TESIS DOCTORAL

IDENTIFICACIÓN Y USO DE BIOMARCADORES PRONÓSTICOS EN EL ICTUS ISQUÉMICO

TERESA GARCÍA BERROCOSO

DIRECTORES:
DR. JOAN MONTANER VILLALONGA
DR. ISRAEL FERNÁNDEZ CADENAS
DRA. PILAR DELGADO MARTÍNEZ

TUTOR:
DR. DAVID GARCÍA DORADO

MEDICINA INTERNA
DEPARTAMENT DE MEDICINA
FACULTAT DE MEDICINA
UNIVERSITAT AUTÒNOMA DE BARCELONA

BARCELONA, 2014

Esta Tesis se ha realizado en el Laboratorio de Investigación Neurovascular del Institut de Recerca del Hospital Universitario Vall d'Hebron, gracias a una ayuda Predoctoral de Formación en Investigación en Salud (PFIS) del Instituto de Salud Carlos III (FI09/00017).

El Laboratorio de Investigación Neurovascular pertenece a la Red de Investigación Cooperativa Neurovascular (RENEVAS) (RD06/0026/0010; RD12/0014/0005) y recibe financiación del Fondo de Investigación Sanitaria (FIS), que ha subvencionado la realización de este estudio (PI08/361; PI11/0176).

La verdad en la ciencia puede ser definida como la mejor hipótesis de trabajo para llegar a la siguiente mejor
(K. Lorenz)

Mujer, 73 años, sin factores de riesgo, hábitos saludables. Evolución de pocas horas. Fallecimiento por ictus isquémico el 23/11/01.

Así empieza la historia de esta Tesis doctoral, sin saber nada sobre las causas, la etiología ni los factores de riesgo del ictus. Sin conocer su incidencia ni las tasas de mortalidad a sus espaldas. Sin imaginar cuánta gente investigando para ponerle remedio. Sólo sintiendo la incertidumbre durante horas y desde la distancia sobre su pronóstico. Pronóstico. Ese término que ligó el pasado con el presente, ese *link* inconsciente sobre el futuro. Algo tendría que ver.

Es difícil resumir todo lo que ha pasado en estos 5 años. Es difícil resumir todo lo que ha pasado en estos 12 años, desde que el ictus se interpuso por primera vez en mi vida, cuando empezaba a estudiar Biología. Es difícil resumir todo lo que ha pasado en estos 30 años, ya que todo ha sido necesario para llegar hasta aquí y a muchos corresponde un pedacito de esta Tesis.

A Cecilia y Pedro, mis abuelos, que aunque ya no están aquí, siempre están presentes. A mi padre, a quien también le "tocó" el ictus, por suerte con otro final, y que me enseñó a elegir. A mi madre, mi hermano y mis tías, por dejarme ser como soy. Todos ellos han hecho posible este camino.

A Joan, por confiar en mí y darme la oportunidad de aprender tantas cosas con tanta gente. Al grupo de *Biomarkers* por hacer posibles los trabajos de esta Tesis y otros tantos.

A *les nenes i els nens* por todos los momentos dentro y fuera del lab, por las conversaciones realistas y surrealistas, por hacerme reír tanto y por hacer más llevadero este proceso (y por las toneladas de galletas y chocolate!). En especial, a Anna, Mireia y Dolors, de las que he aprendido tanto de ciencia como de la vida. Sabéis que siempre digo lo que pienso sin pensarlo, pero aquí quedaría muy largo.

A los que estaban cuando empecé y ya no están en el lab pero que formaron parte de otros grandes momentos que parecen ya muy lejanos. A Mire por haber vuelto con energías renovadas. A Irene por estar al otro lado del teléfono y hacerme desconectar con nuestras conversaciones "filosóficas" (:P).

A Jean-Charles y su grupo, especialmente a Virginie, por hacer que la estancia en Ginebra fuera una delicia y me dieran ganas de quedarme en las montañas.

Y a Javi, por estos 10 años montados en la montaña rusa. Lo conseguimos!. Hasta donde nos lleven los pies. Te quiero.

RESUMEN

El ictus es una enfermedad con una elevada morbimortalidad, suponiendo la segunda causa de muerte a nivel mundial y una de las principales causas de discapacidad en el adulto. Aun así, la heterogeneidad entre los pacientes con ictus dificulta anticipar el pronóstico de la enfermedad. Saber qué pacientes van a empeorar o cuáles se recuperarán sin secuelas ayudaría a facilitar información al paciente y su familia, valorar los riesgos y los beneficios del tratamiento trombolítico o la inclusión en ensayos clínicos y a optimizar los recursos sanitarios en cuanto a atención médica especializada, cuidado intensivo de los pacientes, duración de la estancia intrahospitalaria o terapias de rehabilitación tempranas.

Actualmente, la predicción de la evolución clínica de los pacientes con ictus es poco precisa y suele basarse en la edad y la gravedad inicial del paciente. Aunque se han desarrollado diversos modelos predictivos basados en variables clínicas, en la práctica ninguno de ellos es aplicado al presentar valores de discriminación entre pacientes que, en el mejor de los casos, alcanzan el 70-80 % de precisión. Además, estos modelos suelen incluir variables clínicas que no son accesibles de forma inmediata o que requieren técnicas de neuroimagen. El uso de biomarcadores en la práctica clínica en otras enfermedades ha generado una corriente de investigación en torno a moléculas que puedan predecir la evolución de los pacientes con ictus. En la actualidad, pocos biomarcadores han demostrado tener un valor predictivo suficiente como para que se evalúe su utilidad en amplios estudios multicéntricos; para ello, los biomarcadores deberían añadir valor a las variables clínicas, aumentando la sensibilidad y la especificidad en la discriminación de los pacientes para influir en la toma de decisiones.

Los trabajos incluidos en esta Tesis se centran, por un lado, en sistematizar el conocimiento sobre biomarcadores pronósticos en el ictus isquémico y la aplicación de criterios estadísticos que demuestren el valor añadido necesario para un futuro uso de los marcadores. Con ese propósito hemos desarrollado una página web donde se compila la información sobre las moléculas que se han asociado con el pronóstico del ictus y hemos aplicado técnicas de estadística comparativa y de metaanálisis para evaluar un biomarcador típico como el péptido natriurético de tipo B (BNP). Por otro lado, hemos querido evaluar moléculas involucradas en la fisiopatología del ictus, como las quimiocinas, explorándolas paralelamente a nivel cerebral y circulatorio.

Finalmente, mediante técnicas de identificación masiva, como la proteómica, las librerías de anticuerpos y la transcriptómica, hemos identificado una serie de moléculas que han demostrado su asociación con el pronóstico del ictus en diferentes momentos de la evolución de los pacientes. Además, estas moléculas mejoran, en mayor o menor grado, la predicción basada solamente en variables clínicas.

Valorar éstos u otros biomarcadores en las diferentes complicaciones secundarias al ictus isquémico en las que podrían tomarse decisiones que cambiasen el curso de la enfermedad, como la predicción de las infecciones post-ictus o la transformación hemorrágica, parece una excelente oportunidad de futuro para la aplicación real de los biomarcadores en el ictus.

ABSTRACT

Stroke is a severe disease, being the second cause of death worldwide and one of the main causes of disability. Heterogeneity among stroke patients makes difficult to anticipate the prognosis of the disease. To identify patients who are going to worsen or will recover would help in giving valuable information to the patient and relatives; to evaluate risks and benefits of reperfusion therapies or the inclusion in clinical trials and to optimize public health resources in terms of specialized medical and intensive care, length of in-hospital stay or starting of early rehabilitation programs.

Nowadays, clinical outcome prediction of stroke patients lacks accuracy and is usually based mainly on age and initial neurological severity. Although several predictive models have been developed including clinical variables, none of them is being applied in clinical practice. In the best-case scenario, these models differentiate patients with 70-80 % accuracy. Furthermore they tend to include clinical variables non-easily available or based on neuroimaging techniques. The use of biomarkers in the clinical practice in other diseases generated a trend in research around molecules that can predict stroke patients' outcome. Currently, few biomarkers have demonstrated enough predictive value to be evaluated in large multicenter studies; for that purpose, biomarkers should add value to clinical variables, increasing sensitivity and specificity in the discrimination of patients in order to influence decision-making processes.

The papers comprised in this Thesis focus, on the one hand, on systematizing the knowledge about prognostic biomarkers in ischemic stroke and the application of statistical criteria that demonstrate the needed added value for a future use of the biomarkers. For that purpose we have developed a website to provide a data compilation of molecules that have been associated with stroke prognosis and we have applied comparative statistics and metaanalysis to evaluate a typical biomarker, such as natriuretic peptide type B (BNP). On the other hand, we aimed to evaluate molecules that are involved in the pathophysiology of stroke, such as chemokines, by analyzing them in parallel at both brain and blood levels.

Finally, through massive discovery techniques, such as proteomics, antibody libraries and transcriptomics, we identified several molecules that demonstrated their association with stroke prognosis at different moments during the progression of the disease. Moreover, these molecules improved prediction based solely on clinical variables.

To evaluate utility of these or other biomarkers in some of the secondary complications of ischemic stroke, where decisions can be made to change the evolution of the disease, such as the prediction of post-stroke infections or hemorrhagic transformation, seems an excellent opportunity for a real use of stroke biomarkers in the near future.

INTRODUCCIÓN

1

1.1 El ictus

El ictus se produce por una alteración brusca de la circulación cerebral, que afecta al aporte de oxígeno y nutrientes de las células que conforman el parénquima cerebral. Este trastorno del flujo sanguíneo puede producirse por la rotura (ictus hemorrágico) o por la obstrucción (ictus isquémico) de un vaso cerebral. Dependiendo del tipo de ictus y de la zona cerebral afectada se producen diferentes síntomas, como alteraciones del lenguaje (afasia), del movimiento (paresia) o de la visión (hemianopsia, diplopía).

Los datos más recientes y completos sobre la incidencia del ictus en nuestro entorno indican que se producen 187 casos por cada 100.000 habitantes, con una tasa de hospitalización del 92,4 % (Díaz-Guzmán J *et al.*, 2012). El ictus afecta a todo tipo de personas, independientemente de su género (53 % de hombres) y edad, aunque la incidencia aumenta con los años de vida (con una edad media de 74 años) y, por tanto, el ictus supone un problema sociosanitario en sociedades envejecidas (Hollander M *et al.*, 2003).

El ictus puede considerarse una enfermedad multifactorial, causada por la comorbilidad de diversas patologías, de factores genéticos y ambientales. Las principales enfermedades que suponen un incremento del riesgo para padecer un ictus son la hipertensión arterial, las cardiopatías, la diabetes mellitus y la dislipemia. Éstas, junto con el tabaquismo, el alcoholismo, el sedentarismo y la obesidad son factores que controlados podrían reducir la incidencia del ictus (Di Legge S *et al.*, 2012). Por otro lado, existen evidencias de la influencia de factores genéticos, no modificables, tanto por la presencia de ictus en enfermedades monogénicas (CADASIL, enfermedad de Fabry) como por la asociación de variantes genéticas con subtipos específicos de ictus (Traylor M *et al.*, 2012).

1.1.1 Subtipos de ictus

El tipo mayoritario es el ictus isquémico, representando entre el 80 y el 85 % de los casos, frente al 15-20 % de los ictus hemorrágicos (datos del Sistema Nacional de Salud, 2009). Tanto el ictus isquémico como el hemorrágico pueden clasificarse a diferentes niveles, según la zona cerebral afectada, la extensión, la duración del evento o la etiología subyacente (Figura 1).

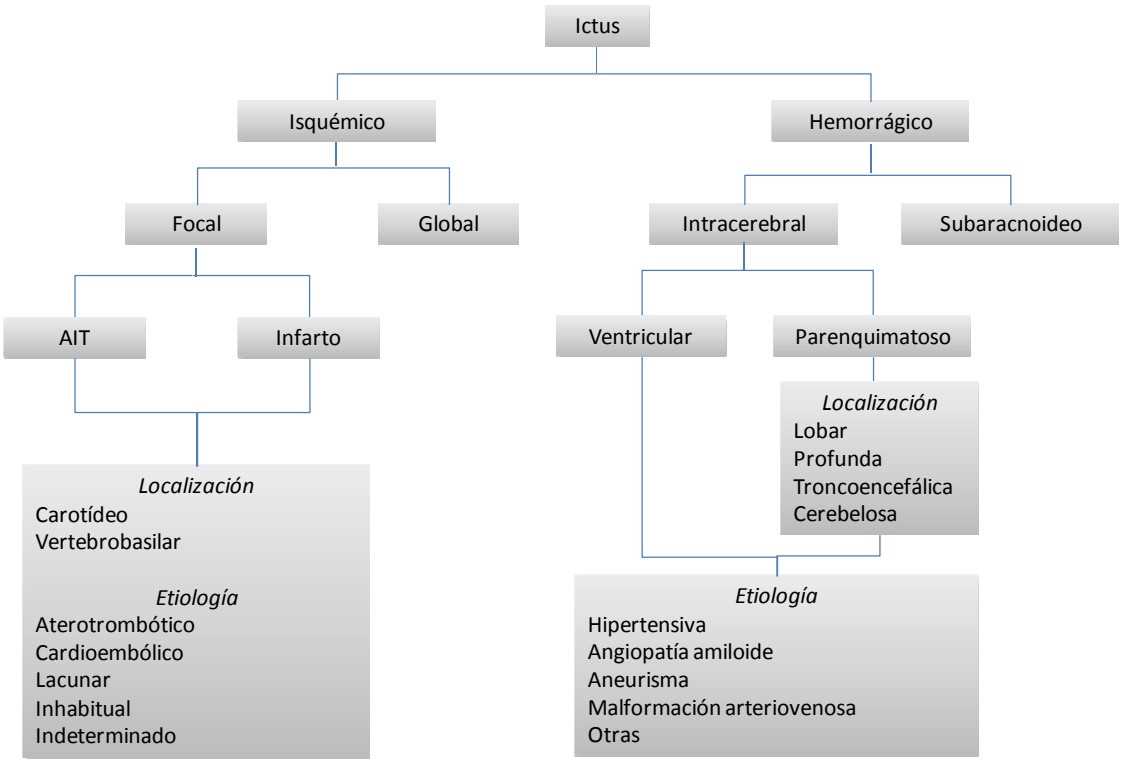


Figura 1. Tipos y subtipos de ictus. Adaptado de Arboix A *et al.*, 2006.
AIT: ataque isquémico transitorio.

El objeto de estudio principal de esta Tesis doctoral es el ictus isquémico, por lo que de aquí en adelante los términos ictus e ictus isquémico se utilizarán indistintamente. El ictus isquémico o infarto cerebral es causado por la interrupción del flujo sanguíneo, produciéndose un déficit neurológico con una duración superior a las 24h. El diagnóstico del ictus isquémico se basa en la anamnesis, la exploración neurológica, los resultados de una analítica sanguínea completa, del estudio cardíaco (electrocardiograma y ecografía transtorácica) y de la exploración vascular (ecografía doppler-dúplex), junto con las pruebas de neuroimagen, bien tomografía computarizada (TC) o resonancia magnética (RM) craneal (Álvarez J, 2006).

Una vez realizado el diagnóstico, es importante conocer la causa de la obstrucción arterial que ha llevado al bloqueo de la circulación. La clasificación etiológica más utilizada actualmente es el sistema TOAST (Adams HP *et al.*, 1993), aunque existen otros sistemas más recientes con modificaciones del TOAST (Amarenco P *et al.*, 2009). Los dos subtipos de ictus más frecuentes son los ictus cardioembólicos, en los que un coágulo o émbolo producido en el corazón viaja por el torrente circulatorio y bloquea una arterial cerebral, y los ictus aterotrombóticos, producidos por la presencia de aterosclerosis que ocluye la luz del vaso o por ruptura de la placa de ateroma que llega en forma de trombo al cerebro. A pesar de los avances en las

técnicas de diagnóstico, en un 25-39 % de los casos la causa subyacente permanece indeterminada (Wolf ME *et al.*, 2012), lo que dificulta el manejo adecuado de los pacientes.

1.1.2 Tratamiento y complicaciones del ictus isquémico

En el ictus isquémico, en respuesta a la pérdida de aporte sanguíneo y la subsiguiente reperfusión, se activa una cascada de procesos celulares y moleculares a nivel tisular (Figura 2) que llevan a la rápida necrosis celular en el centro o *core* del infarto. La zona cerebral inmediata al *core*, el peri-infarto o penumbra, se encuentra hipoperfundida y, por tanto, metabólicamente comprometida aun manteniendo la integridad morfológica y el potencial para ser recuperada si se evita la extensión del infarto (Heiss WD, 2010). Por tanto, el tratamiento agudo del ictus isquémico está enfocado a salvar la zona de peri-infarto, minimizando el daño cerebral producido por la oclusión y, consecuentemente, los déficits neurológicos y funcionales asociados así como las complicaciones secundarias, que determinarán el desenlace de la enfermedad.

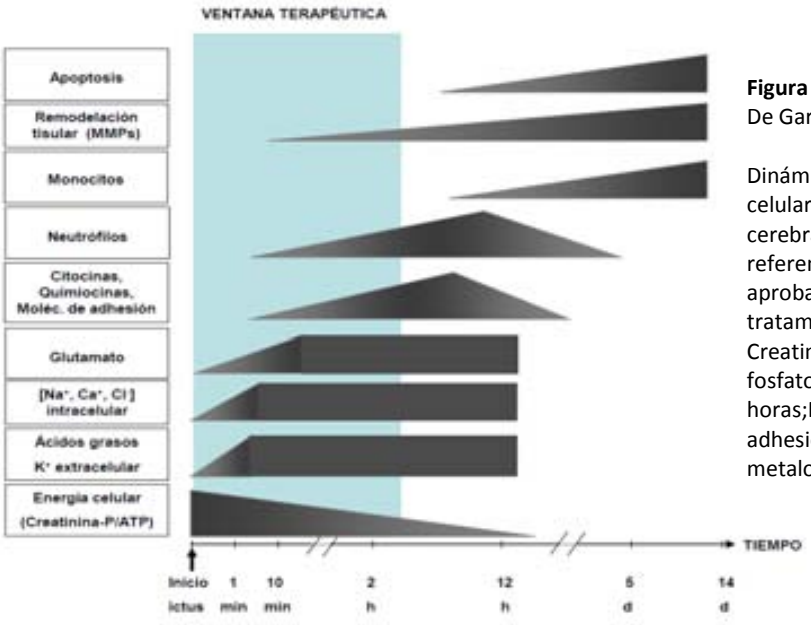


Figura 2. Cascada isquémica.
De García-Berrocoso T *et al.*, 2009.

Dinámica de los eventos moleculares y celulares en el transcurso de la isquemia cerebral. La ventana terapéutica hace referencia al tiempo en el que está aprobada la administración del tratamiento trombolítico.
Creatinina P/ATP: ratio creatinina fosfato/adenosín trifosfato; d: dias; h: horas;M. de adhesión: moléculas de adhesión; min: minutos; MMPs: metaloproteinasas de matriz.

1.1.2.1 Fase aguda

El concepto "tiempo es cerebro" refleja bien la urgencia del tratamiento que permita restaurar el flujo sanguíneo cerebral en la zona afectada para evitar el crecimiento del *core* del infarto. Los fármacos fibrinolíticos o trombolíticos permiten la eliminación del trombo o émbolo que ocluye la arteria e impide la entrada de oxígeno y nutrientes al tejido cerebral.

Actualmente el único tratamiento aprobado para el ictus isquémico durante la fase aguda es la administración de la forma recombinante del activador tisular del plasminógeno (rt-PA). Una

vez que el paciente está estabilizado y dentro de las primeras 4,5 horas tras el inicio de los síntomas (Hacke W *et al.*, 2008), se administra por vía intravenosa una dosis de 0,9 mg/Kg de rt-PA (10 % en forma de bolo durante un minuto y el resto, hasta un máximo de 90 mg, mediante perfusión continua durante una hora). Esta estrategia consigue la recanalización en un 46 % de los casos, aunque hasta un tercio de los pacientes presentan reoclusión arterial tras el tratamiento (Egido JA *et al.*, 2006). La estrecha ventana terapéutica, las estrictas indicaciones y contraindicaciones, la baja tasa de éxito en la reperfusión y la aparición de hemorragias sintomáticas secundarias al tratamiento conllevan que menos del 15 % de los pacientes sean tratados en la fase aguda.

Para aumentar los beneficios del tratamiento trombolítico y disminuir sus efectos secundarios se están estudiando diferentes estrategias que van de la mano de los avances en las técnicas de neuroimagen (Khan R *et al.*, 2013) y que dan lugar a un tratamiento más individualizado. Ejemplos de ello serían la aplicación simultánea de ultrasonidos en la arteria ocluida, que aumenta la tasa de recanalización sin aumentar las transformaciones hemorrágicas (Tsivgoulis G *et al.*, 2010), o el tratamiento endovascular, bien mediante la administración intraarterial del rt-PA o mediante el uso de dispositivos de extracción mecánica del trombo (Pereira VM *et al.*, 2012). Este último parece prometedor al aumentar la tasa de recanalización hasta el 80 % y además podría suponer una segunda oportunidad para aquellos pacientes que no responden al tratamiento intravenoso, aunque requiere de personal muy cualificado y aún se carece de ensayos clínicos de fase III que avalen su eficacia en la práctica clínica (Mullen MT *et al.*, 2012; Broderick JP *et al.*, 2013; Ciccone A *et al.*, 2013). La realización de nuevos ensayos clínicos determinará si dichas técnicas son realmente útiles y si su aplicación es recomendable (Molina CA *et al.*, 2013).

Además del tratamiento para la restauración del flujo sanguíneo, las guías oficiales contemplan el control de la presión arterial, la glucemia, la temperatura corporal, la respiración, la nutrición y la movilización con el fin de reducir posibles complicaciones secundarias (Egido JA *et al.*, 2006). El potencial terapéutico de la neuroprotección, dirigida a salvar el tejido de la zona de penumbra actuando en diferentes niveles de la cascada isquémica, no ha dado aún sus frutos a pesar del exhaustivo estudio a nivel preclínico (Turner RC *et al.*, 2013). Por qué fármacos inhibidores de ciertos procesos fisiopatológicos (como la entrada celular de calcio, la activación de radicales libres o la muerte neuronal), que han funcionado en los modelos experimentales reduciendo el daño cerebral, no son útiles cuando se ensayan en estudios clínicos multicéntricos está aún por determinar (O'Collins VE *et al.*, 2006). Mejorar el éxito en

la translación pasaría por adaptar los modelos preclínicos a la realidad de la enfermedad, tal y como proponen los criterios STAIR (STAIR, 1999), y ampliar la idea de protección a los diferentes componentes de la unidad neurovascular que se ven afectados por la isquemia (Figura 3).

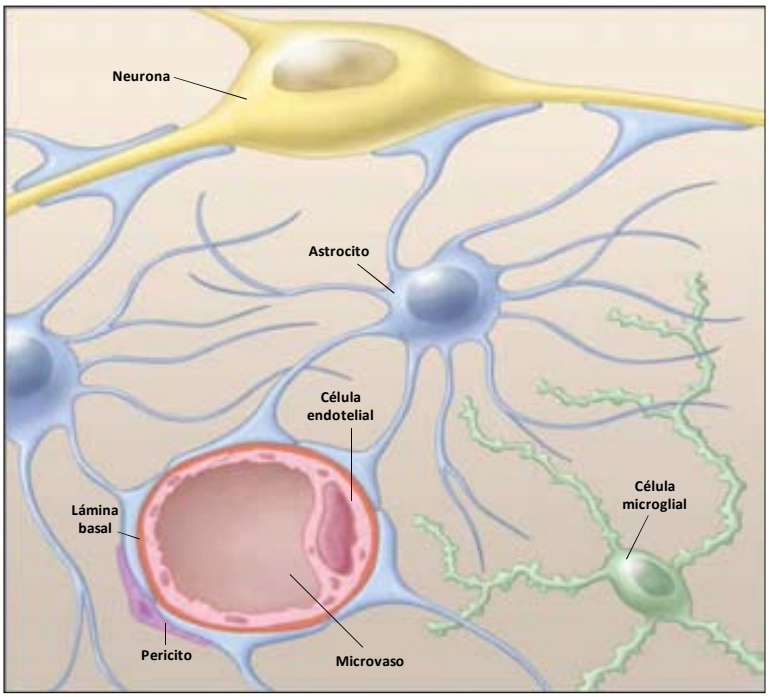


Figura 3. Unidad neurovascular. Adaptado de Del Zoppo GJ, 2006.
Interacciones entre microvasos sanguíneos (compuestos por células endoteliales y la matriz de lámina basal), astrocitos y neuronas, moduladas por otras células de soporte (pericitos, microglia, oligodendrocitos), que dan lugar a una comunicación necesaria para mantener la correcta función del sistema nervioso central.

1.1.2.2 Fase sub-aguda

Una vez superadas las primeras horas, las actuaciones del equipo médico van dirigidas a evitar complicaciones secundarias al ictus. Entre éstas cabe destacar el edema cerebral, la transformación hemorrágica o las infecciones.

Los procesos que se suceden en la cascada isquémica, en particular la generación de estrés oxidativo, la inflamación y la activación de la remodelación tisular, afectan a la unidad neurovascular a nivel estructural y funcional. Estas alteraciones llevan a la pérdida de integridad de la barrera hematoencefálica, aumentando la permeabilidad y permitiendo la extravasación de fluido y componentes circulatorios en el parénquima cerebral que condicionará la aparición de edema vasogénico. Cuando el edema progresa se produce hipertensión intracraneal que, de no ser subsanada, conduce a la desviación de las estructuras de la línea media por efecto de masa entre los 3-5 días tras el ictus. Esto ocurre en torno a un 10 % de los casos, que se denominan *infartos malignos* ya que causan deterioro neurológico

por propagación del infarto y presentan un alto índice de mortalidad (~80 %), superior al del resto de los ictus isquémicos aún afectando a pacientes jóvenes (Hacke W *et al.*, 1996). El tratamiento anti-edema consistiría en reducir el contenido hídrico cerebral mediante la administración de agentes osmóticos (manitol, solución hipertónica), aunque no está claro si podrían darse efectos colaterales deletéreos (Grape S *et al.*, 2012; Grände PO *et al.*, 2012). En el caso de los *infartos malignos* el tratamiento es agresivo y consistiría en una craniectomía descompresiva que mejoraría la supervivencia a expensas de una alta tasa de discapacidad (Kolias AG *et al.*, 2013).

Otra de las complicaciones relacionada con la ruptura de la barrera hematoencefálica es la transformación hemorrágica. Este sangrado secundario en el tejido isquémico puede presentarse en formas diversas, desde pequeñas petequias asintomáticas a hematomas que pueden ejercer efecto de masa, aumentando el daño y la muerte celular (Figura 4).

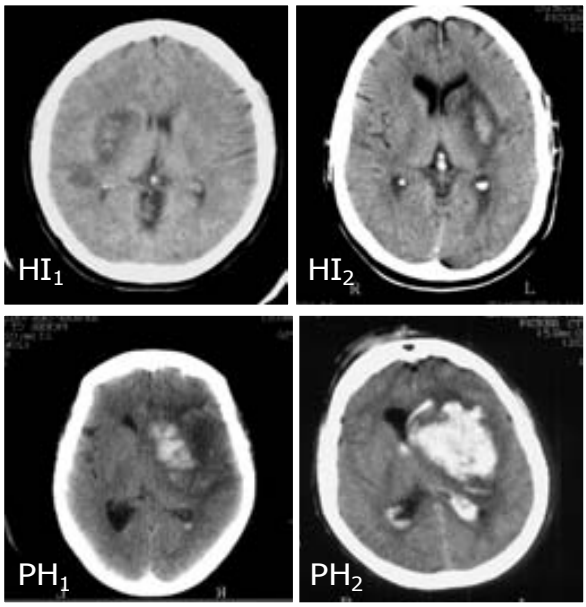


Figura 4. Clasificación de la transformación hemorrágica en el ictus isquémico.
De Montaner J *et al.*, 2003.

Imágenes de tomografía computarizada de los diferentes tipos de transformación hemorrágica en el ictus isquémico, según la clasificación ECASS (Larrue V *et al.*, 1997).
HI: infarto hemorrágico; PH: hematoma parenquimatoso.
HI_1: pequeñas petequias marginales al *core*
HI_2: petequias confluentes en el *core*
PH_1: hematoma ≤30% del *core*; leve efecto de masa
PH_2: hematoma >30% del *core*; efecto de masa

En pacientes que no reciben tratamiento trombolítico, la transformación hemorrágica ocurre en un 8,5 % de los casos, yendo acompañada de deterioro neurológico en el 1,5 % (Lindley RI *et al.*, 2004). Estas frecuencias aumentan considerablemente en los pacientes que reciben algún tipo de terapia trombolítica, llegando hasta el 7 % de hemorragias sintomáticas (Balami JS *et al.*, 2011) por lo cual es muy importante hacer una selección cuidadosa de los pacientes candidatos a este tratamiento. Actualmente poco puede hacerse ante la transformación hemorrágica más allá de intentar restablecer el estado fibrinolítico o, en casos en que exista un hematoma superficial mayor de 30 mL, realizar craniectomía para su extracción (Balami JS *et al.*, 2011).

El daño cerebral ocasionado en la zona del infarto da lugar a la exposición de antígenos propios que generan una respuesta inflamatoria local, con activación de la población celular residente, secreción de citoquinas y quimiocinas e infiltración leucocitaria. Esta actividad leucocitaria tiene un papel dual, amplificando el proceso inflamatorio y, por tanto, contribuyendo al desarrollo de la lesión cerebral, a la vez que realiza funciones beneficiosas de reparación y mantenimiento de la homeostasis en la unidad neurovascular (Ceulemans AG *et al.*, 2010). Acompañando a esta inflamación local también tiene lugar una inflamación sistémica, con el aumento de los niveles circulantes de células de la serie blanca y de reactantes de fase aguda como la proteína C reactiva (CRP) o la interleucina 6 (IL-6) (Dziedzic T, 2008); este estado pro-inflamatorio finalmente decae, debido a un giro desde citoquinas Th1 a Th2 y a la producción de moléculas anti-inflamatorias (Theodorou GL *et al.*, 2008). Tras la respuesta pro-inflamatoria inicial, la respuesta inmune a nivel periférico también disminuye, con la presencia de monocitos desactivados y linfopenia (Esmaeili A *et al.*, 2012; Vogelgesang A *et al.*, 2013). Esta inmunodepresión parece ser una respuesta fisiológica protectora para evitar reacciones auto-inmunitarias frente a los antígenos cerebrales filtrados al torrente sanguíneo, aunque colateralmente contribuye al riesgo de infecciones secundarias al ictus (Dirnagl U *et al.*, 2007). Estas infecciones se producen hasta en un 28-45 % de los casos (Westendorp WF *et al.*, 2011) y se ven facilitadas por la inmovilización de los pacientes, la disfagia, la broncoaspiración o el uso de catéteres urinarios, siendo por tanto mayoritarias las infecciones de las vías respiratoria (9-28 %) y urinaria (10-20 %). Aunque la prevención de estas infecciones secundarias al ictus pueda parecer sencilla mediante el uso de antibióticos, las guías oficiales no lo recomiendan ya que esta medida profiláctica no ha demostrado ser efectiva en cuanto a un mejor desenlace de la enfermedad (Westendorp WF *et al.*, 2012). A día de hoy, las conclusiones se basan en ensayos clínicos aleatorizados de tamaño muestral pequeño; próximamente se conocerán los resultados del ensayo PASS (*Preventive Antibiotics in Stroke Study*) que incluirá 3.200 pacientes holandeses para intentar demostrar los beneficios de la terapia con antibióticos (Nederkoorn PJ *et al.*, 2011; http://www.passamc.nl/index-eng.php).

Otras complicaciones también tienen lugar en los días sucesivos al ictus, como las crisis epilépticas (2-4 %; Camilo O *et al.*, 2004), el delirium (19-33 %; Carin-Levy G *et al.*, 2012), el tromboembolismo venoso (1-4 %) o el síndrome coronario agudo (1-11 %) (Kumar S *et al.*, 2010).

1.1.2.3 Fase crónica

Tras el alta hospitalaria, las personas que han sufrido un ictus deben seguir recibiendo seguimiento asistencial para asegurar su buena progresión y recuperación.

Después del ictus pueden quedar secuelas a nivel motor o sensorial que en ocasiones alteran la capacidad para realizar las actividades básicas de la vida diaria y/o producen complicaciones físicas diversas, como osteoporosis, incontinencia urinaria o dolores, entre otras (Rivero P, 2009). Así mismo también pueden aparecer alteraciones psicológicas y cognitivas, como la depresión (en un 29-36 % de los casos; Hackett ML *et al.*, 2005) o la demencia (7-9 % de casos en el primer año tras el ictus; Leys D *et al.*, 2005). Para evitar estas complicaciones más tardías se están investigando terapias que potencien la neurorreparación endógena. La neurorreparación puede definirse como el conjunto de procesos que tratan de regenerar el tejido cerebral dañado y restablecer los circuitos neuronales para recuperar las funciones motoras y sensoriales perdidas a causa del ictus. Estos mecanismos de reparación, incluyendo la angiogénesis, la neurogénesis, la sinaptogénesis y la oligodendrogénesis, se activan de forma temprana tras el ictus pero se mantienen durante semanas, ampliando la ventana terapéutica de los posibles tratamientos que los potencien o los faciliten (Bacigaluppi M *et al.*, 2008).

Por otro lado, los pacientes que han sufrido un ictus presentan un riesgo mayor de sufrir un nuevo evento vascular, ya sea un ictus recurrente (10 % de riesgo en el primer año y 5 % en los años sucesivos), un evento cardíaco (6 y 4,6 %, respectivamente) o muerte de origen vascular (Kaplan RC *et al.*, 2005). El hecho de que el riesgo sea mayor de forma temprana tras el ictus hace esencial el inicio del tratamiento preventivo lo antes posible. Para ello es importante conocer la etiología del ictus ya que, además de recomendar cambios hacia un estilo de vida más saludable y controlar los factores de riesgo vascular, es necesario el uso de tratamientos específicos que eviten la aparición de nuevos eventos.

El mecanismo de trombosis subyacente es diferente entre los ictus aterotrombóticos, donde se produce la ruptura de una placa de ateroma con activación y agregación plaquetaria, y los cardioembólicos, en los que afectaciones cardíacas dan lugar a estasis sanguíneo que predispone a la formación de trombos. Por tanto, el tratamiento anti-trombótico de prevención secundaria es diferente. En los pacientes con ictus aterotrombótico o lacunar se recomienda el uso de anti-agregantes plaquetarios (ácido acetilsalicílico, clopidogrel, dipiridamol), consiguiendo una reducción del riesgo de recurrencia del 14-23 %. En los casos de

estenosis carotídeas severas, superiores al 70 % del diámetro de la arteria, la endarterectomía o la angioplastia serían el tratamiento de elección. En cambio, en los pacientes que hayan sufrido un ictus de etiología cardioembólica se recomienda el uso de anti-coagulantes, que reducen el riesgo de recurrencia en un 66 % (Fuentes B *et al.*, 2006).

1.2 Pronóstico del ictus isquémico

A pesar de que la población está más envejecida, parece que el control de los factores de riesgo está ayudando a reducir la incidencia del ictus al menos en los países desarrollados, aunque no se aprecian cambios de tendencia en su fatalidad (Carandang R *et al.*, 2006; Feigin VL *et al*., 2009).

La implantación del *Código Ictus*, que se activa de forma extrahospitalaria para poner al equipo médico en alerta ante un posible ictus y facilitar una atención rápida, y la existencia de las Unidades de Ictus especializadas han reducido la estancia y los costes hospitalarios, las complicaciones (principalmente las infecciones; Govan L *et al.*, 2007) y la tasa de mortalidad y dependencia (Álvarez-Sabín J *et al.*, 2004; Ruiz V *et al.*, 2005). Las Unidades de Ictus cuentan con personal especializado que actúa de forma protocolizada y monitorizan de forma semi-intensiva a los pacientes. La especialización a nivel de personal y de material hace que, aunque su beneficio esté probado, actualmente sólo se disponga de 47 Unidades de Ictus a nivel estatal; por ello, la admisión de pacientes debe ser optimizada. Por otro lado, el desarrollo de programas de telemedicina o *Teleictus*, en los que mediante videoconferencia y transmisión de neuroimágenes los hospitales comarcales contactan con hospitales de referencia donde existe guardia de neurología de 24 horas, ha permitido que los pacientes sean evaluados por un especialista que designe el inicio del tratamiento trombolítico, evitando traslados urgentes y costosos. Gracias al *Teleictus* más pacientes se benefician del tratamiento de forma segura y con resultados similares a los que se producen en las Unidades de Ictus (Schwab S *et al.*, 2007; Müller-Barna P *et al.*, 2012).

Junto con el tratamiento especializado en las Unidades de Ictus, las terapias de rehabilitación multidisciplinarias también podrían ayudar a mejorar el pronóstico de los pacientes, sobre todo si se comienza de forma precoz, aunque la divergencia en las variables pronósticas analizadas en los diferentes estudios no permiten extraer conclusiones consistentes (Ali M *et al.*, 2013). En esta fase de cuidado post-ictus, la telemedicina también podría ser aplicada (Rubin MN *et al.*, 2013).

El hecho de que estas aplicaciones no sean accesibles para todos los pacientes, junto con las diferentes circunstancias que tienen lugar antes, durante y después del ictus, hacen que el pronóstico de los pacientes sea muy diverso en cualquiera de sus niveles, incluso en pacientes con ictus de características similares.

1.2.1 Pronóstico neurológico

El estado neurológico de los pacientes con ictus se evalúa mediante escalas de gravedad que permiten cuantificar de forma aproximada el daño ocasionado. La escala más utilizada, por su aplicación en ensayos clínicos, es la NIHSS (*National Institutes of Health Stroke Scale*; Brott T *et al.*, 1989), con 15 ítems que otorgan una puntuación total de entre 0 y 42 puntos, siendo más graves los ictus con mayor puntuación. La escala canadiense (CNS: *Canadian Neurological Scale*; Côté R *et al.*, 1986), la escandinava (SSS: *Scandinavian Stroke Scale*; SSS group, 1985) y la europea (ESS: *European Stroke Scale*; Hantson L *et al.*, 1994) también son utilizadas para la evaluación en la fase aguda (Tabla 1). Existen fórmulas de conversión para equipararlas a la NIHSS (Nilanont Y *et al.*, 2010; Gray LJ *et al.*, 2009).

Escala	Ítems	Puntuación	Tiempo
NIHSS	Nivel de conciencia Visión Función motora facial, superior e inferior Ataxia extremidades Función sensorial Lenguaje (afasia/disartria) Extinción/inatención	**0 – 42 puntos** • 0: No síntomas • 1-4: leve • 5-15: moderado • 16-20: moderado-severo • 21-42: severo	< 8 min
CNS	Nivel de conciencia Orientación Lenguaje Función motora facial, superior e inferior	**0 – 11,5 puntos** • 0: coma • 1-4: severo • 5-7: moderado • ≥ 8: leve	5-10 min
SSS	Conciencia Movimiento ocular Fuerza motora superior e inferior Orientación Lenguaje Parálisis facial Marcha	**0-58 puntos** • 0-25: severo • 26-42: moderado • 43-58: leve	15 min
ESS*	Nivel de conciencia Lenguaje y comprensión Visión Función motora facial, superior e inferior Marcha	**0-100 puntos** Mayor puntuación, mayor severidad	8 min

Tabla 1. Escalas clínicas de gravedad.

CNS: *Canadian Neurological Scale*; ESS: *European Stroke Scale*; NIHSS: *National Institutes of Health Stroke Scale*; SSS: *Scandinavian Stroke Scale*.

* Validada únicamente para ictus con afectación del territorio de la arteria cerebral media.

En los estudios objeto de esta Tesis doctoral se ha utilizado la escala NIHSS, por lo que será la escala neurológica de referencia en los apartados siguientes.

Para valorar la evolución neurológica del paciente no hay un criterio plenamente establecido, aunque se suele considerar una variación en la puntuación de la escala NIHSS de 4 o más puntos, definiendo la mejoría del paciente como la disminución en la puntuación y el empeoramiento como el aumento (Brott TG *et al.*, 1992). Se considera que un paciente permanece estable cuando la variación en el tiempo de la escala NIHSS no alcanza los 4 puntos.

El empeoramiento o deterioro neurológico temprano generalmente tiene lugar en las primeras 48-72 horas tras el inicio de los síntomas y se produce en hasta un 40 % de los casos, según las series de pacientes, el tiempo considerado y la definición. El deterioro puede deberse a la progresión o crecimiento del infarto (que puede estar relacionado con causas hemodinámicas; Tsivgoulis G *et al.*, 2012), el desarrollo de edema, crisis epilépticas o transformaciones hemorrágicas (Thanvi B *et al.*, 2008; Siegler JE *et al.*, 2011).

1.2.2 Pronóstico funcional

Existen varias escalas validadas para estimar el grado de discapacidad o dependencia para las actividades diarias tras el ictus. De forma similar a lo que ocurre con las escalas clínicas, la escala más utilizada para evaluar la funcionalidad de los pacientes tras el ictus es la escala modificada de Rankin (mRS; van Swieten JC *et al.*, 1988), que mide la independencia global de los pacientes, lo que permite comparar pacientes con diferente déficit neurológico y considerando su estado funcional previo. La escala mRS va de 0 a 6 puntos, obteniendo 0 puntos los pacientes que no presentan síntomas, 1 los pacientes con algún síntoma pero sin discapacidad, 2 puntos los pacientes con discapacidad leve, 3-5 puntos los pacientes con discapacidad moderada a severa y 6 puntos los pacientes fallecidos. Otras escalas utilizadas son el índice de Barthel (BI; Mahoney FI *et al.*¸1965), con una puntuación de 0-100 en la evaluación global de 10 ítems diferentes, siendo mayor en aquellos pacientes mejor capacitados para las actividades de la vida diaria, y la GOS (*Glasgow Outcome Scale*; Jennett B *et al.*, 1975), con 5 puntos para los pacientes sin síntomas y 1 punto para los fallecidos. Aunque la mRS y la BI evalúan conceptos diferentes de discapacidad, pueden equipararse (Uyttenboogaart M *et al.*, 2005).

En los estudios objeto de esta Tesis doctoral se ha utilizado la escala mRS, por lo que será la escala funcional de referencia en los apartados siguientes. Por definición de la escala se consideran independientes los pacientes con una mRS ≤ 2, aunque valores de mRS ≤ 1 también sirven para clasificar la funcionalidad de los pacientes (Weisscher N *et al.*, 2008). Las escalas de discapacidad suelen utilizarse para explorar la evolución de los pacientes a largo plazo tras el ictus, ya sea al mes, a los 3 meses, a los 6 meses o al año tras el evento.

En nuestro entorno, de los supervivientes al ictus solamente el 41,5 % de los pacientes es independiente a los 6 meses tras el ictus y el 32,4 % presenta algún grado de discapacidad (datos del Sistema Nacional de Salud, 2009). En la población general, 7,8 de cada 1.000 personas sufren discapacidad como consecuencia de un evento cerebrovascular; esta tasa aumenta exponencialmente con la edad y es mayor en mujeres, con una tasa total de 8,35 por 1.000 frente a 7,25 por 1.000 en los hombres (Figura 5).

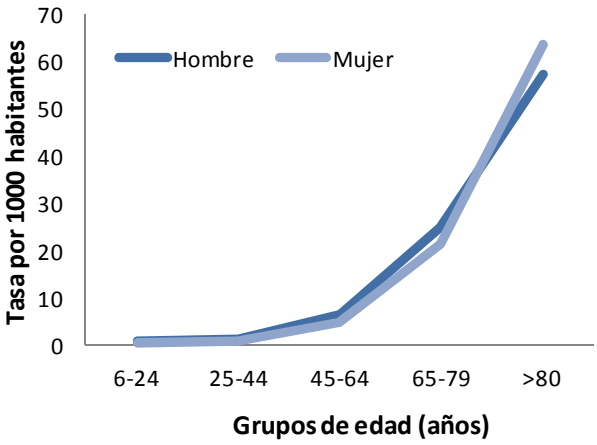


Figura 5. Tasas de población con discapacidad debida a enfermedad cerebrovascular.
Datos del Instituto Nacional de Estadística, 2008.

Tasa por 1.000 habitantes en España de 6 y más años, estratificados por grupos de edad y género.

Entre los residentes en centros, incluyendo residencias para mayores, centros de discapacitados y hospitales geriátricos, el 9,29 % presenta discapacidad secundaria a un evento cerebrovascular (datos del Instituto Nacional de Estadística, 2008).

1.2.3 Pronóstico vital

Además de ser una de las principales causas de discapacidad a nivel mundial, el ictus es la segunda causa de muerte global (Roger VL *et al*., 2012) (Figura 6).

En nuestro entorno, el ictus origina un 62,5 % de las bajas anuales, siendo la segunda causa de muerte por detrás de las enfermedades isquémicas del corazón (pasando a ser la primera causa en las mujeres, entre las que provoca el fallecimiento en un 71,2 %) (datos del Instituto Nacional de Estadística, 2011). La tasa de mortalidad intrahospitalaria aumenta con la edad y

es similar en ambos sexos, produciéndose de forma global en un 11 % de los casos (Díaz-Guzmán J *et al.*, 2012). Los fallecimientos aumentan a un 26,1 % cuando se consideran los 6 meses siguientes al ictus (datos del Sistema Nacional de Salud, 2009).

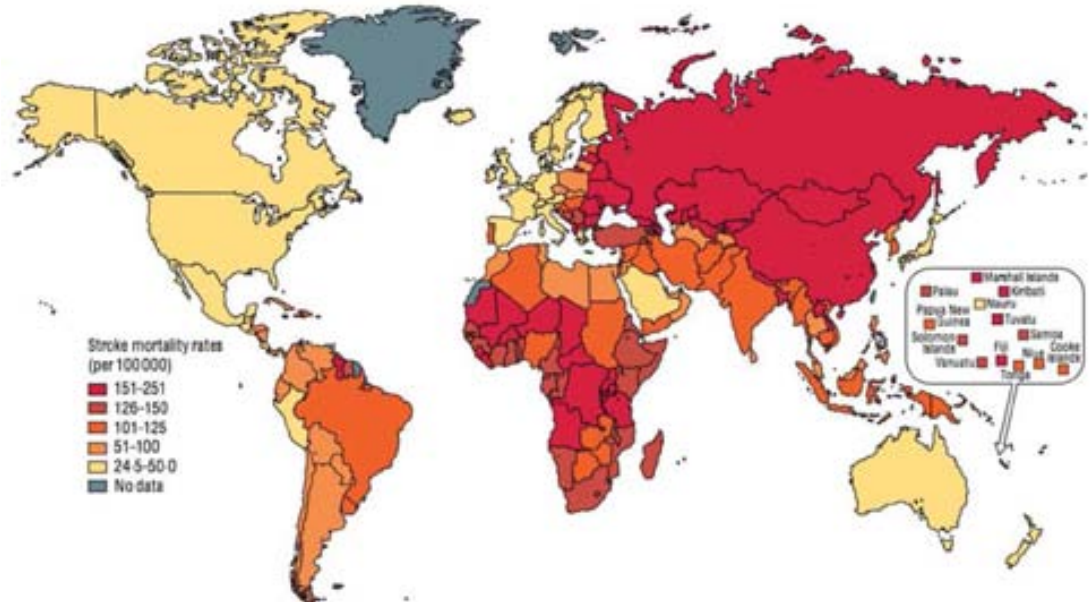


Figura 6. Tasas globales de mortalidad asociada al ictus (ajustadas por edad y género). De Johnston SC *et al.*, 2009.

1.3 Predicción de la evolución de los pacientes con ictus isquémico

Los datos presentados en el apartado anterior reflejan la dramática situación del pronóstico de los pacientes que sufren un ictus. La predicción del pronóstico con tiempo suficiente para intervenir y cambiar el desenlace es uno de los retos en el manejo de estos pacientes. Esta predicción suele depender de la propia experiencia médica y los datos reportados en ensayos clínicos, generando un sesgo principalmente por sobreestimación en la detección de buen pronóstico (Bushnell C, 2011; Saposnik G *et al.*, 2013). La variabilidad presente entre los pacientes, en cuanto a las características clínicas y las del propio ictus, hace evidente la necesidad de predictores multivariable para estimar el pronóstico. En general las variables predictoras deben estar claramente definidas, estandarizadas y ser reproducibles, pudiéndose obtener de los datos demográficos, de la historia clínica, del examen físico, de las características de la enfermedad, de los resultados analíticos o de tratamientos previos, teniendo en cuenta la disponibilidad de los datos en el momento en el que se quiere hacer la predicción (Moons KGM *et al.*, 2009). Estos predictores pueden ser factores determinantes, relacionados con la fisiopatología de la enfermedad, pero no todos los predictores son necesariamente una causa del pronóstico (Moons KGM *et al.*, 2009).

El uso de estos predictores permitiría calcular el riesgo absoluto o la probabilidad de que tenga lugar un mal o un buen pronóstico, que, para influir en la toma de decisiones, debería alcanzar una precisión suficientemente buena (Cucchiara B *et al.*, 2013). Cuál sería el porcentaje de riesgo óptimo está aún por establecer, aunque porcentajes muy extremos (10-90 % de riesgo de mal pronóstico, por ejemplo) parecen más relevantes clínicamente (Whiteley W *et al.*, 2012).

Una estimación fiable del pronóstico permitiría informar al paciente y a sus familiares sobre la situación real, ayudaría a definir los riesgos/beneficios del tratamiento trombolítico u otros más agresivos como la craniectomía, optimizaría la admisión en las Unidades de Ictus o el alta hospitalaria y la selección de pacientes para la inclusión en ensayos clínicos, todo desde una perspectiva más objetiva.

1.3.1 Variables clínicas predictivas

Diversos autores han indagado en el papel predictivo de las variables clínicas para dilucidar el pronóstico de los pacientes con ictus (Counsell C, 2001), enfatizando el interés por este tópico en la práctica clínica. En los últimos años se han generado varios modelos pronósticos para discapacidad y mortalidad en pacientes con ictus que han sido validados y evaluados en términos de precisión, con valores en torno al 80 % en la mayoría de modelos (Tablas resumen en Anexo I). Aunque ninguno ha sido ampliamente aceptado para su aplicación en la práctica clínica, en muchos coinciden como variables predictoras comunes la edad y la gravedad del ictus.

1.4 Biomarcadores en el ictus isquémico

Un biomarcador puede definirse como cualquier característica medible de forma objetiva y que puede ser evaluada como un indicador de procesos biológicos normales o patológicos o de respuesta farmacológica a una intervención terapéutica (*Biomarkers Definitions Working Group*, 2001).

El estudio de los biomarcadores en el ictus es relativamente joven, con un aumento en el número de publicaciones desde la década de los 90, alcanzando actualmente las 700 publicaciones anuales, aunque este número se ha visto disminuido en 2013. Mayoritariamente la investigación en biomarcadores se ha centrado en moléculas presentes en la circulación sanguínea, ya que las muestras de sangre son fácilmente accesibles y su obtención es poco

invasiva (Figura 7). Aún así, no hay que olvidar que otro tipo de fluidos corporales (líquido cefalorraquídeo, orina, saliva) y tejidos (muestras cerebrales obtenidas en necropsias, trombos extraídos mediante tratamiento endovascular, placas de ateroma) pueden ser fuente de estudio de características que podrían funcionar como biomarcadores.

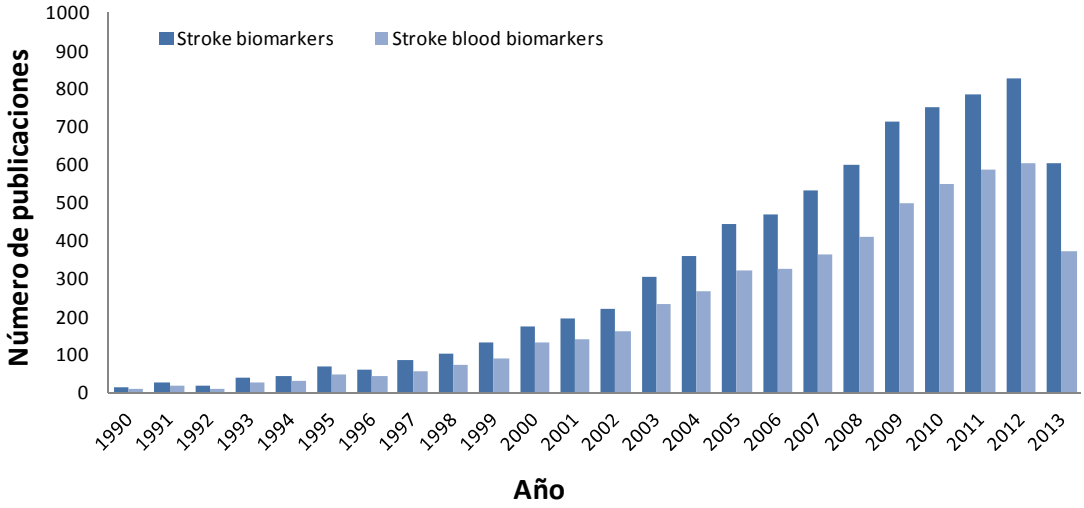


Figura 7. Número de publicaciones anual de biomarcadores en ictus.
Datos obtenidos en la base de búsqueda *Medline-PubMed* utilizando los términos *Stroke biomarkers* o *Stroke blood biomarkers*.

Varias técnicas, como la microdiálisis cerebral *in vivo* (Poca MA *et al.*, 2006) o la microdisección láser para obtener células individuales a partir de tejido cerebral *ex vivo* (Cuadrado E *et al.*, 2007), permiten estudiar estas muestras aislándolas del contexto global donde se ha producido el infarto y, por tanto, son una fuente valiosa de información. Por otro lado, la visualización de estructuras o características anatómicas mediante técnicas de neuroimagen (TC, RM, ultrasonografía) pueden aportar información sobre cambios en la composición o en la función del sistema nervioso.

Para actuar como indicadores, los biomarcadores deben presentar suficiente sensibilidad (capacidad de detectar verdaderos positivos) y especificidad (capacidad de detectar verdaderos negativos) para tener un valor predictivo óptimo en la indicación concreta del marcador. En general, el uso de varios biomarcadores parece una estrategia más factible para mejorar la predicción (Robin X *et al.*, 2009) y, en el caso de los biomarcadores detectables en fluidos biológicos, hoy en día pueden ser combinables en dispositivos de medición rápida que facilitarían su aplicación en la práctica clínica.

En el ámbito de la enfermedad cerebrovascular, los estudios de biomarcadores sanguíneos se han desarrollado en diferentes indicaciones. Biomarcadores como la fosfolipasa A2 asociada a

lipoproteína (Lp-PLA2) o la CRP han demostrado su papel en la predicción del **riesgo** de sufrir un primer ictus o un evento recurrente (Ballantyne CM *et al.*, 2005; Cucchiara BL *et al.*, 2009; Delgado P *et al.*, 2012). En el caso de la Lp-PLA2 incluso se ha aprobado el uso de un test comercializado para aportar información sobre el riesgo de sufrir un evento cardiovascular (PLAC® Test de diaDexus; http://www.plactest.com/index.html). Conociendo el riesgo de sufrir un ictus, podría hacerse más hincapié en las labores de prevención, controlando los factores de riesgo modificables y adquiriendo estilos de vida más saludables, ya que son clave para reducir la morbimortalidad del ictus, con los beneficios socioeconómicos que esto comportaría (Di Legge S *et al.*, 2012).

Otra de las áreas en las que los biomarcadores podrían aportar información muy útil sería en el **diagnóstico** diferencial del ictus respecto a enfermedades cuyos síntomas pueden confundirse con un ictus (conocidas como simuladores o *mimics*; entre otras, tumores cerebrales, crisis epilépticas, migrañas). La discriminación de los verdaderos ictus permitiría reducir el número de traslados urgentes a los hospitales y acelerar el tratamiento, que podría llegar a ser administrado por los servicios de urgencia pre-hospitalarios (Weber JE *et al.*, 2013). Varios biomarcadores han sido propuestos para el diagnóstico del ictus isquémico, aunque en general son estudios con pocos pacientes, que usan población sana como grupo comparativo y sin replicación en cohortes independientes (Whiteley W *et al.*, 2008; Jickling GC *et al.*, 2011). En dos estudios independientes, con más de 1.000 pacientes cada uno, se analizaron paneles de biomarcadores para diferenciar ictus de *mimics*, incluyendo D-dímero, metaloproteinasa-9 (MMP-9), péptido natriurético de tipo B (BNP), caspasa-3, S100B, receptor soluble de productos de glicación avanzada (sRAGE), quimerina y/o secretagogina (Laskowitz DT *et al.*, 2009; Montaner J *et al.*, 2011). En ambos estudios el grupo de pacientes con ictus incluía ictus isquémicos y hemorrágicos, aunque la discriminación entre estos dos **subtipos** de ictus es otra de las indicaciones en las que se ha estudiado el papel de posibles biomarcadores. La proteína gliofibrilar ácida (GFAP) o la S100B, ambas liberadas por necrosis astroglial, podrían funcionar como biomarcadores diagnósticos de ictus hemorrágico en las primeras horas tras el inicio de los síntomas, ya que presentan perfiles cinéticos diferentes con una liberación más temprana en los pacientes con hemorragia intracerebral (Brunkhorst R *et al.*, 2010). De conseguir un test que fuera suficientemente preciso en esta indicación, con la combinación de estos u otros biomarcadores, por ejemplo, se optimizaría el manejo de los pacientes sin tener que realizar la prueba de neuroimagen, método de referencia actual.

En el caso de los ictus isquémicos es importante conocer la **etiología** subyacente para administrar el tratamiento de prevención secundaria (ver apartado 1.1.2.3). Aunque se ha explorado el papel de muchas moléculas en la determinación de la etiología del ictus, como por ejemplo BNP o D-dímero para el ictus cardioembólico, parece que al menos en la fase aguda serían entidades difíciles de diferenciar (García-Berrocoso T *et al.*, 2010).

1.4.1 Biomarcadores asociados con el pronóstico del ictus isquémico

El objeto de estudio de esta Tesis doctoral es profundizar en el conocimiento del papel que pueden desempeñar los biomarcadores en el pronóstico de los pacientes con ictus isquémico.

Como se ha comentado en el apartado 1.3, la predicción de la evolución podría ayudar en la toma de decisiones en el manejo de los pacientes con ictus. Con este fin, se ha postulado que los biomarcadores podrían aportar información objetiva y complementaria a las variables clínicas, mejorando la predicción.

Diversas características estructurales observadas con las técnicas de neuroimagen, como el volumen y localización de la lesión (Phan TG *et al.*, 2010), la hiperdensidad arterial (Paciaroni M *et al.*, 2011), el flujo sanguíneo o perfusión (secuencia PWI) o la difusión de las moléculas de agua en la lesión (secuencias DWI o DTI) (Kruetzelmann A *et al.*, 2011), también se han asociado con la predicción del pronóstico tras el ictus. Las técnicas de neuroimagen funcional, tipo tomografía por emisión de positrones (PET, SPECT) o resonancia magnética funcional (fMRI) también han empezado a aplicarse. Aún así, la integración de esta información tampoco se ha llevado a cabo completamente en la práctica clínica, quizás por las limitaciones técnicas para realizar dichas pruebas en determinados ámbitos sanitarios y porque no está claro qué secuencias y en qué momento deben realizarse ni si mejoran la predicción basada en las variables clínicas (Gale SD *et al.*, 2012).

En el caso de los biomarcadores sanguíneos, revisiones recientes recapitulan los estudios realizados con moléculas candidatas para la predicción del pronóstico neurológico, funcional y/o vital (Whiteley W *et al.*, 2009; Hasan N *et al.*, 2012). De entre las muchas moléculas evaluadas como biomarcadores de pronóstico del ictus, algunas ejercen su función en vías relacionadas con los procesos de la cascada isquémica. Entre estas vías destacan, por el número de biomarcadores asociados, la hemostasia (con factores de coagulación y moléculas implicadas en la generación y lisis de los trombos) y la inflamación (donde juegan un papel importante moléculas de adhesión celular, citoquinas y quimiocinas) (Whiteley W *et al.*, 2009;

Katan M *et al.*, 2011). Además, el conocimiento de las vías metabólicas o las moléculas alteradas podría ayudar a determinar los mecanismos asociados con el desenlace del evento cerebrovascular, facilitando el hallazgo de posibles dianas terapéuticas.

Aunque los biomarcadores pronósticos se han analizado extensamente en el ictus, pocos han demostrado su utilidad clínica al no evaluar si su medición aporta valor predictivo sobre las variables clínicas establecidas (ver apartado 1.3.1). En estudios recientes, los niveles elevados de copeptina (fragmento estable del péptido precursor de la vasopresina) en las primeras 24 horas tras el inicio de los síntomas mejoran la predicción de discapacidad o muerte en los 3 meses subsiguientes al ictus isquémico, por encima de la edad y la gravedad inicial (De Marchis GM *et al.*, 2013).

1.4.2 Descubrimiento de nuevos candidatos

Aunque generalmente se estudian moléculas que son candidatas a biomarcadores por tener una función relacionada con los procesos fisiopatológicos estudiados en la enfermedad de interés, cada vez más las nuevas tecnologías están permitiendo aumentar el conocimiento a nivel molecular de forma masiva, incrementando las posibilidades de descubrir nuevos biomarcadores o dianas terapéuticas. Estas tecnologías de alto rendimiento, que en general se conocen como *ómicas*, realizan un cribado de numerosas biomoléculas al mismo tiempo, evitando el sesgo en la fase de selección, y generan listas de moléculas que se ven alteradas como causa o consecuencia del proceso de estudio. Este cribado o fase de descubrimiento sería el primero de los pasos a seguir en el proceso de desarrollo de nuevos biomarcadores (Figura 8).

Idealmente, una aproximación sistemática en el descubrimiento de biomarcadores implicaría el estudio en todos los niveles moleculares. La **genómica** (el estudio de la secuencia, estructura y función de todos los genes de una muestra concreta) permitiría identificar los genes o sus variantes asociados con el ictus en alguna de sus indicaciones. Diversos estudios de genoma completo (GWAs) se han realizado para identificar genes de susceptibilidad para las etiologías mayoritarias del ictus isquémico y, gracias a la creación de un consorcio internacional, han podido analizarse con potencia estadística suficiente como para identificar algunas variantes génicas asociadas al ictus dependiendo de su etiología (Traylor M et al., 2012). Estudios similares se están llevando a cabo para la identificación de genes y sus variantes asociados al pronóstico de los pacientes con ictus ("GODS Project: Contribución genética al pronóstico funcional y la discapacidad después del ictus").

