gadd45g, Cstad i Cldn20*, menys caracteritzats en el context de l'ictus, han estat proposats com a possibles elements clau implicats en la malaltia, malgrat estudis futurs haurien de verificar i detallar els mecanismes moleculars en que es troben implicats, així com la seva funcionalitat i la seva possible aplicació com a biomarcadors i/o dianes terapèutiques.

Una família de proteïnes que ha destacat en els dos estudis de descobriment de candidats ha estat la família de les CAMK2. Aquestes cinases, regulades pels complexes

calci/calmodulina, juguen un paper clau com a mediadors de les senyals de mort cel·lular desencadenades pel glutamat [282]. Malgrat la seva inhibició farmacològica ha resultat ser neuroprotectora en varis models d'isquèmia, tant *in vivo* com *in vitro*, avui en dia encara hi ha controvèrsia, doncs altres contribucions científiques descriuen efectes absolutament contraris i neurotòxics derivats de la seva inhibició [283–285]. A l'**estudi 4**, els nivells circulants de les CAMK2 han estat avaluats per primer cop com a possibles biomarcadors sanguinis de diagnòstic d'ictus isquèmic, malgrat cap de les isoformes avaluades ha resultat prometedora. Per contra, els nivells circulants de CAMK2B en la fase aguda semblen millorar la capacitat predictiva de l'evolució dels pacients isquèmics als tres mesos després de l'ictus. Aquest fet planteja la necessitat d'estudiar més exhaustivament el valor d'aquesta isoforma com a biomarcador de pronòstic, així com caracteritzar les principals discrepàncies entre aquesta i les altres isoformes de la família.

A l'**estudi 4** també s'han estudiat les proteïnes CMPK i CKB, augmentades substancialment en CSF després de la isquèmia cerebral en rates. La CMPK està involucrada en el metabolisme dels nucleòsids, mentre que la CKB participa en els processos de transducció d'energia i homeòstasis, tots ells essencials per assolir una funció neuronal òptima [286–288]. Ambdues proteïnes han estat estudiades amb anterioritat en el context de la isquèmia cerebral. Pel que fa la CMPK, un estudi previ del nostre mateix grup d'investigació dóna suport a la reducció sobtada dels nivells de CMPK en la regió isquèmica del cervell de ratolins isquèmics, similar al que hem observat en aquesta Tesi Doctoral [289]. El seu rol com a possible biomarcador, però, és poc conegut encara. En aquest estudi hem estat pioners en reportar que els pacients amb ictus isquèmic presenten nivells circulants elevats de CMPK, els quals alhora s'associen estretament a un pitjor pronòstic funcional als 3 mesos després de l'ictus. Per contra, el rol de la CKB com a biomarcador d'ictus ha estat molt més investigat [290, 291]. Malgrat molts estudis, inclòs el presentat com a part d'aquesta Tesi, demostrin que els nivells circulants d'aquesta proteïna s'eleva substancialment després d'un ictus isquèmic, aquest patró també s'ha observat en altres malalties cerebrals, com els traumatismes cranioencefàlics o la meningitis [292–294], de manera que la seva utilitat clínica ha quedat atenuada.

A l'**estudi 5** també hem identificat un sèrie de candidats l'expressió cerebral dels quals s'ha vist alterada per la isquèmia cerebral. Entre els candidats identificats hi ha gens que codifiquen factors de transcripció, com el *Fosb* i l'*Atf3*, les proteïnes dels quals han estat implicades en la regulació dels mecanismes de mort cel·lular programada [295] i la repressió de l'expressió de gens pro-inflamatoris [296–299], respectivament. S'han identificat també gens que formen part dels mecanismes de senyalització intracel·lulars que s'activen en resposta a un esdeveniment isquèmic, com per exemple el *Gadd45g*, i l'*Rgs2,* involucrats en les vies de la MAPK (de l'anglès *mitogen-activated protein kinase*) i

els GPCRs, respectivament. Tanmateix, altres candidats destacats en l'estudi són el gen de la *Ccl3,* que codifica per una quimiocina pro-inflamatòria coneguda per la seva contribució en la progressió de la malaltia [300]; el gen de la *Cldn20*, canvis en l'expressió del qual podrien estar reflectint alteracions en les unions estretes de l'endoteli i condicionar la permeabilitat de la BBB [301]; la proteïna CTNND2, involucrada en les unions cèl·lula-cèl·lula de les terminacions sinàptiques i amb un paper clau com a integrador de les senyalitzacions que desencadenen els neurotransmissors [302, 303]; i els gens *Cstad i 4933427D14Rik*, la funcionalitat dels quals encara és desconeguda.

