INTERNATIONAL DOCTORAL
SCHOOL OF THE USC

Paulo
Ávila Gómez

PhD Thesis

Role of RBM3 in cerebral ischemia

Santiago de Compostela, 2022

Doctoral Programme in Molecular Medicine

TESIS DE DOCTORADO

ROLE OF RBM3 IN CEREBRAL ISCHEMIA

Paulo Ávila Gómez

ESCUELA DE DOCTORADO INTERNACIONAL DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA

PROGRAMA DE DOCTORADO EN MEDICINA MOLECULAR

SANTIAGO DE COMPOSTELA

AÑO 2021

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

DECLARACIÓN DO AUTOR DA TESE

Role of RBM3 in cerebral ischemia

D. **Paulo Ávila Gómez**

Presento a miña tese, seguindo o procedemento axeitado ao Reglamento, e declaro que:

1) A Tese abarca os resultados da elaboración do meu traballo.
2) De ser o caso, na Tese faise referencia ás colaboracións que tivo este traballo.
3) Confirmo que a Tese non incorre en ningún tipo de plaxio doutros autores nin de traballos presentados por min para a obtención doutros títulos.
4) A Tese é a versión definitiva presentada para a súa defensa e coincide a versión impresa coa presentada en formato electrónico.

En Santiago de Compostela, a 19 de diciembre de 2021.

Fdo.: Paulo Ávila Gómez

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

AUTORIZACIÓN DEL DIRECTOR DE LA TESIS

ROLE OF RBM3 IN CEREBRAL ISCHEMIA

D. **José Antonio Castillo Sánchez**
D. **Francisco Campos Pérez**

INFORMA/N:

Que la presente tesis, se corresponde con el trabajo realizado por D. **Paulo Ávila Gómez**, bajo mi dirección, y autorizo su presentación, considerando que reúne los requisitos exigidos en el Reglamento de Estudios de Doctorado de la USC, y que como director de esta no incurre en las causas de abstención establecidas en la Ley 40/2015.

De acuerdo con lo indicado en el Reglamento de Estudios de Doctorado, declara también que la presente tesis doctoral es idónea para ser defendida en base a la modalidad de **Monográfica con reproducción de publicaciones**, en los que la participación del doctorando/a fue decisiva para su elaboración y las publicaciones se ajustan al Plan de Investigación.

En Santiago de Compostela., 17 de diciembre de 2021

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

AUTORIZACIÓN DEL TUTOR DE LA TESIS

ROLE OF RBM3 IN CEREBRAL ISCHEMIA

D. **Juan Bautista Zalvide Torrente**

INFORMA/N:

Que la presente tesis, se corresponde con el trabajo realizado por D. **Paulo Ávila Gómez**, bajo mi tutorización, y autorizo su presentación, considerando que reúne los requisitos exigidos en el Reglamento de Estudios de Doctorado de la USC, y que como director de esta no incurre en las causas de abstención establecidas en la Ley 40/2015.

De acuerdo con lo indicado en el Reglamento de Estudios de Doctorado, declara también que la presente tesis doctoral es idónea para ser defendida en base a la modalidad de **Monográfica con reproducción de publicaciones**, en los que la participación del doctorando/a fue decisiva para su elaboración y las publicaciones se ajustan al Plan de Investigación.

En Santiago de Compostela., 17 de diciembre de 2021

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

ROLE OF RBM3 IN CEREBRAL ISCHEMIA

Tesis doctoral presentada por
PAULO ÁVILA GÓMEZ

Directores
PROF. DR. JOSÉ CASTILLO SÁNCHEZ
CATEDRÁTICO DE NEUROLOGÍA

DR. FRANCISCO CAMPOS PÉREZ
COLABORADOR DOCENTE

Departamento de Psiquiatría, Radiología, Salud Pública, Enfermería y Medicina

Programa de Doctorado en Medicina Molecular
Universidade de Santiago de Compostela

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Agradecimientos

Durante estos últimos años, y desde el día en que entré por las puertas del laboratorio, no he podido evitar hallarme soñando despierto con la idea de alcanzar el grado de Doctor, visualizando la colimación de todo el trabajo realizado en un pequeño ejemplar entre mis manos.

Cinco años más tarde, tras incontables horas de trabajo, carcajadas, llanto, ira, emoción y empeño, y cuando parece que por fin se vislumbra tierra en el horizonte, dicha ilusión ya dista bastante de lo que inicialmente fue. Y es que resulta curioso cómo el marinero, nada más pisar tierra y tras haber navegado durante incontables días en pos de su sueño en alta mar, no puede evitar echar la vista atrás y contemplar la inmensidad del océano que ha logrado atravesar.

Finalmente, me doy cuenta de que el verdadero objetivo de la Tesis, lejos de ser un mero trámite burocrático o la obtención de un galón que poder lucir en la bata, es el camino recorrido durante la propia Tesis. Soy consciente de que mi carácter tozudo y mi empuje han jugado un pequeño papel en la elaboración de este trabajo, pero no me cabe la menor duda de que sin la ayuda de todos y cada uno de los mencionados en las siguientes líneas este trabajo jamás habría podido llegar a forjarse.

En primer lugar, me gustaría agradecerle al Dr. Tomás Sobrino el haber decidido responder aquellos emails insistentes que parecían no aceptar un no por respuesta, y por haberme dado la oportunidad de entrar por la puerta del que sería mi segundo hogar durante todos estos años.

Gracias también a mis directores de tesis, el Dr. Francisco Campos y el Prof. Dr. José Castillo, por haber coordinado,

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

dirigido y guiado en la elaboración de esta Tesis doctoral, así como por haberme permitido trabajar y aprender a su lado.

Fran, gracias por tenderme la mano y permitirme trabajar bajo tu dirección durante todos estos años. Gracias por enseñarme el valor de los detalles pequeños en los trabajos, por tu capacidad de gestión y por estar siempre disponible para revisar cualquier tipo de ocurrencia del referee de turno. Si algo he aprendido de ti es el significado de sacrificarse por un objetivo dado que la ciencia, al igual que muchos otros ámbitos de la vida, no entiende de horarios y requiere un mimo y esfuerzo constantes.

Pepe, gracias de corazón por las jarras de agua fría, los tirones de oreja y las temidas llamadas al despacho. Gracias por tener siempre un momento para hablar, por escuchar y preguntar desde el interés genuino y la preocupación paternal que profesas hacia nosotros, por percibir cambios inapreciables para muchos en nuestro fenotipo habitual y, sobre todo, gracias por haber confiado en aquel chaval inexperto que quiso adentrarse en las neurociencias vasculares hace unos años. Espero de corazón haber sabido estar a la altura.

Gracias al Dr. Ramón Iglesias por toda su ayuda durante la realización de los estudios en resonancia magnética, por su franqueza y naturalidad y por saber aderezar la rectitud necesaria en el trabajo con su característico toque picante.

Gracias al Dr. Pablo Hervella por todo su apoyo en estos años de tesis. Gracias por haberme enseñado a ver oportunidades donde fácilmente se verían paredes, por tu disponibilidad y cercanía dentro y fuera del laboratorio, y por haber podido aprender desinteresadamente de ti siempre que así lo he requerido. Gracias por recordarme que el desempeño

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

científico no está reñido con el carácter afable, y que con trabajo duro y una sonrisa todo acaba saliendo adelante.

Gracias en especial a la Dra. Alba Vieites, mi tutora en la distancia. Gracias por pasarme el testigo de RBM3, por los incontables mensajes de dudas conexión Galicia-Francia y por todo tu apoyo durante la elaboración de esta tesis. De corazón, gracias.

Gracias a la Dra. Uxía Regueiro por haber estado ahí desde el minuto 0. Gracias por las horas de biblioteca, las tardes de Química y los Tilos, y por haberme apoyado siempre desde que entré a formar parte del laboratorio. Quizás haya sido por nuestro background particularmente diferente a la norma o quizás por pura afinidad, pero sea como fuere me alegra haber encontrado a una amiga como tú dentro y fuera de la poyata.

Gracias al Dr. Andrés da Silva por haber sido como un hermano mayor para mí dentro y fuera del laboratorio. Gracias por todas las escapadas, los cafés torrefactos, los consejos entre cañas, las risas al unísono y los piques. Confía en ti tanto como has sabido confiar siempre en mí, porque sé bien que la ciencia agradece tenerte con ella.

Grazas ao Dr. Manuel Rodríguez de Rois pola súa axuda durante todos estes anos. Manoel, o teu semblante xélido non é capaz de soportar moito tempo a calidez da túa ialma. Por toda a túa axuda no laboratorio, polas conversacións profundas a golpe de café sen edulcorar e pola túa calidade como pessoa, grazas, de corazón.

Gracias al Dr. Esteban López por saber siempre enseñar de forma crítica y didáctica, y al Dr. Nacho López por su disposición y ayuda durante estos años. Gracias a las Dras. María Pérez y Clara Correa y al Dr. Héctor Fernández por su apoyo durante estos años y su ayuda durante el aprendizaje de los modelos de

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

experimentación animal, puesto que los inicios, aunque emocionantes, nunca son fáciles. Gracias al Dr. Adrián Posado por haber tenido siempre un momento para echar una mano, para discutir ideas o simplemente para charlar distendidamente. Gracias a los Drs. Alberto Ouro, Daniel Romaus y a la Dra. Maria Luz Alonso por su apoyo en estos últimos meses desde su incorporación al grupo.

Desconozco el porqué de que todavía no figure como doctor, pero me gustaría agradecer también a D. José Ramón Castro Ruibal por su labor gestora, por toda la paciencia que ha tenido y sigue teniendo con nosotros y por su capacidad para mantenerse jovial sin perder un ápice de profesionalidad.

Gracias al casi Dr. Antonio Dopico por haber sido compañero de camino durante estos años de trasiego, por su ayuda durante la Tesis y por compartir las penas derivadas de la elaboración del presente documento. No queda nada ya, Dopi.

Gracias a Ana Bugallo por toda su ayuda dentro y fuera del laboratorio, por su carácter dulce, sus ideas firmes y su total dedicación por sus seres queridos. Anuska, ya sea aprendiendo de tu rectitud en el trabajo o de tu amistad fuera del hospital, gracias por haber estado durante todos estos años y por seguir alegrándole el día a todo aquel que tiene la suerte de cruzarse contigo. Antía, gracias a ti también por haber estado presente en los mejores y peores momentos de la Tesis. Debajo de ese carácter demoledor y personalidad tipo A se esconde una científica reservada con un corazón inmenso y que no duda en sacar las garras cuando la situación lo merece. Gracias también a Maite y Marta por saber quitarle hierro a los ambientes tensos, por su carácter desenfadado y por su apoyo en estos años de peregrinaje académico. Elena, gracias por las conversaciones del café en la sala y los intercambios de recetas, pues han sido de

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

gran ayuda para desconectar por unos minutos de la vorágine científica. A todas vosotras os desearía suerte en la Tesis, pero sé que no os hará falta.

Gracias a María y Lara, las nuevas incorporaciones al laboratorio, por su ayuda durante estos últimos meses. Confío en que podáis disfrutar de este camino tanto como yo lo he hecho.

Me gustaría también agradecer a los Drs. Manuel Rodríguez-Yáñez, Susana Arias, Iria Dequidt, Juan Pías y Rogelio Leira del servicio de Neurología del Hospital de Santiago por haber estado siempre dispuestos a resolver cualquier duda que se me ha presentado durante la elaboración de este trabajo. En especial, me gustaría dar las gracias a la Dra. Clara Domínguez por toda su ayuda durante los trabajos de migraña y depresión propagada, por su preocupación, ánimos y dulzura desde el primer momento. Por último, me gustaría darle las gracias al Dr. Emilio Rodríguez por su ayuda con los estudios de neurología, su amistad y por recordarme que mis casi 10 años de experiencia en el mundo del tenis competitivo no son nada frente a su drive de padel.

I would also like to thank Dr. Christa Rhiner for welcoming me into her group during my stay in Lisbon. Christa, thank you for all your help during these past months, for showing genuine interest in my Thesis project and the collaboration study, for caring about my formation during the stay and for making me feel as part of your team. Muito obrigado a Carolina por me apresentar ao mundo da Drosophila, por me ensinaren o que é dar verdadeiramente o litro, pelas conversas dissecando até cair a noite em Belém, pelos seus ânimos, sua paciência e seu apoio durante toda a estadia, especialmente durante o meu inicio na lingua portuguesa (acho que agora não é tao mau). Obrigado

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

também á Dra. Marta Neto pela súa gentileza e por poder contar sempre com ela. Graças a Anabel pela dose diária de cafeína após do almoço, as longas conversas e as brincadeiras habituais. Obrigado também á futura doutora Mariana Santos pelo apoio na escrita, as noites de café, chá e chatices da Tese e pelos boms momentos conversando de cualquer coisa. Graças também a Rita, María e Margarida pela vossa simpatía e ajuda durante os meus meses no Champalimaud. Obrigado também a Denise, Miguel e Ana pelas cervejas na tarde, a viagem a Mafra e todas as boas recordaçoes em Lisboa. Por último, gracias al Dr. Mario Aguilar por las risas, el intercambio de libros, por seguir el ritmo de las cañas y, sobre todo, por esa mano en la espalda que tanto bien hace. A toda a gente e amigos do Rhiner Lab e Champalimaud, obrigado por me fazer sentir em casa.

Gracias también a Andrea, Marcos, Noel, Lucía y Rocío, mis amigos del Máster en Neurociencias, sin los cuales aquellas tardes cuesta arriba entre apuntes hubiesen alcanzado los 90º. Gracias a toda la tropa de aMIRgos y galenos por las tardes de Conchi, noches de zona vieja y mañanas de Conchi (el MIR no perdona), y en especial a Alfonso, Álvaro, Amparo, Carmen, Lía, Olaya, Tito y Valentín. Santiago no habría sido lo mismo sin vosotros. Gracias a toda mi gente de Pontevedra por los planes en Lérez, el Montaditos de rutina y el sentimiento familiar que siempre ha caracterizado al grupo. Ayer, hoy y siempre: Se lía. Gracias también a mi broski Carlos Calvar, cineasta logrado y romántico empedernido, por hacerme levantar la cabeza del papel y hacerme mirar al cielo de vez en cuando.

Gracias a Diego, Javi y Jairo por los años de la Pere, el piso en A Seca, las tardes de tatami, de Fifty y de caminos hacia la facultad, las mañanas con despertares secuenciados y las comidas en comunión, pero sobre todo por poder seguir

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

compartiendo experiencias juntos. Gracias también a Valen por estar siempre dispuesto para un café, caña o copa con su tranquilidad tan característica. Gracias a Dra. Alejandra Alonso, a Ángela y a Lore por todos los momentos de la carrera y por seguir refrescándonos la memoria año tras año. Gracias también al Dr. José Manuel Pazos Rosales por inculcarme el pensamiento crítico, la pasión por la enseñanza y por motivarme a hacer aquello que realmente me llenaba.

Gracias a mi gente de Ourense, *aos de sempre*, *os siderais*, a los que están cuando uno vuelve sin importar a donde vuelva y que siempre se encargan de que 10 meses parezcan un "hasta mañana". Celia, David, Fran, Guille, Juan, Marcos, Merylu, Nuria, Pablo: esto es tan vuestro como mío. Gracias. Eleniña, gracias por los paseos del 31, las llamadas kilométricas, bromas absurdas, por compartir vivencias desde enanos y por ser la definición de amiga personificada. Patri, Joseja, gracias por las tardes mirando a la plaza, los paseos de soportales y por darle un significado al Avant de las 17:30. Gracias también a mis rufianes del Moruno César, Borja y Jaime. Gracias a todo el grupo de Louro´s in the House por los veranos tan necesarios para desconectar, y en especial a Antón por ser un incondicional por muy fuerte que sea la marea viguesa.

Quiero dar gracias a mi mejor amigo Sergio Pombo por su apoyo a lo largo de todos estos años y por recordarme día a día que el trabajo duro da sus frutos. Gracias por ser un ejemplo de disciplina, dedicación y esfuerzo en todos y cada uno de los aspectos de tu vida y, sobre todo, gracias por estos casi 15 años de amistad inquebrantable. Va por ti, Pombillo.

A mis padres, Jorge y Pilar, gracias por haberme apoyado siempre y por confiar ciegamente en mi por difícil e inestable que parezca mi objetivo. Sé que soy un desastre con el teléfono, pero

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

creedme cuando os digo que me alegráis el día cuando veo vuestro nombre en la pantalla del móvil. Gracias Papá por ser un ejemplo de trabajo duro y superación, y por inculcarme la idea de que quien algo quiere algo le cuesta. Mum, thanks for always being there, for supporting me through thick and thin and for looking after my education since I was a sprout. Now look what you´ve done, Pili!

A mi hermana Carla, gracias por estar. Por estar siempre y por preocuparte. Por enseñarme a apreciar el arte y por tu capacidad polarizante para hacer patentes tus inquietudes. Es un orgullo haberte visto crecer y ver en quién te has convertido.

El último y más importante lugar en este camino lo ocupas tú, Patriña. Este trabajo es tan tuyo como mío, pues tú has estado firme al pie del cañón durante todo este tiempo. Gracias por creer en mí desde el primer día, por apoyarnos en los malos momentos y celebrar en los buenos, por hacerme partícipe de tu pasión por la investigación, por tu inquietud y ansia de conocimiento, por los madrugones, los Media Distancia, las conversaciones eternas, las risas, los agobios, los llantos, los tú puedes, tu sonrisa, tu amistad, cariño y amor, por permitirme crecer a tu lado y seguir aprendiendo de ti y, sobre todo, por el presente y futuro juntos. Te quiero.

Lo que parecía una misión imposible en una balsa destartalada con cuatro troncos ha resultado ser un viaje trepidante acompañado de la mejor tripulación que nadie podría pedir: vosotros. Por todo vuestro apoyo, ayuda y confianza:

Gracias.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Nuestras virtudes y nuestros defectos son inseparables,
al igual que la fuerza y la materia.
Cuando éstas se separan, el hombre deja de existir.

NIKOLA TESLA

He puesto mi corazón y mi alma en mi obra,
y he perdido mi mente en el proceso.

VINCENT VAN GOGH

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INDEX

RESUMO I

ABBREVIATIONS XV

INTRODUCTION 1

1. STROKE **1**

1.1. Epidemiology 2

1.2. Classification of stroke 3

1.3. Risk factors in ischemic stroke 5

1.4. Stroke pathophysiology 10

1.5. Clinical management of ischemic stroke 49

2. TEMPERATURE IN ISCHEMIC STROKE **56**

2.1. Brain and body temperature 57

2.2. Hyperthermia and ischemic stroke 57

2.3. Therapeutic Hypothermia in Ischemic Stroke 62

3. COLD-SHOCK PROTEINS **87**

3.1. RNA-Binding Motif protein 3 88

3.2. Fibroblast growth factor 21 94

3.3. RBM3 and FGF21 in ischemic stroke 105

4. EXPERIMENTAL RESEARCH OF HYPOTHERMIA IN STROKE **108**

4.1. Animal models of ischemic stroke 108

4.2. Therapeutic hypothermia in preclinical research 114

HYPOTHESES AND OBJECTIVES 121

OBJECTIVE 1. 123

Analysis of the circulating levels of RBM3 in ischemic stroke patients subjected to hypothermic treatment 123

1.1 INTRODUCTION **123**

1.2. OBJECTIVES **124**

1.3. MATERIAL AND METHODS **124**

1.4. RESULTS **127**

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

OBJECTIVE 2. 144

The impact of temperature on the circulating levels of RBM3 and FGF21 in a cohort of obese stroke patients 144

2.1. INTRODUCTION **144**

2.2. OBJECTIVES **145**

2.3. MATERIAL AND METHODS **145**

2.4. RESULTS **149**

OBJECTIVE 3. 160

Effect of hypothermia on infarct volume and RBM3 expression in a mice model of cerebral ischemia 160

3.1. INTRODUCTION **160**

3.2. OBJECTIVES **160**

3.3. MATERIAL AND METHODS **161**

3.4. RESULTS **169**

DISCUSSION 176

CONCLUSIONS 188

REFERENCES 190

ANNEX 254

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

RESUMO

O ictus ou infarto cerebral é unha enfermidade cerebrovascular que acontece cando o aporte de sangue arterial ao cerebro vese interrompido por unha oclusión (ictus isquémico) ou ben pola ruptura da parede dun vaso (ictus hemorráxico), o cal impide a correcta chegada de osíxeno e nutrientes ás rexións afectadas. Esta patoloxía ten unha evolución dinámica e dependente do tempo polo que canto maior é a duración destes eventos patolóxicos, maior é o dano celular ao tecido cerebral, maior a perda de función e, como consecuencia, peor é o prognóstico dos doentes.

Diversos factores de risco como poden ser a idade, o sexo ou as variacións xenéticas condicionan a aparición desta enfermidade. A idade é un dos principais condicionantes para padecer esta enfermidade, sendo así que máis do 70% de todos os casos acontecen en persoas maiores de 65 anos. Ademais, o risco de ictus duplícase a partir dos 55 anos. Ademais, outros factores como a hipertensión arterial, enfermidades cardíacas, diabetes, tabaquismo ou a obesidade tamén teñen un impacto importante nesta patoloxía. Malia que a súa incidencia e mortalidade diminuíron nos últimos anos, o ictus sigue sendo a segunda causa de mortalidade e a primeira causa de morbilidade a nivel mundial. Só na Unión Europea, o ictus afecta a máis de

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

un millón de persoas cada ano con case a metade de mortes, o cal reflexa as devastadoras consecuencias desta patoloxía.

Na actualidade, o único tratamento para o ictus isquémico está baseado na recanalización da arteria cerebral ocluída e a reperfusión do tecido afectado, ben sexa mediante o uso de fármacos (como o activador tisular de plasminóxeno), mediante dispositivos mecánicos (trombectomía mecánica) ou mediante unha combinación de ambos. Malia que este tipo de terapia mellora o prognóstico funcional até nun 70% dos casos, o seu uso está limitado a menos dun 10% das persoas debido á súa curta fiestra terapéutica e aos estritos criterios para o seu emprego. É por isto que, a pesares da mellora na atención e manexo clínico, o desenvolvemento e procura de novos fármacos e dianas terapéuticas para os doentes con ictus isquémico sigue sendo unha necesidade a día de hoxe.

Na práctica clínica existen diversos factores cuxa presencia e control resultan cruciais para a mellora ou empeoramento das persoas con ictus isquémico, como poden ser a presión e coagulación sanguínea, a glicemia, o fluxo sanguíneo cerebral, a circulación colateral ou a temperatura. Neste sentido, o presente traballo presta especial atención aos efectos directos e indirectos da temperatura na isquemia cerebral.

Baixo condicións fisiolóxicas, a temperatura corporal é regulada polo hipotálamo mediante respostas integradas dos sistemas endócrino, autónomo e músculoesquelético. Deste xeito, cando a temperatura corporal aumenta, o hipotálamo responde mediante a liberación de mediadores vasoactivos que provocan suor e vasodilatación da pel, permitindo así manter a temperatura corporal dentro duns límites fisiolóxicos. Sen embargo, certas patoloxías como é o caso da isquemia cerebral poden provocar unha resposta térmica que sobrepasa as

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

capacidades reguladoras do hipotálamos, producíndose entón a hipertermia. Neste contexto, a hipertermia pode exacerbar o dano isquémico aumentando a excitotoxicidade, inflamación, alteracións metabólicas, estrés oxidativo e a disfunción da barreira hematoencefálica, a principal liña de defensa do sistema nervioso central. En conxunto, todos estes factores poden agravar o dano isquémico no ictus agudo e comprometer o prognóstico dos doentes. De feito, máis dun 60% dos doentes desenvolve hipertermia (ou febre) durante os primeiros días despois de sufrir un ictus. A relación entre a aparición de hipertermia e o mal prognóstico está amplamente descrita na literatura. Dende o primeiro estudo retrospectivo no ano 1996 que mostrou a asociación de hipertermia co mal prognóstico dos doentes con ictus, o noso grupo publicou múltiples traballos no ámbito preclínico e clínico que reiteraban esta asociación.

Durante as primeiras 24h de hospitalización, a aparición de febre duplica a probabilidade de falecer ao cabo do mes dende o inicio dos síntomas. Nesta fase inicial, o aumento de temperatura normalmente restrínxese á zona infartada como consecuencia da alteración do metabolismo e da resposta inflamatoria, ás veces por afectación directa de núcleos hipotalámicos. Durante fases máis tardías da enfermidade, a hipertermia sistémica (moitas veces consecuencia dun proceso infeccioso) provoca unha resposta inflamatoria xeralizada que aumenta a excitotoxicidade, o dano por radicais libres e a disfunción da barreira hematoencefálica. A hipertermia provoca unha liberación masiva de glutamato, a cal pode aumentar a propagación do dano cerebral por diversos mecanismos que implican a entrada masiva de calcio e a despolarización anormal das membranas celulares. O noso grupo demostrou a presenza dunha maior concentración de glutamato en líquido

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

cefalorraquídeo naqueles individuos que presentaban hipertermia, e que a relación de temperatura con mal prognóstico estaba mediada directamente por este neurotransmisor. Ademais, a cascada inflamatoria iniciada pola hipertermia provoca a liberación de marcadores de dano e piróxenos e diminúe a dispoñibilidade enerxética do tecido isquémico. Estes eventos, xunto cas alteracións no equilibrio iónico do tecido cerebral, provocan unha extensión da lesión isquémica e comprometen o prognóstico funcional.

Neste sentido, a redución da temperatura corporal supón unha das principais estratexias na práctica clínica para o manexo do doente isquémico agudo, e normalmente procúrase diminuír a temperatura corporal mediante a administración de fármacos antipiréticos como poden ser o paracetamol, metamizol ou a aspirina. A pesares de que esta abordaxe pretende recuperar os rangos fisiolóxicos (normotérmicos: 36.5-37.5°C), numerosos estudos preclínicos demostran que a diminución da temperatura corporal até os rangos hipotérmicos (32-34ºC) poden diminuír de forma efectiva os efectos prexudiciais da hipertermia, mellorando así a condición do doente. A redución da temperatura corporal diminúe o edema, morte neuronal, inflamación, dano endotelial, excitotoxicidade, volume de infarto e mellora o prognóstico funcional en modelos animais de isquemia cerebral.

Independentemente do método (superficial, intravascular ou farmacolóxico) e extensión (focal ou sistémico) desta terapia, a hipotermia terapéutica normalmente comprende unha primeira fase de indución até o rango terapéutico (32ºC), unha fase de mantemento e finalmente unha fase de recuperación ou requencemento. Sen embargo, e a pesares de que a hipotermia terapéutica emprégase na práctica clínica diaria en determinadas

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

patoloxías como poden ser o paro cardíaco ou a encefalopatía hipóxico-isquémica en neonatos, o seu usoresulta complicada. A redución da temperatura corporal desencadea unha serie de respostas adversas como poden ser a tiritona, diurese fría, coagulopatías ou as arritmias. Isto, sumado á inmunosupresión e ao risco aumentado de infeccións, dificultan tremendamente o seu emprego en individuos comprometidos como poden ser as persoas con ictus. Até a presente data, ningún dos grandes estudos clínicos en fase III conseguiu resultados satisfactorios no ámbito do ictus isquémico, mentres que o resto de estudos clínicos en fases máis temperás de desenvolvemento obtiveron resultados mixtos, moitas veces comprometidos polos efectos adversos da hipotermia. Non obstante, a hipotermia sigue sendo unha estratexia prometedora cuxo refinamento e entendemento dos mecanismos subxacentes nos vindeiros anos permitirá ampliar as opcións terapéuticas nas enfermidades neurolóxicas agudas.

A pesares de que a hipotermia reduce a actividade metabólica e, consecuentemente, a síntese global de proteínas, a diminución na temperatura corporal provoca o aumento da expresión dun grupo de proteínas denominadas proteínas de choque frío (en inglés: cold-shock proteins; CSPs), as cales participan de forma activa en procesos termorreguladores dirixidos ao mantemento da normotermia corporal. Máis alá das súas capacidades termorreguladoras, estas proteínas tamén contribúen a mecanismos neuroprotectores tanto dependentes coma independentes da hipotermia.

En particular, a proteína *RNA-binding motif 3* (RBM3) exerce os seus efectos neuroprotectores mediante a regulación da síntese de microRNA, regulación de modificacións post-transcripcionais/traducionais, favorecemento da progresión do

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

ciclo celular, inhibición da apoptose ou diminución do estrés no retículo endoplasmático. Ademais, RBM3 tamén favorece os procesos de neuroxénese e sinaptoxénese no adulto, os cales son eventos cruciais nas fases máis tardías da enfermidade cerebrovascular. Recentemente publicamos un traballo no que demostramos que a aplicación de hipotermia sistémica (4h) ou focal (24h) en animais con isquemia cerebral provocaba un aumento significativo na expresión de RBM3 cerebral, e que ademais este tratamento estaba asociado a unha diminución do volume de infarto nestes animais. En persoas, RBM3 foi descrito como un marcador de prognóstico en diversos tipos de cancro, sendo así maiores niveis desta proteína asociáronse a un bo prognóstico dos doentes. Ademais da súa indución mediante a diminución da temperatura, recentemente se descubriu que determinados compostos poden incrementar a expresión de RBM3 mediante mecanismos independentes da hipotermia.

Entre elas, o factor de crecemento de fibroblastos 21 (FGF21) resulta de especial interese polos seus efectos multimodais no organismo e, en particular, na patoloxía cerebrovascular. FGF21 é unha hormona endóxena cuxa expresión está condicionada por elementos como os factores de transcripción, a resposta hormonal, as variacións na dieta e na inxesta de nutrintes, os microRNA ou a temperatura. Neste último caso, FGF21 mantén unha estreita relación cas variacións térmicas, sendo así que a súa expresión é inversamente proporcional ao aumento de temperatura. De forma recíproca, FGF21 participa na regulación da temperatura corporal a través de diversos fenómenos fisiolóxicos como pode ser o "*browning*", ou a conversión de graxa branca en graxa parda. Ademais das súas funcións termorreguladoras, esta hormona participa na regulación dos niveis de glucosa e lípidos en sangue, e favorece mecanismos

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

neuroprotectores como pode ser a diminución do dano endotelial, diminución da inflamación ou favorecemento da sinaptoxénese. O potencial interese neste factor radica nos seus efectos multimodais, na súa capacidade para atravesar a barreira hematoencefálica intacta e no seu efecto potenciador da expresión de outros mediadores neuroprotectores como é o caso de RBM3.

Ambas proteínas son sensibles a variacións de temperatura e exercen efectos neuroprotectores demostrados tanto a nivel experimental coma clínico. Sen embargo, dado o seu recente descubrimento e complexidade dos efectos multimodais, os mecanismos exactos destas proteínas na isquemia cerebral non se coñecen por completo. Estudos previos demostraron que a administración de FGF21 reduce a permeabilidade da barreira hematoencefálica, diminúe o perfil inflamatorio das células microgliais e diminúe o volume de infarto en modelos preclínicos de isquemia cerebral.

Estudos clínicos previos mostraron que aquelas persoas con condicións metabólicamente demandantes coma pode ser a obesidade ou a diabetes presentaban maiores niveis de FGF21, pudendo actuar coma un marcador de prognóstico nestas patoloxías. Sen embargo, a pesares da evidencia preclínica do efecto neuroprotector de RBM3 na isquemia cerebral, o seu papel non fora estudado previamente en humanos.

Polos motivos previamente mencionados, o Obxectivo 1 da presente Tese de doutoramento consistiu en analizar a relación entre os niveis circulantes de RBM3 co prognóstico dos doentes con ictus isquémico, e como esta relación está mediada pola temperatura. Para isto, levamos a cabo un estudo retrospectivo observacional en dúas cohortes independentes de doentes con ictus isquémico: unha pertencente ao Hospital Clínico de

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Santiago de Compostela, e a outra provinte do Hospital Universitario Dr. Josep Trueta de Girona, ambos con aprobación dos seus respectivos comités de ética (Código: 2019/616). Inicialmente, realizamos un estudo preliminar en 20 doentes con ictus isquémico para determinar o tamaño mostral e o tempo óptimo para a determinación dos niveis de RBM3. Os niveis circulantes desta proteína foron analizados no soro mediante kits comerciais de ELISA seguindo as instruccións do proveedor. As mostras foron recollidas ao ingreso, ás 2h, 12h e ás 24h..

Como obxectivos clínicos principais, consideramos primeiramente a hipertermia, definida coma unha temperatura axilar ≥37.5˚C. Pola súa relevancia clínica baseándonos en estudos previos e na literatura, a máxima temperatura ás 24h foi definida como variable. En segundo lugar, o prognóstico dos aos 3 meses definidos como unha puntuación na Escala Rankin modificada (mRS) <3. O índice de severidade dos déficits neurolóxicos foi avaliado ca escala de ictus do Instituto Nacional da Saúde (NIHSS) ao ingreso, 24h, 48h e 72h.

Tras aplicar os criterios de inclusión e exclusión a un total de 301 doentes (172 de Santiago e 129 de Girona), 215 doentes foron finalmente elixibles para o estudo (113 de Santiago e 102 de Girona), sen diferenzas significativas entre ambas cohortes. Nos primeiros 20 doentes, observamos unha asociación inversamente proporcional a temperatura máxima e os niveis de RBM3 nas primeiras 24h. Ademais, a presencia de niveis de RBM3 >300 pg/mL asociouse ca presencia de normotermia ás 24h e 72h. Baseándonos nestes resultados, seleccionamos os niveis de RBM3 ás 24h como punto temporal de referencia para a análise da mostra completa. A análise de correlación mostrou que os individuos normotérmicos presentaban maiores niveis de RBM3 en comparación cos hipertérmicos. Tendo en conta diversas

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

variables relacionadas coa temperatura, avaliamos a relación entre RBM3 e a máxima temperatura nas primeiras 24h <37.5ºC nun modelo de regresión loxística incluindo as variables clínica e estatisticamente significativas no modelo. No presente estudo, observamos unha asociación independente entre a máxima temperatura ás 24h <37.5ºC e os niveis de RBM3 ás 24h ≥300 pg/mL.

Adicionalmente, e debido ao efecto da hipotermia na expresión de RBM3, realizamos unha análise dos niveis desta proteína en doentes sometidos a hipotermia terapéutica obtidos do ensaio clínico fase III EuroHYP-1. O ensaio asignou aleatoriamente aos doentes a tratamento hipotérmico máis atención estándar ou atención estándar soa (controis). No grupo tratado, a hipotermia iniciouse dentro das 6 horas seguintes ao comezo dos síntomas e dentro dos 90 minutos seguintes ao comezo da trombólise (ou dentro dos 90 minutos seguintes ao ingreso no hospital naqueles que non foron tratados con trombólise) utilizando 20 ml/kg de solución salina normal refrixerada (4° C) por vía intravenosa durante 30-60 minutos ou un método de arrefriado superficial preespecificado. A hipotermia mantívose a 34-35° C durante 24 horas mediante un sistema superficial ou endovascular. A partir de entón, o requencemento foi realizado pasivamente a un ritmo de 0,2±0.1° C/hora ata que a temperatura rectal ou da vexiga alcanzou os 36.0° C, tras o cal desconectouse o dispositivo de arrefriado. Todos os participantes recibiron tratamento para ictus agudo e a prevención secundaria segundo as directrices publicadas. Realizáronse análises predefinidos por protocolo en todos os doentes cuxa temperatura corporal mantívose en ≤35.0°C durante polo menos 6 horas durante o arrefriamento activo. Seleccionáronse 31 doentes (14 tratados e 17 controis) para o

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

estudo en función da dispoñibilidade de mostras de sangue. A recollida de mostras para a medición de biomarcadores especificouse previamente en tres puntos temporais: basal (previo á hipotermia); 26 horas (2 horas despois do requencemento) e 72 horas dende o inicio do ictus. Non observamos diferencias significativas entre os niveis de RBM3 e a temperatura corporal, aínda que observamos unha tendencia que mostra maiores niveis no grupo hipotérmico. Os resultados deste estudo inicial supoñen a primeira evidencia clínica do papel de RBM3 no ictus isquémico, e reforzan o interese polo estudo desta proteína na enfermidade cerebrovascular.

Ademais da temperatura, a obesidade e estado metabólico dos doentes condiciona fortemente o prognóstico no ictus isquémico. Sen embargo, malia que a obesidade asociouse tradicionalmente ca aparición de múltiples enfermidades así como un agravante do estado funcional, algúns estudos mostraron que este aumento de peso non ten porqué estar específicamente ligado a un mal prognóstico nos ictus isquémico, un fenómeno que se coñece como "paradoxa da obesidade". Neste sentido, observouse que as persoas con obesidade presentan maiores niveis circulantes de FGF21, o cal está asociado a unha mellora funcional e diminución do dano isquémico en modelos preclínicos de isquemia cerebral.

En vista destes resultados, e tendo en conta a recente descrición de FGF21 como un indutor de RBM3, no Obxectivo 2 investigamos a relación entre os niveis circulantes de RBM3 e FGF21 co prognóstico dos dos doentes, así como o papel que xogan o peso corporal e a temperatura nesta relación. Neste segundo apartado, realizamos un estudo observacional nun rexistro de persoas con ictus isquémico incluídos consecutiva e prospectivamente no noso banco de datos, e que foron admitidos

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

á Unidade de Ictus do Hospital Clínico Universitario de Santiago de Compostela (código 2019/616). O primeiro obxectivo do estudo foi o prognóstico funcional aos 3 meses, dicotomizado esta vez como bo (mRS≤2) e mal (mRS >2) prognóstico. Como variables clínicas, seleccionamos primeiramente a máxima temperatura nas primeiras 24h baseado no estudo previo, e categorizada en hipotermia leve (<36.5ºC), normotermia (36.5-37.5ºC) ou hipertermia (>37.5ºC). A segunda variable clínica definida foi o índice de masa corporal (IMC) e definido polos criterios da Organización Mundial da Saúde: normal (IMC <25 kg/m^2), sobrepeso (IMC 25-30 kg/m^2), obesidade grao I (IMC 30-35 kg/m^2), obesidade grao II (IMC 35-40 kg/m^2) e obesidade grao III (IMC >40 kg/m^2). Como obxectivos secundarios, estudamos a asociación de FGF21 e RBM3 con marcadores de obesidade (leptina e adiponectina) e de inflamación (IL-6, IL-10), así como a súa relación co peso e a temperatura.

Este estudo comprendeu un total de 66 doentes elixibles trala aplicación dos criterios de inclusión e exclusión. Observamos unha clara relación entre os niveis basais de FGF21 e os de RBM3 ás 72h. Ademais, estes niveis diminuíron proporcionalmente conforme a temperatura corporal ía en aumento. En canto ao obxectivo principal do estudo, observamos que a máxima temperatura axilar foi significativamente maior naqueles doentes que tiveron un mal prognóstico. Unha maior concetración de FGF21 ao ingreso, así como de RBM3 ás 72h asociouse cun maior prognóstico aos 3 meses. Ademais, non se atopou asociación entre o mal prognóstico e o IMC. En canto aos marcadores de inflamación e obesidade, atopamos unha correlación positiva entre os niveis de FGF21 e os niveis de IL-6 e leptina ao ingreso, así como unha correlación negativa cos niveis de adiponectina. No caso da leptina, atopamos unha

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

correlación positiva cos niveis basais de FGF21 e IMC, sendo así que a categorización dos doentes como non-obesos (normais e sobrepeso) e obesos (obesidade grao I e II) mostrou unha correlación aínda máis marcada. De forma oposta, os niveis de adiponectina ao ingreso mostraron unha correlación negativa cos niveis de FGF21 só en obesos ao categorizar os doentes segundo o seu IMC. En canto os marcadores inflamatorios, os niveis ao ingreso de IL-6 e FGF21 mostraron unha clara correlación positiva independentemente do IMC dos doentes. En canto a RBM3, observamos unha correlación positiva entre os niveis de RBM3 e os niveis de IL-10 en función da temperatura, independentemente do rango estudado. En conxunto, os resultados derivados deste estudo demostran o potencial de RBM3 e FGF21 como marcadores prognósticos na enfermidade isquémica, sendo así que a súa relación está directamente condicionada tanto pola temperatura coma polo peso corporal dos individuos.

A terceira e última parte da presente Tese de doutoramento (Obxectivo 3) consistiu na optimización dun protocolo de indución de hipotermia sistémica en ratos como tratamento para a isquemia cerebral. O obxectivo desta abordaxe preclínica consistía en replicar o modelo animal en rata previamente optimizado polo noso grupo e trasladalo ao rato pola súa versatilidade para estudos que impliquen manipulación xénica. En ratas, demostramos que 4h de hipotermia sistémica e 24h de hipotermia focal reducen de forma efectiva o volume de infarto en ratas e aumentando de forma significativa a expresión de RBM3. Debido á dificultade técnica para a translacionalidade da hipotermia focal a ratos, no presente estudo comparamos os efectos dun tratamento de hipotermia (32ºC) sistémica de 2h e 4h no volume de infarto en ratos que foron sometidos a cirurxía de

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

oclusión transitoria da arteria cerebral media (tMCAO), así como o seu efecto na expresión de RBM3. Empregáronse ratos macho C57BL6 cun peso comprendido entre os 25-30g. Durante o procedemento, anestesiáronse os animais con sevoflurano (5% de indución e 3% de mantemento nunha mestura de 70% de N_2O e 30% de O_2) e obtivéronse mostras de sangue en condicións basais. A temperatura mantívose a 37 ± 0,5°C mediante unha manta controlada por termostato. O modelo de tMCAO consistiu na introdución dun filamento intraluminal a través da arteria carótida externa até chegar ao territorio da arteria cerebral media. A correcta oclusión confirmouse por medición mediante fluxo Doppler. Tras 50 minutos, retirouse o filamento e o animal foi devolto á súa caixa para recuperarse de forma espontánea. Ás 24h realizouse a análise do volume do infarto mediante resonancia magnética T2 e adquiríronse mostras de sangue previo ao sacrificio para análise para western blot/qPCR ou histoloxía. Os grupos experimentais foron os seguintes: 1) Sham (2h): Normotermia (37ºC) vs hipotermia (32ºC) (n=6); 2) Sham (4h): Normotermia (37ºC) vs hipotermia (32ºC) (n=6); 3) tMCAO (2h): Normotermia (37ºC) vs hipotermia (32ºC) (n=6); 4) tMCAO (4h): Normotermia (37ºC) vs hipotermia (32ºC) (n=6).

O estudo comprendeu un total de 64 animais, dos cales 16 foron excluídos. O tratamento con hipotermia sistémica de 2h produciu unha redución similar no volume de infarto ás 24h comparado co grupo de 4h de tratamento. Ademais, a redución do tempo de exposición de 4h a 2h reduciu a taxa de mortalidade dos animais nun 30%. Non se observaron diferencias nos niveis de RBM3 entre 2h e 4h de tratamento con hipotermia. Os niveis de RBM3 foron maiores nos animais hipotérmicos e, en particular, nos animais que recibiron unha maior exposición ao

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

frío, aínda que estes resultados non foron estatisticamente significativos.

En vista dos resultados obtidos, decidimos realizar un segundo grupo experimental sacrificando aos animais ás 3 horas despois do tratamento co hipotermia durante 4h. O punto temporal e duración foron seleccionados baseado no punto de máxima expresión por western blot no noso estudo previo en ratas, no cal un tratamento de 4h produciu unha maior expresión de RBM3. Os grupos experimentais foron os seguintes: 1) Sham (4h): Normotermia (37ºC) vs hipotermia (32ºC) (n=3); 2) tMCAO (4h): Normotermia (37ºC) vs hipotermia (32ºC) (n=3). Na segunda parte do estudo experimental, o tratamento con hipotermia sistémica durante 4h non produxo diferencias significativas nos niveis de expresión de RBM3 con respecto ao control normotérmico.

En conclusión, a presente tese representa a primeira evidencia clínica do papel de RBM3 como marcador molecular asociado cos efectos protectores da hipotermia en doentes con ictus isquémico, e destaca o potencial desta proteína como unha diana terapéutica na enfermidade cerebrovascular.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

ABBREVIATIONS

ACA anterior cerebral artery
AID activityregulated inhibitors of death
AIF apoptosis-inducing factor
AJs adherens junctions
ALCAM activated leukocyte cell adhesion molecule
Apaf-1 apoptotic protease activating factor 1
AQP4 aquaporin 4
ASIC acid-sensing ion channel
ATF activating transcription factor
ATP adenosine triphosphate
Aβ amyloid β
BAT brown adipose tissue
BBB blood-brain barrier
Bcl-2 B-cell lymphoma 2
BDNF brain-derived neurotrophic factor
BM basement membrane
BMI body mass index
BNIP3 Bcl2 interacting protein 3
BSA bovine serum albumin
CAMKII Ca^{2+}/calmodulin-dependent protein kinase II
CB1 cannabinoid receptor 1
CBF cortical blood flow
CCA common carotid artery
CGBI complete global brain ischemia
CHOP CCAT-enhancer-binding protein homologous protein
CIRP cold-inducible RNA-bidning protein
CNS central nervous system
CV coefficient of variation
Cox cyclooxigenase
CREB cAMP response-element binding protein
CRH corticotropin-releasing hormone
CSD cortical spreading depolarization
CSF cerebrospinal fluid
CSH cold-shock hormone
CSP cold-shock protein
DAMP damage-associated molecular patterns
Dcx doublecortin
Diablo direct inhibitor of apoptosis-binding protein with low pI
DISC death inducing signaling complex
ECA external carotid artery
ECM extracelullar matrix
ECs endotelial cells
eIF2α eukaryotic initiation factor 2α
ELISA enzyme linked immunosorbent assay
eNOS endothelial NO synthase
ER endoplasmic reticulum
ET-1 endothelin-1
FDA Food and Drug Administration

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

FGF fibroblast growth factor
FGF21 fibroblast growth factor 21
GABA g-aminobutyric acid
G-CSF granulocyte-colony stimulating factor
GFAP glial fibrillary acidic protein
GJs gap junctions
GLT-1 glutamate transporter 1
GLUT4 glucose transporter 4
GPx glutathione peroxidase
GRP78 glucose regulated protein 78
HCG human chorionic gonadotropin
HDL high density lipoprotein
HIF hipoxia inducible factor
HMGB1 high mobility group box 1 protein
HPA hypothalamus-pituitary-adrenal axis
HPG hypothalamus-pituitary-gonad axis
HRE
HSP heat-shock protein
HSPBD heparin-sulfate proteoglycan binding domain
ICA: internal carotid artery
ICAM
IFN-γ Interferon-γ
IL Interleukin
iNOS inducible NO synthase
IP3 inositol 3 phosphate
IRE1 inositol-requiring protein 1
LDL low density lipoprotein
MAG myelin-associated glycoprotein
MCA middle cerebral artery
MCAO MCA occlusion
MLKL mixed lineage kinase domain like pseudokinase
MMP matrix metalloproteinase
MNC mononuclear cells
modified Rankin Scale
MPTP mitochondrial permeability transition pores
mRNA messenger RNA
MRS magnetic resonance spectroscopy
MSC mesenchymal stem cells
MSME multi-slice multi-spin-echo sequence
mTORC1 mammalian target of rapamycin complex 1
NF-kβ nuclear factor kappa β
NIHSS National Institute of Health Stroke Scale
NK natural killer
NMDA N-methyl-D-aspartate receptor
nNOS neuronal NO synthase
NO nitric oxide
NVU neurovascular unit
PCA posterior cerebral artery
PDE5 phosphodiesterase 5
PECAM platelet endothelial cell adhesion molecule
PERK protein kinase RNA-like ER kinase
PI3K phosphatidylinositol 3-kinase
PKC atypical protein kinase c
POAH preoptic anterior region of the hypothalamus

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

PPAR Peroxisome proliferator-activated receptor
PTGP pterygopalatine artery
qPCR quantitative polymerase chain-reaction
RBM3 RNA-binding motif protein 3
RGG arginine-glycine-rich domain
RIPK1/3 receptor-interacting protein kinase-1 and 3
RNA ribonucleic acid
RNS reactive nitrogen species
ROS reactive oxygen species
rtPA recombinant tissue plasminogen activator
S100B Ca^{2+}-binding protein B
SDF-1 stromal cell-derived factor-1
SERCA sarco/endoplasmic reticulum Ca^{2+}-ATPases
SGZ subgranular zone
SOD superoxide dismutase
SUMO small ubiquin-like modifier
SVZ subventricular zone
T3 triiodothyronine
TBI traumatic brain injury
TGF-β transforming growth factor β
TIA Transient Ischemic Attack
TJs
TLR Toll-like receptor
TMS transcranial magnetic stimulation
TNF tumoral necrosis factor
TNF-α Tumor necrosis factor α
TOAST Trial of Org 10 172 in Acute Stroke Treatment
tPA tissue plasminogen activator
TRAIL TNF-related apoptosis inducing ligand
TRPM4 transient-receptor potential membrane 4
TWEAK tumor necrosis factor-like weak inducer of apoptosis
UCP1 uncoupling protein 1
UPR unfolded protein response
VCAM vascular cell-adhesion molecule
VEGF vascular endothelial growth factor
WAT white adipose tissue
WB western blot
WHO World Health Organization
XBP1
ZO zona occludens

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

1. STROKE

The first medical use of the word "stroke" dates back to the very beginning of the XVII century, when it was first used to describe a sudden onset of symptoms as if the patient had been "stroke by the hand of God"[1]. Nonetheless, the Greek word "apoplexia" (meaning a sudden, deadly blow) remained the term of choice for over two millennia to describe those neurological deficits that were unrelated to traumatic injuries[2].

A revised definition was provided by the World Health Organization (WHO) in 1970, where stroke was defined as "rapidly developed clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than of vascular origin"[3]. As this description was heavily based on clinical symptoms, the American Heart Association and American Stroke Association elaborated a revised definition that accounted for the advances in the management, nature, timing and imaging techniques, broadening its use for the different subtypes of the aforementioned disease[4].

Physiologically, stroke occurs when the arterial blood supply to the brain is interrupted by the occlusion or rupture of a blood vessel, halting the correct delivery of oxygen and

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

nutrients to the affected regions. The longer this situation is sustained in time, the greater the damage to the cells, the bigger the loss of function and the deadlier the outcome is for the patient.

1.1. Epidemiology

Stroke is a massive global health concern. Although its incidence and mortality has decreased over the past three decades, it is still the second leading cause of death and the first cause of disability worldwide, affecting over 13 million people every year. Among them, roughly 5.5 million people will die, while 1 out of 6 stroke survivors will suffer another stroke within 5 years. Moreover, up to 30% of stroke survivors will be permanently disabled[5].

The risk of suffering a stroke is influenced by both sex and age. Although men present higher mortality rates, women are at a higher risk of suffering a stroke during their lifetime (20-21% vs 14-17%) along with poorer outcomes[6]. Regarding age, the incidence of stroke doubles after 55 years of age, and over 70% of all strokes are older than 65 years[7].

Beyond these variables, the INTERSTROKE study postulated that modifiable risk factors such as hypertension, high cholesterol, current smoking, alcohol consumption, diabetes, stress, obesity, heart disease lack of physical activity, and poor diet were responsible for 90% of all strokes[8]. The incidence rates for stroke vary between geographical regions. In the European Union, stroke affects more than 1 million citizens per year and causes 440,000 deaths, with an estimated cost of €60 billion[9]**.** In Spain, stroke is the second most common cause of death, with an incidence of ~200 people (173 in women vs 202 in

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

men) per 100,000 inhabitants, and an estimated cost of €27,711 a year per patient[10,11].

1.2. Classification of stroke

Stroke is originated from a sudden drop in blood flow to the brain. Depending on the nature of this disturbance, stroke is usually classified in ischemic (derived from a clogged artery) and hemorrhagic (resulting from the rupture and leakage of blood from a vessel) (**Figure 1**).

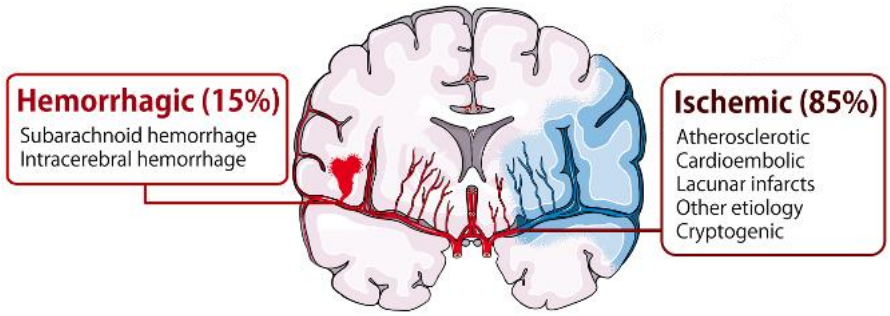


Figure 1. Classification of stroke subtypes. Self-created image using elements with Creative Common license.

1.2.1. Ischemic Stroke

In this type of stroke, a physical barrier (either a clot, embolus or atherosclerosis) impedes the normal circulation of blood to the affected region. Around 85% of strokes are produced by ischemic occlusions, which account for 87% of the casualties. A focal, neurological dysfunction with symptoms lasting less than 1h and no evidence of infarction on brain imaging is known as a transient ischemic attack (TIA). TIA patients are usually propense to a recurrent stroke, with a risk of up to 20% of suffering an additional insult within 3 months, usually with an unfavorable prognosis.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

An extensive, more thorough classification was provided by the Trial of Org 10 172 in Acute Stroke Treatment (TOAST), attending to several aspects such as the etiology, size or affected vascular territory. As such, the TOAST criteria stratify ischemic strokes in five different subgroups: 1) large-artery atherosclerotic stroke, 2) small artery occlusion (lacunar infarcts), 3) cardioembolism, 4) stroke of other etiology, and 5) stroke of undetermined cause (cryptogenic)[12]. Although not rigorously adhered to etiology, this system constitutes a useful tool for clinicians while providing useful information to the patients.

1.2.2. Hemorrhagic stroke

Hemorrhagic stroke is the third most common cause of stroke (15%) after embolism and thrombotic disorders, but is associated with a high mortality rate. Stress to the brain tissue causes the vascular walls to weaken and rupture, leaking blood into the brain parenchyma, ventricular or subarachnoid space. Extravasated blood produces a mass effect as it presses the surrounding tissue, inducing shear stress and altered rheology, which in combination with the decreased blood flow and surrounding toxicity leads to necrosis and cell death in the affected region. Deep perforating vasculopathies are the most common cause of intracerebral hemorrhage, usually as a consequence of an underlying hypertensive condition. Other causes included amyloid angiopathy, ruptured aneurysms and vascular malformations, and anticoagulant-related hemorrhages[13].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

1.3. Risk factors in ischemic stroke

Stroke is a dynamic disease in which the progression and outcome of the injury can be influenced by different conditions. Depending on their nature, risk factors can be classified in non-modifiable (hereditary or predetermined) and modifiable (related to lifestyle and environment)[14–16] **(Figure 2)**. According to the Global Burden of Disease Study, the 5 most prominent risk factors are high systolic blood pressure, high body-mass index, high sodium diet and smoking. Moreover, the study suggests that over 90% of stroke burden worldwide is directly related to modifiable risk factors whose control could prevent over 75% of global stroke burden[17].

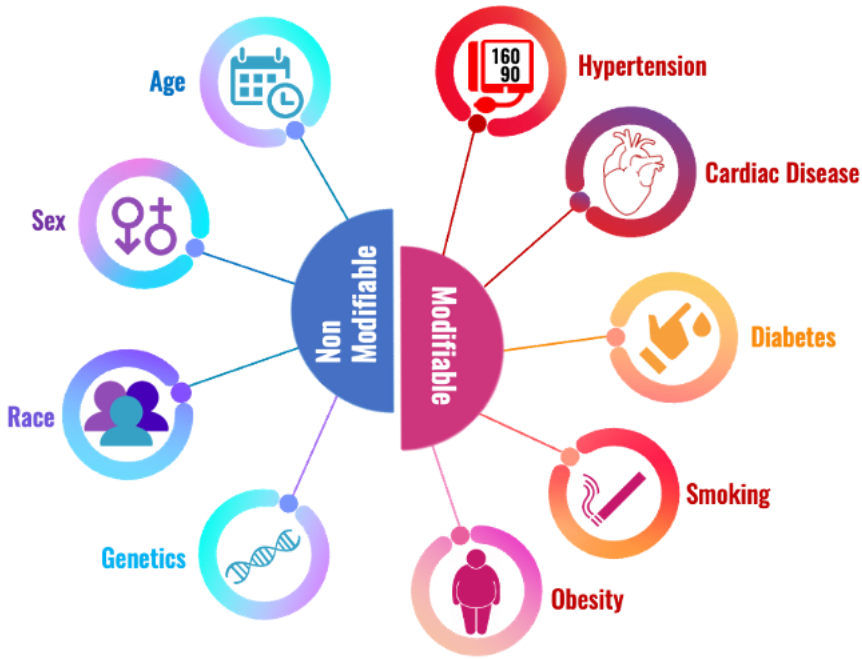


Figure 2. Graphic listing of major risk factors of ischemic stroke. Self-created image using elements with Creative Common license.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

1.3.1. Non-modifiable risk factors

The most determinant factors in this group are age, sex, race and genetic and hereditary factors[17]:

- Age: age ranks first among the non-modifiable risk factors of ischemic stroke. In fact, 95% of strokes occur in people over 45 years old, with about two-thirds occurring in those older than 65. Stroke occurrence more than doubles after 55 years old for every additional 10 years.

- Sex: men have a 19% higher chance of suffering a stroke compared with women. Nonetheless, women are at a higher risk of suffering a stroke during their lifetime (20-21% vs 14-17%) along with poorer outcomes, mainly due to hormonal differences.

- Race: certain aspects such as stroke subtype might also vary depending on racial, ethnic and geographic characteristics.

- Genetic and hereditary factors: different studies have demonstrated an increased risk of stroke within families after adjusting for other risk factors. Rare monogenic disorders such as CADASIL or Fabry's disease increase the predisposition to stroke occurrence.

1.3.2. Modifiable risk factors

Contributing factors to stroke pathophysiology which are susceptible to modification include hypertension, cardiac diseases, diabetes mellitus, smoking or obesity:

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

- Hypertension: Nearly 25% of the population and 50% of the people over 65 years old are hypertensive. Hypertension is the most important modifiable risk factor in ischemic stroke and has been extensively shown to increase the risk of developing all subtypes of ischemic stroke as well as the risk of hemorrhagic transformation[18].

- Cardiac diseases: Certain cardiac diseases increase the risk of suffering a stroke, including atrial fibrillation (one out of six strokes derives from this condition), coronary artery disease (5% of all strokes within two weeks after an acute myocardial infarction), valvulopathies (mitral stenosis, endocarditis and prosthetic heart valves), patent foramen ovale (especially in younger patients with unknown origin), and left ventricular hypertrophy[19].

- Diabetes mellitus: Patients with diabetes mellitus show an increased relative risk compared with healthy population. Moreover, these patients present small cerebral infarcts (predominantly lacunes) in basal ganglia, pons, thalamus and cerebellum[20].

- Smoking: Tobacco increases the risk of stroke in a dose-dependent fashion. A consumption of more than 40 cigarettes a day has twice the relative risk compared to those who smoke less than 10 cigarettes per day[21].

- Obesity: Obesity is a multifactorial, complex and preventable disease defined as an excessive accumulation of fat in the adipose tissue. The Global Burden of Disease from 2013 showed an increase of 27.5% and 47.1% in the prevalence of

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

obesity in adults and children, respectively, from 1980 to 2013[22]. Moreover, the WHO estimated that a total of 39% of the population were overweight, with nearly 13% of the population presenting obesity. The main pathophysiological event in obesity is the energetic imbalance between daily caloric intake and expenditure, resulting in increased body fat in adipose tissue and weight gain[23]. In this regard, adipose tissue has been shown to be heterogeneously composed of different cell types, including pre- and mature adipocytes, fibroblasts, endothelial cells, mast cells, granulocytes, lymphocytes and macrophages[24]. Far from being a mere fat storage, adipose tissue secretes several adipokines and cytokines (pro- or anti-inflammatory), including leptin, adiponectin, resistin and visfatin, as well as interleukin (IL)-4, interferon (IFN)-γ, tumor necrosis factor (TNF-α) or IL-6, among others[25].

- Leptin is a hormone secreted almost exclusively by adipocytes that regulates energy homeostasis through its anorexigenic properties through hypothalamus stimulation[26]. It is also involved in immunoregulatory mechanisms, mainly exerting pro-inflammatory effects such as increased monocyte and T-cell proliferation, increased IL-6 and TNF-α secretion, enhanced Th1 responses and chemokine production by macrophages, along with increased toll-like receptor (TLR) expression[27,28]. Moreover, leptin contributes to atherogenic damage by promoting platelet aggregation and migration, hypertrophy and proliferation of smooth muscle cells from the vascular wall and exacerbated endothelial dysfunction[29]. Although obese patients present particularly

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

high levels of this hormone, its secretion has no impact on energy homeostasis, a condition known as leptin resistance. Leptin resistance may contribute to insulin resistance and lipid accumulation by reducing the lipid oxidation rate in insulin-sensitive organs[30].

- Adiponectin is an anti-inflammatory mediator and the most abundant adipose-specific adipokine[31]. Obese patients show significantly lower levels of adiponectin, which is negatively correlated to visceral fat accumulation[32]. Adiponectin acts through its receptors AdipoR1 (expressed ubiquitously) and AdipoR2 (mainly expressed in the liver). Obesity and insulin resistance decrease the expression of both receptors, leading to increased triglyceride accumulation, inflammation and oxidative stress by decreased peroxisome proliferator-activated receptor (PPAR)-α signaling[30]. On the contrary, adiponectin binding to its receptors promotes IL-10 macrophage production, inhibits TLR-mediated NF-κB activation and promotes the anti-inflammatory M2 phenotype[33]. Finally, adiponectin counteracts the atherogenic response by reducing oxidative stress and attenuating smooth muscle cells proliferation and migration[34].