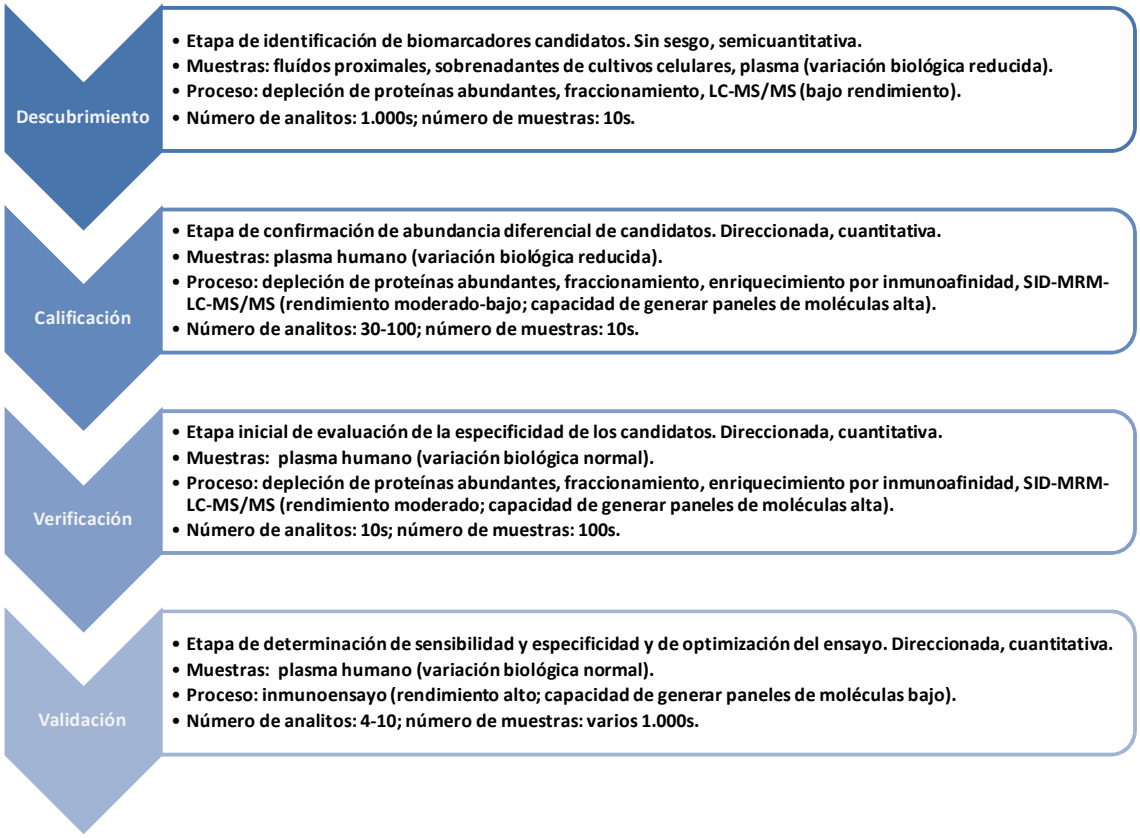


Figura 8. Proceso de desarrollo de nuevos biomarcadores proteicos candidatos. Adaptado de Rifai N, 2006. "Número de analitos" hace referencia al número de proteínas esperadas para ser evaluadas como candidatos en cada fase; "Número de muestras" hace referencia al número de muestras requeridas en cada fase. LC-MS/MS: cromatografía líquida acoplada a espectrometría de masas; SID: dilución de isótopo estable; MRM: proteómica cuantitativa dirigida (*multiple reaction monitoring*).

De la misma manera, varios grupos han abordado el análisis del **transcriptoma** en el ictus (el perfil de expresión de los genes en un determinado momento y bajo determinadas condiciones ambientales), estudiando la expresión en las células circulantes mediante micromatrices de oligonucleótidos o *microarrays* que reconocerán las secuencias de ARN mensajero presentes en la muestra (Sharp FR *et al.*, 2011). Recientemente, el descubrimiento de pequeñas moléculas de ARN circulantes que regulan post-transcripcionalmente la expresión génica, los microARNs o *miR*, ha abierto un nuevo campo de estudio en el ictus con su posible utilización como biomarcadores o como dianas terapéuticas (Wang Y *et al.*, 2013).

El estudio del **proteoma** permite acercarse al fenotipo clínico, complementando así los estudios a nivel genotípico. Las técnicas utilizadas en proteómica, teniendo como punto final la espectrometría de masas, han avanzado rápidamente dando un salto desde los estudios meramente cualitativos a la proteómica comparativa o cuantitativa (Ning M *et al.*, 2012). Los estudios de proteómica en el ictus han incluido desde el análisis de tejido cerebral (Cuadrado E *et al.*, 2010) a estudios del **secretoma** en fluidos, como líquido cefalorraquídeo o fluido

extracelular cerebral (Dayon L *et al.*, 2011) o plasma/suero. Estas últimas son clínicamente más relevantes, aunque presentan la dificultad del amplio rango dinámico en las concentraciones proteicas y suelen requerir pre-tratamiento y, a día de hoy, no han generado listas de proteínas candidatas a biomarcadores de manera similar a las obtenidas tras la exploración de muestras cerebrales (Zhang X *et al.*, 2008; Huang P *et al.*, 2009).

El uso de anticuerpos específicos contra una proteína concreta, aún basándose en la selección de candidatos, también permite explorar el proteoma. Diversas empresas ofrecen librerías de anticuerpos en paneles que facilitan el análisis de la presencia de cientos de proteínas en una muestra dada (p.ej.: DiscoveryMAP® de Myriad RBM, http://rbm.myriad.com/; Ciraplex® de Aushon, http://www.aushon.com/index.php). Por otro lado, el desarrollo de la tecnología de aptámeros, utilizando oligonucleótidos o péptidos que reconocen una diana en base a su estructura (Ni X *et al.*, 2011), ayudará a sobrepasar las limitaciones del uso de anticuerpos en cuanto a producción, estabilidad y plasticidad; actualmente ya existen librerías de aptámeros en las que poder realizar estudios de descubrimiento de nuevos marcadores (p.ej.: SOMAscan™ de SomaLogic, http://www.somalogic.com/) y sus características abrirán el camino a la detección de todo tipo de moléculas mediante técnicas de laboratorio equivalentes a las que se utilizan con los anticuerpos.

Otra ciencia que podría ayudar a definir el fenotipo del ictus isquémico y, por tanto, aportar nuevos candidatos a biomarcadores para las diferentes indicaciones, es la **metabolómica** (estudio de los productos intermedios o finales del metabolismo; Mauri-Capdevila G *et al.*, 2013; Laborde CM *et al.*, 2012).

Integrar la información existente sobre el ictus a todos los niveles moleculares, en lo que se conoce como integrómica, permitirá obtener una visión global de la patología y de las vías metabólicas afectadas. Aún así, no basta con la generación de listas de moléculas identificadas y los datos obtenidos en estos estudios *ómicos* deben interpretarse para poder ser útiles. En esta interpretación deben considerarse ciertas limitaciones: conceptuales (heterogeneidad molecular presente en los pacientes debido a la heterogeneidad propia de la enfermedad; clasificación previa de los pacientes con los diversos criterios disponibles), técnicas (preparación de las muestras; diversas plataformas en cada campo de estudio molecular; los resultados deben ser validados con técnicas alternativas) y bioinformáticas (la exploración de los resultados se realiza de forma mecánica con programas específicos; el uso de tamaños muestrales pequeños confiere una potencia estadística insuficiente; no existe un consenso

respecto a los umbrales de significación o comparación y en consecuencia se generan falsos positivos) (Mellick GD *et al.*, 2010; Abu-Asab MS *et al.*, 2011).

1.5 Valor añadido de los biomarcadores

Para realizar una predicción objetiva del pronóstico en un paciente con ictus, idealmente debería existir un modelo pronóstico multivariable (con los predictores más importantes y que alcanzara una calibración y discriminación aceptables) que hubiera sido validado en cohortes independientes y cuyo impacto en cuanto a la toma de decisiones médicas y al desenlace de la enfermedad pudiera medirse junto a estudios de coste-efectividad (Moons KGM *et al.*, 2009). Para que los biomarcadores puedan tener cabida en esos modelos pronósticos, además deben demostrar su valor añadido a la información obtenida con variables estándares mediante el uso de herramientas estadísticas (Hlatky MA *et al.*, 2009).

1.5.1 Herramientas estadísticas

Para determinar la asociación de un biomarcador con una indicación concreta, p.ej. con el pronóstico del ictus, se utilizan estadísticos sencillos que contrastan la hipótesis de asociación con la de no-asociación con, generalmente, un 95 % mínimo de probabilidad de acierto pre-establecida, generando el conocido valor *p* o significación estadística. Ejemplos de pruebas estadísticas en este nivel serían la *t* de Student para variables con distribución normal o la *U* de Mann-Whitney para estudios no paramétricos. Si la hipótesis de asociación se confirma, este es el primer paso de toda una serie de análisis estadísticos para demostrar el valor del biomarcador como tal.

1.5.1.1 Regresión logística

En el caso del pronóstico, generalmente el estudio de la asociación se refiere a una variable categórica binaria, es decir, si el paciente empeora/no-empeora, es dependiente/independiente o sobrevive/fallece. En este caso los estadísticos de clasificación, como la regresión logística, permiten modelar la probabilidad de que ocurra un evento (buen/mal pronóstico) en función de otro factor, como un biomarcador, de una forma clínicamente interpretable (Hosmer DW Jr *et al.*, 2013). El factor o covariable que forma parte del modelo de regresión logística se asocia de forma independiente al evento y aporta valor en función de su cociente de probabilidad u *odds ratio* (OR), es decir de la capacidad de identificación de verdaderos y falsos positivos. Cuando el modelo de regresión logística es multivariable, el OR del biomarcador estará ajustado por la presencia de los otros factores

independientes y obtendremos una fórmula de predicción en la que cada covariable es ponderada en función de su OR.

Para estimar el valor del biomarcador en la predicción del evento, idealmente los factores por los que se ajuste su OR deberían ser factores de referencia en la predicción del pronóstico, como los comentados en el apartado 1.3.1. A pesar de ser fácilmente interpretable, el OR no permite describir la capacidad de un biomarcador para clasificar pacientes en uno u otro grupo (Pepe MS *et al.*, 2004).

1.5.1.2 Áreas bajo la curva Característica Operativa del Receptor (AUC-ROC)

Una vez que se han establecido las variables que integran el modelo predictivo, el valor global del modelo en cuanto a la discriminación entre categorías puede medirse utilizando las curvas ROC. Estas curvas permiten interpretar de manera sencilla la probabilidad estudiada; p.ej., dados dos individuos, uno que vaya a empeorar y otro que no, el modelo predictivo debería asignarle una probabilidad mayor al individuo que empeorará. La representación de la sensibilidad *versus* la tasa de falsos positivos (1 – especificidad) en todo el rango posible de combinaciones permite la medición del área bajo la curva (AUC) y es esta AUC la que indica la precisión del modelo predictivo (DeLong ER *et al.*, 1988). Con valores de AUC de entre 0,5 (sin valor discriminatorio) y 1 (discriminación perfecta), el modelo predictivo que genere una AUC lo más cercana a 1, será el modelo de elección (Figura 9).

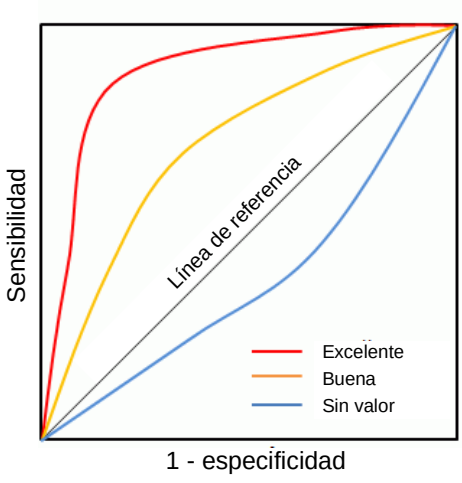


Figura 9. Curva Característica Operativa del Receptor (ROC).

Ejemplos de curvas ROC con mala discriminación (área bajo la curva – AUC- por debajo de la línea de referencia), buena discriminación (AUC = 0,6 – 0,7) y discriminación excelente (AUC ≥ 0,9).

Una vez establecido el mejor modelo predictivo incluyendo las variables clínicas más relevantes, puede compararse el AUC de este modelo con el AUC del mismo modelo con el biomarcador o biomarcadores añadidos, teniendo en cuenta que ambos modelos se han creado en la misma serie de datos (DeLong ER et al., 1988). Una posibilidad a tener en cuenta cuando la aplicación del modelo predictivo requiere una gran especificidad (p.ej., en un

modelo de predicción de buen pronóstico, reconocer perfectamente a los individuos que vayan a evolucionar favorablemente para centrar los esfuerzos en otros pacientes más graves u optimizar el tiempo de estancia intrahospitalaria), es la comparativa del AUC parcial (pAUC) en un rango de especificidad predeterminado (90-100 %). De forma similar podría realizarse para indicaciones que requieran una alta sensibilidad en la discriminación del modelo predictivo y así conocer el valor del biomarcador en un contexto útil clínicamente (Robin X *et al.*, 2011).

Aún siendo uno de los estadísticos comparativos más utilizados a la hora de determinar el valor añadido de los biomarcadores en los modelos predictivos, la medición de la discriminación no parece ser el estadístico más relevante en predicción de eventos y el incremento en el valor de AUC no es tan significativo como el valor de AUC por sí mismo (Pepe MS et al., 2004).

1.5.1.3 Índice de mejoría de la discriminación (IDI)

Por lo comentado en el apartado anterior, se hace necesaria la aplicación de estadísticos comparativos que contribuyan con información adicional a la aportada por el AUC. Una forma de cuantificar la diferencia entre las probabilidades determinadas por el modelo predictivo entre los eventos (mal pronóstico) y los no-eventos (buen-pronóstico; o viceversa, según la indicación del modelo) al añadir los biomarcadores al modelo únicamente clínico es mediante el cálculo del IDI. Por tanto, el cálculo del IDI aporta un valor numérico a la diferencia entre el modelo con y sin biomarcadores, mientras que el AUC solamente indica qué modelo presenta una discriminación mayor. Además, el IDI permite conocer si el modelo con biomarcadores mejora la predicción de los eventos (mayor sensibilidad) o de los no-eventos (mayor especificidad), aportando una idea más global de la discriminación del modelo (Pencina MJ *et al.*, 2008) (Figura 10).

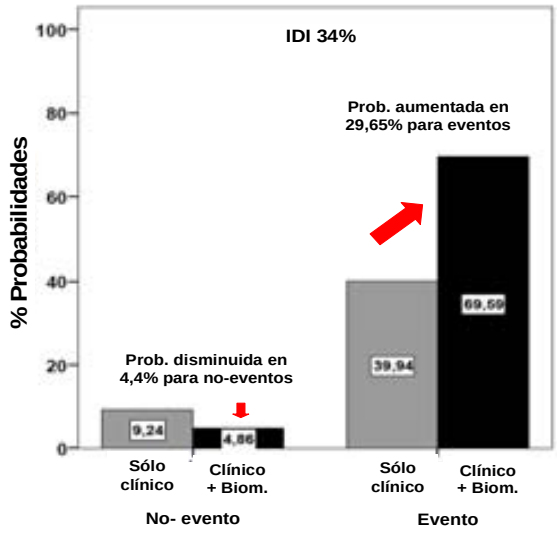


Figura 10. Representación gráfica del estadístico comparativo del índice de mejoría de la discriminación (IDI).

Ejemplo de comparación entre un modelo predictivo únicamente con variables clínicas (barras grises) y un modelo que además incluya biomarcadores (barras negras).

1.5.1.4 Índice de mejoría de la reclasificación (NRI)

Siguiendo un concepto similar al usado en el estadístico IDI, el cálculo del NRI permite conocer el cambio en la probabilidad predicha por el modelo con biomarcadores respecto al modelo solamente clínico. El NRI evalúa el número neto de individuos que se reclasifican correctamente al añadir los biomarcadores al modelo; p.ej., en un modelo de predicción de discapacidad a largo plazo, cuántos pacientes de los que realmente sufrirán una discapacidad son clasificados como eventos por el modelo predictivo incluyendo biomarcadores y no lo eran con el modelo clínico. De la misma manera que el IDI, el cálculo del NRI contempla los movimientos en el número de pacientes tanto para los eventos como para los no-eventos (Pencina MJ *et al.*, 2008).

El NRI puede calcularse con las probabilidades consideradas como una variable continua, donde el incremento de la probabilidad en un punto indica el cambio de categoría, o predefiniendo categorías de riesgo y observando los individuos que cambian de un grupo de riesgo a otro. El uso del NRI categórico está recomendado, en general con un máximo de tres grupos de riesgo (bajo, medio, alto) y que incluyan porcentajes clínicamente relevantes según la indicación del modelo predictivo; el uso del NRI continuo o con más categorías dará como resultado una sobreestimación de la tasa de reclasificación (Pickering JW *et al.*, 2012).

1.5.2 Metaanálisis

Además de realizar toda la evaluación estadística de los biomarcadores identificados para una indicación concreta y así poder conocer su valor real, su translación a la práctica clínica se vería favorecida mediante la publicación de revisiones sistemáticas y análisis sintéticos de la multitud de estudios existentes. Para ello un método básico es el metaanálisis que, mediante métodos estadísticos, permite sintetizar los resultados de estudios independientes, proporcionando estimaciones de los efectos de forma más precisa y conclusiones más contundentes que ayudan a construir la evidencia necesaria para implementar cambios en la práctica médica.

1.5.2.1 Metaanálisis clásico

Una revisión bibliográfica de un tema concreto pretende valorar el grado de evidencia que existe sobre una pregunta concreta utilizando toda la información disponible, pero mantiene un cariz subjetivo en la interpretación. Los metaanálisis aportan objetividad y mejoran cualitativa y cuantitativamente las conclusiones que puedan extraerse de esa revisión

sistemática mediante la aplicación de métodos estadísticos en los que se combinan los datos de estudios independientes y se obtiene un valor promedio del efecto (Burguillo FJ, 2010).

Las conclusiones que pueden extraerse de un metaanálisis están limitadas a la selección de los estudios, la calidad de los estudios originales, la heterogeneidad entre ellos y el sesgo de publicación con la sobrerrepresentación de los estudios positivos (Figura 11). Diferentes métodos estadísticos ayudan a superar cada una de estas limitaciones (Hedges LV *et al.*, 1985).

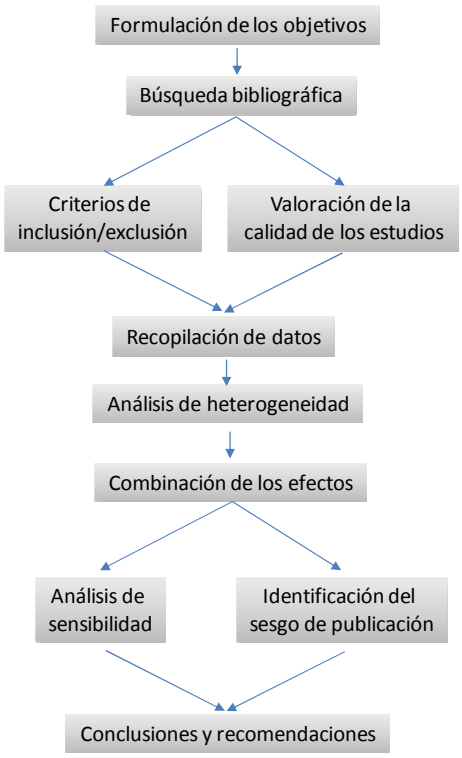


Figura 11. Esquema general de las etapas de un metaanálisis.

1.5.2.2 Metaanálisis de pacientes individuales (IPD)

Para reducir los sesgos y limitaciones del metaanálisis basado en los datos publicados en los artículos científicos se ha postulado el uso del metaanálisis utilizando los datos anónimos de los pacientes que conformaban las cohortes en los estudios originales. Aunque la compilación de los datos es costosa y se necesita de la colaboración de diferentes grupos de investigadores, permite realizar un análisis de mayor calidad, al estandarizar el método de análisis y, además, proporciona mayor información al poder realizar análisis más complejos y con un mayor tamaño muestral (incluyendo análisis de subgrupos particulares) (Riley RD *et al.*, 2010).

Una de las principales ventajas del IPD frente al metaanálisis clásico es la posibilidad de evaluar de forma más fiable las causas de heterogeneidad, ya que permite evaluar las covariables y las interacciones entre ellas a nivel del individuo, y no del estudio, con una potencia estadística suficiente para detectar incluso interacciones moderadas que puedan modificar el efecto observado en los estudios independientes (Marcucci M *et al.*, 2013). Estos IPD se han empezado a realizar con estudios de biomarcadores y, por ejemplo, han demostrado el valor predictivo de los niveles elevados de péptidos natriuréticos postoperatorio en la sucesión de

efectos cardíacos adversos, aunque sin evaluar su valor añadido respecto a factores de riesgo clásicos (Rodseth RN *et al.*, 2013).

Los resultados obtenidos de un IPD pueden ser diferentes a los obtenidos en el análisis combinado de los datos publicados, en general debido a la eliminación de heterogeneidad por la variabilidad en los métodos de análisis entre estudios y la diversidad en las fuentes de información (tanto a nivel de estudios como de pacientes), ya que no siempre se consiguen todos los datos o los nuevos criterios de inclusión permiten considerar más pacientes que los incluidos en el estudio original. Además, en los metaanálisis clásicos no se puede atajar la inadecuada presentación de los resultados, bien de la variable medidora del efecto o de las covariables, o un mal uso de los estadísticos en los estudios independientes (Jones AP *et al.*, 2009).

OBJETIVOS

2

Los objetivos principales de esta Tesis doctoral son:

2.1 Sistematizar la recogida de información existente sobre los biomarcadores pronósticos en el ictus.

2.2 De la información compilada, escoger un biomarcador típicamente asociado con el pronóstico del ictus y evaluar su valor en nuestra cohorte de pacientes aplicando estadísticos comparativos.

2.3 Metaanalizar toda la información recogida de ese biomarcador asociado con el pronóstico del ictus utilizando datos de pacientes individuales y aplicando estadísticos comparativos.

2.4 Estudiar biomarcadores candidatos de la vía inflamatoria, concretamente de la familia de las quimiocinas, en muestras cerebrales y sanguíneas de pacientes con ictus isquémico mediante un panel de anticuerpos.

2.5 Analizar las alteraciones del proteoma tras el ictus isquémico e identificar nuevos biomarcadores pronósticos aplicando estadísticos comparativos.

2.6 Analizar las alteraciones en el transcriptoma asociadas a la evolución de los pacientes con ictus isquémico e identificar nuevos biomarcadores pronósticos aplicando estadísticos comparativos.

COPIA DE LAS PUBLICACIONES 3

3.1 Brain Natriuretic Peptide is associated with worsening and mortality in acute stroke patients but adds no prognostic value to clinical predictors of outcome

(*Cerebrovascular Diseases, 2012, 34, 240-245)*

Original Paper

Cerebrovascular Diseases

Cerebrovasc Dis 2012;34:240–245
DOI: 10.1159/000341858

Received: November 16, 2011
Accepted: July 11, 2012
Published online: September 26, 2012

Brain Natriuretic Peptide Is Associated with Worsening and Mortality in Acute Stroke Patients but Adds No Prognostic Value to Clinical Predictors of Outcome

J. Montaner[a, b] T. García-Berrocoso[a] M. Mendioroz[a] M. Palacios[b] M. Perea-Gainza[c] P. Delgado[a] A. Rosell[a] M. Slevin[d] M. Ribó[b] C.A. Molina[b] J. Alvarez-Sabín[b]

[a]Neurovascular Research Laboratory and [b]Neurovascular Unit, Department of Neurology, Vall d'Hebron University Hospital Research Institute, Universitat Autònoma de Barcelona and [c]Internal Medicine, Clinic Hospital, Barcelona, Spain; [d]School of Healthcare Science, Manchester Metropolitan University, Manchester, UK

Key Words
Biomarkers · Brain natriuretic peptide · Stroke

Abstract
Background: At the present time, the determination of the outcome of stroke patients is based on the analysis of clinical and neuroimaging data. The use of prognostic blood biomarkers could aid in decision-making processes, e.g. admitting patients to specialized stroke units. Although the prognostic role of natriuretic peptides has been studied in heart failure and coronary diseases, the value of brain natriuretic peptide (BNP) is less known within the field of strokes. ***Objective:*** We aimed to study the relationship between plasma levels of BNP and acute neurological worsening or mortality after stroke in a large cohort of patients (investigating both ischemic and hemorrhagic disease). ***Methods:*** Consecutive stroke patients (ischemic and hemorrhagic) admitted to the Stroke Unit of our University Hospital within 24 h of the onset of symptoms were included. Stroke severity was assessed according to the National Institutes of Health Stroke Scale (NIHSS) at admission and at discharge. Neurological worsening was defined as an increase of 4 or more points in the NIHSS score or death during the patient's stay at the Stroke Unit. Blood samples were drawn upon admission to measure plasma levels of BNP (Biosite Inc., San Diego, Calif., USA). Statistical analysis was performed using SPSS 15.0 and R software. ***Results:*** Altogether, 896 patients were included in the study. BNP plasma levels were higher among patients who deteriorated the most over time (n = 112; 90.5 vs. 61.2 ng/l; p = 0.006) or died (n = 83; 118.2 vs. 60.9 ng/l; p < 0.001). Multivariate logistic regression analysis indicated that plasma BNP level was an independent predictor of neurological worsening [BNP >56.7 ng/l; odds ratio (OR) = 1.64; p = 0.04] and death after stroke (BNP >65.3 ng/l; OR = 1.97; p = 0.034). Adding BNP level to other well-known clinical predictors of bad outcome did not significantly increase the predictive value. ***Conclusions:*** Plasma levels of BNP measured during the acute phase of stroke are associated both with early neurological worsening and mortality. However, this biological information does not supply prognostic information which would add to clinical variables, which limits its use as a biomarker. Further investigation and systematic reviews are needed to clarify the role of natriuretic peptides in stroke outcome.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015–9770/12/0343–0240$38.00/0

Accessible online at:
www.karger.com/ced

Dr. Joan Montaner
Neurovascular Research Laboratory, Hospital Universitari Vall d'Hebron
Institut de Recerca (VHIR), Edifici Mediterrània, Pg Vall d'Hebron 119–129
ES–08035 Barcelona (Spain)
Tel. +34 93 489 4073, E-Mail 31862jmv@comb.cat

Introduction

Brain natriuretic peptide (BNP) was originally identified in the brain, but in recent years it was shown to be synthesized mainly in cardiac tissue as a result of myocyte stretch [1]. The prognostic role of natriuretic peptides has been studied in heart failure, coronary diseases and also amongst the general population to predict risk of death and cardiovascular events [2, 3]. However, in ischemic stroke, the prognostic value of BNP has been less studied. Mäkikallio et al. [4] demonstrated a relationship between natriuretic peptides and long-term mortality after a first ischemic stroke in a small series of 51 cases, and Shibazaki et al. [5] showed BNP's relationship to in-hospital mortality after acute ischemic stroke. In our study, we have included a very large number of patients with ischemic and also hemorrhagic stroke to establish the relationship between plasma levels of BNP and acute neurological worsening or mortality after stroke.

Methods

Patients and Protocol

During a 2-year period from 2002 to 2004, a prospective study was conducted on 896 consecutive stroke patients (ischemic and hemorrhagic) admitted to the Stroke Unit of our University Hospital during the first 24 h after the onset of symptoms.

Stroke severity was assessed in each patient according to the National Institutes of Health Stroke Scale (NIHSS) at admission and at discharge. Neurological worsening was defined as an increase of 4 or more points in the NIHSS score or death during the stay of the patient at the Stroke Unit. Risk factors for stroke and stroke subtypes were taken into account.

Laboratory Data

Blood samples were drawn upon arrival at the emergency department, within 24 h of the onset of symptoms, into EDTA tubes centrifuged at 3,000 rpm for 15 min at 4°C and plasma was frozen at –80°C until analysis. Blood testing was performed before any treatments were administered to avoid drug-biomarker interference. Plasma levels of BNP were measured by means of the ELISA technique following the manufacturer's instructions (Biosite Inc., San Diego, Calif., USA). Our study was approved by the ethics committee [PR(AG)89/2003] of the Vall d'Hebron University Hospital and all patients or relatives gave their informed consent.

Statistics

Data were analyzed using the SPSS 15.0 software. In univariate analysis, continuous variables were analyzed by the Student t test or the Mann-Whitney U test, depending on their normality, and the Pearson χ^2 test was used in categorical variables. BNP cut-off level for prediction of both worsening and mortality was established using the best sensitivity and specificity point in a receiver operator characteristics (ROC) curve. A forward-step multivariate logistic regression analysis was applied to variables that had significant statistical differences in univariate analysis for worsening or mortality to establish their individual predictive value. Odds ratio (OR) and 95% confidence intervals (CI) were calculated for each variable. The areas under the ROC curve (AUC), for a model with or without the biomarker, were compared and the integrated discrimination improvement (IDI) and net reclassification improvement (NRI) tests were calculated with R software (Hmisc and PredictABEL packages) to assess the added value of the biomarker to the clinical models to predict outcome [6]. In the case of the NRI test, prespecified clinically relevant thresholds of predicted risk (<10 and >90%) were used to calculate reclassification. In all cases, $p < 0.05$ was considered significant at a 95% CI.

Results

Of the 896 patients, 48% were female, mean age was 72 (SD 12) years, 23% had had a stroke previously, 13.4% had ischemic heart disease, 23.3% had atrial fibrillation, 57.3% were hypertensive and 26.3% were diabetics. Ischemic stroke cases comprised 84.6% (7.7% of whom received rt-PA treatment) and the remainder suffered from intracerebral hemorrhage.

In the univariate analysis, patients who had suffered a previous ischemic stroke ($p = 0.048$), who had hypertension ($p = 0.02$) or atrial fibrillation ($p = 0.048$), elder patients ($p = 0.037$) or patients that had higher baseline NIHSS ($p < 0.001$) had the worst outcome. Regarding mortality, patients who had had a previous ischemic stroke ($p = 0.019$), who had atrial fibrillation ($p < 0.001$), elder patients ($p = 0.02$) or patients with a higher baseline NIHSS ($p < 0.001$) had increased odds (table 1).

BNP plasma level showed no differences between ischemic and hemorrhagic strokes. Regarding prognosis, BNP was higher among all patients who later on worsened ($n = 112$; 90.5 vs. 61.2 ng/l; $p = 0.006$) or died ($n = 83$; 118.2 vs. 60.9 ng/l; $p < 0.001$). Other baseline clinical characteristics are also shown in the online supplementary table (for all online supplementary material, see www.karger.com/doi/10.1159/000341858).

By means of ROC curves, a cut-off point of 56.7 ng/l was selected for worsening, with a sensitivity of 68.8%, a specificity of 47.9%, a positive predictive value (PPV) of 90.8% and a negative predictive value (NPV) of 16.9%. Regarding mortality, a cut-off point of 65.3 ng/l showed a sensitivity of 73.5%, a specificity of 51.2%, a PPV of 94.6% and an NPV of 14.2%. Selected cut-off points showed good discrimination regarding worsening and mortality in all NIHSS scores in both ischemic and hemorrhagic strokes (fig. 1).

The multivariate logistic regression analysis performed in the whole cohort confirmed that BNP plasma

Table 1. Association between baseline characteristics and in-hospital outcome after stroke

	Worsening (n = 112)			Mortality (n = 83)		
	no	yes	p value	no	yes	p value
Males, n (%)	55 (13.9)	57 (12.9)	0.663	46 (11.6)	37 (8.4)	0.125
HTA, n (%)	32 (9.5)	69 (15.1)	**0.02**	24 (7.1)	49 (10.7)	0.08
Diabetes mellitus, n (%)	67 (11.4)	34 (16.4)	0.064	53 (9)	20 (9.7)	0.781
Dislypemia, n (%)	79 (13.1)	22 (11.7)	0.626	55 (9)	18 (9.7)	0.769
Previous ischemic stroke, n (%)	72 (11.5)	31 (17)	**0.048**	50 (8)	25 (13.7)	**0.019**
Ischemic cardiopathy, n (%)	90 (13.1)	11 (10.2)	0.392	68 (9.9)	5 (4.7)	0.082
Atrial fibrillation, n (%)	70 (11.5)	31 (17)	0.048	41 (6.7)	32 (17.6)	**<0.001**
Smoker, n (%)	87 (13.1)	14 (11.1)	0.547	65 (9.7)	8 (6.4)	0.239
Alcohol, n (%)	95 (12.7)	6 (13)	0.951	70 (9.3)	3 (6.7)	0.79
BNP >56.7 ng/l, n (%)	35 (9.2)	77 (16.9)	**0.001**	–	–	–
BNP >65.3 ng/l, n (%)	–	–	–	22 (5.4)	61 (14.2)	**<0.001**
Median age (IQR)	74 (66–81)	76 (70–82.5)	**0.037**	74 (66–81)	77.5 (71–84)	**0.02**
Median baseline NIHSS (IQR)	6 (2–13)	12 (6–18)	**<0.001**	6 (2–12)	16 (10–21)	**<0.001**

Univariate analysis performed separately for worsening and mortality. Median and interquartile range (IQR) for continuous variables and count (n) with percentage (%) for categorical variables. All variables with a p value <0.05 are marked in bold.

Table 2. Multivariate logistic regression analysis

	p value	OR (95% CI)
Worsening		
BNP >56.7 ng/l	0.04	1.637 (1.023–2.619)
Previous stroke	0.048	1.677 (1.005–2.799)
Diabetes mellitus	0.043	1.635 (1.015–2.632)
Baseline NIHSS	<0.001	1.094 (1.061–1.128)
Mortality		
BNP >65.3 ng/l	0.034	1.972 (1.053–3.695)
Previous stroke	0.031	1.981 (1.064–3.686)
Atrial fibrilation	0.029	1.926 (1.07–3.468)
Baseline NIHSS	<0.001	1.147 (1.103–1.193)

Independent predictors for both worsening and mortality analysis.

level was an independent predictor of neurological worsening (BNP >56.7 ng/l; OR = 1.64; p = 0.04) and death after stroke (BNP >65.35 ng/l; OR = 1.97; p = 0.034). Other predictors of bad outcome were a previous stroke, stroke severity (baseline NIHSS), diabetes mellitus and atrial fibrillation (table 2).

However, the AUC were similar for a model with or without BNP for both worsening (AUC 0.706, 95% CI 0.655–0.758 vs. AUC 0.700, 95% CI 0.647–0.753) and mortality (AUC 0.806, 95% CI 0.753–0.859 vs. AUC 0.796, 95% CI 0.74–0.851) (fig. 2). Neither the IDI nor the NRI tests showed any added value of the biomarker to the clinical models to predict outcome (data not shown).

Discussion

Our study demonstrates that plasma BNP level is an independent prognostic factor for early mortality and a newly defined factor for neurological worsening after an acute stroke. BNP increase has been associated with the intensity of brain ischemia reflecting the magnitude of brain injury [4, 7]. On the other hand, an acute blood pressure elevation has been well documented during the acute phase of stroke and it might indeed increase BNP released from the heart, as BNP plays a role in the hemodynamic regulation during this period [8]. Since a high BNP level might reflect severe brain damage and produce disturbances in the autonomic cardiovascular system, its role in providing an indication of worsening prognosis seems feasible.

Regarding mortality, our results are in accordance with the findings by Jensen et al. [9] and Mäkikallio et al. [4], which reported higher levels of natriuretic peptides among patients who died after 6 months of stroke or

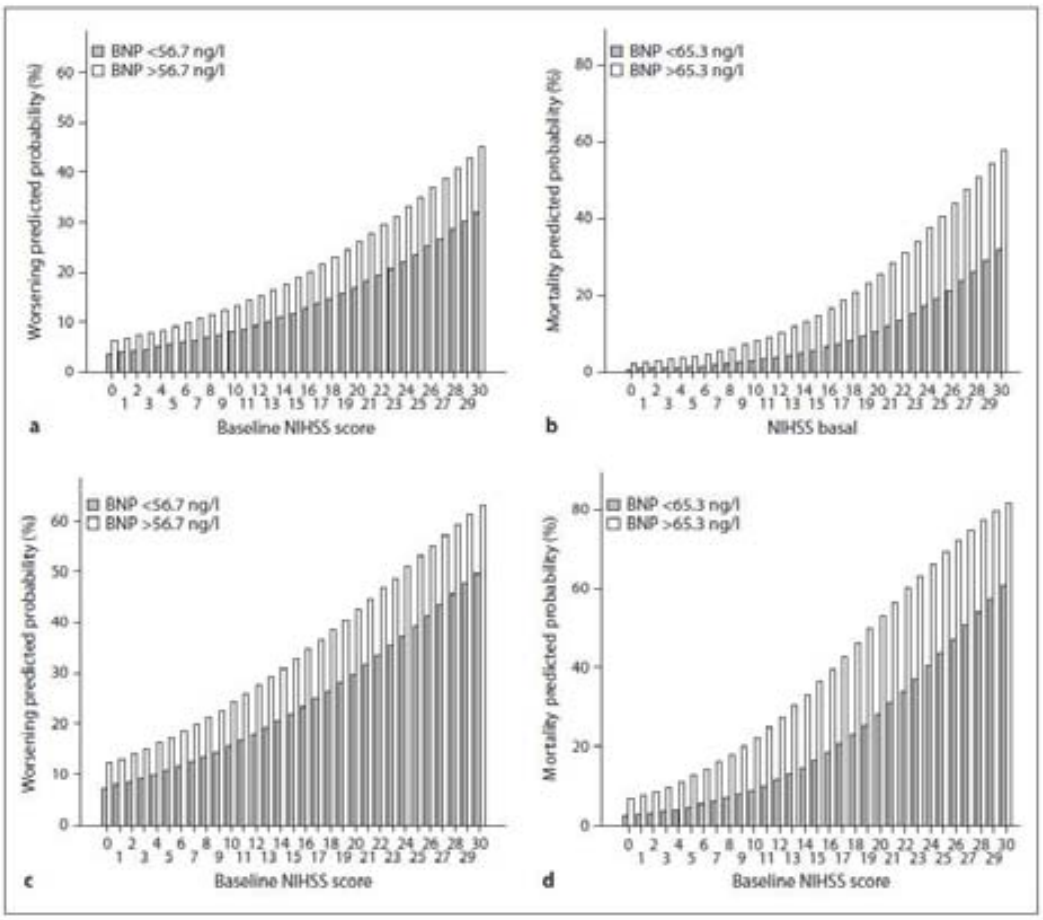


Fig. 1. BNP levels showed a good discrimination regarding outcome. Relation between BNP selected cut-off points and predicted probability of worsening (**a**, ischemic strokes; **c**, hemorrhagic strokes) or mortality (**b**, ischemic strokes; **d**, hemorrhagic strokes) across all the span of NIHSS scores.

within a 4-year follow-up, respectively. We extended our results to the acute phase of stroke, confirming findings by Shibazaki et al. [5]. Interestingly, we also confirmed that the prognostic value of BNP is not only useful in ischemic but in hemorrhagic stroke, as alluded to in other studies [10], and we extended the findings to the acute phase of brain hemorrhage.

Other reports have not been able to show the relationship between the N-terminal prohormone of BNP (NT-proBNP) and 3-month mortality [11]. NT-proBNP showed a significant influence on the outcome in the univariate analysis, but its effect was outweighed if other important predictors of outcome such as initial NIHSS and lesion volume were taken into account. The fact that we used only in-hospital mortality may explain these differences, although this may also be a limitation of our study because we obtained no outcome data for periods longer than 3 months.

The AUC has been the 'metric of choice' to quantify new biomarker performance. Two new metrics, the IDI and NRI, have been rapidly adopted to quantify the added value of a biomarker to an existing test [6]. Applying the test to our data, it may be concluded that the plasma level of BNP measured during the acute phase of stroke does not add prognostic information to the clinical data, i.e. it seems of limited use as a prognostic biomarker.

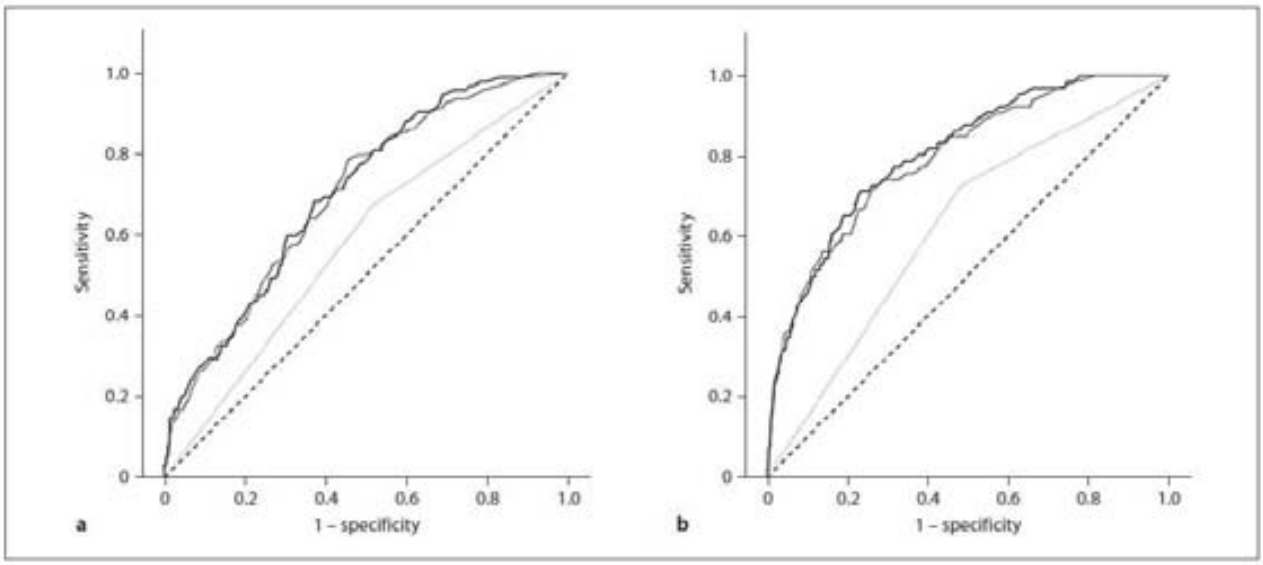


Fig. 2. ROC curves from predictive models for worsening (**a**) and mortality (**b**). Black line: model including both clinical and BNP variables; dark grey line: model including only clinical variables; light grey line: model including only BNP variable; dashed line: reference line.

Some of the methodological differences need to be noted between our work and previous studies where NT-proBNP instead of BNP was used. Although both BNP and NT-proBNP assays have a nearly identical test performance in ruling out severely reduced left ventricular ejection fraction and in their prediction of all-cause mortality or heart failure [12], direct comparisons of both biomarkers have not yet been made in the field of strokes. Another limitation of our study is that it was designed to use this marker as a short-term prognostic tool, without differentiating stroke subtypes or medical conditions that might also affect BNP plasma levels. In fact, a recent report shows that the addition of BNP to multivariate models increased their predictive performance for functional outcome and mortality only amongst those with cardioembolic stroke [13].

Because having very early information about the prognosis of a stroke patient might influence in the rationalization and decision-making regarding their admission to a specialized stroke unit or participation in a clinical trial, future identification of biomarkers that add prognostic information to clinical and radiological data would be ideal. In addition to clinical parameters such as the NIHSS [14], some markers such as copeptin have been shown to be of value. This was also the case with BNP in our study (fig. 1), but when more strict tests were applied to the combination of a clinical model containing not only stroke severity scales but also other vascular risk factors related to outcome, the biomarker was found to not offer further prognostic information. Therefore, all new prognostic biomarkers in the field should be carefully statistically evaluated and need to be replicated in multicenter studies before their implementation in clinical practice.

Regarding the translation of biomarker measurements to clinical practice, the use of point-of-care tools based on biosensors is being implemented in order to obtain reliable and rapid results. These devices allow the detection of biomarkers in whole blood, without the necessity for sample preparation, within a 15-minute time frame [15]. Models combining clinical data with biomarker information in an algorithm that is easy to interpret for clinicians would be mandatory to implement the use of biomarkers in daily practice.

In conclusion, BNP is clearly an independent predictor of poor outcome in stroke, but does not add any value to known clinical models of prognosis. In view of these controversial results, further investigation including systematic review is needed to clarify the real role of BNP in stroke outcome.

Acknowledgements

Thanks go to the emergency department nurses, on-call neurologists and all technicians of the core laboratory and especially to Anna Penalba for coordinating sample recruitment. We thank all participating stroke patients and their relatives for their continuous support in studies on stroke biomarkers.

Disclosure Statement

This study was partially funded by grants from the Fondo de Investigaciones Sanitarias (FIS 08/361 & 11/176). The Neurovascular Research Laboratory received a research grant from Biosite Inc., and it participates in the Spanish Stroke Research Network (RENEVAS, RD06/0026/0010) and the European Stroke Network (EUSTROKE 7FP Health F2-08-202213). J.M. has received honoraria for speaker's presentations. P.D. and A.R. were supported by Miguel Servet senior research contracts (CP09/00136 and CP09/00265, respectively) and T.G.-B. by a predoctoral fellowship (FI09/00017) from the Carlos III Health Institute.

References

1 Levin ER, Gardner DG, Samson WK: Natriuretic peptides. N Engl J Med 1998;339:321–328.
2 de Lemos JA, McGuire DK, Drazner MH: B-type natriuretic peptide in cardiovascular disease. Lancet 2003;362:316–322.
3 Wang TJ, Larson MG, Levy D, Benjamin EJ, Leip EP, Omland T, Wolf PA, Vasan RS: Plasma natriuretic peptide levels and the risk of cardiovascular events and death. N Engl J Med 2004;350:655–663.
4 Mäkikallio AM, Mäkikallio TH, Koperlainen JT, Voulteenaho O, Tapanainen JM, Ylitalo K, Sotaniemi KA, Huikuri HV, Myllylä VV: Natriuretic peptides and mortality after stroke. Stroke 2005;36:1016–1020.
5 Shibazaki K, Kimura K, Okada Y, Iguchi Y, Uemura J, Terasawa Y, Aoki J: Plasma brain natriuretic peptide as an independent predictor of in-hospital mortality after acute ischemic stroke. Intern Med 2009;48:1601–1606.
6 Pencina MJ, D'Agostino RB Sr, D'Agostino RB Jr, Vasan RS: Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. Stat Med 2008;27:157–172.
7 Nogami M, Shiga J, Takatsu A, Endo N, Ishiyama I: Immunohistochemistry of atrial natriuretic peptide in brain infarction. Histochem J 2001;33:87–90.
8 Nakagawa K, Yamaguchi T, Seida M, Yamada S, Imae S, Tanaka Y, Yamamoto K, Ohno K: Plasma concentrations of brain natriuretic peptides in patients with acute ischemic stroke. Cerebrovasc Dis 2005;19:157–164.
9 Jensen JK, Mickley H, Bak S, Korsholm L, Kristensen SR: Serial measurements of N-terminal pro-brain natriuretic peptide after acute ischemic stroke. Cerebrovasc Dis 2006;22:439–444.
10 James ML, Blessing R, Phillips-Bute BG, Bennett E, Laskowitz DT: S100B and brain natriuretic peptide predict functional neurological outcome after intracerebral haemorrhage. Biomarkers 2009;14:388–394.
11 Etgen T, Baum H, Sander K, Sander D: Cardiac troponins and N-terminal pro-brain natriuretic peptide in acute ischemic stroke do not relate to clinical prognosis. Stroke 2005;36:270–275.
12 Richards M, Nicholls MG, Espiner EA, Lainchbury JG, Troughton RW, Elliott J, Frampton CM, Crozier IG, Yandle TG, Doughty R, MacMahon S, Sharpe N, Christchurch Cardioendocrine Research Group, Australia-New Zealand Heart Failure Group: Comparison of B-type natriuretic peptides for assessment of cardiac function and prognosis in stable ischemic heart disease. J Am Coll Cardiol 2006;47:52–60.
13 Rost NS, Biffi A, Cloonan L, Chorba J, Kelly P, Greer D, Ellinor P, Furie KL: Brain natriuretic peptide predicts functional outcome in ischemic stroke. Stroke 2012;43:441–445.
14 Katan M, Fluri F, Morgenthaler NG, Schuetz P, Zweifel C, Bingisser R, Müller K, Meckel S, Gass A, Kappos L, Steck AJ, Engelter ST, Müller B, Christ-Crain M: Copeptin: a novel, independent prognostic marker in patients with ischemic stroke. Ann Neurol 2009;66:799–808.
15 Santamarina E, Penalba A, Garcia-Berrocoso T, Delgado P, Quintana M, González-Alujas T, Ribó M, Maisterra O, Molina CA, Evangelista A, Alvarez-Sabin J, Montaner J: Biomarker level improves the diagnosis of embolic source in ischemic stroke of unknown origin. J Neurol 2012, E-pub ahead of print.

Supplemental table 1: Brain Natriuretic Peptide values regarding baseline clinical characteristics.

	BNP (ng/L)		
	No	Yes	p-value
Sex (male)	82.4 (33.4-175.8)	49.8 (0-135.4)	**<0.001**
HTA	58 (21.6-153.7)	69.4 (21.6-176.4)	0.296
Diabetes mellitus	61.3 (20.3-161.6)	72.7 (26.1-164.2)	0.168
Dislypemia	70.1 (22.4-162.5)	50.1 (0-153.7)	0.154
Previous ischemic stroke	66.7 (21.5-162.9)	65.4 (24-139.1)	0.574
Ischemic cardiopathy	60 (18.9-146.9)	133.2 (48-232.5)	**<0.001**
Atrial fibrillation	47.9 (0-120)	157.5 (77.9-252.7)	**<0.001**
Smoker	74.2 (27.7-176.1)	28 (0-78.3)	**<0.001**
Alcohol	67 (22.5-100.5)	31 (0-100.5)	**0.011**
Atherotrombotic	73.4 (24.5-176.4)	44.3 (0-105.9)	**0.001**
Cardioembolic	44.6 (0-101.4)	143.9 (63.5-236.5)	**<0.001**
Age	Spearman Rho=0.36		**<0.001**
NIHSS baseline	Spearman Rho=0.185		**<0.001**
ICH	69 (20.8-169.2)	55.8 (22.6-126.7)	0.234
Mortality	60.9 (20.3-154.5)	118.2 (48.2-226.6)	**<0.001**
Worsening	61.2 (20.8-154.6)	90.5 (35.7-211.8)	**0.006**

BNP values given in ng/L. HTA, hypertension; ICH, intracranial hemorrhage; NIHSS, National Institutes of Health Stroke Scale for assessing stroke severity. All variables with a p-value < 0.05 were marked in bold.

3.2 B-type natriuretic peptides and mortality after stroke: A systematic review and meta-analysis

(*Neurology, 2013, 81, 1976-1985*)

B-type natriuretic peptides and mortality after stroke

A systematic review and meta-analysis

Teresa García-Berrocoso
Dolors Giralt
Alejandro Bustamante, MD
Thorleif Etgen, MD
Jesper K. Jensen, MD
Jagdish C. Sharma, FRCP
Kensaku Shibazaki, MD
Ayhan Saritas, MD
Xingyong Chen
William N. Whiteley, MRCP
Joan Montaner, PhD

Correspondence to Dr. Montaner: 31862jmv@comb.es

ABSTRACT

Objective: To measure the association of B-type natriuretic peptide (BNP) and N-terminal fragment of BNP (NT-proBNP) with all-cause mortality after stroke, and to evaluate the additional predictive value of BNP/NT-proBNP over clinical information.

Methods: Suitable studies for meta-analysis were found by searching MEDLINE and EMBASE databases until October 26, 2012. Weighted mean differences measured effect size; meta-regression and publication bias were assessed. Individual participant data were used to estimate effects by logistic regression and to evaluate BNP/NT-proBNP additional predictive value by area under the receiver operating characteristic curves, and integrated discrimination improvement and categorical net reclassification improvement indexes.

Results: Literature-based meta-analysis included 3,498 stroke patients from 16 studies and revealed that BNP/NT-proBNP levels were 255.78 pg/mL (95% confidence interval [CI] 105.10–406.47, $p = 0.001$) higher in patients who died; publication bias entailed the loss of this association. Individual participant data analysis comprised 2,258 stroke patients. After normalization of the data, patients in the highest quartile had double the risk of death after adjustment for clinical variables (NIH Stroke Scale score, age, sex) (odds ratio 2.30, 95% CI 1.32–4.01 for BNP; and odds ratio 2.63, 95% CI 1.75–3.94 for NT-proBNP). Only NT-proBNP showed a slight added value to clinical prognostic variables, increasing discrimination by 0.028 points (integrated discrimination improvement index; $p < 0.001$) and reclassifying 8.1% of patients into correct risk mortality categories (net reclassification improvement index; $p = 0.003$). Neither etiology nor time from onset to death affected the association of BNP/NT-proBNP with mortality.

Conclusion: BNPs are associated with poststroke mortality independent of NIH Stroke Scale score, age, and sex. However, their translation to clinical practice seems difficult because BNP/NT-proBNP add only minor predictive value to clinical information. *Neurology*® 2013;81:1976–1985

GLOSSARY

AUC = area under the receiver operating characteristic curve; **BNP** = B-type natriuretic peptide; **CI** = confidence interval; **IDI** = integrated discrimination improvement; **IPD** = individual participant data; **IQR** = interquartile range; **NIHSS** = NIH Stroke Scale; **NRI** = net reclassification improvement; **NT-proBNP** = N-terminal fragment of B-type natriuretic peptide; **OR** = odds ratio; **WMD** = weighted mean difference.

Early prediction of fatal outcome after stroke might improve decision-making processes, such as the use of more aggressive therapies or the inclusion of selected patients in clinical trials. Clinical models to predict death after stroke perform with reasonable accuracy,[1,2] but the addition of blood biomarkers might lead to better predictive models.[3,4]

B-type natriuretic peptide (BNP), a vasoactive hormone with natriuretic, diuretic, and vasodilator activity, is synthesized mainly in cardiac tissue as a result of myocyte stretch.[5] Activation of BNP generates an inactive N-terminal fragment (NT-proBNP)[6] and both peptides have been associated with unfavorable clinical outcome and mortality in stroke patients.[7–9] Two recent

Editorial, page 1970

Supplemental data at www.neurology.org

From the Neurovascular Research Laboratory (T.G.-B., D.G., A.B., J.M.), Vall d'Hebron Institute of Research, Universitat Autònoma de Barcelona, Spain; Department of Neurology (T.E.), Klinikum Südostbayern-Klinikum Traunstein, Germany; Department of Psychiatry and Psychotherapy (T.E.), Technische Universität München, Germany; Department of Cardiology (J.K.J.), Odense University, Denmark; Stroke Medicine (J.C.S.), Lincoln County Hospital, University of Nottingham, UK; Department of Stroke Medicine (K.S.), Kawasaki Medical School, Kurashiki City, Okayama, Japan; Department of Emergency Medicine (A.S.), School of Medicine, Duzce University, Turkey; Department of Neurology (X.C.), Fujian Provincial Hospital, Fujian Medical University, Fuzhou, China; and Centre for Clinical Brain Sciences (W.N.W.), University of Edinburgh, UK.

Go to Neurology.org for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

studies[10,11] show that, although BNP has a statistically significant association with mortality and functional outcome, it does not add prognostic information to simple clinical variables. However, this new information should be carefully managed, because both studies were small and BNP-related peptides are important predictors of poor outcome in other diseases.[12,13]

Because there is still uncertainty about whether BNP could be useful in stroke practice to predict mortality, first we conducted a systematic review and a literature-based meta-analysis to examine the association of BNP/NT-proBNP levels with all-cause mortality after a cerebrovascular event. Second, we performed an individual participant data (IPD) analysis to further explore the role of natriuretic peptides and to assess whether their addition to clinical information could have additional predictive value and/or could improve mortality risk stratification.

METHODS

Standard protocol approvals, registrations, and patient consents. Methods for this study were specified in advance and registered in the PROSPERO database (CRD42012003284).[14] We report it in reference to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) statement.[15]

Eligibility criteria and search strategy. We included studies that reported original data and i) recruited patients with ischemic stroke, TIA, and/or hemorrhagic stroke; ii) measured BNP or NT-proBNP; and iii) recorded whether patients died during follow-up.

We searched MEDLINE and EMBASE databases up to October 26, 2012 without restriction (appendix e-1 on the *Neurology*® Web site at www.neurology.org). Reference lists from identified articles and published reviews were hand-searched.

Two independent reviewers thoroughly checked titles and/or abstracts yielded by search and also screened potentially relevant articles in full text after removing duplicates. Disagreements were resolved by discussion.

Data collection. Two independent reviewers extracted data using a simple, standardized template. Appendix e-2 shows items included in that template. If data were missing, the corresponding author was contacted by e-mail.

Three independent reviewers assessed the quality of each included article using a 15-point quality score (appendix e-3), which does not apply for inclusion of the article in the meta-analysis. Corresponding authors from included articles were invited to participate sharing individual records using a preplanned dataset including baseline variables for IPD analysis (see appendix e-4).

Statistical analysis. STATA 10.0 (StataCorp, College Station, TX) and SPSS 15.0 (SPSS Inc., Chicago, IL) were used for statistical analyses, unless otherwise stated.

Literature-based meta-analysis. The strength of association of BNP/NT-proBNP with death was measured by weighted mean differences (WMDs) between death and survival groups with a random effects model. Cochrane Q and I^2 statistics measured heterogeneity. WMD not including zero on its confidence interval (CI) and with a z test p value <0.05 were considered significant; forest plot summarized these size effects. We used meta-regression to adjust our study level meta-analysis for the potential confounders. We used Duval-Tweedie nonparametric "trim-and-fill" test[16] to assess publication bias.

IPD analysis. A whole compiled database including all individual information was used as a unique cohort to perform a complete statistical analysis. We assessed publication bias with Duval-Tweedie test for both BNP and NT-proBNP separately. BNP and NT-proBNP levels had a nonnormal distribution (Kolmogorov-Smirnov test with $p < 0.05$), so we applied Mann-Whitney U or Kruskal-Wallis tests and reported median and interquartile range (IQR). Pearson χ^2 test assessed differences among categorical variables. Correlations with stroke severity measured by NIH Stroke Scale (NIHSS) at admission and age were performed by Spearman rank.

Data from all different cohorts were normalized by standardized z scores in the unique database. We constructed forward stepwise multivariate logistic regression models with all clinical variables associated with mortality at $p < 0.05$ (baseline NIHSS score, age, and sex). Odds ratio (OR), 95% CI, and p value were given. Biomarker levels, using the highest quartile cutoff point, were added to the logistic regression model including clinical variables. To gain results consistency, we performed bootstrap calculation for OR and 95% CI in a random sample with replacement of the total number of patients, using a modified version of Car R-package.[17]

The method of DeLong et al.[18] allowed us to compare the areas under the receiver operating characteristic curve (AUCs) from models including biomarkers with AUCs from clinical model only using MedCalc 12.3 software. Using R software (Hmisc and PredictABEL packages), net reclassification improvement (NRI) and integrated discrimination improvement (IDI) indexes assessed the added value of the biomarker to the clinical model to predict mortality.[19] For the NRI test, prespecified clinically relevant thresholds of predicted risk (<10% and >90%) were used to calculate reclassification of patients.

In all cases, a p value <0.05 was considered significant at a 95% confidence level. For multitesting, we applied Bonferroni correction.

RESULTS

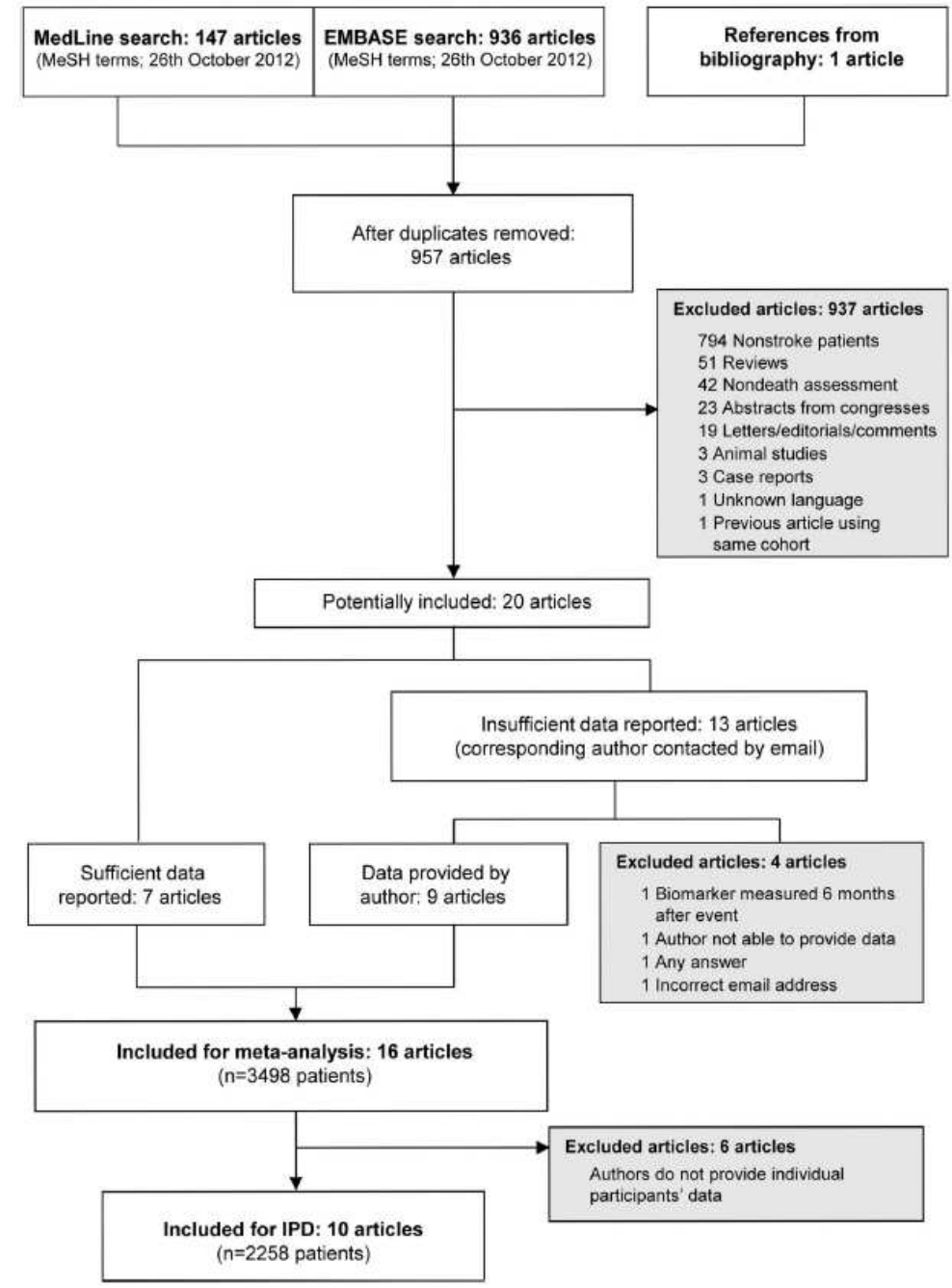
Study selection and characteristics. Our search strategy yielded 957 references, 20 of which were considered for full screening. Finally, 16 articles met inclusion criteria and were included in the meta-analysis (figure 1).