La desregulació de tots aquests gens i proteïnes en aquests processos biològics suggereix el seu possible paper com a elements importants en el desencadenament de la malaltia, la modulació dels quals podria inclús esdevenir una bona diana terapèutica. De fet, alguns dels candidats proposats ja han estat avaluats com a tal. Per exemple, l'augment de l'expressió de *Atf3* intracerebral ha resultat ser neuroprotector [304], mentre que la seva supressió provoca infarts més grans i accentua l'empitjorament neurològic dels animals afectats per la isquèmia [296]. De forma similar, l'administració de CCL3 en animals isquèmics ha mostrat també un augment substancial del seu volum d'infart [305], malgrat estratègies orientades a inhibir l'expressió d'aquesta quimiocina encara manquen. Paral·lelament a la seva modulació, tenint en compte que tots aquests candidats es troben alterats en una fase molt primerenca de la malaltia, és inevitable pensar que algun d'ells pugui ser també explorat en un futur pel seu potencial com a biomarcador. Amb tal finalitat, per exemple, la CCL3 ja ha estat estudiada: els pacients isquèmics presenten nivells elevats d'aquesta quimiocina en circulació, els quals correlacionen alhora amb la discapacitat funcional dels pacients afectats a curt termini [306].

En cas de corroborar en un futur el paper d'algun dels candidats identificats en aquesta Tesi Doctoral com a biomarcadors d'ictus, també valdria la pena pensar en la seva possible combinació en panells que permetin la mesura de múltiples marcadors simultàniament, fet que permetria incrementar l'especificitat i la sensibilitat tant sigui pel diagnòstic com pel pronòstic de l'ictus isquèmic. A més, tenint en compte la millora exponencial de les tècniques *–òmiques* i la seva factible aplicabilitat a la pràctica clínica, aquestes combinacions de múltiples biomarcadors podrien aconseguir-se fins i tot mitjançant les tècniques emprades aquí o inclús en dispositius de detecció immediata.

4.4 Combinació de teràpies neuroprotectores per al tractament de l'ictus isquèmic

Durant les últimes dècades, un gran nombre d'estudis han invertit els seus esforços en identificar noves estratègies neuroprotectores per tal de mitigar o fins i tot revertir la

progressió de l'ictus isquèmic. La gran majoria d'aquestes aproximacions, però, han estat orientades a la modulació única d'algun dels principals mecanismes o motius alterats en la fisiopatologia isquèmica, com ara l'excitotoxicitat causada pel glutamat, la sobrecàrrega de calci, l'estrès oxidatiu, la resposta inflamatòria, la mort cel·lular programada, etc. [83, 125, 126].

Malgrat molts d'aquests tractaments hagin mostrat eficàcia a nivell experimental, el fracàs absolut de la seva translació a la clínica ha resultat ser una prova conceptual que suggereix que la modulació de només un d'aquests processos patològics podria no ser suficient per atenuar el desencadenament d'aquesta malaltia tan complexa i multifactorial. És per això que la idea de combinar diferents fàrmacs que actuïn simultàniament en diferents processos patològics podria resultar una estratègia terapèutica efectiva per obtenir efectes sinèrgics que permetin combatre eficaçment l'ictus isquèmic [307, 308].

Hi ha qui pensa, però, que l'administració de més d'un fàrmac en combinació podria augmentar considerablement els efectes secundaris d'aquests [309]. No obstant, nombroses evidències suggereixen que la teràpia combinada podria inclús reduir les dosis individuals dels fàrmacs, fet que permetria reduir la toxicitat secundària d'aquests mantenint, o fins i tot incrementant, l'efecte neuroprotector desitjat.

Aquest concepte de teràpia combinada, de fet, ja està plenament implementat, per exemple, en el tractament del càncer, on agents quimioterapèutics es combinen amb altres tipus de fàrmacs per obtenir una major reducció del tumor i mitigar el potencial de metàstasis [310]. En el context de l'ictus, malgrat el concepte es va proposar ja fa molts anys [308], són comptats els estudis de neuroprotecció que fins al moment han descrit potencials efectes sinèrgics de fàrmacs en combinació. La gran majoria han estat destinats a testar els efectes de l'administració conjunta d'estratègies farmacològiques de reperfusió amb agents neuroprotectors [311]. En aquest sentit, a l'**estudi 2** d'aquesta Tesi Doctoral n'hem descrit un possible cas: la combinació d'un inhibidor de la proteïna VAP-1/SSAO amb el rt-PA, amb l'objectiu de protegir la BBB millorant la seguretat de l'agent trombolític i disminuint el risc de patir TH associades a aquest.