Although obesity has been extensively associated with increased risk of developing several diseases including type II diabetes mellitus, coronary heart disease or cancer, a growing number of studies from the last decade has found an association between obesity, improved outcome and lower mortality rate, a phenomenon termed the **obesity paradox**[35]. A potential

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

explanation of these findings could be the protective role of the adipose tissue through variations in the inflammatory profile. A recent study from our group reported that although obese patients showed higher pro-inflammatory levels (i.e., IL-6) during the hyperacute phase, these levels decreased at 7 days concurring with an increase in anti-inflammatory mediators such as IL-10, a phenomenon that was not observed in non-obese patients[36]. Another possible explanation could be the catabolic state present in the patient during the post-stroke phase, leading to additional weight loss that would be reflected in an attenuated inflammatory state. Nonetheless, these assumptions are derived mostly from observational studies and thus need further validation studies.

1.4. Stroke pathophysiology

Following the interruption in proper tissue irrigation, a myriad of fast-developing mechanisms take place within seconds[37]. As time passes by and brain insult subsides, a series of reparatory mechanisms are triggered in ordered to compensate for the damaged areas. To describe the molecular and cellular mechanisms underlying ischemic stroke in detail, these events have been allocated to four temporal phases: hyper acute (first minutes to first 2-3 hours), acute (first 2-3 hours to days), subacute (first weeks) and delayed (first months after the onset) **(Figure 3)**.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

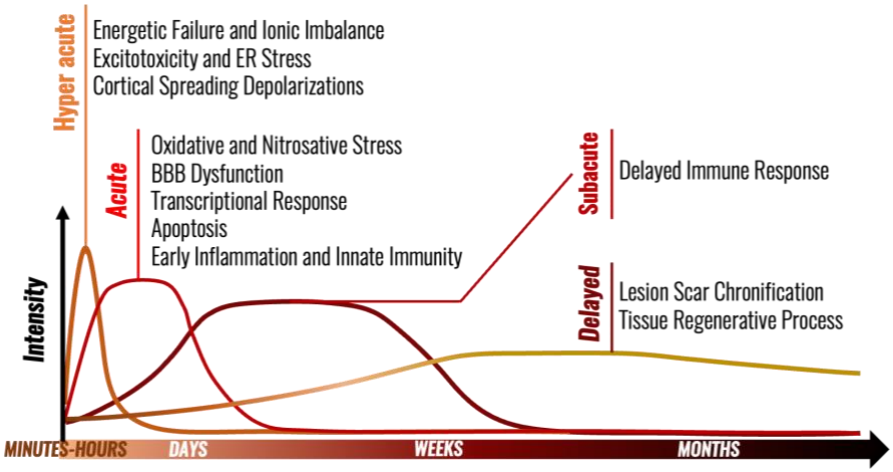


Figure 3. Dynamic profile of the evolution of stroke-derived injury and repair events. Adapted from Dirnagel et al. 2014 using Creative Common elements.

1.4.1. Hyperacute phase

This initial phase comprises cellular and biochemical changes arising from the sudden occlusion of the vessel, from the first minutes to the initial 2-3 hours after injury. The major events during the hyperacute phase concern, energetic failure and ionic imbalance, excitotoxicity, endoplasmic reticulum stress and cortical spreading depolarizations:

1.4.1.1. Energetic failure and ionic imbalance

The human brain critically depends on a continuous supply of oxygen and glucose as its main source of energy. Brain activity accounts for nearly 20% of total glucose (around 5.6 mg of glucose per 100 g) and oxygen consumption[38]. While energetically demanding, this allows for the maintenance of neuronal and non-neuronal functions by means of adenosine triphosphate (ATP) production, as well as physiological production of neurotransmitters.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

ATP generation is the result of the work of an orchestrated cellular machinery that begins with the conversion of pyruvate (the glycolysis output of glucose metabolism) into Acetil-CoA by the pyruvate dehydrogenase. Subsequently, Acetil-CoA is led to the mitochondrial matrix and into the Krebs cycle, generating NADH and $FADH_2$ as byproducts. These reductor agents are then used in the electron transport chain to generate a proton gradient, ultimately producing a substantial amount of ATP by oxidative phosphorylation. ATP serves a critical purpose as fuel for the correct function of ion pumps, particularly for the Na^+/K^+ and Ca^{2+}/ATP pump, aiding in the regulation of homeostatic conditions across membranes[39].

During an ischemic stroke, the lack of blood supply leads to hypoxia and scarce glucose availability, which corner the brain into seeking alternative metabolic substrates for energy production such as the ketone bodies, lactate, β-hydroxybutyrate or acetoacetate[40]. These metabolic pathways are drastically less efficient than oxidative phosphorylation, which in turn is reflected in altered excitability due to ion disturbance.

In this scenario, the Na^+/K^+ pump is unable to compensate for the increase in intracellular Na^+ concentration, leading to Cl^- and water influx, endothelial swelling and rupture of the cell membranes. Additionally, low ATP availability also affects other transporters such the Ca^{2+}/ATPase and Na^+/Ca^{2+} exchanger, increasing intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and affecting neuronal excitability[39].

1.4.1.2. Excitotoxicity

Ca^{2+} plays a pivotal role in neurotransmitters release and propagation of electrical signals across brain tissue. Under normal conditions, a constant but limited amount Ca^{2+} enters the

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

cell and mediates in several homeostatic processes such as cellular proliferation and differentiation[41]. However, the stroke-induced restriction in ATP directly affects the Ca^{2+} extrusion mechanisms (i.e. Ca^{2+}/ATPase and Na^{+}/Ca^{2+} exchanger), increasing $[Ca^{2+}]i$. This unbridled influx promotes the binding of Ca^{2+} to the vesicular synaptogamin at the presynaptic terminal, triggering vesicle fusion and the consequent release of neurotransmitters such as glutamate[42].

Glutamate is considered the major source of excitotoxicity and early cell death during stroke onset. Glutamate promotes the hyper excitability of ionotropic (N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate) and metabotropic receptors in the post-synaptic terminal. Among them, the NMDA receptors play a critical role in Ca^{2+} overload and the propagation of glutamate excitotoxicity. The NMDAr acts as gated regulators of cell depolarization, participating in processes such as learning, memory or synaptic plasticity[43]. In physiological conditions, Mg^{2+} blocks the NMDAr subunits, impeding the entrance of ions into the cell. Excessive glutamate binding removes this blockade, favoring the uncontrolled entry of Ca^{2+} and Na^{+}, thus increasing both the $[Ca^{2+}]_i$ and glutamate release. The entry of Ca^{2+} through the NMDAr activates calpain, a cytosolic protease that drives the degradation of the Na^{2+}/Ca^{2+} exchanger, further enhancing Ca^{2+} overload and promoting cell death pathways. Simultaneously, uncontrolled Ca^{2+} influx via NMDAr activates the Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII), which phosphorylates both NMDA and AMPA receptors, facilitating intracellular Ca^{2+} accumulation and thoroughly favoring excitotoxicity[39].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

NMDAr have been shown to play disparate roles depending on their location. On the one hand, Ca^{2+} binding to synaptic NMDAr has been linked to activation of prosurvival pathways such as the cyclic adenosine monophosphate (cAMP) response-element binding protein (CREB), which induces the production of neuroprotective mediators such as the activity-regulated inhibitors of death (AID) and brain-derived neurotrophic factor (BDNF)[44]. On the other hand, extrasynaptic NMDAr contribute to excitotoxicity by mechanisms that generally interfere with the synaptic protective pathways. These include inhibition of the extracellular receptor kinase (ERK)1/2, Jacob-mediated dephosphorylation of CREB or the recently discovered transient-receptor potential membrane 4 (TRPM4)-NMDAr complex, which promote cell damage and trigger programmed cell death[43,45] **(Figure 4).**

To sum up, excitotoxicity is mainly a glutamate-driven phenomenon where Ca^{2+} plays a paramount role in both its initiation and perpetuation. Nonetheless, Ca^{2+} overload also poses a vexing threat to other organelles.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

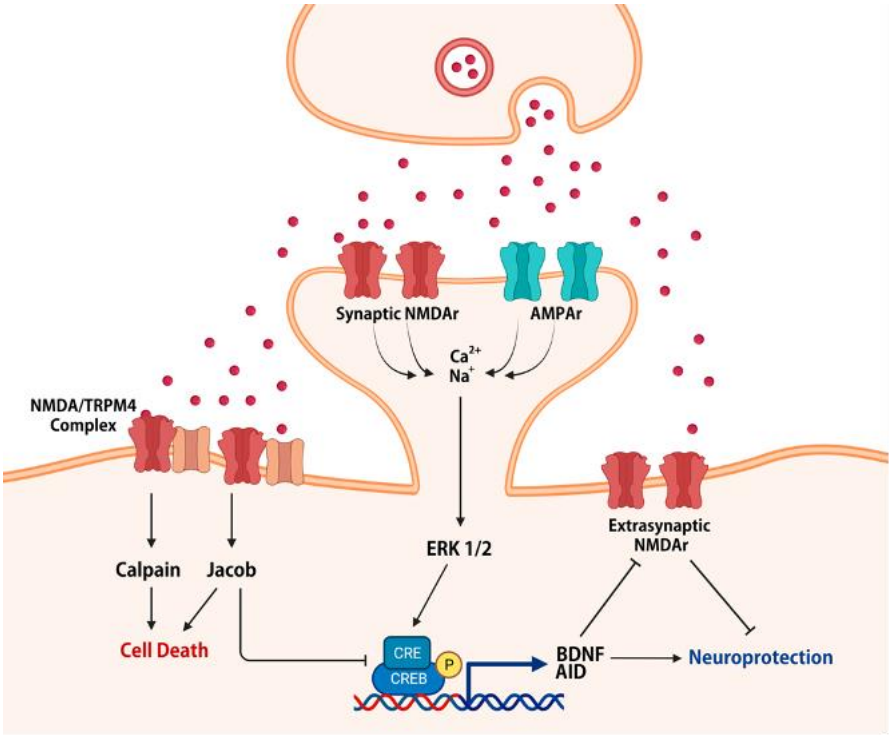


Figure 4. Glutamate receptors and signaling in the brain. Self-created image using elements with BioRender software.

1.4.1.3. Unfolded Protein Response

The endoplasmic reticulum (ER) is a multifaceted organelle which regulates protein synthesis, protein folding and cell trafficking, processes which are highly dependent on Ca^{2+} signaling. ER regulates their function by coordinating both the release and reuptake of Ca^{2+} into its lumen, mainly by the action of the second messenger inositol 1, 4, 5-triphosphate (IP3) and the sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA), respectively. Under normal conditions, culling of unfit proteins is mediated by ER-associated chaperones such as the glucose regulated protein 78 (GRP78), which are normally associated with inactive inositol-requiring protein 1 (IRE1), protein kinase

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

ribonucleic acid (RNA)-like ER kinase (PERK) or activating transcription factor-6 (ATF6)[46]. During stroke, the energetic failure deranges Ca^{2+} homeostasis by impairing Ca^{2+} pumps, increasing $[Ca^{2+}]_i$ and producing misfolded proteins, leading to ER stress. In this scenario, GRP78 detaches from its receptors and binds to the unfolded proteins, sequestering them in the ER and initiating the unfolded protein response (UPR)[47].

Upon activation, PERK undergoes autophosphorylation and dimerization, and proceeds to phosphorylate eukaryotic initiation factor 2α (eIF2α). While eIF2α phosphorylation attenuates global protein translation, it specifically promotes chaperone production and upregulates activating transcription factor 4 (ATF4), which turns on UPR genes involved in the antioxidative stress response[48]. In a similar fashion, IRE1 also dimerizes and autophosphorylates after GRP78 disassociation. IRE1 subsequently splices X-box binding protein 1 (XBP1), while ATF6 undergoes cleavage in the Golgi. Both proteins work hand to hand to promote the expression of genes involved in protein folding and degradation[47].

Although the aforementioned mechanisms promote prosurvival pathways, an overwhelming stress to the UPR can lead to apoptotic cell death mechanisms initiated by IRE1 (via JNK/AP-1/Gadd153) or ATF4 (via CCAT-enhancer-binding protein homologous protein (CHOP))[49] **(Figure 5).**

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

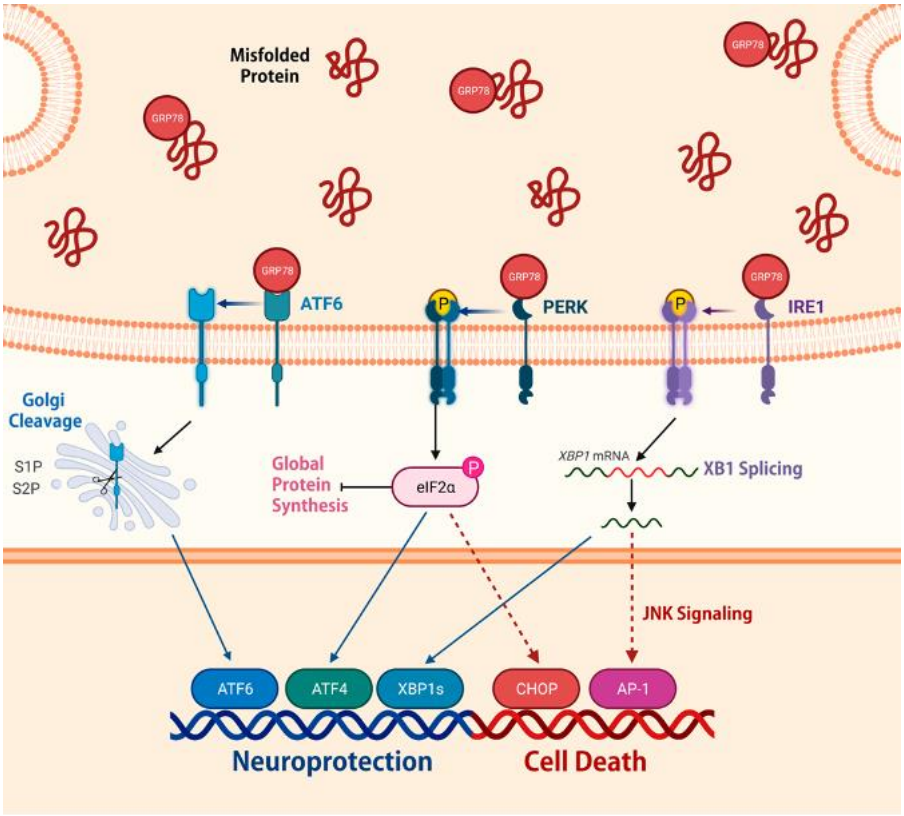


Figure 5. Unfolded Protein Response in ischemic stroke. The accumulation of misfolded proteins inside the ER triggers a series of mechanisms aimed at restoring the correct function of the ER. A derailed or maintained response can also promote damage signaling and activate cell death pathways. Self-created image using elements with BioRender software.

1.4.1.4. Cortical Spreading Depolarizations

Persistent ATP and oxygen depletion lead to ionic imbalance and near-complete breakdown of ion gradients across membranes, a phenomenon known as cortical spreading depolarization (CSD). CSD is a slowly propagating wave (2-5 mm/min) that induces massive glial and neuronal depolarization, disrupting ion homeostasis and producing a transient depression in electrical activity. This phenomenon

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

dramatically derails the energetic availability of the affected tissue, as ion pumps and other ATP-dependent mechanisms are necessary for the restoration of homeostasis[50].

As a consequence, regional blood flow transiently augments to compensate for the increased energetic demands (spreading hyperemia), followed by a sustained hypoperfusion. CSD has also been shown to play a role in the already challenged ischemic tissue following stroke, as this phenomenon has been observed in clusters in nearly 90% of patients with middle cerebral artery (MCA) infarctions[51]. In this setting, extracellular K^+ dramatically rises as a consequence of impaired Na^+/K^+-ATPase activity. This triggers CSD initiation and propagation, which in turn increases metabolic demands of glucose and oxygen in grey matter[52].

Unlike in the well-nourished tissue, the high levels of K^+ and low nitric oxide (NO) availability in the ischemic regions lead to a decrease in regional blood known as spreading ischemia, whose vasoconstrictive response is directly proportional to the severity of the injury. The ion changes induced by CSD generate intracellular hyperosmolality, leading to water influx and causing cytotoxic edema[53] **(Figure 6)**.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

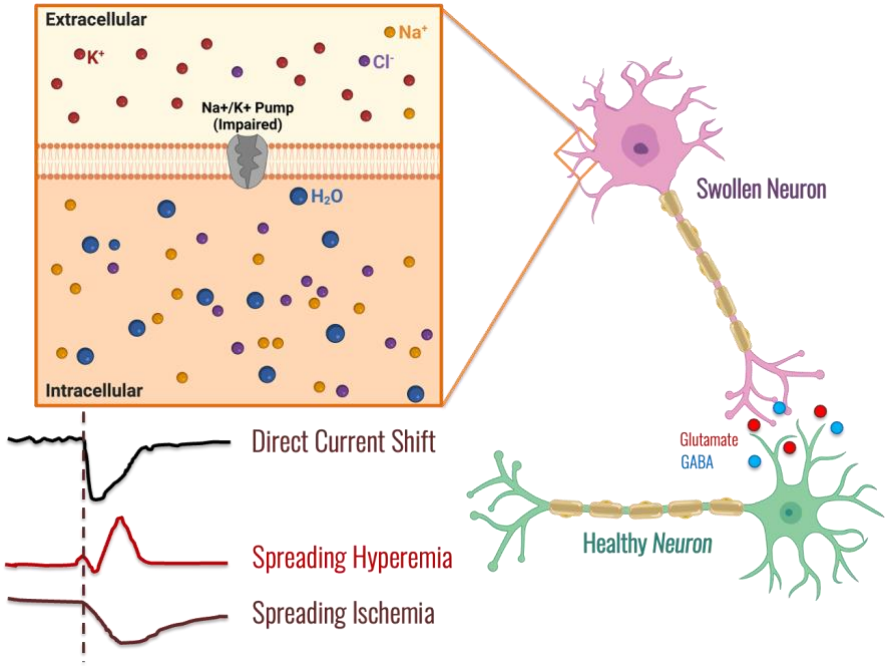


Figure 6. Ionic imbalance and cytotoxic edema in CSD. Self-created image using elements with BioRender software.

Moreover, the propagation of CSD triggers the release of damaging mediators and neurotransmitters such as glutamate, acetylcholine or g-aminobutyric acid (GABA), which further promote excitotoxicity, facilitate organelle swelling and result in increased infarct size[54].

1.4.2. Acute Phase

Following the initial injury events, a series of events develop after the initial 2-3 hours that set the scenario for the subsequent damaging mechanisms. This phase comprises oxidative and nitrosative stress, blood brain barrier (BBB) dysfunction and macrophage infiltration, transcriptional

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

response, cell death mechanisms, early inflammation and innate immunity responses.

1.4.2.1. Oxidative and nitrosative stress

During energetic cellular processes such as oxidative phosphorylation or immune crosstalk upon xenobiotic recognition, a group of small molecules known as reactive oxygen species (ROS) are generated as a by-product of the partial reduction of oxygen. ROS comprise radical and non-radical compounds such as oxygen ions, peroxides or free radicals, which under physiological conditions engage in neuroprotective pathways by participating in cell signaling, immune defense or breakdown of toxic cellular debris[55]. Among them, oxygen superoxide (O^{2-}) is the most abundant species. O^{2-} is converted to H_2O_2 by the superoxide dismutase (SOD), which is then released to act as an intracellular messenger. In addition, H_2O_2 is also generated as a by-product of lipid metabolism in peroxisomes. H_2O_2 can then be quickly degraded to O_2 and H_20 by the catalase, the primary antioxidant enzyme of the peroxisome, or reduced by the glutathione peroxidase (GPx)[56]. Other antioxidant mechanisms include low-molecular weight molecules such as uric acid, coenzyme Q or lipoic acid[57].

During ischemia, the increased production of ROS along with the depleted pool of oxidative scavengers impairs the antioxidant system, ultimately leading to the accumulation of ROS termed as oxidative stress. In particular, the brain is remarkably vulnerable to oxidative damage due to its rich content of unsaturated lipids and iron, its demanding oxygen consumption and a relatively shallow antioxidant activity[58]. ROS actively damages the brain parenchyma by inducing lipid peroxidation, DNA fragmentation, impaired cytoskeletal

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

assembly or by inhibiting enzymatic activity, as well as promoting excitotoxicity by inducing Ca^{2+} release from intracellular compartments[59].

This damage is further increased by the production of reactive nitrogen species (RNS) such as the NO radical (NO) by the endothelial (eNOS), inducible (iNOS) and neuronal (nNOS) nitric oxide synthases. In physiological conditions, NO plays a major role in the regulation of neuronal signaling, vessel dilation and smooth muscle relaxation. Nonetheless, NO enhances oxidative damage in ischemia by reacting with O^{2-} to generate the peroxynitrite anion ($ONOO^-$), a formidable oxidizing agent capable of permeabilizing lipid bilayers, inhibiting SOD and triggering cell death pathways[60].

1.4.2.2. Endothelial Dysfunction and Macrophage Infiltration

The BBB plays a crucial role in the maintenance and protection of the central nervous system (CNS), shielding the brain parenchyma from pathogens and toxins while allowing the selective entrance of oxygen and nutrients. Ionic and neurotransmitter balance, as well as proper electrochemical signaling is also regulated by a number of channels and transporters expressed on the BBB. Altogether, this dynamic barrier preserves the necessary parenchymal microenvironment necessary for neuronal function[61].

The restrictive nature of the BBB is a direct consequence of the particular properties of its blood vessels. The apical (luminal) surface of BBB vessels presents a continuous layer of endothelial cells (ECs) with no fenestrations and a paucity of transcytosis across membranes. BBB ECs are highly polarized and possess heightened mitochondrial presence compared with their

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

peripheral counterparts, which provides the increased energetic output necessary for receptor-mediated regulation of ion homeostasis and molecular permeability[62].

This cellular continuum is further reinforced by the junctional complex, an intercellular scaffold construct composed of three different protein structures: tight junctions (TJs), adherens junctions (AJs) and GAP junctions (GJs). TJs are primarily comprised of occludins, junction adhesion molecules and claudins, along with the accessory zonula occludens proteins (ZO), which binds these proteins to the actin cytoskeleton. These proteins effectively cover microvessels and limit passive diffusion of polar molecules and proteins across membranes. AJs are mainly composed of cadherins, which provide structural support via cell-cell adhesion and aid in permeability regulation, and catenins, linker proteins responsible of binding the former to the cytoskeleton. Finally, GJs are composed of connexin proteins and surveil intercellular communication[63].

The basolateral (abluminal) surface of the vessels is surrounded by two basement membranes (BM), the inner vascular BM and the outer, glia limitans perivascularis. The structures present a variety of extracellular matrix components including type IV collagen, laminin, fibronectin, hyaluronan, heparin sulfate proteoglycans and other glycoproteins, and provide both a structural anchor and a functional separation between cells[61,62]. Pericytes are vascular mural cells embedded in the BM with contractile and phagocytic capability that also contribute to vascular permeability. Anchored to the basolateral membrane by their end-foot processes, astrocytes also regulate blood flow and contribute to membrane permeability by releasing bioactive compounds such as NO or vascular

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

endothelial growth factor (VEGF), or via aquaporin 4 (AQP4), an astrocyte end-feet water channel that facilitates water movement across membranes. The interaction between ECs and pericytes, along with the structural and physiological support from astrocytes, neurons, immune cells and extracellular matrix, derives in the formation of a sophisticated dynamic complex known as the neurovascular unit (NVU)[64].

Under physiological conditions, only low molecular weight (<400-600 Da) lipophilic compounds, positively charged molecules or blood gases (such as O_2 and CO_2) can circulate across the BBB. Other molecules can attain said transport by endogenous transport systems such as solute carriers (i.e. glucose, amino acids and organic anions and cations), receptor-mediated transcytosis (i.e. insulin, leptin or iron) or diapedesis (leukocytic movement with intact TJs)[65]. During stroke, the compromised delivery of oxygen and glucose impacts the normal functioning of ionic pumps. Na^+ concentration builds up inside the cell, driving water influx and generating cytotoxic edema, endothelial cell swelling via AQP4 and BBB breakdown. Simultaneously, $[Ca^{2+}]_i$ concentration dramatically rises as a consequence of the impaired ATP-ases, resulting in glutamatergic excitotoxicity via AMPA/NMDA receptors and oxidative stress damage (by ROS compounds such as O^{2-}), which constrict pericytes and produce micro-occlusions in the surrounding vessels. In addition, extracellular proteases such as the matrix metalloproteinases (MMPs) directly contribute to BBB disruption by degrading TJs and basal lamina components, increasing paracellular permeability and allowing the entrance of toxic compounds and macromolecules that, by osmotic gradient, further contribute to BBB leakage and cell death[39,66]. The aforementioned events contribute to a reactive response in

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

damaged endothelial cells, which release danger signals and express adhesion molecules that drive the subsequent immune infiltration into the brain parenchyma (**Figure 7**).

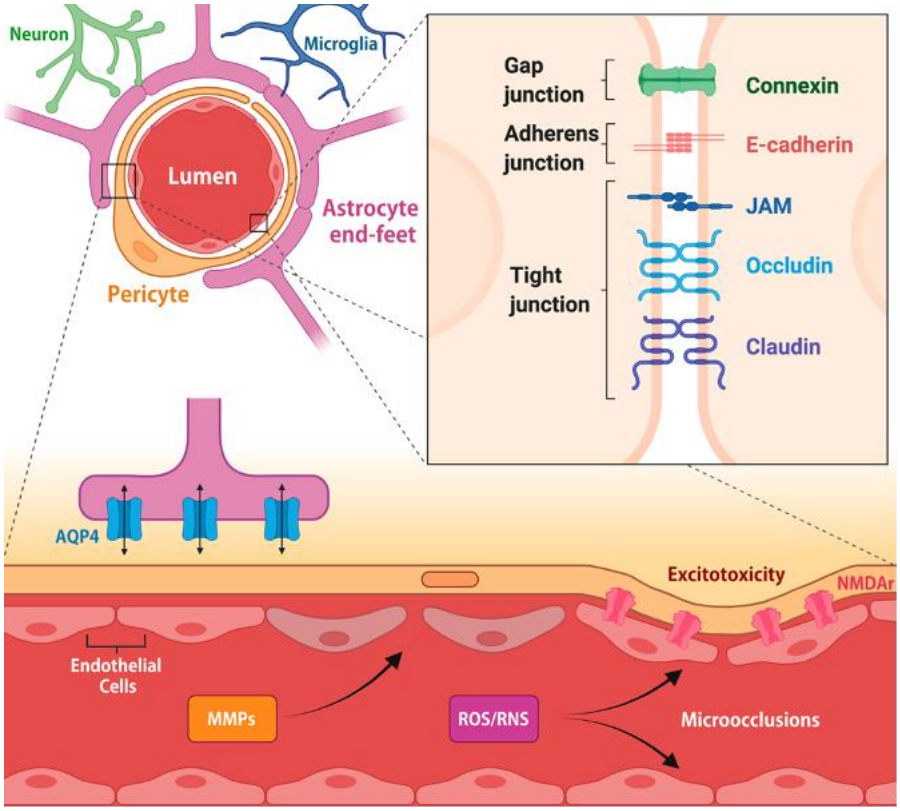


Figure 7. Neurovascular unit, junctional proteins and endothelial dysfunction. Self-created image using elements with BioRender software.

1.4.2.3. Transcriptional Response

Proper oxygen delivery is of utmost importance to guarantee the survival of many animal species including humans. In this context, hypoxia-inducible factors (HIFs) represent the main regulators of low oxygen conditions, especially the HIF-1 isoform. HIF-1 is composed of two protein

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

subunits, α and β: while HIF-1 β is constitutively expressed, HIF-1 α is particularly unstable in the presence of oxygen, promoting a continuous cycle of being synthesis, ubiquitination and degradation in the proteasome[67]. However, the hypoxic conditions derived from cerebral ischemia allow for the stabilization and translocation of the α subunit to the nucleus, undergoing dimerization with HIF-1 β and binding to hypoxia responsive elements (HREs) expressed by target genes that regulate cell proliferation (insulin-like growth factor 2), energy metabolism (GLUT1) and angiogenesis (VEGF)[68]. Nonetheless, its activation can also be detrimental and contribute to ischemic damage, as HIF-1 activation has been shown to stabilize p53, a pro-apoptotic factor, and to upregulate the BCL2 Interacting Protein 3 (BNIP3), which mediates membrane permeabilization and cytochrome C release via mitochondrial permeability transition pores (mPTP), resulting in mitochondrial dysfunction[69].

Besides the aforementioned factors, other transcriptional regulators activated under hypoxia include nuclear factor kappa β (NF-kβ), CREB or the Signal Transducer and Activator of Transcription 3 (STAT3). These factors participate in hypoxia adaptation either by directly regulating HIF-1 α or via independent mechanisms such as p53 inhibition, VEGF induction or SOD upregulation[70,71].

1.4.2.4. Cell death mechanisms in ischemic stroke

In eukaryotic organisms, excessive ROS and intracellular Ca^{2+} are usually regulated and controlled by mitochondria under physiological conditions. Nonetheless, the deranged metabolism and disturbed energetic balance following stroke leads to cytosolic Ca^{2+} accumulation, membrane

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

depolarization and excitotoxic cell death. As damage builds up, the accumulated edema causes rupturing of the cellular membranes and degradation of the nuclear content. This dead-end road is known as necrosis, and is particularly characterized by morphology changes such loss of membrane integrity and cytosolic vacuolation[72].

Depending on the intensity of the ischemic injury, cells might be able to overcome the demise and survive the energetic failure, leading to a form of programmed cell death known as apoptosis. This process comprises two major pathways, the intrinsic (mitochondrial) and extrinsic (receptor) pathway, mediated by death signaling to the mitochondria or plasma membrane, respectively. Irrespective of their origin, both pathways can be mediated with or without the presence of caspases[39].

The intrinsic pathway is primarily dependent on the expression levels of B-cell lymphoma 2 (Bcl-2) family proteins, which can be either anti (i.e., Bcl-2, Bid) or pro (i.e., Bak, Bax, tBid) apoptotic. During ischemia, Ca^{2+} accumulation promotes the translocation and cleave of proapoptotic members of the Bcl-2 family, which force the opening of mPTP on the dysfunctional mitochondria, with the subsequent release of cytochrome C. In the presence of dATP, cytochrome C binds to the apoptotic protease activating factor 1 (Apaf-1) and procaspase-9 to form an apoptosome. This triggers a downstream of proteolytic enzymes that activate caspase-9 and the subsequent caspase-3 and -7, producing nuclear damage and DNA fragmentation. Additionally, pore formation allows for the release and translocation of other apoptogenic factors such as the apoptosis-inducing factor (AIF) or the direct inhibitor of apoptosis-binding protein with low pI (Diablo), which produce DNA

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

fragmentation and inhibit anti-apoptotic mechanisms, respectively, in caspase-independent processes[39,73].

The extrinsic pathway is driven by ligand binding to death receptors on the plasmatic membrane of targeted cells. Upon activation, death receptors of the TNF receptor superfamily such as the TNF-related apoptosis-inducing ligand (TRAIL) or CD95 (APO-1/Fas) promote the formation of the death inducing signaling complex (DISC), activating both caspase-8 and -10. Caspase 8 ultimately leads to activation of caspase-3 and -7, producing DNA condensation, DNA fragmentation and, eventually, cell death. Interestingly, activation of caspase-8 results in the cleavage and mitochondrial translocation of Bid, contributing to the intrinsic apoptotic pathway[39] **(Figure 8).**

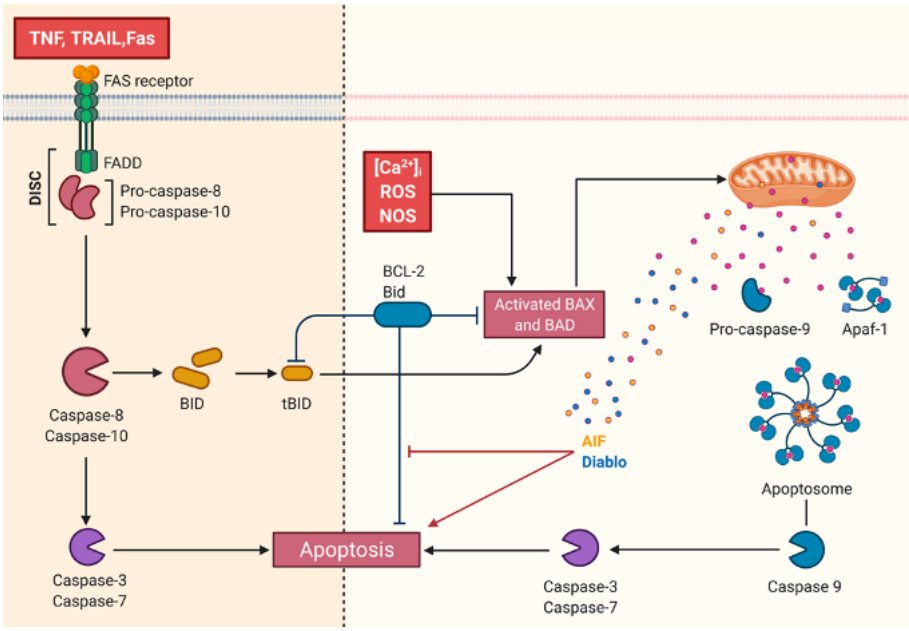


Figure 8. Intrinsic and extrinsic pathways in apoptosis cell death. Self-created image using elements with BioRender software.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Overall, cells undergo either necrotic or apoptotic mechanisms based on the severity of the insult. However, necrotic cell deaths can also undergo apoptotic mechanisms under certain conditions, a phenomenon known as necroptosis. Necroptotic cells characteristically present both necrotic (lysosomal rupture) and apoptotic (caspase-3 cleavage and cytochrome C release) features, and rely on the activation of the receptor-interacting protein kinase-1 and 3 (RIPK1/3) and its substrate mixed lineage kinase domain like pseudokinase (MLKL), along with the inhibition of caspase-8. In this scenario, RIPK1/3 autophosphorilate and form a complex known as the necrosome, which then phosphorylates and translocates MLKL to the plasmatic membrane. MLKL induces pore formation, leading to extravasation of intracellular content and release of danger signals that ultimately lead to cell death[74].

1.4.2.5. Early Inflammation and Innate Immunity

Although certain features of inflammation may have a beneficial effect during later stages of ischemic stroke, inflammation is rapidly initiated after the halt in blood circulation, generating shear stress, hypoxia and oxidative damage to the endothelium, causing cell death. Injured and damaged cells release damage-associated molecular patterns (DAMPs), which marks the initiation of the inflammatory cascade. DAMPs include purines (ATP, UTP), high mobility group box protein 1 (HMGB1), heat-shock proteins (HSPs) and β-amyloid (Aβ), among others. Upon release, these mediators bind to pattern recognition receptors such as the TLRs, especially TLR2 and 4 on brain resident immune cells such as microglia or astrocytes[75].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Microglia are hematopoietic brain-resident cells and the first responders to acute ischemic damage within the CNS. Under homeostatic conditions, resting microglia continuously extend and retract their processes in order to survey the parenchyma. Damage from ischemia induces the activation of both macrophages and microglia, which undergo morphological changes and adapt a pro-inflammatory profile known as M1 (also known as classical pathway). M1 macrophages and microglia exacerbate ischemic damage by releasing TNF-α, ROS, RNS and pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-23. During later stages, the anti-inflammatory cues promote the M2 phenotype (alternative pathway, anti-inflammatory), which counteracts inflammation by releasing IL-10 and growth factors such as the transforming growth factor β (TGF-β)[76].

Apart from microglia, astrocytes also participate in brain surveillance and represent the most abundant cells within the parenchyma. After ischemia, these cells proliferate and undergo morphological modifications that result in reactive gliosis (astrogliosis) and an increase in the glial fibrillary acidic protein (GFAP). Reactive astrocytes contribute to ischemic damage by modulating the release of proinflammatory cytokines, secreting iNOS and enhancing leukocyte recruitment by chemotactic signaling. Moreover, activated astrocytes also express pro-apoptotic mediators such as the tumor necrosis factor-like weak inducer of apoptosis (TWEAK), which promote apoptotic cell death and release of pro-inflammatory cytokines[77]. Similar to microglia, astrocytes can also engage in anti-inflammatory mechanisms during later stages of stroke upon proper stimulation.

Following microglial and astrocyte activation, blood-borne immune cells are directed to the endothelium and guided into

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

the brain parenchyma. Endothelial cells first respond to the inflammatory input by the deployment of selectins, a family of three different adhesion molecules specialized in platelet (P-selectin), endothelial (P- and E-selectins) and vascular leukocytic (L-selectin) interactions. Ischemic injury triggers the release of P-selectin from granule storages within the endothelium (Weibel-Palade bodies) and platelets (α-granules). On the contrary, E-selectin is not stored, and its release is dependent on P-selectin or cytokine activation[78]. E and P-selectin loosely bind to glycoproteins expressed on the surface of circulating leukocytes, slowing them down and promoting cluster formation on the endothelial surface. Moreover, L-selectin on the leukocytic surface drives the recruitment to the site of injury[79].

This platform is reinforced by the interplay of integrins, membrane heterodimeric glycoproteins expressed by leukocytes. Integrins activation is biphasic, starting with a conformational change induced by molecular messengers (inside-out signaling) followed by their interaction and binding (outside-in signaling) to their specific endothelial ligands such as VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intercellular adhesion molecule-1), PECAM-1 (platelet endothelial cell adhesion molecule) and ALCAM (activated leukocyte cell adhesion molecule). Integrins further reinforce the leukocyte cluster by preventing their detachment due to changes in blood flow, facilitating the diapedesis and transmigration of leukocytes through the vessel wall and into the injured brain[80].

Among the infiltrated leukocytes, neutrophils activate within minutes after cerebral ischemia and contribute to early endothelial damage by releasing inflammatory mediators such as proteinases (i.e., MMP-8 and MMP-9), chemokines (CCL2-4), pro-inflammatory cytokines (IL-6, IL-8, TNF-α) and iNOS.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Neutrophil activation also induces intravascular clogging by limiting tissue perfusion and enhancing leukocyte recruitment, creating a positive feedback loop[81].

Other innate immune populations include mast cells, blood monocytes and macrophages. While mast cells contribute to BBB disruption and edema by releasing peptidases and vasoactive molecules, monocytes and macrophages play a more convoluted role. Upon infiltration, they mature into microglia and macrophage-like cells which can both acquire a pro- or anti-inflammatory phenotype based on the condition of the surrounding medium. A subset of these cells matures into dendritic cells, which play a key role in the transition from innate to the latter adaptive immunity[75].

Beyond the role of immune cells in the initiation of brain inflammation, other pathways such as the coagulation cascade and the complement system also have deleterious consequences for the parenchyma. The coagulation cascade refers to the successive release of coagulation factors after an injury. These factors promote clot formation *in situ* with the aim of protecting the damaged tissue while the reparative mechanisms take place. During stroke, these coagulation factors promote the upregulation of adhesion molecules through NF-kβ and directly activate the complement system (i.e., C3 and C5 components), leading to loss of BBB integrity and enhanced inflammation[82]. Finally, the complement system represents the humoral branch of innate immunity, and comprises three different activation pathways known as the classical, alternative and lectin pathways. The initiation of any of these pathways results in degranulation and increased ROS and proinflammatory cytokines production[83] (**Figure 9**).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

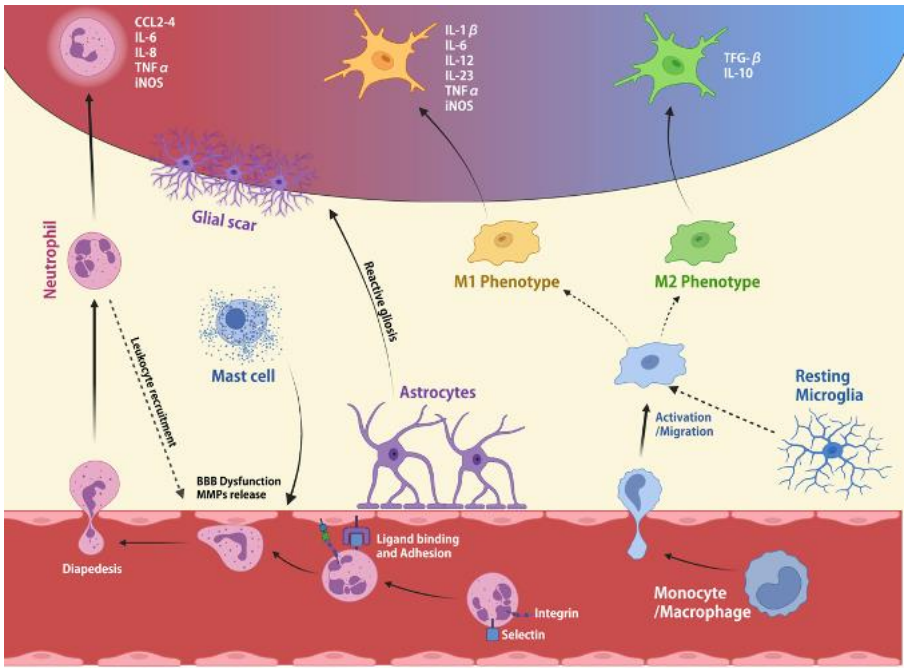


Figure 9. Early inflammation and innate immune response following an ischemic stroke. Self-created image using elements with Creative Common license.

1.4.3. Subacute Phase:

1.4.3.1. Adaptive immunity:

While innate immunity is immediately triggered after blood flow interruption, adaptive immunity comprises slower response mechanisms that take place days after injury. Injured cells locally release antigenic mediators that diffuse through the dysfunctional BBB and reach the systemic circulation, initiating adaptive mechanisms through two major routes known as the cellular and the humoral pathways. Both pathways usually involve antigen presentation, where peptides originated from protein degradation interact with major histocompatibility complex (MHC) molecules. While MHC class I molecules are found in all nucleated body cells, MHC class II

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

proteins are restricted to macrophages, dendritic cells and B lymphocytes[84].

Cellular immunity is mainly mediated by T-lymphocytes (or T cells). These cells can either express CD8 (CD8+) or CD4 (CD4+) glycoproteins on their surface, which will determine their subsequent role in the response. CD8+ cells are the first adaptive immune cells to migrate to the ischemic region and can be seen within the first hours following stroke[82]. After binding to MHC class I-antigen complexes, they exert cytotoxic effects by inducing perforin-mediated membrane permeabilization, apoptosis via Fas/caspase pathways and releasing pro-inflammatory mediators including TNF, IL-2 and IFNγ. In contrast, CD4+ cells turn into helper T cells (Th), a moniker that alludes to their supportive role and lack of cytotoxic function in the immune response. Th cells can be divided into effector or regulatory T cells (Treg). Effector cells develop different inflammatory profiles depending on the incoming stimuli. IL-12 secretion induces Th type 1 effector cells (Th1) differentiation, which promote cytotoxicity by secreting TNF and IFNγ. Th type 2 effector cells (Th2) turnover is initiated by the presence of IL-4. These cells secrete anti-inflammatory cytokines including IL-4, IL-5, IL-10, IL-13, promoting humoral and mucosal immunity and participating in neuroprotective mechanisms[85].

Besides these subtypes, natural killer (NK) and natural killer T (NKT) cells are also included in this group. While both modulate inflammation via cytokine production, NK cells do not require antigen presentation. Treg development is mediated by TGF-β, which in turn inhibits Th1 and Th2 response. These cells partake in neuroprotective mechanisms that include IL-16 release, attenuation of NF-kB-related mechanisms and suppression of neutrophil MMP-9 release[82]. Moreover, Treg-

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

induced immunosuppression via IL-10 and TGF-β release plays a pivotal role in compensatory mechanisms against the exacerbated inflammatory state after stroke. γδT cells are a subset of effector lymphocytes capable of recognizing non-peptide antigens that participate both in neuroprotective (growth factors secretion) and damaging (cytolysis, IL-17 secretion) mechanisms in cerebral ischemia depending on the molecular stimulants present in their milieu[84].

Humoral immunity is mainly mediated by B cells via antibody production. These cells can influence the outcome of cerebral ischemia by limiting the production of pro-inflammatory cytokines (TNF and IFNγ), restricting lymphocyte infiltration and reducing infarct volume. Moreover, B-cells could also counterbalance inflammation by enhancing Treg activity and promoting phagocytosis of cellular debris in the ischemic injury[85].

1.4.4. Delayed Phase

After several weeks, the acute and subacute injury events fade in favor of regenerative and repair signaling mechanisms. This phase is characterized by the chronification of the lesion scar and the initiation of tissue repair processes.

1.4.4.1. Lesion scar

As a response to ischemic stroke, activated astrocytes rapidly proliferate and migrate towards the damaged tissue. This is accompanied by an upregulation in GFAP, Ca^{2+}-binding protein B (S100B), nestin and vimentin[86–88]. Similar to the reactive phenotyping of microglia, activated astrocytes can be further classified into A1 and A2 subtypes. A1 astrocytes are neuroinflammatory and upregulate genes that are detrimental to

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

synapse formation, while ischemia-induced A2 reactive astrocytes promote neuronal growth and synapse repair by upregulating neurotrophic factors and thrombospondins[89].

Reactive astrocytes align and intertwine with activated microglia and macrophages to form a defense line that secludes the injured tissue and restricts its expansion, resulting in a glial scar. As the lesion progresses over time, microglia tend to be superseded by fibroblasts arising from the injured vessels and meninges. These cells act by secreting large amounts of extracellular matrix (ECM) proteins such as fibronectin, laminin and chondroitin sulphate proteoglycans, forming the fibrotic scar. This barrier not only allows for the compartmentalization of the injury, but provides a dynamic scaffold for development and adult neurogenesis[90,91]. Moreover, scar formation stimulates revascularization, maintains ion and fluid homeostasis and contributes to free radical scavenging. Nonetheless, this very barrier has also been suggested to impede axonal regeneration by the release of several growth-inhibitory molecules from reactive astrocytes, affecting brain plasticity and compromising functional outcome[92].

1.4.4.2. Tissue reparation

Once damaging and pro-inflammatory mechanisms begin to subside, the steady reparation mechanisms start to act. These processes are mainly comprised in angiogenesis, neurogenesis and neuroplasticity.

- **Angiogenesis:** Within a few days after ischemic stroke, angiogenic signals are released promoting the neovascularization of the injured region. Angiogenesis involves endothelial cell proliferation and migration, tubule

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

of formation, branching and anastomosis of the new vessels. This process is initiated by the secretion of angiopoietin 1 and 2 from endothelial and perivascular cells, which promotes pericyte detachment and endothelial cells proliferation. This process is tightly regulated by grow factors such as VEGF or fibroblast growth factor (FGF) that promote vascular ramification and make the newly formed vessels highly permeable. Additionally, vascular development is accompanied by extracellular matrix remodeling via MMPs release[93]. Although angiogenesis is generally considered a neuroprotective mechanism, the characteristic permeability of the *de novo* formed vessels and protease activity can lead to increased secondary damage after ischemia by increasing BBB breakdown, vascular leakage and brain edema, which highlights the necessity of an exhaustive regulation of repair mechanisms after stroke[94].

- **Neurogenesis:** Although limited, the adult brain exhibits ongoing neurogenesis mechanisms in two focal regions: the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ) of the hippocampus. Under physiological conditions, neuroblasts from the SVZ and SGZ migrate to the olfactory bulb and hippocampus and differentiate into GABAergic interneurons and glutamatergic granular neurons, respectively[95,96]. Nonetheless, cerebral ischemia stimulates neurogenesis and redirect neuroblast migration to the infarcted region[97]. However, whether this response makes a significant contribution to brain repair is still discussed, as new-born neuroblasts have been shown to die prematurely upon differentiation. Moreover, the ability of the surviving

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

differentiated neuroblasts to functionally integrate in neural circuits is still a vexed debate. Both neurogenesis and the aforementioned angiogenesis are not isolated events but interdependently regulated phenomena in post-stroke recovery[98]. Neuroblast tropism is driven through perivascular routes that provide the tissue with the much-required oxygen and nutrients. Vascular growth stimulates neurogenesis through metabolic mediators and growth factors such as stromal cell-derived factor-1 (SDF-1), VEGF and MMPs, facilitating progenitor cells migration to the ischemic region. Simultaneously, incoming progenitor cells release of angiopoietin-2 and VEGFR2 enhance angiogenesis, resulting in a positive feedback loop[99].

- **Neuroplasticity:** Neuroplasticity refers to the ability of the nervous system to respond to extrinsic and intrinsic stimuli by modifying its anatomy, function and neural connections[100]. Stroke causes a sudden interruption of neuronal connections that lead to impaired functions known as diaschisis, particularly caused by glutamate excitotoxicity. These changes are experience and learning dependent, and comprise rewiring of brain circuitry, activation of parallel pathways by modulating GABAergic inhibition and training-induced synaptogenesis. In this regard, cognitive and physical neurorehabilitation have been shown to improve dendrite and axonal function by upregulating BDNF production and to facilitate synaptic plasticity by modulating AMPA/NMDA glutamate response[99]. Moreover, physical exercise promotes interhemispheric connectivity from the ipsilateral

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

hemisphere to the contralateral M1 motor region and enhances motor map reorganization[101].

1.4.5. Therapeutic opportunities in ischemic stroke

Different strategies were studied to reduce the ischemic lesion in the different phases of injury evolution. These strategies are classified in neuroprotective and reparative therapies.

1.4.5.1. Neuroprotective strategies

The events that develop during the earliest period following an ischemic stroke greatly determine the progression and proportions of the ischemic injury. Owing to this fact, most therapeutic approaches have been focused on the hyperacute and acute phases of cerebral ischemia to prevent the unraveling of irreversible damaging mechanisms. In spite of the encouraging preclinical results, neuroprotection is still a hard-reaching goal due to a daunting failure in translationality from animals to the daily clinical practice. Nonetheless, the fast-developing advances in drug development and the improved understanding of the underlying mechanisms in multiple therapeutic targets makes neuroprotection an encouraging line of research in stroke.

- **Ca^{2+} blockers and antagonists:** Intracellular Ca^{2+} overload plays a major role in stroke pathogenesis by contributing to ionic imbalance and excitotoxicity. Many therapeutic strategies have been directed towards stopping the inward Ca^{2+} currents, especially through the blockage of Ca^{2+}-gated channels. **Nimodipine** is a well-researched blocker of L-type voltage-gated Ca^{2+} channels that reduces Ca^{2+} influx and exerts potent vasodilator properties. Nonetheless, although

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

the vasoactive effects of this drug have proven useful in the prevention of hemorrhagic vasospasm, nimodipine administration has not shown benefits in the treatment of cerebral ischemia. In fact, a recent meta-analysis of 7731 patients and 34 trials using Ca^{2+} antagonists showed that nimodipine worsened the outcome of ischemic stroke patients at higher doses[102,103]. **Verapamil** is another L-type Ca^{2+} channel blocker with similar vasoactive properties that has shown promising results in combined therapy approaches (i.e., thrombectomy), although the limited sample size of the studies demands further research [104]. Currently, there is an ongoing phase I study in recruitment phase for the validation of this drug as a neuroprotective agent for stroke patients (NCT03347786). Alternative approaches against Ca^{2+} dysregulation include Mg^{2+} (Ca^{2+} binding competitor and NMDA blocker) and acid-sensing ion channel (ASIC) blockers, but none of them have demonstrated any benefits in clinical trials[105,106].

- **Excitability modulators:** Glutamate is the major excitatory neurotransmitter of the CNS and the main driver of ischemic excitotoxicity. As glutamate exerts its damaging mechanisms by binding to its receptors, most therapeutic strategies targeting excitotoxicity have been focused on NMDA and AMPA receptors[58]. NMDA antagonists such as **selfotel**, **dextromethorphan** and **gavestinel** showed promising results in animal models of focal cerebral ischemia but failed to show positive outcomes in clinical trials[107]. Similarly, AMPA antagonists such as **ZK200755** and **YM872/zonampanel** have shown good preclinical results but poor clinical evidence[108]. Moreover, beyond glutamate

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

antagonism lies the concept of glutamate grabbing, consisting of a reduction in peripheral blood glutamate to force its diffusion from the brain through the BBB and into the systemic circulation[109]. GABA agonists have also been evaluated in the setting of ischemic stroke due to its inhibitory action, counteracting glutamate´s excitatory activity. Among them, **diazepam** and **clomethiazole** are the most extensively researched agonists. While both showed reduced infarct volume in rodents and primates none of them were able to achieve significant results in randomize clinical trials. A recent meta-analysis of 2838 patients from 5 different clinical trials did not find any differences in the outcome of patients treated with GABA agonists[110].

- **Glutamate grabbers:** one of the key features of ischemic excitotoxicity is the overwhelming and uncontrolled release of glutamate into the brain parenchyma. The concept of glutamate grabbing (or scavenging) emerged as a solution for this phenomenon, and it is based on the reduction of blood glutamate levels by the administration of the glutamate oxaloacetate transaminase 1 (**GOT1**), which catalyzes the conversion of oxaloacetate and glutamate to aspartate and α-ketoglutarate. This reduction in blood glutamate in turn promotes the diffusion of brain glutamate into the bloodstream, thus ameliorating its excitotoxic effects during ischemia[109]. In this regard, our group demonstrated that a combined administration of oxaloacetate and recombinant GOT1 effectively reduced blood and parenchymal glutamate levels, decreased infarct volume and promoted functional recovery in a rat model of cerebral ischemia[111]. In patients, we found that a higher GOT1 activity

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

and lower glutamate levels were associated with a better outcome at 3 months, which marked the first clinical evidence of the neuroprotective potential of this strategy[112]. Based on these findings, we performed a 3-step translational work that involved an *in vitro* drug-repositioning study, an *in vivo* study of experimental cerebral ischemia and a randomized, double-blind Phase IIb clinical trial. Our work demonstrated that the administration of **riboflavin** (vitamin B_2), a novel glutamate grabber, induced a marked decreased in blood glutamate levels and reduced injury volume in rats. In patients, we demonstrated the safety and feasibility of **riboflavin** administration in stroke patients, where those patients who received riboflavin showed reduced levels of blood glutamate and a trend for functional recovery after stroke. Altogether, our results show that glutamate scavenging is a promising and safe approach for the treatment of acute ischemic stroke patients.

- **Anesthetics and anticonvulsants:** used in the daily clinical practice, drugs such as inhaled gases (sevo/isoflurane), **ketamine** or **pentobarbital** have also been studied in preclinical models with positive results[102,113]. However, these drugs have failed to show additional improvement in the outcome of patients upon administration, possibly owing to the associated side-effects derived from anesthesia induction and maintenance in already compromised patients.

- **Antioxidants:** several antioxidant agents have been tested in ischemic stroke aimed at reducing secondary damage. The antioxidant **NXY-059** (disufentom sodium) showed a dramatic reduction in infarct volume both in rodents and

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

primates, but failed to prove effective in the SAINT I and II clinical studies[55]. Overall, this drug showed low solubility, low BBB penetrance and a lower antioxidant capacity compared to other compounds such as vitamin E. **Tirilazad mesylate** and **ebselen** are inhibitors of lipid peroxidation with free radical scavenging activity that have shown limited to no clinical evidence in the recent years[114]. Finally, **citicoline**, a precursor in the phosphatidylcholine synthesis, was tested due to its antioxidant and phospholipid synthesis stimulatory activity. Although preclinical studies showed promising results, larger clinical trials failed to reproduce these outcomes[108].

- **Anti-inflammatory drugs:** Early and delayed inflammation are also appealing processes susceptible to be modulated. These processes provide a larger therapeutic window, increasing their usability. The goal in anti-inflammatory or immunomodulatory therapies is to restrict lymphocyte infiltration and promote anti-inflammatory phenotypes in the glia.

  Minocycline is a second-generation, semi-synthetic tetracycline with over 30 years of clinical use. This gram-positive and gram-negative bacteria antagonist has also been shown to dampen the neuroinflammatory cascade by inhibiting microglial activation. Although a small number of preclinical studies showed improved post-stroke outcomes, recent clinical trials showed no differences with the placebo group[115].

  IL-1 is a pro-inflammatory cytokine that induces matrix degradation and promotes pro-apoptotic mechanisms. A preclinical cross-laboratory stroke trial designed to

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

investigate the efficacy of IL-1 receptor antagonist in different research laboratories across Europe showed promising results in preclinical animal models. Conversely, a subsequent randomized phase 2 clinical trial showed an enhanced reduction in plasma inflammatory markers of ischemic stroke patients, but failed to demonstrate an improvement in the outcome of patients[116]. Therapies targeting lymphocyte infiltration of activated lymphocytes have also been tested. **Natalizumab**, a monoclonal antibody against α4-β1 integrin (CD49a) used in the treatment of multiple sclerosis, was tested in a phase II clinical trial but failed to demonstrate its effectiveness in stroke patients. Moreover, natalizumab proved effective in a prospective multicenter animal study in a permanent distal MCA occlusion model, but not in a temporary proximal occlusion model[108,117].

Fingolimod is a sphingosine-1-phosphate receptor modulator that regulates lymphocyte growing, differentiation and migration[118]. A recent open-label trial demonstrated the safety of fingolimod for its administration within 72h of the onset, showing decreased microvascular permeability and improved outcome. Nonetheless, further clinical trials are necessary to validate these results[119].

Other compounds aimed at reducing leukocyte adhesion and infiltration by specifically blocking CD11b or ICAM-1 showed promising results in preclinical models but lacked compelling clinical evidence[120].

Targeting the complement system has recently gained attention as an attractive therapeutic target due to emerging data supporting a pivotal role in neuroinflammation and neurodegeneration. **Pexelizumab** is a single-chain anti-C5

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

monoclonal antibody that blocks C5 cleavage and the generation of C5a and the membrane attack complex. A 2003 phase II clinical trial with the objective of using this drug to reduce complement-mediated tissue damage and inflammation showed no significant improvement in cognition or neurological deficits. Recently, a phase III clinical trial was planned to assess the efficacy and safety profile of pexelizumab (NCT00048308), but although the study has already been completed no further details have been updated[115].

1.4.5.2. Reparative therapies

The target of these therapies is the ischemic tissue that has already been damaged. Traditionally based on the subacute or delayed phase, an increasing number of studies has suggested the benefits of an earlier application of reparative therapies during the acute phase.

In this group of therapies are englobed pharmacological approaches and neurotrophic factors aiming to stimulate endogenous neurogenesis, angiogenesis and synaptic plasticity, and cell-based therapies.

- **Small drugs:** multiple small drugs have been tested for the improvement of stroke outcome, particularly due to its reduced sized, increased BBB diffusion and facilitated access to the injured region[121]. Among them, monoaminergic drugs have been the most extensively studied compounds as of today. Amphetamine administration showed promising preliminary results but failed to demonstrate an enhanced benefit in the STARS study, a randomized, double-blind, placebo-controlled clinical trial[122,123]. Administration of

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

levodopa, a booster of dopaminergic neurotransmission, improved the outcome and primary endpoints when combined with physical therapy, although the study is still pending replication[124]. Nonetheless, smaller studies which tested alternative dopamine agonists did not yield positive results[125]. Serotonin is another monoaminergic neurotransmitter involved in neural repair memory consolidation. In this regard, selective serotonin reuptake inhibitors (SSRIs) have shown promising results both in preclinical animal models and randomized clinical trials, decreasing post-stroke depression and improving long-term motor and cognitive functions[126,127]. Modulatory drugs of norepinephrine and acetylcholine neurotransmission have also been researched. Norepinephrine transmission is critical for neuronal activity amplification and executive function, while cholinergic pathways modulate procedural memory and operant conditioning. Compelling evidence from preclinical results shows the potential of these therapies, although results derived from human studies are sparse and require further replication[128]. Regarding glutamate and GABA modulators, the repurposed drug sildenafil, a phosphodiesterase 5 (PDE 5) inhibitor initially used in erectile dysfunction, improved the outcome in stroke animal models and enhanced angiogenic and neurogenic mechanisms. This compound has also shown favorable results as a restorative post-stroke agent in human studies, but further research is still necessary to validate its efficacy[129,130].

- **Monoclonal Antibodies:** Owing to the highly specific binding to targeted receptors, monoclonal antibody therapy

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

has been extensively used in patient care in several diseases. In stroke, the focus of this therapy has been placed on the neutralization of axonal growth inhibitors, particularly in myelin-associated glycoprotein (MAG), oligo-myelin glycoprotein and Nogo-A, whose expression is exacerbated after ischemia and directly interferes with neurite outgrowth. The safety of these compounds has been successfully tested, but the results concerning is therapeutic applicability are yet to be conducted[131].

- **Growth factors**: Growth factors are of particular interest due to their natural capacity to promote angiogenesis, synaptic plasticity and cell survival. Granulocyte colony-stimulating factor (G-CSF), erythropoietin, human chorionic gonadotropin (hCG) or VEGF, among others, have been tested both in the preclinical and clinical setting. Although G-CSF demonstrated immunomodulatory and anti-apoptotic effects on preclinical studies, a subsequent multi-center study and a recent meta-analysis did not find favorable effects compared with the placebo in ischemic stroke patients[132–134]. Erythropoietin systemic administration has proven effective in preclinical animal models of acute ischemic stroke[135]. In human studies, the safety of erythropoietin administration alone or in combination with hCG was assessed in the BETAS safety trial[136]. Nonetheless, the recent REGENESIS study (a randomized, placebo-controlled, double-blind study) found increased mortality in the treatment arm due to intracerebral hemorrhage complications, and thus was put on hold[137]. Similarly, observational studies found an association between VEGF circulating levels and microbleeding[138].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

- **Cell-based therapies:** Therapies focused on cellular approaches have drawn increased attention due to its immunomodulatory and growth promoting effects, and their ability to influence stroke repair through multiple pathways as opposed to pharmacological approaches that act on a single mechanism. Multiple cell lines have been examined, including modified tumor cells, embryonic stem cells, umbilical cord and placenta cells and bone marrow stromal cells, among others[139]. In particular, mesenchymal stromal cells (MSCs) and marron-derived mononuclear cells (MNCs) are the most studied approaches. A meta-analysis of 46 preclinical studies concerning MSCs application provided robust results, showing improved outcomes in 44 of 46 studies with a consistent effect size[140]. Regarding human studies, a recent systematic review and meta-analysis of 9 randomized clinical trials and seven non-randomized clinical trials showed that stem cell-based therapies improved the outcome of patients. Nonetheless, fever, headache and increased stroke recurrence was reported as frequent cell therapy side-effects, and the included trials were still at an early development stage, which highlights the necessity of further studies to verify the efficacy of this therapeutic approach[141].