Table 1 presents the characteristics of included studies.[7–11,20–30] The quality of the 16 included articles was moderate (7 points, IQR 9–11; minimum 5, maximum 13). Only 25% of articles specified that measurement of biomarker was blind to clinical data; only one article reported sample size calculation (table e-1).

Synthesis of results from literature-based meta-analysis. The included articles recruited a total of 3,498 stroke patients. Although BNP/NT-proBNP mean levels were higher in nonsurvivors than survivors, all studies showed large deviations (table 1) and a high heterogeneity between them ($Q = 274.92$, $I^2 = 94.5\%$, df 15, $p < 0.001$).

On average, BNP/NT-proBNP levels were 255.78 pg/mL higher in those patients who died

Figure 1 Flow diagram



IPD = individual participant data.

(95% CI 105.10–406.47, $p = 0.001$) (WMDs for each individual study and all-studies average are given in figure 2). This association was not affected by any of the study level confounders that were considered, including BNP vs NT-proBNP, stroke severity, age, plasma or serum measurement, time of sample collection, sex, and quality score. However, there exists uncertainty about the value of this result because study bias was detected by loss of significance when the Duval-Tweedie test was applied (pooled WMD 121.55 pg/mL, 95% CI −33.56 to 276.66, $p = 0.125$).

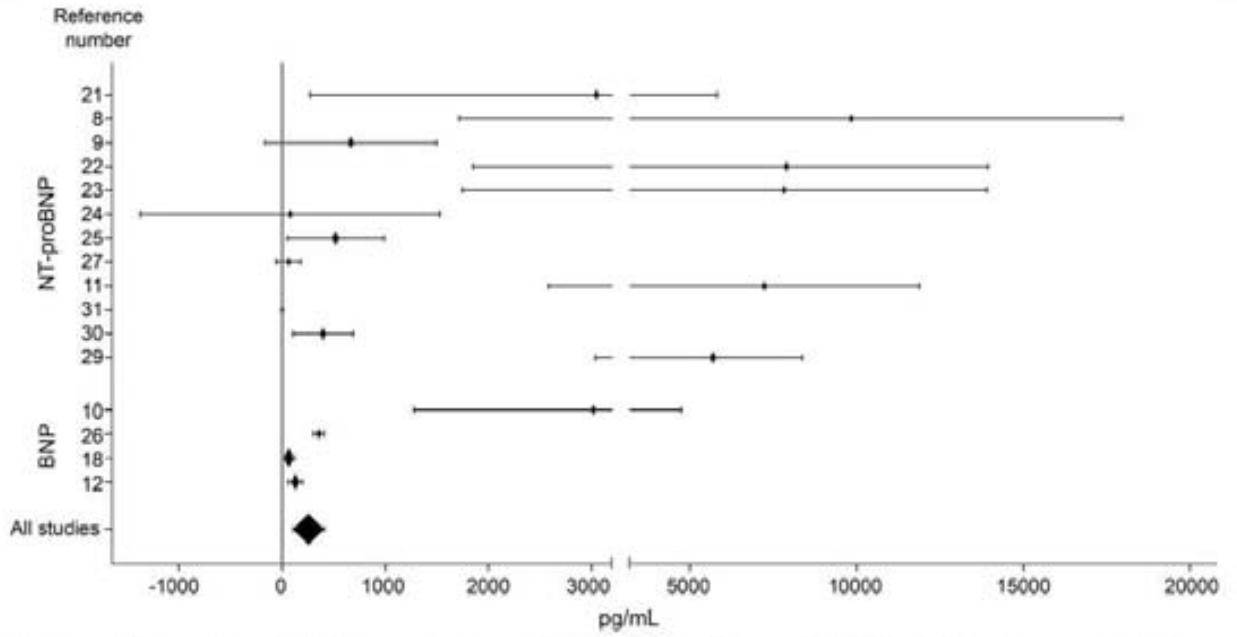
Synthesis of results from IPD analysis. We obtained individual information from 2,258 stroke patients from 10 included articles.[8–11,20,21,24,25,27,30] In the compiled dataset, we found NT-proBNP levels almost 10

Table 1 Overview of included articles: Main data collected for each of the 16 included articles

Ref.	Marker	Method	Blood collection	Sample size	Time of death	Death, %	NIHSS adm.	Age, y	Males, %	Biomarker in deceased patients	Biomarker in alive patients
20	2	Elecsys2010	<12 h	174	90 d	10.9	9.8 ± 7.9	67.7 ± 11.6	59.2	4,053.8 ± 6,138.3	1,009.4 ± 1,741.6
7	2	Radioimmunoassay	2 d	51	4 y	43.1	12.9 ± 19.8	67 ± 10	43.1	11,909.1 ± 19,352.1	2,070.3 ± 2,083.0
8	2	Elecsys2010	Adm.	250	6 mo	9	9 ± 7.9	68.6 ± 12.8	53.2	1,491.1 ± 1,847.5	825.9 ± 2,692.5
21	2	Modular Analytics E170	5 d (mean)	114[a]	120 d	11.4	11.2 ± 5.9	72.9 ± 12.6	48.2	8,956.0 ± 11,097.3	1,060.3 ± 2,070.6
22	2	—	48 h (median)	72[a]	In-hospital	25	—	63 ± 13	40	11,898 ± 12,741	4,073 ± 5,691
23	2	Modular Analytics E170	<5 h	41[a]	In-hospital	12.2	7.5 ± 6.2	78.3 ± 6.8	36	950.1 ± 1,567.1	873.2 ± 1,415.7
9	1	Immunoassay (Shionogi)	<24 h	335	In-hospital (1 mo)	6	9 ± 8.1	72.3 ± 12.3	62.7	731.5 ± 1,070.9	213.1 ± 384.5
24	2	Modular Analytics E170	3 d (mean)	125[a]	1 y	16	13.4 ± 20.1	73 ± 12	49.6	8,289.5 ± 10,570.4	1,060.5 ± 2,071.1
25	1	Triage (Biosite)	<12 h	60	In-hospital	26.7	10.6 ± 6.3	60.7 ± 14.1	41.2	172.2 ± 226.4	111.0 ± 146.6
26	2	Immulite 2500 (Siemens)	Adm.	92[a]	In-hospital	30.4	—	65.6 ± 11	44.6	1.8 ± 0.9	0.7 ± 0.8
27	1	Immunoassay (Shionogi)	<24 h	221[a]	In-hospital	10.9	12.1 ± 8.9	76.5 ± 10.9	54.7	714.1 ± 716.3	320.0 ± 380.7
10	2	Elecsys2010	<24 h	270[a]	90 d	11	6 ± 7.0	74.4 ± 12.5	41.8	6,852.6 ± 7,541.0	1,152.4 ± 1,777.9
28	2	Immunoassay (Biomedica Gruppe)	<48 h	569	6 mo	5.8	—	67.9 ± 15	54	379.6 ± 200.4	251.5 ± 167.1
29	2	COBAS h232 (Roche)	Adm.	106	In-hospital	21.7	15.5 ± 6.5	69.2 ± 11.9	58.5	508.3 ± 121.3	153.4 ± 63.4
11	1	Immunoassay (Biosite)	<24 h	896[a]	In-hospital	9.3	8.2 ± 7.0	72 ± 12	52	186.1 ± 282.3	123.8 ± 219.7
30	2	—	<72 h	122	In-hospital	18.8	9.8 ± 5.1	71.5 ± 9.8	56.6	4,642.3 ± 4,097.5	1,626.0 ± 2,370.4

Abbreviations: Adm. = at admission; BNP = B-type natriuretic peptide; NIHSS = NIH Stroke Scale; NT-proBNP = N-terminal fragment of BNP; Ref. = reference.
Data represent mean ± SD for NIHSS score, age, and biomarker levels. Biomarker levels given as pg/mL. Marker: 2 = NT-proBNP; 1 = BNP. — = information not available.
[a]Study including more than one type of event (i.e., ischemic and hemorrhagic and/or TIA).

Figure 2 Forest plot



Weighted mean differences (diamonds) for B-type natriuretic peptide (BNP) and N-terminal fragment of BNP (NT-proBNP) levels between death and survival groups. Lines represent 95% confidence interval. Sizes of diamonds are proportional to sample size.

times higher than BNP levels (401 pg/mL [IQR 126–1,375] vs 77.8 pg/mL [IQR 27.8–191]; $p < 0.001$). Therefore, analyses for the 2 peptides were performed separately. Neither BNP nor NT-proBNP changed the degree of association when the Duval-Tweedie test was applied. There was therefore no evidence of publication bias (data not shown). However, for BNP (with only 3 studies), the association with mortality did not reach statistical significance when WMDs were considered (pooled WMD 113.75 pg/mL, $p = 0.164$).

We assessed the association of BNP and NT-proBNP levels with several demographic and clinical characteristics. Higher levels of both peptides were found in women (119 [43–244.9] vs 59.9 [19.4–180.4] pg/mL, $p < 0.001$ for BNP; and 414.4 [139.5–1,822.5] vs 279.1 [59.2–1463.1] pg/mL, $p < 0.001$ for NT-proBNP), nonsmokers (89.4 [32–216.2] vs 54.4 [17.2–192.5] pg/mL, $p = 0.001$ for BNP; 507.4 [109.9–1,911.3] vs 279.1 [84.6–820.3] pg/mL, $p < 0.001$ for NT-proBNP), and patients with atrial fibrillation (207.6 [108–356] vs 44.6 [14.6–113.3] pg/mL, $p < 0.001$ for BNP; 1,864.8 [816.1–4368] vs 164.9 [59.2–524.3] pg/mL, $p < 0.001$ for NT-proBNP). Both BNP and NT-proBNP levels positively correlated with NIHSS score at admission ($r = 0.228$, $p < 0.001$ for BNP; $r = 0.272$, $p < 0.001$ for NT-proBNP) and age ($r = 0.340$, $p < 0.001$; $r = 0.477$, $p < 0.001$, respectively).

Because our systematic search was focused only on ischemic events, we further analyzed only ischemic stroke patients regarding mortality, and after exclusion of patients with hemorrhagic stroke or TIA, the sample size was 957 patients with BNP measurement and 880 patients for NT-proBNP. Table 2 presents demographic characteristics for each individual study and for pooled data. After data normalization, we divided patients into quartiles for both BNP and NT-proBNP and when the highest quartile was compared with the others, BNP was independently associated with poststroke mortality, giving a pooled adjusted OR of 2.30 (95% CI 1.32–4.01, $p = 0.003$) after logistic regression adjustment for NIHSS score, age, and sex. NT-proBNP was also associated with mortality after stroke (adjusted OR = 2.63, 95% CI 1.75–3.94, $p < 0.001$). These results indicate a double risk of death for those patients in the highest quartile for both peptides.

By using these cutoff points, only NT-proBNP showed a slight added value to model with clinical variables alone (NIHSS score, age, sex), showing an increase in AUC ($p = 0.029$). Regarding discrimination improvement measured with the IDI index, both markers showed a small increase (0.018 points for BNP and 0.028 points for NT-proBNP). However, when reclassification of patients into predefined categories of risk of death was analyzed, again only the addition of NT-proBNP to simple clinical variables showed added value, classifying 8.1% of patients (NRI index; $p = 0.003$) into more accurate risk categories than a model based on clinical variables alone (table e-2 and graphical representation in figure 3).

Table 2 Demographic characteristics of ischemic stroke patients of each included cohort and pooled data for IPD

Factor	Cohort 1[30] (n = 174)	Cohort 2[8] (n = 250)	Cohort 3[32,34] (n = 104)	Cohort 4[4,27] (n = 410)	Cohort 5[25] (n = 23)	Cohort 6[10] (n = 230)	Cohort 7[11] (n = 524)	Cohort 8[30] (n = 122)	Pooled data (n = 1,837)
Age, y	68.5 (61-77)	69 (59-79)	74 (66.5-83)	75 (66-82)	67 (47.5-73)	76 (69-84)	76 (67-82)	72 (66-79)	74 (65-81)
NIHSS score at admission	8 (4-14)	6 (3-13)	9 (7-15)	8 (2-16)	8 (5.5-14)	4 (2-10)	7 (4-15)	10.5 (6-14)	7 (3-15)
Sex, male	59.2 (103)	53.2 (133)	47.1 (49)	61.0 (250)	47.8 (11)	48.7 (112)	50.8 (266)	56.6 (69)	54.1 (993)
Smokers	41.4 (72)	50.4 (126)	44.2 (46)	47.1 (193)	17.4 (4)	24.7 (56)	16.3 (83)	—	34.2 (560)
Arterial hypertension	70.7 (123)	55.2 (138)	61.5 (64)	65.4 (268)	43.5 (10)	56.5 (130)	58.0 (296)	75.4 (92)	61.5 (1,121)
Diabetes mellitus	29.9 (52)	11.7 (29)	14.4 (15)	27.1 (111)	0 (0)	13.5 (31)	27.3 (139)	33.6 (41)	23.0 (418
Dyslipidemia	25.9 (45)	23.2 (57)	23.1 (24)	27.1 (111)	0 (0)	38.1 (86)	25.1 (128)	—	26.6 (451)
Atrial fibrillation	—	0 (0)	28.8 (30)	49.5 (203)	0 (0)	28.4 (65)	25.1 (128)	45.9 (56)	29.2 (482)
Previous stroke	—	20.4 (51)	19.2 (20)	26.8 (110)	0 (0)	27.8 (64)	21.0 (108)	—	23.0 (353
TOAST		—			—	—		—	
Atherothrombotic	15.7 (26)		40.4 (42)	6.8 (28)			21.2 (103)		17.1 (199
Cardioembolic	44.6 (74)		30.8 (32)	52.0 (213)			38.1 (185)		43.2 (504)
Lacunar	16.9 (28)		28.8 (30)	12.2 (50)			19.8 (96)		17.5 (204)
Undetermined	18.1 (30)		0 (0)	21.2 (87)			21.0 (102)		18.8 (219
Death	10.9 (19)	28.8 (72)	14.4 (15)	7.1 (29)	4.3 (1)	13.2 (30)	8.6 (45)	18.9 (23)	12.8 (234)

Abbreviations: IPD = individual participant data; NIHSS = NIH Stroke Scale; TOAST = Trial of Org 10172 in Acute Stroke Treatment.
Data represent median (interquartile range) for NIHSS score and age. All other variables represented as percentage (sample size). — = information not available. Only ischemic stroke patients were considered. Sample size of each cohort was given. Articles sharing cohorts were considered together in a unique database to avoid double counting of subjects.

Furthermore, for those patients who died, we explored BNP/NT-proBNP blood levels regarding the time that passed from stroke to death and no statistical difference was found (data not shown). The association with mortality was subanalyzed considering ischemic stroke etiologies. BNP/NT-proBNP levels were higher in cardioembolic strokes but only when alive patients were considered; we found differences in circulating levels between dead and alive patients in cardioembolic etiology, but also in lacunar (for BNP) and atherothrombotic (for NT-proBNP) strokes (figure e-1). Time of sample collection did not affect the association of BNP or NT-proBNP level with mortality (figure e-2).

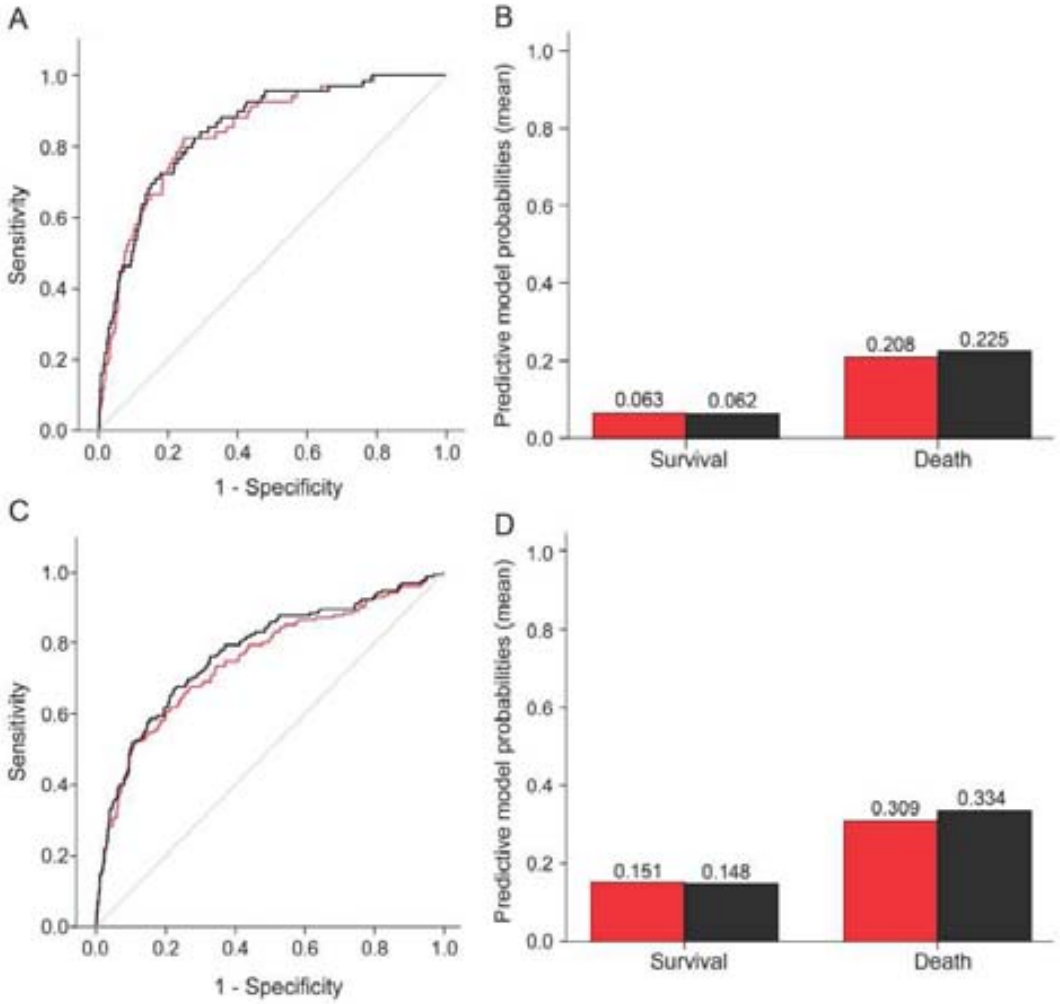
We found similar results when nonnormalized data were used, for both predictive models (in which the highest quartile corresponds to BNP >216.40 pg/mL and NT-proBNP >1,453.45 pg/mL) and subanalyses (data not shown).

DISCUSSION Our meta-analyses confirmed the association of BNPs with mortality after stroke, independent of age, sex, and baseline neurologic impairment. However, although this association was statistically significant, these biomarkers did not lead to better prediction of death than clinical information alone.

The design used in this study has allowed us to perform a thorough analysis. Our literature-based meta-analysis performed after systematic review, with close to 3,500 patients, gave the statistical power needed to estimate whether a real association exists between BNP/NT-proBNP and death. Our results confirmed the presence of higher circulating BNP/NT-proBNP levels in those patients who died after a cerebrovascular event.[8,9,30] However, we found moderate quality of reporting (7 of 15 points), high heterogeneity among included studies (94.5%), and evidence of publication bias. Given this, the results of the literature-based meta-analysis should be carefully interpreted. Publication of negative studies has generally less acceptation and impact, and perhaps the lack of published negative studies in the field of biomarkers affects the results of meta-analyses; the publication of negative results may contribute first to give a more realistic point of view and second to save resources for other researchers.

A strong point in our design was the IPD analysis. We considered several covariates at patient level, being the least biased and most reliable means of addressing questions not resolved in independent studies.[31] We performed IPD analysis following a one-stage approach[32] (using the compiled database as a unique cohort). The IPD analysis showed both BNP and NT-proBNP as independent predictors of mortality after stroke, even after adjustment by typical confounders (such as NIHSS score, sex, and age). To have BNP/NT-proBNP levels in the highest quartile

Figure 3 Graphical comparison of predictive models for mortality



Comparison of the predictive model with only clinical variables (NIHSS score at admission, age, sex) and the model also including BNP (A, B) or NT-proBNP (C, D). Biomarkers were included in the models using the cutoff points above the fourth quartile after data normalization. (A, C) Receiver operating characteristic curves for both clinical data model only (red line) and model including biomarker (black line), which are not distinguishable. (B, D) Bars indicating the mean of probabilities of death for each predictive model for survival and death groups (graphical representation of integrated discrimination improvement index). Addition of biomarkers (black bars) to clinical models (red bars) does not help to detect false positives (does not reduce the probability of death in the survival group) or true positives (does not increase the probability of death in the death group). BNP = B-type natriuretic peptide; NIHSS = NIH Stroke Scale; NT-proBNP = N-terminal fragment of BNP.

doubled the risk of death after ischemic stroke compared with other quartiles.

Furthermore, one-stage IPD allows the performance of more complete statistical analysis in large cohorts (more than 2,000 patients in our case). We detected differences between BNP and NT-proBNP levels: NT-proBNP showed almost 10-fold higher concentration. This contrast might be attributable mainly to differences in the metabolism of these 2 peptides, which implies different half-lives, the longer corresponding to NT-proBNP,[33] and thus it justifies the analysis of each molecule separately.

Our main interest was to assess whether these biomarkers could improve the information given by clinical information alone by applying different statistical metrics, such as IDI and NRI.[19] In our IPD analysis, only NT-proBNP showed a marginal additional value over clinical data. Based on these results, we would not recommend the use of BNPs alone at the bedside to predict stroke outcome. Because currently there is not consensus about how much a marker must add to clinical models in terms of discrimination and reclassification improvements, with our results, only NT-proBNP might be considered in a future panel of prognostic biomarkers, including other known (interleukin-6, copeptin, etc.) or still unknown blood outcome biomarkers, in which each biomarker potentially increases discrimination and reclassification, reaching outstanding accuracy.

Recently, the possibility has been suggested that the prognostic value of natriuretic peptides is dependent on stroke etiology.[28] We detected higher levels of both BNP and NT-proBNP in cardioembolic strokes only when alive patients were considered; moreover, circulating BNP and NT-proBNP were not exclusively associated with mortality in cardioembolic, but also in other causes of stroke. In addition, recently, high levels of BNP have been associated with cardioembolic etiology and with infarct volume in stroke patients,[34] reinforcing the heart-brain link and the suggestion that cardiovascular factors, in particular cardiac failure, adversely influence acute stroke outcome.[35] In our meta-analyses, we have focused on the relationship between BNP/NT-proBNP and stroke mortality and their role as useful biomarkers; therefore, the potential role of BNP/NT-proBNP in the detection of cardioembolic etiology,[36] mainly to define cryptogenic strokes,[37] needs further exploration and we suggest following a similar approach to the one presented here.

We believe that the implementation of these types of statistical analyses together with the proposal of minimal quality request (like other guidelines stated for publication[16]) in biomarker studies would minimize the risk of bias and facilitate translation into clinical practice. Finding a way to predict poor outcome in those patients with stroke would provide information for patients and relatives, would help to evaluate the risk and benefits of acute treatments or inclusion into clinical trials, and would optimize the allocation into specialized stroke units.

Our meta-analysis presents some limitations. First, there were differences in methodology among the included articles, for both the method of analysis and time of blood collection, which could influence our results. Second, our systematic review focused on ischemic stroke, thus studies assessing mortality in hemorrhagic stroke or TIA were missed. Nevertheless, some of the included articles considered a mixed population, with ischemic as well as hemorrhagic strokes and/or TIA, which could not be separated in our literature-based meta-analysis but were excluded in the IPD. Third, for subanalysis of mortality by stroke etiology, our conclusions should be carefully interpreted because of limited sample size: stroke etiology classification by TOAST (Trial of Org 10172 in Acute Stroke Treatment) was available in only half of the patients in the IPD analysis, including 278 patients with NT-proBNP measurement and 934 for BNP.

Finally, our IPD analysis, although considering several covariates, does not include some pathologies related to increased levels of BNP/NT-proBNP such as cardiopathies different from atrial fibrillation[38] or renal disease.[39] In addition, we could not access data regarding specific causes of death after stroke, which could influence the level of association of BNP/NT-proBNP.

In conclusion, both our literature-based meta-analysis and IPD analysis reinforce the role of BNP/NT-proBNP as independent predictors of all-cause mortality after stroke. However, these natriuretic peptides add minor predictive value to clinical information for the prediction of death. Their role in the clinical identification of an ischemic stroke of cardioembolic origin is uncertain and needs more research.

Comment: Natriuretic peptides as predictive biomarkers of stroke outcome

B-type natriuretic peptide (BNP), along with an inactive N-terminal peptide fragment (NT-proBNP), is secreted by cardiac ventricular myocytes in response to excessive myocardial stretching. The plasma half-life of these peptides is 0.3 and 2.0 hours, respectively, so a single measurement mostly reflects recent cardiac stress that may, for example, reflect sympathetic stimulation in response to acute stroke. Results from a variety of clinical studies indicate that these peptides may be useful as biomarkers for a variety of both cardiac and cerebrovascular events. In a carefully performed meta-analysis, García-Berrocoso and colleagues[1] evaluate the relationship of BNP and NT-proBNP levels with mortality following acute stroke. Although individuals in the highest quartile for either of the 2 peptides had twice the risk of death compared to the lower quartiles, only the NT-proBNP measures added slightly (additional 8.1% of patients) to the typical clinical measures for predicting stroke mortality, including age, sex, and NIH Stroke Scale score. The results do not go far enough to establish a relationship between the cause of death and the highest natriuretic levels. Some suggestive data from the ARISTOTLE trial indicate that cardiac death has an important role, but more careful studies that focus on the usefulness of measuring BNP or NT-proBNP within the first 24 hours after acute stroke, along with an analysis of the explicit causes of death, are needed.[2]

1. García-Berrocoso T, Giralt D, Bustamante A, et al. B-type natriuretic peptides and mortality after stroke: a systematic review and meta-analysis. Neurology 2013;81:1976–1985.
2. Hijazi Z, Wallentin L, Siegbahn A, et al. N-terminal pro-B-type natriuretic peptide for risk assessment in patients with atrial fibrillation: insights from the ARISTOTLE Trial (Apixaban for the Prevention of Stroke in Subjects with Atrial Fibrillation). J Am Coll Cardiol 2013;61:2274–2284.

Bruce M. Coull, MD

From the University of Arizona Health Science Center, Tucson.

Study funding: No targeted funding reported.
Disclosure: B. Coull serves/has served on scientific advisory boards for NIH, NINDS, Astra Zeneca, Neurobiological Technologies, and Boehringer-Ingelheim; and receives research support from NIH/NINDS. Go to Neurology.org for full disclosures.

AUTHOR CONTRIBUTIONS

Study concept and design (T.G.-B., D.G., W.N.W., J.M.), analysis and interpretation of data (T.G.-B., D.G., A.B.), drafting and revising the manuscript (T.G.-B., D.G., A.B., T.E., J.K.J., J.C.S., K.S., A.S., W.N.W., X.C., J.M.), acquisition of data (T.E., J.K.J., J.C.S., K.S., A.S., W.N.W., X.C., J.M.), statistical analyses (T.G.-B., D.G.), study supervision or coordination (T.G.-B., J.M.). T.G.-B. and J.M. have full access to all of the data and take full responsibility for the data, the analyses, and interpretation. All authors reviewed and approved the final report.

ACKNOWLEDGMENT

The authors are grateful to Natalia Rost, MD, MPH (Boston, MA), Emina Hajdinjak, MD (Maribor, Slovenia), and Pedro J. Modrego (Zaragoza, Spain) for their kind comments during protocol preparation.

STUDY FUNDING

No targeted funding reported.

DISCLOSURE

T. García-Berrocoso is supported by a predoctoral fellowship (FI09/00017) from the Instituto de Salud Carlos III. Neurovascular Research Laboratory takes part in the Spanish stroke research network INVICTUS (RD12/0014/0005) and is supported on stroke biomarkers research by FIS 11/0176. D. Giralt, A. Bustamante, T. Ergen, J. Jensen, J. Sharma, K. Shibazaki, A. Saritas, and X. Chen report no disclosures. W. Whiteley was supported by the Chief Scientist's Office (CAF/06/30) and is now funded by a UK Medical Research Council Clinician Scientist Fellowship (G0902303). J. Montaner reports no disclosures. Go to Neurology.org for full disclosures.

Received March 21, 2013. Accepted in final form July 29, 2013.

REFERENCES

1. Saposnik G, Kapral MK, Liu Y, et al. IScore: a risk score to predict death early after hospitalization for an acute ischemic stroke. Circulation 2011;123:739–749.
2. O'Donnell MJ, Fang J, D'Uva C, et al. The PLAN Score: a bedside prediction rule for death and severe disability following acute ischemic stroke. Arch Intern Med 2012; 172:1548–1556.
3. Whiteley W, Chong WL, Sengupta A, Sandercock P. Blood markers for the prognosis of ischemic stroke: a systematic review. Stroke 2009;40:e380–e389.
4. Whiteley W, Jackson C, Lewis S, et al. Inflammatory markers and poor outcome after stroke: a prospective cohort study and systematic review of interleukin-6. PLoS Med 2009;6:e1000145.
5. deLemos JA, McGuire DK, Drazner MH. B-type natriuretic peptide in cardiovascular disease. Lancet 2003;362:316–322.
6. Levin ER, Gardner DG, Samson WK. Natriuretic peptides. N Engl J Med 1998;339:321–328.
7. Mäkikallio AM, Mäkikallio TH, Korpelainen JT, et al. Natriuretic peptides and mortality after stroke. Stroke 2005;36:1016–1020.
8. Jensen JK, Mickley H, Bak S, Korsholm L, Kristensen SR. Serial measurements of N-terminal pro-brain natriuretic peptide after acute ischemic stroke. Cerebrovasc Dis 2006;22:439–444.
9. Shibazaki K, Kimura K, Okada Y, et al. Plasma brain natriuretic peptide as an independent predictor of in-hospital mortality after acute ischemic stroke. Intern Med 2009;48:1601–1606.
10. Whiteley W, Wardlaw J, Dennis M, et al. The use of blood biomarkers to predict poor outcome after acute transient ischemic attack or ischemic stroke. Stroke 2012;43:86–91.
11. Montaner J, García-Berrocoso T, Mendioroz M, et al. Brain natriuretic peptide is associated with worsening and mortality in acute stroke patients but adds no prognostic value to clinical predictors of outcome. Cerebrovasc Dis 2012;34:240–245.
12. Januzzi JL Jr, Maisel AS, Silver M, Xue Y, DeFilippi C. Natriuretic peptide testing for predicting adverse events following heart failure hospitalization. Congest Heart Fail 2012;18:S9–S13.
13. Coutance G, Le Page O, Lo T, Hamon M. Prognostic value of brain natriuretic peptide in acute pulmonary embolism. Crit Care 2008;12:R109.
14. García-Berrocoso T, Giralt D, Bustamante A, Montaner J. B-type natriuretic peptides (BNP and NT-proBNP) blood levels as biomarkers for all-cause mortality after stroke: a systematic review, meta-analysis and pooled-data analysis. PROSPERO 2012:CRD42012003284. Available at: http://www.crd.york.ac.uk/PROSPERO/display_record.asp?ID=CRD42012003284#.Umou6HDWMil. Accessed October 2013.
15. Moher D, Liberati A, Tetzlaff J, Altman DG; PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. PLoS Med 2009;6:e1000097.
16. Duval S, Tweedie R. Trim and fill: a simple funnel-plot-based method of testing and adjusting for publication bias in meta-analysis. Biometrics 2000;56:455–463.
17. Efron B, Tibshirani R. An introduction to the bootstrap. In: Cox DR, Hinkley DV, Reid N, Rubin DB, Silverman BW, editors. Monographs on Statistics and Applied Probability. New York: Chapman & Hall/CRC; 1993.
18. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics 1988;44:837–845.
19. Pencina MJ, D'Agostino RB, D'Agostino RB, Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. Stat Med 2008;27:157–172.
20. Etgen T, Baum H, Sander K, Sander D. Cardiac troponins and N-terminal pro-brain natriuretic peptide in acute ischemic stroke do not relate to clinical prognosis. Stroke 2005;36:270–275.
21. Sharma JC, Ananda K, Ross I, Hill R, Vassallo M. N-terminal probrain natriuretic peptide levels predict short-term poststroke survival. J Stroke Cerebrovasc Dis 2006;15:121–127.
22. Koenig MA, Puttgen HA, Prabhakaran V, Reich D, Stevens RD. B-type natriuretic peptide as a marker for heart failure in patients with acute stroke. Intensive Care Med 2007;33:1587–1593.
23. Modrego PJ, Boned B, Berlanga JJ, Serrano M. Plasmatic B-type natriuretic peptide and C-reactive protein in hyperacute stroke as markers of CT-evidence of brain edema. Int J Med Sci 2008;5:18–23.
24. Idris I, Hill R, Ross I, Sharma JC. N-terminal probrain natriuretic peptide predicts 1-year mortality following acute stroke: possible evidence of occult cardiac dysfunction among patients with acute stroke. Age Ageing 2010; 39:752–755.
25. Cakir Z, Saritas A, Emet M, Aslan S, Akoz A, Gundogdu F. A prospective study of brain natriuretic peptide levels in three subgroups: stroke with hypertension, stroke without hypertension, and hypertension alone. Ann Indian Acad Neurol 2010;13:47–51.
26. Üstündağ M, Orak M, Güloğlu C, Öztürk E, Tamam Y, Kale E. The role of serum ferritin, pro-brain natriuretic peptide and homocysteine levels in determining ischaemic stroke subtype, severity and mortality. J Emerg Med 2010; 17:13–21.
27. Shibazaki K, Kimura K, Iguchi Y, Aoki J, Sakai K, Kobayashi K. Plasma brain natriuretic peptide predicts death during hospitalization in acute ischaemic stroke and transient ischaemic attack patients with atrial fibrillation. Eur J Neurol 2011;18:165–169.
28. Rost N, Biffi A, Cloonan L, et al. Brain natriuretic peptide predicts functional outcome in ischemic stroke. Stroke 2012;43:441–445.
29. Hajdinjak E, Klemen P, Grmec Š. Prognostic value of a single prehospital measurement of N-terminal pro-brain natriuretic peptide and troponin T after acute ischaemic stroke. J Int Med Res 2012;40:768–776.

30. Chen X, Zhan X, Chen M, et al. The prognostic value of combined NT-pro-BNP levels and NIHSS scores in patients with acute ischemic stroke. Intern Med 2012;51:2887–2892.
31. Stewart LA, Parmar MKB. Meta-analysis of the literature or of individual patient data: is there a difference? Lancet 1993;341:418–422.
32. Riley RD, Lambert PC, Abo-Zaid G. Meta-analysis of individual participant data: rationale, conduct, and reporting. BMJ 2010;340:c221.
33. McCullough PA, Omland T, Maisel AS. B-type natriuretic peptides: a diagnostic breakthrough for clinicians. Rev Cardiovasc Med 2003;4:72–80.
34. Kim SH, Lee JY, Park SH, et al. Plasma B-type natriuretic peptide level in patients with acute cerebral infarction according to infarction subtype and infarction volume. Int J Med Sci 2013;10:103–109.
35. Sharma JC, Ross I, Vassallo M. Cardio-protection in acute stroke. Int J Stroke 2007;2:288–290.
36. Montaner J, Perea-Gainza M, Delgado P, et al. Etiologic diagnosis of ischemic stroke subtypes with plasma biomarkers. Stroke 2008;39:2280–2287.
37. Santamarina E, Penalba A, García-Berrocoso T, et al. Biomarker level improves the diagnosis of embolic source in ischemic stroke of unknown origin. J Neurol 2012;259:2538–2545.
38. Vuolteenaho O, Ala-Kopsala M, Ruskoaho H. BNP as a biomarker in heart disease. Adv Clin Chem 2005;40:1–36.
39. Srisawasdi P, Vanavanan S, Charoenpunichkit C, Kroll MH. The effect of renal dysfunction on BNP, NT-proBNP, and their ratio. Am J Clin Pathol 2010;133:14–23.

B-type natriuretic peptides and mortality after stroke: A systematic review and meta-analysis

Teresa García-Berrocoso, Dolors Giralt, Alejandro Bustamante, Thorleif Etgen, Jesper Jensen, Jagdish Sharma, Kensaku Shibazaki, Ayhan Saritas, Xingyong Chen, William Whiteley, Joan Montaner.

Supplemental files

Appendices

Appendix e-1. Electronic search strategy.

1. cerebrovascular disorders/ or basal ganglia cerebrovascular disease/ or exp brain ischemia/ or carotid artery diseases/ or carotid artery thrombosis/ or carotid stenosis/ or cerebrovascular accident/ or exp brain infarction/ or exp hypoxia-ischemia, brain/ or exp intracranial arterial diseases/ or exp "intracranial embolism and thrombosis"/
2. ((brain or cerebr$ or cerebell$ or vertebrobasil$ or hemispher$ or intracran$ or intracerebral or infratentorial or supratentorial or middle cerebr$ or mca$ or anterior circulation) adj5 (isch?emi$ or infarct$ or thrombo$ or emboli$ or occlus$ or hypoxi$)).tw.
3. (isch?emi$ adj6 (stroke$ or apoplex$ or cerebral vasc$ or cerebrovasc$ or cva or attack$)).tw.
4. 1 or 2 or 3
5. brain natriuretic peptide.mp. or exp Natriuretic Peptide, Brain/
6. b-type natriuretic peptide.mp.
7. (brain natriuretic peptide or nesiritide or b-type natriuretic peptide or bnp gene product or bnp-32 or brain natriuretic peptide-32 or natrecor or natriuretic factor-32 or natriuretic peptide type-b or type-b natriuretic peptide or ventricular natriuretic peptide, b-type).mp. [mp=title, abstract, original title, name of substance word, subject heading word, protocol supplementary concept, rare disease supplementary concept, unique identifier]
8. NT-pro BNP.mp.
9. 5 or 6 or 7 or 8
10. Incidence/ or exp mortality/ or follow up studies/ or mortality/ or prognos$.tw. or predict$.tw. or course.tw. or rankin.tw. or Glasgow outcome scale.tw. or NIHSS.tw.
11. (prognos$ or outcome$ or follow-up or predict$).tw,sh.
12. exp Prognosis/
13. Disease Progression/
14. ((clinical or natural$ or disease$) adj (progress$ or course$ or histor$)).tw,sh.
15. Time Factors/
16. 10 or 11 or 12 or 13 or 14 or 15
17. 4 and 9 and 16

Appendix e-2. Data collected for systematic review and meta-analysis.

- PubMed unique identifier (PMID).
- First author, year and journal of publication.
- Biological material (e.g. plasma, serum) and time from stroke symptoms onset to sample collection.
- Biomarker being analyzed (i.e. BNP or NT-proBNP) and method of analysis.
- When used, cut-off point for biomarker, with sensitivity and specificity percentages.
- Time of death assessment and mortality rate.
- Mean values and standard deviation (SD) for neurological scale NIHSS scores at admission and age. Male sex rate.
- Total sample size and sample size for both death and survival groups.
- Mean values and SD for biomarker in both death and survival groups.

Items included in our standardized template form to be completed from articles selected for meta-analysis.
NIHSS: National Institutes of Health Stroke Scale.

Appendix e-3. Quality score (adapted from QUADAS[e1] and Quality[e2] questionnaires).

1. Diagnosis of stroke is based on expert clinical opinion supported by neuroimaging.
2. All patients (including controls, in case) have expert opinion + neuroimaging.
3. The biomarker is not used to determine the end-point.
4. Specify that biomarker measurement is blinded to clinical data.
5. Specify that clinical data collection is blinded or collected before biomarker measurement.
6. All patients who entered into study complete it or withdrawals are explained.
7. Biomarker cut-off is previously established, based on literature or pilot study.
8. Specify any or none disclosure.
9. Report if the study is prospective.
10. Definition of time period, follow-up end and median follow-up time of the study (at least two items).
11. Clinical end-points to be measured are defined prior to analysis.
12. Rationale for used sample size.

13. Reporting a list of candidate variables and estimated effect (Odd Ratio/Hazard Ratio) with 95% CI for all variables in multivariate analysis.
14. Specify the assay method and provide or reference a detailed protocol.
15. Cases are not representing a selected subgroup of patients (e.g. only atrial fibrillation patients).

Appendix e-4. Individual participants' information compiled for IPD analysis.

- BNP/NT-proBNP blood levels and blood collection time.
- Death/survival and time from onset to death assessment.
- Age, sex, NIHSS and glucose blood levels at admission.
- Type of stroke, TOAST etiological classification.
- Presence/absence of several risk factors for stroke: hypertension, diabetes mellitus, dyslipidemia, atrial fibrillation, smoking, alcohol intake, previous stroke.
- Presence/absence of thrombolytic treatment (rt-PA) administration.

Items requested to corresponding authors from articles included in meta-analysis.
NIHSS: National Institutes of Health Stroke Scale; TOAST: trial of ORG 10172 in acute stroke treatment; rt-PA: recombinant tissue Plasminogen Activator.
All information was gathered in an Excel database for each anonymized individual patient included in each original published article. Finally, data from all articles was compiled in a single whole database, including a study identifier (first author and year of publication). When articles from the same research group shared some patients from the same cohort [e3, e4] or the whole cohort was followed up any longer, [e5, e6] after authors' agreement, both studies were considered together as a unique cohort in the whole database to avoid double counting of subjects. The provided data remain entirely under the principal investigator property and was only used for this analysis.
In order to have more consistency, some variables were transformed when needed:
- All BNP/NT-proBNP values were given as pg/mL. To convert NT-proBNP levels expressed in pmol/L to pg/mL, values were multiplied by 8.457.
- Time from symptoms onset to blood sample collection was given as hours.
- Time from onset to death assessment was given as days.
- Neurological severity was assessed by NIHSS. When SSS (Scandinavian Stroke Scale) was used, scores were transformed to NIHSS by the formula:
NIHSS = 25.68-0.43*SSS.[e7]

Tables

Table e-1. Quality score report for each included study.

Ref.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Quality score
e8	+	+	+	-	-	+	+	+	+	-	+	-	+	+	+	11
e9	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	12
e10	+	+	+	-	+	-	-	+	+	+	+	-	-	+	+	10
e6	+	+	+	-	-	+	-	-	+	+	+	-	+	+	+	10
e11	?	?	+	-	-	+	+	-	-	-	+	-	+	-	-	5
e12	+	+	+	-	-	+	?	+	-	?	+	-	-	+	+	8
e4	+	+	+	-	-	-	-	-	+	+	+	-	+	+	+	9
e5	+	+	+	+	-	-	-	+	-	-	+	-	-	+	+	8
e13	?	?	+	-	-	+	?	+	+	-	+	-	-	+	+	7
e14	+	+	+	+	-	+	?	-	-	+	+	-	+	+	+	10
e3	+	+	+	-	-	-	-	-	+	+	+	-	+	+	-	8
e15	+	+	+	+	+	-	?	+	+	+	+	-	+	+	+	12
e16	+	+	+	-	+	-	?	+	+	+	+	-	+	+	+	11
e17	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	13
e18	?	?	+	-	-	+	-	+	+	?	?	-	+	+	+	7
e19	+	-	+	-	-	-	-	+	-	-	+	-	+	+	+	7

1-15 referred to items evaluated for quality assessment (see Appendix 2). +: yes; -: no; ?: insufficient information.

Only positive answers accounted for quality score (i.e. negative answers to items did not penalize).

Table e-2. Additive predictive value of BNP/NT-proBNP over clinical variables for ischemic stroke mortality.

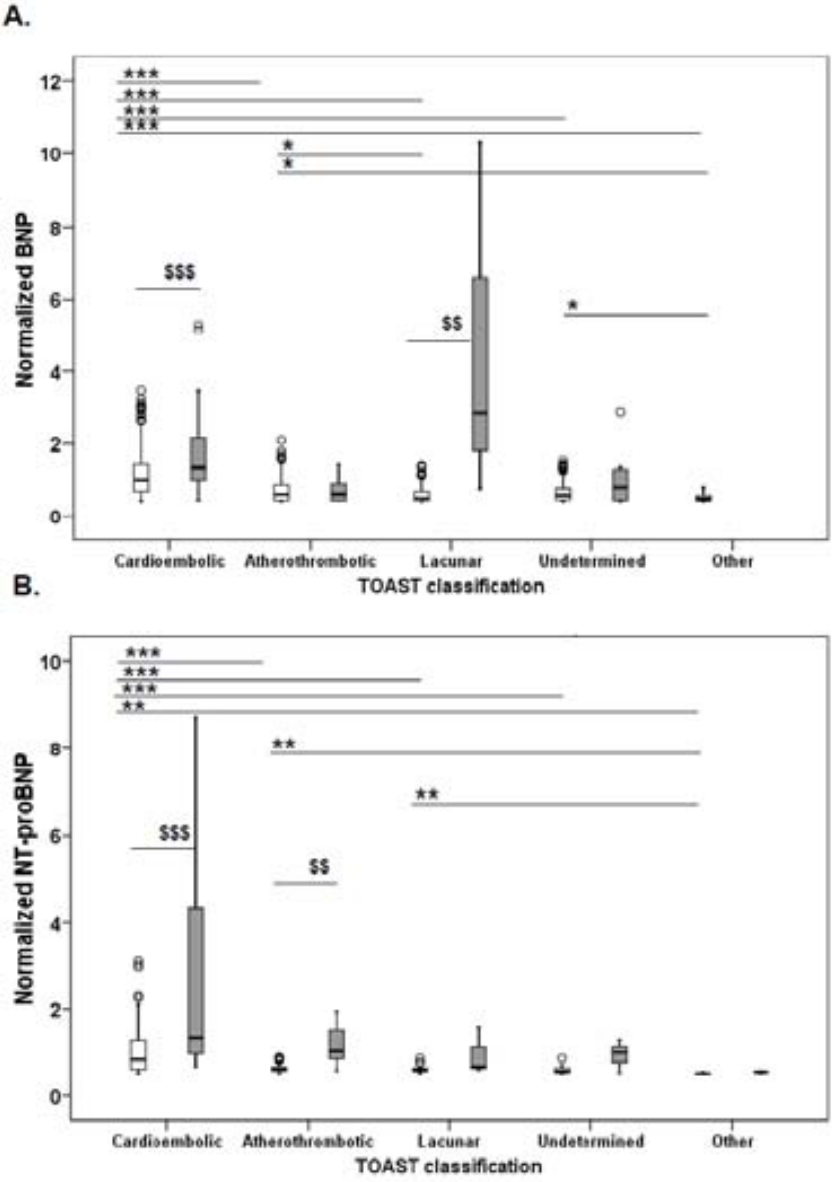
		Model - All-cause mortality			
		Only clinical	**Clinical + BNP**	**Only clinical**	**Clinical + NT-proBNP**
Logistic regression	NIHSS admission	1.17 (1.13-1.22), p<0.001	1.17 (1.12-1.21), p<0.001	1.11 (1.08-1.13), p<0.001	1.10 (1.07-1.13), p<0.001
	Age	1.03 (1.01-1.06), p=0.015	1.03 (1.00-1.05), p=0.076	1.05 (1.03-1.07), p<0.001	1.04 (1.02-1.06), p<0.001
	BNP	-	2.30 (1.32-4.01), p=0.003	-	-
	NT-proBNP	-	-	-	2.63 (1.75-3.94), p<0.001
Categorical NRI	NRI events	-	-1.4%	-	-1.9%
	NRI non-events	-	0.6%	-	10.0%
	NRI	-	-0.9% (-7.4-5.7)	-	8.1% (2.8-13.3)
	p-value	Ref.	0.794	Ref.	0.003
IDI statistics	IDI events	-	0.017	-	0.024
	IDI non-events	-	0.001	-	0.003
	IDI	-	0.018 (0.003-0.034)	-	0.028 (0.013-0.043)
	p-value	Ref.	0.020	Ref.	<0.001
ROC curves	AUC	0.840 (0.815-0.863)	0.848 (0.823-0.870)	0.752 (0.722-0.781)	0.774 (0.745-0.802)
	p-value	Ref.	0.285	Ref.	0.029

Logistic regression models gave odd-ratios (OR) adjusted by gender with 95% CI and p-value for each included variable. Biomarkers were added to clinical logistic regression model using highest quartile cut-off point. Bootstrapping gives 95% CI for OR of BNP (1.35-4.29) and NT-proBNP (1.79-4.04), which increases consistency to our regression results.

NRI: net reclassification improvement index (risk categories used: ≤10%, 10-90% and >90%); percentage of reclassification given for both events (i.e. patients who died) and non-events and for the sum of both (with 95% CI) when biomarker was added to clinical predictive model. IDI: integrated discrimination improvement index; index given for both events and non-events and for the sum of both (with 95% CI). AUC: area under ROC Curve; area with 95%CI given for each model. Clinical model always used as reference model to compare. NIHSS: National Institutes Health Stroke Scale.

Figures

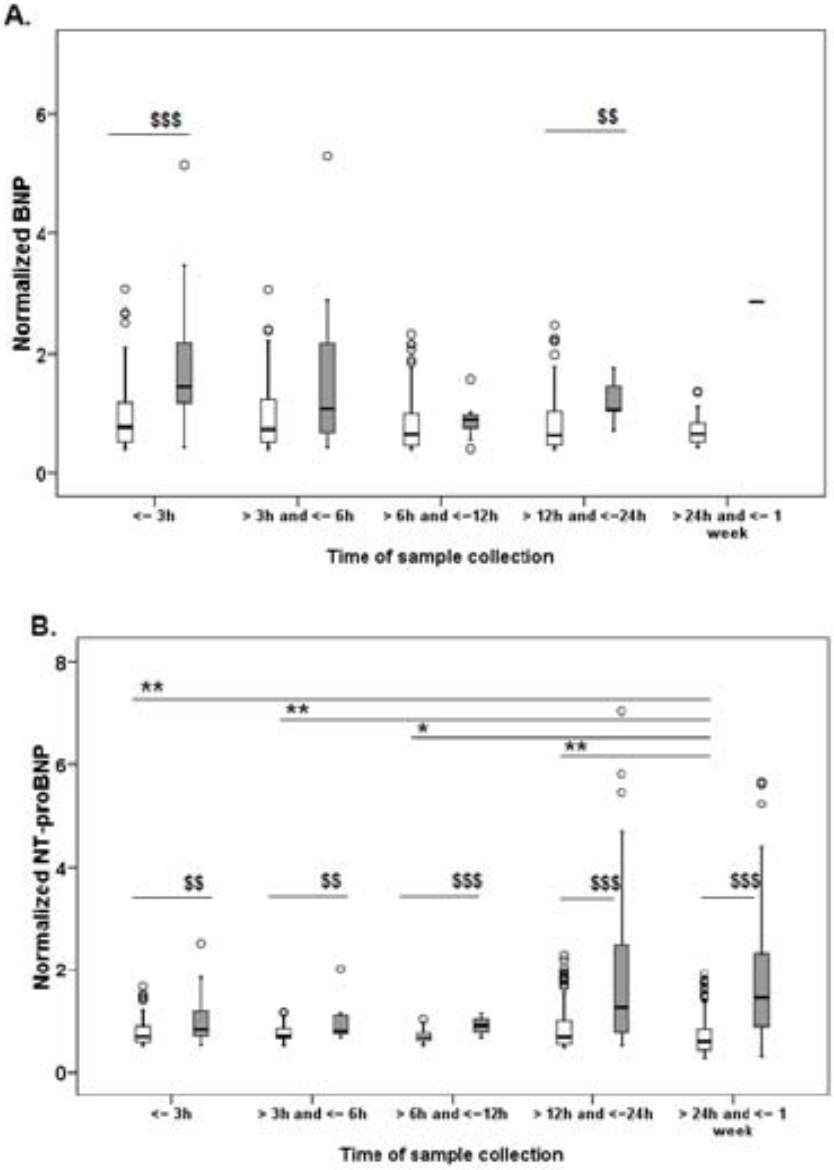
Figure e-1. Mortality association of BNP/NT-proBNP depending on ischemic stroke etiologies.



Median and interquartile range for normalized BNP (A) and NT-proBNP (B) in alive (white boxes) and dead (grey boxes) patients, depending on TOAST etiology.

* p<0.05; ** p<0.01; *** p<0.001 (among etiologies; alive patients); $ p<0.05; $$ p<0.01; $$$ p<0.001 (between alive and dead patients). All p-values given after Bonferroni correction. There were not differences among etiologies for dead patients.

Figure e-2. Mortality association of BNP/NT-proBNP depending on time of sample collection.



Median and interquartile range for normalized BNP (A) and NT-proBNP (B) in alive (white boxes) and dead (grey boxes) patients, depending on time of sample collection.

* p<0.05; ** p<0.01; *** p<0.001 (among times; alive patients); $ p<0.05; $$ p<0.01; $$$ p<0.001 (between alive and dead patients). All p-values given after Bonferroni correction. There were not differences among times for dead patients.

e-References

e1. Whiteley W, Tseng MC, Sandercock P. Blood biomarkers in the diagnosis of ischemic stroke. A systematic review. Stroke 2008;39:2902-2909.

e2. Whiteley W, Chong WL, Sengupta A, Sandercock P. Blood markers for the prognosis of ischemic stroke: a systematic review. Stroke 2009;40:e380-e389.

e3. Shibazaki K, Kimura K, Iguchi Y, Aoki J, Sakai K, Kobayashi K. Plasma brain natriuretic peptide predicts death during hospitalization in acute ischaemic stroke and transient ischaemic attack patients with atrial fibrillation. Eur J Neurol 2011;18:165-169.

e4. Shibazaki K, Kimura K, Okada Y, et al. Plasma brain natriuretic peptide as an independent predictor of in-hospital mortality after acute ischemic stroke. Inter Med 2009;48:1601-1606.

e5. Idris I, Hill R, Ross I, Sharma JC. N-terminal probrain natriuretic peptide predicts 1-year mortality following acute stroke: possible evidence of occult cardiac dysfunction among patients with acute stroke. Age Ageing 2010;39:752-755.

e6. Sharma JC, Ananda K, Ross I, Hill R, Vassallo M. N-terminal probrain natriuretic peptide levels predict short-term poststroke survival. J Stroke Cerebrovasc Dis 2006;15:121-127.

e7. Gray LJ, Ali M, Lyden PD, Bath PMW. Interconversion of the National Institutes of Health Stroke Scale and Scandinavian Stroke Scale in acute stroke. J Stroke Cerebrovasc Dis 2009;18:466-468.

e8. Etgen T, Baum H, Sander K, Sander D. Cardiac troponins and N-terminal pro-brain natriuretic peptide in acute ischemic stroke do not relate to clinical prognosis. Stroke 2005;36:270-275.

e9. Mäkikallio AM, Mäkikallio TH, Korpelainen JT, et al. Natriuretic peptides and mortality after stroke. Stroke 2005;36:1016-1020.

e10. Jensen JK, Mickley H, Bak S, Korsholm L, Kristensen SR. Serial measurements of N-terminal pro-brain natriuretic peptide after acute ischemic stroke. Cerebrovasc Dis 2006;22:439-444.

e11. Koenig MA, Puttgen HA, Prabhakaran V, Reich D, Stevens RD. B-type natriuretic peptide as a marker for heart failure in patients with acute stroke. Intensive Care Med 2007;33:1587-1593.

e12. Modrego PJ, Boned B, Berlanga JJ, Serrano M. Plasmatic b-type natriuretic peptide and C-reactive protein in hyperacute stroke as markers of Ct-evidence of brain edema. Int J Med Sci 2008;5:18-23.
e13. Cakir Z, Saritas A, Emet M, Aslan S, Akoz A, Gundogdu F. A prospective study of brain natriuretic peptide levels in three subgroups: stroke with hypertension, stroke without hypertension, and hypertension alone. Ann Indian Acad Neurol 2010;13:47-51.
e14. Üstündağ M, Orak M, Güloğlu C, Öztürk E, Tamam Y, Kale E. The role of serum ferritin, pro-brain natriuretic peptide and homocysteine levels in determining ischaemic stroke subtype, severity and mortality. Hong Kong J Emerg Med 2010;17:13-21.
e15. Whiteley W, Wardlaw J, Dennis M, et al. The use of blood biomarkers to predict poor outcome after acute transient ischemic attack or ischemic stroke. Stroke 2012;43:86-91.
e16. Rost N, Biffi A, Cloonan L, et al. Brain natriuretic peptide predicts functional outcome in ischemic stroke. Stroke 2012;43:441-445.
e17. Hajdinjak E, Klemen P, Grmec Š. Prognostic value of a single prehospital measurement of N-terminal pro-brain natriuretic peptide and troponin T after acute ischaemic stroke. J Int Med Res 2012;40;768-776.
e18. Montaner J, García-Berrocoso T, Mendioroz M, et al. Brain natriuretic peptide is associated with worsening and mortality in acute stroke patients but adds no prognostic value to clinical predictors of outcome. Cerebrovasc Dis 2012;34:240-245.
e19. Chen X, Zhan X, Chen M, et al. The prognostic value of combined NT-pro-BNP levels and NIHSS scores in patients with acute ischemic stroke. Intern Med 2012;51:2887-2892.

3.3 From brain to blood: New biomarkers for ischemic stroke prognosis

(*Journal of Proteomics, 2013, 94, 138-148*)

JOURNAL OF PROTEOMICS 94 (2013) 138–148

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot

From brain to blood: New biomarkers for ischemic stroke prognosis

Teresa García-Berrocoso[a], Anna Penalba[a], Cristina Boada[a], Dolors Giralt[a], Eloy Cuadrado[a,1], Núria Colomé[b], Loïc Dayon[c,2], Francesc Canals[b], Jean-Charles Sanchez[c], Anna Rosell[a], Joan Montaner[a,*]

[a]Neurovascular Research Laboratory, Vall d'Hebron Institute of Research (VHIR), Universitat Autònoma de Barcelona, Barcelona, Spain
[b]Proteomics Laboratory, Vall d'Hebron Institute of Oncology (VHIO), Universitat Autònoma de Barcelona, Barcelona, Spain
[c]Translational Biomarker Group, Human Protein Science Department, University of Geneva, Geneva, Switzerland

ARTICLE INFO

Article history:
Received 12 August 2013
Accepted 14 September 2013
Available online 20 September 2013

Keywords:
Orbitrap
Stroke
Brain
Biomarker
Prognosis
Mortality

ABSTRACT

Despite being ischemic stroke a leading cause of death and functional disability, there are no other accurate tools to predict outcome of patients beyond clinical variables such as age and stroke severity. In this scenario, defining protein changes associated with acute ischemic brain damage might help to identify new biomarker candidates for stroke prognosis. By means of mass spectrometry-based proteomics, we identified 51 proteins which levels were altered in the infarcted area of the human brain after stroke. Among 8 selected protein candidates, circulating levels of gelsolin, dihydropyrimidinase-related protein 2 and cystatin A were independent predictors of poor outcome. Logistic regression models including these innovative biomarkers significantly improved the predictive value with respect to the only use of clinical variables in both discrimination and reclassification analyses. Our results indicate that early blood determination of these three biomarkers might predict outcome of patients and might help in decision-making processes related to ischemic stroke management.

Biological significance
Circulating levels of gelsolin, dihydropyrimidinase-related protein 2 and cystatin A, proteins found altered in human brain after cerebral ischemia, demonstrate potential usefulness as biomarkers for long-term stroke prognosis.

1. Introduction

Ischemic stroke is the second cause of death and a major cause of disability worldwide [1]. The prediction of this fatal outcome could help in several decision-making processes, such as the early admission of patients to specialized stroke units, where an optimal care can be provided. Moreover in these units, patients might receive prophylactic antibiotic treatment or be included in clinical trials oriented to neuroprotection or neurorepair strategies.

Several clinical scores have been proposed to predict both functional outcome [2,3] and mortality [4,5]. Unfortunately, those have moderate sensitivity. In this scenario, the search for blood biomarkers for stroke prognosis is becoming increasingly

* Corresponding author.
E-mail address: 31862jmv@comb.es (J. Montaner).
[1] Current address: Netherlands Institute for Neuroscience, Department of Astrocyte Biology & Neurodegeneration, Amsterdam, The Netherlands.
[2] Current address: Molecular Biomarkers Core, Nestlé Institute of Health Sciences, Lausanne, Switzerland.

1874-3919/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jprot.2013.09.005

popular (reviewed in [6]) and in some cases, such as copeptin [7], blood biomarkers started to show some added value.

In this study, we aimed to discover new molecules associated with ischemic brain damage, broadening our previous proteomic approach carried out by our group using brain tissue from deceased stroke patients [8]. From the list of proteins that were altered after stroke, some candidates were further evaluated for their potential role in improving the prediction of outcome by using comparative statistical metrics. Three of our candidates demonstrated their potential use as long-term prognostic biomarkers.

2. Materials and methods

2.1. Sample collection

2.1.1. Brain tissue samples

Brain sample preparation and demographic data were previously published [8]. Briefly, tissue samples from ischemic ipsilateral (infarct core and peri-infarct area) and healthy contralateral hemispheres of 3 ischemic stroke patients were obtained within the first 6 h after death. Cortical brain areas from 3 patients who died because of non-inflammatory and non-neurological diseases were used as controls. All samples were snap frozen in liquid nitrogen and immediately stored at −80 °C until homogenization.