A banda de la combinació amb agents trombolítics, però, també s'ha demostrat augmentar considerablement els efectes beneficiosos a través de l'administració de més d'un agent neuroprotector. Per exemple, s'han demostrat efectes sinèrgics terapèutics per l'ictus a través de la combinació de la Memantina, un antagonista no competitiu del receptor NMDA, i el Clenbuterol, un agonista del receptor β2-adrenèrgic [312]. De fet, la combinació d'ambdós fàrmacs va permetre fins i tot ampliar la finestra terapèutica del Clenbuterol, fet altament rellevant per a la possible translació clínica d'aquests tractaments. De manera similar, la combinació de Resveratrol, un potent agent antioxidant, amb l'àcid Valproic o

l'MS-275, ambdós dirigits a la inhibició farmacològica de les histones desacetilases (HDAC) implicades en la regulació de la transcripció gènica en resposta a l'ictus, també han mostrat caràcter terapèutic en experimentació animal [313]. Ambdós exemples fan pensar, doncs, en la possibilitat de sumar efectes protectors a través de la modulació simultània de múltiples efectors de l'ictus isquèmic, com podrien ser, per exemple, els candidats identificats al llarg d'aquesta Tesi Doctoral.

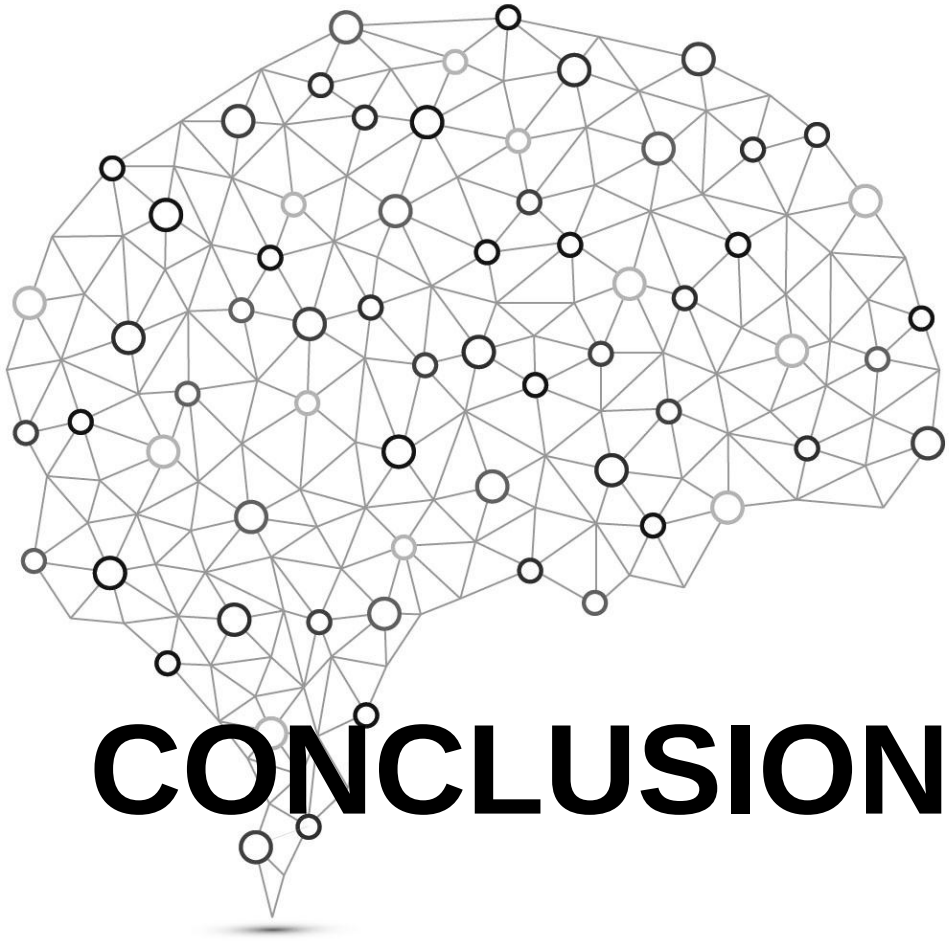
A l'**estudi 6** s'ha utilitzat una metodologia innovadora per identificar i estudiar en detall l'efecte neuroprotector sinèrgic de diversos fàrmacs en combinació. S'ha desenvolupat un model matemàtic que mimetitza *in silico* la patologia de l'ictus isquèmic, a través del qual s'han explorat milers de combinacions de fàrmacs en la cerca d'aquelles amb millor capacitat terapèutica per a combatre la malaltia. Dues de les combinacions seleccionades (CA i CB)[1] han estat avaluades i corroborades en un model d'isquèmia en ratolí. Els efectes neuroprotectors de les combinacions han resultat ser molt superiors als dels tractaments de cadascun dels fàrmacs individuals. Molecularment, la literatura descriu que els dos fàrmacs de la combinació CA (D1 i D2) estan implicats en la regulació dels neurotransmissors excitatoris, malgrat la D2 també actua prevenint la disfunció de la BBB, l'estrès oxidatiu i la resposta inflamatòria, i afavorint la supervivència neuronal a través de l'estimulació de la producció de factors neurotròfics. En quant a la combinació CB, es pensa que la D3 pot actuar majoritàriament modulant l'excitotoxicitat produïda pel glutamat, mentre que la D4 presenta propietats anti-apoptòtiques i antiinflamatòries destacables. En base a aquesta informació, pensem que la combinació d'aquests fàrmacs pot resultar terapèuticament eficaç donada la seva possible complementarietat funcional en l'atenuació de diversos mecanismes fisiopatològics implicats en la malaltia. Per a corroborar aquestes troballes inicials, però, encara hi ha molt camí per recórrer. L'avaluació d'aquests fàrmacs en altres estudis experimentals que utilitzin també diferents espècies, soques, sexes, edats i comorbiditats, tal i com suggereixen els criteris STAIR, és molt recomanable [131]. També, estudiar diferents dosis i temps d'administració per delimitar la finestra terapèutica en el que els tractaments mostren capacitat neuroprotectora és essencial per a poder traslladar en un futur els resultats a la pràctica clínica, així com assegurar la compatibilitat d'aquests agents múltiples amb les actuals estratègies de reperfusió.