1.4.5.3. Multifactorial therapies

One of the main limitations of most therapeutic strategies is that they are designed to target a single mechanism irrespective of their effect on secondary pathways. Nonetheless, multifactorial therapies have been shown to exert neuroprotective effects by targeting several mechanisms at once.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Melatonin is a hormone mainly secreted by the pineal gland that actively participates in circadian rhythm regulation. Several preclinical studies have shown potent antioxidant activity by this hormone, as melatonin can scavenge peroxynitrite anions and hydroxyl radicals. It has also been shown to participate in Ca^{2+} and NO regulation, and to promote anti-apoptotic and pro-survival signaling pathways. In clinical trials, multiple sclerosis patients who received melatonin showed a marked reduction in pro-inflammatory cytokines including TNF, IL-6 and IL-10. Regarding cerebral ischemia, there are currently several registered clinical trials aimed at validating the safety and therapeutic potential of melatonin in ischemic stroke patients[142].

1.4.5.4. Brain stimulation and physical therapy

The brain is the most energetically demanding organ in the human organism, accounting for over 20% of total oxygen and glucose metabolism[38]. In this regard, several therapies aimed at improving electrical brain function and promote neural circuitry repair have been tested. These strategies comprise repetitive transcranial magnetic stimulation (TMS), theta burst stimulation, transcranial alternating current stimulation or vagal nerve stimulation, which are therapies that have yielded both favorable and poor outcome results.

Physical therapy is oriented to the improvement of motor and integrated cognitive functions after an ischemic stroke. Exercise-derived effects include enhanced dendritic arborization, increased proBDNF maturation, increased synaptic plasticity and improved interhemispheric connectivity[101]. In this regard, constraint-induced movement therapy (which promotes the use of the affected limb while restricting the non-affected one) has shown positive results in several clinical trials[143–145]. Key

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

findings from these studies found that activity-based therapies may require hyperacute interventions and high-intensity training regimens for the treatment to be significantly effective. Combined strategies using brain stimulation and physical therapy could further enhance the outcome of patients compared with standalone approaches.

1.5. Clinical management of ischemic stroke

1.5.1. The concept of ischemic penumbra

The sudden blockage of blood flow in any given vascular territory rapidly starts a myriad of pathophysiological events particular to stroke such as altered ion homeostasis, excitototoxicity, inflammation and cell death. The center of the vascular occlusion is known as the core, which is clinically defined as the area with a regional blood flow <20%. The ischemic core is particularly compromised by the extreme energy demands and uncontrolled cellular death, which renders the salvageability of this region virtually impossible. Beyond the core lies the ischemic penumbra, regions where the restriction in blood flow is not sufficient to derail energy metabolism and thus neurons, although metabolically stressed, remain viable for a longer period of time. Finally, the areas beyond the penumbral tissue are known as oligemic regions, characterized by their normal functioning in spite of the reduced blood supply[85]. Although salvageable, penumbral neurons will still perceive damage and undergo cell death if blood flow is not sufficiently reestablished, which is why most of the current therapeutic approaches are focused on the potential recovery of this area (**Figure 10**).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

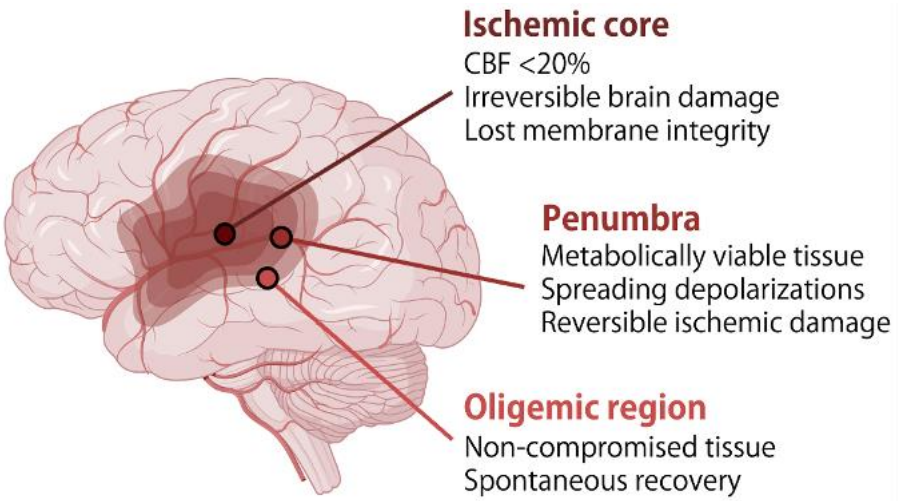


Figure 10. Tissue viability in ischemic stroke. The occlusion of a vessel leads to decreased perfusion and ischemia in the affected tissue. This damage is graded, with a near-complete damage in the core (central to the occlusion) and a milder effect in the penumbra or surrounding (oligemic) regions. Although penumbral tissue is potentially salvageable, its outcome is dependent on the amount of time elapsed until cortical blood flow (CBF) is restored. Self-created image using elements with BioRender software.

1.5.2. CBF reestablishment

As ischemic stroke is mechanistically produced by a reduction in blood supply to the brain, the primary goal during clinical management is the removal of the occluding artifact. As previously mentioned, stroke is a dynamic disease in which time equals salvageable brain tissue, which highlights the importance of a rapid intervention to ameliorate the damage. Currently, there are two available strategies to achieve said reperfusion: intravascular thrombolysis and mechanical recanalization (**Figure 11**).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

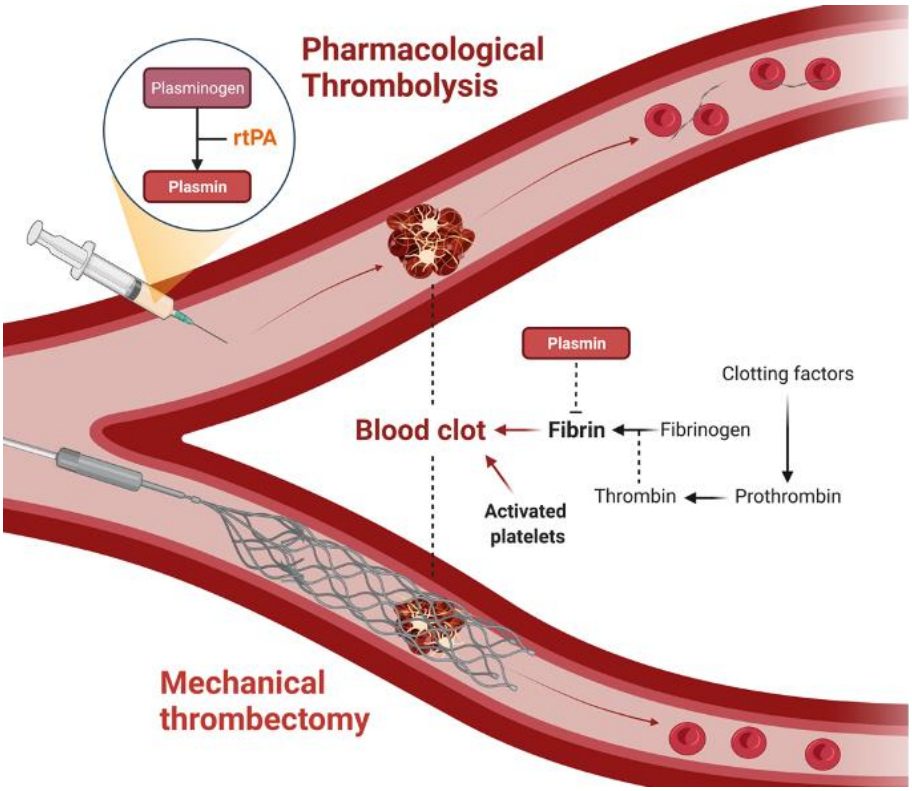


Figure 11. Schematic representation of the pharmacological and mechanical recanalization approaches in ischemic stroke. Self-created image using elements with BioRender software.

1.5.2.1. Intravascular thrombolysis

Thrombolytic therapy consists in the intravascular administration of agents capable to dissolve the thrombus *in situ.*

- **Intravenous:** Tissue plasminogen activator (tPA) is an endogenous serine protease with thrombolytic activity, and the only Food and Drug Administration (FDA) drug with approval for the treatment of acute ischemic stroke[146,147]. This enzyme exerts its activity through the cleavage of the zymogen plasminogen into plasmin, an

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

active protease. Plasmin then tears down the hydrogen bonds on the fibrin fibers that hold platelets together in the blood clot, resulting in clot dissolution. In 1983, a recombinant version of tPA (rtPA) with enhanced pharmacological properties was designed as a treatment for thrombosis. The drug received approval in 1995 based on clinical evidence that showed that its administration within the first 3 hours after a stroke improved clinical outcome at 3 months[148]. This therapeutic window was later extended up to 4.5 hours, which is nowadays the timepoint reference for its administration in the clinical practice[149,150]. Due to the restrictive eligibility criteria and the narrow therapeutic window, it is estimated that less than 10% of ischemic stroke patients are actually apt for rtPA administration, of which less than 50% achieve complete arterial reperfusion[151].

- **Intraarterial:** This type of administration provides a more direct access to the thrombus, thus reducing dose delivery and systemic circulation of fibrinolytic agents, thus reducing bleeding[149]. Aimed at ameliorating the associated side-effects of thrombolytic therapy such as bleeding or hepatic damage, alternative forms of the tPA have been developed for the treatment of ischemic stroke. The drugs included in this type of therapy include **Urokinase**, **Reteplase** or **Tenecteplase**, which are modified analogs that present a lower risk of hemorrhagic transformation, longer half-life and milder neurotoxic effects. In spite of the benefits of the aforementioned isoforms, evidence from large clinical trials is still scarce

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

thus limiting its potential therapeutic application in the setting of ischemic stroke[13].

1.5.2.2. Mechanical Thrombectomy

The restrictive conditions for thrombolytic drugs administration caused a shift from intraarterial thrombolysis with rtPA to mechanical thrombectomy techniques. The main objective of mechanical thrombectomy is the removal of the clot from the occluded vessel by means of a semi-invasive intravascular catheter (usually introduced via femoral artery).

Patients who benefit from this therapy include rtPA-non responders and those ineligible for thrombolytic therapy. As of today, a total of 4 mechanical devices have received approval from the FDA for the treatment of acute ischemic stroke treatment within 8 hours from the onset with or without rtPA co-administration. First generation devices comprise the mechanical embolus removal in cerebral ischemia (MERCI Retriever; Stryker Neurovascular) and Penumbra Aspiration System (Penumbra Inc.). Second generation devices include the Solitaire Stent Retriever (ev3 Neurovascular) and Trevo Stent Retriever (Stryker Neurovascular)[147]. These devices differ in size and shape, and present higher recanalization rates and a longer therapeutic window than rtPA (initially 8h recently extended up to 24h upon reasonable criteria)[152].

Nonetheless, mechanical thrombectomy comes with its own risks as these devices require anesthesia and proper personnel formation for its application. Moreover, thrombectomy might cause vascular trauma, thrombus fragmentation and distal embolization. For the aforementioned reasons, endovascular thrombectomy is usually restricted to specialized centers[152].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

1.5.3 Factors related to brain ischemia

There are several factors that will modulate the appearance and evolution of damage: blood pressure, aggregation and coagulation state, glycemia, CBF and collateral circulation, temperature.

1.5.3.1 Blood Pressure

Arterial hypertension is a common phenomenon in patients that have suffered an ischemic stroke. While hypertension increases the risk of hemorrhagic transformation, hypotension is associated to poor outcome possibly due to tissue hypoperfusion. Results from clinical studies are sparse, as permissive hypertension is recommended to ensure the correct perfusion of the brain while avoiding vessel-damaging values[153].

1.5.3.2 Antiaggregation and anticoagulation

Platelet antiaggregants and anticoagulation agents are frequently used to help stabilize the CBF, irrespective of the success of the reperfusion. Acetylsalicilic acid is an irreversible inhibitor of cyclooxygenase I (COX1) and the most common antiagreggant in the clinical practice. Inhibition of COX1 reduces the synthesis of prostaglandins and thromboxane, key contributors to platelet aggregation. Additionally, anticoagulation therapy with heparin is frequently considered in order to avoid venous thromboembolisms, common complications in ischemic stroke. Both strategies are started in the acute or subacute phase based on the relative risk of hemorrhagic transformation and recurrent stroke[149,154].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

1.5.3.3. Glycemia

Ischemic sroke patients usually present high glucose levels whether they are diabetic or not. Hyperglycemia is associated with poor outcome by inducing anaerobic metabolism, lactic acidosis and free radical production, leading to endothelial dysfunction and increased infarct[155]. Similarly, fasting or insulin-derived hypoglycemia leads to alternative metabolism pathways, causing structural damage with selective cortical neuron necrosis and preservation of glial cells and increasing mortality rate. Currently, in the clinical practice, glucose-free solutions are given to hyperglycemic patients, while glucose saline is generally avoided in diabetic patients[156]. Moreover, punctual glucose measurements have been falling in disuse in favor of glucose oscillations, as the latter have been shown to possess a higher predictive value[157].

1.5.3.4. CBF and collateral circulation

CBF is critically dependent on two variables, which are the gradient of cerebral perfusion pressure and the vascular resistances[158]. Cerebral perfusion pressure is defined as the difference between the average pressure of the cerebral arteries, intracranial pressure and venous pressure[159]. Under physiological conditions, the autoregulatory mechanisms of brain circulation protect the brain of sudden changes in blood pressure. In this regard, collateral circulation (blood support from adjacent regions) plays a key role in CBF autoregulation. Some of the most notable examples of collateral circulation include the anastomoses between carotid arteries, between the external carotid and vertebral arteries, Heubner´s leptomeningeal anastomoses and the circle of Willis, the cornerstone of intracranial circulation[160]. During cerebral

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

ischemia, these autoregulatory mechanisms disappear and the CPP becomes dependent on blood pressure. The impaired collateral circulation cannot compensate for the reduction in blood pressure or vascular resistance, leading to reduced CBF to the ischemic tissue and increased infarct size[75].

1.5.3.5. Temperature

Hyperthermia or fever (derived or not from an infectious process) have been demonstrated to dramatically worsen the outcome of patients with ischemic stroke during the acute phase[161–167]. Conversely, as most of these damaging mechanisms are temperature-dependent, antipyretic drugs such as **aspirin**, **paracetamol** or **metamizole** are used in the clinical practice as therapeutic strategies for temperature management[168]. Nonetheless, hypothermic temperature ranges can also be detrimental for the outcome of patients due to associated side-effects such as shivering, increased risk of respiratory infection and arrhythmia, among others, and so require an exhaustive control within physiological values[169].

2. TEMPERATURE IN ISCHEMIC STROKE

The intimate relationship between temperature and neurological injury has been exhaustingly studied throughout history. In his book "*De partibus animalium"*, Aristotle asserted the importance of the brain in thermal regulation as "the brain is capable of keeping the heart cool enough for optimal mental activity"[170]. Galen of Pergamon stated that fever originated from the heart as a consequence of inflammation, degrading the humors and driving them into the brain[171]. In the XIX century, the German internist Hermann Nothnagel described the deleterious effects of temperature on thrombotic events, stating that "the sick

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

succumbs if temperature rises by the second or third day, reaching up to 40 ºC. Besides those cases where the thermometric curve presents a quick decrease, in severe cerebral softening there is no such initial decrease in temperature"[172].

Nowadays, data derived from preclinical animal studies and large clinical trials has allowed for a better understanding of the pathogenic mechanisms derived from temperature variations and their impact on the progression of acute neurological diseases, including ischemic stroke.

2.1. Brain and body temperature

Brain temperature is approximately 1.5°C higher than rectal temperature in healthy individuals[173].Conversely, the increase in temperature experienced by stroke patients heightens this difference, with variations of up to 2ºC between body temperature and that of cerebral intraventricular regions[174–176].

Advances in neuroimaging techniques such as magnetic resonance spectroscopy (MRS) have helped elucidate how temperature influences stroke progression[177]. Thanks to this technology, we now know that temperature is higher in the injured hemisphere than in the contralateral region, and that this increase is even more pronounced in the penumbra. Moreover, systemic hyperthermia has been shown to occur subsequently to the increase in brain temperature, which suggests the implication of different initiating mechanisms[178,179].

2.2. Hyperthermia and ischemic stroke

Under physiological conditions, body temperature is accurately regulated by the hypothalamus through endocrine, autonomic and musculoskeletal integrated responses. When body temperature rises, the hypothalamus responds by releasing

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

vasoactive mediators that induce sweating and skin vasodilation, maintaining body temperature within the physiological ranges. However, certain disorders such as ischemic stroke induce a thermal response that overrules the hypothalamical regulatory capacity, leading to hyperthermia.

In this setting, hyperthermia can exacerbate the ischemic injury by promoting excitotoxicity, inflammation, metabolic dysregulations, oxidative stress and BBB dysfunction, causing increased infarct size and worsening the outcome of patients[180]. Furthermore, hyperthermia can be also aggravated by the action of exogen pyrogens that mediate inflammation through the synthesis of prostaglandins[181]. The relationship between brain and body temperature with acute ischemic stroke has been extensively demonstrated throughout a robust number of studies[172,182].

In fact, more than 60% of patients develop hyperthermia during the first days after an ischemic stroke[162]. This rate is directly dependent on the definition of hyperthermia (37-38ºC), registration period (from admission up to 1 week), method of measurement (tympanic, oral, axillary or rectal), type of stroke, recording frequency (every 1, 4, 6, 8 or 24h) and efficacy of the attention protocols. Treatment of hyperthermia, independently from its origin, has been found to improve patient comfort and short and long-term outcome. In the following lines we will discuss different aspects about this issue.

2.2.1 Etiology of hyperthermia in stroke

Based on its origin, hyperthermia can be divided in central (neurogenic) or systemic hyperthermia. Neurogenic hyperthermia develops as a consequence of damaging stimuli to the hypothalamic thermoregulatory center or the pontine centers

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

during the acute phase, which is usually caused by large infarcts or smaller local infarcts in these particular regions[183]. Moreover, the increased secretion of pro-inflammatory cytokines and leukocyte migration into the ischemic tissue leads to hypothalamic and hippocampal neuronal stimulation through their specific receptors, increasing body temperature and producing hyperthermia[181,184–186]. Systemic hyperthermia is a frequent consequence of infectious processes (particularly those of urinary or respiratory origin), which could be attributed to the immunosuppressed state induced by the ischemic injury in later stages of the disease[187].

2.2.2. Hyperthermia and poor prognosis in stroke

The deleterious effects of hyperthermia on the progression of the ischemic injury have been thoroughly demonstrated in global and focal animal models of cerebral ischemia, where both approaches shared a marked increase in infarct volume[188–191]. The first retrospective work on the effects of hyperthermia in stroke was carried out by Hindfelt et al. in 1996, in which the increase in body temperature was associated with a poor outcome[192]. Subsequently, Castillo et al. published the first prospective study in this line of research, showing that hyperthermia was an independent predictor of morbidity and mortality at 6 months in ischemic stroke patients[161]. Further research demonstrated that stroke patients, particularly in the earlier phases of the disease, were nearly 10 times more prone to show early neurological deterioration per each 1ºC increase in axillary temperature[193]. These results were confirmed in later retrospective and prospective studies that showed a clear association between hyperthermia and higher disability, poor outcome and death rate in ischemic stroke patients[194–197].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Up to this day, three different meta-analyses have been published regarding the impact of hyperthermia and fever in stroke outcome. The work of Hajat et al., conducted in 3790 stroke patients found a 9% increase in morbidity and a 1% increase in mortality in those patients that developed hyperthermia[198]. In 2008, Greer et al. found a mortality and poor outcome relative risk of of 1.5 and 2.2, respectively in a series of 14431 patients with stroke and other brain injuries[163]. In addition, this association was also confirmed by the Virtual International Stroke Trials Archive (VISTA) study, conducted on data from 5305 patients in acute stroke trials. Interestingly, their work demonstrated an early time-dependent association where, the later the hyperthermia occurs within the first week, the worse the outcome is for stroke patients[199]. Finally, Prasad and Krishnan´s meta-analysis on six cohorts involving 2986 patients found that the appearance of fever within the first 24h of hospitalization doubled the odds of mortality during the first month after stroke onset[200].

2.2.3. Mechanisms of ischemic brain damage by hyperthermia

During the first hours after an ischemic stroke, the initiation of hyperthermia occurs locally to the infarcted area as a consequence of the altered metabolism and the inflammatory response[172]. As it has been previously mentioned, large and strategically located infarcts near hypothalamic regions can induce systemic hyperthermia through proinflammatory and excitotoxic signaling. Nonetheless, such scenarios only account for less than 4% of the cases in which the presence of hyperthermia was associated with a poor clinical outcome[178].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

During later stages of the disease, systemic hyperthermia (usually derived from infectious processes) initiates an inflammatory cascade that enhances excitotoxicity, free radical damage and BBB dysfunction. These events, along with the ionic imbalance and propagation of damage by CSDs, increase the extension of the ischemic lesion and hinders the room for neurological recovery[162].

Hyperthermia significantly increases glutamate release in animal models of cerebral ischemia, which could augment the frequency of spreading depolarizations in the peri-infarct region and increase the infarct volume[201]. Moreover, spreading depolarizations have also been shown to increase brain temperature in a dose-dependent manner due to the increased metabolism and homeostasis imbalance[202].

Castillo et al. found that hyperthermic patients had a significantly higher concentration of glutamate in cerebrospinal fluid (CSF) than those in the normothermic range, and that the association of hyperthermia with poor clinical outcome was directly dependent on the effect of glutamate[203].

The inflammatory cascade involves the interplay of several activated cells which release proinflammatory cytokines that act as endogenous pyrogens. In this regard, prospective study of 229 ischemic stroke patients found an association between hyperthermia and higher levels of IL-6, TNF-α and ICAM-1, higher infarct volumes and worse outcome at 3 months. Moreover, higher levels of these inflammatory markers at 48h (but not on admission) were also correlated with a poor outcome[204].

Furthermore, inflammation might also contribute to stroke injury by increasing regional metabolism in the energetically depleted ischemic tissue. This sudden, enhanced demand would

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

accelerate the consumption of the scarce oxygen and energy supplies, increasing the conversion of salvageable tissue into infarcted territory[179]. Moreover, hyperthermia has also been shown to increase intracranial pressure, free radical release and BBB permeability, further promoting ischemic damage[205–207].

2.3. Therapeutic Hypothermia in Ischemic Stroke

Hypothermia is defined as a reduction in core body temperature under the threshold of adult normothermia, which is considered to be at 36.5ºC. Based on this definition, therapeutic hypothermia comprises the controlled reduction in body temperature to induce a therapeutic effect on the patient[208]. Depending on the target temperature, therapeutic hypothermia is stratified into ultra-mild (<36->35ºC), mild (34–32°C), moderate (31–28°C), deep (27–11°C) and profound (<10°C).

2.3.1. Origins of Hypothermia: landmarks and discoveries

Egyptians used hypothermia as a treatment for traumatic brain injury (TBI) as described in the Edwin Smith Papyrus, the oldest known treatise on medicine and surgery dating back to 3000 bc[209]. Snow was first recommended by Hippocrates for the treatment of tetanus and later for the treatment of hemorrhages. Interestingly, the Greek philosopher noted that cold may have acute, regional effects as he advised to "apply the cold not to the actual spot from which the bleeding occurs or is expected, but round about. Swelling and pain in the joints unassociated with ulceration, gout and spasms are mostly relieved and reduced by cold douches and the pain thus dispelled. A moderate numbness relieves pain"**.** The study of hypothermia during the 18th century was marked by the research

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

of John Hunter and James Currie on resuscitation, whole body hypothermia and rewarming in animals and humans, respectively, which led to important discoveries such as the ability of living beings to generate heat and the effect of the peripheral vasodilator response after cold exposure. By the end of this century, hypothermia was used by Sir William Osler in the treatment of typhoid fever, and by Dr. Charles Phelps for pain alleviation in traumatic head injuries[210].

A few years forward, the work of Dr. Temple Fay served as the setting stone for future research into the mechanisms of hypothermia neuroprotection, and is credited by many as one of the most notable contributors to modern-day therapeutic hypothermia. Fay´s initial observations on the effect of heat on metastatic progression and cell proliferation led him to translate his work from bench to bedside, first in cancer patients and later on TBI with an overall improvement in pain, remission of symptoms and survival rate. In spite of the technical limitations and concern of the nursing staff, Fay´s work allowed for the development of special blankets for hypothermia induction (by means of pumped cold solutions), more accurate rectal thermometers and tailored head wraps for focal head hypothermia[211].

Nonetheless, his research on hypothermia caught the attention of Nazi scientists from the Dachau concentration camp. As a consequence, the Germans managed to acquire a copy of Fay´s work and conducted their own research on prisoners from their camps. The immorality and lack of ethics in their experiments tainted the image of hypothermia as a therapeutic resource, and most studies in this line of research were consequently discontinued for years[210]. As time progressed and the stigma from the Nazi horrors faded away, animal and clinical

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

research resumed. A.G. Kanaar encouraged the use of hypothermia in tourniquets as a means of protecting the tissue from necrosis until the pulse was restored[212].

In 1954, Rosomoff and Holaday showed that hypothermia reduced cerebral metabolism and regional blood flow, and accelerated the initiation of reparative mechanisms[213]. Following Dr. Fay´s research and motivated by the success of Wilfred G. Bigelow and his colleagues in the use of hypothermia for open cardiac surgery, several neurosurgeons began treating multiple cerebrovascular injuries under hypothermic conditions[211]. Between 1955 and 1958, a total of 100 patients (including 83 intracranial aneurysms) received intraoperative hypothermia with promising but inconclusive results[214]. In 1959 Drs. Williams and Spencer published a series of 27 cases of cardiac arrest that detailed a marked improvement in survival rates in those patients that received hypothermia[210].

From the 60s and until the 90s, the interest in hypothermia was progressively lost in view of numerous works demonstrating high complication rates and worsened survival due to prolonged, deep hypothermia procedures. During the following years, the advances in surgical procedures, the improved treatment of coagulopathies and the accumulating evidence on cold mechanisms derived from animal research thawed the cold shoulder the medical community had given to therapeutic hypothermia[211]. As a consequence, the largest number of studies on hypothermia are condensed into the last two decades. Hypothermia has been successfully used in the treatment of patients with severe TBI, and more recently in anoxic brain injury after out-of-hospital cardiac arrest. The accumulated evidence over the years secured a niche for therapeutic hypothermia in the post-resuscitation treatment

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

guidelines and reignited the long-lost interest in this line of research.

2.3.2. Clinical use of therapeutic hypothermia: state of the art

In spite of the notable results from recent years, the use of hypothermia in the clinic is still limited. Ultra-mild to moderate hypothermia is currently restricted to the treatment of cardiac arrest and perinatal asphyxia encephalopathy[215–217]. Furthermore, deep hypothermia is advised in programmed surgeries that require a temporal interruption in CBF (such as cardiac surgeries or intracranial aneurysms in adults, or congenital heart conditions in neonates) in order to reduce ischemic/reperfusion injury[218]. The effectiveness of hypothermia in the treatment of such diseases has reinvigorated the idea of the potential application of this therapy in other acute CNS injuries with shared pathophysiological features such as stroke, TBI or epileptic seizures.

Germane to this, several studies have demonstrated the ability of mild to moderate hypothermia to decrease edema, neuronal death, inflammation, infarct volume and improve the functional outcome of animal models in the setting of cerebral ischemia[219,220]. Moreover, these results have been backed and validated in various meta-analyses, concurring on the promising potential of therapeutic hypothermia in ischemic stroke[221–223].

Nonetheless, the translation of preclinical research to the human clinic is still challenging. Multiple pilot studies and Phase I clinical trials with different cooling methods demonstrated the feasibility and safety of mild to moderate hypothermia application in patients, even in those subjected to thrombolysis[224–233]. However, associated side-effects such as

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

bradycardia, blood coagulopathies or increased risk of pneumonia are common findings shared by all the aforementioned works.

Based on the feasibility and safety of mild to moderate hypothermia, some of the previous phase I studies were derived into larger Phase II or III. The Intravascular Cooling in the Treatment of Stroke 2/3, (ICTuS 2/3; NCT01123161) was a two-staged clinical trial composed of an initial phase II study of 400 patients followed by an efficacy phase III study with 1200 additional patients. The aim of the trial was to demonstrate the improvement in the outcome of patients when thrombolytic therapy was administered in combination with therapeutic hypothermia. First registered in 2010, the USA-based study was prematurely interrupted during the phase II trial due to the frequent development of pneumonia, and has been on hiatus since 2017[234]. The South Korean study known as HELMET (NCT02985060) was a phase II clinical trial aimed at validating the safety and feasibility of mild hypothermia induced by means of cold saline infusions and maintained through surface cooling in patients with successful recanalization after mechanical thrombectomy. Nonetheless, the status of the study is currently unknown and has not received an update since its registration in the NIH.

The most recent randomized clinical trial is the European multicenter, randomized phase III clinical trial of hypothermia plus best medical treatment vs. best medical treatment alone for acute stroke, known as EuroHYP1 (NCT01833312). The EuroHYP-1 was the largest prospective clinical trial on Therapeutic Hypothermia for stroke patients ever conducted in Europe, with an expected sample size of 1500 ischemic stroke patients. The aim of the study was to validate whether mild to

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

moderate hypothermia (34-35ºC) initiated in the first 6 hours within symptoms onset and maintained over 24 hours improved the functional outcome after three months[235]. Following a recruitment period of 52 months, in March 2018, recruitment was closed with a total of 98 patients included, 49 per group (therapeutic hypothermia vs standard treatment). Although the study did not find neuroimaging or outcome differences between groups, patients who received hypothermia showed reduced levels of GFAP at 72h and increased levels of inflammatory markers at 24h. Nonetheless, the study could not demonstrate the effectiveness of targeted hypothermia to 34-35ºC on ischemic stroke outcome, and the small sample size did not allow to draw further conclusions from the study[236].

In the last 5 years, evidence from the most recently published studies has yielded mixed results regarding the benefits of therapeutic hypothermia. In 2016, Su et al compared the effect of hypothermia in patients with massive cerebral hemispheric infarction in a randomized controlled trial. In this study, hypothermia was induced with cold saline and maintained at 33-34ºC from 24 to 72h after the initiation of the therapy. The results showed that 87.5% of patients who received hypothermia had a good outcome at 6 months compared to the 40% from the control group, with no significant differences in mortality rate[237].

In 2017, the Cooling for Ischemic Stroke Trial (COOLIST) aimed at demonstrating the feasibility and safety of cooling at 34, 34.5 and 35ºC by means of the administration of a bolus of cold saline followed by surface cooling during 24h. Similar to ICTuS-2, the study was prematurely terminated due to the rigorous guidelines for patient wellbeing, as 8 out of the 22 participants developed pneumonia following hypothermia. Nonetheless, the study demonstrated that, unlike 34ºC and

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

34.5ºC, a target temperature of 35ºC was a feasible objective for therapeutic hypothermia in acute ischemic stroke[238].

Finally, a recent randomized clinical trial investigated the safety and therapeutic potential of hypothermia (33°C ± 1°C) after hemicraniectomy in 50 patients with malignant stroke. Similar to previous works, the study was terminated early for safety reasons due to the higher rate of mortality and severe side-effects in the hypothermic group [239]. Finally, a recent prospective, single-center RCT compared the effectiveness of hypothermia (surface cooling and endovascular administration) in patients with large cerebral infarctions after craniectomy. Patients who received hypothermia showed a trend for improvement in neurological outcomes at 6 months, although these results did not reach statistical significance[240].

In brief, therapeutic hypothermia is a potentially effective tool in the treatment of ischemic stroke. However, the associated-side effects, the limited knowledge on the involved mechanisms and the technical difficulties in its clinical management have difficulted its availability as a therapeutic strategy in ischemic stroke.

2.3.3. Phases of the treatment

The use of therapeutic hypothermia involves three critical phases: induction, maintenance and rewarming. Although effective, this therapy is critically dependent on an exhaustive monitoring and tight control of the physiological changes derived from its use.

- **Induction:** the first step of the procedure involves the early initiation of hypothermia by means of the cooling method (or a combination of different methods) of choice to a pre-

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

selected target temperature. Currently, there is no consensus for the optimal temperature range in therapeutic hypothermia in acute ischemic stroke, although evidence from the most recent systematic review and meta-analysis showed the median to be at 33ºC (ranging from 32ºC to 35ºC)[241]. The induction causes a rapid and dramatic drop in body temperature, and as such it is the most convoluted and risky situation, both for patient and clinician. As cooling progresses, the thermoregulatory response to cold takes place by several mechanisms that include vasoconstriction, shivering, renal dysfunction with associated electrolytic imbalances, alterations in blood viscosity and coagulation. These physiological responses are usually addressed by the administration of anxiolytic (buspirone) and opioids (meperidine), with the use of sedation reserved for severe cases[169,242,243].

- **Maintenance:** the second step consists in the stabilization of the patient at the target temperature for hours or days depending on the injury, patient state and progression of the therapy. Patients are usually more stable during this phase, which is reflected in lower occurrence of hemodynamic, electrolyte and volume disturbances. Nonetheless, application of hypothermia even for short periods can increase the risk of infection due to alterations in drug metabolism. Strict monitoring and registration of vital signs at least every 1h is required depending of the hemodynamic status of the patient to ensure the success of the therapy[208].

- **Rewarming:** the last step of the procedure involves the cessation of cooling and steady return to normothermia.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Hypothermia notably silences metabolic rate, alters myocardial sensitivity to K^+, promotes insulin resistance and directly affects blood circulatory mechanisms. As a consequence, a rapid rewarming could aggravate the condition of the patients by the rebound effect, an initiation of damaging cardiac, metabolic and circulatory complications including accidental hyperthermia, reflex vasodilation and massive brain swelling [169,208,243]. Rewarming can be either active or passive, with a general consensus of a 0.2-0.3ºC increase per hour. To avoid the aforementioned, rewarming may last up to 72h depending on the depth of hypothermia, allowing the body to recover homeostasis spontaneously[241].

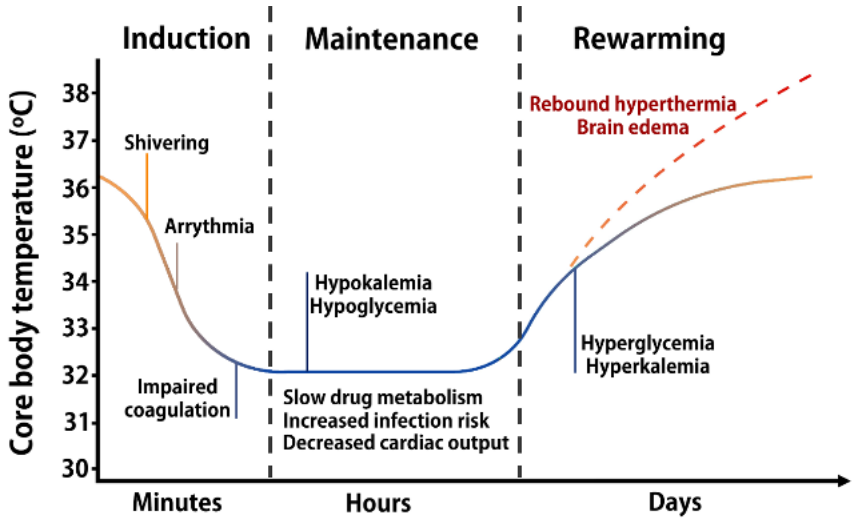


Figure 11. Phases of therapeutic hypothermia and physiological changes associated. Adapted from Andersen et al. 2015 with Creative Common elements.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

2.3.4. Cooling methods

The promising but impactful effects of hypothermia on patients´ condition has led researchers to develop several methods and devices to obtain the optimal therapeutic effect while minimizing the associated side-effects. Depending on degree of invasiveness, these methods are generally divided in surface or intravascular cooling methods. Both approaches can be additionally combined with pharmacological administration to further enhance the therapy.

2.3.4.1. Surface cooling

This approach aims at maximizing the physiological exchange of heat through the skin and mucosa. Ice packs, cold-water immersions, special cooling blankets, trans-nasal evaporation, esophageal cooling and focal cooling with helmet or neck devices are among the most used approaches in this modality.

- **Ice packs and cold-water immersion:** traditionally the earliest and easiest means of reducing body temperature. In particular, cold-water immersions allow for a rapid reduction of core body temperature (nearly 10ºC/h). Nonetheless, these techniques provide poor control of body temperature and their clinical use is limited[244].

- **Cooling blankets and pads:** cooling blankets emerged as a more-refined alternative to ice packs and cold-water immersions. These devices work by circulating a cooled fluid or air through blankets or pads surrounding the patient´s body. A feedback system by means of a temperature probe at the digestive tract of the patient allows

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

for an acutely regulated monitoring. Currently available systems include the Curewrap blankets and CritiCool feedback system (MTRE, Israel), Thermogard XP and STx Surface Pad System (Zoll, USA) Kool-Kit blankets plus Blanketrol III monitoring (Cincinnati Sub-Zero, USA), Gaymar Medi-Therm III system (by Stryker, USA) or the cooling pads of Artic Sun (Medivance, USA)[245].

- **Trans-nasal evaporative cooling:** is based in the combined administration of a nebulized liquid coolant and oxygen in the nasopharyngeal cavity of the patient. Upon contact with the mucosa and evaporation, the coolant absorbs heat from the nasopharyngeal mucous, rapidly reducing intracavity temperature. The most notable commercial device of trans-nasal evaporation is the Rhino Chill system (BeneChill, USA). The portability of these devices allows for an out-of-hospital administration within minutes from the onset of symptoms[246].

- **Esophageal cooling:** this method is a more invasive and direct approach to surface hypothermia that consists in the introduction of a silicone tube into the esophagus. The tube is divided into two compartments: an internal cold and warm water circuit, and a central chamber connected to a gastric port. The circulation of water increases the heat exchange in the mucosa, allowing for a controlled regulation of body temperature. This system was designed to be connected to cooling devices such as the Gaymar Medi-Therm III system or the Blanketrol III, originally designed for blankets or pad cooling[247]. More recently, the EnsoETM (Attune Medical, Chicago, IL) has been developed as an

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

improved version the previous models. This system works by replacing the standard silicone tube with an esophageal heat transfer device (routinely placed after endotracheal intubation) showing improved temperature modulation capability, reduced pharmacological cost and less risk of shivering than traditional modalities of esophageal cooling[248].

- **Head and neck cooling helmets:** These devices are physical adaptations of cooling blankets to the anatomical features of the head, and as such share the same heat exchanging mechanisms. Although useful in neonates due to their thinner skull and smaller head, this approach is not translational to adult mammals, as maintained focal cooling might induce systemic hypothermia beyond local cooling[249,250].

In brief, surface cooling methods are non-invasive approaches to therapeutic hypothermia. Nonetheless, some of the associated side-effects including shivering, skin vasoconstriction and abrasion and the steady cooling rates hampers its use in the clinic. Moreover, in obesity (a major risk factor of stroke) this type of approach is not viable as obese individuals present a low surface/volume ratio that renders this therapy ineffective.

2.3.4.2. Intravascular cooling methods

These approaches include the intravascular injection of cold solutions and intravascular cooling devices. Contrary to surface devices, intravascular methods present a fast-cooling

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

rate, which implies mandatory sedation and anesthesia due to their invasive nature.

- **Cold fluid injections:** these approaches involve intravenous injection of cold saline and Ringer solution into the systemic circulation to decrease body temperature. In 2016, Wang et al successfully adapted this technique to induce selective brain cooling in Rhesus monkeys by injecting cold Ringer solution into the MCA with a microcatheter[251]. Although intravenous injections have been shown to effectively induce systemic hypothermia, this effect is merely transitory. As a consequence, this approach could prove useful during the induction process, transitioning to a more stable method during the remaining time of the procedure[245,252].

- **Cooling catheters:** Based on heat loss by convection, catheters provide fast cooling rates and allow for an accurate monitoring of body temperature. Currently, the Thermoguard XP temperature management system (Zoll) and the InnerCool RTx with Accutrol catheter (Philips) are the two available devices on the market. The Thermoguard XP system uses central venous catheters placed in the subclavian, internal jugular or femoral veins to circulate cool or warm saline through a closed loop. The Innercool RTx induces vascular cooling through the Accutrol, a brand-specific catheter with a built-in temperature sensor. Both systems present an auto-feedback mechanism that allow for a tightly regulated control of temperature during the three phases of the hypothermia protocol. Nonetheless, although these devices reduce the risk of shivering, their outcome is

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

similar to surface devices, possibly due to the increased risk of infection and thrombosis[253,254].

2.3.4.3. Pharmacological cooling: clinical practice

Antipyretic therapy is used to reduce patient discomfort, morbidity and metabolic demand, ant to prevent the development of fever. Common antipyretic drugs (namely **aspirin**, **paracetamol**, **metamizol** and **ibuprofen**) have been tested in ischemic stroke with no clear effect on mortality or morbidity attenuation[168].

Up to this day, the **Paracetamol** (Acetaminophen) in Stroke (PAIS) trial from 2005 is the only large phase III clinical trial allowed on pharmacologically-induced hypothermia in acute stroke patients. The study comprehended a series of 1400 patients with ischemic stroke with less than 12 hours from the onset who received a daily dose of 6g of paracetamol. Although the treated group showed a positive trend towards improved outcome, the results were not significant and hypothermia was not successfully induced[255].

2.3.5. Temperature assessment during hypothermia

Therapeutic hypothermia is a promising strategy in neurological injuries that requires a delicate and well-rounded balance of multiple physiological parameters. In fact, hypothermia is accompanied by a myriad of adverse side-effects for each 1ºC down in temperature, including pneumonia, alteration of platelet function, thrombocytopenia, cardiac arrhythmia, arterial hypo- or hypertension, increased of amylase and lipase levels and alterations of blood coagulation, among others[256].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

In this endeavor, several temperature assessment methods have been developed to ensure proper delivery of hypothermia within the therapeutic ranges. These methods can be either invasive, semi-invasive or non-invasive.

- **Invasive measurement:** this modality is performed by means of thermal probes directly placed into the pulmonary or femoral artery, or into the brain parenchyma, these methods are among the most reliable means for temperature determination. Nonetheless, these procedures are invasive by nature and so their application to the human clinic is heavily restricted and usually involves maintained sedation[257].

- **Semi-invasive measurement:** sites for temperature registry in this approach include the rectum, oral cavity, esophagus, urinary bladder or trachea. Although monitoring at these sites provides a more reliable measurement than surface methods, they usually require sedation and intubation, which increases the probability of medical complications[257].

- **Non-invasive measurement:** Non-invasive methods allow to determine temperature at the ears, axilla, forehead, fingers and, more recently, brain. While the application of any of these approaches lacks any type of risk due to their non-invasiveness, temperature readings from these sites can only provide information on surface temperature, which is usually dependent on room temperature and regional blood flow[257]. Nonetheless, the recent advances in non-invasive measurements have derived into the development of improved devices that allow for reliable temperature

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

monitoring. One such advance is the magnetic resonance thermometry (MRT), a safe and non-invasive volumetric method based on the specific shifts of water molecules within a tissue that allows for the determination of relative changes and absolute temperature in specific tissues *in vivo.* In this regard, our group recently demonstrated that MRT accurately measures focal brain temperature compared with invasive thermo probes in a rat model of cerebral ischemia[258]. More recently, a disposable, non-invasive temperature sensor based has been studied as an alternative approach to invasive methods. This device is based on the zero-heat and heat flux sensor approach (Double Sensor), in which a mathematical model is used to calculate the core body temperature from temperatures measured on a perfectly insulated small skin area such as the forehead. In a recent study on cardiac arrest patients under targeted temperature management, the Double Sensor approach was tested against esophagus thermometry showing similar results, which could demonstrate the applicability of this method to other acute neurological injuries such as ischemic stroke[259].

To sum up, temperature measurement is a critical step for therapeutic hypothermia that requires an accurate and constant regulation. The method of choice must be selected to guarantee the integrity of the patient while providing reliable temperature measurements.

2.3.6. Physiological response to hypothermia: associated side-effects

The induction of hypothermia triggers the initiation of a systemic thermoregulatory response to maintain the body

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

within normothermic ranges. Although it is a natural response of the body, this adaptive mechanism can compromise the management of patients and must be correctly addressed to ensure the success of the treatment.

This complex response is first initiated at the skin and various internal organs by the stimulation of thermal receptors known as transient receptor potential channels (TRPs) located in free nerve endings of somatosensory fibers, and whose cell bodies are resident to the trigeminal ganglia and dorsal horn of the spine. TRPs vary in their temperature activation threshold, sending a myriad of different afferent stimuli to the brain and into the POAH, which works in tandem with the dorsomedial region of the hypothalamus to deliver an integrated and sophisticated systemic response[260]. These signals are transmitted through efferent pathways to preganglionic neurons involved in skin vasoconstriction, cardiovascular adaptations and metabolic changes[261]. Clinically speaking, although these responses arise naturally and conform a physiological adaptation, they pose a threat to both the integrity and success of the therapy, and are so considered as associated side-effects of therapeutic hypothermia.

- **Metabolic rate:** hypothermia has been shown to decrease brain oxygen consumption and glucose metabolism by 5% per each Celsius degree[262]. The reduced demands of oxygen, nutrients and energetic requirements of the tissues enhances insulin resistance and glucagon secretion while reducing insulin secretion[263].

- **Effects of hypothermia in electrolyte balance:** a reduction in body temperature modifies ion diffusion, ion pumps and

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

transporter activity. Upon hypothermia, this shift in ion dynamics increases K^+ efflux from the vessels and into the extravascular compartment, which in turn reduces serum K^+. Low K^+ availability can directly impair cardiac function and initiate arrhythmias including atrial fibrillation, ventricular fibrillation and atrioventricular block, both during induction and rewarming[263,264].

- **Shunting and vasoconstriction:** the first line of defense against hypothermia is the induction of peripheral vasoconstriction to enhance heat loss through the skin. The amount of heat released is dictated by the shunting or opening of precapillary sphincters, a band composed of smooth muscle fibers and pericytes located in the base of capillaries. Hypothermia triggers vasoconstriction by inducing shunting of precapillary sphincters, increasing vascular resistance and fluid sequestration. This response creates a temperature gradient between core and periphery, allowing to maintain heat in the core of the body while avoiding peripheral heat dissipation[260].

- **Musculoskeletal response and shivering**: when the previous mechanisms are not sufficient to compensate for the progressive reduction in body temperature, a second physiological response is started in the musculoskeletal system. Efferent signals from the preoptic anterior region of the hypothalamus (POAH) travel through descending pathways into the spinal cord, activating γ and α-motor neurons and triggering a rapid and repeated contraction of skeletal muscle fibers, which leads to heat production. Nonetheless, this response is highly inefficient in terms of

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

cost-effectiveness, demanding large amounts of ATP to maintain heat production. As a consequence, this response is particularly vexing in a disease such as stroke, where the brain tissue is already subjected to a near-total depletion of energetic resources. Moreover, shivering is an uncomfortable phenomenon is most patients that usually requires the use of sedatives to control it, which difficult the clinical management of the patient[260,265].

- **Altered cardiac function:** hypothermia directly impairs cardiac function by two different mechanisms. On the one hand, a reduction in body temperature prompts the slowdown of heart rate from the normal 60-70 beats per min (36ºC) to 40-45 beats per min (32ºC). The effect of hypothermia on electrolyte balance leads to decreased ionic permeability and an impairment in cardiomyocytes repolarization, slowing down impulse conductivity and extending action potentials. On the other hand, hypothermia decreases blood output to the tissues, but the reduced metabolism during cold exposure compensates for the restricted supply of nutrients and oxygen[266].

- **Altered diuresis:** In spite of the decrease in heart rate, hypothermia increases mean arterial pressure and systemic vascular resistance as a consequence of peripheral vasoconstriction. This results in increased venous return, enhanced secretion of atrial natriuretic peptide and decreased production of antidiuretic hormone, leading to increased diuresis. In the setting of hypothermia, "cold diuresis" can induce hypovolemia, hypotension and

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

increase blood viscosity, further aggravating the condition of the patient[210,266].

- **Altered hematologic function**: platelet function is disrupted below 35ºC, while the coagulation cascade tumbles down at temperatures less than 33ºC. Hypothermia directly impairs the synthesis and activity of clotting enzymes and inhibitors of the plasminogen activator. These phenomena lead to decreased blood coagulation, increased occurrence of coagulopathies and increased rate of hemorrhagic transformations[266]. Interestingly, hypothermia can also increase blood viscosity by means of its effect on peripheral vasoconstriction, which increases intravascular hydrostatic pressure and, as a consequence, capillary permeability[263].

- **Immunosuppression and altered immune response:** the restricted metabolism derived from hypothermia application modulates the signaling activity of several immune cells, including white blood cells. Hypothermia induction, even for short periods, leads to immunosuppression and increase the risk of infections (especially pneumonia). Nonetheless, in the setting of ischemic injury, the reduced inflammatory response following hypothermia could have beneficial effects in stroke outcome[266].

- **Altered liver function and drug metabolism:** hypothermia causes a marked decrease of tubular secretion and reabsorption and directly impairs enzymatic activity. Although there are additional routes of drug elimination, hypothermia can directly modulate drug metabolism

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

through impaired liver function. In this regard, hypothermia has been shown to impair cytochrome p450 activity, reducing drug clearance and potentially promoting overdose[263,267].

2.3.7. Therapeutic hypothermia: underlying mechanisms

The effects of therapeutic hypothermia are pleiotropic, affecting multiple steps from cellular signaling to behavioral and physiological systemic responses (**Figure 12**). In this regard, animal models have helped unveil the underlying mechanisms to the beneficial effects of hypothermia. Although therapeutic hypothermia represents a promising tool in brain neuroprotection, a further understanding of its mechanisms of action will allow for the development of novel therapeutic strategies based on its beneficial effects.

- **Energetic failure and ionic gradients:** hypothermia reduces metabolic rate, ATP consumption and, as a consequence, energetic demands from the compromised tissue. In the setting of cerebral ischemia, hypothermia promotes the activity of ion pumps to reestablish ion gradients across membranes without the need of alternative, less-effective pathways such as anaerobic metabolism[268,269].

- **CBF and microvascular injury:** hypothermia induces a reduction in CBF and BBB permeability, reducing albumin and water infiltration and, as a consequence, halting brain edema and decreasing intracranial pressure[266,270,271]. Moreover, hypothermia hampers astrocyte and microglial activation with the consequent decrease in MMPs secretion

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

and endothelial damage. Interestingly, the reduction in CBF is directly proportional to that of temperature. This is justified by the modulatory effect of hypothermia on the local secretion of vasoactive molecules such as the vasoconstrictors endothelin and thromboxane A2 or the vasodilator prostaglandin I2. Following ischemia, the production rate of thromboxane A2 is particularly increased leading to an imbalance in vessel dilation/constriction and resulting in vasoconstriction, hypoperfusion and thrombotic injury in damaged brain regions. Hypothermia can correct this imbalance by attenuating the increased vasoconstrictive response[266,269,272].

- **Excitotoxicity:** hypothermia can acutely regulate neurotransmitter release by different mechanisms. The most notable mechanism is through reduced ATP demand and consumption, which favors ionic homeostasis across membranes and hampers uncontrolled neurotransmitter release. The major neuroexcitatory neurotransmitter is glutamate, whose action is mainly dependent on NMDA and AMPA signaling. Hypothermia creates a marked reduction in brain glycine levels, a coactivator of NMDA channels, thus decreasing Ca^{2+} influx and hyperexcitability. Moreover, cerebral ischemia promotes the expression of GluR2-deficient AMPA receptors that facilitate the massive entrance of Ca^{2+}. Hypothermia acts by counterbalancing this altered expression, thus reducing Ca^{2+} influx trough AMPA channels[269,273]. Beyond neuronal excitotoxicity, hypothermia can also attenuate glutamate excitotoxicity by its inhibitory effect on connexin 43 (which promotes astrocytic ATP and glutamate release) and upregulation of the glutamate

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

transporter 1 (GLT-1), which facilitates removal of glutamate from the synaptic space by astrocytes[274].

- **ER Stress and post-translational response:** in healthy tissue, hypothermia enhances ER stress and triggers the UPR, which reduces global protein synthesis and promotes pro-apoptotic signaling by upregulating CHOP transcription. In this regard, ER stress can be subjected to post-translational regulation by the Small Ubiquitin-like Modifier (SUMO). Similar to ubiquitination, SUMO is a post-translational protein modification that involves several cellular processes that include genome stabilization, ubiquitination antagonism, regulation of protein transport, among others[275]. Targets of this pathway generally include transcription factors and nuclear proteins that modulate gene expression, usually resulting in the suppression of gene transcription. Physiological stressors such as ischemia promote protein SUMOylation as a counterbalance to cell damage, providing ischemic tolerance by downregulating gene expression and attenuating ER stress due to the accumulation of misfolded proteins. In this regard, hypothermia can also promote SUMO post-translational modifications, increasing SUMOylated protein translocation to the nucleus and shielding cells from death mechanisms such as apoptosis. Additionally, under maintained ER stress conditions, hypothermia can also limit CHOP signaling and pro-apoptotic mechanisms[276,277].

- **Cold-shock response:** although hypothermia and ischemia reduce global protein synthesis, a small subset of proteins show an increase in their expression following these

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

damaging stimuli known as cold-shock proteins, or CSPs, and cold-shock hormones (CSH). Emerging evidence suggests that these proteins act as molecular mediators in a broad set of biochemical and molecular changes, which may boost the neuroprotective effects of therapeutic hypothermia[278]. **Their origin, function and therapeutic capabilities will be discussed in the following section.**

- **Mitochondrial dysfunction:** hypothermia reduces mitochondrial permeabilization by reducing mPTP formation on its membrane, thus impeding the release of cytochrome C. Cerebral ischemia directly damages complexes I, II, and III of the mitochondrial respiratory chain, leading to impaired electron transport and aberrant superoxide generation. Hypothermia reduces the imbalance between the enzymatic complex I and III in the respiratory chain, thus stabilizing mitochondrial function[279].

- **Oxidative and nitrosative stress:** hypothermia protects against oxidative stress by decreasing the production of oxygen and nitrogen reactive species. This effect is secondary to the improved energetic balance, mitochondrial function and decreased glutamate excitotoxicity derived from the cooling process[58,266].

- **Apoptosis:** hypothermia causes an inhibition of apoptotic signaling by modulating gene expression and transcription. Besides the aforementioned effects of cooling on CHOP signaling, hypothermia blocks p53 signaling, downregulating pro-apoptotic (BAX, AIF) and enhancing anti-apoptotic Bcl-2 expression. Moreover, hypothermia

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

directly inhibits caspase signaling by blocking caspases 3, 6, 7, 8, 9 and cytochrome C, thus impeding apoptosis[269,280].

- **Inflammation:** as inflammation is strongly dependent on brain and body temperature. Hypothermia promotes immunosuppression and a shift from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 profile in activated microglia. Moreover, hypothermia has been shown to decreases the release of pro-inflammatory molecules including IL-1β, IL-6, IL-12, IL-18, IL-23, TNF-α and iNOS, while increasing the expression of anti-inflammatory cytokines to inhibit the inflammatory response[281]. Nonetheless, hypothermia can also hinder the expression of anti-inflammatory cytokines such as IL-10, which could be counterproductive to the resolution of the inflammatory milieu[282].

- **Tissue repair:** most works regarding the therapeutic application of hypothermia have been focused on the neuroprotective effects of cooling on the acute injury mechanisms. Therefore, the effects of hypothermia on the delayed phase of stroke are scarce and less explored. Hypothermia seems to promote neuronal synaptogenesis, although its effect on neurogenesis is not fully understood[283]. Some studies suggest the detrimental role in progenitor cell survival, while others indicate the protective role of cold preserving these cells[284]. A recent work in TBI found improved neuronal survival and maturation in the dentate gyrus of rats treated with mild hypothermia, suggesting a potential therapeutic role in endogenous repair mechanisms. Moreover, hypothermia has also been shown to play a role

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

in vascular and tissue remodeling by preventing excessive gliosis and reducing VEGF expression, although the precise implications of these findings on cerebral ischemia are yet not clear[285].

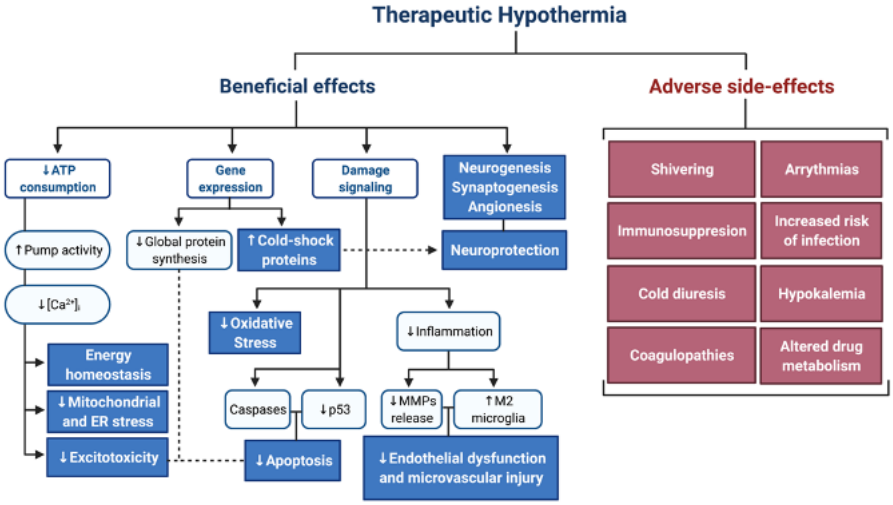


Figure 12. Beneficial and adverse side-effects of therapeutic hypothermia in ischemic stroke. Self-created image using elements with BioRender software.

3. COLD-SHOCK PROTEINS

Beyond the aforementioned traditional neuroprotective mechanisms underlying therapeutic hypothermia, a plethora of mechanisms and adaptive events is mediated by CSPs and CSHs. Both protein families activate thermogenic regulatory mechanisms and participate in the maintenance of body normothermia, and have shown neuroprotective effects both mediated and beyond hypothermia. These results have generated a growing interest in these molecular targets for its potential application on the clinic, as the majority of the compelling preclinical evidence on therapeutic hypothermia has proven ineffective in human-based studies.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Nonetheless, the multi-faceted action of these proteins requires a deep understanding of their cellular mechanisms and signaling pathways to elucidate their impact on the neurological injury, particularly during the earlier acute phase. Based on the recent and robust evidence on their neuroprotective effect in cerebral ischemia and other acute neurological injuries, **the present Thesis will focus on the RNA-binding motif 3 protein (RBM3) and the fibroblast growth factor 21 (FGF21).**

3.1. RNA-Binding Motif protein 3

Glycine-rich RNA-binding proteins (GRPs) are a family of proteins involved in post-transcriptional modifications and RNA metabolism that exert their function by direct binding to single or double stranded RNA or messenger RNAs (mRNAs), which are important during the different phases of cell regulation and gene transcription. In particular, the RBM3 protein was initially discovered as a first responder to cold exposure in mammals. This CSP plays major roles in cell regulation and proliferation, mRNA stability and protection against damaging processes including hypoxia, hyperthermia or excitotoxicity[286].

3.1.1. Structure and functional expression

The RBM3 gene encodes a 157-amino acid protein with an estimated molecular weight of 17kDa, and has been mapped to the short arm of the X-chromosome at Xp11.23[287]. This protein features two highly conserved RNA recognition motifs (RRM) at the N-terminus (known as RNP1 and RNP2) and a less conserved arginine-glycine-rich domain (RGG) located at the C-terminus of its structure. The RMM domain is involved in

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

mRNA capping and splicing, while the RGG domain participates regulates post-transcriptional modifications of pre-MRNA including cleavage and polyadenylation[288–290]**.** This gene is widely conserved across animal species and particularly in mammals, which could be explained by the role of CSPs in thermal maintenance during hibernation[287].

Besides its recruited expression upon stressful stimuli, RBM3 upregulation is mostly restricted to early postnatal period (coinciding with the peak in synaptogenesis) and decreases over time in the majority of brain regions, except in areas such as the SVZ, the SGZ or the rostral migratory stream where cell proliferation and migration remains active[291,292].Interestingly, the recently updated Human Protein Atlas showed a similar expression pattern of body RBM3 between fetal and adult humans under physiological conditions, which suggests that RBM3 could be involved in mechanisms beyond cell regulation, proliferation and neuroprotection during adulthood[293]. The spatial distribution of RBM3 varies among species. RBM3 is relatively low or absent in the heart and thyroid in humans. Hibernating animals such as squirrels or bears show an enhanced expression of RBM3 in the muscles, liver, heart, testis (mainly in Sertoli cells) and brain[287,294–296]. At the subcellular level, this protein is mainly expressed in the nucleus, regulating gene transcription and post-translational modifications.

Nonetheless, RBM3 can shuttle to the cytoplasm, ER and dendrites during stressful conditions such as oxidative damage and ER stress[297–299].

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

3.1.2. Responsive expression

Exogenous and endogenous stressors actively modulate the expression of this protein, including hypoxia, inflammation or temperature variations.

- **Hypoxia:** Preclinical studies have shown that severe (1%), moderate (5%) and mild (8%) hypoxia effectively upregulate RBM3 expression by a mitochondrial and HIF-independent pathway[300]. Moreover, the hypoxia-induced increase in RBM3 levels has also been shown to stimulate neural stem cell proliferation and viability, even at very mild hypoxic levels (18%)[301]. This response appears to be time-dependent, as pregnant mice exposed to severe systemic hypoxia promotes the overexpression of HIF-dependent genes while downregulating RBM3 in the placenta and developing brain of the newborns[302].
- **Drugs:** Certain drugs and multimodal mediators can also upregulate RBM3 expression. In fact, compounds such as the neurotoxin **domoic acid**, **FGF21** or **melatonin** have been shown to upregulate RBM3 expression in rodents[303,304]. RBM3 has also been shown to form a protein complex with the first 60 nucleotides of the COX-2 mRNA by which the lipopolysaccharide inflammatory response is regulated[305]. Conversely, the administration of metformin and the adenosine monophosphate (AMP) analog AICAR have been shown to inhibit RBM3 expression both *in vivo* and *in vitro*[306].