2.1.2. Blood samples

Consecutive patients with an acute ischemic stroke admitted to the emergency department of the Vall d'Hebron Hospital (Barcelona, Spain) within the first 4.5 h after symptoms onset, were recruited from February 2007 to September 2009. Stroke onset was defined as the last time the patient was known to be asymptomatic. All patients received thrombolytic treatment.

All 60 patients included in this study underwent a standardized protocol of clinical and neuroradiological assessments as previously described by our group [9]. Stroke severity was assessed by using the National Institutes of Health stroke scale (NIHSS) score [10]. Functional outcome was evaluated at third month after the event at the outpatient clinic by means of the modified Rankin Scale (mRS) by a trained neurologist; patients with a mRS score from 0 to 1 were categorized as "good outcome" and patients with mRS from 2 to 6 as "poor outcome". Further analysis includes all cause in-hospital death as endpoint.

Peripheral blood samples were drawn at admission before any treatment was given. Plasma or sera were immediately separated by centrifugation at 3500 rpm for 15 min at 4 °C and stored at −80 °C.

2.1.3. Ethics statement

The local ethical committee of our institution approved both studies (human brain tissue and blood sampling), and written consent was obtained from all subjects or relatives in accordance with the Declaration of Helsinki.

2.2. Proteomics analysis

2.2.1. 2D-DIGE

Sample preparation for 2D-DIGE was previously published [8]. Briefly, cyanine stained brain tissue homogenates from each area were fractionated by 2-D gel electrophoresis. Fluorescence images of each gel were analyzed and relative quantification of protein abundance was performed. For those proteins differentially expressed between brain areas, gel spots were excised and in-gel digestion was performed using trypsin.

2.2.2. Protein identification by MALDI-TOF/TOF MS

Identification of the proteins was carried out as described in detail in our previous article [8]. Briefly, MALDI MS analysis of tryptic peptides was performed on an Ultraflex TOF–TOF Instrument (Bruker, Bremen, Germany). Peak lists derived from the MS spectra were used for peptide mass fingerprint protein identification. TOF/TOF fragmentation spectra of selected precursor ions were used for MS/MS ion search to corroborate identifications. Mascot algorithm (Matrix Sciences, London, UK) was used to search the MSDB database for human proteins. Criterion for positive identification was a significant Mascot probability score (score >64; $p < 0.05$).

2.2.3. Protein identification by RP-LC ESI LTQ-OT MS

Twenty of the unidentified protein spots that were not identified by MALDI-TOF/TOF MS were analyzed with RP-LC ESI MS using a LTQ-OT XL (Thermo Electron, San Jose, CA, USA) equipped with a NanoAcquity system from Waters (Mildford, MA, USA). Tryptic peptides were trapped on a home-made 5 μm 200 Å Magic C18 AQ (Bruker-Michrom, Auburn, CA, USA) 100 μm × 20 mm pre-column and separated on a home-made 5 μm 100 Å Magic C18 AQ (Bruker-Michrom) 75 μm × 150 mm column with a gravity-pulled emitter. The analytical separation was run for 30 min using a gradient of water/formic acid 99.9%/0.1% (solvent A) and acetonitrile/formic acid 99.9%/0.1% (solvent B). The gradient was run as follows: 0–1 min 95% A and 5% B, then to 65% A and 35% B at 25 min, and 20% A and 80% B at 30 min at a flow rate of 220 nL/min. For MS survey scans, the OT resolution was set to 60,000 and the ion population was set to 5×10^5 with an m/z window from 400 to 2000. Five precursor ions were selected for CID in the LTQ. For this, the ion population was set to 1×10^4 (isolation width of 2 m/z). The normalized collision energies were set to 35% for CID.

Protein identification peak lists were generated from raw data using the embedded software from the instrument vendor (extract_MSN.exe). The monoisotopic masses of the selected precursor ions were corrected using an in-house written Perl script [11]. The corrected mgf files were searched against UniProtSP database (v56.9 of 03-Mar-2009) using Mascot (version 2.2.2). *Homo sapiens* taxonomy was specified for database searching. The parent ion tolerance was set to 10 ppm and fragment mass tolerance was 0.6 Da. Variable amino acid modifications were oxidized methionine. Carbamidomethylation of cysteines was set as a fixed modification. Trypsin was selected as the enzyme, with one potential missed cleavage. Significant threshold and ions score cut-off were 0.05. For all analyses, only proteins matching at least two different peptide sequences were kept.

2.3. Verification with immunoassays

A replication study of the selected candidates was performed by ELISA commercial kits in blood samples, either plasma or serum.

Brain glycogen phosphorylase (PYGB), dihydropyrimidinase-related protein 2 (DRP2) and gelsolin (GELS) ELISAs were from Cusabio Biotech Co. (Newark, DE, USA). Rho GDP-dissociation inhibitor-alpha (GDIR1), septin-5 (SEPT5), calcyclin-binding protein (CYBP), contactin-1 (CNTN1) and cystatin A (CYTA) ELISA kits were purchased from USCN Life Science Inc. (Wuhan, P.R. China). All ELISAs were performed according to the manufacturer's instructions by trained technicians blinded to clinical details.

Each sample was assayed twice and the mean value of both measurements was used. All samples with intra-assay CV higher than 20% were excluded from the analysis. The mean inter-assay CV was lower than 30% for all proteins assessed.

2.4. Statistical analysis

Whole analysis was performed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA), unless contrary was stated. Those variables normally distributed (Kolmogorov–Smirnov test with $p > 0.05$) were analyzed by Student's t test or ANOVA; mean and SD values were given. For variables with non-normal distribution Mann–Whitney U or Kruskal–Wallis test were used; median and interquartile range were reported. Differences among categorical variables were assessed by Pearson chi-squared test.

A Classification Tree (CRT; Gini method) analysis was used to find cut-off points for each individual biomarker. The minimum number of patients used for the nodes was established at 15 for parent nodes and 5 for terminal nodes.

Forward stepwise multivariate logistic regression models for both third month poor outcome and in-hospital mortality were constructed with all clinical variables associated with the end-point at $p < 0.1$. Odds ratio (OR) and 95% confidence interval (CI) were adjusted by clinical variables that are known to be related to stroke prognosis (i.e. age, NIHSS at admission and gender, when needed) [2,5,12]. Using cut-off points, baseline levels of individual biomarkers or the combination of them were added to the correspondent clinical model to find a new predictive model.

The AUC from models with individual or combined biomarkers plus clinical variables were compared with AUC from only clinical model by DeLong's method [13] using MedCalc 12.3 software (MedCalc Software, Ostend, Belgium). Using R software (Hmisc and PredictABEL packages), net reclassification improvement (NRI) and integrated discrimination improvement (IDI) indexes were calculated to assess the added value of the biomarker or combination of biomarkers to the clinical models to predict outcome [14,15]. In the case of NRI test, pre-specified clinically relevant thresholds of predicted risk (<10% and >90% risk) were used to calculate the reclassification of patients into risk outcome groups when biomarkers were added to the model [16].

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

3. Results

3.1. Differential brain protein profile

A summarized workflow chart of the whole study is shown in Fig. 1. Brain homogenates obtained from the contralateral hemisphere, peri-infarct and infarct core from 3 stroke patients and 3 controls were used to create a 2-D protein map. On average, 1442 ± 231 protein spots were detected in the gels and 132 spots displayed at least a 1.5-fold change in their relative expression between the different brain tissue areas. As previously published by our group, of these, 42 spots (corresponding to 39 unique proteins) were successfully identified by MALDI-TOF/TOF MS [8].

New proteomic technologies have opened new possibilities to identify undetected peptides [17]. By using state-of-the-art RP-LC coupled to an ESI LTQ-OT MS, which presents higher accuracy and resolution, we were able to identify 20 previously unidentified protein spots. These 20 spots corresponded to 12 new unique proteins (Table 1). From this list, 5 proteins were increased in the infarct core (fibrinogen gamma chain, FIBG; GELS; CYTA; immunoglobulin gamma chain, IGHG1; CNTN1) and 7 were decreased in the infarct core (citrate synthase, CISY; glutathione S-transferase Mu2, GSTM2; alpha-aminoadipic semialdehyde dehydrogenase, AL7A1; phosphoserine aminotransferase, SERC; CYBP; L-lactate dehydrogenase A chain, LDHA; alcohol dehydrogenase, ADH) (Fig. S1). Altogether, 51 proteins were recognized as differently expressed among the different brain areas.

3.2. Potential blood biomarkers

To identify potential blood biomarkers, 8 candidates were selected from different functional categories based on their potential scientific relevance and the availability of commercial immunoassays at that moment: GELS, CYTA, CNTN1 and CYBP (from the 12 new identified proteins) and SEPT5, PYGB, GDIR1 and DRP2 (from the list of 39 previously identified proteins, [8]).

The candidate proteins were analyzed in blood samples from 60 ischemic stroke patients that were followed until the third month after the ischemic event. The median age was 77 years (65.5–81), 50% were male and, in average, they had a moderate neurological impairment (NIHSS score at admission was 14.7 ± 5.9).

From the 8 candidates, PYGB, SEPT5 and CYBP were non-detectable either in plasma or in serum with the used immunoassays. Finally, plasma samples were used to assay GELS and CYTA and, on the other hand, GDIR1, DRP2 and CNTN1 were performed in serum samples.

Additionally, we explored factors that could influence blood levels of our biomarker candidates. In patients who suffered a previous stroke, GDIR1 and DRP2 were elevated and GELS was reduced ($p = 0.002$, $p = 0.004$ and $p = 0.021$, respectively). Besides CYTA was elevated in hypertensive patients ($p = 0.032$) (Table S1).

3.3. Long-term outcome

Forty-three out of the 60 patients (71.7%) showed a poor functional outcome three months after the ischemic event. These patients were older ($p < 0.001$), had higher mRS and NIHSS scores at admission ($p = 0.063$ and $p = 0.006$, respectively) and had higher occurrence of diabetes mellitus ($p = 0.024$) (Table 2).

With regard to biomarkers, patients presenting poor outcome had higher baseline concentration of GELS (20.7 vs.

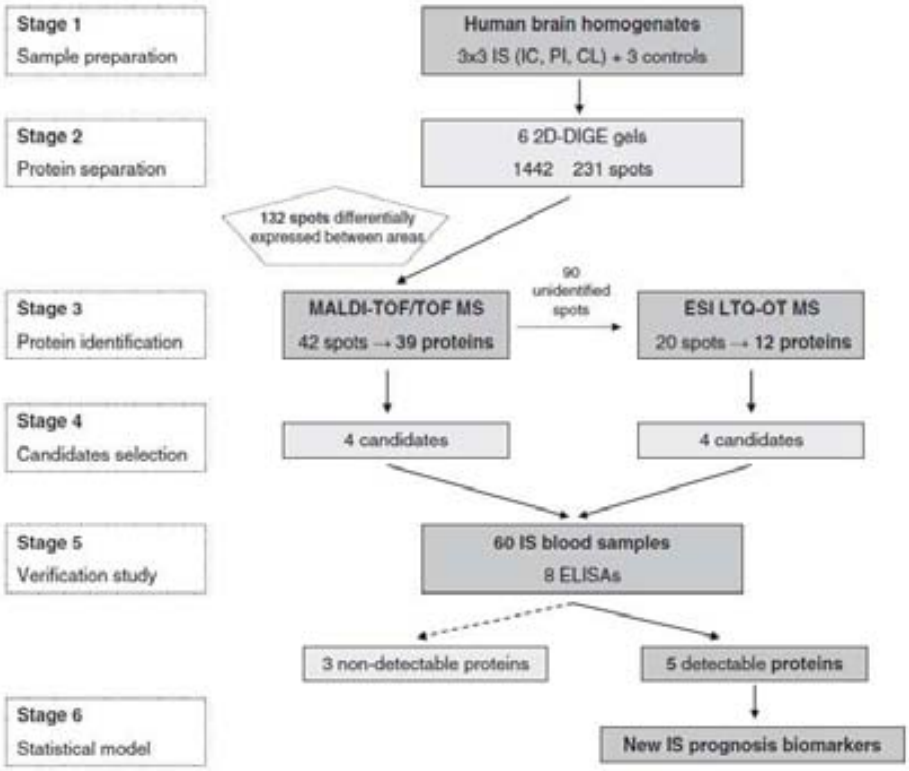


Fig. 1 – Workflow chart. Schematic description of the different stages of our study. IS: ischemic stroke patients; IC: infarct core brain area; PI: peri-infarct brain area; and CL: healthy contralateral brain area.

Table 1 – Human brain identified proteins. List of identified proteins from human brain homogenates by ESI LTQ-OT mass spectrometry of differential 2D-DIGE spots.

Spot no.	Protein name	Accession no.	MW (Da)	pI	Mascot score	Peptides
Blood coagulation						
1074	Fibrinogen gamma chain (FIBG)	P02679	52,106	5.37	716	23
Carbohydrate metabolism						
1272	Citrate synthase (CISY), mitochondrial	O75390	51,908	8.45	567	19
Cell adhesion molecules						
104	Contactin-1 (CNTN1)	Q12860	114,104	5.62	548	16
Cytoskeleton						
292	Gelsolin (GELS)	P06396	86,043	5.9	284	8
Glycolysis						
1668	L-lactate dehydrogenase A chain (LDHA)	P00338	36,950	8.44	181	8
Immunity						
1027	Ig gamma chain C region (IGHG1)	P01857	36,596	8.46	455	11
Oxidoreductase						
2162	Alcohol dehydrogenase (ADH)	P00325	36,892	6.32	433	16
1023	Alpha-aminoadipic semialdehyde dehydrogenase (AL7A1)	P49419	55,845	6.44	461	11
Proteinase inhibitor						
1746	Cystatin A (CYTA)	P01040	11,000	5.38	284	7
Transferase						
1957	Glutathione S-transferase Mu2 (GSTM2)	P28161	25,899	6	240	10
1441	Phosphoserine aminotransferase (SERC)	Q9Y617	40,796	7.56	180	4
Ubiquitination						
1844	Calcyclin-binding protein (CYBP)	Q9HB71	26,308	8.28	267	15

Spot No.: Protein gel spot numbers corresponding to the 2D-DIGE (8). Proteins are listed and grouped according to functional classification. Accession no.: Accession number in Uni-Prot/Swiss-Prot. MW: Theoretical molecular weight in Da. pI: Theoretical isoelectrical point. Peptides: Number of peptides matching to the identified protein.

18.2 ng/mL, p = 0.042) and CYTA (26.9 vs. 22.6 ng/mL p = 0.052) and lower DRP2 (0.48 vs. 1.26 ng/mL, p = 0.073) than those patients with a better long-term prognosis (Table S1). We determined individual cut-off points associated with long-term poor outcome for each biomarker: GELS 19.87 ng/mL (71.8% sensitivity, 76.5% specificity), CYTA 31.76 ng/mL (26.8% sensitivity, 100% specificity) and DRP2 2.09 ng/mL (97.1% sensitivity, 28.6% specificity) (Table 2).

We also performed a multivariate logistic regression analysis of the data. The significant variables were adjusted for NIHSS score at admission and gender. With the logistic regression model we confirmed diabetes mellitus and age as independent predictors for poor outcome at third month. When baseline GELS >19.87 ng/mL or DRP2 <2.09 ng/mL were added to this clinical model, these biomarkers remained independent predictors of poor outcome. Moreover, the combination of both GELS and DRP2 or the combination of them with CYTA >31.76 ng/mL were also independent predictors of poor outcome (Table 3).

The addition of the biomarkers to the predictive model gradually improved the AUC of the clinical data from 0.867 to 0.957 (p = 0.091). Furthermore, the use of the three-biomarker combination significantly enhanced the discrimination of the model (measured by IDI index) up to a 28% when compared to clinical model only (Fig. 2A). The combination of GELS, DRP2 and CYTA also allowed a better classification of the patients into predicted risk categories (by NRI analysis) with an 85.7% reclassification compared to clinical variables (Fig. 2B, Table 3).

3.4. *In-hospital mortality*

Those patients who died as a consequence of stroke (n = 8, 13.3%) had higher NIHSS scores at admission (p = 0.002) and had suffered more frequently a previous stroke (p = 0.065) (Table 2).

Regarding our candidate biomarkers, only CYTA was elevated in those patients who suffered a fatal stroke (32.3 vs. 24.6 ng/mL, p = 0.112) (Table S1). We identified a cut-off point of 34.35 ng/mL for CYTA (62.5% sensibility, 96% specificity) that was significantly associated with in-hospital mortality (Table 2).

The multivariate logistic regression model, adjusted by age and gender, confirmed NIHSS score on admission and history of a previous stroke as independent predictors of early death. When baseline CYTA >34.35 ng/mL was added to the clinical model, it remained also as an independent predictor (Table 4).

The predictive model including CYTA improved the AUC of the clinical data from 0.877 to 0.970 (p = 0.048). Moreover, CYTA enhanced the discrimination of the model by 34% (measured by IDI index) (Fig. 2C) and reclassifying correctly a 39% of patients into the appropriate predictive risk categories (by NRI analysis) (Fig. 2D, Table 4).

4. Discussion

The technical combination of 2D-DIGE with both MALDI-TOF/TOF MS and RP-LC LTQ-OT MS platforms has allowed us to describe the proteome changes in the human brain after stroke. In a previous work, we recognized changes in 39 proteins but 90 differentially expressed spots remained unknown [8]. In the present study, we have identified 20 of those unidentified spots that correspond to 12 new proteins.

These 12 proteins have been identified for the first time in the brain of patients who died because of an ischemic stroke. They are involved in different cellular processes, such cell metabolism, for which CISY, LDHA, ADH and AL7A1 enzymes showed lower levels within the infarcted area. Furthermore, processes related with neurorepair were also altered as evidenced by changes in proteins involved in regulation of axonal growth (CNTN1 [18,19]) or neuronal remodeling (SERC [20]), mainly by regulating cytoskeletal organization (CYBP [21,22]).

Apart from these intracellular proteins, we have also found increased levels of FIBG within the infarct core; this blood protein can reach the brain parenchyma by leakage of the blood–brain barrier following ischemia, where FIBG might bind and activate microglia cells leading to inflammation and axonal damage [23]. Furthermore, high circulating levels of FIBG have been associated with ischemic stroke [24], having higher levels in those patients with poor outcome [25]. This finding is a good example of the crosstalk between the brain and the bloodstream after ischemic stroke.

Three out of the 8 selected candidate molecules were not found in our collection of blood samples from stroke patients. To the best of our knowledge no other studies have reported PYGB, SEPT5 and CYBP presence in the bloodstream after cerebral ischemia.

We have profoundly explored the role of the 5 proteins that were present in blood as potential prognostic biomarkers for stroke. GDIR1, which participates in vesicle trafficking, was found down-expressed in ischemic areas maybe supporting the idea of an increased exocytosis after brain ischemia [8]. Circulating levels of GDIR1 were lower in those patients with poor outcome at third month after stroke, although the association was not sufficiently strong to consider it as a biomarker. On the other hand, we have found higher levels of CNTN1 in human ischemic brain areas. The increase of this brain-specific adhesion molecule might be reflecting a process of neurite outgrowth and neurorecovery after ischemia [26]. Despite the fact that we detected CNTN1 in the blood samples of patients, no association was found with stroke outcome. Further exploration would be needed to assess its potential role as a diagnostic biomarker.

Interestingly, we have found three molecules associated with poor stroke outcome: GELS, DRP2 and CYTA. GELS is expressed in both cytoplasmic and secreted forms. The main function of GELS is to bind actin in a calcium-dependent manner in order to regulate the dynamics of actin polymerization within the cell (cytoplasmic form) or to scavenge actin leaking into the bloodstream after tissue injury (plasmatic form). In addition, other roles for GELS have been described, such as inflammation and apoptosis modulation [27]. In this regard we observed increased GELS within the brain of stroke patients. Cytoplasmic GELS is mainly located into neuronal growth cones, where actin instability is related to axon remodeling processes [28]. Furthermore, GELS can be cleaved by caspases [27] and metalloproteinases [29], two families

Table 2 – Univariate analysis. Clinical characteristics and factors associated with long-term outcome (mRS at third month after stroke) and in-hospital mortality.

Factors	Good outcome (N = 17)	Poor outcome (N = 43)	p-value	Survivor (N = 52)	Exitus (N = 8)	p-value
Age median (IQR)	64 (61–71)	78 (75–82)	**<0.001**	76.5 (63–81)	77.5 (77–82)	0.231
NIHSS at admission mean ± SD	11.4 ± 5.2	16.0 ± 5.7	**0.006**	13.8 ± 5.4	20.5 ± 5.8	**0.002**
Previous mRS median (IQR)	0 (0–0)	0 (0–1)	0.063*	0 (0–0)	0 (0–1)	0.481
Glucose at admission (mg/dL) mean ± SD	139.2 ± 40.9	149.2 ± 58.3	0.570	150.9 ± 57.0	122.5 ± 27.7	0.175
Minutes to t-PA treatment mean ± SD	191.2 ± 58.1	189.4 ± 69.8	0.925	193.0 ± 63.3	169.4 ± 84.7	0.351
Gender (male) % (n)	52.9 (9)	48.8 (21)	0.774	50.0 (26)	50.0 (4)	1.000
Smokers % (n)	17.6 (3)	7.1 (3)	0.341	11.8 (6)	0 (0)	0.583
Arterial hypertension % (n)	58.8 (10)	76.7 (33)	0.209	71.2 (37)	75.0 (6)	1.000
Diabetes mellitus %(n)	5.9 (1)	37.2 (16)	**0.024**	30.8 (16)	12.5 (1)	0.420
Dyslipidemia % (n)	41.2 (7)	39.5 (17)	0.907	42.3 (22)	25.0 (2)	0.457
Atrial fibrillation % (n)	17.6 (3)	37.2 (16)	0.142	30.8 (16)	37.5 (3)	0.699
Heart disease % (n)	23.5 (4)	23.3 (10)	1.000	25.0 (13)	12.5 (1)	0.667
Previous stroke % (n)	5.9 (1)	16.3 (7)	0.420	9.6 (5)	37.5 (3)	0.065*
TOAST	–	–	0.365	–	–	0.391
-Atherothrombotic % (n)	17.6 (3)	23.3 (10)	–	21.2 (11)	25.0 (2)	–
-Cardioembolic % (n)	35.3 (6)	48.8 (21)	–	42.3 (22)	62.5 (5)	–
-Undetermined % (n)	47.1 (8)	27.9 (12)	–	36.5 (19)	12.5 (1)	–
GELS >19.87 ng/mL % (n)	23.5 (4)	71.8 (28)	**0.001**	–	–	–
CYTA >31.76 ng/mL % (n)	0 (0)	26.8 (11)	**0.024**	–	–	–
DRP2 <2.09 ng/mL % (n)	71.4 (10)	97.1 (33)	**0.021**	–	–	–
CYTA>34.35 ng/mL % (n)	–	–	–	4.0 (2)	62.5 (5)	**<0.001**

mRS: modified Rankin Scale; t-PA: thrombolytic treatment; IQR: interquartile range; TOAST: etiology stroke subtype classification; NIHSS: National Institutes of Health stroke scale. Statistically significant differences between groups are expressed as bold p-values and statistical trend ($p < 0.1$) are marked with *.

of proteins up-regulated in stroke. These cleavages could increase the possibilities of detecting GELS fragments in the blood.

The fact that plasmatic and cytoplasmic GELS differ only by an additional sequence of 24 amino-acids in the amino-terminal part of the protein, makes nowadays impossible to distinguish both forms by means of immuno-based techniques [30]. Thus, the protein levels detected in blood samples could be representing either secreted plasmatic form or cytoplasmic form released by cell lysis after injury [31,32]. In our study, elevated circulating GELS levels have been found as independent predictors of long-term poor outcome after ischemic stroke, following adjustment by potential clinical confounders. In contrast, lower plasma levels of GELS have been found in patients with subarachnoid hemorrhage [33] and patients who died after traumatic brain injury [34], intracerebral hemorrhage [35] or ischemic stroke [36]. In all these studies GELS levels were several orders of magnitude higher than ours perhaps reflecting different isoforms identified depending on the detection assay.

Regarding DRP2, we found previously lower levels within the ischemic brain areas [8] and we have found herein lower circulating DRP2 levels independently associated with poor outcome at third month after stroke. DRP2 is mainly associated with the regulation of microtubule dynamics and endocytosis, in the context of axonogenesis and neuronal polarity [37,38]. In animal models of cerebral ischemia, DRP2 has been found both decreased [39] and increased [40] within ischemic area. These controversial results may be due to the existence of different isoforms, cleaved-forms and phosphorylation states of DRP2 [38]. In stroke patients, DRP2 has also been identified in cerebral microdialysate but never in cerebrospinal fluid [41,42]; to our knowledge, this is the first study showing that DRP2 is detectable in serum from stroke patients.

In addition to GELS and DRP2, three of our explored proteins are also related to neuritogenesis; a process where cytoskeletal dynamism is essential [43]. A hypothetical molecular pathway involving all these proteins (GELS, DRP2, GDIR1, CNTN1 and CYBP) and their potential relationship with neuritogenesis after ischemia is shown in Fig. S2. It is conceivable that changes in these important processes, neurogenesis and neurorepair, might influence the functional recovery after stroke, leading to worse outcomes in patients, as suggested by our findings.

Our last candidate was CYTA (or stefin A), a cytoplasmatic inhibitor which main function is to regulate cysteine proteinases involved in the lysosomal cell death pathway. Furthermore, when cysteine proteinases are released from lysosomes, they are able to degrade metalloproteinases and thus they are involved in extracellular matrix remodeling; as a consequence, an inhibitor as CYTA could stabilize and permit the activity of metalloproteinases, which are well-known players in ischemic stroke [44]. In former studies, CYTA was found in neutrophils [45] and was related to markers of neutrophil degranulation [46], which could be a source of metalloproteinases after thrombolytic treatment in ischemic stroke [47]. In cerebral ischemia, the external administration of CYTA has been associated with infarct volume reduction in an animal model [48]. In our study, we have found increased levels of CYTA within infarct areas, which could be a compensatory effect to the action of cysteine proteases after ischemia or maybe could reflect CYTA role in other pathological processes. Regarding circulating CYTA levels in our cohort of stroke patients, higher concentration predicts worse outcome.

Table 3 – Predictive models comparative for third month poor outcome.

		Model for long-term poor outcome (mRS 2–6)				
		Only clinical	Clinical + GELS	Clinical + DRP2	Clinical + combination GELS & DRP2	Clinical + combination GELS,DRP2 & CYTA
Logistic regression (OR adj)	Age	1.11 (1.0–1.2), **0.009**	1.10 (1.0–1.2), **0.019**	1.24 (1.1–1.4), **0.005**	1.24 (1.0–1.4), **0.010**	1.20 (1.0–1.4), **0.025**
	DM	9.34 (1.0–88.2), 0.051*	14.8 (1.2–186), **0.036**	51.5 (0.8–3470), 0.066*	167.0 (1.8–15825), **0.028**	219.9 (1.2–40602), **0.043**
	NIHSS admission	1.12 (1.0–1.3), 0.105	1.08 (0.9–1.2), 0.344	1.05 (0.9–1.2), 0.617	1.01 (0.8–1.2), 0.948	1.02 (0.8–1.2), 0.864
	Gender (female)	0.71 (0.2–3.1), 0.643	0.74 (0.1–3.9), 0.727	0.65 (0.1–5.2), 0.688	0.93 (0.1–8.4), 0.947	0.54 (0.0–8.1), 0.653
	GELS	–	8.9 (1.7–47.8), **0.010**	–	–	–
	DRP2	–	–	51.4 (1.7–1535.9), **0.023**	–	–
	GELS & DRP2	–	–	–	14.2 (2.1–98.1), **0.007**	–
	GELS, DRP2 & CYTA	–	–	–	–	31.2 (2.7–359.2), **0.006**
Categorical NRI	NRI events	–	7.1%	14.7%	14.3%	21.4%
	NRI non-events	–	14.3%	14.3%	50.0%	64.3%
	NRI	–	21.4% (−4.8–47.6)	29.0% (4–54.0)	64.3% (22.3–106.3)	85.7% (39.3–132.2)
	p-value	Ref.	0.109	**0.023**	**0.003**	**<0.001**
IDI statistics	IDI events	–	0.023	0.036	0.037	0.052
	IDI non-events	–	0.062	0.168	0.170	0.226
	IDI	–	0.085 (−0.01–0.18)	0.204 (0.07–0.34)	0.207 (0.08–0.33)	0.278 (0.13–0.43)
	p-value	Ref.	0.082*	**0.002**	**0.001**	**<0.001**
ROC curves	AUC	0.867 (0.755–0.941)	0.910 (0.802–0.970)	0.914 (0.797–0.975)	0.931 (0.812–0.985)	0.957 (0.845–0.995)
	p-value	Ref.	0.246	0.248	0.128	0.091*

For logistic regression models, OR_{adj} (95% CI) and p-values were given. Biomarkers were added to clinical logistic regression model using cut-off point. NRI: net reclassification improvement index (risk categories used: ≤10%, 10–90% and >90%); percentage of reclassification given for both events (i.e. patients at poor outcome group) and non-events and for the sum of both (with 95% CI). IDI: integrated discrimination improvement index; index given for both events and non-events and for the sum of both (with 95% CI). AUC: area with 95% CI given for each model. Clinical model always used as reference model. Statistically significant results expressed as bold p-values; * as statistical trend.

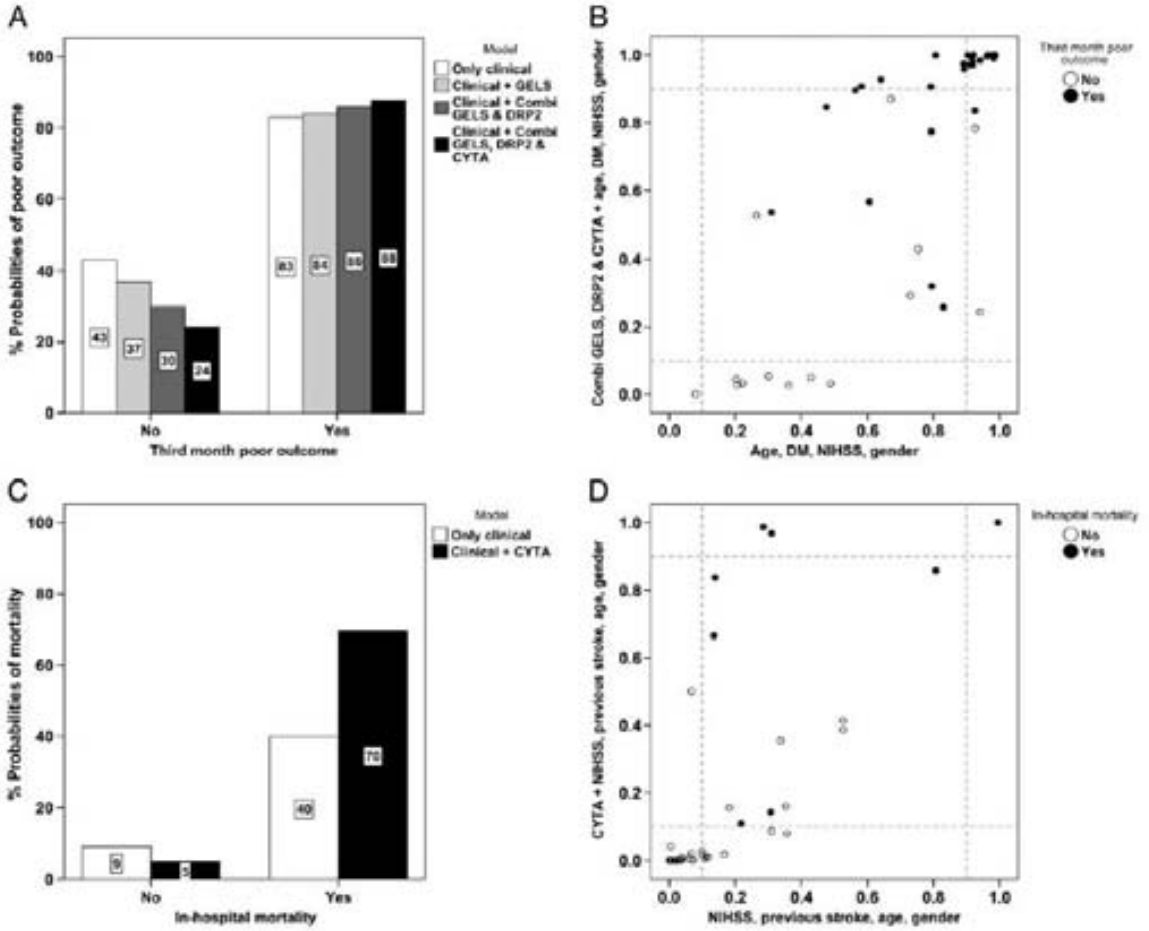


Fig. 2 – Predictive clinical and biological models. Graphical representation of IDI (A, C) and NRI (B, D) indexes for predictive models regarding third month poor outcome (A, B) and in-hospital mortality (C, D). (A, C) Bars show the mean percentage of probability for each predictive model of poor outcome (A) or in-hospital mortality (C). (B, D) Scatter dot graph showing logistic regression probabilities for poor outcome from only clinical model (x axis) and model with biomarkers (y axis). Discontinuous lines represent pre-specified risk categories (10% and 90%). In all cases, biomarkers were added to regression model using cut-off points. GELS: gelsolin; DRP2: dihydropyrimidinase-related protein 2; CYTA: cystatin A; DM: diabetes mellitus; NIHSS: National Institutes of Health stroke scale.

Finally, we have constructed a predictive model for long-term outcome after ischemic stroke including blood levels of GELS, DRP2 and CYTA. The three biomarkers improved discrimination and classified patients better than clinical variables alone, thus demonstrating their added value and their plausible use in clinical practice to predict stroke outcome.

Our results are limited by the small sample size used in both the discovery phase in brain tissue and in the verification phase with blood samples, although following the typical pipeline for biomarker development [49]. Nevertheless, our results, showing the same trend in both brain and blood, make our list of proteins a promising exploratory path for new biomarker candidates in the field of cerebral ischemia. If validated in a larger independent cohort also including non-thrombolyzed patients, our prognostic biomarker candidates (GELS, DRP2 and CYTA) might be included in a biomarkers' panel together with other good candidates, such as copeptin [7] or interleukin-6 [50]. With this type of panels, a quicker and easier assessment of prognosis could be done to help in the risk and benefit balance of treatment options. Withdrawing and withholding treatments are an ethical concern and, in this context, any predictive variable, either clinical factors or biomarkers, must be reliable enough to make decisions. Nowadays there is no consensus in how good a biomarker or biomarkers' panel should be to be good enough. Once ours or other candidates were validated as prognostic biomarkers with demonstrated added value, they should be evaluated in terms of the profit while accepting some risk due to false-positive or false-negative decisions. It is out of our purpose to determine here the minimal acceptable false rates, which needs a more global consensus.

Table 4 – Predictive models comparative for in-hospital mortality.

		Model for in-hospital mortality	
		Only clinical	Clinical + CYTA
Logistic regression (OR adj)	NIHSS	1.43 (1.1–1.9), **0.018**	1.91 (1.0–3.5), **0.040**
	Previous stroke	26.0 (1.3–512.4), **0.032**	187.8 (0.8–45514), 0.062[#]
	Age	1.05 (0.9–1.2), 0.532	1.19 (0.7–1.9), 0.463
	Gender (female)	2.19 (0.3–16.9), 0.453	1.28 (0.1–25.0), 0.871
	CYTA	–	316.3 (2.2–46067), **0.024**
Categorical NRI	NRI events	–	25%
	NRI non-events	–	14%
	NRI	–	39% (2.4–75.6)
	p-value	Ref.	**0.037**
IDI statistics	IDI events	–	0.297
	IDI non-events	–	0.039
	IDI	–	0.336 (0.068–0.603)
	p-value	Ref.	**0.014**
ROC curves	AUC	0.877 (0.767–0.948)	0.970 (0.888–0.997)
	p-value	Ref.	**0.048**

For logistic regression models, OR_{adj} (95% CI) and p-value were given. CYTA was added to clinical logistic regression model using cut-off point. NRI: net reclassification improvement index (risk categories used: ≤10%, 10–90% and >90%); percentage of reclassification given for both events (i.e. patients who died) and non-events and for the sum of both (with 95% CI). IDI: integrated discrimination improvement index; index given for both events and non-events and for the sum of both (with 95% CI). AUC: area with 95% CI given for each model. Clinical model used as reference model. Statistically significant results expressed as bold p-values; [#] as statistical trend.

In conclusion, our proteomic approach provided new insights into the pathophysiological pathways involved in human brain ischemia. Moreover some of the proteins altered by the ischemia showed up as good biomarker candidates for the prognosis of stroke and we demonstrated its added value to typical clinical marker.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.09.005.

Acknowledgments

We thank all study collaborators, residents, neurologists and nurses of the Stroke Unit and Neurology ward from the Vall d'Hebron University Hospital. Funding: T.G-B. is supported by a predoctoral fellowship (FI09/00017) and A.R. is a senior researcher of the Miguel Servet Program, both from the *Instituto de Salud Carlos III*. Neurovascular Research Laboratory takes part in the Spanish stroke research network *INVICTUS* (RD12/0014/0005) and is supported on stroke biomarker research by FIS 11/0176. The Proteomics Laboratory at VHIO belongs to the Spanish network of proteomics facilities ProteoRed-ISCIII. Any of the sponsors was involved either in the design, the analysis or the writing of this study. Author contributions: T.G-B. helped in performing the immunoassays, did the statistical analysis together with D.G. and wrote the article. A.P. and C.B. performed the immunoassays. E.C., N.C., L.D. and A.R. prepared the samples for mass spectrometry analysis and critically reviewed the article content. J.M. planned the whole project. F.C. J-C.S. and J.M. agreed the experimental design, supervised the experiments and critically reviewed the article content. All authors approved the final manuscript. Competing interests: T.G-B. and J.M. are inventors in the European patent EP12382411.2, which includes the biomarkers discovered in this study. All other authors declare that they have no competing interests.

REFERENCES

[1] Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, et al. American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics — 2012 update: a report from the American Heart Association. Circulation 2012;125:e2-220.

[2] Strbian D, Meretoja A, Ahlhelm FJ, Pitkäniemi J, Lyrer P, Kaste M, et al. Predicting outcome of IV thrombolysis-treated ischemic stroke patients: the DRAGON score. Neurology 2012;78:427–32.

[3] Ntaios G, Faouzi M, Ferrari J, Lang W, Vemmos K, Michel P. An integer-based score to predict functional outcome in acute ischemic stroke: the ASTRAL score. Neurology 2012;78:1916–22.

[4] O'Donnell MJ, Fang J, D'Uva C, Saposnik G, Gould L, McGrath E, et al. Investigators of the Registry of the Canadian Stroke Network. The PLAN score: a bedside prediction rule for death and severe disability following acute ischemic stroke. Arch Intern Med 2012;172:1548–56.

[5] Saposnik G, Kapral MK, Liu Y, Hall R, O'Donnell M, Raptis S, et al. Investigators of the Registry of the Canadian Stroke Network, Stroke Outcomes Research Canada (SORCan) Working Group. IScore: a risk score to predict death early after hospitalization for an acute ischemic stroke. Circulation 2011;123:739–49.

[6] Whiteley W, Chong WL, Sengupta A, Sandercock P. Blood markers for the prognosis of ischemic stroke: a systematic review. Stroke 2009;40:e380–9.
[7] De Marchis GM, Katan M, Weck A, Fluri F, Foerch C, Findling O, et al. Copeptin adds prognostic information after ischemic stroke: results from the CoRisk study. Neurology 2013;80:1278–86.
[8] Cuadrado E, Rosell A, Colomé N, Hernández-Guillamon M, García-Berrocoso T, Ribó M, et al. The proteome of human brain after ischemic stroke. J Neuropathol Exp Neurol 2010;69:1105–15.
[9] Mendioroz M, Fernández-Cadenas I, Rosell A, Delgado P, Domingues-Montanari S, Ribó M, et al. Osteopontin predicts long-term functional outcome among ischemic stroke patients. J Neurol 2011;258:486–93.
[10] Brott T, Bogousslavsky J. Treatment of acute ischemic stroke. N Engl J Med 2000;343:710–22.
[11] Scherl A, Tsai YS, Shaffer SA, Goodlett DR. Increasing information from shotgun proteomic data by accounting for misassigned precursor ion masses. Proteomics 2008;8:2791–7.
[12] König IR, Ziegler A, Bluhmki E, Hacke W, Bath PMW, Sacco RL, et al. Weimar C, on behalf of the Virtual International Stroke Trials Archive (VISTA) Investigators. Stroke 2008;39:1821–6.
[13] DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. Biometrics 1988;44:837–45.
[14] Pencina MJ, D'Agostino Sr RB, D'Agostino Jr RB, Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. Stat Med 2008;27:157–72.
[15] Pickering JW, Endre ZH. New metrics for assessing diagnostic potential of candidate biomarkers. Clin J Am Soc Nephrol 2012;7:1355–64.
[16] Whiteley W, Wardlaw J, Dennis M, Lowe G, Rumley A, Sattar N, et al. The use of blood biomarkers to predict poor outcome after acute transient ischemic attack or ischemic stroke. Stroke 2012;43:86–91.
[17] Scigelova M, Makarov A. Advances in bioanalytical LC-MS using the Orbitrap™ mass analyzer. Bioanalysis 2009;1:741–54.
[18] Grumet M. Nr-CAM: a cell adhesion molecule with ligand and receptor functions. Cell Tissue Res 1997;290:423–8.
[19] Mikami T, Yasunaga D, Kitagawa H. Contactin-1 is a functional receptor for neuroregulatory chondroitin sulfate-E. J Biol Chem 2009;284:4494–9.
[20] Tabatabaie L, Klomp LW, Berger R, de Koning TJ. L-Serine síntesis in the central nervous system: a review on serine deficiency disorders. Mol Genet Metab 2010;99:256–62.
[21] Schneider G, Nieznanski K, Jozwiak J, Slomnicki LP, Redowicz MJ, Filipek A. Tubulin binding protein, CacyBP/SIP, induces actin polymerization and may link actin and tubulin cytoskeletons. Biochim Biophys Acta 1803;2010:1308–17.
[22] Schneider G, Filipek A. S100A6 binding protein and Siah-1 interacting protein (CacyBP/SIP): spotlight on properties and cellular function. Amino Acids 2011;41:773–80.
[23] Davalos D, Ryu JK, Merlini M, Baeten KM, Le Moan N, Petersen MA, et al. Fibrinogen-induced perivascular microglial clustering is required for the development of axonal damage in neuroinflammation. Nat Commun Nat Commun 2012;3:1227.
[24] Jood K, Danielson J, Ladenvall C, Blomstrand C, Jern C. Fibrinogen gene variation and ischemic stroke. J Thromb Haemost 2008;6:897–904.
[25] del Zoppo GJ, Levy DE, Wasiewski WW, Pancioli AM, Demchuk AM, Trammel J, et al. Hyperfibrinogenemia and functional outcome from acute ischemic stroke. Stroke 2009;40:1687–91.
[26] Cho H, Shimazaki K, Takeuchi K, Kobayashi S, Watanabe K, Oguro K, et al. Biphasic changes in F3/contactin expression in the gerbil hippocampus after transient ischemia. Exp Brain Res 1998;122:227–34.
[27] Silacci P, Mazzolai L, Gauci C, Stergiopulos N, Yin HL, Hayoz D. Gelsolin superfamily proteins: key regulators of cellular functions. Cell Mol Life Sci 2004;61:2614–23.
[28] Endres M, Fink K, Zhu J, Stagliano NE, Bondada V, Geddes JW, et al. Neuroprotective effects of gelsolin during murine stroke. J Clin Invest 1999;103:347–54.
[29] Park SM, Hwang IK, Kim SY, Lee SJ, Park KS, Lee ST. Characterization of plasma gelsolin as a substrate for matrix metalloproteinases. Proteomics 2006;6:1192–9.
[30] Haverland N, Pottiez G, Wiederin J, Ciborowski P. Immunoreactivity of anti-gelsolin antibodies: implications for biomarker validation. J Transl Med 2010;8:137.
[31] Bucki R, Levental I, Kulakowska A, Janmey PA. Plasma gelsolin: function, prognostic value and potential therapeutic use. Curr Protein Pept Sci 2008;9:541–51.
[32] Pottiez G, Haverland N, Ciborowski P. Mass spectrometric characterization of gelsolin isoforms. Rapid Commun Mass Spectrom 2010;24:2620–4.
[33] Chou SHY, Lee PS, Konigsberg RG, Gallacci D, Chiou T, Arai K, et al. Plasma type gelsolin is decreased in human blood and cerebrospinal fluid after subarachnoid hemorrhage. Stroke 2011;42:3624–7.
[34] Xu JF, Liu WG, Dong XQ, Yang SB, Fan J. Change in plasma gelsolin level after traumatic brain injury. J Trauma 2010;72:491–6.
[35] Zhao DQ, Wang K, Zhang HD, Li YJ. Significant reduction of plasma gelsolin levels in patients with intracerebral hemorrhage. Clin Chim Acta 2013;415:202–6.
[36] Guo XC, Luo BY, Li XF, Yang DG, Zheng XN, Zhang K. Plasma gelsolin levels and 1-year mortality after first-ever ischemic stroke. J Crit Care 2011;26:608–12.
[37] Arimura N, Menager C, Fukata Y, Kaibuchi K. Role of CRMP-2 in neuronal polarity. J Neurobiol 2004;58:34–47.
[38] Hensley K, Venkova K, Christov A, Gunning W, Park J. Collapsin response mediator protein-2: an emerging pathologic feature and therapeutic target for neurodisease indications. Mol Neurobiol 2011;43:180–91.
[39] Chung MA, Lee JE, Lee JY, Ko MJ, Lee ST, Kim HJ. Alteration of collapsin response mediator protein-2 expression in focal ischemic rat brain. Neuroreport 2005;16:1647–53.
[40] Chen A, Liao WP, Lu Q, Wong WSF, Wong PTH. Upregulation of dihydropyrimidinase-related protein 2, spectrin α II chain, heat shock cognate protein 70 pseudogene 1 and tropomodulin 2 after focal cerebral ischemia in rats — a proteomics approach. Neurochem Int 2007;50:1078–86.
[41] Maurer MH, Berger C, Wolf M, Fütterer CD, Feldmann Jr RE, Schwab S, et al. The proteome of human brain microdialysate. Proteome Sci 2003;1:7.
[42] Dayon L, Turck N, García-Berrocoso T, Walter N, Burkhard PR, Vilalta A, et al. Brain extracellular fluid protein changes in acute stroke patients. J Proteome Res 2011;10:1043–51.
[43] da Silva JS, Dotti CG. Breaking the neuronal sphere: regulation of the actin cytoskeleton in neuritogenesis. Nat Rev Neurosci 2002;3:694–704.
[44] Ray S, Lukyanov P, Ochieng J. Members of the cystatin superfamily interact with MMP-9 and protect it from autolytic degradation without affecting its gelatinolytic activities. Biochim Biophys Acta 2003;1652:91–102.
[45] Brzin J, Kopitar M, Turk V. Protein inhibitors of cysteine proteinases. I. Isolation and characterization of stefin, a cytosolic protein inhibitor of cysteine proteinases from human polymorphonuclear granulocytes. Hoppe Seylers Z Physiol Chem 1983;364:1475–80.
[46] Buttle DJ, Burnett D, Abrahamson M. Levels of neutrophil elastase and cathepsin B activities, and cystatins in human sputum: relationship to inflammation. Scand J Clin Lab Invest 1990;50:509–16.

[47] Cuadrado E, Ortega L, Hernández-Guillamon M, Penalba A, Fernández-Cadenas I, Rosell A, et al. Tissue plasminogen activator (t-PA) promotes neutrophil degranulation and MMP-9 release. J Leukoc Biol 2008;84:207–14.
[48] Seyfried D, Han Y, Zheng Z, Day N, Moin K, Rempel S, et al. Cathepsin B and middle cerebral artery occlusion in the rat. J Neurosurg 1997;87:716–23.
[49] Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat Biotechnol 2006;24:971–83.
[50] Whiteley W, Jackson C, Lewis S, Lowe G, Rumley A, Sandercock P, et al. Inflammatory markers and poor outcome after stroke: a prospective cohort study and systematic review of interleukin-6. PLoS Med 2009;6:e1000145.

Supplementary data

Fig. S1. Clustering heat-map. The hierarchical clustering algorithm EPCLUST (http://www.bioinf.ebc.ee/EP/EP/EPCLUST/) was used. Left panel shows the cluster of the identified proteins according to the mean of their expression versus internal standard pool in each brain area. Right panel shows the cluster of the significant comparison between brain areas after normalizing the values by the standard pool ($p<0.05$); those pair areas with non-significant comparison were marked in black color. In both cases, pink color indicates increased protein abundance and blue color indicates decreased protein abundance. IC, infarct core; PI, peri-infarct; CL, contralateral; C, control.

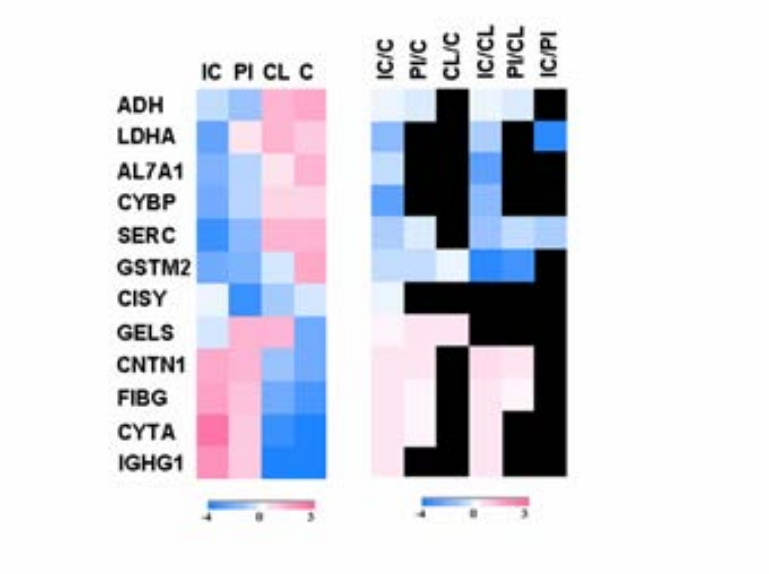
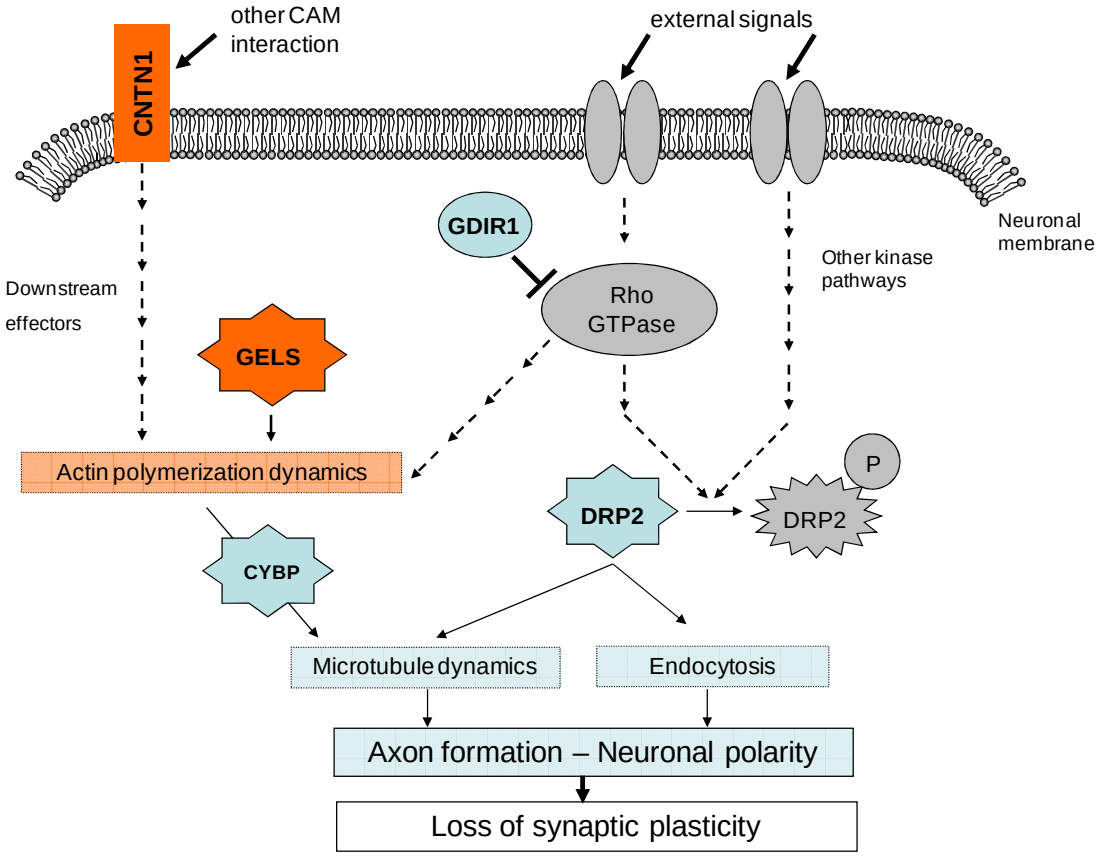


Fig. S2. Impaired neuritogenesis after ischemia. Hypothetical scheme of molecular processes involved in axonogenesis and that might be impaired after ischemic stroke. Orange color indicates increased levels and blue color indicates decreased levels of proteins. Cytoskeletal remodeling is essential for axon formation and it involves both actin microfilaments, which act first, and microtubules. Furthermore there is an interconnection between both cytoskeletons in which some proteins, as CYBP, are involved. In our hypothesis, higher CNTN1 and GELS levels might lead to changes in actin dynamics. On the other hand, the reduced levels of GDIR1, which are in part responsible for the decreased levels of the active DRP2, and the reduction of the intermediary CYBP, might cause a final reduction in the axon formation processes. Consequently, there is a loss of synaptogenesis and synaptic plasticity, which could explain a worse functional outcome after stroke.



Supplementary Table S1. Demographic-related blood biomarker levels.

Factors	GELS (ng/mL)			CYTA (ng/mL)			GDIR1 (ng/mL)		
	Yes	No	p-value	Yes	No	p-value	Yes	No	p-value
Gender (male)	20.0±5	19.8±4	0.842	24.8±6	26.6±9	0.384	18.3±15	16.4±12	0.602
Smoker	21.1±5	19.9±4	0.512	24.7±5	25.9±8	0.722	15.2±10	17.7±14	0.680
Hypertension	19.6±4	20.7±3	0.376	27.0±8	22.3±5	**0.032**	17.5±13	17.3±15	0.971
Diabetes mellitus	18.9±3	20.3±5	0.267	27.3±8	25.0±7	0.295	19.1±16	16.9±13	0.619
Dyslipidemia	19.4±4	20.4±4	0.384	24.6±8	26.4±7	0.366	16.0±12	18.2±15	0.565
Atrial fibrillation	20.6±5	19.7±4	0.473	26.8±6	25.1±8	0.438	14.4±11	18.8±15	0.281
Heart disease	19.1±5	20.2±4	0.386	27.8±9	25.0±7	0.235	23.5±15	15.7±13	0.078[#]
Previous stroke	16.6±4	20.4±4	**0.021**	28.0±11	25.2±7	0.314	30.6±14	15.2±12	**0.002**
Atherothrombotic	19.2±3	20.1±4	0.512	28.0±10	24.9±7	0.183	20.4±16	16.6±13	0.398
Cardioembolic	20.0±5	19.9±4	0.913	25.4±6	25.8±9	0.846	14.0±10	20.3±15	0.078[#]
Third month mRS 2-6	20.7±4	18.2±3	**0.042**	26.9±8	22.6±5	0.052[#]	15.8±13	21.5±13	0.159
In-hospital death	18.4±5	20.2±4	0.308	32.3±12	24.6±6	0.112[#]	19.0±17	17.1±13	0.727

Table S1 (continued).

Factors	DRP2 (ng/mL)			CNTN1 (ng/mL)		
	Yes	No	p-value	Yes	No	p-value
Gender (male)	0.70 (0.38-1.54)	0.56 (0.33-0.78)	0.232	37.9±17	35.8±14	0.631
Smoker	0.43 (0.36-0.67)	0.65 (0.34-1.43)	0.542	39.1±16	36.4±16	0.714
Hypertension	0.66 (0.37-0.90)	0.48 (0.34-2.05)	0.598	37.7±17	34.7±10	0.461
Diabetes mellitus	0.64 (0.39-0.78)	0.66 (0.34-1.43)	0.826	34.0±11	38.0±17	0.321
Dyslipidemia	0.49 (0.33-0.88)	0.66 (0.41-1.57)	0.242	35.3±16	38.0±15	0.550
Atrial fibrillation	0.60 (0.28-0.74)	0.69 (0.36-1.54)	0.205	41.1±17	35.0±14	0.202
Heart disease	0.80 (0.38-2.10)	0.64 (0.33-0.90)	0.160	37.9±21	36.5±13	0.788
Previous stroke	1.85 (1.48-1.98)	0.55 (0.33-0.90)	**0.004**	51.1±23	34.9±13	0.142
Atherothrombotic	0.78 (0.45-1.33)	0.64 (0.32-1.15)	0.353	32.6±14	38.2±16	0.283
Cardioembolic	0.52 (0.28-0.78)	0.78 (0.39-1.76)	0.057[#]	41.0±18	33.5±12	0.106
Thirds month mRS 2-6	0.48 (0.33-0.92)	1.26 (0.40-2.35)	0.073[#]	36.2±15	39.0±16	0.604
In-hospital death	0.90 (0.34-1.76)	0.66 (0.33-1.48)	0.589	44.2±18	35.6±15	0.176

Biomarker blood levels related to demographic, clinical and risk factors (Yes: to have the factor; No: not to have the factor). GELS, CYTA, GDIR1 and CNTN1 are expressed as mean ± SD; DRP2 is expressed as median (lower-upper quartile). Statistically significant differences are expressed as bold p-value and statistical trends are marked with [#]. mRS: modified Rankin scale.

4 RESULTADOS Y DISCUSIÓN

4.1 Evolución de los pacientes con ictus: papel pronóstico de los biomarcadores sanguíneos

Predecir cómo van a evolucionar los pacientes tras un ictus y tomar decisiones médicas en consecuencia es uno de los retos de esta enfermedad que supone una de las principales causas de muerte y discapacidad a nivel mundial (Roger VL *et al.*, 2012). A día de hoy, estas predicciones son poco precisas, basándose en la experiencia previa del médico y en los datos reportados a partir de ensayos clínicos, que no suelen reflejar la realidad de la práctica médica diaria. Para anticipar el pronóstico de los pacientes con ictus, el uso de escalas o modelos clínicos parece una alternativa interesante siempre que sean sencillos, fáciles de recordar, estén compuestos por variables fácilmente accesibles e incluso que puedan ser aplicados por personal no especializado. Además, estos modelos deben alcanzar valores de precisión y discriminación aceptables en la predicción de parámetros pronósticos clínicamente relevantes y deben ser validados en cohortes externas de gran tamaño.

Como puede verse en el Anexo I, la mayoría de los modelos clínicos existentes incluyen muchas variables, algunas de las cuales además no pueden ser evaluadas de forma inmediata a la admisión de los pacientes, retrasando la predicción y, por tanto, las actividades médicas dirigidas a evitar un mal desenlace de la enfermedad. Para la aplicación de estos modelos complejos sería necesaria la implantación en programas computacionales que incluyeran formularios fácilmente cumplimentables y que ofrecieran interpretaciones sencillas; la necesidad de esta tecnología haría difícil su uso en países en vías de desarrollo o subdesarrollados donde el ictus es una enfermedad incipiente (Feigin VL *et al.*, 2009).

La edad y la gravedad inicial del ictus son las variables predictoras principales, incluidas en todos los modelos, y que por sí solas (modelo de Weimar C *et al.*, 2004 y König R *et al.*, 2008) alcanzan una discriminación con AUCs de 0,8, muy similar a la obtenida con modelos más complejos evaluados en la misma cohorte (Weimar C *et al.*, 2002 y GSS collaboration, 2004). Por otro lado, estos factores no modificables explican la mayoría del mal pronóstico a largo plazo, por encima de complicaciones tempranas, como neumonía o edema, que afectan al desenlace de la enfermedad y que podrían ser tratados de forma precoz o incluso profiláctica (Grube MM *et al.*, 2013). Por tanto, parece que el uso de estas variables tan fácilmente accesibles sería suficiente para la predicción de la evolución de los pacientes con ictus; lamentablemente, su uso exclusivo genera una infraestimación del mal pronóstico (Saposnik G *et al.*, 2011-2). Esto podría deberse a la heterogeneidad que existe entre los pacientes aun considerando sólo estas sencillas variables, ya que existe un envejecimiento diferencial entre

los individuos e incluso entre los órganos del mismo individuo y afectaciones neurológicas de grado similar pueden deberse a ictus de cariz muy diferente.

Una manera de alcanzar mayor objetividad en el pronóstico de los pacientes con ictus sería el uso de marcadores biológicos. La medición de distintos parámetros mediante técnicas de neuroimagen ha mostrado su utilidad como herramienta pronóstica (Arsava EM, 2012), aunque su aplicación requiere del manejo de algoritmos complejos y no es una técnica ampliamente disponible. Por todo ello, el uso de biomarcadores moleculares ha sido ampliamente estudiado en el campo del pronóstico del ictus (Whiteley W *et al.*, 2009; Katan M *et al.*, 2011; Hasan N *et al.*, 2012). Con la idea de mantener actualizada la información disponible en el estudio de biomarcadores pronósticos del ictus hemos creado la página web http://stroke-biomarkers.com/ (Anexo II), donde estamos recopilando todas las moléculas estudiadas en relación al pronóstico del ictus. La búsqueda sistemática de los artículos publicados en inglés o en español se realiza en la base de datos PubMed, con más de 23 millones de referencias (http://www.ncbi.nlm.nih.gov/pubmed). Los términos de búsqueda empleados hacen referencia a diferentes conceptos pronósticos, entre ellos, mortalidad, discapacidad o mejoría. De los 701 artículos hallados en Noviembre de 2013, solamente 102 artículos fueron considerados susceptibles para su inclusión en la indicación de pronóstico del ictus. De este número de artículos potenciales, 19 moléculas cumplen el requisito de publicación mínima contemplado, es decir, que al menos se haya estudiado la molécula en relación al pronóstico del ictus en tres estudios independientes o en cohortes de más de 200 pacientes (Tabla 2). Con este criterio creemos que los marcadores compilados en la web tendrán una asociación plausible con el pronóstico, si bien pocos de los biomarcadores que cumplen este requisito han sido estudiados en relación a su valor añadido sobre variables clínicas pronósticas o en cohortes independientes de gran tamaño.

