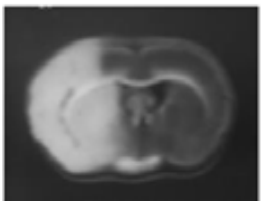
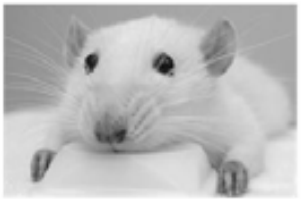
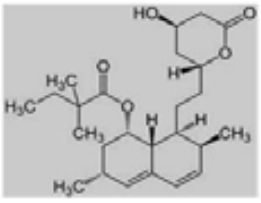
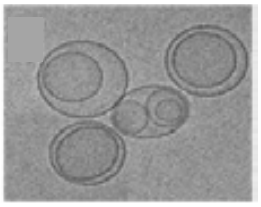
TESI DOCTORAL

EFICÀCIA I SEGURETAT DE LA SIMVASTATINA ADMINISTRADA EN LA FASE AGUDA DE LA ISQUÈMIA CEREBRAL EXPERIMENTAL

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Aquesta tesi ha estat realitzada al Laboratori de Recerca Neurovascular de l'Institut de Recerca de la Vall d'Hebron amb el suport de l'ajuda Predoctoral de Formació en Investigació en Salut (PFIS) de l'Institut de Salut Carlos III (FI 10/00508).

El Laboratori de Recerca Neurovascular pertany a la xarxa espanyola de recerca neurovascular INVICTUS (RD12/0014/0005) i rep financiació del Fondo de Investigación Sanitaria (FIS).

Els estudis presentats a continuació han estat possibles també, gràcies al suport i col·laboració del Servei d'Estabulari del VHIR, així com al servei de Plataforma d'Imatge Molecular i a la Unitat d'Alta Tecnologia.

La ciencia és el gran antídot

contra el verí de l'entussiasme i la superstició

(Adam Smith)

No ha estat un camí gens fàcil, ni les coses han anat sempre com estava previst, però finalment ja sóc aquí!! Escrivint les darreres pàgines d'aquesta tesi!

No m'agradaria estendre'm massa, que sinó hi ha membres del Tribunal que comenten que no pot ser que l'apartat d'agraïments sigui més llarg que el dels resultats de la Tesi. I tenen raó. Però penso que, arribat aquest moment, és inevitable mirar enrere i reconèixer la importància dels que ens han recolzat, animat i confiat en nosaltres quan més ho hem necessitat.

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I a tu, Pau, moltíssimes gràcies per estar sempre allà. Senzillament, moltes gràcies per fer-me la vida tan divertida i tan dolça.

Llistat d'abreviatures

ACK buffer: de l'anglès, *Ammonium-chlorid-kaliumhydrogencarbonat* buffer

ATP: Adenosina trifosfat

BHE: Barrera hemato-encefàlica

CEEA: Comitè Ètic d' Experimentació Animal

CFU's: en anglès, cèl·lules formadores de colònies

CL: Hemisferi contralateral (o no infartat) del cervell

CRP: en anglès, Proteïna C reactiva

DIGE: de l'anglès, *Difference in Gel Electrophoresis*

EE: en anglès, coeficient d'encapsulació

EPC's: en anglès, cèl·lules endotelials progenitores

FDA: de l' angles, *Food and Drug Administration*

HEPES: de l'anglès*, 4-(2-hydroxy ethyl)-1-piperazineethanesulfonic acid*

HBSS-Hepes: de l'anglès, *Hanks' balanced salt solution – Hepes*

HCL: àcid clorhídric

HDL: en anglès, lipoproteines d'alta densitat

HI: en angles, infarts hemorràgics

HMG-CoA: hidroximetil-glutaril-coenzim-A

IP: Hemisferi ipsilateral (o infartat) del cervell

ITU: Infeccions del tracte urinari

IVIS: de l'anglès, *In Vivo Imaging System*

LCR: Líquid céfalo-raquidi

LDL: en anglès, lipoproteines de baixa densitat

MALDI-TOF MS : de l'anglès, *Matrix-Assisted Laser Desorption/Ionization-Time-of-flight mass spectrometry*

MCA: en angles, artèria cerebral mitja

MCAO: de l' anglès, *Middle Crerebral Artery Occlusion*

MMPs: en anglès, metaloproteïnases de matriu

N. Vague: Nervi Vague

NIHSS: de l'anglès *National Institutes of Health Stroke Scale*

NINDS: de l'anglès, *National Institute of Neurological Disorders and Stroke*

NO: en angles, òxid nítric

NOS: en anglès, òxid nítric sintasa

OGD: en anglès, deprivació d'oxigen i glucosa

PAI-1: en anglès, inhibidor de l'activador del plasminogen

PH: en anglès, hematomes parenquimatosos

PMNs: Polimorfonuclears

rt-PA: en anglès, activador tisular del plasminògen recombinant

ROS: de l'anglès*, reactive oxygen species*

STAIR: de l'anglès, *Stroke Treatment Academic Industry Roundtable*

SNC: Sistema Nerviós Central

SNS: Sistema Nerviós Simpàtic

SSF: Sèrum salí fisiològic

SSH: Sèrum salí heparinitzat

SUVs: en anglès, petites vesícules unilamelars

TH: Transformacions hemorràgiques

TOAST: de l'anglès, Trial *of Org 10172 in Acute Stroke Treatment*

UHPLC: de l' anglès, *Ultra High Performance Liquid Chromatography*

WB:de l'anglès, *Western Blot*

1. INTRODUCCIÓ

1.1 L'ictus

1.1.1 Aspectes epidemiològics i clínics

L'ictus o infart cerebral és un trastorn neurològic produït per una alteració del flux sanguini d'una determinada regió cerebral. Aquesta alteració del flux pot ser transitòria (si es restableix espontàniament o mitjançant accions terapèutiques) o permanent. Com a conseqüència, poden aparèixer tot un seguit de símptomes neurològics com ara: afàsia (trastorn de la parla), hemiparèsia (afectació de la capacitat motora d'un costat del cos), pèrdua de consciència, vertigen, pèrdua de visió o trastorns del llenguatge, entre altres. Segons la zona de l'encèfal afectada, apareixeran uns o altres dèficits, que seran reversibles, o no, en funció del temps d'oclusió i de l'evolució clínica del pacient (Díez-Tejedor *et al.* 2001).

La importància d'investigar aquesta malaltia rau en que és una de les primeres causes de mortalitat, juntament amb les malalties cardíaques i el càncer, en el món occidental. Segons dades obtingudes de la World Health Organitzation, www.who.org, l'ictus és responsable de 5,5 milions de morts anuals arreu del món i de 5 milions de supervivents amb discapacitats neurològiques. A l'estat espanyol, es calcula que la seva incidència anual és de 200 casos per cada 100.000 habitants i és considerada la primera causa de mort en dones i la segona en homes(Díaz-Guzmán *et al.* 2012). Per altra banda, és també la primera causa d'incapacitat i invalidesa a llarg termini degut a les seqüeles sensitives i motores que provoca, i això acaba suposant un cost econòmic, tant sanitari com social, molt elevat (Bonita 1992). Cal remarcar que, tot i el millor control dels factors de risc cardiovascular, l'envelliment de la població ha suposat un increment en la incidència i la prevalença de l'ictus en els últims anys. S'espera que aquesta tendència continuï en un futur, ja que es calcula que la població mundial major de 65 anys continuarà augmentant en 9 milions de persones l'any (Mukherjee *et al.* 2001).

Actualment, el control dels factors de risc que poden determinar l'aparició de la patologia és una de les eines més importants per a la seva prevenció. Aquests factors de risc poden classificar-se en modificables o fixes. Alguns factors de risc modificables són comuns amb altres malalties cardiovasculars tals com la hipertensió arterial, la diabetis, la dislipèmia, l'obesitat, el tabaquisme, l'alcoholisme i el sedentarisme; mentre que altres són menys freqüents i més específics de l'ictus (Di Legge *et al*. 2012), com ara la fibril·lació auricular o els atacs isquèmics transitoris. Els factors de risc fixes inclourien l'edat, el sexe i un component genètic. Alguns factors de risc genètic es presenten com a malalties monogèniques (malaltia de

Fabry o CADASIL (*Cerebral Autosomal-Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy*) però en la majoria de casos es creu que la relació genètica de la malaltia és resultat de l'efecte multiplicador de diferents al·lels patogènics, cadascun dels quals confereix un lleuger augment del risc (Markus 2012).

1.1.2 Subtipus d'ictus

De manera general, l'ictus es pot classificar en dos grans grups: ictus hemorràgic i ictus isquèmic. En el primer es produeix una extravasació de sang al parènquima com a conseqüència del trencament d'una artèria, mentre que en el segon, el que es produeix és una disminució del flux arteriala causa d'un coàgul o una trombosi **(Figura 1)**.

L'ictus hemorràgic o hemorràgia cerebral apareix com a conseqüència del trencament d'un vas sanguini; suposa un 15-20% del total dels ictus i té una taxa de mortalitat molt elevada. Els ictus d'aquest tipus es classifiquen en hematomes intracerebrals (extravasació de sang cap al parènquima cerebral) o hemorràgies subaracnoïdals (extravasació de sang directament a l'espai subaracnoïdal). L'etiologia més freqüent és la hipertensió arterial. El seu pronòstic és molt dramàtic i actualment no es disposa de cap tractament efectiu. Tot i la gravetat de l'ictus hemorràgic, aquest subtipus d'ictus és el menys estudiat i encara es desconeixen els mecanismes moleculars que intervenen en la seva fisiopatologia (Montaner 2010).

L'ictus isquèmic és més comú que l'hemorràgic isuposa aproximadament el 80% dels infarts cerebrals. Aquest apareix com a conseqüència de l'oclusió d'una artèria de calibre mitjà o gran per un mecanisme embòlic (per coàguls, generalment procedents del cor) o trombòtic (per plaques d'ateroma que redueixen la llum del vas i faciliten la producció del trombus). La disminució de l'aportació sanguínia cerebral pot ser total (isquèmia global) o parcial (isquèmia focal). La isquèmia cerebral focal és la més freqüent i es divideix segons la duració de l'episodi en: atac isquèmic transitori (AIT) quan es tracta d'un episodi d'inici agut i etiologia isquèmica de disfunció neurològica focal amb una duració menor a les 24 hores (generalment entre 1-2 hores), o infart cerebral.

Existeixen diferents criteris per a classificar els ictus: segons etiologia i mecanisme d'acció, durada dels símptomes o lloc de l'oclusió arterial y territori del cervell afectat. Però una de les classificacions més usada és la basada en l'etiologia. Aquesta classificació és coneguda com a

"criteris TOAST" (*Trial of Org 10172 in Acute Stroke Treatment*) (Adams *et al.* 1993) i ordena els ictus isquèmics en 5 subgrups:

1. **Aterotrombòtic**: infart degut a una estenosi >50% d'una artèria intracranial o extracranial, i absència de cardiopatia embolígena. Infarts generalment mitjans o grans, de topografia cortical o subcortical i amb aterosclerosi de localització carotídia o vertebrobasilar.
2. **Cardioembòlic:** infart mitjà o gran que s'ha produït per obstrucció d'una artèria cerebral per material embòlic d'origen cardíac. Topografia habitualment cortical.
3. **Lacunar o patologia de vas petit d'origen no aterotrombòtic:** infart de mida petita (<1.5 cm de diàmetre) produït en el territori de distribució d'una artèria perforant cerebral.
4. **De causa inhabitual:** infart en el què s'ha descartat l'origen aterotrombòtic, cardioembòlic o lacunar i en el qual s'ha identificat una causa menys freqüent. Se solen produir per malalties sistèmiques com ara infeccions, neoplàsies, alteracions metabòliques o de coagulació, o per aneurismes, migranyes o malformacions arteriovenoses, entre d'altres.
5. **D'origen indeterminat:** infart en què després d'un estudi diagnòstic exhaustiu, s'han descartat els subtipus aterotrombòtic, cardioembòlic, lacunar i de causa inhabitual, o bé s'han identificat vàries etiologies. Són infarts petits, mitjans o grans amb localització cortical o subcortical, o bé en el territori vertebrobasilar o carotidi.

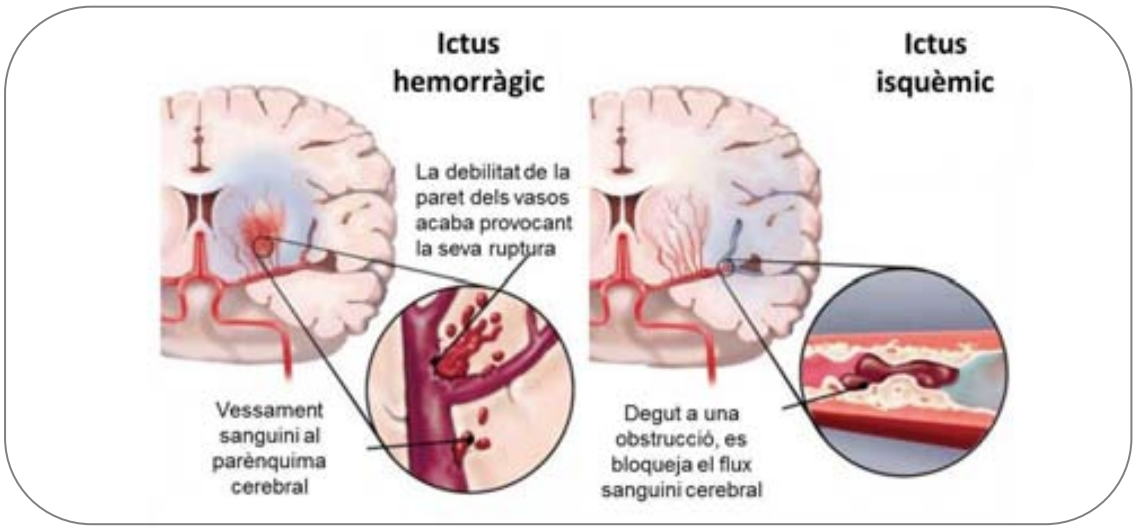

Figura 1. Esquema dels tipus d'ictus: isquèmic i hemorràgic. Adaptat de ®Heart and Stroke foundation of Canada.

Pel que fa al pronòstic, l'estat neurològic dels pacients d'ictus s'avalua a partir d'escales objectives que mesuren la gravetat del dany produït per la lesió. L'escala més utilitzada és la NIHSS (*National Institutes of Health Stroke Scale*), que amb una puntuació de 0 a 42 punts de menys a més gravetat permet valorar el dèficit neurològic i la millora o empitjorament a curt termini (dies, setmanes) (Brot *et al.* 1989). Una variació en el temps menor de 4 punts es considera estabilitat del pacient, un increment de 4 punts o més indica empitjorament i una baixada de 4 o mes punts indica milloria neurològica.

1.1.3 Fisiopatologia de l'ictus

La conseqüència immediata de l'oclusió d'una artèria cerebral, ja sigui per un coàgul, una trombosi o una hipoperfusió sistèmica, és la restricció del flux cerebral sanguini. La falta d'oxigen i glucosa al teixit cerebral desencadena una sèrie de mecanismes (tant a nivell cel·lular com a nivell tissular) que acaben ocasionant la mort neuronal.

Cal remarcar, per això, que la reducció del flux sanguini cerebral no es dóna de forma homogènia en tot el territori afectat, sinó que existeix un nucli central anomenat *core de l'infart*, que és la zona directament irrigada per l'artèria obstruïda i on es produeix una lesió tissular, i una zona circumdant que s'anomena *penombra isquèmica* (en estudis de neuroimatge s'observa hipoperfosa). Aquesta segona zona és de gran interès ja que tot i trobar-se compromesa metabòlicament, es considera que és potencialment salvable (Astrup *et al.* 1977) **(Figura 2)**. A la zona de penombra les neurones són funcionalment inactives però encara són viables (De Keyser *et al*. 1999a, 1999b). Ara bé, si el flux sanguini no es restableix ràpidament, el teixit acaba morint i contribuint a la progressió de l'infart. Els factors que determinen aquesta progressió de la penombra a l'infart són el grau de circulació arterial col·lateral, la duració de la isquèmia i l'estat funcional i metabòlic previ de la cèl·lula. La possibilitat de recuperar el teixit danyat, disminuint així el deteriorament neurològic, ha fet de la penombra isquèmica una diana rellevant i el focus de nombroses investigacions.

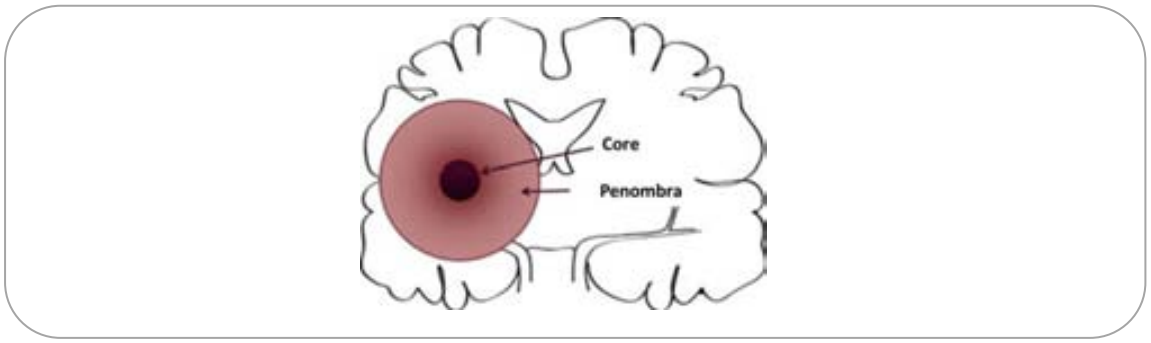

Figura 2. Esquema on es mostra l'àrea de penombra isquèmica, zona on es considera que poden revertir-se els danys ocasionats per la isquèmia. Adaptat de Coultrap *et al.* 2011.

Al *core* de l'infart, on la gravetat de la isquèmia és màxima, es produeix una depleció energètica casi total, així com una pèrdua de la funció de membrana i del gradient iònic, edematització i destrucció cel·lular. A la zona de la *penombra*, on existeix un flux residual i el dèficit d'energia és menor, la isquèmia ocasiona la pèrdua de funcions cel·lulars donant pas a diverses alteracions bioquímiques que tenen efectes nocius diversos i que constitueixen l'anomenada *cascada isquèmica.*

1.1.3.1 La cascada isquèmica

Com a conseqüència de la restricció del flux cerebral sanguini (ja sigui ocasionat per un coàgul, una trombosi o una hipoperfusió sistèmica) l'aportació d'oxigen i glucosa per a mantenir l'homeòstasi cel·lular és insuficient. Aquesta situació provoca múltiples processos bioquímics que afecten tant les neurones, com la glia i la microcirculació **(Figura 3).**

En els primers moments de la isquèmia, el contingut energètic de les cèl·lules es redueix sobtadament provocant alteracions en tots els mecanismes dependents de l'ATP com la bomba Na^+/K^+ ATP-asa, i té conseqüències tan greus com que la cèl·lula perdi la seva capacitat per mantenir l'homeòstasi iònica. Això desencadena una despolarització (pèrdua de l'activitat elèctrica neuronal) i una entrada massiva de Ca^{2+}, Na^+ i aigua (formació d'edema cel·lular) (Siesjö 1990).

Seguidament, i com a conseqüència de l'alteració de la permeabilitat cel·lular, es produeix una alliberació massiva de neurotransmissors a l'espai extracel·lular (*excitotoxicitat*). D'entre tots els neurotransmissors alliberats, cal destacar el Glutamat, que juntament amb una sobrecàrrega de Ca^{2+} a l'interior de la cèl·lula, provoca una sèrie d'efectes tòxics mediats per

receptors específics. La sobrecàrrega de Ca^{2+} incrementa la permeabilitat de la membrana i activa enzims lítics que destrueixen components cel·lulars (proteases, lipases, endonucleases). A més, facilita la síntesi d'òxid nítric (NO) i l'activitat de l'òxid nítric sintasa (NOS), la formació de radicals lliures derivats del mateix, i també desacobla la fosforilació oxidativa, comprometent encara més la disponibilitat energètica (Castillo i Rodriguez 2004, Puyal *et al.* 2013). Finalment, el Ca^{2+} activa diversos factors de transcripció que poden afavorir l'activació de la cascada d'apoptosi.

L'acidosi, l'entrada de Ca^{2+} i l'estrès oxidatiu*,* són processos comuns a totes les cèl·lules del parènquima cerebral afectades per la isquèmia, tot i que cada tipus cel·lular es veu afectat d'una manera determinada. Els astròcits, per exemple, pateixen un procés d'hipertròfia i proliferació conegut amb el nom de *gliosi reactiva* que té un paper molt important tant en l'establiment de la lesió definitiva com en la reparació tissular. Per altra banda, les cèl·lules glials, en no tenir estructures post-sinàptiques, són menys sensibles al dany per mecanismes d'excitotoxicitat mentre que la micròglia (cèl·lules inflamatòries residents al cervell) i l'oligodendròglia (cèl·lules productores de mielina) contribueixen al dany isquèmic produint citocines i radicals lliures.

Per últim, cal destacar que tant la isquèmia com la reperfusió posterior indueixen una resposta inflamatòria que s'inicia a la microcirculació. Tant les cèl·lules endotelials que es troben a la perifèria de la zona isquèmica, com les neurones, els astròcits i la micròglia són activades mitjançant l'alliberació de citocines (IL-1, TNF entre d'altres), responsables d'iniciar aquesta resposta inflamatòria (Hallenbeck 1996). Aquestes dues citocines indueixen una segona resposta inflamatòria, molt més persistent i mediada per altres citocines (IL-6 i IL-8), quimiocines i molècules d'adhesió. Al mateix temps, es creu que la sobreexpressió de les molècules d'adhesió a les cèl·lules endotelials de la barrera hematoencefàlica (BHE) indueix la infiltració de neutròfils al teixit cerebral (Rosell *et al.* 2006). Aquesta infiltració incrementa el dany tissular en la fase aguda ja que estimula l'alliberament de substàncies vasoconstrictores i d'enzims proteolítics que trenquen la BHE i permeten el flux d'aigua i eritròcits, contribuint així, en la formació de l'edema cerebral i de la transformació hemorràgica (Castillo *et al.* 2001, Castellanos *et al*. 2007).

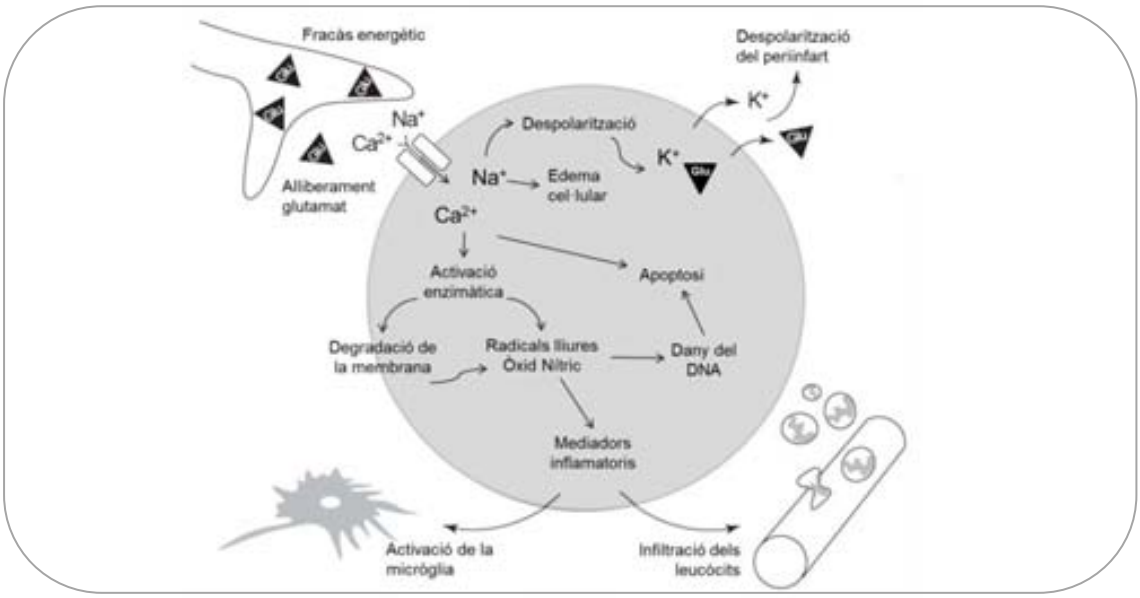

Figura 3. Esquema-resum dels processos fisiopatològics (cascada isquèmica) que tenen lloc en el teixit cerebral isquèmic. Adaptat de Dirnagl *et al.* 1999.

1.1.4 Pronòstic i complicacions associades a l' ictus

1.1.4.1 Transformacions hemorràgiques

Les transformacions hemorràgiques (TH) consisteixen en un sagnat localitzat habitualment a l'àrea infartada que s'ocasiona després de produir-se l'ictus. Són un fenomen complex i multifactorial ocasionat quan el flux cerebral sanguini es restableix en una vasculatura danyada (que ha patit la disrupció de la BHE). De fet, els desajustaments metabòlics produïts per la isquèmia cerebral són els responsables de debilitar també la BHE (Warach i Latour 2004). Lin i col.laboradors (2007) varen descriure que fins el 88% dels pacients d'ictus tenien incrementada la permeabilitat microvascular dins les primeres 3 hores des de l'inici dels símptomes i que a més, aquesta permeabilitat semblava significativament incrementada en els pacients que més tard patirien una TH (Lin *et al*. 2007). Les TH acaben afectant entre un 10 i un 40% dels pacients que han patit un ictus (Terruso *et al*. 2009, Beslow *et al*. 2011), i s'associen amb un increment de la morbiditat i mortalitat (Fiorelli *et al.* 1999). A més, com s'ha comentat a l'apartat anterior, les TH són una de les complicacions més freqüents després de l'administració del trombolític rt-PA (en l'apartat 1.3.1 es parlarà més extensament del tractament amb rt-PA). Es considera que l'administració del trombolític pot incrementar fins a10 vegades la probabilitat de desencadenar una TH (NINDS, 1995) degut a varis mecanismes. En primer lloc, el fet de promoure la reperfusió mitjançant la degradació dels coàguls de fibrina

ja es considera un desencadenant per a l'aparició de TH (Ueda *et al.* 1995) i en segon lloc, l'increment que se li atribueix en l'expressió de metaloproeteïnasses de matriu, com la MMP9, MMP2 i MMP3, així com l'efecte que té sobre receptors específics (PDGFRα i LRP) (Suzuki *et al.* 2011).

Per altra banda, Jickling *et al.* (2014) consideren que cal distingir 2 tipus de TH; les que s'ocasionen de seguida, anomenades *early* o TH primarenques (18-24h) i les que s'ocasionen de manera tardana (a partir de les 18-24h). Aquest mateix grup descriu en el seu estudi que per a cada un dels 2 tipus hi intervenen mediadors diferents. Mentre que les *early* TH són mediades per metaloproteïnases de matriu 9 (MMP9) i *reactive oxygen species (ROS)* de la sang, i per MMP2 del cervell, en les TH més tardanes hi intervenen MMP9 i MMP3 del cervell, altres proteasses, remodeladors vasculars (VEGF, Ang1, HMGB1) i la pròpia neuroinflamació. **(Figura 4).**

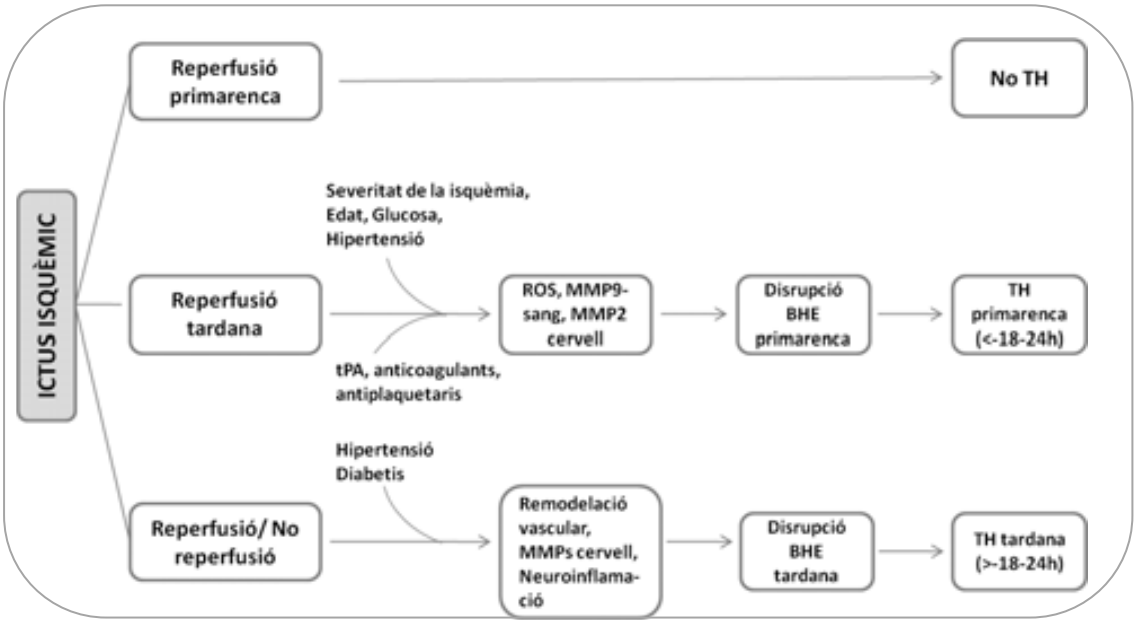

Figura 4. Esquema on es mostren els factors que intervenen en la formació de transformacions hemorràgiques (TH). Adaptat de Jickling *et al.* 2014.

Per tal de definir la severitat de les TH, els neuròlegs usen una classificació que les divideix en 4 grups segons l'aparença radiològica (Pessin *et al.* 1990, Montaner *et al.* 2009). Primerament les classifiquen en: infarts hemorràgics (HI=hemorràgies heterogènies que ocupen una porció de l'infart) o hematomes parenquimatosos (PH= hematomes homogenis amb efecte massa)

(Taula 1 i Figura 5). D'entre els 4 tipus, només la PH-2 s'ha pogut associar a un empitjorament de l'estat neurològic (Fiorelli *et al.* 1999).

Tipus d'hemorràgies	Aparença radiològica
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. Adaptat de Martí-Fàbregas *et al.* 2007.

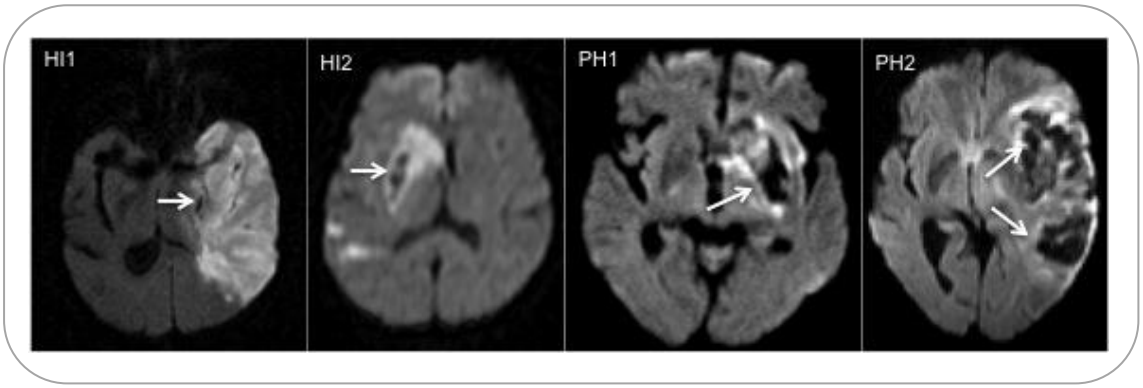

Figura 5. Imatges de ressonància magnètica on es mostren els diferents tipus de TH. Les regions hemorràgiques es veuen com a zones de molt baixa intensitat (marcades amb una fletxa). Adaptat de Perchyonok *et al.* 2002.

A part dels tipus de TH esmentats, existeix el que s'anomena hematoma parenquimatós remot (PH-r), que engloba qualsevol sagnat intracranial localitzat fora de la zona infartada. Tot i que aquest últim tipus de sagnat sol aparèixer igualment després de l'administració d'rt-PA (Winkler *et al.* 2002), s'ha descrit que s'associa amb la presència prèvia d'altres alteracions tals com coagulopaties, estructures vasculars anòmales o angiopaties (Kidwell *et al*. 2003, Derex *et al.* 2004). Les PH-r poden presentar-se com a úniques o mútiples. Cal dir, per això,

que les PH-r tenen una incidència molt baixa (1-3% dels pacients que pateixen un ictus) (Trouillas i von Kummer 2006).

Per altra banda, un gran nombre de factors clínics s'han associat amb l'aparició de TH després de produir-se la isquèmia cerebral. Entre ells, la severitat de la isquèmia i la mida de l'infart cerebral són els factors que millor s'han correlacionat amb les TH (Terruso *et al*. 2009). Altres factors en estudi són l'edat avançada, la hipertensió, els nivells de glucosa en sang i l'ús d'antiplaquetaris (Hacke *et al.* 2004, Castellanos *et al.* 2003)

Pel que fa al tractament i prevenció de les TH, cal dir que en l'actualitat encara no existeix cap fàrmac eficaç. Tot i que molts compostos han demostrat ser capaços de reduir la incidència de TH en models animals, cap d'ells ha mostrat ser eficaç quan s'ha administrat a pacients amb ictus. Les dianes terapèutiques que s'han considerat fins al moment són la reducció de *ROS,* la inhibició de MMPs i la modulació de factors capaços de reduir la permeabilitat de la BHE. El fetque fàrmacs molt prometedors en models animals hagin fracassat en la translació als pacients encara continua sent una incògnita. Malgrat això, sí que es creu que alguns models animals no són capaços de simular l'efectivitat d'un fàrmac en humans i és per això que es recomana l'ús de varis models animals per a l'avaluació de compostos que previnguin les TH (Fagan *et al.* 2013).

1.1.4.2 Infeccions

Les infeccions, igual que les TH, són considerades una de les majors complicacions associades a l'ictus. S'ha descrit que un 30% dels pacients que han patit un ictus es veuen afectats, a més, per algun tipus d'infecció, sobretot pneumònies i infeccions del tracte urinari (ITU) (Westendorp *et al*. 2011). L'estreta relació entre els ictus i les posteriors infeccions pot explicar-se per diversos mecanismes, alguns dels quals poden considerar-se conseqüència directa de la isquèmia, mentre que d'altres no. L'edat o la presència de malalties concomitants, com la fibril·lació atrial o la insuficiència cardíaca congestiva, han mostrat associació amb una major incidència d'infeccions (Aslanyan *et al.* 2004, Ovbiagele *et al.* 2006).

Primerament cal tenir en compte que com a conseqüència de l'ictus, els pacients experimenten una sèrie de símptomes que faciliten l'aparició de les infeccions: immobilitat, disfàgia (que incrementa el risc d'aspiracions), alteració en el reflex de la tos i disfunció de la bufeta de l'orina. A més, aquests símptomes poden agreujar-se més per la dificultat o

impossibilitat que tenen aquests pacients per comunicar-se i/o cooperar degut a l'estat de coma o a l'afàsia (Johnsen *et al.* 2012).

Per altra banda, els processos invasius com catèters urinaris, vies i tubs per a la ventilació mecànica o per a l'alimentació són una via molt accessible per a l'entrada de patògens a l'organisme. Cal destacar els catèters urinaris permanents ja que se'ls ha associat amb un risc d'ITU d'entre 3-10% per cada dia de cateterització, arribant a un 100% després de 30 dies (Maki *et al.* 2001, Gould *et al.* 2010).

Un altre factor a destacar és l'estat d'immunodepressió al què es veuen sotmesos els pacients que han patit lesions al Sistema Nerviós Central (SNC), com ara un ictus (Emsley and Hopkins 2008, Chamorro *et al.* 2007). Bàsicament, el dany al SNC ocasiona una alteració en el balanç entre el propi SNC i el sistema immunitari. A partir de moduladors com ara les interleucines, la lesió del SNC activa l'eix hipotàlem-pituïtari-adrenal així com el sistema nerviós simpàtic i el parasimpàtic. Aquesta activació ocasiona l'alliberament de noradrenalina, glucocorticoides i acetilcolina, queindueixen una resposta antiinflamatòria sistèmica o estat d'immunodepressió. A més, la disrupció de la integritat de les barreres immunològiques també contribueix al desenvolupament de la infecció (Meisel *et al.* 2005) **(Figura 6).**

La importància de les infeccions que es desenvolupen posteriorment a l'ictus rau en que s'han associat a un pitjor pronòstic (Emsley *et al.* 2008) ia una major mortalitat (Westendorp *et al.* 2011). De fet, nombrosos estudis han remarcat la pneumònia com a principal causa de mort durant la fase post-aguda de la isquèmia cerebral, convertint-se així en el factor responsable de la mort d'1 de cada 3 casos (Heuschmann *et al*. 2004, Katzan *et al.* 2003).

Pel que fa als dos tipus d' infeccions més freqüents, s'ha descrit que la pneumònia afecta a 1.2-22% dels pacients durant l'hospitalització, sobretot durant les primeres 24 hores i poques vegades més enllà dels 4 dies (Indredavik *et al.* 2008) i també que la major part d'aquestes pneumònies són degudes a aspiracions (Johnston *et al.* 1998). A més, s'han pogut identificar factors independents que predisposen a patir aquest tipus d'infeccions. Els més destacats són l'edat (major de 65 anys), i la severitat de l'episodi (ja sigui tenint en compte el dèficit neurològic o el volum de l'infart). I també altres factors relacionats amb els anteriors com són l'estat de dependència a l'admissió, la dificultat de parla, la severitat de la incapacitat després de l'ictus, el deteriorament cognitiu, la disfàgia, el tipus d'ictus (no lacunars), i les comorbiditats presents (malaltia coronària, malaltia pulmonar obstructiva crònica) (Ovbiagele *et al.* 2006).

Per altra banda, el risc a patir una ITU varia substancialment depenent de l'estudi però Westendorp *et al.* (2011) van observar que la incidència era d'un 10%, així com que aquest tipus d'infeccions solien ocasionar-se entre els 15-17 dies després de patir la isquèmia. Tot i que la major part d'aquestes infeccions estaven associades a l'ús de sondes urinàries, també es va veure que sondats o no, els pacients amb ictus tenien més del doble de probabilitats de patir aquest tipus d'infecció si es comparaven amb la resta de pacients hospitalitzats (Ersoz *et al.* 2007).

Actualment, els efectes adversos que ocasionen les infeccions estan ben demostrats, i és per això que s'estan estudiant mesures per tal de poder-los evitar o, si més no, reduir. D'entre aquestes mesures cal destacar l'ús preventiu d'antibiòtics. Fins al moment, degut a l'heterogenicitat i al baix nombre de pacients inclosos, els resultats en diferents estudis són controvertits. Mentre que alguns estudis mostraren una reducció significativa en la incidència d'infeccions pulmonars en els pacients que rebien antibiòtics (Chamorro *et al*. 2005), altres no veieren diferències (Shwarz *et al.* 2008, Hams *et al*. 2008). Cal destacar, per això, la publicació d'un metanàlisi posterior on s'inclogueren 5 estudis i conclogué que el tractament antibiòtic precoç disminueix la incidència d'infeccions en la fase aguda de l'ictus, malgrat no aporta una milloria significativa en l'evolució neurològica ni en la reducció de la mortalitat (Westendorp et *al.* 2012). Altres mesures a tenir en compte serien la reducció en l'ús de catèters permanents, l'inici immediat de la rehabilitació i de la mobilització dels pacients i la valoració del reflex de deglució mitjançant tests de salivació abans de donar-los aigua o menjar (Salat *et al.* 2012).

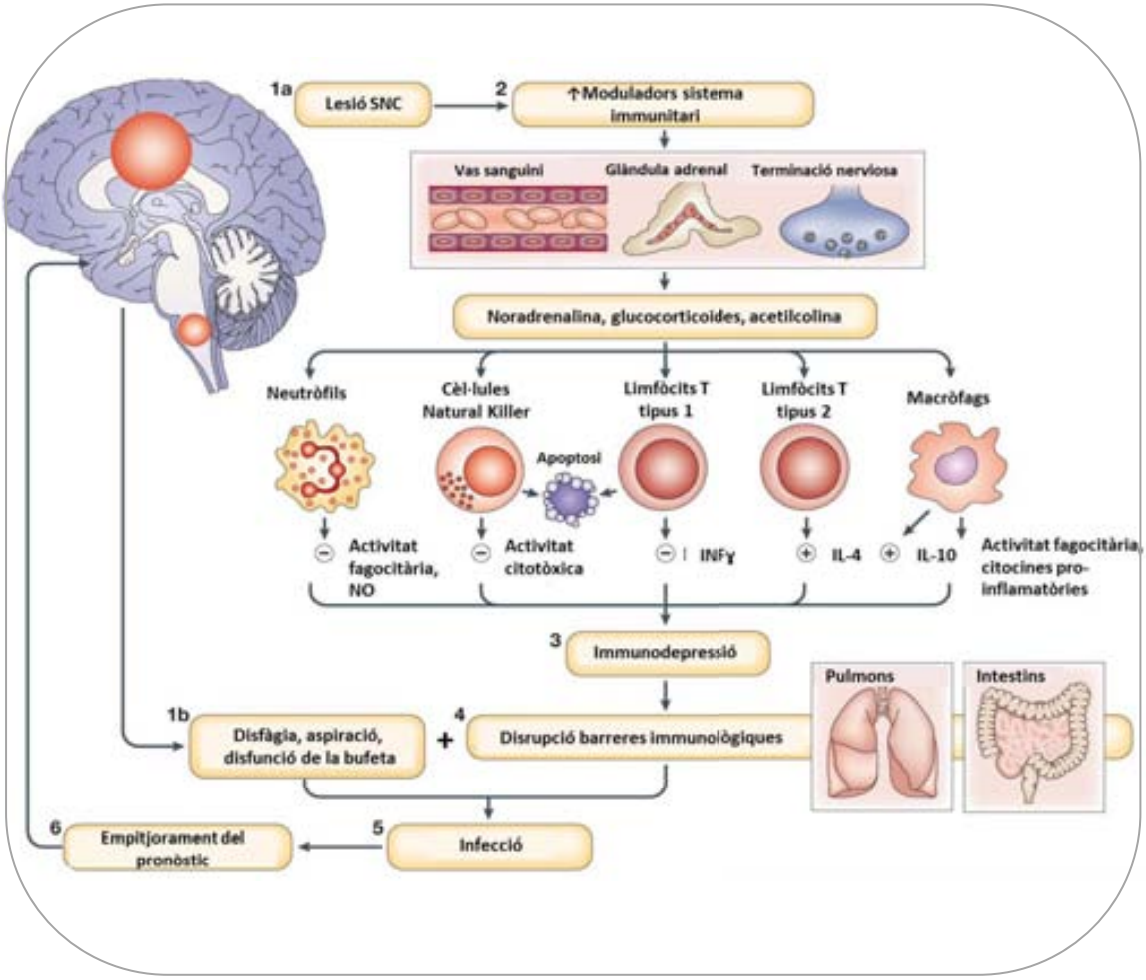

Figura 6. Esquema que resumeix els esdeveniments fisiopatològics que condueixen la lesió del SNC fins a la infecció i l'empitjorament de l'evolució del pacient. Adaptat de Meisel *et al.* 2005.

1.2 Models animals d'isquèmia cerebral

L'ús de models experimentals ha esdevingut imprescindible per a l'estudi de la isquèmia cerebral humana. Degut a que la patologia és molt variada en les seves manifestacions i causes, aquesta diversitat només pot abordar-se des dels models experimentals, on el control d'aquestes variables és possible.

Els models experimentals engloben els models *in vitro* i els models *in vivo.* Pel que fa als models *in vitro*, un dels més rellevants consisteix en sotmetre un tipus cel·lular determinat a una deprivació d'oxigen i glucosa (OGD) (Golden i Choi 1993). Aquests primers models permeten fer estudis de tipus molecular, avaluar vies de senyalització i valorar la resposta d'un tipus cel·lular determinat a un fàrmac. Els models *in vivo*, per altra banda, pretenen reproduir el més estrictament possible els fenòmens biològics implicats en la malaltia que es vol estudiar, permetent estudiar la causa o l'evolució d'una patologia i, sobretot, l'estudi de possibles tractaments dels què podrien beneficiar-se els pacients en un futur. Per qüestions econòmiques, de major acceptació ètica i de facilitat a l'hora d'estabular i manipular, els animals més utilitzats en la investigació de la isquèmia cerebral són els rosegadors, tant les rates com els ratolins.

Tot i l'existència de múltiples models *in vitro,* el fet de recórrer als models *in vivo* per a l'estudi de la isquèmia cerebral humana és essencial ja que aquests models permeten l'estudi histopatològic, bioquímic i fisiològic del teixit cerebral isquèmic, així com l'avaluació del teixit en els primers moments de dany isquèmic o en el moment de la reperfusió. A més, permeten la valoració de l'efecte d' un determinat fàrmac sobre el dèficit neurològic així com sobre la resta de teixits de l'organisme no implicats directament en la isquèmia cerebral.

Els models animals d'isquèmia cerebral usats en els estudis d'aquesta Tesi Doctoral s'engloben dins dels tipus de models anomenats induïts, ja que és el propi investigador qui indueix la isquèmia en animals sans que parteixen d'unes condicions fisiològiques idèntiques entre ells. Aquests models pretenen evitar l'arribada d'oxigen i glucosa al teixit cerebral, intentant reproduir el dany cerebral ocasionat després de l'ictus en humans.

Pel que fa a la classificació dels models animals usats en rosegadors **(Figura 7)**, existeixen dos tipus bàsics: global i focal. La isquèmia global reprodueix la lesió cerebral d'una parada cardíaca i els models d' isquèmia focal, en canvi, es centren en l'oclusió de l'artèria cerebral mitja (MCA). Aquests últims es poden classificar en models transitoris o permanents depenent

de si es permet o no la reperfusió del teixit isquèmic. A més, cada un d'aquests tipus de models es pot realitzar mitjançant vàries tècniques, com s'especifica en la **Figura 4**.

A l'hora d'escollir un model animal experimental, cal tenir molt en compte l'objectiu de l'estudi per tal d'adaptar el model a les variables que més interessin: temps de durada de la isquèmia, sexe, soca i espècie animal, presència de malalties concomitants, i variabilitat i mortalitat associades al model.

Per als nostres estudis, hem utilitzat un model d'isquèmia focal per oclusió de l'artèria cerebral mitja (en anglès, *MCAO*) i s'han usat dues tècniques diferents per a ocloure la MCA: un coàgul sanguini format amb sang d'una rata donant (model embòlic), o un filament de niló amb la punta arrodonida (model del filament). Com es discutirà més endavant, cada un d'aquests dos models comporta una sèrie d'avantatges però també d'inconvenients. El model embòlic, per exemple, simula millor la patologia humana però va lligat a una gran variabilitat pel que fa al volum de l'infart dels animals. El model del filament, per altra banda, és molt més reproduïble però consisteix en la introducció d'un cos estrany a l'artèria de l'animal i, a part, no és útil en cas que es vulguin administrar tractaments trombolítics.

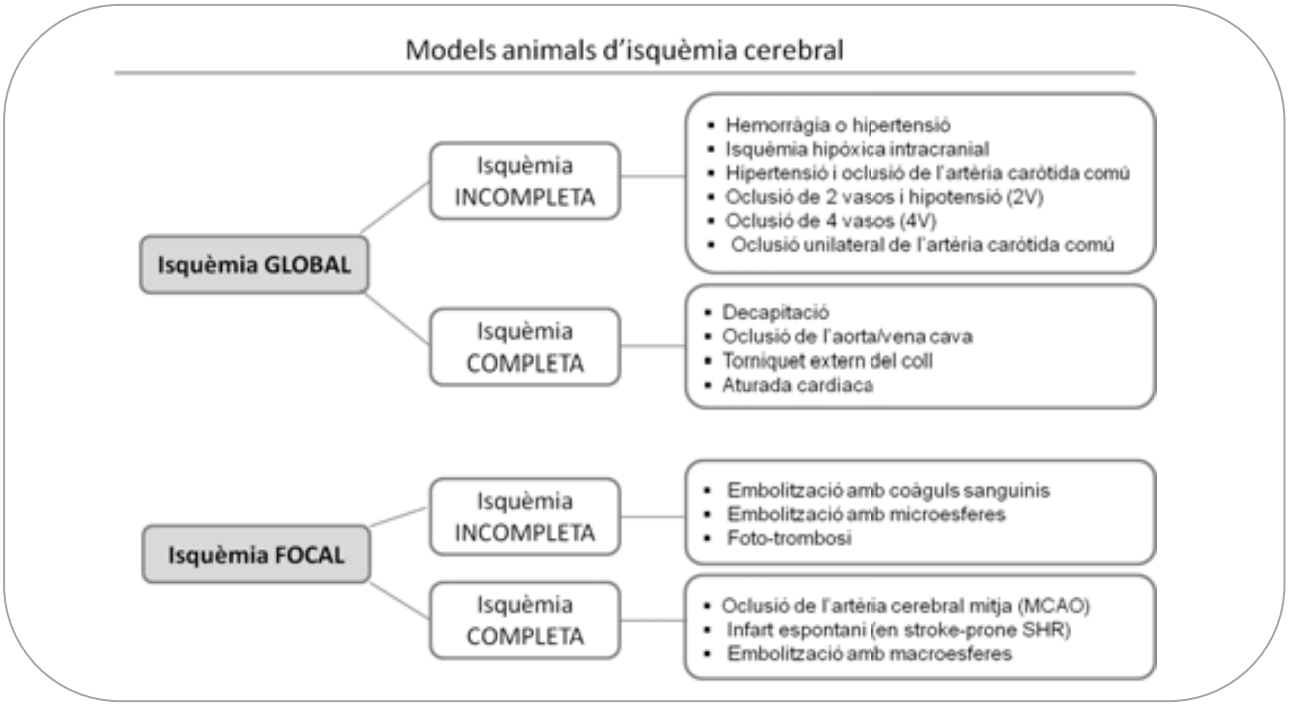

Figura 7. Esquema general dels tipus de models animals existents per a l'estudi de la isquèmia cerebral en rosegadors. Adaptat de Liu i McCullough, 2011.