- **Temperature:** Temperature (and in particular, hypothermia) is the probably the most notable modifier of RBM3 expression in mammals. Danno et al. conducted the first study which confirmed that mild hypothermia (32°C)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

maintained for 24h induced the overexpression of RBM3 in mammalian cells. Subsequent studies confirmed that the expression of this protein peaks in the ranges of mild to moderate hypothermia (34-28ºC), exponentially decreasing under deep cooling (25º-15ºC). Conversely, the expression of RBM3 decreases after heat exposure (37-39ºC) both *in vitro* and *in vivo*[307]. Age might also be an important determinant of RBM3 expression. Chip et al. found a higher baseline (37ºC) expression and a near threefold upregulation of RBM3 after cold exposure (32ºC) in murine brain cortices[286]. Similarly, Jackson TC et al. found that mild hypothermia (33ºC) exposure for 48h produced a marked increase in RBM3 levels in primary rat cortical neurons but not in mature neurons[304]. It is worth noting how this protein is particularly sensitive to temperature variations, as a minimal drop of 1ºC from 37ºC to 36ºC can effectively increase its expression in neuronal tissue. Larrayoz et al. reported a 30-40% increase in RBM3 protein levels after cold exposure (8ºC) for 15 minutes in neonate rats. In adult rats, a 3 hours induction of profound (8ºC) hypothermia was needed to induce a 10% increase in baseline RBM3 levels[308]. Newborns have a higher surface/volume ratio compared with adults and a relatively large head, which makes them highly susceptible to hypothermia and heat loss[309].

3.1.3. Molecular and cellular functions of RBM3

The major functions of RBM3 comprise regulation of post-transcriptional, translational changes and microRNA synthesis, stemness and cell progression, abolition of apoptosis, ER stress and neurogenesis processes (**Figure 13**).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

- **Post-transcriptional and post-translational regulation:** RBM3 has been shown to alter mRNA expression of COX-2, IL-8 and VEGF in macrophages and cancerous cells, modulating alternative polyadenylation and ensuring correct stabilization and translation of RNA during mitosis[310,311]. Moreover, RBM3 participates in mRNA alternative splicing and enhances global protein translation by binding to 60S ribosomal subunits, promoting active polysome formation or by modulating the phosphorylation of various initiation factors[297,312].

- **Regulation of microRNA synthesis:** microRNA (miRNAs) are small, non-coding RNA molecules of 19-23 nucleotides which are involved in several cellular, developmental and metabolic mechanisms. These molecules modulate gene expression in a post-transcriptional manner by binding to the 3′-untranslated region of mRNAs, inducing mRNAs translational silencing or repression. The relationship between RBM3 and miRNAs is particularly convoluted due to the apparently disparate results from experimental studies. In particular, RBM3 has been shown to upregulate and downregulate miR125b expression[312,313]. RBM3 overexpression has also been shown to enhance the expression of miR383 and to promote the pre-miRNA association with the Dicer complex[314,315]. Conversely, reduced levels of RBM3 were associated with increased expression of miR-142–5p and miR-143 following hyperthermia, and that these molecules might be involved in protective mechanisms against hyperthermia[316].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

- **Cell stemness and cycle progression:** The Wnt/B-catenin pathway is one of the major regulatory pathways of progenitor and stem cell self-renewal[317]. RBM3 has been shown to enhance B-catenin signaling by suppressing GSK3B, a protein kinase associated with inflammation and apoptosis signaling in several diseases including stroke[310,318,319]. RBM3 expression is crucial for cell progression during mitosis, as absence of this protein induces cell arrest at G2/M and triggers pro-apoptotic mechanisms[320]. Furthermore, a recent work on neural stem cells showed that overexpression of RBM3 reduced cell blockage at the G0/G1 phase and promoted cell transit into the S phase[301].

- **Apoptosis:** RBM3 upregulation directly inhibits PARP cleavage in neuron-like cells exposed to staurosporine, a potent inductor of apoptosis through both caspase-dependent and independent pathways. In addition, the overexpression of RBM3 has been shown to inhibit pro-apoptotic Bax and Bad, as well as caspases-3, -8, and -9[321,322].

- **ER stress:** the UPR is triggered as a mean of rescuing cells subjected to ER stress due to an excessive accumulation of misfolded proteins. In this regard, RBM3 has been shown to suppress the phosphorylation of PERK and eIF2α, decreasing CHOP signaling and halting apoptosis[323].

- **Neurogenesis and plasticity:** adult neurogenesis is canonically restricted to the SVZ and SGZ, areas in which RBM3 expression is constitutively expressed, both in rodents and humans. In fact, RBM3 colocalizes with neural stem cell marker nestin and neuroblast marker doublecortin (Dcx),

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

which highlights its role in stem cell neurogenesis and proliferation[286,313]. A recent work by Zhu et al. demonstrated that RBM3 promotes neurogenesis in a niche-selective manner. While RBM3 overexpression promoted neuronal differentiation in both regions, its effect on cell proliferation was limited to the SGZ[324]. Finally, RBM3 has been suggested to be a regulator of synaptic function through translational mechanisms, as knockdown of RBM3 in hippocampal newborn rat neurons altered synaptic vesicle dynamics and results in abnormal neuronal activity patterns[325].

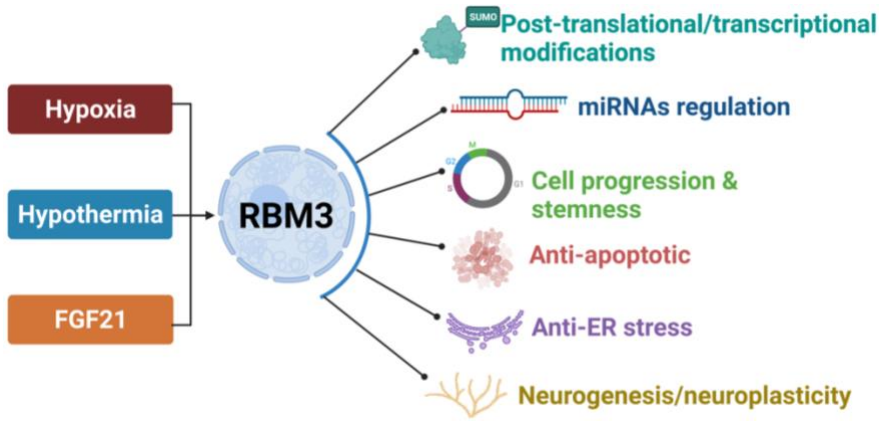


Figure 13. Cellular functions of RBM3. Self-created image with BioRender software.

3.2. Fibroblast growth factor 21

Fibroblast growth factors (FGFs) are mostly paracrine hormones with a heparin-sulfate proteoglycan binding domain (HSPBD) which limits their activity to their immediate surroundings[326]. Interestingly, evolution has caused the loss of this heparin binding function in a subset of FGFs (FGF19, FGF21, FGF23), allowing them to circulate freely upon release. This modification

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

has demanded the coordinated use of transmembrane proteins such as the klotho (a-klotho and/or b-klotho) family as coreceptors, favoring their interaction with ligands and providing stability as a molecular scaffold[327,328].

3.2.1. Structure and expression

FGF21 is an integral hormone of relatively small weight (19.4 kDa) located in chromosome 19 (19q13.33), with three exons coding for a 208 amino acids protein. The mammalian FGF21 gene is highly conserved among in mammals, with minimal differences between humans and other primates including chimpanzees or gorillas, and a homology of over 80% between human and rodent FGF21. Beyond mammalian homology, this gene is also found in other vertebrates, including reptiles or fish[329]. As a non-heparin binding protein, FGF21 requires the obligatory aid of B-Klotho as a coreceptor for ligand interaction and activation of tyrosine kinase receptors (FGFRs)[330,331]. This coreceptor increases the affinity of FGF21 for other FGF isoforms such as FGFR1c, FGFR2c and FGFR3c, ensuring tissue specificity signaling[330]. Although FGF21 is predominantly expressed in the liver, it is also present in the pancreas, adipose tissue, muscles, heart and at some neuronal populations of the hypothalamus[332,333]. Conversely, B-klotho expression in the brain is only abundant in young individuals (i.e., infants, toddlers), with near negligible levels in adult brain regions such as the frontal cortex or the hypothalamus[332–335].

3.2.2. Regulation of FGF21 expression

Several mechanisms regulate the expression of FGF21 in different target tissues. These mechanisms include transcription

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

factors, hormones, starvation and overeating, diet, ER stress, microRNAs and temperature:

- **Transcription factors**: The expression of FGF21 is tightly regulated by PPAR receptors, which bind to the transcriptional regulatory unit at the promoter region of FGF2[336]. The PPAR family is composed of PPARα, PPARβ/δ, and PPARγ. Among them, the α and γ subtypes can upregulate FGF21 expression. While PPARα promotes FGF21 expression in the liver, PPARγ does so in adipose tissue, promoting fat cell secretion of FGF21 and accelerated glucose consumption[337]. Interestingly, FGF21 can also upregulate PPARα and PPARγ, creating a cyclic positive loop that further enhances its expression[338].

- **Hormones**: a physiological link was found between the expression of FGF21 and the activity of different hormones. Glucagon stimulates FGF21 secretion from the liver and increases its circulatory levels, and is critical for the activation of the PPARα/FGF21 axis. In this regard, glucagon receptor-deficient mice who were injected with a lipid infusion of PPARα natural ligands failed to increase FGF21 gene expression. Interestingly, although glucagon and insulin have opposing effects on glucose and energy metabolism, they can act synergistically to modulate FGF21 expression. Nonetheless, these hormones have shown heterogeneous results in terms of FGF21 expression, with some studies reporting increased expression against experimental data that indicates the opposite[339]. The relationship between these hormones is complex and their effect on FGF21 appears to be tissue-dependent. Thyroid

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

hormones can also modulate FGF21 expression. In fact, the thyroid hormone triiodothyronine (T3) has been shown to increase hepatic secretion of FGF21 by a dose-dependent mechanism. This effect is absent in PPARα-null animals, which suggests that this receptor mediates the effects of T3[340].

- **Starvation and overeating**: FGF21 serum levels of healthy individuals remain stable over the course of a day (21–5300 pg/mL; mean, 450 pg/mL) in spite of particular interindividual variations. Nonetheless, disturbances and abnormal dietary events such as starvation or overnutrition can modulate FGF21 expression[341]. In this regard, starvation has been shown to upregulate FGF21 in the liver by both PPARα-dependent and independent mechanisms. Starvation accordingly enhances FGFR1c/b-klotho signaling complexes in the liver, pancreas, hypothalamus and adipose tissue[329]. It is worth mentioning that the increase of FGF21 by fasting or starvation is strictly dependent on its duration, as serum FGF21 is remains unchanged in humans who fasted for 2 days but increased in those individuals who fasted for 1 week[341,342]. In contrast, plasma FGF21 levels have been shown to be increased in patients with obesity, type 2 diabetes or non-alcoholic liver disease, which could be attributed to the heightened levels of triglycerides in these individuals[343]. An increase in fat cell FGF21 has been reported in adipocytes undergoing differentiation in a PPARγ-dependent manner[344].

- **Diet and nutrients**: both circulating and local hepatic FGF21 expression are modulated by dietary macronutrients.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Carbohydrate-rich diets have been shown to upregulate FGF21 expression. For instance, a lipogenic high-carbohydrate diet rich in glucose increased liver FGF21 mRNA levels in mice, but this effect was not found in animals who received a fat-rich diet. Besides glucose, different sugars including sucrose, fructose or saccharin have been shown to enhance FGF21 expression in the liver, both in rodents and humans. On par with the effect of carbohydrates, a diminished intake in dietary protein appears to modulate FGF21 expression. Low-protein diets have shown to induce the expression of FGF21 in rodents and humans, reportedly by the UPR through the PERK/eIF2α/ATF4 axis. Finally, the evidence on fat-rich diets is intricate and requires further study. While some works reported an increase in weight, adipose tissue, food intake and FGF21 levels in mice fed with a high-fat diet, other studies did not find these differences when compared to a low-fat intake[337].

- **ER stress:** a robust body of work has demonstrated the pivotal role of the ER in the homeostatic regulation in various metabolic conditions including insulin resistance, type 2 diabetes or obesity. Recent evidence has shown that FGF21 is upregulated following stress to the ER by the activation of ATF4 and CHOP. In particular, ATF-4 activation prompts the upregulation of FGF21 due to its conserved binding sites on the FGF21 promoter, while CHOP helps stabilize FGF21 transcripts as it lacks binding sites on said promoter region. In brief, the upregulation of FGF21 by the UPR is considered to be a counterbalancing mechanism to the activated eIF2α/ATF4/CHOP pathway[345].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

- **miRNA:** miRNAs have also been implicated in metabolic disorders such as diabetes. The miR-577 has been shown to directly target the 3′UTR region of FGF21, blocking its expression. Diabetic children present higher circulating levels of this miR-577, and this miRNA has been shown to block the FGF21-induced secretion of insulin[346,347]. In addition, the miR-212 has also been reported to downregulate FGF21 expression. This miRNA is particularly implicated in the development of insulin resistance and non-alcoholic fatty liver disease. In this regard, anti-miR-212 administration resulted in increased FGF21 expression and reduced lipid synthesis in liver HepG2 cells. Moreover, higher miR-212 and lower FGF21 levels were found in mice who received a high-fat diet[348,349].

- **Temperature:** FGF21 and body temperature are intimately tied and reciprocally regulate each other. This finding was initially reported by Inagaki et al., who found that inducing an overexpression of FGF21 mRNA in the liver induced torpor in fasting transgenic mice[336]. In rodents, a 4h treatment at 4°C produced a marked increase in FGF21 mRNA levels in BAT but not in the blood at this timepoint[350]. A subsequent work by Hondares et al found that cold exposure (4ºC) for 6h, 24h or 30 days effectively increased FGF21 mRNA levels in brown adipose tissue (BAT), while the effect on its circulating levels was only evident at 24h and showed a robust increase after chronic cooling (30 days)[351]. Therefore, acute cold stress induces the secretion and local action of FGF21 within the tissue but does not reach the systemic circulation until 24h. Opposing results regarding

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

the origin of FGF21 circulating levels after cold exposure have led to the development of a liver FGF21–brain–BAT axis, in which FGF21 is first secreted by the liver, which then travels to BAT to aid in the protection against cold stress. In humans, cold exposure (19ºC) for 12 hours caused a sustained increase in FGF21 plasma levels compared with thermoneutral controls (24ºC), which was validated in a separate cohort in a subsequent study by the same group[352,353]. In addition, systemic FGF21 levels are profoundly impacted by circadian rhythms, peaking during the first hours and reaching nadir levels by the end of the day and after cold exposure[352–354].

3.2.3. Molecular functions of FGF21

The earliest studies on FGF21 were mainly focused on its role in glycemic control, lipid and body weight regulation or improved insulin response, which are frequent events in metabolic diseases including obesity. Nonetheless, a novel wave of studies has helped elucidate the active contribution of FGF21 against oxidative and endothelial damage, apoptosis or inflammation. The present work will delve into the major functions of FGF21 while addressing novel mechanisms of this protein (**Figure 14**).

- **Glucose metabolism:** insulin is one of the most important contributors to glucose metabolism. This hormone enhances glucose absorption through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which in turn upregulates glucose transporter 4 (GLUT4)[355]. In this regard, although FGF21 can also modulate blood glucose balance through this pathway, most of its glucose-inhibiting signaling takes place

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

by phosphorylating the atypical protein kinase C (PKC)[356]. This factor has also been shown to enhance glucose uptake by binding to and upregulating GLUT1. Notably, FGF21 and insulin appear to work in tandem in several glucose regulation mechanisms. First, insulin can activate PKC, upregulating FGF21 and promoting its action on GLUT1[357]. Second, FGF21 can downregulate the mammalian target of rapamycin complex 1 (mTORC1), an inhibitor of PI3K and major contributor to insulin resistance, which leads to improved insulin sensitivity[358]. Third, FGF21 can promote Akt phosphorylation via insulin, increasing the number of insulin receptors and improving insulin resistance[359]. Additionally, FGF21 can contribute to blood glucose regulation by upregulating adiponectin expression in adipocytes via PPARγ, or by inhibiting glucagon secretion from the pancreatic islets, among others[360,361]. Notably, FGF21 can also contribute to gluconeogenesis. Once released, this hormone is able to cross the BBB and stimulating the hypothalamus, favoring the synthesis and release of corticosterone from the adrenal glands to increase blood glucose levels and upregulate gluconeogenesis-related genes[362,363]. Providing its ability to reach the intact CNS, FGF21 and its analogues (**LY2405319**, **PF-05231023** or **BMS- 986036**) have been shown to improve insulin resistance, reducing blood glucose and insulin in preclinical studies, although their effect on blood glycemia was not significant in human clinical trials[364–366].

- **Lipid Metabolism:** one of the classic roles of FGF21 involves lipid regulation. This hormone is able to effectively reduce the plasmatic levels of different lipids, including total

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

cholesterol, low-density lipoproteins (LDL) and triglycerides, as well as increasing circulating high-density lipoprotein (HDL) and adiponectin[367]. In rodents, a continued administration of FGF21 induced weight loss, reduced plasma triglycerides and LDL and increased HDL levels in diet-induced and transgenic obese mice, with similar results in diabetic monkeys[368,369]. FGF21 also contributes to the alleviation of damage in fatty liver disease. Mice who received a 5% alcohol diet over a period of 4 weeks showed signs of liver damage, steatosis and increased levels of FGF21 as a compensatory mechanism[370]. Conversely, the administration of recombinant FGF21 reduced inflammation and alleviated hepatic steatosis. Furthermore, a recent work on alcoholic steatohepatitis showed that **B1344**, an analog of FGF21, reduced liver inflammation and immune cell infiltration, suppressed hepatocyte death and halted a further progression of the disease[371].

- **Energy metabolism and thermoregulation:** adipose tissue plays a pivotal role in energy homeostasis and thermoregulation. After satisfying the required metabolic demands, all the energetic surplus is stored in the form of triglycerides in the white adipose tissue (WAT), which is reflected in weight gain[372]. In contrast, BAT is a mitochondrial abundant tissue and a key contributor to thermogenesis by the uncoupling protein 1 (UCP1)[373]. UCP1 activation uncouples biological oxidation from ATP synthesis, which converts the resulting energy into heat, thus increasing body temperature. In this regard, FGF21 has been shown to upregulate UCP1 expression and to promote the conversion of WAT into BAT, a phenomenon known as

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

"browning"[374]. Moreover, the contraction of skeletal muscles promotes FGF21 transport to WAT, promoting "browning" and increasing energy consumption[375]. Nonetheless, the role of FGF21 in energy balance appears to be restricted to altered metabolism conditions and not to metabolically healthy individuals.

- **Cardiac function:** a recent body of works have suggested a cardioprotective role for this protein. For instance, FGF21 has been shown to inhibit the apoptotic factor C-caspase 3 via PI3K/Akt signaling, which is reflected in increasing ATP availability and suppressed apoptosis signaling in cardiomyocytes. Moreover, FGF21 intervenes in the PERK-eIF2α-ATF4- CHOP axis during ER stress, reducing protein misfolding and inhibiting cardiomyocyte death. Owing to its antioxidant capacity, FGF21 promotes the upregulation of antioxidant genes including Ucp2, Ucp3 or Sod2 in the heart, thus protecting this tissue against oxidative damage. Finally, FGF21 signaling through PPARγ has been shown to protect against cardiac hypertrophy and to accelerate fatty acid metabolism, while this condition was aggravated in FGF21-knockout mice[376].

- **FGF21 in the Brain:** although the specifics concerning FGF21 expression are yet unknown, several studies have demonstrated that this hormone is able to diffuse across the BBB, activating central regulatory pathways and engaging in neuroprotective mechanisms[377]. As previously mentioned, FGF21 can bind to B-Klotho and FGFRs expressed in certain brain regions (more prominently in hypothalamic nuclei) to activate the hypothalamus-pituitary-adrenal axis (HPA) and

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

hypothalamic-pituitary-gonad axis (HPG), which regulate the levels of corticotropin-releasing hormone (CRH)[378]. Increased CRH signaling triggers the release of several adrenal cortex hormones, which increase the output to the liver and upregulate the expression of gluconeogenesis genes, thus regulating blood glycemia. Moreover, studies in obese mice have demonstrated that CRH secretion stimulates BAT sympathetic activity and upregulate UCP1 expression, increasing BAT lipolysis, energy consumption and weight loss. FGF21 can also exert neuroprotective effects upon brain injury[379]. Furthermore, FGF21 can alleviate cognitive deficits related to TBI such as spatial memory impairment, hippocampal nerve damage and altered dendritic morphology. In addition, FGF21 has been reported to promote synaptic plasticity, dendrite proliferation and to suppress Tau and Aβ accumulation in the brain, thus protecting against neurodegenerative diseases such as Alzheimer[5,380–382].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

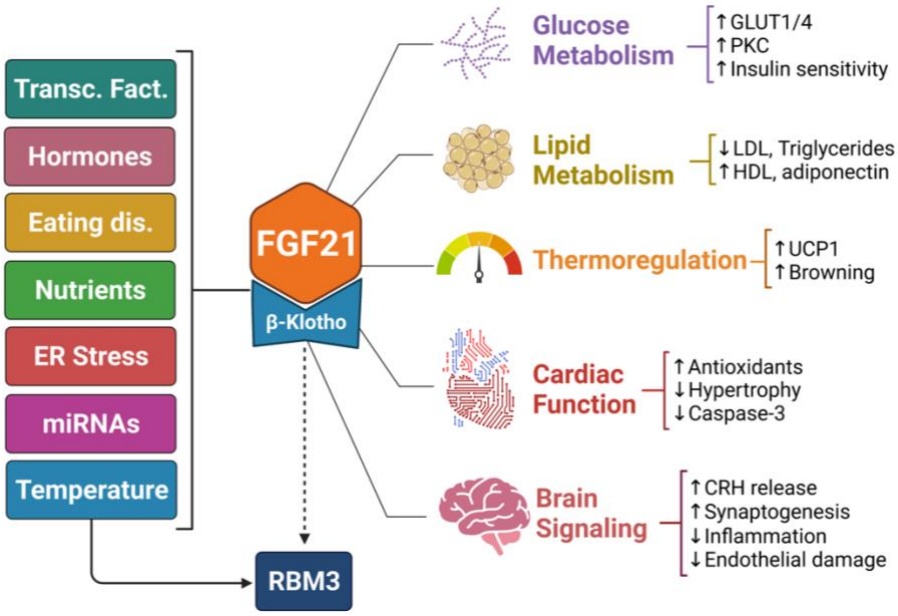


Figure 14. Multimodal effects of FGF21 in different target tissues. Self-created image using elements with BioRender software.

3.3. RBM3 and FGF21 in ischemic stroke

Both RBM3 and FGF21 are responsive to variations in temperature and exert neuroprotective effects. Nonetheless, due to their novelty and multifaceted participation in different pathways, the exact mechanisms and implications of these proteins in the setting of ischemic stroke are not fully understood.

FGF21 has shown promising results in the treatment of cerebral ischemia. Administration of a recombinant form of FGF21 has been shown to reduce BBB disruption and improve long-term neurological scores in type 2 diabetes mellitus mice subjected to MCA occlusion (MCAO)[383]. As previously mentioned in this work, the number of patients who can benefit from recanalization therapy is limited owing to its restrictive

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

therapeutic window and inclusion criteria. Nonetheless, a recent body of evidence has shown promising results in delayed recanalization for those patients who were not eligible for this type of therapy[384]. One of the upsides of this delay is the increase in endogenous FGF21 in the penumbra after recanalization, which is reflected in decreased neuronal apoptosis, reduced glutamate excitotoxicity and improved neurological outcome via FGF21/FGFR1/PI3K/caspase-3 signaling[385,386]. Furthermore, intraperitoneal administration of recombinant FGF21 ameliorated M1 polarization, reduced NF-κB signaling and increased the activity of PPAR-γ in a mice model of MCAO[387].

Not much is known regarding the role of RBM3 in the particular setting of ischemic stroke. In 2019, Zhu et al showed that hypoxia-induced RBM3 expression promoted neurogenesis and protected against apoptosis in the SGZ and SVZ after hypoxia/ischemic injury[324]. More recently, our group demonstrated that exposure to both systemic and focal mild hypothermia (32ºC) produced a remarkable increase in RBM3 expression which was associated with a reduction in infarct volume compared with normothermic controls in an animal model of MCAO[388] **(Figure 15)**. In another subset of experiments, and as a tentative approach to study the neuroprotective effect of RBM3 while bypassing the associated side-effects of hypothermia, we induced an upregulation and downregulation of RBM3 in the ipsilateral ischemic hemisphere prior to MCAO using adeno-associated virus vectors. While this approach did not show differences in infarct volume by RBM3 expression, animals showed a trend toward improved outcome in the rotarod functional test. Finally, a recent work on rat primary cortical neurons showed a protective effect against apoptosis by increasing the number of stress granules, translational

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

aggregates formed during the acute phase of ischemic stroke that suppress the generation of ROS[389].

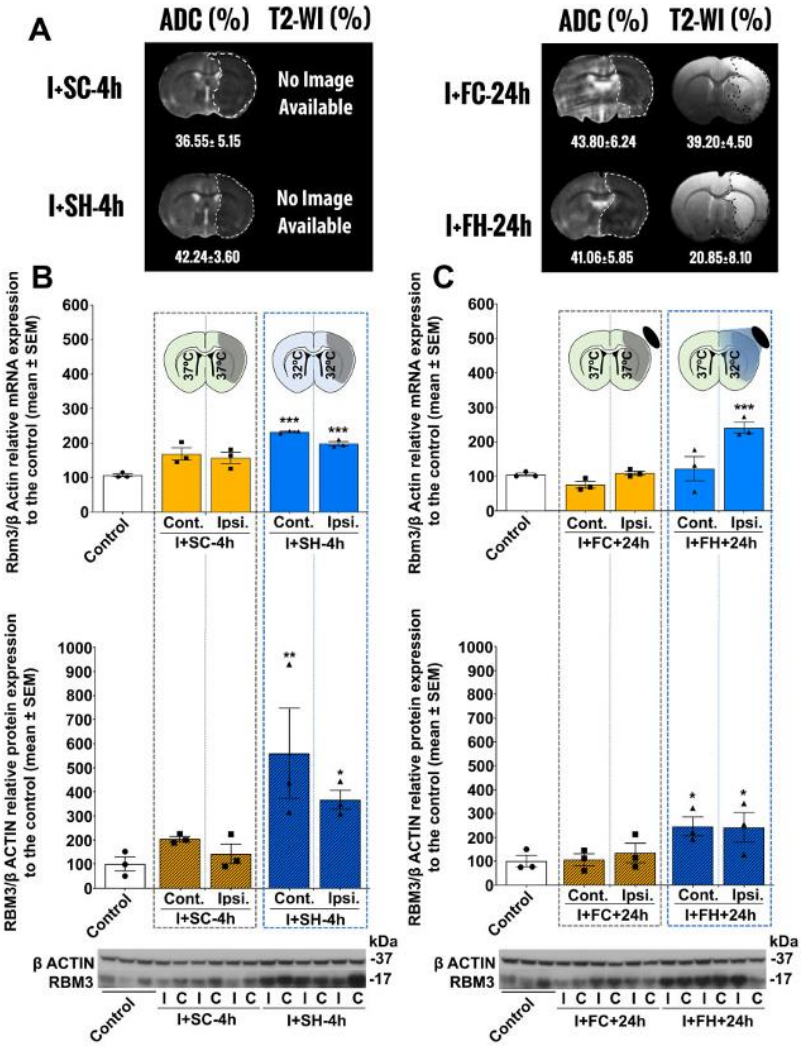


Figure 15. Hypothermia reduces ischemic lesion volume and enhances RBM3 expression. (A) MRI assessments of ischemic injury. (B) Brain RBM3 mRNA and protein levels in ischemic animals subjected to systemic normothermia or hypothermia C) Above: Brain RBM3 mRNA and protein levels in ischemic animals subjected to focal normothermia or hypothermia. Self-created image with GraphPad Prism software.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

The relationship between both molecules is of the utmost interest to the present work. Both proteins are novel neuroprotective candidates with a sensitive and marked response to cold stress adaptation. Moreover, FGF21 has been recently described as a potent modulator of RBM3 expression in an age- and temperature-dependent manner. Altogether, the above stated evidence invigorates the research on these molecules as promising protective targets in ischemic stroke.

4. EXPERIMENTAL RESEARCH OF HYPOTHERMIA IN STROKE

The use of animal models as a versatile tool for stroke research has improved our understanding of the pathophysiologic mechanisms of the ischemic injury, allowing for the development and validation of potential therapeutic compounds. Animal models are frequently classified into induced (the condition is experimentally induced), spontaneous (naturally occurring diseases), negative (growth inhibition or non-responsive upon stimuli) and orphan (naturally occurring diseases not yet described in humans) models.

4.1. Animal models of ischemic stroke

In the field of cerebral ischemia, animal models are classified into 2 different groups according to the extension of the injured region: global ischemia and focal cerebral ischemia[390].

- **Global ischemia** is characterized by a critical reduction in cerebral blood flood in the whole brain. The induced ischemia can be either incomplete (four vessel occlusion (4-VO) and 2-VO models) or complete (complete global brain ischemia (CGBI)). As whole blood flow reduction is not a common feature of human ischemic stroke, this model is

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

usually focused on preclinical research on cardiac arrest and resuscitation.

- **Focal ischemia** consists of a reduction of blood flow in specific vascular territories, thus allowing for a bonafide replication of human brain stroke. These models comprise mechanical or embolization occlusion of a particular vessel, and replicate several pathophysiological events such as energetic failure, excitotoxicity, CSD, inflammation and cell death. Moreover, focal ischemic models can be either transient or permanent attending to the duration and state of the occlusion. As the MCA is the most commonly affected territory (near 50% of all cases) in human strokes, most models of focal ischemia are based on MCAO[391].

4.1.1. Animal models of focal cerebral ischemia

At present, this model is mainly divided into five types: endovascular occlusion of the MCA with an intraluminal filament, embolic occlusion, transcranial occlusion, endothelin-1 occlusion and photothrombotic occlusion. The main advantages and disadvantages of the mentioned models are summarized in **Table 1.**

4.1.1.1. MCA occlusion with intraluminal filament

Initially described by Koizumi in 1986 and later modified by Longa et al in 1989, this model consists of the occlusion of the MCA by means of the introduction of a round tipped monofilament through the internal carotid artery. This approach can be performed either permanently or transiently and does not require access to the vessel via craniotomy[392].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

4.1.1.2. Embolic occlusion

Most focal ischemic strokes are produced by thromboembolisms. This model can be classified into thromboemboli and sphere-induced embolus. In addition, thromboemboli can be further classified into spontaneous clots (autogenous or allogeneic) and induced thrombi (thrombin-induced clots or photothrombotic).

- Intraluminal thromboembolism: this model was described by Kudo et al in 1982, and later revised by Busch et al. in 1997. This model replaces the filament with the injection of autologous blood clots through the internal carotid artery, and usually compromises MCA and anterior choroidal artery territories[393,394].

- MCA thromboembolism: published in 2007 by Orset et al., this model involves the local microinjection of thrombin into the MCA, inducing the formation of a blood clot in situ[395].

- Embolism by artificial spheres: described by Zivin et al. in 1987, this model is based on the injection of artificial macro (300-400 μm diameter) or microspheres (15-50 μm) into the CCA, ICA or MCA, inducing diffuse microembolization and widespread infarcts[396].

4.1.1.3. Transcranial occlusion

This approach is based in the direct electrocoagulation or ligation of the proximal MCA, with or without combined occlusion of other major arteries. The MCA electrocoagulation model was initially described by Tamura et al., while the ligation approach was first published by Chen et al

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

in 1986. Both settings require craniotomy and allow for temporary or permanent occlusions[397,398]. The thromboembolism model previously described can also be adscribed to this category[395].

4.1.1.4. Endothelin-1 occlusion

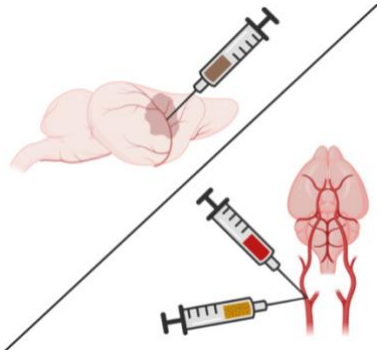
Endothelin-1 (ET-1) is a potent vasoconstrictive peptide initially described in 1987 and later proposed by Sharkey et al. in 1993 as an animal model of focal ischemia. ET-1 can be injected into the exposed MCA or applied by stereotaxic injection into other brain regions including the cortex or striatum[399].

4.1.1.5. Photothrombosis model

Watson et al. developed this model in 1985 with the aim of producing reproductible cortical infarcts without the need of craniotomy. This model is a 2-step procedure that involves the injection of a photosensitive dye (usually rose Bengal) into the systemic circulation followed by an irradiation with a laser beam. Irradiation over the territory of the MCA leads to the photooxygenation of the dye, producing platelet adhesion and aggregation and occluding the vessel[400].

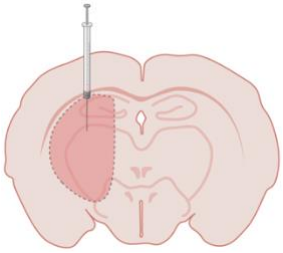
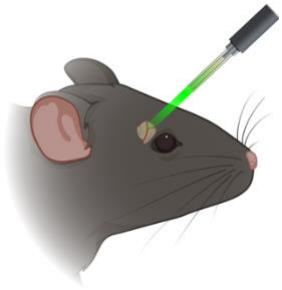
USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Table 1. Pros and cons of the different focal cerebral ischemia animal models in rodents. Self-created image using elements with with BioRender.com. (cont).

	ADVANTAGES	DISADVANTAGES
 MCA occlusion with intraluminal filament *Longa et al. 1985*	No craniotomy Highly reproductible Ischemic penumbra Controllable reperfusion	Not useful for the study of thrombolytic therapies
 **Embolic Occlusion:** • **Intraluminal** *(Kudo et al., 1982)* • **Thrombin** *(Orset et al., 2007)* • **Microspheres** *(Zivin et al., 1987)*	Useful for the study of thrombolytic therapies (intraluminal and thrombin)	Low reproducibility Spontaneous recirculation Not suitable for transient occlusion and thrombolysis (microspheres)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Table 1 (cont.). Pros and cons of the different focal cerebral ischemia animal models in rodents. Self-created image using elements with BioRender.com

Transcranial Occlusion *Tamura et al., 1981; Chen et al., 1986*	Smaller infarcts Lower mortality Highly reproductible	Destroys dura Intracranial infection Not useful for the study of thrombolytic therapies
 **Endothelin-1 Occlusion** *Sharkey et al., 1993*	Highly reproductible Flexible selection of infarct regions	Affected by anesthetics Not useful for the study of thrombolytic therapies
 **Photothrombosis model** *Watson et al., 1985*	Highly reproductible Less trauma Improved long-term survival	Absence of penumbra Poor responses to rtPA

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

An excellent stroke model should be reproductible, suitable for different trials on different species and should allow to accurately control the degree of the ischemic injury. Moreover, as the endpoint for stroke research is the development of therapeutic strategies for human disease, ischemic animal models should be able to simultaneously replicate common clinical complications (hypertension, hypoglycemia) while being sensitive to the currently available clinical treatment (rtPA)[401]. Rodents, particularly rats and mice, are the most used species in stroke research. These animals present several advantages such as the well-characterized injury mechanisms, the availability of genetically modified strains, its ethical acceptability and a lower economical and logistic cost. Although their physiology and cerebral vasculature is similar to that of humans there are still some limitations. Rodents are lysencephalic and have significantly lower white matter content compared with humans, which demand a mandatory reproduction in larger gyrencephalic animals such as cats, dogs or primates[402]. Nonetheless, animal models have exponentially improved our understanding of the disease and represent a key contributor in stroke research. As there is no universal model for ischemic stroke, the specific animal model should be selected based on the objective of the study, the involved injury mechanisms and the comorbidities related to the disease[403].

4.2. Therapeutic hypothermia in preclinical research

The advances and refinement in preclinical stroke animal models have allowed for the development of innovative strategies that allow for a better understanding of the physiological effects of cold exposure in the setting of neurological diseases. Attending to the reach of the protocol,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

these methods can be either systemic (general hypothermic procedures with whole-body side-effects) or focal (a more specific and regional-oriented approach aimed at diminishing the adverse effects of whole-body cooling).

4.2.1. Systemic hypothermia

General hypothermia involves the exposure of the whole-body of the animal to the cold exposure at the desired temperature. The most common approaches for this strategy are physical and pharmacological cooling.

4.2.1.1. Physical cooling

This modality comprises the use of ice pads, cooling blankets, water immersion or infusion of cooled saline, among others. Although the use of these methods allows for a rapid reduction in body temperature, this same effect hinders the maintenance of target temperature. Moreover, the uneven contact between the body of the animal and the cooling surface can lead to unintentional swings in body temperature, thus affecting the general procedure. To bypass these inconveniences, a strict monitoring through a thermo-regulated pad can help overcome some of the technical difficulties. Furthermore, physical hypothermia usually demands a combined application with general anesthesia during the entirety of the procedure to counteract the associated physiological reactions such as shivering or peripheral vasoconstriction. Finally, intravenous cooling allows for a more controlled and rapid intervention but requires further preparation and implies additional risks due to its invasive nature[280].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

4.2.1.2. Pharmacological cooling

Thermoregulation is a complex physiological mechanism that involves the interaction of multiple receptors and their specific ligands. The thermoregulatory control of the organism is primarily directed by the POAH, which integrates multiple afferent stimuli from peripheral and central receptors. In the setting of hypothermia, the body responds to the change in temperature with internal mechanisms that include shivering, vasoconstriction or arteriovenous shunting, among others[404]. By means of different drug compounds, these thermoregulatory mechanisms can be bypassed while attaining the desired therapeutic benefit from decreased body temperature.

Different systems have been targeted in preclinical research including the cannabinoid and opioid system, transient receptor potential vanilloid 1 (TRPV1) channel, neurotensin receptors, thyroxine derivatives, gases and adenosine nucleotides.

- **Cannabinoid and opioid system:** modulation of the cannabinoid system involves the arachidonic acid and its receptors, particularly the cannabinoid receptor 1 (CB1) for its role in thermoregulation. Although different agonists of CB1 showed a marked reduction in core temperature up to 4ºC, its use was associated with altered cardiac function. Similarly, agonists of κ and δ-opioid receptors also exert effects beyond lowering body temperature such as a drop in blood pressure and increased risk of arrhythmias, thus truncating its translation to the clinic[404].

- **Neurotensin:** analogs of this molecule have shown a reduction in body temperature up to 5ºC. In spite of its potent hypothermic effect, its translation to the clinic

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

remains a difficult feat due to its dramatic effects on glucose, insulin and glucagon function[404].

- **Gaseous agents:** several gaseous agents have been tested including xenon or helium or with mixed results and concerns over their interaction with tPA. Hydrogen sulfide is another hypothermic agent that acts by inhibiting cytochrome C oxidase and reducing cell metabolism with neuroprotective effects in animal models of stroke. Nonetheless, recent studies found a role for this gas in reactive inflammation in high doses, which demands the critical need of toxicity studies in humans[404].

- **Adenosine nucleotides:** the thermoregulatory roles of ATP and AMP have been thoroughly studied. Although both molecules initially showed promising hypothermic effects, subsequent studies showed that the decrease in body temperature was not associated with improved neuroprotection, highlighting the particularly excitotoxic effect of ATP during cerebral ischemia[404].

- **Thyroxine derivatives:** as thyroidal hormones participate in physiological thermoregulation, the hypothermic potential of two thyronamines (decarboxylated derivatives) was tested in an animal model of MCAO. Intraperitoneal injection of thyronamines T0AM and T1AM showed a prolonged reduction of body temperature (from 36ºC to 31ºC) during 6-10h without triggering shivering, piloerection or other physiological responses to hypothermia, and produced a significant decrease in infarct volume at 24h. For their therapeutic effect and limited side-

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

effects, these agents are among the most promising candidates to reach clinical translation[404].

- **TRPV1 channels:** TRPV receptors are nonspecific cation channels involved in temperature sensing both in the peripheral and CNS, as well as in pain, touch, taste or osmolarity responses. In stroke, capsaicin, dihydrocapsaicin and its analog rinvanil have shown specific binding to the TRPV1 channel. These ligands modulate the thermoregulatory set point of the POAH, inducing hypothermia without the development of the shivering response[404,405].

4.2.2. Focal hypothermia

In spite of the potent neuroprotective effect of systemic hypothermia in the neurological injury, the associated side-effects such coagulation disorders, shivering or immunosuppression can impact the outcome of the therapy. In this regard, restricting the exposure range of the cooling source to the target tissue could overcome these adversities while maintaining the desired beneficial effects. Focal approaches include the following:

- **Whole head cooling with a silicone tube**: described by Taniguchi et al. and later modified by Liu et al. in 2012, this method involves the circulation of cold water through a silicone tubing driven by a pump[406,407]. Although effective, this approach derived into systemic hypothermia.

- **Thermoelectric regulation by a Peltier device**: initially described by Imoto et al in 2006, this method involves the

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

cortical implantation of a thermoelectric artifact, allowing for a rapid decrease in brain temperature[408].

- **Focal cooling through a metallic coil**: Clark and Colbourne in 2007 designed a metallic coil placed in the cranium surface through which cold water was circulated to induce focal hypothermia[409]. A modified revision of this approach was used by our group to induce focal hypothermia, showing an effective reduction in brain temperature and infarct volume in a rat model of ischemic stroke[258].

- **Heat dissipation device**: a heat-dissipating device was created by D'ambrosio et al. in 2013 that allowed for a subtle reduction in brain temperature in chronic conditions such as in chronic spontaneous seizures[410].

- **Intravascular focal cooling**: Mattingly et al. purposed this method in 2016, where focal hypothermia was induced by through the common carotid artery in swine[411]. Although this approach reduced brain temperature by 12ºC in the ipsilateral region, core temperature was also reduced by 4ºC.

Focal hypothermia presents multiple benefits compared with the systemic approach, such as the potential avoidance of adverse side-effects. Nonetheless, this method comes with some disadvantages such as the undesired transition into systemic cooling over long exposure periods in some of the described applications. Focal brain hypothermia usually requires longer cooling periods to achieve a similar benefit to systemic exposures. Moreover, the required cold depth to achieve the desired therapeutic effect might be limited to superficial

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

structures such as the cortex and thus fail to address broader and deeper insults such as in the case of cerebral ischemia. Finally, the viability of focal approaches is dependent on the animal species. Larger rodents such as rats allow for a finer-tuned and specific delivery of cold to the desired regions, as we have previously demonstrated in recent works in this regard. On the contrary, the reduced size of mice and their enhanced sensibility to temperature variations could trigger a systemic effect and hinder the applicability of these approaches.

As there is currently no ideal method for therapeutic hypothermia, the cooling approach must be carefully selected based on the injury, scope of the study and manageability of the possible adverse effects.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

HYPOTHESES AND OBJECTIVES

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

HYPOTHESES AND OBJECTIVES

Hypothermia is one of the most promising therapeutic strategies against the cerebral ischemia. Nonetheless, its translational use remains hampered owing to its associated adverse-effects (pneumonia, arrhythmia or coagulopathies). In this regard, cold-shock proteins have been shown to underly the beneficial effects of cooling, and could become a valuable therapeutic target without the adverse effects of hypothermia. In previous experimental studies we have already demonstrated that an increase in the expression of RBM3 was associated to the effect of hypothermia as well as to the reduced infarct volume in ischemic animal models. In order to validate these previous findings and demonstrate the potential application of RBM3 as a therapeutic target for stroke, the present work provides clinical evidence on the effects of hypothermia on the expression of RBM3 and the outcome of patients, and explores the influence of temperature on FGF21 (a novel RBM3 inductor) and RBM3 levels in obese stroke patients. Moreover, we studied the protective effect of hypothermia and RBM3 in a mice model of cerebral ischemia to evaluate future strategies to induce a RBM3-mediated protective effect.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

To further explore the aforementioned questions, the following hypotheses will be addressed in this Thesis:

A) RBM3 IS BE A BIOMARKER OF GOOD PROGNOSIS IN STROKE PATIENTS.

B) THE EXPRESSION OF RBM3 AND FGF21 IS INFLUENCED BY TEMPERATURE, AND THIS RELATIONSHIP COULD DETERMINE THE OUTCOME OF STROKE PATIENTS.

C) HYPOTHERMIA REPRODUCES THE PREVIOUSLY REPORTED INCREASES IN BRAIN RBM3 IN MICE SUBJECTED TO CEREBRAL ISCHEMIA SURGERY.

In order to verify these concepts, the present work will focus on the following objectives:

1. ***TO ANALYZE THE CIRCULATING LEVELS OF RBM3 IN ISCHEMIC STROKE PATIENTS SUBJECTED TO HYPOTHERMIC TREATMENT.***
2. ***TO DETERMINE THE IMPACT OF TEMPERATURE ON THE CIRCULATING LEVELS OF RBM3 AND FGF21 IN A COHORT OF OBESE STROKE PATIENTS.***
3. ***TO STUDY THE EFFECT OF HYPOTHERMIA ON INFARCT VOLUME AND RBM3 EXPRESSION IN A MICE MODEL OF CEREBRAL ISCHEMIA***

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

OBJECTIVE 1. Analysis of the circulating levels of RBM3 in ischemic stroke patients subjected to hypothermic treatment

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

The present objective partially reproduces the content from the following publication, of which the PhD is co-author:

Publication 1: Cold stress protein RBM3 responds to hypothermia and is associated with good stroke outcome
-Year: 2020

-**Authors**: Paulo Ávila-Gómez[1*] Alba Vieites-Prado[1*] Antonio Dopico-López[1] Saima Bashir[2], Héctor Fernández-Susavila[1], Carme Gubern[2], María Pérez-Mato[1,3], Clara Correa-Paz[1], Ramón Iglesias-Rey[1], Tomás Sobrino[1], Alejandro Bustamante[4], Sven Wellmann[5], Joan Montaner[4], Joaquín Serena[2], José Castillo[1], Pablo Hervella[1] and Francisco Campos[1]

-**Affiliation**:

1. Clinical Neurosciences Research Laboratory, Health Research Institute of Santiago de Compostela (IDIS), Hospital Clínico Universitario, Santiago de Compostela 15706, Spain
2. Stroke Unit, Department of Neurology, Hospital Universitari Dr. Josep Trueta de Girona, IDIBGI, Girona 17007, Spain
3. Neuroscience and Cerebrovascular Research Laboratory, Department of Neurology and Stroke Center, La Paz University Hospital.Hospital La Paz Institute for Health Research (IdiPAZ), Autonomous University of Madrid, 28046, Madrid, Spain
4. Neurovascular Research Laboratory, Vall d'Hebron Institute of Research (VHIR), Universitat Autónoma de Barcelona, Barcelona 08035, Spain

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

5. Department of Neonatology, University Children's Hospital Regensburg (KUNO), University of Regensburg, 93049 Regensburg, Germany

-**Publisher and ISSN**:
Oxford University Press, 2632-1297

-**Quality indexes**:
Impact factor: Not yet released
h-Index: Not yet released
Quartile: Not yet released

-**PhD contribution**:
The PhD contributed to the analysis of the human samples.

- **Content present in this Thesis**: Objective 1

- **Reference**:
Paulo Ávila-Gómez, Alba Vieites-Prado, Antonio Dopico-López, Saima Bashir, Héctor Fernández-Susavila, Carme Gubern, María Pérez-Mato, Clara Correa-Paz, Ramón Iglesias-Rey, Tomás Sobrino, Alejandro Bustamante, Sven Wellmann, Joan Montaner, Joaquín Serena, José Castillo, Pablo Hervella, Francisco Campos, Cold stress protein RBM3 responds to hypothermia and is associated with good stroke outcome, Brain Communications, Volume 2, Issue 2, 2020, fcaa078, https://doi.org/10.1093/braincomms/fcaa078

- **Editorial Approval**: See **Annex**

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

OBJECTIVE 1.
Analysis of the circulating levels of RBM3 in ischemic stroke patients subjected to hypothermic treatment

1.1 INTRODUCTION

Hypothermia is one of the most promising neuroprotective strategies in the treatment of ischemic stroke[162,172,222,258]. Nonetheless, its translationality from bench to bedside remains hampered by adverse effects including shivering, hypotension, arrhythmia and increased risk of pneumonia, which usually demand the use of anesthetics[169]. Moreover, although target-specific approaches such as brain focal hypothermia have shown promising results in experimental studies, their clinical translation remains difficult owing to the thicker human skull, which effectively insulates the brain from external temperature variations. In spite of these limitations, the recent advance in the understanding of the underlying mechanisms of hypothermia has allowed for the discovery of promising neuroprotective targets for reducing ischemic lesions[271].

For instance, the global downregulation in protein synthesis following hypothermia is concurrent with the upregulation of CSPs. In particular, RBM3 has acquired a significant amount of academic interest due to its critical role in the protective effect of therapeutic hypothermia[335]. RMB3 is a glycine-rich protein (17 kDa) that promotes global protein synthesis at 32°C by accelerating ribosome assembly, stabilizing mRNA, and

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

decreasing microRNA expression[312]. Moreover, a recent work by our group demonstrated that hypothermia (either systemic or focal) enhanced the expression of this protein in the brain, and that this treatment caused a profound reduction in infarct volume in a rat model of cerebral ischemia[388]. Nonetheless, although hypothermia is a promising therapeutic strategy in ischemic stroke, the precise role of RBM3 in ischemic stroke patients has not been explored as of today.

1.2. OBJECTIVES

To analyze the relationship of circulating RBM3 with the outcome of ischemic stroke patients, and how this effect is mediated by temperature.

1.3. MATERIAL AND METHODS

1.3.1. Study Design

This is a retrospective observational study performed on two independent cohorts: one from the Clinical Hospital of Santiago de Compostela, and the other from Dr. Josep Trueta University Hospital in Girona, following approval by the respective ethics committees (Code: 2019/616). The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and to the STROBE Guidelines for Observational Studies. The recruitment period took place from January to December 2019. Sample size was calculated using EPIDAT statistical software based on the difference of RBM3 values at 24 hours in relation to outcome at 3 months. In the first place, we conducted a preliminary study in 20 patients with acute IS to determine the sample size and the time needed to analyze the RBM3 levels. RBM3 levels were analyzed on admission (baseline) and 2, 12 and 24 hours after admission for

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

IS patients. Minimum sample (n=198) size was calculated accepting an alpha level of 5% and a power of 80%. Then, two independent cohorts of 301 ischemic stroke patients were considered for the study (129 patients from Girona and 172 patients from Santiago). Patients were admitted to the Stroke Unit of their respective hospital and treated in accordance with the guidelines of the Cerebrovascular Diseases Study group of the Spanish Society of Neurology.

1.3.2. Outcomes

The first clinical outcome considered in this work was hyperthermia, defined as an axillary temperature ≥37.5°C. Axillary temperature was carefully measured by the nursing staff of the Stroke Unit and collected every 6 hours. As a variable, the highest temperature was selected in the first 24 hours. The second clinical outcome in this study was the patient´s good outcome at 3 months, defined as a modified Rankin Scale Score (mRS) <3. Stroke severity was assessed using the National Institute of Health Stroke Scale (NIHSS) score at admission, 24, 48 and 72 hours. Both NIHSS and mRS were evaluated by internationally accredited neurologists.

1.3.3. RBM3 determinations in ischemic stroke patients submitted to hypothermia

Human blood samples for RBM3 analysis were obtained from the phase III EuroHYP-1 trial of therapeutic cooling in ischemic stroke patients[235]. The trial randomized patients to hypothermic treatment plus standard care or standard care alone (controls). In the hypothermic arm, cooling was started within 6 hours of symptom onset and within 90 minutes of starting thrombolysis (or within 90 minutes of hospital admission in those not treated

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

with thrombolysis) using 20 ml/kg refrigerated normal saline (4°C) IV over 30–60 minutes or a pre-specified surface cooling method. Cooling was maintained at 34°C –35°C for 24 hours using a surface or endovascular technique. Thereafter, patients were passively rewarmed at a rate of 0.2±0.1°C/hour until rectal or bladder temperature reached 36.0°C, after which the cooling device was disconnected. All patients received treatment for acute IS and secondary prevention according to published guidelines. Pre-defined per-protocol analyses were performed in all patients whose body temperature was maintained at ≤35.0°C for at least 6 hours during active cooling. We selected 31 patients (14 treated and 17 controls) for the study based on blood sample availability. Sample collection for biomarker measurement was pre-specified at three time-points: basal (pre-cooling baseline); 26 hours (2 hours after rewarming) and 72 hours after stroke onset.

1.3.3. RBM3 analysis in human blood samples

Serum RBM3 levels were measured using commercial enzyme linked immunosorbent assay (ELISA) kits following manufacturer instructions (Biotez RBM3 ELISA, Berlin, Germany). Blood samples were collected in test tubes, centrifuged at 3,000 g for 15 minutes, frozen immediately, and stored at -80°C. RBM3 levels were measured using commercial ELISA kits following manufacturer instructions (Biotez RBM3 ELISA). RBM3 was analyzed over the range 31.25 pg/mL to 2000 pg/mL, with a sensitivity of 10 pg/mL. Intra- and inter-assay coefficients of variation were 1.9% and 2.7%, respectively.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

We defined percentage variation in RBM3 levels in the first 24 hours as

$$\Delta RBM3 = \frac{(RBM3\ at\ 24h - RBM3\ on\ admission)}{RBM3\ at\ 24\ h} * 100$$

1.3.4. Statistical Analysis

All preclinical data were analyzed in GraphPad Prism 5.01 as the mean and standard error of the mean. Clinical data were analyzed blind in SPSS 21.0 (IBM, USA) as percentages for categorical variables and as the mean ± standard deviation (SD) or median and range (25th-75th percentiles) for continuous variables depending on their adjustment to normality, assessed by Kolmogorov-Smirnov and Lilliefors tests. Bivariate correlations were performed using Pearson's or Spearman correlations depending on the distribution of the variable. Differences between patients according to outcome group were determined by ANOVA and chi square tests. Logistic regression analysis was adjusted by the clinically significant variables identified in the bivariate analysis and the results shown as odds ratios (ORs) with 95% confidence intervals (95% CI).

1.4. RESULTS

1.4.1. Description of the sample

We studied two independent cohorts adding to a total of 301 patients (172 from Santiago and 129 from Girona), of whom 215 were finally eligible after applying for the inclusion and exclusion criteria (**Tables 2 and 3**).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Table 2. Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
• Ischemic stroke < 6 hours post-onset (all Girona patients had thrombolysis) • Confirmation by neuroimaging • Previous mRS score ≤ 1 • Stroke Unit neurologist care since inclusion • Signed informed consent	• Wake-up stroke • Loss to follow-up ≥ 3 months • No blood sample at admission and/or at 24 hours • Systemic hypothermia treatment • Comorbidity with life expectancy < 6 months

Table 4. Number of patients included and excluded per center (Girona and Santiago).

	TOTAL SAMPLE	GIRONA	SANTIAGO
Patients included	301	129	172
Patients excluded			
Wake-up stroke	7	0	7
Loss to follow-up ≥ 3 months	35	12	23
No blood sample at admission and/or at 24 hours	40	15	25
Systemic hypothermia treatment	0	0	0
Comorbidity with life expectancy < 6 months	4	0	4
Eligible patients	215	102	113

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

The characteristics of the stroke patients from Girona and Santiago (listed in **Table 4**) showed significant differences regarding the percentage of atrial fibrillation (22.5 vs 37.2%, p=0.025); classification of subtype of acute ischemic stroke (p<0.0001); NIHSS at 48 hours (9 [2,20] vs 3 [0,13], p p<0.0001); NIHSS at discharge (6 [1,17] vs 2 [0,10], p=0.006); previous mRS (0 [0,1] vs 0 [0,0]), p=0.008); mRS at 3 months (3 [1,5] vs 1 [0,2], p<0.0001) and percentage of patients with good outcome at three months (47.1 % vs 66.4%, p=0.006). Due to the differences found in the sample, the results were adjusted by center in the following analyses. RBM3 levels both at admission and after 24 hours did not show significant differences between the two centers.

Table 4. Sample description. *P values between hospital samples. IS patient characteristics in both cohorts showed a negative correlation between basal RBM3 levels and body temperature at admission (Spearman coefficient -0.150, P=0.028) and at 24 hours (Spearman coefficient -0.662, P<0.0001).

	Total sample n = 215	Girona n = 102	Santiago n = 113	*P* *
Age, years	75.9 ± 10.2	76.6 ± 12.4	75.2 ± 7.8	0.786
Women, %	45.1	44.1	46.0	0.681
History of hypertension, %	63.3	65.7	61.1	0.576
History of diabetes, %	25.6	23.5	27.4	0.535
Smoking, %	14.9	17.6	12.4	0.339
Dyslipidemia, %	33.5	38.2	29.2	0.193
Atrial fibrillation, %	30.2	22.5	37.2	0.025
Ischemic heart disease, %	7.4	7.8	7.1	0.517
Previous stroke, %	10.7	12.7	8.8	0.384
Peripheral arterial disease, %	5.6	4.9	6.2	0.772
Systolic blood pressure, mmHg	151.3 ± 28.8	153.9 ± 28.2	149.1 ± 29.3	0.221
Diastolic blood pressure, mmHg	80.6 ± 17.6	81.2 ± 18.0	80.1 ± 17.3	0.624
Glycemia, mg/dL	147.3 ± 62.4	138.6 ± 57.5	155.1 ± 65.7	0.053
Leukocytes, x 10^3/mL	9.2 ± 3.1	9.0 ± 3.1	9.4 ± 3.1	0.373

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Fibrinogen, mg/dL	491.0 ± 152.6	502.6 ± 156.9	480.6 ± 148.5	0.292
Platelets, x 10^3/mL	222.1 ± 71.3	222.8 ± 72.1	221.6 ± 70.9	0.899
Axillary temperature on admission, ˚C	36.4 ± 0.6	36.4 ± 0.4	36.3 ± 0.7	0.162
Maximum temperature in first 24 hours, ˚C	37.3 ± 0.7	37.3 ± 0.6	37.2 ± 0.7	0.338
Temperature in first 24 hours ≥37.5˚C, %	36.7	33.3	39.8	0.395
Systemic fibrinolysis, %	74.0	100	50.4	<0.0001
Endovascular treatment, %	10.7	1.0	19.5	<0.0001
TOAST				<0.0001
- Atherothrombotic, %	20.0	14.7	24.8	
- Cardioembolic, %	45.6	52.0	39.8	
- Lacunar, %	9.3	0	17.7	
- Indeterminate, %	25.1	33.4	17.7	
Previous Rankin	0 [0, 1]	0 [0, 1]	0 [0, 0]	0.008
NIHSS on admission	16 [9, 20]	16 [10, 20]	14 [8, 17]	0.139
NIHSS at 24 hours	8 [2, 25]	11 [2, 21]	7 [2, 20]	0.163
NIHSS at 48 hours	6 [2, 17]	9 [2, 20]	3 [0, 13]	<0.0001
NIHSS at discharge	4 [0, 13]	6 [1, 17]	2 [0, 10]	0.006
Rankin at 3 months	2 [0, 5]	3 [1, 5]	1 [0, 2]	<0.0001
Good outcome at 3 months, %	57.2	47.1	66.4	0.006
RBM3 on admission, pg/mL	288.1 ± 192.9	283.1 ± 209.6	292.5 ± 177.3	0.722
RBM3 at 24 hours, pg/mL	471.1 ± 412.3	412.9 ± 397.1	523.5 ± 420.1	0.050

1.4.2. Relationship between circulating RBM3 and body temperature

Our first objective was to study the relationship between body temperature and RBM3 expression. To this aim, we began by studying 20 patients with acute IS to determine sample size and RBM3 sampling times: levels were analyzed on admission (baseline) and 2, 24 and 72 hours after admission for IS patients.

The bivariate analysis showed a lack of correlation between the basal levels of RBM3 and the body temperature at admission (Pearson´s coefficient=-0.100, p=0.215). However, a strong negative correlation was found between the RBM3 levels at 24

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

hours and the maximum temperature measured in the first 24 h (Pearson´s coefficient=-0.574, p<0.0001), and this correlation was similar for the two hospitals included in the study. The bivariate correlation showed similar results when analyzing the maximum temperature in the first 24 h with ΔRBM3 (Pearson´s coefficient=-0.529, p<0.0001).

The strong correlation between the RBM3 expression and the temperature was further demonstrated for patients with a maximum body temperature below 37.5 ˚C. The analysis of RBM3 levels on admission and after 2, 24 and 72 hours showed an association between normothermia (maximum temperatures below 37.5 ˚C) and high levels of RBM3 (>300 pg/mL) at 24 and 72 hours, but not with the circulating levels upon admission and at 2 hours (**Figure 16**).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

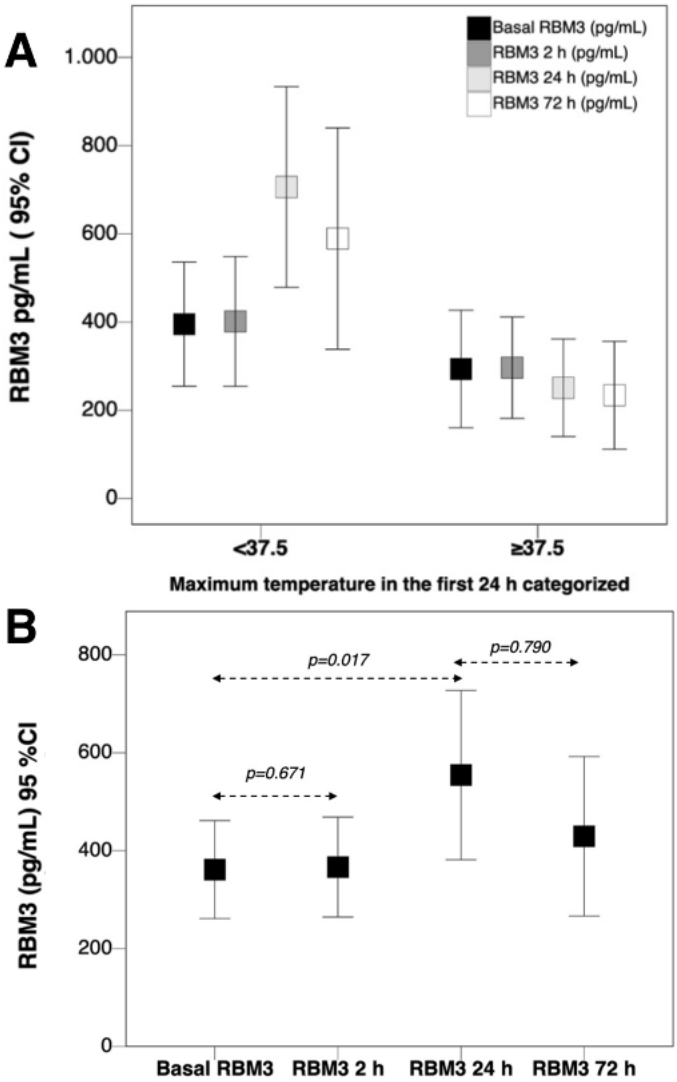


Figure 16. Optimal RBM3 determination timepoints in baseline study. (A) Correlation between hyperthermia (maximum temperature in the first 24 hours ≥37.5ºC) and mean RBM3 levels on admission and at 2, 24 and 72 hours. (B) Mean RBM3 levels on admission and after 2, 24 and 72 hours in the first 20 IS patients included in the study. Self-created image using SPSS 21.0 (IBM, USA).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

In view of these preliminary results, we selected RBM3 levels on admission and at 24 hours after stroke onset for the whole sample. Subsequently, we evaluated the levels of RBM3 at 24 hours and ΔRBM3 for the final 215 patients included in the study. The correlation analysis showed that normothermic (<37.5 ºC) patients had higher RBM3 values at 24 hours compared with hyperthermic (≥37.5 ºC) patients (**Figure 17A**). Interestingly, the mean variation between the RBM3 levels determined upon admission and 24 hours after stroke onset (ΔRBM3) was positive in normothermic patients vs negative in hyperthermic patients (**Figure 17B**).