Altrament, és interessant comentar que l'estratègia utilitzada per obtenir combinacions de fàrmacs incorporava també un llindar mínim d'eficàcia teòrica, obtinguda a través del nostre model virtual de l'ictus, que tota combinació havia de superar. Aquest llindar es va establir en base a l'eficàcia neuroprotectora teòrica que mostraven en el nostre model matemàtic una sèrie de fàrmacs que no han resultat eficaços terapèuticament en assajos

[1] Els noms de medicaments no es divulguen en aquest manuscrit per qüestions de confidencialitat relacionades amb el procés de protecció de la propietat intel·lectual.

clínics amb pacients amb ictus isquèmics. Alguns d'aquests fàrmacs són, per exemple, l'Enlimomab i el Natalizumab, dos inhibidors de la infiltració leucocitària prèviament mencionats; la Simvastatina [314], una estatina amb múltiples efectes pleiotròpics en l'ictus; el magnesi [315], un regulador del receptor d'NMDA; el Diazepam [316], un agonista GABAergic; o el Nalmefen [317], un antagonista dels receptors opioides, entre molts d'altres. Pensem que establir aquest llindar va ser un bon punt de partida per limitar les combinacions obtingudes a aquelles amb una alta probabilitat d'eficàcia i seleccionar les que teòricament siguin més prometedores que els tractaments testats fins al moment. Caldrà veure, però, si estudis experimentals futurs segueixen reportant tan bons resultats per a les dues combinacions de fàrmacs seleccionades com els aconseguits fins ara i permeten fer el pas a futures fases clíniques.

També, cal destacar que l'estratègia utilitzada en aquest estudi ha estat basada en el reposicionament de fàrmacs, és a dir, en la cerca de noves indicacions terapèutiques per fàrmacs que ja s'utilitzen per a altres indicacions mèdiques. Així, els fàrmacs de les combinacions proposades com a neuroprotectores ja han estat aprovats per a l'ús clínic amb anterioritat, malgrat per altres indicacions lluny de la neuroprotecció. Aquest fet permet accelerar exponencialment la translació cap a la clínica dels èxits assolits un cop corroborats en futures investigacions, doncs el procés d'avaluació de la seguretat i toxicitat d'aquests fàrmacs en persones ja s'han dut a terme anteriorment. A més, el fet de trobar i validar aplicacions mèdiques noves per a fàrmacs ja comercialitzats fa que els processos de desenvolupament i sortida al mercat d'aquests es vegin reduïts en temps i costos, tant econòmics com de gestió.