1.3 Tractaments i estratègies terapèutiques en l' ictus isquèmic

1.3.1 Tractament trombolític amb activador recombinant del plasminogen tissular (rt-PA)

Malgrat tots els esforços realitzats per a trobar un tractament efectiu per administrar durant la fase aguda de la isquèmia cerebral, actualment l'únic fàrmac aprovat per la FDA (*Food and Drug Administration*) continua sent, des de la dècada dels 90, el trombolític rt-PA. En un primer moment, gràcies a l'estudi del *National Institute of Neurological Disorders and Stroke* (NINDS, 1995) es va aprovar el seu ús dins les primeres 3 hores des de l'inici dels símptomes i uns anys més tard, es va ampliar la finestra terapèutica a 4.5 hores gràcies als resultats d'un altre estudi (ECASS III, Bluhmki *et al.* 2009).

Amb l'administració dels fàrmacs trombolítics, que han demostrat ser eficaços administrats tant intravenosament com intraarterialment (Bourekas *et al.* 2004), es pretén dissoldre el coàgul de fibrina. L'rt-PA junt amb el plasminogen endogen, forma un complex que s'uneix a la fibrina i la degrada dissolent l'èmbol i produint una recanalització de l'artèria **(Figures 8 i 9)**. Tot i estar més que demostrada l'eficàcia d'aquest tractament, també se li atribueixen una sèrie d'inconvenients. Per començar, el fàrmac té una finestra terapèutica molt estreta (4.5h), la qual és responsable que menys d'un 10% dels pacients puguin beneficiar-se del tractament, ja sigui pel temps que triguen a arribar a l'hospital o pel temps que es requereix per arribar a un diagnòstic complert (Hacke *et al.* 2008). A més, el temps de vida mitja en humans del rt-PA és únicament de 5-10 minuts degut a que el fàrmac s'uneix ràpidament a l'inhibidor de l'activador del plasminogen (PAI-1) i aquests complexes es degraden ràpidament al fetge.

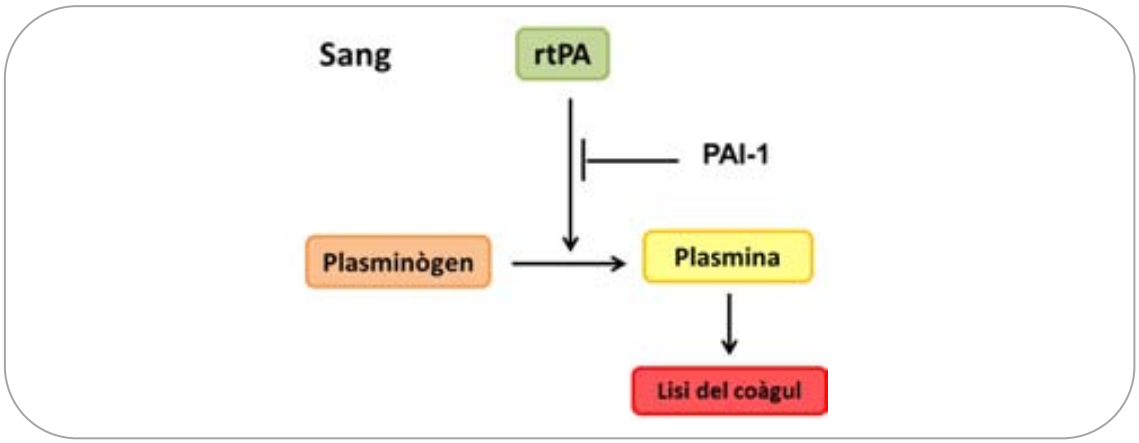

Figura 8. Mecanisme d'acció de l'rt-PA. Adaptat de Eng H Lo 2004.

Per altra banda, a l'rt-PA també se li han atribuït efectes adversos tan greus com són transformacions hemorràgiques (TH) o efectes neurotòxics degut a l'estimulació dels receptors del glutamat (Adibhatla and Hatcher, 2008). L'hemorràgia intracranial és una de les complicacions més temudes després de l'administració del trombolític ja que s'associa amb un increment en la taxa de mortalitat (Del Zoppo *et al*. 2009, Wahlgren *et al*. 2007). Cal diferenciar les TH simptomàtiques, que són aquelles que impliquen directament una clara deterioració clínica del pacient, de les no simptomàtiques (Berger *et al*. 2001). Malgrat les dades sobre la incidència d'aparició de TH varien molt en funció de l'estudi, prendrem coma referència els resultats de Tanne *et al*. (2002),que observaren que tot i tractant els pacients dins la finestra terapèutica establerta, un 6% presentaven hemorràgies simptomàtiques i un altre 7% hemorràgies assimptomàtiques.

És per tot això que avui en dia es continua fent esforços per tal que el tractament en la fase aguda sigui més beneficiós. S'estan cercant diferents estratègies com el disseny de noves mol·lècules, l'administració conjunta del trombolític amb altres fàrmacs neuroprotectors o antioxidants, o la combinació del trombolític amb la tromboectomia intraarterial.

Aquesta última opció és la que s'està duent a la pràctica en l'actualitat als hospitals del nostre país. La trombectomia consisteix en l'extracció o disrupció del coàgul mitjançant dispositius mecànics. Els dispositius capturen o aspiren el coàgul i el treuen de la circulació cerebral, augmentant així la taxa de recanalització i el dèficit neurològic (Lally *et al*. 2013). Actualments'està intentant obtenir dispositius més eficaços en termes de millora funcional ja que no s'ha pogut comprovar que els dispositius actuals aportin majors beneficis que l'administració de l'rt-PA intraarterial (Broderick*et al*. 2013, Molina *et al*. 2013).

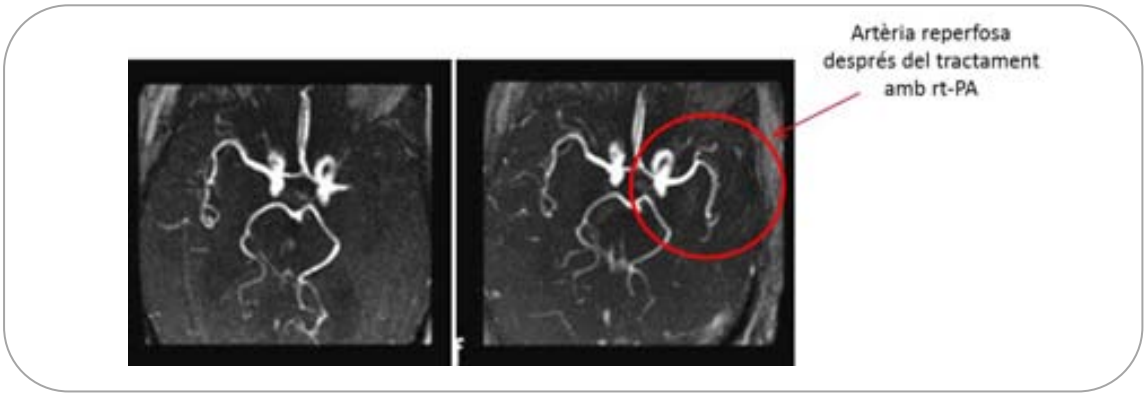

Figura 9. Imatge on es mostra la recanalització d'una artèria després de l'administració d'rt-PA. Fotografia adaptada de Bammer *et al.* 2005.

1.3.2 Neuroprotecció

A part dels tractaments de reperfusió, actualment s'està treballant molt tant en l'estudi de teràpies neuroprotectores com neuroreparadores. Primerament, doncs, caldria diferenciar els termes **neuroprotecció** i **neuroreparació**. Els tractaments **neuroprotectors** tenen com a finalitat interferir en algun punt de la cascada isquèmica, bloquejant molècules clau dels diferents mecanismes de dany cerebral i prevenint la mort cel·lular en la penombra isquèmica. Per tant, queden incloses dins d'aquesta nomenclatura aquelles molècules que inhibeixen processos fisiopatològics com l'entrada de calci a les cèl·lules, l'activació dels radicals lliures o la mort neuronal. Per altra banda, la **neuroreparació** és una altra estratègia terapèutica que consisteix en potenciar la recuperació funcional espontània. Anomenem neuroreparació al conjunt de processos que es produeixen de forma espontània, que es donen més tardanament en la isquèmia cerebral i que, des de les zones del peri-infart, intenten regenerar el teixit danyat i restablir els circuits neuronals per recuperar les funcions sensorials i motores perdudes (Bacigaluppi i Hermann 2008).

Malgrat la recerca és àmplia en ambdues estratègies, nosaltres ens centrarem en els neuroprotectors, concretament en l'administració d'estatines en la fase aguda de la isquèmia cerebral **(Figura 10).**

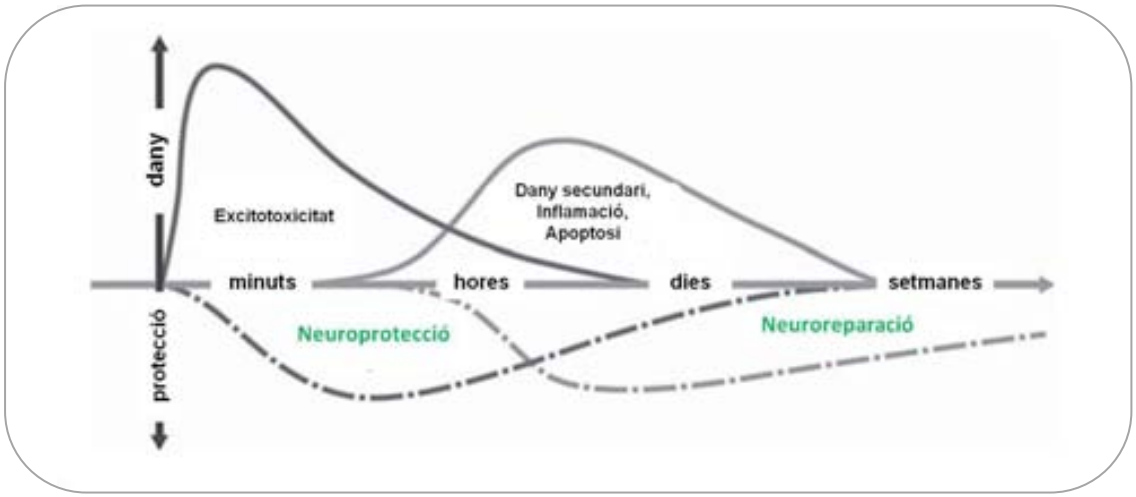

Figura 10. Esquema del balanç entre els processos de dany i reparació que es donen a la zona infartada. Adaptat de Enders *et al.* 2008.

1.3.2.1 Fracassos en la translació de fàrmacs neuroprotectors

Malgrat la gran quantitat de fàrmacs que s'estan estudiant, un dels grans problemes amb què ens trobem avui dia en el camp de la neuroprotecció és que tot i l'elevat nombre de tractaments que han demostrat ser eficaços en models animals d'isquèmia cerebral (reduint considerablement el volum de l'infart i millorant l'evolució funcional), cap d'ells ha demostrat eficàcia en assaigs clínics multicèntrics (O'Collins *et al. 2006)*. En la mateixa direcció, varis autors (Kidwell *et al.* 2001 i Green *et al*. 2003) han reportat que després de testar un gran nombre d'agents que havien mostrattenir efectes sobre els mecanismes d'excitotoxicitat i d'alliberació de radicals lliures en estudis experimentals, cap d'ells ha mostrat eficàcia en cap dels 30 assajos clínics duts a terme. Es creu que hi ha múltiples causes que poden explicar aquests fracassos, entre les quals trobem el fet d'administrar medicaments que actuen sobre mecanismes que s'activen just en el moment de produir-se la isquèmia en pacients que ja tenen ben establerts els danys de la isquèmia (Karatas *et al.* 2009).

1.3.2.2 Criteris STAIR

Un altre dels motius importants que podria explicar aquests fracassos és la baixa qualitat metodològica d'alguns estudis pre-clínics i és per això que el grup de treball STAIR (*Stroke Treatment Academic Industry Roundtable*) va recollir en una guia pràctica les possibles causes i solucions d'aquest problema. A partir de la publicació d'aquesta guia (STAIR 1999), s'han anat fent actualitzacions (Fisher *et al*. 2009) i noves aportacions (RIGOR 2012). Alguns dels punts més destacats a tenir en compte a l'hora de realitzar estudis pre-clínics de fàrmacs neuroprotectors són la bona elecció del model i la soca animal així com considerar les diferències que poden existir entre sexes i la necessitat d'incloure animals amb comorbiditats (hipertensió, diabetis). A més, aquestes guies insisteixen molt en la importància de la transparència dels estudis. És a dir, que els estudis experimentals han de ser randomitzats i cecs, s'han de definir bé els criteris d'inclusió i exclusió dels animals, mostrar els càlculs de la mida de la mostra i la potència dels resultats així com revelar possibles conflictes d'interès. També remarquen la necessitat de demostrar l'eficàcia del fàrmac almenys, en dos laboratoris diferents i la importància de publicar tots els resultats, ja siguin positius o negatius.

Podríem dir, doncs, que és imprescindible treballar de manera rigorosa i tenint en compte aquests punts si el que pretenem és fer una recerca translacional que arribi a ser beneficiosa algun dia per als pacients d'ictus.

1.3.1.3 Noves estratègies i fàrmacs neuroprotectors

En l'actualitat hi ha un gran nombre de fàrmacs que estan essent objecte d'investigació (Glutamat oxaloacetat transaminasa, Prostaglandin E1, receptor Sigma-1, Fingolimod, Agonistes de receptors d'opioides, Cinnamophilin, Dichlorobenzamil, Sulfat magnesi, Àcid Arúndic, Antioxidants..) (Broussalis 2012). Alguns d'aquests fàrmacs pretenen ampliar el temps en què la penombra de l'infart és viable mentre que altres pretenen reduir el dany per reperfusió o millorar els efectes de reparació endògens. En tot cas, es creu que la combinació de la reperfusió seguida de l'administració d'un fàrmac neuroprotector podria ser una bona opció per inhibir el dany ocasionat per la reperfusió i protegir el teixit en risc (Broussalis 2012).

L'administració de cèl·lules mare també és considerada una tècnica prometedora ja que en models animals s'ha pogut comprovar que són capaces de migrar cap a l'àrea lesionada i exercir funcions tan interessants com la secreció de factors neurotròfics, estimular la revascularització, incrementar la plasticitat i regular la resposta inflamatòria (Kaneko *et al*. 2011).

Finalment, una altra estratègia que està mostrant resultats molt interessants és la hipotèrmia. Actualment s'està duent a terme un estudi a nivell internacional (Euro-hyp-1) (van der Worp HB*et al.* 2014) en el què els pacients se sotmeten, dins les 6 hores primeres des de l'inici dels símptomes i durant 24 hores a temperatures entre 34-35ºC mitjançant administració intravenosa de sèrum salí refrigerat o ús de superfícies hipotèrmiques. Amb l'aplicació d'aquesta tècnica es pretén actuar simultàniament sobre múltiples mecanismes de mort cel·lular tals com la depleció energètica, disrrupció de la BHE, formació de radicals liures, excitotoxicitat o inflamació, contràriament a altres fàrmacs estudiats que només actuaven sobre alguna d'aquestes fases. L'estudi està actualment en procés i el protocol ben establert (http://www.eurohyp1.eu.).

1.4 Estatines

La família de les estatines engloba una gran varietat de fàrmacs que poden classificar-se en: naturals (Lovastatina i Paravastatina) i sintètiques (Atorvastatina , Rosuvastatina, Fluvastatina, Pitavastatina i Simvastatina) o també en: lipofíliques, que poden travessar la BHE (Lovastatina, Pitavastatina, Atorvastatina, Fluvastatina i Simvastatina), o hidrofíliques (Paravastatina, i Rosuvastatina) (Vaughan 2004). En aquesta tesi ens centrarem en la simvastatina **(Figura 11)**, estatina lipofílica obtinguda sintèticament a partir d'un producte de fermentació de l'*Aspergillus terrus* (Alberts 1990).

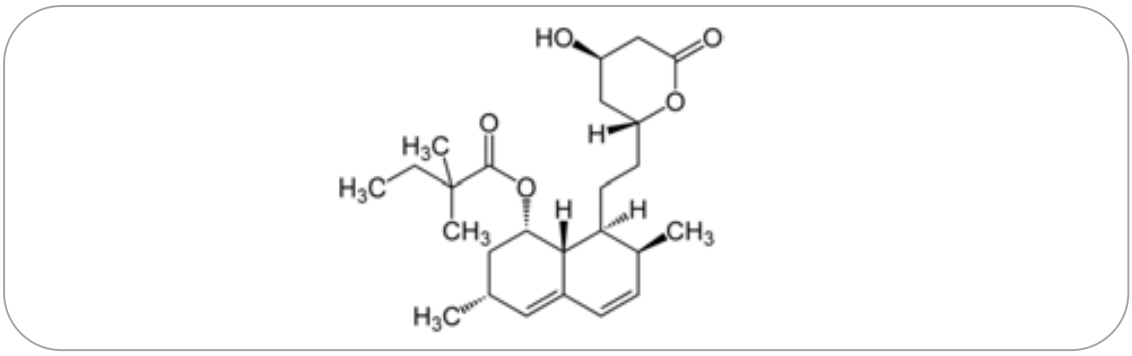

Figura 11. Estructura química de la simvastatina

Des del punt de vista farmacològic, les estatines s'enquadren dins els inhibidors de l'hidroximetil-glutaril-coenzim-A reductasa (HMG-CoA). Aquest enzim catalitza la conversió d'HMG-CoA a mevalonat, que és un metabòlit clau en la biosíntesi del colesterol i està descrit que aquest bloqueig es produeix gràcies a la gran semblança estructural entre aquests fàrmacs i l'HMG-CoA (Endo *et al.* 1976). Malgrat les estatines continuen essent usades en clínica bàsicament per a disminuir el colesterol en pacients hipercolesterolèmics, ja fa anys que s'ha vist que podrien tenir altres aplicacions clíniques com prevenció de malalties cardiovasculars (Nakamura *et al.* 2006), prevenció secundària de l'ictus (Elissa *et al.* 2014), possible eficàcia en esclerosi múltiple (Ciurleo *et al.* 2014) traumatisme cerebral (Jansen *et al*. 2013), disminució de la incidència d'Alzheimer (Hoglund i Blennow 2007; Li *et al.* 2007) i Parkinson (Huang *et al*. 2007; Wahner *et al.* 2008). Aquesta gran aplicabilitat és deguda a que a partir de la inhibició de la HMG-CoA, es produeixen 2 tipus d'efectes: els derivats de la interacció en el metabolisme del colesterol i els efectes pleiotròpics (independents del metabolisme del colesterol) (**Figura 12)**. Alguns d'aquests efectes s'anumeren a continuació:

1. Efectes derivats de la interacció sobre el metabolisme del colesterol

- Disminució dels nivells de colesterol total i LDL (Opie *et al.* 2005)
- Disminució de la densitat de partícules LDL (Packard*et al*. 2000)
- Disminució dels nivells d' apolipoproteïna B (Robinson *et al.* 2012)
- Augment moderat de cHDL i reducció dels triglicèrids plasmàtics (Xu *et al*. 2014)

2. Efectes pleiotròpics: beneficis addicionals no atribuïbles a la reducció de cLDL

- Reducció de la prenilació de les proteïnes G (Rho, Rac, Racl, Rab i Ras), implicades en la regulació de la pressió arterial i la contracció de la musculatura vascular (Liao 2002).
- Efectes sobre la funció endotelial mitjançant l'augment de la biodisponibilitat de l'NO, molècula responsable de la inhibició de mecanismes proinflamatoris i dels efectes antioxidants sobre les lipoproteïnes (Laufs *et al.* 1998). També inhibeixen l'expressió d'endotl·lina-1, un potent vasoconstrictor (Ohkita *et al.* 2006).
- Propietats antitrombòtiques a través de la disminució de l'agregació plaquetària (Laufs *et al.* 2000).
- Estabilització de la placa d'ateroma mitjançant la reducció del tromboxà A2 per part de les plaquetes i la disminució de la infiltració de cèl·lules inflamatòries. A més, incrementen la síntesi de col·lagen (Noyes and Thompson 2014).
- Acció antiinflamatòria. Inhibició de vàries citoquines proinflamatòries i també de la CRP (Proteïna C reactiva), considerada un marcador d'inflamació així com un factor pronòstic de risc de malaltia cardiovascular (Min *et al.* 2014).
- Propietats immunomoduladores: inhibeixen l'activació de monòcits i limfòcits T, així com l'expressió de mediadors com l'INFγ (Ciurleo *et al*. 2014).

- Propietats antioxidants. La pròpia acció hipolipomiant redueix l'estrès oxidatiu però a més, les estatines tenen mecanismes antioxidants propis que inhibeixen la producció del radical anió superòxid i disminueixen la producció de radicals lliures (Moon *et al*. 2014).
- Propietats de protecció vascular mitjançant l'expressió de l'NO, la inhibició de la CRP i la supressió de l'activació de MMPs (relacionades en la isquèmia cerebral amb la degradació de la làmina basal, la resposta neuroinflamatòria, la mort cel·lular, l'edema vasogènic i les complicacions hemorràgiques) (Kurzepa *et al*. 2014).
- Promoció de l'angiogènesi mitjançant un increment en la circulació de cèl·lules endotelials progenitores (EPC's) (Martí-Fàbregas *et al*. 2013).

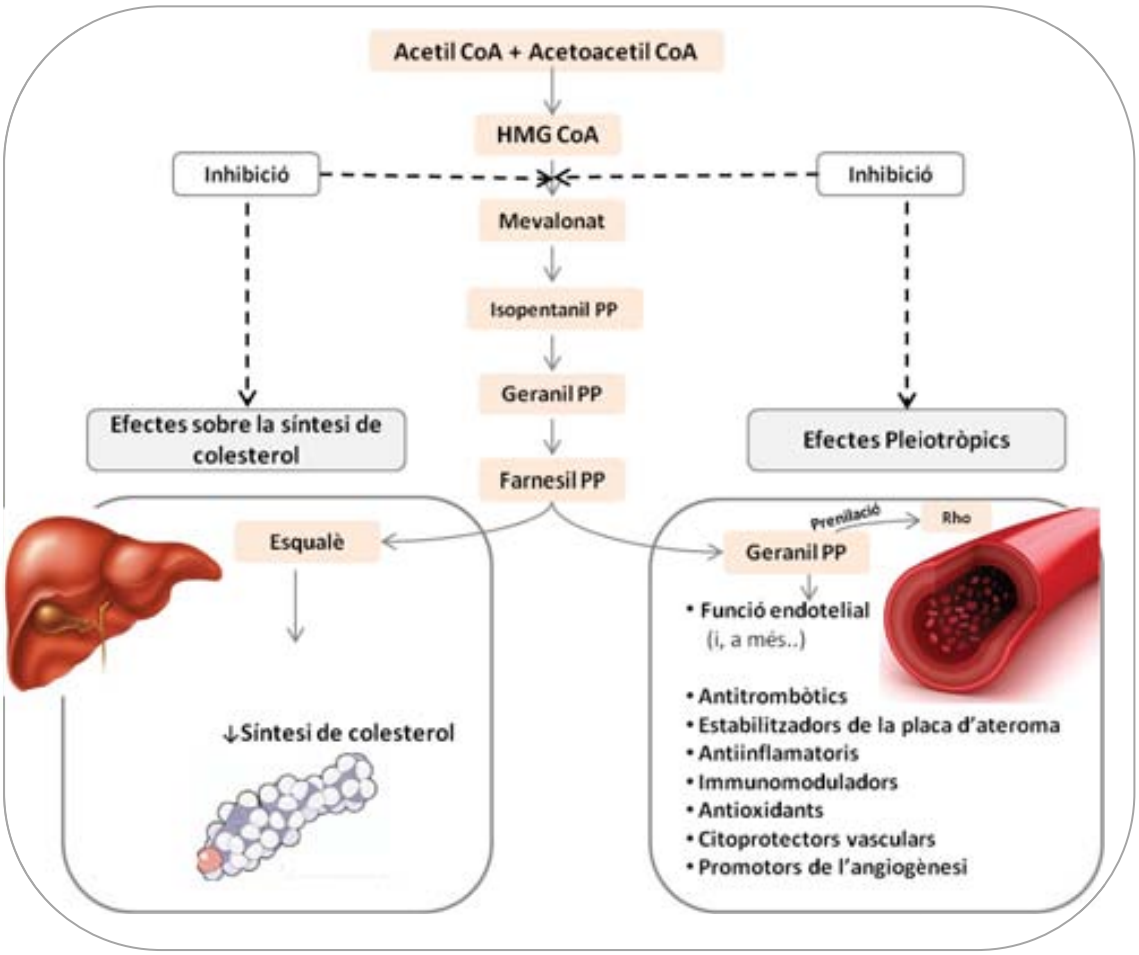

Figura 12. Mecanisme d'acció de les estatines. Adaptat de Kausik *et al.* 2005

1.4.1 Estatines en malalties neurovasculars

Veient totes les propietats que tenen, no és d'estranyar que les estatines s'hagin estudiat tan extensament en el camp de la isquèmia cerebral, on s'ha demostrat que poden ser útils per vàries d'aquestes accions. Es creu que el major benefici de les estatines en la isquèmia cerebral vindria donat per l'increment de la disponibilitat d'NO, molècula capaç de regular la perfusió cerebral i de millorar la funció endotelial (Atochin i Huang 2011). Però també s'ha demostrat que els pacients d'ictus podrien beneficiar-se d'altres propietats de les estatines tals com: efectes antioxidants, inhibició de respostes inflamatòries, efectes immunomoduladors, regulació de les EPCs i establització de les plaques d'ateroma.

Cal destacar que diversos estudis observacionals han pogut demostrar que els pacients que prenien estatines abans de patir un ictus tenien una millor evolució neurològica durant la fase

aguda del mateix (Jonsson and Asplund 2001, Martí-Fàbregas 2004). Aquestes dades van desembocar, fa uns anys, en el disseny i posada a punt per part de la Unitat Neurovascular de l'Hospital de la Vall d' Hebron d'un assaig clínic pilot utilitzant simvastatina com a tractament de fase aguda de l'ictus(estudi MISTICS). Aquest estudi pilot va ser el primer en administrar la simvastatina en la fase aguda (40 mg/Kg durant la primera setmana i 20 mg/kg fins als 90 dies) i va demostrar un sorprenent benefici clínic sobre els pacients, amb millores significatives a favor de la simvastatina ja el tercer dia des de l'inici del tractament respecte dels pacients que rebien placebo (46.4% vs 17.9%, p=0.022) (Montaner 2007). El benefici produït per les estatines es va poder observar també en la puntuació de la NIHSS, on,en el grup que rebia simvastatina, una major proporció de pacients presentavauna millorianeurològica el dia 90 (descens de més de 8 punts en la NIHSS o recuperació total: 15 vs 4 pacients, p=0.011). Només va sorprendre que en el grup de simvastatina existia també una tendència a presentar una major proporció d'infeccions (OR= 2.4, 1.06-5.4). A partir d'aquests resultats, i per tal de poder-los generalitzar, es decidí que s'havien de reproduir en una altra cohort de pacients i també que s'havia de valorar la interacció i possibles efectes sinèrgics de la simvastatina amb l'únic tractament en fase aguda de l'ictus (rt-PA).

El primer punt, (la replicació en una altra cohort) es decidí abordar-lo amb un segon assaig clínic anomenat STARS, actualment en procés: http://clinicaltrials.gov/ct2/show/NCT01073007. L'STARS és un assaig multicèntric, aleatoritzat i doble cec que pretén avaluar l'eficàcia del tractament amb simvastatina (40 mg/Kg, iniciat dins les primeres 12 hores i durant 3 mesos) sobre l'evolució neurològica dels pacients (als 7 dies i als 3 mesos des de l'inici dels símptomes).

El segon punt (possibles efectes sinèrgics dela simvastatina amb l'rt-PA) s'abordà amb l'ús d'un model d'isquèmia cerebral en rates (objectiu de la present tesi).

1.4.2 Estatines en isquèmia cerebral experimental

A nivell experimental, i tal com es desenvoluparà posteriorment a l'apartat de resultats del metanàlisi (Article 1), existeix un gran nombre de publicacions on s'avaluaven les principals estatines (majoritàriament Simvastatina, Atorvastatina i Rosuvastatina). Gran part d'aquests treballs, cal destacar, fan referència a un tractament pre-isquèmia que implica, lògicament, l'obtenció de millors resultats pel que fa a l'eficàcia si es compara amb l'administració posterior a la isquèmia (Danton i Dietrich 2004). Tot i així, considerem més interessants els treballs que avaluen l'efecte de les estatines administrades posteriorment a la isquèmia,

sobretot si tenim en compte que és prioritari i necessari trobar un neuroprotector que resulti eficaç administrat el més aviat possible després de produir-se l'oclusió del vas. A més, varis estudis clínics ja han pogut demostrar que el fet d'estar en tractament amb estatines redueix el risc de patir un ictus (Prinz i Endres 2011, Amarenco i Labreuche 2009) i millora l'estat funcional en cas de patir-lo (Biffi *et al.* 2011).

Per altra banda, les publicacions en models animals han permès demostrar alguns mecanismes d'acció i entendre quina influència poden tenir les estatines en la isquèmia cerebral. Per exemple, Endres *et al. (*2004) i Asahi *et al;* (2005) demostraren que els beneficis obtinguts amb el tractament a base d'estatines eren deguts a la seva capacitat per incrementar els nivells d'òxid nítric sintasa endotelial (eNOS) i d'rtPA endogen. Hong *et al*. (2006) i Cui *et al*. (2009) reportaren que la neuroprotecció exercida per les estatines estaria relacionada amb els efectes anti-oxidants. I Sironi *et al.* (2006) que les estatines actuaven sobre els mecanismes d'inflamació i de resposta immunitària.

Malgrat la majoria de treballs s'hagin realitzat en rosegadors o en conills, és sorprenent la variabilitat que hi ha entre ells pel que fa als models animals escollits per induir la isquèmia cerebral (model del filament o embòlic, model transitori o permanent), així com l'ampli rang de dosis utilitzat, la durada del tractament o les diferents vies d'administració avaluades. En cap de tots aquests treballs, per això, s'ha estudiat l'eficàcia de la simvastatina administrada únicament durant la fase aguda de la isquèmia i conjuntament amb l'rt-PA (únic tractament aprovat en l'actualitat) tot usant el model més semblant a la clínica humana (MCAO embòlic).

1.5 Noves estratègies per a facilitar el pas de medicaments a través de la barrera hemato-encefàlica

A l'hora de buscar tractaments per a la isquèmia cerebral cal tenir molt en compte l'existència de la BHE que, a part d'actuar com a barrera immunològica i metabòlica per a protegir el teixit cerebral, impedeix el pas de certes substàncies a l'interstici cerebral.
La BHE és una barrera de transport estructural i bioquímica, altament especialitzada. Consta de vasos formats per cèl·lules endotelials, connectades per unions estretes i rodejades pels peus dels astròcits **(Figura 13).**

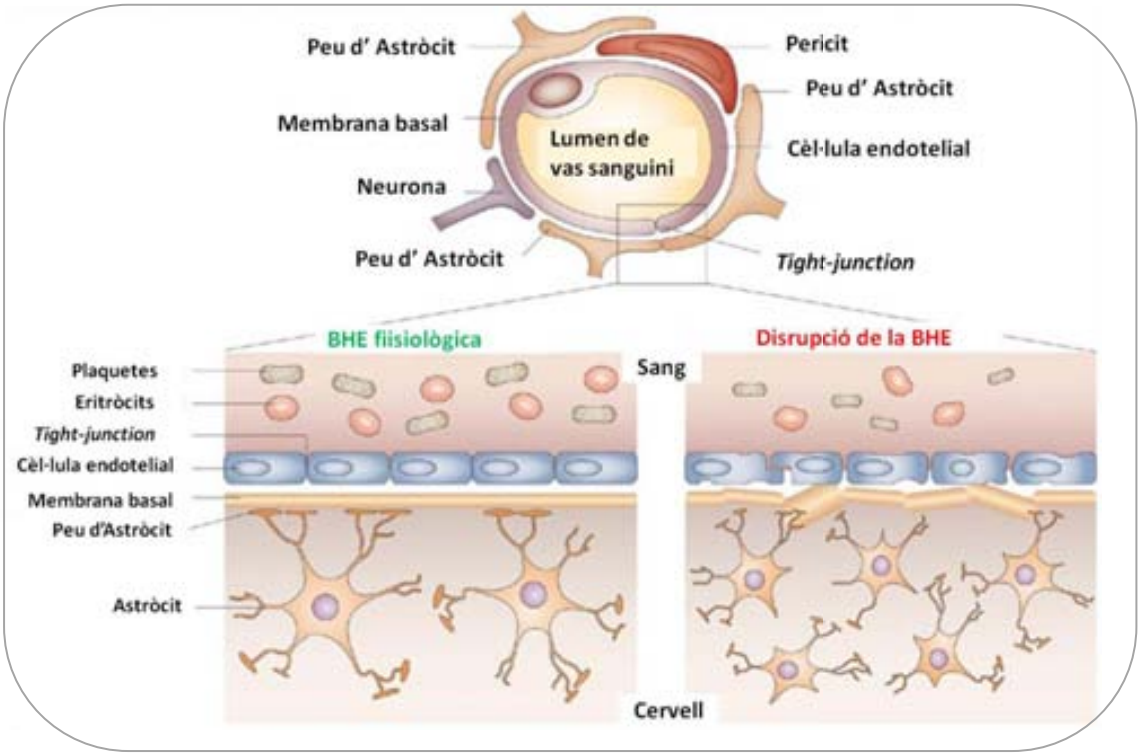

Figura 13. Estructura de la BHE, en condicions fisiològiques i després de patir una disrupció. Adaptat de Gerstner 2009.

De fet, a part de la BHE (que es troba a l'interfase entre l'encèfal i els vasos sanguinis) hi ha dues barreres més que limiten el transport de medicaments al parènquima cerebral: la barrera sang-líquid cefaloraquidi (localitzada al plexe coroideu) i la barrera sang-aracnoides (que és la capa aracnoides de les meninges) (Taies *et al*. 2014).

Tot això fa que molts fàrmacs que són útils per al tractament de trastorns sistèmics, resultin ineficaços a l'hora de tractar patologies del SNC. Els neuropèptids, les proteïnes i els antineoplàsics són exemples d'agents que tenen dificultats per a traspassar la BHE. De fet, està descrit que l'administració d'agents terapèutics al SNC no resulta eficaç per al 98% de molècules de mida petita i per al 100% de mida més gran (Pardridge 2005).

Tot i que algunes molècules petites, els pèptids i certes proteïnes administrades sistèmicament aconsegueixen arribar al parènquima cerebral tot creuant la BHE (Banks 2008), calen dosis elevadíssimes per tal d'aconseguir els nivells terapèutics al SNC i això pot comportar molts efectes adversos a la resta de l'organisme.

Com s'ha explicat en punts anteriors, cal destacar que la isquèmia cerebral ocasiona canvis en la integritat de la BHE que varien en funció del mecanisme, la durada i la gravetat de l'episodi isquèmic. Durant la fase aguda de l'accident cerebrovascular, la BHE experimenta un canvi ràpid durat les primeres 3 hores, el màxim de permeabilitat se situa cap a les 48 hores i es creu

que disminueix a partir del quart dia (Taheri *et al.* 2009). En tot cas, els canvis en la permeabilitat de la BHE poden representar una oportunitat per a l'administració de fàrmacs al SNC ja que està descrit que podria facilitar el pas de partícules de gran mida (~150kD), fet impensable en condicions no patològiques (Zehender *et al.* 2011, Brouns i De Deyn 2009).

Un dels principals reptes en el tractament de les malalties del SNC és, doncs, el desenvolupament de mètodes eficaços que permetin la difusió de medicaments a través de la BHE. És per això que s'han investigat una àmplia varietat d'estratègies per assolir aquest objectiu **(Figura 14).**

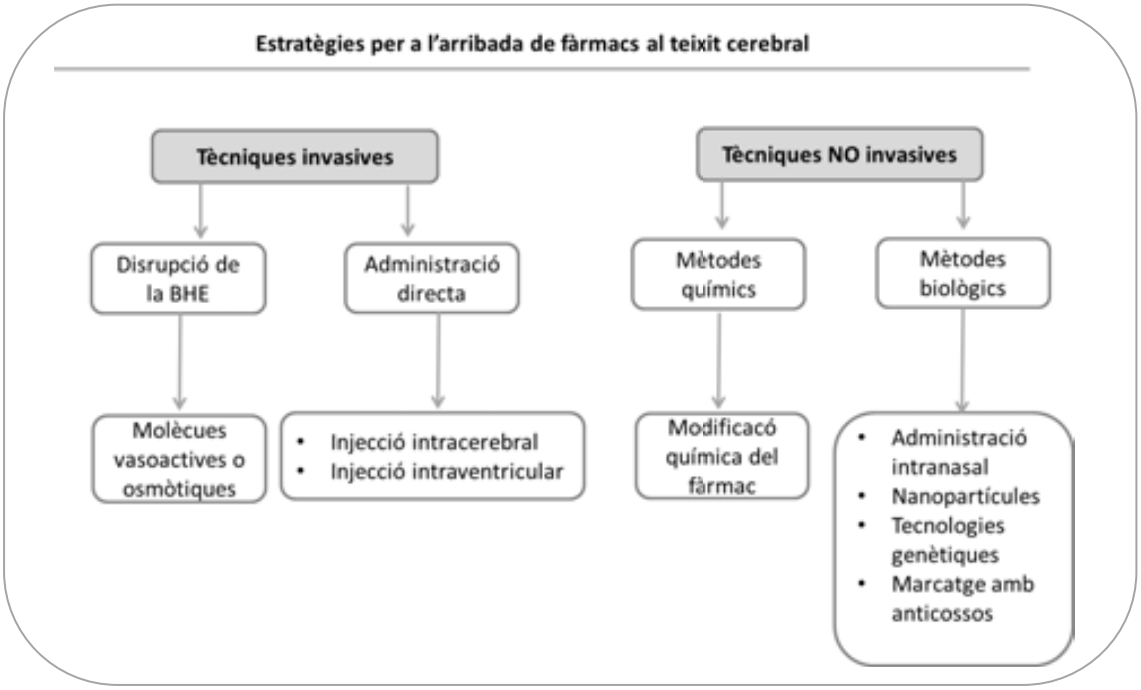

Figura 14. Representació esquemàtica de les diferents estratègies existents per a l'administració de fàrmacs al cervell. Adaptat de Loch-Neckel i Koepp 2010.

L' obertura de la BHE mitjançant l'administració de **solucions hiperosmòtiques** o **molècules vasoactives** ha demostrat ser un mètode poc específic per a l'alliberació de fàrmacs ja que provoca un augment en cervell de molècules potencialment tòxiques (Joshi *et al.* 2007).

Una altra possibilitat és l'**administració** dels agents terapèutics directament **als ventricles cerebrals** o mitjançant **injeccions intraparenquimatoses,** però si es requereixen dosis múltiples, ambdues tècniques resulten molt invasives, d'elevat risc i també molt cares, pel fet de requerir processos quirúrgics complexes.

Per altra banda, també s'ha plantejat l'ús d'**anticossos monoclonals**que reconeixin receptors específics en les cèl·lules endotelials proporcionant una vectorització eficaç per a l'alliberació

dels medicaments. Desafortunadament, per això, el medicament que s'ha d'alliberar ha de reconèixer un mecanisme de transport específic de la BHE i tenir una alta activitat intrínseca per al receptor i a més, és difícil trobar antígens específics (Groothuis 2000).
Les **tecnologies genètiques** també han permès avanços importants mitjançant l'ús de pèptids endògens, proteïnes modificades o anticossos monoclonals vinculats als transports endògens de la BHE (Pardridge 2007).

La **modificació química dels fàrmacs** és una altra posssible solució per a facilitar el seu pas a través de la BHE. Aquest mètode sol aplicar-se per a substituir les propietats físico-químiques del compost com ara incrementar la seva lipofilicitat o minimitzar el potencial d'unió al grup hidrògen. Això permet que mitjançant tècniques d'esterificació o de midonització, per exemple, els medicaments que contenen grups hidroxil o aminoàcids, puguin incrementar la seva solubilitat i, per tant, facilitar la seva entrada al SNC.

Es creu que hi ha vàries vies implicades en l'arribada dels compostos al SNC **administrades intranasalment:** nervis (Trigènim i Olfactori), vascultura, líquid cefalo-raquidi (LCR) i sistema limfàtic i que predomina una o bé una altra depenent de les característiques del compost administrat (osmoloaritat, PH) (Dhuria *et al.* 2010), i, fins i tot d'algunes particularitats a l'hora de realitzar l'administració (volum administrat o posició del cap). Malgrat tot, l'arribada al SNC de partícules administrades per via intranasal està condicionada a la seva mida i lipofilicitat (més difícil com més grans i més hidrofíliques siguin les partícules) (Gizurarson *et al.* 1996 i Dufes *et al.* 2003). És per això que els estudis reporten que només un baix nombre de fàrmacs s'han pogut detectar al SNC després de l'administració intranasal i tot i així, a baixes concentracions (tant a LCR com en regions del parènquima cerebral) (Illum 2000).

A continuació, exposem amb més detall el mètode escollit en els nostres estudis per tal de facilitar l'arribada de simvastatina a la zona isquèmica del cervell.

1.5.1 Administració del fàrmac en nanopartícules

El terme nanopartícules engloba tant les nanoesferes com les nanocàpsules. Aquestes estructures es poden obtenir a partir de polímers o lípids i estan caracteritzades per tenir una mida que varia de 10-1000 nm. Una gran avantatge és que les nanopartícules permeten incorporar quantitats relativament grans de medicaments, compostos o proteïnes. D'aquesta manera, l'encapsulació de fàrmacs en nanopartícules pot millorar la seva biodisponibilitat,

augmentar la difusió a través de membranes biològiques o protegir-los contra la inactivació enzimàtica (Loch-Neckel *et al.* 2007).

A més, el fet que la superfície de les nanopartícules es pogui modificar, així com unir-se a grups funcionals, facilita el seu pas a través de la BHE, i, per tant, l'arribada dels medicaments a les cèl·lules i teixits del SNC. L' ús de nanopartícules afavoreix, per tant, l' accés de medicaments que normalment no penetren en la BHE, ja que l'encapsulació del fàrmac en aquests sistemes permet l'emmascarament de les seves característiques fisicoquímiques (Hanson i Frey 2008), així com una major arribada dels medicaments que, per les seves característiques, ja serien capaços de penetrar la BHE.

En el cas dels liposomes (englobats en la categoría de nanocàpsules), cal dir que són vesícules esfèriques compostes per una bicapa a base de fosfolípids anfifílics i colesterol i un interior aquós. Aquestes estructures permeten l'encapsulació de medicaments tant a l'interior de la bicapa com a la seva interfase, depenent de les característiques del compost a encapsular. Està descrit que per tal d'alliberar els fàrmacs, aquests sistemes s'uneixen o interaccionen amb la membrana de la cèl·lula diana (Guzmán *et al.* 1996) i també que són capaços d'alliberar de manera perllongada compostos que són poc solubles en aigua (Bawarski *et al.* 2008).

Actualment l'encapsulació de medicaments en nanopartícules està essent àmpliament avaluada en la patologia del càncer. En aquest camp es considera que l'encapsulació podria disminuir significativament els efectes adversos dels anticancerígens facilitant l'alliberació del fàrmac únicament a la zona del tumor en comptes de fer-ho de manera sistèmica (Matsmura 2014). El clar avenç d'aquestes tècniques terapèutiques en certs tipus de càncers ha permès que a dia d'avui hi hagi varis estudis clínics finalitzats mostrant resultats molt favorables (Plummer et al. 2011, Su et al. 2012).

Pel que fa al camp de la investigació de l' ictus, la nanoneurociència (Nair *et al*. 2012) es limita, de moment, als estudis experimentals. Karatas *et al.* (2009) van demostrar, per exemple, que un inhibidor de caspasa-3 (proteÏna pro-apoptòtica) que per les seves característiques no era capaç de travessar la BHE per sí sol, encapsulat en liposomes recoberts amb polietilenglicol induïa una reducció del volum d'infart, una milloria del dèficit neurològic i una disminució de l'activitat de caspasa-3 en ratolins sotmesos a un model de MCAO. Per altra banda,Reddy i Labhasetwar (2009) avaluaren l'eficàcia de l'encapsulació de superòxid dismutasa (SOD) (potent neutralitzant de radicals lliures). L'encapsulació de SOD en nanopartícules permeté salvar les dificultats del compost (curt temps de vida mitja i incapacitat de creuar la BHE) i la

seva administració conduí a una major supervivència i milloria neurològica en les rates sotmeses a un model de MCAO.

Malgrat es considera que el camp de la nanotecnologia té grans perspectives de futur en les malalties neurològiques, tant pel que fa al diagnòstic com per al seu tractament (Nair *et al*. 2012), encara hi ha molts aspectes a resoldre abans no puguin usar-se en clínica humana. Ens referim a l'estudi de les característiques físiques, químiques i biològiques d'aquestes nanopartícules, així com a les característiques de la seva administració (dosi, via, edat del pacient), tots ells factors decisius a l'hora de determinar l'absorció, distribució pels diferents teixits, metabolisme o toxicitat dels compostos encapsulats (Lanone i Boczkowski 2006, Zhao i Castranova 2011). Amb tot això, doncs, es considera imprescindible resoldre totes aquestes incògnites així com fer estudis de toxicitat, a curt i a llarg plaç, abans de ser un mètode aprovat per a ús clínic.

2. OBJECTIUS

2.1 OBJECTIU GENERAL

Avaluar l'efecte neuroprotector de l'administració d'estatines (simvastatina) durant la fase aguda de la isquèmia cerebral en un model experimental en rata, profunditzant en els aspectes relacionats amb l'optimització tant de la seva eficàcia com de la seva seguretat.

2.2 OBJECTIUS ESPECÍFICS

1. Realitzar una revisió sistemàtica i posterior anàlisi estadística (metanàlisi) sobre l'efecte neuroprotector de les estatines en models animals d'isquèmia cerebral.
2. Comprovar en el nostre laboratori, l'eficàcia de la simvastatina administrada en la fase aguda de la isquèmia cerebral utilitzant un model embòlic en rata.
3. Estudiar mitjançant anàlisi proteòmica els mecanismes pels quals la simvastatina podria exercir un efecte neuroprotector en administrar-se durant la fase aguda de la isquèmia cerebral.
4. Comprovar la seguretat, en termes d'incidència de transformacions hemorràgiques, de la simvastatina en administrar-se en combinació amb l'rt-PA, en un model embòlic en rates hipertenses.
5. Posar a punt un model experimental d'isquèmia cerebral en rata per tal d'avaluar l'efecte de l'administració de simvastatina sobre l'aparició d'infeccions respiratòries secundàries.
6. Explorar nous mètodes d'administració de simvastatina (encapsulació en liposomes) per tal d'optimitzar la seva arribada al teixit cerebral lesionat i potenciar, així, el seu efecte neuroprotector .

3. MATERIALS I MÈTODES

3.1 Característiques dels animals utilitzats

Tots els processos que han implicat l'ús d'animals al llarg d'aquesta tesi han sigut aprovats pel Comitè Ètic d' Experimentació Animal (CEAA) de l'Institut de recerca de la Vall d' Hebron amb els números de Registre següents: 02/09, 16/12, 01/13 i 58/13.

Primerament cal remarcar que tots els estudis inclosos en aquesta tesi s'han dut a terme amb rates. El fet de treballar amb rosegadors comporta una sèrie d'avantatges tals com una major acceptació ètica, facilitat per a estabular i manipular i relativament baix cost econòmic. Tenint en compte la malaltia en estudi, un altre punt fort dels rosegadors és que tenen una anatomia cerebro-vascular força semblant als humans ja que també tenen polígon de willis. El fet de triar la rata envers el ratolí rau en la mida de l'animal. Considerant el model animal proposat, les petites dimensions del ratolí haurien dificultat encara més les cirurgies per a la inducció de la isquèmia cerebral.

Un altre punt a destacar és que totes les rates usades són de sexe masculí. Degut a que el nivell d'estròg ens està essent objecte d'estudi com a factor protector per a la incidència o l'evolució de la isquèmia cerebral, l'ús de femelles hauria suposat l'adició d'una nova variable.

En tots els estudis, s' inclogueren animals d'entre 250-310 grams de pes de dues soques diferents:

- Wistar (control normotenses), de la casa comercial Charles River

- SHR (de l'anglès *Spontaneous Hypertensive Rats*), dels laboratoris Harlan. Aquesta soca es va desenvolupar fa més de 30 anys a partir de rates Wistar Kyoto. Es comprovà que sotmetent aquests animals a estrès crònic, desenvolupaven hipertensió espontàniament entre les 7 i les 15 setmanes d'edat. Així doncs, aquestes rates presenten un increment en la resistència vascular perifèrica que provoca una alteració en la regulació central de la pressió sanguínia. Tenint en compte que la hipertensió és un dels factors de risc per a l'aparició de TH després de l'ictus, vam creure interessant usar una soca on l'aparició d'hemorràgies fos freqüent i ens permetés veure l'efecte de l'administració dels diferents tractaments sobre la incidència d'aquesta important complicació secundària.

3.2 **Tractaments administrats**

3.2.1 Simvastatina/ Vehicle Simvastatina

En tots els estudis, s'administrà una única dosi de simvastatina (per via subcutània, intravenosa o encapsulada en liposomes) 15 minuts després de l'oclusió.