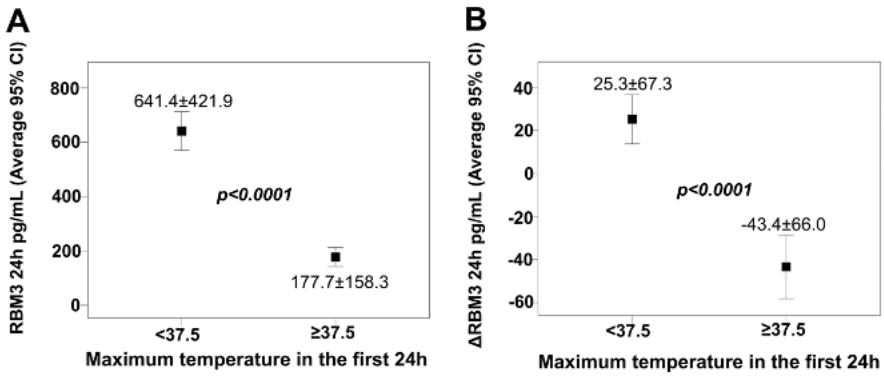


Figure 17. Association of RBM3 with the outcome and temperature (**A**) RBM3 levels at 24 h and (**B**) ΔRBM3 in normothermic (<37.5˚C) and hyperthermic (≥37.5˚C) IS patients. ΔRBM3 was defined as % variation of RBM3 at 24 h respect to the basal levels. Self-created image using SPSS 21.0 (IBM, USA).

The analysis of the area under the curve (AUC) of the ROC curves showed values of 0.852 (95% CI: 0.801-0.903, p<0.0001) for RBM3 levels at 24 hours and 0.802 (95% CI: 0.740-0.864, p<0.0001) for ΔRBM3. RBM3 levels at 24 hours ≥300 pg/mL were associated with temperatures <37.5˚C with a sensitivity of 75% and a specificity of 85%, while the increase in ΔRBM3 ≥10% are

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

associated with temperatures <37.5˚C with a sensitivity of 70% and a specificity of 81%.

In addition to the RBM3 levels at 24 h and ΔRBM3, other variables also showed significant relationship with the maximum temperature at 24 h (**Table 5**), such as: glycemia (136.5 ± 48.0 mg/dL vs 165.8 ± 78.3 mg/dL, p=0.001); leukocytes (8.6 ± 2.7 vs 10.4 ± 3.4 x 103/mL, p<0.0001); fibirinogen (461.7 ± 122.2 mg/mL vs 541.4 ± 184.3 mg/mL, p<0.0001); temperature at admission (36.2 ± 0.5 vs 36.6 ± 0.7 ˚C, p<0.0001); systemic fibrinolysis (81.6 vs 60.8 %, p=0.001); endovascular treatment (7.4 vs 16.5 % , p=0.042); Previous Rankin (0 [0, 0] vs 0 [0, 1], p=0.004) and NIHSS at admission (12 [8, 18] vs 17 [10, 20], p<0.0001).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Table 5. Clinical and biochemical characteristics in normothermic vs hyperthermic patients.

	<37.5ºC n = 136	≥37.5ºC n = 79	*P*
Age, years	76.4 ± 10.6	74.9 ± 9.7	0.301
Women, %	48.5	41.8	0.495
History of hypertension, %	64.7	60.8	0.562
History of diabetes, %	25.7	25.3	0.940
Smoking, %	11.0	21.5	0.057
Dyslipidemia, %	31.6	36.7	0.457
Atrial fibrillation, %	36.8	39.2	0.771
Ischemic heart disease, %	5.9	10.1	0.287
Previous stroke, %	10.3	11.4	0.822
Peripheral arterial disease, %	6.6	3.8	0.542
Systolic blood pressure, mmHg	155.4 ± 29.6	144.4 ± 26.3	0.057
Diastolic blood pressure, mmHg	82.3 ± 18.0	77.8 ± 16.7	0.072
Glycemia, mg/dL	136.5 ± 48.0	165.8 ± 78.3	0.001
Leukocytes, x 10^3/mL	8.6 ± 2.7	10.4 ± 3.4	<0.0001
Fibrinogen, mg/dL	461.7 ± 122.2	541.4 ± 184.3	<0.0001
Platelets, x 10^3/mL	215.9 ± 60.2	233.0 ± 86.5	0.090
Axillary temperature at admission, ºC	36.2 ± 0.5	36.6 ± 0.7	<0.0001
Systemic fibrinolysis, %	81.6	60.8	0.001
Endovascular treatment, %	7.4	16.5	0.042
TOAST			0.056
- Atherothrombotic, %	15.4	27.8	
- Cardioembolic, %	44.9	46.8	
- Lacunar, %	13.2	2.5	
- Indeterminate, %	26.5	22.8	
Previous Rankin	0 [0, 0]	0 [0, 1]	0.004
NIHSS at admission	12 [8, 18]	17 [10, 20]	<0.0001
NIHSS at 24 hours	4 [1, 12]	16 [6, 21]	<0.0001
NIHSS at 48 hours	4 [0, 12]	14 [3, 20]	<0.0001
NIHSS at discharge	2 [0, 9]	10 [2, 18]	<0.0001
Rankin at 3 months	1 [0, 3]	3 [1, 4]	<0.0001
RBM3 at admission, pg/mL	320.7 ± 194.6	231.8 ± 177.6	0.001
RBM3 at 24 hours, pg/mL	641.4 ± 421.9	177.7 ± 158.3	<0.0001
RBM3 at 24 hours ≥300 pg/mL, %	74.3	15.2	<0.0001
ΔRBM3, %	25.2 ± 67.3	-43.3 ± 66.0	<0.0001
ΔRBM3 ≥10%, %	76.5	19.0	<0.0001

Subsequently, we evaluated ORs for the association between RBM3 and maximum temperature at 24 hours <37.5°C by performing a logistic regression analysis using the variables identified as clinically and statistically significant in the model.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

The analysis revealed an independent association between maximum temperature at 24 hours <37.5°C and RBM3 levels at 24 hours ≥300 pg/mL (obtained from the ROC curve), with an adjusted OR of 15.83 (95% CI: 6.71–37.39; *P*<0.0001)), after adjusting for center, glycemia, leukocytes, fibrinogen, systemic fibrinolysis, endovascular treatment, previous mRS and NIHSS at admission (**Table 6**).

Table 6. Multivariate analysis. Dependent variable: maximum temperature in the first 24 hours<37.5 ˚C, not adjusted and adjusted by clinically relevant variables found in our study.

	OR not adjusted	CI 95%	p	OR adjusted	CI 95%	p
Center (cat)	1.32	0.76 - 2.31	0.325	1.22	0.43 - 3.50	0.708
Glycemia	0.99	0.99 - 0.99	0.002	0.99	0.99 - 1.00	0.177
Leukocytes	0.82	0.74 - 0.90	<0.0001	0.79	0.69 - 0.93	0.004
Fibrinogen	0.99	0.99 - 0.99	<0.0001	0.99	0.99 - 1.00	0.070
Systemic fibrinolysis (cat)	2.86	1.53 - 5.36	0.001	2.81	0.91 - 8.74	0.074
Endovascular treatment (cat)	0.40	0.17 - 0.97	0.042	0.49	0.12 - 1.97	0.317
Previous Rankin	0.76	0.58 - 0.99	0.040	0.94	0.65 - 1.37	0.764
NIHSS on admission	0.91	0.87 - 0.96	<0.0001	0.92	0.86 - 0.98	0.014
RBM3 at 24 hours ≥300 pg/mL	***16.11***	***7.81 - 33.26***	***<0.0001***	***15.83***	***6.71 - 37.39***	***<0.0001***

OR: Odds Ratio; Cat: categorized

1.4.3. Relationship between the expression of RBM3 and the outcome at 3 months

The second outcome of this work was to analyze the relationship between the RBM3 levels and the clinical outcome of ischemic stroke patients. We observed a clear relationship between RBM3

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

levels at 24 hours and functional outcome at 3 months measured on the mRS (**Figure 18A**). Following the same trend observed with temperature, mean ΔRBM3 was positive in those patients who had a good outcome vs negative in those with poor outcome (**Figure 18B**).

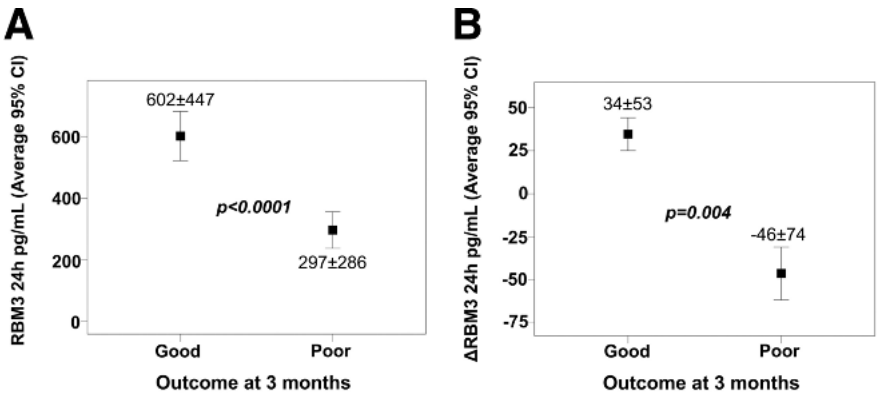


Figure 18. RBM3 is associated with a good outcome and is temperature-dependent. A) RBM3 levels at 24 h and **(B)** ΔRBM3 in IS patients with poor and good clinical outcome at 3 months. ΔRBM3 was defined as % variation of RBM3 at 24 h respect to the basal levels. Self-created image using SPSS 21.0 (IBM, USA).

The predictive potential of RBM3 levels at 24 hours and ΔRBM3 for the functional outcome of IS patients were also tested The AUC of the ROC curves was found to be lower for RBM3 levels at 24 hours (AUC:0.699; 95% CI 0.628-0.770, p<0.0001) compared with the values obtained for ΔRBM3 (AUC:0.836; 95% CI: 0.779-0.893; p<0.0001). RBM3 levels at 24 hours ≥300 pg/mL were associated with good clinical outcome with a sensitivity of 66.7% and a specificity of 66.3%, while the increase in ΔRBM3 ≥10% were associated with good outcome with a sensitivity of 82% and a specificity of 80.4%.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

The independent variables that were associated with poor functional outcome at 3 months were (**Table 7**): age (77.6 ± 9.4 vs 74.5 ± 10.7, p=0.028); temperature at admission (36.5 ± 0.7 ˚C vs 36.3 ± 0.4˚C, p=0.009); maximum temperature at 24 h (37.6 ± 0.7˚C vs 37.1 ± 0.5˚C, p<0.0001); subtype of IS (p<0.001); previous mRS (0 [0,2] vs 0 [0,0], p<0.0001); NIHSS at admission (17 [12,21] vs 11 [8,17],p<0.0001), NIHSS at 24 hours (20 [12,22] vs 2 [1,8],p<0.0001), NIHSS at 48 hours (18 [13,22] vs 2 [1,3],p<0.0001), NIHSS at discharge (6[1,17] vs 2 [0,7],p<0.0001); RBM3 at 24 hours (296.5 ± 286 pg/mL vs 601± 447 pg/mL, p<0.0001); RBM3 at 24 ≥300 pg/mL (33.7% vs 66.7%, p<0.0001); ΔRBM3 (-46.2 ± 73.8 vs 34.6 ± 53.3, p<0.0001) and ΔRBM3≥10 (19.6% vs 82.1 %, p<0.0001).

Table 7. Clinical and biochemical characteristics in patients with poor vs good outcome at 3 months

	Poor outcome n = 92	Good outcome n = 123	*P*
Age, years	77.6 ± 9.4	74.5 ± 10.7	0.028
Women, %	53.3	40.7	0.073
History of hypertension, %	59.8	65.9	0.393
History of diabetes, %	29.3	22.8	0.343
Smoking, %	15.2	14.6	0.527
Dyslipidemia, %	40.2	28.5	0.081
Atrial fibrillation, %	40.2	35.8	0.570
Ischemic heart disease, %	6.5	8.1	0.795
Previous stroke, %	10.9	10.6	0.557
Peripheral arterial disease, %	5.6	9.8	0.164
Systolic blood pressure, mmHg	150.6 ± 26.2	151.9 ± 30.8	0.755
Diastolic blood pressure, mmHg	80.6 ± 17.1	80.7 ± 18.0	0.980
Glycemia, mg/dL	154.4 ± 64.7	141.9 ± 60.3	0.150
Leukocytes, × 10^3/mL	9.6 ± 2.9	9.0 ± 3.2	0.186
Fibrinogen, mg/dL	501.4 ± 152.7	483.2 ± 152.6	0.388
Platelets, × 10^3/mL	217.6 ± 58.9	225.5 ± 79.4	0.421
Axillary temperature at admission, ºC	36.5 ± 0.7	36.3 ± 0.4	0.009
Maximum temperature in first 24 hours, ºC	37.6 ± 0.7	37.1 ± 0.5	<0.0001

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Temperature in first 24 hours <37.5ºC, %	45.7	76.4	<0.0001
Systemic fibrinolysis, %	70.7	76.4	0.351
Endovascular treatment, %	9.8	11.4	0.825
TOAST			0.001
- Atherothrombotic, %	13.0	25.2	
- Cardioembolic, %	53.5	39.8	
- Lacunar, %	2.2	14.6	
- Indeterminate, %	31.5	20.3	
TOAST categorized			<0.0001
- Not cardioembolic, %	15.2	39.8	
- Probably cardioembolic, %	84.8	60.2	
Previous Rankin	0 [0, 2]	0 [0, 0]	<0.0001
NIHSS at admission	17 [12, 21]	11 [8, 17]	<0.0001
NIHSS at 24 hours	20 [12, 22]	2 [1, 8]	<0.0001
NIHSS at 48 hours	18 [13, 22]	2 [1, 13]	<0.0001
NIHSS at discharge	6 [1, 17]	2 [0, 7]	<0.0001
Rankin at 3 months	4 [3, 6]	1 [0, 1]	<0.0001
RBM3 at admission, pg/mL	308.6 ± 207.8	272.7 ± 180.3	0.177
RBM3 at 24 hours, pg/mL	296.5 ± 286.1	601.6 ± 447.1	<0.0001
RBM3 at 24 hours ≥300 pg/mL, %	33.7	66.7	<0.0001
ΔRBM3	-46.2 ± 73.8	34.6 ± 53.3	<0.0001
ΔRBM3 ≥10%, %	19.6	82.1	<0.0001

In addition, logistic regression analysis revealed that an ΔRBM3 value ≥10 (obtained from the ROC curve) was an independent marker of good outcome at 3 months (OR: 26.52; 95% CI 9.32-75.44; p<0.0001), after adjusting for after adjusting for center, age, temperature in the first 24 h, subtype of IS, previous Rankin and NIHSS on admission (**Table 8**).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Table 8. Multivariate analysis. Dependent variable: Clinical outcome at 3 months, not adjusted and adjusted by clinically relevant variables found in our study.

	OR not adjusted	CI 95%	p	OR adjusted	CI 95%	p
Center (cat)	0.45	0.26 - 0.78	0.005	0.84	0.25 - 1.98	0.682
Age	0.97	0.94 - 0.99	0.030	1.00	0.96 - 1.05	0.955
Temperature in the first 24 h <37.5˚C (cat)	3.86	2.15 - 6.92	<0.0001	0.61	0.22 - 1.70	0.341
Cardioembolic vs non-cardioembolic	3.69	1.88 - 7.24	<0.0001	2.05	0.75 - 5.58	0.162
Previous Rankin	0.33	0.22 - 0.51	<0.0001	0.37	0.22 - 0.64	<0.0001
NIHSS on admission	0.84	0.79 - 0.89	<0.0001	0.87	0.81 - 0.95	0.001
ΔRBM3 ≥10%	***18.87***	***9.45 - 37.68***	***<0.0001***	***26.52***	***9.32 - 75.44***	***<0.0001***

OR: Odds Ratio; Cat: categorized

1.4.4. Blood RBM3 levels in cooled IS patients

In the 31 patients from the EuroHYP-1 trial we found no significant differences in RBM3 levels between those treated with hypothermia (n=14) or placebo (n=17), although rewarming levels tended to be higher in those that received active treatment (p=0.131) (**Figure 19A**). Similar results were found (p=0.153) when including only those patients who met pre-defined criteria for per-protocol analyses (**Figure 19B**).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

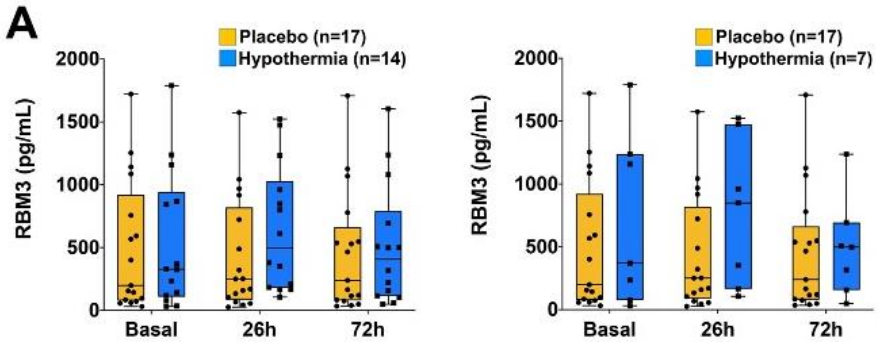


Figure 19. RBM3 levels in patients treated with hypothermia vs placebo. RBM3 levels across the pre-specified collection time-points: basal (before cooling), 26 hours (2 hours after starting rewarming) and 72 hours after stroke onset. Left panel includes all 31 patients and the right panel the systemic hypothermia group only, comprising patients fulfilling pre-defined criteria for per-protocol analyses (n=7). Self-created image using SPSS 21.0 (IBM, USA).

We observed the same trend (p=0.278) towards higher RBM3 levels in cooled patients when comparing ΔRBM3 values between baseline and rewarming (2 hours after completing the 24-hour treatment, i.e. 26 hours) with those in placebo patients (**Figure 20A**) Similar results (p=0.601) were found in patients fulfilling pre-defined criteria for per-protocol analyses (**Figure 20B**).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

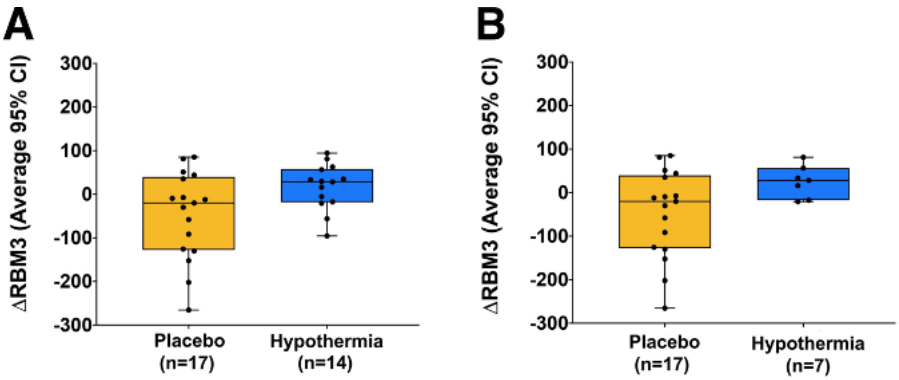


Figure 20. RBM3 levels in patients treated with hypothermia vs placebo. Boxplots represent the median (interquartile range) of ΔRBM3 values between baseline and rewarming time-points in the systemic hypothermia and placebo arms (left, all cases; right, only patients fulfilling pre-defined criteria for per-protocol analyses). Self-created image using SPSS 21.0 (IBM, USA).

No significant correlation was found between RBM3 levels and body temperature except for a negative correlation between RBM3 after rewarming (26 hours) and temperature at 24 hours (R=-0.414, *p*=0.029) (**Figure 21A**). When both groups were analyzed independently, similar results were found in the hypothermic group (R=-0.565, *P*=0.44) for RBM3 after rewarming and body temperature at 24 hours) (**Figure 21B**). This correlation was not present (neither at other time-points) in the placebo group.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

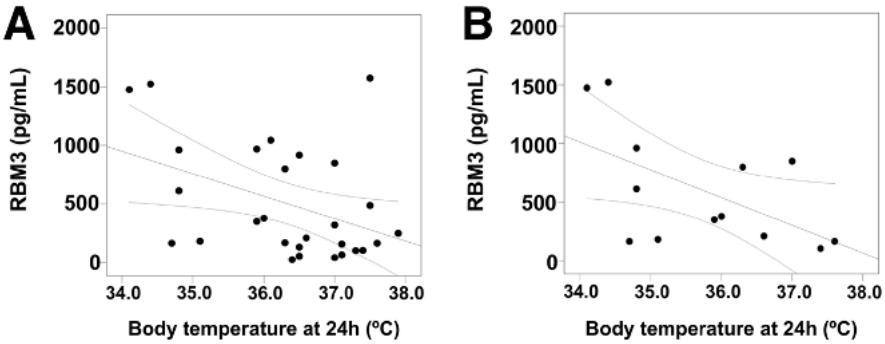


Figure 21. RBM3 levels in patients treated with hypothermia vs placebo. Correlation between RBM3 levels at rewarming and body temperature at 24 hours in all patients (left) and those treated with systemic hypothermia (right). Self-created image using SPSS 21.0 (IBM, USA).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

OBJECTIVE 2.
The impact of temperature on the circulating levels of RBM3 and FGF21 in a cohort of obese stroke patients

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

OBJECTIVE 2.
The impact of temperature on the circulating levels of RBM3 and FGF21 in a cohort of obese stroke patients

2.1. INTRODUCTION

Obesity is a complex, chronic and multifactorial disease with a steadily growing burden in the recent years[412]. The increased risk of cerebral and cardiovascular events, the deterioration in quality of life and scarce treatment options, along with a tremendous economic impact convey the pandemic proportions of this health issue[413,414]. Nonetheless, although obesity has traditionally been associated with the appearance of a myriad of pathological conditions[415,416], some studies have found that this increase in body weight is not necessarily associated with poor prognosis in obese patients who have suffered an ischemic event, a phenomenon known as the **obesity paradox**[417–421]. In this regard, a recent prospective, case-control study from our group showed that obese patients did not have a worse functional outcome than non-obese subjects, and even showed a trend in the improvement of their recovery from neurological symptoms[36].

From a molecular perspective, it has been previously reported that obese patients present higher circulating levels of FGF21[422]. This multimodal function protein, which is mainly expressed in the liver and adipose tissue, acts as a regulator of

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

mechanisms such as lipidic metabolism, torpor and glucose homeostasis[329]. In rodent models, the administration of a recombinant form of FGF21 has been shown to attenuate the obesity-related cognitive decline and to improve functional recovery after cerebral ischemia[387,423,424]. FGF21 also exerts anti-atherosclerotic effects by increasing the expression of the angiotensin-converting enzyme 2 and decreasing macrophage infiltration[425]. Furthermore, FGF21 acts as an inductor of RBM3, a CSP with neuroprotective effects that has been shown to promote brain activity restoration, reduced synaptic loss and anti-apoptotic mechanisms[286,304,325,335,389]. Interestingly, both proteins have been shown to be tightly influenced by temperature variations, as a decrease in body temperature following cold exposure has been shown to cause a marked increase in the expression of both FGF21 and RBM3[278].

2.2. OBJECTIVES

Based on the existing evidence, the present work focuses on the association between circulating levels of RBM3 and FGF21 and the outcome of acute ischemic stroke patients, and the role of body weight and temperature in this response.

2.3. MATERIAL AND METHODS

2.3.1. Study Design

This is an observational study conducted on a registry of patients with ischemic stroke included consecutively and prospectively to our maintained databank, who were admitted to the Stroke Unit of the University Clinical Hospital of Santiago de Compostela (Spain). The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and approved by the Research Ethics Committee of

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Santiago (Project identification code 2019/616). Informed consent was obtained from each patient or from their relatives after full explanation of the procedures.
Inclusion criteria were: 1) Available blood samples on admission and at 72 h, 2) registered temperature measurements 3) registered body mass index (BMI), 4) mRS measurement at 3 months.

The following exclusion criteria were considered: 1) chronic inflammatory disease, 2) previous disability (defined as a score ≥2 in the mRS)), 3) cancer, 4) severe systemic condition that determines a life expectancy lower than 6 months, 5) infectious disease in the last 15 days and 6) continuous anti-inflammatory drugs intake in the last 15 days.

2.3.2. Clinical Variables

All patients were admitted to the Stroke unit and were treated under the Spanish Neurological Society protocol by trained neurologists experienced in cerebrovascular diseases [156]. Patients with alimentation difficulties were evaluated and followed by the Nutrition Unit from the Endocrinology Department following the protocol in our Stroke Unit. Axillary temperature was registered every 6 h by the nursing staff. Based on our previous study, we selected maximum temperature at 24 hours as an independent factor associated with the outcome of patients at 3 months[166]. BMI was calculated by means of the following formula: weight (kg)/height (m^2), and classified according to the WHO cut-off points[414]. Stroke subtypes were registered following the TOAST criteria[12]. The intensity of the neurological deficit was determined by the National Institute of Health Stroke Scale (NIHSS) upon admission to the Stroke Unit and the mRS was used to evaluate the functional deficit. Both scales were

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

measured on admission, at discharge, and at 3 months of follow-up by a certified neurologist.

2.3.3. Blood Samples and Biomarker Assays

For the molecular determinations, venous blood samples were collected in Vacutainer tubes (Becton Dickinson, San Jose, CA, USA) on admission and 72h from stroke onset, according to the ethical protocol approved for our biobank. Blood samples were centrifuged for 10 minutes at 3,000 x g, and the serum was immediately aliquoted, frozen, and stored at −80 °C until analysis. Serum levels of RBM3, FGF21, obesity (leptin and adiponectin) and inflammatory markers (IL-6 and IL-10) were measured by ELISA technique following manufacturer's instructions. For the RBM3 kit (Biotez RBM3 ELISA, Berlin, Germany), the minimum assay sensitivity was 10 pg/mL with an inter and intra-assay coefficient of variation (CV) of 2.6% and 1.9%, respectively. The FGF21 assay kit (Biovendor, Brno, Czech Republic) had a minimum assay sensitivity of 7 pg/mL, with an intra-assay CV of 2% and an inter-assay CV of 3.3%. Leptin (Abnova Corporation, Taipei, Taiwan) and adiponectin (Proteintech Group, Manchester, UK) assay kits had an inter and intra-assay CV<8%. IL-6 and IL-10 were measured by using the IMMULITE 1000 immunodiagnostic system (Siemens Healthcare Global, Los Angeles, CA, USA).

2.3.4. Endpoints

The main endpoint of the present study was the functional outcome at 3 months by the mRS, dichotomized into good outcome (mRS≤2) and poor outcome (mRS >2). Temperature was categorized into mild hypothermia (<36.5 ºC), normothermia (36.5-37.5 ºC) and hyperthermia (>37.5 ºC). Body weight was

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

categorized as follows: Normal weight (BMI <25 kg/m^2), overweight (BMI 25-30 kg/m^2), grade I obesity (BMI 30-35 kg/m^2), obesity grade II (BMI 35-40 kg/m^2 and grade III obesity (BMI >40 kg/m^2)[414].

As secondary endpoints, we studied the association of FGF21 and RBM3 with obesity (leptin, adiponectin) and inflammation (IL-6, IL-10) biomarkers, and their relationship with body weight and temperature.

2.3.5. Statistical Analysis

Categorical variables were expressed with frequency and percentage and as mean (standard deviation [S.D]) or median and interquartile range (25th and 75th percentiles) for the continuous variables, depending on their adjustment to normality. The normal distributions of the sample and the continuous variables were determined using the Kolmogorov-Smirnov test followed by a Lilliefors correction. Statistical inference was then carried out with the chi-square test, t-student or Mann-Whitney according to the nature of the contrast variable and its adjustment to normality. Bivariate correlations were performed using Pearson's or Spearman correlations depending on the distribution of the variable.

The association of RBM3 and FGF21 (on admission and at 72 hours), maximum temperature at 24h and BMI with the outcome at 3 months was evaluated with logistic regression analysis models. Each model was adjusted for the independent variables in the bivariate analysis. Results were expressed as adjusted odds ratios (ORs) their respective confidence interval of 95%. A P value <0.05 was considered significant through all tests. All analyses were conducted in SPSS Statistics 20.0 (IBM, Chicago, IL, USA) for Mac by a researcher blinded to randomization.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

2.4. RESULTS

2.4.1. Sample Description

A total of 66 patients from our biobank who met the inclusion criteria were included in this study. Description of the sample is shown in **Table 9**.

Table 9. Description of the 66 stroke patients analyzed in the present section.

	Variable
Age (years)	67.6 ± 14.6
Female gender (%)	45.5
Arterial hypertension (%)	47.0
Diabetes (%)	28.0
Dyslipemia (%)	37.9
Smoking (%)	22.7
Alcohol consumption (%)	15.2
Atrial fibrillation (%)	33.3
Weight:	
- Normal (BMI <25 kg/m^2) (%)	19.7
- Overweight (BMI 25-30 kg/m^2) (%)	30.3
- Obesity grade I (BMI 30-35 kg/m^2) (%)	31.8
- Obesity grade II (BMI 35-40 kg/m^2) (%)	18.2
Axillary temperature on admission (ºC)	35.7 ± 0.6
Maximum temperature in the first 24 h (ºC)	36.9 ± 0.6
Maximum temperature in the first 24 h (ºC) categorized (%):	
- < 36.5ºC	31.8
- 36.5 - 37.5ºC	48.5
- >37.5ºC	19.7
TOAST (%):	
- Atherothrombotic	10.6
- Cardioembolic	37.9
- Lacunar	6.1
- Indeterminate	45.4
Reperfusion treatment (%)	42.0
NIHSS on admission	9 [3 - 15]
mRS at 3 months	2 [1 - 4]
Outcome at 3 months (%):	
- Good	51.6
- Poor	48.4

The analysis of body weight showed that 31.8% of the subjects were grade I obese patients closely followed by the overweight subjects (30.3%). Normal body weight patients

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

amounted to 19.7% of the sample, while the remainder 18.2% had grade II obesity. Moreover, no patients among the studied sample had grade III obesity. The mean maximum axillary temperature during the first 24 hours was 36.9±0.6 ºC.

Higher levels of FGF21 were found at admission, while the peak for RBM3 expression was at 72h after stroke onset. Based on this data, we performed a correlation analysis to determine the association between the serum levels of FGF21 and RBM3. Pearson bivariate correlation analysis performed for the two measurements in both markers showed a positive correlation between circulating levels of FGF21 and RBM3 upon admission (r=0.409; p=0.001). Furthermore, the highest relationship was observed between basal levels of FGF21 and RBM3 levels at 72h (r= 0.799; p<0.0001) (**Table 10**).

Table 10. Correlation of FGF21 and RBM3 on admission and at 72h.

		Pearson' coefficient	p
Basal RBM3	Basal FGF21	0.409	0.001
	FGF21 72 h	-0.015	0.906
RBM3 72 h	Basal FGF21	0.799	<0.0001
	FGF21 72 h	0.040	0.747

Based on these results, baseline levels of FGF21 and the RBM3 levels at 72h were used for the next analysis (**Figure 22**).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

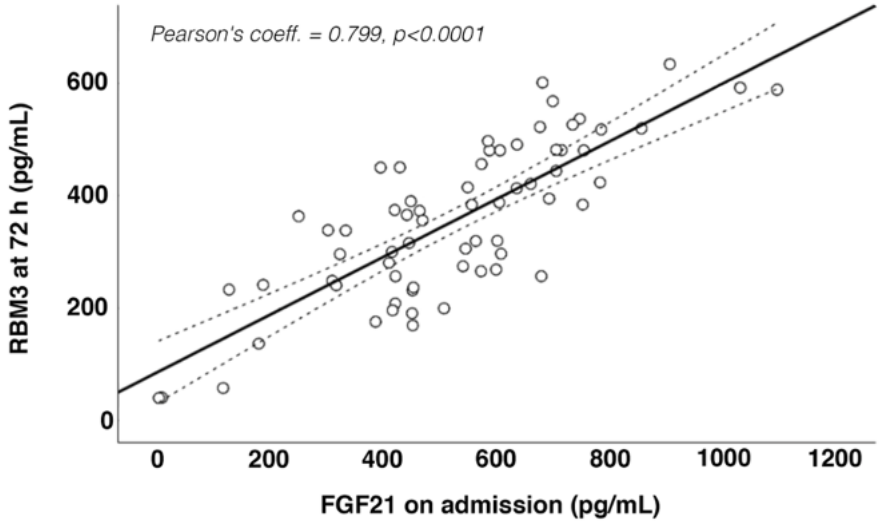


Figure 22. Association of baseline FGF21 and RBM3 at 72h. Self-created image using SPSS 21.0 (IBM, USA).

2.4.2. Analysis of the association between RBM3 and FGF21 with temperature and body weight

Patients with lower body temperature at 24h showed higher levels of FGF21 on admission (625.1 ±178.8 pg/mL) compared with normothermic patients (516.5 ± 207.5 pg/mL), while subjects who developed hyperthermia showed the lowest levels on admission (359.9 ± 191.4 pg/mL) (**Figure 23A**). A similar profile was found for RBM3 at 72 hours for the three temperature ranges (**Figure 23B**). A negative correlation was found between the maximum temperature at 24h and FGF21 on admission (r=-0.735; p<0.0001) and RBM3 at 72h (r=-0.474; p<0.0001).

Regarding body weight, serum levels of FGF21 on admission and RBM3 at 72h were directly proportional to the BMI. Grade II obesity patients had the highest circulating levels of FGF21 (729.6 ± 200.9 pg/mL) and RBM3 (408.8 ± 133.9 pg/mL), while patients with a BMI <25 presented the lowest values

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

(FGF21=311.7 ± 192.9 pg/mL; RBM3= 285.5 ± 122.2 pg/mL) (**Figure 23C and D**).

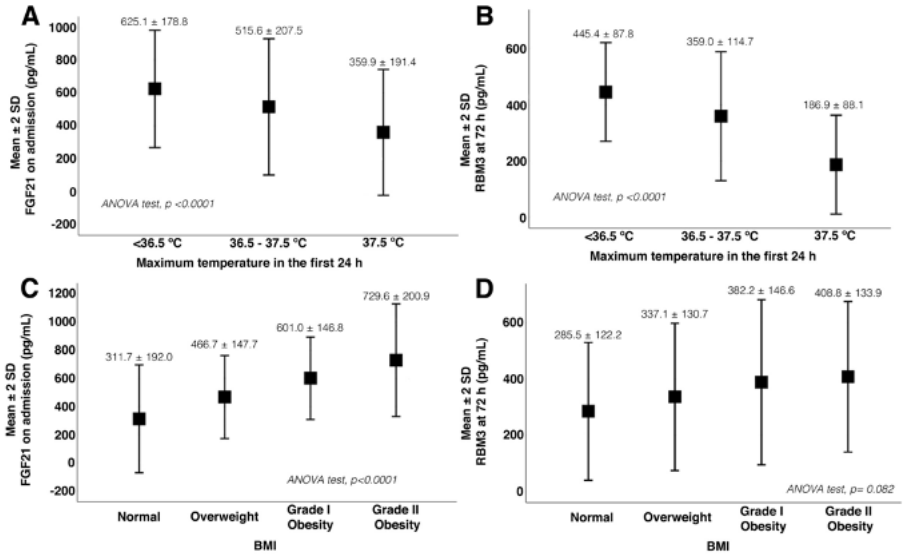


Figure 23. Analysis of serum FGF21 and RBM3 and their association with temperature (a, b) and BMI (c, d). Self-created image using SPSS 21.0 (IBM, USA).

2.4.3. Influence of serum RBM3 and FGF21 on functional outcome

A univariate analysis was performed to evaluate the differences between groups based on the outcome (**Table 11**). No differences were found for BMI or dyslipidemia. Maximum axillary temperature was significantly higher in the poor outcome group (37.2±0.7 ºC) compared to those patients who had a good outcome (36.4±0.6 ºC) (p<0.0001).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Table 11. Univariate analysis of the simple.

	Good outcome n = 34	Poor outcome n = 32	p
Age (years)	59.3 ± 13.0	76.4 ± 10.5	<0.0001
Female gender (%)	41.2	50.0	0.319
Arterial hypertension (%)	38.2	56.3	0.111
Diabetes (%)	15.6	41.2	0.021
Dyslipemia (%)	38.2	37.5	0.576
Smoking (%)	35.3	9.4	0.012
Alcohol consumption (%)	17.6	12.5	0.407
Atrial fibrillation (%)	20.6	46.9	0.022
Body mass index (kg/m^2)	30.2 ± 5.6	29.1 ± 5.1	0.358
Weight:			0.941
- Normal (BMI <25 kg/m^2) (%)	17.6	21.9	
- Overweight (BMI 25-30 kg/m^2) (%)	29.3	31.3	
- Obesity grade I (BMI 30-35 kg/m^2) (%)	32.4	31.3	
- Obesity grade II (BMI 35-40 kg/m^2) (%)	20.6	15.6	
Maximum temperature first 24 h (ºC)	36.4 ± 0.6	37.2 ± 0.7	<0.0001
Maximum temperature first 24 h (ºC) categorized (%):			<0.0001
- < 36.5ºC	61.8	21.9	
- 36.5 - 37.5ºC	35.3	34.4	
- >37.5ºC	2.9	43.8	
TOAST (%):			0.069
- Atherothrombotic	11.8	9.4	
- Cardioembolic	20.6	56.3	
- Lacunar	8.8	3.1	
- Indeterminate	58.7	31.3	
Reperfusion treatment (%)	32.3	53.1	0.072
NIHSS on admission	3 [1 - 9]	14 [10 - 19]	<0.0001
RBM3 at 72 h (pg/mL)	458.9 ± 95.1	252.6 ± 96.0	<0.0001
FGF21 at admission	639.6 ± 179.9	409.7 ± 193.2	<0.0001

Regarding the main biomarkers of the study, higher levels of FGF21 on admission were shown on those patients with good functional outcome at 3 months compared to those with a poor

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

outcome (639.6±179.9 pg/mL versus 409.7±193.2 pg/mL; p<0.0001) (**Figure 24A**). In a similar way, patients who had a good outcome showed higher RBM3 levels at 72h as opposed to the lower levels in the poor outcome group (458.9±95.1 pg/mL versus 252.6±96.0 pg/mL; p<0.001) (**Figure 24B**).

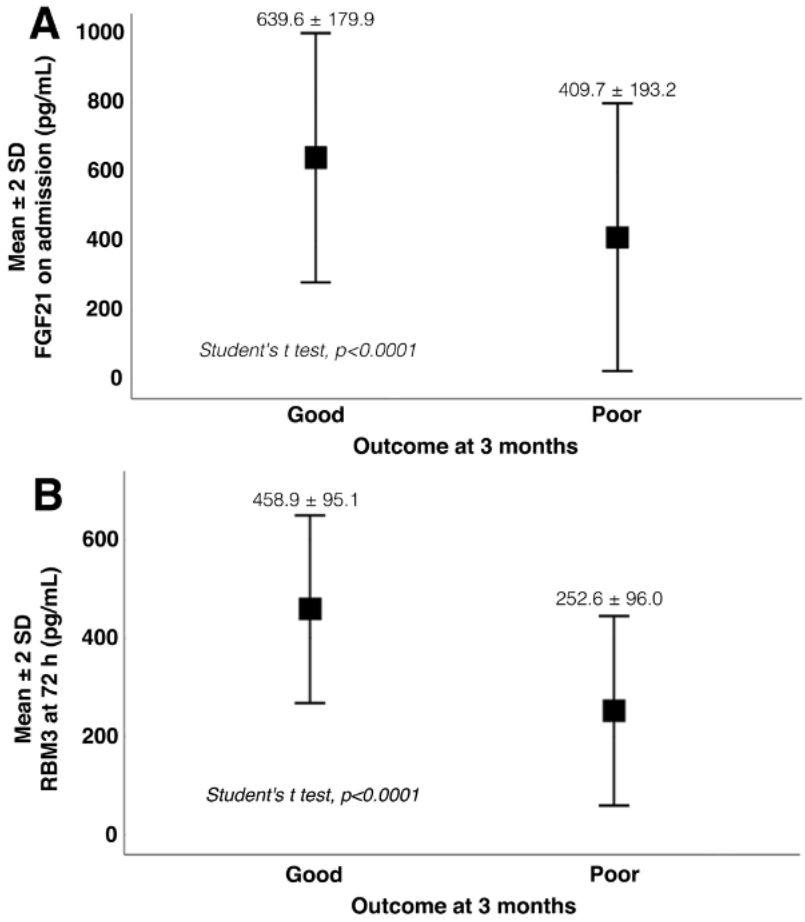


Figure 24. Analysis of serum FGF21 (a) and RBM3 (b) and their association with the outcome of patients. Self-created image using SPSS 21.0 (IBM, USA).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Subsequently, we performed logistic regression models to evaluate the association of clinically relevant variables (age, diabetes, dyslipemia, smoking, atrial fibrillation, body temperature and NIHSS on admission) with the outcome at 3 months. FGF21 on admission was independently associated with good functional outcome at 3 months (OR 0.99, CI 95% 0.99-0.99; p=0.032). A similar association was found for RBM3, as patients with higher levels at 72h had a better outcome at 3 months (OR 0.97, CI 95% 0.95-0.99, p=0.029). In line with our hypothesis, BMI was not associated with the poor outcome of patients at 3 months (OR 0.92, CI 95% 0.79-1.09, p=0.348).

2.4.4. Influence of RBM3 and FGF21 on weight and inflammatory-related markers

Circulating levels of inflammation (IL-6 and IL-10) and obesity (leptin and adiponectin) biomarkers were also analyzed for this study. The levels of the mentioned molecules are described in **Table 12**.

Table 12. Levels of biomarkers on admission and at 72h.

	Basal	72 h	p
IL-6 (pg/mL)	39.71 ± 20.63	43.34 ± 28.00	0.035
IL-10 (pg/mL)	11.92 ± 6.85	13.01 ± 5.38	<0.0001
Leptin (ng/mL)	89.82 ± 31.23	83.82 ± 28.77	<0.0001
Adiponectin (μg/mL)	36.81 ± 17.91	30.84 ± 15.95	<0.0001

On admission, FGF21 levels were positively correlated with IL-6 and leptin (r=0.551, p<0.0001; r=0.677, p<0.0001) and negatively correlated with adiponectin (r= -0.536, p<0.0001). Regarding RBM3, a significant correlation was found with IL-10 at 72h (r= 0.560, p<0.0001). Based on the relationship between FGF21 levels and body weight, we first analyzed its association

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

with leptin and adiponectin. Leptin levels upon admission were positively correlated with baseline FGF21 (r=0.677, p<0.0001) and BMI (r=0.720, p<0.0001). When we categorized the patients by non-obese (normal weight and overweight, n=33) and obese (grade I and II obesity, n=33), a stronger correlation was observed in obese patients (r=0.762, p<0.0001) (**Figure 25**).

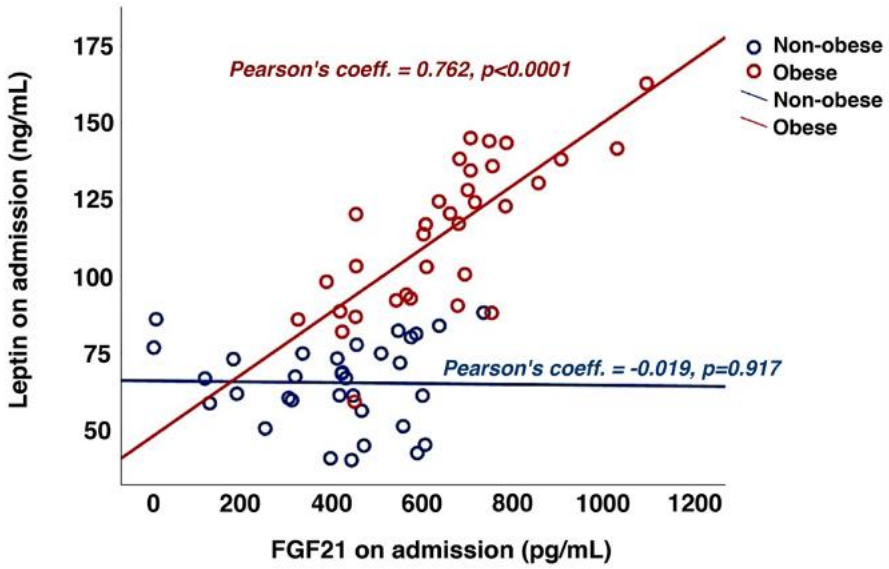


Figure 25. Association between baseline FGF21 and baseline leptin in obese and non-obese subjects. Self-created image using SPSS 21.0 (IBM, USA).

On baseline, adiponectin levels were negatively correlated with FGF21 and BMI (r=-0.536, p<0.0001; r=-0.763, p<0.0001, respectively). Unlike leptin, when the sample was categorized by weight the relationship between baseline levels of FGF21 and adiponectin was only demonstrated for the non-obese patients (r=-0.575; p<0.0001) (**Figure 26**).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

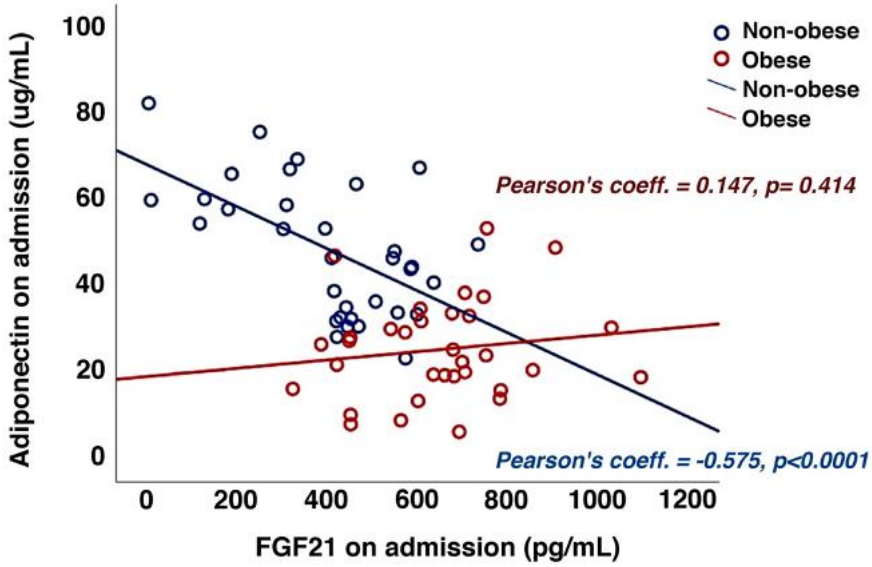


Figure 26. Association between baseline FGF21 and baseline adiponectin in obese and non-obese subjects. Self-created image using SPSS 21.0 (IBM, USA).

Regarding the inflammatory biomarkers, baseline IL-6 levels were significantly associated with baseline FGF21 (r= 0.551, p<0.0001) and body weight (r= 0.632, p<0.0001). When the sample was arranged by weight, a similar trend was found between baseline FGF21 and IL-6 regardless of the BMI, although the association was only significant in obese patients (r= 0.367, p= 0.036) (**Figure 27**).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

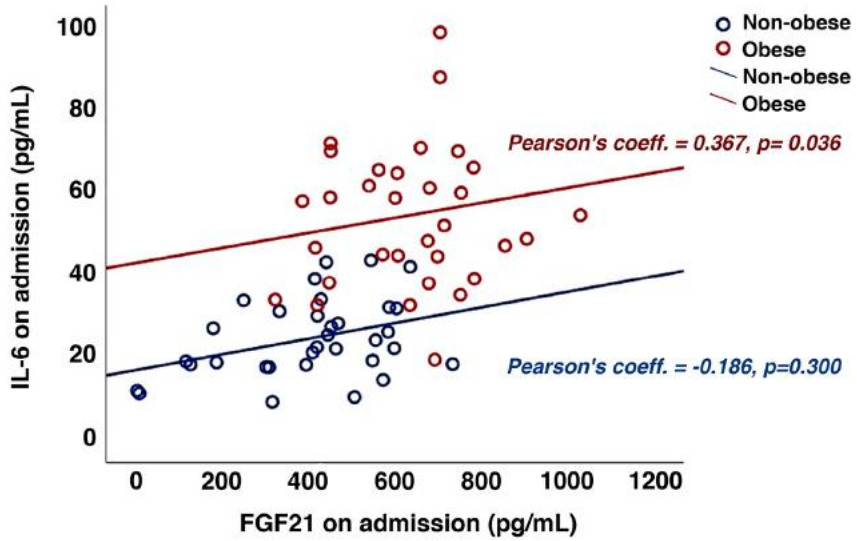


Figure 27. Association between baseline FGF21 and baseline IL-6 in obese and non-obese subjects. Self-created image using SPSS 21.0 (IBM, USA).

Finally, IL-10 levels at 72 h were positively associated with RBM3 circulating levels at 72 h (r= 0.560, p<0.0001). Similar to RBM3, a negative association was found with the maximum temperature during the first 24h (r= -0.417, p<0.0001). When patients were categorized based on their maximum temperature during the first 24h, a positive correlation was found between IL-10 and RBM3 levels at 72h irrespective of the temperature range (**Figure 28**).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

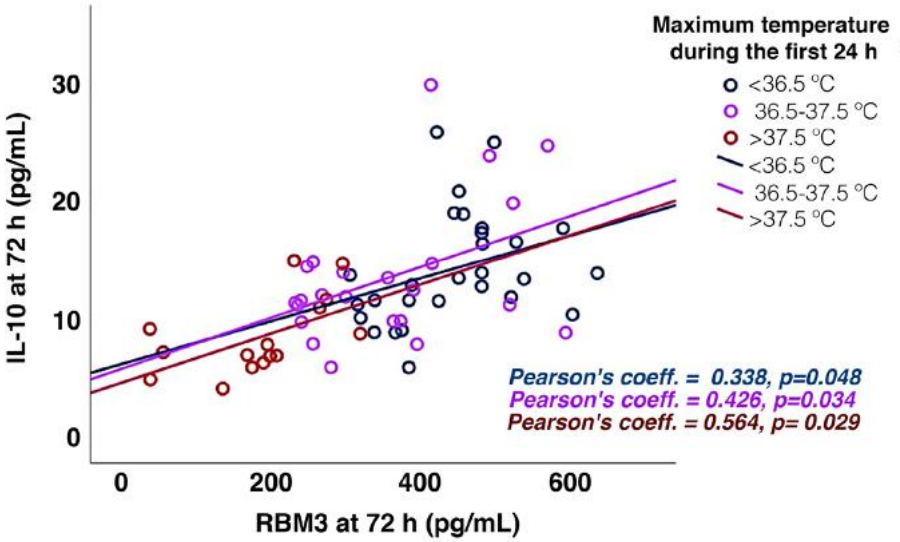


Figure 28. Association of the circulating levels of RBM3 and IL-10 at 72 h with the maximum temperature during the first 24 h. Self-created image using SPSS 21.0 (IBM, USA).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

OBJECTIVE 3. Effect of hypothermia on infarct volume and RBM3 expression in a mice model of cerebral ischemia

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

OBJECTIVE 3. Effect of hypothermia on infarct volume and RBM3 expression in a mice model of cerebral ischemia

3.1. INTRODUCTION

CSPs have emerged as promising neuroprotective candidates in therapeutic hypothermia for neurological diseases. In this regard, our group has demonstrated that RBM3 levels increase in the brain of those animals that were exposed to a cold stimulus following MCAO, and that this treatment was reflected in a reduction in infarct volume[388]. Moreover, we found that focal brain hypothermia for 24h was a safe alternative to a systemic exposure for 4h, with reductions in infarct volume and a similar increase in RBM3 expression. Based on these results, we wanted to optimize this approach in mice due to the feasibility for genetically modified approaches in order to develop protective strategies based on RBM3 modulation.

3.2. OBJECTIVES

The objective of the experimental study of the present work was to study the effect of hypothermia on RBM3 in the setting of cerebral ischemia by means of the optimization of a protocol for the induction of systemic hypothermia in a mouse model of transient MCAO (tMCAO).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

3.3. MATERIAL AND METHODS

3.3.1. Animals

All experimental protocols were approved by the local Animal Care Committee according to the European Union (EU) rules (86/609/CEE, 2003/65/CE and 2010/63/EU, Code: 15010/2019/004). Male C57BL6 mice with a weight of 25-30 g were used for the experiments. Animals were housed in stable environmental conditions (environmental temperature of 23 ºC, relative humidity of 40% and a light-dark cycle of 12h), and access to food and water *ad libitum.* Animals were anesthetized with sevoflurane (5% induction and 3% maintenance in a mixture of 70% N_2O and 30% O_2). Temperature was maintained at 37 ± 0.5°C by means of an electronic thermostat-controlled pad (Neos Biotec) through the experimental procedures and until complete recovery from anesthesia (normal motor activity display). Animal heads were located over a porexpan plate to avoid direct contact between the pad and head.

3.3.2. Mouse model of cerebral ischemia

Transient focal ischemia (50min) was induced by intraluminal occlusion of the MCA, following the method described by Longa et al[426]. Briefly, a midline incision was made in the neck, exposing the carotidal system. The right common carotid artery (CCA) and the external ramification of the CCA (ECA) were permanently ligated, while the internal ramification of the CCA (ICA) was only ligated temporarily (**Figure 29**).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

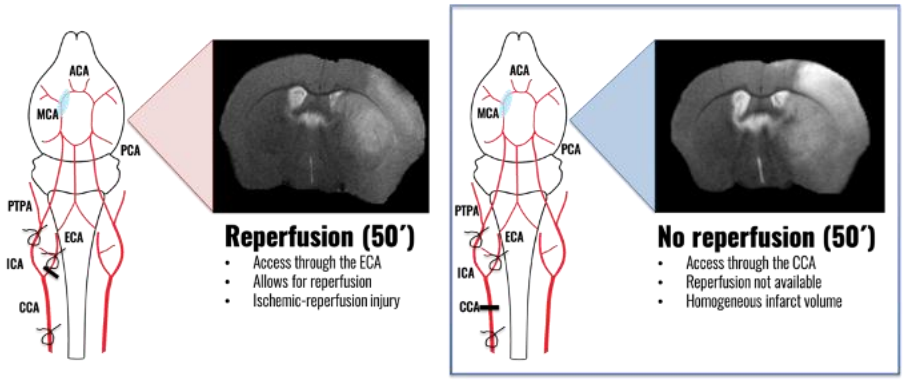


Figure 29. Schematic representation of MCA occlusion with intraluminal filament with T2 infarct volume at 24 hours. Initially, reperfusion of the CCA was also tested but later discontinued as it increased ischemic reperfusion injury and did not produce homogeneous and reproductible infarcts between animals. CCA: common carotid artery; ECA: external carotid artery; ICA: internal carotid artery; PTGP: pterygopalatine artery; PCA: posterior cerebral artery; MCA: middle cerebral artery; ACA: anterior cerebral artery. Self-created image using elements with Creative Common license.

Figure 30 shows a schematic representation of the general procedure. Initially, a commercial suture with a silicon-rubber coated head (Doccol, USA) of 230 μm in diameter and 1.5 mm in length was introduced through the ECA and the ICA by removing temporary the ICA ligature. The suture was placed in the origin of the middle cerebral artery (MCA) blocking the blood flow. The left common carotid artery was left untied to prevent excessive infarct volume and higher mortality. Cortical perfusion was monitored by laser Doppler flowmetry through by means of an optical fiber (MT B500-0 L120, Perimed) placed in the parietal bone right below the temporal muscle, near the sagittal crest. Once the artery was occluded, brain ischemia was maintained for 50 minutes and controlled by doppler signal. Subsequently, the filament was removed and animals were returned to their cages. At 24 hours, infarct volume was assessed

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

by T2 imaging and blood samples were acquired for molecular determinations.

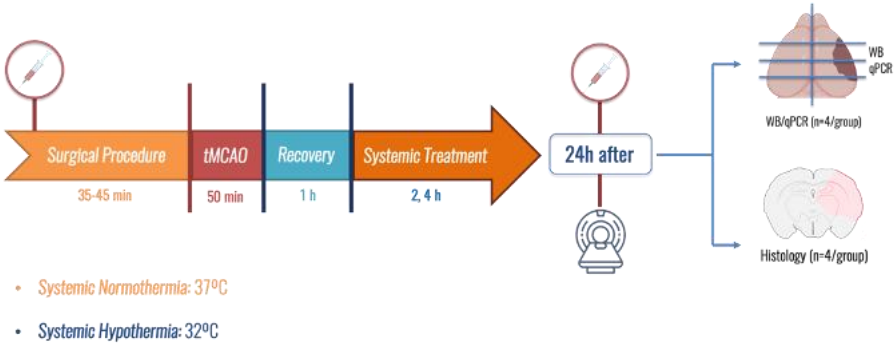


Figure 30. Schematic representation of the surgery. Blood samples were acquired at the beginning of the surgery and before sacrifice at 24 hours. A schematic representation of the brain dissection for western blot (WB)/quantitative polymerase chain-reaction (PCR) analysis is shown on the right side of the figure. Self-created image using elements with Creative Common license.

3.3.3. Exclusion criteria

To guarantee the homogeneity of the animal sample before hypothermic treatments, the following exclusion criteria were used: 1) relative cerebral blood flow reduction lower than 70% when the MCA was occluded, measured by laser Doppler; 2) infarct volume higher than 45% respect to the ipsilateral hemisphere measured in T2; 3) absence of reperfusion or long time reperfusion (more than 10 min until recovery of at least 50% of the basal cerebral blood flow) after filament removing and 5) unable to complete the treatment. All excluded or deceased animals were replaced until the total number of animals indicated for each group was completed.

3.3.4. Experimental groups

After MCA reperfusion, the animals accomplishing the basal study criteria were randomized to the treatment groups by

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

means of computer-generated random numbers. Four main experimental groups were studied (**Figure 31**):

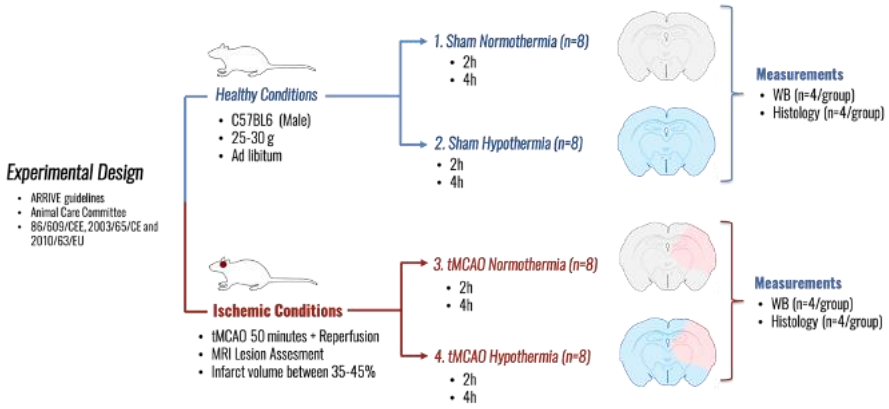


Figure 31. Schematic representation of the experimental design for all studies. Mice were divided in healthy and ischemic conditions. After surgery, animals underwent systemic treatment (normothermia or hypothermia) for 2 or 4 hours depending on group allocation. At 24 hours, animals were randomly assigned to be processed for WB/qPCR studies or for histology studies (n=4/group). Self-created image using elements with Creative Common license.

- Healthy conditions (Sham): Normothermia (37ºC) + Mild hypothermia (32 ºC) (n=6/group). Treatment duration: 2/4 h. One hour after artery reperfusion, anesthetized animals were maintained under normothermic or mild hypothermic conditions (target rectal temperature of 32 and 37ºC, respectively) for 2 or 4 hours. Then, the animals were awoken from anesthesia.

- Ischemic conditions (tMCAO): Normothermia (37ºC) + Mild hypothermia (32 ºC) (n=6/group). Treatment duration: 2/4 h One hour after artery reperfusion, anesthetized animals were maintained under normothermic or mild hypothermic conditions (target rectal temperature of 32 and 37ºC, respectively) for 2 or 4 hours. Then, animals were awoken

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

from anesthesia. Brain ischemic lesion was determined at 24h by means of T2 images. Blood samples were obtained at the beginning of the surgery and at 24 hours after MRI imaging for molecular determinations.