La aparición de guías creadas por la comunidad científica para mejorar la calidad en las publicaciones mediante el consenso de información mínima que debe ser reportada en las investigaciones biológicas y biomédicas (Taylor CF *et al.*, 2008) facilitará la consecución de proyectos de calidad en el campo de los biomarcadores y su translación a la práctica clínica. Aunque aún no existe consenso sobre los requisitos mínimos para que un biomarcador pueda llegar a ser ensayado en estudios de coste-efectividad en el ámbito sanitario, parece necesario realizar estudios estadísticos sobre su asociación independiente y su valor añadido mediante test que demuestren su potencial de discriminación y reclasificación de pacientes, así como, en las etapas finales del desarrollo de nuevos biomarcadores, su estudio en cohortes amplias.

Tabla 2. Biomarcadores sanguíneos asociados con el pronóstico del ictus (estudio(s) con al menos 200 pacientes).

Código	Molécula	Asociación	Referencias ejemplo
P22303	**Acetilcolinesterasa**	Hidrolasa de la acetilcolina. Pacientes que fallecerán en el primer año tras el ictus presentan baja actividad colinesterásica.	Ben Assayag E, 2010
Q15848	**Adiponectina**	Adipocina. Asociada con discapacidad funcional al 3r mes tras el ictus isquémico.	Whiteley W, 2012
P02768	**Albúmina**	Transportador. La albúmina sérica está disminuida en aquellos pacientes que morirán tras el ictus (1m).	Shibazaki K, 2009; Shibazaki K, 2011
P09429	**Amfoterina**	Proteína asociada a la cromatina. Asociada a mal pronóstico (discapacidad, mortalidad) en el primer año post-ictus.	Huang JM, 2013; Schulze J, 2013
P06276	**Butirilcolinesterasa**	Hidrolasa de la acetilcolina. Pacientes que fallecerán en el primer año tras el ictus presentan baja actividad colinesterásica.	Ben Assayag E, 2010
P01185	**Copeptina**	Fragmento del precursor de la vasopresina. Su uso como biomarcador añade información predictiva en el pronóstico de discapacidad y mortalidad a largo plazo.	De Marchis GM, 2013; Tu WJ, 2013; Zhang JL, 2013
1N86	**D-dímero**	Producto de degradación de fibrina. Niveles elevados asociados con discapacidad (3m, 1a) o fallecimiento (1m, 3m, 1a).	Shibazaki K, 2009; Whiteley W, 2012; Tu WJ, 2013
Q99988	**Factor de diferenciación del crecimiento-15**	Citocina. Independientemente asociada con discapacidad a largo plazo, pero no aporta información a la escala NIHSS.	Gröschel K, 2012
P04275	**Factor von Willebrand**	Glicoproteína hemostásica. Niveles elevados en pacientes que presentarán discapacidad funcional al 3r mes tras el ictus.	Whiteley W, 2012
P02671	**Fibrinógeno**	Precursor de la fibrina. Niveles circulantes elevados se han asociado con discapacidad (3m) y mortalidad (1m) post-ictus.	Shibazaki K, 2011; Whiteley W, 2012
9085	**Homoarginina**	Aminoácido no proteico. Niveles bajos asociados con mal pronóstico a corto y largo plazo, aunque no parece añadir información sobre las variables clínicas.	Choe CU, 2013
778	**Homocisteína**	Aminoácido azufrado. La hiperhomocisteinemia se ha asociado con empeoramiento neurológico (5d) y fallecimiento (intrahospitalario, 3m). Su asociación con discapacidad funcional (1m, 3m) no está consensuada.	Üstündağ M, 2010; Men X, 2013; Tu WJ, 2013
P05231	**Interleucina-6**	Citocina. Elevada en pacientes con mal pronóstico neurológico y funcional.	Castellanos M, 2008; Whiteley W, 2012; Rodríguez-Yáñez M, 2013
P14780	**Metaloproteinasa-9**	Proteasa de la matriz extracelular. Su papel como biomarcador pronóstico de discapacidad al 3r mes no está claramente establecido.	Whiteley W, 2012; Rodríguez-Yáñez M, 2013
Q13740	**Molécula de adhesión celular leucocito-activada**	Immunoglobulina. Niveles circulantes de ALCAM/CD166 elevados en pacientes que fallecerán tras el ictus (4a).	Smedbakken L, 2011
P26022	**Pentraxina-3**	Reactante de fase aguda. Niveles elevados se asocian a mortalidad post-ictus a largo plazo.	Ryu WS, 2012
P16860	**Péptido natriurético B**	Hormona vasodilatadora. Niveles incrementados de BNP o NT-proBNP (fragmento aminoterminal) se asocian a mal pronóstico funcional y vital a largo plazo.	Etgen T, 2005; Rost NS, 2012; Whiteley W, 2012
P02741	**Proteína C-reactiva**	Reactante de fase aguda. Niveles altos se asocian a mal pronóstico neurológico, funcional y vital.	Men X, 2013; Tu WJ, 2013; Vangilder RL, 2013
P45379	**Troponina T**	Contracción músculo cardíaco. Niveles elevados se han asociado a mal pronóstico funcional y muerte a largo plazo.	Etgen T, 2005; Smedbakken L, 2011; Whiteley W, 2012

Códigos de las proteínas en *Uniprot* (http://www.uniprot.org/), de las moléculas orgánicas en *PubChem* (http://pubchem.ncbi.nlm.nih.gov/)y de los péptidos en *Protein Data Bank* (http://www.rcsb.org/pdb/home/home.do).

NIHSS: *National Institutes of Health Stroke Scale*.

Con la intención de posibilitar tanto discusiones en torno al consenso de valores óptimos de discriminación y reclasificación u otros requisitos de los marcadores así como poder establecer redes multinacionales para el ensayo de biomarcadores prometedores, en 2011 se creó el grupo de estudio *International Biomarker in Cerebrovascular Diseases (IBCD)*, que cuenta con expertos internacionales de diferentes disciplinas en el estudio de la enfermedad cerebrovascular. Asentar las bases de este consorcio será uno de los pasos necesarios para el avance en estudios de biomarcadores de mayor calidad y dirigidos a responder preguntas clínicamente relevantes, que permitan ver en un futuro la aplicación de biomarcadores en el ictus.

4.2 Péptido natriurético de tipo B (BNP): evaluación de su papel real como biomarcador pronóstico en el ictus

Como puede verse en la Tabla 2, uno de los biomarcadores típicamente asociado al mal pronóstico del ictus es el péptido natriurético de tipo B, tanto su forma hormonal activa (BNP) como el fragmento amino-terminal inactivo que se libera equimolarmente en su formación (NT-proBNP). El BNP es liberado desde las células cardíacas en respuesta a un aumento de la presión arterial y genera un efecto vasodilatador gracias a su función natriurética y diurética (deLemos JA *et al.*, 2003). Los estudios existentes de BNP/NT-proBNP en relación con el pronóstico del ictus se han realizado en cohortes generalmente pequeñas y principalmente analizando su asociación con mortalidad post-ictus, en diferentes plazos de tiempo.

En el artículo 1 de esta Tesis se ha estudiado el papel de BNP activo en la predicción de deterioro neurológico en la fase aguda del ictus y mortalidad temprana tras el evento cerebrovascular. En la cohorte considerada, de 896 pacientes con ictus (incluyendo ambas entidades, isquémico y hemorrágico), se demostró la asociación independiente de valores altos de BNP con cerca de dos veces mayor riesgo de mal pronóstico tras el ictus. Los modelos predictivos con variables clínicas (ictus previo, gravedad inicial y diabetes mellitus o fibrilación auricular) mostraron una predicción similar al modelo que además incluía el biomarcador, con discriminaciones medidas utilizando el AUC del 70 % para deterioro neurológico y de 80 % para mortalidad intrahospitalaria. La comparación de las AUC y los test estadísticos de mejoría de la discriminación (IDI) y de la reclasificación (NRI) mostraron que, incluso existiendo una asociación independiente, el BNP no aporta valor añadido a las variables clínicas siendo poco útil su uso como biomarcador.

Estos resultados nos llevaron a la realización de una revisión sistemática y metaanálisis del papel de BNP como biomarcador pronóstico de mortalidad, que ha constituido el artículo 2 de esta Tesis. De los 20 artículos existentes en Octubre de 2012, 16 publicaciones analizando la asociación de BNP o NT-proBNP con mortalidad tras el ictus fueron incluidas. El metaanálisis de los estudios concluyó que niveles elevados de péptido natriurético se asociaban con mortalidad, si bien los artículos incluidos presentaban una calidad moderada y existía heterogeneidad entre ellos, además de sesgo de publicación. Para realizar un análisis menos sesgado y con mayor fiabilidad, recopilamos los datos individuales anonimizados tras contactar con los autores de todos los artículos, alcanzando un tamaño muestral superior a 2.000 pacientes. El análisis tras la normalización de los resultados de cada cohorte confirmó la asociación, independiente de la edad, el género y la gravedad inicial, de los valores elevados de BNP/NT-proBNP con el doble de riesgo de mortalidad. Los estadísticos comparativos mostraron un ligero valor añadido únicamente para NT-proBNP, con una mejora en la discriminación del 3 % y una mejora de la reclasificación del 8 %.

Los resultados de ambos estudios nos permiten considerar los péptidos natriuréticos como marcadores circulantes asociados con el pronóstico, pero sin valor añadido por si solos, y por tanto, difícilmente aplicables en la práctica clínica. En el caso de NT-proBNP, quizás su medición dentro de un panel de biomarcadores podría aportar valor para alcanzar precisión suficiente en términos de sensibilidad y especificidad para la predicción del pronóstico de los pacientes con ictus.

Estos resultados corroboran la necesidad de, una vez establecida la asociación de un biomarcador con una indicación concreta, realizar estudios en cohortes de tamaño muestral considerable. El uso del metaanálisis parece una alternativa interesante para alcanzar potencial estadístico suficiente y aumentar la precisión en los efectos definidos, al reducir los errores debidos al azar, el sesgo y los factores confusores. Como hemos visto, las principales limitaciones de los metaanálisis basados en la literatura son: el sesgo de publicación, ya que estudios con resultados destacables tienden a ser publicados frente a estudios de poco impacto o en los que la asociación es negativa, creando una sobreestimación del efecto, y la heterogeneidad entre estudios. El metaanálisis de pacientes individuales (IPD) permite superar ésta última al unificar los criterios y utilizar métodos de análisis que permiten corregir la presencia de confusores a nivel del paciente y realizar otros sub-análisis, aún siendo más costosos en cuanto a tiempo y manejo de los datos (Lewington S *et al.*, 2012). IPDs que consideren datos de estudios no publicados evitarían el sesgo de publicación, aunque la

obtención de la información es aún más compleja y la calidad de los resultados no ha sido valorada por revisores críticos.

Por otro lado, el uso de estadísticos comparativos que valoren el papel predictivo de las moléculas prometedoras es imprescindible para que lleguen a ser biomarcadores reales en la era de la medicina basada en la evidencia, aunque aún está por determinar qué porcentajes de mejora en la predicción serían suficientes. Mientras tanto, otros marcadores pronósticos típicos como IL-6 podrán ser evaluados utilizando la misma estrategia para definir su papel como biomarcador (Bustamante A *et al.*, 2013).

4.3 Biomarcadores candidatos por función, como las quimiocinas, no siempre tienen valor pronóstico

Aunque por definición los biomarcadores no tienen por qué estar relacionados con la causa o consecuencia de un proceso, una aproximación al descubrimiento de biomarcadores es el estudio de moléculas involucradas en la fisiopatología de una enfermedad. En el caso del ictus, el estudio de las vías fisiológicas alteradas tras el evento isquémico parece un campo prometedor para el desarrollo de biomarcadores. En concreto, las vías de apoptosis e inflamación, que se activan pronto tras la isquemia pero se mantienen alteradas lo suficiente para ser exploradas, han generado candidatos interesantes, como caspasa-3 (Rosell A *et al.*, 2008) e IL-6 o CRP (Whiteley W *et al.*, 2012).

Para estudiar una parte de la vía inflamatoria, en esta Tesis hemos considerado la superfamilia de las quimiocinas (Anexo III). Las quimiocinas son pequeñas citoquinas secretadas, cuya principal función es actuar como mediadoras de procesos inmunoinflamatorios ejerciendo un estímulo quimiotáctico que permite el reclutamiento de leucocitos efectores en el lugar de la lesión. Esta acción ayuda a la eliminación de los residuos del daño tisular pero a su vez amplifica la respuesta inflamatoria, dando lugar a un papel dual de las quimiocinas tanto beneficioso como perjudicial. Además, otras funciones relacionadas con procesos angiogénicos o de supervivencia neuronal contribuyen a la acción benéfica de las quimiocinas (Mackay CR, 2001; Ceulemans AG *et al.*, 2010).

Con la intención de ampliar el conocimiento sobre la función de las quimiocinas tras la isquemia cerebral humana, en este estudio hemos explorado la presencia a nivel tisular y circulatorio de un panel de 9 quimiocinas, algunas de las cuales no habían sido estudiadas previamente en el ámbito del ictus. El uso de la técnica de microdisección láser nos ha

permitido describir una concentración mayor de CCL1 y CCL2 en las neuronas que en los vasos sanguíneos cerebrales y la reducción en los niveles de CCL5 y CCL22 en la zona del *core* del infarto. En el plasma, los niveles de las quimiocinas estudiadas permanecen estables hasta 90 días tras el ictus, excepto en el caso de CCL22 que mostró una reducción significativa a las 24h de la admisión en el hospital. Los niveles a las 24h de CCL22 y CCL17, no exploradas previamente en pacientes con ictus, se correlacionaban negativamente con la gravedad neurológica de forma mantenida en el tiempo; es decir, niveles más altos de estas quimiocinas se relacionaban con un mejor estado neurológico. El hecho de que CCL17 y CCL22 atraigan selectivamente linfocitos cooperadores de tipo 2 (o Th2; Imai T *et al.*, 1999) que contrarresten la respuesta Th1 a la presencia de antígenos cerebrales, genera un estado anti-inflamatorio que podría explicar su asociación con la mejoría neurológica (Hendrix S *et al.*, 2007). Aún así, en la fase hiperaguda del ictus, dentro de las 4,5 primeras horas, ninguna de las quimiocinas estudiadas presentó potencia estadística suficiente para ser utilizada como biomarcador pronóstico en el ictus.

Los resultados generados muestran como las quimiocinas estudiadas en este trabajo se comportan de forma diferente, aún perteneciendo a la misma subfamilia (8 de las quimiocinas son CC), por lo que el papel pronóstico de otras quimiocinas no puede descartarse si bien los pocos estudios publicados al respecto (resumidos en la Tabla 1 del artículo en el Anexo 3 de esta Tesis) no son concluyentes. Estas discordancias podrían ser debidas a las diferencias en el diseño de los estudios existentes, donde, según el momento de medición de las quimiocinas desde el inicio de los síntomas, la balanza del efecto de las quimiocinas podría inclinarse más hacia el lado beneficioso o perjudicial. Sin embargo, este rol dual junto con las diferencias de expresión en cuanto a tipo celular o en presencia del estímulo isquémico hacen que las quimiocinas se presenten como posibles dianas terapéuticas modulables mediante el uso de anticuerpos monoclonales (Chan AC *et al.*, 2010).

Aunque los resultados de este trabajo no permiten valorar si estas quimiocinas podrían tener un papel como biomarcadores pronósticos en tiempos más tardíos desde el inicio de los síntomas, no parecen útiles en las primeras horas, cuando la toma de decisiones en cuanto al tratamiento fibrinolítico, la inclusión en las unidades de ictus u otras medidas son relevantes (Jauch EC *et al.*, 2013).

4.4 Análisis de muestras cerebrales como base para encontrar biomarcadores pronósticos específicos

Con el fin de descubrir nuevas moléculas que puedan funcionar como biomarcadores pronósticos en la fase aguda del ictus isquémico, hemos llevado a cabo experimentos con diseños diferentes pero que tienen en común el uso de técnicas del mundo de las *ómicas*.

En la primera aproximación quisimos determinar proteínas que se vieran alteradas por la isquemia a nivel cerebral para generar una lista de candidatos utilizando técnicas de proteómica basadas en electroforesis bidimensional diferencial en gel (2D-DIGE). Inicialmente, el análisis de homogenados cerebrales de las zonas del *core*, el peri-infarto y el contralateral, así como de cerebros control, utilizando espectrometría de masas de tipo MALDI-TOF, generó una lista de 39 proteínas (Cuadrado E *et al.* 2010). Los avances en el desarrollo de espectrómetros de masas, con instrumentos más resolutivos, precisos y rápidos (Thevis M *et al.*, 2012), nos han permitido ampliar el conocimiento del proteoma cerebral humano tras el ictus isquémico.

El artículo 3 de esta Tesis doctoral incluye la lista de 12 nuevas proteínas diferencialmente expresadas en el experimento de 2D-DIGE que han podido ser identificadas al utilizar un espectrómetro de tipo Orbitrap, incrementando la lista de proteínas alteradas tras la isquemia cerebral. Del total de 51 proteínas identificadas en los homogenados cerebrales, se seleccionaron 8 candidatos que fueron analizados en muestras de sangre de pacientes con ictus mediante ELISA. Los niveles circulantes de gelsolina (GELS), proteína relacionada con la dihidropirimidinasa 2 (DRP-2) y cistatina-A (CYTA) demostraron su valor como biomarcadores en la predicción de dependencia funcional 3 meses después del ictus al mejorar tanto la discriminación (hasta un 28 % al considerar los 3 biomarcadores) como la reclasificación de los pacientes en grupos de riesgo (hasta el 85,7 %) respecto a edad, género, gravedad inicial y presencia de diabetes mellitus. Por otro lado, niveles elevados de CYTA en plasma predicen mortalidad intrahospitalaria, mejorando la discriminación en un 34 % y la reclasificación en un 39 % respecto a edad, género, gravedad inicial y antecedentes de ictus.

Las funciones de GELS y DRP-2 en la remodelación del citoesqueleto los relacionan con procesos de neuritogénesis. Cómo los cambios en GELS, DRP-2 y otras de las proteínas identificadas afectarían al proceso de neuritogénesis podría explicarse por la implicación del citoesqueleto de actina, que actúa primero, y de los microtúbulos en la formación de axones neuronales. Hipotéticamente, los niveles elevados de contactina-1 (CNTN1) y GELS podrían

generar cambios en la dinámica del citoesqueleto de actina. La polimerización de la actina se vería contrarrestada por una reducción en la formación de microtúbulos, al estar disminuidas otras proteínas en el *core* del infarto. Esta inhibición en el proceso de formación de axones generaría fallos en la sinaptogénesis y en la plasticidad sináptica y, por tanto, podrían explicar la asociación hallada con mal pronóstico funcional (para referencia, Figura S2 del artículo 3).

Por otro lado, el papel de CYTA en relación al mal pronóstico en los pacientes con ictus podría explicarse por su acción como inhibidor de proteasas de cisteína que, junto a su asociación con la degranulación de neutrófilos, favorecería la acción de metaloproteinasas de matriz (Ray S *et al.*, 2003), cuyo papel es de sobra conocido en la isquemia cerebral (Morancho A *et al.*, 2010).

Los resultados de este trabajo corroboran que el uso de un panel de varios biomarcadores cuyas funciones metabólicas fueran diferentes sería una herramienta de mayor utilidad en la práctica clínica (Robin X *et al.*, 2009). El hecho de ir añadiendo biomarcadores al modelo predictivo permite aumentar la discriminación de un 8 a un 28 % y la reclasificación de los pacientes de un 21 a un 86 % en grupos de riesgo relevantes clínicamente, niveles de precisión nada desdeñables de ser verificados en otras cohortes de mayor tamaño muestral. Hasta ahora, el único biomarcador que ha demostrado valor añadido respecto a variables clínicas pronósticas en una cohorte de cerca de 800 pacientes, copeptina (De Marchis GM *et al.*, 2013), ha alcanzado porcentajes de reclasificación del 12 % para pronóstico funcional, aunque utilizando porcentajes predefinidos de riesgo bajo (máximo 15 %) divididos en 4 categorías. Conocer el valor añadido de copeptina considerando grupos de riesgo más extremos y en combinación con otros biomarcadores sería muy interesante en el futuro del pronóstico del ictus. Qué y cuántos biomarcadores podrían formar parte de un panel predictivo aplicable clínicamente para anticiparse a la evolución de los pacientes con ictus está aún por determinar, si bien, los resultados de GELS, DRP-2 y CYTA son prometedores.

Además, la lista de proteínas identificadas podría ser estudiada para hallar nuevos biomarcadores para ésta u otra indicación. Por otro lado, al ser moléculas que se han hallado alteradas en muestras cerebrales podrían ser exploradas como dianas terapéuticas con la intención de mejorar el pronóstico de los pacientes con ictus. El trabajo de este artículo 3 se ha basado en el análisis de homogenados cerebrales, muestras en las que no se puede discernir la contribución de cada uno de los componentes celulares que conforman la heterogeneidad tisular que define al cerebro humano. Esta heterogeneidad parece ser uno de los factores que dificulta el estudio a nivel molecular del cerebro, ya que una misma molécula puede ser

expresada diferencialmente frente al estímulo isquémico según el tipo celular. Con la intención de superar esta dificultad y profundizar aún más en el conocimiento del proteoma cerebral tras el ictus isquémico, hemos llevado a cabo un estudio en el que se combina la microdisección láser con proteómica cuantitativa basada en espectrometría de masas (Anexo IV). El análisis de las neuronas microdisecadas ha permitido identificar 41 proteínas que se encuentran en mayor proporción en la zona del *core* del infarto que en el contralateral, representando principalmente vías catalíticas relacionadas con la obtención de energía e involucradas en modificaciones estructurales necesarias en procesos de neurorreparación. Próximamente, el análisis de los vasos sanguíneos obtenidos de las mismas muestras nos permitirá conocer si se producen el mismo tipo de alteraciones que en las neuronas o si la isquemia afecta de forma diferente a estos dos tipos celulares de la unidad neurovascular, obteniendo una lista de proteínas candidatas a dianas terapéuticas o biomarcadores específicos.

4.5 Proteómica y transcriptómica en el ictus: nuevos candidatos para la predicción de la evolución

De forma paralela, hemos explorado los cambios a nivel del proteoma y del transcriptoma en muestras sanguíneas obtenidas en las primeras horas después del ictus y su asociación con el deterioro neurológico temprano. En ambos estudios hemos seguido la misma estrategia de combinar muestras de diferentes individuos con un diseño experimental de *sub-pooling* con la intención de reducir la variabilidad interindividual y encontrar candidatos a biomarcadores pronósticos generales, que luego serán replicados en muestras independientes (Sham P *et al.*, 2002; Kendziorski C *et al.*, 2005; Zhang W *et al.*, 2007).

En el artículo del Anexo 5 de esta Tesis analizamos 9 *pools* de plasma, 3 por cada grupo de evolución (empeoramiento, estabilidad y mejoría), mediante una librería de 177 anticuerpos. Las 35 proteínas que se vieron alteradas por el ictus isquémico pertenecían principalmente a las vías de inflamación, respuesta inmune, coagulación y apoptosis, todas ellas presentes en el proceso fisiopatológico del ictus isquémico (Jickling GC *et al.*, 2011). De las proteínas seleccionadas para la replicación, beta-defensina 2 (BD-2) y el receptor de la interleucina 4 (IL-4R) en su forma soluble predecían el empeoramiento en las primeras 24 – 48 horas de forma independiente. Al incluir los niveles de BD-2 e IL-4R en un modelo clínico con la edad, el género, la gravedad inicial y la presencia de diabetes mellitus como variables predictivas, la discriminación de los pacientes que empeoraban mejoró en un 9 – 10 %, según el tiempo

considerado. La combinación de los dos biomarcadores mejoraba la reclasificación de los pacientes en grupos de riesgo extremos entre un 19 % a las 24 h y un 28 % a las 48 h.

BD-2 e IL-4R parecen estar relacionadas con el proceso inflamatorio que tiene lugar en las primeras horas tras el evento isquémico. Aunque esta vía es una de las candidatas en la búsqueda de biomarcadores para el ictus (como hemos visto en el artículo del Anexo 3), estas dos moléculas no habían sido exploradas previamente en este contexto y parecen interesantes para ser estudiadas en cohortes más grandes, donde su valor añadido podrá ser evaluado con mayor precisión. Aunque las mejoras en la identificación de los pacientes que empeorarán en los primeros días tras el ictus no son tan impactantes como las conseguidas en el artículo 3 para discapacidad funcional, BD-2 e IL-4R podrían formar parte de un panel de marcadores tempranos para evaluar la respuesta inflamatoria post-ictus y su relación con el pronóstico a corto plazo. Este panel también podría incluir la medición de la actividad de la enzima quitotriosidasa, también relacionada con la respuesta inflamatoria. Resultados recientes de nuestro grupo muestran cómo una reducción de la actividad quitotriosidasa a nivel basal permite discriminar un 6 % mejor y reclasificar un 11,6 % de los pacientes que mejoran neurológicamente en las primeras 48 h (Bustamante A *et al.*, 2013-2). La idea de combinar biomarcadores que detecten empeoramiento con otros que identifiquen mejoría permitiría hacer una mejor predicción del estado neurológico de los pacientes al alcanzar una sensibilidad y una especificidad elevadas.

Paralelamente, en el estudio de transcriptómica, donde hemos analizado los cambios de expresión en la fase hiperaguda del ictus isquémico a nivel de leucocitos circulantes, 16 *pools* de ARN fueron construidos en función del grupo de evolución neurológica, de forma similar al estudio anterior (Anexo VI). Un total de 85 genes mostraron cambios en su expresión en aquellos pacientes que mejoraban o empeoraban neurológicamente, cuando la edad y la gravedad inicial eran consideradas como covariables en el análisis de las micromatrices de oligonucleótidos. Los genes afectados estaban asociados con procesos de señalización y muerte celular y vías metabólicas, aunque algunos no tienen función conocida. 30 de estos genes fueron seleccionados para la fase de replicación en muestras individuales. De los genes replicados el 80 % estaban relacionados con mejoría del estado neurológico y de estos *DCAKD* y *PMS2* se asociaron de forma significativa a la mejoría a las 24 horas después del ictus. Los resultados de esta replicación han sido examinados utilizando la herramienta computacional PanelomiX (Robin X *et al.*, 2013), que determinó los puntos de corte óptimos de *DCAKD*, *PMS2* y *CCL4* para la predicción de mejoría neurológica a las 24 h después del ictus. El panel con los

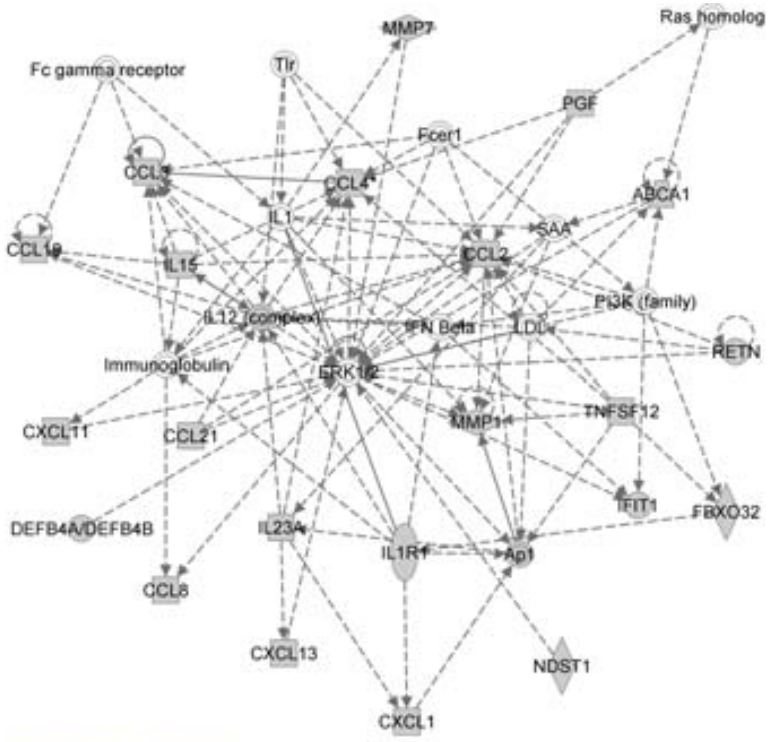
tres biomarcadores junto con las variables clínicas de gravedad, edad y género alcanzó una precisión medida por el AUC del 79,3 %, superior al 69,6 % alcanzada únicamente con las variables clínicas. Además, el panel de tres biomarcadores mejora la discriminación en un 10 % y la reclasificación de los pacientes en un 21 %.

DCAKD tiene actividad quinasa y PMS2 es una endonucleasa con actividad reparadora que está involucrada en el cambio de isotipo de las inmunoglobulinas (Péron S *et al.*, 2008). Cómo el aumento de la expresión de estos genes en los leucocitos circulantes puede asociarse a una mejoría en el estado neurológico después del ictus está aún por determinar. El hecho de que la mayoría de los genes identificados estén relacionados con mejoría (aumentada su expresión en los pacientes que mejoran o disminuida en los que empeoran) podría explicarse por ser un fenotipo menos complejo que el empeoramiento, donde pueden contribuir diferentes causas. En el caso de la mejoría, se han implicado mecanismos de recuperación de la perfusión, bien por fibrinolisis endógena (Barber PA *et al.*, 1998) o por circulación colateral (Bang OY *et al.*, 2008), y los procesos de neuroprotección endógena, donde la restauración del flujo sanguíneo también juega un papel importante (Gursoy-Ozdemir Y *et al.*, 2012). Encontrar moléculas que puedan discriminar con fiabilidad qué pacientes van a mejorar tras el ictus permitiría reducir la sobreestimación del buen pronóstico existente (Bushnell C, 2011) y optimizar los recursos clínicos en cuanto a admisión en las unidades de ictus o el tiempo de ingreso.

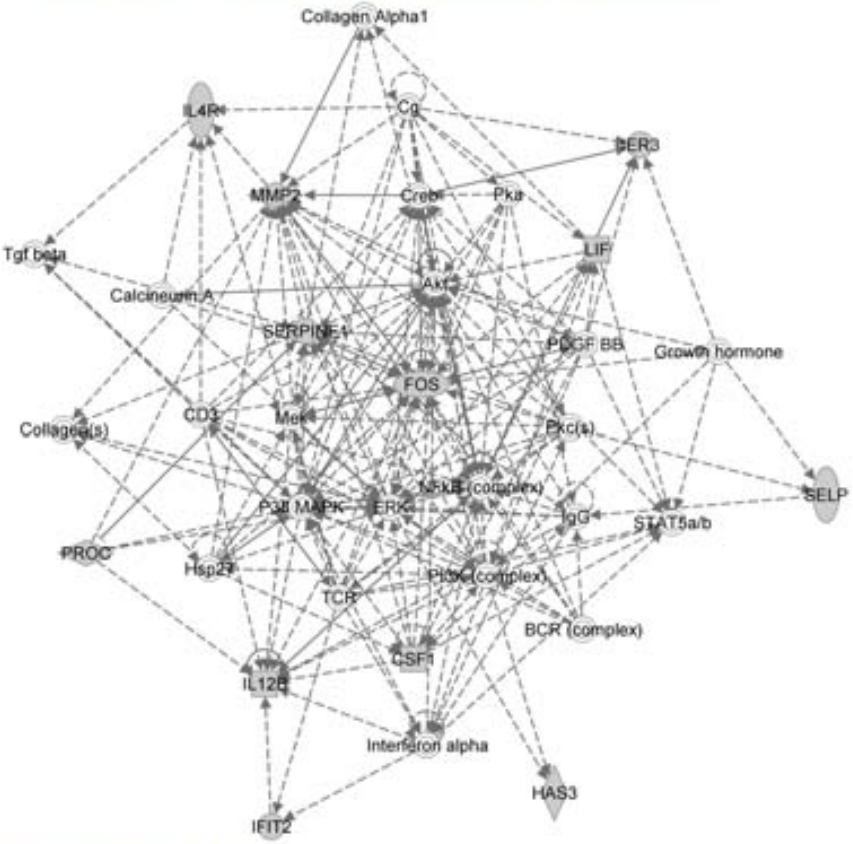
La combinación de los resultados de ambos estudios, de proteómica y de transcriptómica, ha generado una lista con un total de 119 moléculas que mostraron cambios en la fase aguda del ictus isquémico. Aunque solamente CCL4 coincide en ambos estudios, el análisis funcional de estas moléculas mediante el uso del programa Ingenuity Pathways Analysis (IPA; para referencia ver Anexo VI) ha identificado interacciones entre las moléculas asociadas con empeoramiento neurológico (un total de 76) en 7 redes, principalmente relacionadas con tráfico de células del sistema inmune y respuesta inflamatoria (Figura 12). Las vías canónicas asociadas con mayor significación son las de adhesión de leucocitos y diapédesis (-log (valor p) = 16) y la de regulación de producción de citoquinas (-log (valor p) = 10).

Figura 12. Principales vías moleculares de interacción entre las proteínas y genes alterados en relación al empeoramiento neurológico en la fase aguda del ictus isquémico.
Las moléculas en gris pertenecen a la lista de 76 proteínas y genes alterados; las líneas continuas indican una interacción directa y las líneas discontinuas, interacciones indirectas.

Movimiento celular – Tráfico de células del sistema inmune



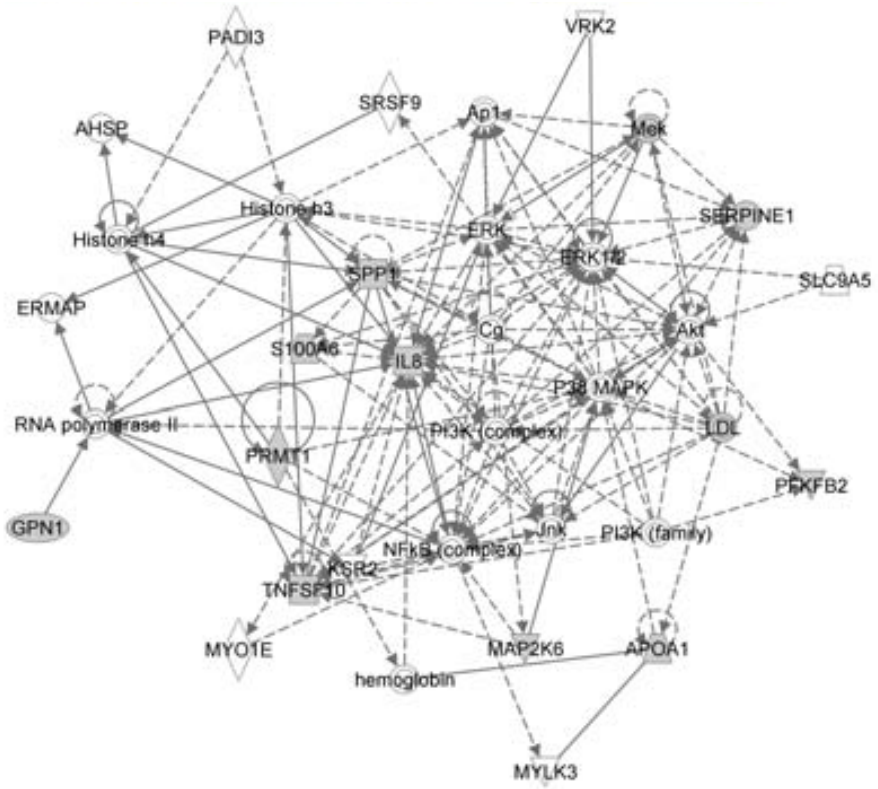
Respuesta inflamatoria – Señalización e interacción intercelular



Sin embargo, el estudio funcional con IPA considerando los 43 genes y proteínas asociados con mejoría neurológica en la fase aguda del ictus isquémico ha identificado como principal vía alterada la del metabolismo de carbohidratos (Figura 13). Las funciones de desarrollo tisular y desarrollo del sistema cardiovascular son las más significativas (-log (valor *p*) = 5). Estas funciones quizás indican una capacidad mayor de regeneración en aquellos pacientes que mejoran su estado de forma rápida, para la cual es necesaria energía que se obtiene de procesos metabólicos basados en la glucosa.

Figura 13. Principales vías moleculares de interacción entre las proteínas y genes alterados en relación a la mejoría neurológica en la fase aguda del ictus isquémico.
Las moléculas en gris pertenecen a la lista de 43 proteínas y genes alterados; las líneas continuas indican una interacción directa y las líneas discontinuas, interacciones indirectas.

Metabolismo de carbohidratos – Desarrollo tisular



Con estos resultados, las diferencias tempranas halladas a nivel molecular entre los pacientes que empeorarán o mejorarán neurológicamente en los primeros días tras el ictus podrían servir para explorar nuevas dianas terapéuticas que regulen estas vías y ayuden en la prevención de un desenlace fatídico del ictus.

4.6 Futuro de los biomarcadores pronósticos en el ictus isquémico

Idealmente, biomarcadores que demuestren su asociación independiente con alguna de las variables pronósticas (deterioro o mejoría neurológica, discapacidad funcional, mortalidad) y además aporten valor predictivo, podrían utilizarse en la práctica clínica formando parte de modelos con otras variables sencillas. Estudios como TABASCO (*Tel Aviv Brain Acute Stroke Cohort*; Assayag EB *et al.*, 2012), en el que se está recopilando información clínica, de neuroimagen y de biomarcadores sanguíneos para hallar asociaciones con diferentes variables de evaluación del pronóstico en una cohorte prospectiva de más de 1.000 pacientes, generarán información para poder construir modelos predictivos que incluyan biomarcadores.

Las moléculas identificadas en los estudios que forman esta Tesis han mostrado su valor añadido a modelos clínicos sencillos cuando son analizadas de forma temprana para la predicción de la evolución de los pacientes con ictus (Tabla 3). Si éstos u otros biomarcadores serán aplicables en la práctica clínica diaria en un futuro pasa por que sean validados en cohortes independientes y muestren su valor coste-efectivo. Además, en el caso de las moléculas que puedan ayudar en la predicción del pronóstico a corto plazo (como el deterioro neurológico temprano) aquéllas que demuestren su utilidad clínica deberían ser incorporadas en dispositivos de medición rápida (conocidos como POCs o *point-of-care devices*). Gracias al desarrollo de la nanotecnología se está avanzando mucho en el diseño de estos dispositivos y en su capacidad para la medición de marcadores moleculares de distinta índole (proteínas, ARN, ADN) en muestras reales de pacientes (Olasagasti F *et al.*, 2012).

Tabla 3. Resumen de las moléculas identificadas con valor pronóstico.

Molécula	Tiempo medición	Variable pronóstica	Incremento AUC	IDI	NRI
BD-2	< 4,5 h	Deterioro neurológico (24h)	11 %	9,5 %	19 %
IL-4R	< 4,5 h	Deterioro neurológico (24h)			
DCAKD	< 4,5 h	Deterioro neurológico (24h)	9,7 % *	10,3 %	21,4 %
PMS2	< 4,5 h	Deterioro neurológico (24h)			
CCL4	< 4,5 h	Deterioro neurológico (24h)			
Cistatina-A	< 4,5 h	Muerte intrahospitalaria	9,3 %	33,6 %	39 %
Gelsolina	< 4,5 h	Discapacidad funcional (3m)	9 %	27,8 %	85,7 %
DRP-2	< 4,5 h	Discapacidad funcional (3m)			
Cistatina-A	< 4,5 h	Discapacidad funcional (3m)			

Moléculas (proteínas y *tránscritos*) identificados en los estudios de proteómica y transcriptómica.
Incremento AUC: diferencia en el área bajo la curva ROC entre el modelo con y sin biomarcadores; IDI: índice de mejoría de la discriminación; NRI: índice de mejoría de la reclasificación (utilizando porcentajes predeterminados de 10 y 90 % de riesgo).
* El modelo para el panel de tránscritos se obtuvo con PanelomiX, un algoritmo ligeramente diferente al utilizado en la regresión logística utilizada para calcular el IDI y el NRI.

La aplicación clínica de biomarcadores pronósticos podría ayudar a determinar qué pacientes podrían beneficiarse más de terapias más agresivas o de nuevos tratamientos en el futuro. La realización de ensayos clínicos con tratamientos que han fallado en pacientes considerados de forma general (p.ej. PAIS, *Paracetamol (Acetaminophen) In Stroke*; Den Hertog HM *et al.*, 2009), podrían ser beneficiosos para un subgrupo concreto de pacientes (en el ejemplo, pacientes con fiebre; De Ridder IR *et al.*, 2013). En este aspecto, los biomarcadores pronósticos aportarían información valiosa.

¿Cómo mejorar el descubrimiento de biomarcadores pronósticos? Un aspecto a tener en cuenta es que las lesiones que se producen a nivel cerebral como consecuencia de la interrupción del flujo sanguíneo no son estáticas. Los procesos que se llevan a cabo en la fase aguda varían con el paso del tiempo, dando lugar a procesos celulares y moleculares diferentes en los días sucesivos. En los estudios de biomarcadores habría que considerar en qué momento se está midiendo el marcador molecular y a qué plazo quiere predecirse el desenlace. Actualmente la mayoría de estudios para la predicción de la discapacidad funcional o la mortalidad al tercer mes o al año de haber sufrido el ictus se están realizando con la medición del biomarcador en muestras sanguíneas obtenidas en las primeras horas tras la isquemia. Conocer si las moléculas que se alteran de forma temprana tienen un papel relevante en la predicción a largo plazo porque se mantienen alteradas de forma continuada o porque inician procesos claves que darán lugar a uno u otro desenlace debería considerarse un campo de estudio para avanzar en el desarrollo de biomarcadores pronósticos. En el primer caso, el hecho de poder medir los niveles moleculares en un rango más amplio de tiempo también facilitaría su aplicación clínica, al no requerir de dispositivos de medición rápida y quizás poder incorporarse a las mediciones de rutina en sangre. Por otro lado, también debería considerarse el estudio de moléculas que varíen en tiempos más cercanos al de evaluación, cuando puede ocurrir otra serie de procesos que modifiquen el desenlace de la enfermedad.

Los acontecimientos que se suceden antes, durante y después del ictus generan una gran heterogeneidad en la evolución de los pacientes. Esta heterogeneidad se manifiesta principalmente en las diferentes causas de mal pronóstico. Desarrollar biomarcadores para la detección precoz de los diferentes tipos de complicaciones permitiría anticiparse a ellas y, lo que es más importante, incluso poder prevenirlas. Un claro ejemplo de ello serían los biomarcadores destinados a predecir infecciones post-ictus; copeptina, procalcitonina y CRP parecen buenos candidatos para tal propósito (Fluir F *et al.*, 2012). El tratamiento profiláctico de las infecciones con antibióticos no ha demostrado mejorar el pronóstico en el conjunto

general de pacientes con ictus (Chamorro A *et al.*, 2005). El ensayo STRAWINSKI (*STRoke Adverse outcome is associated WIth NoSocomial Infections*; Ulm L *et al.*, 2013) utilizará los niveles circulantes de procalcitonina para la toma de decisiones frente a la administración de antibióticos profilácticos en aquellos pacientes con mayor riesgo de desarrollar una infección y determinar cómo este tratamiento afecta al pronóstico a largo plazo. Si los resultados de este ensayo son positivos, la aplicación de los biomarcadores en la práctica clínica podría empezar a ser una realidad.

De forma similar, otra aplicación en la que los biomarcadores podrían ser utilizados para cambiar el pronóstico sería en la predicción de transformación hemorrágica secundaria al tratamiento trombolítico. La metaloproteinasa de matriz 9 (MMP-9; Montaner J *et al.*, 2003) y la fibronectina celular (Castellanos M *et al.*, 2007) se han asociado de forma bastante específica con el desarrollo de hematoma parenquimatoso tras la administración de rt-PA. Si estos biomarcadores podrían utilizarse como guía en la toma de decisiones respecto a la conveniencia o no de realizar el tratamiento fibrinolítico para evitar esta complicación secundaria está aún por determinar.

Parece que el futuro del pronóstico en el ictus pasa por desgranar las diferentes causas de complicación y ya existen modelos clínicos predictivos en algunos casos, como las transformaciones hemorrágicas secundarias al tratamiento (modelo HAT, Lou M *et al.*, 2008; modelo GRASPS, Menon BK *et al.*, 2012; modelo SEDAN, Strbian D *et al.*, 2012-2) o las infecciones (modelo A^2DS^2, Hoffmann S *et al.*, 2012; modelo AIS-APS, Ji R *et al.*, 2013). El hecho de que algunos de estos modelos incluyan variables de neuroimagen, que retrasarían la toma de decisiones, y que algunas variables sean compartidas entre los modelos para predecir diferentes complicaciones hace aún más interesante el desarrollo de biomarcadores específicos para cada tipo de complicación.

CONCLUSIONES/*CONCLUSIONS* 5

5.1 La página http://stroke-biomarkers.com/ permitirá el acceso a información actualizada sobre los biomarcadores en el ictus. Decenas de moléculas han sido asociadas con el pronóstico del ictus, aunque pocas han sido evaluadas en cuanto a su valor añadido sobre las variables clínicas.

5.2 Siendo un biomarcador típicamente asociado con mal pronóstico, en nuestra cohorte de casi 900 pacientes, los niveles plasmáticos de BNP en la fase aguda del ictus se asocian de forma independiente a empeoramiento neurológico y mortalidad intrahospitalaria, aunque BNP no añade valor predictivo sobre las variables clínicas.

5.3 El metaanálisis de 16 artículos demuestra la asociación de los niveles elevados de BNP y su fragmento amino-terminal (NT-proBNP) con la mortalidad post-ictus, aunque existe sesgo de publicaciones. El análisis con los datos individuales de 2.258 pacientes muestra que concentraciones aumentadas de los péptidos natriuréticos doblan el riesgo de fallecimiento. Sólo NT-proBNP añade un mínimo valor predictivo respecto a las variables clínicas, dificultando su traslación a la práctica clínica como biomarcador único.

5.4 La combinación de la microdisección láser con un panel de anticuerpos contra 9 quimiocinas diferentes nos ha permitido detectar niveles mayores de CCL1 y CCL2 en neuronas que en vasos sanguíneos de cerebros de pacientes fallecidos por ictus. Además CCL5 y CCL22 están reducidas en la zona del *core* del infarto. En muestras sanguíneas, CCL2, CCL17 y CCL22 muestran una correlación negativa con la gravedad neurológica. Las quimiocinas estudiadas presentan una potencia estadística mínima como biomarcadores pronósticos del ictus.

5.5 Mediante estudios de proteómica de homogenados cerebrales de pacientes fallecidos por ictus identificamos gelsolina, DRP-2 y cistatina-A circulantes como biomarcadores predictores de discapacidad funcional a largo plazo y, además, de cistatina-A circulante como predictor de mortalidad intrahospitalaria. El estudio del proteoma plasmático ha mostrado a BD-2 y la forma soluble de IL-4R como predictores de empeoramiento en la fase aguda del ictus. Todos ellos aportan valor en la discriminación y reclasificación de los pacientes sobre las variables clínicas.

5.6 El análisis de expresión génica de los leucocitos circulantes nos permitió identificar un panel de 10 tránscritos relacionados con la evolución de los pacientes con ictus. Un 80 % de estas moléculas se asocian a mejoría neurológica. Un panel incorporando *DCAKD*, *PMS2* y *CCL4* aumenta un 10% la precisión en la predicción de mejoría neurológica.

5.1 *The website http://stroke-biomarkers.com/ will allow to have updated information about stroke biomarkers. Tens of molecules have been associated with stroke prognosis, although few have been evaluated in terms of added value over clinical variables.*

5.2 *Being a biomarker typically associated with poor outcome, in our cohort of near 900 patients, plasmatic levels of BNP in the acute phase of stroke were independently associated with neurological worsening and in-hospital mortality, although BNP does not add predictive value over clinical variables.*

5.3 *A meta-analysis of 16 articles demonstrates the association of high BNP and the amino-terminal peptide (NT-proBNP) levels with death after stroke, although there is publication bias. The analysis with 2,258 individual patients' data shows that higher concentration of natriuretic peptides double the risk of death in stroke patients. Only NT-proBNP shows a minor added predictive value to clinical variables, thus making difficult its translation as a unique biomarker into clinical practice.*

5.4 *Laser microdissection technique coupled to the use of an antibodies array against 9 different chemokines has allowed to detect higher CCL1 and CCL2 levels in neurons than blood vessels in brains from patients who died following stroke. Furthermore CCL5 and CCL22 are less represented in the core of the infarct area. In blood samples, CCL2, CCL17 and CCL22 show a negative correlation with neurological severity. The studied chemokines are statistically underpowered when their role as stroke prognostic biomarkers was evaluated.*

5.5 *Proteomics of brain homogenates from patients who died following stroke have identified circulating gelsolin, DRP-2 and cystatin-A as predictive biomarkers of long-term functional disability and also of circulating cystatin-A as in-hospital death predictor. Proteomics of plasma samples have shown BD-2 and the soluble form of IL-4R as prognostic biomarkers of worsening in the acute phase of stroke. All these biomarkers contribute to discrimination and reclassification of patients over clinical variables.*

5.6 *Gene expression analysis of circulating leukocytes has allowed us to identify a panel of 10 transcripts related to stroke patients' outcome. An 80 % of these molecules are associated with neurological improvement. A panel including DCAKD, PMS2 and CCL4 increase a 10 % the precision of predicting neurological improvement.*

BIBLIOGRAFÍA

6

A

Abu-Asab MS, Chaouchi M, Alesci S, Galli S, Laassri M, Cheema AK, Atouf F, VanMeter J, Amri H. Biomarkers in the age of omics: time for a systems biology approach. *OMICS* 2011; 15: 105-112.

Adams HP Jr, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE 3rd. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in acute stroke treatment. *Stroke* 1993; 24: 35-41.

Ali M, English C, Bernhardt J, Sunnerhagen KS, Brady M, on behalf of the VISTA-Rehab Collaboration. More outcomes than trials: a call for consistent data collection across stroke rehabilitation trials. *Int J Stroke* 2013; 8: 18-24.

Allen CMC. Predicting the outcome of acute stroke: a prognostic score. *J Neurol Neurosurg Psychiatry* 1984; 47: 475-480.

Álvarez J, Rovira A, Molina C, Serena J, Moltó JM, por el comité *ad hoc* del Grupo de Estudio de Enfermedades Cerebrovasculares de la SEN. Guía para la utilización de métodos y técnicas diagnósticas en el ictus. En: Díez E, ed. Guía para el diagnóstico y tratamiento del ictus, nº 3. Guías oficiales de la Sociedad Española de Neurología (SEN). Barcelona: Prous Science, 2006.

Álvarez-Sabín J, Molina C, Montaner J, Arenillas J, Pujadas F, Huertas R, Mouriño J, Ribó M, Santamarina E, Quintana M. Beneficios clínicos de la implantación de un sistema de atención especializada y urgente del ictus. *Med Clin (Barc)* 2004; 122: 528-531.

Amarenco P, Bogousslavsky J, Caplan LR, Donnan GA, Hennerici MG. Classification of stroke subtypes. *Cerebrovasc Dis* 2009; 27: 493-501.

Arboix A, Díaz J, Pérez-Sempere A, Álvarez J, por el comité *ad hoc* del Grupo de Estudio de Enfermedades Cerebrovasculares de la SEN. Ictus: tipos etiológicos y criterios diagnósticos. En: Díez E, ed. Guía para el diagnóstico y tratamiento del ictus, nº 3. Guías oficiales de la Sociedad Española de Neurología (SEN). Barcelona: Prous Science, 2006.

Arsava EM. The role of MRI as a prognostic tool in ischemic stroke. *J Neurochem* 2012; 123: 22-28.

Assayag EB, Korczyn AD, Giladi N, Goldbourt U, Berliner AS, Shenhar-Tsarfaty S, Kliper E, Hallevi H, Shopin L, Hendler T, Baashat DB, Aizenstein O, Soreq H, Katz N, Solomon Z, Mike A, Usher S, Hausdorff JM, Auriel E, Shapira I, Bornstein NM. Predictors for poststroke outcomes: the Tel Aviv Brain Acute Stroke Cohort (TABASCO) study protocol. *Int J Stroke* 2012; 7: 341-347.

B

Bacigaluppi M, Hermann DM. New targets of neuroprotection in ischemic stroke. *ScientificWorldJournal* 2008; 8: 698-712.

Balami JS, Chen RL, Grunwald IQ, Buchan AM. Neurological complications of acute ischaemic stroke. *Lancet Neurol* 2011; 10: 357-371.

Ballantyne CM, Hoogeveen RC, Bang H, Coresh J, Folsom AR, Chambless LE, Myerson M, Wu KK, Sharrett AR, Boerwinkle E. Lipoprotein-associated phospholipase A2, high-sensitivity C-reactive protein, and risk for incident ischemic stroke in middle-aged men and women in the Atherosclerosis Risk in Communities (ARIC) study. *Arch Intern Med* 2005; 165: 2479-2484.

Bang OY, Saver JL, Buck BH, Alger JR, Starkman S, Ovbiagele B, Kim D, Jahan R, Duckwiler GR, Yoon SR, Viñuela F, Liebeskind DS; UCLA Collateral Investigators. Impact of collateral flow on tissue fate in acute ischaemic stroke. *J Neurol Neurosurg Psychiatry* 2008; 79: 625-629.

Barber PA, Davis SM, Infeld B, Baird AE, Donnan GA, Jolley D, Lichtenstein M. Spontaneous reperfusion after ischemic stroke is associated with improved outcome. *Stroke* 1998; 29:2522-2528.

Ben Assayag E, Shenhar-Tsarfaty S, Ofek K, Soreg L, Bova I, Shopin L, Berg RM, Berliner S, Shapira I, Bornstein NM, Soreg H. Serum cholinesterase activities distinguish between stroke patients and controls and predict 12-month mortality. *Mol Med* 2010; 16: 278-286.

Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 2001; 69: 89-95.

Broderick JP, Palesch YY, Demchuk AM, Yeatts SD, Khatri P, Hill MD, Jauch EC, Jovin TG, Yan B, Silver FL, von Kummer R, Molina CA, Demaerschalk BM, Budzik R, Clark WM, Zaidat OO, Malisch TW, Goyal M, Schonewille WJ, Mazighi M, Engelter ST, Anderson C, Spilker J, Carrozzella J, Ryckborst KJ, Janis LS, Martin RH, Foster LD, Tomsick TA, Interventional Management of Stroke (IMS) III investigators. Endovascular therapy after intravenous t-PA versus t-PA alone for stroke. *N Engl J Med* 2013; 368: 893-903.

Brott T, Adams HP Jr, Olinger CP, Marler JR, Barsan WG, Biller J, Spilker J, Holleran R, Eberle R, Hertzberg V, Rorick M, Moomaw CJ, Walker M. Measurements of acute cerebral infarction: a clinical examination scale. *Stroke* 1989; 20: 864-870.

Brott TG, Haley EC Jr, Levy DE, Barsan W, Broderick J, Sheppard GL, Spilker J, Kongable GL, Massey S, Reed R. Urgent therapy for stroke. Part I. Pilot study of tissue plasminogen activator administered within 90 minutes. *Stroke* 1992; 23: 632-640.

Brunkhorst R, Pfeilschifter W, Foerch C. Astroglial proteins as diagnostic markers of acute intracerebral hemorrhage – Pathophysiological background and clinical findings. *Transl Stroke Res* 2010; 1: 246-251.

Burguillo FJ. El meta-análisis como herramienta de investigación en ciencias de la salud: Medicina basada en la evidencia. VII Congreso SEMTSI, 2010; Salamanca.

Bushnell C. Another score to predict ischemic stroke mortality?. *Circulation* 2011; 123: 712-713.

Bustamante A, Ugarriza I, Giralt D, Espadaler M, Garcia-Berrocoso T, Montaner J. Interleukin 6 in the prediction of functional outcome after ischemic stroke: a literature-based and individual participant data meta-analysis. PROSPERO 2013:CRD42013004434. Available from: http://www.crd.york.ac.uk/PROSPERO_REBRANDING/display_record.asp?ID=CRD42013004434

C

Camilo O, Goldstein LB. Seizures and epilepsy after ischemic stroke. *Stroke* 2004; 35: 1769-1775.

Carandang R, Seshadri S, Beiser A, Kelly-Hayes M, Kase CS, Kannel WB, Wolf PA. Trends in incidence, lifetime risk, severity, and 30-day mortality of stroke over the past 50 years. *JAMA* 2006; 296: 2939-2946.

Carin-Levy G, Mead GE, Nicol K, Rush R, van Wijck F. Delirium in acute stroke: screening tools, incidence rates and predictors: a systematic review. *J Neurol* 2012; 259: 1590-1599.

Castellanos M, Sobrino T, Millán M, García M, Arenillas J, Nombela F, Brea D, Perez de la Ossa N, Serena J, Vivancos J, Castillo J, Dávalos A. Serum cellular fibronectin and matrix metalloproteinase-9 as screening biomarkers for the prediction of parenchymal hematoma after thrombolytic therapy in acute ischemic stroke: a multicenter confirmatory study. *Stroke* 2007; 38: 1855-1859.

Castellanos M, Sobrino T, Pedraza S, Moldes O, Pumar JM, Silva Y, Serena J, García-Gil M, Castillo J, Dávalos A. High plasma glutamate concentrations are associated with infarct growth in acute ischemic stroke. *Neurology* 2008; 71: 1862-1868.

Ceulemans AG, Zgavc T, Kooijman R, Hachimi-Idrissi S, Sarre S, Michotte Y. The dual role of the neuroinflammatory response after ischemic stroke: modulatory effects of hypothermia. *J Neuroinflammation* 2010; 7: 74.

Chamorro A, Horcajada JP, Obach V, Vargas M, Revilla M, Torres F, Cervera A, Planas AM, Mensa J. The Early Systemic Prophylaxis of Infection After Stroke study: A randomized clinical trial. *Stroke* 2005; 36: 1495-1500.

Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. *Nat Rev Immunol* 2010; 10: 301-316.

Choe CU, Atzler D, Wild PS, Carter AM, Böger RH, Ojeda F, Simova O, Stockebrand M, Lackner K, Nabuurs C, Marescau B, Streichert T, Müller C, Lüneburg N, De Deyn PP, Benndorf RA, Baldus S, Gerloff C, Blankenberg S, Heerschap A, Grant PJ, Magnus T, Zeller T, Isbrandt D, Schwedhelm E. Homoarginine levels are regulated by L-arginine:glycine amidinotransferase and affect stroke outcome: results from human and murine studies. *Circulation* 2013; 128: 1451-1461.

Ciccone A, Valvassori L, Nichelatti M, Sgoifo A, Ponzio M, Sterzi R, Boccardi E, for the SYNTHESIS Expansion Investigators. Endovascular treatment for acute ischemic stroke. *N Engl J Med* 2013; 368: 904-913.

Côté R, Hachinski VC, Shurvell BL, Norris JW, Wolfson C. The Canadian Neurological Scale: a preliminary study in acute stroke. *Stroke* 1986; 17: 731-737.

Counsell C, Dennis M. Systematic review of prognostic models in patients with acute stroke. *Cerebrovasc Dis* 2001; 12: 159-170.

Counsell C, Dennis M, McDowall M., Warlow C. Predicting outcome after acute and subacute stroke. Development and validation of new prognostic models. *Stroke* 2002; 33: 1041-1047.

Cuadrado E, Rosell A, Álvarez-Sabín J, Montaner J. Captura por microdisección láser: una nueva herramienta para el estudio de la isquemia cerebral. *Rev Neurol* 2007; 44: 551-555.

Cuadrado E, Rosell A, Colomé N, Hernández-Guillamon M, García-Berrocoso T, Ribó M, Alcazar A, Ortega-Aznar A, Salinas M, Canals F, Montaner-Villalonga J. The proteome of human brain after ischemic stroke. *J Neuropath Exp Neur* 2010; 69: 1105-1115.

Cucchiara BL, Messe SR, Sansing L, MacKenzie L, Taylor RA, Pacelli J, Shah Q, Kasner SE. Lipoprotein-associated phospholipase A2 and C-reactive protein for risk-stratification of patients with TIA. *Stroke* 2009; 40: 2332-2336.

Cucchiara B, Montaner J. Blood biomarkers for predicting outcome after stroke: reading the tea leaves. *Neurology* 2013; 80: 1270-1271.

D

Dayon L, Turck N, García-Berrocoso T, Walter N, Burkhard PR, Vilalta A, Sahuquillo J, Montaner J, Sanchez JC. Brain extracellular fluid protein changes in acute stroke patients. *J Proteome Res* 2011; 10: 1043-1051.

De Marchis GM, Katan M, Weck A, Fluri F, Foerch C, Findling O, Schuetz P, Buhl D, El-Koussy M, Gensicke H, Seiler M, Morgenthaler N, Mattle HP, Mueller B, Christ-Crain M, Arnold M. Copeptin adds prognostic information after ischemic stroke: results from the CoRisk study. *Neurology* 2013; 80: 1278-1286.

De Ridder IR, de Jong FJ, den Hertog HM, Lingsma HF, van Gemert HMA, Schreuder AHCML, Ruitenberg A, Maasland EL, Saxena R, Oomes P, van Tuijl J, Koudstaal PJ, Kappelle LJ, Algra A, van der Worp HB, Dippel DWJ. Paracetamol (Acetaminophen) in stroke 2 (PAIS 2): Protocol for a randomized, placebo-controlled, double-blind clinical trial to assess the effect of high-dose paracetamol on functional

outcome in patients with acute stroke and a body temperature of 36.5ºC or above. *Int J Stroke* 2013; doi: 10.1111/ijs.12053.

Del Zoppo GJ. Stroke and neurovascular protection. *N Engl J Med* 2006; 354: 553-555.

Delgado P, Chacón P, Penalba A, Pelegri D, García-Berrocoso T, Giralt D, Santamarina E, Ribó M, Maisterra O, Alvarez-Sabín J, Rosell A, Montaner J. Lipoprotein-associated phospholipase A(2) activity is associated with large-artery atherosclerotic etiology and recurrent stroke in TIA patients. *Cerebrovasc Dis* 2012; 33: 150-158.