Vehicle Simvastatina: Cada mL de vehicle es va composar d'H_2O destil·lada (75%), etanol absolut (10%) i NaOH 0.1 M (15%).

Simvastatina (Grupo Uriach)**:** La dosi utilitzada va ser de 20 mg/Kg i, tenint en compte que el pes dels animals (~300 g), cada animal va rebre 6 mg de simvastatina dissolts en 1 mL de vehicle.

Per tal de preparar el medicament a administrar, la simvastatina s'afegí al volum de vehicle adequat. La simvastatina és un compost difícil de dissoldre en aigua, és per això que és imprescindible la presència d'etanol en el vehicle. Per tal de millorar encara més la dissolució del fàrmac, la barreja de vehicle i simvastatina s'escalfà al bany a 50ºC durant 2 hores. Una vegada aconseguida la dissolució, es neutralitzà el pH a 7.2 amb l'ajuda d'àcid clorhídric (HCl) (Banes-Berceli *et al*. 2006). Aquest ajustament és totalment imprescindible per tal que la simvastatina sigui bio-activa, fet que a nivell d'estructura química implica l'obertura de l'anell de lactona (Sirtori 1993) **(Figura 15).** Una vegada finalitzat el procés de preparació, es van fer alíquotes d'1mL i es van conservar al congelador a -80ºC. Cada alíquota es descongelà lentament en gel abans del seu ús i en cap cas s' utilitzà el fàrmac recongelat.

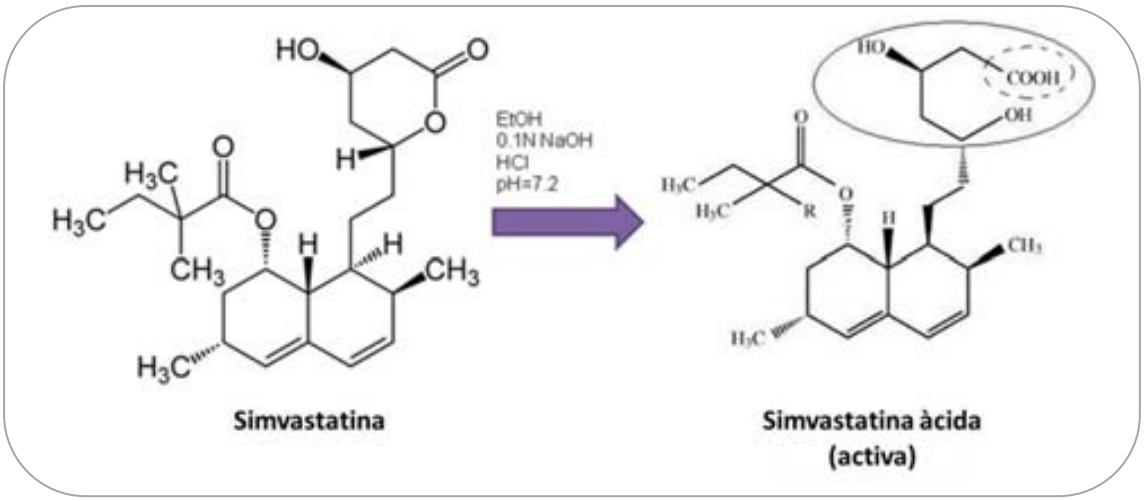

Figura 15. Reacció química en el procés d'activació de la simvastatina.

3.2.2 Encapsulació en liposomes

El procés d'encapsulació de la simvastatina en liposomes va ser realitzat pel grup de recerca de l'Institut Català de Nanotecnologia de la UAB. Després d'una llarga posada a punt, els liposomes escollits es composaren de lípids DLPC, colesterol i DSPG dissolts en una mescla de cloroform:metanol:aigua.

En un baló de 10 mL es van addicionar els lípids i la mescla de dissolvents. A continuació, s'evaporà el dissolvent orgànic en un rota-evaporador al buit i a 50ºC per tal d'obtenir una pel·lícula de lípid al baló. Seguidament es va hidratar el film de lípid amb 2 mL de medi de liposomes amb una solució de simvastatina activada o amb un medi equivalent però sense el fàrmac. Una vegada hidratat el film, es va ultrasonificar durant 15 min en un bany d'ultrasons d'on s'obtingué una suspensió de liposomes. Finalment es van passar els liposomes per un *extruder* (Lipex Biomembranes Vancouver, Canada) amb una membrana de 200 nm **(Figura 16 A)** per tal d' obtenir petites vesícules unilamelars (SUVs).

La distribució de la mida de les partícules es va determinar mitjançant *dynamic light scattering* i l'estabilitat de les partícules expressada com a potencial zeta (ζ), mitjançant Malvern Zetasizer (Malvern Instruments, UK). Finalment, la morfologia i la lamelaritat dels liposomes s'examinaren amb un microscopi electrònic de transmissió (cryo-TEM).

El coefficient d' encapsulació (EE) de la simvastatina va ser calculat a partir de la següent equació $EE(\%) = \left(\left[\left([C]_{total} - C_{free}\right)\right]/C_{total}\right] \times 100$, on C_{total} és la concentració inicial de simvastatina i C_{free} és la concentració de simvastatina no encapsulada.

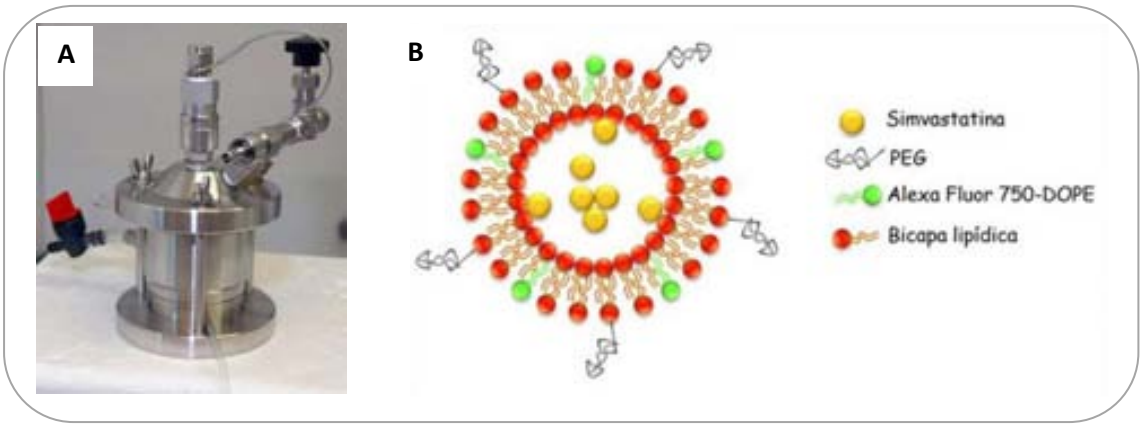

Figura 16. Fabricació i disseny dels liposomes. A) Extruder amb el què es permet adaptar els liposomes a la mida necessària després de passar-los per un filtre (en el nostre cas, de 200nm). B) Esquema de les parts fonamentals dels liposomes dissenyats per al nostre projecte.

En el sub-estudi que es realitzà per tal d'escollir entre les diferents composicions de liposomes tenint en compte la seva biodisponibilitat en plasma i capacitat d'arribada al cervell, s'usaren liposomes de 3 tipus: tipus 1, 2 i 3. Una vegada es comprovà que els liposomes tipus 2 eren els que ens aportaven majors avantatges, en els següents sub-estudis s'usaren, únicament, aquest tipus de liposomes **(Figura 16 B).**

Els liposomes es ressuspengueren en vehicle i s'hi addicionà la mateixa quantitat de simvastatina (prèviament activada) que l'usada en l'administració de simvastatina en forma lliure (6mg de simvastatina en 1 mL se solució). Tenint en compte el coeficient d'encapsulació (EE), aproximadament un 60% d'aquests 6 mg de simvastatina s'administrà dins els liposomes mentre que el 40% s'administrà en forma lliure, dissolta en el vehicle.

3.2.3 rt-PA/ vehicle rt-PA

rt-PA (Actilyse, Boehringer): S'administrà a una dosi de 9 mg/Kg. Per a preparar-lo, es reconstituí la pols amb el reconstituent a una concentració de 2mg/mL, s'aliquotà i es guardà al congelador a -80ºC fins pocs minuts abans de l'administració.

Vehicle rt-PA: Es va utilitzar el reconstituent del fàrmac i per a cada alíquota s'hi afegí L-arginina (0.4M) i Tween 80 (0.24%). També s'aliquotà i es congelà fina la seva utilització.

3.3 Procediments quirúrgics

3.3.1 Cirurgia *Capping*

El *capping* és una cirurgia que consisteix en la trepanació del crani en una localització molt concreta, per això es tenen en compte unes coordenades a partir del bregma (on s'ajunten les sutures cranials). L'objectiu d'aquesta cirurgia és poder introduir, posteriorment, una sonda làser que permetrà mesurar el flux cerebral sanguini de la zona irrigada per l'artèria cerebral mitja (ACM). El registre del flux sanguini cerebral serveix per assegurar-nos que l'oclusió s'ha realitzat correctament i que tots els animals inclosos en l'estudi han patit una isquèmia d'intensitat semblant.

Els animals van ser sotmesos al *capping* entre 24 i 48 hores abans de la isquèmia cerebral. Anestesiats prèviament amb Isoflorà, es van col·locar en una taula estereotàxica on van ser immobilitzats per les orelles i els incisius. Mitjançant una broca d'1 mm de diàmetre, es realitzà una trepanació a 1 mm del bregma (direcció caudal) i a 5 mm cap a la dreta **(Figura 17 A).** Realitzant la trepanació en aquestes coordenades, havíem comprovat prèviament, que estàvem registrant el flux cerebral sanguini de la regió irrigada per l'ACM. Una vegada realitzada la perforació, s'introduí un tub PE-50 (0.5mm de diàmetre intern) d'1 cm de longitud aproximadament **(Figura 17 B)** i es procedí a la fixació del mateix amb cola *superglue* i ciment dental. Finalment, es procedí a la sutura de la incisió i a la neteja amb iode.

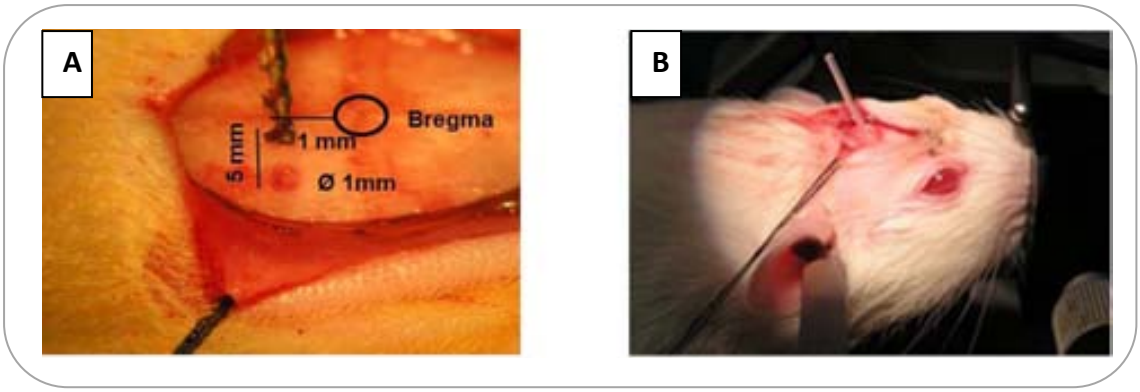

Figura 17. Fotografies realitzades durant la cirurgia *Capping*. A) Imatge presa a través del microscòpic on es mostren les coordenades utilitzades per a fer la trepanació. B) Fotografia després de col·locar el tub de polietilè per on s'introdueix la sonda làser.

3.3.2 MCAO

Per tal d'acomplir els objectius proposats en aquesta tesi, s'han realitzat 2 models animals diferents d'isquèmia cerebral en rata: model embòlic (MCAOe) i model transitori del filament (MCAOt). La diferència principal entre els dos models rau en què en el primer es realitza l'oclusió de l' ACM amb un coàgul format a partir de sang d'una rata donant i en el segon, amb un filament de niló. A cada un dels dos models s'hi associen certes avantatges i certs inconvenients. El model embòlic, per exemple, es considera que és el model que millor reprodueix la patologia en humans. A més, permet la reperfusió de l'artèria oclosa mitjançant l'adminitració d' rt-PA. Per contra, és un model que comporta una elevada mortalitat i una gran variabilitat entre animals d'un mateix grup (sobretot si no s'administra el trombolític, ja que els animals poden o no reperfondre). Per altra banda, el model transitori en què s'introdueix un filament de niló per ocloure l'ACM permet establir un temps d'isquèmia determinat i això facilita l'homogeneïtat pel que fa al volum d'infart dels animals. Aquest segon model, per això, pot considerar-se més artificial pel fet d'induir la isquèmia amb un cos estrany.

Formació de coàguls per al model MCAOe

Els coàguls que posteriorment s'introduïren fins la part més distal de la MCA induint la isquèmia als animals van ser formats a partir de sang arterial (extreta de l'artèria femoral mitjançant un procediment quirúrgic) d'una rata donant. Es realitzaren dos protocols diferents segons la soca utilitzada:

- Rates normotenses (Wistar)

Es realitzà l'extracció de sang i es procedí a fer una barreja de sang (150 µL) i trombina reconstituïda (10 µL a concentració 0.1 UI/ µL). La barreja s'introduí en un catèter de polietilè de 0.5 mm de diàmetre amb l'ajuda d'una xeringa. Es va mantenir el tub dins un incubador a 37ºC durant 2 hores i després es deixà a temperatura ambient fins poc abans de la cirurgia, quan la sang ja coagulada s'extragué i es netejà amb l'ajuda d'una xeringa amb abundant sèrum salí fisiològic (SSF). Per a induir la isquèmia es van utilitzar 2 coàguls d' 1,5 cm per a cada rata.

- Rates hipertenses (SHR)

Es realitzà l'extracció de sang i s'introduí en un catèter de polietilè de 0.5 mm de diàmetre amb l'ajuda d'una xeringa. Es va mantenir el tub a temperatura ambient durant 2 hores i després es deixà a 4ºC fins poc abans de la cirurgia, quan s'extragueren els coàguls i es netejaren amb abundant SSF. Per a induir la isquèmia es va utilitzar 1 sol coàgul de 3 cm per a cada rata. El fet de canviar el protocol per a treballar amb les rates hipertenses es justifica per l'elevada mortalitat amb què ens trobàrem aplicant el protocol anterior en aquesta nova soca.

Procés quirúrgic per a induir la isquèmia mitjançant MCAO

Sota anestèsia amb Isoflorà (2%), es realitzà una dissecció medial a nivell del coll i s'exposà la bifurcació carotídia dreta separant les glàndules submaxilars i els músculs omohioideu i esternohioideu. Les artèries caròtida comú (ACC) i caròtida externa (ACE) van ser dissecades i posteriorment es van termocoagular les dues primeres branques de l'ACE. Per sobre d'aquestes dues ramificacions es col·locaren dues lligadures i es seccionà l'ACE deixant un monyó. A continuació es dissecà l'artèria caròtida interna (ACI) fins veure l'artèria pterigopalatina. Aquesta última va ser lligada per la seva part més distal **(Figura 18)**.

Arribats a aquest punt, es col·locaren dos clips microvasculars (un sobre l' ACC i l'altre sobre l'ACI) per tal de poder interrompre el flux sanguini cerebral momentàniament i poder introduir el coàgul exogen. Es realitzà una petita incisió a l'extrem distal del monyó i s'introduí, o bé el coàgul preparat prèviament i mitjançant una xeringa (eMCAO) o bé el filament de niló (tMCAO). L'oclusió de l'ACM es produeix quan el coàgul sanguini o el filament de niló introduït arriba fins a la part més distal de l'artèria, al polígon de Willis. La realització correcta del procés es comprovà amb l'observació del descens brusc de la perfusió cerebrocortical registrada amb la sonda làser- Doppler **(Figura 19 A).** S'establí com a criteri d'inclusió, que només els animals que mostressin un descens del flux ≥75% respecte el flux basal serien inclosos als corresponents estudis **(Figura 19 B).** En el cas dels animals sotmesos a un model tMCAO, on es comprova també la reperfusió després de la retirada del filament, havien de complir, a més, que el flux després de la reperfusió fos ≥75% respecte el flux basal.

Cal remarcar que en el cas del model tMCAO després de provar diferents tipus de filaments: (fabricats manualment i comprats en una casa comercial) es van seleccionar els filaments de la casa comercial Doccol Company amb referència: 403723PK10.

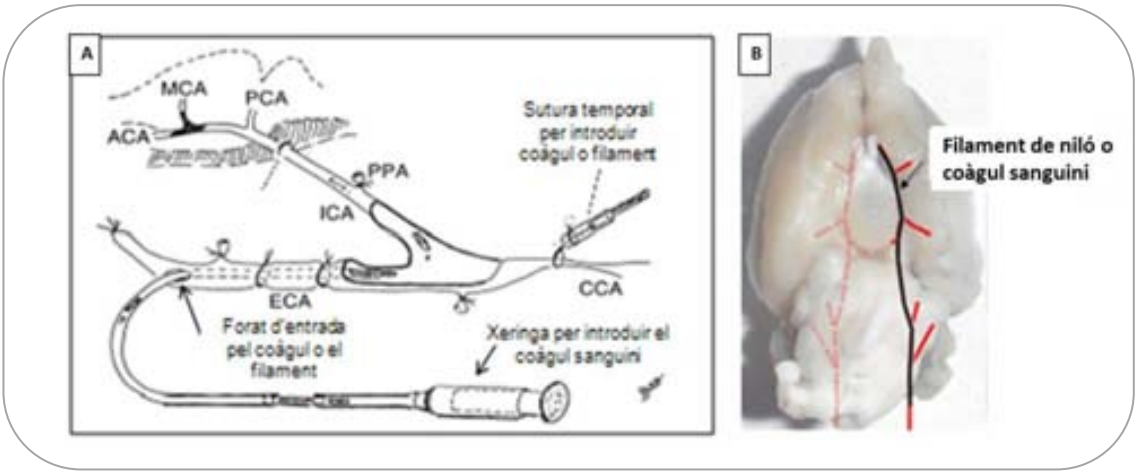

Figura 18. Esquema del model MCAO. A) Esquema on es mostra els passos a seguir per a realitzar una MCAO. B) Recorregut que ha de fer el coàgul sanguini (eMCAO) o el filament de niló (tMCAO) per tal d'ocluir la part distal de l'ACM.

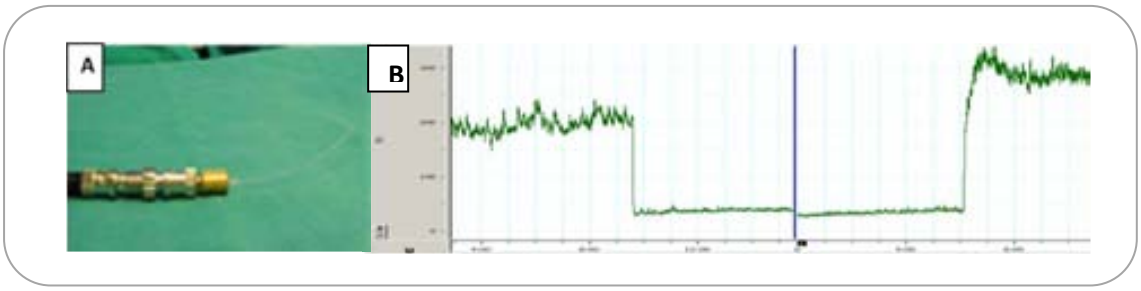

Figura 19. Monitorització del flux sanguini cerebral. A) Sonda del làser Doppler B) Gràfica del flux sanguini cerebral mostrant una correcta oclusió i reperfusió de l' ACM.

3.4 Càlcul del volum d'infart

La lesió induïda per la isquèmia és un paràmetre fonamental per a valorar l'eficàcia d'un fàrmac. En els estudis inclosos en aquesta tesi es van usar dues tècniques diferents:

3.4.1 Tinció amb TTC

Una vegada extret el cervell, es realitzaren seccions coronals de 2mm de gruix amb l'ajuda d'una guillotina. Les seccions cerebrals es van submergir en TTC (triphenyletrazolium chloride, Sigma-Aldrich) al 2.5% durant 15 minuts a temperatura ambient. Després de la tinció es podien diferenciar clarament zones pàl·lides i zones tenyides. Les zones tenyides corresponien al teixit viu, on el compost (TTC) s'havia reduït enzimàticament i s'havia tornat vermell a causa de l'activitat de diverses deshidrogenases, mentre que el compost es mantingué blanc a les zones necròtiques (corresponents a l'infart) ja que en aquesta àrea, els enzims quedaren desnaturalitzats o degradats.

Figura 20. Imatges dels talls coronals del cervell després de la tinció amb TTC

3.4.2 Tinció amb Tionina

Sota anestèsia general, els animals van ser perfosos primerament amb sèrum salí heparinitzat (SSH) i després amb sèrum salí fisoplògic (SSF) per tal d' eliminar tota la sang de dins els vasos. Amb aquesta finalitat, l'administració es realitzà a través d'un catèter intracardíac connectat a una bomba d'infusió. Una vegada extret el cervell se submergí en Paraformaldehid (PFA) al 4% durant 24 hores i posteriorment en sacarosa al 30% durant 24 hores més per tal de crioprotegir el teixit. Transcorregudes les 48 hores, el cervell es va incloure en OCT i es conservà a -80ºC fins a ser tallat al criòstat en seccions de 30 µm de gruix. El volum d'infart es calculà tenint en compte 15 seccions diferents del cervell. Per a realitzar la tinció amb Tionina

es fabricà un buffer a base d' aigua destil·lada (180mL), acetat sòdic 1M (9 mL), àcid acètic glacial 1M (21 mL) i 18 mL de 0.5% de solució de tionina (0.5g de tionina en 100 mL d'aigua destil·lada). Després de realitzar la tinció i els posteriors rentats amb etanol, aquesta tècnica mostrava la zona isquèmica amb una major pal·lidesa que el teixit sa i, a més augments, permetia observar el dany neuronal.

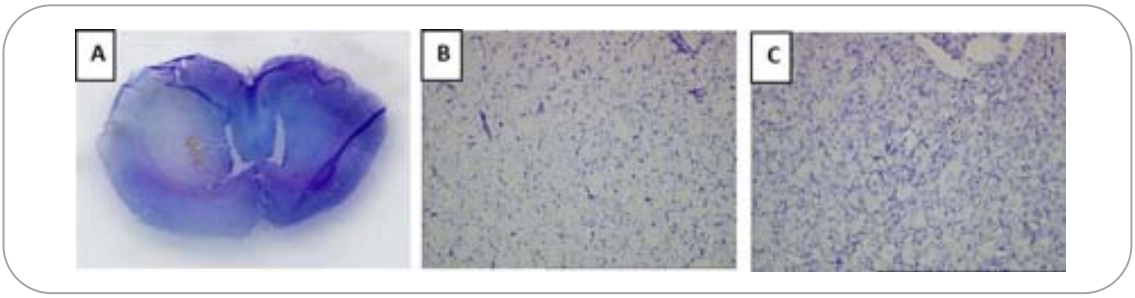

Figura 21. Imatges d'un tall de cervell després de ser tenyit amb tionina. A) Imatge macroscòpica on es pot diferenciar l'àrea infartada amb una tonalitat més pàl·lida. B) i C) Micrografies (magnificació 10X) on s'observa un menor número de neurones en l'hemisferi IP (B) que en el CL (C).

La valoració de l'infart mitjançant la tinció amb TTC resultà molt més ràpida i senzilla. A més, una vegada calculat el volum de l'infart, aquesta tinció permet el posterior anàlisi d'altres marcadors biològics mitjançant la realització d'homogenzats cerebrals. Tot i així, cal tenir en compte que la utilització de la tinció amb TTC pot alterar, i per tant, impedir, l'anàlisi posterior basat en tècniques colorimètriques.

La tinció amb tionina, en canvi, és molt més tediosa i amb més complicacions pel fet d'haver de tallar el teixit al criòstat però té l'avantatge que permet la realització de tècniques immunològiques i/o histològiques amb els talls que no s'han tenyit, ja que només es seleccionen uns quants talls per tal de determinar el volum de l' infart.

Independentment de la tècnica usada, en ambdós casos es calculà l'àrea infartada (respecte l'àrea de l'hemisferi IP) mitjançant el software Image J (rsbweb.nih.gov), i s'obtingué el percentatge del volum infartat sumant totes les àrees afectades d'un mateix cervell.

3.5 Valoració del dèficit neurològic

Les conseqüències neurològiques de la isquèmia cerebral van ser avaluades mitjançant el Test de Bederson modificat (Pérez-Asensio *et al.* 2005) on 0 és la puntuació esperable per a un animal naïve i 9 la màxima puntuació per a un animal sotmès al procés d'isquèmia cerebral **(Taula 2).** En el nostre estudi avaluar la situació neurològica va ser un dels principals *endpoints* per tal de valorar l'efecte del tractament. Per això es realitzà el test als 90 minuts de la isquèmia i just abans del sacrifici (a les 24 hores en rates hipertenses i a les 24 i 48 hores en normotenses).

Test de Bederson modificat (0-9)		
Desplaçament-exploració	Normal	0
	Desplaçament sense exploració	1
	Desplaçament només en estimular o no es desplaça gens	2
Deriva a l' esquerra	Mai	0
	En elevar la cua	1
	Espontàniament	2
	Baldufa (gir sense desplaçament)	3
Subjecció de l'extremitat anterior esquerra	No ho permet	0
	Ho permet però amb resistència	1
	No oposa resistència	2
Reflex de paracaigudes	Simètric	0
	Asimètric	1
	Pota anterior esquerra retreta cap al cos	2

Taula 2. Escala modificada de Bederson . Adaptada de Perez-Asensio *et al.* 2005

3.6 Anàlisi proteòmica d'homogenat cerebral

Primerament, els animals van ser perfosos per tal de retirar tota la sang dels vasos cerebrals. Per a aconseguir-ho, s'administrà SSH (10 min) i SSF (10 min) amb l'ajuda d'una bomba d'infusió i es procedí a l'extracció dels cervells. Cada cervell es dividí en 4 parts, descartant prèviament l'hipocamp: còrtex IP (ipsilateral) , còrtex CL (contralateral), estriat IP i estriat CL. Cada una d'aquestes àrees es va sotmetre a una congelació ràpida mitjançant nitrogen líquid i posteriorment les mostres van ser enviades al servei de Proteòmica del VHIO (Vall d'Hebron Institut d'Oncologia). Allà, les mostres es van homogenitzar amb DIGE lisis buffer (7M urea, 2M tiourea, 4% CHAPS, 30mM Tris, pH=8.5) per tal de realitzar l'extracció proteica. Seguidament, les mostres es van sonicar i es van centrifugar a 12000g durant 5 minuts a 4ºC. Els sobrenedants es van purificar mitjançant una precipitació modificada (2-D-Clean up kit, Amersham Biosciences) i van ser dissolts en DIGE *lysis buffer*. La concentració de les proteïnes es va determinar usant un kit; Bio-Rad RCDC Protein Assay (Bio-Rad).

Les mostres per a cada una de les condicions (córtex o estriat i IP o CL) i un pool van ser analitzades mitjançant tecnologia 2D DIGE (de l'anglès, *Difference in Gel Electrophoresis*). El pool, en el què hi havia la mateixa quantitat de proteïna per a cada una de les mostres, va ser usat com a control intern per a realitzar comparacions quantitatives. Per evitar biaixos relacionats amb el marcatge de les mostres, les mostres per a cada grup van ser marcades alternativament amb CY3 i CY5. L'electroforesis en dues dimensions es va realitzar amb els reactius i l'aparell de GE Healthcare.

Els spots presents en 4 de les 4 mostres de la mateixa condició, amb una $p<0.05$ (test ANOVA) i un fold change >1.3 entre comparacions, van ser seleccionats per a la seva identificació mitjançant MALDI-TOF MS (*Matrix-Assisted Laser Desorption/Ionization-Time-of-flight mass spectrometry*). Les proteïnes escollides es van picar amb un aparell anomenat Spot Picker (GE Healthcare) i la identificació es realitzà amb el software MASCOT (Matrix Science) fent servir bases de dades de proteïnes específica de rata. Finalment, la identificació de les proteïnes es va confirmar amb una segona fragmentació mitjançant un analitzador TOF-TOF i una trampa iònica.

3.7 Western Blot per a les mostres de l'estudi de proteòmica

Per tal de validar els resultats obtinguts mitjançant l'anàlisi proteòmica, les mateixes mostres d'homogenat cerebral van ser analitzades també per tècnica de Western Blot.

Trenta-cinc μg de cada mostra van ser carregats a un gel d'electroforesi del8% de poliacrilamida. Una vegada separades, les proteïnes van ser transferides a una membrana PVDF mitjançant un Mini Trans-Blot®Electrophoretic Transfer Cell (Laboratoris Bio-Rad) durant 1h a 100V. Per evitar unions no-específiques, es va bloquejar la membrana amb llet no grassa al 10% prèviament a la incubació *overnight* a 4ºC amb els anticossos primaris: mouse anti-Guanin nucleotide-bindig-protein (Go protein alpha, 1:3000, número de catàleg: ab78218; Abcam), mouse anti-Heat shock protein 60 (HSP-60, 1:100, número de catàleg: sc-376240; Santa Cruz Biotechnology), mouse anti- heat shock protein 75 (HSP-75, 1:200, número de catàleg: sc-135944; Santa Cruz Biotechnology), mouse anti-Heat shock protein 70 (HSP-70, 1:1000, Calbiochem), rabbit anti-Hemoglobin α (H-80, 1:1000, número de catàleg: sc-21005; Santa Cruz Biotechnology), rabbit anti-Dynamin (H-300, 1:200, número de catàleg: sc-11362, Santa Cruz Biotechnology), rabbit anti-DRP-2 (1:200, número de catàleg: sc-30228; Santa Cruz Biotechnology), rabbit anti-Alpha Fodrin (Spectrin α, 1:1000, número de catàleg: ab75755; Abcam) en tampó de bloqueig. Posteriorment, els anticossos secundaris: anti-rabbit-HRP or anti-mouse-HRP (Chemicon), es van diluir 1: 2000 en tampó de bloqueigi van ser incubats durant 1 hora a temperatura ambient. Abans i després de les incubacions, les membranes van ser esbaldides 3 vegades (10 minuts cada una) amb PBS. El procés de revelatge es realitzà amb un reactiu de quimioluminescència: Luminol (Amersham, GE) i s'obtingueren les imatges mitjançant un analitzador d'imatges (Las-3000, Fujifilm, CT, USA). Finalment, les bandes escanejades del Western Blot van ser quantificades amb l'ajuda d'un software anomenat Image Quantity One (Laboratoris Bio-Rad).

3.8 Valoració de les transformacions hemorràgiques (TH)

En l'estudi per a avaluar la influència del tractament amb simvastatina sobre l'aparició de transformacions hemorràgiques (TH), es van usar 3 tècniques diferents:

3.8.1 Qualificació visual

Una vegada el cervell va ser tallat en seccions de 2mm (amb l'ajuda d'una guillotina) i tenyit amb TTC es va procedir a una valoració macroscòpica de les hemorràgies. L' escala utilitzada va ser la mateixa que s'usa en clínica humana **(Figura 22).**

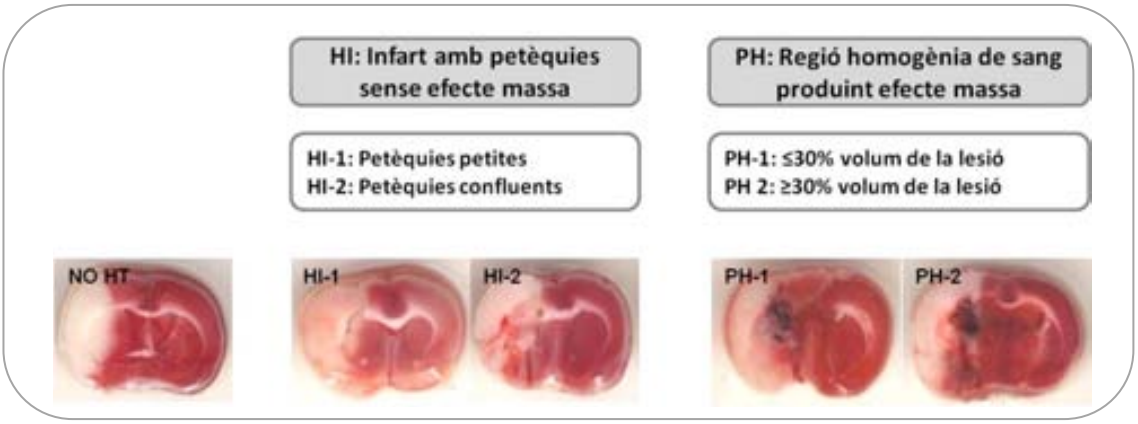

Figura 22. Escala utilitzada per a la valoració de TH i exemples obtinguts de cada una de les categories.

3.8.2 Quantificació de l'àrea PH

Només en el cas dels animals que mostraren una TH tipus PH (PH-1 o PH-2), es va procedir a la quantificació de l'àrea que ocupava l'hemorràgia mitjançant el mateix software utilitzat per al càlcul de volums d'infart (Image J).

Figura 23. Imatge on es mostra l'àrea quantificada d'una hemorràgia tipus PH-1.

3.8.3 Western Blot

Paral·lelament a la valoració macroscòpica de les hemorràgies, es va fer una valoració més quantitativa del contingut d'hemoglobina (Hgb) mitjançant Western Blot a partir d'homogenat cerebral. Cada animal es va representar amb dues mostres (hemisferi IP o isquèmic i hemisferi contralateral CL o no isquèmic), així l' hemisferi contralateral va servir de control intern per a cada un dels animals.

Per a la realització dels Western Blots es seguí el mateix protocol exposat a l'apartat 3.7. En aquest cas els anticossos primaris utilitzats van ser: anti α Hemoglobina (Santa Cruz, 1:1000) i anti Actina (1:10.000). I els anticossos secundaris: Goat anti rabbit (1:2000) i anti mouse (1:2000)

3.9 Tècniques per a la valoració d e l'estat d' immunodepressió/infecció

3.9.1 Citometria de Flux

Per tal d'avaluar el recompte diferencial de leucòcits en plasma (percentatge de limfòcits, polimorfonuclears (PMN's) i monòcits), es processaren mostres de sang d'animals naïve, sham i isquèmics 48 hores després de la cirurgia. Amb aquesta finalitat, just abans de la perfusió, es va recollir sang dels animals per punció cardíaca. Mig mL d'aquesta sang es va recollir en un tub amb EDTA i es va posar en gel. Es va procedir a la lisi dels eritròcits mitjançant l'addició de 5mL d'ACK buffer (Ammonium-chlorid-kaliumhydrogencarbonat) i a la neteja de la mostra amb 10 mL d'HBSS-Hepes (Hanks' balanced salt solution – Hepes). Es centrifugà la mostra a 1500 rpm durant 5 minuts i es repetí el procés lisi-neteja-centrífugació 3 vegades. Es descartà el sobrenedant obtingut en la tercera centrifugació i es ressuspengué el *pellet* resultant de cada mostra amb 100 µL de FACS buffer (BD Biosciences).

Una vegada processada la mostra, es va procedir al bloqueig de la mateixa incubant-la amb 0.25µg (0.5 µL) de Mouse anti-Rat CD32 (eBioscience) durant 10 minuts. Seguidament es va incubar amb els 2 anticossos durant 20 minuts: 0.4 µg (2 µL) d'Anti-Rat Granulocyte Marker PE (eBioscience) + 1 µg (2 µL) FITC Mouse Anti-Rat CD11b (eBioscience).

A part del processament de les mostres, es van utilitzar 3 mostres més que serviren com a controls cada dia que es realitzà l'experiment. Un primer control consistia en l'adició (a 30uL de mostra) d'un anticòs específic per a detectar la viabilitat cel·lular: to-Pro (eBioscience). Tenint en compte que el to-Pro és un colorant capaç de penetrar dins la cèl·lula només en cas que aquesta tingui la membrana trencada, aquest primer control ens permeté, per una banda, calcular el percentatge aproximat de cèl·lules no viables i, per l'altra, fer un recompte leucocitari diferencial a partir de les cèl·lules vives únicament (to-Pro negatives). Els altres 2 controls consistiren en l'adició, per separat, de cada un dels dos anticossos (Anti-Rat Granulocyte Marker PE i FITC Mouse Anti-Rat CD11b) amb el seu respectiu anticòs secundari. Cada un dels 2 anticossos s'afegí, també, a 30uL de mostra. Aquests 2 controls ens serviren per establir bé els límits del què consideraríem marcatge positiu o negatiu de cada un dels 2 anticossos utilitzats.

Una vegada preparades totes les mostres i els controls, es procedí a la lectura de les mostres a baixa velocitat. El citòmetre de flux utilitzat era un FacScalibur (Becton Dickinson ^{TM}) i es van

adquirir 10.000 events to-Pro negatius per cada mostra. Els resultats obtinguts s'analitzaren posteriorment amb el programa FCS Express (De Novo software).

3.9.2 Valoració histològica del teixit pulmonar

Sota anestèsia general i en condicions de màxima esterilitat (campana de flux laminar i material quirúrgic estèril), es va procedir a l'extracció dels pulmons. Primerament es va separar el lòbul dret i l'esquerre. El lòbul esquerre es va conservar en formaldehid 4% (Sigma-Aldrich) durant 48 hores i es va portar al servei d'Anatomia Patològica de l'Hospital Vall d'Hebron. Allí, els pulmons es van incloure en parafina i es van realizar talls de 4µm de gruix amb l'ajuda d'un micròtom. Els talls es tenyiren amb Hematoxilina-Eosina i l'avaluació histològica es va realizar de manera cega per una anotomopatòloga del servei d'Anatomia Patològica de l'Hospital. Els paràmetres avaluats van ser els següents: congestió vascular, edema, hemorràgia, infiltració de pilimorfonuclears (PMN's) a l'interstici pulmonar, presència de necrosi i/o fibrina a l'interior dels alvèols o dels bronquíols i presencia de cèl·lules mononuclears.

3.9.3 Cultius bacteriològics d'homogenat pulmonar i sang

El recompte d' Unitats formadores de colònies (CFU's) en plaques d'agar sang va ser considerat un dels paràmetres més importants per a avaluar les infeccions induïdes per la isquèmia cerebral. Es van realitzar recomptes de CFU's tant de sang com de pulmó. Per cada animal, es van sembrar 100µL de sang pura en plaques d'agar sang (Biomérieux). A més, es realitzà una impromta amb el pulmó dret i s'homogenitzà tot el lòbul dret en 1 mL de PBS estèril 1X. Amb aquest homogenat pulmonar es realitzaren dilucions seriades de manera que es van sembrar 100µL d'homogenat pur, 100µL d'homogenat a dilució 1/10 i 100µL d'homogenat a dilució 1/100. Les plaques sembrades es van mantenir a l'incubador a 37ºC i es realitzaren els recomptes de CFU's després de 24 i 48 hores de la sembra. A l'hora de donar els resultats, en el cas dels homogenats pulmonars, es van tenir en compte les plaques que mostraven entre 30 i 300 CFU's i les dades es corregiren segons la dilució escollida.

3.10 Tècnica IVIS per a l' estudi de distribució de liposomes

El sistema Xenogen IVIS® Spectrum (*In Vivo Imaging System*) està situat a l'estabulari i pertany al servei d'experimentació animal del nostre Institut. És un equip d'imatge òptica i baix soroll de fons que permet, entre altres coses, adquirir imatges *ex vivo* de diferents òrgans on es poden localitzar i quantificar partícules prèviament marcades amb fluorescència.

En el nostres estudi, vam analitzar el cervell i altres teixits (melsa, fetge, ronyons, pulmons, teixit adipós, múscul i pell) d'animals que prèviament havien rebut liposomes intravenosament. Per tal de poder localitzar aquests liposomes, el servei de nanotecnologia de la UAB va conjugar fluoròfors amb una elevada longitud d'ona Alexa-Fluor 750 (Life Technologies) a la membrana dels mateixos. Per tal d'incorporar el fluoròfor a la membrana dels liposomes, aquest es conjugà amb succinimidyl ester i DOPE-NH_2 i s'incorporà a la composició lipídica.

Amb les imatges adquirides amb l'IVIS, vam poder saber on s'acumulava la fluorescència, és a dir, on anaven a parar els liposomes marcats una vegada administrats. Posteriorment a l'adquisició d'imatges, es va usar un software associat a l'equip (Living Image 4.3.1), que va permetre la quantificació de la senyal observada.

En tots els casos, les dades corresponents a la senyal emesa pels liposomes es van corregir tenint en compte el background (autofluorescència o senyal emesa pel mateix òrgan sense administració de liposomes) i per la diferent senyal emesa segons el tipus de liposoma. Els resultats d'aquests experiments es van presentar en *radiant efficiency,* considerada una unitat arbitrària .

3.11 Tècnica UHPLC per a la determinació de simvastatina en homogenat cerebral

Les mostres usades per a determinar la presència de simvastatina en cervell consistiren en hemisferis cerebrals (IP i CL per separat) prèviament perfosos, homogeneïtzats amb metanol i aigua (1:1).

Per tal de realitzar l'extracció de simvastatina, 100 uL de cada una de les mostres s'addicionaren a 100 uL d'estàndard intern i a 150 uL d'acetonitril. L'estàndard intern consisteix en simvastatina adulterada comercial (Alsachim) i ens permet corregir els valors obtinguts de simvastatina evitant els errors deguts al processament de la mostra. La barreja dels tres compostos es va vortejar bé i seguidament se li afegiren 50 uL de format d'amoni 5M (pH=4). Posteriorment, es procedí a vortejar novament les mostres i a centrifugar-les (13000 G, 10 minuts, 4ºC). Cent uL de sobrenedant es van transferir a tubs d' injecció, on es diluïren amb 100 uL de fase mòbil (acetat d'amoni 0.5mM pH 4.5 i acetronitril, 70:30). Els tubs van ser vortejats novament abans d'injectar les mostres al cromatògraf (UHPLC System Acquity (Waters)). La separació es realitzà mitjançant una columna analítica de Phenyl (Acquity BEH 2.1 x 100 mm) a un flux de 0.25 mL/min i la detecció es va fer amb un sistema MS/MS quadrupole Xevo TQ MS (Waters).

Els resultats d' aquest experiment s'expressaren com a ràtios entre l'àrea corresponent a la quantitat de simvastatina i l àrea corresponent a la quantitatde l'estàndard intern.

4. RESULTATS

ARTICLE 1:

Evidence for the efficacy of statins in animal stroke models: a meta- analysis. *J. Neurochem.* 2012 Jul;122(2):233-43

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SYSTEMATIC REVIEW

Evidence for the efficacy of statins in animal stroke models: a meta-analysis

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Abstract

Protective effects of statins have been well documented for stroke therapy. Here, we used a systematic review and meta-analysis to assess these evidences. We identified 190 studies using statin treatment in stroke animal models by electronic searching. From those, only studies describing ischemic occlusive stroke and reporting data on infarct volume and/or neurological outcome were included in the analysis (41 publications, 1882 animals). The global estimate effect was assessed by Weighted Mean Difference meta-analysis. Statins reduced infarct volume by 25.12% (20.66%–29.58%, $P < 0.001$) and consistently, induced an improvement on neurological outcome (20.36% (14.17%–26.56%), $P < 0.001$). Stratified analysis showed that simvastatin had the greatest effect on infarct volume reduction (38.18%) and neurological improvement (22.94%), whereas bigger infarct reduction was observed giving the statin as a pre-treatment (33.5%) compared with post-treatment (16.02%). The use of pentobarbital sodium, the timing of statin administration, the statement of conflict of interest and the type of statin studied were found to be independent factors in the meta-regression, indicating their influence on the results of studies examining statin treatment. In conclusion, this meta-analysis provides further evidences of the efficacy of statins, supporting their potential use for human stroke therapy.

Keywords: animal models, ischemic stroke, meta-analysis, meta-regression, statin, systematic review.

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The benefits of statins on ischemic stroke have been well documented since their introduction into clinical therapy in the late 1980s, as an efficacious and safe treatment for cardiovascular disease (Grundy 1988). On average, statins can lower LDL cholesterol by 1.8 mmol/l (70 mg/dl), which translates into a 60% decrease in the number of ischemic heart diseases events and a 17% reduced risk of stroke after long-term treatment (Wald *et al.* 2003). Aside their lipid-lowering properties (Endo *et al.* 1976), pleiotropic effects of statins (George *et al.* 2002) may exert neuroprotection as it has been extensively pointed out in the last two decades by using animal stroke models. On the top of these properties, statins increase the expression of the nitric oxide synthase (NOS), specifically its endothelial isoform (eNOS), which improves endothelial function and increases cerebral perfusion in the ischemic penumbra (Endres & Laufs 2004, Yamada *et al.* 2000, Endres *et al.* 1998). Statins can exert neuroprotection by modifying anti-oxidative pathways via inhibition of NADPH oxidase- derived superoxide (Hong *et al.* 2006, Cui *et al.* 2009) and additionally, statins may alter inflammatory and immune responses after stroke. These can modulate NF-κB activity and the expression of

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Abbreviations used: CI, confidence interval; df, degrees of freedom; eNos, endothelial isoform; WMD, Wekghted Mean Difference.

inflammatory mediators like IL1β, monocyte chemoattractant protein-1 (MCP-1), or tumor necrosis factor alpha (TNF-α) (Sironi *et al.* 2006, Romano *et al.* 2000a, Kawai *et al.* 2011) and attenuate up-regulation of adhesion molecules like P-selectin, ICAM-1, CD18, and CD11b integrins (Romano *et al.* 2000b, Pruefer *et al.* 1999, Mayanagi *et al.* 2008)

Moreover, the use of statins has widely been explored either as pre-treatment in stroke prevention or as post-treatment after ischemia for neuroprotection of the injured ischemic brain. Their benefits in experimental stroke have been described and compared using several types of statins, such as atorvastatin, pitavastatin, simvastatin, (Hayashi *et al.* 2005, Laufs *et al.* 2000), pravastatin (Trinkl *et al.* 2006, Berger *et al.* 2008), lovastatin (Endres et al. 1998), or rosuvastatin (Laufs *et al.* 2002) and reported in both transient or permanent cerebral ischemia models (for details, see table 1).

Here, we conducted a systematic review and meta-analysis to further investigate the described benefits of different statins. We sought to assay whether these drugs globally offer clear advantages in ischemic stroke models that might be translated into human stroke therapy.

Experimental procedures

Search strategy and data extraction

To identify pre-clinical studies investigating the use of statin on animal stroke, we performed an electronic searching in PubMed (all publications until November 30, 2011) using the search terms of [STATIN] AND [ISCHEMIA] OR [ISCHAEMIA] AND [BRAIN]; [STATIN] AND [STROKE] and [STATIN] AND [CEREBRAL INFARCTION], limited to 'animal species'. Titles and abstracts from identified studies were screened by three independently investigators (DG, DS, and LG-B) to decide whether they were eligible for data extraction. Studies were considered eligible when they described animal models of ischemic occlusive stroke, implying the middle cerebral artery or its branches, and in which data of statin treatment on infarct size and/or on neurological outcome were reported. Discrepancies in extraction were resolved by discussion among the three investigators.

Our pre-determined primary end-points were infarct volume and neurological deficit outcome. From each study, we identified individual comparisons where primary outcomes were measured in a group of animals receiving a specific statin at a specific time(s), and compared with the outcome in a control group. Data regarding the number of animals used, the mean and the standard deviation (SD) or the standard error of mean (SEM) from treated and control groups, were extracted. Where neurological outcome was measured serially, only the final measure was used. Data from studies with multiple treatment groups investigating dose response or time course, were extracted from each group as individual values. Where data required for meta-analysis were missing or unpublished, we contacted authors to request additional information. Data presented graphically rather than reported in the text, were obtained using digital ruler software (Universal Desktop Ruler). Studies in which data required for meta-analysis were not presented or obtainable were excluded from the analysis.

Moreover, information on study design including species, age, weight, sex, dose, method of ischemia induction, type of statin, dose, time and route of administration, time of assessment of outcome, methods of both neurological and infarct volume evaluation and quality of the study, were collected from each study.

Study quality

Study quality was assessed based on a quality 10 points-checklist modified from the CAMARADES study (Macleod *et al.* 2004), comprising (1) peer review publication, (2) randomized assignment to study treatment, (3) blinded treatment administration, (4) blinded assessment of outcome, (5) statement of control of temperature, (6) sample size calculation, (7) statement of compliance with animal welfare regulations, (8) avoidance of anesthetics with marked intrinsic neuroprotective properties (ketamine), (9) statement of potential conflicts of interest, and (10) the use of accurate/suitable/adequate animal models (i.e. use of aged or co-morbid animals).

Data Analysis

Extracted data were analyzed as previously described (Sena *et al.* 2010a) using MS access from CAMARADES group. From each comparison, the effect size was calculated by applying the formula: Effect (%) = 100 x ((outcome control) – (outcome treatment))/(outcome control) on infarct volume or neurological outcome of extracted data. For the purpose of meta-analysis, we considered neuro-behavioral scales as continuous scales even when they were ordinal. Weighted Mean Difference meta-analysis (WMD) and its 95% confidence interval (CI) with random effects model to avoid heterogeneity (DerSimonian R and Laird N), were used to assay differences of the global estimate effect on infarct volume and neurological outcome. Stratified analysis was conducted to explore the influence of the quality of the study, types of stroke models (permanent or transient), dose, timing of first dose (pre-treatment or post-treatment), or type of statin on estimates of effect size. Differences of mean effect sizes were assessed partitioning heterogeneity using χ^2 distribution with n-1 degrees of freedom (df). *P*-values <0.003 were considered significant using Bonferroni correction. Correlation between drug administration timing and statin effect on infarct volume reduction was assessed using Spearman's rho correlation coefficient.