CONCLUSIONS

CONCLUSIONS

- Donada la rellevància de la neuroinflamació en la fisiopatologia de l'ictus, s'ha revisat a la literatura el possible paper dual com a biomarcadors i dianes terapèutiques de mediadors inflamatoris prèviament estudiats, tals com les molècules d'adhesió i les citocines i quimiocines pro-inflamatòries circulants.

- S'ha demostrat que la inhibició de la proteïna d'adhesió vascular VAP-1/SSAO, prèviament identificada com un bon biomarcador de TH associades a l'rt-PA, és una estratègia prometedora per tal d'evitar l'aparició de TH associades a l'administració d'aquest agent trombolític.

- S'ha identificat per primer cop el rol de la CCL23 com a possible biomarcador plasmàtic de diagnòstic de dany cerebral i pronòstic de la discapacitat funcional i mortalitat associada l'ictus isquèmic. També, l'administració de la CCL23 recombinant ha mostrat evidencies preliminars de millorar l'estat neurològic dels animals afectats per la isquèmia.

- L'estudi del proteoma del CSF de rata durant la fase aguda de la isquèmia cerebral ens ha permès identificar 7 candidats potencialment alterats per la isquèmia cerebral. Entre ells, destaquen la CKB i la CMPK com a biomarcadors de diagnòstic de l'ictus i la CAMK2B i la CMPK com a biomarcadors de pronòstic de l'evolució dels pacients isquèmics. Futurs estudis determinaran el seu potencial per a esdevenir plausibles dianes terapèutiques de l'ictus isquèmic.

- Mitjançant l'estudi de transcriptoma i proteoma del cervell isquèmic de ratolins durant la fase aguda de la isquèmia, i a través d'un anàlisis bioestadístic integratiu d'ambdós conjunts de dades, s'han identificat, replicat i explorat al llarg del temps 10 candidats involucrats en la isquèmia cerebral. Entre ells, destaquen els gens *Rgs2, Gadd45g, Cldn20* i *Cstad* i la proteïna CTNND2, la rellevància funcional i el rol com a biomarcador o com a diana terapèutica dels quals és encara desconeguda en el context de l'ictus isquèmic.

- S'ha caracteritzat un model matemàtic que simula la patologia de l'ictus isquèmic a través del qual s'han identificat dues combinacions de fàrmacs que han mostrat *in silico* un efecte terapèutic sinèrgic prometedor. L'eficàcia d'ambdues combinacions terapèutiques s'ha validat exitosament en un model murí d'isquèmia cerebral proximal transitòria com a teràpies per l'ictus isquèmic.

CONCLUSIONS

- Given the relevance of neuroinflammation in the pathophysiology of stroke, the potential dual role of previously studied inflammatory mediators as biomarkers and therapeutic targets has been carefully reviewed in the literature.