Additionally, based on the peak expression at 3h after cold exposure observed in our previous study[388], we analyzed the expression of RBM3 in a new subset of experiments. The experimental groups were the following:

- Healthy conditions (Sham): Normothermia (37ºC) + Mild hypothermia (32 ºC) (n=3/group). Treatment duration: 4 h. One hour after artery reperfusion, anesthetized animals were maintained under normothermic or mild hypothermic conditions (target rectal temperature of 32 and 37ºC, respectively) for 4 hours. Then, animals were awoken from anesthesia. Three hours after recovery, animals were anesthetized, sacrificed and processed for subsequent analyses.

- Ischemic conditions (tMCAO): Normothermia (37ºC) + Mild hypothermia (32 ºC) (n=3/group). Treatment duration: 4 h. One hour after artery reperfusion, anesthetized animals were maintained under normothermic or mild hypothermic conditions (target rectal temperature of 32 and 37ºC, respectively) for 4 hours. Then, animals were awoken from anesthesia. Three hours after recovery, animals were anesthetized, sacrificed and processed for subsequent analyses.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

3.3.5. MRI assessment and MRI data analysis

MRI studies were conducted on a 9.4 T MR system (Bruker Biospin, Germany) with 440 mT/m gradients, using a combination of a linear birdcage resonator of 7 cm of diameter for signal transmission and a 2x2 arrayed surface coil for signal detection. A quadrature volume coil (7 cm of diameter) was also used in some cases under particular experiment conditions. A quadrature volume coil (7 cm of diameter) was also used in some cases under particular experiment conditions.

To analyze the lesion volume, T2 weighted images were acquired 24 hours after the induction of ischemia (from which T2 relaxation maps were also calculated) using a multi-slice multi-spin-echo sequence (MSME) with the following acquisition parameters: TE=9ms, TR=3s, 16 echos and NA=2. The geometry parameters were 14 consecutive slices of 1 mm thickness with a FOV of 19.2x19.2 mm2 and an in-plane resolution of 0.1 mm/pixel (192x192 points matrix).

All images were processed with ImageJ (Rasband WS, USA) by using an independent computer workstation. Infarct volumes were determined from T2 weighted images (with an average between the echoes 4th to 8th) by a blinded researcher. Edema formation was determined by means of the quantification of the midline deviation represented as the ratio between ipsilateral and contralateral hemispheric volume. Lesion volume in mm^3 was calculated as lesion volume in mm3/MD. Lesion volume in % of ipsilateral hemisphere was calculated as [(lesion volume in mm^3/MD)/ipsilateral hemisphere in mm^3]x100.

3.3.6. Sacrifice and tissue processing

To ensure correct preservation of nucleic acids and proteins, and similar anesthetic dosages before sample collection, all animals

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

undergoing RNA or protein extraction were sacrificed following the same methodology. Briefly, Animals were anesthetized with 5% sevoflurane in a mixture of 70% N_2O and 30% O_2 for approximately 1 minute, enough to halt consciousness and block voluntary motor response. The animals were then sacrificed by decapitation with a guillotine (Fine Science Tools, USA). Mice heads were rapidly positioned on an ice plate, and brains were dissected and positioned in sterile Petri plates for section in two transversal slices, in which the ventral region and lateral cortex were removed. Finally, brain hemispheres were separated by cutting through the interhemispheric line. All brain pieces (approx. 40 mg/each) were inserted into sterile RNase-free cryovials and homogenized in liquid N_2. Frozen samples were stored at -80 ºC for subsequent analysis by WB.

For histological studies, animals were deeply anesthetized and transcardially perfused with 20 mL of phosphate saline buffer (PBS) 0.1M pH 7.4 and 20 mL of 4% formaldehyde. Brains were carefully removed from the skull snf post fixed by immersion in 4% formaldehyde overnight. Then, the brains were washed in PBS and cryoprotected in a 30% sucrose solution in 0.05% sodium azide PBS. Brains were finally snap frozen using isopentane cooled with liquid nitrogen and stored until sectioning. Whole brains were included in OCT resine (Tissue-Tek, Japan) and sectioned in 10 µm thickness slices in a cryostat (Tissue-Tek, Japan).

3.3.7. RBM3 quantification by WB

RBM3 expression was quantified by WB. Cold buffer lysis was added to brain slices in an approximate 10:1 (volume:weight) proportion and the tissue disrupted using a TissueLyser II (Qiagen, Switzerland); lysates were then centrifuged for 30

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

minutes at 20,000 g and the supernatants collected for WB. Lysate protein content was quantified using the Micro BCA™ Protein Assay kit (Thermo Fisher Scientific, USA). The requisite volume of cell lysate containing a total 40 μg protein was subjected to SDS-PAGE in 12% Criterion™ TGX™ Precast Midi Protein Gel (BioRad, USA) using a fixed voltage of 200V. Proteins were then electrotrasferred to a PVDF membrane (Millipore, Ireland) using a Trans-Blot semi-dry system (Bio-Rad, USA) with a limited voltage of 25V and 180 milliamps for 2 hours.

Post-blot membranes were blocked for 1 hour with 3% bovine serum albumin (BSA) in tris-chloride buffer containing 0.1% Tween 20 (TBST). Once blocked, target proteins were detected by incubation with primary antibodies against RBM3 (rabbit monoclonal anti-RBM3 ab134946, Abcam, UK) and β-actin (rabbit polyclonal anti-β-actin ab8227, Abcam, UK) diluted 1:1000 and 1:5000 respectively in TBST with 3% BSA. Primary antibodies were incubated overnight at room temperature with agitation, then the membranes were washed three times in PBST to remove excess primary antibody. Secondary antibody was HRP-conjugated goat anti-rabbit IgG (Dako, Denmark) diluted to 1:5000 in TBST with 3% BSA and incubated for 1 hour at room temperature with agitation. Finally, after washing in TBST, HRP activity was revealed with Pierce™ ECL Western Blotting Substrate (Thermo Fisher, USA) and detected with the ChemiDoc MP Imaging System (Bio-Rad, USA). WB results were analyzed using ImageJ (Rasband WS, USA) by measuring the mean gray value of protein bands delimited in ROIs. Relative expression of RBM3 to β-actin was calculated for each sample, and each group sample average was normalized to the control.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

3.3.8 Statistical Analysis

All preclinical data were analyzed in GraphPad Prism 5.01 as the mean and standard error of the mean. One- or two-way ANOVA followed by post-hoc Bonferroni evaluation was used to determine significant difference between multiple groups, with P set at <0.05. Investigators responsible for the assessment of outcomes (infarct size, behavior, and molecular biology analyses) were blinded to treatment groups (coded animals, samples, and MRI images). Investigators performing surgeries could not be blinded to the hypothermic treatment (systemic and focal) due to the differences in the experimental procedure. Sample size was established based on our previous study where we have already evaluated the effect of systemic and focal hypothermia in the same experimental ischemic animal model.

3.4. RESULTS

3.4.1. Effect of hypothermia on infarct volume

The first objective of the experimental study was to determine the effect of systemic hypothermia previously tested in rats[258] (4h of hypothermia at 32ºC) on infarct volume at 24h in mice who were submitted to cerebral ischemia surgery.

A total of 64 animals were included in the study, of which 16 were excluded. Ten animals died from respiratory complications during systemic treatment, while the remainder 6 died before T2 acquisition at 24h. In ischemic animals, hypothermia caused a marked reduction in mortality rate at 4h (Hypothermic 57.14% vs Normothermic 45.45%). When the exposure time was reduced from 4h to 2h, mortality rate was reduced to 33.33% in hypothermic animals vs 0% normothermic (**Figure 32A**).

Regarding the effect of hypothermia on the progression of the ischemic injury, cool exposure at 32º for 4h effectively

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

reduced infarct volume compared with normothermic controls (35.26 ± 1.46% vs 41.66 ± 1.26%; $p<0.001$). Similarly, ischemic animals who were submitted to systemic hypothermia treatment for 2h after the ischemic injury had a significantly lower infarct volume compared with their normothermic counterparts (33.23 ± 0.43% vs 40.03 ± 1.24%; $p<0.01$) (**Figure 32B**).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

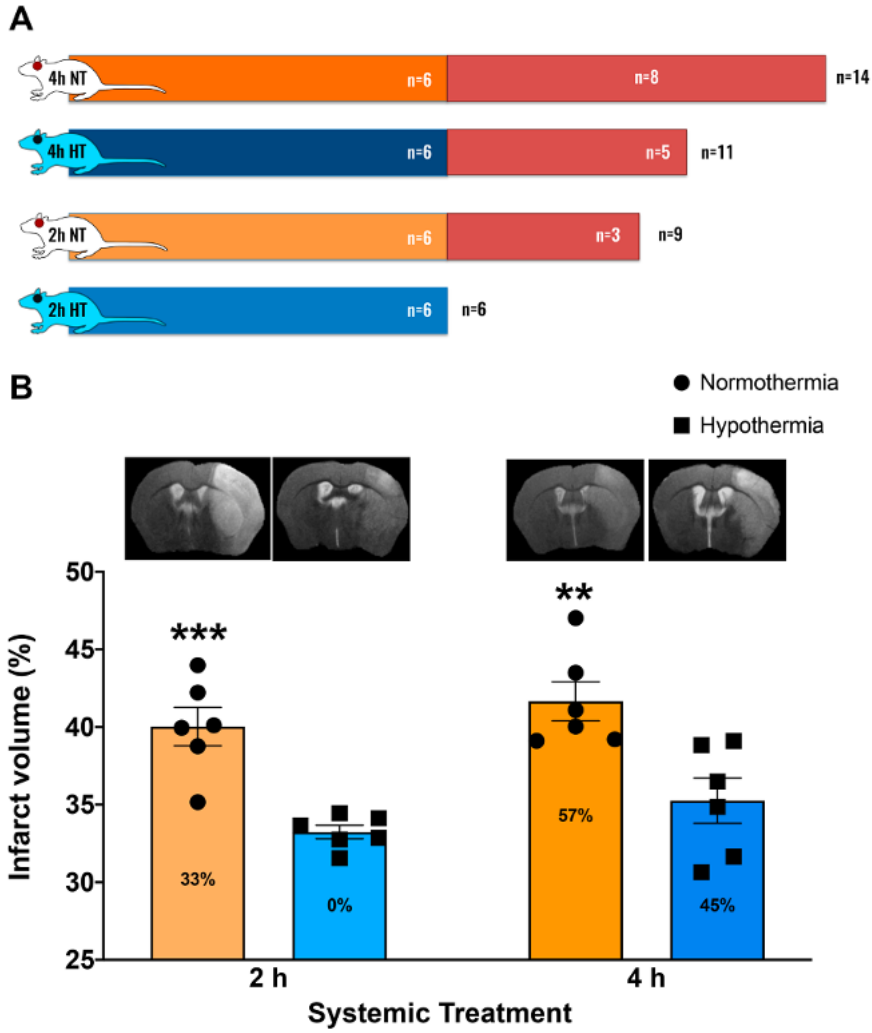


Figure 32. Effect of systemic hypothermia on survival and infarct volume in ischemic animals. (**A**) Animals included in the study after brain ischemia. A total of 32 ischemic animals were included in the study. (**B**) Quantitative analysis of lesion volumes on T2 at 24 hours after 2 or 4 hours of normothermia or hypothermia. Exclusion percentages are shown inside the bars for each respective group. Data are shown as mean ± SEM (n=6/group). Self-created image with GraphPad software.

These results show that 2h of systemic hypothermia are enough to produce a significant reduction in infarct volume with

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

an advantageous lower mortality rate compared with 4h of cold exposure.

3.4.2. Effect of hypothermia on RBM3 expression

Once determined that systemic hypothermia was an effective approach for the reduction of infarct volume in ischemic mice, we wanted to determine if this reduction in infarct volume was related with an increase in brain RBM3.

In sham mice who were not conditioned by cerebral ischemia, those animals who received a 2h treatment of systemic hypothermia had lower levels of RBM3 compared with normothermic mice (Contralateral: 88,8 ± 18.83%; Ipsilateral: 75,27 ± 6.22% vs 100%). Ischemic animals had substantially lower RBM3 levels compared with non-injured animals (Contralateral: 53,59 ± 10.53%; Ipsilateral: 62,74 ± 11.01% vs 100%). Nonetheless, hypothermia induced an increase in RBM3 expression (Contralateral: 71.17 ± 13.66%; Ipsilateral: 84,20 ± 18.34%) compared with normothermic controls (Contralateral: 53,59 ± 10.53%; Ipsilateral: 62,74 ± 11.01%) among injured animals (**Figure 33**).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

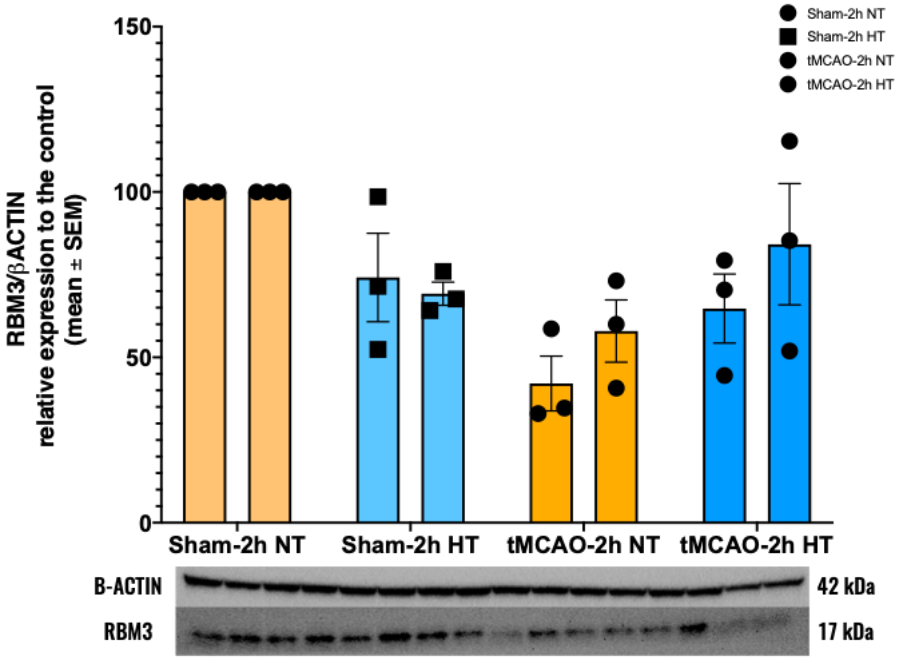


Figure 33. Effect of hypothermia on RBM3 expression at 24 hours. Comparison between sham and ischemic animals who underwent normothermia or hypothermia during 2 hours. Data are shown as mean ± SEM (n=3/group). Self-created image with GraphPad software.

In view of these results, we wanted to determine if there were differences in terms of RBM3 expression between 2h and 4h of cold exposure at 32ºC. In the 2h group, RBM3 levels were slightly higher in hypothermic animals compared with normothermic ischemic mice (Contralateral: 104.35 ± 12.48%; Ipsilateral: 108,99 ± 34.83% vs 100%), although these results did not reach statistical significance. A similar, non-significant trend was found for the 4h treatment group between normothermic (Contralateral: 135.62 ± 66.32%; Ipsilateral: 120.03± 28.34%) and hypothermic animals (Contralateral: 133.47 ± 81.31%; Ipsilateral: 104.93± 13.59%).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Overall, a higher expression of RBM3 was found in hypothermic animals and, in particular, in those animals who had a longer exposure to cold induction. Nonetheless, none of these results reached statistical significance (**Figure 34**).

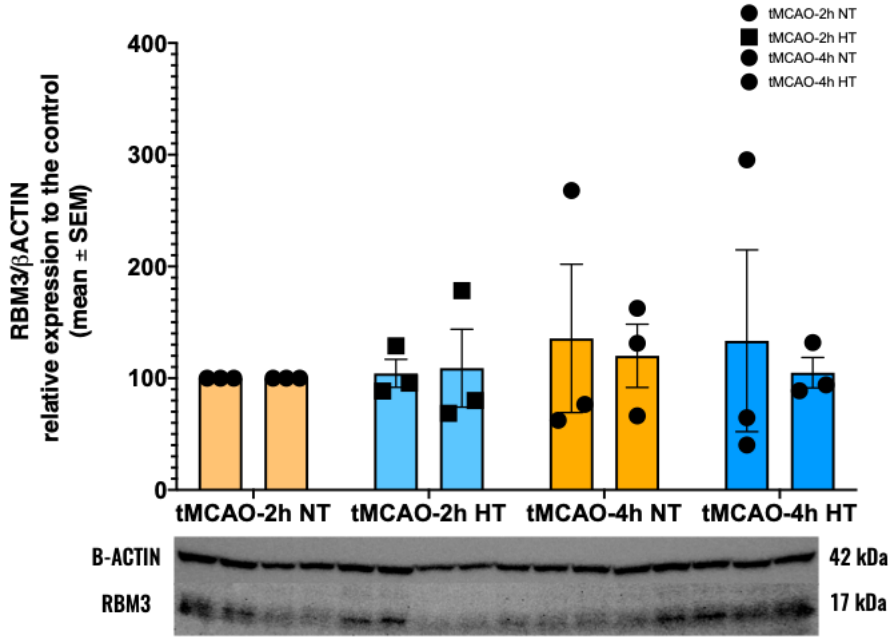


Figure 34. Effect of hypothermia on RBM3 expression at 24 hours. Comparison between systemic treatments during 2 and 4 hours in ischemic animals. Data are shown as mean ± SEM (n=3/group). Self-created image with GraphPad software.

The previous experiments were performed at 24h as a necessity for the infarct assay by T2 sequences via MRI. Nonetheless, previous experiments from our group showed that, in rats, the peak expression for RBM3 after systemic hypothermia was found at 3h following cold exposure[388]. To maximize protein acquisition, we carried out the sacrifice at 3 hours after the systemic treatment. In our experimental conditions, hypothermia did not increase the expression of RBM3 in those animals who were subjected to cerebral ischemia.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Strikingly, the expression of RBM3 was lower in hypothermic animals (Contralateral: 97.01 ± 5.75%; Ipsilateral: 93.04± 5.75%) than in normothermic mice (Contralateral: 107.46 ± 1.20%; Ipsilateral: 105.14± 5.47%) (**Figure 35**).

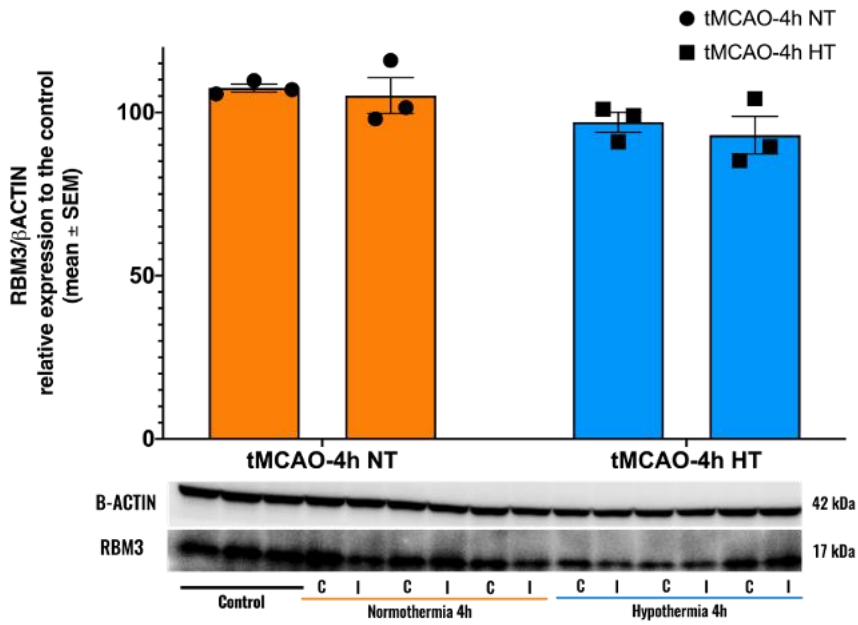


Figure 35. Effect of hypothermia on RBM3 expression at 3 hours on ischemic animals. Comparison between systemic treatments 4 hours in ischemic animals. Data are shown as mean ± SEM (n=3/group). C: Contralateral; I: Ipsilateral. Self-created image with GraphPad software.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

DISCUSSION

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

DISCUSSION

OBJECTIVE 1. TO ANALYZE THE CIRCULATING LEVELS OF RBM3 IN ISCHEMIC STROKE PATIENTS SUBJECTED TO HYPOTHERMIC TREATMENT

Therapeutic hypothermia is one of the most promising neuroprotective strategies in neurological injuries. However, the use of hypothermia in acute neurological disorders entails a series of associated adverse effects that can directly impair the condition of the patients. Moreover, as most acute conditions require sedation *per se*, the introduction of an external stressor such as a cold stimulus requires the administration of anxiolytics or sedatives to stabilize the patient and ensure his survival. Furthermore, although recent evidence suggests that mild hypothermia is the ideal risk-reward depth in therapeutic cooling, the optimal duration and approach for this strategy remains uncertain. To overcome these challenges, focal hypothermia has been proposed as an alternative to whole-body cooling. This approach focuses on the induction of a local decrease in temperature in a given target tissue to attain the therapeutic effect while avoiding compromising side-effects such as shivering or coagulopathies. In this regard, we have recently demonstrated the feasibility and safety of a focal brain hypothermic protocol in cerebral ischemia, where a 24h focal cooling effectively reduced infarct volume in awake animals with tMCAO[258].

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Nonetheless, in spite of the invigorating preclinical results, its applicability in the clinical setting is still debatable. The adult human skull is considerably thicker (≃6.5 mm average) compared with adult rats (≃0.71 mm average) or mice (≃0.34 mm average), which demands dramatically longer and deeper exposures to the cooling inductor to reach the therapeutic range[427–429]. In contrast, the skull thickness of human newborns falls around the ≃1.3 mm, which could be one of the reasons why this strategy has proven effective in conditions such as neonatal hypoxic-ischemic encephalopathy[430,431]. Moreover, a recent systematic review showed that 90% of focal brain hypothermia studies on experimental stroke have been performed under general anesthesia, re-introducing the systemic paradigm into an initially specific approach[432].

Traditionally, the therapeutic effect of acute cooling has been linked to the marked decrease in metabolic rate and reduction of excitotoxicity mechanisms, among others. In fact, this relationship has been thoroughly demonstrated by our group in the setting of ischemic stroke, where hyperthermia and higher circulating levels of glutamate were related to a worse outcome, both in experimental and clinical studies[166,433,434]. Nonetheless, the recent technological advances and improvement in the clinical management of the adverse responses to cold exposure have allowed to delve into novel and promising targets in therapeutic hypothermia beyond the aforementioned mechanisms.

One of these novel targets are CSPs, which have received increasing attention due to their multimodal beneficial effects and their unique behavior following cold exposure. A reduction in core body temperature is usually accompanied by a generalized reduction in protein synthesis in order to preserve cell integrity. Nonetheless, the expression of CSPs is increased

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

following cold stress as they are intrinsic to cold tolerance, playing a key role as a relay defense line when thermogenesis responses are incapable of maintaining normothermia[278]. Among them, the cold-inducible RNA-binding protein (CIRP) and RBM3 are the most documented members of this family in mammals. CIRP was the first CSPs to be described in mammals and was initially found to regulate cell growth *in vitro*[435]. Nonetheless, in vivo studies in animal models of cerebral ischemia found that CIRP contributes to microglial activation, increased TNF-α expression via NFκB and increased infarct volume[299]. Conversely, RBM3 has been shown to exert multimodal neuroprotective functions in neurological injuries, such as inhibition of apoptosis, attenuation of ER stress or protection against neurodegenerative conditions[278]. Nonetheless, the precise role of this neuroprotective candidate in the setting of ischemic stroke had never been assessed before in the clinical setting.

The initial section of the present Thesis represents the first clinical evidence of the association of RBM3 expression and hypothermia in ischemic stroke patients. We found an enhanced expression of RBM3 serum levels at 2h following admission to the Stroke Unit, increasing in the following hours and peaking at 24h. Moreover, no significant differences were found between both centers. Our study failed to demonstrate a relationship between baseline RBM3 levels and temperature. Conversely, we found a strong association between the levels of RBM3 at 24h and temperature, where RBM3 levels were significantly higher in those patients with lower temperatures. Accordingly, hyperthermic patients presented lower levels of both RBM3 and ΔRBM3 at 24h, and we demonstrated that RBM3 levels at 24 hours higher than 300 pg/mL were independently associated

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

with a 16 times higher probability of presenting a lower body temperature. Moreover, the relationship between temperature and RBM3 levels was maintained independently of all factors associated with hyperthermia.

Hyperthermia is a well-described predictor of poor outcome in ischemic stroke, a risk factor for hemorrhagic transformation and a critical trigger in several damaging mechanisms such as glutamate excitotoxicity or inflammation during the acute phase of ischemic stroke[164,166,167,433,434]. In our study, we found that an increase in ΔRBM3 ≥10% was independently associated with a 27 times higher probability of presenting a good outcome at 3 months. The fact that the association of RBM3 with a good outcome was stronger than with temperature itself suggests that RBM3 could be mediating its neuroprotective effects by mechanisms beyond hypothermia. In this regard, a recent study found that RBM3, but not mild hypothermia, protected against apoptosis in neuroblastoma cells[436]. Several clinical studies are in agreement with our results, as higher levels of RBM3 have been associated with prolonged overall survival in intestinal-type gastric cancer, good outcome in invasive breast cancer and colon cancer, and improved survival and response to treatment in metastatic colorectal cancer, and none of these benefits seem to be mediated by temperature[437–440].

Subsequently, we analyzed the levels of RBM3 in blood samples from patients who had been submitted to hypothermic treatment in the EuroHYP-1 clinical trial in ischemic stroke. Although the results did not reach statistical significance, the trend observed in this small subset of 17 patients allowed to confirm the association between RBM3 and hypothermia. The lack of statistical significance could be attributed to the small sample size and restricted eligibility for this type of therapy in

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

the setting of acute ischemic stroke. The EuroHYP-1 was the largest phase III clinical trial ever conducted in Europe and was expected to include up to 1500 patients[236]. Unfortunately, less than 7% of the stablished sample size was enrolled into the study owing to the difficulties of the patients to complete the cooling protocol, the derived adverse effects and the incompatibility between blood sampling and hypothermic induction. As promising as it is, therapeutic hypothermia is not yet a reality in routine management of ischemic stroke, and alternative approaches need to be sought to optimize its application and benefits from its therapeutic effects. Altogether, our results demonstrate that RBM3 is a molecular marker associated with the protective effect of hypothermia, emerging as a promising neuroprotective target in the treatment of ischemic stroke.

OBJECTIVE 2. THE IMPACT OF TEMPERATURE ON THE CIRCULATING LEVELS OF RBM3 AND FGF21 IN A COHORT OF OBESE STROKE PATIENTS

Based on these previous results, the following step was to sought out potential modulators of RBM3 expression that could enhance its expression without relying on hypothermia. A promising candidate for this endeavor was FGF21. This protein had been previously shown to protect against ischemic damage both in preclinical and clinical trials. FGF21 is predominantly expressed in adipose tissue and the liver, and its administration has also proven effective in obesity-related disorders. Moreover, it had recently been shown that although it is intimately related to thermoregulatory processes, it is also capable of inducing a notable overexpression of RBM3 independent of hypothermia[441]. For the aforementioned reasons, our objective was to analyze the relationship of FGF21 and RBM3 in the outcome of ischemic

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

stroke patients, with a special focus on the role of body weight and temperature.

In our cohort, we found a clear correlation between baseline FGF21 and RBM3 at 72h. Furthermore, the circulating levels of both markers decreased as temperature rose. Previous studies have reported that the expression of these proteins is tightly related to temperature, showing a remarkable increase following cold exposure[352,388,441]. In contrast, exposure to hyperthermic temperatures has been shown to blunt the expression of RBM3[287,316]. In this regard, lower body temperatures could influence RBM3 expression either directly or indirectly via FGF21 signaling. Regarding the impact of body weight on this association, we found a strong correlation between baseline FGF21, RBM3 at 72h and BMI. Moreover, the highest and lowest levels of both proteins were found in obese and lean patients, respectively. Beyond its neuroprotective effects, FGF21 plays a key role in thermoregulation during cold stress by inducing non-shivering thermogenesis through WAT browning[442]. In fact, along with the liver and pancreas, fat tissue is one of the main sources of endogenous FGF21[329]. Diet-induced and genetically obese mice have been reported to present a higher expression of FGF21 both in WAT and liver[443,444].

In agreement with our results, clinical studies have previously shown that BMI and FGF21 levels are strongly correlated, and that a decrease in body weight leads to a reduction in plasmatic FGF21[344,422,445]. Nonetheless, these findings do not apply to RBM3, as its expression is not directly related to body weight or fat tissue. In this regard, a recent study in hibernating squirrels found that CIRP, but not RBM3, was upregulated in BAT, liver and skeletal muscle[446]. These findings suggest that the hypothermia-mediated effects of RBM3 are

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

eminently neuroprotective and do not participate in endocrine adaptations, and that the reported increase in RBM3 expression related to body weight is in fact attributed to the increased levels of FGF21.

Regarding the main endpoint of our study, both RBM3 and FGF21 were independently associated with a better outcome at 3 months after an ischemic stroke. As we have previously mentioned, a higher expression of RBM3 has been clinically linked to an improved outcome in different diseases, and the present work has demonstrated its potential as a marker of good prognosis in ischemic stroke patients[438,440,447,448].

In spite of the beneficial effects of FGF21, increased levels of this protein have been reported in patients with metabolically stressful conditions such as obesity, type 2 diabetes mellitus or hypertension[344,449,450]. These findings have prompted speculation about the existence of a FGF21-resistance similar to that of insulin or leptin in obese patients[451]. The presence of FGF21 has been previously associated with atherosclerosis, coronary heart disease and worsened cardiovascular mortality[452–455], and has also been studied as a marker of transplantation in hepatic injury. A recent analysis of two randomized control trials found an association between baseline FGF21 levels and severity, prolonged hospitalization and mortality of pneumonic patients at 30 days[456]. Finally, a recent prospective study found that higher plasma FGF21 was a marker of poor prognosis in acute ischemic stroke patients[457]. Nonetheless, all patients included in both studies had a mean BMI <25 kg/m^2, which could indicate that the beneficial effect of FGF21 found in our sample is primarily mediated by weight.

Our findings suggest that the therapeutic effect of FGF21 in obesity could be mainly mediated by leptin. Previous evidence

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

has shown that acute administration of leptin increased circulating levels of FGF21[458]. In stroke, both markers have been shown to effectively protect against cerebral ischemia by reducing brain edema, infarct volume and macrophage infiltration, along with improving the associated neurological deficits[383,459,460]. Furthermore, FGF21 has been shown to protect against atherosclerosis and hypoxia injury by inducing the expression of adiponectin via PPARγ receptor in adipose tissue[461–463]. Nonetheless, in our study adiponectin was only related to FGF21 levels in non-obese patients.

Regarding the inflammatory markers, IL-6 is a well described biomarker of poor stroke outcome[464,465]. The chronic inflammatory state in obesity could explain the increased levels of IL-6 and their association with FGF21, as roughly 30% of circulating IL-6 is derived from adipose tissue[466]. Finally, we believe that the association of RMB3 and IL-10 is an original contribution to the neuroprotective mechanism associated with low temperatures. While hypothermia has been shown to increase RBM3 expression, it also reduces IL-10 levels[388,467]. Although both molecules have proven neuroprotective, the exact pathways by which these molecules interact are yet to be elucidated.

The present study has some limitations. This is a retrospective study that was conducted on a small sample with a short follow-up period, thus not allowing for stronger statistical analyses. Additional samples from different timepoints (i.e., 48h, 7 days) would allow for a better protein expression profile in serum. Moreover, the use of BMI as an indicator of obesity could affect the analysis as muscle, water and fat percentage is not taken into consideration in its formula. Finally, our study did not include any grade III obesity patients,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

but the trend in our sample suggests that FGF21 and RBM3 would increase accordingly.

In summary, our study shows the influence of FGF21 and RBM3 on the obesity paradox from a molecular point of view, and that this effect is mainly mediated by temperature and body weight.

OBJECTIVE 3. EFFECT OF HYPOTHERMIA ON INFARCT VOLUME AND RBM3 EXPRESSION IN A MICE MODEL OF CEREBRAL ISCHEMIA

The objective of the preclinical study was the optimization of a protocol for the induction of systemic hypothermia in mice as a treatment for tMCAO, similar to our previously published protocol in rats[258,388]. In this work, we have compared the exposure of injured and non-injured mice to cold exposure (32ºC) during 2h or 4h. Our initial results showed a near 30% decrease in the mortality rate between both groups, irrespective of the temperature. Moreover, the hypothermic treatment produced a similar reduction in infarct volume at 24h both with 2h and 4h of treatment. These preliminary results suggested that, in a mice model of tMCAO, 2 hours of mild hypothermia were sufficient to reduce infarct volume while ensuring mice survival thorough the experimental groups. Similar results have been reported in the literature regarding the neuroprotective effect of cold exposure in murine models of cerebral ischemia, with times ranging from 2h up to 6h in the moderate to mild hypothermic range[281,468–471]. Overall, the threshold for a bonafide therapeutic effect following hypothermia in mice appears to be positioned around 2h of exposure. A systematic review and meta-analysis from 2007 showed that across species, the optimal therapeutic effect for cooling was achieved at 31ºC with a median duration of 180 minutes[222]. The next step consisted of the analysis of RBM3

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

expression in the mice brain following cold exposure. We initially started by optimizing and performing total RBM3 protein expression by means of WB. Strikingly, hypothermic animals showed lower levels of RBM3 in the control group. In ischemic animals, 2h of hypothermia induced an increase in RBM3 expression, although none of these results reached statistical significance. Moreover, no differences in RBM3 expression were found between 2h and 4h of cold exposure.

In our previous work, 4h of hypothermia induction following cerebral ischemia induced a robust increase in RBM3 expression, and these results were replicated in a focal hypothermic approach maintained for 24h[388]. Our main assumption for these differences was attributed to the fact that rats in our previous work were sacrificed at 3h, which coincided with the peak in RBM3 expression following hypothermia. Nonetheless, the first group of animals needed to be sacrificed at 24h in order to acquire the T2 images for infarct volume determination. For the subsequent experimental group, we adapted the same experimental protocol as in our previous work in rats (4h of systemic treatment and sacrifice 3h afterwards). Once again, no differences in RBM3 expression were found between normothermic and hypothermic animals.

Several factors could explain these unexpected findings. First, temperature was not monitored during the rewarming phase, as animals were allowed to spontaneously recover in their cages following cold exposure. Rewarming is a delicate phase of therapeutic hypothermia where a fast recovery in body temperature can have detrimental effects to the general procedure[169]. Previous studies have shown that RBM3 expression increases following hypothermia and slowly reverts to baseline levels during rewarming in a time-dependent

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

fashion[472,473]. Moreover, the increase in temperature during uncontrolled rewarming can lead to the development of rebound pyrexia and fever, which could be reflected in a decrease in RBM3 expression[474]. Second, the implied differences between species. Rats have a nearly 10 times higher weight than mice (300 g-rat vs 25-g mouse), better heat insulation and reduced thermal conductance, which allows rats to remain more thermostable both during hypothermic and normothermic temperatures (i.e., during rewarming)[475]. Third, and based on the previous reasons, the temporal profile for RBM3 expression in mice in our experimental conditions is probably deviated from our previous findings in rats. Following studies in RBM3 expression following hypothermia would require the elaboration of an accurate temporal profile with an exhaustive control of temperature during the rewarming phase to maximize protein expression.
Although the sample size of the study was based on our previous experiments, it is nonetheless relatively small and thus could also artifact the observed results. The analysis by qPCR and histology could not be performed within the timeframe of the present work mainly due to logistic and temporal limitations derived from the COVID pandemic. These complimentary analyses would further reinforce our current results and help elucidate the basis of the observed findings.

Beyond the use of hypothermia, future approaches to overexpress RBM3 independent of the hypothermic conundrum are particularly attractive in the setting of ischemic stroke. In this regard, FGF21 is a novel inductor of RBM3 expression and a promising therapeutic target in ischemic stroke. In experimental studies, FGF21 administration has been shown to have a neuroprotective effect in several conditions including type 2 diabetes mellitus, obesity, ischemia/reperfusion injury, hypoxia

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

and ischemic stroke[476]. Moreover, its low binding affinity for heparans allows it to diffuse across the intact BBB, making it an attractive target in neurological diseases[377]. Nonetheless, endogenous FGF21 has a short half-life and is particularly sensitive to proteolytic degradation *in vivo,* which has led to the development of analogues that overcome these technical downsides. Up to this day, four different analogues of FGF21 have been tested in six randomized clinical trials, reporting increased HDL and adiponectin, reduced levels of LDL and triglycerides and an improvement in glycemic control and insulin sensitivity[477].

Overall, the present thesis provides the first clinical evidence of the role of RBM3 as a molecular marker associated with the protective effect of hypothermia in ischemic stroke patients, and highlights the potential application of FGF21 as a promising therapeutic target in the disease.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

CONCLUSIONS

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

CONCLUSIONS

1. RBM3 is an independent marker of good outcome in ischemic stroke patients, and its effect is directly mediated by body temperature.

2. FGF21 is a novel inductor of RBM3 expression. Both hypothermia and increased levels of FGF21 were correlated with higher levels of RBM3.

3. While the effects of RBM3 are clearly related to temperature, its relationship with body weight appears to be strictly mediated by the modulatory effect of FGF21.

4. Both RBM3 and FGF21 were independently associated with a better prognosis at 3 months in ischemic stroke patients.

5. A systemic induction of hypothermia effectively reduced infarct volume in a mice model of cerebral ischemia.

6. The induction of systemic cooling for 2h was enough to reduce infarct volume while improving overall survival compared with the 4h protocol.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

CONCLUSIÓNS

1. RBM3 é un marcador independente de bo prognóstico en doentes con ictus isquémico, e o seu efecto está directamente mediado pola temperatura corporal.

2. FGF21 é un novo inductor da expresión de RBM3. Tanto a hipotermia coma os niveis aumentados de FGF21 correlacionaron cunha maior expresión de RBM3.

3. Mentres que os efectos de RBM3 están claramente relacionados ca temperatura, a súa relación co peso corporal parece estar mediada únicamente polo efecto modulatorio de FGF21.

4. Tanto RBM3 coma FGF21 asociáronse de forma independente cun mellor prognóstico aos 3 meses en doentes con ictus isquémico.

5. Unha indución sistémica de hipotermia diminúe de forma efectiva o volume de infarto nun modelo de isquemia cerebral en rato.

6. Unha aplicación de hipotermia de 2h foi suficiente para reducir o volume de infarto á vez que mellorou a taxa de supervivencia comparado co protocolo de 4h.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

REFERENCES

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

REFERENCES

1. Pound P, Bury M, Ebrahim S. From apoplexy to stroke. *Age Ageing* 1997; 26: 331–337.
2. Clarke E. Apoplexy in the Hippocratic Writings. *Bull Hist Med* 1963; 37: 301–314.
3. Aho K, Harmsen P, Hatano S, Marquardsen J, Smirnov VE, Strasser T. Cerebrovascular disease in the community: results of a WHO Collaborative Study. *Bull World Health Organ* 1980; 58: 113.
4. Sacco RL, Kasner SE, Broderick JP, Caplan LR, Connors JJ, Culebras A, Elkind MSV, George MG, Hamdan AD, Higashida RT, Hoh BL, Janis LS, Kase CS, Kleindorfer DO, Lee JM, Moseley ME, Peterson ED, Turan TN, Valderrama AL, et al. An updated definition of stroke for the 21st century: a statement for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke* 2013; 44: 2064–2089.
5. Johnson CO, Nguyen M, Roth GA, Nichols E, Alam T, Abate D, Abd-Allah F, Abdelalim A, Abraha HN, Abu-Rmeileh NM, Adebayo OM, Adeoye AM, Agarwal G, Agrawal S, Aichour AN, Aichour I, Aichour MTE, Alahdab F, Ali R, et al. Global, regional, and national burden of stroke, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Neurol* 2019; 18: 439–458.
6. Reeves MJ, Bushnell CD, Howard G, Gargano JW, Duncan

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

PW, Lynch G, Khatiwoda A, Lisabeth L. Sex differences in stroke: epidemiology, clinical presentation, medical care, and outcomes. *Lancet Neurol* 2008; 7: 915–926.

7. Kelly-Hayes M. Influence of age and health behaviors on stroke risk: lessons from longitudinal studies. *J Am Geriatr Soc*; 58 Suppl 2. Epub ahead of print October 2010. DOI: 10.1111/J.1532-5415.2010.02915.X.
8. O'Donnell MJ, Denis X, Liu L, Zhang H, Chin SL, Rao-Melacini P, Rangarajan S, Islam S, Pais P, McQueen MJ, Mondo C, Damasceno A, Lopez-Jaramillo P, Hankey GJ, Dans AL, Yusoff K, Truelsen T, Diener HC, Sacco RL, et al. Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries (the INTERSTROKE study): a case-control study. *Lancet (London, England)* 2010; 376: 112–123.
9. Luengo-Fernandez R, Violato M, Candio P, Leal J. Economic burden of stroke across Europe: A population-based cost analysis. *Eur stroke J* 2020; 5: 17–25.
10. Díaz-Guzmán J, Egido JA, Gabriel-Sánchez R, Barberá-Comes G, Fuentes-Gimeno B, Fernández-Pérez C. Stroke and transient ischemic attack incidence rate in Spain: the IBERICTUS study. *Cerebrovasc Dis* 2012; 34: 272–281.
11. Oliva-Moreno J, Peña-Longobardo LM, Mar J, Masjuan J, Soulard S, Gonzalez-Rojas N, Becerra V, Casado MÁ, Torres C, Yebenes M, Quintana M, Alvarez-Sabín J. Determinants of Informal Care, Burden, and Risk of Burnout in Caregivers of Stroke Survivors: The CONOCES Study. *Stroke* 2018; 49: 140–146.
12. Adams HP, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Treatment. *Stroke* 1993; 24: 35–41.
13. Kuriakose D, Xiao Z. Pathophysiology and Treatment of Stroke: Present Status and Future Perspectives. *Int J Mol Sci* 2020; 21: 1–24.
14. Elkind MS, Sacco RL. Stroke risk factors and stroke prevention. *Semin Neurol* 1998; 18: 429–440.
15. Allen CL, Bayraktutan U. Risk factors for ischaemic stroke. *Int J Stroke* 2008; 3: 105–116.
16. Bang OY, Ovbiagele B, Kim JS. Nontraditional Risk Factors for Ischemic Stroke: An Update. *Stroke* 2015; 46: 3571–3578.
17. Feigin VL, Roth GA, Naghavi M, Parmar P, Krishnamurthi R, Chugh S, Mensah GA, Norrving B, Shiue I, Ng M, Estep K, Cercy K, Murray CJL, Forouzanfar MH. Global burden of stroke and risk factors in 188 countries, during 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet Neurol* 2016; 15: 913–924.
18. Buonacera A, Stancanelli B, Malatino L. Stroke and Hypertension: An Appraisal from Pathophysiology to Clinical Practice. *Curr Vasc Pharmacol* 2017; 17: 72–84.
19. Wolf PA, Abbott RD, Kannel WB. Atrial fibrillation as an independent risk factor for stroke: the Framingham Study. *Stroke* 1991; 22: 983–988.
20. Kuller LH, Shemanski L, Psaty BM, Borhani NO, Gardin J, Haan MN, O'Leary DH, Savage PJ, Tell GS, Tracy R. Subclinical disease as an independent risk factor for cardiovascular disease. *Circulation* 1995; 92: 720–726.
21. Wolf PA, D'agostino RB, Kannel WB, Bonita R, Belanger AJ. Cigarette Smoking as a Risk Factor for Stroke: The Framingham Study. *JAMA* 1988; 259: 1025–1029.
22. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C, Mullany EC, Biryukov S, Abbafati C, Abera

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

SF, Abraham JP, Abu-Rmeileh NME, Achoki T, Albuhairan FS, Alemu ZA, Alfonso R, Ali MK, Ali R, Guzman NA, et al. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet (London, England)* 2014; 384: 766–781.

23. Morton GJ, Meek TH, Schwartz MW. Neurobiology of food intake in health and disease. *Nat Rev Neurosci* 2014; 15: 367–378.
24. Ferrante AW, Jr. The Immune Cells in Adipose Tissue. *Diabetes Obes Metab* 2013; 15: 34.
25. Schmidt FM, Weschenfelder J, Sander C, Minkwitz J, Thormann J, Chittka T, Mergl R, Kirkby KC, Faßhauer M, Stumvoll M, Holdt LM, Teupser D, Hegerl U, Himmerich H. Inflammatory Cytokines in General and Central Obesity and Modulating Effects of Physical Activity. *PLoS One*; 10. Epub ahead of print 17 March 2015. doi: 10.1371/journal.pone.0121971.
26. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998; 395: 763–770.
27. Matarese G, Moschos S, Mantzoros CS. Leptin in immunology. *J Immunol* 2005; 174: 3137–3142.
28. Mancuso P. The role of adipokines in chronic inflammation. *ImmunoTargets Ther* 2016; 5: 47–56.
29. Beltowski J. Leptin and atherosclerosis. *Atherosclerosis* 2006; 189: 47–60.
30. Yadav A, Kataria MA, Saini V, Yadav A. Role of leptin and adiponectin in insulin resistance. *Clin Chim Acta* 2013; 417: 80–84.
31. Okamoto Y, Kihara S, Funahashi T, Matsuzawa Y, Libby

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

P. Adiponectin: a key adipocytokine in metabolic syndrome. *Clin Sci (Lond)* 2006; 110: 267–278.
32. Ouchi N, Kihara S, Funahashi T, Matsuzawa Y, Walsh K. Obesity, adiponectin and vascular inflammatory disease. *Curr Opin Lipidol* 2003; 14: 561–566.
33. Luo Y, Liu M. Adiponectin: a versatile player of innate immunity. *J Mol Cell Biol* 2016; 8: 120–128.
34. Li R, Lau WB, Ma XL. Adiponectin resistance and vascular dysfunction in the hyperlipidemic state. *Acta Pharmacol Sin* 2010; 31: 1258.
35. Oesch L, Tatlisumak T, Arnold M, Sarikaya H. Obesity paradox in stroke – Myth or reality? A systematic review. *PLoS One*; 12. Epub ahead of print 1 March 2017. doi: 10.1371/journal.pone.0171334.
36. Rodríguez-Castro E, Rodríguez-Yáñez M, Arias-Rivas S, Santamaría-Cadavid M, López-Dequidt I, Hervella P, López M, Campos F, Sobrino T, Castillo J. Obesity Paradox in Ischemic Stroke: Clinical and Molecular Insights. *Transl Stroke Res* 2019; 10: 639–649.
37. Dirnagl U, Endres M. Found in translation: Preclinical stroke research predicts human pathophysiology, clinical phenotypes, and therapeutic outcomes. *Stroke* 2014; 45: 1510–1518.
38. Mergenthaler P, Lindauer U, Dienel GA, Meisel A. Sugar for the brain: the role of glucose in physiological and pathological brain function. *Trends Neurosci* 2013; 36: 587–597.
39. Sekerdag E, Solaroglu I, Gursoy-Ozdemir Y. Cell Death Mechanisms in Stroke and Novel Molecular and Cellular Treatment Options. *Curr Neuropharmacol* 2018; 16: 1396–1415.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

40. Prins ML. Cerebral metabolic adaptation and ketone metabolism after brain injury. *J Cereb Blood Flow Metab* 2008; 28: 1–16.
41. Bano D, Nicotera P. Ca2+ signals and neuronal death in brain ischemia. *Stroke* 2007; 38: 674–676.
42. Kawamoto EM, Vivar C, Camandola S. Physiology and pathology of calcium signaling in the brain. *Front Pharmacol* 2012; 3 APR: 61.
43. Lai TW, Zhang S, Wang YT. Excitotoxicity and stroke: identifying novel targets for neuroprotection. *Prog Neurobiol* 2014; 115: 157–188.
44. Hardingham GE, Bading H. Synaptic versus extrasynaptic NMDA receptor signalling: implications for neurodegenerative disorders. *Nat Rev Neurosci 2010 1110* 2010; 11: 682–696.
45. Yan J, Peter Bengtson C, Buchthal B, Hagenston AM, Bading H. Coupling of NMDA receptors and TRPM4 guides discovery of unconventional neuroprotectants. *Science*; 370. Epub ahead of print 9 October 2020. doi: 10.1126/science.aay3302.
46. Sanderson TH, Gallaway M, Kumar R. Unfolding the unfolded protein response: unique insights into brain ischemia. *Int J Mol Sci* 2015; 16: 7133–7142.
47. Su Y, Li F. Endoplasmic reticulum stress in brain ischemia. *Int J Neurosci* 2016; 126: 681–691.
48. Kemp K, Poe C. Stressed: The Unfolded Protein Response in T Cell Development, Activation, and Function. *Int J Mol Sci 2019, Vol 20, Page 1792* 2019; 20: 1792.
49. Bartoszewska S, Collawn JF. Unfolded protein response (UPR) integrated signaling networks determine cell fate during hypoxia. *Cell Mol Biol Lett 2020 251* 2020; 25: 1–20.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

50. Charles A, Brennan K. Cortical spreading depression-new insights and persistent questions. *Cephalalgia* 2009; 29: 1115–24.
51. Dohmen C, Sakowitz OW, Fabricius M, Bosche B, Reithmeier T, Ernestus RI, Brinker G, Dreier JP, Woitzik J, Strong AJ, Graf R. Spreading depolarizations occur in human ischemic stroke with high incidence. *Ann Neurol* 2008; 63: 720–728.
52. Østergaard L, Dreier JP, Hadjikhani N, Jespersen SN, Dirnagl U, Dalkara T. Neurovascular Coupling during Cortical Spreading Depolarization and -Depression. *Stroke* 2015; 46: 1392–1401.
53. Dreier JP, Reiffurth C. The stroke-migraine depolarization continuum. *Neuron* 2015; 86: 902–922.
54. Dreier JP, Lemale CL, Kola V, Friedman A, Schoknecht K. Spreading depolarization is not an epiphenomenon but the principal mechanism of the cytotoxic edema in various gray matter structures of the brain during stroke. *Neuropharmacology* 2018; 134: 189–207.
55. Allen CL, Bayraktutan U. Oxidative stress and its role in the pathogenesis of ischaemic stroke. *Int J Stroke* 2009; 4: 461–470.
56. Ighodaro OM, Akinloye OA. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria J Med* 2018; 54: 287–293.
57. Pagano G, Pallardó F V., Lyakhovich A, Tiano L, Fittipaldi MR, Toscanesi M, Trifuoggi M. Aging-Related Disorders and Mitochondrial Dysfunction: A Critical Review for Prospect Mitoprotective Strategies Based on

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Mitochondrial Nutrient Mixtures. *Int J Mol Sci* 2020; 21: 1–16.

58. Chamorro Á, Dirnagl U, Urra X, Planas AM. Neuroprotection in acute stroke: targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. *Lancet Neurol* 2016; 15: 869–881.
59. Orellana-Urzúa S, Rojas I, Líbano L, Rodrigo R. Pathophysiology of Ischemic Stroke: Role of Oxidative Stress. *Curr Pharm Des* 2020; 26: 4246–4260.
60. Manzanero S, Santro T, Arumugam T V. Neuronal oxidative stress in acute ischemic stroke: sources and contribution to cell injury. *Neurochem Int* 2013; 62: 712–718.
61. Daneman R, Prat A. The blood-brain barrier. *Cold Spring Harb Perspect Biol*; 7. Epub ahead of print 1 January 2015. doi: 10.1101/cshperspect.a020412.
62. Obermeier B, Daneman R, Ransohoff RM. Development, maintenance and disruption of the blood-brain barrier. *Nat Med* 2013; 19: 1584–1596.
63. Stamatovic SM, Johnson AM, Keep RF, Andjelkovic A V. Junctional proteins of the blood-brain barrier: New insights into function and dysfunction. *Tissue barriers*; 4. Epub ahead of print 2 January 2016. doi: 10.1080/21688370.2016.1154641.
64. Nian K, Harding IC, Herman IM, Ebong EE. Blood-Brain Barrier Damage in Ischemic Stroke and Its Regulation by Endothelial Mechanotransduction. *Front Physiol*; 11. Epub ahead of print 22 December 2020. doi: 10.3389/fphys.2020.605398.
65. Banks WA. Characteristics of compounds that cross the blood-brain barrier. *BMC Neurol* 2009; 9: S3.
66. Jiang X, Andjelkovic A V., Zhu L, Yang T, Bennett MVL,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Chen J, Keep RF, Shi Y. Blood-brain barrier dysfunction and recovery after ischemic stroke. *Prog Neurobiol* 2018; 163–164: 144–171.

67. Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* 1997; 272: 22642–22647.
68. Chen R, Lai UH, Zhu L, Singh A, Ahmed M, Forsyth NR. Reactive oxygen species formation in the brain at different oxygen levels: The role of hypoxia inducible factors. *Front Cell Dev Biol* 2018; 6: 132.
69. Shi H. Hypoxia inducible factor 1 as a therapeutic target in ischemic stroke. *Curr Med Chem* 2009; 16: 4593–4600.
70. Nakayama K. cAMP-response element-binding protein (CREB) and NF-κB transcription factors are activated during prolonged hypoxia and cooperatively regulate the induction of matrix metalloproteinase MMP1. *J Biol Chem* 2013; 288: 22584–22595.
71. Wu Y, Xu J, Xu J, Zheng W, Chen Q, Jiao D. Study on the mechanism of JAK2/STAT3 signaling pathway-mediated inflammatory reaction after cerebral ischemia. *Mol Med Rep* 2018; 17: 5007.
72. Shubin A V., Demidyuk I V., Komissarov AA, Rafieva LM, Kostrov S V. Cytoplasmic vacuolization in cell death and survival. *Oncotarget* 2016; 7: 55863.
73. Broughton BRS, Reutens DC, Sobey CG. Apoptotic mechanisms after cerebral ischemia. *Stroke*; 40. Epub ahead of print 1 May 2009. doi: 10.1161/strokeaha.108.531632.
74. Datta A, Sarmah D, Mounica L, Kaur H, Kesharwani R, Verma G, Veeresh P, Kotian V, Kalia K, Borah A, Wang X,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Dave KR, Yavagal DR, Bhattacharya P. Cell Death Pathways in Ischemic Stroke and Targeted Pharmacotherapy. *Transl Stroke Res* 2020; 11: 1185–1202.

75. Anrather J, Iadecola C. Inflammation and Stroke: An Overview. *Neurotherapeutics* 2016; 13: 661.
76. Zhao SC, Ma LS, Chu ZH, Xu H, Wu WQ, Liu F. Regulation of microglial activation in stroke. *Acta Pharmacol Sin* 2017; 38: 445.
77. Boulamery A, Desplat-Jégo S. Regulation of neuroinflammation: What role for the tumor necrosis factor-like weak inducer of apoptosis/Fn14 pathway? *Front Immunol* 2017; 8: 1534.
78. Subramaniam M, Koedam JA, Wagner DD. Divergent fates of P- and E-selectins after their expression on the plasma membrane. *https://doi.org/101091/mbc48791* 2017; 4: 791–801.
79. Edwards DN, Bix GJ. The inflammatory response after ischemic stroke: Targeting β2 and β1 integrins. *Front Neurosci* 2019; 13: 540.
80. Petrovic-Djergovic D, Goonewardena SN, Pinsky DJ. Inflammatory Disequilibrium in Stroke. *Circ Res* 2016; 119: 142.
81. Gideon HP, Phuah J, Junecko BA, Mattila JT. Neutrophils express pro- and anti-inflammatory cytokines in granulomas from Mycobacterium tuberculosis-infected cynomolgus macaques. *Mucosal Immunol 2019 126* 2019; 12: 1370–1381.
82. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. *Nat Med* 2011; 17: 796–808.
83. Ma Y, Liu Y, Zhang Z, Yang GY. Significance of Complement System in Ischemic Stroke: A

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Comprehensive Review. *Aging Dis* 2019; 10: 429.
84. Qin X, Akter F, Qin L, Cheng J, Guo M, Yao S, Jian Z, Liu R, Wu S. Adaptive Immunity Regulation and Cerebral Ischemia. *Front Immunol* 2020; 11: 689.
85. Iadecola C, Buckwalter MS, Anrather J. Immune responses to stroke: mechanisms, modulation, and therapeutic potential. *J Clin Invest* 2020; 130: 2777–2788.
86. Pekny M, Nilsson M. Astrocyte activation and reactive gliosis. *Glia* 2005; 50: 427–434.
87. Farina C, Aloisi F, Meinl E. Astrocytes are active players in cerebral innate immunity. *Trends Immunol* 2007; 28: 138–145.
88. Cekanaviciute E, Buckwalter MS. Astrocytes: Integrative Regulators of Neuroinflammation in Stroke and Other Neurological Diseases. *Neurotherapeutics* 2016; 13: 685–701.
89. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, Bennett ML, Münch AE, Chung WS, Peterson TC, Wilton DK, Frouin A, Napier BA, Panicker N, Kumar M, Buckwalter MS, Rowitch DH, Dawson VL, Dawson TM, et al. Neurotoxic reactive astrocytes are induced by activated microglia. *Nat 2017 5417638* 2017; 541: 481–487.
90. Dityatev A, Seidenbecher CI, Schachner M. Compartmentalization from the outside: the extracellular matrix and functional microdomains in the brain. *Trends Neurosci* 2010; 33: 503–512.
91. Kwok JCF, Dick G, Wang D, Fawcett JW. Extracellular matrix and perineuronal nets in CNS repair. *Dev Neurobiol* 2011; 71: 1073–1089.
92. Fernández-Klett F, Priller J. The fibrotic scar in neurological disorders. *Brain Pathol* 2014; 24: 404–413.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

93. Zhu H, Zhang Y, Zhong Y, Ye Y, Hu X, Gu L, Xiong X. Inflammation-Mediated Angiogenesis in Ischemic Stroke. *Front Cell Neurosci* 2021; 15: 57.
94. Rust R. Insights into the dual role of angiogenesis following stroke. *J Cereb Blood Flow Metab* 2020; 40: 1167–1171.
95. Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med* 2002; 8: 963–970.
96. Machalinski B. Tissue regeneration in stroke: cellular and trophic mechanisms. *Expert Rev Neurother* 2014; 14: 957–967.
97. Kreuzberg M, Kanov E, Timofeev O, Schwaninger M, Monyer H, Khodosevich K. Increased subventricular zone-derived cortical neurogenesis after ischemic lesion. *Exp Neurol* 2010; 226: 90–99.
98. Moskowitz MA, Lo EH, Iadecola C. The science of stroke: mechanisms in search of treatments. *Neuron* 2010; 67: 181–198.
99. Moon S, Chang MS, Koh SH, Choi YK. Repair Mechanisms of the Neurovascular Unit after Ischemic Stroke with a Focus on VEGF. *Int J Mol Sci 2021, Vol 22, Page 8543* 2021; 22: 8543.
100. Carey L, Walsh A, Adikari A, Goodin P, Alahakoon D, De Silva D, Ong KL, Nilsson M, Boyd L. Finding the Intersection of Neuroplasticity, Stroke Recovery, and Learning: Scope and Contributions to Stroke Rehabilitation. *Neural Plast*; 2019. Epub ahead of print 2019. doi: 10.1155/2019/5232374.
101. Xing Y, Bai Y. A Review of Exercise-Induced Neuroplasticity in Ischemic Stroke: Pathology and

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Mechanisms. *Mol Neurobiol* 2020; 57: 4218–4231.
102. Weinberger JM. Evolving therapeutic approaches to treating acute ischemic stroke. *J Neurol Sci* 2006; 249: 101–109.
103. Ginsberg MD. Neuroprotection for ischemic stroke: past, present and future. *Neuropharmacology* 2008; 55: 363–389.
104. Fraser JF, Maniskas M, Trout A, Lukins D, Parker L, Stafford WL, Alhajeri A, Roberts J, Bix GJ. Intra-arterial verapamil post-thrombectomy is feasible, safe, and neuroprotective in stroke. *J Cereb Blood Flow Metab* 2017; 37: 3531–3543.
105. Saver JL, Starkman S, Eckstein M, Stratton SJ, Pratt FD, Hamilton S, Conwit R, Liebeskind DS, Sung G, Kramer I, Moreau G, Goldweber R, Sanossian N. Prehospital Use of Magnesium Sulfate as Neuroprotection in Acute Stroke. *N Engl J Med* 2015; 372: 528–536.
106. Leng TD, Si HF, Li J, Yang T, Zhu M, Wang B, Simon RP, Xiong ZG. Amiloride Analogs as ASIC1a Inhibitors. *CNS Neurosci Ther* 2016; 22: 468–476.
107. Jia M, Njapo SAN, Rastogi V, Hedna VS. Taming glutamate excitotoxicity: strategic pathway modulation for neuroprotection. *CNS Drugs* 2015; 29: 153–162.
108. Karsy M, Brock A, Guan J, Taussky P, Yashar M, Park MS. Neuroprotective strategies and the underlying molecular basis of cerebrovascular stroke. *Neurosurg Focus* 2017; 42: E3.
109. Castillo J, Loza MI, Mirelman D, Brea J, Blanco M, Sobrino T, Campos F. A novel mechanism of neuroprotection: Blood glutamate grabber. *J Cereb Blood Flow Metab* 2016; 36: 292–301.
110. Liu J, Zhang J, Wang LN. Gamma aminobutyric acid

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

(GABA) receptor agonists for acute stroke. *Cochrane database Syst Rev*; 10. Epub ahead of print 30 October 2018. doi: 10.1002/14651858.cd009622.pub5.