Multivariable meta-regression and related biases were performed using STATA software as describe elsewhere (Frantzias *et al.* 2011). Meta-regression was conducted to explore potential confounders among the following co-variates: number of dose, timing of first dose (pre-treatment or post-treatment), type of statin, effect of anesthesia, co-morbidity, and statement of conflict interest. Funnel plots were used to assess publication bias. Asymmetry was detected in the plots by visual inspection and when any bias was observed, a new corrected effect size was calculated using ''trim and fill'' by the Egger regression method (Sterne *et al.* 2001).

Results

Design of the study

Forty-seven of 190 total identified studies investigating the statin effects on animal stroke models met our inclusion

Table 1 Pre- or post-treatment with different type of statin in animal models of focal ischemia

	Model	Dose (mg/Kg/day)	Administration way	Species	Phase	Reference
Pre-treatment						
Atorvastatin	Embolic MCAO	20	sc (14d)	SV/129-C57BL/6 mice	Acute	Asahi et al. 2005
	pMCAO	10	sc (7d or 14d)	C57BL/6J mice	Acute/ subacute	Chen et al. 2005
		3	po (7d)	Retired breeder male Wistar rats	Acute/ Chronic	Chen et al. 2006
	tMCAO	10	sc (3d)	SD rats	Acute	Hong et al. 2006
		10	sc (14d)	SV/129 (wt and eNOS$^{-/-}$) mice	Acute	Laufs et al. 2000
		10	po (14d)	Wistar rats	Acute	Hayashi et al. 2005
		10	sc (14d)	129/SV wt mice	Acute	Gertz et al. 2003
	SHR-SP	20	po (4 w)	Spontaneously Hypertensive Stroke-Prone Rats		Nagotani et al. 2005
		2 or 20	po (11w)	Spontaneously Hypertensive Stroke-Prone Rats		Tanaka et al. 2007
Simvastatin	Embolic MCAO	20	sc (14d)	SV/129-C57BL/6 mice	Acute	Asahi et al. 2005
		100	po (14d)	SD rats (hyper- or normotermia)	Acute	Shabanzadeh et al. 2008
		100	ip (2w)	SD rats	Acute	Shabanzadeh et al. 2005
	pMCAO	20	sc (3d)	SD rats	Acute	Sironi et al. 2003 Sironi et al. 2006
		10	po (14d)	Long Evans rats	Acute/ Chronic	Yrjänheikki et al. 2005
	tMCAO	20	sc (14d)	SV/129-C57BL/6 (wt and eNOS$^{-/-}$) mice	Acute	Laufs et al. 2000
		1	ip (14d)	diabetic swiss albino mice	Acute	Çakmak et al. 2007
		0.2, 2 or 20	sc (14d)	SV/129, C57BL/6 and eNOS$^{-/-}$ mice	Acute	Endres et al. 1998
		20	sc (3d)			
		10	po (14d)	Wistar rats	Acute	Hayashi et al. 2005
Simvastatin In combination with Dipyridamole and Aspirin		0.1, 1, 10 or 20 (Sim) 60 or 30 (Dip) 10 (ASA)	ip (14d) po (3d) po (3d)	C57BL/6 wt and eNOS$^{-/-}$	Acute	Kim et al. 2008
Rosuvastatin	tMCAO	10 mg/Kg/d	ip (3d)	C57BL/6J, C57BL/6J ob/ob and lean mice	Acute	Mayanagi et al. 2008
		5mg	ip (14d)	SD rats	Acute/ subacute	Engelhorn et al. 2006
		2mg	ip (14d)	129/SV wt mice	Acute	Laufs et al.2002
Pitavastatin	tMCAO	10	po (14d)	Wistar rats	Acute	Hayashi et al. 2005
	SHR-SP	10 or 20	po (4 w)	Spontaneously Hypertensive Stroke-Prone Rats		Nagotani et al. 2005
Pravastatin	tMCAO	20	diet (4w)	Wistar rats	Acute	Trinkl et al. 2006

Table 1 (*Continued*)

	Model	Dose (mg/Kg/day)	Administration way	Species	Phase	Reference
Cerivastatin	SHR-SP	2	po (10w)	Spontaneously Hypertensive Stroke-Prone Rats		Kawashima et al. 2003
Mevastatin	tMCAO	2 or 20	continuons infusion (7, 14 or 28d)	129-SV/eVTAcBr mice	Acute	Amin-Hanjani et al. 2001
Lovastatin	tMCAO	0.2, 2 or 20 20	sc (14d) sc (3d)	SV/129, C57BL/6 and eNOS$^{-/-}$) mice	Acute	Endres et al. 1998
Post-treatment						
Atorvastatin	tMCAO	10	sc (2d)	SD rats	Acute	Sironi et al. 2003
		1, 3 or 8	po (7d)	Wistar rats	Acute/ subacute	Chen et al. 2003
	Embolic MCAO	20	sc (2d)	Wistar rats	Acute/ subacute	Zhang et al. 2007 Zhang et al. 2005
		20	sc (2d)	Wistar rats	Acute	Ding et al. 2006 Liu et al. 2006
Simvastatin	pMCAO	10	po (7d)	Long Evans rats	Acute/ Chronic	Yrjänheikki et al. 2005
		20	sc (2d)	SD rats	Acute	Sironi et al. 2003
	Embolic MCAO	1	po (7d)	Wistar rats	Acute/ subacute	Zacharek et al. 2009
		20 or 40	sc (single dose)	Wistar rats	Acute	Nagaraja et al. 2006
		40	sc (single dose)	SD rats (hyper- or normotermia)	Acute/ Chronic	Shabanzadeh et al. 2008
Rosuvastatin	tMCAO	0.2 or 2 20	iv (5d q24h) ip (5d q24h)	129/SV wt mice	Acute	Prinz et al. 2008
		0.5 or 5 or 20	ip (single dose)	C57BL/6 mice	Acute	Kilic Ü et al. 2005
		20	ip (single dose)	C57BL/6 and eNOS$^{-/-}$ mice	Acute	Kilic E et al. 2005
Pravastatin	tMCAO	50	continuons infusion (6d)	Wistar rats	Acute/ subacute	Sugiura et al. 2007
		0.1, 0.5, 1 or 2	ip (4d)	Wistar rats	Acute/ subacute	Berger et al. 2008
Fluvastatin	pMCAO	5	po (3m)	Wistar rats	Chronic	Shimamura et al. 2007

MCAO, Middle Cerebral Artery; permanent (pMCAO), transient (tMCAO), or embolic (embolic MCAO) stroke model; SHR-SP, Spontaneously Hypertensive Stroke-Prone Rats. Dose (expressed in mg/Kg/day) and way of administration (subcutaneous, sc; oral, po; intraperitoneal, ip). Different administration times are indicated in brackets (hour, h; day, d; week, w; m, month). *SD, Sprague-Dowley.* Acute, Subacute, or Chronic phases refer the studied time point of the ischemic lesion and/or neurological outcome.

criteria of occlusive ischemia model and examination of infarct volume and/or neurological outcome to be analyzed (fig. 1). Six of those were excluded from the analysis because data were neither reported nor obtainable. Then, 136 comparisons of the final 41 included publications were extracted in the analysis (supplementary table 1); 99 in 1882 animals reported data as infarct volume and 37 in 796 animals as neurological score. All the studies were in mice or rats, except for one performed in rabbit (supplementary table 1).

Meta-analysis

Treatment with statins reduced the infarct volume by 25.12% (95% confidence interval (CI), 20.66%–29.58%, $P < 0.001$) as compared with the control group. Consistent with this, a considerable improvement in the neurological outcome

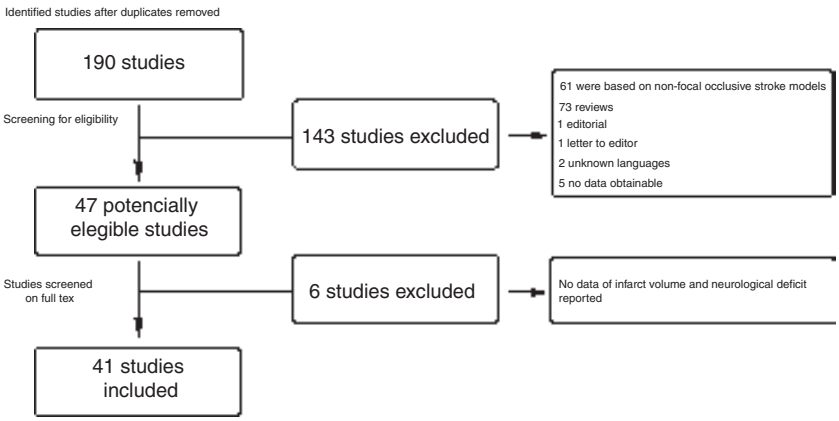

Fig. 1 Flow diagram of the study selection.

was observed (20.36% (14.17%–26.56%), $P < 0.001$), (fig. 2).

Reported study quality

Overall, the median quality (range scale 0-10) of the 41 included studies was modest (4, interquartile range, 4–6) with a score that ranged from 1 to 6. No studies scored 0 or high quality rating (7–10 points). Twenty-seven studies (65.9%) reported temperature controlling during surgical procedure, but only 17 studies (17.5%) reported randomization of the assignment to study treatment and 12 studies (29.3%) reported that the treatment was concealed to investigators during outcome assessment. Moreover, any study reported blinded treatment administration.

When the effect size on infarct volume reduction and neurological improvement were examined relative to study quality score, a significant heterogeneity over the scoring range was observed (fig. 3). Although no significant differences were found in theses parameters between low-scored studies and higher-scored studied, the effect size on infarct volume decreased by 12% in comparisons with maximun score (6). Similarly, comparisons with a low quality score of 2 and 3 showed higher efficacy on neurological outcome (23% and 36% effect, respectively) than comparisons scoring 4-5 (15%) and 6 (13%).

Stratified meta-analysis

Effect size on infarct volume was significantly higher when the statin was administered as a pre-treatment (33.57%; 95% CI, 28.47%–38.53%) compared with post-treatment (16.02% (11.63%–20.42%); $\chi^2 = 408$, df=1, $P < 0.001$) and it was accompanied by a better neurological improvement (26.52% (15.05%–37.99%) vs. 14.37% (7.26%–21.48%),; $\chi^2 = 17$, df=1, $P < 0.001$), (fig. 4). The median and the interquartil range timing for statin pre- or post-treatment administration were 14days (5–14d) and 4h (1.13–12h), respectively. Statin efficacy was correlated with the timing of administration ($r^2 = 0.334$, $P < 0.001$), being more effective as soon as it was administered. There was a decrease of the efficacy to reduce infarct volume of 0.5% for every day delay to pre-treatment and of 0.06% for every hour (1.4% per day) delay to post-treatment.

The analysis according to the route of administration showed better results when the drug was administered orally

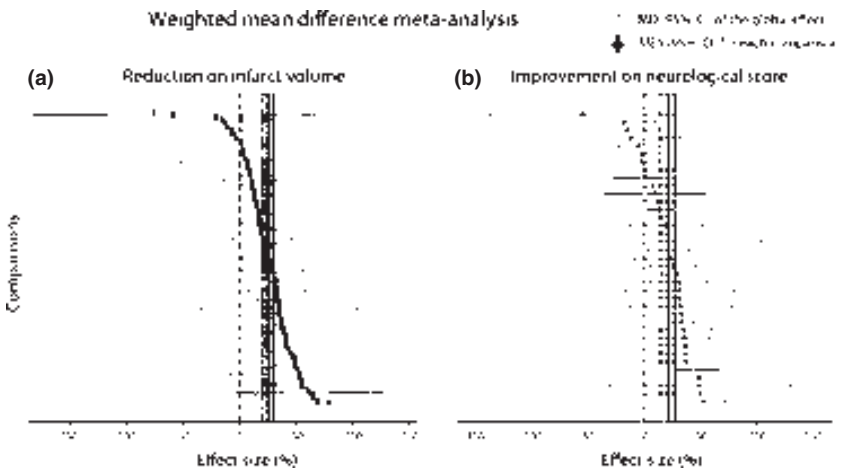

Fig. 2 Meta-analysis of effect of statins on infarct volume reduction (a) and neurological improvement (b). The horizontal lines represent the mean estimated effect size (MES) and the 95% confidence intervals (CI) for each individual comparison according to their effect on infarct volume (a) and neurological score (b). The mean difference (MD) and the 95% CI of the global estimate are represented as solid and dashed vertical lines, respectively.

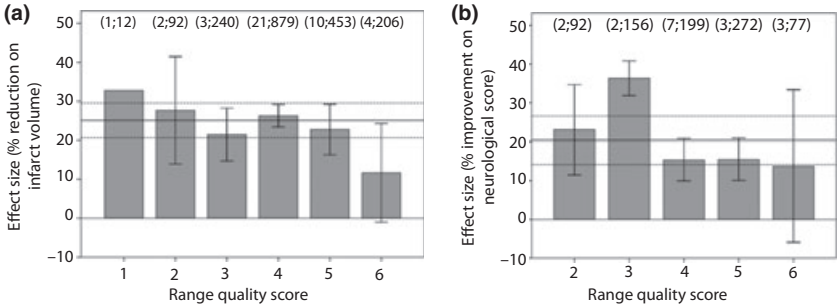

Fig. 3 Effect on infarct volume reduction (a) and neurological score improvement (b) regarding reported quality score. The horizontal lines represent the mean (solid line) and the 95% confidence intervals (dashed lines) of the global estimate. Each bar represents the mean +/- SEM for the individual estimates. The number of both studies and animals contributing to each individual estimate are indicated in brackets.

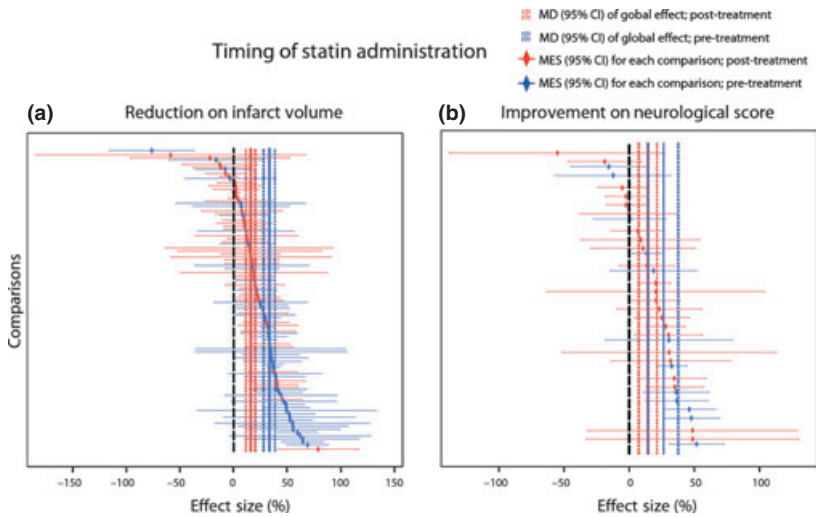

Fig. 4 Effect size on infarct volume reduction (a) and neurological score improvement (b) stratified by statin pre- (blue color) or post-treatment (red color). The horizontal lines represent the mean estimated effect size (MES) and the 95% confidence intervals (CI) for each individual comparison. The mean difference (MD) and the 95% CI of the global estimate are represented as solid and dashed vertical lines, respectively.

(infarct volume reduction of 34.50% (26.65%–42.35%)) as compared to intraperitoneally (16.96% (10.62%–23-31%)), whereas the effect was similar in those animals receiving the statin subcutaneously (28.17% (20.39%–35-95%); $\chi^2 = 219$, df=2, $P < 0.001$) (fig. 5a–c). In terms of effect size on neurological outcome, oral administration led to higher favorable improvement (28.35% (22.74%–33.97%) than either subcutaneous (9.76% (-0.91%– 20.42%)) or intraperitoneal administration (19.66% (3.32%–36.00%), ($\chi^2 = 21$, df=2, $P < 0.001$), (fig. 5d–f).

The global estimated benefits of statins on infarct volume were similar when comparing permanent [25.48% (17.66%–33.29%)] or transient (24.70% (20.38%–29.02%)) cerebral ischemia. However, there was a high statistical significant heterogeneity between studies ($\chi^2 = 283$, df=1, $P < 0.001$). Regarding neurological improvement, better outcome was seen in animals that underwent transient ischemia (9.11% (-4.86%–23.08%) vs. 23.05% (16.63%–39.46%), $P < 0.001$), (fig. 6). Significant inter-study heterogeneity was also observed ($\chi^2 = 77$, df=1, $P < 0.001$).

Analysis of effect size on infarct volume stratifying by the number of statin dose, repeated or single dose, showed significantly larger reduction in animals receiving multiple dose (30.08% (25.49%-34.68%) vs. 13.72%(8.76%–18.69%), $P < 0.001$), (fig. 7).

By type of statin, simvastatin was significantly associated with a greater infarct size reduction [38.18% (33-23%–43-14%)] with respect to atorvastatin [23.71% (17.05%–30.38%)] and rosuvastatin [13.88% (6.54–21.21%); $\chi^2 = 345$, df=2, $P < 0.001$], (fig. 8a–c). Neurological improvement was similar in animals receiving simvastatin [22.94% (12.16%–33.73%)] and artovastatin [19.24% (10.52%–27.96%)] and, in contrast, no effect size was observed in rosuvastatin treated-animals [-0.27% (-18.9%–18.35%)], (data not shown). The heterogeneity was high in that analysis ($\chi^2 = 25$, df=2, $P < 0.001$). Furthermore, we sought to analyze the efficiency on the infarct volume reduction regarding administration of low versus high-dose of different statins. Rosuvastatin, atorvastatin, and simvastatin triggered higher effects at high-dose (20.13% (9.25%–31.01%), 27.40%(18.07%–36.73%)

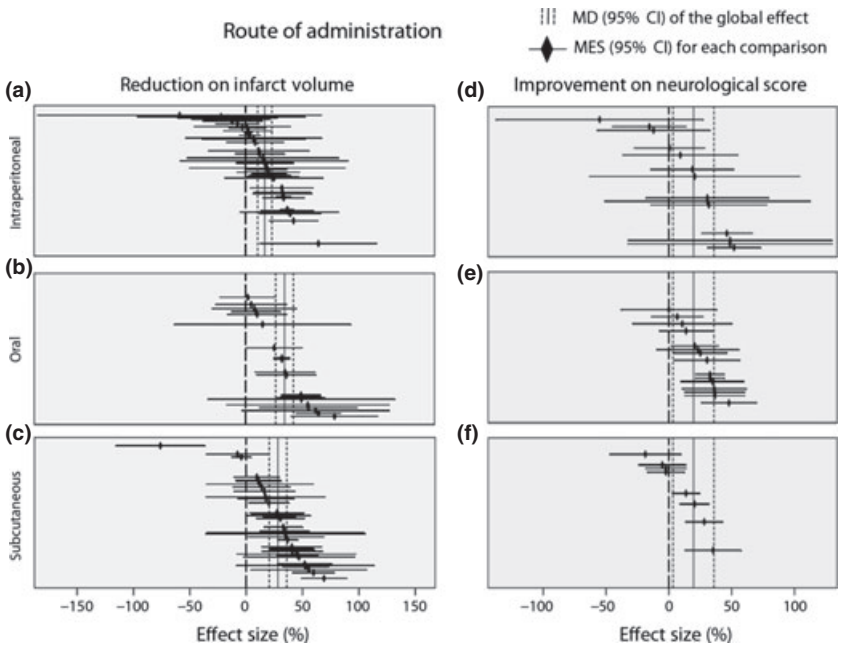

Fig. 5 Effect size on infarct volume reduction (a–c) and neurological score improvement (d–f) stratified by route of statin administration. The horizontal lines represent the mean estimated effect size (MES) and the 95% confidence intervals (CI) for each individual comparison according to their effect on infarct volume (a–c) and neurological score (d–f). The mean difference (MD) and the 95% CI of the global estimate are represented as solid and dashed vertical lines, respectively.

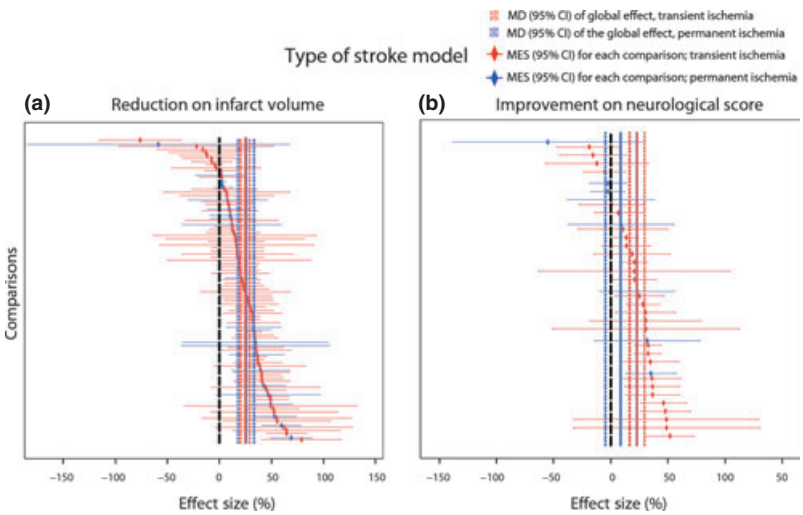

Fig. 6 Effect size on infarct volume reduction (a) and neurological score improvement (b) stratified by permanent (blue color) or transient (red color) cerebral ischemia model. The horizontal lines represent the mean estimated effect size (MES) and the 95% confidence intervals (CI) for each individual comparison. The mean difference (MD) and the 95% CI of the global estimate are represented as solid and dashed vertical lines, respectively.

and 40.46% (33.4%–47.52%), respectively) compared with low-dose (11.36% (2.13%–20.59%), 16.55% (1.93%–31.17%), and 36.10% (28.85%–43.35%; (χ^2 = 632, df=1, $P < 0.001$), (χ^2 = 489, df=1, $P < 0.001$), (χ^2 = 637, df=1, $P < 0.001$); $p < 0.001$) (fig. 8d–f).

Meta-regression analysis

Meta-regression was conducted for infarct volume including data that assayed the effect of simvastatin, atorvastatin, or rosuvastatin, to further explore meta-analysis heterogeneity. The analysis identified four factors: type of anesthetics (pentobarbital sodium vs. others), timing of first dose (pre-treatment or post-treatment), statement of conflict of interest, and type of statin to account for 60.9% of between-study variance (τ^2 = 98.5, adjusted r^2 = 0.609). Studies that used pentobarbirtal sodium as anesthetic reported 18.4% (4.6%-32.1%) lower efficacy than those studies that used a different anesthetic. Pre-treatment with statin reduced the infarct volume, an additional 9% (0.3%-17.7%) compared with post-treatment. Studies that stated conflict of interest showed an efficacy of 12.6% (0.6%-24.6%) less than those that did not. Relative to type of statin, administration of rosuvastatin reduced the infarct volume by 11.7% (25%-20.8%) less compared with either atorvastatin or rosuvastatin.

Bias publication

Finally, we sought to identify whether small studies' effects may contribute to publication bias in those analysis performed by statin-type (fig. 9). Funnel plot evidenced

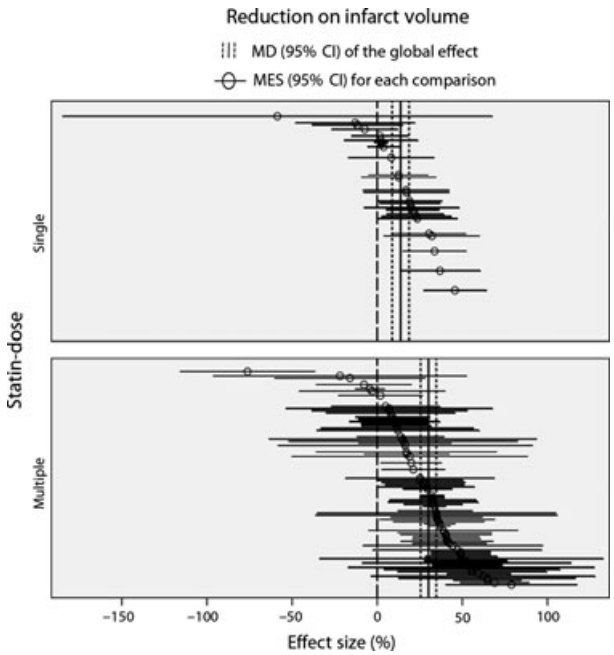

Fig. 7 Effect size on infarct volume reduction stratified by single or multiple doses of statin administration. The horizontal lines represent the mean estimated effect size (MES) and the 95% confidence intervals (CI) for each individual comparison according to their effect on infarct volume. The mean difference (MD) and the 95% CI of the global estimate are represented as solid and dashed vertical lines, respectively.

asymmetry in atorvastatin data (Egger regression, $P < 0.001$) while visual inspection of both simvastatin and rosuvastatin funnel plots indicated obvious symmetric inverted funnel shapes (Egger regression, $P = 0.939$ and $P = 0.679$, respectively).

Discussion

Our systematic review and meta-analysis reinforces the value of statin treatment in cerebral ischemia and points to simvastatin as the statin that provides the highest neuroprotective effect to the injured brain. The estimated effect was higher when statins were given as pre-treatment rather than post-treatment. Effectiveness was greater when drug was given orally, and multiple-dose instead of a single-dose treatment provided better neurological outcomes. However, statin treatment led to similar neuroprotection in either transient or permanent cerebral ischemia.

Concerning the study quality, we did not find differences on statin efficacy across the range of scores, but studies with a lower quality showed a trend toward better outcomes. Therefore, the global estimated effect may be overstated in low-scored studies. Another point of note is the reduction of the neuroprotective effect, by more than 10%, when titles stated conflict of interest, or the fact that the articles did not report blinding administration of the treatment, revealing the importance of these study-quality issues in their contribution to publication bias.

It is also interesting to note the finding that the use of the anesthetic pentobarbital markedly decreased (18%) the neuroprotective effect of statins. The influence of the use of pentobarbital on the outcome of animal stroke models has been recently described in a systematic review and meta-analysis on the efficacy of tPA treatment (Sena et al. 2010a). However, in contrast with our findings, this study showed a better neurological outcome when pentobarbital was used. No interactions between statin and pentobarbital have been described so far, but since pentobarbital is a strong cytochrome P450 inducer (Waxman & Azaroff 1992) and statins are predominantly metabolized by the cytochrome

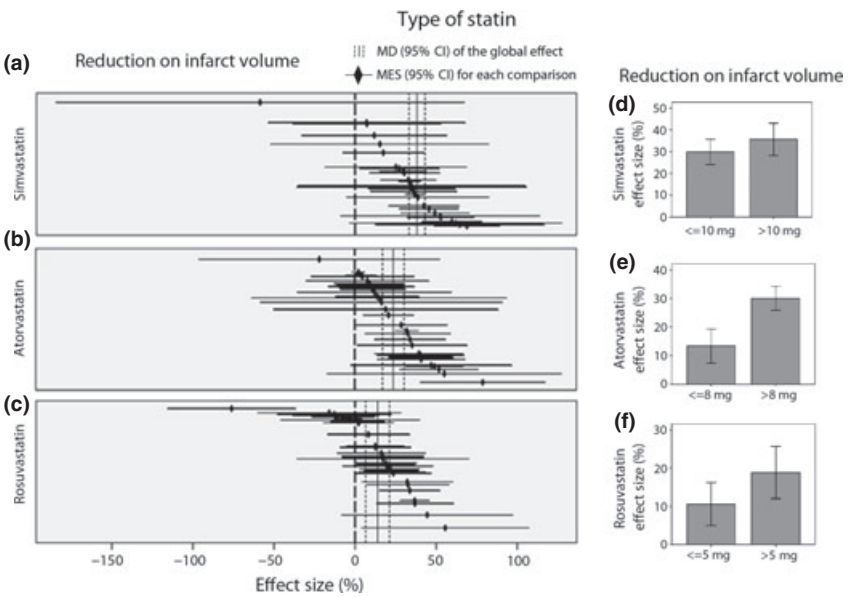

Fig. 8 Effect size on infarct volume reduction stratified by type of statin: simvastatin (a), atorvastatin (b), and rosuvastatin (c). The horizontal lines represent the mean estimated effect size (MES) and the 95% confidence intervals (CI) for each individual comparison. The mean difference (MD) and the 95% CI of the global estimate are represented as solid and dashed vertical lines, respectively. Bar plots represent the mean +/- SEM for the individual estimates comparing low and high-dose of simvastatin (d), atorvastatin (e), or rosuvastatin (f).

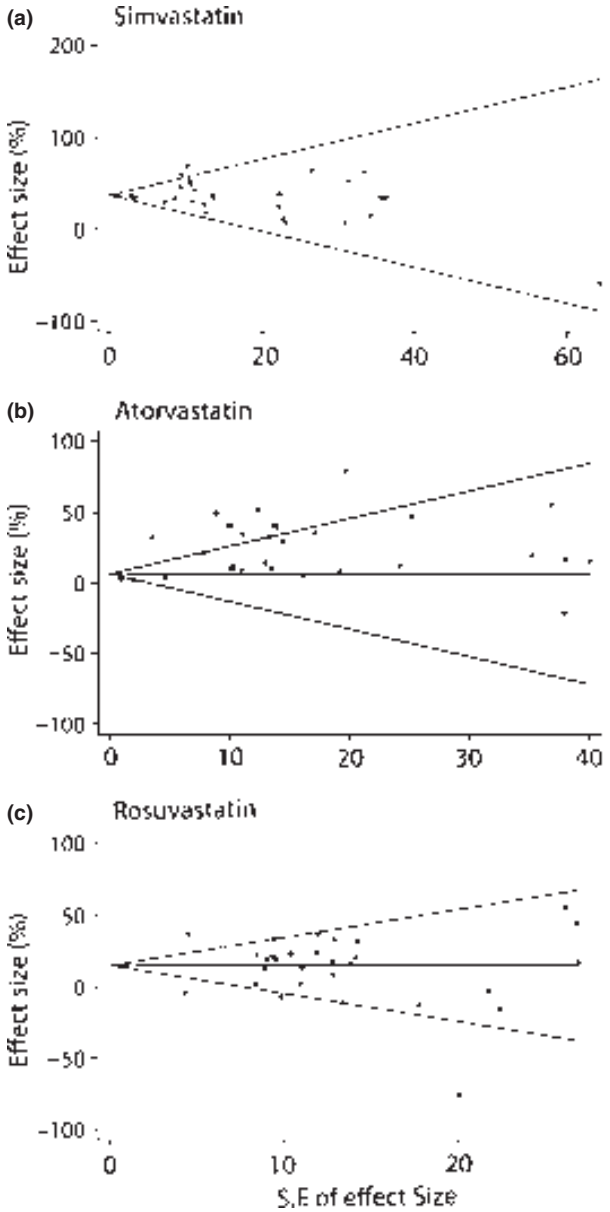

Fig. 9 Funnel plot for simvastatin (a), atorvastatin (b), and rosuvastatin (c).

P450 system (Bellosta *et al.* 2004), statin plasma concentrations might be reduced.

Experiments with animal models usually start with a pre-treatment protocol owing to the fact that therapeutic manipulations generally work better when administered before or immediately after the ischemic insult (Danton & Dietrich 2004). Nevertheless, that is not the best approach when we aim to look for a neuroprotectant drug to be administered for acute stroke patients that arrive to the Emergency Departments after the brain artery occlusion. In accordance, we found that statin pre-treatment yielded a 2-fold significant reduction on the infarct lesion (33.57%; 52 comparison) as compared with post-treatment (16.02%; 53 comparison). Reliable clinical evidences have demonstrated that the use of statin preventive therapy reduces the stroke risk (Prinz & Endres 2011, Amarenco & Labreuche 2009) and improves the functional outcome after ischemic stroke (Biffi *et al.* 2011). On the other hand, clinical studies using statin therapy in the acute phase of the ischemic stroke are scarce. A recent systematic review concluded that insufficient data are available from randomized trials to establish whether statins are safe and effective in cases of acute ischemic stroke (Squizzato *et al.* 2011). Indeed, to our best knowledge, the MISTICS is the only pilot clinical trial, up to date, examining the simvastatin effect on neurological outcome (Montaner *et al.* 2008), which showed an improvement among simvastatin-treated patients by the third day of stroke onset (46.4% vs. 17.9%, $P = 0.022$). The post-treatment effectiveness found in this study has been similarly reported in a recently published meta-analysis (Baryan *et al.* 2012). The post-treatment effect, albeit being smaller than the pre-treatment effect, supports the therapeutic potential of statin treatment in the acute stroke.

To compare the efficacy by type of statin, we examined the neuroprotective effect associated with simvastatin, atorvastatin, or rosuvastatin administration. We did not include pravastatin, pitavastatin, and perivastatin in the analysis because of the small group data. We observed a higher estimate of efficacy for simvastatin, followed by atorvastatin and rosuvastatin. The least benefit found for rosuvastatin treatment could be explained by its selective uptake into hepatocytes (Nezasa *et al.* 2003), reducing the systemic bioavailability and its effective dose.

In spite of the well-known efficacy of atorvastatin, we detected a substantial publication bias that was not observed for either simvastatin or rosuvastatin. For atorvastatin, the estimated effect of 23.71% on infarct volume including 32 comparisons, decreased to 4.82% when it was adjusted for publication bias with an input of 15 studies missing. Therefore, the existence of negative or less efficient atorvastatin-treatment unpublished results might be implied.

As expected, multiple doses of statins, well tolerated and safe in long-term exposure (Maron *et al.* 2000), triggered higher positive effects than a single-dose treatment. Of note, however, in all the studies in which a multiple-dose regimen was used, statins were administered before the ischemic insult. In keeping with this, the increase in statin efficacy might be related not to the dose regimen treatment, but to the use of a pre-treatment protocol. As we found that pre-treatment with statins additionally reduced the infarct volume by 9% and that the factor 'single vs. multiple-dose' was not independently identified in the regression analysis, this assumption may be plausible.

To clarify the influence of the dosage on the effect of each statin, we sought to analyze the estimate by high versus low-dose of statin given. All the statins evaluated

(simvastatin, atorvastatin, and rosuvastatin), had individually bigger effects on the reduction of the infarct volume at higher dose, as shown by the global estimated effect. Nevertheless, the eventual use of higher-statin doses in stroke therapy needs to be considered because of potential toxic side effects (STAIR 1999). In this regard, hydrophilic statins such as rosuvastatin, are less toxic than lipophilic statins such as atorvastatin and simvastatin, owing to the already mentioned first-pass effect into the liver (Nezasa et al. 2003). Moreover, although lipophilic statins can easily cross the blood-brain barrier and potentially exert neuroprotection, the administration of high doses might trigger harmful effects (Wood *et al.* 2010).

Statin effectiveness exerted by oral route was superior compared to either intraperitoneal or subcutaneous administration. This difference might be related to the first-pass hepatic metabolism, after absorption from the gastrointestinal tract, where several bioactive metabolites may be produced. However, this finding should be interpreted with caution, because the regression analysis did not identify the route of administration as an independent variable.

In addition, we found that statin neuroprotective effect was present regardless of the cerebral ischemia model used. Restoration of cerebral blood flow achieved after transient ischemia allows a better delivery of the drug to the injured area, as well as increasing the possibility of rescuing the ischemic penumbra (Nagahiro *et al.* 1998). In that sense, we expected to find more favorable outcomes in transient than in permanent cerebral ischemia. On the contrary, the statin treatment has been extensively described in both models (table 1 and supplementary table 1). It has been well demonstrated that statins can act at several different levels (i.e. improving cerebral perfusion, anti-oxidant, and anti-inflammatory properties) and exert neuroprotection in both models. Nevertheless, as our analysis showed a high inter-study heterogeneity, and the method of ischemia induction was not found to be independent in the meta-regression, this result must be taken with caution and we cannot make any inferences on the effect of statin by transient or permanent ischemic model.

Extrapolating the findings from these preclinical studies to clinical ones is difficult. For example, a small fraction of the studies reported detrimental effect of statin on stroke outcomes in our analysis: 17% and 9.8% of the studies reported a negative effect size on infarct volume reduction and neurological deficit, respectively. Such an effect is usually caused by the phenomenon of ''file drawer problem'' (Sena *et al.* 2010b) by which negative results, as concluded to be of no interest, are unpublished. Therefore, statin effectiveness maybe be overstated in our meta-analysis. In fact, the funnel plot analysis revealed atorvastatin efficacy was overstated. Other limitations of inferring clinical conclusions from this analysis are the fact that the included studies were performed on rodent stroke models, which do not recapitulate all the features of human stroke and may introduce important confounders, such as anesthesia and surgical trauma (Iadecola & Anrather 2011). The lack of co-morbidities in animal models represents another way in which it fails to mimic human stroke. In our meta-analysis, most of the studies were performed in young healthy rodents and only five studies used animals with comorbidities, whereas stroke patients are old population with secondary complications (hypertension, high cholesterol). Thus, a great effort is needed to improve the quality of neuroprotection experimental research and extrapolating data from meta-analysis of animal model must be taken with caution.

The main limitation of our analysis is that we only performed an electronic searching strategy in PubMed. Even though it represents an effective strategy to search for studies, combination of other electronic sources, such as EMBASE and BIOSIS, and handsearching should be performed to adequately identify all reports. Thus, we cannot discard the fact that we have missed unpublished or awaiting publication reports in the meta-analysis, which might contribute to publication bias on the reported effectiveness of statins.

Overall, there is enough evidence to confirm the benefits of statins in ameliorating brain damage after cerebral ischemia. In addition, our meta-analysis supports a neuroprotective effect when statins are administered either as pre-treatment or post-treatment. According to this data, the ideal clinical trial should be conducted with simvastatin, given orally at high and repeated doses, as soon as possible following the stroke onset. A clinical trial designed with these characteristics is ongoing (STARS trial) and might help the translation of this information into the acute clinical practice.

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Supporting information

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

Appendix S1. Publications included in the meta-analysis

Table S1. Characteristic of studies and comparisons included in the analysis.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

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ARTICLE 2:

Brain proteomics identifies potential simvastatin targets in acute phase of stroke in a rat embolic model.*J Neurochem*. 2014 Jul;130(2):301-12

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ORIGINAL ARTICLE

Brain proteomics identifies potential simvastatin targets in acute phase of stroke in a rat embolic model

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Abstract

Finding an efficient neuroprotectant is of urgent need in the field of stroke research. The goal of this study was to test the effect of acute simvastatin administration after stroke in a rat embolic model and to explore its mechanism of action through brain proteomics. To that end, male Wistar rats were subjected to a Middle Cerebral Arteria Occlusion and simvastatin (20 mg/kg s.c) (n = 11) or vehicle (n = 9) were administered 15 min after. To evaluate the neuroprotective mechanisms of simvastatin, brain homogenates after 48 h were analyzed by two-dimensional uorescence Difference in Gel Electrophoresis (DIGE) technology. We confirmed that simvastatin reduced the infarct volume and improved neurological impairment at 48 h after the stroke in this model. Considering our proteomics analysis, 66 spots, which revealed significant differences between groups, were analyzed by matrix-assisted laser desorption/ionization-time of ight mass spectrometry allowing the identification of 27 proteins. From these results, we suggest that simvastatin protective effect can be partly explained by the attenuation of the oxidative and stress response at blood–brain barrier level after cerebral ischemia. Interestingly, analyzing one of the proteins (HSP75) in plasma from stroke patients who had received simvastatin during the acute phase, we confirmed the results found in the pre-clinical model.

Keywords: acute phase treatment, embolic cerebral ischemia, proteomics, rat, simvastatin, stroke.

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Stroke is the third leading cause of death and the most common cause of permanent disability among adults worldwide (Roger *et al.* 2012). Nowadays, the only existing treatment in acute phase of stroke approved by the Food and Drug Administration is the thrombolytic rt-PA (NINDS rt-PA Stroke Study Group 1995). Owing to its known side-effects (NINDS rt-PA Stroke Study Group 1997), new treatments with wider therapeutic window, which can be easily and rapidly administered and are available before the patients' arrival in emergency units, are needed for neuroprotection. Many studies have demonstrated beneficial effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) in decreasing the incidence of stroke in patients at high risk of cardiovascular disease (Jonsson and

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Abbreviations used: BBB, blood–brain barrier; DIGE, difference in gel electrophoresis; DRP2, dihydropyrimidinase-related protein 2; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MCAO, middle cerebral arteria occlusion.

Asplund 2001; Martí-Fàbregas *et al.* 2004; Greisenegger *et al.* 2004; Yoon *et al.* 2004), although such effects have not been attributed to their cholesterol-lowering therapeutic actions. Alternatively, pleiotropic effects such as increasing endothelial nitric oxide (Laufs *et al.* 1998; Endres *et al.* 1998), reduction of oxidative stress (Balduini *et al.* 2001), inhibition of platelet activation (Rosenson 1999) and anti-inflammatory actions (Jain and Ridker 2005) seem to be implicated on statins' protective role after stroke.

In this study, we have focused on the benefits that statins can offer when administered during the acute phase of stroke. Although different animal models have previously been used to study the utility of statins on brain ischemia (García-Bonilla *et al.* 2012), the mechanisms of neuroprotection are less clear and are mainly attributed to endothelial nitric oxide synthase up-regulation (Laufs *et al.* 1998; Amin-Hanjani *et al.* 2001 and Laufs *et al.* 2000). We aimed to explore the neuroprotective effects of simvastatin in an embolic model of stroke, as it closely resembles the nature of human stroke and has yet to be adequately explored. Moreover, the effects of simvastatin on different brain areas were evaluated by means of proteomics. Therefore, this will be the first systematic study using hypothesis-free system biology tools to test the global effect of simvastatin administered in the hyperacute phase of the ischemic event. Our data are likely to corroborate the idea of stroke patients being able to benefit from simvastatin treatment at very early time points, in an ambulance, for instance, before arriving at the hospital.

Materials and methods

All procedures were approved by the Ethics Committee of the Vall d'Hebron Research Institute (protocol number 02/09) and were conducted in compliance with the Spanish legislation and in accordance with the Directives of the European Union. Experiments were performed in male Wistar rats (300–325 g; Charles River Laboratories Inc., Wilmington, MA, USA). Rats were kept in a climate-controlled environment on a 12-h light/12-h dark cycle. Food and water were available *ad libitum* and analgesia (Magnesic methamizol, Boehringer Ingelheim, España) was given to all rats every 24 h from the first until the last day of the experiment protocol to minimize their pain and discomfort.

Animal model

Infarction in the territory of the middle cerebral artery (MCA) was induced trough a clot injection as described in detail elsewhere (Zhang *et al.* 1997). Animals were anesthetized under spontaneous respiration with 2% isoflurane (Abbot Laboratories, Kent, UK) in oxygen during surgery and body temperature was maintained at 37°C. Arterial blood from a donor rat was withdrawn to form two identical clots (length: 1.5 cm; diameter: 0.3 mm, each). Continuous laser–Doppler flowmetry (Moor Instruments, Devon, UK) was used to monitor regional cerebral blood flow and only animals that exhibited a reduction > 75% in regional cerebral blood flow during middle cerebral arteria occlusion (MCAO) were included in the study.

Experimental design

A total of 77 animals were needed to obtain all data. For the study of simvastatin efficacy 55 animals were used. From these, 19 animals were excluded after applying the following criteria: inappropriate occlusion of the middle cerebral artery after embolization (n = 10); spontaneous reperfusion within 10 min of occlusion (n = 4), death during the following minutes after the occlusion (n = 2), or poor cerebral blood flow register (n = 3). Sixteen animals died before the completion of the experimental protocol (12 during the first 24 h and 4 between 24 and 48 h after the occlusion).

For the proteomic study, four animals per group were evaluated. To that end, 22 animals were needed. Seven animals were excluded following the same criteria: inappropriate occlusion (n = 3), spontaneous reperfusion (n = 3), and death just after the occlusion (n = 1). Seven animals died during the experimental protocol (six during the first 24 h and one at 48 h post-ischemia). All animals were subjected to MCAO and randomly allocated to experimental groups (simvastatin or vehicle) using a computer-generated randomization list. One mL of Simvastatin solution (20 mg/kg (diluted in vehicle); Uriach Laboratories, Barcelona, Spain) or 1 mL of vehicle was subcutaneously injected 15 min after occlusion in a blinded manner. Simvastatin was prepared by opening the lacton ring and activating it. In short, simvastatin was dissolved in vehicle [distilled H_2O (75%), absolute ethanol (10%), and 0.1 M NaOH (15%)], incubated at 50°C for 2 h and pH adjusted at 7.2.

Neurological deficit

Rats were assessed by a blind experimenter using a 9-point neurological deficit scale (modified Bederson test), as previously described (Perez-Asensio *et al.* 2005). 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) parachute reflex (symmetrical = 0, asymmetrical = 1, contralateral forelimb retracted = 2), and (iv) resistance to left forepaw stretching (stretching not allowed = 0, stretching allowed after some attempts = 1, no resistance = 2). Neurological score was assessed at 90 min, 24 and 48 h after occlusion.

Infarct volume

Infarct volume was evaluated by an investigator blinded to the treatment using thionin stain. Animals were killed and transcardially perfused with Paraformaldehyde 4%. Brains were removed and fixed during 24 h in Paraformaldehyde 4% at 4°C and immersed in sucrose for another 24 h also at 4°C. Afterward, brains were embedded in OCT and placed at 80°C. Coronal brain sections (30 μm) were cryostat-cut, mounted on slides and stained with thionin. Non-injured areas were stained in deep blue, whereas ischemic areas remained pale. Images of the stained slices were captured using a CanoScan 4200F scanner (Canon USA Inc., New York, NY, USA) and infarct, contralateral (CL) and ipsilateral (IP) areas were outlined and quantified using an image analysis system (Scion Image v4.02, Scion Corporation, Frederick, MD, USA). Infarct volume was measured by integration of infarcted areas and expressed as a percentage of the IP hemisphere. Edema correction of infarct volume was evaluated taking into account the following equation: volume correction = (infarct volume × CL volume)/IP volume.

Brain tissue samples

To maintain brain tissue structure for the proteomic study, whole brains from a second group of rats were used to obtain brain homogenates as described below. Owing to infarct volume, calculation was not possible in this set of animals, only those that showed a reduction > 75% in regional cerebral blood flow and a neurological score > 3 at 90 min and ≥ 2 at 24 and 48 h were included in the study. The inclusion criteria applied guaranteed that all the rats included in the study suffered an ischemic episode. Rats were transcardially perfused with cold saline and brains were removed and divided into four areas after discarding the hippocampus: cortex IP, cortex CL, striatum IP, and striatum CL. Each separated area separately was snap frozen in liquid nitrogen.

Frozen samples were homogenized using cold difference in gel electrophoresis (DIGE) lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris, pH 8.5), for protein extraction. After sonication and centrifugation at 12 000 *g* for 5 min at 4°C, the cleared protein extract was further purified by a modified trichloroacetic acid–acetone precipitation (2-D-CleanUp kit, Amersham Biosciences, Munich, Germany) and re-dissolved in DIGE lysis buffer. Protein concentration was determined using the Bio-Rad RCDC Protein Assay (Bio-Rad, Hercules, CA, USA).

Two-dimensional fluorescence difference in gel electrophoresis

Samples of each experimental condition (cortex or striatum and IP or CL) and a pool consisting of equal protein amounts of each of these samples were analyzed by two-dimensional fluorescence DIGE technology. The pool was prepared to be used as an internal standard for quantitative comparisons as described (Alban *et al.* 2003). Samples were labeled with Cy3 or Cy5 cyanine dyes; internal standard pooled sample was labeled with Cy2 dye, by the addition of 400 pmol of Cy dye in 1 μL of anhydrous *N*,*N*-dimethylformarnide per 50 μg of protein. To avoid possible bias introduced by labeling efficiency, the samples from each group were alternatively labeled with both Cy3 and Cy5 dyes. After 30-min incubation on ice in the dark, the reaction was quenched with 10 mM lysine and further incubated for 10 min. Samples were finally combined according to the experimental design, at 50 μg of protein per Cy dye per gel, and diluted 2-fold with Isoelectric Focosing IEF sample buffer (7 M urea, 2 M thiourea, 4% wt/vol CHAPS, 2% dithiothreitol, 2% pharmalytes pH 3–10 and 0.002% bromophenol blue). The two-dimension electrophoresis was performed using GE Healthcare reagents and equipment (Buckinghamshire, UK). First dimension isoelectric focusing was performed on immobilized pH gradient strips (24 cm; linear gradient pH 3–10) using an Ettan-immobilized pH gradientphor system (GE Healthcare Europe, Freiburg, Germany). First, strips were incubated overnight in 450 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% wt/vol CHAPS, 1% pharmalytes pH 3–10, 100 mM DeStreak and 0.002% bromophenol blue). Then, samples were applied via cup loading near the acidic end of the strips. After focusing at a global voltage of 67 kV, strips were equilibrated first for 15 min in 6 mL of reducing solution [6 M urea, 100 mM Tris–HCl pH 8, 30% vol/vol glycerol, 2% wt/vol sodium dodecyl sulfate (SDS), 5 mg/mL dithiothreitol, and 0.002% bromophenol blue] and then in 6 mL of alkylation solution (6 M urea, 100 mM Tris–HCl pH 8, 30% vol/vol glycerol, 2% wt/vol SDS, 22.5 mg/mL iodoacetamide, and 0.002% bromophenol blue) for 15 min, on a rocking platform. Second-dimensional SDS–polyacrylamide gel electrophoresis were run by overlaying the strips on 12.5% isocratic Laemmli gels (24 × 20 cm), casted in low fluorescence glass plates on an Ettan DALT six system (GE Healthcare Europe). Gels were run at 20°C at constant power 2.5 W per gel for 30 min followed by 17 W per gel until the bromophenol blue tracking front reached the end of the gel. Fluorescence images of the gels were acquired on a Typhoon 9400 scanner (GE Healthcare). Cy2, Cy3, and Cy5 images were scanned at 488/520, 532/580, and 633/670 nm excitation/emission wavelengths, respectively, at a 100 μm resolution. Image analysis and statistical quantification of relative protein abundances were performed using Progenesis SameSpots software (Non-linear Dynamics Limited, Newcastle upon Tyne, UK).