DeLemos JA, McGuire DK, Drazner MH. B-type natriuretic peptide in cardiovascular disease. *Lancet* 2003; 362: 316-322.

DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: A nonparametric approach. *Biometrics* 1988; 44: 837-845.

Den Hertog HM, van der Worp HB, van Gemert HM, Algra A, Kappelle LJ, van Gijn J, Koudstaal PJ, Dippel DW; PAIS Investigators. The Paracetamol (Acetaminophen) In Stroke (PAIS) trial: a multicentre, randomised, placebo-controlled, phase III trial. *Lancet Neurol* 2009; 8: 434-440.

Di Legge S, Koch G, Diomedi M, Stanzione P, Sallustio F. Stroke prevention: Managing modifiable risk factors. *Stroke Res Treat* 2012; 2012: 391538

Díaz-Guzmán J, Egido JA, Gabriel-Sánchez R, Barberá-Comes G, Fuentes-Gimeno B, Fernández-Pérez C, on behalf of the IBERICTUS study investigators of the stroke project of the spanish cerebrovascular diseases study group. Stroke and transient ischemic attack incidence rate in Spain: The IBERICTUS study. *Cerebrovasc Dis* 2012; 34: 272-281.

Dirnagl U, Klehmet J, Braun JS, Harms H, Meisel C, Ziemssen T, Prass K, Meisel A. Stroke-induced immunodepression: experimental evidence and clinical relevance. *Stroke* 2007; 38: 770-773.

Dziedzic T. Clinical significance of acute phase reaction in stroke patients. Front Biosci 2008; 13: 2922-2927.

E

Egido JA, Alonso M, Martínez E, Díez E, por el comité *ad hoc* del Grupo de Estudio de Enfermedades Cerebrovasculares de la SEN. Guía para el tratamiento del infarto cerebral agudo. En: Díez E, ed. Guía para el diagnóstico y tratamiento del ictus, nº 3. Guías oficiales de la Sociedad Española de Neurología (SEN). Barcelona: Prous Science, 2006.

Esmaeili A, Dadkhahfar S, Fadakar K, Rezaei N. Post-stroke immunodeficiency: effects of sensitization and tolerization to brain antigens. *Int Rev Immunol* 2012; 31: 396-409.

Etgen T, Baum H, Sander K, Sander D. Cardiac troponins and N-terminal pro-brain natriuretic peptide in acute ischemic stroke do not relate to clinical prognosis. *Stroke* 2005; 36: 270-275.

F

Feigin VL, Lawes CMM, Bennett DA, Barker-Collo SL, Parag V. Worldwide stroke incidence and early case fatality reported in 56 population-based studies: a systematic review. *Lancet Neurol* 2009; 8: 355-369.

Fluir F, Morgenthaler NG, Mueller B, Christ-Crain M, Katan M. Copeptin, procalcitonin and routine inflammatory markers – predictors of infection after stroke. *PLoS ONE* 2012; 7: e48309.

Fuentes B, Díez E, Gil A, Gil A, Matías J, por el comité *ad hoc* del Grupo de Estudio de Enfermedades Cerebrovasculares de la SEN. Guía para el tratamiento preventivo de la isquemia cerebral. En: Díez E, ed.

Guía para el diagnóstico y tratamiento del ictus, nº 3. Guías oficiales de la Sociedad Española de Neurología (SEN). Barcelona: Prous Science, 2006.

G

Gale SD, Pearson CM. Neuroimaging predictors of stroke outcome: Implications for neurorehabilitation. *NeuroRehabilitation* 2012; 31: 331-344.

García-Berrocoso T, Mendioroz M. Biomarcadores de isquemia cerebral transitoria. En: Montaner J, ed. Ataque isquémico transitorio. Avances en patología neurovascular. Barcelona: Marge Médica Books, 2009.

García-Berrocoso T, Fernández-Cadenas I, Delgado P, Rosell A, Montaner J. Blood biomarkers to identify ischemic stroke etiologies. *Therapy* 2010; 7: 337-353.

German Stroke Study collaboration. Predicting outcome after acute ischemic stroke: An external validation of prognostic models. *Neurology* 2004; 62: 581-585.

Giralt-Steinhauer E, Rodríguez-Campello A, Cuadrado-Godia E, Ois Á, Jiménez-Conde J, Soriano-Tárraga C, Roquer J. External validation of the DRAGON score in an elderly Spanish population: prediction of stroke prognosis after IV thrombolysis. *Cerebrovasc Dis* 2013; 36: 110-114.

Gompertz P, Pound P, Ebrahim S. Predicting stroke outcome: Guy's prognostic score in practice. *J Neurol Neurosurg Psychiatry* 1994; 57: 932-935.

Govan L, Langhorne P, Weir CJ. Does the prevention of complications explain the survival benefit of organized inpatient (stroke unit) care?: Further analysis of a systematic review. *Stroke* 2007; 38: 2536-2540.

Grände PO, Romner B. Osmotherapy in brain edema: a questionable therapy. *J Neurosurg Anesthesiol* 2012; 24: 407-412.

Grape S, Ravussin P. PRO: Osmotherapy for the treatment of acute intracranial hypertension. *J Neurosurg Anesthesiol* 2012; 24: 402-406.

Gray LJ, Ali M, Lyden PD, Bath PMW, for the Virtual International Stroke Trials Archive Collaboration. Interconversion of the National Institutes of Health Stroke Scale and Scandinavian Stroke Scale in acute stroke. *J Stroke Cerebrovasc Dis* 2009; 18: 466-468.

Gröschel K, Schnaudigel S, Edelmann F, Niehaus CF, Weber-Krüger M, Haase B, Lahno R, Seegers J, Wasser K, Wohlfahrt J, Vollmann D, Stahrenberg R, Wachter R. Growth-differentiation factor-15 and functional outcome after acute ischemic stroke. *J Neurol* 2012; 259: 1574-1579.

Grube MM, Koennecke HC, Walter G, Meisel A, Sobesky J, Nolte CH, Wellwood I, Heuschmann PU, on behalf of the Berlin Stroke Register (BSR). Influence of acute complications on outcome 3 months after ischemic stroke. *PloS One* 2013; 8: e75719.

Gursoy-Ozdemir Y, Yemisci M, Dalkara T. Microvascular protection is essential for successful neuroprotection in stroke. *J Neurochem* 2012; 123: 2-11.

H

Hacke W, Schwab S, Horn M, Spranger M, De Georgia M, von Kummer R. 'Malignant' middle cerebral artery territory infarction: clinical course and prognostic signs. *Arch Neurol* 1996; 53: 309-315.

Hacke W, Kaste M, Bluhmki E, Brozman M, Dávalos A, Guidetti D, Larrue V, Lees KR, Medeghri Z, Machnig T, Schneider D, von Kummer R, Wahlgren N, Toni D, for the ECASS Investigators. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N Engl J Med* 2008; 359: 1317-1329.

Hackett ML, Yapa C, Parag V, Anderson CS. Frequency of depression after stroke: a systematic review of observational studies. *Stroke* 2005; 36: 1330-1340.

Hantson L, De Weerdt W, De Keyser J, Diener HC, Franke C, Palm R, Van Orshoven M, Schoonderwalt H, De Klippel N, Herroelen L. The European Stroke Scale. *Stroke* 1994; 25: 2215-2219.

Hasan N, McColgan P, Bentley P, Edwards RJ, Sharma P. Towards the identification of blood biomarkers for acute stroke in humans: a comprehensive systematic review. *Br J Clin Pharmacol* 2012; 74: 230-240.

Hedges LV, Olkin I. Statistical methods for meta-analysis. Orlando: Academic Press, Inc., 1985.

Heiss WD. The concept of the penumbra: can it be translated to stroke management?. *Int J Stroke* 2010; 5: 290-295.

Hendrix S, Nitsch R. The role of T helper cells in neuroprotection and regeneration. *J Neuroimmunol* 2007; 184: 100-112.

Hlatky MA, Greenland P, Arnett DK, Ballantyne CM, Criqui MH, Elkind MSV, Go AS, Harrell FE, Hong Y, Howard BV, Howard VJ, Hsue PY, Kramer CM, McConnell JP, Normand SLT, O'Donnell CJ, Smith SC Jr, Wilson PWF, on behalf of the American Heart Association expert panel on subclinical atherosclerotic diseases and emerging risk factors and the Stroke Council. *Circulation* 2009; 119: 2408-2416.

Hoffmann S, Malzahn U, Harms H, Koennecke HC, Berger K, Kalic M, Walter G, Meisel A, Heuschmann PU for the Berlin Stroke Register and the Stroke Register of Northwest Germany. Development of a clinical score (A^2DS^2) to predict pneumonia in acute ischemic stroke. *Stroke* 2012; 43: 2617-2623.

Hollander M, Koudstaal PJ, Bots ML, Grobbee DE, Hofman A, Breteler MMB. Incidence, risk, and case fatality of first ever stroke in the elderly population. The Rotterdam Study. *J Neurol Neurosurg Psychiatry* 2003; 74: 317-321.

Hosmer DW Jr, Lemeshow S, Sturdivant RX. Introduction to the logistic regression model. En: Hosmer DW, ed. Applied logistic regression, 3ª ed. Hoboken, New Jersey: John Wiley & Sons, Inc., 2013.

Huang JM, Hu J, Chen N, Hu ML. Relationship between plasma high-mobility group box-1 levels and clinical outcomes of ischemic stroke. *J Crit Care* 2013; 28: 792-797.

Huang P, Lo LH, Chen YC, Lin RT, Shiea J, Liu CK. Serum free haemoglobin as a novel potential biomarker for acute ischemic stroke. *J Neurol* 2009; 256: 625-631.

I

Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, Yoshie O. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int Immunol* 1999; 11: 81-88.

J

Jauch EC, Saver JL, Adams HP Jr, Bruno A, Connors JJ, Demaerschalk BM, Khatri P, McMullan PW Jr, Qureshi AI, Rosenfield K, Scott PA, Summers DR, Wang DZ, Wintermark M, Yonas H; American Heart Association Stroke Council; Council on Cardiovascular Nursing; Council on Peripheral Vascular Disease; Council on Clinical Cardiology. Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke* 2013; 44: 870-947.

Jennett B, Bond M. Assessment of outcome after severe brain damage. *Lancet* 1975; 1: 480-484.

Ji R, Shen H, Pan Y, Wang P, Liu G, Wang Y, Li H, Wang Y, on behalf of China National Stroke Registry Investigators. Novel risk score to predict pneumonia after acute ischemic stroke. *Stroke* 2013; 44: 1303-1309.

Jickling GC, Sharp FR. Blood biomarkers of ischemic stroke. *Neurotherapeutics* 2011; 8: 349-360.

Johnston KC, Connors AF, Wagner DP, Knaus WA, Wang XQ, Clarke Haley E Jr, for the randomized trial of tirilazad mesylate in acute stroke (RANTTAS) investigators. A predictive risk model for outcomes of ischemic stroke. *Stroke* 2000; 31: 448-455.

Johnston KC, Connors AF, Wagner DP, Haley EC Jr. Predicting outcome in ischemic stroke. External validation of predictive risk models. *Stroke* 2003; 34: 200-202.

Johnston SC, Mendis S, Mathers CD. Global variation in stroke burden and mortality: estimates from monitoring, surveillance, and modelling. *Lancet Neurol* 2009; 8: 345-354.

Jones AP, Riley RD, Williamson PR, Whitehead A. Meta-analysis of individual patient data versus aggregate data from longitudinal clinical trials. *Clin Trials* 2009; 6: 16-27.

K

Kaplan RC, Tirschwell DL, Longstreth WT Jr., Manolio TA, Heckbert SR, Lefkowitz D, El-Saed A, Psaty BM. Vascular events, mortality, and preventive therapy following ischemic stroke in the elderly. *Neurology* 2005; 65: 835-842.

Katan M, Elkind MSV. Inflammatory and neuroendocrine biomarkers of prognosis after ischemic stroke. *Expert Rev Neurother* 2011; 11: 225-239.

Kendziorski C, Irizarry RA, Chen KS, Haaq JD, Gould MN. On the utility of pooling biological samples in microarray experiments. *Proc Natl Acad Sci USA* 2005; 102: 4252-4257.

Kent DM, Selker HP, Ruthazer R, Bluhmki E, Hacke W. The Stroke-Thrombolytic predictive instrument: A predictive instrument for intravenous thrombolysis in acute ischemic stroke. *Stroke* 2006; 37: 2957-2962.

Khan R, Nael K, Erly W. Acute stroke imaging: what clinicians need to know. *Am J Med* 2013; 126: 379-386.

Kolias AG, Kirkpatrick PJ, Hutchinson PJ. Decompressive craniectomy: past, present and future. *Nat Rev Neurol* 2013; 9: 405-415.

König IR, Ziegler A, Bluhmki E, Hacke W, Bath PMW, Sacco RL, Diener HC, Weimar C, on behalf of the Virtual International Stroke Trials Archive (VISTA) investigators. Predicting long-term outcome after acute ischemic stroke. A simple index works in patients from controlled clinical trials. *Stroke* 2008; 39: 1821-1826.

Kruetzelmann A, Köhrmann M, Sobesky J, Cheng B, Rosenkranz M, Röther J, Schellinger PD, Ringleb P, Gerloff C, Fiehler J, Thomalla G. Pretreatment Diffusion-Weighted Imaging lesion volume predicts favorable outcome after intravenous thrombolysis with tissue-type plasminogen activator in acute ischemic stroke. *Stroke* 2011; 42: 1251-1254.

Kumar S, Selim MH, Caplan LR. Medical complications after stroke. *Lancet Neurol* 2010; 9: 105-118.

Kwok CS, Potter JF, Dalton G, George A, Metcalf AK, Ngeh J, Nicolson A, Owusu-Agyei P, Shekhar R, Walsh K, Warburton EA, Myint PK, on behalf of the Anglia Stroke Clinical Network Evaluation Study (ASCNES) group. The SOAR stroke score predicts inpatient and 7-day mortality in acute stroke. *Stroke* 2013; 44: 2010-2012.

L

Laborde CM, Mourino-Alvarez L, Akerstrom F, Padial LR, Vivanco F, Gil-Dones F, Barderas MG. Potential blood biomarkers for stroke. *Expert Rev Proteomics* 2012; 9: 437-449.

Larrue V, von Kummer R, del Zoppo G, Bluhmki E. Hemorrhagic transformation in acute ischemic stroke. Potential contributing factors in the European Cooperative Acute Stroke Study. *Stroke* 1997; 28: 957-960.

Laskowitz DT, Kasner SE, Saver J, Remmel KS, Jauch EC, BRAIN study group. Clinical usefulness of a biomarker-based diagnostic test for acute stroke: The Biomarker Rapid Assessment in Ischemic Injury (BRAIN) study. *Stroke* 2009; 40: 77-85.

Lee J, Morishima T, Kunisawa S, Sasaki N, Otsubo T, Ikai H, Imanaka Y. Derivation and validation of in-hospital mortality prediction models in ischaemic stroke patients using administrative data. *Cerebrovasc Dis* 2013; 35: 73-80.

Lewington S, Bragg F, Clarke R. A review on metaanalysis of biomarkers: promises and pitfalls. *Clin Chem* 2012; 58: 1192-1204.

Leys D, Hénon H, Mackowiak-Cordoliani MA, Pasquier F. Poststroke dementia. *Lancet Neurol* 2005; 4: 752-759.

Lindley R, Wardlaw JM, Sandercock PAG, Rimdusid P, Lewis SC, Signorini DF, Ricci S. Frequency and risk factors for spontaneous hemorrhagic transformation of cerebral infarction. *J Stroke Cerebrovasc Dis* 2004; 13: 235-246.

Liu G, Ntaios G, Zheng H, Wang Y, Michel P, Wang DZ, Fang J, Papavasileiou V, Liu L, Dong K, Wang C, Zhao X, Wang Y. External validation of the ASTRAL score to predict 3- and 12-month functional outcome in the China National Stroke registry. *Stroke* 2013; 44: 1443-1445.

Lou M, Safdar A, Mehdiratta M, Kumar S, Schlaug G, Caplan L, Searls D, Selim M. The HAT score: a simple grading scale for predicting hemorrhage after thrombolysis. *Neurology* 2008; 71: 1417-1423.

M

Mackay CR. Chemokines: immunology's high impact factors. *Nat Immunol* 2001; 2: 95-101.

Mahoney FI, Barthel DW. Functional evaluation: The Barthel Index. *Md State Med J* 1965; 14: 61-65.

Marcucci M, Smith CT, Douketis JD, Tosetto A, Baglin T, Cushman M, Eichinger S, Palareti G, Poli D, Tait RC, Iorio A. Patient-level compared with study-level meta-analyses demonstrate consistency of D-dimer as predictor of venous thromboembolic recurrences. *J Clin Epidemiol* 2013; 66: 415-425.

Mauri-Capdevila G, Jove M, Suarez-Luis I, Portero-Otin M, Purroy F. La metabolómica en el ictus isquémico, nuevos biomarcadores diagnósticos y pronósticos. *Rev Neurol* 2013; 57: 29-36.

Mellick GD, Silburn PA, Sutherland GT, Siebert GA. Exploiting the potential of molecular profiling in Parkinson's disease: current practice and future probabilities. *Expert Rev Mol Diagn* 2010; 10: 1035-1050.

Men X, Li J, Zhang B, Zhang L, Li H, Lu Z. Homocysteine and C-reactive protein associated with progression and prognosis of intracranial branch atheromatous disease. *PLoS One* 2013; 8: e73030.

Menon BK, Saver JL, Prabhakaran S, Reeves M, Liang L, Olson DWM, Peterson ED, Hernandez AF, Fonarow GC, Schwamm LH, Smith EE. Risk score for intracranial hemorrhage in patients with acute ischemic stroke treated with intravenous tissue-type plasminogen activator. *Stroke* 2012; 43: 2293-2299.

Molina CA, Chamorro A, Rovira A, de Miguel A, Serena J, Roman LS, Jovin TG, Davalos A, Cobo E. REVASCAT: a randomized trial of revacularization with SOLITAIRE FR® device vs. best medical therapy in the treatment of acute stroke due to anterior circulation large vessel occlusion presenting within eight-hours of symptom onset. *Int J Stroke* 2013; doi: 10.1111/ijs.12157.

Montaner J, Molina CA, Monasterio J, Abilleira S, Arenillas JF, Ribó M, Quintana M, Alvarez-Sabín J. Matrix metalloproteinase-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis in human stroke. *Circulation* 2003; 107: 598-603.

Montaner J, Mendioroz M, Ribó M, Delgado P, Quintana M, Penalba A, Chacón P, Molina C, Fernández-Cadenas I, Rosell A, Alvarez-Sabín J. A panel of biomarkers including caspase-3 and D-dimer may differentiate acute stroke from stroke-mimicking conditions in the emergency department. *J Intern Med* 2011; 270: 166-174.

Moons KGM, Royston P, Vergouwe Y, Grobbee DE, Altman DG. Prognosis and prognostic research: what, why and how?. *BMJ* 2009; 338: b375.

Morancho A, Rosell A, García-Bonilla L, Montaner J. Metalloproteinase and stroke infarct size: role for anti-inflammatory treatment?. *Ann N Y Acad Sci* 2010; 1207: 123-133.

Mullen MT, Pisapia JM, Tilwa S, Messé SR, Stein SC. Systematic review of outcome after ischemic stroke due to anterior circulation occlusion treated with intravenous, intra-arterial, or combined intravenous + intra-arterial thrombolysis. *Stroke* 2012; 43: 2350-2355.

Müller-Barna P, Schwamm LH, Haberl RL. Telestroke increases use of acute stroke therapy. *Curr Opin Neurol* 2012; 25: 5-10.

Muscari A, Puddu GM, Santoro N, Zoli M. A simple scoring system for outcome prediction of ischemic stroke. *Acta Neurol Scand* 2011; 124: 334-342.

Myint PK, Clark AB, Kwok CS, Davis J, Durairaj R, Dixit AK, Sharma AK, Ford GA, Potter JF. A simple 8-point score strongly predicts early outcomes in acute stroke. *Int J Stroke* 2013; Jul 9. doi: 10.1111/ijs.12088.

N

Nederkoorn PJ, Westendorp WF, Hooijenga IJ, de Haan RJ, Dippel DW, Vermeij FH, Dijkgraaf MG, Prins JM, Spanjaard L, van de Beek D. Preventive antibiotics in stroke study: rationale and protocol for a randomised trial. *Int J Stroke* 2011; 6: 159-163.

Ni X, Castanares M, Mukherjee A, Lupold SE. Nucleic acid aptamers: clinical applications and promising new horizons. *Curr Med Chem* 2011; 18: 4206-4214.

Nilanont Y, Komoltri C, Saposnik G, Côté R, Di Legge S, Jin Y, Prayoonwiwat N, Poungvarin N, Hachinski V. The Canadian Neurological Scale and the NIHSS: development and validation of a simple conversion model. *Cerebrovasc Dis* 2010; 30: 120-126.

Ning M, Lopez M, Cao J, Buonanno FS, Lo EH. Application of proteomics to cerebrovascular disease. *Electrophoresis* 2012; 33: 3582-3597.

Ntaios G, Faouzi M, Ferrari J, Lang W, Vemmos K, Michel P. An integer-based score to predict functional outcome in acute ischemic stroke: The ASTRAL score. *Neurology* 2012; 78: 1916-1922.

O

O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells DW. 1,026 experimental treatments in acute stroke. *Ann Neurol* 2006; 59: 467-477.

O'Donnell MJ, Fang J, D'Uva C, Saposnik G, Gould L, McGrath E, Kapral MK, for the investigators of the registry of the Canadian Stroke Network. The PLAN score. A bedside prediction rule for death and severe disability following acute ischemic stroke. *Arch Intern Med* 2012; 172: 1548-1556.

Olasagasti F, Ruiz de Gordoa JC. Miniaturized technology for protein and nucleic acid point-of-care testing. *Transl Res* 2012; 160: 332-345.

P

Paciaroni M, Agnelli G, Floridi P, Alberti A, Acciarresi M, Venti M, Alagia MG, Fiacca A, Gallina MC, Guercini G, Pantaleoni R, Leone F, Pieroni A, Caso V. Hyperdense middle cerebral and/or internal carotid arteries in acute ischemic stroke: rate, predictive factors and influence on clinical outcome. *Cerebrovasc Dis* 2011; 32: 239-245.

Papavasileiou V, Milionis H, Michel P, Makaritsis K, Vemmou A, Koroboki E, Manios E, Vemmos K, Ntaios G. ASTRAL score predicts 5-year dependence and mortality in acute ischemic stroke. *Stroke* 2013; 44: 1616-1620.

Park TH, Saposnik G, Bae HJ, Lee SJ, Lee KB, Lee J, Park JM, Choi JC, Kim DE, Cho YJ, Kim JT, Cha JK, Lee J, Yu KH, Lee BC, Yoon BW. The iScore predicts functional outcome in Korean patients with ischemic stroke. *Stroke* 2013; 44: 1440-1442.

Pencina MJ, D'Agostino RB Sr, D'Agostino RB Jr, Vasan RS. Evaluating the added predictive ability of a new marker: From area under the ROC curve to reclassification and beyond. *Statist Med* 2008; 27: 157-172.

Pepe MS, Janes H, Longton G, Leisenring W, Newcomb P. Limitations of the odds ratio in gauging the performance of a diagnostic, prognostic, or screening marker. *Am J Epidemiol* 2004; 159: 882-890.

Pereira VM, Narata AP, Gonzalez AM, Sztajzel R, Lovblad KO. Use of stentrievers in acute stroke: tips, tricks, and current results. *Tech Vasc Interv Radiol* 2012; 15: 68-77.

Péron S, Metin A, Gardès P, Alyanakian MA, Sheridan E, Kratz CP, Fischer A, Durandy A. Human PMS2 deficiency is associated with impaired immunoglobulin class switch recombination. *J Exp Med* 2008; 205: 2465-2472.

Phan TG, Chen J, Donnan G, Srikanth V, Wood A, Reutens DC. Development of a new tool to correlate stroke outcome with infarct topography: A proof-of-concept study. *NeuroImage* 2010; 49: 127-133.

Pickering JW, Endre ZH. New metrics for assessing diagnostic potential of candidate biomarkers. *Clin J Am Soc Nephrol* 2012; 7: 1355-1364.

Poca MA, Sahuquillo J, Vilalta A, De los Rios J, Robles A, Exposito L. Percutaneous implantation of cerebral microdialysis catheters by twist-drill craniostomy in neurocritical patients: Description of the technique and results of a feasibility study in 97 patients. *J Neurotrauma* 2006; 23: 1510-1517.

R

Ray S, Lukyanov P, Ochieng J. Members of the cystatin superfamily interact with MMP-9 and protect it from autolytic degradation without affecting its gelatinolytic activities. *Biochem Biophys Acta* 2003; 1652: 91-102.

Reid JM, Gubitz GJ, Dai D, Reidy Y, Christian C, Counsell C, Dennis M, Phillips SJ. External validation of a six simple variable model of stroke outcome and verification in hyper-acute stroke. *J Neurol Neurosurg Psychiatry* 2007; 78: 1390-1391.

Reid JM, Gubitz GJ, Dai D, Kydd D, Eskes G, Reidy Y, Christian C, Counsell CE, Dennis M, Phillips SJ. Predicting functional outcome after stroke by modeling baseline clinical and CT variables. *Age Ageing* 2010; 39: 360-366.

Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat Biotechnol* 2006; 24: 971-983.

Riley RD, Lambert PC, Abo-Zaid G. Meta-analysis of individual participant data: rationale, conduct, and reporting. *BMJ* 2010; 340: c221.

Rivero P, por el grupo de trabajo de la Guía de Práctica Clínica para el manejo de pacientes con ictus en atención primaria. Guía de Práctica Clínica para el manejo de pacientes con ictus en atención primaria. *Plan de calidad para el Sistema Nacional de Salud del Ministerio de Sanidad y Política Social. Unidad de evaluación de tecnologías sanitarias de la agencia Laín Entralgo de la comunidad de Madrid* 2009; UETS Nº 2007/5-2.

Robin X, Turck N, Hainard A, Lisacek F, Sanchez JC, Müller M. Bioinformatics for protein biomarker panel classification: what is needed to bring biomarker panels into *in vitro* diagnostics?. *Expert Rev Proteomics* 2009; 6: 675-689.

Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, Müller M. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 2011; 12: 77.

Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, Müller M. PanelomiX: A threshold-based algorithm to create panels of biomarkers. *Transl Proteomics* 2013; 1: 57-64.

Rodríguez-Yáñez M, Castellanos M, Sobrino T, Brea D, Ramos-Cabrer P, Pedraza S, Castiñeiras JA, Serena J, Dávalos A, Castillo J, Blanco M. Interleukin-10 facilitates the selection of patients for systemic thrombolysis. *BMC Neurol* 2013; 13: 62.

Rodseth RN, Biccard BM, Chu R, Lurati Buse GA, Thabane L, Bakhai A, Bolliger D, Cagini L, Cahill TJ, Cardinale D, Chong CP, Cnotliwy M, Di Somma S, Fahrner R, Lim WK, Mahla E, Le Manach Y, Manikandan R, Pyun WB, Rajagopalan S, Radovic M, Schutt RC, Sessler DI, Suttie S, Vanniyasingam T, Waliszek M, Devereaux PJ. Postoperative B-type natriuretic peptide for prediction of major cardiac events in patients undergoing noncardiac surgery: systematic review and individual patient meta-analysis. *Anesthesiology* 2013; 119: 270-283.

Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, Bravata DM, Dai S, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Soliman EZ, Sorlie PD, Sotoodehnia N, Turan TN, Virani SS, Wong ND, Woo D, Turner MB. Heart disease and stroke statistics--2012 update: A report from the american heart association. *Circulation* 2012; 125: e2-e220.

Rosell A, Cuadrado E, Alvarez-Sabín J, Hernández-Guillamon M, Delgado P, Penalba A, Mendioroz M, Rovira A, Fernández-Cadenas I, Ribó M, Molina CA, Montaner J. Caspase-3 is related to infarct growth after human ischemic stroke. *Neurosci Lett* 2008; 430: 1-6.

Rost NS, Biffi A, Cloonan L, Chorba J, Kelly P, Greer D, Ellinor P, Furie KL. Brain natriuretic peptide predicts functional outcome in ischemic stroke. *Stroke* 2012; 43: 441-445.

Rubin MN, Wellik KE, Channer DD, Demaerschalk BM. Systematic review of telestroke for post-stroke care and rehabilitation. *Curr Atheroscler Rep* 2013; 15: 343.

Ruiz V, Ramón N, Juan Ó, Tembl J. Unidades de ictus: más supervivencia. Una revisión sistemática. *Med Clin (Barc)* 2005; 124: 22-29.

Ryu WS, Kim CK, Kim BJ, Kim C, Lee SH, Yoon BW. Pentraxin 3: a novel and independent prognostic marker in ischemic stroke. *Atherosclerosis* 2012; 220: 581-586.

S

Saposnik G, Raptis S, Kapral MK, Liu Y, Tu JV, Mamdani M, Austin PC, on behalf of the investigators of the registry of the Canadian Stroke Network and the Stroke Outcomes Research Canada working group. The iScore predicts poor functional outcomes early after hospitalization for an acute ischemic stroke. *Stroke* 2011; 42: 3421-3428.

Saposnik G, Kapral MK, Liu Y, Hall R, O'Donnell M, Raptis S, Tu JV, Mamdani M, Austin PC, on behalf of the investigators of the registry of the Canadian Stroke Network and the Stroke Outcomes Research Canada (SORCan) working group. IScore: A risk score to predict death early after hospitalization for an acute ischemic stroke. *Circulation* 2011; 123: 739-749.

Saposnik G, Cote R, Mamdani M, Raptis S, Thorpe KE, Fang J, Redelmeier DA, Goldstein LB. JURaSSIC: Accuracy of clinician vs risk score prediction of ischemic stroke outcomes. *Neurology* 2013; 81: 448-455.

Scandinavian Stroke Study group. Multicenter trial of hemodilution in ischemic stroke. Background and study protocol. *Stroke* 1985; 16: 885-890.

Schulze J, Zierath D, Tanzi P, Cain K, Shibata D, Dressel A, Becker K. Severe stroke induces long-lasting alterations of high-mobility group box 1. *Stroke* 2013; 44: 246-248.

Schwab S, Vatankhah B, Kukla C, Hauchwitz M, Bogdahn U, Fürst A, Audebert HJ, Horn M, on behalf of the TEMPiS group. Long-term outcome after thrombolysis in telemedical stroke care. *Neurology* 2007; 69: 898-903.

SCOPE (Stroke Complications and Outcomes Prediction Engine) collaborations; IST, Lewis SC, Sandercock PA, Dennis MS. Predicting outcome in hyper-acute stroke: validation of a prognostic model in the Third International Stroke Trial (IST3). *J Neurol Neurosurg Psychiatry* 2008; 79: 397-400.

Sham P, Bader JS, Craig I, O'Donovan M, Owen M. DNA pooling: a tool for large-scale association studies. *Nat Rev Genet* 2002; 3: 862-871.

Sharp FR, Jickling GC, Stamova B, Tian Y, Zhan X, Liu D, Kuczynski B, Cox CD, Ander BP. Molecular markers and mechanisms of stroke: RNA studies of blood in animals and humans. *J Cereb Blood Flow Metab* 2011; 31: 1513-1531.

Shibazaki K, Kimura K, Okada Y, Iguchi Y, Uemura J, Terasawa Y, Aoki J. Plasma brain natriuretic peptide as an independent predictor of in-hospital mortality after acute ischemic stroke. *Intern Med* 2009; 48: 1601-1606.

Shibazaki K, Kimura K, Iguchi Y, Aoki J, Sakai K, Kobayashi K. Plasma brain natriuretic peptide predicts death during hospitalization in acute ischaemic stroke and transient ischaemic attack patients with atrial fibrillation. *Eur J Neurol* 2011; 18: 165-169.

Siegler JE, Martin-Schild S. Early neurological deterioration (END) after stroke: the END depends on the definition. *Int J Stroke* 2011; 6: 211-212.

Smedbakken L, Jensen JK, Hallén J, Atar D, Januzzi JL, Halvorsen B, Aukrust P, Ueland T. Activated leukocyte cell adhesion molecule and prognosis in acute ischemic stroke. *Stroke* 2011; 42: 2453-2458.

Smith EE, Shobha N, Dai D, Olson DWM, Reeves MJ, Saver JL, Hernandez AF, Peterson ED, Fonarow GC, Schwamm LH. Risk score for in-hospital ischemic stroke mortality derived and validated within the Get With the Guidelines – stroke program. *Circulation* 2010; 122: 1496-1504.

Solberg OG, Dahl M, Mowinckel P, Stavem K. Derivation and validation of a simple risk score for predicting 1-year mortality in stroke. *J Neurol* 2007; 254: 1376-1383.

Strbian D, Meretoja A, Ahlhelm FJ, Pitkäniemi J, Lyrer P, Kaste M, Engelter S, Tatlisumak T. Predicting outcome of IV thrombolysis-treated ischemic stroke patients: The DRAGON score. *Neurology* 2012; 78: 427-432.

Strbian D, Engelter S, Michel P, Meretoja A, Sekoranja L, Ahlhelm FJ, Mustanoja S, Kuzmanovic I, Sairanen T, Forss N, Cordier M, Lyrer P, Kaste M, Tatlisumak T. Symptomatic intracranial hemorrhage after stroke thrombolysis: the SEDAN score. *Ann Neurol* 2012; 71: 634-641.

Strbian D, Seiffge DJ, Breuer L, Numminen H, Michel P, Meretoja A, Coote S, Bordet R, Obach V, Weder B, Jung S, Caso V, Curtze S, Ollikainen J, Lyrer PA, Eskandari A, Mattle HP, Chamorro A, Leys D, Bladin C, Davis SM, Köhrmann M, Engelter ST, Tatlisumak T. Validation of the DRAGON score in 12 stroke centers in anterior and posterior circulation. *Stroke* 2013; 44: 2718-2721.

Stroke Therapy Academic Industry Roundtable (STAIR). Recommendations for standards regarding preclinical neuroprotective and restorative drug development. *Stroke* 1999; 30: 2752-2758.

T

Taylor CF, Field D, Sansone SA, Aerts J, Apweiler R, Ashburner M, Ball CA, Binz PA, Bogue M, Booth T, Brazma A, Brinkman RR, Clark AM, Deutsch EW, Fiehn O, Fostel J, Ghazal P, Gibson F, Gray T, Grimes G, Hancock JM, Hardy NW, Hermjakob H, Julian RK Jr, Kane M, Kettner C, Kinsinger C, Kolker E, Kuiper M, Le Novère N, Leebens-Mack J, Lewis SE, Lord P, Mallon AM, Marthandan N, Masuya H, McNally R, Mehrle A, Morrison N, Orchard S, Quackenbush J, Reecy JM, Robertson DG, Rocca-Serra P, Rodriguez H, Rosenfelder H, Santoyo-Lopez J, Scheuermann RH, Schober D, Smith B, Snape J, Stoeckert CJ Jr, Tipton K, Sterk P, Untergasser A, Vandesompele J, Wiemann S. Promoting coherent minimum reporting guidelines for biological and biomedical investigations: the MIBBI project. *Nat Biotechnol* , 2008; 26: 889-896.

Thanvi B, Treadwell S, Robinson T. Early neurological deterioration in acute ischaemic stroke: predictors, mechanism and management. *Postgrad Med J* 2008; 84: 412-417.

Theodorou GL, Marousi S, Ellul J, Mougiou A, Theodori E, Mouzaki A, Karakantza M. T helper 1 (Th1)/Th2 cytokine expression shift of peripheral blood CD4+ and CD8+ T cells in patients at the post-acute phase of stroke. *Clin Exp Immunol* 2008; 152: 456-463.

Thevis M, Volmer DA. Recent instrumental progress in mass spectrometry: advancing resolution, accuracy, and speed of drug detection. *Drug Test Anal* 2012; 4: 242-245.

Traylor M, Farrall M, Holliday EG, Sudlow C, Hopewell JC, Cheng YC, Fornage M, Ikram MA, Malik R, Bevan S, Thorsteinsdottir U, Nalls MA, Longstreth W, Wiggins KL, Yadav S, Parati EA, Destefano AL, Worrall BB, Kittner SJ, Khan MS, Reiner AP, Helgadottir A, Achterberg S, Fernandez-Cadenas I, Abboud S, Schmidt R, Walters M, Chen WM, Ringelstein EB, O'Donnell M, Ho WK, Pera J, Lemmens R, Norrving B, Higgins P, Benn M, Sale M, Kuhlenbäumer G, Doney AS, Vicente AM, Delavaran H, Algra A, Davies G, Oliveira SA, Palmer CN, Deary I, Schmidt H, Pandolfo M, Montaner J, Carty C, de Bakker PI, Kostulas K, Ferro JM, van Zuydam NR, Valdimarsson E, Nordestgaard BG, Lindgren A, Thijs V, Slowik A, Saleheen D, Paré G, Berger K, Thorleifsson G; Australian StrokeGenetics Collaborative, Wellcome Trust Case Control Consortium 2 (WTCCC2), Hofman A, Mosley TH, Mitchell BD, Furie K, Clarke R, Levi C, Seshadri S, Gschwendtner A, Boncoraglio GB, Sharma P, Bis JC, Gretarsdottir S, Psaty BM, Rothwell PM, Rosand J, Meschia JF, Stefansson K, Dichgans M, Markus HS; International Stroke Genetics Consortium. Genetic risk factors for ischaemic stroke and its subtypes (the METASTROKE collaboration): a meta-analysis of genome-wide association studies. *Lancet Neurol* 2012; 11: 951-962.

Tsivgoulis G, Eggers J, Ribo M, Perren F, Saqqur M, Rubiera M, Sergentanis TN, Vadikolias K, Larrue V, Molina CA, Alexandrov AV. Safety and efficacy of ultrasound-enhanced thrombolysis: a comprehensive review and meta-analysis of randomized and nonrandomized studies. *Stroke* 2010; 41: 280-287.

Tsivgoulis G, Apostolidou N, Giannopoulos S, Sharma VK. Hemodynamic causes of deterioration in acute ischemic stroke. Perspectives in Medicine 2012; 1: 177-184.

Tu WJ, Dong X, Zhao SJ, Yang DG, Chen H. Prognostic value of plasma neuroendocrine biomarkers in patients with acute ischaemic stroke. *J Neuroendocrinol* 2013; 25: 771-778.

Turner RC, Dodson SC, Rosen CL, Huber JD. The science of cerebral ischemia and the quest for neuroprotection: navigating past failure to future success. A review. *J Neurosurg* 2013; 118: 1072-1085.

U

Ulm L, Ohlraun S, Harms H, Hoffmann S, Klehmet J, Ebmeyer S, Hartmann O, Meisel C, Anker SD, Meisel A. STRoke Adverse outcome is associated WIth NoSocomial Infections (STRAWINSKI): procalcitonin ultrasensitive-guided antibacterial therapy in severe ischaemic stroke patients – rationale and protocol for a randomized controlled trial. *Int J Stroke* 2013; 8: 598-603.

Üstündağ M, Orak M, Güloğlu C, Öztürk E, Tamam Y, Kale E. The role of serum ferritin, pro-brain natriuretic peptide and homocysteine levels in determining ischaemic stroke subtype, severity and mortality. *Hong Kong J of Emergency Medicine* 2010; 17: 13-21.

Uyttenboogaart M, Stewart RE, Vroomen PCAJ, De Keyser J, Luijckx GJ. Optimizing cutoff scores for the Barthel index and the modified Rankin scale for defining outcome in acute stroke trials. *Stroke* 2005; 36: 1984-1987.

Uyttenboogaart M, Stewart RE, Vroomen PC, Luijckx GJ, De Keyser J. Utility of the stroke-thrombolytic predictive instrument. *J Neurol Neurosurg Psychiatry* 2008; 79: 1079-1081.

V

Van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJ, van Gijn J. Interobserver agreement for the assessment of handicap in stroke patients. *Stroke* 1988; 19: 604-607.

Vangilder RL, Davidov DM, Stinehart KR, Huber JD, Turner RC, Wilson KS, Haney E, Davis SM, Chantler PD, Theeke L, Rosen CL, Crocco TJ, Gutmann L, Barr TL. C-reactive protein and long-term ischemic stroke prognosis. *J Clin Neurosci* 2013; Aug 23. doi: 10.1016/j.jocn.2013.06.015.

Vogelgesang A, Becker KJ, Dressel A. Immunological consequences of ischemic stroke. *Acta Neurol Scand* 2013; Jul 15. doi: 10.1111/ane.12165.

W

Wang Y, Lim LLY, Heller RF, Fisher J, Levi CR. A prediction model of 1-year mortality for acute ischemic stroke patients. *Arch Phys Med Rehabil* 2003; 84: 1006-1111.

Wang Y, Wang Y, Yang GY. MicroRNAs in cerebral ischemia. *Stroke Res Treat* 2013; 2013: 276540.

Weber JE, Ebinger M, Rozanski M, Waldschmidt C, Wendt M, Winter B, Kellner P, Baumann A, Fiebach JB, Villringer K, Kaczmarek S, Endres M, Audebert HJ, STEMO-Consortium. Prehospital thrombolysis in acute stroke: results of the PHANTOM-S pilot study. *Neurology* 2013; 80: 163-168.

Weimar C, Ziegler A, König IR, Diener HC, on behalf of the German Stroke Study collaborators. Predicting functional outcome and survival after acute ischemic stroke. *J Neurol* 2002; 249: 888-895.

Weimar C, König IR, Kraywinkel K, Ziegler A, Diener HC, on behalf of the German Stroke Study collaboration. Age and National Institutes of Health Stroke Scale score within 6 hours after onset are accurate predictors of outcome after cerebral ischemia. Development and external validation of prognostic models. *Stroke* 2004; 35: 158-162.

Weisscher N, Vermeulen M, Roos YB, de Haan RJ. What should be defined as good outcome in stroke trials; a modified Rankin score of 0-1 or 0-2?. *J Neurol* 2008; 255: 867-874.

Westendorp WF, Nederkoorn PJ, Vermeij JD, Dijkgraaf MG, van de Beek D. Post-stroke infection: A systematic review and meta-analysis. *BMC Neurol* 2011; 11: 110.

Westendorp WF, Vermeij JD, Vermeij F, Den Hertog HM, Dippel DWJ, van de Beek D, Nederkoorn PJ. Antibiotic therapy for preventing infections in patients with acute stroke. *Cochrane Database Syst Rev* 2012; 1: CD008530.

Whiteley W, Tseng MC, Sandercock P. Blood biomarkers in the diagnosis of ischemic stroke: A systematic review. *Stroke* 2008; 39: 2902-2909.

Whiteley W, Chong WL, Sengupta A, Sandercock P. Blood markers for the prognosis of ischemic stroke: a systematic review. *Stroke* 2009; 40: e380-e389.

Whiteley W, Wardlaw J, Dennis M, Lowe G, Rumley A, Sattar N, Welsh P, Green A, Andrews M, Sandercock P. The use of blood biomarkers to predict poor outcome after acute transient ischemic attack or ischemic stroke. *Stroke* 2012; 43: 86-91.

Williams GR, Jiang JG. Development of an ischemic stroke survival score. *Stroke* 2000; 31: 2414-2420.

Wolf ME, Sauer T, Alonso A, Hennerici MG. Comparison of the new ASCO classification with the TOAST classification in a population with acute ischemic stroke. *J Neurol* 2012; 259: 1284-1289.

Z

Zhang JL, Yin CH, Zhang Y, Zhao LB, Fu HJ, Feng JC. Plasma copeptin and long-term outcomes in acute ischemic stroke. *Acta Neurol Scand* 2013; 128: 372-380.

Zhang N, Liu G, Zhang G, Fang J, Wang Y, Zhao X, Guo L, Wang Y. A risk score based on Get With the Guidelines – stroke program data works in patients with acute ischemic stroke in China. *Stroke* 2012; 43: 3108-3109.

Zhang W, Carriquiry A, Nettleton D, Dekkers JCM. Pooling mRNA in microarray experiments and its effect on power. *Bioinformatics* 2007; 23: 1217-1224.

Zhang X, Guo T, Wang H, He W, Mei H, Hong M, Yu J, Hu Y, Song S. Potential biomarkers of acute cerebral infarction detected by SELDI-TOF-MS. *Am J Clin Pathol* 2008; 130: 299-304.

Citas de páginas web

Academic medical center. Department of Neurology, H2-233, Amsterdam, The Netherlands. PASS: Preventive Antibiotics in Stroke Study. Fecha de actualización: 13 de Diciembre de 2013 [Fecha de consulta: 27 de Diciembre de 2013]. Disponible en: http://www.passamc.nl/index-eng.php

Aushon BioSystems, Billerica, Massachusetts, USA. Aushon: Shaping the future of ELISA. Fecha de actualización: 02 de Octubre de 2013 [Fecha de consulta: 27 de Diciembre de 2013]. Disponible en: http://www.aushon.com/index.php

diaDexus, Inc, South San Francisco, California, USA. PLAC Test for Lp-PLA_2. Fecha de actualización: 27 de Diciembre de 2013 [Fecha de consulta: 27 de Diciembre de 2013]. Disponible en: http://www.plactest.com/index.html

Myriad RBM, Austin, Texas, USA. Myriad RBM, Innovative biomarker solutions. [Fecha de consulta: 27 de Diciembre de 2013]. Disponible en: http://rbm.myriad.com/

National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA. PubChem Compound Database. Fecha de actualización: 21 de Noviembre de 2013 [Fecha de consulta: 05 de Enero de 2014]. Disponible en: http://pubchem.ncbi.nlm.nih.gov/

National Center for Biotechnology Information (NCBI), Bethesda, Maryland, USA. PubMed Database. Fecha de actualización: 19 de Diciembre de 2013 [Fecha de consulta: 06 de Enero de 2014]. Disponible en: http://www.ncbi.nlm.nih.gov/pubmed

Neurovascular Research Laboratory. Vall d'Hebron Research Institute (VHIR), Barcelona. Fecha de actualización: 20 de Diciembre de 2013 [Fecha de consulta: 27 de Diciembre de 2013]. Disponible en: http://stroke-biomarkers.com/

Research Collaboratory for Structural Bioinformatics (RCSB), USA. Protein Data Bank (PDB). Fecha de actualización: 31 de Diciembre de 2013 [Fecha de consulta: 05 de Enero de 2014]. Disponible en: http://www.rcsb.org/pdb/home/home.do

Somalogic, Inc., Boulder, Colorado, USA. SomaLogic: Deeper biology, Better outcomes. Fecha de actualización: 03 de Diciembre de 2013 [Fecha de consulta: 27 de Diciembre de 2013]. Disponible en: http://www.somalogic.com/

UniProt Consortium: European Bioinformatics Institute (EMBL-EBI), UK; Swiss Institute of Bioinformatics (SIB), Switzerland; Protein Information Resource (PIR), USA. UniProt. Fecha de actualización: 11 de Diciembre de 2013 [Fecha de consulta: 05 de enero de 2014]. Disponible en: http://www.uniprot.org/

ANEXOS

7

Tabla 2A. Modelos pronósticos validados para discapacidad tras el ictus.

Modelos pronósticos de discapacidad							
Modelo	**Variables**	**Escala**	**Tiempo**	**Puntuación**	**N Original**	**N Validación**	**Precisión**
ASTRAL (Ntaios G, 2012; Liu G, 2013; Papavasileiou V, 2013)	**Recogidas en admisión** Edad (en lustros) Gravedad (NIHSS) Tiempo síntomas > 3h Visión Glucosa <3,7 o >7,3 mmol/L Nivel de conciencia	mRS Malo: mRS > 2	3 m 1 a 5 a	Máximo: 8 + edad + NIHSS Tabla de riesgo con código de colores	1645 34 % malo	1659 + 653 + 3755 + 1520 40% (1 a) malo	AUC-ROC Original: 0,850 Validación: 0,902 + 0,82 0,81 (1 a) 0,89 (5 a)
BOAS (Muscari A, 2011)	**Recogidas en < 3 d** Edad ≥ 78 a Parálisis superior Gravedad (NIHSS ≥ 10) Administración oxígeno Catéter urinario	mRS Malo: mRS > 2	9 m	1 punto por factor Malo: ≥ 2 puntos Bueno: 0-1 punto	221 58,4 % malo	100 64 % malo	AUC-ROC Original: 0,891 Validación: 0,845
DRAGON (Strbian D, 2012; Strbian D, 2013; Giralt-Steinhauer E, 2013)	**Recogidas en < 4,5 h** Signo TC temprano Discapacidad previa (mRS>1) Edad Glucosa > 8 mmol/L Tiempo al rt-PA > 90 min Gravedad (NIHSS)	mRS Bueno: mRS 0-2 Malo: mRS 3-6 Miserable: mRS 5-6	3 m	0-10 puntos	1319 39,5 % malo	333 + 4519 + 297 43% + 48% malo	AUC-ROC Original: 0,84 Validación: 0,80 + 0,84 + 0,84 (pacientes con rt-PA)
G score (Allen CMC, 1984; Gompertz P, 1994)	**Recogidas en ~1 semana** Parálisis superior completa Disfunción cerebral + hemiplejía + hemianopia Somnolencia Edad Nivel de conciencia Hemiparesisa	Bueno: indep. actividad dia Malo: dependiente o muerto (Original) BI <13/20 (Validación)	2 m 6 m	Máximo: 7 puntos (Validación)	137 36 % malo (2 m) 38 % malo (6 m) (pacientes < 76 a)	314 69% malo (6 m)	AUC-ROC: Validación: 0,64

Tabla 2A (continuación)

Modelos pronósticos de discapacidad							
Modelo	**Variables**	**Escala**	**Tiempo**	**Puntuación**	**N Original**	**N Validación**	**Precisión**
iScore (Saposnik G, 2011; Park TH, 2013)	**Recogidas en admisión** Edad Sexo Gravedad (CNS) Subtipo ictus FA Insuf. cardíaca Cáncer Diálisis renal Discapacidad previa Glucosa ≥ 7,5 mmol/L	mRS Moderado-severo: mRS ≥ 3	Alta 3 m	Máximo: 245 + edad Muy bajo: < 105 Bajo: 106-120 Medio: 121-145 Alto: 146-175 Muy alto: >175	3818 43,2 % discapacidad	4635 + 4061 33% + 37% (3m) discapacidad	AUC-ROC Original: 0,787 Validación: 0,679 (incluye muerte a los 30 d) 0,819 (3 m)
(Johnston KC, 2000; Johnston KC, 2003)	**Recogidas en ~1 semana** Edad Gravedad (NIHSS) Volumen infarto (cm^3) Etiología lacunar Ictus previo DM Discapacidad previa (GOS)	BI, GOS, NIHSS Bueno: BI > 95, GOS =1, NIHSS ≤ 1 Malo: BI ≤ 60, GOS > 2, NIHSS ≥ 20, o muerte	3 m	-	256 Bueno: 55% (BI), 47% (GOS), 43% (NIHSS) Malo: 31% (BI); 34% (GOS), 16%(NIHSS)	299 Bueno: 13% (BI), 10% (GOS), 7% (NIHSS) Malo: 14% (BI), 15% (GOS), 8% (NIHSS)	AUC-ROC: Original: Bueno: 0,84 (BI), 0,84 (GOS), 0,87 (NIHSS) Malo: 0,88 (BI), 0,87 (GOS), 0,79 (NIHSS) Validación: Bueno: 0,83 (BI), 0,81 (GOS), 0,85 (NIHSS) Malo: 0,84 (BI), 0,89 (GOS), 0,75 (NIHSS) (pacientes de ensayo clínico)

Tabla 2A (continuación)

Modelos pronósticos de discapacidad							
Modelo	**Variables**	**Escala**	**Tiempo**	**Puntuación**	**N Original**	**N Validación**	**Precisión**
PLAN (O'Donnell MJ, 2012)	**Recogidas en admisión** Discapacidad previa Cáncer Insuficiencia cardíaca FA Nivel de conciencia Edad (en décadas) Debilidad inferior Debilidad superior Afasia o inatención	mRS Malo: mRS 5-6	Alta	Máximo: 25 puntos Insignificante: <6 Muy bajo: 6-9 Bajo: 10-12 Medio: 13-15 Alto: 16-19 Muy alto: 20-25	4943 14,9% malo	4904 16,4 % malo	AUC-ROC: Validación: 0,88
(Reid JM, 2010)	**Recogidas en admisión** Edad Independencia previa GCS verbal Función motora superior Marcha	mRS Independencia: mRS ≤ 2	6 m	-	538 42,4% independencia	530 + 1330 -	AUC-ROC Original: 0,876 Validación: 0,787 + 0,773
SSV (Counsell C, 2002; Reid JM, 2007; SCOPE, 2008)	**Recogidas en ~4 días /adm.** Edad Vivir solo Independencia previa GCS verbal Función motora superior Marcha	OHS, mRS Independiente: OHS < 3, mRS ≤ 2	6 m	-	530 9,6 % indep.	538 + 1330 + 538 + 537 6,1% + 3,7% + 42% + 33% indep.	AUC-ROC Validación: 0,839 + 0,840 + 0,792 + 0,82

Tabla 2A (continuación)

Modelos pronósticos de discapacidad							
Modelo	**Variables**	**Escala**	**Tiempo**	**Puntuación**	**N Original**	**N Validación**	**Precisión**
s-TPI (Kent DM, 2006; Uyttenboogaart M, 2008)	**Recogidas en < 4,5 h** Tto rt-PA Edad DM Gravedad (NIHSS) Sexo Ictus previo Presión sistólica Tiempo al rt-PA	mRS Bueno: mRS ≤ 1	3 m	-	2184 36 % bueno (pacientes de ensayos clínicos)	301 35 % bueno (pacientes con rt-PA)	AUC-ROC Original: 0,793 Validación: 0,80
s-TPI (Kent DM, 2006; Uyttenboogaart M, 2008)	**Recogidas en < 4,5 h** Edad Gravedad (NIHSS) Glucosa ASPECT *score* (opcional)	mRS Catastrófico: mRS ≥ 5	3 m	-	2184 22 % catastrófico (pacientes de ensayos clínicos)	301 26 % catastrófico (pacientes con rt-PA)	AUC-ROC Original: 0,784 Validación: 0,78
(Weimar C, 2002; GSS collab, 2004)	**Recogidas en < 72 h** Complicaciones neuro Fiebre > 38ºC Infarto lenticuloestriado DM Ictus previo Sexo Edad Discapacidad previa (mRS) Debilidad superior Gravedad (NIHSS)	BI Malo: BI < 95 o muerte	100 d	-	1754 32,1 % malo	1470 35,8% malo	AUC-ROC Original: 0,88
(Weimar C, 2004; König IR, 2008)	**Recogidas en < 6 h** Edad Gravedad (NIHSS)	BI Malo: BI < 95 o muerte	100 d	Normograma	1079 28,8 % malo	1307 + 5048 34% + 61% malo (2ª cohorte, ensayos clínicos)	AUC-ROC Original: 0,856 Validación: 0,808

Tabla 2B. Modelos pronósticos validados para mortalidad tras el ictus.

Modelos pronósticos de mortalidad						
Modelo	**Variables**	**Tiempo**	**Puntuación**	**N Original**	**N Validación**	**Precisión**
GWTG-Stroke (Smith EE, 2010; Zhang N, 2012)	**Recogidas en admisión** Edad Modo llegada al hospital FA Infarto previo Enf. coronaria Estenosis carotidea > 50% DM Enf. vascular periférica Hipertensión Dislipidemia Tabaquismo Admisión en horario oficina Gravedad (NIHSS) (opcional)	Intrahospitalaria	0 -204 puntos	164993 5,51 % muertes	109995 + 7015 5,5% + 2,9% muertes	AUC-ROC Validación: 0,72 + 0,73 0,85 + 0,87 (NIHSS)
iScore (Saposnik G, 2011-2; Park TH, 2013)	**Recogidas en admisión** Edad Sexo Gravedad (CNS) Subtipo ictus FA Insuf. cardíaca Cáncer Diálisis renal Discapacidad previa Glucosa ≥ 7,5 mmol/L	30 d 3 m	Máximo: 245 + edad Muy bajo: < 105 Bajo: 106-120 Medio: 121-145 Alto: 146-175 Muy alto: >175	8223 12,2 % muertes	4039 + 3270 + 4061 13% + 12% + 7% (3 m) muertes	AUC-ROC Original: 0,850 Validación: 0,851 + 0,79 0,861 (3 m)

Tabla 2B (continuación)

Modelos pronósticos de mortalidad						
Modelo	**Variables**	**Tiempo**	**Puntuación**	**N Original**	**N Validación**	**Precisión**
iScore (Saposnik G, 2011-2)	**Recogidas en admisión** Edad Sexo Gravedad (CNS) Subtipo ictus FA Insuf. cardíaca IM previo Tabaquismo Cáncer Diálisis renal Discapacidad previa Glucosa ≥ 7,5 mmol/L	1 a	Máximo: 205 + edad Muy bajo: <90 Bajo: 91-105 Medio: 106-120 Alto: 121-140 Muy alto: >140	8223 22,5 % muertes	4039 + 3270 22,9% + 24,4% muertes	AUC-ROC Original: 0,823 Validación: 0,84 + 0,782
(Lee J, 2013)	**Recogidas en admisión** Edad Sexo IM FA Dislipidemia Hipertensión Enf. vascular periférica Enf. pulmonar crónica Enf. tejido conectivo Enf. hígado Enf. renal Cáncer metastático Discapacidad (JCS, BI, mRS) Admisión en horario oficina	7 d 30 d	-	10774 2,6 % (7d) muertes 4,5% (30d) muertes	10671 2,4% (7d) muertes 4,4% (30d) muertes	AUC-ROC Original: 0,906 (7d) 0,893 (30d) Validación: 0,901 (7d) 0,872 (30d)

Tabla 2B (continuación)

Modelos pronósticos de mortalidad						
Modelo	**Variables**	**Tiempo**	**Puntuación**	**N Original**	**N Validación**	**Precisión**
ISSS (Williams GR, 2000)	**Recogidas en 7 d** Edad Gravedad (SSS) Discapacidad (RDRS) Ictus previo	1 a	-	226 -	227 -	AUC-ROC Validación: 0,86 (pacientes de ensayo clínico)
PLAN (O'Donnell MJ, 2012)	**Recogidas en admisión** Discapacidad previa Cáncer Insuficiencia cardíaca FA Nivel de conciencia Edad (en décadas) Debilidad inferior Debilidad superior Afasia o inatención	30 d 1 a	Máximo: 25 puntos Insignificante: <6 Muy bajo: 6-9 Bajo: 10-12 Medio: 13-15 Alto: 16-19 Muy alto: 20-25	4943 11,5 % muertes 22,0 % muertes	4904 13,5 % muertes 23,8 % muertes	AUC-ROC: Validación: 0,87 Validación: 0,84
SOAR (Myint PK, 2013; Kwok CS, 2013)	**Recogidas en admisión** Edad Sexo Tipo de ictus OCSP Discapacidad previa (mRS)	Intrahospitalaria	0 – 7 puntos	12355 -	3547 -	AUC-ROC Original: 0,81 Validación: 0,80
(Solberg OG, 2007)	**Recogidas en < 24 h** Edad (en décadas) Gravedad (CNS ≤ 3,5)	1 a	Muy bajo: 0-11,70 Bajo: 11,71-17,40 Medio: 17,41-24,21 Alto: 24,22-45,20 Muy alto: 45,21-70	442 -	295 -	AUC-ROC Original: 0,747 Validación: 0,709 (pacientes > 60 a)

Tabla 2B (continuación)

Modelos pronósticos de mortalidad						
Modelo	**Variables**	**Tiempo**	**Puntuación**	**N Original**	**N Validación**	**Precisión**
SSV (Counsell C, 2002; SCOPE, 2008)	**Recogidas en admisión** Edad Vivir solo Independencia previa GCS verbal Función motora superior Marcha	30 d	-	530 16,4 % superviv.	538 + 1330 + 537 16% + 7% + 79 % supervivencia	AUC-ROC Validación: 0,880 + 0,870 + 0,73
(Wang Y, 2003)	**Recogidas en < 24 h** Nivel de conciencia Disfagia Incontinencia urinaria Afectación bilateral Hipertermia Enf. coronaria Enf. vascular periférica DM	1 a	0 - 35 puntos Alto: ≥ 10 puntos Bajo: < 10 puntos	223 21,5 % muertes	217 -	AUC-ROC Original: 0,92
(Weimar C, 2002; GSS collab, 2004)	**Recogidas en < 72 h** Fiebre > 38ºC Edad Gravedad (NIHSS)	100 d	-	1754 9,5 % muertes	1470 7,7 % muertes	AUC-ROC Original: 0,87
(Weimar C, 2004; König IR, 2008)	**Recogidas en < 6 h** Edad Gravedad (NIHSS)	100 d	Normograma	1079 11,5 % muertes	1307 + 5419 10,7% + 18% muertes (2ª cohorte, pacientes de ensayos clínicos)	AUC-ROC Original: 0,832 Validación: 0,706

ASPECTS: *Alberta Stroke Program Early CT Score*; AUC-ROC: area bajo la curva de Característica Operativa del Receptor; BI: índice de Barthel; CNS: *Canadian Neurological Scale*; DM: diabetes mellitus; FA: fibrilación auricular; GCS: *Glasgow Coma Scale*; GOS: *Glasgow Outcome Scale*; IM: infarto de miocardio; JCS: *Japan Coma Scale*; mRS: escala de Rankin modificada; NIHSS: *National Institutes of Health Stroke Scale*; OCSP: *Oxfordshire Stroke Project Classification*; OHS: *Oxford Handicap Scale*; RDRS: *Rapid Disability Rating Scale*; SSS: *Scandinavian Stroke Scale*; TC: tomografía computacional; Tto. rt-PA: tratamiento con la forma recombinante del activador tisular del plasminógeno.