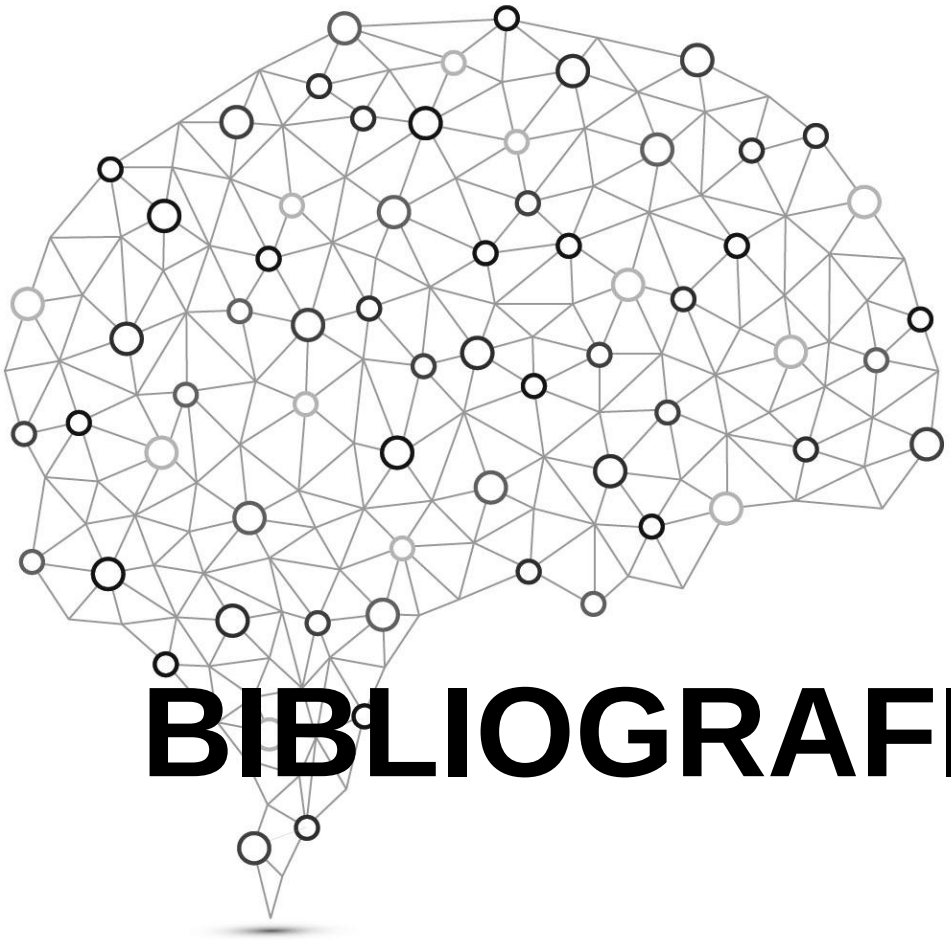
- The inhibition of the vascular adhesion protein VAP-1/SSAO, a previously identified promising biomarker of rt-PA-associated hemorrhagic transformations, has shown evidences for neuroprotection by reducing the incidence of hemorrhagic transformations after the administration of this trombolytic agent.

- CCL23 has been identified for the first time as a possible biomarker for the diagnosis of brain damage and the prognosis of stroke-related functional disability and long-term mortality. Besides, the administration of recombinant CCL23 has shown preliminary evidences of improving the neurological state of animals after cerebral ischemia.

- The study of the rat CSF proteome during the acute phase of cerebral ischemia has identified 7 candidates altered after cerebral ischemia. Among them, CKB and CMPK stand out as biomarkers for stroke diagnosis and CAMK2B and CMPK as biomarkers for stroke prognosis. Future studies will determine their potential to become plausible therapeutic targets for ischemic stroke.

- Through the study of the transcriptome and the proteome of the mouse ischemic brain during the acute phase of ischemia, and their integrative biostatistical analysis, 10 stroke-associated elements have been identified. Outstanding among them are the genes *Rgs2, Gadd45g, Cldn20* and *Cstad* and the CTNND2 protein, their role as a biomarker or therapeutic target for stroke has to be future explored.

- We have characterized a mathematical model that simulates *in silico* the ischemic stroke pathology, through which two drug combinations have been identified and tested for their promising synergistic therapeutic effect. Their efficacy has been proved in a mouse model of transient cerebral ischemia as new therapies for ischemic stroke.



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Formació acadèmica universitària

- **Doctorat en Bioquímica, Biologia Molecular i Biomedicina** (en curs). Universitat Autònoma de Barcelona, Espanya. Neurovascular Research Laboratory (LIN), Vall d'Hebrón Institut de Recerca (VHIR), Barcelona.

- **Màster oficial en Investigació Biomèdica** (2013-2014). Universitat Pompeu Fabra, Barcelona, Espanya. Unitat d'investigació en múscul i aparell respiratori (URMAR), Institut Hospital del Mar d'Investigacions Mèdiques (IMIM), Parc de Recerca Biomèdica de Barcelona (PRBB), Barcelona.

- **Grau en Bioquímica** (2009-2013). Universitat de Barcelona, Barcelona, Espanya. Immunology, pathophysiology and Experimental Therapies laboratory, Molecular biomedical research, University of Gent, Belgium.

Producció científica

- **Simats A**; García-Berrocoso T; Ramiro L; Giralt D; Gill N; Penalba A; Bustamante A; Rosell A; Montaner J. *Characterization of the rat cerebrospinal fluid proteome following acute cerebral ischemia using an aptamer-based proteomic technology.* Scientific Reports. Accepted for publication: April 2018.