111. Pérez-Mato M, Ramos-Cabrer P, Sobrino T, Blanco M, Ruban A, Mirelman D, Menendez P, Castillo J, Campos F. Human recombinant glutamate oxaloacetate transaminase 1 (GOT1) supplemented with oxaloacetate induces a protective effect after cerebral ischemia. *Cell Death Dis*; 5. Epub ahead of print January 2014. doi: 10.1038/cddis.2013.507.
112. Campos F, Sobrino T, Ramos-Cabrer P, Castellanos M, Blanco M, Rodríguez-Yáñez M, Serena J, Leira R, Castillo J. High blood glutamate oxaloacetate transaminase levels are associated with good functional outcome in acute ischemic stroke. *J Cereb Blood Flow Metab* 2011; 31: 1387–1393.
113. Matchett GA, Allard MW, Martin RD, Zhang JH. Neuroprotective effect of volatile anesthetic agents: molecular mechanisms. *Neurol Res* 2009; 31: 128–134.
114. Shirle R, Ord ENJ, Work LM. Oxidative Stress and the Use of Antioxidants in Stroke. *Antioxidants 2014, Vol 3, Pages 472-501* 2014; 3: 472–501.
115. Mallah K, Couch C, Borucki DM, Toutonji A, Alshareef M, Tomlinson S. Anti-inflammatory and Neuroprotective Agents in Clinical Trials for CNS Disease and Injury: Where Do We Go From Here? *Front Immunol* 2020; 11: 2021.
116. Smith CJ, Hulme S, Vail A, Heal C, Parry-Jones AR, Scarth S, Hopkins K, Hoadley M, Allan SM, Rothwell NJ, Hopkins SJ, Tyrrell PJ. SCIL-STROKE (subcutaneous interleukin-1 receptor antagonist in ischemic stroke): A

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

randomized controlled phase 2 trial. *Stroke* 2018; 49: 1210–1216.

117. Campbell BCV, De Silva DA, Macleod MR, Coutts SB, Schwamm LH, Davis SM, Donnan GA. Ischaemic stroke. *Nat Rev Dis Prim 2019 51* 2019; 5: 1–22.
118. Campos F, Qin T, Castillo J, Seo JH, Arai K, Lo EH, Waeber C. Fingolimod reduces hemorrhagic transformation associated with delayed tissue plasminogen activator treatment in a mouse thromboembolic model. *Stroke* 2013; 44: 505–511.
119. Fu Y, Zhang N, Ren L, Yan Y, Sun N, Li YJ, Han W, Xue R, Liu Q, Hao J, Yu C, Shi FD. Impact of an immune modulator fingolimod on acute ischemic stroke. *Proc Natl Acad Sci U S A* 2014; 111: 18315–18320.
120. Veltkamp R, Gill D. Clinical Trials of Immunomodulation in Ischemic Stroke. *Neurotherapeutics* 2016; 13: 791–800.
121. Feeney DM, Gonzalez A, Law WA. Amphetamine, haloperidol, and experience interact to affect rate of recovery after motor cortex injury. *Science* 1982; 217: 855–857.
122. Crisostomo EA, Duncan PW, Propst M, Dawson D V., Davis JN. Evidence that amphetamine with physical therapy promotes recovery of motor function in stroke patients. *Ann Neurol* 1988; 23: 94–97.
123. Walker-Batson D, Smith P, Curtis S, Unwin H, Greenlee R. Amphetamine Paired With Physical Therapy Accelerates Motor Recovery After Stroke. *Stroke* 1995; 26: 2254–2259.
124. Scheidtmann K, Fries W, Müller F, Koenig E. Effect of levodopa in combination with physiotherapy on functional motor recovery after stroke: a prospective, randomised, double-blind study. *Lancet (London, England)*

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

2001; 358: 787–790.

125. Cramer SC, Dobkin BH, Noser EA, Rodriguez RW, Enney LA. Randomized, placebo-controlled, double-blind study of ropinirole in chronic stroke. *Stroke* 2009; 40: 3034–3038.
126. Chollet F, Tardy J, Albucher JF, Thalamas C, Berard E, Lamy C, Bejot Y, Deltour S, Jaillard A, Niclot P, Guillon B, Moulin T, Marque P, Pariente J, Arnaud C, Loubinoux I. Fluoxetine for motor recovery after acute ischaemic stroke (FLAME): a randomised placebo-controlled trial. *Lancet Neurol* 2011; 10: 123–130.
127. McCann SK, Irvine C, Mead GE, Sena ES, Currie GL, Egan KE, Macleod MR, Howells DW. Efficacy of antidepressants in animal models of ischemic stroke: A systematic review and meta-analysis. *Stroke* 2014; 45: 3055–3063.
128. Regenhardt RW, Das AS, Stapleton CJ, Chandra R V., Rabinov JD, Patel AB, Hirsch JA, Leslie-Mazwi TM. Blood pressure and penumbral sustenance in stroke from large vessel occlusion. *Front Neurol* 2017; 8: 317.
129. Zhang R, Wang Y, Zhang L, Zhang Z, Tsang W, Lu M, Zhang L, Chopp M. Sildenafil (Viagra) induces neurogenesis and promotes functional recovery after stroke in rats. *Stroke* 2002; 33: 2675–2680.
130. Silver B, McCarthy S, Lu M, Mitsias P, Russman AN, Katramados A, Morris DC, Lewandowski CA, Chopp M. Sildenafil treatment of subacute ischemic stroke: a safety study at 25-mg daily for 2 weeks. *J Stroke Cerebrovasc Dis* 2009; 18: 381–383.
131. Woods D, Jiang Q, Chu X-P. Monoclonal antibody as an emerging therapy for acute ischemic stroke. *Int J Physiol Pathophysiol Pharmacol* 2020; 12: 95.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

132. England TJ, Abaei M, Auer DP, Lowe J, Jones DRE, Sare G, Walker M, Bath PMW. Granulocyte-colony stimulating factor for mobilizing bone marrow stem cells in subacute stroke: The stem cell trial of recovery enhancement after stroke 2 randomized controlled trial. *Stroke* 2012; 43: 405–411.
133. Ringelstein EB, Thijs V, Norrving B, Chamorro A, Aichner F, Grond M, Saver J, Laage R, Schneider A, Rathgeb F, Vogt G, Charissé G, Fiebach JB, Schwab S, Schäbitz WR, Kollmar R, Fisher M, Brozman M, Skoloudik D, et al. Granulocyte colony-stimulating factor in patients with acute ischemic stroke: results of the AX200 for Ischemic Stroke trial. *Stroke* 2013; 44: 2681–2687.
134. England TJ, Sprigg N, Alasheev AM, Belkin AA, Kumar A, Prasad K, Bath PM. Granulocyte-Colony Stimulating Factor (G-CSF) for stroke: an individual patient data meta-analysis. *Sci Reports 2016 61* 2016; 6: 1–7.
135. Jerndal M, Forsberg K, Sena ES, MacLeod MR, O'Collins VE, Linden T, Nilsson M, Howells DW. A systematic review and meta-analysis of erythropoietin in experimental stroke. *J Cereb Blood Flow Metab Off J Int Soc Cereb Blood Flow Metab* 2010; 30: 961.
136. Cramer SC, Fitzpatrick C, Warren M, Hill MD, Brown D, Whitaker L, Ryckborst KJ, Plon L. The beta-hCG+erythropoietin in acute stroke (BETAS) study: A 3-center, single-dose, open-label, noncontrolled, phase IIa safety trial. *Stroke* 2010; 41: 927–931.
137. Cramer SC, Hill MD. Human choriogonadotropin and epoetin alfa in acute ischemic stroke patients (REGENESIS-LED trial). *Int J Stroke* 2014; 9: 321–327.
138. Dassan P, Brown MM, Gregoire SM, Keir G, Werring DJ.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Association of cerebral microbleeds in acute ischemic stroke with high serum levels of vascular endothelial growth factor. *Arch Neurol* 2012; 69: 1186–1189.

139. Park YJ, Niizuma K, Mokin M, Dezawa M, Borlongan C V. Cell-Based Therapy for Stroke: Musing with Muse Cells. *Stroke* 2020; 2854–2862.
140. Vu Q, Xie K, Eckert M, Zhao W, Cramer SC. Meta-analysis of preclinical studies of mesenchymal stromal cells for ischemic stroke. *Neurology* 2014; 82: 1277.
141. Li Z, Dong X, Tian M, Liu C, Wang K, Li L, Liu Z, Liu J. Stem cell-based therapies for ischemic stroke: a systematic review and meta-analysis of clinical trials. *Stem Cell Res Ther* 2020; 11: 252.
142. Sadanandan N, Cozene B, Cho J, Park YJ, Saft M, Gonzales-Portillo B, Borlongan C V. Melatonin—A Potent Therapeutic for Stroke and Stroke-Related Dementia. *Antioxidants 2020, Vol 9, Page 672* 2020; 9: 672.
143. Cramer SC. The EXCITE trial: A major step forward for restorative therapies in stroke. *Stroke* 2007; 38: 2204–2205.
144. Duncan PW, Sullivan KJ, Behrman AL, Azen SP, Wu SS, Nadeau SE, Dobkin BH, Rose DK, Tilson JK. Protocol for the Locomotor Experience Applied Post-stroke (LEAPS) trial: a randomized controlled trial. *BMC Neurol* 2007; 7: 39.
145. Dromerick AW, Lang CE, Birkenmeier RL, Wagner JM, Miller JP, Videen TO, Powers WJ, Wolf SL, Edwards DF. Very Early Constraint-Induced Movement during Stroke Rehabilitation (VECTORS): A single-center RCT. *Neurology* 2009; 73: 195.
146. Gravanis I, Tsirka SE. Tissue-type plasminogen activator as a therapeutic target in stroke. *Expert Opin Ther Targets* 2008; 12: 159–170.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

147. Lin MP, Sanossian N. Reperfusion therapy in the acute management of ischemic stroke. *Cardiol Clin* 2015; 33: 99–109.
148. Marler JR. NINDS clinical trials in stroke: Lessons learned and future directions. *Stroke* 2007; 38: 3302–3307.
149. Dorado L, Millán M, Dávalos A. Reperfusion Therapies for Acute Ischemic Stroke: An Update. *Curr Cardiol Rev* 2014; 10: 327.
150. Hill MD, Goyal M, Demchuk AM, Fisher M. Ischemic Stroke Tissue-Window in the New Era of Endovascular Treatment. *Stroke* 2015; 46: 2332–2334.
151. Goyal M, Demchuk AM, Menon BK, Eesa M, Rempel JL, Thornton J, Roy D, Jovin TG, Willinsky RA, Sapkota BL, Dowlatshahi D, Frei DF, Kamal NR, Montanera WJ, Poppe AY, Ryckborst KJ, Silver FL, Shuaib A, Tampieri D, et al. Randomized Assessment of Rapid Endovascular Treatment of Ischemic Stroke. *N Engl J Med* 2015; 372: 1019–1030.
152. Nogueira RG, Jadhav AP, Haussen DC, Bonafe A, Budzik RF, Bhuva P, Yavagal DR, Ribo M, Cognard C, Hanel RA, Sila CA, Hassan AE, Millan M, Levy EI, Mitchell P, Chen M, English JD, Shah QA, Silver FL, et al. Thrombectomy 6 to 24 Hours after Stroke with a Mismatch between Deficit and Infarct. *N Engl J Med* 2018; 378: 11–21.
153. McManus M, Liebeskind DS. Blood Pressure in Acute Ischemic Stroke. *J Clin Neurol* 2016; 12: 137–146.
154. Hankey GJ. The benefits of aspirin in early secondary stroke prevention. *Lancet (London, England)* 2016; 388: 312–314.
155. Lindsberg PJ, Roine RO. Hyperglycemia in Acute Stroke. *Stroke* 2004; 35: 363–364.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

156. Alonso de Leciñana M, Egido JA, Casado I, Ribó M, Dávalos A, Masjuan J, Caniego JL, Martínez Vila E, Díez Tejedor E, Fuentes Secretaría B, Álvarez-Sabin J, Arenillas J, Calleja S, Castellanos M, Castillo J, Díaz-Otero F, López-Fernández JC, Freijo M, Gállego J, et al. Guidelines for the treatment of acute ischaemic stroke. *Neurologia* 2014; 29: 102–122.
157. González-Moreno EI, Cámara-Lemarroy CR, González-González JG, Góngora-Rivera F. Glycemic variability and acute ischemic stroke: the missing link? *Transl Stroke Res* 2014; 5: 638–646.
158. Dávalos A, Toni D, Iweins F, Lesaffre E, Bastianello S, Castillo J. Neurological Deterioration in Acute Ischemic Stroke. *Stroke* 1999; 30: 2631–2636.
159. Bousser MG, Ferro JM. Cerebral venous thrombosis: an update. *Lancet Neurol* 2007; 6: 162–170.
160. Brozici M, Van der Zwan A, Hillen B. Anatomy and Functionality of Leptomeningeal Anastomoses: A Review. *Stroke* 2003; 34: 2750–2762.
161. Castillo J, Martinez F, Leiro R, Prieto JM, Lema M, Noya M. Mortality and Morbidity of Acute Cerebral Infarction Related to Temperature and Basal Analytic Parameters. *Cerebrovasc Dis* 1994; 4: 66–71.
162. Castillo J, Dávalos A, Marrugat J, Noya M. Timing for fever-related brain damage in acute ischemic stroke. *Stroke* 1998; 29: 2455–2460.
163. Greer DM, Funk SE, Reaven NL, Ouzounelli M, Uman GC. Impact of fever on outcome in patients with stroke and neurologic injury: a comprehensive meta-analysis. *Stroke* 2008; 39: 3029–3035.
164. Millán M, Grau L, Castellanos M, Rodríguez-Yáñez M,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Arenillas JF, Nombela F, Pérez De La Ossa N, López-Manzanares L, Serena J, Castillo J, Dávalos A. Body temperature and response to thrombolytic therapy in acute ischaemic stroke. *Eur J Neurol* 2008; 15: 1384–1389.

165. Polderman KH. Induced hypothermia and fever control for prevention and treatment of neurological injuries. *Lancet (London, England)* 2008; 371: 1955–1969.
166. Blanco M, Campos F, Rodríguez-Yáñez M, Arias S, Fernández-Ferro J, Gómez-Sánchez JC, Castillo J. Neuroprotection or Increased Brain Damage Mediated by Temperature in Stroke Is Time Dependent. *PLoS One* 2012; 7: e30700.
167. Leira R, Sobrino T, Blanco M, Campos F, Rodríguez-Ýñez M, Castellanos M, Moldes O, Millán M, Dávalos A, Castillo J. A higher body temperature is associated with haemorrhagic transformation in patients with acute stroke untreated with recombinant tissue-type plasminogen activator (rtPA). *Clin Sci (Lond)* 2012; 122: 113–119.
168. Malik A, Begh R, Roze S, Green K, Lindson N. Cooling therapy for acute stroke. *Cochrane Database Syst Rev*; 2019. Epub ahead of print 19 June 2019. DOI: 10.1002/14651858.CD013351.
169. Andresen M, Gazmuri JT, Marín A, Regueira T, Rovegno M. Therapeutic hypothermia for acute brain injuries. *Scand J Trauma Resusc Emerg Med* 2015; 23: 42.
170. Gross CG. Aristotle on the Brain. *Neurosci* 1995; 1: 245–250.
171. Mattern S. Galen and his patients. *Lancet* 2011; 378: 478–479.
172. Campos F, Blanco M, Barral D, Agulla J, Ramos-Cabrer P, Castillo J. Influence of temperature on ischemic brain: basic and clinical principles. *Neurochem Int* 2012; 60: 495–

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

505.
173. Covaciu L, Rubertsson S, Ortiz-Nieto F, Ahlström H, Weis J. Human brain MR spectroscopy thermometry using metabolite aqueous-solution calibrations. *J Magn Reson Imaging* 2010; 31: 807–814.
174. Mellergard P, Nordstrom CH. Intracerebral Temperature in Neurosurgical Patients. *Neurosurgery* 1991; 28: 709–713.
175. Schwab S, Spranger M, Aschoff A, Steiner T, Hacke W. Brain temperature monitoring and modulation in patients with severe MCA infarction. *Neurology* 1997; 48: 762–767.
176. Ginsberg MD, Busto R. Combating hyperthermia in acute stroke: a significant clinical concern. *Stroke* 1998; 29: 529–534.
177. Marshall I, Karaszewski B, Wardlaw JM, Cvoro V, Wartolowska K, Armitage PA, Carpenter T, Bastin ME, Farrall A, Haga K. Measurement of regional brain temperature using proton spectroscopic imaging: validation and application to acute ischemic stroke. *Magn Reson Imaging* 2006; 24: 699–706.
178. Karaszewski B, Wardlaw JM, Marshall I, Cvoro V, Wartolowska K, Haga K, Armitage PA, Bastin ME, Dennis MS. Measurement of brain temperature with magnetic resonance spectroscopy in acute ischemic stroke. *Ann Neurol* 2006; 60: 438–446.
179. Karaszewski B, Wardlaw JM, Marshall I, Cvoro V, Wartolowska K, Haga K, Armitage PA, Bastin ME, Dennis MS. Early brain temperature elevation and anaerobic metabolism in human acute ischaemic stroke. *Brain* 2009; 132: 955–964.
180. Boulant JA. Role of the preoptic-anterior hypothalamus in thermoregulation and fever. *Clin Infect Dis*; 31 Suppl 5.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Epub ahead of print 2000. doi: 10.1086/317521.
181. Badjatia N. Fever control in the neuro-ICU: why, who, and when? *Curr Opin Crit Care* 2009; 15: 79–82.
182. Wang H, Wang B, Normoyle KP, Jackson K, Spitler K, Sharrock M, Miller CM, Best C, Llano D, Du R. Brain temperature and its fundamental properties: a review for clinical neuroscientists. *Front Neurosci*; 8. Epub ahead of print 2014. doi: 10.3389/fnins.2014.00307.
183. Young AB, Ott LG, Beard D, Dempsey RJ, Tibbs PA, McClain CJ. The acute-phase response of the brain-injured patient. *J Neurosurg* 1988; 69: 375–380.
184. Wong ML, Licinio J. Localization of interleukin 1 type I receptor mRNA in rat brain. *Neuroimmunomodulation* 1994; 1: 110–115.
185. Kinoshita K, Chatzipanteli K, Vitarbo E, Truettner JS, Alonso OF, Dietrich WD, Selman WR, Lee SM, Kelly DF. Interleukin-1beta messenger ribonucleic acid and protein levels after fluid-percussion brain injury in rats: importance of injury severity and brain temperature. *Neurosurgery* 2002; 51: 195–203.
186. Dietrich WD, Chatzipanteli K, Vitarbo E, Wada K, Kinoshita K. The role of inflammatory processes in the pathophysiology and treatment of brain and spinal cord trauma. *Acta Neurochir Suppl* 2004; 89: 69–74.
187. Chamorro Á, Urra X, Planas AM. Infection after acute ischemic stroke: a manifestation of brain-induced immunodepression. *Stroke* 2007; 38: 1097–1103.
188. Dietrich WD, Busto R, Valdes L, Loor Y. Effects of normothermic versus mild hyperthermic forebrain ischemia in rats. *Stroke* 1990; 21: 1318–1325.
189. Chen H, Chopp M, Welch KMA. Effect of mild

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

hyperthermia on the ischemic infarct volume after middle cerebral artery occlusion in the rat. *Neurology* 1991; 41: 1133–1135.

190. Morikawa E, Ginsberg MD, Dietrich WD, Duncan RC, Kraydieh S, Globus MYT, Busto R. The significance of brain temperature in focal cerebral ischemia: histopathological consequences of middle cerebral artery occlusion in the rat. *J Cereb Blood Flow Metab* 1992; 12: 380–389.
191. Kim Y, Busto R, Dietrich WD, Kraydieh S, Ginsberg MD. Delayed postischemic hyperthermia in awake rats worsens the histopathological outcome of transient focal cerebral ischemia. *Stroke* 1996; 27: 2274–2281.
192. Hindfelt B. The prognostic significance of subfebrility and fever in ischaemic cerebral infarction. *Acta Neurol Scand* 1976; 53: 72–79.
193. Antoni D, José C, Pumar JM, Noya M. Body Temperature and Fibrinogen Are Related to Early Neurological Deterioration in Acute Ischemic Stroke. *Cerebrovasc Dis* 1997; 7: 64–69.
194. Grau AJ, Buggle F, Schnitzler P, Spiel M, Lichy C, Hacke W. Fever and infection early after ischemic stroke. *J Neurol Sci* 1999; 171: 115–120.
195. Wang Y, Lim LLY, Levi C, Heller RF, Fisher J. Influence of admission body temperature on stroke mortality. *Stroke* 2000; 31: 404–409.
196. Audebert HJ, Rott MM, Eck T, Haberl RL. Systemic inflammatory response depends on initial stroke severity but is attenuated by successful thrombolysis. *Stroke* 2004; 35: 2128–2133.
197. Seo WK, Yu SW, Kim JH, Park KW, Koh SB. The impact of

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

hyperthermia and infection on acute ischemic stroke patients in the intensive care unit. *Neurocrit Care* 2008; 9: 183–188.

198. Hajat C, Hajat S, Sharma P. Effects of poststroke pyrexia on stroke outcome : a meta-analysis of studies in patients. *Stroke* 2000; 31: 410–414.
199. Saini M, Saqqur M, Kamruzzaman A, Lees KR, Shuaib A. Effect of hyperthermia on prognosis after acute ischemic stroke. *Stroke* 2009; 40: 3051–3059.
200. Prasad K, Krishnan PR. Fever is associated with doubling of odds of short-term mortality in ischemic stroke: an updated meta-analysis. *Acta Neurol Scand* 2010; 122: 404–408.
201. Chen Q, Chopp M, Bodzin G, Chen H. Temperature modulation of cerebral depolarization during focal cerebral ischemia in rats: correlation with ischemic injury. *J Cereb Blood Flow Metab* 1993; 13: 389–394.
202. Schiefecker AJ, Kofler M, Gaasch M, Beer R, Unterberger I, Pfausler B, Broessner G, Lackner P, Rhomberg P, Gizewski E, Hackl WO, Mulino M, Ortler M, Thome C, Schmutzhard E, Helbok R. Brain temperature but not core temperature increases during spreading depolarizations in patients with spontaneous intracerebral hemorrhage. *J Cereb Blood Flow Metab* 2018; 38: 549–558.
203. Castillo J, Dávalos A, Noya M. Aggravation of acute ischemic stroke by hyperthermia is related to an excitotoxic mechanism. *Cerebrovasc Dis* 1999; 9: 22–27.
204. Leira R, Rodríguez-Yáñez M, Castellanos M, Blanco M, Nombela F, Sobrino T, Lizasoain I, Dávalos A, Castillo J. Hyperthermia is a surrogate marker of inflammation-mediated cause of brain damage in acute ischaemic stroke.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

J Intern Med 2006; 260: 343–349.

205. Wang CX, Shuaib A. Critical role of microvasculature basal lamina in ischemic brain injury. *Prog Neurobiol* 2007; 83: 140–148.
206. White MG, Luca LE, Nonner D, Saleh O, Hu B, Barrett EF, Barrett JN. Cellular mechanisms of neuronal damage from hyperthermia. *Prog Brain Res* 2007; 162: 347–371.
207. Wang CX, Stroink A, Casto JM, Kattner K. Hyperthermia exacerbates ischaemic brain injury. *Int J Stroke* 2009; 4: 274–284.
208. So HY. Therapeutic hypothermia. *Korean J Anesthesiol* 2010; 59: 299.
209. Kochanek PM, Jackson TC. The Brain and Hypothermia-From Aristotle to Targeted Temperature Management. *Crit Care Med* 2017; 45: 305–310.
210. Karnatovskaia L V., Wartenberg KE, Freeman WD. Therapeutic hypothermia for neuroprotection: history, mechanisms, risks, and clinical applications. *The Neurohospitalist* 2014; 4: 153–163.
211. Bohl MA, Martirosyan NL, Killeen ZW, Belykh E, Zabramski JM, Spetzler RF, Preul MC. The history of therapeutic hypothermia and its use in neurosurgery. *J Neurosurg* 2018; 130: 1006–1020.
212. Kanaar AG. Refrigeration in the treatment of trauma, with review of crymotherapy. *Curr Res Anesth Analg* 1946; 25: 177–190.
213. ROSOMOFF HL, HOLADAY DA. Cerebral Blood Flow and Cerebral Oxygen Consumption During Hypothermia. *https://doi.org/101152/ajplegacy1954179185* 1954; 179: 85–88.
214. DRAKE CG, BARR HW, COLES JC, GERGELY NF. The Use of Extracorporeal Circulation and Profound

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Hypothermia in the Treatment of Ruptured Intracranial Aneurysm. *J Neurosurg* 1964; 21: 575–581.

215. Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, Fanaroff AA, Poole WK, Wright LL, Higgins RD, Finer NN, Carlo WA, Duara S, Oh W, Cotten CM, Stevenson DK, Stoll BJ, Lemons JA, Guillet R, et al. Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *N Engl J Med* 2005; 353: 1574–1584.
216. Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, Smith K. Treatment of Comatose Survivors of Out-of-Hospital Cardiac Arrest with Induced Hypothermia. *http://dx.doi.org/101056/NEJMoa003289* 2009; 346: 557–563.
217. Shankaran S. Neonatal encephalopathy: treatment with hypothermia. *J Neurotrauma* 2009; 26: 437–443.
218. Mackensen GB, McDonagh DL, Warner DS. Perioperative hypothermia: use and therapeutic implications. *J Neurotrauma* 2009; 26: 341–358.
219. Florian B, Vintilescu R, Balseanu AT, Buga AM, Grisk O, Walker LC, Kessler C, Popa-Wagner A. Long-term hypothermia reduces infarct volume in aged rats after focal ischemia. *Neurosci Lett* 2008; 438: 180–185.
220. Chang YP, Liao PT, Shen EY, Liu IH. Protective effect against focal cerebral ischemia injury in acute phase of a novel invasive device for regional hypothermia. *J Chin Med Assoc* 2015; 78: 67–75.
221. Miyazawa T, Tamura A, Fukui S, Hossmann KA. Effect of mild hypothermia on focal cerebral ischemia. Review of experimental studies. *Neurol Res* 2003; 25: 457–464.
222. Van Der Worp HB, Sena ES, Donnan GA, Howells DW,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Macleod MR. Hypothermia in animal models of acute ischaemic stroke: a systematic review and meta-analysis. *Brain* 2007; 130: 3063–3074.

223. Dumitrascu OM, Lamb J, Lyden PD. Still cooling after all these years: Meta-analysis of pre-clinical trials of therapeutic hypothermia for acute ischemic stroke. *J Cereb Blood Flow Metab* 2016; 36: 1157–1164.
224. Kammersgaard LP, Rasmussen BH, Jorgensen HS, Reith J, Weber U, Olsen TS. Feasibility and Safety of Inducing Modest Hypothermia in Awake Patients With Acute Stroke Through Surface Cooling: A Case-Control Study. *Stroke* 2000; 31: 2251–2256.
225. Schwab S, Georgiadis D, Berrouschot J, Schellinger PD, Graffagnino C, Mayer SA. Feasibility and safety of moderate hypothermia after massive hemispheric infarction. *Stroke* 2001; 32: 2033–2035.
226. Georgiadis D, Schwarz S, Kollmar R, Schwab S. Endovascular cooling for moderate hypothermia in patients with acute stroke: first results of a novel approach. *Stroke* 2001; 32: 2550–2553.
227. Steiner T, Friede T, Aschoff A, Schellinger PD, Schwab S, Hacke W. Effect and feasibility of controlled rewarming after moderate hypothermia in stroke patients with malignant infarction of the middle cerebral artery. *Stroke* 2001; 32: 2833–2835.
228. De Georgia MA, Krieger DW, Abou-Chebl A, Devlin TG, Jauss M, Davis SM, Koroshetz WJ, Rordorf G, Warach S. Cooling for Acute Ischemic Brain Damage (COOL AID): a feasibility trial of endovascular cooling. *Neurology* 2004; 63: 312–317.
229. Els T, Oehm E, Voigt S, Klisch J, Hetzel A, Kassubek J.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Safety and therapeutical benefit of hemicraniectomy combined with mild hypothermia in comparison with hemicraniectomy alone in patients with malignant ischemic stroke. *Cerebrovasc Dis* 2006; 21: 79–85.

230. Hemmen TM, Raman R, Guluma KZ, Meyer BC, Gomes JA, Cruz-Flores S, Wijman CA, Rapp KS, Grotta JC, Lyden PD. Intravenous thrombolysis plus hypothermia for acute treatment of ischemic stroke (ICTuS-L): final results. *Stroke* 2010; 41: 2265–2270.
231. Horn CM, Sun CHJ, Nogueira RG, Patel VN, Krishnan A, Glenn BA, Belagaje SR, Thomas TT, Anderson AM, Frankel MR, Schindler KM, Gupta R. Endovascular Reperfusion and Cooling in Cerebral Acute Ischemia (ReCCLAIM I). *J Neurointerv Surg* 2014; 6: 91–95.
232. Piironen K, Tiainen M, Mustanoja S, Kaukonen KM, Meretoja A, Tatlisumak T, Kaste M. Mild hypothermia after intravenous thrombolysis in patients with acute stroke: a randomized controlled trial. *Stroke* 2014; 45: 486–491.
233. Poli S, Purrucker J, Priglinger M, Ebner M, Sykora M, Diedler J, Bulut C, Popp E, Rupp A, Hametner C. Rapid Induction of COOLing in Stroke Patients (iCOOL1): a randomised pilot study comparing cold infusions with nasopharyngeal cooling. *Crit Care*; 18. Epub ahead of print 2014. doi: 10.1186/s13054-014-0582-1.
234. Lyden P, Hemmen T, Grotta J, Rapp K, Ernstrom K, Rzesiewicz T, Parker S, Concha M, Hussain S, Agarwal S, Meyer B, Jurf J, Altafullah I, Raman R, Hess MJ, Mullin A, Muranevici G, Piantadosi B, Jimenez-Maggiora G, et al. Results of the Intravascular Cooling in the Treatment of Acute Stroke 2 Trial (ICTuS-2). *Stroke* 2016; 47: 2888.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

235. van der Worp HB, Macleod MR, Bath PMW, Demotes J, Durand-Zaleski I, Gebhardt B, Gluud C, Kollmar R, Krieger DW, Lees KR, Molina C, Montaner J, Roine RO, Petersson J, Staykov D, Szabo I, Wardlaw JM, Schwab S. EuroHYP-1: European multicenter, randomized, phase III clinical trial of therapeutic hypothermia plus best medical treatment vs. best medical treatment alone for acute ischemic stroke. *Int J Stroke* 2014; 9: 642–645.
236. van der Worp HB, Macleod MR, Bath PM, Bathula R, Christensen H, Colam B, Cordonnier C, Demotes-Mainard J, Durand-Zaleski I, Gluud C, Jakobsen JC, Kallmünzer B, Kollmar R, Krieger DW, Lees KR, Michalski D, Molina C, Montaner J, Roine RO, et al. Therapeutic hypothermia for acute ischaemic stroke. Results of a European multicentre, randomised, phase III clinical trial. *Eur stroke J* 2019; 4: 254–262.
237. Su Y, Fan L, Zhang Y, Zhang Y, Ye H, Gao D, Chen W, Liu G. Improved Neurological Outcome With Mild Hypothermia in Surviving Patients With Massive Cerebral Hemispheric Infarction. *Stroke* 2016; 47: 457–463.
238. Geurts M, Petersson J, Brizzi M, Olsson-Hau S, Luijckx GJ, Algra A, Dippel DWJ, Kappelle LJ, Van Der Worp HB. COOLIST (Cooling for Ischemic Stroke Trial). *Stroke* 2017; 48: 219–221.
239. Neugebauer H, Schneider H, Bösel J, Hobohm C, Poli S, Kollmar R, Sobesky J, Wolf S, Bauer M, Tittel S, Beyersmann J, Woitzik J, Heuschmann PU, Jüttler E. Outcomes of Hypothermia in Addition to Decompressive Hemicraniectomy in Treatment of Malignant Middle Cerebral Artery Stroke: A Randomized Clinical Trial. *JAMA Neurol* 2019; 76: 571–579.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

240. Fan L, Su Y, Zhang Y, Ye H, Chen W, Liu G. Decompressive craniectomy combined with mild hypothermia in patients with large hemispheric infarction: a randomized controlled trial. *BMC Neurol* 2021; 21: 1–8.
241. Kuczynski AM, Marzoughi S, Al Sultan AS, Colbourne F, Menon BK, van Es ACGM, Berez AL, Goyal M, Demchuk AM, Almekhlafi MA. Therapeutic Hypothermia in Acute Ischemic Stroke-a Systematic Review and Meta-Analysis. *Curr Neurol Neurosci Rep*; 20. Epub ahead of print 1 May 2020. doi: 10.1007/s11910-020-01029-3.
242. Mokhtarani M, Mahgoub AN, Morioka N, Doufas AG, Dae M, Shaughnessy TE, Bjorksten AR, Sessler DI. Buspirone and meperidine synergistically reduce the shivering threshold. *Anesth Analg* 2001; 93: 1233–1239.
243. Moore EM, Nichol AD, Bernard SA, Bellomo R. Therapeutic hypothermia: benefits, mechanisms and potential clinical applications in neurological, cardiac and kidney injury. *Injury* 2011; 42: 843–854.
244. Liu L, Yenari MA. Clinical application of therapeutic hypothermia in stroke. *Neurol Res* 2009; 31: 331.
245. Vaity C, Al-Subaie N, Cecconi M. Cooling techniques for targeted temperature management post-cardiac arrest. *Crit Care*; 19. Epub ahead of print 14 December 2015. doi: 10.1186/s13054-015-0804-1.
246. Straus D, Prasad V, Munoz L. Selective therapeutic hypothermia: a review of invasive and noninvasive techniques. *Arq Neuropsiquiatr* 2011; 69: 981–987.
247. Naiman M, Shanley P, Garrett F, Kulstad E. Evaluation of advanced cooling therapy's esophageal cooling device for core temperature control. *Expert Rev Med Devices* 2016; 13: 423–433.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

248. Khan I, Haymore J, Barnaba B, Armahizer M, Melinosky C, Bautista MA, Blaber B, Chang WT, Parikh G, Motta M, Badjatia N. Esophageal Cooling Device Versus Other Temperature Modulation Devices for Therapeutic Normothermia in Subarachnoid and Intracranial Hemorrhage. *Ther Hypothermia Temp Manag* 2018; 8: 53–58.
249. Wang H, Olivero W, Lanzino G, Elkins W, Rose J, Honings D, Rodde M, Burnham J, Wang D. Rapid and selective cerebral hypothermia achieved using a cooling helmet. *J Neurosurg* 2004; 100: 272–277.
250. Poli S, Purrucker J, Priglinger M, Diedler J, Sykora M, Popp E, Steiner T, Veltkamp R, Bösel J, Rupp A, Hacke W, Hametner C. Induction of cooling with a passive head and neck cooling device: effects on brain temperature after stroke. *Stroke* 2013; 44: 708–713.
251. Wang B, Wu D, Dornbos D, Shi J, Ma Y, Zhang M, Liu Y, Chen J, Ding Y, Luo Y, Ji X. Local cerebral hypothermia induced by selective infusion of cold lactated ringer's: a feasibility study in rhesus monkeys. *Neurol Res* 2016; 38: 545–552.
252. Froehler MT, Ovbiagele B. Therapeutic hypothermia for acute ischemic stroke. *https://doi.org/101586/erc09129* 2014; 8: 593–603.
253. Lyden PD, Allgren RL, Ng K, Akins P, Meyer B, Al-Sanani F, Lutsep H, Dobak J, Matsubara BS, Zivin J. Intravascular Cooling in the Treatment of Stroke (ICTuS): early clinical experience. *J Stroke Cerebrovasc Dis* 2005; 14: 107–114.
254. Gillies MA, Pratt R, Whiteley C, Borg J, Beale RJ, Tibby SM. Therapeutic hypothermia after cardiac arrest: a retrospective comparison of surface and endovascular cooling techniques. *Resuscitation* 2010; 81: 1117–1122.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

255. den Hertog HM, van der Worp HB, van Gemert HMA, Algra A, Kappelle LJ, van Gijn J, Koudstaal PJ, Dippel DW. The Paracetamol (Acetaminophen) In Stroke (PAIS) trial: a multicentre, randomised, placebo-controlled, phase III trial. *Lancet Neurol* 2009; 8: 434–440.
256. Jaramillo A, Illanes S, Díaz V. Is hypothermia useful in malignant ischemic stroke? Current status and future perspectives. *J Neurol Sci* 2008; 266: 1–8.
257. Sund-Levander M, Grodzinsky E. Assessment of body temperature measurement options. *Br J Nurs* 2013; 22: 942–950.
258. Vieites-Prado A, Iglesias-Rey R, Fernández-Susavila H, Da Silva-Candal A, Rodríguez-Castro E, Gröhn OHJ, Wellmann S, Sobrino T, Castillo J, Campos F. Protective Effects and Magnetic Resonance Imaging Temperature Mapping of Systemic and Focal Hypothermia in Cerebral Ischemia. *Stroke* 2016; 47: 2386–2396.
259. Janke D, Kagelmann N, Storm C, Maggioni MA, Kienast C, Gunga HC, Opatz O. Measuring Core Body Temperature Using a Non-invasive, Disposable Double-Sensor During Targeted Temperature Management in Post-cardiac Arrest Patients. *Front Med* 2021; 8: 468.
260. Sessler DI. Thermoregulatory defense mechanisms. *Crit Care Med*; 37. Epub ahead of print 2009. doi: 10.1097/ccm.0b013e3181aa5568.
261. Seebacher F. Responses to temperature variation: integration of thermoregulation and metabolism in vertebrates. *J Exp Biol* 2009; 212: 2885–2891.
262. Prakash O, Jonson B, Bos E, Meij S, Hugenholtz PG, Hekman W. Cardiorespiratory and metabolic effects of profound hypothermia. *Crit Care Med* 1978; 6: 340–346.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

263. Wood T, Thoresen M. Physiological responses to hypothermia. *Semin Fetal Neonatal Med* 2015; 20: 87–96.
264. Mirzoyev SA, McLeod CJ, Bunch TJ, Bell MR, White RD. Hypokalemia during the cooling phase of therapeutic hypothermia and its impact on arrhythmogenesis. *Resuscitation* 2010; 81: 1632–1636.
265. Nakamura K, Morrison SF. Central efferent pathways for cold-defensive and febrile shivering. *J Physiol* 2011; 589: 3641–3658.
266. Polderman KH. Mechanisms of action, physiological effects, and complications of hypothermia. *Crit Care Med*; 37. Epub ahead of print 2009. doi: 10.1097/ccm.0b013e3181aa5241.
267. Zhou J, Poloyac SM. The effect of therapeutic hypothermia on drug metabolism and drug response: cellular mechanisms to organ function. *Expert Opin Drug Metab Toxicol* 2011; 7: 803.
268. Erecinska M, Thoresen M, Silver IA. Effects of hypothermia on energy metabolism in Mammalian central nervous system. *J Cereb Blood Flow Metab* 2003; 23: 513–530.
269. González-Ibarra FP, Varon J, López-Meza EG. Therapeutic hypothermia: Critical review of the molecular mechanisms of action. *Front Neurol* 2011; FEB: 4.
270. Kiyatkin EA, Sharma HS. Permeability of the blood-brain barrier depends on brain temperature. *Neuroscience* 2009; 161: 926–939.
271. Yenari MA, Han HS. Neuroprotective mechanisms of hypothermia in brain ischaemia. *Nat Rev Neurosci* 2012; 13: 267–278.
272. Urbano LA, Oddo M. Therapeutic hypothermia for traumatic brain injury. *Curr Neurol Neurosci Rep* 2012; 12:

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

580–591.
273. Wright A, Vissel B. The essential role of AMPA receptor GluA2 subunit RNA editing in the normal and diseased brain. *Front Mol Neurosci* 2012; 0: 34.
274. Li YH, Zhang CL, Zhang XY, Zhou HX, Meng LL. Effects of mild induced hypothermia on hippocampal connexin 43 and glutamate transporter 1 expression following traumatic brain injury in rats. *Mol Med Rep* 2015; 11: 1991–1996.
275. Li G, Liu X, Su Z, Zhang D. Hypothermia exerts early neuroprotective effects involving protein conjugation of SUMO-2/3 in a rat model of middle cerebral artery occlusion. *Mol Med Rep* 2017; 16: 3217–3223.
276. Wang L, Ma Q, Yang W, MacKensen GB, Paschen W. Moderate hypothermia induces marked increase in levels and nuclear accumulation of SUMO2/3-conjugated proteins in neurons. *J Neurochem* 2012; 123: 349.
277. Lee YJ, Mou Y, Klimanis D, Bernstock JD, Hallenbeck JM. Global SUMOylation is a molecular mechanism underlying hypothermia-induced ischemic tolerance. *Front Cell Neurosci*; 8. Epub ahead of print 4 December 2014. doi: 10.3389/fncel.2014.00416.
278. Jackson TC, Kochanek PM. A New Vision for Therapeutic Hypothermia in the Era of Targeted Temperature Management: A Speculative Synthesis. *Ther Hypothermia Temp Manag* 2019; 9: 13.
279. Talma N, Kok WF, de Veij Mestdagh CF, Shanbhag NC, Bouma HR, Henning RH. Neuroprotective hypothermia - Why keep your head cool during ischemia and reperfusion. *Biochim Biophys Acta* 2016; 1860: 2521–2528.
280. Sun YJ, Zhang ZY, Fan B, Li GY. Neuroprotection by

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

therapeutic hypothermia. *Front Neurosci* 2019; 13: 586.

281. Lee JH, Wei ZZ, Cao W, Won S, Gu X, Winter M, Dix TA, Wei L, Yu SP. Regulation of therapeutic hypothermia on inflammatory cytokines, microglia polarization, migration and functional recovery after ischemic stroke in mice. *Neurobiol Dis* 2016; 96: 248–260.
282. Huet O, Kinirons B, Dupic L, Lajeunie E, Mazoit JX, Benhamou D, Vicaut E, Duranteau J. Induced mild hypothermia reduces mortality during acute inflammation in rats. *Acta Anaesthesiol Scand* 2007; 51: 1211–1216.
283. Peretti D, Bastide A, Radford H, Verity N, Molloy C, Martin MG, Moreno JA, Steinert JR, Smith T, Dinsdale D, Willis AE, Mallucci GR. RBM3 mediates structural plasticity and protective effects of cooling in neurodegeneration. *Nature* 2015; 518: 236–239.
284. Yenari MA, Han HS. Influence of therapeutic hypothermia on regeneration after cerebral ischemia. *Front Neurol Neurosci* 2013; 32: 122–128.
285. Rey-Funes M, Dorfman VB, Ibarra ME, Peña E, Contartese DS, Goldstein J, Acosta JM, Larráyoz IM, Martínez-Murillo R, Martíinez A, Loidl CF. Hypothermia prevents gliosis and angiogenesis development in an experimental model of ischemic proliferative retinopathy. *Invest Ophthalmol Vis Sci* 2013; 54: 2836–2846.
286. Chip S, Zelmer A, Ogunshola OO, Felderhoff-Mueser U, Nitsch C, Bührer C, Wellmann S. The RNA-binding protein RBM3 is involved in hypothermia induced neuroprotection. *Neurobiol Dis* 2011; 43: 388–396.
287. Danno S, Nishiyama H, Higashitsuji H, Yokoi H, Xue JH, Itoh K, Matsuda T, Fujita J. Increased transcript level of

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

RBM3, a member of the glycine-rich RNA-binding protein family, in human cells in response to cold stress. *Biochem Biophys Res Commun* 1997; 236: 804–807.

288. Godin KS, Varani G. How Arginine-Rich Domains Coordinate mRNA Maturation Events. *http://dx.doi.org/104161/rna424869* 2007; 4: 69–75.
289. Gonatopoulos-Pournatzis T, Cowling VH. Cap-binding complex (CBC). *Biochem J* 2014; 457: 231–242.
290. Zhan X, Qian B, Cao F, Wu W, Yang L, Guan Q, Gu X, Wang P, Okusolubo TA, Dunn SL, Zhu JK, Zhu J. An Arabidopsis PWI and RRM motif-containing protein is critical for pre-mRNA splicing and ABA responses. *Nat Commun 2015 61* 2015; 6: 1–12.
291. Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Prog Neurobiol* 2013; 106–107: 1–16.
292. Pilotte J, Kiosses W, Chan SW, Makarenkova HP, Dupont-Versteegden E, Vanderklish PW. Morphoregulatory functions of the RNA-binding motif protein 3 in cell spreading, polarity and migration. *Sci Reports 2018 81* 2018; 8: 1–19.
293. Petryszak R, Keays M, Tang YA, Fonseca NA, Barrera E, Burdett T, Füllgrabe A, Fuentes AMP, Jupp S, Koskinen S, Mannion O, Huerta L, Megy K, Snow C, Williams E, Barzine M, Hastings E, Weisser H, Wright J, et al. Expression Atlas update—an integrated database of gene and protein expression in humans, animals and plants. *Nucleic Acids Res* 2016; 44: D746–D752.
294. Williams DR, Epperson LE, Li W, Hughes MA, Taylor R, Rogers J, Martin SL, Cossins AR, Gracey AY. Seasonally

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

hibernating phenotype assessed through transcript screening. *Physiol Genomics* 2005; 24: 13–22.

295. Wellmann S, Truss M, Bruder E, Tornillo L, Zelmer A, Seeger K, Bü Hrer C. The RNA-binding protein RBM3 is required for cell proliferation and protects against serum deprivation-induced cell death. *Pediatr Res* 2010; 67: 35–41.
296. VB F, AV G, Ø T, NC S, C C, H W, J Y, LC S, MK S, BM B. Modulation of gene expression in heart and liver of hibernating black bears (Ursus americanus). *BMC Genomics*; 12. Epub ahead of print 5 April 2011. doi: 10.1186/1471-2164-12-171.
297. Smart F, Aschrafi A, Atkins A, Owens GC, Pilotte J, Cunningham BA, Vanderklish PW. Two isoforms of the cold-inducible mRNA-binding protein RBM3 localize to dendrites and promote translation. *J Neurochem* 2007; 101: 1367–1379.
298. Thandapani P, O'Connor TR, Bailey TL, Richard S. Defining the RGG/RG motif. *Mol Cell* 2013; 50: 613–623.
299. Zhu X, Bührer C, Wellmann S. Cold-inducible proteins CIRP and RBM3, a unique couple with activities far beyond the cold. *Cell Mol Life Sci 2016 7320* 2016; 73: 3839–3859.
300. Wellmann S, Bührer C, Moderegger E, Zelmer A, Kirschner R, Koehne P, Fujita J, Seegar K. Oxygen-regulated expression of the RNA-binding proteins RBM3 and CIRP by a HIF-1-independent mechanism. *J Cell Sci* 2004; 117: 1785–1794.
301. Yan J, Goerne T, Zelmer A, Guzman R, Kapfhammer JP, Wellmann S, Zhu X. The RNA-Binding Protein RBM3 Promotes Neural Stem Cell (NSC) Proliferation Under Hypoxia. *Front Cell Dev Biol* 2019; 7: 288.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

302. Trollmann R, Rehrauer H, Schneider C, Krischke G, Huemmler N, Keller S, Rascher W, Gassmann M. Late-gestational systemic hypoxia leads to a similar early gene response in mouse placenta and developing brain. *Am J Physiol - Regul Integr Comp Physiol* 2010; 299: 1489–1499.
303. Ryan JC, Morey JS, Ramsdell JS, Van Dolah FM. Acute phase gene expression in mice exposed to the marine neurotoxin domoic acid. *Neuroscience* 2005; 136: 1121–1132.
304. Jackson TC, Manole MD, Kotermanski SE, Jackson EK, Clark RSB, Kochanek PM. Cold stress protein RBM3 responds to temperature change in an ultra-sensitive manner in young neurons. *Neuroscience* 2015; 305: 268–278.
305. Cok SJ, Acton SJ, Sexton AE, Morrison AR. Identification of RNA-binding proteins in RAW 264.7 cells that recognize a lipopolysaccharide-responsive element in the 3-untranslated region of the murine cyclooxygenase-2 mRNA. *J Biol Chem* 2004; 279: 8196–8205.
306. Laustriat D, Gide J, Barrault L, Chautard E, Benoit C, Auboeuf D, Boland A, Battail C, Artiguenave F, Deleuze JF, Bénit P, Rustin P, Franc S, Charpentier G, Furling D, Bassez G, Nissan X, Martinat C, Peschanski M, et al. In Vitro and In Vivo Modulation of Alternative Splicing by the Biguanide Metformin. *Mol Ther Nucleic Acids* 2015; 4: e262.
307. Danno S, Itoh K, Matsuda T, Fujita J. Decreased expression of mouse Rbm3, a cold-shock protein, in Sertoli cells of cryptorchid testis. *Am J Pathol* 2000; 156: 1685–1692.
308. Larrayoz IM, Rey-Funes M, Contartese DS, Rolón F, Sarotto A, Dorfman VB, Loidl CF, Martínez A. Cold Shock Proteins Are Expressed in the Retina Following Exposure to Low Temperatures. *PLoS One*; 11. Epub ahead of print 1

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

August 2016. doi: 10.1371/journal.pone.0161458.

309. Karlsson H. Skin to skin care:heat balance. *Arch Dis Child Fetal Neonatal Ed*; 75. Epub ahead of print 1996. doi: 10.1136/fn.75.2.f130.
310. Sureban SM, Ramalingam S, Natarajan G, May R, Subramaniam D, Bishnupuri KS, Morrison AR, Dieckgraefe BK, Brackett DJ, Postier RG, Houchen CW, Anant S. Translation regulatory factor RBM3 is a proto-oncogene that prevents mitotic catastrophe. *Oncogene 2008 2733* 2008; 27: 4544–4556.
311. Hu W, Liu Y, Yan J. Microarray Meta-Analysis of RNA-Binding Protein Functions in Alternative Polyadenylation. *PLoS One* 2014; 9: e90774.
312. Dresios J, Aschrafi A, Owens GC, Vanderklish PW, Edelman GM, Mauro VP. Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alters microRNA levels, and enhances global protein synthesis. *Proc Natl Acad Sci* 2005; 102: 1865–1870.
313. Pilotte J, Cunningham BA, Edelman GM, Vanderklish PW. Developmentally regulated expression of the cold-inducible RNA-binding motif protein 3 in euthermic rat brain. *Brain Res* 2009; 1258: 12–24.
314. Pilotte J, Dupont-Versteegden EE, Vanderklish PW. Widespread Regulation of miRNA Biogenesis at the Dicer Step by the Cold-Inducible RNA-Binding Protein, RBM3. *PLoS One* 2011; 6: e28446.
315. Wang S, Chen X, Qiao T. Long non-coding RNA MIR4435-2HG promotes the progression of head and neck squamous cell carcinoma by regulating the miR-383-5p/RBM3 axis. *Oncol Rep* 2021; 45: 1–10.
316. Wong JJL, Au AYM, Gao D, Pinello N, Kwok CT, Thoeng

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

A, Lau KA, Gordon JEA, Schmitz U, Feng Y, Nguyen T V., Middleton R, Bailey CG, Holst J, Rasko JEJ, Ritchie W. RBM3 regulates temperature sensitive miR-142–5p and miR-143 (thermomiRs), which target immune genes and control fever. *Nucleic Acids Res* 2016; 44: 2888–2897.

317. Angers S, Moon RT. Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol 2009 107* 2009; 10: 468–477.
318. Matsuda A, Ogawa M, Yanai H, Naka D, Goto A, Ao T, Tanno Y, Takeda K, Watanabe Y, Honda K, Taniguchi T. Generation of mice deficient in RNA-binding motif protein 3 (RBM3) and characterization of its role in innate immune responses and cell growth. *Biochem Biophys Res Commun* 2011; 411: 7–13.
319. Venugopal A, Subramaniam D, Balmaceda J, Roy B, Dixon DA, Umar S, Weir SJ, Anant S. RNA binding protein RBM3 increases β-catenin signaling to increase stem cell characteristics in colorectal cancer cells. *Mol Carcinog* 2016; 55: 1503–1516.
320. Ehlén Å, Brennan DJ, Nodin B, O'Connor DP, Eberhard J, Alvarado-Kristensson M, Jeffrey IB, Manjer J, Brändstedt J, Uhlén M, Pontén F, Jirström K. Expression of the RNA-binding protein RBM3 is associated with a favourable prognosis and cisplatin sensitivity in epithelial ovarian cancer. *J Transl Med* 2010; 8: 1–12.
321. Ferry AL, Vanderklish PW, Dupont-Versteegden EE. Enhanced survival of skeletal muscle myoblasts in response to overexpression of cold shock protein RBM3. *Am J Physiol - Cell Physiol* 2011; 301: 392–402.
322. Zhuang RJ, Ma J, Shi X, Ju F, Ma SP, Wang L, Cheng BF, Ma YW, Wang M, Li T, Feng ZW, Yang HJ. Cold-Inducible

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Protein RBM3 Protects UV Irradiation-Induced Apoptosis in Neuroblastoma Cells by Affecting p38 and JNK Pathways and Bcl2 Family Proteins. *J Mol Neurosci* 2017; 63: 142–151.

323. Rzechorzek NM, Connick P, Patani R, Selvaraj BT, Chandran S. Hypothermic Preconditioning of Human Cortical Neurons Requires Proteostatic Priming. *EBioMedicine* 2015; 2: 528–535.
324. Zhu X, Yan J, Bregere C, Zelmer A, Goerne T, Kapfhammer JP, Guzman R, Wellmann S. RBM3 promotes neurogenesis in a niche-dependent manner via IMP2-IGF2 signaling pathway after hypoxic-ischemic brain injury. *Nat Commun 2019 101* 2019; 10: 1–14.
325. Sertel SM, von Elling-Tammen MS, Rizzoli SO. The mRNA-Binding Protein RBM3 Regulates Activity Patterns and Local Synaptic Translation in Cultured Hippocampal Neurons. *J Neurosci* 2021; 41: 1157–1173.
326. Ornitz DM, Itoh N. The Fibroblast Growth Factor signaling pathway. *Wiley Interdiscip Rev Dev Biol* 2015; 4: 215–266.
327. Chen G, Liu Y, Goetz R, Fu L, Jayaraman S, Hu MC, Moe OW, Liang G, Li X, Mohammadi M. α-Klotho is a non-enzymatic molecular scaffold for FGF23 hormone signalling. *Nature* 2018; 553: 461–466.
328. Lee S, Choi J, Mohanty J, Sousa LP, Tome F, Pardon E, Steyaert J, Lemmon MA, Lax I, Schlessinger J. Structures of β-klotho reveal a 'zip code'-like mechanism for endocrine FGF signalling. *Nature* 2018; 553: 501–505.
329. Fisher FM, Maratos-Flier E. Understanding the Physiology of FGF21. *Annu Rev Physiol* 2016; 78: 223–241.
330. Kurosu H, Choi M, Ogawa Y, Dickson AS, Goetz R,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Eliseenkova A V., Mohammadi M, Rosenblatt KP, Kliewer SA, Kuro-O M. Tissue-specific expression of betaKlotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21. *J Biol Chem* 2007; 282: 26687–26695.

331. Adams AC, Cheng CC, Coskun T, Kharitonenkov A. FGF21 Requires βklotho to Act In Vivo. *PLoS One*; 7. Epub ahead of print 27 November 2012. doi: 10.1371/journal.pone.0049977.
332. Tacer KF, Bookout AL, Ding X, Kurosu H, John GB, Wang L, Goetz R, Mohammadi M, Kuro-o M, Mangelsdorf DJ, Kliewer SA. Research resource: Comprehensive expression atlas of the fibroblast growth factor system in adult mouse. *Mol Endocrinol* 2010; 24: 2050–2064.
333. Bookout AL, De Groot MHM, Owen BM, Lee S, Gautron L, Lawrence HL, Ding X, Elmquist JK, Takahashi JS, Mangelsdorf DJ, Kliewer SA. FGF21 regulates metabolism and circadian behavior by acting on the nervous system. *Nat Med* 2013; 19: 1147–1152.
334. Nelson BT, Ding X, Boney-Montoya J, Gerard RD, Kliewer SA, Andrews MT. Metabolic hormone FGF21 is induced in ground squirrels during hibernation but its overexpression is not sufficient to cause torpor. *PLoS One*; 8. Epub ahead of print 2 January 2013. doi: 10.1371/journal.pone.0053574.
335. Jackson TC, Kotermanski SE, Kochanek PM. Infants Uniquely Express High Levels of RBM3 and Other Cold-Adaptive Neuroprotectant Proteins in the Human Brain. *Dev Neurosci* 2018; 40: 325–336.
336. Inagaki T, Dutchak P, Zhao G, Ding X, Gautron L, Parameswara V, Li Y, Goetz R, Mohammadi M, Esser V,

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Elmquist JK, Gerard RD, Burgess SC, Hammer RE, Mangelsdorf DJ, Kliewer SA. Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. *Cell Metab* 2007; 5: 415–425.

337. Lin W, Zhang T, Zhou Y, Zheng J, Lin Z. Advances in Biological Functions and Clinical Studies of FGF21 . *Diabetes, Metab Syndr Obes Targets Ther* 2021; 14: 3281–3290.
338. Dutchak PA, Katafuchi T, Bookout AL, Choi JH, Yu RT, Mangelsdorf DJ, Kliewer SA. Fibroblast growth factor-21 regulates PPARγ activity and the antidiabetic actions of thiazolidinediones. *Cell* 2012; 148: 556–567.
339. Habegger KM, Stemmer K, Cheng C, Müller TD, Heppner KM, Ottaway N, Holland J, Hembree JL, Smiley D, Gelfanov V, Krishna R, Arafat AM, Konkar A, Belli S, Kapps M, Woods SC, Hofmann SM, D'Alessio D, Pfluger PT, et al. Fibroblast growth factor 21 mediates specific glucagon actions. *Diabetes* 2013; 62: 1453–1463.
340. Adams AC, Astapova I, Fisher FM, Badman MK, Kurgansky KE, Flier JS, Hollenberg AN, Maratos-Flier E. Thyroid hormone regulates hepatic expression of fibroblast growth factor 21 in a PPARalpha-dependent manner. *J Biol Chem* 2010; 285: 14078–14082.
341. Gälman C, Lundåsen T, Kharitonenkov A, Bina HA, Eriksson M, Hafström I, Dahlin M, Åmark P, Angelin B, Rudling M. The Circulating Metabolic Regulator FGF21 Is Induced by Prolonged Fasting and PPARα Activation in Man. *Cell Metab* 2008; 8: 169–174.
342. Hafström I, Ringertz B, Gyllenhammar H, Palmblad J, Harms-Ringdahl M. Effects of fasting on disease activity, neutrophil function, fatty acid composition, and leukotriene biosynthesis in patients with rheumatoid

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

arthritis. *Arthritis Rheum* 1988; 31: 585–592.
343. Li H, Fang Q, Gao F, Fan J, Zhou J, Wang X, Zhang H, Pan X, Bao Y, Xiang K, Xu A, Jia W. Fibroblast growth factor 21 levels are increased in nonalcoholic fatty liver disease patients and are correlated with hepatic triglyceride. *J Hepatol* 2010; 53: 934–940.
344. Zhang X, Yeung DCY, Karpisek M, Stejskal D, Zhou ZG, Liu F, Wong RLC, Chow WS, Tso AWK, Lam KSL, Xu A. Serum FGF21 levels are increased in obesity and are independently associated with the metabolic syndrome in humans. *Diabetes* 2008; 57: 1246–1253.
345. Erickson A, Moreau R. The regulation of FGF21 gene expression by metabolic factors and nutrients. *Horm Mol Biol Clin Investig*; 30. Epub ahead of print 1 April 2017. doi: 10.1515/hmbci-2016-0016/pdf.
346. Wente W, Efanov AM, Brenner M, Kharitonenkov A, Köster A, Sandusky GE, Sewing S, Treinies I, Zitzer H, Gromada J. Fibroblast growth factor-21 improves pancreatic beta-cell function and survival by activation of extracellular signal-regulated kinase 1/2 and Akt signaling pathways. *Diabetes* 2006; 55: 2470–2478.
347. Rottiers V, Näär AM. MicroRNAs in metabolism and metabolic disorders. *Nat Rev Mol Cell Biol* 2012; 13: 239–251.
348. Tang Y, Banan A, Forsyth CB, Fields JZ, Lau CK, Zhang LJ, Keshavarzian A. Effect of alcohol on miR-212 expression in intestinal epithelial cells and its potential role in alcoholic liver disease. *Alcohol Clin Exp Res* 2008; 32: 355–364.
349. Xiao J, Bei Y, Liu J, Dimitrova-Shumkovska J, Kuang D, Zhou Q, Li J, Yang Y, Xiang Y, Wang F, Yang C, Yang W.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

miR-212 downregulation contributes to the protective effect of exercise against non-alcoholic fatty liver via targeting FGF-21. *J Cell Mol Med* 2016; 20: 204–216.