II. Página web: http://stroke-biomarkers.com/

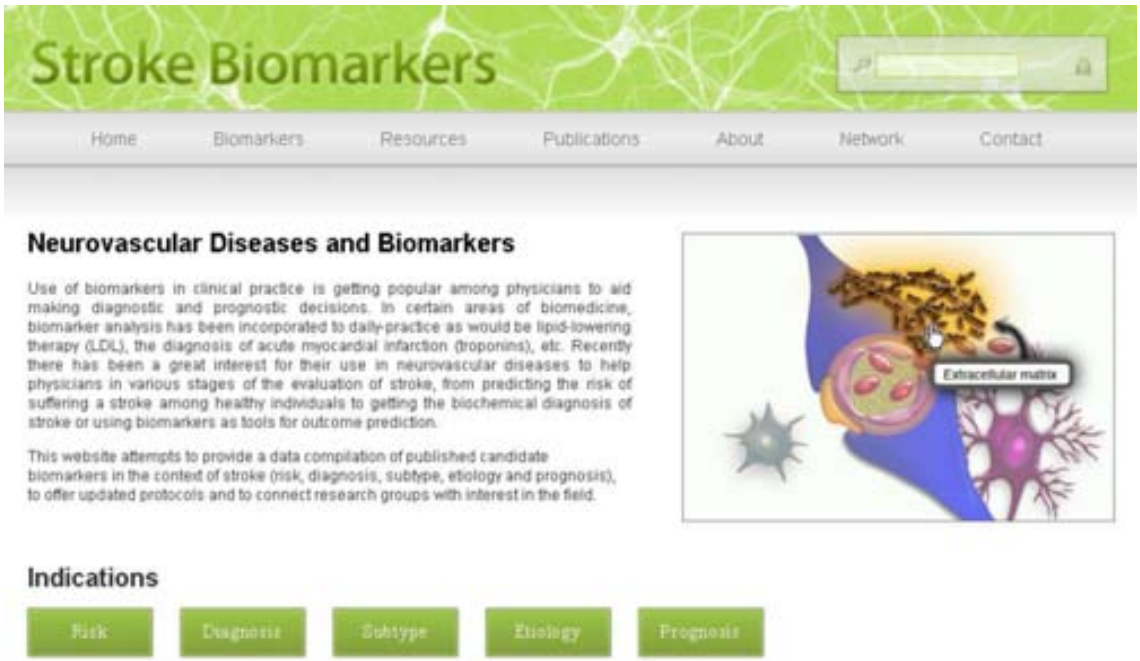


Figura 16. Interfaz de la página inicial de la web http://stroke-biomarkers.com/ .

La web se ha desarrollado por el Laboratorio de Investigación Neurovascular con el objetivo de proporcionar una compilación de los datos publicados sobre biomarcadores candidatos en diferentes indicaciones del ictus: riesgo, diagnóstico, subtipos, etiología y pronóstico.

Además, ofrece información actualizada relacionada con el tema, en cuanto a publicaciones interesantes y guías, y permite la conexión entre grupos de investigación con interés en este campo.

III. Artículo: Chemokines after human ischemic stroke: from neurovascular unit to blood using protein arrays

(*Translational Proteomics, 2014, under review*)

Chemokines after human ischemic stroke: from neurovascular unit to blood using protein arrays

Teresa García-Berrocoso[a], Dolors Giralt[a], Víctor Llombart[a], Alejandro Bustamante[a], Anna Penalba[a], Alan Flores[b], Marc Ribó[b], Carlos A Molina[b], Anna Rosell[a], Joan Montaner[a,b,§]

[a]Neurovascular Research Laboratory, Vall d'Hebron University Hospital Research Institute (VHIR), Universitat Autònoma de Barcelona, Barcelona, Spain
[b]Neurovascular Unit, Department of Neurology, Vall d'Hebron University Hospital, Barcelona, Spain

KEYWORDS

Biomarker
Chemokines
Ischemic stroke
CCL22
Microdissection
Temporal profile

ABSTRACT

Chemokines act mainly guiding leukocyte migration along the endothelium. Together with other pleiotropic effects, such as angiogenesis or neuronal survival, chemokines have a role in both damage and repair in brain tissue after ischemic stroke. We studied the presence of chemokines directly in neurons and brain blood vessels that were obtained by means of laser microdissection from human ischemic brains. Using multiple ELISA Searchlight® array we evaluated 9 chemokines (CCL1 - 5, CCL11, CCL17, CCL22 and CXCL8) in those microdissected samples. We found higher levels of CCL1 and CCL2 in neurons than in vessels; CCL5 and CCL22 were decreased in the infarcted areas.

The same ELISA array was performed in plasma samples from stroke patients. We explored the temporal profile of circulating chemokines from admission to 90 days after the cerebrovascular event, finding that only CCL22 showed significant changes along time and that these changes negatively correlated with neurological severity. When neurological outcome was assessed in the hyperacute phase of stroke no associations were found.

From our study, we can conclude that these chemokines do not seem to have a role as outcome biomarkers. Further studies need to assess which mechanisms are underlying the association of chemokines with the neurological state at distinct time points as the differences found here could be reflecting the dual role of chemokines in neuroinflammation.

[§]Corresponding author:
Joan Montaner
Neurovascular Research Laboratory, Vall d'Hebron Institute of Research (VHIR), Vall d'Hebron University Hospital, Pg. Vall d'Hebron 119-129, 08035, Barcelona, Spain. +34 93 489 40 73. 31862jmv@comb.es

Introduction

Cell death after cerebral ischemia activates a series of molecular mechanisms that lead to the production of inflammatory mediators, such as cytokines and chemokines, in order to attract leukocytes to the site of tissue injury [1]. There, leukocytes amplify the signal of cytokines, thus increasing brain inflammation and contributing to stroke severity, by their participation in tissue damage and growing of the infarct core [2]. On the other hand, the physiological function of leukocytes is phagocytosis and clearance of dying cells and debris. In that context, a dual role has been hypothesized, with neuroinflammation being both deleterious and restorative and thus, an interesting pathway to be therapeutically modulated [3].

In that scenario, chemokines act mainly recruiting effector leukocytes to the ischemic brain. From the 4 known subfamilies, CC and CXC chemokines seem the most relevant in cerebral ischemia, as they recruit neutrophils and monocytes, which present phagocytic activity [4]. This is evidenced by the high number of studies of chemokines in cerebral ischemia, mainly showing an increased expression within the ischemic brain, although non-concluding remarks can be obtained regarding its plausible role as biomarkers in the diagnosis or prognosis of stroke (Table 1).

The response to inflammation within the brain involves all cellular components of the neurovascular unit, both as a producers of and responders to inflammatory molecules. As examples, endothelial cells express cell adhesion molecules that facilitate leukocytes infiltration in response to chemokines; glial cells can secrete chemokines after ischemic stimulus and neurons suffer the deleterious effects of inflammation in the injured tissue [reviewed in 5]. On the other hand, chemokines are also involved in other biological functions affecting neurovascular unit components, such as angiogenesis or neuronal survival [6].

Considering all these precedents, we aimed to study the expression of chemokines by several components of the neurovascular unit after human stroke. For that purpose, we have combined two precise techniques: a multiple ELISA array of nine chemokines from CC and CXC families and laser microdissection to obtain neurons and blood brain vessels from patients who died following an ischemic stroke. Moreover, in order to assess the plausible use of chemokines as biomarkers or therapeutic targets in stroke field, we evaluated their temporal profile in blood samples and their association with stroke severity and outcome.

Materials and methods

Brain tissue samples

Four deceased patients who had an ischemic stroke secondary to middle cerebral artery (MCA) occlusion within the previous 4 days (range, 40 – 100 hours) were included in this part of the study (Supplementary Table 1). Brain tissue sampling from infarcted core and healthy contralateral areas was performed within the first hours after death according to our previously published procedure [7]. All samples were snap frozen in liquid nitrogen and immediately stored at -80ºC until use.

Clinical protocol and blood samples

Differential diagnosis of stroke was based on clinical examination by an expert neurologist and supported by computed tomography. In all cases, stroke onset was defined as the last time the patient was known to be asymptomatic.

Patients from the placebo arm of the MISTICS study [8] were considered for exploring blood temporal profile. From that cohort, 20 patients with a cortical ischemic stroke admitted to the emergency department within the first 3 to 12 hours after symptoms onset were included in the study. Any patient had known inflammatory (infectious and immunologic) or malignant disease and none of them received thrombolytic treatment. Peripheral blood samples were drawn from each patient at admission, day 1, 3, 7 and 90. Complete temporal profile was achieved from 15 patients.

On the other hand, we wanted to analyze the presence of chemokines in the hyperacute phase of stroke and their relationship with outcome. We included 36 ischemic stroke patients admitted within the first 4.5 hours after onset and in which blood samples were obtained before all them received thrombolytic treatment [standard dose of 0.9 mg/Kg recombinant tissue-plasminogen activator (rt-PA)]. In this cohort, consecutive patients were selected from the cohort to balance the sample size in all outcome groups (improvement, stability or worsening of the neurological state during in-hospital stay).

Table 1. CC and CXC chemokines in cerebral ischemia.

Chemokine	Ischemic stroke patients	Animal models of stroke	*In vitro* models of stroke	NVU producing cell
CCL1	Not known	Not known	Not known	Not known
CCL2	Increased in acute human CSF [23], whereas controversial results were found in blood with respect to controls [24-27]. Stable circulating levels after stroke [26], with higher blood levels in those patients with poor long-term outcome [26,28] and recurrence [29]. However, higher levels 3 days after stroke were associated with neurological improvement [30].	Higher concentration in infarcted hemisphere [31,32]. KO animal showed decreased infarct volume [33,34] and reduction of BBB leakage [35], while CCL2 overexpression increased infarct volume and leukocytes infiltration [36]. On the other hand, CCL2 attract neuroblasts to infarcted zone [37,38]. CCL2 expression has a role in ischemic tolerance [39].	Increased expression in microvessel endothelial culture after 24h OGD [40] and after reperfusion in BBB [41].	Neuron (first [39,42]), astrocyte, endothelial cell. Increased CCL2 expression in LMD-active vessels in human brain after stroke [40].
CCL3	Higher serum levels 1-3 days after stroke [26], although no differences where found when compared to asymptomatic carotid stenosis [43]. Regarding outcome, there is not a concluding association [26,30].	Higher concentration in infarcted hemisphere [32], being maintained 14 days after ischemia [44]. Exogenous CCL3 increased infarct volume [45].	Not known	Neuron, astrocyte, microglia (constitutive).
CCL4	No differences in serum levels when compared to asymptomatic carotid stenosis [43].	Higher expression in ischemic brain [46,47].	OGD-injured microvascular endothelial culture secretes CCL4 [48].	Not known
CCL5	Stable circulating levels after stroke [26. No differences in concentration when compared to controls [26], but higher levels when compared with patients with asymptomatic carotid stenosis [43].	KO animals showed smaller infarcts [49]. In immature rats there is a limited induction of CCL5 1-14 days after ischemia [46].	Not known	Not known
CCL7	Not known	Higher expression in ischemic hemisphere [50].	Not known	Not known
CCL9	Not known	Higher expression in ischemic cortex [51].	Not known	Not known
CCL11	Not known	No expressed in brain after ischemia [46].	Not known	Not known
CCL17	Not known	Not known	Not known	Not known
CCL20	Not known	Increased levels after brain ischemia-reperfusion [52]. Use of a neutralizing antibody markedly reduced infarct volume [53].	Not known	Astrocyte.
CCL21	Not known	Increased expression in ischemic brains [54].	Not known	Neuron.
CCL22	Not known	Not known	Not known	Not known

Table 1 (continued).

Chemokine	Ischemic stroke patients	Animal models of stroke	*In vitro* models of stroke	NVU producing cell
CXCL1	Higher CSF levels [55], but not concluding association in serum [55,56].	Increased expression in infarcted brain [47,57,58]. There is controversy regarding circulating levels [57,58].	Not known	Astrocyte, microglia.
CXCL2	Not known	Increased expression in the infarcted hemisphere [58].	Not known	Not known
CXCL4	Increased in plasma from stroke patients and higher levels associated with disability [59]. CXCL4 levels disminished along time [60].	Not known	Not known	Not known
CXCL5	Higher levels in CSF but not in serum [61].	Not known	Not known	Not known
CXCL8	Higher circulating levels than in controls [56], being maintained for a week [62].	CXCL8 inhibition reduced infarct size and neurological deficit [63].	Not known	Not known
CXCL10	Higher levels in ischemic brain [64].	Increased expression in infarcted brain [47,65], which is maintained along time [66].	Not known	Neuron (first), glia.
CXCL11	Not known	Increased expression in infarcted brain [47].	Not known	Not known
CXCL12	Plasma levels slightly increased with time after stroke onset [67] and peak 24h later [68], although there are not clear differences with controls [67,69]. Controversial results regarding correlation with initial severity [67,70].	Expression increases in infarct core and penumbra [47,71]. Over-expression of CXCL12 after ischemia induced a delayed neuroprotection [72].	Not known	Endothelial cell, astrocyte, microglia, neuron.
CXCL13	Not known	Increased expression in infarcted brain [47].	Not known	Not known
CXCL16	Higher circulating levels when compared to controls [73]. Higher plasma levels in the subacute phase of stroke patients who will die due to cardiovascular disease [74].	Not known	Not known	Endothelial cell, microglia, astrocyte.

Those chemokines in bold are chemokines included in our array; for the rest of known CC and CXC chemokines that are not in the list, we did not find any publication in cerebral ischemia.
BBB: blood brain barrier; CSF: cerebrospinal fluid; KO: knock-out; LMD: laser microdissection; NVU: neurovascular unit; OGD: oxygen and glucose deprivation.

Neurological severity was assessed by using the National Institutes of Health Stroke Scale (NIHSS) [9]; all included patients had a NIHSS score from 2 to 23, ranging from mild to severe neurological impairment. We defined neurological improvement as a decrease in NIHSS score by ≥ 4 points during in-hospital stay [10]. Clinical and neuroimaging data were blinded to the results of chemokines array.

In all cases, plasma was immediately separated by centrifugation at 1,500 *g* for 15 min at 4ºC and stored at -80ºC until use.

The local ethical committee approved both studies (i.e., human brain tissue and blood sampling), and written consent was obtained from all patients or relatives in accordance with the Helsinki declaration.

Immuno-laser microdissection

Frozen brain samples were embedded in Tissue-Tek OCT (Sakura Finetek Europe, The Netherlands) and 10 µm-thick sections were cut using a cryostat (Leica CM3050 S; Leica Microsystems, Germany). Sections were mounted on 2 µm PEN-membrane slides (MicroDissect GmbH, Germany) and stored at -80ºC. Neurons were stained using a mouse anti-NeuN antibody (1:50; Chemicon, USA) and brain microvessels were stained using *Ulex europeaus* Agglutinin I (UEA I) lectin (1:20; Sigma-Aldrich, U.S.A.) following the procedure as previously described [11].

Laser microdissection (LMD) was performed on a LMD6000 microscope (Leica). Cells were dissected

into dry 0.2 mL tube caps at a power of 41 – 45 KW and a speed of 14 ns using a 20x objective. Approximate total areas of 2,000,000 μm^2 of each cell type (5,000 – 7,000 cells) were pooled from several dissections from both infarct and contralateral brain tissue. Cells were recovered in 140 µL of cold lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij-35, 0.02% NaN_3 and 1% Triton X-100) containing protease inhibitors (1 mM PMSF and 7 µg/mL aprotinin), vortexed for 5 min, centrifuged at 12,000 *g* for 10 min at 4ºC and stored at -80ºC until use. Total protein content of the samples was determined by bicinchoninic acid assay (microBCA, Pierce, USA), yielding on average 83.6 µg/mL.

Multiplexed SearchLight® chemokines protein array

A multiplexed sandwich ELISA (SearchLight® Human Chemokine Array, Aushon Biosystems, USA) was used for the simultaneous quantitative measurement of 9 chemokines in both LMD-cells and plasma samples: CC or β-chemokines (I-309/CCL1, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, Eotaxin/CCL11, TARC/CCL17 and MDC/CCL22) and CXC or α-chemokines (IL-8/CXCL8). The chemiluminescent signal detected with a cooled CCD camera (Pierce, USA) was analyzed with ArrayVision 8.0 software (Imaging Research, USA). The sensitivity limit for each molecule was: CCL1 (0.8 pg/mL), CCL2 (0.8 pg/mL), CCL3 (3.1 pg/mL), CCL4 (0.8 pg/mL), CCL5 (0.4 pg/mL), CCL11 (0.5 pg/mL), CCL17 (0.4 pg/mL), CCL22 (0.2 pg/mL) and CXCL8 (0.2 pg/mL) as provided by the manufacturer. For LMD-samples, all values below the limit of detection were assigned with the corresponding limit value.

Manufacturer's instructions were followed and the assay was conducted in a blinded manner. LMD and plasma samples (with exception of temporal profiles) were assayed twice and the mean value of both measurements was given. For LMD-cell samples the resulting chemokine protein concentration was finally corrected by the total protein content and values are given as pg /mg. Plasma results were expressed as pg/mL.

Statistical analyses

Whole analysis was performed with SPSS 15.0 software (SPSS Inc, USA).

Shapiro-Wilk test was used to define normally distributed variables ($p>0.05$), due to small sample sizes. Normal distribution was analyzed by Students' *t* test or ANOVA and means and SD values were given. Different time points of temporal profiles were compared by ANOVA of repeated measures and paired-*t* test, while correlations with other continuous variables were assessed by Pearson test.

Non-normal distribution was assessed by Mann-Whitney U or Kruskal-Wallis tests and medians and interquartile ranges (IQR) were reported. Temporal profiles were compared by Friedman and Wilcoxon tests and correlations were analyzed by Spearman test.

Pearson chi-squared test was used to compare categorical variables.

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

For sample size and statistical power calculation we compared medians by using Ene 3.0 free software (GlaxoSmithKline S.A., Spain; http://sct.uab.cat/estadistica/es).

Results

Chemokines in human brain after stroke

From the nine chemokines assayed, CCL3, CCL4 and CCL17 were not detected in LMD-cell samples. Among the 6 studied chemokines, CCL1 and CCL2 were found at higher levels in neurons than in blood vessels ($p=0.029$ in both cases), without differences between infarcted and contralateral areas. Interestingly, CCL5 was decreased within the vessels and CCL22 within the neurons of the infarcted tissue (both cases with a $p=0.057$) (Figure 1).

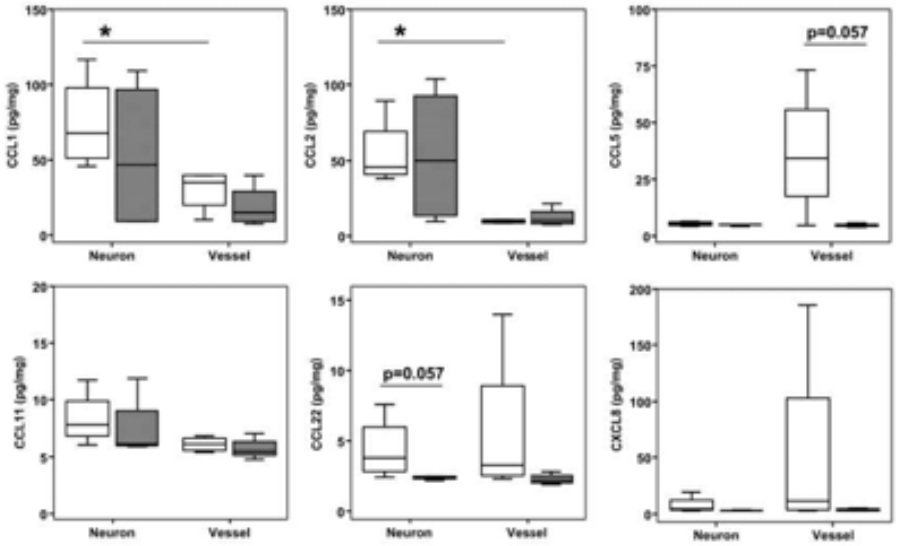


Figure 1. Chemokines levels in human brain microdissected-samples. Results of 6 chemokines are given in pg/mg of total protein for both neurons and blood vessels. Box-plots represent median and interquartile range. White boxes correspond to contralateral area (CL) and grey boxes correspond to infarcted area (IC). * p<0.05.

Blood detection of chemokines after stroke

All 9 chemokines were detected in plasma samples of ischemic stroke patients. No differences regarding demographic and clinical data were found between both studied cohorts as shown in Supplementary Table 2.

We explored the temporal profile of the circulating levels of these chemokines in a non-rt-PA treated cohort in order to avoid a possible influence in concentration due to thrombolytic treatment. Most chemokines showed stable circulating levels throughout time. As an exception, CCL22 levels presented a significant decrease during the acute phase (p=0.004) and a peak 7 days after the event (p=0.01) (Figure 2). This reduction on CCL22 levels within the first days after stroke was negatively correlated with stroke severity at different time points: the lower the CCL22 levels, the higher the NIHSS score. Similar negative correlations with stroke severity were found for CCL2 and CCL17 as well (Table 2).

Chemokines and stroke outcome

In view of these associations with neurological severity, we studied the plausible role of these chemokines as early outcome biomarkers in the hyperacute phase of stroke.

Only CCL3 showed a trend to be higher in those patients who improved within 24 h (p=0.098) (Table 3). None of the chemokines that showed a negative correlation with stroke severity were found associated with early outcome in rt-PA treated patients. Calculations of the sample size needed to achieve statistical significant association at an 80% of power revealed large number of patients in each outcome group (Table 3).

Discussion

Extensive research regarding the role of chemokines in both physiological and pathological states of the central nervous system has been published and reviewed. Although some chemokines are constitutively expressed at low level in the brain in order to maintain homeostasis (like Fractalkine/CX3CL1 in neurons or CXCL12 in astrocytes), their expression is mainly induced after brain injury in resident cells, activated local cells and infiltrated leukocytes leading to an inflammatory state that could be either detrimental or beneficial [12]. The more remarkable chemokines that have been described in pathological states include CCL2 - 5 and CXCL8 [13].

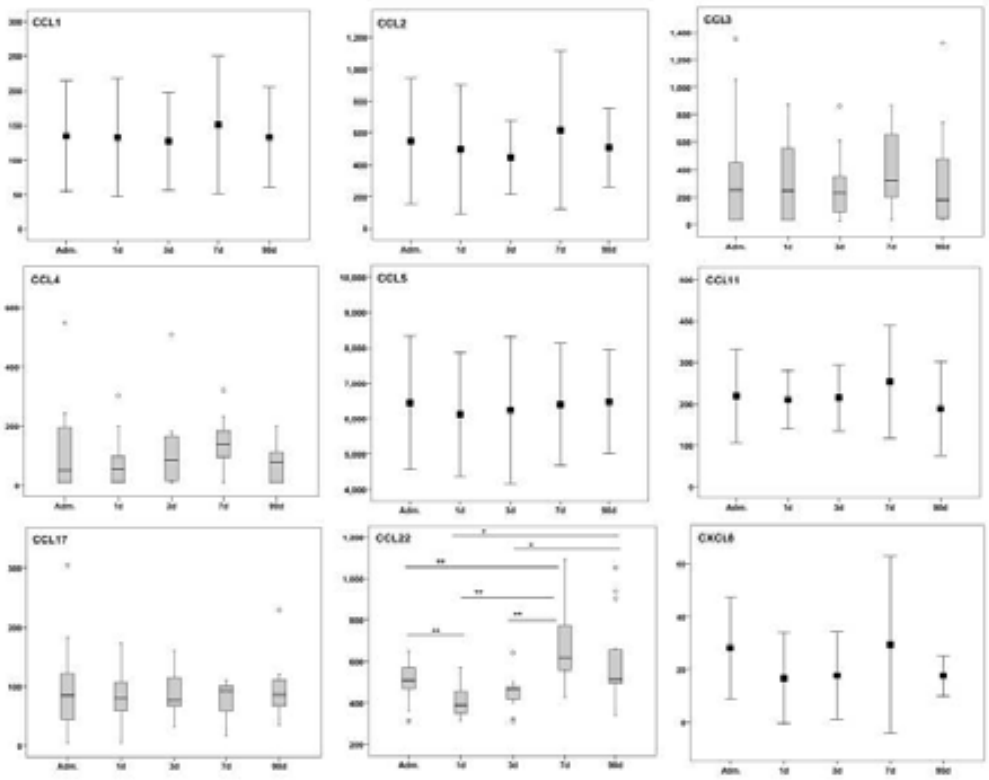


Figure 2. Chemokines temporal profiles in plasma samples from ischemic stroke patients. Results are given in pg/mL, from admission (Adm.) to day 90 after the event. Box-plots represent median and interquartile range; error bars represent mean ± SD. * p<0.05, ** p≤0.01.

Table 2. Correlations between blood levels of chemokines and severity of stroke.

Chemokine	NIHSS Adm.	NIHSS 1d	NIHSS 3d	NIHSS 5d	NIHSS 7d
CCL1 Adm.	R= -0.168; p=0.478	R= -0.020; p=0.932	R= -0.092; p=0.701	R= 0.065; p=0.790	R= 0.041; p=0.867
CCL1 1d	R= -0.116; p=0.635	R= -0.108; p=0.660	R= -0.165; p=0.500	R= -0.301; p=0.224	R= -0.276; p=0.268
CCL1 3d	R= 0.359; p=0.132	R= 0.143; p=0.560	R= 0.293; p=0.224	R= 0.103; p=0.685	R= 0.146; p=0.563
CCL1 7d	R= -0.224; p=0.357	R= -0.057; p=0.815	R= -0.079; p=0.747	R= -0.103; p=0.676	R= -0.083; p=0.736
CCL2 Adm.	R= -0.271; p=0.248	R= -0.434; p=0.056$	R= -0.339; p=0.143	**R= -0.500; p=0.029**	R= -0.450; p=0.053$
CCL2 1d	R= -0.260; p=0.282	R= -0.193; p=0.429	R= -0.399; p=0.091$	R= -0.369; p=0.132	R= -0.297; p=0.232
CCL2 3d	R= -0.222; p=0.360	R= -0.184; p=0.451	R= -0.438; p=0.061$	R= -0.402; p=0.098$	R= -0.343; p=0.163
CCL2 7d	R= -0.104; p=0.671	R= -0.174: p=0.476	R= -0.206; p=0.397	R= -0.214; p=0.380	R= -0.215; p=0.377
CCL3 Adm.	R= 0.056; p=0.815	R= -0.106; p=0.656	R= 0.077; p=0.747	R= -0.064; p=0.795	R= -0.042; p=0.863
CCL3 1d	R= -0.001; p=0.997	R= -0.227; p=0.350	R= -0.194; p=0.426	R= -0.355; p=0.148	R= -0.325; p=0.188
CCL3 3d	R= -0.034; p=0.890	R= 0.003; p=0.990	R= 0.181; p=0.457	R= 0.131; p=0.604	R= 0.188; p=0.455
CCL3 7d	R= -0.012; p=0.960	R= -0.039; p=0.874	R= -0.133; p=0.586	R= -0.205; p=0.400	R= -0.231; p=0.341
CCL4 Adm.	R= 0.012; p=0.959	R= -0.108; p=0.649	R= -0.007; p=0.977	R= -0.075; p=0.762	R= -0.055; p=0.823
CCL4 1d	R= 0.108; p=0.660	R= -0.067; p=0.785	R= -0.020; p=0.934	R= -0.023; p=0.928	R= -0.025; p=0.921
CCL4 3d	R= -0.099; p=0.687	R= -0.135; p=0.581	R= 0.135; p=0.581	R= 0.276; p=0.268	R= 0.297; p=0.232
CCL4 7d	R= -0.087; p=0.723	R= -0.252; p=0.298	R= -0.114; p=0.642	R= -0.145; p=0.553	R= -0.195; p=0.425
CCL5 Adm.	R= 0.239; p=0.310	R= 0.138; p=0.561	R= 0.201; p=0.396	R= 0.006; p=0.981	R= 0.014; p=0.955
CCL5 1d	R= 0.321; p=0.180	R= 0.070; p=0.776	R= 0.244; p=0.314	R=0.112; p=0.657	R= 0.165; p=0.512
CCL5 3d	**R = 0.584; p=0.009**	R= 0.423; p=0.071$	R= 0.334; p=0.163	R= 0.188; p=0.456	R= 0.183; p=0.467
CCL5 7d	R= 0.356; p=0.135	R=0.385; p=0.104	R= 0.281; p=0.244	R=0.183; p=0.454	R= 0.188; p=0.441
CCL11 Adm.	R= 0.083; p=0.726	R= -0.118; p=0.620	R= -0.130; p=0.585	R= -0.095; p=0.699	R= -0.043; p=0.862
CCL11 1d	R= 0.262; p=0.279	R= 0.255; p=0.293	R= 0.084; p=0.732	R= 0.316; p=0.202	R=0.308; p=0.214
CCL11 3d	R= 0.147; p=0.548	R= 0.012; p=0.961	R= 0.002; p=0.994	R= 0.021; p=0.935	R= -0.014; p=0.955
CCL11 7d	R= -0.035; p=0.886	R= -0.095; p=0.700	R= -0.110; p=0.654	R= -0.147; p=0.549	R= -0.153; p=0.532
CCL17 Adm.	R=-0.286; p=0.221	**R= -0.451; p=0.046**	R= -0.311; p=0.183	R= -0.281; p=0.244	R= -0.266; p=0.272
CCL17 1d	R= -0.441; p=0.059$	**R= -0.510; p=0.026**	**R= -0.534; p=0.019**	**R= -0.476; p=0.046**	R= -0.466; p=0.051$
CCL17 3d	R= 0.273; p=0.259	R= 0.075; p=0.761	R= 0.080; p=0.744	R= -0.046; p=0.856	R= -0.057; p=0.822
CCL17 7d	R= 0.287; p=0.234	R= 0.262; p=0.279	R= 0.283; p=0.241	R= 0.260; p=0.282	R= 0.242; p=0.318
CCL22 Adm.	R= -0.074; p=0.755	R= -0.208; p=0.380	R= -0.054; p=0.820	R= -0.075; p=0.761	R= -0.077; p=0.753
CCL22 1d	R= -0.335; p=0.161	**R= -0.576; p=0.010**	**R= -0.562; p=0.012**	**R= -0.522; p=0.026**	**R= -0.532; p= 0.023**
CCL22 3d	**R= -0.486; p=0.035**	**R= -0.676; p=0.001**	**R= -0.694; p=0.001**	**R= -0.587; p=0.010**	**R= -0.561; p=0.015**
CCL22 7d	R= -0.142; p=0.561	R= -0.192; p=0.432	R= -0.065; p=0.791	R= -0.070; p=0.775	R= -0.128; p=0.603
CXCL8 Adm.	R= 0.115; p=0.631	R= 0.022; p=0.927	R=0.216; p=0.361	R= 0.057; p=0.818	R=0.114; p=0.642
CXCL8 1d	R= 0.060; p=0.808	R= -0.062; p=0.802	R= -0.133; p=0.588	R= -0.139; p=0.582	R= -0.123; p=0.626
CXCL8 3d	R= -0.164; p=0.502	R= -0.123; p=0.615	R= -0.036; p=0.883	R= 0.009; p=0.971	R= 0.017; p=0.945
CXCL8 7d	**R= 0.555; p=0.014**	R= 0.292; p=0.226	R= 0.439; p=0.060$	R= 0.257; p=0.288	R= 0.249; p=0.303

Correlation coefficients (R) and p-values are given for each comparison. Significant correlations were in bold; $ stands for statistical trend (p<0.1). Adm.: at admission; NIHSS: National Institutes of Health stroke scale.

In the context of cerebral ischemia it has been hypothesized an inter-relationship between the different components of the neurovascular unit that contribute to the post-ischemic inflammatory state [5]. However, as far as we know, the study of inflammation in ischemic brains has been performed mainly in a local context, by studying individual components of the neurovascular unit, as neurons and endothelial cells, in cell cultures from immortalized human cell lines. Some *in vivo* models, usually performed in rodents, have been used as well, although they manifest differences in the immune system with regards to a more specialized defense in humans [14,15].

Here, we presented our results of the levels of chemokines in individual neurons and brain vessels but isolated by LMD from a global context as can be human brains that suffered an ischemic event. Moreover, the method presented here couples contact-free LMD to the immunofluorescence detection of the cells of interest in fresh-frozen tissues, thus granting the obtaining of pure populations of individual cells and good-quality proteins for further analyses. In this way, LMD allows a semi-quantitative measurement of the chemokines content in these isolated microvessels and neurons instead of a simple qualitative histological comparison between brain areas.

Although being an important part of the neurovascular unit, as a connector between vessels and neurons and having a role in neuroinflammation, we did not microdissected astrocytes or other glial cells in that study. Their complex shape, with cell bodies and processes, make difficult their pure isolation from the whole parenchyma of human brain pieces and therefore the measurement of chemokines' expression might be overestimated.

The use of an antibodies array combining different components of the chemokine family of proteins has allowed us to assess the levels of nine chemokines both in brain and in blood at the same time in the same cohort of patients. This array included some CC chemokines that, at least to our knowledge, have never been studied in cerebral ischemia, such as CCL1, CCL17 or CCL22, together with more studied chemokines in this field as CCL2.

CCL22 was underexpressed in the infarct core of damaged tissue after cerebral ischemia and it showed a reduction in the circulating levels 24h after stroke symptoms onset. Moreover, lower circulating levels were associated with sustained stroke severity. Altogether, these results seemed to indicate that a decrease in the expression of CCL22 is related to poor outcome in stroke patients. On the other hand, CCL17 was not detected in LMD-cells but it showed a similar association regarding to low circulating levels and stroke severity. Interestingly, both CCL17 and CCL22 co-localize in the same chromosomal loci, are similar in their sequence and share CCR4 as a receptor [16]. CCR4 is expressed in leukocytes of the Th2 type, thus being CCL17 and CCL22 amplifiers of the immune response of type II [17]. A systemic shift of the immune system towards anti-inflammatory type II response has been described in the post-acute phase of cerebral ischemia [18]. This anti-inflammatory state leads to immunodepression that could be a protective adaptive reaction to suppress the aggressive Th1 response to brain antigen exposure to the immune system, thus being beneficial, although increasing the risk of detrimental secondary infections [19,20]. In that context, the modulation of the immune system by the use of monoclonal antibodies could be of interest in stroke as well as in other diseases with an inflammatory background [reviewed in 21].

Our results showed a very faint power for CCL17 and CCL22 to discriminate those patients who will improve within the first 24 – 48 hours after stroke, thus not seeming plausible prognostic biomarkers in the hyperacute phase of stroke, when the patient arrives to the hospital and quick decision-making is needed in order to start a more exhaustive management to avoid secondary complications.

However, the results of our study might inspire new lines of investigation around the

Table 3. Chemokines plasma levels and neurological improvement in the acute phase of stroke.

Chemokine	Neurological improvement at 24h			Statistical power	
	NO (N=24)	YES (N=12)	p-value	Power	Sample size for each group
CCL1	67.8 (57.5 – 80.1)	87.2 (64.5 – 100.7)	0.409	13.2%	188
CCL2	443.7 (361.9 – 605.2)	486.1 (416.6 – 604.4)	0.514	9.7%	306
CCL3	135.6 (96.6 – 155.4)	185.5 (137.6 – 282.1)	0.098$^{\$}$	39.1%	50
CCL4	244.2 (184.5 – 276.9)	191.7 (177.3 – 328.7)	0.619	7.2%	719
CCL5	NA	NA	-	-	-
CCL11	191.6 (165.5 – 240.3)	176.1 (151.0 – 201.3)	0.271	19.9%	153
CCL17	98.7 (56.1 – 120.9)	88.1 (82.3 – 118.1)	0.963	2.8%	75835
CCL22	433.0 (396.2 – 620.0)	531.7 (475.4 – 612.4)	0.367	14.8%	215
CXCL8	2.0 (2.0 – 4.5)	6.2 (2.0 -19.2)	0.464	10.5%	328

Neurological improvement considered as a decrease of 4 or more points in NIHSS score from admission to 24 hours (identical results were obtained for improvement at 48 hours; data not shown). All chemokine values are given as pg/mL. $^{\$}$ stands for statistical trend ($p<0.1$). Statistical power calculations based on the results obtained for improvement at 24 hours; sample size needed in each group (improvement and no-improvement) to reach statistical significance at an 80% of power. CCL5 values were over the corresponding curve in this plate (non-available (NA) results). NIHSS: National Institutes of Health stroke scale.

modulation of CCL17 and/or CCL22 or even CCR4.

Our study stands with several limitations. We cannot dismiss the possible presence of some astrocyte end-feet in our vessel samples, as in brain their interrelationship form the tight blood-brain-barrier. Regarding blood samples, we could not study the relationship of chemokines with neurological outcome in the MISTIC cohort due to the limited number of worsening/improvement cases. Moreover, the sample size used for the study in the hyperacute phase is relatively small, but the sample size calculations showed very large number of samples for most of the studied chemokines to get any significant results. Further studies are needed to answer if CCL17 or CCL22 could have a role as outcome biomarkers at some later point than hyperacute phase. Other chemokines not included in SearchLight® array might be of interest in stroke field, as some of them that has not been studied in human stroke (CCL7, CCL9, and CXCL2). Novel multiplexed immunoassays based on fluorescently encoded microspheres might increase the screening of circulating inflammatory molecules in stroke patients while using very few amount of sample [22].

Conclusions

In conclusion, we described the presence of some chemokines in neurons and brain blood vessels and how these chemokines behave in plasma following ischemic stroke. The results found depending on the patients' cohort seem to indicate a different role for these chemokines along time with regards to neurological state. On the other hand, the studied chemokines does not seem of interest as outcome biomarkers, at least in the hyperacute phase. Further investigation is needed to assess if chemokines could be therapeutic targets to modulate neuroinflammation after ischemic stroke.

Authors' contributions

TG-B carried out the immunoassays of brain samples, participated in the design of the study and the statistical analysis and drafted the manuscript. DG performed the statistical analysis. VL did the microdissection of brain samples. AP carried out the immunoassays of blood samples. AF, MR and CAM coordinated the recruitment of patients and compiled clinical data. AB and AR critically reviewed the article content. JM conceived of the study, designed the experiments and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank to all study collaborators, residents, neurologists and nurses of the Stroke Unit and Neurology Ward from the Vall d'Hebron Hospital and

especially to all the patients who participated in the study.

Funding: A.R. is supported by Miguel Servet senior research contract (CP09/00265) and T.G-B by a predoctoral fellowship (FI09/00017) from the *Instituto de Salud Carlos III*. V.L is supported by a predoctoral fellowship from Vall d'Hebron Institute of Research. Neurovascular Research Laboratory takes part in the Spanish stroke research network *INVICTUS (RD12/0014/0005)* and is supported on stroke biomarkers research by FIS 11/0176.

Conflict of interest: All authors declare no-competing interests and founders were neither involved in the study design, collection and analysis of the data nor in the writing or the submission.

References

1. Mehta SL, Manhas N, Raghubir R. Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Res Rev 2007;54:34-66.
2. Barone FC, Feuerstein GZ. Inflammatory mediators and stroke: new opportunities for novel therapeutics. J Cereb Blood Flow Metab 1999;19:819-34.
3. Ceulemans AG, Zgavc T, Kooijman R, Hachimi-Idrissi S, Sarre S, Michotte Y. The dual role of the neuroinflammatory response after ischemic stroke: modulatory effects of hypothermia. J Neuroinflammation 2010;7:74.
4. Kochanek PM, Hallenbeck JM. Polymorphonuclear leukocytes and monocytes/macrophages in the pathogenesis of cerebral ischemia and stroke. Stroke 1992;23:1367-79.
5. Del Zoppo GJ. Inflammation and the neurovascular unit in the setting of focal cerebral ischemia. Neuroscience 2009;158:972-82.
6. Mackay CR. Chemokines: immunology's high impact factors. Nat Immunol 2001;2:95-101.
7. Rosell A, Ortega-Aznar A, Alvarez-Sabin J, Fernandez-Cadenas I, Ribo M, Molina CA, Lo EH, Montaner J. Increased brain expression of matrix metalloproteinase-9 after ischemic and hemorrhagic human stroke. Stroke 2006;37:1399-406.
8. Montaner J, Chacón P, Krupinski J, Rubio F, Millán M, Molina CA, Hereu P, Quintana M, Alvarez-Sabín J. Simvastatin in the acute phase of ischemic stroke: a safety and efficacy pilot trial. Eur J Neurol 2008;15:82-90.
9. Brott T, Bogousslavsky J. Treatment of acute ischemic stroke. N Engl J Med 2000;343:710-22.
10. Brott TG, Haley EC Jr, Levy DE, Barsan W, Broderick J, Sheppard GL, Spilker J, Kongable GL, Massey S, Reed R. Urgent therapy for stroke. Part I. Pilot study of tissue plasminogen activator administered within 90 minutes. Stroke 1992;23:632-40.
11. Cuadrado E, Rosell A, Penalba A, Slevin M, Alvarez-Sabín J, Ortega-Aznar A, Montaner J. Vascular MMP-9/TIMP-2 and neuronal MMP-10 up-regulation in human brain after stroke: A combined laser microdissection and protein array study. J Proteome Res 2009;8:3191-7.
12. Jaerve A, Müller HW. Chemokines in CNS injury and repair. Cell Tissue Res 2012;349:229-48.
13. Biber K, Zuurman MW, Dijkstra IM, Boddeke HWGM. Chemokines in the brain: neuroimmunology and beyond. Curr Opin Pharmacol 2002;2:63-8.
14. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. J Immunol 2004;172:2731-8.
15. Shibata K, Nomiyama H, Yoshie O, Tanase S. Genome diversification mechanism of rodent and lagomorpha chemokine genes. Biomed Res Int 2013;2013:856265.
16. Mantovani A, Gray PA, Van Damme J, Sozzani S. Macrophage-derived chemokine (MDC). J Leukoc Biol 2000;68:400-4.
17. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, Yoshie O. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 1999;11:81-8.
18. Theodorou GL, Marousi S, Ellul J, Mougiou A, Theodori E, Mouzaki A, Karakantza M. T helper 1 (Th1)/Th2 cytokine expression shift of peripheral blood CD4+ and CD8+ T cells in patients at the post-acute phase of stroke. Clin Exp Immunol 2008;152:456-63.
19. Dirnagl U, Klehmet J, Braun JS, Harms H, Meisel C, Ziemssen T, Prass K, Meisel A. Stroke-induced immunodepression: experimental evidence and clinical relevance. Stroke 2007;38:770-3.
20. Becker KJ. Modulation of the postischemic immune response to improve stroke outcome. Stroke 2010;41:S75-8.
21. Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. Nat Rev Immunol 2010;10:301-16.
22. Sudduth TL, Schmitt FA, Nelson PT, Wilcock DM. Neuroinflammatory phenotype in early Alzheimer's disease. Neurobiol Aging 2013;34:1051-9.
23. Losy J, Zaremba J. Monocyte chemoattractant protein-1 is increased in the cerebrospinal fluid of patients with ischemic stroke. Stroke 2001;32:2695-6.
24. Sánchez-Moreno C, Dashe JF, Scott T, Thaler D, Folstein MF, Martin A. Decreased levels of plasma vitamin C and increased concentrations of inflammatory and oxidative stress markers after stroke. Stroke 2004;35:163-8.
25. Arakelyan A, Petrkova J, Hermanova Z, Boyajyan A, Lukl J, Petrek M. Serum levels of the MCP-1 chemokine in patients with ischemic stroke and myocardial infarction. Mediators Inflamm 2005;2005:175-9.
26. Zaremba J, Ilkowski J, Losy J. Serial measurements of levels of the chemokines CCL2, CCL3 and CCL5 in serum of patients with acute ischemic stroke. Folia Neuropathol 2006;44:282-9.
27. Chen S, Martens-Lobenhoffer J, Weissenborn K, Kielstein JT, Lichtinghagen R, Deb M, Li N, Tryc AB, Goldbecker A, Dong Q, Bode-Böger SM, Worthmann H. Association of dimethylarginines and mediators of inflammation after acute ischemic stroke. J Neuroinflammation 2012;9:251.

28. Worthmann H, Tryc AB, Goldbecker A, Ma YT, Tountopoulou A, Hahn A, Dengler R, Lichtinghagen R, Weissenborn K. The temporal profile of inflammatory markers and mediators in blood after acute ischemic stroke differs depending on stroke outcome. Cerebrovasc Dis 2010;30:85-92.
29. Kuriyama N, Mizuno T, Kita M, Nagakane Y, Hosomi A, Harada S, Takeda K, Ozasa K, Yamada K, Tokuda T, Watanabe Y, Nakagawa M. Predictive markers of blood cytokine and chemokine in recurrent brain infarction. J Interferon Cytokine Res 2009;29:729-34.
30. Alvarez-Sabín J, Penalba A, Quintana M, Krupinski J, García-Bonilla L, Montaner J. Trifusal and aspirin have different effects on inflammatory biomarkers measured in patients with acute ischemic stroke. Cerebrovasc Dis 2009;28:371-7.
31. Yamagami S, Tamura M, Hayashi M, Endo N, Tanabe H, Katsuura Y, Komoriya K. Differential production of MCP-1 and cytokine-induced neutrophil chemoattractant in the ischemic brain after transient focal ischemia in rats. J Leukoc Biol 1999;65:744-9.
32. Jiang L, Newman M, Saporta S, Chen N, Sanberg C, Sanberg PR, Willing AE. MIP-1alpha and MCP-1 induce migration of human umbilical cord blood cells in models of stroke. Curr Neurovasc Res 2008;5:118-24.
33. Schilling M, Strecker JK, Schabitz WR, Ringelstein EB, Kiefer R. Effects of monocyte chemoattractant protein 1 on blood-borne cell recruitment after focal cerebral ischemia in mice. Neuroscience 2009;161:806-12.
34. Strecker JK, Minnerup J, Gess B, Ringelstein EB, Schäbitz WR, Schilling M. Monocyte chemoattractant protein-1-deficiency impairs the expression of IL-6, IL-1β and G-CSF after transient focal ischemia in mice. PLoS One 2011;6:e25863.
35. Strecker JK, Minnerup J, Schütte-Nütgen K, Gess B, Schäbitz WR, Schilling M. Monocyte chemoattractant protein-1-deficiency results in altered blood-brain barrier breakdown after experimental stroke. Stroke 2013;44:2536-44.
36. Chen Y, Hallenbeck JM, Ruetzler C, Bol D, Thomas K, Berman NEJ, Vogel SN. Overexpression of monocyte chemoattractant protein 1 in the brain exacerbates ischemic brain injury and is associated with recruitment of inflammatory cells. J Cereb Blood Flow Metab 2003;23:748-55.
37. Yan YP, Sailor KA, Lang BT, Park SW, Vemuganti R, Dempsey RJ. Monocyte chemoattractant protein-1 plays a critical role in neuroblast migration after focal cerebral ischemia. J Cereb Blood Flow Metab 2007;27:1213-24.
38. Andres RH, Choi R, Pendharkar AV, Gaeta X, Wang N, Nathan JK, Chua JY, Lee SW, Palmer TD, Steinberg GK, Guzman R. The CCR2/CCL2 interaction mediates the transendothelial recruitment of intravascularly delivered neural stem cells to the ischemic brain. Stroke 2011;42:2923-31.
39. Stowe AM, Wacker BK, Cravens PD, Perfater JL, Li MK, Hu R, Freie AB, Stüve O, Gidday JM. CCL2 upregulation triggers hypoxic preconditioning-induced protection from stroke. J Neuroinflammation 2012;9:33.
40. Slevin M, Krupinski J, Rovira N, Turu M, Luque A, Baldellou M, Sanfeliu C, de Vera N, Badimon L. Identification of pro-angiogenic markers in blood vessels from stroked-affected brain tissue using laser-capture microdissection. BMC Genomics 2009;10:113.
41. Dimitrijevic OB, Stamatovic SM, Keep RF, Andjelkovic AV. Effects of the chemokine CCL2 on blood-brain barrier permeability during ischemia-reperfusion injury. J Cereb Blood Flow Metab 2006;26:797-810.
42. Che X, Ye W, Panga L, Wu DC, Yang GY. Monocyte chemoattractant protein-1 expressed in neurons and astrocytes during focal ischemia in mice. Brain Res 2001;902:171-7.
43. Montecucco F, Lenglet S, Gayet-Ageron A, Bertolotto M, Pelli G, Palombo D, Pane B, Spinella G, Steffens S, Raffaghello L, Pistoia V, Ottonello L, Pende A, Dallegri F, Mach F. Systemic and intraplaque mediators of inflammation are increased in patients symptomatic for ischemic stroke. Stroke 2010;41:1394-404.
44. Küry P, Schroeter M, Jander S. Transcriptional response to circumscribed cortical brain ischemia: spatiotemporal patterns in ischemic vs. remote non-ischemic cortex. Eur J Neurosci 2004;19:1708-20.
45. Takami S, Minami M, Nagata I, Namura S, Satoh M. Chemokine receptor antagonist peptide, viral MIP-II, protects the brain against focal cerebral ischemia in mice. J Cereb Blood Flow Metab 2001;21:1430-5.
46. Bona E, Andersson AL, Blomgren K, Gilland E, Puka-Sundvall M, Gustafson K, Hagberg H. Chemokine and inflammatory cell response to hypoxia-ischemia in immature rats. Pediatr Res 1999;45:500-9.
47. Ramos-Cejudo J, Gutiérrez-Fernández M, Rodríguez-Frutos B, Expósito Alcaide M, Sánchez-Cabo F, Dopazo A, Díez-Tejedor E. Spatial and temporal gene expression differences in core and periinfarct areas in experimental stroke: a microarray analysis. PLoS One 2012;7:e52121.
48. Wang J, Li PT, Du H, Hou JC, Li WH, Pan YS, Chen HC. Tong Luo Jiu Nao injection, a traditional Chinese medicinal preparation, inhibits MIP-1β expression in brain microvascular endothelial cells injured by oxygen-glucose deprivation. J Ethnopharmacol 2012;141:151-7.
49. Terao S, Yilmaz G, Stokes KY, Russell J, Ishikawa M, Kawase T, Granger DN. Blood cell-derived RANTES mediates cerebral microvascular dysfunction, inflammation, and tissue injury after focal ischemia-reperfusion. Stroke 2008;39:2560-70.
50. Wang X, Li X, Yaish-Ohad S, Sarau HM, Barone FC, Feuerstein GZ. Molecular cloning and expression of the rat monocyte chemotactic protein-3 gene: a possible role in stroke. Brain Res Mol Brain Res 1999;71:304-12.
51. White RE, Palm CJ, Xu L, Ling EB, Ginsburg M, Daigle Jr BJ, Han R, Patterson AJ, Altman RB, Giffard RG. Mice lacking the β2 adrenergic receptor have a unique genetic profile before and after focal brain ischemia. ASN Neuro 2012;4:e00096.
52. Ohta H, Terao Y, Shintani Y, Kiyota Y. Therapeutic time window of post-ischemic mild hypothermia and the gene expression associated with the neuroprotection in rat focal cerebral ischemia. Neurosci Res 2007;57:424-33.

53. Terao Y, Ohta H, Oda A, Nakagaito Y, Kiyota Y, Shintani Y. Macrophage inflammatory protein-3alpha plays a key role in the inflammatory cascade in rat focal cerebral ischemia. Neurosci Res 2009;64:75-82.
54. Biber K, Sauter A, Brouwer N, Copray SC, Boddeke HW. Ischemia-induced neuronal expression of the microglia attracting chemokine Secondary Lymphoid-tissue Chemokine (SLC). Glia 2001;34:121-33.
55. Losy J, Zaremba J, Skrobanski P. CXCL1 (GRO-alpha) chemokine in acute ischaemic stroke patients. Folia Neuropathol 2005;43:97-102.
56. Ormstad H, Aass HC, Lund-Sørensen N, Amthor KF, Sandvik L. Serum levels of cytokines and C-reactive protein in acute ischemic stroke patients, and their relationship to stroke lateralization, type, and infarct volume. J Neurol 2011;258:677-85.
57. Benakis C, Vaslin A, Pasquali C, Hirt L. Neuroprotection by inhibiting the c-Jun N-terminal kinase pathway after cerebral ischemia occurs independently of interleukin-6 and keratinocyte-derived chemokine (KC/CXCL1) secretion. J Neuroinflammation 2012;9:76.
58. Copin JC, da Silva RF, Fraga-Silva RA, Capettini L, Quintao S, Lenglet S, Pelli G, Galan K, Burger F, Braunersreuther V, Schaller K, Deruaz M, Proudfoot AE, Dallegri F, Stergiopulos N, Santos RA, Gasche Y, Mach F, Montecucco F. Treatment with Evasin-3 reduces atherosclerotic vulnerability for ischemic stroke, but not brain injury in mice. J Cereb Blood Flow Metab 2013;33:490-8.
59. Tombul T, Atbas C, Anlar O. Hemostatic markers and platelet aggregation factors as predictive markers for type of stroke and neurological disability following cerebral infarction. J Clin Neurosci 2005;12:429-34.
60. Kurabayashi H, Tamura J, Naruse T, Kubota K. Possible existence of platelet activation before the onset of cerebral infarction. Atherosclerosis 2000;153:203-7.
61. Zaremba J, Skrobanski P, Losy J. The level of chemokine CXCL5 in the cerebrospinal fluid is increased during the first 24 hours of ischaemic stroke and correlates with the size of early brain damage. Folia Morphol (Warsz) 2006;65:1-5.
62. Grau AJ, Reis A, Buggle F, Al-Khalaf A, Werle E, Valois N, Bertram M, Becher H, Grond-Ginsbach C. Monocyte function and plasma levels of interleukin-8 in acute ischemic stroke. J Neurol Sci 2001;192:41-7.
63. Villa P, Triulzi S, Cavalieri B, Di Bitondo R, Bertini R, Barbera S, Bigini P, Mennini T, Gelosa P, Tremoli E, Sironi L, Ghezzi P. The interleukin-8 (IL-8/CXCL8) receptor inhibitor reparixin improves neurological deficits and reduces long-term inflammation in permanent and transient cerebral ischemia in rats. Mol Med 2007;13:125-33.
64. Chaitanya GV, Eeka P, Munker R, Alexander JS, Babu PP. Role of cytotoxic protease granzyme-b in neuronal degeneration during human stroke. Brain Pathol 2011;21:16-30.
65. Kuboyama K, Harada H, Tozaki-Saitoh H, Tsuda M, Ushijima K, Inoue K. Astrocytic P2Y(1) receptor is involved in the regulation of cytokine/chemokine transcription and cerebral damage in a rat model of cerebral ischemia. J Cereb Blood Flow Metab 2011;31:1930-41.
66. Wang X, Ellison JA, Siren AL, Lysko PG, Yue TL, Barone FC, Shatzman A, Feuerstein GZ. Prolonged expression of interferon-inducible protein-10 in ischemic cortex after permanent occlusion of the middle cerebral artery in rat. J Neurochem 1998;71:1194-204.
67. Kim SJ, Moon GJ, Cho YH, Kang HY, Hyung NK, Kim D, Lee JH, Nam JY, Bang OY. Circulating mesenchymal stem cells microparticles in patients with cerebrovascular disease. PLoS One 2012;7:e37036.
68. Sobrino T, Pérez-Mato M, Brea D, Rodríguez-Yáñez M, Blanco M, Castillo J. Temporal profile of molecular signatures associated with circulating endothelial progenitor cells in human ischemic stroke. J Neurosci Res 2012;90:1788-93.
69. Wurster T, Stellos K, Geisler T, Seizer P, Andia ME, Schuster A, May AE, Melms A, Gawaz M, Bigalke B. Expression of stromal-cell-derived factor-1 (SDF-1): a predictor of ischaemic stroke?. Eur J Neurol 2012;19:395-401.
70. Kim YS, Baek W, Kim MK, Kim HY, Lee KY, Lee YJ, Kim SH, Chang DI, Koh SH. Association between serum stromal cell-derived factor-1α and long-term outcome of acute ischemic stroke. Eur Neurol 2012;67:363-9.
71. Stumm RK, Rummel J, Junker V, Culmsee C, Pfeiffer M, Krieglstein J, Hollt V, Schulz S. A dual role for the SDF-1/CXCR4 chemokine receptor system in adult brain: isoform-selective regulation of SDF-1 expression modulates CXCR4-dependent neuronal plasticity and cerebral leukocyte recruitment after focal ischemia. J Neurosci 2002;22:5865-78.
72. Yoo J, Seo JJ, Eom JH, Hwang DY. Effects of stromal cell-derived factor 1α delivered at different phases of transient focal ischemia in rats. Neuroscience 2012;209:171-86.
73. Wang KD, Liu ZZ, Wang RM, Wang YJ, Zhang GJ, Su JR, Kang XX. Chemokine CXC Ligand 16 serum concentration but not A181V genotype is associated with atherosclerotic stroke. Clin Chim Acta 2010;411:1447-51.
74. Ueland T, Smedbakken LM, Hallén J, Atar D, Januzzi JL, Halvorsen B, Jensen JK, Aukrust P. Soluble CXCL16 and long-term outcome in acute ischemic stroke. Atherosclerosis 2012;220:244-9.

•Chemokines have a dual role in neuroinflammation: protective and detrimental.

•Their association with the pathophysiology of stroke is not clear.

•LMD combined with an antibodies array has allowed us to study chemokines in the NVU.

•CCL17 and CCL22 are associated with severity, but are not useful as outcome biomarkers

•Therapeutical immunomodulation seem promising although further studies are needed.

Supplementary Tables

Supplementary Table 1. Demographic and clinical data from patients included for brain microdissection study.

Patient ID	Gender	Age (years)	Lesion location (hemisphere)	Risk factors	Time from onset to death (hours)	Post-mortem interval (hours)	rt-PA treatment
N22	Male	67	Left MCA	AHT, DLP	62	7	Yes
N33	Male	80	Right MCA	MI, AF, AHT, DLP	100	4.5	No
N35	Male	84	Left MCA	AF, AHT, DLP	40	7.5	No
N36	Female	73	Left MCA	DLP	44	4	Yes

Brain samples were obtained from both infarcted core and contralateral areas for each patient.

AF: atrial fibrillation; AHT: arterial hypertension; DLP: dislypidemia; MCA: middle cerebral artery; MI: myocardial infarction; rt-PA: recombinant tissue-Plasminogen Activator.

Supplementary Table 2. Demographic and clinical factors from both ischemic stroke patients' cohorts.

Factors	MISTIC cohort (N=20)	rt-PA cohort (N=36)	p-value
Age, years median (IQR)	74.1 (68.5 – 79.4)	78.5 (67.5 – 83.5)	0.259
NIHSS at admission median (IQR)	9.5 (8.0 – 14.5)	15 (8.5 – 19)	0.109
Gender (Male) % (n)	50 (10)	58.3 (21)	0.548
Arterial hypertension % (n)	50 (10)	54.3 (19)	0.759
Diabetes mellitus % (n)	20 (4)	25.7 (9)	0.749
Dyslipidemia % (n)	20 (4)	34.3 (12)	0.262
Atrial fibrillation % (n)	30 (6)	45.7 (16)	0.252
Ischemic cardiopathy % (n)	5 (1)	20 (7)	0.234
Previous stroke % (n)	5 (1)	17.1 (6)	0.402
TOAST			0.767
- Atherothrombotic % (n)	25 (5)	17.1 (6)	
- Cardioembolic % (n)	55 (11)	54.3 (19)	
- Undetermined % (n)	20 (4)	25.7 (9)	

IQR: interquartile range; NIHSS: National Institutes of Health stroke scale; TOAST: etiologic classification of ischemic stroke.

IV. Immuno-laser microdissection coupled to label-free proteomics for the analysis of human brain cells after cerebral ischemia.

(*Póster presentado en European SummerSchool "Advanced Proteomics", Brixen, Italia, Agosto de 2013)*

Immuno-laser microdissection coupled to label-free proteomics for the analysis of human brain cells after cerebral ischemia

Teresa Garcia-Berrocoso[1], Virginie Licker[2], Alexandre Hainard[3], Victor Llombart[1], Jean-Charles Sanchez[3], Joan Montaner[1]

1. Neurovascular Research Laboratory, Vall d'Hebron Institute of Research, Universitat Autònoma de Barcelona, Barcelona, Spain
2. Neuroproteomics group, Human protein science department, University Medical Center, University of Geneva, Geneva, Switzerland
3. Translational biomarker group, Human protein science department, University Medical Center, University of Geneva, Geneva, Switzerland

BACKGROUND AND OBJECTIVE

Cerebral infarction or stroke is due to a lack of nutrients and oxygen supply to the brain parenchyma that compromises the metabolism of the cells. As these cells die, the infarcted area expands and leads to impaired neurological and functional status of stroke patients. To know which pathophysiological processes underlie cerebral ischemia, our group firstly described changes in the proteome of human brain after stroke (*Cuadrado E, J Neuropathol Exp Neurol, 2010*). In the present study, our aim is to further analyze the proteome of the different components of the neurovascular unit, such as neurons and brain vessels, after ischemic stroke.

METHODS

Brain slices from infarcted (n=6), peri-infarcted (n=3) and healthy contralateral areas (n=6) of patients who died following an ischemic stroke were visualized by immunofluorescence staining with NeuN (*Chemicon*). By means of laser microdissection (LMD6000, *Leica*) we obtained about 2,500 neurons from each sample (**Figure 1**). Cells were lysed by sonication in 60 µL of 0.1% Rapigest SF surfactant (*Waters*). After trypsin digestion, acidification and C18 columns purification, samples were analyzed in a ESI LTQ-OT MS (*Thermo Electron*). Gas phase fractionation (GPF) with 4 injections of each sample with 400-520, 515-690, 685-979 and 974-2000 m/z ranges was used for precursor ion selection. Mascot was used for protein identification, considering a minimum score of 20 and at least 2 spectra per peptide. *Progenesis LC-MS v4.0* software was used for quantification and statistical comparison analysis. Further biological analyses were performed with *PANTHER* and *Ingenuity Pathway Analysis* (*IPA*).

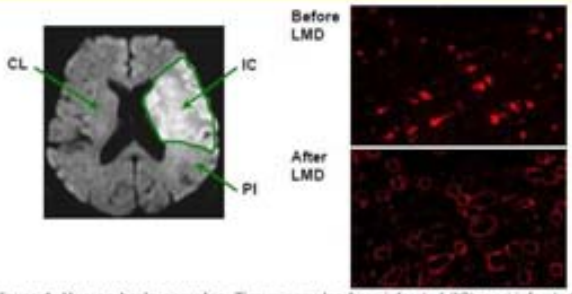


Figure 1. Human brain samples. Tissue samples from infarcted (IC), peri-infarcted (PI) and healthy contralateral (CL) areas were used for laser microdissection (LMD) of NeuN immunolabeled neurons.