- Bustamante A; Ning M; García-Berrocoso T; Penalba A; Boada C; **Simats A**; Pagola J; Ribó M; Molina C; Lo E; Montaner J. *Usefulness of adamts13 to predict response to recanalization therapies in acute ischemic stroke*. Neurology, 2018, 90(12):e995-e1004

- **Simats A**; García-Berrocoso T; Penalba A; Giralt D; Llovera G; Yinghua J; Ramiro L; Bustamante A; Martinez-Saez E; Canals F; Xiaoying W; Liesz A; Rosell A; Montaner J. *CCL23: A new CC chemokine involved in human brain damage.* Journal of Internal Medicine. 2018, 283(5):461-475

- Sun P; Hernandez-Guillamon M; Campos-Martorell M; **Simats A**; Montaner J; Unzeta M; Solé M. *Simvastatin blocks soluble SSAO/VAP-1 release in experimental models of cerebral ischemia: possible benefits for stroke-induced inflammation control.* Biochim Biophys Acta - Mol Basis Dis. 2018, 1864(2):542-553.

- García-Berrocoso T; Llombart V; Colàs-Campàs L; Hainard A; Licker V; Penalba A; Ramiro L; **Simats A**; Bustamante A; Martínez-Saez E; Canals F; Sanchez J.C; Montaner J. *Single cell immuno-laser microdissection coupled to label-free proteomics to reveal the proteotypes of human brain cells after ischemia.* Mol Cell Proteomics. 2018, 17(1):175-189

- Bustamante A, García-Berrocoso T, Penalba A, Giralt D, **Simats A**, Muchada M, Zapata E, Rubiera M, Montaner J. *Sepsis biomarkers reprofiling to predict stroke-associated infections. J Neuroimmunol.* 2017, 312:19-23.

- N. Percie Du Sert, A. Alfieri, S. M. Allan, H. Vo Carswell, G. A. Deuchar, T. D. Farr, P. Flecknell, L. Gallagher, C. L. Gibson, M. J. Haley, M. R. Macleod, B. W. Mccoll, C. Mccabe, A. Morancho, L. D. Moon, M. J. O 'neill, I. Pérez De Puig, A. Planas, C. I. Ragan, A. Rosell, L. A. Roy, K. O. Ryder, **A. Simats**, E. S. Sena, M. D. Tricklebank, R. C. Trueman, L. Whitfield, R. Wong, and I. M. Macrae. *The IMPROVE Guidelines (Ischaemia Models: Procedural Refinements Of in Vivo Experiments). J Cereb Blood Flow Metab.* 2017, 37(11):3488-3517.

- Llombart V; Trejo S; Bronsoms S; Morancho A; Ma F; Faura J; García-Berrocoso T; **Simats A**; Rosell A; Canals F; Hernández-Guillamón M; Montaner J. *Profiling and*

identification of new proteins involved in brain ischemia using MALDI-imaging-mass-spectrometry. Journal of proteomics. 2017, 152:243-253.

- Bustamante A; **Simats A**; Vilar-Bergua A; García-Berrocoso T; Montaner J. *Blood/brain biomarkers of inflammation after stroke and their association with outcome: from C-reactive protein to damage-associated molecular patterns.* The Journal of the American Society for Experimental NeuroTherapeutics. 2016, 13(4):671-684.

- **Simats A**; García-Berrocoso T; Montaner J*. Natalizumab: a new therapy for acute ischemic stroke?* Expert Review of Neurotherapeutics. 2016 Sep, 16(9):1013-21.

- Campos-Martorell M, Cano-Saabia M, **Simats A**, Hernández-Guillamon M, Rosell A, Maspoch D, Montaner J. *Charge effect of a liposomal delivery system encapsulating simvastatin to treat experimental ischemic stroke in rats.* International Journal of Nanomedicine. 2016, 11:3035-3048.

- Llombart V, García-Berrocoso T, Bech-Serra JJ, **Simats A**, Bustamante A, Giralt D, Reverter-Branchat G, Canals F, Hernández-Guillamon M, Montaner J*. Characterzation of secretomes from a human blood brain barrier endothelial cells in-vitro model after ischemia by stable isotope labeling with aminoacids in cell culture (SILAC).* Journal of Proteomics. 2015, 133:100-112.

- **Simats A**; García-Berrocoso T; Montaner J. *Neuroinflammatroy biomarkers: From stroke diagnosis and prognosis to therapy*. Biochim. Biophys. Acta. 2016, 1862(3):411-424.