350. Chartoumpekis D V., Habeos IG, Ziros PG, Psyrogiannis AI, Kyriazopoulou VE, Papavassiliou AG. Brown adipose tissue responds to cold and adrenergic stimulation by induction of FGF21. *Mol Med* 2011; 17: 736–740.
351. Hondares E, Iglesias R, Giralt A, Gonzalez FJ, Giralt M, Mampel T, Villarroya F. Thermogenic Activation Induces FGF21 Expression and Release in Brown Adipose Tissue *. *J Biol Chem* 2011; 286: 12983–12990.
352. Lee P, Brychta RJ, Linderman J, Smith S, Chen KY, Celi FS. Mild cold exposure modulates fibroblast growth factor 21 (FGF21) diurnal rhythm in humans: relationship between FGF21 levels, lipolysis, and cold-induced thermogenesis. *J Clin Endocrinol Metab*; 98. Epub ahead of print January 2013. doi: 10.1210/jc.2012-3107.
353. Lee P, Linderman JD, Smith S, Brychta RJ, Wang J, Idelson C, Perron RM, Werner CD, Phan GQ, Kammula US, Kebebew E, Pacak K, Chen KY, Celi FS. Irisin and FGF21 are cold-induced endocrine activators of brown fat function in humans. *Cell Metab* 2014; 19: 302–309.
354. Yu H, Xia F, Lam KSL, Wang Y, Bao Y, Zhang J, Gu Y, Zhou P, Lu J, Jia W, Xu A. Circadian rhythm of circulating fibroblast growth factor 21 is related to diurnal changes in fatty acids in humans. *Clin Chem* 2011; 57: 691–700.
355. Huang X, Liu G, Guo J, Su ZQ. The PI3K/AKT pathway in obesity and type 2 diabetes. *Int J Biol Sci* 2018; 14: 1483–1496.
356. Kong LJ, Feng W, Wright M, Chen Y, Dallas-Yang Q, Zhou YP, Berger JP. FGF21 suppresses hepatic glucose

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

production through the activation of atypical protein kinase Cι/λ. *Eur J Pharmacol* 2013; 702: 302–308.
357. Woo YC, Xu A, Wang Y, Lam KSL. Fibroblast growth factor 21 as an emerging metabolic regulator: clinical perspectives. *Clin Endocrinol (Oxf)* 2013; 78: 489–496.
358. Gong Q, Hu Z, Zhang F, Cui A, Chen X, Jiang H, Gao J, Chen X, Han Y, Liang Q, Ye D, Shi L, Chin YE, Wang Y, Xiao H, Guo F, Liu Y, Zang M, Xu A, et al. Fibroblast growth factor 21 improves hepatic insulin sensitivity by inhibiting mammalian target of rapamycin complex 1 in mice. *Hepatology* 2016; 64: 425–438.
359. Yu D, Ye X, Wu Q, Li S, Yang Y, He J, Liu Y, Zhang X, Yuan Q, Liu M, Li D, Ren G. Insulin sensitizes FGF21 in glucose and lipid metabolisms via activating common AKT pathway. *Endocrine* 2016; 52: 527–540.
360. Li K, Li L, Yang M, Liu H, Boden G, Yang G. The effects of fibroblast growth factor-21 knockdown and over-expression on its signaling pathway and glucose-lipid metabolism in vitro. *Mol Cell Endocrinol* 2012; 348: 21–26.
361. Cho YM, Kim DH, Lee KH, Jeong SW, Kwon OJ. The IRE1α-XBP1s pathway promotes insulin-stimulated glucose uptake in adipocytes by increasing PPARγ activity. *Exp Mol Med 2018 508* 2018; 50: 1–15.
362. Mai K, Andres J, Biedasek K, Weicht J, Bobbert T, Sabath M, Meinus S, Reinecke F, Möhlig M, Weickert MO, Clemenz M, Pfeiffer AFH, Kintscher U, Spuler S, Spranger J. Free Fatty Acids Link Metabolism and Regulation of the Insulin-Sensitizing Fibroblast Growth Factor-21. *Diabetes* 2009; 58: 1532–1538.
363. Fisher FM, Estall JL, Adams AC, Antonellis PJ, Bina HA, Flier JS, Kharitonenkov A, Spiegelman BM, Maratos-Flier

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

E. Integrated Regulation of Hepatic Metabolism by Fibroblast Growth Factor 21 (FGF21) in Vivo. *Endocrinology* 2011; 152: 2996–3004.

364. Gaich G, Chien JY, Fu H, Glass LC, Deeg MA, Holland WL, Kharitonenkov A, Bumol T, Schilske HK, Moller DE. The Effects of LY2405319, an FGF21 Analog, in Obese Human Subjects with Type 2 Diabetes. *Cell Metab* 2013; 18: 333–340.
365. Talukdar S, Zhou Y, Li D, Rossulek M, Dong J, Somayaji V, Weng Y, Clark R, Lanba A, Owen BM, Brenner MB, Trimmer JK, Gropp KE, Chabot JR, Erion DM, Rolph TP, Goodwin B, Calle RA. A Long-Acting FGF21 Molecule, PF-05231023, Decreases Body Weight and Improves Lipid Profile in Non-human Primates and Type 2 Diabetic Subjects. *Cell Metab* 2016; 23: 427–440.
366. Charles ED, Neuschwander-Tetri BA, Pablo Frias J, Kundu S, Luo Y, Tirucherai GS, Christian R. Pegbelfermin (BMS-986036), PEGylated FGF21, in Patients with Obesity and Type 2 Diabetes: Results from a Randomized Phase 2 Study. *Obesity* 2019; 27: 41–49.
367. Dong JQ, Rossulek M, Somayaji VR, Baltrukonis D, Liang Y, Hudson K, Hernandez-Illas M, Calle RA. Pharmacokinetics and pharmacodynamics of PF-05231023, a novel long-acting FGF21 mimetic, in a first-in-human study. *Br J Clin Pharmacol* 2015; 80: 1051–1063.
368. Kharitonenkov A, Wroblewski VJ, Koester A, Chen YF, Clutinger CK, Tigno XT, Hansen BC, Shanafelt AB, Etgen GJ. The metabolic state of diabetic monkeys is regulated by fibroblast growth factor-21. *Endocrinology* 2007; 148: 774–781.
369. Li X, Ge H, Weiszmann J, Hecht R, Li Y sheng, Véniant MM, Xu J, Wu X, Lindberg R, Li Y. Inhibition of lipolysis

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

may contribute to the acute regulation of plasma FFA and glucose by FGF21 in ob/ob mice. *FEBS Lett* 2009; 583: 3230–3234.

370. Liu Y, Zhao C, Xiao J, Liu L, Zhang M, Wang C, Wu G, Zheng MH, Xu LM, Chen YP, Mohammadi M, Chen SY, Cave M, McClain C, Li X, Feng W. Fibroblast growth factor 21 deficiency exacerbates chronic alcohol-induced hepatic steatosis and injury. *Sci Reports 2016 61* 2016; 6: 1–13.
371. Cui A, Li J, Ji S, Ma F, Wang G, Xue Y, Liu Z, Gao J, Han J, Tai P, Wang T, Chen J, Ma X, Li Y. The Effects of B1344, a Novel Fibroblast Growth Factor 21 Analog, on Nonalcoholic Steatohepatitis in Nonhuman Primates. *Diabetes* 2020; 69: 1611–1623.
372. Villarroya F, Cereijo R, Villarroya J, Giralt M. Brown adipose tissue as a secretory organ. *Nat Rev Endocrinol* 2017; 13: 26–35.
373. Kwon MM, O'Dwyer SM, Baker RK, Covey SD, Kieffer TJ. FGF21-Mediated Improvements in Glucose Clearance Require Uncoupling Protein 1. *Cell Rep* 2015; 13: 1521–1527.
374. Fisher FF, Kleiner S, Douris N, Fox EC, Mepani RJ, Verdeguer F, Wu J, Kharitonenkov A, Flier JS, Maratos-Flier E, Spiegelman BM. FGF21 regulates PGC-1α and browning of white adipose tissues in adaptive thermogenesis. *Genes Dev* 2012; 26: 271–281.
375. Kruse R, Vienberg SG, Vind BF, Andersen B, Højlund K. Effects of insulin and exercise training on FGF21, its receptors and target genes in obesity and type 2 diabetes. *Diabetologia* 2017; 60: 2042–2051.
376. Planavila A, Redondo-Angulo I, Villarroya F. FGF21 and Cardiac Physiopathology. *Front Endocrinol (Lausanne)*

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

2015; 6: 133.

377. Hsuchou H, Pan W, Kastin AJ. The fasting polypeptide FGF21 can enter brain from blood. *Peptides* 2007; 28: 2382–2386.
378. Liang Q, Zhong L, Zhang J, Wang Y, Bornstein SR, Triggle CR, Ding H, Lam KSL, Xu A. FGF21 maintains glucose homeostasis by mediating the cross talk between liver and brain during prolonged fasting. *Diabetes* 2014; 63: 4064–4075.
379. Owen BM, Ding X, Morgan DA, Coate KC, Bookout AL, Rahmouni K, Kliewer SA, Mangelsdorf DJ. FGF21 acts centrally to induce sympathetic nerve activity, energy expenditure, and weight loss. *Cell Metab* 2014; 20: 670–677.
380. Sa-nguanmoo P, Tanajak P, Kerdphoo S, Satjaritanun P, Wang X, Liang G, Li X, Jiang C, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. FGF21 improves cognition by restored synaptic plasticity, dendritic spine density, brain mitochondrial function and cell apoptosis in obese-insulin resistant male rats. *Horm Behav* 2016; 85: 86–95.
381. Sun Y, Wang Y, Chen ST, Chen YJ, Shen J, Yao WB, Gao XD, Chen S. Modulation of the astrocyte-neuron lactate shuttle system contributes to neuroprotective action of fibroblast growth factor 21. *Theranostics* 2020; 10: 8430–8445.
382. Shahror RA, Linares GR, Wang Y, Hsueh SC, Wu CC, Chuang DM, Chiang YH, Chen KY. Transplantation of mesenchymal stem cells overexpressing fibroblast growth factor 21 facilitates cognitive recovery and enhances neurogenesis in a mouse model of traumatic brain injury. *J Neurotrauma* 2020; 37: 14–26.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

383. Jiang Y, Lin L, Liu N, Wang Q, Yuan J, Li Y, Chung KK, Guo S, Yu Z, Wang X. FGF21 Protects against Aggravated Blood-Brain Barrier Disruption after Ischemic Focal Stroke in Diabetic db/db Male Mice via Cerebrovascular PPARγ Activation. *Int J Mol Sci*; 21. Epub ahead of print 1 February 2020. doi: 10.3390/ijms21030824.
384. Kelly AG, Holloway RG. Guideline: The AHA/ASA made 217 recommendations for early management of acute ischemic stroke in adults. *Ann Intern Med* 2018; 168: JC63.
385. Leng Y, Wang Z, Tsai LK, Leeds P, Fessler EB, Wang J, Chuang DM. FGF-21, a novel metabolic regulator, has a robust neuroprotective role and is dramatically elevated in neurons by mood stabilizers. *Mol Psychiatry* 2015; 20: 215.
386. Zheng W, Matei N, Pang J, Luo X, Song Z, Tang J, Zhang JH. Delayed recanalization at 3 days after permanent MCAO attenuates neuronal apoptosis through FGF21/FGFR1/PI3K/Caspase-3 pathway in rats. *Exp Neurol*; 320. Epub ahead of print 1 October 2019. doi: 10.1016/j.expneurol.2019.113007.
387. Wang D, Liu F, Zhu L, Lin P, Han F, Wang X, Tan X, Lin L, Xiong Y. FGF21 alleviates neuroinflammation following ischemic stroke by modulating the temporal and spatial dynamics of microglia/macrophages. *J Neuroinflammation* 2020; 17: 1–17.
388. Ávila-Gómez P, Vieites-Prado A, Dopico-López A, Bashir S, Fernández-Susavila H, Gubern C, Pérez-Mato M, Correa-Paz C, Iglesias-Rey R, Sobrino T, Bustamante A, Wellmann S, Montaner J, Serena J, Castillo J, Hervella P, Campos F. Cold stress protein RBM3 responds to hypothermia and is associated with good stroke outcome. *Brain Commun*; 2. Epub ahead of print 1 July 2020. doi:

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

10.1093/braincomms/fcaa078.

389. Si W, Li Z, Huang Z, Ye S, Li X, Li Y, Kuang W, Chen D, Zhu M. RNA Binding Protein Motif 3 Inhibits Oxygen-Glucose Deprivation/Reoxygenation-Induced Apoptosis Through Promoting Stress Granules Formation in PC12 Cells and Rat Primary Cortical Neurons. *Front Cell Neurosci* 2020; 14: 287.
390. Li Y, Zhang J. Animal models of stroke. *Anim Model Exp Med* 2021; 4: 204–219.
391. Ng YS, Stein J, Ning MM, Black-Schaffer RM. Comparison of clinical characteristics and functional outcomes of ischemic stroke in different vascular territories. *Stroke* 2007; 38: 2309–2314.
392. Morris GP, Wright AL, Tan RP, Gladbach A, Ittner LM, Vissel B. A Comparative Study of Variables Influencing Ischemic Injury in the Longa and Koizumi Methods of Intraluminal Filament Middle Cerebral Artery Occlusion in Mice. *PLoS One*; 11. Epub ahead of print 1 February 2016. doi: 10.1371/journal.pone.0148503.
393. Kudo M, Aoyama A, Ichimori S, Fukunaga N. An animal model of cerebral infarction. Homologous blood clot emboli in rats. *Stroke* 1982; 13: 505–508.
394. Busch E, Krüger K, Hossmann KA. Improved model of thromboembolic stroke and rt-PA induced reperfusion in the rat. *Brain Res* 1997; 778: 16–24.
395. Orset C, Macrez R, Young AR, Panthou D, Angles-Cano E, Maubert E, Agin V, Vivien D. Mouse model of in situ thromboembolic stroke and reperfusion. *Stroke* 2007; 38: 2771–2778.
396. Zivin JA, DeGirolami U, Kochhar A, Lyden PD, Mazzarella V, Hemenway CC, Henry ME. A model for

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

quantitative evaluation of embolic stroke therapy. *Brain Res* 1987; 435: 305–309.
397. Tamura A, Graham DI, McCulloch J, Teasdale GM. Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. *J Cereb Blood Flow Metab* 1981; 1: 53–60.
398. Chen ST, Hsu CY, Hogan EL, Maricq H, Balentine JD. A model of focal ischemic stroke in the rat: reproducible extensive cortical infarction. *Stroke* 1986; 17: 738–743.
399. Sharkey J, Ritchie IM, Kelly PAT. Perivascular microapplication of endothelin-1: a new model of focal cerebral ischaemia in the rat. *J Cereb Blood Flow Metab* 1993; 13: 865–871.
400. Watson BD, Dietrich WD, Busto R, Wachtel MS, Ginsberg MD. Induction of reproducible brain infarction by photochemically initiated thrombosis. *Ann Neurol* 1985; 17: 497–504.
401. Fluri F, Schuhmann MK, Kleinschnitz C. Animal models of ischemic stroke and their application in clinical research. *Drug Des Devel Ther* 2015; 9: 3445–3454.
402. MacRae I. Preclinical stroke research--advantages and disadvantages of the most common rodent models of focal ischaemia. *Br J Pharmacol* 2011; 164: 1062–1078.
403. Kumar A, Aakriti, Gupta V. A review on animal models of stroke: An update. *Brain Res Bull* 2016; 122: 35–44.
404. Liu K, Khan H, Geng X, Zhang J, Ding Y. Pharmacological hypothermia: a potential for future stroke therapy? *Neurol Res* 2016; 38: 478–490.
405. Feketa V V., Marrelli SP. Systemic Administration of the TRPV3 Ion Channel Agonist Carvacrol Induces

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Hypothermia in Conscious Rodents. *PLoS One* 2015; 10: e0141994.

406. Taniguchi T, Morikawa E, Mori T, Matsui T. Neuroprotective efficacy of selective brain hypothermia induced by a novel external cooling device on permanent cerebral ischemia in rats. *Neurol Res* 2005; 27: 613–619.
407. Liu Q, Cai Y, Lin W, Turner GH, An H. A magnetic resonance (MR) compatible selective brain temperature manipulation system for preclinical study. *Med Devices (Auckl)* 2012; 5: 13–22.
408. Imoto H, Fujii M, Uchiyama J, Fujisawa H, Nakano K, Kunitsugu I, Nomura S, Saito T, Suzuki M. Use of a Peltier chip with a newly devised local brain-cooling system for neocortical seizures in the rat. Technical note. *J Neurosurg* 2006; 104: 150–156.
409. Clark DL, Colbourne F. A simple method to induce focal brain hypothermia in rats. *J Cereb Blood Flow Metab* 2007; 27: 115–122.
410. D'Ambrosio R, Eastman CL, Darvas F, Fender JS, Verley DR, Farin FM, Wilkerson HW, Temkin NR, Miller JW, Ojemann J, Rothman SM, Smyth MD. Mild passive focal cooling prevents epileptic seizures after head injury in rats. *Ann Neurol* 2013; 73: 199–209.
411. Mattingly TK, Denning LM, Siroen KL, Lehrbass B, Lopez-Ojeda P, Stitt L, Pelz DM, Das S, Ang LC, Lee DH, Lownie SP. Catheter based selective hypothermia reduces stroke volume during focal cerebral ischemia in swine. *J Neurointerv Surg* 2016; 8: 418–422.
412. Mancini MC, De Melo ME. The burden of obesity in the current world and the new treatments available: focus on liraglutide 3.0 mg. *Diabetol Metab Syndr* 2017; 9: 44.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

413. Cawley J, Meyerhoefer C. The medical care costs of obesity: an instrumental variables approach. *J Health Econ* 2012; 31: 219–230.
414. Blüher M. Obesity: global epidemiology and pathogenesis. *Nat Rev Endocrinol* 2019; 15: 288–298.
415. Bhardwaj P, Brown KA. Obese Adipose Tissue as a Driver of Breast Cancer Growth and Development: Update and Emerging Evidence. *Front Oncol*; 11. Epub ahead of print 30 March 2021. DOI: 10.3389/FONC.2021.638918.
416. Formica V, Morelli C, Riondino S, Renzi N, Nitti D, Di Daniele N, Roselli M, Tesauro M. Obesity and common pathways of cancer and cardiovascular disease. *Endocr Metab Sci* 2020; 1: 100065.
417. Andersen KK, Olsen TS. The obesity paradox in stroke: lower mortality and lower risk of readmission for recurrent stroke in obese stroke patients. *Int J Stroke* 2015; 10: 99–104.
418. Andersen KK, Olsen TS. Body Mass Index and Stroke: Overweight and Obesity Less Often Associated with Stroke Recurrence. *J Stroke Cerebrovasc Dis* 2013; 22: e576–e581.
419. Antonopoulos AS, Oikonomou EK, Antoniades C, Tousoulis D. From the BMI paradox to the obesity paradox: the obesity-mortality association in coronary heart disease. *Obes Rev* 2016; 17: 989–1000.
420. Hallan DR. Obesity and Mechanical Thrombectomy. *Cureus*; 13. Epub ahead of print 13 January 2021. doi: 10.7759/cureus.12671.
421. Gago-Dominguez M, Sobrino T, Torres-Español M, Calaza M, Rodríguez-Castro E, Campos F, Redondo CM, Castillo J, Carracedo Á. Obesity-related genetic determinants of

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

stroke. *Brain Commun*; 3. Epub ahead of print 5 April 2021. doi: 10.1093/braincomms/fcab069.

422. Crujeiras AB, Gomez-Arbelaez D, Zulet MA, Carreira MC, Sajoux I, De Luis D, Castro AI, Baltar J, Baamonde I, Sueiro A, MacIas-Gonzalez M, Bellido D, Tinahones FJ, Martinez JA, Casanueva FF. Plasma FGF21 levels in obese patients undergoing energy-restricted diets or bariatric surgery: a marker of metabolic stress? *Int J Obes (Lond)* 2017; 41: 1570–1578.
423. Chen S, Chen ST, Sun Y, Xu Z, Wang Y, Yao SY, Yao WB, Gao XD. Fibroblast growth factor 21 ameliorates neurodegeneration in rat and cellular models of Alzheimer's disease. *Redox Biol*; 22. Epub ahead of print 1 April 2019. DOI: 10.1016/J.REDOX.2019.101133.
424. Jiang Y, Liu N, Wang Q, Yu Z, Lin L, Yuan J, Guo S, Ahn BJ, Wang XJ, Li X, Lo EH, Sun X, Wang X. Endocrine Regulator rFGF21 (Recombinant Human Fibroblast Growth Factor 21) Improves Neurological Outcomes Following Focal Ischemic Stroke of Type 2 Diabetes Mellitus Male Mice. *Stroke* 2018; 49: 3039–3049.
425. Tabari FS, Karimian A, Parsian H, Rameshknia V, Mahmoodpour A, Majidinia M, Maniati M, Yousefi B. The roles of FGF21 in atherosclerosis pathogenesis. *Rev Endocr Metab Disord*; 20. Epub ahead of print 2019. doi: 10.1007/s11154-019-09488-x.
426. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 1989; 20: 84–91.
427. De Boer HH (Hans., Van der Merwe AE (Lida., Soerdjbalie-Maikoe V (Vidija). Human cranial vault thickness in a contemporary sample of 1097 autopsy cases:

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

relation to body weight, stature, age, sex and ancestry. *Int J Legal Med* 2016; 130: 1371.

428. O'Reilly MA, Muller A, Hynynen K. Ultrasound insertion loss of rat parietal bone appears to be proportional to animal mass at sub-megahertz frequencies. *Ultrasound Med Biol* 2011; 37: 1930.
429. Soleimanzad H, Gurden H, Pain F. Optical properties of mice skull bone in the 455- to 705-nm range. *J Biomed Opt* 2017; 22: 010503.
430. Shankaran S, Pappas A, McDonald SA, Vohr BR, Hintz SR, Yolton K, Gustafson KE, Leach TM, Green C, Bara R, Huitema CMP, Ehrenkranz RA, Tyson JE, Das A, Hammond J, Peralta-Carcelen M, Evans PW, Heyne RJ, Wilson-Costello DE, et al. Childhood Outcomes after Hypothermia for Neonatal Encephalopathy. *N Engl J Med* 2012; 366: 2085.
431. Guillet R, Edwards AD, Thoresen M, Ferriero DM, Gluckman PD, Whitelaw A, Gunn AJ. Seven- to eight-year follow-up of the CoolCap trial of head cooling for neonatal encephalopathy. *Pediatr Res 2012 712* 2011; 71: 205–209.
432. Liddle LJ, Kalisvaart ACJ, Abrahart AH, Almekhlafi M, Demchuk A, Colbourne F. Targeting focal ischemic and hemorrhagic stroke neuroprotection: Current prospects for local hypothermia. *J Neurochem* 2021; 00: 1–17.
433. Campos F, Sobrino T, Vieites-Prado A, Pérez-Mato M, Rodríguez-Yáñez M, Blanco M, Castillo J. Hyperthermia in Human Ischemic and Hemorrhagic Stroke: Similar Outcome, Different Mechanisms. *PLoS One* 2013; 8: e78429.
434. Campos F, Pérez-Mato M, Agulla J, Blanco M, Barral D, Almeida Á, Brea D, Waeber C, Castillo J, Ramos-Cabrer P. Glutamate excitoxicity is the key molecular mechanism

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

which is influenced by body temperature during the acute phase of brain stroke. *PLoS One*; 7. Epub ahead of print 28 August 2012. doi: 10.1371/journal.pone.0044191.

435. Nishiyama H, Itoh K, Kaneko Y, Kishishita M, Yoshida O, Fujita J. A glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth. *J Cell Biol* 1997; 137: 899–908.
436. Yang HJ, Shi X, Ju F, Hao BN, Ma SP, Wang L, Cheng BF, Wang M. Cold Shock Induced Protein RBM3 but Not Mild Hypothermia Protects Human SH-SY5Y Neuroblastoma Cells From MPP +-Induced Neurotoxicity. *Front Neurosci*; 12. Epub ahead of print 3 May 2018. doi: 10.3389/fnins.2018.00298.
437. Ye F, Jin P, Cai X, Cai PP, An H. High RNA-Binding Motif Protein 3 (RBM3) Expression is Independently Associated with Prolonged Overall Survival in Intestinal-Type Gastric Cancer. *Med Sci Monit* 2017; 23: 6033–6041.
438. Kang SH, Cho J, Jeong H, Kwon SY. High RNA-binding Motif Protein 3 Expression Is Associated with Improved Clinical Outcomes in Invasive Breast Cancer. *J Breast Cancer* 2018; 21: 288–296.
439. Jang HH, Lee HN, Kim SY, Hong S, Lee WS. Expression of RNA-binding Motif Protein 3 (RBM3) and Cold-inducible RNA-binding protein (CIRP) Is Associated with Improved Clinical Outcome in Patients with Colon Cancer. *Anticancer Res* 2017; 37: 1779–1785.
440. Siesing C, Sorbye H, Dragomir A, Pfeiffer P, Qvortrup C, Pontén F, Jirström K, Glimelius B, Eberhard J. High RBM3 expression is associated with an improved survival and oxaliplatin response in patients with metastatic colorectal cancer. *PLoS One*; 12. Epub ahead of print 1 August 2017.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

doi: 10.1371/journal.pone.0182512.
441. Jackson TC, Janesko-Feldman K, Carlson SW, Kotermanski SE, Kochanek PM. Robust RBM3 and β-klotho expression in developing neurons in the human brain. *J Cereb Blood Flow Metab* 2019; 39: 2355–2367.
442. Cuevas-Ramos D, Mehta R, Aguilar-Salinas CA. Fibroblast growth factor 21 and browning of white adipose tissue. *Front Physiol* 2019; 10: 37.
443. Muise ES, Azzolina B, Kuo DW, El-Sherbeini M, Tan Y, Yuan X, Mu J, Thompson JR, Berger JP, Wong KK. Adipose fibroblast growth factor 21 is up-regulated by peroxisome proliferator-activated receptor gamma and altered metabolic states. *Mol Pharmacol* 2008; 74: 403–412.
444. Badman MK, Kennedy AR, Adams AC, Pissios P, Maratos-Flier E. A very low carbohydrate ketogenic diet improves glucose tolerance in ob/ob mice independently of weight loss. *Am J Physiol Endocrinol Metab*; 297. Epub ahead of print November 2009. doi: 10.1152/ajpendo.00357.2009.
445. Dostálová I, Kaválková P, Haluzíková D, Lacinová Z, Mráz M, Papežová H, Haluzík M. Plasma concentrations of fibroblast growth factors 19 and 21 in patients with anorexia nervosa. *J Clin Endocrinol Metab* 2008; 93: 3627–3632.
446. Logan SM, Storey KB. Cold-inducible RNA-binding protein Cirp, but not Rbm3, may regulate transcript processing and protection in tissues of the hibernating ground squirrel. *Cell Stress Chaperones* 2020; 25: 857.
447. Jonsson L, Bergman J, Nodin B, Manjer J, Pontén F, Uhlén M, Jirström K. Low RBM3 protein expression correlates with tumour progression and poor prognosis in malignant melanoma: an analysis of 215 cases from the Malmo Diet

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

and Cancer Study. *J Transl Med* 2011; 9: 114–114.

448. Jögi A, Brennan DJ, Rydén L, Magnusson K, Fernö M, Stål O, Borgquist S, Uhlen M, Landberg G, Påhlman S, Pontén F, Jirström K. Nuclear expression of the RNA-binding protein RBM3 is associated with an improved clinical outcome in breast cancer. *Mod Pathol 2009 2212* 2009; 22: 1564–1574.
449. Chavez AO, Molina-Carrion M, Abdul-Ghani MA, Folli F, DeFronzo RA, Tripathy D. Circulating fibroblast growth factor-21 is elevated in impaired glucose tolerance and type 2 diabetes and correlates with muscle and hepatic insulin resistance. *Diabetes Care* 2009; 32: 1542–1546.
450. Semba RD, Crasto C, Strait J, Sun K, Schaumberg DA, Ferrucci L. Elevated serum fibroblast growth factor 21 is associated with hypertension in community-dwelling adults. *J Hum Hypertens* 2013; 27: 397–399.
451. Fisher FM, Chui PC, Antonellis PJ, Bina HA, Kharitonenkov A, Flier JS, Maratos-Flier E. Obesity Is a Fibroblast Growth Factor 21 (FGF21)-Resistant State. *Diabetes* 2010; 59: 2781.
452. Mraz M, Bartlova M, Lacinova Z, Michalsky D, Kasalicky M, Haluzikova D, Matoulek M, Dostalova I, Humenanska V, Haluzik M. Serum concentrations and tissue expression of a novel endocrine regulator fibroblast growth factor-21 in patients with type 2 diabetes and obesity. *Clin Endocrinol (Oxf)* 2009; 71: 369–375.
453. Lin Z, Wu Z, Yin X, Liu Y, Yan X, Lin S, Xiao J, Wang X, Feng W, Li X. Serum levels of FGF-21 are increased in coronary heart disease patients and are independently associated with adverse lipid profile. *PLoS One*; 5. Epub ahead of print 2010. doi: 10.1371/journal.pone.0015534.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

454. Lenart-Lipińska M, Matyjaszek-Matuszek B, Gernand W, Nowakowski A, Solski J. Serum fibroblast growth factor 21 is predictive of combined cardiovascular morbidity and mortality in patients with type 2 diabetes at a relatively short-term follow-up. *Diabetes Res Clin Pract* 2013; 101: 194–200.
455. Ye D, Li H, Wang Y, Jia W, Zhou J, Fan J, Man K, Lo C, Wong C, Wang Yu Y, Lam KSL, Xu A. Circulating Fibroblast Growth Factor 21 Is A Sensitive Biomarker for Severe Ischemia/reperfusion Injury in Patients with Liver Transplantation. *Sci Rep*; 6. Epub ahead of print 25 January 2016. doi: 10.1038/srep19776.
456. Ebrahimi F, Wolffenbuttel C, Blum CA, Baumgartner C, Mueller B, Schuetz P, Meier C, Kraenzlin M, Christ-Crain M, Betz MJ. Fibroblast growth factor 21 predicts outcome in community-acquired pneumonia: secondary analysis of two randomised controlled trials. *Eur Respir J*; 53. Epub ahead of print 1 February 2019. doi: 10.1183/13993003.00973-2018.
457. Zheng X, Zhu Z, Guo D, Zhong C, Xu T, Peng Y, Wang A, Peng H, Ju Z, Geng D, Zhang Y, He J. Prognostic value of plasma fibroblast growth factor 21 among patients with acute ischemic stroke. *Eur J Neurol* 2021; 28: 844–851.
458. Asrih M, Veyrat-Durebex C, Poher AL, Lyautey J, Rohner-Jeanrenaud F, Jornayvaz FR. Leptin as a Potential Regulator of FGF21. *Cell Physiol Biochem* 2016; 38: 1218–1225.
459. Greco SJ, Hamzelou A, Johnston JM, Smith MA, Ashford JW, Tezapsidis N. Leptin boosts cellular metabolism by activating AMPK and the sirtuins to reduce tau phosphorylation and β-amyloid in neurons. *Biochem*

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Biophys Res Commun 2011; 414: 170–174.

460. Busch HJ, Schirmer SH, Jost M, Van Stijn S, Peters SLM, Piek JJ, Bode C, Buschmann IR, Mies G. Leptin augments cerebral hemodynamic reserve after three-vessel occlusion: Distinct effects on cerebrovascular tone and proliferation in a nonlethal model of hypoperfused rat brain. *J Cereb Blood Flow Metab* 2011; 31: 1085–1092.
461. Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y. PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes* 2001; 50: 2094–2099.
462. Lin Z, Pan X, Wu F, Ye D, Zhang Y, Wang Y, Jin L, Lian Q, Huang Y, Ding H, Triggle C, Wang K, Li X, Xu A. Fibroblast growth factor 21 prevents atherosclerosis by suppression of hepatic sterol regulatory element-binding protein-2 and induction of adiponectin in mice. *Circulation* 2015; 131: 1861–1871.
463. Liu C, Schö Nke M, Zhou E, Li Z, Kooijman S, Tte M, Boon R, Larsson M, Wallenius K, Dekker N, Barlind L, Peng X-R, Wang Y, Rensen PCN. Pharmacological treatment with FGF21 strongly improves plasma cholesterol metabolism to reduce atherosclerosis. *Cardiovasc Res*. Epub ahead of print 8 March 2021. doi: 10.1093/cvr/cvab076.
464. Vila N, Castillo J, Dávalos A, Chamorro Á. Proinflammatory cytokines and early neurological worsening in ischemic stroke. *Stroke* 2000; 31: 2325–2329.
465. Castillo J, Álvarez-Sabín J, Martínez-Vila E, Montaner J, Sobrino T, Vivancos J. Inflammation markers and prediction of post-stroke vascular disease recurrence: the

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

MITICO study. *J Neurol* 2009; 256: 217–224.

466. Berti L, Irmler M, Zdichavsky M, Meile T, Böhm A, Stefan N, Fritsche A, Beckers J, Königsrainer A, Häring HU, de Angelis MH, Staiger H. Fibroblast growth factor 21 is elevated in metabolically unhealthy obesity and affects lipid deposition, adipogenesis, and adipokine secretion of human abdominal subcutaneous adipocytes. *Mol Metab* 2015; 4: 519–527.
467. Russwurm S, Stonans I, Schwerter K, Stonane E, Meissner W, Reinhart K. Direct influence of mild hypothermia on cytokine expression and release in cultures of human peripheral blood mononuclear cells. *J Interferon Cytokine Res* 2002; 22: 215–221.
468. Sutcliffe IT, Smith HA, Stanimirovic D, Hutchison JS. Effects of moderate hypothermia on IL-1 beta-induced leukocyte rolling and adhesion in pial microcirculation of mice and on proinflammatory gene expression in human cerebral endothelial cells. *J Cereb Blood Flow Metab* 2001; 21: 1310–1319.
469. Liu L, Liu X, Wang R, Yan F, Luo Y, Chandra A, Ding Y, Ji X. Mild focal hypothermia regulates the dynamic polarization of microglia after ischemic stroke in mice. *https://doi.org/101080/0161641220181454090* 2018; 40: 508–515.
470. Burnsed JC, Chavez-Valdez R, Hossain MS, Kesavan K, Martin LJ, Zhang J, Northington FJ. Hypoxia-Ischemia and Therapeutic Hypothermia in the Neonatal Mouse Brain – A Longitudinal Study. *PLoS One*; 10. Epub ahead of print 16 March 2015. DOI: 10.1371/JOURNAL.PONE.0118889.
471. Koo E, Sheldon RA, Lee BS, Vexler ZS, Ferriero DM. Effects of therapeutic hypothermia on white matter injury from

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

murine neonatal hypoxia–ischemia. *Pediatr Res 2017 823* 2017; 82: 518–526.

472. Tong G, Ahnert U, Schmitt K. RBM3: Possible biomarker for therapeutic hypothermia. *Resuscitation* 2016; 106: e83.
473. Rosenthal LM, Leithner C, Tong G, Streitberger KJ, Krech J, Storm C, Luise Schmitt KR. RBM3 and CIRP expressions in targeted temperature management treated cardiac arrest patients—A prospective single center study. *PLoS One* 2019; 14: e0226005.
474. Makker P, Kanei Y, Misra D. Clinical Effect of Rebound Hyperthermia After Cooling Postcardiac Arrest: A Meta-Analysis. *Ther Hypothermia Temp Manag* 2017; 7: 206–209.
475. Hankenson FC, Marx JO, Gordon CJ, David JM. Effects of Rodent Thermoregulation on Animal Models in the Research Environment. *Comp Med* 2018; 68: 425.
476. Dordoe C, Chen K, Huang W, Chen J, Hu J, Wang X, Lin L. Roles of Fibroblast Growth Factors and Their Therapeutic Potential in Treatment of Ischemic Stroke. *Front Pharmacol* 2021; 12: 853.
477. Shao W, Jin T. Hepatic hormone FGF21 and its analogues in clinical trials. *Chronic Dis Transl Med*. Epub ahead of print 20 October 2021. doi: 10.1016/j.cdtm.2021.08.005.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

ANNEX

Editorial Approval:

OXFORD UNIVERSITY PRESS LICENSE
TERMS AND CONDITIONS

Feb 10, 2022

This Agreement between Mr. Paulo Ávila-Gómez ("You") and Oxford University Press ("Oxford University Press") consists of your license details and the terms and conditions provided by Oxford University Press and Copyright Clearance Center.

License Number	5245420277197
License date	Feb 10, 2022
Licensed content publisher	Oxford University Press
Licensed content publication	Brain Communications
Licensed content title	Cold stress protein RBM3 responds to hypothermia and is associated with good stroke outcome
Licensed content author	Ávila-Gómez, Paulo; Vieites-Prado, Alba
Licensed content date	Jun 4, 2020
Type of Use	Thesis/Dissertation
Institution name	
Title of your work	Role of RBM3 in cerebral ischemia

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Publisher of your work	University of Santiago de Compostela
Expected publication date	Feb 2022
Permissions cost	0.00 USD
Value added tax	0.00 USD
Total	0.00 USD
Title	Role of RBM3 in cerebral ischemia
Institution name	University of Santiago de Compostela
Expected presentation date	Feb 2022
Portions	Text: Description of the clinical studies in the Matherial and Methods, Results and Supplemental section. The text has been rearranged and partially rewritten. -Figures: 2, 5, 6, S2, S3, S4 -Tables: 1, 2, S3, S4, S7, S8, S9, S10
Requestor Location	Mr. Paulo Ávila-Gómez Virgen del Camino 1 5ºB Pontevedra, Pontevedra 15701 Spain Attn: Mr. Paulo Ávila-Gómez
Publisher Tax ID	GB125506730
Total	0.00 USD

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Other first-author publications derived from the Thesis period

Ávila-Gómez P, Hervella P, Da Silva-Candal A, Pérez-Mato M, Rodríguez-Yáñez M, López-Dequidt I, Pumar JM, Castillo J, Sobrino T, Iglesias-Rey R, Campos F. Temperature-Induced Changes in Reperfused Stroke: Inflammatory and Thrombolytic Biomarkers. J Clin Med. 2020 Jul 4;9(7):2108. doi: 10.3390/jcm9072108. CLINICAL MEDICINE. ISSN: 2077-0383 Impact Factor: 4.242 (Q1).

Ávila-Gómez P, Hervella P, Dopico-López A, da Silva-Candal A, Bugallo-Casal A, López-Amoedo A, Candamo M, Sobrino T, Iglesias-Rey R, Castillo J, Campos F. RBM3 and FGF21 mediate in the good outcome in stroke obesity paradox (In preparation)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Conflicts of interest

The author and Directors agreed to present this Thesis and declare no conflicts of interest.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Research stay

During the development of the Thesis, the PhD student performed a stay in an international laboratory with the group of Dr. Christa Rhiner at the Champalimaud Centre of the Unknown in Lisbon, Portugal, from the first of July to the 15th of October 2021.

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Funding
This study was partially supported by the Instituto de Salud Carlos III (PI17/00540, the European Union program FEDER and the European Regional Development Fund-ERDF, and the Xunta de Galicia (GAIN_IN607D2020/03).

During the development of this Thesis, the doctoral candidate benefited from a predoctoral scholarship granted by the Health Research Institute of Santiago de Compostela Foundation. During this period, Dr. Francisco Campos, Director of the Thesis, has been the beneficiary of the Miguel Servet contract, of the Instituto de Salud Carlos III (CP14/00154).

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

XUNTA DE GALICIA
CONSELLERÍA DE SANIDADE
Secretaría Xeral Técnica

Comité de Ét ca de la Invest gación
Sant ago Lugo

galicia

DICTAMEN DEL COMITÉ DE ÉTICA DE LA INVESTIGACIÓN DE SANTIAGO-LUGO

Mª Mercedes Rodicio García, Vicesecretaria del Comité de Ét ca de la Invest gación de Sant ago-Lugo,

CERTIFICA:

Que este Comité evaluó en su reunión del día 18 de febrero de 2020 el estudio:

Título: Papel de la proteína RBM3 en la isquemia cerebral
Versión: V23/01/2020
Promotor/a: Francisco Campos Pérez
Invest gador/a: Francisco Campos Pérez
Código de Registro: 2019/616

Y que este Comité, tomando en consideración la pert nencia del estudio, el conocimiento disponible, los requisitos legales aplicables y los Procedimientos Normalizados de Trabajo del Comité, emite un dictamen **FAVORABLE** para la realización del citado estudio.

Xacobeo 2021

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

XUNTA DE GALICIA
CONSELLERÍA DE SANIDADE
Secretaría Xeral Técnica

Comité de Ét ca de la Invest gación
Sant ago Lugo

galicia

Y HACE CONSTAR QUE:

1.- El Comité Territorial de Ét ca de la Invest gación de Sant ago-Lugo cumple tanto en su composición como en sus PNTs los requisitos legales vigentes.

2.- La composición actual del Comité Territorial de Ét ca de la Invest gación de Sant ago-Lugo es:

Presidente
Juan Manuel Vázquez Lago. Médico especialista en Medicina Prevent va y Salud Pública. Área de Gest ón Integrada de Sant ago.

Vicepresidenta
Pilar Rodríguez Ledo. Médico especialista en Medicina Familiar y Comunitaria. Área de Gest ón Integrada de Lugo.

Secretaria
Ana Estany Gestal. Licenciada en Farmacia. Fundación Inst tuto de Invest gación Sanitaria de Sant ago de Compostela.

Vicesecretaria
María Mercedes Rodicio García. Médico especialista en Pediatría. Área de Gest ón Integrada de Lugo.

Vocales
María José Alfaro Águila-Real. Paciente experta. Lugo
Francisco Campos Pérez. Biólogo. Fundación Inst tuto de Invest gación Sanitaria de Sant ago de Compostela.
Ricardo García Mart nez. Licenciado en Derecho. Área de Gest ón Integrada de Lugo.
Jaime Gulín Dávila. Farmacéut co especialista en Farmacia Hospitalaria. Área de Gest ón Integrada de Lugo.
Guillermo José Prada Ramallal Médico especialista en Farmacología Clínica. Área de Gest ón Integrada de Sant ago. Fundación Inst tuto de Invest gación Sanitaria de Sant ago de Compostela.
Jesús Prego Domínguez. Enfermero. Área de Gest ón Integrada de Sant ago.
Lorenzo Armenteros del Olmo. Médico especialista en Medicina Familiar y Comunitaria. Área de Gest ón Integrada de Lugo.
Carlos Rodríguez Moreno. Médico especialista en Farmacología Clínica. Área de Gest ón Integrada de Sant ago.

Para que conste donde proceda, y a pet ción de quien proceda, en Sant ago de Compostela,

La Vicesecretaria del Comité Territorial de Ét ca de la Invest gación de Sant ago Lugo,

Xacobeo 2021

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

XUNTA DE GALICIA
CONSELLERÍA DE SANIDADE
Secretaría Xeral Técnica

Comité de Ét ca de la Invest gación
Sant ago Lugo

galicia

NOTA: Se le recuerda que en el caso de que en este estudio se recluten pacientes, el equipo invest gador debe tener disponible el Documento de Consent miento Informado (Hojas de Información y Hojas de Firma) tanto en *galego* como en castellano en el momento de comenzar el reclutamiento.

Xacobeo 2021

USC
UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

XUNTA DE GALICIA
CONSELLERÍA DO MEDIO RURAL

Departamento Territorial
Edificio Administrativo de Servizos Múltiples
Vicente Ferrer, 2
15071 – A Coruña

RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTOS DE EXPERIMENTACIÓN ANIMAL

Expediente núm.: 15010/2019/004 **Data de inicio:** 05.02.2019
Persoa interesada: Francisco Campos Pérez **Procedemento:** resolución de autorización
Forma de inicio: solicitude da persoa interesada

ANTECEDENTES

A persoa interesada, como representante do centro CIMUS (Universidade de Santiago de Compostela), presentou con data 05.02.2019 unha solicitude para a realización do proxecto de experimentación animal (entrada no Rexistro Electrónico da Xunta de Galicia 2019/246024), cuxos datos se detallan a continuación:

Denominación do proxecto: *Nanopartículas biomiméticas para a administración dirixida de nanomedicinas*
Nome do centro usuario: Animalario do CIMUS
Persoa responsable do proxecto: Francisco Campos Pérez
Establecemento onde se realizarán os procedementos do proxecto (ou lugar xeográfico no caso de traballos de campo): Animalario do CIMUS
Clasificación do proxecto : Tipo I ☐ Tipo II ☐ Tipo III ☒

CONSIDERACIÓNS LEGAIS E TÉCNICAS

1 O Real decreto 53/2013, de 1 de febreiro (BOE 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece no seu artigo 33 as condicións de autorizacións dos proxectos con animais de experimentación.

2 O artigo 88 da Lei 39/2015, de 1 de outubro, do procedemento administrativo común das administracións públicas (BOE 236, do 2 de outubro de 2015) establece que a resolución que poña fin o procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas deste.

3 O Servizo de Gandaría da Coruña revisou a documentación achegada na solicitude e o resultado favorable da avaliación do proxecto, realizada polo órgano habilitado da Sección de Experimentación Animal do Comité de Bioética da Universidade de Santiago de Compostela.

Esta xefatura territorial é competente para ditar unha resolución, de conformidade co Decreto 149/2018, do 5 de decembro, polo que se establece a estrutura orgánica da

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

XUNTA DE GALICIA
CONSELLERÍA DO MEDIO RURAL

Departamento Territorial
Edificio Administrativo de Servizos Múltiples
Vicente Ferrer, 2
15071 – A Coruña

Consellería do Medio Rural e se modifica parcialmente o Decreto 177/2016, do 15 de decembro, polo que se fixa a estrutura orgánica da Vicepresidencia e das consellerías da Xunta de Galicia (DOG 235, do 11 de novembro).

De acordo con todo o indicado, RESOLVO:

1 Autorizar o proxecto solicitado.
2 O mencionado proxecto precisa someterse a unha avaliación retrospectiva tras finalizar a súa autorización.
3 A autorización deste proxecto terá unha duración de dous anos e unha vez transcorrido este tempo deberá ser autorizado de novo.

A citada autorización é unicamente válida nas condicións que figuran no expediente. Ante calquera cambio significativo no proxecto que poida ter efectos negativos sobre o benestar dos animais, deberá solicitar a confirmación da autorización ao Servizo Provincial de Gandaría.

Esta autorización poderá ser suspendida, no caso de que o proxecto non se leve a cabo de acordo coas condicións de autorización e retirala, previo expediente tramitado ao que se lle dará audiencia.

Contra a presente resolución, que non lle pon fin á vía administrativa, poderá interpoñer un recurso de alzada ante o conselleiro de Medio Rural. O prazo comezará a contar dende o día seguinte ao da recepción desta resolución. Todo isto, segundo o disposto nos artigos 121 e 122 da citada Lei 39/2015.

Mediante este escrito notifícaselle ao CIMUS da USC esta resolución segundo o esixido no artigo 40.1 da antedita Lei 39/2015.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

VICERREITORÍA DE INVESTIGACIÓN
E INNOVACIÓN
Oficina de Investigación e Tecnoloxía
Servizo de Convocatorias e Recursos Humanos de I+i
Edificio CACTUS – Campus Vida
15782 Santiago de Compostela
Tel. 981 547 040 - Fax 981 547 077
Correo electrónico: otiinfo@usc.es
http://mailsd.usc.es

JOSÉ MANUEL CIFUENTES MARTÍNEZ, PRESIDENTE DEL COMITÉ DE BIOÉTICA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA, cuya Sección de Experimentación animal ha sido designada como Órgano Habilitado para la evaluación de proyectos de experimentación animal por resolución de la Xunta de Galicia, con fecha 11 de noviembre de 2013, de acuerdo con lo exigido por el RD 53/2013 de 1 de febrero, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia,

INFORMA:

Que el proyecto de investigación titulado: "**Nanopartículas biomiméticas para la administración dirigida de nanomedicinas**" del que es investigador responsable **D. Francisco Campos Pérez**, ha sido examinado por el Comité de Bioética de esta Universidad, Sección de Experimentación Animal, llegando a las siguientes conclusiones:

Con respecto a su finalidad, se trata de un proyecto de investigación traslacional o aplicada cuyo objetivo fundamentale el desarrollo de nanosistemas biomiméticos basados en cubiertas celulares (extraídas de células madre mesenquimales y/o plaquetas) para dirigir nanomedicinas a su lugar de acción (diana).

- Con respecto a los requisitos de las 3Rs,
  - No cabe la posibilidad de reemplazo ya que no se han encontrado otros métodos o estrategias de ensayo que permitan llevar a cabo los experimentos propuestos en este trabajo.
  - La experimentación se realizará en un centro registrado como usuario de animales de experimentación por lo que la manipulación, manejo y supervisión de los animales durante todo el proyecto será llevada a cabo por personas capacitadas. El grupo investigador lo componen personas con capacitaciones A, B y C, lo que asegura su preparación para garantizar el bienestar animales durante todos los procedimientos (requisito de refinamiento).
  - Finalmente, con respecto al requisito de reducción, se considera que el número de animales a utilizar es el mínimo imprescindible para la obtención de los resultados.

- La clasificación de los procedimientos en función de su grado de severidad es de "leve pa ra los procedimientos 1, 3 y "severo" para los procedimiento 2 y 4.
- Con respecto al balance de los daños y los beneficios, los procedimientos se efectúan bajo analgesia y anestesia previa a la administración de sustancias e intervenciones quirúrgicas por lo que se minimiza el dolor, angustia y sufrimiento. Los métodos de sacrificio descritos (sobredosis de anestesia) se encuentran entre los indicados por el propio RD 53/2013.
- Se han examinado las situaciones y excepciones previstas en el punto e) del artículo 34. 2 encontrando que ninguna de ellas es aplicable en este proyecto.
- El el proyecto se clasifica como tipo III y por tanto debe ser sometido a evaluación retrospectiva. Este Comité considera que dicha evaluación debería efectuarse a los dos años de la concesión de la autorización.

VICERREITORÍA DE INVESTIGACIÓN
E INNOVACIÓN
Oficina de Investigación e Tecnoloxía
Servizo de Convocatorias e Recursos Humanos de I+i
Edificio CACTUS – Campus Vida
15782 Santiago de Compostela
Tel. 981 547 040 - Fax 981 547 077
Correo electrónico: ctiinfo@usc.es
http://maisd.usc.es

Por todas estas razones, este Comité acordó emitir un **INFORME FAVORABLE.**

En la evaluación de este proyecto NO HA EXISTIDO CONFLICTO DE INTERESES.

Lugo, 17 de abril de 2018

AXENCIA GALEGA DA CALIDADE ALIMENTARIA

Avda. Do Camiño Francés, 10, Baixo
15703 Santiago de Compostela
A Coruña

Xacobeo 21·22

MÓDULOS FUNDAMENTAIS OU TRONCAIS E ESPECÍFICOS CORRESPONDENTES Á CATEGORÍA "A" COIDADO DOS ANIMAIS – ORDE ECC/566/2015, DE 20 DE MARZO

MÓDULOS FUNDAMENTAIS OU TRONCAIS

1.- Lexislación nacional (1 hora).

2.- Ética, benestar animal e as "tres erres", nivel 1 (2 horas).

3.- Bioloxía básica e adecuada, nivel 1 (3 horas).

4.- Coidado, saúde e manexo dos animais, nivel 1 (5 horas).

5.- Recoñecemento do dolor, o sufrimento e a angustia (3 horas).

6.- Métodos incruentos de sacrificio, nivel 1 (2 horas)

MÓDULOS ESPECÍFICOS DA CATEGORÍA "A"

1.-Bioloxía básica e adecuada, nivel 2 (3 horas)

.....

MÓDULOS FUNDAMENTALES O TRONCALES

1.- Legislación nacional (1 hora).

2.- Ética, bienestar animal y las "tres erres", nivel 1 (2 horas).

3.- Biología básica y adecuada, nivel 1 (3 horas).

4.- Cuidado, salud y manejo de los animales, nivel 1 (5 horas).

5.- Reconocimiento del dolor, el sufrimiento y la angustia (3 horas).

6.- Métodos incruentos de sacrificio, nivel 1 (2 horas)

MÓDULOS ESPECÍFICOS DE LA CATEGORÍA "A"

1.-Biología básica y adecuada, nivel 2 (3 horas)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

AXENCIA GALEGA DA CALIDADE ALIMENTARIA

Avda. Do Camiño Francés, 10, Baixo
15703 Santiago de Compostela
A Coruña

Xacobeo 21·22

Certificado de capacitación en materia de protección de animais utilizados, criados ou subministrados con fins de experimentación e otros fins científicos, incluíndo a docencia conforme coa Orde ECC/566/2015 de 20 de marzo.

Certificado de capacitación en materia de protección de animales utilizados, criados o suministrados con fines de experimentación y otros fines científicos, incluyendo la docencia conforme con la Orden ECC/566/2015 de 20 de marzo.

1. IDENTIFICACIÓN

1.1. Apelidos / *Apellidos / Surname:*
ÁVILA GÓMEZ

1.2. Nome / *Nombre / First names:*			DNI /*DNI / Identity card number:*
PAULO			**44657095L**
1.3. Categoría/*Categoría/Category:*	**"a"**	1.4. Especies/*Especies/Species* **ROEDORES**	1.5. Válido ata/ *válido hasta/expires:* **12/07/2029**

2. Nº DO CERTIFICADO / *Nº DEL CERTIFICADO / CERTIFICATE NUMBER*

A308

3.ORGANISMO QUE EXPIDE O CERTIFICADO / *ORGANISMO QUE EXPIDE EL CERTIFICADO / BODY ISSUING THE CERTIFICATE:*

3.1. Nome e enderezo do organismo que expide o certificado / *Nombre y dirección del organismo que expide el certificado / Name and address of the body issuing the certificate:*

AXENCIA GALEGA DA CALIDADE ALIMENTARIA
CONSELLERÍA DO MEDIO RURAL – XUNTA DE GALICIA
Rúa do Camiño Francés, 10 baixo
15781 Santiago de Compostela
A Coruña (España)

3.2. Teléfono / *Teléfono / Telephone:*	3.3. Fax / *Fax / Fax:*	3.4. Correo electrónico / *Correo electrónico / Email:*
981 546 657	981 546 651	formacion.cmrm@xunta.es
3.5. Data / *Fecha / Date:*	3.6. Lugar / *Lugar / Place:* **Santiago de Compostela**	

3.7. Nome e sinatura / *Nombre y firma / Name and signature*

Asdo.: **Xiana María Perales Arroyo**
Xefa do Departamento de Formación
(asinado dixitalmente)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

AXENCIA GALEGA DA CALIDADE ALIMENTARIA

Avda. Do Camiño Francés, 10, Baixo
15703 Santiago de Compostela
A Coruña

MÓDULOS FUNDAMENTAIS OU TRONCAIS E ESPECÍFICOS CORRESPONDENTES Á CATEGORÍA "B" EUTANASIA DOS ANIMAIS – ORDE ECC/566/2015, DE 20 DE MARZO

MÓDULOS FUNDAMENTAIS OU TRONCAIS

1.- Lexislación nacional (1 hora).

2.- Ética, benestar animal e as "tres erres", nivel 1 (2 horas).

3.- Bioloxía básica e adecuada, nivel 1 (3 horas).

4.- Coidado, saúde e manexo dos animais, nivel 1 (5 horas).

5.- Recoñecemento do dolor, o sufrimento e a angustia (3 horas).

6.- Métodos incruentos de sacrificio, nivel 1 (2 horas)

MÓDULOS ESPECÍFICOS DA CATEGORÍA "B"

1.-Bioloxía básica e adecuada, nivel 2 (3 horas)

2.- Métodos incruentos de sacrificio, nivel 2 (3 horas)

.....

MÓDULOS FUNDAMENTALES O TRONCALES

1.- Legislación nacional (1 hora).

2.- Ética, bienestar animal y las "tres erres", nivel 1 (2 horas).

3.- Biología básica y adecuada, nivel 1 (3 horas).

4.- Cuidado, salud y manejo de los animales, nivel 1 (5 horas).

5.- Reconocimiento del dolor, el sufrimiento y la angustia (3 horas).

6.- Métodos incruentos de sacrificio, nivel 1 (2 horas)

MÓDULOS ESPECÍFICOS DE LA CATEGORÍA "B"

1.-Biología básica y adecuada, nivel 2 (3 horas)

2.- Métodos incruentos de sacrificio, nivel 2 (3 horas)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

AXENCIA GALEGA DA CALIDADE ALIMENTARIA

Avda. Do Camiño Francés, 10, Baixo
15703 Santiago de Compostela
A Coruña

Xacobeo 21·22

Certificado de capacitación en materia de protección de animais utilizados, criados ou subministrados con fins de experimentación e otros fins científicos, incluíndo a docencia conforme coa Orde ECC/566/2015 de 20 de marzo.

Certificado de capacitación en materia de protección de animales utilizados, criados o suministrados con fines de experimentación y otros fines científicos, incluyendo la docencia conforme con la Orden ECC/566/2015 de 20 de marzo.

1. IDENTIFICACIÓN		
1.1. Apelidos / *Apellidos / Surname:* **ÁVILA GÓMEZ**		
1.2.Nome / *NoCmbre / First names:* **PAULO**		DNI /*DNI / Identity card number:* **44657095L**
1.3. Categoría/*Categoría/Category:* **"b"**	1.4. Especies/*Especies/Species:* **ROEDORES**	1.5. Válido ata/ *válido hasta/expires:* **12/07/2029**

2. Nº DO CERTIFICADO / *Nº DEL CERTIFICADO / CERTIFICATE NUMBER*

B587

3.ORGANISMO QUE EXPIDE O CERTIFICADO / *ORGANISMO QUE EXPIDE EL CERTIFICADO / BODY ISSUING THE CERTIFICATE:*

3.1.Nome e enderezo do organismo que expide o certificado / *Nombre y dirección del organismo que expide el certificado / Name and address of the body issuing the certificate:*

CONSELLERÍA DO MEDIO RURAL – XUNTA DE GALICIA
AXENCIA GALEGA DA CALIDADE ALIMENTARIA
Avda. Do Camiño Francés, 10 baixo
15781 Santiago de Compostela
A Coruña (España)

3.2.Teléfono / *Teléfono / Telephone:*	3.3.Fax / *Fax / Fax:*	3.4.Correo electrónico / *Correo electrónico / Email:*
981 546 657	981 546 651	formacion.cmrm@xunta.es
3.5.Data / *Fecha / Date:*	3.6.Lugar / *Lugar / Place:* **Santiago de Compostela**	

3.7.Nome e sinatura / Nombre y firma / Name and signature

Asdo.: Xiana María Perales Arroyo
Xefa do Departamento de Formación
(asinado dixitalmente)

AXENCIA GALEGA DA CALIDADE ALIMENTARIA

Avda. Do Camiño Francés, 10, Baixo
15703 Santiago de Compostela
A Coruña

Xacobeo 21·22

MÓDULOS FUNDAMENTAIS OU TRONCAIS E ESPECÍFICOS CORRESPONDENTES Á CATEGORÍA "C" REALIZACIÓN DOS PROCEDEMENTOS – ORDE ECC/566/2015, DE 20 DE MARZO

MÓDULOS FUNDAMENTAIS OU TRONCAIS

1.- Lexislación nacional (1 hora).

2.- Ética, benestar animal e as "tres erres", nivel 1 (2 horas).

3.- Bioloxía básica e adecuada, nivel 1 (3 horas).

4.- Coidado, saúde e manexo dos animais, nivel 1 (5 horas).

5.- Recoñecemento do dolor, o sufrimento e a angustia (3 horas).

6.- Métodos incruentos de sacrificio, nivel 1 (2 horas)

MÓDULOS ESPECÍFICOS DA CATEGORÍA "C"

1.- Bioloxía básica e adecuada, nivel 2 (3 horas)

2.- Procedementos minimamente invasores sen anestesia, nivel 1 (5 horas)

3.- Procedementos minimamente invasores sen anestesia, nivel 2 (10 horas)

4.- Anestesia para procedementos menores (5 horas)

5.- Anestesia avanzada para intervencións cirúrxicas ou procedementos prolongados (8 horas)

6.- Principios de cirurxía (5 horas)

.....

MÓDULOS FUNDAMENTALES O TRONCALES

1.- Legislación nacional (1 hora).

2.- Ética, bienestar animal y las "tres erres", nivel 1 (2 horas).

3.- Biología básica y adecuada, nivel 1 (3 horas).

4.- Cuidado, salud y manejo de los animales, nivel 1 (5 horas).

5.- Reconocimiento del dolor, el sufrimento y la angustia (3 horas).

6.- Métodos incruentos de sacrificio, nivel 1 (2 horas)

MÓDULOS ESPECÍFICOS DE LA CATEGORÍA "C"

1.- Biología básica y adecuada, nivel 2 (3 horas)

2.- Procedimientos mínimamente invasivos sin anestesia, nivel 1 (5 horas)

3.- Procedimientos mínimamente invasivos sin anestesia, nivel 2 (10 horas)

4.- Anestesia para procedementos menores (5 horas)

5.- Anestesia avanzada para intervenciones quirúrgicas o procedimientos prolongados (8 horas)

6.- Principios de cirugía (5 horas)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

AXENCIA GALEGA DA CALIDADE ALIMENTARIA

Avda. Do Camiño Francés, 10, Baixo
15703 Santiago de Compostela
A Coruña

Xacobeo 21·22

Certificado de capacitación en materia de protección de animais utilizados, criados ou subministrados con fins de experimentación e otros fins científicos, incluíndo a docencia conforme coa Orde ECC/566/2015 de 20 de marzo.

Certificado de capacitación en materia de protección de animales utilizados, criados o suministrados con fines de experimentación y otros fines científicos, incluyendo la docencia conforme con la Orden ECC/566/2015 de 20 de marzo.

1. IDENTIFICACIÓN

1.1. Apelidos / *Apellidos / Surname:*

ÁVILA GÓMEZ

1.2. Nome / *Nombre / First names:*	DNI /*DNI / Identity card number:*
PAULO	**44657095L**

1.3. Categoría/*Categoría/Category:*	1.4. Especies/Especies/Species:	1.5. Válido ata/ *válido hasta/expires:*
"C"	***ROEDORES***	**12/07/2029**

2. Nº DO CERTIFICADO / *Nº DEL CERTIFICADO / CERTIFICATE NUMBER*

C619

3.ORGANISMO QUE EXPIDE O CERTIFICADO / *ORGANISMO QUE EXPIDE EL CERTIFICADO / BODY ISSUING THE CERTIFICATE:*

3.1. Nome e enderezo do organismo que expide o certificado / *Nombre y dirección del organismo que expide el certificado / Name and address of the body issuing the certificate:*

CONSELLERÍA DO MEDIO RURAL – XUNTA DE GALICIA
AXENCIA GALEGA DA CALIDADE ALIMENTARIA
Avda. Do Camiño Francés, 10 baixo
15781 Santiago de Compostela
A Coruña (España)

3.2. Teléfono / *Teléfono / Telephone:*	3.3. Fax / *Fax / Fax:*	3.4. Correo electrónico / *Correo electrónico / Email:*
981 546 657	981 546 651	formacion.cmrm@xunta.es

3.5. Data / *Fecha / Date:*	3.6. Lugar / *Lugar / Place:*
	Santiago de Compostela

3.7. Nome e sinatura / *Nombre y firma / Name and signature*

Asdo.: Xiana María Perales Arroyo
Xefa do Departamento de Formación
(asinado dixitalmente)

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Stroke is the second leading cause of death worldwide with an ever-increasing incidence. Despite the advances in experimental research and clinical management, the only available drug for ischemic stroke is the tissue plasminogen activator. Although hypothermia is a promising therapeutic strategy against cerebral ischemia, its associated adverse-effects hamper its clinical application. In this regard, cold shock proteins could become a valuable tool without said downsides. The present work provides clinical evidence on the effects of hypothermia on the expression of RBM3 and the outcome of patients, and explores the influence of temperature on FGF21 (a novel RBM3 inductor) and RBM3 levels in obese stroke patients. Moreover, we studied the protective effect of hypothermia and RBM3 in a mice model of cerebral ischemia.