Protein identification by mass spectrometry

To investigate treatment effects on protein expression in ischemic animals, brain samples obtained from ischemic IP and CL hemispheres of both cerebral cortex and striatum from simvastatin-treated ischemic animals (Simv) and vehicle-treated ischemic animals (Veh) as control, were resolved by two-dimensional DIGE gels. The samples were analyzed by pairs of simvastatin and control samples mixed with the pooled internal standard. These pairs of samples were used for comparisons in the differential protein expression analysis. The selected comparisons for protein expression analysis were as follows: ipsilateral hemisphere from simvastatin-treated animals (Simv-IP) versus vehicle-treated animals (Veh-IP); contralateral hemisphere from simvastatin-treated animals (Simv-CL) versus vehicle-treated animals (Veh-CL); ipsilateral versus contralateral hemisphere from vehicle-treated animals (Veh-IP and Veh-CL, respectively); and ipsilateral versus contralateral hemisphere from simvastatin-treated animals (Simv-IP and Simv-CL, respectively). All comparisons were made in both cerebral cortex (C) and striatum (S). The differential protein expression analysis of two-dimensional DIGE gels was performed in samples from four different animals. Spots present in four of four animal samples per group, with significant ANOVA test ($p < 0.05$) and an averaged ratio > 1.3 between comparisons, were considered and selected for protein identification by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS).

Protein spots were excised from the gel using an automated Spot Picker (GE Healthcare). In-gel trypsin digestion was performed as described (Shevchenko *et al.* 1996) using autolysis stabilized trypsin (Promega, Madison, WI, USA). Tryptic digests were purified using ZipTip microtiter plates (Millipore, Cork, Ireland). MALDI-TOF MS analysis of tryptic peptides was performed on an Ultraflex TOF-TOF Instrument (Bruker, Bremen, Germany). Samples were prepared using Prespotted AnchorChip (PAC96) target with alpha-cyano-4-hydroxycinnamic acid matrix for 96 sample spots and 24 calibration spots (Bruker Daltonics). The spectra were processed using Flex Analysis 3.2 software (Bruker Daltonics). Identification of the proteins was carried out by peptide-mass fingerprinting data from MALDI-TOF MS. Database searches were performed using the MASCOT program (Matrix Science, London, UK). Protein identification was confirmed by TOF-TOF post-source decay fragmentation spectra or by ion trap mass spectrometry as described (Esselens *et al.* 2008).

Validation by western blot

With the same samples previously used in DIGE analysis, validation of DIGE and MS results of selected proteins in brain

homogenates was performed by western blot. Briefly, equal protein amounts (35 μg) were resolved by 5% or 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Separated proteins were transferred to a polyvinylidene difluoride membrane using a mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad Laboratories) for 1 h at 100 V. Non-specific bindings were blocked with 10% non-fat milk and 0.05% tween-phosphate buffer saline before membranes were incubated overnight at 4°C with the following antibodies: mouse anti-Guanin nucleotide-binding-protein (Go protein alpha, 1 : 3000, catalog no.ab78218; Abcam, Cambridge, UK), mouse anti-heat-shock protein 60 (HSP60, 1 : 100, catalog no. sc-376240; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-HSP75 (1 : 200, catalog no sc-135944; Santa Cruz Biotechnology), mouse anti-HSP70 (1 : 1000; Calbiochem, Dramstadt, Germany), rabbit anti-Hemoglobin α (H-80, 1 : 1000, catalog no. sc-21005; Santa Cruz Biotechnology), rabbit anti-Dynamin (H-300, 1 : 200, catalog no. sc-11362, Santa Cruz Biotechnology), rabbit anti-DRP-2 (1 : 200, catalog no. sc-30228; Santa Cruz Biotechnology), rabbit anti-Alpha Fodrin (Spectrin α, 1 : 1000, catalog no. ab75755; Abcam) in 10% non-fat milk. Secondary antibodies (Chemicon International, Inc, Rockford, IL, USA) anti-rabbit-horseradish peroxidase or anti-mouse-horseradish peroxidase, were diluted 1 : 2000 in 10% non-fat milk and incubated at 25°C for 1 h. Before and after incubations, membranes were washed three times (10 min each) with 0.05% tween-phosphate buffer saline. The substrate reaction was developed with chemiluminescent reagent Luminol (Amersham Biosciences, GE Healthcare) and visualized with a luminescent image analyzer (Las-3000; Fujifilm Medical Systems, Stamford, CT, USA). Scanned western blots were quantified with the image Quantity One software package (Bio-Rad Laboratories). All protein levels were corrected by a loading control detected using the antibody mouse anti-Actin (1 : 20 000, catalog no. A1978, Sigma-Aldrich, St Louis, MO, USA) except for Guanin Go. Levels, which was corrected using anti-mouse-αTubulin (1 : 1000, catalog no,T6199; Sigma-Aldrich).

HSP75 protein level in stroke patients' plasma

A pilot study was performed to explore in human plasma from stroke patients the changes found in HSP75 in the proteomic study. Blood samples from stroke patients included in a clinical trial (Montaner *et al.* 2008) who received simvastatin or placebo at 3–12 h from symptom onset at an initial dose of 40 mg/day for the first week were used. Samples were drawn in citrate tubes just before treatment administration and plasma fractions were obtained by centrifugation (1500 g during 15 min at 4°C) and stored at 80°C until analysis was performed. A sandwich enzyme immunoassay (Human HSP75 kDa, mitochondrial TRAP1/HSP75 ELISA kit; Cusabio Biotech CO., LTD, Wuhan, China) was performed according to manufacturer's instructions. Optical density was detected with a microplate reader (Synergy Mx, Biotek, China). Each sample was assayed twice. If intra-assay coefficient of variation (CV) was < 20%, the mean value of both measurements was used in the statistical analysis, whereas samples with a CV > 20% were excluded from the analysis. Only patients with a complete temporal profile: basal time (before treatment administration), third day and seventh day after stroke, were included in the analysis (placebo = 5 patients, simvastatin = 6 patients). All results were expressed as a percentage of basal values. Clinical and demographic characteristics of the included patients were taken into consideration and are shown in Table S1 (Supporting Information).

Statistical analysis

Statistical significance for intergroup differences was assessed by ANOVA followed by Bonferroni post hoc test using Progenesis SameSpots software (Non-linear Dynamics Limited). Statistical significance for quantified data from western blots was analyzed by Student's *t*-test using GraphPad Prism_v5 software. For non-parametric data, Mann–Whitney test, Kruskal–Wallis test, and Friedman test followed by Dunn's Multiple Comparison test were assayed. Bars represent mean ± SD for parametric data and box plots represent median (IQR) for non-parametric data. For all data, a $p < 0.05$ was considered statistically significant at a 95% confidence level.

Results

Infarct lesion, neurological deficit, and mortality

Animals treated with simvastatin 15 min after the occlusion showed a 43% reduction on the infarct lesion measured 48 h post-ischemia compared to vehicle-treated animals [21.95 (15.44, 42.95)% for vehicle vs.12.92 (10.24, 19.63)% for simvastatin, $p < 0.05$] (Fig. 1a, b).

Neurological deficit assessed by the Bederson test, at 90 min post-occlusion, showed no differences between groups. At 24 h, simvastatin-treated rats scored lower [6 (5, 7) for vehicle vs. 5.75 (4, 7) for simvastatin], although differences were not significant. However, at 48 h post-occlusion, rats treated with simvastatin showed a 35% significant improvement in neurological behavior [5.50 (4.25, 6) vs. 3 (1.25, 5.75), $p < 0.05$] (Fig. 1c).

Mortality rates were high, as already reported for this MCAO model (Lapergue *et al.* 2010; Hernández-Guillamon *et al.* 2010), but no significant differences were found between vehicle- and simvastatin-treatment groups (43.75% vs. 42.86%, respectively) Fig. 1d.

Protein expression profile in simvastatin-treated animals

Brain samples of each experimental condition (cortex or striatum of IP or CL hemispheres) were analyzed following the experimental design showed in the flow chart (Fig. 2). In the two-dimensional gels we detected 186 protein spots present in all samples (Fig. 3a). We found a total of 74 spots with significant expression differences which were further analyzed by MALDI-TOF MS. Among them, 38 spots and 27 different proteins were identified, as detailed in Table S2 Supporting Information. For expression analysis, only spots present in all samples (4 per treatment group), showing a significant *p* value ($p < 0.05$) and fold change> 1.3 between groups were selected for protein identification by MALDI-TOF MS (Fig. 3b). The effect of simvastatin on the ischemic brain was analyzed by the comparison of the Simv-IP group with respect to the Veh-IP group, in cerebral cortex (Fig. 4A; Simv-IP vs. Veh-IP) and striatum (Fig. 4B; Simv-IP vs.

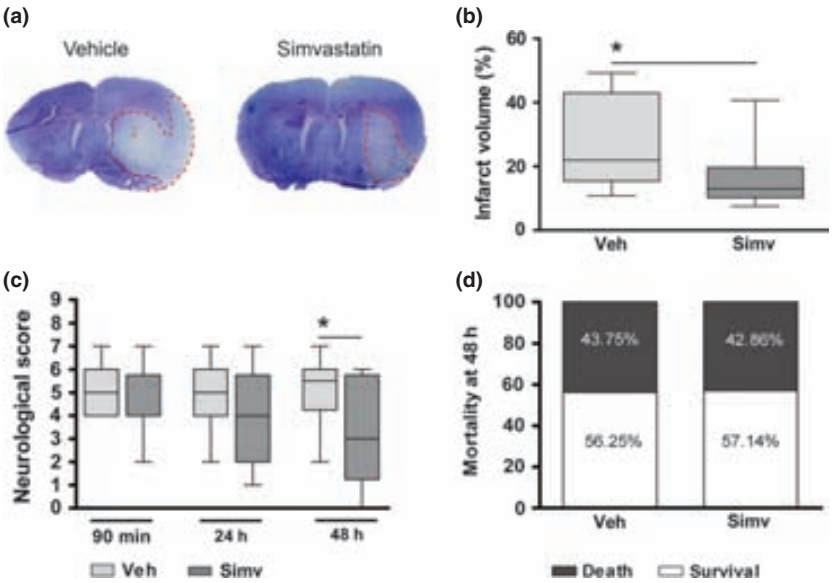

Fig. 1 (a) Representative images of infarcted brain from vehicle-treated rat and simvastatin-treated rat, stained with thionin 48 h after middle cerebral arteria occlusion (MCAO). (b) Animals receiving simvastatin showed a significant reduction on infarct volume when compared with animals receiving vehicle ($p < 0.05$). Bars represented the mean ± SD.; vehicle ($n = 9$), simv ($n = 11$). (c) Neurological test assessment showed no differences between animals 90 min after the ischemia. At 24 h, simvastatin group showed less deficit ($p = 0.09$). A statistical difference between vehicle- and simvastatin-treated animals ($p < 0.05$) was observed at 48 h post-ischemia, (d) Mortality at 48 h after ischemia showed no differences between groups. *$p < 0.05$.

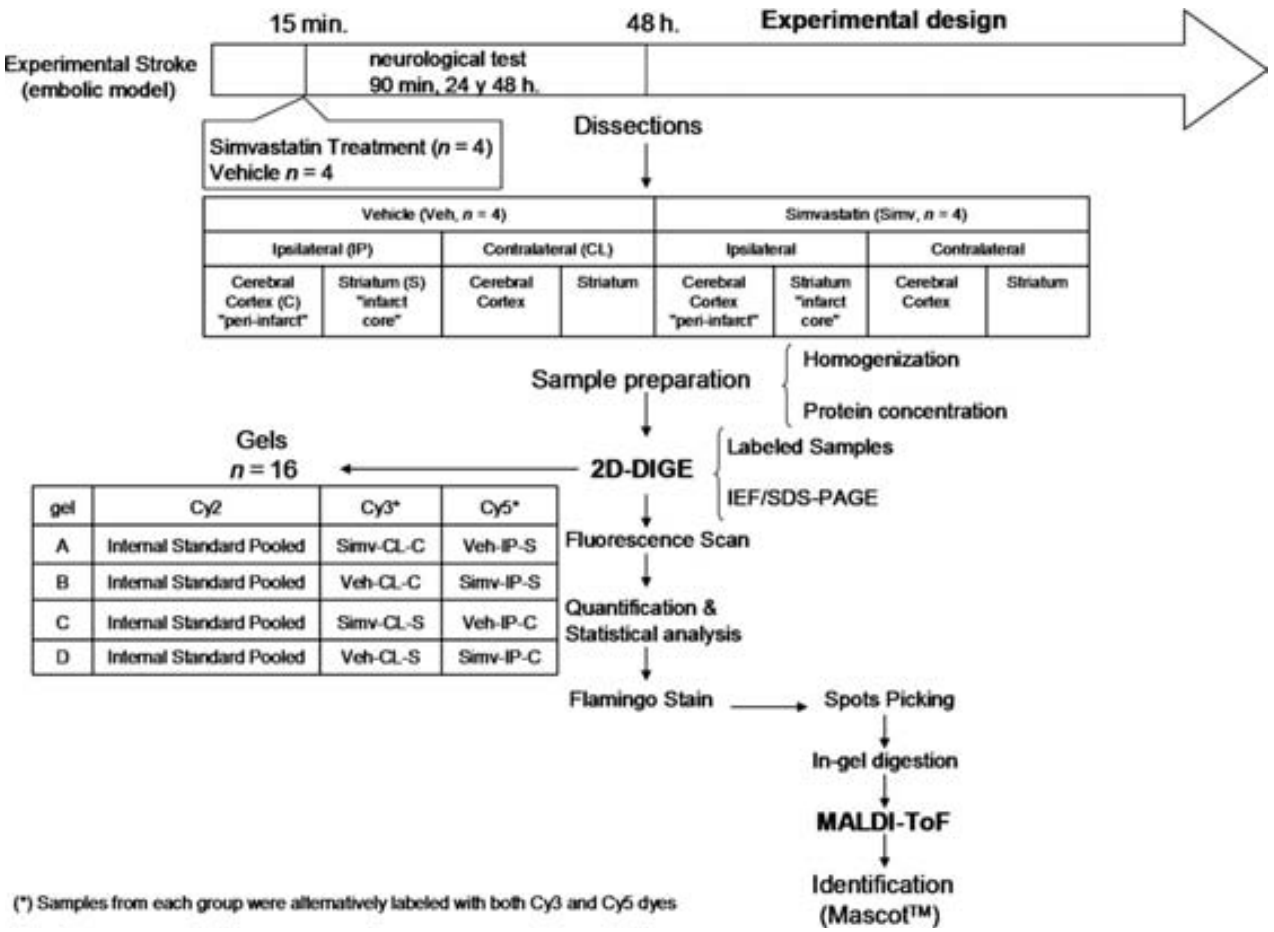

Fig. 2 Flow chart of the experimental model. Preparation and quantification of the sample and identification strategy. A to D correspond to the indicated pair of simvastatin and control samples labeled with Cy3- and Cy5-dyes, mixed with internal standard and run on the respective two-dimensional gel.

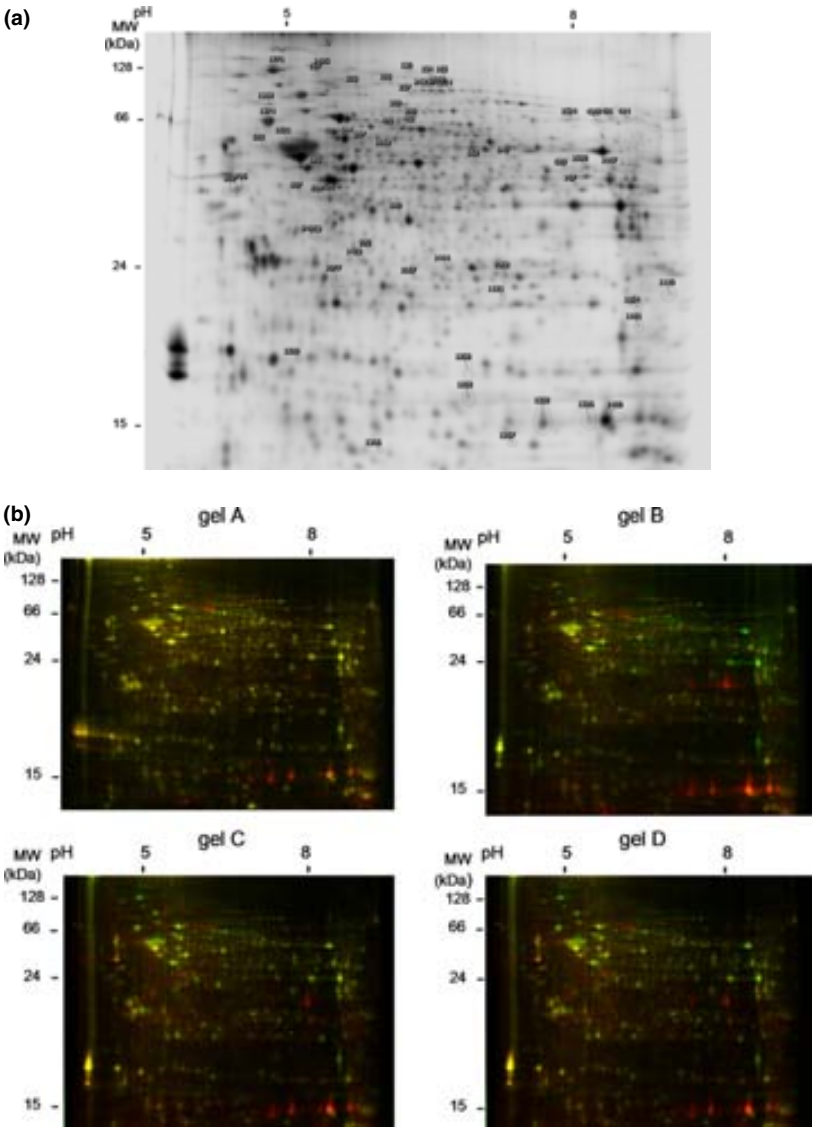

Fig. 3 Representative two-dimensional difference in gel electrophoresis (DIGE) gels of brain homogenates. (a) Representative pooled internal standard DIGE gel Cy2 uorescence image with labeled protein spots selected for matrix-assisted laser desorption/ionization-time of ight (MALDI-TOF) MS analysis. The range of the horizontal dimension is isoelectric point (from pI = 3 to 10); the range of the vertical dimension is molecular weight (from 150 to 15 kDa). (b) Superimposed images in pseudocolor from Cy3 (green)- and Cy5 (red)-labeled samples run on two-dimensional DIGE gels. The images shown are representative of each pair of Cy-labeled samples ($n = 4$) run on the respective gel A to D (see Fig. 2).

Veh-IP). The differential protein expression analysis in cerebral cortex detected nine spots with significant differences and five proteins were successfully identified. The results showed higher induction of the alpha subunit of guanine nucleotide-binding protein Go (2 isoforms), and decreased levels of mitochondrial 60 kDa HSP60 and fumarate hydratase (2 isoforms d). In striatum, eight spots were detected with significant differences and two proteins were identified; microtubule-associated protein RP/EB showed higher levels in the Simv-IP group, whereas the 75 kDa HSP75 presented lower levels in this group of animals.

The effect of simvastatin on non-ischemic tissue was analyzed when compared the Simv-CL group with the Veh-CL control group, in cerebral cortex (Fig. 4A; Simv-CL vs. Veh-CL) and striatum (Fig. 4B; Simv-CL vs. Veh-CL). The differential protein expression analysis in cerebral cortex detected four spots corresponding to two proteins. The result showed higher levels of the beta subunit of mitochondrial ATP synthase, and decreased expression of fumarate hydratase in Simv-CL group. In striatum, four spots corresponding to two proteins were detected; the 70 kDa HSP70 presented higher levels in Simv-CL, whereas phosphoglycerate mutase 1 presented lower levels in the same group.

The effect of ischemia in vehicle-treated animals was studied by comparing the changes in protein expression of the Veh-IP group with respect to the Veh-CL control. In cerebral cortex, 16 spots were detected and eight proteins were successfully identified (Fig. 4A; Veh-IP vs. Veh-CL). The results showed higher levels of hemoglobin alpha and beta, and serine protease inhibitor A3N (Serpin A3N), and decreased levels of dynamin 1 (detected in three isoforms), pyruvate carboxylase, and hexokinase 1. In striatum, 10 spots were detected and eight proteins were identified (Fig. 4B;

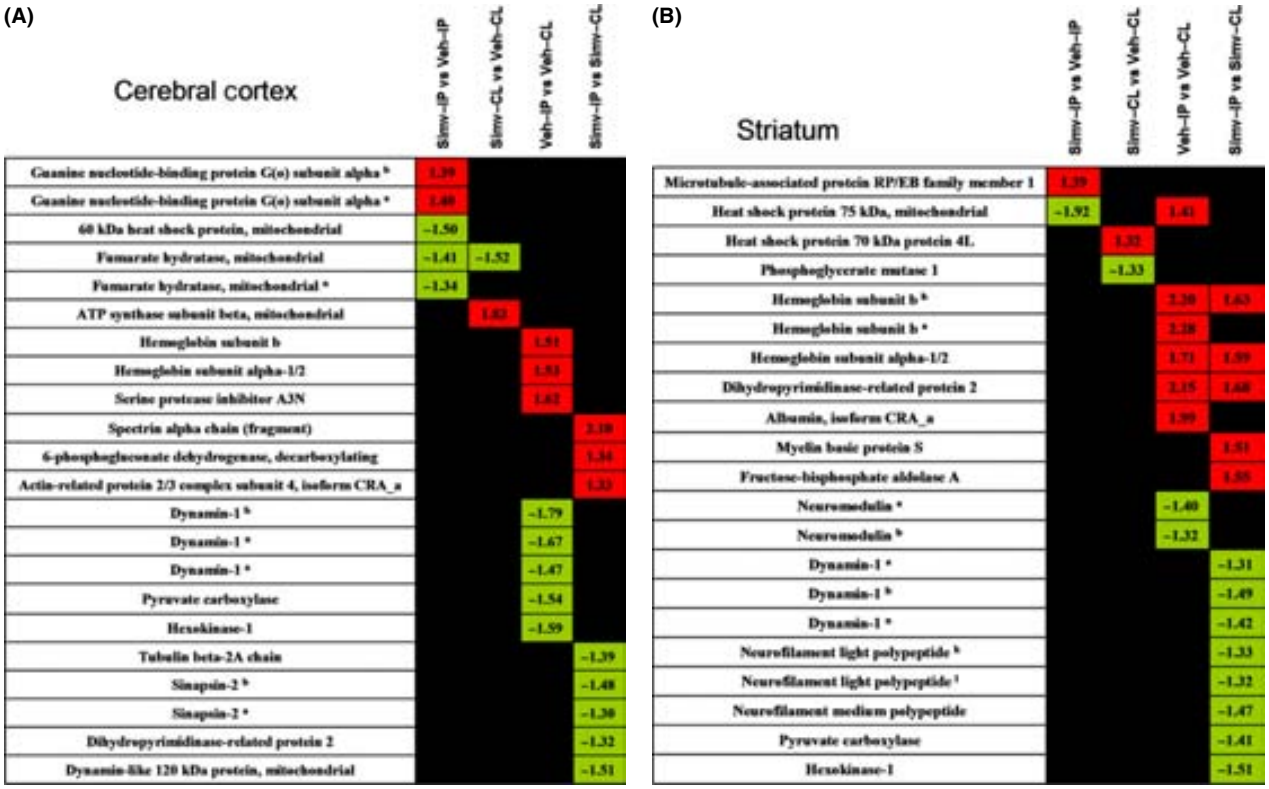

Fig. 4 Changes on protein expression in brain samples from (A) cerebral cortex (the peri-infarct region) and (B) striatum (the infarct core region), of ipsilateral (IP) hemisphere or contralateral (CL) hemisphere as control, from simvastatin-treated ischemic animals (Simv) and vehicle-treated ischemic animals (Veh). Only changes statistically significant by ANOVA and Bonferroni post-test ($p < 0.05$) of identified proteins were represented. Color indicates increased (red) or decreased (green) protein expression for each comparison, and the numbers indicate the increased (ratio) or decreased (-ratio[exp]-1) change for each comparison. (a), (b) and (n) correspond to acid, basic and neutral isoform, respectively. (h) and (l) indicate higher and lower molecular weight isoform, respectively.

Veh-IP vs. Veh-CL). Among them, albumin, hemoglobin alpha and beta (2 isoforms), dihydropyrimidinase-related protein 2 (DRP2), and HSP75 were detected presenting higher levels in Veh-IP, whereas neuromodulin/GAP43 (2 isoforms) was identified with decreased levels.

Finally, the effect of simvastatin was studied in the ischemic groups by comparing the protein changes in the Simv-IP group with respect to the Simv-CL control. In cerebral cortex, 15 spots were detected and eight proteins successfully identified (Fig. 4A; Simv-IP vs. Simv-CL). The results showed higher induction of alpha spectrin (fragment), 6-phosphogluconate dehydrogenase decarboxylating and actin-related protein 2/3 complex subunit 4, and decreased levels of DRP2, synapsin 2 (2 isoforms), beta tubulin 2A and dynamin-like 120 kDa protein. In striatum, 20 spots were detected and 13 proteins were identified (Fig. 4B; Simv-IP vs. Simv-CL). Hemoglobin alpha and beta, DRP2, in addition to myelin basic protein S (MBP-S) and fructose-bisphosphate aldolase A, were detected showing higher levels in Simv-IP; whereas dynamin 1 (3 isoforms), neurofilament L (NF-L, 2 isoforms), neurofilament M (NF-M), pyruvate carboxylase, and hexokinase 1 presented lower levels.

Once the proteins were identified, they were classified and grouped into functional role according to data base of Pathway Commons, Pather, and NCBI (Fig. 5). Proteins showed specific functions in the nervous system (DRP2, dynamin 1, dynamin-like 120 kDa protein, MBP-S, neuromodulin, and synapsin 2); another six were cytoskeleton proteins (actin-related protein 2/3 complex subunit 4,

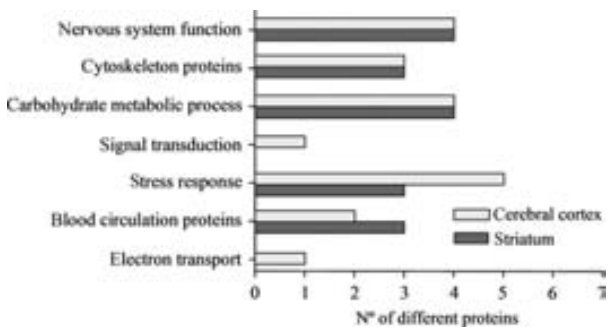

Fig. 5 Functional distribution of identified proteins with changes in protein expression using functional categories, according to brain localization.

microtubule-associated protein RP/EB, NF-L, NF-M, alpha spectrin, and beta tubulin 2A); five were stress–response-related proteins (HSP60, HSP70, HSP75, fumarate hydratase and Serpin A3N); five belonged to the carbohydrate metabolic process (6-phosphogluconate dehydrogenase decarboxylating, fructose-bisphosphate aldolase A, hexokinase 1, phosphoglycerate mutase 1, and pyruvate carboxylase); three proteins were from blood circulation (albumin and hemoglobin alpha and beta); one was a signal-transduction protein (the guanine nucleotide-binding protein Go);and one was an electron transport protein (the ATP synthase).

Then, the changes observed in some of the proteins identified by MALDI-TOF MS were also evaluated by western blot. Among the 27 proteins differentially expressed between groups, eight (corresponding to 13 comparisons) were chosen regarding antibody availability, fold change and functional role. From these 13 comparisons, seven were found in the same expression-direction although differences did not reach statistical significance, whereas the other six could not be validated (Figures S1 and S2).

Levels of HSP75 in stroke plasma samples

Next, to study the clinical impact of our study performed using a rat stroke model, we tested the levels of one of the proteins, HSP75, which showed relevant lower levels in simvastatin-treated ischemic brains. In our cohort of stroke patients we observed that, while patients who received placebo did not show significant differences at several time points in terms of HSP75 plasma concentration, patients receiving simvastatin after stroke, presented lower levels at third and seventh day after the stroke compared to basal HSP75 level ($p < 0.05$) (Fig. 6).

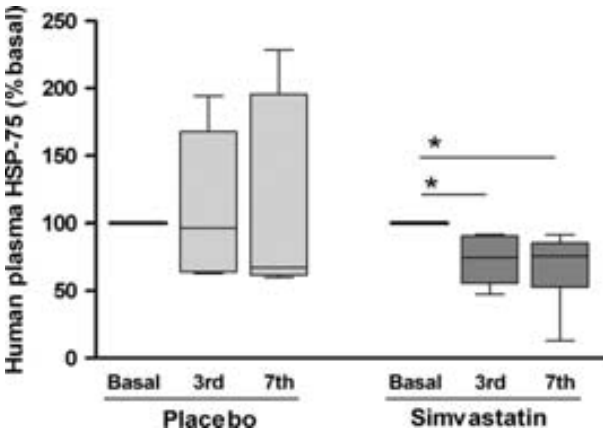

Fig. 6 Analysis of HSP75 in stroke patient's plasma by ELISA assay. Blood was drawn at basal time (before treatment administration) third and seventh day after stroke onset. Graph represents the increase or decrease in HSP75 concentration in plasma respect to basal time in each group. Only patients treated with simvastatin, showed a significant decrease in plasma concentration when comparing both third and seventh day respect to basal time ($p = 0.028$ and $p = 0.028$, respectively, after Bonferroni correction, $p = 0.056$ in both cases). Each point represents a stroke patient. Horizontal bars represent the mean (placebo, $n = 5$, simvastatin $n = 6$). *$p < 0.05$.

Discussion

Our results clearly show neuroprotective effects of simvastatin on brain ischemia when administered in a single dose (20 mg/kg) during the acute phase. The dose was selected according to previously published meta-analysis (García-Bonilla *et al.* 2012) which determined that the typical range in *in vivo* rodent studies was 10–100 mg/kg/day. Although the dose chosen differs with the one used in human studies (40 mg/day), we are of the opinion that more studies are needed to clarify the extrapolation of statin doses between human and rodent studies.

Despite our efforts in reducing the pain and discomfort of the rats subjected to the embolic stroke, the mortality associated to this experimental model was around 40%, as shown in other studies (Hernández-Guillamon *et al.* 2010). This high mortality rate, which is considered a limitation of this study, is on account of the severe brain injury and some secondary complications such as weight loss.

Previous studies, compiled in a recent meta-analysis (García-Bonilla *et al.* 2012), reflected statins' neuroprotection effect in both infarct size reduction and neurological deficit improvement in the short term. However, to our knowledge, only a recent study (Pirzad Jahromi *et al.* 2012), has tested simvastatin efficacy after ischemia in an embolic model, the one that best resembles human stroke (Carmichael 2005). Previous investigations conducted with the intraluminal model showed a lesion reduction of 15% administrating a very low dose of simvastatin (1 mg/kg) (Chen *et al.* 2003) and up to 45.62% or 30.41% infarct reductions when higher doses of simvastatin were administered (20 and 40 mg/kg), respectively (Laufs *et al.* 1998; Nagaraja *et al.* 2006). This beneficial effect was explained, at least in part, by one of the main strengths of statins: their ability to up-regulate endothelial nitric oxide synthase expression and activity, promoting a cerebral blood flow increase that leads to a cerebral infarct size reduction (Kureishi *et al.* 2000). It has also been reported that statins interfere with the coagulation system by up-regulating tissue-type plasminogen activator (tPA) and down-regulating plasminogen activator inhibitor (PAI-1) (Essig *et al.* 1998). Moreover, statins alter the inflammatory response to ischemia, by minimizing the inflammatory components of the ischemic cascade and reperfusion injury. They also modulate the immune responses by the recruitment, differentiation and secretory activity of monocytes, macrophages, and T cells (Palinski and Tsimikas 2002).

Because of statins pleiotropic effects, other proteomic approaches have been carried out so as to explore their effect in different cells or tissues. Despite our study being the first, to our knowledge, to explore statins' neuroprotective effect after

cerebral ischemia, the conclusions of some of these other papers may also contribute to the understanding of this mechanism. Pienaar *et al.* (2009), for instance, evaluated rats' substantia nigra and reported that statins may prevent the increase in oxygen-free radical productions. Gu *et al.* (2012), after studying the translocation of lipid-raft-related proteins in endothelial cells, revealed an up-regulation on anti-oxidative proteins, as well as a down-regulation on proteins associated with inflammation and cell adhesion. Brioschi *et al.* (2013) analyzed endothelial cells and demonstrated that statins influenced the thrombogenic response of the vessel wall as well as thrombotic factors in blood.

In our study, however, we sought to study the brain proteomic changes after a stroke embolic model and explore the effect that simvastatin may exert on different ischemic brain regions (striatum and cortex) and in healthy tissue (contralateral brain hemisphere).

Apart from the neuroprotection study, brains of four animals per treatment group were analyzed through proteomics technique. Although this low sample size can be assumed as a limitation of the study, we could observe appealing results. The time point of the analysis (48 h after the ischemia) was chosen in accordance with neuroprotection study results, which showed that infarct lesion was well established and that simvastatin had exerted its protection at this time point. Interestingly, we have found different protein expression changes considering the analyzed area, possibly because of the distinct effects that neuroprotectants are described to induce on cortex and striatum after submitting the rats to an eMCAO (Wang *et al.* 2001). In addition, regarding simvastatin effect on infarct size, we cannot rule out that the differences on protein expression were in partly explained by the smaller infarct lesion.

First, our data show simvastatin influencing cell signaling by up-regulating the levels of the guanine nucleotide-binding protein Go, and reducing cellular stress by decreasing the HSP60 and the fumarate hydratase. As described by others (Worley *et al.* 1986 and Neves *et al.* 2002), guanine nucleotide-binding protein Go seems to play an important role in signal transduction by modulating adenilate cyclase (Huff *et al.* 1985) and coupling to ion channels (Pfaffinger *et al.* 1985) whereas HSP60 is reported to be at high levels after many stressors, and to be indicative of mitochondrial biogenesis (Yin *et al.* 2008). Thus, both actions would be helpful for recovery from ischemic damage in our system. Our results are in accordance with other studies which conclude that simvastatin therapy is associated with significant reduction of serum HSPs antibody titers in general (Moohebati *et al.* 2011) and plasma HSP60 antibody titers in particular (Ghayour-Mobarhan *et al.* 2005). These studies claim that HSPs levels decrease may be modulated by simvastatin through its immunomodulatory properties.

Second, the effect of simvastatin into the striatum, showed an increase of the microtubule-associated protein RP/EB (MAP1) and a decrease in the HSP75 concentration. Both processes are indicative of cell reparation and lower stress induction, respectively, and are useful for tissue preservation. MAP1, a structural microtubule-associated protein, was found to be highly expressed in developing neurons playing an important role in neurite and axon extension (Tortosa *et al.* 2013). Regarding HSP75, it has been previously published that levels of mitochondrial HSP75 are high during ischemia conditions (Kiang and Tsokos 1998). Other authors, however, showed that Hsp75 levels decreases reactive oxygen species production and preserves mitochondrial membrane potential during glucose deprivation in astrocyte cell cultures (Volobouéva *et al.* 2008). Overall, as simvastatin-treated rats showed a less severe brain injury, we can infer that lower HSPs levels are needed to exert neuroprotection. Moreover, an ischemic insult involving immunological mechanisms such as autoimmune reaction against HSPs could be mitigated through immunomodulatory statin effect on lymphocyte function (Guisasola *et al.* 2009).

Third, the effect of simvastatin in contralateral hemispheres revealed that only four proteins show significant differences regarding its concentration levels, in both cerebral cortex and striatum regions. These results may indicate a limited effect of simvastatin administered acutely in the non-ischemic tissue, although we noted that simvastatin induces HSP70 expression in healthy striatum.

On the other hand, when we studied the effect of ischemia in simvastatin-treated animals we detected 15 and 20 spots in cerebral cortex and striatum, respectively. In cerebral cortex, the increase of spectrin fragment may reflect possible neuroregeneration and repair in response to the degeneration caused by the ischemia (Aikman *et al.* 2006), whereas 6-phosphogluconate dehydrogenase decarboxylating up-regulation is indicative of glycolisis (Belfiore *et al.* 1975). Moreover, the increased levels of actin-related protein 2/3 complex subunit 4 would show cell reparation (Hetrick *et al.* 2013). In addition, the decrease in functional and structural neuronal proteins, such as synapsin 2, dynamin-like 120 kDa protein, and beta tubulin 2A, would indicate neuronal damage. Furthermore, the decreased concentration of DRP2 could show less significant ischemic injury, although some controversy exists about this protein as it has been reported to be up or down-regulated depending on its phosphorilation status (Zhou *et al.* 2008). In striatum, we identified blood circulation proteins, in addition to DRP2, fructose-bisphosphate aldolase A, and MBP-S. The latter may indicate developing of glial cells (Lashgari *et al.* 1990). In addition, the decrease in functional or structural neuronal proteins, such as dynamin 1, NF-L and –M, and proteins from carbohydrate metabolic process, such as pyruvate carboxylase and hexokinase 1, would point out again to neuronal damage (Julien and Biebuyck 1990).

Finally, we studied the effect of ischemia in un-treated animals. Sixteen and 10 spots in cerebral cortex and striatum, respectively, were detected and identified as blood circulation

proteins, hemoglobin alpha and beta, or albumin, in addition to the decrease of proteins essential in the CNS function or in the carbohydrate metabolic process as dymanin 1 and neuromodulin, or pyruvate carboxylase and hexokinase 1. Accordingly with previous proteomics approaches (Chen *et al.* 2007; Koh 2010) after cerebral ischemia in rat, in striatum it was detected increased levels of DRP2, a protein which was reported to be involved in neuronal differentiation and axonal guidance (Quinn *et al.* 1999) by promoting microtubule formation (Fukata *et al.* 2002) and HSP75, a stress response protein involved in mitochondria biogenesis (Bellizzi *et al.* 2009).

In summary, when comparing the effect of ischemia in simvastatin-treated animals respect to the un-treated animals, we found that in the infarct core region (striatum) the blood circulation proteins were significantly decreased or absent, such as hemoglobin beta or albumin (additional information is presented in Supporting information) as well as HSP75. Thus, the most important finding of this study was simvastatin capacity to attenuate stroke-induced changes in blood–brain barrier (BBB) permeability and to protect against cell stress and signal transduction stimulation. This statement is consistent with other *in vitro* (Kahles *et al.* 2007) and in vivo studies which concluded that statin BBB protection was associated with the abolition of isoprenylation processes (Law *et al.* 2006). In addition, it has experimentally been proved (Nagaraja *et al.* 2006) that rats receiving simvastatin 30 min after MCA occlusion had reduced volumes of AIB leakage at 6 h post-ischemia.

Moreover, when comparing this effect in the cortex region, the blood circulation proteins were absent, as well as Serpin A3N, an inflammatory response protein (Law *et al.* 2006). Simvastatin also decreased DRP2, a characteristic protein of ischemic damage (Chen *et al.* 2007). In addition to the protection against cell stress, simvastatin demonstrated again a protection against BBB damage in both regions, striatum and cortex (additional information is presented in Supporting information). All in all, BBB protection could lead to a milder inflammatory process as well as in a neurological improvement, as we could see in our experimental study.

Regarding global protein changes previously observed in brain rodents after an ischemic event, they seem to depend on the animal stroke model used and on evaluation time point. It is likely that the earlier the brains are evaluated, the more differences can be found. Chen *et al.* (2007) and Koh (2011) for instance, reported proteins up-regulation more than 3-fold 24 h after submitting rats to a permanent MCAO. Other studies performed in mice (Hori *et al.* 2012; Föcking *et al.* 2006) reported expression changes of 1.5-2 fold when evaluating at 1, 6, or 24 h after the ischemia. Nevertheless, and in accordance with our results, some proteins show up in most of the studies such as HSP's or DRP-2.

Furthermore, the validation study by western blot was performed with the same samples used in the proteomics study. This second assay tested only eight of the 27 proteins found by proteomics analysis and reflected that 53.85% of the analyzed comparisons coincided with previous results, meaning that they presented similar expression patterns than those found in the proteomic study, although no statistical significance was reached. This poor validation percentage could be attributed to the small sample size ($n = 4$) and also to the incapability of western blot technique to distinguish between different protein species (isoelectric point) of the same protein.

Considering the consistent fold change found in the proteomic assay, we moved one step forward with the analysis of HSP75 in plasma samples of stroke patients treated with simvastatin in the acute phase of stroke. Accordingly with our proteomics data, simvastatin-treated patients appear to have a more homogenous distribution and lower HSP75 plasma levels after stroke than placebo-treated patients. Related to the well-documented role of HSPs in maintaining cellular homeostasis in response to stress (Bellizzi *et al.* 2009) it has been published that both mitochondrial and cytosolic inducible HSPs are up-regulated during ischemic conditions (Kregel 2002). Moreover, and in agreement with our results, it has also been demonstrated that simvastatin therapy is associated with significant reductions in serum HSPs (Moohebati *et al.* 2011) owing to statins immunosupressive properties.

We assume this substudy entails some limitations such as the small sample size or the fact of analyzing only the HSP75 protein in human samples. Moreover, a better gender-matched between simvastatin and placebo-treated patients would have been useful to rule out the possibility of HSP75 levels being influenced by gender or other demographic factors.

All in all, however, it should be emphasized the importance of the concordant results found in both experimental and clinical assays, support embolic cerebral ischemia model as capable of reproducing human stroke conditions.

In conclusion, our study identifies, for the first time through proteomic techniques, the key proteins that are likely involved in the neuroprotective mechanisms of simvastatin. Although simvastatin seems to be a promising neuroprotective drug, clinical trials are needed to demonstrate its beneficial effects also in stroke patients in the hyperacute phase. In addition, the use of surrogate markers such as HSP75 may be useful in future studies.

Acknowledgments and conflict of interest disclosure

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2013) under grant agreements no. 201024 and no. 202213 (European Stroke Network). Neurovascular Research Laboratory takes part in the Spanish stroke research network INVICTUS (RD12/0014/0005) and the Proteomics Laboratory at VHIO belongs to the Spanish networked platform ProteoRed-ISCIII. This study was partially funded by projects FIS 11/0176 on stroke biomarkers research and EC07/90195 on increasing safety and efficacy of simvastatin neuroprotection.

All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. Demographic and clinic characteristics of the stroke patients included in HSP75 plasma analysis.

Table S2. List of proteins identified by MALDI-TOF MS from the selected spots.

Figure S1. Identified protein candidates validated by western blot.

Figure S2. Validation of differential gel electrophoresis (DIGE) results by WB.

Figure S3. Proteomic data represented with bars to show simvastatin effect on BBB protection.

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ARTICLE 3:

Combining statins with tissue plasminogen activator treatment after experimental and human stroke: a safety study on hemorrhagic transformation. *CNS Neurosci Ther.* 2013 Nov;19(11):863-70

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ORIGINAL ARTICLE

Combining Statins with Tissue Plasminogen Activator Treatment After Experimental and Human Stroke: A Safety Study on Hemorrhagic Transformation

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SUMMARY

Aims: Statins may afford neuroprotection against ischemic injury, but it remains controversial whether combined treatment with tissue plasminogen activator (tPA) after stroke increases the risk of hemorrhagic transformation (HT), the major tPA-related complication. We evaluated the safety of combining statin with tPA administration during the acute phase of both experimental and human stroke. **Methods:** The occurrence and severity of HT, infarct volume, and neurological outcome were evaluated in spontaneous hypertensive rats (SHR) subjected to embolic middle cerebral arterial occlusion (MCAO), which received vehicle or simvastatin (20 mg/kg), 15 min after ischemia and tPA (9 mg/kg) 3 h after ischemia. Additionally, HT rate was evaluated in stroke patients who were treated with tPA (0.9 mg/kg) within 3 h after symptom onset, considering whether or not were under statins treatment when the stroke occurred. **Results:** In the experimental study, no differences in HT rates and severity were found between treatment groups, neither regarding mortality, neurological deficit, infarct volume, or metalloproteinases (MMPs) brain content. In the clinical study, HT rates and hemorrhage type were similar in stroke patients who were or not under statins treatment. **Conclusion:** This study consistently confirms that the use of statins does not increase HT rates and severity when is combined with tPA administration.

Introduction

Stroke remains as a major cause of death and disability worldwide that contributes to the rising costs of health care [1]. Nowadays, the thrombolytic treatment with the tissue plasminogen activator (tPA) agent is the only existing therapy for the acute phase of stroke [2]. While high effectiveness on stroke outcome has been attributed to its use, tPA-treated stroke patients have a 10-fold higher risk of suffering an intracranial hemorrhage than untreated patients, which is fatal in about 3% of those patients [2–4]. Thus, the occurrence of hemorrhagic transformation (HT) after tPA treatment represents the main concern in the stroke acute therapy.

Animal studies have extensively shown that treatment with statin, including simvastatin, reduces the infarct volume and ameliorates the neurological deficit after experimental stroke [5,6]. These particular benefits in cerebral ischemia have not been attributed to the statin cholesterol lowering effects. Instead, pleiotropic effects, such as endothelial nitric oxide increase [7], reduction of oxidative stress [8], block of platelet activation [9], and antiinflammatory actions [10], have been implicated in its protective role in stroke. These properties suggest statins as a safe and promising cotreatment for reperfusion therapies in the acute phase of stroke [11,12]. Statin-treatment advantages have also been shown in the clinical field. Statin pretreatment decreases the incidence of stroke patients at risk of cardiovascular disease [13,14]. Statin treatment before or early after an ischemic stroke triggers more favorable outcome in patients [15,16]. However, clinical and experimental studies investigating the risk of HT after combined tPA treatment with statin are scarce. Preclinical studies in animals have demonstrated statins safety when combined with tPA [17,18] although none of them have studied the effect of simvastatin and tPA combination as a posttreatment. Conversely, a recent clinical study has raised caution reporting an increased risk of HT among those tPA-treated patients that were under statins at the moment of having a stroke [19]. Therefore, special attention in evaluating the HT after coadministering statin and tPA is required before planning future clinical trials. In this study, we aimed to test both experimentally and clinically, whether the

combined treatment of statin plus tPA is a safe therapy in the acute stroke setting.

Methods

Experimental Study

All procedures were approved by the Animal Ethics Committee of the Vall d'Hebron Research Institute (02/09 CEEA) and were conducted in compliance with the Spanish legislation, in accordance with the Directives of the European Union. Experiments were performed in male spontaneous hypertensive rats (SHR) weighing 300–325 g (Harlan Laboratories, UK). Rats were kept in a climate-controlled environment on a 12-h light/12-h dark cycle. Food and water were available *ad libitum*. All rats were subjected to an embolic ischemia consisting of a blood embolus placement at the origin of the MCA via a midline neck incision [20]. Arterial blood from a donor rat was withdrawn to form single clots (length: 3 cm; diameter: 0.3 mm) as described [21]. Just before surgery, noninvasively systolic blood pressure was measured with a tail cuff (LE 5002 Storage pressure meter Harvard apparatus, USA) to ensure the hypertension condition of all rats. Continuous laser–Doppler flowmetry (Moor Instruments, Devon, UK) was used to monitor regional cerebral blood flow (rCBF), and only animals that exhibited a reduction >75% during MCAO when compared with rCBF baseline were included in the study. After the surgery, analgesia was administered to the rats (Metamizol, Boehringer Ingelheim, St Cugat del Vallès, Spain).

Animals were anesthetized under spontaneous respiration with 2% isoflurane (Abbot Laboratories, Kent, UK) in oxygen during surgery, and body temperature was maintained at 37°C. All animals were euthanized and transcardially perfused 24 h after the ischemia. Heparine solution followed by saline solution was injected using an infusion pump.

Pilot Study

The aim of our pilot study was to establish a reliable embolic model on hypertensive rats to obtain an adequate rate of HT, our primary endpoint, without increasing the mortality rate. As the time of tPA administration is a critical variable [22], we tested tPA administration at 1.5 and 3 h after MCAO to select the most suitable timing of administration. Animals were euthanized 24 h postischemia, and brains were carefully harvested. Triphenyltetrazolium chloride (TTC) staining was performed to measure the infarct volume, and visual score (0–4) was assessed to evaluate HT (0 = No hemorrhage, 1 = HI-1:Petechial hemorrhage occupying <30% of infarcted area, 2 = HI-2:Petechial hemorrhage occupying >30% of infarcted area, 3 = PH-1:Homogenous blood area occupying <30% of infarcted area, 4 = PH-2:Homogenous blood area occupying >30% of infarcted area).

Experimental Design

Animals subjected to MCAO were randomly allocated to experimental groups (simvastatin+tPA or vehicle+tPA) using a computer-generated randomization list. One mililiter of simvastatin solution (20 mg/kg (diluted in vehicle); Uriach Laboratories, Barcelona, Spain) or vehicle (distilled H_2O (75%), absolute ethanol (10%) and 0.1 M NaOH (15%)) was subcutaneously injected 15 minutes after occlusion in a blinded manner, and subsequently, rats were allowed to wake up. Animals were reanesthetized 3 h after embolization to receive tPA (Actylise, Boehringer Laboratories) which was slowly infused using an automatic injector in a 9 mg/kg dose. Regional cerebral blood flow (rCBF) was also monitored during the thrombolytic infusion. This timing for tPA administration ensures a high rate of HT without increasing the mortality rate, as established in our pilot study. A total of 50 animals were included in this study. Twelve animals were excluded after applying the following criteria: inappropriate occlusion of the MCA after embolization (n = 4); spontaneous reperfusion before tPA administration (n = 6) and death before or just after tPA administration (n = 2).

Neurological Deficit and Infarct Volume Evaluation

Neurological status was assessed using a 9-point neurological deficit scale in a blinded manner at both 90 min and 24 h after MCAO. This scale consists of four consecutive tests, as previously described [23].