- Vandendriessche B; Goethals A; **Simats A**; Van Hamme E; Brouckaert P; Cauwels A. *MAPK-activated protein kinase 2-deficiency causes hyperacute tumor necrosis factor-induced inflammatory shock*. BMC Physiology. 2014, 14(1):5.

- Bustamante A; Garcia-Berrocoso T; Llombart V; **Simats A**; Giralt D; Montaner J. *Overcoming the major hurdles in using neuroendocrine hormones as prognostic biomarkers in the setting of acute stroke.* Expert Review of Neurotherapeutics. 2014, 14(12):1391-1403.

Participació en projectes d'investigació

- *Stroke Bio-targets: Nuevas estrategias para descubrir biomarcadores de isquemia cerebral y su aplicación como dianas terapéuticas en el ictus*. PI15/00354 (01/01/2016-31/12/2018) Investigador principal (IP): Joan Montaner.

- *INVICTUS* (Redes Temáticas RETICS, RD12/0014/0005). Fondo de Investigación Sanitaria, Instituto Carlos III (01/01/2013-31/12/2016). Investigador principal (IP): Joan Montaner

- *Genetic Architecture of Human Brain Ischemia*. National institute of Health (NIH), EEUU (01/04/2016-31/03/2019). Investigador principal (IP): Joan Montaner

- *Protective effect of R-Tech Ueno VAP-1-inhibitor molecule on an "in vivo" stroke model*. Projecte de financiació privada del laboratori. (2015-2016) Vall d'Hebrón Institut de Recerca (VHIR) - Tech Ueno-R, Ltd., Japan. Investigador principal (IP): Joan Montaner

- PRESTIGE-AF: PREvention of STroke in Intracerebral hemorrhaGE survivors with Atrial Fibrillation. Unió Europea, Fundació Intsitut de Recerca de l'Hospital Universitari Vall d'Hebron (01/01/2018-30/11/2022).

- PROOF: Penumbral Rescue by Normobaric O=O Administration in Patients With Ischaemic Stroke. GA-Nr.733379. Unió Europea, Institut de Recerca de l'Hospital Universitari Vall d'Hebron.

Formació científica: cursos i congressos

- Participació en les **Jornades anuals de l'Institut de Recerca de Vall d'Hebron** (VHIR) (13th December, 2017). Títol de la presentació en format pòster: *Characterization of the cerebrospinal fluid proteome following acute cerebral ischemia using an aptamer-based proteomic technology.*

- Assistència i presentació d'una comunicació en format pòster a la conferencia **ENABLE The 1st European PhD and Postdoc Symposium** (ENABLE network), 2017, Barcelona. Títol de la presentació en format pòster: *Characterization of the cerebrospinal fluid proteome following acute cerebral ischemia using an aptamer-based proteomic technology.*

- Assistència i presentació d'una comunicació oral al **28th Symposium on Cerebral Blood Flow, Metabolism and Function** (1st-4th April, 2017, Berlin). Títol de la comunicació: *CCL23: a chemokine early present in brain after cerebral ischemia that plays a role as a blood biomarker for brain damage and stroke outcome.*

- Participació en el congres anual de la **Red temàtica de Investigación en Enfermedades Vasculares** Cerebrales, RETICS-INVICTUS+ (27th-28th March, 2017, Santiago de Compostela) (RD16/0019).

- **Annual Proteomics Symposium – Applying proteomics to life science** (11th November 2016, Barcelona). Organitzat pel Centre de Regulació Genòmica (CRG) i la Societat Catalana de Biologia.

- Assistència i presentació d'una comunicació en format pòster al **X simposi de neurobiología** (6th-7th October, 2016, Barcelona). Societat Catalana de Biologia. Títol de la comunicació: CCL23: *A new chemokine present in brain after cerebral ischemia might play a role as a blood biomarker for brain damage and stroke outcome.*

- **Curso de educación continuada en el Laboratorio Clínico** (Any acadèmic 2015-2016). Societat Espanyola de Bioquímica clínica i patologia molecular (SEQC), acreditada pel consell Català de formació mèdica continuada.

- **5th Hands-on Course in ultrafast Sample Treatment for Proteomics** (22nd-24th of June, 2015). PROTEOMASS Scientific Society, Faculty of Science and Technology of the New University of Libon, Portugal.

- **How to improve you scientific presentations** (11th-12th of May, 2015). Fundació Dr. Antoni Esteve I IRB Barcelona, Barcelona.

- **Acreditació com a investigador usuari d'animals d'experimentació** (2013). Universitat de Barcelona.