Infarct volume was measured using 2,3,5- triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining as described [24]. TTC images were captured using a Cano Scan 4200F, and infarct volume was measured using ImageJ software by integration of infarcted areas. Infarct volume data were expressed as a percentage of the ipsilateral hemisphere, and edema was evaluated taking into account the following equation: edema = (infarct volume × contralateral volume)/ipsilateral volume.

Assessment of Hemorrhagic Transformation (HT)

Three different methods were used to evaluate HT severity: (1) classification of HT by visual score, (2) determination of the area of parenchymal hemorrhage (PH), and (3) extravasated hemoglobin (Hb) quantification by Western blot. The antibody used was rabbit anti-Hemoglobinα (H-80, 1:1000, catalog no. sc-21005; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Brain samples used for HT evaluation were obtained after homogenized TTC stained from transcardially perfused rats.

MMP Zymography

Standard gel zymography was used to measure levels of MMP-2 and MMP-9 in both blood and brain samples of ischemic rats. Blood samples were drawn just before tPA administration (3 h postischemia) and before euthanasia (24 h postischemia), collected in EDTA tubs, and immediately centrifuged at 1000 ***g*** for 10 min to obtain plasma supernatants. For brain samples, frozen coronal brain sections stained with TTC were used to obtain brain homogenates, where describe in detail elsewhere [25]. We previously confirmed that TTC-stain procedure did not modify the MPP-2 and MPP-9 activation status. Both for blood and homogenate brain samples, total protein concentrations were determined with the BCA assay.

Clinical Study

We performed a prospective study of patients with acute ischemic stroke who had been admitted to the emergency room within 3 h after symptom onset. A total of 653 consecutive patients were selected from a large database originally created to study thrombolytic treatment implication on biological and clinical variables. All evaluated patients suffered from a nonlacunar stroke involving the vascular territory of the MCA or the basilar artery. All underwent urgent carotid ultrasound and transcranial Doppler examinations and received tPA (Actylise, Boehringer Laboratories) in a standard 0.9 mg/kg dose (10% bolus, 90% continuous infusion for 1 h). From the 653 patients, 138 were under statins treatment (any kind) at the moment of the stroke, and 515 were not.

Clinical Protocol

A detailed history of vascular risk factors was obtained from each patient with especial attention on the drugs they were taking before stroke onset. To identify possible mechanisms of cerebral infarction, we performed a set of diagnostic tests and classified the cohort according to previously defined etiologic subgroups [26]. Our cohort included 311 cardioembolic strokes, 127 atherothrombotic strokes, 183 patients with an undetermined etiology, 18 dissections, and 14 with uncommon strokes or incomplete study.

Computed Tomography (CT)

All patients underwent CT within the first 3 h of stroke onset. CT was repeated at 24–48 h (or earlier in the case of rapid neurological deterioration) to evaluate the presence of HT. The CT scans were reviewed by a neuroradiologist with extensive experience in acute stroke. The presence and HT type were defined according to previously published criteria [27]. Hemorrhagic infarction (HI) was defined as a petechial infarction without a space-occupying effect, and parenchymal hemorrhage (PH) was defined as hemorrhage with a mass effect. CT-based HT subtypes were defined as: HI-1, for small petechiae along the margins of the infarct; HI-2, for more confluent petechiae within the infarcted area; PH-1, when hematoma involved ≤30% of the infarcted area with some slight space-occupying effect; PH-2, when hematoma involved >30% of the infarcted area with substantial mass effect; or PH-R, when the clot was remote from the infarcted area.

Statistical Analysis

Experimental data were analyzed using GraphPad Prism_v5 software (Graph Pad Prism Software Inc, San Diego, CA, USA) and clinical data using SPSS 15.0 (IBM, New York, NY, USA). Statistical significance for intergroup differences was assessed by Student's test and ANOVA followed by Bonferroni post hoc test for parametric data. For nonparametric data, Mann–Whitney test or Kruskal–Wallis test followed by Dunn's multiple comparison test was assayed. To analyze percentages (mortality rate, hemorrhage incidence, and hemorrhage subtypes in animal study and also patient characteristics), Pearson's chi-square test was used. Bars represent mean ± SD for parametric data, and box plots represent median (IQR) for nonparametric data. A P value < 0.05 was considered statistically significant at a 95% confidence level.

Results

Pilot Study

Surprisingly, we found that SHR without receiving tPA after MCAO showed similar infarct volume than those which received tPA at 1.5 and 3 h postischemia ($P = 0.8$) (Figure 1A). However, we observed significant differences regarding HT rates ($P = 0.016$), although, after Dunn's correction, only when no tPA-treated animals and 3 h tPA-treated animals were compared, the difference reached statistically significance ($P = 0.018$). (Figure 1B). HT's incidence was 57.14% for animals nontreated with tPA, 90% for animal treated at 1.5 h, and 92.30% for those treated at 3 h. Mortality rate was not different among groups.

Simvastatin Efficacy

All animals included in the study showed high arterial systolic pressure and similar weight and occlusion percentage, without significant differences between simvastatin and vehicle-treated groups. Neurological outcome at 90 min (basal) and 24 h postischemia also showed no differences between treatment groups (Table S1).

Furthermore, we observed large infarct volumes and different types of intracranial hemorrhages in SHR subjected to embolic stroke, as shown in Figure 2A. No differences were detected on infarct volume percentages between groups: 46.33 (27.58–54.71)%, vehicle+tPA vs. 50(20.09–57.86)%, simvastatin+tPA; $P = 0.91$ (Figure 2B). Correspondingly, rats treated with

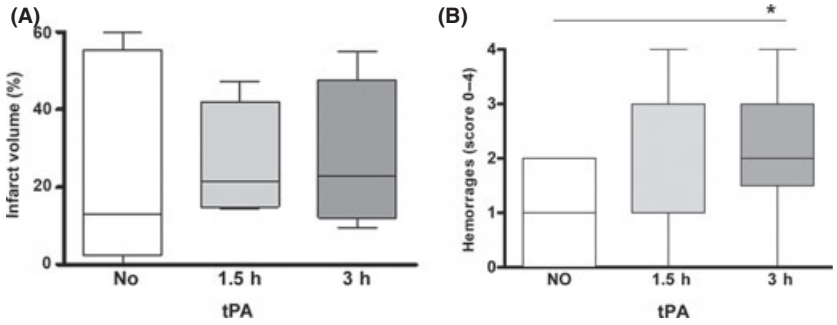

Figure 1 Pilot study. (**A**) Percentage of infarct volume at 24 h of the ischemia expressed as a percentage of the ipsilateral hemisphere, considering no tPA administration (No), tPA administration at 1.5 (1.5 h), and at 3 h (3 h) after the occlusion. (**B**) Visual hemorrhage score assessed after TTC staining and taking into account tPA administration (n = 9–11 each group).

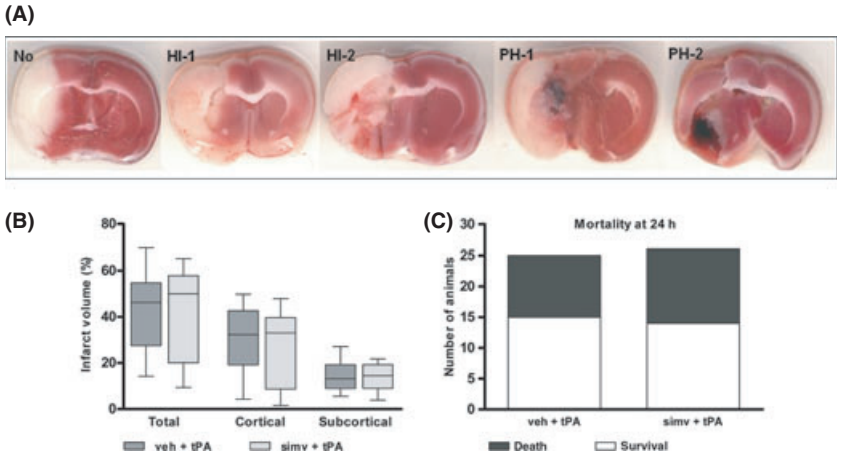

Figure 2 Simvastatin efficacy. (**A**) Representative images of TTC staining where TTC-unstained areas (in white) correspond to infarcted brain tissue from vehicle and tPA-treated rats. Hemorrhages are also evidently seen on the same TTC-stained slices and classified according to ECASS criteria (**B**) Effect of treatments on infarct volume. Total, cortical, and subcortical size infarcts expressed as percentage of total brain volume in rats subjected to MCAO. Vehicle+tPA (n = 15), Simvastatin+tPA (n = 13) (**C**) Graph showing mortality. Bars represent the number of animals that survived or died after 24 h of MCAO.

vehicle+tPA had similar edema volume than those treated with simvastatin+tPA (114.1 ± 74.92 mm^3 vs. 121.6 ± 89.77 mm^3; $P = 0.89$). Furthermore, the mortality rate was high. Twenty-two animals died within 24 h after MCAO, and it was comparable between groups: 10 animals died in the vehicle group (38.46%) and 12 in the simvastatin group (41.37%); $P = 0.77$ (Figure 2C). Regarding CBF reperfusion during the 20 min period of tPA infusion, only few animals showed CBF recovering: 20% of the animals from vehicle+tPA group and 30.85% from simvastatin+tPA group, $P = 0.51$.

Simvastatin Safety

HT was observed in most of the animals independently of treatment allocation at 24 h postischemia (vehicle+tPA = 86.60% vs. simvastatin+tPA = 92.85%, $P = 0.99$). The most frequent hemorrhage identified was PH type (vehicle+tPA = 60% vs. simvastatin+tPA = 71.4%), followed by HI type (vehicle+tPA = 26.6% vs. simvastatin+tPA = 21.4%), but no differences in the incidence of hemorrhage subtype were found among groups ($P = 0.875$) (Figure 3A). Animals treated with simvastatin tended to have smaller PH area as compared with vehicle group (8.03 ± 5.69 mm^2 vs. 20.03 ± 17.42 mm^2, $P = 0.09$), as shown in Figure 3B. In addition, levels of Hb in ipsilateral hemispheres were not different between groups (18.98 OD ratio [8.62–27.45] vs. 15.46 OD ratio [4.45–23.95], $P = 0.40$) (Figure 3C,D).

Gelatinases Brain and Plasma Content After Simvastatin Treatment

Band intensities of pro-forms of MMP-9 and MMP-2 protein were quantified in gel zymography of brain homogenates to measure gelatinases brain level (Figure 4A). Twenty-four hours after ischemia, pro-MMP-9 levels in brain homogenates were significantly increased in ipsilateral compared with contralateral hemispheres in both vehicle (13.80 [9.20–32.74] A.U vs. 2.79 [0.01–7.2] A.U, $P = 0.0014$) and simvastatin (20.05 [6.25–30.54] vs. 2.11 [0.01–9.16], $P = 0.0007$) groups. Even so, levels were similar in ipsilateral hemispheres of both groups, $P = 0.52$ (Figure 4B).

On the other hand, we observed a significant increase in pro-MMP-9 OD in the ipsilateral hemisphere of animals that presented hemorrhages (45.00 [34.73–76.26] for HI and 50.21 [6.66–167.30] for PH) in comparison with animals that did not (12.86

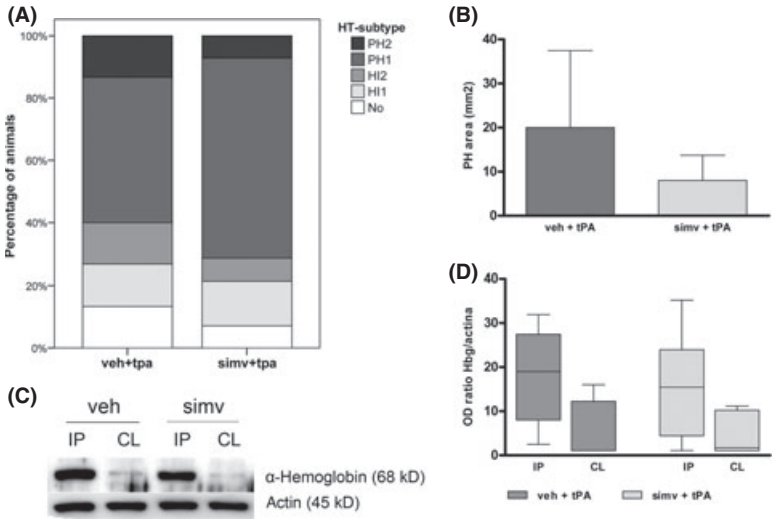

Figure 3 Simvastatin safety. (**A**) Animals' classification according to hemorrhage visual score criteria. Vehicle+tPA (n = 15), Simvastatin+tPA (n = 13) (**B**) Quantification of PH areas. Vehicle+tPA (n = 9), Simvastatin+tPA (n = 9). (**C**) Representative Western blot for hemoglobin and loading control (β-actin) in ipsilateral (IP) and in contralateral (CL) hemispheres. (**D**). Vehicle+tPA (n = 15), Simvastatin+tPA (n = 13).

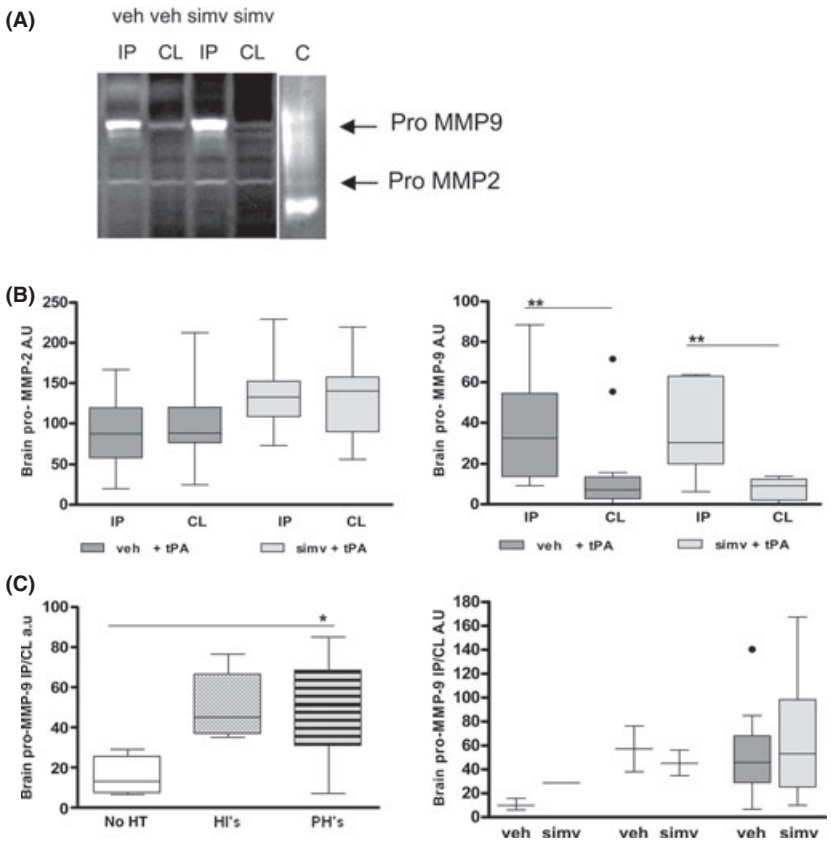

Figure 4 Matrix Metalloproteinases (MMPs) levels in brain homogenates. (**A**) Representative zymography. (**B**) Levels of MMP-9 proform in ipsilateral (IP) and contralateral (CL) hemispheres. (**C**) Quantification of pro-MMP-9 regarding the hemorrhage type. (**D**) Pro-MMP-9 regarding treatment and hemorrhage type (**E**) Quantification of MMP-2 proform levels. C, loading control. In all graphs of this figure, Vehicle+tPA (n = 15), Simvastatin+tPA (n = 13).

[6.14–28.71], $P = 0.03$) (Figure 4C). Importantly, we did not found differences between treatment groups (no HT: 9.86 [6.14–15.85] for vehicle and 28.71 [28.71–28.71] for simvastatin; HI's: 57.13 [38.00–76.26] for vehicle and 45.80 [34.73–56.11] for simvastatin; PH's: 45.06 [6.66–140.40] for vehicle and 53.00 [9.99–167.3]) for simvastatin (Figure 4D).

Regarding MMP-2 pro-form, similar levels were found in the ipsilateral and contralateral hemispheres within the same group (vehicle: 87.34 [19.96–166.80] vs. 88.06 [24.88–212.60], $P = 0.59$; simvastatin: 132.6 [73.44–229.60] vs. 140.4 [56.30–219.70], $P = 0.77$) and between animals that received simvastatin compared with vehicle treatment ($P = 0.66$) (Figure 4E).

Plasma zymography quantification (Figure 5A) revealed that both pro-MMP-2 and pro-MMP-9 levels were statistically higher at 3 h postischemia compared with 24 hours. (MMP-2 OD ratios: 121.70 [98.54–175.80] vs. 102.00 [68.72–125.00], $P = 0.02$; MMP-9: 135.00 [108.20–249.50] vs. 104.8 [56.76–132.20], $P = 0.006$) (Figure 5B,D). However, no differences were obtained between groups at studied time points (Figure 5C,E).

Statins Treatment and Hemorrhage Incidence in Stroke Patients

Among the recruited patients, only 138 were under statins (21.10%) and 515 were not (78.90%). Importantly, neither differences on HT incidence between both groups ($P = 0.74$) nor among hemorrhage subtype ($P = 0.49$) were found, as shown in Figure 6.

Demographic and clinic characteristics of the stroke population regarding statins treatment were considered (Table S2).

Discussion

As it has been previously published, statins pleiotropic effects could be beneficial even in the acute phase of stroke [28]. It has been demonstrated that statins are able to upregulate endothelial nitric oxide synthase exercising an endothelial protection and to inhibit the upregulation of inducible nitric oxide synthase (mediated by cytokines in astrocytes and macrophages during ischemia and reperfusion), mechanism which results in a cerebral infarct volume decrease [29,30]. Thereon, the main goal of this study was to address the safety concerns related to HT after the combined therapy of statin with tPA. This represents a controversial issue as some studies suggested that statins may reduce HT rates when administered together with tPA [11,12], while others affirmed that the prior statin treatment in stroke patients that received thrombolytic therapy was associated with an increased in HT rate [19] and with a nonfavorable outcome [31,32]. To solve the controversia, a meta-analysis [33] which included 31 randomized controlled trials has been recently published and concluded that statin therapy was not associated with significant increase in ICH.

In our study, we performed a physio-pathological and reliable embolic stroke model in SHR to study the frequency and magnitude of intracranial hemorrhage after cerebral ischemia [34]. The higher vascular resistance in addition to the lower vascular

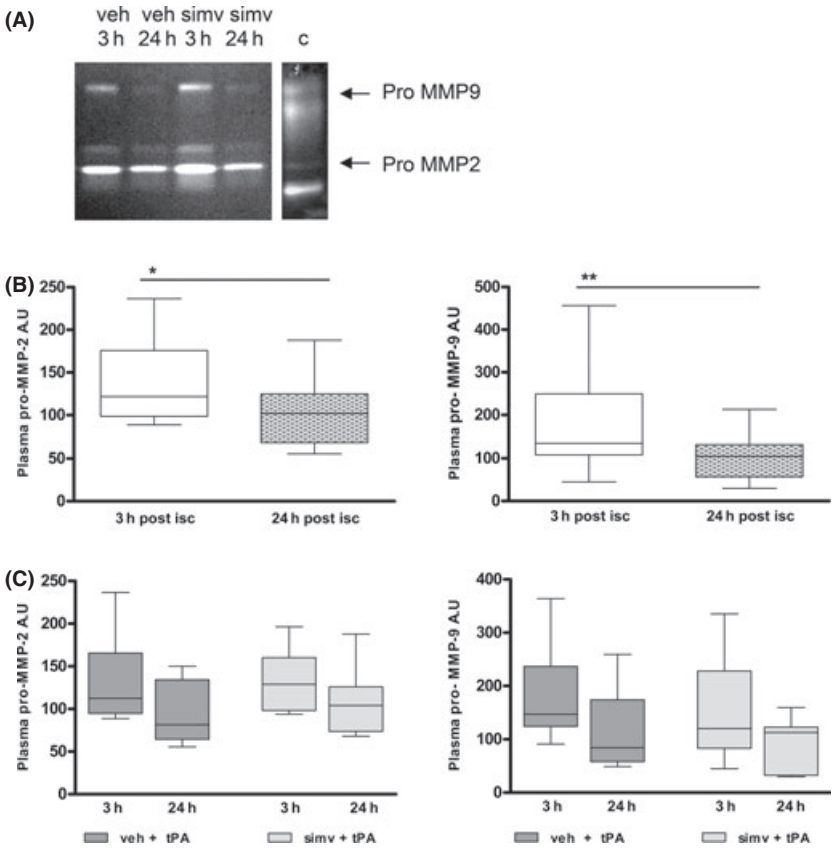

Figure 5 Matrix Metalloproteinases (MMPs) plasma levels at 3 and 24 h after ischemia. (**A**) Representative gel zymography. (**B**) Pro-MMP-2 plasma levels at early and late times of ischemia. (**C**) Pro-MMP-2 plasma expression comparing treatment groups (**D**). Pro-MMP-9 plasma levels at early and late times of ischemia. (**E**) Pro-MMP-9 plasma expression comparing treatment groups. C, positive control. In all graphs of this figure, Vehicle+tPA (n = 11), Simvastatin+tPA (n = 11).

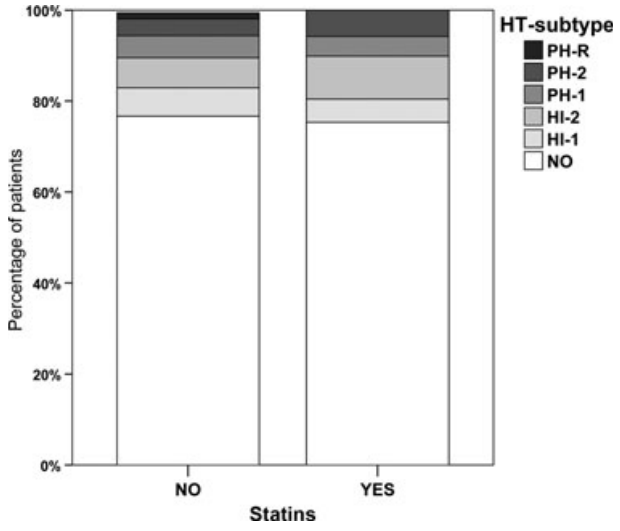

Figure 6 Statins safety in stroke patients. Representation of hemorrhage types distribution in tPA-treated patients who were under statin treatment at the moment of the stroke and those who were not. No statins (n = 515, 78.9%); statins (n = 138, 21.1%).

compliance in hypertensive rats induces larger infarcts, intensifies the vasogenic edema, and aggravates the blood brain barrier (BBB) disruption after ischemia. Consequently, it produces higher frequency of HT in comparison with normotensive animals, in which HT are less frequent [35]; making this an ideal model to study tPA-induced bleeding complications.

We found that acute simvastatin treatment did not increase the frequency of HT after thrombolytic therapy in SHR. Indeed, the extent of HT evaluated by extravasated hemoglobin showed no differences between vehicle and simvastatin-treated group, and the estimation of PH area indicated even smaller bleedings in animals receiving simvastatin. Regarding MMP data, our results are consistent with previous publications. Stroke produced an acute increase in MMP-9 levels in the ischemic hemisphere at 24 h post-ischemia, which is associated with tPA-related cerebral hemorrhage [36]. Importantly, combined thrombolytic therapy with simvastatin did not further increase MMP-9 levels according to the incidence of HT found, thus supporting the safety of simvastatin when combined with tPA therapy. On the other hand, plasma levels of both MMPs were similarly elevated at 3 h postischemia, preceding tPA administration, in either rats treated with simvastatin or vehicle. A transient elevation in MMP-9 plasma levels during the early reperfusion has been reported in SHR stroke [37], and it has been recently associated with blood-cerebrospinal fluid barrier disruption in SHR [38]. By contrast, we did not observe an ischemic neuroprotective effect exerted by simvastatin treatment in our model. Previous studies performing different stroke models (ligation, intraluminal filament) or using other species and strains (rabbit, mouse, normotensive rats) have shown that simvastatin reduces infarct volume and improves neurological deficit either administered before [39] or after stroke [40]. Therefore, we can

plausible infer that the selected model, with large infarct volumes and hemorrhagic conversions, may abolish the neuroprotective effect of simvastatin in SHR.

In accordance with the results from our pilot study, a recent meta-analysis has also reported that tPA has not significant effect in the infarct volume reduction or in the neurological outcome when administered to ischemic SHR [41]. Their hypothesis is that dilatation of arterioles in response to nitric oxide is mitigated in SHR, which might impair reperfusion after MCAO [42]. To our knowledge, there is only one published study using the embolic stroke model in rats that evaluates the effect of the combined acute treatment of statins and tPA [17]. This work reports that the combined treatment extents the therapeutic window of tPA to 6 h after stroke without increasing HT incidence, and in addition, induces neuroprotection by reducing the infarct volume and improving the neurological outcome. However, the study was performed on normotensive rats, and both their HT rates and infarct volumes were lower than ours. Another study [18] suggests that simvastatin treatment attenuates mechanisms involved in tPA-induced hemorrhage as they demonstrated that the pretreatment with simvastatin significantly decreased tPA-induced hemorrhage incidence when rabbits were subjected to an embolic occlusion. Thus, the protective effect of the statin plus tPA combined treatment assessed in animal stroke models with comorbidities, such us hypertension, has not been demonstrated yet.

Furthermore, the prospective clinical study carried out in our center supports the experimental findings, because statins did not influence HT incidence and severity in stroke patients treated with tPA. Our findings are in the same direction as previous reports that showed that prior statin treatment is an independent predictor of a favorable clinical outcome without an increased frequency of ICH on patients receiving tPA [43]. Contrary, other authors defended that prior statin use is associated with a higher frequency of any intracerebral hemorrhage (ICH) after intraarterial thrombolysis [19]. As these former authors support [19], we also reckon that the different results between the studies can be due to the patient's selection, time to treatment, or treatment modalities (e.g., additional mechanical recanalization). Moreover, it is unknown whether the use of different thrombolytic agents (urokinase and tPA) and different administration routes (systemically or locally) may influence bleeding risk in patients with acute stroke under statin treatment. Additionally, as potential risk factors for ICH (glucose levels, blood pressure) have not been analyzed, the increased risk of ICH found in statin patients group may be the result of those patients being sicker, and therefore, more prone to bleeding compared with patients without statin pretreatment. In this aspect, the patients under statins treatment included in our clinical study showed a significant increase in the leading risk factors related to HT (diabetes mellitus, hypertension) and spite of that, no increase in bleeding complications was identified, reinforcing the safety of the combined treatment. Therefore, only clinical trials using statins in the acute phase of stroke, such as the ongoing STARS trial (clinicaltrials.gov, identifier: NCT 01073007), will clearly demonstrate the safety and efficacy of the combination of statins plus tPA. If a true effect of statins is demonstrated, treatments might be initiated even before hospital arrival to reach a broader number of stroke patients.

Limitations

Although the animal model chosen might be the most suitable to study HT, we accept this model entails some limitations such as high variability due in part to the different animal response after tPA administration. Moreover, the severity of the experimental model could have not allowed us to see differences regarding HT occurrence between groups or even simvastatin efficacy on infarct volume reduction and neurological improvement.

Despite clinical study supports experimental data, we are aware of the limitations due to the different methodology employed (hypertensive rats receiving a single dose of simvastatin during the acute phase of the stroke vs. hypertension-controlled patients receiving statins chronically before the stroke onset). Regarding the hypertension issue, we wanted to clarify that we use this model in SHR to force high ICH rates that was our main endpoint and not to mimic hypertension related with stroke in humans.

Finally, simvastatin was selected in our experimental study because, as we recently reported in a meta-analysis study [44], it triggers the highest effect associated with stroke neuroprotection among other statins. Nonetheless, other types of statin in combination with tPA may exert similar effects.

In conclusion our study shows that statins administration in the acute phase of stroke (in an experimental/animal study) and as a pretreatment (in a clinical/human study) combined with tPA does not raise HT risk in comparison with administration of tPA alone, supporting the safety of the combined treatment.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Table S1. Physiological parameters, percentage of occlusion and neurological score of animals included in the experimental study.

Table S2. Demographic and clinic characteristics of the stroke population regarding statins treatment included in the clinic study.

ARTICLE 4:

Rat Middle Cerebral Artery occlusion is not a suitable model for the study of stroke-induced spontaneous infections. ***PLoS One.*** **2014 Jun 12;9(6):e99169.**

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PLOS ONE

Rat Middle Cerebral Artery Occlusion Is Not a Suitable Model for the Study of Stroke-Induced Spontaneous Infections

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Abstract

Background: Infections related to stroke-induced immunodepression are an important complication causing a high rate of death in patients. Several experimental studies in mouse stroke models have described this process but it has never been tested in other species such as rats.

Methods: Our study focused on the appearance of secondary systemic and pulmonary infections in ischemic rats, comparing with sham and naive animals. For that purpose, male Wistar rats were subjected to embolic middle cerebral artery occlusion (eMCAO) or to transient MCAO (tMCAO) inserting a nylon filament. Forty-eight hours after ischemia, blood and lung samples were evaluated.

Results: In eMCAO set, ischemic rats showed a significant decrease in blood-peripheral lymphocytes (naive = 58.8±18.1%, ischemic = 22.9±16.4%) together with an increase in polymorphonuclears (PMNs) (naive = 29.2±14.7%, ischemic = 71.7±19.5%), while no change in monocytes was observed. The increase in PMNs counts was positively correlated with worse neurological outcome 48 hours after eMCAO ($r = 0.55$, $p = 0.043$). However, sham animals showed similar changes in peripheral leukocytes as those seen in ischemic rats (lymphocytes: 40.1±19.7%; PMNs: 51.7±19.2%). Analysis of bacteriological lung growth showed clear differences between naive (0±0 CFU/mL; log10) and both sham (3.9±2.5 CFU/mL; log10) and ischemic (4.3±2.9 CFU/mL; log10) groups. Additionally, naive animals presented non-pathological lung histology, while both sham and ischemic showed congestion, edema or hemorrhage. Concordant results were found in the second set of animals submitted to a tMCAO.

Conclusions: Inflammatory and infection changes in Wistar rats subjected to MCAO models may be attributed not only to the brain ischemic injury but to the surgical aggression and/or anaesthetic stress. Consequently, we suggest that stroke-induced immunodepression in ischemic experimental models should be interpreted with caution in further experimental and translational studies, at least in rat stroke models that entail cervicotomy and cranial trepanation.

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Introduction

Stroke not only produces local brain injury but also causes a systemic inflammatory response followed by an immunodepression process which predisposes patients to infections. This immunodepression state is thought to be induced through a sympathetic nervous system over-action which contributes to an increase of catecholamines and glucocortocoids [1]. Thus, the prolongation of this immune suppression may increase the chance of infection [2], predisposing stroke patients to pneumonia and sepsis [3]. On the other hand, it is well known that stroke prognosis depends on secondary complications incidence [4], recognizing infection as an independent risk factor for adverse outcome [3,5]. However, the precise mechanisms by which this association operates remain poorly understood. The incidence and prognostic impact of infections have been recently evaluated in a meta-analysis on this subject, which concluded that infections complicated acute stroke in 30% of patients [3]. This high incidence on stroke patients seems to be a result of an impaired immune function [6,7] complicated with stroke-facilitated aspiration, due to patient

dysphagia, immobilization and the need of invasive procedures like insertion of intravenous lines and urinary catheters [8]. Pneumonia is the most common infection [3,9,10] and it is the leading cause of death within the first 48 hours after stroke [11,12].

Experimental studies with different animal models have been crucial in understanding several aspects related to the physiopathology of the infection during the acute phase of stroke. Some studies in mouse models of cerebral ischemia (suture MCAO model) have demonstrated that nervous system damage results in spontaneous bacterial infections because of a deterioration of cell-mediated immune responses [8,13,14]. Others have shown that when a harmless concentration of *Streptococcus peumoniae* is intranasally applied, only mice with stroke shift to severe infection [15]. However, studies of stroke-induced immunodepression and spontaneous infections associated with brain ischemia have not been previously reported in rats. Therefore, we aimed to study secondary systemic and respiratory tract infections in rats using the MCAO model induced by blood-clot administration in rats, the model that best resembles clinical stroke [16].

Materials and Methods

Ethics statement

All procedures were approved by the Animal Ethics Committee of the Vall d'Hebron Research Institute (02/09 and 58/13 CEEA) and were conducted in compliance with the Spanish legislation and in accordance with the Directives of the European Union. In all experiments, male Wistar rats (weighing 275 to 310 g; Charles River Laboratories) were used. Rats were kept in a climate-controlled environment on a 12-hour light/12-hour dark cycle. Food and water were available ad libitum during all experimental process and all efforts were made to minimize suffering.

Embolic MCAO model

Infarction in the territory of the middle cerebral artery (MCA) was induced by embolic occlusion (embolic MCAO) as previously described [17]. Animals were anesthetized under spontaneous respiration with 2% isoflurane (Abbot Laboratories, Kent, UK) in oxygen during surgery and body temperature was maintained at 37°C. Arterial blood from a donor rat was withdrawn to form 2 clots (length: 1.5 cm; diameter: 0.3 mm) and they both were used for embolization of the right MCA. Cranial trepanation was performed the day before MCAO surgery to attach a laser–Doppler probe (Moor Instruments, Devon, UK) and monitor regional cerebral blood flow. Only animals that exhibited a reduction >75% in regional cerebral blood flow during MCAO were included in the study. Sham animals were submitted to both trepanation and surgery procedures, but no blood clots were injected into arteries. Three doses of analgesia (magnesic metamizol) were administrated just after cranial trepanation, MCAO surgery and 24 hours after it. All animals were euthanized 48 hours after the surgery. Naive animals were not submitted to any procedure and were sacrificed the same day as ischemic and sham ones.

A total of 59 rats were used for this study. Nine of them were naive rats, 13 were sham and 37 were submitted to embolic MCAO. Eleven of the ischemic animals were excluded after applying the following criteria: inappropriate occlusion of the MCA after embolization (n = 6); spontaneous reperfusion within the next ten minutes after occlusion (n = 3) or sudden death during the surgery process (n = 2). Blood samples were drawn through transcardiac puncture 48 hours after embolic MCAO or after sham surgery. Afterwards, rats were transcardially perfused with sterile PBS and lungs were carefully removed in aseptic conditions for posterior evaluation. From the total of 26 rats submitted to embolic MCAO and included in the study, 10 of them died before the experimental protocol finished (eight during the first 24 hours and two of them between 24 and 48 hours after the occlusion). No animals died in the sham group.

Intraluminal tMCAO model

In order to ratify our findings, experiments were repeated in a new set of rats submitted to a transient MCAO model using an intraluminal filament as described previously [18]. In brief, after the surgical exposure of the bifurcation of the external carotid artery and the internal carotid artery on the right side, a silicone-coated nylon monofilament (Doccol Corporation, reference number: 403723PK10) was introduced to occlude the MCA. After occlusion, animals were allowed to recover from anesthesia. Reperfusion was induced 90 minutes later and to that end, animals were re-anaesthetized. Only animals that exhibited a reduction > 75% in regional cerebral blood flow after filament placement and a recovery of >75% after filament removal were included in the study. Sham-operated animals were submitted to the same processes except for the filament introduction. Naive animals were not submitted to any procedure and were sacrificed the same day as ischemic and sham ones. Analgesia protocol and methods employed for sample collection were exactly the same as the ones used in the previous rat set submitted to an eMCAO model.

From the total of 10 rats submitted to tMCAO, two of them were excluded due to bad reperfusion and another one died before the experimental protocol finished. No animals died in the sham group.

Infarct volume and neurological deficit evaluation

Infarct volume was measured using 2,3,5- triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining as described [19]. TTC images were captured using a Cano Scan 4200F and infarct volume was measured using Image J software by integration of infarcted areas. Infarct volume data was expressed as a percentage of the ipsilateral hemisphere and edema was evaluated taking into account the following equation: edema = (infarct volume×contralateral volume)/ipsilateral volume.

Rats were assessed using a 9-point neurological deficit scale, as previously described [18]. 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) parachute reflex (symmetrical = 0, asymmetrical = 1, contralateral forelimb retracted = 2), and (IV) resistance to left forepaw stretching (stretching not allowed = 0, stretching allowed after some attempts = 1, no resistance = 2). Neurological score was assessed in a blinded manner at 90 minutes, 24 hours and 48 hours after occlusion.

Flow cytometry

Blood-leukocyte populations (monocytes, polymorphonuclears (PMNs) and lymphocytes) were evaluated by flow cytometry technique. With this aim, 500 µl of blood were incubated with Ammonium-chlorid-kaliumhydrogencarbonat (ACK) buffer (room temperature, 5 minutes) to lysate erythrocytes and later the pellet was rinsed with Hanks' balanced salt solution - Hepes (HBSS-Hepes, 500 g, 5 minutes at 4°C). The same process (lysis-cleanliness-centrifugation) was repeated thrice. Cells were resuspended in 100 µl of FACS buffer, blocked with 0.25 µg purified

mouse anti-Rat CD32 (eBioscience, San Diego, Ca, USA) on ice for 10 minutes and stained with 0.4 μg anti-Granulocyte Marker-PE (GrM-PE) (eBioscience) and 1 μg anti-Rat CD11b- FITC (eBioscience) antibodies for 20 minutes on ice. A specific antibody to detect cell viability, To-Pro-3 (eBioscience) was used to assess samples preservation. A total of ten-thousand events per sample were acquired using FacScalibur (Becton Dickinson, USA). To discriminate leukocyte types, the following populations were defined: PMNs were CD11b-FITC$^+$ and GrM-PE$^+$; monocytes CD11b-FITC$^+$ and GrM-PE$^-$ and lymphocytes CD11b-FITC$^-$ and GrM-PE$^-$. Data were analyzed with FCS Express version 3 (De Novo Software, USA) and expressed as percentage of total leukocytes.

Lung histology

Lungs were carefully removed and separated into right and left lung under sterile conditions. Left lung was immersed in formaldehyde 4% (Sigma, St. Louis, MO, USA) for 48 hours, embedded in paraffin wax and sectioned with a microtome in 4 μm thick sections. Hematoxilyn & Eosin (HE) stain was carried out to perform lung tissue histological evaluation by an investigator blinded for study group.

Microbiological analysis

Lung imprints were performed pressing 4 times on blood-agar plates' surface with a piece of sterile right lung. Blood was withdrawn by cardiac puncture in sterile conditions and lungs were collected, minced and homogenized also under sterile conditions. One-hundred μL of all specimens were serially diluted in sterile PBS up to 200 μL of final solution, which was plated onto blood agar plates (Biomérieux, Marcy l'Etoile, France). After 24 and 48 hours of incubation at 37°C, agar plates were analyzed for growth colonies. Results of lung imprints were expressed as the number of observed colonies. Results of both blood and lung homogenate were expressed as a logarithmic function (log10).

CXCL-1 measurement

For the measurement of blood CXCL-1 cytokine, a simple ELISA kit was purchased from R&D systems and used according to the manufacturer's protocol.

Statistical analysis

Data were analyzed using GraphPad Prism_v5 software. Statistical significance for intergroup differences was tested by Student's t-test and ANOVA followed by Bonferroni post hoc test for parametric data. For non-parametric data, Mann Whitney and Kruskal-Wallis test followed by Dunn's Multiple Comparison test was performed. Correlations between parameters were tested by Pearson (parametric data) or Spearman tests (non- parametric data). For parametric data, bars represent mean± SD and for non- parametric data, box plots represent median (Interquartil range). A p value<0.05 was considered statistically significant at a 95% confidence level.

Results

Infarct volume and neurological deficit

Infarct volumes of a subgroup of rats submitted to an eMCAO were calculated and the mean was $40.62\pm10.09\%$ ($n=6$), whereas no infarct lesion was observed in sham animals in any case ($n=13$). Mean neurological score at 48 hours was 4.43 ± 2.9 for ischemic animals and 0.28 ± 0.82 for sham animals.

Changes in peripheral leukocytes count are correlated with neurological outcome after stroke

As shown in figures 1A and 1B, striking differences were detected through flow cytometry analysis among animal groups. Leukocyte subpopulations could be properly separated and lymphocytes clearly distinguished (Figure 1C).

Considering experimental groups, results obtained in flow cytometry assays are represented in Figure 2. Cerebral ischemia triggered an increase of PMNs in blood ($71.7\pm19.5\%$) as compared to sham ($51.7\pm19.2\%$) $p<0.05$; and naive rats ($29.2\pm14.7\%$); $p<0.01$; ($n=$ 9-14 per group) (Figure 2A) and conversely, decreased blood lymphocytes ($22.90\pm16.4\%$) were found when compared either to sham ($40.1\pm19.7\%$) or naive groups ($58.8\pm18.1\%$), although only when comparing ischemic vs. naive the difference was significant, $p<0.001$ (Figure 2B). No differences in monocytes percentage were found among groups (naive: 2.07[0.79, 9.99] %; sham: 2.38[0.78, 5.48] %; ischemia: 2.32[0, 10.02] %), as shown in Figure 2C.

Interestingly, higher percentages of blood PMNs were positively correlated with a worse neurological outcome 48 hours after MCAO ($p<0.05$, $r=0.55$); (Figure 2D), but no correlation was observed at other time points. Neither lymphocyte nor monocyte counts were correlated with neurological outcome.

Bacteriological analysis

Bacterial growth from lung homogenates plated on agar was detected in both sham and MCAO groups, as shown in figure 3A. In this sense, the number of CFU, was similar in sham rats (3.9 ± 2.5; CFU/mL; log10) and in ischemic rats (4.3 ± 2.9; CFU/mL; log10, $p=0.95$) while naive lung homogenates did not show bacterial growth (Figure 3B). Contrarily, no CFUs were detected after plating blood samples of any of the groups. Regarding lung imprints (Figure 3C), CFU growth also appeared in both sham (43.40 ± 38.09; CFU/mL) and ischemic (50.67 ± 76.25; CFU/mL, $p=0.55$) groups. However, no CFU were seen in naive lung homogenates, then indicating no previous pulmonary infection to the surgical procedure (figure 3D).

Lung histology evaluation

As expected, all naive rats (4/4) showed normal lung histology consisting of definite alveoli with thin walls lined by two types of cells: pneumocyte type I, the predominant cell type, and pneumocyte type II, which are large and cuboidal and show short microvilli on their cell surface. The interalveolar septa appeared basically thin, although normal focal areas of thick septum could be observed. In lung tissue from naive rats we also observed that the BALT (bronchus-associated lymphoid tissue) which corresponds to the lung immune system, was generally located in bronchi bifurcation. (Figure 4A–B).On the other hand, both sham ($n=8$) and ischemic ($n=8$) groups showed altered lung histology involving acute vascular congestion (10/16), oedema (6/16) and intra-alveolar haemorrhage (4/16), in a diffused or patch distribution and affecting one or more pulmonary lobes. We observed lungs with lobular pneumonia and/or acute bronchopneumonia (4/16), characterized by PMN infiltration and associated to cellular necrosis and parenchymal fibrin inside alveoli or bronchioles. Some of these rats (3/16) showed also a mononuclear cellularity increase. In addition, in all these rats, although the number of BALT was strongly variable, they were always associated to segmented bronchi and without germinal centre, which is indicative of a reactive change (Figures 4C–F).

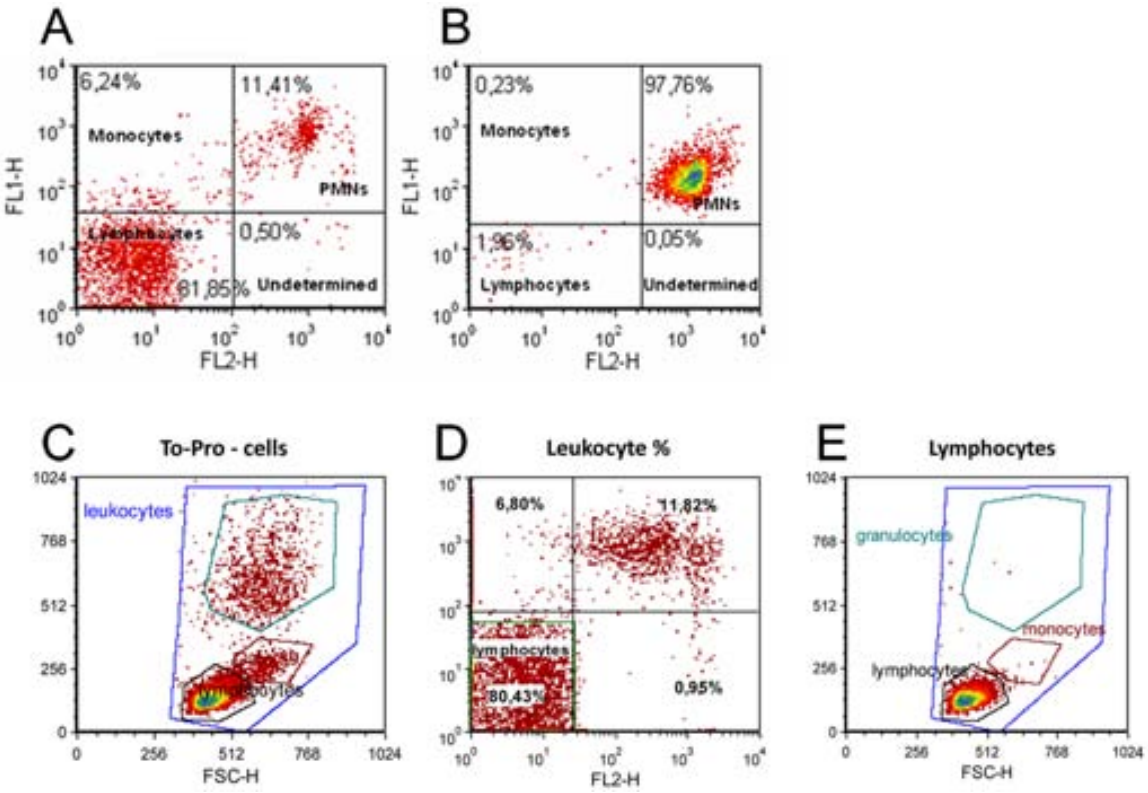

Figure 1. On the upper row: cytometry plots of representative (A) naive and (B) ischemic rats with high neurological score. On the lower row: Strategy used for Lymphocyte identification. (C) Leukocytes distribution considering their granularity (SSC-H) and their size (FSC-H) (D) Leukocyte distribution according to antibody's labelling. FL1-H represents Fit-C and FL2-H, PE. As shown, lymphocytes are CD11b-FITC$^-$ and GrM-PE$^-$ (E) Lymphocytes view after gating them from graph.
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Replication study using an intraluminal rat tMCAO model

Results obtained in this second set of experiments using a transient model with a nylon filament were equivalent to those derived from eMCAO model.The mean infarct volume of ischemic rats submitted to a tMCAO (n = 7) was 40.54±12.56%, and no lesion was detected in sham animals (n = 7). The mean neurological score was 4.14±1.07 for ischemic rats and 0 for sham rats.

Regarding blood leukocyte counts, we observed that ischemic animals showed an increase in blood PMNs (63.78±22.28%) as compared to sham (38.88±26.65%) or naive (22±6.5%) groups. In this case, only the comparison between naive and ischemic groups reached significance after Bonferroni correction ($p<0.01$) (Figure 5A). Also in agreement with the data previously obtained, a clear decrease in lymphocyte counts were found in ischemic animals (33.06±20.59%) when compared either to sham (55.94±25.11%) or naive animals (71.87±7.55%), being significant only the comparison between naive and ischemic groups ($p<0.01$) (Figure 5B). No differences in monocyte percentages were detected among the three groups (naive: 4.45 [1.08, 7.6] %, sham: 4.23[1.3, 10.24] %, ischemia: 1.25[0.25, 60.7] %) (Figure 5C).

In relation to bacteriological analysis, CFU growth was observed only after plating sham and ischemic lung homogenate samples. We could detect significant differences when comparing naive to sham animals (0 vs. 1.88±2.16 CFU/mL log10, $p<0.05$) and also naive to ischemic animals (0 vs. 2.5±1.81 CFU/mL; log10, $p<0.01$) (Figure 5D). No CFUs were obtained after plating blood samples of any of the groups. Considering lung imprints, we only found differences between naive and ischemic rats (0 vs. 81.57±97.43 CFU/mL, $p<0.01$), while sham animals showed a more moderate CFU growth (20.86±35.06 CFU/mL) (Figure 5E).

CXCL-1 measurement

Plasma levels of CXCL1 were evaluated 48 hours after ischemia or sham surgery and also in naive rats (n = 7/each group). Sham animals showed upregulated levels (15.79 [4.02, 45.98] pg/mL) as compared with naive rats (3.9 [2.34, 15.53] pg/mL), although a more pronounced release was seen in ischemic animals (30.75 [19.16, 123.4] pg/mL). After correction, only significance was reached when comparing naive and ischemic animals ($p<0.001$) (Figure 5F). Correlation between PMNs mobilization and CXCL1 plasma levels was also evaluated and we found a clear significance ($r = 0.66$, $p = 0.001$) when animals of all groups were included (n = 28) and a trend towards significance ($r = 0.71$, $p = 0.08$) when only ischemic animals were evaluated (n = 7) (Data not shown).

Discussion

Experimental stroke induces systemic inflammatory responses followed by global immunosuppression [7] that can make ischemic animals more prone to infections. In this study, we aimed to evaluate such stroke-induced infections using a MCAO rat model.

Blood analysis showed differences between sham and ischemic animals in circulating PMNs. According to previous studies, it has been well characterized that brain injury produces a secondary inflammatory response, accompanied by an increase on systemic neutrophils, the most representative PMN subtype [20,21]. Interestingly, we observed a positive correlation between circulat-

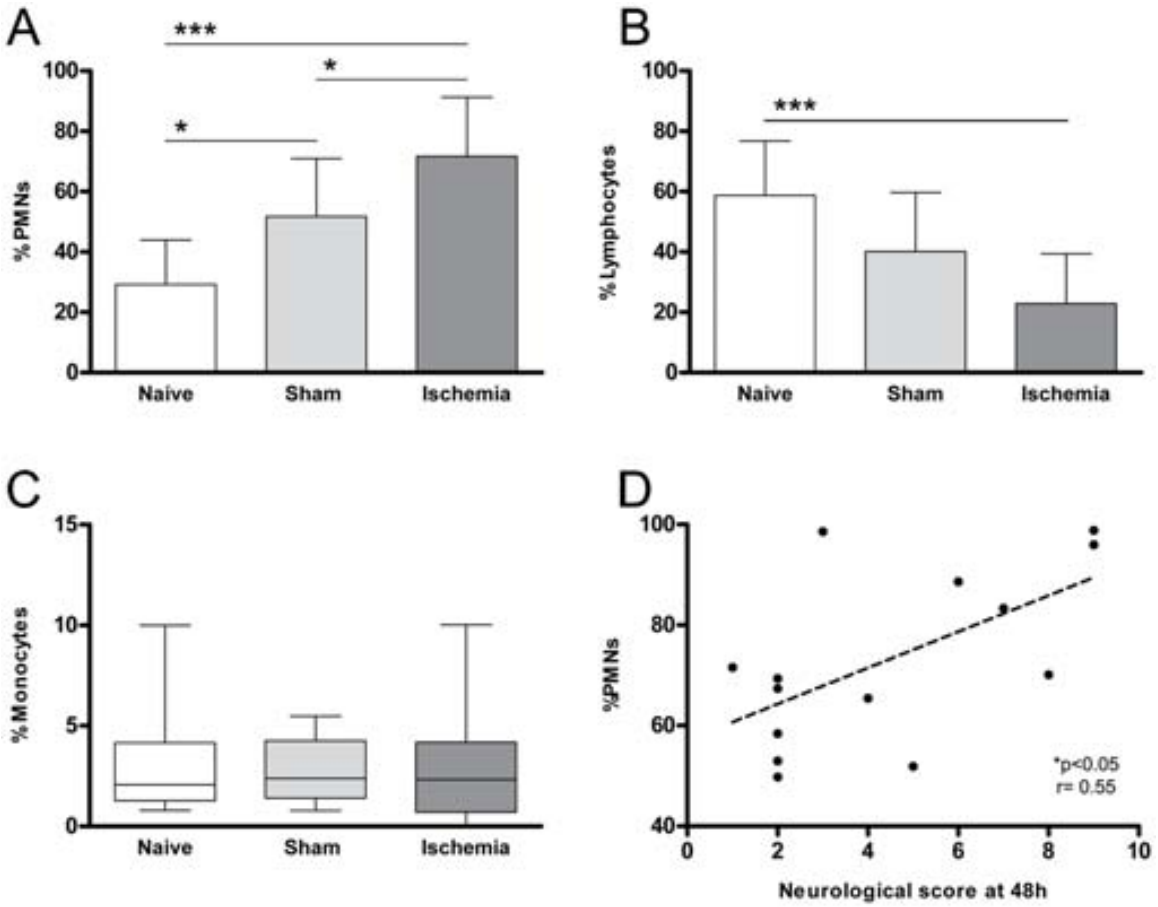

Figure 2. Leukocyte percentage assessed by flow cytometry of naive, sham or ischemic animals 48 hours after surgery. (A) Peripheral PMNs percentage: a significant increase was observed in sham and in ischemic animals with respect to the naive group ($p<0.05$). (B) Lymphocyte percentage. A significant decrease was detected between naive and ischemic animals ($p<0.001$) but no differences were shown between sham and ischemic animals. (C) Monocyte percentage. (D) Correlation between the percentage of systemic PMNs and the neurological score detected at 48 hours post-ischemia ($p<0.05$, $r=0.55$). Naive ($n=9$), sham ($n=13$), ischemia ($n=14$). *$p<0.05$, ***$p<0.001$.
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ing PMNs and neurological deficit at 48 hours. In agreement with our results, other publications have reported an association between early elevation of systemic neutrophil count in stroke patients and ischemia severity [20]. On the other hand, similar to other publications performed with mice [13] or humans [8,22], we detected a lymphopenia on our ischemic rats. Though we did not analyze lymphocyte subpopulations after stroke, we assume that it would have been of great interest as it seems to be a controversial issue. While most previous publications are in agreement showing a significant reduction in blood T-cells after stroke [23–26], the fact of whether or not B cells, NK cells, Tregs and monocytes are altered after cerebral ischemia is still on debate [26–29].

Remarkably, our results showed that leukocyte changes were in the same direction in both sham and ischemic groups. To explain this, we support that both isoflurane and sham surgery might disturb leukocyte responses, a hypothesis recently demonstrated in a published study [30]. This work reported that surgical stress and anaesthesia exert evident effects on both early and late cellular responses in the bone marrow after submitting mice to an experimental stroke. They also emphasized that volatile anaesthetics might affect leukocyte responses by altering neutrophil adherence to blood vessels and inducing lymphocyte apoptosis. Despite a significant decrease in lymphocyte percentage could be observed in ischemic compared to naive animals, no correlation was found considering the infarct size stated by others [31]. Our hypothesis is that all the disturbing factors entailed in these stroke animal models (anaesthesia, surgical stress, inflammation) may alter the association between infarct size and both the infection and the immunodepression severity.

Pneumonia and bacteraemia are the most common infections in stroke experimental studies [8], although bacteraemia appears less frequently and later than pneumonia. In human stroke, urinary tract infection has also a high incidence [3,32] but it is not as common in animals as is in patients, probably because animals do not need to be catheterized. Regarding our bacteria colonies growth, surprisingly but in agreement with other studies [33], none of the animals have shown signs of blood infection. Contrarily, other studies detected bacteraemia after a transient MCAO in mice [8,13,14]. Although it has been reported that susceptibility to poststroke infections is species and strain dependent [34], we reckon that another explanation for the discrepancy in the results might be the short time our animals were under study before euthanasia (48 hours versus 3, 5 or 15 days reported by other studies). Furthermore, regarding our CFU/mL lung counts at 48 hours (4.3 ± 2.9 CFU/mL log10), we consider they are in accordance with other publications [35] which reported around 3.5 CFU/mL log10 at 24 hours and 6 CFU/mL log10 at 72 hours after stroke in mice. However the variability

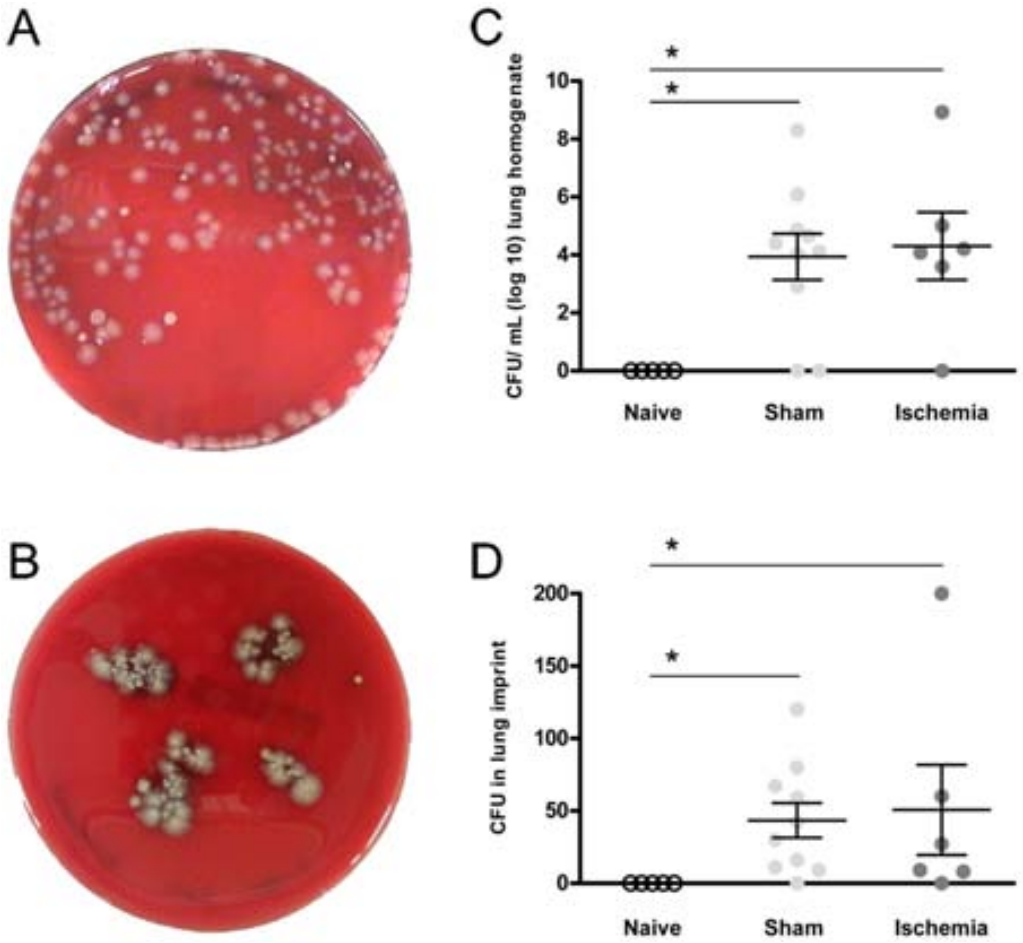

Figure 3. Bacterial growth 48 hours after plating (A) 200 μL of lung homogenate and (B) lung imprint. (C) Number of colonies (CFU) found in lung homogenates expressed as log 10. (D) Number of colonies in lung imprints. In the graphs, each dot represents an animal: naive (n = 5), sham (n = 10) and ischemia (n = 6).
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in number and type of colonies found in different spontaneous infection studies has been demonstrated to be totally related with the environment where the animals are placed [33,34].

Lung evaluation showed normal histology in naive rats whereas both sham and ischemic animals presented non-specific lesions such as distension of alveolar units, thickened alveolar septa, cellular consolidated areas, presence of inflammatory infiltrates, oedema and haemorrhage. The severity of these lesions was extraordinarily variable among animals of the same group in both sham and ischemic animals. Again, surgery stress, anaesthesia and inflammatory response due to the cranial trepanation could also explain these non-specific lung lesions found in both sham and ischemic animals.

Compiling our results, we have found many unexpected changes in sham animals in the present study. The animal (species and strain) or the model we have performed to induce ischemia could have had a big influence in our final results. To our knowledge, this is the first study inducing ischemia in rats by embolic MCAO and assessing bacterial analysis and lung histology thus we can speculate that the strong severity of the model (in part due to the cranial trepanation and the prolonged anaesthesia) could contribute to an important systemic inflammatory response. Despite our efforts in reducing the pain and discomfort of the rats subjected to an embolic stroke, the mortality associated to this experimental model is around 40%, as similarly shown in other studies [17] and can be attributed to the severe brain injury and to some secondary complications such as the loss of weight. Investigation of immunity and infection response at further time points (5–7 days after MCAO) would be, undoubtedly interesting. Nonetheless, such mortality rate during the first 48 hours limits the potential of studying longer time points that may allow us to assess more evident differences between sham and ischemic animals and avoid the effects of the surgery. Besides, although the embolic model is believed to be the most resembling stroke model to human pathology, it leads to a considerable variability among animals in terms of infarct volume and outcome which makes more difficult to reach to conclusions.

On the other hand, the fact of obtaining similar results after submitting the rats to a tMCAO model introducing a nylon filament and submitting them to an embolic MCAO model makes us consider the cranial trepanation and the cervicotomy as responsibles for the immunodepression state detected also in sham animals. In this direction, some authors concluded that even being sterile, brain surgeries may contribute to a general immunodepression [36] induced by stress and injury [37]. Considering this statement, our sham rats (submited to a cranial trepanation) could also be considered a model of brain surgery. Additionally, cervicotomy performed in both ischemic and sham animals,

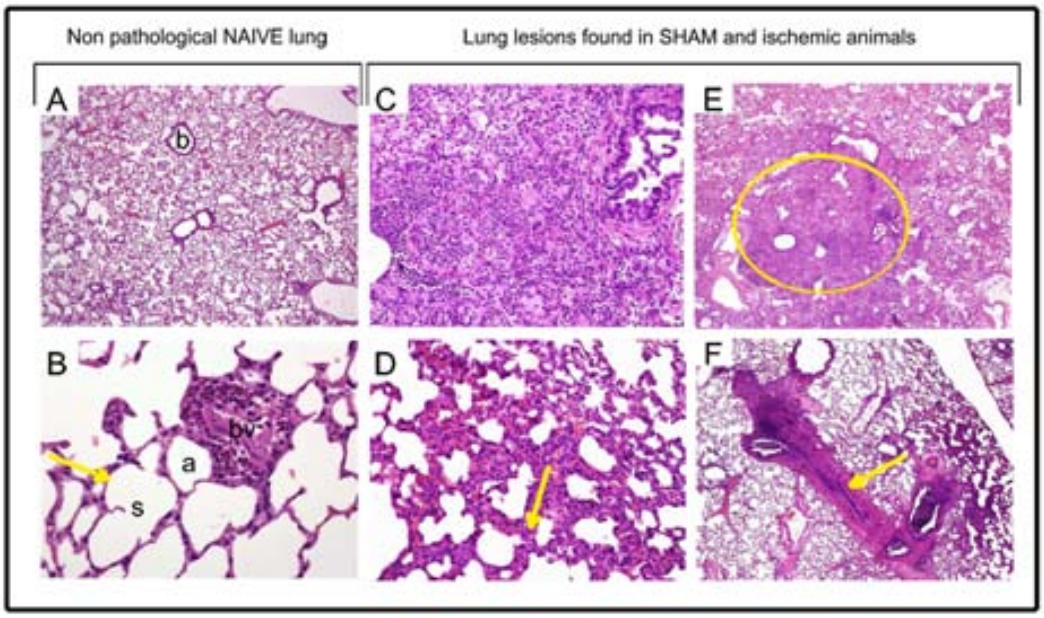

Figure 4. Representative images of lung histology after HE stain. (A) Naive rat lung showing a normal architecture (100x magnification), (b) indicates normal bronchioles. (B) Naive rat lung with alveolar sacs (s), expanded and non-filled alveoli (a), thin septa (arrow), and blood vessels (bv), (400x magnification). (C–F) Lung lesions observed in both sham and ischemic rats (C) Interstitial inflammatory cellular infiltration (100x magnification). (D) Thickened septa (arrow) and inflammatory cellular infiltration (400x magnification). (E) Patchy areas of cellular consolidation (circle), (100x magnification). (F) Acute bronchiolitis (arrow) surrounded by acute pneumonia (400x magnification).
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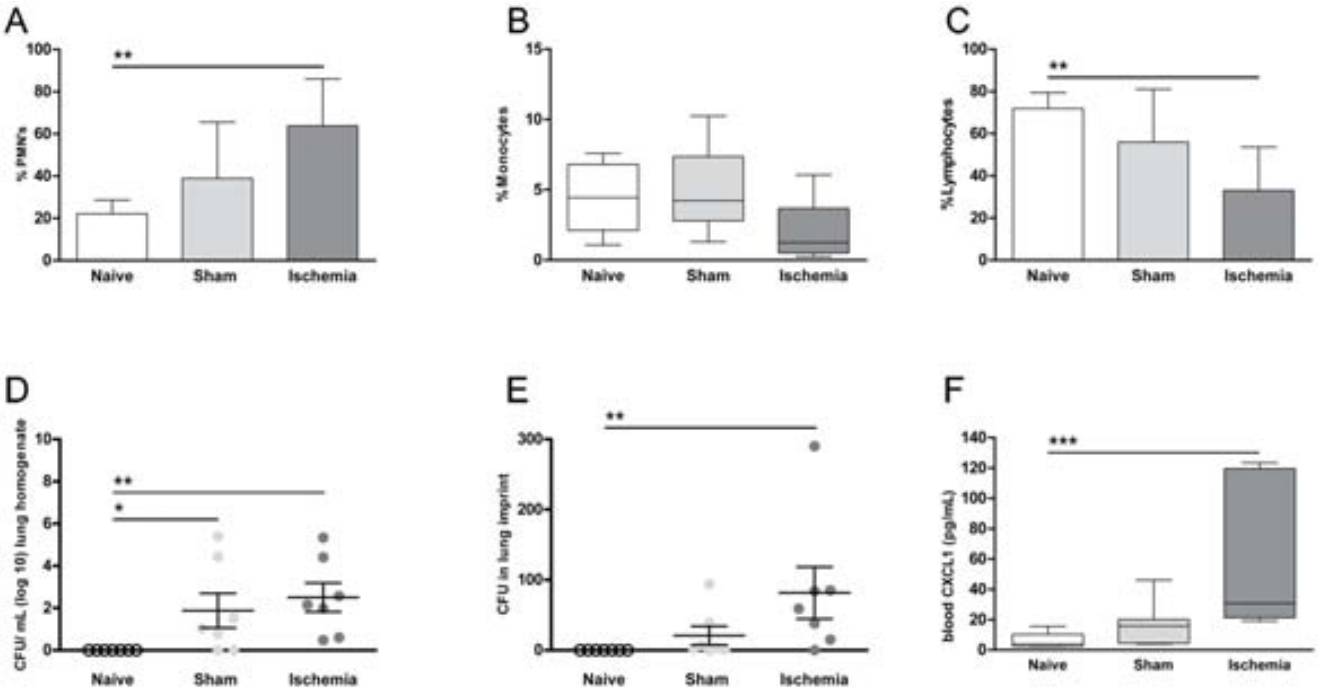

Figure 5. Leukocyte percentages, bacteriological analysis and measurement of CXCL1 plasma levels of a new set of animals submitted to a tMCAO model (90 min). Animals were evaluated at 48 hours after ischemia or sham surgery. (A) Peripheral PMNs percentage: a significant increase was observed in ischemic animals with respect to the naive group ($p<0.01$). (B) Lymphocyte percentage. A significant decrease was detected between naive and ischemic animals ($p<0.01$) but no differences were shown between sham and ischemic animals. (C) Monocyte percentage. (D) Number of colonies (CFU) found in lung homogenates expressed as log 10. Significant differences were detected when comparing naive to sham groups ($p<0.05$) and naive to ischemia groups ($p<0.01$). (E) Number of colonies in lung imprints. Only the comparison between naive and ischemic animals reached significance ($p<0.01$). (F) CXCL1 plasma levels in naive, sham and ischemic rats. In agreement with peripheral PMNs percentage data, a significant increase was found in ischemic animals with respect to naive ones ($p<0.001$). In all graphs, naive ($n=7$), sham ($n=7$) and ischemia ($n=7$).
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which can envolve iatrogenic manipulation of Vagus nerve, may also contribute to immune system alterations.

In agreement with Campbell *et al.* [38] who reported that after an acute injury in rodent brain, central nervous system triggers CXC chemokine expression (which is associated with leukocyte mobilisation), CXCL1 blood levels appeared elevated in both sham and ischemic animals in our study (although only the comparison between naive and ischemic animals reached significance). Moreover, good correlation was found between PMNs mobilization and CXCL1 blood levels. With these findings we can affirm; firstly, that both surgical stress and volatile anaesthesia exerted effects on chemokine response, as stated by Denes *et al.* [30]. Secondly, that cerebral ischemia *per se* triggers an additional chemokine effect. Thirdly, that in this animal model, as found previously in others [30,36] chemokines such as CXCL1 are contributing to PMNs mobilization.

Consequently, an important result to highlight from our study is that including a sham animal group in stroke-induce infection studies is extremely important to identify surgery-related responses and avoid misinterpretation of the data from ischemic animals. Additionally, it might be of great importance to check the sanitary status of the animals (presence of opportunistic and not only pathogenic microorganisms) previously to the surgery if the aim of the study is to evaluate spontaneous infections.

In conclusion, our study could not discriminate ischemic from sham animals in terms of infection evaluation after embolic MCAO. Therefore, we cannot affirm that changes observed in ischemic animals are the consequence of the ischemic insult. The initial hypothesis of our study was that stroke-induced immunodepression in the rat would make ischemic animals more prone to respiratory infections. Considering very recent publications which report some neuroprotectants to ameliorate stroke-induced peripheral immunodepression [35], we thought that having a rat model of stroke-induced immunodepression would be of great importance to test potential drugs for immunomodulatory therapeutic strategies. But unexpectedly, we found that in almost all evaluated parameters, sham animals were more similar to ischemic than to naive animals. Thus, our caution is that both embolic and intraluminal nylon suture MCAO in rats are not suitable models to study infections after stroke. All in all, the differences on immunological parameters depending on the species and strain and the influence of anaesthetics and surgical stress have to be taken into account in further experimental and translational studies for immunomodulatory therapeutic strategies, at least in rat stroke models which entail cervicotomy and cranial trepanation.

Author Contributions

Conceived and designed the experiments: MC MHG AR LGB JM. Performed the experiments: MC MAM JG. Analyzed the data: MC MAM DS. Contributed reagents/materials/analysis tools: MAM JG. Wrote the paper: MC MHG LGB AR DS JM.

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ARTICLE 5:

Characterization of simvastatin encapsulation into liposomal delivery system after ischemic stroke in rats. ***(Manuscript en preparació. No publicat ni valorat per peer review)***

Characterization of simvastatin encapsulation into liposomal delivery system to treat experimental ischemic stroke in rats

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ABSTRACT

Background and Aims: Although statins beneficial effects on stroke have been widely demonstrated both in experimental studies and in clinical trials the aim of this study is to produce and characterize liposomal systems encapsulating simvastatin to improve such effects.

Methods: In order to select a liposome composition with higher capacity to reach the brain, male Wistar rats were submitted to MCAOt surgery and treated (i.v) with fluorescent-labelled liposomes with different net surface charges: liposome type 1 (LIPO1), liposome type 2 (LIPO2) and liposome type 3 (LIPO3). Brain, blood, liver, lung, spleen and kidneys were evaluated *ex-vivo* 90 minutes after the liposomes administratation by the imaging system Xenogen IVIS® Spectrum. In a second sub-study, simvastatin arrival into the brain comparing free or encapsulated simvastatin (i.v) administration was assessed. For that purpose, simvastatin levels in brain homogenates from MCAOt rats after 2 or 4 h of receiving the treatment, were detected through UHPLC.

Results: Whereas LIPO1 were not detected in brain or plasma 90 minutes after their administration, LIPO2 and LIPO3 were able to reach the brain and accumulate specifically in the infarcted area. Moreover, LIPO2 exhibited higher bioavailability in plasma 4 hours after being administered. The detection of simvastatin by UHPLC confirmed its capability to cross the blood brain barrier (BBB) either when administered as a free drug or encapsulated into liposomes.

Conclusions: This study confirms that liposomes' charge is critical to promote its accumulation in the brain infarct area after MCAOt. Furthermore, simvastatin can be delivered in spite of being encapsulated. Thus, simvastatin encapsulation might be a promising strategy in order to ensure the drug arrival to the brain while increasing its bioavailability and reducing possible secondary effects.

INTRODUCTION

Despite all efforts, stroke still remains a major cause of death and disability worldwide that contributes to the rising costs of healthcare (Roger *et al*. 2012). Nowadays, the thrombolytic treatment with the Tissue Plasminogen Activator (tPA) agent is the only existing therapy for the acute phase of stroke (NINDS, 1995). Thus, the need of finding efficient neuroprotectant drugs with few side effects is becoming an urgency.

Many studies have shown beneficial effects of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) when administered before or after ischemic stroke in animals (García-Bonilla *et al.* 2013, Campos-Martorell *et al.* 2014) as well as in decreasing the incidence of stroke in patients (Jonsson and Asplund 2001, Martí-Fàbregas *et al*. 2004). However, statin benefits in cerebral ischemia have not been attributed to cholesterol-lowering therapeutic actions. Alternatively, pleiotropic effects seem to be implicated in its protective role in stroke (Laufs *et al.* 1998, Balduini *et al.* 2001, Rosenson 1999, Jain and Ridker 2005). On the other hand, high-dose statins therapy in humans have been associated with infrequent but undesirable side-effects such as myopathy, rhabdomiolysis or elevated hepatic transaminases (Wang *et al.* 2008).

In this direction, the use of drug delivery systems such as liposomes is being seriously considered in order to reduce secondary effects by site-selected delivery of the drug. In spite of simvastatin being able to cross blood brain barrier (BBB) *per se* due to its lipophilic condition and its reduced molecular weight (418 kDa), it exhibits poor bioavailability (Romana *et al.* 2014).

Furthermore, it has been described that BBB disruption occurs at an early stage after cerebral occlusion, allowing macromolecules to pass through the spaces between capillary endothelial cells (Ishii *et al.* 2013). Accordingly, nanoparticles might also be able to leak into brain parenchyma during the acute phase of cerebral ischemia. Interestingly, Sahagun and colleagues suggested that both size and charge of drug carriers are important in determining net brain permeation (Sahagun *et. al.* 1990).

Consequently, our hypothesis is that the bioavailability of simvastatin will be enhanced when it is encapsulated into liposomes, optimizing the opportunities to reach the ischemic brain and increasing the neuroprotective effect, while making the drug safer.

To that end, in this study we explored and characterized the tissue retention patterns of liposomes presenting different net surface charges. Additionally, we assessed how simvastatin encapsulated into the best candidate liposome was able to reach the rat ischemic brain compared to the free delivery of the drug.

MATERIALS AND METHODS

Ethics Statement

All procedures were approved by the Ethics Committee of the Vall d'Hebron Research Institute (protocol numbers 01/13 and 58/13) and were conducted in compliance with the Spanish legislation and in accordance with the Directives of the European Union. In all experiments, male Wistar rats (270 to 300 g; Charles River Laboratories Inc., Wilmington, MA, USA) were used. Rats were kept in a climate-controlled environment on a 12-hour light/12-hour dark cycle. Food and water were available *ad libitum* and analgesia (Buprenorfina, 0.05 mg/Kg s.c, Divasa Farma-Vic S.A, Barcelona, Spain) was

given to all rats in order to minimize their pain and discomfort.

Intraluminal tMCAO

Infarction in the territory of the middle cerebral artery (MCA) was induced by introducing an intraluminal filament as described previously (García-Bonilla *et al.* 2011). Animals were anesthetized under spontaneous respiration with 2% isoflurane (Abbot Laboratories, Kent, UK) in oxygen during surgery and body temperature was maintained at 37℃. In brief, after the surgical exposure of the bifurcation of the external carotid artery and the internal carotid artery on the right side, a silicone-coated nylon monofilament (Doccol Corporation, reference number: 403723PK10) was introduced to occlude the MCA. After occlusion, animals were allowed to recover from anesthesia. Reperfusion was induced 90 minutes later and to that end, animals were re-anaesthetized. Cranial trepanation was performed the day before MCAO surgery to attach a laser–Doppler probe (Moor Instruments, Devon, UK) and monitor regional cerebral blood flow. Only animals that exhibited a reduction >75% in regional cerebral blood flow after filament placement and a recovery of >75% after filament removal were included in the study. Analgesia was administered just after cranial trepanation. Naïve rats were not submitted to any surgical procedure.

Experimental design

A total of 107 animals were needed to complete the study. In order to characterize different liposome composition, 50 animals were used. Among them, 12 animals were excluded after applying the following criteria: uncompleted reperfusion after filament removal (n=4), poor cerebral blood flow register (n=3), presence of subarachnoid hemorrhage or other hemorrhage subtype (n=4) and death before experimental protocol finished (n=1). In the second sub-study in which simvastatin was determined through UHPLC (Ultra high performance liquid chromatography) a total of 57 rats were used. Among them, 16 animals were excluded following the same criteria: uncompleted reperfusion after filament removal (n=6), poor cerebral blood flow register (n=6), presence of subarachnoid hemorrhage (n=3) and death before experimental protocol finished (n=1).

Neurological evaluation

Rats were assessed using a 9-point neurological deficit scale (modified Bederson test), as previously described (Pérez-Asensio *et al*. 2005). 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) parachute reflex (symmetrical=0, asymmetrical=1, contralateral forelimb retracted=2), and (IV) resistance to left forepaw stretching (stretching not allowed=0, stretching allowed after some

attempts=1, no resistance=2). Neurological score was assessed in a blinded manner at 90 minutes and only animals which exhibited a score higher than 3 were included in the study.

Liposomes synthesis and characterization

Three differently charged liposomes (LIPO1, LIPO2 and LIPO3) were prepared using the thin film hydratation method with some modifications (Lowery *et al*. 2011, Afergan *et al*. 2010). Before its encapsulation, simvastatin was activated by opening the lactone ring. In short, simvastatin was dissolved in vehicle [distilled H_2O (75%), absolute ethanol (10%) and 0.1M NaOH (15%)], incubated at 50ºC for 2 hours and pH adjusted at 7.2.

Simvastatin encapsulation efficiency was calculated according to the equation, $EE(\%) = \left(\frac{[[(C]_{total} - C_{free}))}{C_{total}}\right] \times 100$ where C_{total} is the initial simvastatin concentration and C_{free} is the concentration of non-encapsulated simvastatin. To quantify C_{free}, liposomes were centrifugated and aliquots of supernatant were analyzed by UV-Vis spectroscopy using a Nanodrop ND-1000 (Thermo Scientific, USA). Mean absorbance values were introduced in a standard calibration curve for the estimation of C_{free} simvastatin.

Figures 5A and 5B show a scheme and a cryo-TEM image of liposomes encapsulating simvastatin, respectively.

Liposomes distribution (IVIS)

To monitor the distribution in the rat body, liposomes were fluorescent labelled with Alexa Fluor 750 (catalog no: A-20011, Life Technologies, NY, USA). To that aim, Alexa Fluor 750 (AF750) succinimidyl ester and DOPE-NH_2 were conjugated as previously described (Papagiannaros *et al.* 2013). Only conjugated Alexa Fluor 750 was detected by Thin-layer chromatography (Rf=0.6), indicating that conjugation was complete. The fluorescent labelled AF750 liposomes were prepared by incorporating AF750-DOPE into the lipid mixture (0.01 molar ratio).

Alexa Fluor-labelled liposomes were intravenously injected into MCAO (n=3-4 per liposome charge) rats 90 minutes after the surgery. In MCAO animals, filament was removed also after 90 minutes of occlusion. Thus, labelled-liposomes were administered in ischemic rats just when reperfusion started. All rats were euthanized 90 minutes after labelled-liposomes administration and brain, liver, spleen, kidney, lung and plasma were removed from all animals. All organs were entire maintained on ice until analyzed *ex vivo* with an imaging system (Xenogen IVIS® Spectrum; PerkinElmer®, Massachusetts, USA). Brains were analyzed considering both the entire organ and slicing it into 2-mm thick coronal sections in order to explore the exact localization of the liposomes. Captured images were afterwards analyzed using Living Image 4.3.1 software (PerkinElmer®, Massachusetts, USA). Liposomes signal data were in all cases

corrected twice: subtracting the signal detected in non-liposomes administered animal (background) as well as applying a correction factor in order to normalize fluorescent signal depending on the day and on the liposome charge. Fluorescent signal data from all tissues were obtained considering the mean between anterior and posterior view. Data are expressed as radiant efficiency, considered a calibrated measurement of the photon emission from the subject.

Liposomes kinetics in plasma

Naïve rats received Alexa-Fluor labelled-liposomes (LIPO2 (n=3) or LIPO3 (n=3)) and blood samples were collected from tail vein after 1.5, 4 or 24 hours in EDTA tubs. Fifty uL of plasma were placed in well plates and analyzed with Xenogen IVIS® Spectrum. Fluorescent signal was quantified with Living Image software and results expressed as radiant efficiency.

Determination of simvastatin in brain tissue (UHPLC)

The aim of this sub-study was to explore simvastatin capability of crossing blood-brain-barrier and to assess its possible enhancement after encapsulating it into liposomes. MCAO rats received either free or encapsulated simvastatin into LIPO2 (previously chosen in the former sub-study) and were euthanized 2 (n= 16) or 4 hours (n=16) after drug intravenously administration. Naïve animals (n=3) without receiving treatment were also evaluated to correct background signal. Under anaesthesia, animals were transcardially perfused with heparine solution followed by saline solution, both injected with an infusion pump. Brains were removed, divided into ipsilateral (IP) and contralateral (CL) hemispheres and weighted. Each hemisphere was homogenated separately with a buffer composed of methanol and distilled water (1:1). Each gram of brain tissue was homogenated in 5mL of the aforementioned buffer and sonicated. Homogenate samples were stored at -80ºC until analysis was performed in all samples at the same time.

Simvastatin extraction was performed as previously described (Zhang *et al.* 2010). One hundred uL of each sample was mixed with 100 uL of Internal standard (IS) solution ($[^2H_6]$-Simvastatin hydroxy acid ammonium salt, catalog no: c3520, Alsachim, Illkirch Graffenstaden, France) and 150uL of acetronitrile. Then, the mixture was vortexed at 1200 rpm for 30 seconds, 50uL of 5M ammonium formate buffer (pH 4) were added. Samples were vortexed again and centrifuged at 13000G at 4ºC for 10 minutes. One hundred uL of supernatant was transferred into the injection tube where it was diluted with 100 uL of mobile phase (ammonium acetate 0.5mM pH 4.5:acetronitrile) (70:30). Tubes were vortexed again before samples were injected.

Chromatographic detection was performed using an UPLC System Acquity (Waters) with ACQ-binary solvent manager and CQ-sample manager. The separation was made in a ACQUITY BEH 2.1x100 mm Phenyl analytical column with precolumn filter. Mobile phase was composed of acetonitrile and 0.5mM ammonium acetate

buffer pH 4.5 using gradient elution, initial mobile phase composition being acetonitrile, ammonium acetate buffer (30:70). Thereafter, the concentration was changed within 1.5 minutes to 30% of ammoniumacetate buffer and subsequently to 5% of the buffer within 5.25 min. Flow rate was 0.25 ml/min. The analytical column was kept at 35º C by column oven and solutions were stored in the autosampler at 4ºC. The full loop injection mode was set up to inject 5µl using 10µl injection loop. Acetonitrile was used as a strong wash and 70% ammonium acetate buffer in acetonitrile was used as a weak wash solvent.

Detection was performed by a MS/MS triple quadrupole system Xevo TQ MS (Waters) with electrospray ionization (ESI) in negative mode for active simvastatin (called simvastatin from now on) and simvastatin acid-d6 (IS), using transitions m/z 435 to 114.7 and 435 to 318 for simvastatin and 441 to 319.9 for IS. The ranges of simvastatin quantification were 4 to 40 ng/mL in brain homogenate.

Statistical analysis

Data were analyzed using GraphPad Prism_v5 software. Statistical significance for intergroup differences was tested by Student's t-test and ANOVA followed by Bonferroni post hoc test for parametric data. For non-parametric data, Mann Whitney and Kruskal-Wallis test followed by Dunn's Multiple Comparison test was performed. For parametric data, bars or symbols represent mean± SD and for non-parametric data, box plots or symbols represent median (Interquartile range). Two way ANOVA was used in order to compare the liposomes signal by IVIS in plasma over time. A p value <0.05 was considered statistically significant at a 95% confidence level.

RESULTS

Liposomes characterization

Series of liposomal formulations with different lipid membrane composition and net surface charges were prepared to determine the optimal formulation to ensure the liposomes permeation to ischemic brain. Figure 1 shows cryoTEM captures of 3 different liposome types. In all cases, a 5% of CHOL-PEG was included to achieve uniform samples and fluorescent Alexa Fluor 750 dye was added to the lipid mixture to obtain fluorescent labelled liposomes for its *in vivo* tracking. All formulations were formed by small unilamellar vesicles (SUVs) with a mean particle size ranging from 154.60 to 171.07 nm.

Cerebral distribution of liposomes

Alexa fluor-labelled liposomes were detected in the whole brain 90 minutes after their administration in MCAO animals only when LIPO2 or LIPO3 were used (Figure 2A).

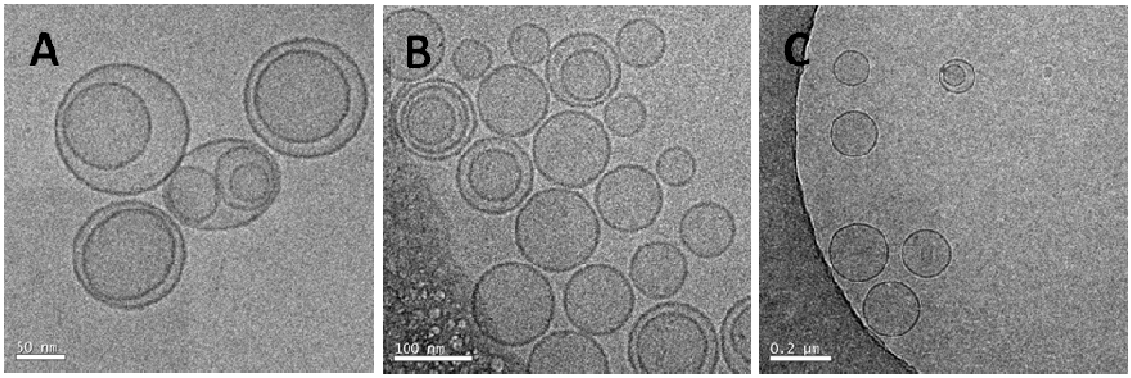

Figure 1. **CryoTEM images of liposomes A)** LIPO2 **B)** LIPO1 **C)** LIPO3

After correction, ischemic rats administered with LIPO2 also presented a significant signal increase in the brain only when compared with the signal emited by LIPO1 (1.320e+009 [3.040e+008, 2.490e+009] vs 3,430e+007 [5,550e+006, 7,890e+007], p<0.05) (Figure 2C). Similar quantifications were obtained when brains were sliced into coronal sections (data not shown). Remarkably, when liposomes were administered, signal tended to concentrate in the IP hemisphere, mostly in the infarcted region, rather than in the CL side (Figure 2B).

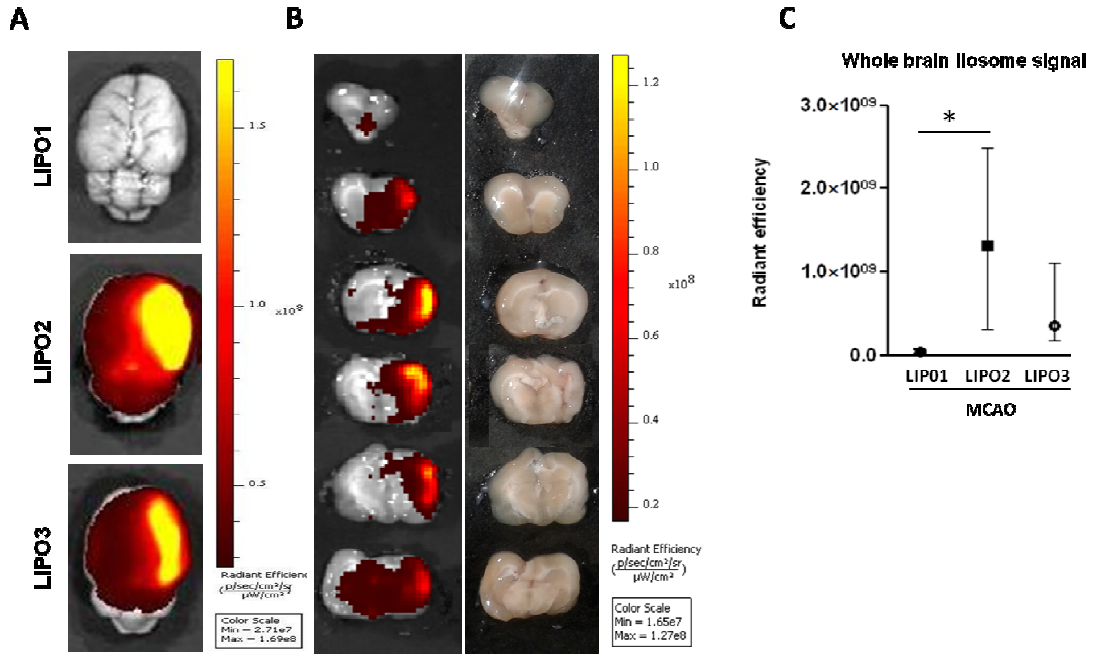

Figure 2. Representative *ex vivo* rat brain images and fluorescent signal quantifications captured by an imaging system (Xenogen IVIS® Spectrum). **A)** Whole brain of representative ischemic rats after 90 minutes of receiving different net surface charged liposomes. **B)** Representative sliced rat brain after being submitted to a MCAO and receiving LIPO2. Fluorescent signal co-localize with infarcted area. **C)** Quantifications of liposome-emitted signal in MCAO rat brain expressed as radiant efficiency. Images are adjusted to the scale positioned beside. Data present 4 different experiments in which 3-4 animals per group were included. Symbols indicated median (Interquartile range) and significant differences are indicated as *p<0.05.

Liposomes distribution in other tissues

Ninety minutes after liposomes administration, different organs were analyzed by IVIS so as to explore liposomes affinity to different tissues depending on their net surface charge. Liver, spleen, kidney, and lung were examined in ischemic animals (n=10). Statistics did not show any significant difference regarding different charged-liposomes when these 4 tissues were evaluated (Figure 3).

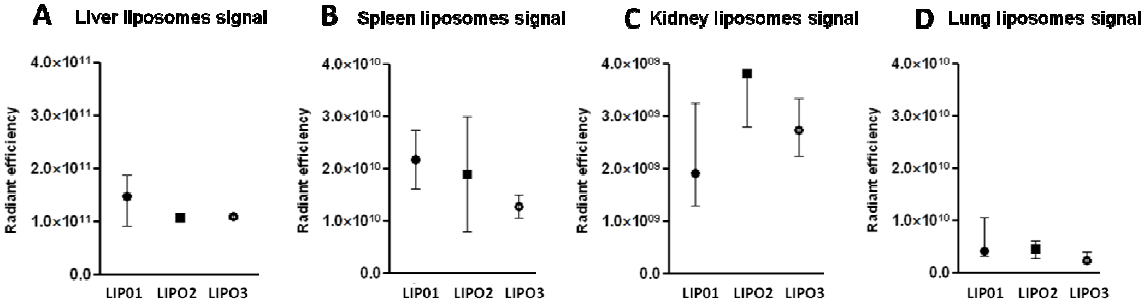

Figure 3. Fluorescent signal quantifications of images captured by Imaging system (Xenogen IVIS® Spectrum) considering differentially-charged liposomes distribution within the body in MCAO rats. A) Liposomes accumulation in liver. **B)** In spleen. **C)** In kidney. **D)** In lung. In each group, 3-4 animals were included. Symbols indicated mean ± SD or median (Interquartile range) depending on normality data distribution. Significant differences are as indicated: *p<0.05, **p<0.01.

Plasma liposomes bioavailability

Plasma collected 90 minutes after liposomes administration exhibited unexpected differences between groups. Ischemic rats treated with LIPO2 showed a significant higher fluorescent signal in plasma compared to animals treated with LIPO1 (1.100e+008 [5.210e+007, 1.250e+008] *vs.* 3.995e+006 [1.840e+006, 6.020e+006], p<0.05) (Figure 4A, 4B). Although fluorescent signal emitted by LIPO2 was higher than those emitted by LIPO3 in ischemic animals, differences did not reach statistic significance.

In order to further study the consequences of administrating LIPO2 or LIPO3, the plasma bioavailability of these two groups was studied in more detail at different time points. Strikingly, 90 minutes after administration, LIPO2 exhibited significantly elevated signal as compared with LIPO3 (1.437e+008 ± 8.737e+006 *vs.* 6.353e+007 ± 9.278e+006, p<0.001). In the same direction, signal detected for LIPO2 was also higher 4 hours after administration (3.767e+007 ± 1.386e+007 *vs*. 1.470e+007 ± 4.987e+006, p<0.05) whereas almost no signal was observed at 24 hours in none of the groups (Figure 4C, 4D).

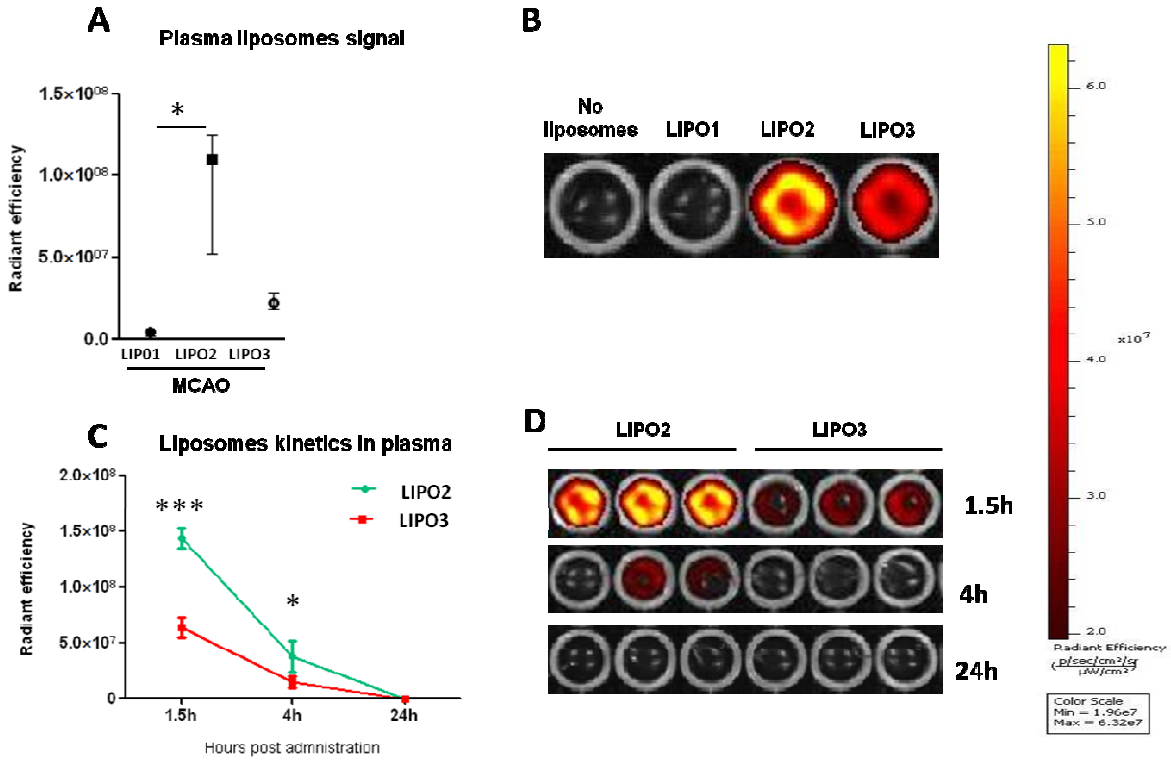

Figure 4. Liposomes presence in plasma. A) Liposomes signal in plasma. Plasma was collected from ischemic rats 90 minutes after receiving liposomes. Symbols indicated median (Interquartile range) and significant differences are indicated as *p<0.05. **B)** Representative plasma image of one experiment. **C)** Liposomes kinetics in plasma. Blood was collected from naïve rats after 1.5, 4 and 24 hours of receiving LIPO2 or LIPO3. Symbols indicated mean ± SD. **D)** Image representing data on graph C. Images are adjusted to the scale positioned beside. In all experiments, 3-4 animals per group were considered. Significant differences are represented as *p<0.05, **p<0.01 and ***p<0.001.

Simvastatin detection in brain

Considering the first sub-study results, LIPO2 were selected as the optimal candidates for further experiments. Consequently, in order to study simvastatin arrival into the brain, LIPO2 were characterized after being loaded with the drug (LIP4S) (Figure 5A). When loaded with simvastatin, by cryo-TEM analysis, it was also observed that liposomes encapsulating simvastatin, still presenteded a small unilamellar vesicle (SUV) morphology (Figure 5B).

Analyzing brain hemisphere homogenates by UHPLC technique, we were able to detect simvastatin as well as simvastatin hydroxy acid ammonium salt, used as an Internal Standard (IS). Each compound presented a different peak, easily distinguishable, when a sample was analyzed (Figure 5C, 5D).

Through UHPLC technique we could demonstrate that simvastatin was capable of crossing the BBB in both administration forms (free and into liposomes) since it was detectable in rat brains after 2 and 4 hours of administration (Figure 6B). In general terms, higher simvastatin levels were detected when rats were euthanized at 2 hours, compared with 4 hours post treatment. This difference was clearly evidenced when free simvastatin levels

were compared between IP hemispheres (IP. 2h: 2.6 [2.3, 3.8] *vs.* IP. 4h: 0.85 [0.3, 1.4], p<0.05) (Figure 6A), as well as when encapsulated simvastatin levels were compared (IP. 2h: 3.35 [1.3, 4.7] *vs.* IP. 4h: 0.9 [0.5, 1.0], p<0.05) (Figure 6B). Interestingly, ischemic brains showed higher simvastatin accumulation in the IP hemisphere than in the CL hemisphere when free simvastatin was administered (2h: IP isch.: 2.6 [2.3, 3.8] *vs.* CL isch.: 1.8 [0.0, 2.4], p<0.05) (Figure 6A).

Regarding the comparison between free or encapsulated administration, we can state that no differences were observed at none of the evaluated time points (Figure 6 B).

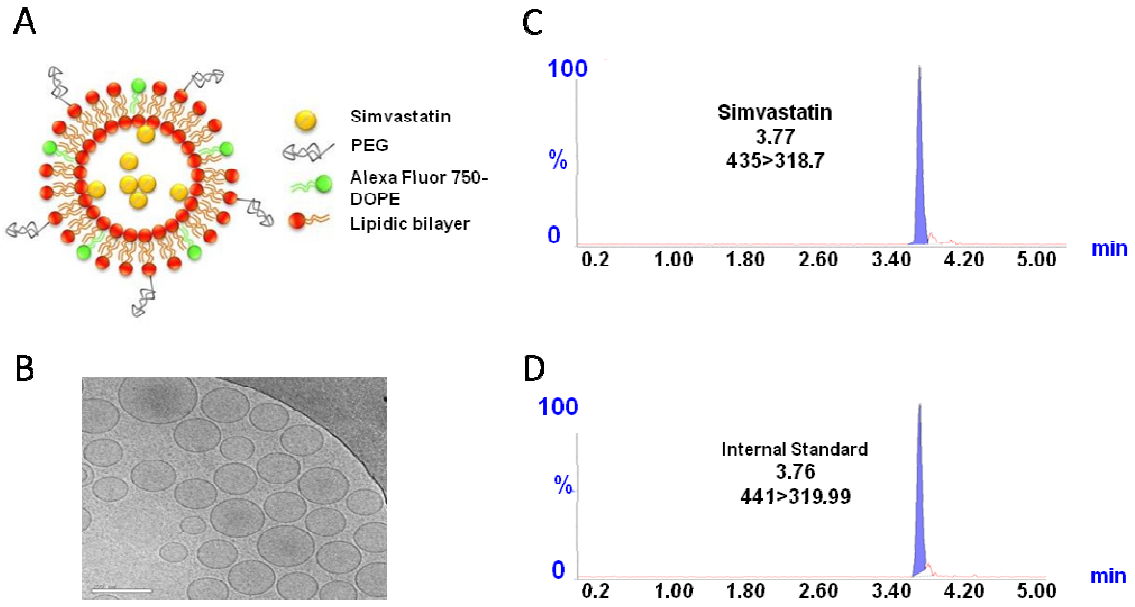

Figure 5. Simvastatin detection after its encapsulation into LIPO2. **A)** Scheme of LIPO2 compounds used in detection experiments **B)** CryoTEM image of liposomes with encapsulated simvastatin **C, D)** Chromatography graphs showing peaks corresponding to **C)** simvastatin, and **D)** Internal Standard (Simvastatin hydroxy acid ammonium salt). For each compound analyzed, retention time (expressed in minutes) and transition are presented.

DISCUSSION

As previously described (Ishiii *et al*. 2013) the use of a liposomal drug delivery system is a promising strategy for avoiding side effects and enhancing drug efficiency by changing the distribution of the intact drug. Liposomes main advantages entail characteristics such as biocompatibility or nontoxicity, ability to protect their cargo from degradation by plasma enzymes and capability of transporting their load across biological membranes (Elbayoumi and Torchilin 2010). Until present, liposomes have been used clinically as cargo systems for therapeutic drug delivery of

chemotherapeutic agents, antibiotics, and antifungals (Spuch and Navarro 2011) showing an increase in some drugs safety and efficacy. Taking into account the difficulty for neuroprotectants to reach the target zone due to BBB presence, we considered that the development of a suitable liposomal carrier to encapsulate neuroactive compounds was highly encouraging.

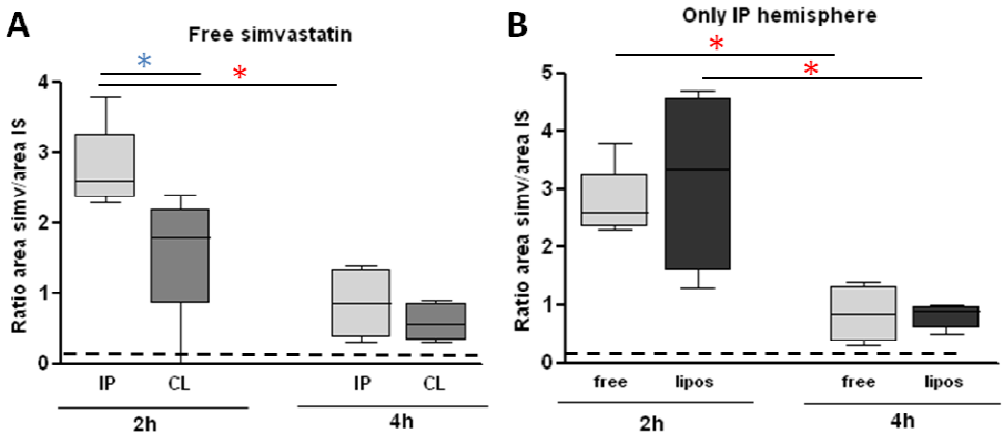

Figure 6. Active simvastatin detection in brain tissue through UHPLC technique after rats being treated intravenously with either free simvastatin or simvastatin encapsulated into LIPO2. All rats were treated with the same simvastatin dose (20mg/kg). Rats were submitted to MCAO surgery, treated 90 minutes later and killed 2 or 4 hours after treatment administration. **A)** Detection of simvastatin when rats were treated with free simvastatin and euthanized 2 or 4 hours later. **B)** Detection of simvastatin in ipsilateral (IP) hemispheres when rats were treated with free or encapsulated simvastatin and killed 2 or 4 hours later. In all experiments, 3-5 animals per group were included. Box plots indicated median (Interquartile range). Significant differences are represented as *p<0.05 after applying Mann Whitney test (in red: comparisons between 2 and 4 hours, in blue: comparisons between IP and CL hemispheres). Broken lines represent background signal detected through UHPLC technique when brains of naïve animals without receiving treatment were analyzed.

Aggarwal and colleagues (2009) previously stated that nanoparticle properties such as size, shape, solubility, surface modifications and zeta potential (surface charge) can determine their distribution within the body. For instance, the presence of polyethylene glycol (PEG) on the surface of the liposomal carrier has been shown to extend blood-circulation time while reducing mononuclear phagocyte system uptake (Immordino *et al.* 2006), and, consequently, decreasing their recognition by the liver and spleen (Liu *et al.* 1997). On the other hand, plasma proteins are described to bind the surface of nanoparticles immediately upon their introduction into a physiological environment (Aggarwal *et al*. 2009) forming a nanoparticle-protein complex which affect the internalization process of nanoparticles into macrophages and the overall distribution thorough the body (Moghimi *et al*. 2001, Owens and Peppas 2006). Accordingly, Gessner *et al.* (2002) postulated plasma protein absorption as a key factor for the *in vivo* organ distribution of intravenously administered colloidal drug carriers and concluded that it was strongly

influenced by nanoparticle surface characteristics.

All in all, we were able to produce and characterize liposomes with different zeta potential and to show that surface liposome charge contributes to define liposome behavior and distribution, supporting the aforementioned hypothesis.

When we analyzed the liposome detection in ischemic rat brain, we showed that LIPO2 and LIPO3 were able to cross BBB. Remarkably, higher intragroup variability was obtained probably attributable to the lesion severity.

Furthermore, we demonstrated that LIPO2 and LIPO3 were prone to accumulate in the ischemic core of the brain. In this sense, we reckon that the BBB disruption in this area (Kuroiwa *et al.* 1995, Belayev *et al.* 1996), as well as the inflammatory processes around the ischemic lesion (Yuan *et al.* 1994) might be the responsibles of such interesting accumulation. Thus, agreeing with the statement of Schroeder and colleagues (2014) who consider that the great interest in nanoliposomal systems stems from their ability to accumulate in sites of increased vascular permeability.

In order to examine whether LIPO2 or LIPO3 were the most proper delivery carriers, a plasma kinetics study was performed, analyzing blood liposomes signal from naïve animals at different time points. LIPO2 showed a more intense fluorescence both at 90 minutes and at 4 hours from being administered.

Taking as a whole, several factors seem to affect liposomes distribution and, undoubtedly, their surface charge is a very determinant one to be taken into consideration. Moreover, our experiments of simvastatin analysis in rat brain after its IV administration through UHPLC corroborated the capability of the compound of crossing the BBB *per se,* as other authors previously stated (Saheki *et al.* 1994, Vuletic *et al.* 2006). Simvastatin is lipophilic in nature, therefore, easily diffuses across enterocytes cell membranes. Interestingly, highest treatment accumulation was observed in IP hemispheres of ischemic animals, probably due to BBB disruption. As other studies reported (Misra *et al.* 2003), with BBB weakened, systemically administered drugs can undergo enhanced extravasation rates in the cerebral endothelium, leading to increased parenchymal drug concentrations. Moreover, we could detect more simvastatin in the rat brain at 2 than at 4 hours after being administered, accordingly with Bellosta and colleagues (2004) who reported simvastatin elimination half-life (t ½ (h)) being 2-3 hours. Furthermore, it should be emphasized that simvastatin is an inactive hydrophobic lactone prodrug which is commonly administered by oral route. After being orally administered, it is described to be metabolized *in vivo* to several more polar and pharmacologically active compound (Vickers *et al.* 1990, Mauro 1993) by both liver and plasma esterases (Lippert *et al*.2012). Given that some stroke patients suffer from disphagia, we considered that parenteral statin administration would represent an improvement for them. This is the reason why, in our study, simvastatin was intravenously administered and

activated *in vitro* before its administration. Our aim was to optimize simvastatin arrival to the brain and, consequently, its effect.

On the one hand, although our data showed similar levels of simvastatin in ischemic area after free or encapsulated simvastatin administration, thus not demonstrating an enhancing in drug delivery, we could state, at least, that liposomes do not impede simvastatin brain penetration. In this direction, variability among infarct sizes and small sample size could have negatively interfered with our data. At this point, we assume the impossibility to calculate infarct volumes as a clear limitation of the study. Correlation between infarct severity and liposome accumulation in brain would be undoubtedly of high interest.

On the other hand, simvastatin encapsulation might represent a clear advantage if we consider Romana and colleagues data (2014), who reported that nano-formulations such as liposomes might solve statin problems including adverse effects on muscles and liver or poor bioavailability. Although statins are reported to be extremely well tolerate (Ballard and Thomson 2013) and the frequency of clinically significant side effects associated is low (Kashani *et al.* 2006) some studies have described elevated transaminases and muscle pain when statins where chronically administered (de Denus *et al.* 2004, Baigent *et al.* 2005).

Since we could demonstrate that liposomes are able to accumulate in the infarct area and also that simvastatin can be delivered after being encapsulated, we believe that further *in vivo* studies are needed in order to explore encapsulated simvastatin neuroprotection effect as well as other beneficial effects such as liver or muscle toxicity reduction in chronic treatments.

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Disclosure/Conflict of interest

Liposomes' composition as well as its manufacturing process were not disclosed in this manuscript in preparation due to protection of intellectual property reasons.

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5. DISCUSSIÓ

5.1 Les estatines redueixen el volum d'infart i milloren el dèficit neurològic en animals sotmesos a models d'isquèmia cerebral

Tenint en compte la gran quantitat i variabilitat de treballs publicats que feien referència a l'estudi de les estatines usades en models animals d'isquèmia cerebral, vam creure necessari realitzar un metanàlisi (Article 1: García-Bonilla *et al. J Neurochem.*2012) per tal d'analitzar-ne els resultats conjuntament i poder-ne extreure algunes conclusions.

El fet que hi hagi tants estudis experimentals publicats és sinònim de que molts investigadors confien en la varietat d'efectes beneficiosos que són capaços de desencadenar aquests fàrmacs (efectes pleiotròpics) deixant de banda el seu conegut efecte hipocolesterolèmic. De fet, des dels anys 80, les estatines s'usen ja en la pràctica clínica com a tractament per a les malalties cardiovasculars (Grundy 1988), per a la prevenció de malalties vasculars (Nakamura *et al.* 2006), i per a la prevenció secundària de l'ictus (Laloux 2013, Kikuchi *et al.* 2012).

Malgrat el seu ús en pacients, els estudis en models animals continuen sent necessaris per tal d'estudiar diferents aspectes que requereixen l'aplicació de tècniques que no es poden dur a terme en humans (ex: avaluació del teixit cerebral per entendre els mecanismes d'acció de les estatines). Tanmateix, és necessari tenir molta precaució a l'hora d'extrapolar els resultats obtinguts en els estudis pre-clínics i és per això que cal tenir en compte la qualitat dels treballs publicats.

En referència a la qualitat dels estudis analitzats al metanàlisis, es va observar que els estudis que tenien una puntuació inferior, eren els que mostraven millors resultats pel que fa a l'eficàcia del tractament. Això demostra la importància d'administrar els tractaments de manera randomitzada i cega per tal de disminuir els biaixos que es poden ocasionar. Cal remarcar, també, la mala pràctica de no publicar els resultats negatius pel fet de semblar poc interessants. És precisament per això, que en el metanàlisi realitzat vam decidir incloure un *funnel plot* (eina estadística que permet corregir els biaixos deguts a la no publicació de resultats negatius). Cal tenir en compte que, malgrat el gran esforç realitzat durant anys per tal de desenvolupar models animals cada vegada més semblants a la patologia en humans, els models experimentals no són capaços de reproduir totes les característiques pròpies de la malaltia i, a més, comporten la introducció d'una sèrie de factors que poden potenciar els danys ocasionats per la pròpia isquèmia: l'estrès, la inflamació derivada de la cirurgia o l'anestèsia (Iadecola i Anrather 2011). La manca d'estudis realitzats en animals amb

comorbiditats (diabetis, hipertensió, edat avançada) també contribueix, en gran mesura, a que els models animals s'allunyin de la realitat de la patologia en humans.

Una de les conclusions més importants que podem extreure del metanàlisi realitzat és que d'entre les estatines avaluades, la simvastatina és la que mostrava major efecte neuroprotector (tenint en compte la reducció del volum d'infart i la milloria del dèficit neurològic), seguida de l'atorvastatina i finalment de la rosuvastatina. Cal dir que altres estatines també utilitzades en la pràctica clínica (pravastatina, fluvastatina, lovastatina, mevastatina, cerivastatina, pitavastatina i perivastatina) no van ser analitzades ja que hi havia un reduït nombre de publicacions, fet que impossibilitava l'anàlisi estadística. El fet que la rosuvastatina fos la que mostrava menys efecte neuroprotector podria atribuir-se a l'absorció selectiva que pateix a nivell dels hepatòcits (Nezasa *et al*. 2003) i que ocasiona una disminució en la seva biodisponibilitat. Pel que fa a l'atorvastatina, malgrat estar demostrats experimentalment els seus efectes com a neuroprotector (Asashi *et al.* 2005, Ding *et al*. 2006), en corregir les dades obtingudes pel biaix de les publicacions, veiérem reduïda la seva eficàcia respecte l'obtinguda amb la simvastatina.

A més, i com era d'esperar, vam poder comprovar que l'efecte de les estatines era superior quan aquestes s'administraven com a pre-tractament (abans d'induir la isquèmia cerebral) que com a post-tractament (després de produir-se la isquèmia cerebral), en concordança amb Danton i Dietrich (2004) que descrigueren que els tractaments solen funcionar millor quan s'administren abans de la isquèmia cerebral o immediatament després que aquesta es produeixi. La reducció de l'infart, per exemple, era dues vegades superior quan el tractament s'administrava abans que després de la isquèmia. De fet, ha quedat ben demostrat que el tractament amb estatines redueix el risc de patir un ictus (Prinz i Endres 2011, Amarenco i Labreuche 2009), i també l'eficàcia de les estatines en la prevenció d'un segon episodi (estudi SPARCL, Amarenco *et al.* 2009). És per això que el tractament amb estatines ja consta en les guies terapèutiques per a la prevenció secundària de l'ictus. Tanmateix, els resultats del metanàlisi van revelar que, malgrat mostrar una menor eficàcia respecte el pre-tractament, el post-tractament també aportava millories significatives en la reducció del volum de l'infart.

Un altre fet a destacar és que l'administració de múltiples dosis mostrà millors resultats que l'administració única. Cal puntualitzar, per això, que l'administració en múltiples dosis no va resultar ser un factor independent en la regressió logística. A més, en la majoria d'estudis on s'administraren múltiples dosis, aquests es realitzaren com a pre-tractament. És molt

probable, doncs, que aquest increment en l'eficàcia fos degut a l'administració prèvia a la isquèmia i no al tipus de dosificació establerta.

Per altra banda, per a tots els tipus d'estatina es va observar que l'eficàcia era major quan s'administraven dosis altes, tenint en compte el rang de dosificació establert per a cada tipus d'estatina. És interessant destacar que les dosis usades en els estudis pre-clínics són significativament superiors a les que s'usen en clínica humana. Mentre el rang usat per a la simvastatina en models animals sol ser de 10-100mg/Kg/dia (Jin *et al.* 2013), en humans és de 40mg/dia. Creiem que hi ha varis factors que poden explicar aquesta diferència en l'ús de les dosis. Per començar, no es coneix l'equivalència de dosis entre rosegadors i humans. De fet, Bjorkhem-Bergman *et al.* (2010) van arribar a la conclusió que el metabolisme de les estatines era diferent entre humans i rosegadors i que per això les dosis que podrien considerar-se tòxiques en humans, no tenien perquè ser-ho en rosegadors. Així mateix, cal tenir en compte que el metabolisme dels rosegadors és molt més accelerat que el dels humans. Cal destacar, també, que el tractament amb estatines en humans sol ser crònic (això pot equivaldre a molts anys), mentre que en animals, l'estudi que s' allarga més en el temps ho fa durant 14 dies o 1 mes. Per tot això creiem que la precaució d'administrar dosis baixes per evitar possibles efectes secundaris pot ser menys estricta en rosegadors. Altrament, en el nostre estudi, en el què es volia administrar la simvastatina com a neuroprotector, calia tenir compte que només un baix percentatge del medicament administrat seria capaç de travessar la BHE i d'arribar a la regió d'interès, mentre que en humans, les dosis d'estatines s'han establert per a altres fins terapèutics (aconseguir un efecte hipocolesterolèmic o com a prevenció secundària de malalties cardiovasculars). En tot cas, tot i que podem entendre que el fet d'administrar dosis tan elevades del medicament en els estudis pre-clínics pot considerar-se una limitació (Thelen *et al.* 2006), considerem que calen estudis més exhaustius per tal d'extrapolar correctament les dosis d'estatines, tal com recomanen les guies STAIR (Fisher et al. 2009).

Pel que fa a la via d'administració del fàrmac, la via oral va resultar més eficaç que la via subcutània o intraperitoneal. Pensem que una explicació podria ser que el medicament administrat oralment, passa a ser metabolitzat al fetge (Corsini *et al.* 1999) , on es produeixen importants metabòlits de les estatines (Bellosta *et al*. 2004). En tot cas, aquests resultats faciliten una possible translació a la clínica ja que les estatines en l'actualitat només s'administren per via oral. Tanmateix, cal prendre's aquestes dades amb molta cautela ja que la via d'administració no constà com a variable independent després de realitzar l'ànalisi de metaregressió.

Finalment, tenint en compte el model emprat per a induir la isquèmia cerebral als animals, no vam veure que hi hagués diferències en l'efectivitat de les estatines comparant els models transitoris (que permeten la reperfusió de l'artèria oclosa) amb els models permanents. Aquest resultat es podria atribuir a dos fets. Per una banda a que les estatines poden tenir efecte a diferents nivells (propietats antiinflamatòries i antioxidants, i millora de la perfusió cerebral) i que, per tant, podrien estar exercint efectes diferents segons el model. I, per l'altra, que les estatines actuessin sobre els mecanismes que tenen en comú els dos models.

En tot cas, aquest treball ens va servir per a confirmar l'evidència que les estatines exerceixen un efecte neuroprotector en administrar-se en models animals d'isquèmia cerebral i ens va permetre establir les millors característiques per a realitzar un estudi clínic en pacients que es trobessin en la fase aguda de l'ictus: administració oral de vàries dosis de simvastatina, i tan aviat com fos possible després de produir-se l'episodi isquèmic. Es va dissenyar, doncs, un estudi en humans amb aquestes característiques: estudi STARS (es pot consultar informació a la següent pàgina web: http://clinicaltrials.gov/show/NCT01073007). Esperem que un futur aquest estudi pugui servir per aplicar tota aquesta informació en la pràctica clínica i, sobretot, per a millorar la qualitat de vida dels pacients.

5.2 L' administració de simvastatina durant la fase aguda de la isquèmia cerebral és eficaç en un model embòlic en rata

Malgrat l'elevat nombre d'articles ja publicats demostrant l'efecte beneficiós de les estatines, cap d'ells estudiava l'efecte que podia tenir la seva administració en la fase aguda de la isquèmia cerebral en un model embòlic. Per la nostra experiència, i tot i les complicacions que comporta (elevada mortalitat i variabilitat), creiem que el model embòlic és el que millor reprodueix la patologia humana. El fet d'administrar un coàgul sanguini que després pot dissoldre's o no, simula millor la patologia humana que la inserció d'un filament de niló.

Tenint en compte les conclusions del metanàlisi, vam escollir la simvastatina d'entre totes les estatines perquè era la que millors resultats presentava. Pel que fa al temps d'administració, vam escollir un temps curt després de l'oclusió (15 minuts), tot i que l'equivalència d'aquest temps en humans no es fàcilment extrapolable. La nostra idea era que si podíem demostrar la seguretat i també l'eficàcia de l'administració de simvastatina després de la isquèmia cerebral, la simvastatina seria un medicament susceptible de ser administrat en cas de sospita d'ictus, a la mateixa ambulància, durant el trasllat del pacient cap a l'hospital, i abans del tractament amb l'rt-PA. La via d'administració escollida va ser la subcutània, malgrat la que havia demostrat ser més eficaç en el metanàlisi va ser l'oral. Pensàrem que evitar l'administració oral podria representar una avantatge afegida per a aquells pacients que pateixen problemes de disfàgia o baix nivell de consciència després de l'ictus.

Els resultats del nostre estudi realitzat en un model embòlic en rata (Article 2: Campos-Martorell *et al*. *J Neurochem.* 2014) indicaven que la simvastatina administrada durant la fase aguda de la isquèmia cerebral era capaç de reduir significativament tant el volum de l'infart com el dèficit neurològic. És per això que una vegada comprovat l'efecte neuroprotector de la simvastatina, vam creure convenient estudiar els mecanismes moleculars pels quals podria produir-se aquest efecte beneficiós mitjançant l'anàlisi proteòmica d'homogenats cerebrals de rata.

5.3 L' administració de simvastatina en la fase aguda de la isquèmia cerebral estimula la reparació cel·lular, disminueix l'estrès oxidatiu i atenua la permeabilitat de la BHE.

Tot i la gran quantitat d'efectes atribuïts a les estatines, l'objectiu d'aquest sub-estudi (inclòs en l'Article 2: Campos-Martorell *et al. J Neurochem.* 2014) era comprovar els mecanismes d'acció pels quals la simvastatina administrada en la fase aguda de la isquèmia cerebral exerciria un efecte neuroprotector. L'anàlisi proteòmica per tècnica DIGE va resultar ser un bon mètode per tenir una visió global, però a l'hora detallada, dels canvis proteics induïts pel tractament. Les diferències es van analitzar a les 48 hores de la isquèmia, temps en el qual la simvastatina havia mostrat exercir el seu efecte neuroprotector.

Un fet a destacar és que vam trobar diferències en l'expressió de proteïnes en funció de l'àrea cerebral que analitzàrem (còrtex o estriat). Podem justificar aquest fenomen tenint en compte que el còrtex es considera la zona "salvable" en els models d'oclusió de l'ACM, és a dir, la zona on es produeixen alteracions en la funcionalitat cel·lular i on s'esperaria veure recuperació després de l'administració d'un neuroprotector. Mentres que l'estriat, per contra, es considera el *core* de l'infart, és a dir, l'àrea de màxima gravetat de la isquèmia on es produeix una destrucció cel·lular. Malgrat al *core* és on esperaríem veure menys canvis després de l'administració del tractament (Joo *et al.* 2013), els nostres resultats indicaven que la simvastatina exercia efecte en ambdues zones.

De manera general, i tenint en compte els resultats de l'article 2, podem establir que el tractament amb simvastatina en el nostre model, desencadena 3 processos principals diferenciats: reparació cel·lular (representat per la sobreexpressió de molècules com Guanin Go i MAP RB) (Neves *et al.* 2002, Tortosa *et al.* 2013) disminució de l'estrès oxidatiu (nivells reduïts de HSP60, HSP75 i Fumarat hidratasa) (Yin *et al.* 2008, Kiang and Tsokos 1998) i atenuació de la permeabilitat de la BHE (nivells reduïts o absents de proteïnes de la circulació sanguínia com hemoglobina o albúmina) (Obermeier *et al.* 2013). Vam comprovar que els dos primers processos tenien lloc tant a la zona del córtex com de l'estriat, mentre que el tercer, que feia referència a l'atenuació de la permeabilitat de la BHE, es localitzava només a la zona de l'estriat de l'hemisferi IP en rates tractades amb simvastatina.

Una vegada obtinguts els resultats, calia validar-los i/o replicar-los i és per això que, mitjançant tècnica Western Blot (WB), es van analitzar les mateixes mostres usades en l'anàlisi proteòmica. Tot i detectar que prop del 54% de les proteïnes analitzades mostraven el mateix patró en ambdues tècniques, les diferencies no van resultar estadísticament significatives.

Conclòs l'estudi, podem assumir algunes limitacions. En primer lloc, el reduït número de mostres (4 mostres per grup) i, en segon, el fet que la tècnica de WB no permet la distinció de les diferents isoformes una proteïna, a diferència de la tècnica per proteòmica. Els gels de poliacrilamida i els agents reductors usats en la tècnica de WB mantenen les proteïnes en un estat de desnaturalització (sense estructura terciària ni quaternària), fet que permet la separació de les diferents proteïnes, únicament per pes molecular (Burnette 1981), i no per punt isoelèctric. Aquesta podria ser la causa per la què no hauríem validat exactament la mateixa isoforma de la proteïna amb les dues tècniques. I podria explicar, doncs, el baix percentatge de significació trobada en aquesta tècnica, en comparació amb els resultats obtinguts en l'anàlisi proteòmica.

Per tal d'avaluar la possible rellevància clínica dels nostres resultats, es va escollir una proteïna candidata que mostrava una funció biològica important (sobreexpressió en resposta a l'estrès) així com una reducció significativa dels seus nivells en els animals tractats amb simvastatina. Es tracta de la HSP75 (*Heat shock protein 75*), una proteïna de resposta a l'estrès, implicada en la biogènesi mitocondrial i que també actua com a antagonista de ROS així com exercint un efecte anti-apoptòtic (Hua *et a*l.2007; Pridgeon *et al.* 2007). En la mateixa direcció que els nostres resultats, Kiang i Tsokos (1998) reportaren nivells sobreexpressats d'aquesta proteïna en condicions d'isquèmia així com altres autors observaren que el tractament amb simvastatina era capaç de reduir els nivells d'HSPs (Moohebati *et al.* 2011, Ghayour-Mobarhan *et al.* 2005) i ho atribuïen a les seves propietats immunomoduladores.

Els nivells en plasma d'HSP-75 es van analitzar mitjançant ELISA simple en mostres de pacients en diferents moments després de la isquèmia cerebral (basal, tercer dia i setè dia). Aquestres mostres s'obtingueren d'un assaig clínic pilot dut a terme pel nostre laboratori, estudi MISTICS (Montaner *et al.* 2008) en el què pacients amb ictus reberen placebo o simvastatina (una primera dosi dins les primeres 12 hores des de l'inici dels símptomes i continuaren amb el tractament fins als 90 dies). Els resultats obtinguts d'aquesta anàlisi mostraren que els pacients tractats amb simvastatina, tal i com vèiem en l'estudi amb rates, mostraven nivells significativament reduïts d'HSP75 respecte els pacients que havien rebut placebo.

La monitorització d'un biomarcador com aquest podria servir en un futur per a optimitzar l'efecte neuroprotector de les estatines, permetent l'ajustament de la dosi del tractament en funció de l'efecte observat. Cal remarcar que aquest resultat no només recolzava la hipòtesi que la simvastatina era capaç de disminuir l'estrès oxidatiu, sinó també que el model embòlic era capaç de reproduir les condicions de l'ictus en humans.

5.4 L' administració del tractament combinat simvastatina + rt-PA en la fase aguda de la isquèmia cerebral és segura pel que fa a la incidència de TH.

Una vegada comprovada l'eficàcia del nostre tractament administrat durant la fase aguda de la isquèmia cerebral, era essencial comprovar-ne la seva seguretat (Article 3: Campos-Martorell *et al. CNS Neurosci Ther.* 2013). Tal com s'ha esmentat a la introducció del present treball, les TH, juntament amb les infeccions, es consideren les complicacions secundàries més freqüents de l'ictus isquèmic (Terruso *et al*. 2009, Beslow *et. al*. 2011).

Convé destacar que la literatura ens advertí que l'efecte de les estatines sobre la incidència de TH en pacients amb ictus era un tema controvertit. Estudis epidemiològics havien reportat un increment en la incidència de TH en aquells individus amb nivells baixos de colesterol (Iribarren *et al.* 1995, Sturgeon *et al.* 2007) i a partir d'aquí, s'hipotetitzà que el colesterol era un factor important per al manteniment de la integritat de la BHE i, conseqüentment, que nivells reduïts del mateix, implicaven un major risc de disrupció de la BHE i d'aparició de TH (Bjorkhem*et al.* 2004). De manera paral·lela, Collins *et al.* (2004) observaren una incidència de TH dues vegades superior en pacients en tractament amb estatines al moment de patir un ictus. I en l'assaig clínic SPARCL (Amarenco *et al.* 2006), la meitat dels pacients que reberen dosis elevades d'atorvastatina com a prevenció secundària de l'ictus mostraren una incidència significativament superior a patir TH que els que no havien rebut tractament. Contràriament, un metanàlisi que avaluava 23 assajos clínics demostrà que no es podien associar les estatines amb un major risc de TH (Hackam *et al.* 2011), així com un altre metanàlisi, encara més recent (McKinney i Kostis 2012) en el què s'hi inclogueren prop de 100.000 pacients que havien rebut estatines i 100.000 més que no n'havien rebut, també conclogué que el tractament amb estatines no estava associat amb la incidència de TH.

Malgrat no totes les TH s'ocasionen degut al tractament amb rt-PA, sí és cert que les TH són una de les complicacions més freqüents després de l'administració del trombolític rt-PA, sobretot si el trombolític s'administra de manera tardana (Adibhatla i Hatcher 2008). Així, doncs, per tal d'obtenir una incidència de TH adequada en el nostre estudi experimental, en primer lloc, es van tractar tots els animals amb rt-PA, i en segon lloc, es va escollir de realitzar l'estudi en una soca de rates espontàniament hipertenses (SHR). L'elevada resistència vascular i la baixa distensibilitat vascular de les rates d'aquesta soca són responsables d'induir infarts cerebrals majors que els de les rates normotenses, d'intensificar l'edema vasogènic i d'agreujar la disrupció de la BHE després de la isquèmia (Barone *et al*. 1992). És per això que

aquestes rates mostren una incidència molt més elevada de TH i es consideren un model molt adequat per a l'estudi de les complicacions associades a l'rt-PA (Yamakawa *et al*. 2003).

Per tal d'escollir el moment més adequat per a l'administració del trombolític, vam realitzar un estudi pilot previ en el que buscàrem l'equilibri entre una elevada incidència de TH i una taxa de mortalitat acceptable. Vam observar que les rates que rebien l'rt-PA a les 3 hores de la isquèmia presentaven una major incidència de TH que les que rebien el tractament al cap de 90 minuts o que les que no en rebien. Curiosament, per això, no vam trobar diferències significatives entre els grups pel que fa al volum d'infart. El fet de no veure l'efecte de l'rt-PA en aquestes rates podria explicar-se, segons Hacke *et. al* (2008) pel fet que en aquesta soca la dilatació de les arterioles, en resposta a l'òxid nítric, sol estar molt atenuada. Això, doncs, dificultaria clarament la reperfusió una vegada administrat l'rt-PA.

Tenint en compte els resultats obtinguts, es pot concloure que un model que és ideal per a un estudi en concret (ex: estudi de d'incidència de THs) no té perquè ser-ho per a valorar altres paràmetres (ex: neuroprotecció). En resum, els animals inclosos en aquest estudi que havien rebut simvastatina durant la fase aguda de la isquèmia no van mostrar una major incidència de TH respecte els que van rebre vehicle, però tampoc van mostrar una reducció en el volum d'infart o una milloria neurològica, tal com cabria esperar. Nosaltres atribuïm aquest fet a l'agressivitat del model escollit. Els infarts, així com l'afectació neurològica, o la incidència de mortalitat, eren majors que els observats en les rates normotenses, és per això que el temps d'estudi s'escurçà a les 24 hores en comptes de les 48 hores. Amb tot això, plantegem dues hipòtesis per a la no observació d'eficàcia del tractament en estudi. Una podria ser que l'efecte de la simvastatina no fos suficientment potent com per actuar en casos d'isquemia cerebral severa. L'altra, que l'eficàcia de la simvastatina en el nostre model es veiés a partir de temps més llargs i no a les 24 hores (temps escollit en aquet estudi).

Finalment, i per tal de recolzar els nostres resultats experimentals, es va analitzar, mitjançant un estudi retrospectiu, la presència de TH en malalts que havien patit un ictus (i que estaven en tractament amb simvastatina o no) i que posteriorment havien rebut rt-PA. D'aquesta manera vam poder demostrar en la nostra cohort que el fet d'estar prenent estatines era un factor independent de bon pronòstic sense tenir efecte sobre l'aparició de TH. No vam observar diferències entre el grup tractament i el grup control ni en la incidència ni en la gravetat de les TH, reforçant així els resultats d'altres estudis prèviament publicats (Álvarez-Sabín *et al.* 2007).

5.5 Els models d'oclusió de l'artèria cerebral mitja (MCAO) realitzats en rata no són adequats per a l'estudi d'infeccions respiratòries.

Una vegada demostrada la seguretat de la simvastatina en relació a l'aparició de TH (Article 3: Campos-Martorell *et al. CNS Neurosci Ther*. 2013), el següent pas era l'avaluació de l'administració de simvastatina en la fase aguda de la isquèmia sobre l'aparició secundària d'infeccions respiratòries. El possible efecte de les estatines sobre les infeccions després de l'ictus ens interessava especialment ja que l'estudi pilot anomenat MISTICS realitzat prèviament al laboratori (Montaner 2007) mostrà resultats inesperats. Aquest estudi conclogué que els pacients que havien rebut simvastatina poc després de patir un ictus evidenciaven un millor pronòstic ja a partir del tercer dia però, per altra banda, també presentaven una tendència a patir més infeccions respiratòries.

A nivell experimental, altres grups ja havien demostrat en models en ratolí, que la isquèmia cerebral induïa una resposta inflamatòria sistèmica seguida d'un estat d'immunodepressió (Offner *et al.* 2006) i que aquest estat facilitava l'aparició d'infeccions bacterianes espontànies (Prass *et al.* 2003, Liesz *et al.* 2009, Meisel *et al*. 2004). Així doncs, calia posar a punt un model d'isquèmia cerebral en rata que ens permetés valorar quin paper jugava la simvastatina en les infeccions secundàries a la isquèmia i ens servís per a recolzar o descartar els resultats obtinguts prèviament en l'assaig clínic pilot (MISTICS).

Inesperadament, no vam observar diferències entre els animals sotmesos a una isquèmia cerebral (utilitzant 2 models d'isquèmia experimental diferents: embòlic i del filament) i els animals *sham* (sotmesos a la cirurgia però sense induir la isquèmia amb el filament o el coàgul sanguini) en el recompte diferencial de leucòcits, lesions pulmonars i creixement de colònies en cultivar els homogenats de teixit pulmonar. Considerem que hi ha múltiples causes que poden explicar per què els animals *sham* també mostraven una alteració en el seu sistema immunitari.

Primer de tot, la nostra cirurgia *sham* implicava una trepanació cranial per tal de registrar el flux sanguini cerebral. I com prèviament reportaren altres grups (Asadullah *et al.* 1995), tota cirurgia cerebral va acompanyada d'un procés inflamatori i d'un estat d'immunodepressió. A més, també estava descrit que l'administració d'anestèsics volàtils afecta la resposta dels leucòcits ja que, per una banda altera l'adhesió dels neutròfils als vasos sanguinis i, per l'altra, indueix l'apoptosi dels limfòcits (Loop *et al.* 2005). Sorprenentment, el fet de realitzar una incisió a nivell cervical (tant en la cirurgia *sham* com en la isquèmia) també podria influir en la

inducció de l'estat d'immunodepressió. Això és degut a que el nervi Vague (N. Vague) recorre juntament amb l'artèria Caròtida (artèria implicada en la nostra cirurgia), a la zona cervical. Tanmateix, la manipulació d'aquest nervi és pràcticament inevitable durant la cirurgia. En aquesta direcció, Kessler *et al.* (2012) reportaren que la senyal del N. Vague és un component essencial en la modulació de la resposta immunitària. Per altra banda, Wong *et al.* (2011) descrigueren que una activació del sistema nerviós simpàtic (SNS), per exemple a causa d'un ictus, acaba induint la secreció de citoquines immunodepressores per part de les cèl·lules iNKT del fetge (innervades pel SNS). Així doncs, tenint en compte que Berthoud *et al.* (2002) demostraren una relació anatòmica entre el N. Vague i el fetge, podem deduir que l'estimulació iatrogènica del N. Vague podria contribuir a l'estat d'immunodepressió degut a la secressió de citoquines a nivell del fetge. A banda d'això, Campbell *et al.* (2005) descrigueren que una lesió al SNC (on s'inclouen les cirurgies *sham* i d'isquèmia) indueix l'expressió a nivell hepàtic de citoquines CC i CXC associades amb la mobilització de leucòcits.

Pel que fa a les rates isquèmiques, a part de patir la immunodepressió provocada per la pròpia cirurgia, pateixen, a més, la immunodepressió deguda a la pròpia isquèmia, induïda per una sobre-activació del SNS caracteritzada per un increment en l'alliberació de catecolamines i glucocorticoides a nivell de les glàndules adrenals (Meisel *et al.* 2005). Conseqüentment, les rates sotmeses a una isquèmia cerebral presentaren una major immunodepressió que les rates *sham*, fet que es traduïa amb un menor percentatge de limfòcits i un major percentatge de PMN's.

Una manera d'explicar les diferències observades en el recompte diferencial de leucòcits era avaluar els nivells plasmàtics de quimiocines tals com la CXCL1, molècula associada a la mobilització de leucòcits (Asadullah *et al.* 1995, Denes *et al.* 2011). És per això que analitzarem mitjançant ELISA simple, els nivells plasmàtics d'aquesta molècula. Campbell *et al.* (2005), mostraren una sobreexpressió d'aquesta proteïna en els animals amb lesions cerebrals agudes. En la mateixa direcció, nosaltres en detectàrem majors nivells en plasma de rates *sham* i isquèmiques que en rates naïve (malgrat només la comparació entre naïves i isquèmiques va ser significativa), així com una correlació positiva entre els nivells de CXCL1 i el percentatge de PMN's.

Així, doncs, podem concloure que els nivells elevats de PMNs trobats en plasma de rates *sham* i isquèmiques poden ser conseqüència d'una sobreexpressió de citoquines (almenys de CXCL1) a nivell hepàtic.

Finalment convé ressaltar que el fet d'obtenir resultats concordants entre les rates sotmeses a un model embòlic (eMCAO) i les que s'usà el mecànic (tMCAO), apunta com a responsables de la immunodepressió dels animals *sham*: la trepanació cranial, la cervicotomia i l'anestèsia amb isoflorà.

En conjunt, aquest estudi no ens va permetre tenir un model animal per tal d'avaluar possibles neuroprotectors amb efecte immunomodulador en rata (objectiu establert en un principi) ja que vam concloure que part dels canvis detectats en els animals isquèmics eren deguts als efectes de la pròpia cirurgia, i no a la isquèmia en sí. Però, per contra, aquest treball (Article 4: Campos-Martorell *et al*. *PLos One* 2014) ens va mostrar la importància que té la consideració de factors com l'espècie, la soca o el model animal escollit, així com l'efecte de l'anestèsia o de l'estrès induït per la cirurgia, a l'hora d'extrapolar els resultats obtinguts.

En el moment que vam arribar a la conclusió que els models d'oclusió de l'ACM en rata no eren adequats per a l'estudi d'infeccions, es va publicar un estudi en ratolins (Jin *et al*. 2013) que mostrava que la simvastatina administrada després de la isquèmia, no només disminuïa la susceptibilitat de patir infeccions pulmonars espontànies, sinó que també reduïa l'atròfia esplènica associada al procés d'immunodepressió post-ictus. Aquest article, juntament amb altres publicacions que estudiaven el possible benefici de les estatines en pacients amb sepsis (Pasin *et al.* 2013), recolza la hipòtesi que en futurs assaigs clínics amb simvastatina, les infeccions respiratòries no haurien de ser un efecte secundari limitant per a l'ús d'aquesta droga en la fase aguda de l'ictus.

5.6 L' encapsulació de simvastatina en liposomes és una eina prometedora per a optimitzar l'arribada del fàrmac al teixit cerebral lesionat

Tenint en compte la dificultat de molts neuroprotectors per tal d'arribar a la regió isquèmica degut a la presència de la BHE, vam creure molt interessant el desenvolupament i caracterització de liposomes capaços de transportar simvastatina i alliberar-la a l'àrea d'interès (Article 5, adjuntat a l'apartat de Resultats malgrat no estar publicat ni sotmès a revisió per *peer review*). Tal com es descrigué prèviament, l'ús de liposomes, caracteritzats per la seva biocompatibilitat i no-toxicitat (Elbayoumi i Torchilin 2010) és una estratègia prometedora per tal d'incrementar l'eficàcia del medicament i de reduir-ne els possibles efectes secundaris que se'n deriven (Ishiii *et al*. 2013).

L'últim treball d'aquesta tesi (Article 5) ens demostra, tal i com altres autors descrigueren (Aggarwal *et al*. 2009), que la càrrega i composició dels liposomes és un factor clau per a determinar la seva distribució. La literatura és molt controvertida a l'hora d'establir quina és la càrrega més adequada de les nanopartícules per a aconseguir que la màxima quantitat possible de liposomes penetri al teixit cerebral a través de la BHE. Bàsicament, considerem que aquest fet pot atribuir-se a la gran quantitat de modificacions que es poden dur a terme (adhesió de molècules i/o anticossos, mida, propietats de la droga encapsulada). Tot això fa que els liposomes usats en diferents estudis difereixin, no només en la càrrega, sinó en molts altres factors, i aquest fet impossibiliti la comparació entre publicacions.

En el nostre estudi comparàrem la distribució i l'arribada al teixit cerebral de tres tipus de liposomes que diferien en la càrrega. Podem afirmar, doncs, que els LIPOS1 eren els que es comportaven d'una manera clarament diferenciada, ja que no es van poder detectar en teixit cerebral i es detectaven en molt baixa senyal en plasma. Altres autors han suggerit que aquest tipus de liposomes són capaços d'absorbir una gran quantitat de proteïnes plasmàtiques, i això els provoca una inestabilitat que es tradueix amb una ràpida degradació dels liposomes en sang i, conseqüentment, amb la impossibilitat dels mateixos de creuar la BHE.

Pel que fa als altres dos tipus de liposomes estudiats (LIPOS2 i LIPOS3) vam comprovar que sí eren capaços d'arribar al teixit cerebral. És lògic pensar que els liposomes penetren més fàcilment a les zones del cervell on s'hi ha produït una disrupció de la BHE (Dirnagl *et al*. 1999 i Schroeder *et al.* 2014) i és per això que observàrem una acumulació de liposomes a l'hemisferi IP en les rates isquèmiques tractades amb liposomes amb fluorescència. Aquest fet el vam poder corroborar també amb els experiments en què les rates eren sacrificades a les 2 o a les 4

hores d'haver rebut simvastatina lliure o encapsulada en liposomes i l'homogenat cerebral va ser analitzat mitjançant UHPLC. Independentment de la forma en què rebien el tractament, la detecció de simvastatina va ser superior en l'hemisferi IP que en el CL.

Amb tot això podem deduir que els liposomes (tant els LIPOS2 com els LIPOS) arriben al teixit cerebral per diferents vies depenent de l'estat de la BHE. Mentre que en l'hemisferi IP els liposomes s'acumulen a l'àrea isquèmica aprofitant l'increment de permeabilitat vascular induït pel trencament de la BHE (Schroeder *et al.* 2014), en l'hemisferi CL de les rates isquèmiques, els liposomes requereixen de mecanismes d'interacció amb les cèl·lules endotelials (tals com endocitosi) per tal de penetrar a través de la BHE (Kreuter 2001).

Cal tenir en compte que la simvastatina, en ser una molècula relativament petita i de naturalesa lipofílica (Vuletic *et al.* 2006), és capaç de penetrar la BHE per sí sola per mecanismes de difusió passiva. En canvi, en encapsular-se en liposomes, aquests requereixen interaccionar amb les cèl·lules endotelials per tal d'alliberar la droga. En tot cas, els nostres resultats mostren que les concentracions de simvastatina alliberades al teixit cerebral són similars independentment de la forma en què s' hagi administrat la droga intravenosament.

Malgrat no haver observat que l'encapsulació impliqui un increment en l'arribada de simvastatina a la zona d'interès respecte a l'administració en forma lliure, sí podem afirmar que els liposomes són capaços de creuar la BHE (estudis amb fluorescència) així com d'alliberar la droga al teixit cerebral (estudis de detecció mitjançant UHPLC). Així, doncs, el nostre estudi conclou que els liposomes permeten que la simvastatina arribi i s'alliberi al cervell.

El proper pas consistiria en la realització d'estudis d'eficàcia *in vivo* en els quals es comprovaria si el fet d'encapsular el medicament implicaria una major reducció del volum d'infart i una millor evolució neurològica. Per altra banda, i independentment de si els l'encapsulació de simvastatina en liposomes suposa un increment en l'eficàcia, seria molt interessant avaluar si l'encapsulació del medicament és capaç de reduir els efectes secundaris associats a la seva administració crònica (miopaties i hepatotoxicitat). En aquesta direcció, altres autors (Gwinn *et al.* 2011) han mostrat que medicaments amb un elevat número d'efectes secundaris associats, mantenen la seva eficàcia al mateix temps que incrementen la seva seguretat en ser encapsulats en liposomes. Per aquest mateix motiu, els liposomes ja s'han usat clínicament per al transport d'agents quimioterapèutics, antibiòtics i antifúngics (Spuch *et al.* 2011).

5.7 Què pot aportar aquesta tesi

Des del nostre punt de vista, el baix cost del medicament estudiat (simvastatina) i la simplicitat de l'acció que es proposa (usar un tractament que ja existeix per a la prevenció de malalties cardiovasculars administrant-lo amb una altra indicació: com a neuroprotector durant la fase aguda de l'ictus) són punts forts a l'hora de considerar la seva translació a la clínica.

Tenint en compte tots els resultats obtinguts en la realització d'aquesta tesi doctoral, podem afirmar que en models experimentals d'isquèmia cerebral, l'administració de simvastatina en la fase aguda exerceix efectes neuroprotectors que es tradueixen en una reducció de la mida de l'infart i en una millora del dèficit neurològic. Contràriament, cal recalcar dos fets observats, relacionats entre sí. Primerament, que l'efecte neuroprotector no es conserva quan la severitat de l'infart és molt important (en l'estudi on s'usaren rates hipertenses que presentaven un volum d'infart més gran no es veié que la simvastatina exercís cap efecte). I, per altra banda, que el tractament no sembla tenir efecte sobre la reducció de la mortalitat (en cap dels estudis realitzats es comprovà aquest fet).

Amb tot això, la conclusió final d'aquesta tesi és que l'administració de simvastatina durant la fase agda de l'ictus, per exemple a l'ambulància, durant el trasllat del pacient a l'hospital, podria aportar beneficis als pacients (una millor evolució neurològica). A més, aquesta intervenció no suposaria un risc pel que fa a l'aparició de transformacions hemorràgiques, entenent que el pacient podria rebre igualment tractament trombolític en cas que complís els criteris establerts. Tanmateix, caldria comprovar si els pacients amb ictus més severs podrien o no beneficiar-se del tractament així com els pacients amb hemorràgies cerebrals.

Finalment, creiem que serien necessaris més estudis experimentals per tal de demostrar si l'encapsulació de simvastatina en liposomes, a més de permetre l'arribada i l'alliberament del fàrmac al lloc de la lesió, també podria comportar una major eficàcia i una reducció dels possibles efectes adversos tal com la bibliografia descriu per altres fàrmacs. De complir-se això, l'encapsulació de simvastatina en liposomes podria esdevenir una eina prometedora per al tractament de l'ictus isquèmic en la fase aguda.

6. CONCLUSIONS

Les conclusions d'aquesta tesi són les següents:

- Tenint en compte la literatura publicada, les estatines exerceixen un efecte neuroprotector quan s'administren en models d'isquèmia cerebral en rosegadors. A més, aquest efecte és superior quan les estatines s'administren prèviament a la isquèmia, en múltiples dosis i quan l'estatina administrada és la simvastatina.
- L'administració de simvastatina 15 minuts després d'induir la isquèmia mitjançant un model embòlic en rata, va resultar ser un neuroprotector eficaç en el nostre laboratori.
- L'efecte neuroprotector que exerceix la simvastatina administrada durant la fase aguda de la isquèmia cerebral podria explicar-se per la seva capacitat per a disminuir l'estrès oxidatiu, estimular els fenòmens de reparació cel·lular i atenuar la permeabilitat de la BHE.
- L'administració de simvastatina juntament amb el trombolític rt-PA és segura pel que fa a l'aparició de transformacions hemorràgiques.
- Malgrat no es va poder avaluar l'efecte que tindria l'administració de simvastatina sobre la incidència d'infeccions respiratòries, sí es va poder demostrar que els models d'oclusió de l'artèria cerebral mitja en rata no són útils per a aquest tipus d'estudi ja que bona part dels canvis observats són atribuïbles a la pròpia cirurgia i no a la isquèmia cerebral en sí.
- L' administració de simvastatina encapsulada en liposomes permet l'arribada del farmac al seu lloc d'acció, al cervell isquemic, convertint-se en una estratègia prometedora per tal d'augmentar l'eficàcia i la seguretat d'aquest fàrmac.

7. BIBLIOGRAFIA

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