RESULTS

A total of 520 proteins were identified and quantified in neurons from ischemic stroke patients. From these, 41 proteins were found elevated in the infarcted as compared with the contralateral areas ($p \leq 0.05$, fold-change ≥ 2, and peptide count ≥ 2). Any protein showed decreased levels in the infarcted area with these criteria. Most proteins had catalytic activity or were involved in binding processes, mainly with nucleic acids and in membrane trafficking, as shown in *PANTHER* analysis (**Figures 2A & 2B**). *IPA* networks revealed 29 of these proteins to be involved in assembly, organization and movement of cells, in cytoskeleton conformation and in synaptogenesis and axonogenesis processes (**Figure 3**). When peri-infarcted area was also considered in a paired analysis, 4 of these proteins showed a progressive reduction in their level from infarcted to peri-infarcted and to contralateral areas (**Figure 4**).

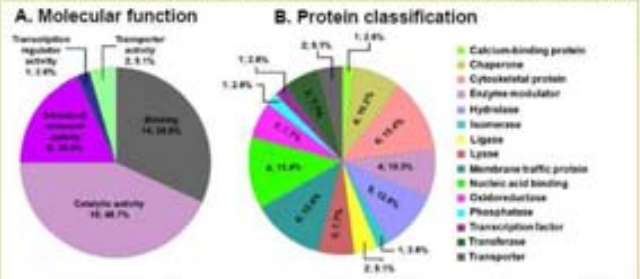


Figure 2. Neuronal biological processes increased after ischemia. Molecular function (A) and protein classification (B) found in *PANTHER* analysis for the 41 proteins elevated in the infarcted area (number of proteins and corresponding percentage are shown).

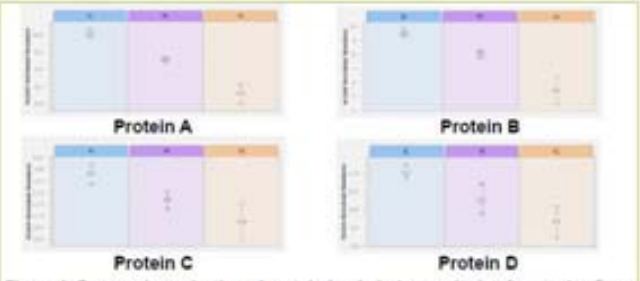


Figure 4. Progressive reduction of protein levels in human brain after stroke. From the 41 proteins, 4 proteins showed progressive decrease through infarcted (IC) to peri-infarcted (PI) to contralateral (CL) areas. ($p \leq 0.05$, fold-change ≥ 2, and peptide count ≥ 2)

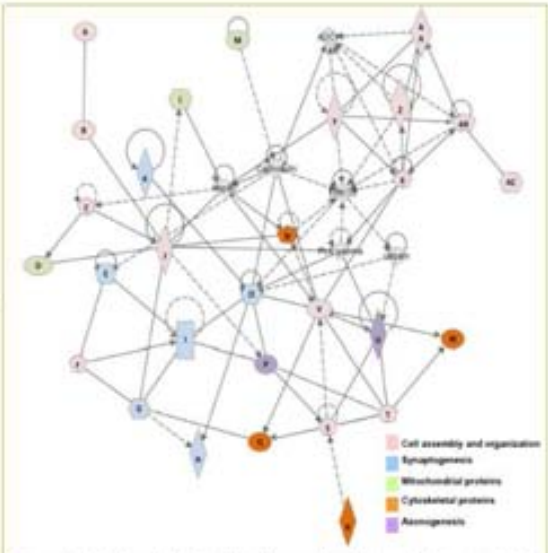


Figure 3. *IPA* network including 29 of these 41 proteins, which are involved in cellular movement, assembly and organization.

CONCLUSIONS

We described changes in protein levels of human neurons after cerebral ischemia, finding neurorepair processes stimulated in the infarcted area. After validation, these results could contribute to the molecular knowledge of stroke pathology and might highlight new therapeutical targets or potential biomarkers for the diagnosis or prognosis of stroke.

V. Artículo: Role of beta-defensin-2 and interleukin-4 receptor as stroke outcome biomarkers

(*Journal of Neurochemistry, 2014, doi: 10.1111/jnc.12649*)

Role of beta-defensin-2 and interleukin-4 receptor as stroke outcome biomarkers

Teresa García-Berrocoso MSc[1], Dolors Giralt BSc[1], Alejandro Bustamante MD[1], Victor Llombart MSc[1], Marta Rubiera MD, PhD[2], Anna Penalba[1], Cristina Boada MSc[1], Marc Espadaler[1], Carlos Molina MD, PhD[2], Joan Montaner MD, PhD[1,2,*]

[1]Neurovascular Research Laboratory, Vall d'Hebron Institute of Research (VHIR), Universitat Autònoma de Barcelona, Barcelona, Spain

[2]Neurovascular Unit, Department of Neurology, Vall d'Hebron University Hospital, Barcelona, Spain

Abstract

Acute ischemic stroke is a complex disease with huge interindividual evolution variability that makes challenging the prediction of an adverse outcome. Our aim was to study the association of bloodstream signatures to early neurological outcome after stroke, by combining a sub-pooling of samples strategy with protein array discovery approach.

Plasma samples from 36 acute stroke patients (<4.5h from onset) were equally pooled within outcome groups: worsening, stability and improvement (n=3 pools of 4 patients each, for each outcome group). These 9 pools were screened using a 177 antibodies library and 35 proteins were found altered regarding outcome classification (p<0.1). Processes of inflammation, immune response, coagulation and apoptosis were regulated by these proteins. Ten representative candidates, mainly cytokines and chemokines, were assayed for replication in individual baseline plasma samples from 80 new stroke patients: β-defensin-2 (BD-2), MIP-3b, PAI-1 active, BCA-1, Exodus-2, interleukin-4 receptor (IL-4R), IL-12p40, LIF, MIP-1b and TWEAK. Multivariate logistic regression analysis showed BD-2 (ORadj 4.87 [1.13-20.91] p=0.033) and IL-4R (ORadj 3.52 [1.03-12.08] p=0.045) as independent predictors of worsening at 24h after adjustment by clinical variables. Both biomarkers improve the prediction by 19% as compared to clinical information, suggesting a potential role for risk stratification in acute thrombolysed stroke patients.

Keywords: biomarkers, cerebral ischemia, immune response, outcome, prognosis, stroke

* Corresponding author: Dr. Joan Montaner, Neurovascular Research Laboratory, Vall d'Hebron Institute of Research (VHIR), Vall d'Hebron University Hospital, Pg. Vall d'Hebron 119-129, 08035, Barcelona, Spain. +34 93 489 40 73, 31862jmv@comb.es

Abbreviations

END: early neurological deterioration; IDI: integrated discrimination improvement index; NRI: net reclassification improvement index; rt-PA: recombinant tissue-plasminogen activator; NIHSS: National Institutes of Health stroke scale; BD-2: β-defensin 2; PAI-1: plasminogen activator inhibitor 1; MIP: macrophage inflammatory protein; BCA-1: β cell-attracting chemokine 1; IL: interleukin; LIF: leukemia inhibitor factor; TWEAK: tumor necrosis factor-related weak inducer of apoptosis; CV: coefficient of variation; IQR: interquartile range; ROC: receiver operator characteristic; OR_{adj}: adjusted odd ratio; CI: confidence interval; AUC: area under the ROC curve

Stroke is the second cause of death and one of the main causes of disability worldwide (Roger V.L., 2012). Early neurological deterioration (END) is an important concern during acute stroke management and in some series it occurs up to 40% (Lin L.C., 2012; Arenillas J.F., 2002). Although there is not an international consensus about END, one consistently used definition is the increase of 4 or more points in the National Institutes of Health stroke scale (NIHSS) within the first 48-72 hours (Alawneh J.A., 2009). Several causes of complication such as hemorrhagic transformation, arterial reocclusions or malignant edema, may appear in the first hours after stroke contributing to END. Although nowadays there does not exist a specific therapeutic treatment to solve neurological deterioration, the admission of stroke patients into specialized stroke units have demonstrated to prevent END and thus to reduce the rate of poor outcome after ischemic stroke (Roquer J., 2008). Therefore the prediction of END is one of the challenges in stroke, as an accurate identification of patients more prone to worsen might help to optimize the admission to the scarce stroke units.

Some acute neuroimaging factors have been associated with END, such as hypodensity or hyperdense middle cerebral artery sign on computerized tomography or large diffusion weighted image in magnetic resonance (Alawneh J.A., 2009). As neuroimaging techniques are not broadly accessible, the use of blood biomarkers could be a more feasible option. For that proposal, the exploration of molecules which could anticipate the development of END is becoming increasingly popular. Some candidates have been explored in that context, such as interleukin-6 (IL-6) (Vila N., 2000) or b-type natriuretic peptide (Montaner J., 2012), although their added value to clinical prognostic models is still unclear (Montaner J., 2012; Whiteley W., 2009).

The complexity of blood and the interindividual variability make difficult to validate differential protein expression to distinguish among disease stages. In order to obtain a common signature of protein changes which are associated to a specific stage, pooled-blood samples are being employed in other diseases (Ernoult E., 2010; Fragnoud R., 2012) and are recommended for high throughput proteomics (Barker P.E., 2006). Moreover multiple sub-pools, which are generated by random distribution of individual samples, can be performed in order to estimate variation within population (Karp N.A., 2009).

In order to go in depth in the physiopathology of ischemic stroke we performed the first exploratory study of the plasma proteome by screening an antibodies library with a sub-pooled samples approach. Nowadays there is an assortment of different antibodies libraries in multiplexed arrays available in the market that allow the study of hundreds of proteins involved in different pathways while using few amount of patients' samples. We have used the SearchLight® library, which included 177 antibodies covering the exploration of several cellular processes in a multiplex ELISA-based manner.

We aimed to discover a common signature of protein expression changes for those patients who have an early poor outcome after stroke. Furthermore, after replication of our results in

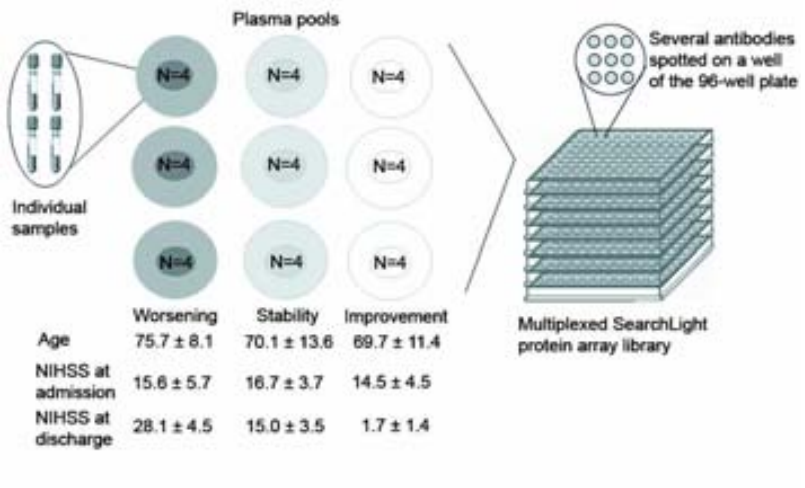


Figure 1. Schematic design of the discovery phase.
Each pool was prepared with 4 individual plasma samples from ischemic stroke patients with similar clinical characteristics. Three different pools were screened for each outcome group (worsening, stability, improvement) in 177 antibodies from Searchlight® multiple ELISA library. Median and IQR were given for age and NIHSS score at admission and at discharge.

individual stroke samples, we assessed the added value of our biomarker candidates to clinical predictive models by means of comparative statistical metrics such as Integrated Discrimination Improvement (IDI) and Net Reclassification Improvement (NRI) indexes.

Materials and Methods

Patients and protocol

We recruited patients who were admitted to the emergency department of the Vall d'Hebron University Hospital (Barcelona, Spain) from 2004 to 2010 with an acute ischemic stroke within the first 4.5 hours after symptoms onset. This large cohort of 395 patients was used to select candidates for the different phases of derivation and replication of this study. Stroke diagnosis was performed based on a standardized protocol of clinical and neuroradiological assessments as previously described by our group (Mendioroz M., 2011). All patients received intravenous recombinant tissue-plasminogen activator (rt-PA) in a standard 0.9 mg/Kg dose (10% bolus, 90% continuous infusion during 1h).

Stroke severity was assessed by using NIHSS (Brott T., 2000). We defined neurological improvement as a decrease in NIHSS score by 4 or more points, neurological stability as changes in NIHSS score of 3 or less points and neurological deterioration as death or an increase in NIHSS score by 4 or more points at 24 h or 48 h (Brott T.G., 1992). Clinical data was blinded to biomarker measurement.

Before administration of any treatment, peripheral blood samples were drawn from each patient in EDTA collection tubes. Plasma was immediately separated by centrifugation at 1500 *g* for 15 min at 4ºC and was stored at -80ºC until use.

The local ethical committee approved the study and written consent was obtained from all patients or relatives in accordance with the Helsinki declaration.

Discovery phase

Pooling strategy

A scheme of our technical approach is shown in Fig 1. From our cohort, we randomly selected 36 ischemic stroke patients to perform pooled plasma samples of each outcome group, which were balanced according to age, gender, NIHSS score at admission and etiology subtype. Individual plasma samples were ice-thawed and equal volumes (0.5 mL) of 4 different samples from

patients with similar clinical characteristics were mixed by agitation during 2 hours at 4ºC in order to obtain a pool. In total 9 pools were prepared for analysis, including 3 pools for each outcome group:

1. Worsening: patients with neurological deterioration during in-hospital stay.
2. Stability: patients without changes in neurological state during in-hospital stay.
3. Improvement: patients with neurological improvement during in-hospital stay.

Screening by multiplexed SearchLight® antibodies array library

A library of 177 anti-human antibodies was screened with multiplexed sandwich-ELISAs from SearchLight® platform (Aushon BioSystems). We screened the complete library, with all the antibodies that the company offers in a multiplex system, which is based on chemiluminiscent detection of molecules whose respective capture-antibodies were combined in 96-well plates. The molecules which are included in the library represent different biological processes from gene ontology pathways (Supporting Information Table I).

Replication study

Representative biomarkers were selected by their illustration of the discovery phase findings regarding biological processes, the possibility of being combined in multiplexed ELISA and/or their statistical significance. Ten candidates were combined in SearchLight® custom-arrays, including β-defensin 2 (BD-2), plasminogen activator inhibitor 1 (PAI-1) active-form, macrophage inflammatory protein (MIP) 3b, MIP-1b, β cell-attracting chemokine 1 (BCA-1), exodus-2, interleukin (IL) 4 receptor (IL-4R) and IL-12p40 and leukemia inhibitor factor (LIF). Tumor necrosis factor-related weak inducer of apoptosis (TWEAK) was analyzed by a single ELISA commercial kit (eBioscience).

These candidates were tested in individual plasma samples from 80 new stroke patients fulfilling similar inclusion criteria than the derivation cohort. To achieve enough statistical power in the comparison among outcome groups, patients who worsened were first randomly selected from our cohort and afterward groups of patients who remained stable or improved were balanced in accordance to clinical variables.

In both phases (discovery and replication) biomarker results were blinded to clinical data. Each sample was assayed twice and the mean value was used, removing those results with either the intrassay coefficient of variation (CV) or interassay CV higher than 30%.

Statistical analysis

SPSS statistical package 15.0 was used, unless contrary is stated.

Normality for continuous biomarker levels or clinical variables was assessed by Shapiro-Wilk test for the Discovery phase and Kolmogorov-Smirnov test for the Replication study. Those normally distributed variables ($p>0.05$) were analyzed by Student's t test or ANOVA and mean and SD values are given whereas for variables with non-normal distribution Mann–Whitney U or Kruskal–

Wallis test were used and median and interquartile range (IQR) are reported.

In the univariate analysis, intergroup differences were assessed by Pearson chi-squared test for categorical variables. Cut-off points with the optimal accuracy (both sensitivity and specificity) to predict outcome were obtained from receiver operator characteristic (ROC) curves for each individual biomarker. To build predictive models, all clinical variables which were associated with outcome at $p<0.1$ in the univariate analysis were included in a forward stepwise multivariate logistic regression analysis. For those independent variables, odds ratio (OR_{adj}) and 95% confidence interval (CI) were adjusted by NIHSS at admission, age and sex. Afterward biomarkers alone or in combination were added by Enter method to clinical models.

The areas under the ROC curve (AUC) from models that include biomarkers were compared with AUC from only clinical model by DeLong's method (DeLong E.R., 1988) with MedCalc 12.4 software. Using R software (Hmisc and PredictABEL packages), net reclassification improvement (NRI) and integrated discrimination improvement (IDI) indexes were calculated to assess the added value of the biomarkers to the clinical predictive model (Pencina M.J., 2008; Pickering J.W., 2012). In the case of NRI test, pre-specified clinically relevant thresholds of predicted risk (≤10% and >90%) were used to calculate reclassification of patients into risk outcome groups (Whiteley W., 2012).

In all cases a $p\leq0.05$ was considered significant at a 95% confidence level.

Results

Discovery phase: proteome screening

Nine pooled-plasma samples from stroke patients who worsened, remained stable or improved during in-hospital stay were screened in a 177 antibodies library (Supporting Information Table I). A schematic view of the design of the study and clinical data which corresponds to the whole derivation cohort (N=36) is shown (Fig 1). No difference was found for age neither for NIHSS at admission regarding outcome.

From 177 analyzed proteins, 12 proteins were non-detectable in our pooled-plasma samples. In total, 35 proteins were found to be altered ($p<0.1$) regarding outcome classification. Inflammation and immune response were the main modified pathways, with changes in the expression of chemokines, cytokines and their receptors, as well as other cellular and systemic processes (Fig 2). From these 35 proteins, 29 were associated with worsening (such as metalloproteinases, several components of the MIP family…), being either elevated in

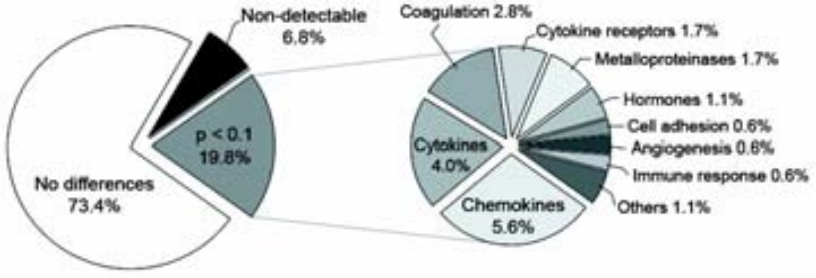


Figure 2. General proteome changes according to neurological stroke outcome. Results of the 9 pooled-plasma samples screened in SearchLight® library. Thirty-five proteins were found altered ($p<0.1$) when outcome groups were statistically compared. Pathways and cellular processes based on gene ontologies classification are detailed.

those patients who worsened or decreased in those patients who improved, and only 6 proteins were associated with improvement (Apo A-1, TRAIL, C-peptide, IL-8, PAI-1 active-form and OPN), conversely (Table 1).

Replication phase in 10 candidates

There were no differences in demographic characteristics between the derivation (N=36) and the replication (N=80) cohorts (Supporting Information Table II).

Table 1. Biomarker levels in pooled-plasma samples regarding in-hospital outcome classification

	Worsening			**Improvement**		
Biomarker	**Yes**	**No**	**p-value**	**Yes**	**No**	**p-value**
Apo A-1 (μg/mL)	201.7 ± 56.4	303.2 ± 37.2	**0.013**	299.6 ±33.3	254.2 ± 74.2	n.a.
BCA-1 (pg/mL)	44.8 ± 22.1	34.7 ± 15.0	n.a.	23.3 ± 7.4	45.4 ± 15.6	0.058
BD-2 (ng/mL)	1.9 ± 0.6	1.0 ± 0.4	**0.043**	1.2 ± 0.5	1.3 ± 0.8	n.a.
C-peptide (ng/mL)	2.0 ± 0.4	1.8 ± 0.6	n.a.	2.3 ± 0.4	1.7 ± 0.5	0.095
D-dimer (μg/mL)	320.5 (228.1-412.9)	73.7 (59.6-122.5)	**0.046**	69.2 (61.0-73.7)	125.3 (122.5-228.1)	n.a.
Eotaxin-2 (pg/mL)	188. 6 ± 68.6	125.3 ± 34.9	0.099	116.1 ± 45.1	161.6 ± 55.3	n.a.
Exodus-2 (pg/mL)	74.1 ± 12.1	51.9 ± 8.8	**0.038**	43.7 ± 8.4	64.0 ± 11.2	0.073
Fibrinogen (mg/mL)	18.6 ± 5.2	7.5 ± 2.1	0.056	6.6 ± 0.9	13.5 ±6.7	n.a.
IL-1R-I (ng/mL)	2.3 ± 0.5	2.2 ± 0.3	n.a.	2.0 ± 0.0	2.3 ± 0.4	0.071
IL-12p40 (pg/mL)	7.8 (7.3-66.0)	11.0 (4.7-13.4)	n.a.	4.7 (2.8-7.8)	12.2 (7.8-22.4)	0.071
IL-15 (pg/mL)	5.3 (4.7-15.4)	4.6 (3.8-5.4)	n.a.	3.8 (3.2-4.5)	5.3 (4.2-6.4)	0.071
IL-23 (pg/mL)	1436.9 (730.4-1916.6)	41.4 (16.3-47.2)	n.a.	16.3 (9.1-31.3)	75.1 (36.5-1436.9)	0.071
IL-4R (pg/mL)	677.9 ± 104.8	563.0 ± 180.2	n.a.	442.6 ± 115.4	680.7 ± 118.9	**0.025**
IL-8 (pg/mL)	11.6 ± 1.9	19.0 ± 5.2	0.055	19.7 ± 4.9	15.0 ± 5.7	n.a.
ITAC (pg/mL)	32.1 (23.4-49.7)	15.8 (12.4-26.8)	n.a.	12.4 (12.0-13.9)	29.4 (16.2-65.0)	**0.039**
LIF (pg/mL)	0.8 (0.7-4.2)	0.3 (0.3-0.3)	**0.016**	0.3 (0.3-0.3)	0.7 (0.3-0.8)	0.088
MCP-1 (pg/mL)	559.8 (504.7-669.2)	537.0 (480.7-795.7)	n.a.	480.7 (458.5-492.7)	673.9 (559.8-795.7)	0.071
MCP-2 (pg/mL)	11.4 (10.9-18.3)	9.2 (6.6-12.5)	n.a.	6.6 (6.5-8.1)	11.9 (10.5-25.2)	**0.039**
M-CSF (pg/mL)	58.0 (31.6-61.7)	39.5 (7.3-68.0)	n.a.	7.3 (6.2-20.2)	61.7 (45.9-68.0)	0.092
MIP-1a (pg/mL)	6.4 ± 4.2	5.8 ± 3.9	n.a.	2.8 ± 2.4	7.6 ± 3.3	0.066
MIP-1b (pg/mL)	65.4 (56.7-100.7)	49.7 (45.0-63.3)	n.a.	45.0 (40.1-46.1)	64.3 (52.3-66.7)	**0.020**
MIP-3b (pg/mL)	399.6 (350.2-1115.3)	240.7 (178.8-271.3)	**0.020**	178.8 (178.7-221.7)	288.0 (271.3-399.6)	**0.039**
MMP-1 (ng/mL)	8.6 ± 3.1	9.5 ± 5.2	n.a.	5.6 ± 2.8	10.9 ± 4.1	0.089
MMP-2 (ng/mL)	205.2 ± 23.3	166.6 ± 28.1	0.082	165.5 ± 34.1	186.5 ± 31.1	n.a.
MMP-7 (ng/mL)	2.0 (1.7-4.5)	1.8 (1.5-2.7)	n.a.	1.5 (1.3-1.7)	2.4 (1.8-3.0)	0.071
OPN (ng/mL)	35.7 ± 6.8	45.2 ± 10.1	n.a.	52.6 ± 7.6	37.0 ± 5.4	**0.014**
PAI-1 active (ng/mL)	1.7 ± 1.7	5.0 ± 2.1	**0.049**	5.4 ± 2.3	3.2 ± 2.4	n.a.
PAI-1 total (ng/mL)	67.0 (62.0-107.8)	37.1 (32.8-42.0)	0.071	40.5 (36.7-41.3)	62.0 (33.6-93.8)	n.a.
PD-1 (pg/mL)	671.2 (433.8-863.2)	196.7 (172.3-333.3)	n.a.	172.3 (156.9-188.2)	356.8 (196.4-671.2)	0.071
PLGF (pg/mL)	3.1 (3.0-25.8)	3.9 (1.9-4.9)	n.a.	1.9 (1.4-2.5)	4.8 (3.1-9.3)	0.071
Protein C (μg/mL)	2.8 ± 0.6	2.2 ± 0.2	0.062	2.4 ± 0.1	2.4 ± 0.6	n.a.
P-Selectin (ng/mL)	588.9 ± 189.0	313.2 ± 76.6	**0.014**	313.8 ± 62.7	450.7 ± 203.7	n.a.
Resistin (ng/mL)	25.9 ± 5.8	27.1 ± 12.4	n.a.	17.6 ± 3.5	31.3 ± 9.5	0.051
TRAIL (pg/mL)	59.8 (61.6-60.6)	69.8 (62.1-78.0)	0.071	62.1 (59.1-65.5)	66.1 (59.8-78.0)	n.a.
TWEAK (pg/mL)	1068.7 ± 106.4	881.8 ± 82.0	**0.021**	830.2 ± 49.9	1001.1 ± 112.1	**0.044**

List of 35 proteins that were found associated (p<0.1) to outcome in at least one analysis (worsening vs. stability/improvement or improvement vs. stability/worsening). Those normally distributed proteins were expressed as mean ± SD and those non-normally distributed proteins were described as median (IQR). Statistically significant differences are expressed as bold p-value; n.a.: non-associated (p>0.1).

Our ten selected proteins were mainly cytokines and chemokines which were associated with worsening: BD-2, MIP-3b, BCA-1, exodus-2, IL-4R, IL-12p40, LIF, MIP-1b and TWEAK. Nevertheless PAI-1 active-form was associated with improvement in the discovery phase. Results from MIP-3b and PAI-1 active-form were excluded from further analysis because high CV interassay.

We have explored the influence of several clinical factors on the level of our candidate biomarkers, without substantial findings (Supporting Information Table III). Regarding outcome, BD-2 and IL-4R levels were elevated in those patients who worsened within 24h (p=0.046 and p=0.062, respectively) and 48h after stroke symptoms onset (p=0.041 and p=0.031, respectively) (Fig 3A to D). Interestingly, this association with worsening occurs only in those patients who received rt-PA earlier (within the first 3 hours from onset) and had a less established infarct (p=0.058 for BD-2 and p=0.024 for IL-4R) than those patients who received rt-PA beyond 3 hours (p=0.399 for BD-2 and p=0.909 for IL-4R) (Fig 4A to B).

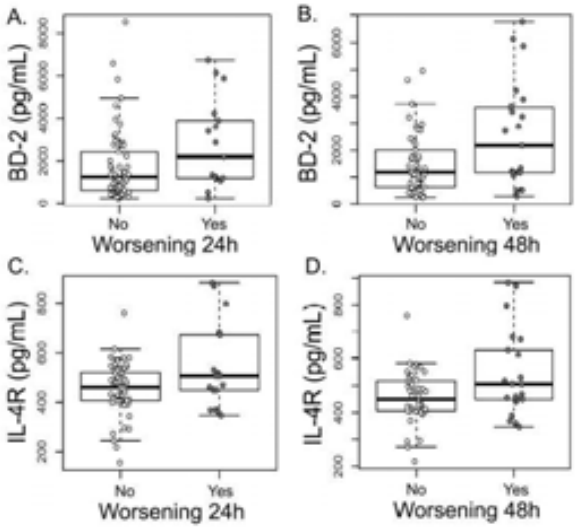


Figure 3. Blood level of biomarker candidates regarding in-hospital outcome.
Graphs represent baseline biomarkers levels in relation to worsening at 24h (3A and 3C) and 48h (3B and 3D) from stroke symptoms onset (N=80 patients). Box-plot represents median and interquartile range, with overlapping dot-plot to show the distribution of the values

New biomarkers for acute stroke prognosis

In our replication cohort 21.5% of patients worsened within 24h and 31.8% of patients worsened at 48h, with 40% of worsening due to hemorrhagic transformation (that was symptomatic in 75% of the cases). Patients who worsened were more likely to have diabetes mellitus, higher NIHSS at admission and to have suffered a previous stroke (Table 2). A cut-off of 1.15 ng/mL for BD-2 (82% sensitivity, 48% specificity) and of 503.40 pg/mL for IL-4R (53% sensitivity, 72% specificity) discriminated between patients who worsened or not at 24h. At 48h identical cut-off points remained discriminative with 76% sensitivity, 49% specificity for BD-2 and 52% sensitivity, 73% specificity for IL-4R. Any association was found between BD-2 or IL-4R and hemorrhagic transformation as a specific cause of worsening (data not shown).

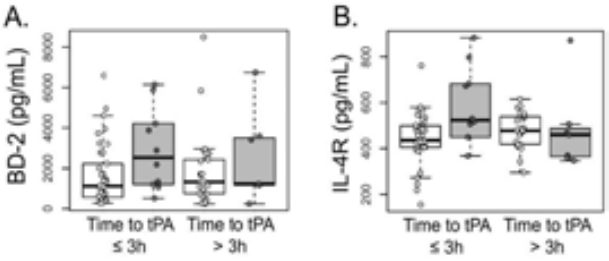


Figure 4. Blood level of biomarker candidates depending on time to treatment and outcome.
Graphs represent baseline biomarkers levels in relation to time from symptoms onset to rt-PA treatment, differentiating between patients who worsened (grey) or not (white) at 24h. Box-plot represents median and interquartile range, with overlapping dot-plot to show the distribution of the values.

After including all associated variables into the multivariate logistic regression analysis, only NIHSS at admission (OR_{adj} 1.10 [95% CI 1.00-1.22] p=0.050) and diabetes mellitus

Table 2. Univariate analyses for worsening at 24h and 48h in the replication cohort.

	Worsening 24h			**Worsening 48h**		
Factors	**Yes (N=17)**	**No (N=62)**	**p-value**	**Yes (N=21)**	**No (N=45)**	**p-value**
Age, years median (IQR)	79.0 (67.0-82.0)	78.0 (72.0-82.0)	0.797	78.0 (67.0-82.0)	78.0 (72.0-82.0)	0.767
NIHSS at admission mean ± SD	17.2 ± 5.4	14.7 ± 6.8	0.155	16.9 ± 5.1	13.8 ± 7.0	0.075*
Previous mRS median (IQR)	0 (0-0)	0 (0-0)	0.359	0 (0-0)	0 (0-0)	0.518
Gender (Male) % (n)	52.9 (9)	51.6 (32)	0.923	47.6 (10)	60.0 (27)	0.345
Smokers % (n)	6.7 (1)	16.7 (10)	0.445	16.7 (3)	11.4 (5)	0.681
Glucose (mg/dL) median (IQR)	120.0 (99.0-170.5)	118.0 (94.0-146.0)	0.382	127.5 (97.0-158)	113.5 (88.5-138.0)	0.139
Arterial hypertension % (n)	70.6 (12)	66.1 (41)	0.729	76.2 (16)	60.0 (27)	0.199
Diabetes mellitus %(n)	47.1 (8)	19.4 (12)	**0.029**	47.6 (10)	11.1 (5)	**0.003**
Dyslipidemia % (n)	41.2 (7)	29.0 (18)	0.340	38.1 (8)	33.3 (15)	0.705
Atrial fibrillation % (n)	35.3 (6)	35.5 (22)	0.988	33.3 (7)	35.6 (16)	0.860
Ischemic cardiopathy % (n)	17.6 (3)	22.6 (14)	1.000	14.3 (3)	28.9 (13)	0.197
Early signs % (n)	13.3 (2)	12.3 (7)	1.000	21.1 (4)	10.0 (4)	0.416
Previous stroke % (n)	23.5 (4)	19.4 (12)	0.738	38.1 (8)	17.8 (8)	0.073*
Minutes to treatment mean ± SD	206.8 ± 114.0	175.4 ± 60.4	0.289	192.5 ± 101.6	177.5 ± 68.4	0.489
Vessel localization			0.297			0.215
- MCA % (n)	70.6 (12)	87.1 (54)		76.2 (16)	88.9 (40)	
TOAST			0.203			0.113
- Atherothrombotic % (n)	41.2 (7)	19.4 (12)		42.9 (9)	15.6 (7)	
- Cardioembolic % (n)	41.2 (7)	48.4 (30)		33.3 (7)	53.3 (24)	
- Undetermined % (n)	11.8 (2)	29.0 (18)		19.0 (4)	26.7 (12)	
BD-2 > 1.15 ng/mL % (n)	82.4 (14)	51.6 (32)	**0.023**	76.2 (16)	51.1 (23)	0.054*
IL-4R > 503.40 pg/mL % (n)	52.9 (9)	27.9 (17)	0.052*	52.4 (11)	27.3 (12)	**0.048**

mRS, modified Rankin scale; TOAST, etiology stroke subtype classification; NIHSS, National Institutes of Health Stroke Scale; IQR, interquartile range. Statistically significant differences between groups are expressed as bold p-value; * stands for statistical trend (p<0.1).

(OR_{adj} 5.17 [1.47-18.13] p=0.010) were clinical independent predictors of worsening within the first 24h after symptoms onset. When plasma levels above the cut-off for both BD-2 (OR_{adj} 4.87 [1.13-20.91] p=0.033) or IL-4R (OR_{adj} 3.52 [1.03-12.08] p=0.045) were added separately or in combination (OR_{adj} 3.81 [1.43-10.14] p=0.008) to the clinical predictive model, the discriminative power of the predictive model increased from an AUC of 0.719 (95% CI 0.606-0.815) to an AUC of 0.829 (0.727-0.905) (p=0.056) (Table 3). Moreover further statistical analyses showed how the combination of both biomarkers increased significantly the discrimination between patients who worsened or who did not (IDI index 0.095, p=0.033). Regarding patient reclassification into higher risk categories, BD-2 alone reclassified better both the events and non-events (NRI index 28.2%, p=0.009) than the combination of both biomarkers (NRI index 19%, p=0.020) (Table 3). Similar results were obtained when worsening was assessed at 48h from stroke symptoms onset, when the combination of both BD-2 and IL-4R allow the reclassification of 27.5% of patients (p=0.020) (Supporting Information Table IV).

Discussion

In this study we firstly described the plasma protein profile which is associated with early neurological outcome after ischemic stroke. Furthermore a methodological improvement was attempted both technically (by introducing the pooling strategy) and statistically (by using comparative metrics). As a result two new outcome biomarkers (BD-2 and IL-4R) were discovered.

We consider the use of pooling strategies highly suitable for the discovery of new candidates to become biomarkers. Pooling reduces the biological variability, as it is assumed that the expression in the pooled-sample averages the expression of the individual samples which were contained in the pool (Kendziorski C., 2005). Another good point of pooling is the reduced costs, both in

number of assays and number of biological samples. The use of a sub-pooling strategy to gain accuracy by including some variability within each group (Zhang W., 2007) and the replication of the discovery findings in individual samples (Sham P., 2002; Walker L.C. 2010) contribute to a more desirable approach.

Following this design, we found 35 altered proteins which are involved in biological and cellular processes with a known role in ischemic stroke, such as inflammation, apoptosis or the coagulation cascade (Mehta S.L., 2007; Jickling G.C., 2011). Some of these proteins have been previously associated to stroke outcome, confirming the validity of our strategy: fibrinogen (del Zoppo G.J., 2009), D-dimer (Welsh P., 2009), protein C (Mendioroz M., 2009), resistin (Efstathiou S.P., 2007), MMP-2 (Montaner J., 2001), MCP-1/CCL2 (Worthmann H., 2010) and MIP-1a/CCL3 (Zaremba J., 2006).

We have only detected one protein, OPN, which discloses an inversely associated relation in our study and in previous literature; while we found higher levels in those patients

Table 3. Comparison between predictive models with only clinical variables and models including biomarkers for worsening at 24h.

		Model – Worsening 24h			
		Only Clinical	**Clinical + BD-2**	**Clinical + IL-4R**	**Clinical + Combination BD-2 & IL-4R**
Logistic regression (OR adj)	NIHSS admission	1.1 (1.0-1.2), **0.050**	1.1 (1.0-1.2), **0.049**	1.1 (1.0-1.2), **0.030**	1.1 (1.0-1.3), **0.028**
	DM	5.2 (1.5-18.1), **0.010**	5.1 (1.4-19.3), **0.015**	5.2 (1.4-19.3), **0.013**	5.4 (1.3-21-3), **0.017**
	Age	1.0 (0.9-1.0), 0.690	1.0 (0.9-1.0), 0.882	1.0 (0.9-1.0), 0.834	1.0 (0.9-1.0), 0.991
	Gender (female)	1.5 (0.4-5.0), 0.513	1.1 (0.3-4.1), 0.850	1.4 (0.4-5.0), 0.565	1.1 (0.3-4.2), 0.860
	BD-2	-	4.9 (1.1-21.0), **0.033**	-	-
	IL-4R	-	-	3.5 (1.0-12.1), **0.045**	-
	BD-2 + IL-4R	-	-	-	3.8 (1.4-10.1), **0.008**
Categorical NRI	NRI events	-	11.8%	11.8%	5.9%
	NRI non-events	-	16.4%	6.5%	13.1%
	NRI	-	28.2% (6.9 – 49.4)	18.3% (-0.8 – 37.5)	19% (3.0 – 35.0)
	p-value	Ref.	**0.009**	0.061*	**0.020**
IDI statistics	IDI events	-	0.046	0.034	0.076
	IDI non-events	-	0.012	0.007	0.019
	IDI	-	0.058 (-0.002 – 0.119)	0.041 (-0.024 – 0.106)	0.095 (0.007 – 0.182)
	p-value	Ref.	0.059*	0.213	**0.033**
ROC curves	AUC	0.719 (0.606-0.815)	0.788 (0.681-0.872)	0.774 (0.665-0.861)	0.829 (0.727-0.905)
	p-value	Ref.	0.123	0.299	0.056*

All logistic regression models were adjusted by NIHSS at admission, age and gender; OR_{adj} (95% CI) and p-value were given. Biomarkers were added to clinical logistic regression model using cut-off point: BD-2 > 1.15 ng/mL and IL-4R > 503.40 pg/mL. NRI: Net Reclassification Improvement index (risk categories used: ≤10%, 10-90% and >90%); percentage of reclassification given for both events (i.e. patients who worsened at 24h) and non-events and for the sum of both (with 95% CI). IDI: Integrated Discrimination Improvement index; index given for both events and non-events and for the sum of both (with 95% CI). AUC: Area Under the ROC Curve; area with 95%CI given for each model. Clinical model always used as reference model to compare. Statistically significant results expressed as bold p-values; * statistical trend.

who improved, in a previous study from our group OPN was oppositely associated to long-term poor prognosis (Mendioroz M., 2011). Moreover, some molecules that have been associated with short-term prognosis in our study, such as C-peptide (O'Neill P.A., 1991), P-selectin (Bath P.M.W., 1998) and IL-8 (Zeng L., 2013), have been studied in other cohorts without any association regarding stroke prognosis. Although following different approaches, the possibility of false-positive results could not be overlooked. On the other hand those proteins typically associated to poor prognosis, such IL-6 (Smith C.J., 2004) and CRP (Montaner J., 2006), were not associated in our pooled cohort. This could be related to the dilution effect which was commented above, a plausible explanation since neither IL-6 nor CRP have shown a great association with outcome in individual studies (Whiteley W., 2009).

Nonetheless our discovery experiment provides a list of interesting candidates which have not been previously explored in the context of stroke prognosis. From them, we chose 10 candidates (BCA-1/CXCL13, BD-2, Exodus-2/CCL21, IL-12p40, IL-4R, LIF, MIP-1b, MIP-3b, PAI-1 active form and TWEAK) to be tested in our replication cohort. BD-2 and IL-4R were found as independent predictors of neurological worsening in the acute phase of ischemic stroke, within 24 and 48 hours after symptoms onset, mainly when the infarct has not been fully established.

Human beta-defensins play a role in immune-inflammatory responses, mainly acting as antimicrobial peptides and also as chemoattractants. BD-2 is mainly expressed in the respiratory tract epithelia, but it can also be expressed by monocytes and macrophages and, at brain level, by capillary endothelial cells and astrocytes. BD-2 expression is inducible by cytokines, such as TNF-a or IL-1b, and bacteria (Schröder J.M., 1999). Moreover, *in vitro* and *in vivo* models have shown an increase in expression and the release of BD-2 after hypoxic/ischemic stimuli (Nickel D., 2012; Liu K.X. 2009). In stroke patients, only the copy number variant of BD-2 gene (*DEFB4*) has been studied and reflects a higher protein plasma concentration in those patients with more gene copies (Tiszlavicz Z., 2012). Thus, the higher levels of BD-2 which were associated with worsening might be reflecting the inflammatory state that is produced after stroke.

IL-4 is a multifunctional cytokine which is required for the development of Th2 cells and thus with anti-inflammatory properties. To exert this effect, IL-4 binds to membrane-bound IL-4R; however, there exists a soluble form of IL-4R (mainly produced by MMPs-mediated proteolysis (Jung T., 1999)) which can block or prolong IL-4 effects depending on its concentration (Jung T., 1999-2). Together with the higher expression of MMPs detected in patients who worsened, the association of soluble IL-4R with poor outcome is also in accordance with the pro-inflammatory state that is suggested by BD-2.

We also wanted to prove that, apart from being independent predictors, both biomarkers add value to clinical information. Clinical variables typically associated with neurological deterioration are stroke severity at admission and age, as non-modifiable factors, together with risk factors such as

diabetes mellitus or arterial hypertension. The clinical variables that were independently associated with early worsening in our cohort comprise NIHSS at admission and diabetes mellitus, after being adjusted by age and gender. The deleterious effect of diabetes mellitus on stroke outcome has been previously observed by several groups, as recently reviewed (Desilles J.P., 2013). When this clinical model was considered, comparison of AUCs showed how BD-2 and IL-4R, alone or in combination, improved the measure of discrimination of clinical variables, which changes from acceptable to excellent discrimination between patients who worsened and those who did not. However, some authors suggested that the comparison of AUCs is not the best way to know the additional value of biomarkers (Pepe M.S., 2004). Therefore we have employed statistical tools which are being applied to know the capacities of biomarkers regarding discrimination (IDI) and patients' reclassification into risk categories (NRI). These two tests are based on the risk prediction models (i.e. logistical regression models) and the probability of an event for each patient (Pickering J.W., 2012). Both IDI and NRI can be calculated per separate for events (i.e. worsening) and non-events (i.e. non-worsening) to better interpret how the biomarker is adding value, if it is by better recognizing the events or if it is by reducing the rate of false positives for non-events. Regarding our results for IDI test, we found an improvement in discrimination by the identification of real events for BD-2 and particularly for the combination of both BD-2 and IL-4R. This may be explained because BD-2 cut-off point had great sensitivity and IL-4R increased specificity, thus the combination of both biomarkers gained statistical power. Regarding reclassification of the patients into extreme risk categories, BD-2 alone showed the best performance at 24h, reclassifying correctly a 28.2% of patients, mainly by reducing false positives. At 48h, the combination of both BD-2 and IL-4R achieved this non-negligible reclassification rate. If confirmed in prospective studies as prognostic biomarkers, the measurement of plasma levels of BD-2 and IL-4R in the acute phase of stroke might help in decision-making processes, such as in giving information to patients and relatives, optimization of inclusion in specialized stroke units, evaluation of treatment benefits or inclusion into clinical trials.

Our study stands with several limitations. The results of our discovery phase have not been corrected in accordance to multiple testing; we consider this phase merely exploratory and the limited sample size has not statistical power enough to sustain this kind of correction. Moreover, sample size of our replication cohort is relatively small and limits the assessment of biomarkers association with specific worsening causes that could influence outcome, as has been suggested in larger cohorts, although non-modifiable factors are the main force (Grube M.M., 2013); thus an independent larger study might conduct sub-analysis by specific causes and to assess the value of these biomarkers in all types of stroke patients since the results of the present study can only be generalized to stroke patients who underwent thrombolysis. In the future, BD-2 and IL-4R might be further explored in a prospective cohort that is being recruited in our hospital and that will collect a

more complete information regarding outcome. Furthermore, different detection methods (simple ELISA, Luminex arrays or others) should be explored in order to confirm our results. Finally, molecules which have been found altered in our discovery phase but have not been included in the replication due to the impossibility of performing multiplex ELISA as well as molecules that are not included in the discovery array might be of interest, such as the recently described stroke outcome biomarker copeptin (De Marchis G.M., 2013) or others.

Acknowledgements

T.G-B is supported by a predoctoral fellowship (FI09/00017) from the *Instituto de Salud Carlos III*. V.L is supported by a predoctoral fellowship from Vall d'Hebron Institute of Research. Neurovascular Research Laboratory takes part in the Spanish stroke research network *INVICTUS* (RD12/0014/0005) and is supported on stroke biomarkers research by FIS 11/0176. All authors declare no conflicts of interest.

We would like to thank to all study collaborators, residents, neurologists and nurses of the Stroke Unit and Neurology Ward from the Vall d'Hebron Hospital and especially to all the patients who participated in the study.

References

Alawneh J.A., Moustafa R.R. and Baron J.C. (2009) Hemodynamic factors and perfusion abnormalities in early neurological deterioration. Stroke 40, e443-e450.

Arenillas J.F., Rovira A., Molina C.A., Grivé E., Montaner J. and Alvarez-Sabín J. (2002) Prediction of early neurological deterioration using diffusion- and perfusion-weighted imaging in hyperacute middle cerebral artery ischemic stroke. Stroke 33, 2197-2203.

Barker P.E., Wagner P.D., Stein S.E., Bunk D.M., Srivastava S. and Omenn G.S. (2006) Standards for plasma and serum proteomics in early cancer detection: a needs assessment report from the National Institute of Standards and Technology-National Cancer Institute Standards, methods, assays, reagents and technologies workshop, August 18-19, 2005. Clin. Chem. 52, 1669-1674.

Bath P.M.W., Blann A., Smith N. and Butterworth R.J. (1998) Von Willebrand factor, P-selectin and fibrinogen levels in patients with acute ischaemic and haemorrhagic stroke, and their relationship with stroke sub-type and functional outcome. Platelets 9, 155-159.

Brott T.G., Haley E.C. Jr., Levy D.E., Barsan W., Broderick J., Sheppard G.L., Spilker J., Kongable G.L., Massey S., Reed R. and Marler J.R. (1992) Urgent therapy for stroke. Part I. Pilot study of tissue plasminogen activator administered within 90 minutes. Stroke 23, 632-640.

Brott T. and Bogousslavsky J. (2000) Treatment of acute ischemic stroke. N. Engl. J. Med. 343, 710-722.

De Marchis G.M., Katan M., Weck A., Fluri F., Foerch C., Findling O., Schuetz P., Buhl D., El-Koussy M., Gensicke H., Seiler M., Morgenthaler N., Mattle H.P., Mueller B., Christ-Crain M. and Arnold M. (2013) Copeptin adds prognostic information after ischemic stroke: Results from the CoRisk study. Neurology 80, 1278-1286.

del Zoppo G.J., Levy D.E., Wasiewski W.W., Pancioli A.M., Demchuk A.M., Trammel J., Demaerschalk B.M., Kaste M., Albers G.W. and Ringelstein E.B. (2009) Hyperfibrinogenemia and functional outcome from acute ischemic stroke. Stroke 40, 1687-1691.

DeLong E.R., DeLong D.M. and Clarke-Pearson D.L. (1988) Comparing the areas under two or more correlated receiver operating characteristic curves: A nonparametric approach. Biometrics 44, 837-845.

Desilles J.P., Meseguer E., Labreuche J., Lapergue B., Sirimarco G., Gonzalez-Valcarcel J., Lavallée P., Cabrejo L., Guidoix C., Klein I., Amarenco P. and Mazighi M. (2013) Diabetes mellitus, admission glucose, and outcomes after stroke thrombolysis: A registry and systematic review. Stroke 44, 1915-1923.

Efstathiou S.P., Tsiakou A.G., Tsioulos D.I., Panagiotou T.N., Pefanis A.V., Achimastos A.D. and Mountokalakis T.D. (2007) Prognostic significance of plasma resistin levels in patients with atherothrombotic ischemic stroke. Clin. Chim. Acta 378, 78-85.

Ernoult E., Bourreau A., Gamelin E. and Guette C. (2010) A proteomic approach for plasma biomarker discovery with iTRAQ labelling and OFFGEL fractionation. J. Biomed. Biotechnol. 2010, 927917.

Fragnoud R., Yugueros-Marcos J., Pachot A. and Bedin F. (2012) Isotope coded protein labeling analysis of plasma specimens from acute severe dengue fever patients. Proteome Science 10, 60.

Grube M.M., Koennecke H-C., Walter G., Meisel A., Sobesky J., Nolte C.H., Wellwood I. and Heuschmann P.U. (2013) Influence of acute complications on outcome 3 months after ischemic stroke. PLoS ONE 8, e75719.

Jickling G.C. and Sharp F.R. (2011) Blood biomarkers of ischemic stroke. Neurotherapeutics 8, 349-360.

Jung T., Schrader N., Hellwig M., Enssle K.H. and Neumann C. (1999) Soluble human interleukin-4 receptor is produced by activated T cells under the control of metalloproteinases. Int. Arch. Allergy Immunol. 119, 23-30.

Jung T., Wagner K., Neumann C. and Heusser C.H. (1999) Enhancement of human IL-4 activity by soluble IL-4 receptors in vitro. Eur. J. Immunol. 29, 864-871.

Karp N.A. and Lilley K.S. (2009) Investigating sample pooling strategies for DIGE experiments to address biological variability. Proteomics 9, 388-397.

Kendziorski C., Irizarry R.A., Chen K.S., Haaq J.D. and Gould M.N. (2005) On the utility of pooling biological samples in microarray experiments. Proc. Natl. Acad. Sci. USA 102, 4252-4257.

Lin L.C., Lee T.H., Chang C.H., Chang Y.J., Liou C.W., Chang K.C., Lee J.D., Peng T.Y., Chung J., Chen S.C. and Yang J.T. (2012) Predictors of clinical deterioration during hospitalization following acute ischemic stroke. Eur. Neurol. 67,186-192.

Liu K.X., Chen S.Q., Zhang H., Guo J.Y., Li Y.S. and Huang W.Q. (2009) Intestinal ischaemia/reperfusion upregulates β-defensin-2 expression and causes acute lung injury in the rat. Injury 40, 950-955.

Mehta S.L., Manhas N. and Raghubir R. (2007) Molecular targets in cerebral ischemia for developing novel therapeutics. Brain Res. Rev. 54, 34-66.

Mendioroz M., Fernández-Cadenas I., Alvarez-Sabín J., Rosell A., Quiroga D., Cuadrado E., Delgado P., Rubiera M., Ribó M., Molina C. and Montaner J. (2009) Endogenous activated protein C predicts hemorrhagic transformation and mortality after tissue plasminogen activator treatment in stroke patients. Cerebrovasc. Dis. 28, 143-150.

Mendioroz M., Fernández-Cadenas I., Rosell A., Delgado P., Domingues-Montanari S., Ribó M., Penalba A., Quintana M., Alvarez-Sabín J. and Montaner J. (2011) Osteopontin predicts long-term functional outcome among ischemic stroke patients. J. Neurol. 258, 486-493.

Montaner J., Alvarez-Sabín J., Molina C., Anglés A., Abilleira S., Arenillas J., González M.A. and Monasterio J. (2001) Matrix metalloproteinase expression after human cardioembolic stroke: temporal profile and relation to neurological impairment. Stroke 32, 1759-1766.

Montaner J., Fernandez-Cadenas I., Molina C.A., Ribó M., Huertas R., Rosell A., Penalba A., Ortega L., Chacón P. and Alvarez-Sabín J. (2006) Poststroke C-reactive protein is a powerful prognostic tool among candidates for thrombolysis. Stroke 37, 1205-1210.

Montaner J., García-Berrocoso T., Mendioroz M., Palacios M., Perea-Gainza M., Delgado P., Rosell A., Slevin M., Ribó M., Molina C.A. and Alvarez-Sabín J. (2012) Brain natriuretic peptide is associated with worsening and mortality in acute stroke patients but adds no prognostic value to clinical predictors of outcome. Cerebrovasc. Dis. 34, 240-245.

Nickel D., Busch M., Mayer D., Hagemann B., Knoll V. and Stenger S. (2012) Hypoxia triggers the expression of human β defensin 2 and antimicrobial activity against Mycobacterium tuberculosis in human macrophages. J. Immunol. 188, 4001-4007.

O'Neill P.A., Davies I., Fullerton K.J. and Bennett D. (1991) Stress hormone and blood glucose response following acute stroke in the elderly. Stroke 22, 842-847.

Pencina M.J., D'Agostino R.B., D'Agostino R.B. and Vasan R.S. (2008) Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. Stat. Med. 27, 157-172.

Pepe M.S., Janes H., Longton G., Leisenring W. and Newcomb P. (2004) Limitations of the Odds Ratio in gauging the performance of a diagnostic, prognostic, or screening marker. Am. J. Epidemiol. 159, 882-890.

Pickering J.W. and Endre Z.H. (2012) New metrics for assessing diagnostic potential of candidate biomarkers. Clin. J. Am. Soc. Nephrol. 7, 1355-1364.

Roger V.L., Go A.S., Lloyd-Jones D.M., Benjamin E.J., Berry J.D., Borden W.B., Bravata D.M., Dai S., Ford E.S., Fox C.S., Fullerton H.J., Gillespie C., Hailpern S.M., Heit J.A., Howard V.J., Kissela B.M., Kittner S.J., Lackland D.T., Lichtman J.H., Lisabeth L.D., Makuc D.M., Marcus G.M., Marelli A., Matchar D.B., Moy C.S., Mozaffarian D., Mussolino M.E., Nichol G., Paynter N.P., Soliman E.Z., Sorlie P.D., Sotoodehnia N., Turan T.N., Virani S.S., Wong N.D., Woo D. and Turner M.B. (2012) Heart disease and stroke statistics--2012 update: A report from the american heart association. Circulation 125, e2-e220.

Roquer J., Rodríguez-Campello A., Gomis M., Jiménez-Conde J., Cuadrado-Godia E., Vivanco R., Giralt E., Sepúlveda M., Pont-Sunyer C., Cucurella G. and Ois A. (2008) Acute stroke unit care and early neurological deterioration in ischemic stroke. J. Neurol. 255, 1012-1017.

Schröder J.M. and Harder J. (1999) Human beta-defensin-2. Int. J. Biochem. Cell. Biol. 31, 645-651.

Sham P., Bader J.S., Craig I., O'Donovan M. and Owen M. (2002) DNA pooling: a tool for large-scale association studies. Nat. Rev. Genet. 3, 862-871.

Smith C.J., Emsley H.C.A., Gavin C.M., Georgiou R.F., Vail A., Barberan E.M., del Zoppo G.J., Hallenbeck J.M., Rothwell N.J., Hopkins S.J. and Tyrrell P.J. (2004) Peak plasma interleukin-6 and other peripheral markers of inflammation in the first week of ischaemic stroke correlate with brain infarct volume, stroke severity and long-term outcome. BMC Neurol. 4, 2.

Tiszlavicz Z., Somogyvári F., Szolnoki Z., Sztriha L.K., Németh B., Vécsei L. and Mándi Y. (2012) Genetic polymorphisms of human β-defensins in patients with ischemic stroke. Acta Neurol. Scand. 126, 109-115.

Vila N., Castillo J., Dávalos A. and Chamorro A. (2000) Proinflammatory cytokines and early neurological worsening in ischemic stroke. Stroke 31, 2325-2329.

Walker L.C., Thompson B.A., Waddell N.; kConFab Investigators, Grimmond S.M. and Spurdle A.B. (2010) Use of DNA-Damaging agents and RNA pooling to assess expression profiles associated with *BRCA1* and *BRCA2* mutation status in familial breast cancer patients. PLoS Genet. 6, e1000850.

Welsh P., Barber M., Langhorne P., Rumley A., Lowe G.D. and Stott D.J. (2009) Associations of inflammatory and haemostatic biomarkers with poor outcome in acute ischaemic stroke. Cerebrovasc. Dis. 27, 247-253.

Whiteley W., Chong W.L., Sengupta A. and Sandercock P. (2009) Blood markers for the prognosis of ischemic stroke: a systematic review. Stroke 40, e380-e389.

Whiteley W., Wardlaw J., Dennis M., Lowe G., Rumley A., Sattar N., Welsh P., Green A., Andrews M. and Sandercock P. (2012) The use of blood biomarkers to predict poor outcome after acute transient ischemic attack or ischemic stroke. Stroke 43, 86-91.

Worthmann H., Tryc A.B., Goldbecker A., Ma Y.T., Tountopoulou A., Hahn A., Dengler R., Lichtinghagen R. and Weissenborn K. (2010) The temporal profile of inflammatory markers and mediators in blood after acute ischemic stroke differs depending on stroke outcome. Cerebrovasc. Dis. 30, 85-92.

Zaremba J., Ilkowski J. and Losy J. (2006) Serial measurements of levels of the chemokines CCL2, CCL3 and CCL5 in serum of patients with acute ischemic stroke. Folia Neuropathol. 44, 282-289.

Zeng L., Liu J., Wang Y., Wang L., Weng S., Chen S. and Yang G.Y. (2013) Cocktail blood biomarkers: prediction of clinical outcomes in patients with acute ischemic stroke. Eur. Neurol. 69, 68-75.

Zhang W., Carriquiry A., Nettleton D. and Dekkers J.C.M. (2007) Pooling mRNA in microarray experiments and its effect on power. Bioinformatics 23,1217-1224.

Role of beta-defensin-2 and interleukin-4 receptor as stroke outcome biomarkers

Teresa García-Berrocoso, Dolors Giralt, Alejandro Bustamante, Victor Llombart, Marta Rubiera, Anna Penalba, Cristina Boada, Marc Espadaler, Carlos Molina, Joan Montaner

Supporting Information

Tables

Table I. SearchLight® antibodies library list.

Angiogenesis Factors	Ang-2 (Angiopoietin 2)
	BMP-9 (Bone Morphogenetic Protein 9)
	ER (Epiregulin)
	FGF basic (Fibroblast Growth Factor basic)
	Fibronectin
	HB-EGF (Heparin-Binding Epidermal Growth Factor)
	HGF (Hepatocyte Growth Factor)
	KGF (Keratinocyte Growth Factor)
	PDGF-AA (Platelet-Derived Growth Factor AA)
	PDGF-AB (Platelet-Derived Growth Factor AB)
	PDGF-BB (Platelet-Derived Growth Factor BB)
	PLGF (Placental Growth Factor)
	TPO (Thrombopoietin)
	VEGF (Vascular Endothelial Growth Factor)
	VEGF-C (Vascular Endothelial Growth Factor C)
	VEGF-D (Vascular Endothelial Growth Factor D)
	VEGF-R1 (Vascular Endothelial Growth Factor Receptor 1)
	VEGF-R2 (Vascular Endothelial Growth Factor Receptor 2)
Cell Adhesion Molecules	E-Cadherin
	Endoglin
	E-Selectin
	ICAM-1 (Intercellular Adhesion Molecule1)
	ICAM-3 (Intercellular Adhesion Molecule 3)
	L-Selectin
	PECAM-1 (Platelet Endothelial Cell Adhesion Molecule)
	P-Selectin
	TSP-1 (Thrombospondin-1)
	TSP-2 (Thrombospondin-2)
	VCAM-1 (Vascular Cell Adhesion Molecule 1)
Chemokines	BCA-1/CXCL13 (B Cell-Attracting Chemokine)
	ENA-78 (Epithelial Cell-Derived Neutrophil-Activating Peptide 78)
	Eotaxin
	Eotaxin-2/CCL24
	Eotaxin-3
	Exodus-2/CCL21/SLC
	GROα (Growth-Regulated Protein alpha)
	GROg (Growth-Regulated Protein gamma)
	HCC-4/CCL-16 (Hemofiltrate CC Chemokine 4)
Chemokines	I-309
	IL-8 (Interleukin 8)
	IP-10 (Interferon gamma-Induced Protein 10 kDa)
	I-TAC (Interferon-Inducible T-cell Alpha Chemoattractant)
	Lymphotactin
	MCP-1 (Monocyte Chemotactic Protein 1)
	MCP-2 (Monocyte Chemotactic Protein 2)
	MCP-3 (Monocyte Chemotactic Protein 3)
	MCP-4 (Monocyte Chemotactic Protein 4)
	MDC (Macrophage-Derived Chemokine)
	MIG (Monokine Induced by Gamma Interferon)
	MIP-1α (Macrophage Inflammatory Protein 1 alpha)
	MIP-1β (Macrophage Inflammatory Protein 1 beta)
	MIP-1δ (Macrophage Inflammatory Protein 1 delta)
	MIP-3α (Macrophage Inflammatory Protein 3 alpha)
	MIP-3β (Macrophage Inflammatory Protein 3 beta)
	MIP-4/PARC (Macrophage Inflammatory Protein 4)
	MPIF-1 (Myeloid Progenitor Inhibitory Factor 1)
	NAP-2 (Neutrophil Activating Peptide 2)
	RANTES (Regulated upon Activation, Normal T cell Expressed and presumably Secreted)
	SDF-1β (Stromal Cell-Derived Factor 1 beta)
	TARC (Thymus and Activation-Regulated Chemokine)
Coagulation	D-Dimer
	Fibrinogen
	PAI-1 Active (Plasminogen Activator Inhibitor 1 Active
	PAI-1 Total (Plasminogen Activator Inhibitor 1 Total)
	Protein C
	Tissue Factor
	Thrombomodulin
	Von Willebrand Factor
Cytokines	CD40L (Cluster of Differentiation 40 Ligand)
	GM-CSF (Granulocyte Macrophage Colony-Stimulating Factor)
	G-CSF (Granulocyte Colony-Stimulating Factor)
	FasL (Fas Ligand)
	IFNα (Interferon alpha)
	IFNg (Interferon gamma)
	IL-1α (Interleukin 1 alpha)
	IL-1β (Interleukin 1 beta)
Cytokines	IL-1ra (Interleukin 1 Receptor Antagonist
	IL-2 (Interleukin 2)
	IL-3 (Interleukin 3)
	IL-4 (Interleukin 4)
	IL-5 (Interleukin 5)
	IL-6 (Interleukin 6)
	IL-7 (Interleukin 7)
	IL-9 (Interleukin 9)
	IL-10 (Interleukin 10)
	IL-11 (Interleukin 11)
	IL-12p40 (Interleukin 12 p40 homodimer)
	IL-12p70 (Interleukin 12 p70 hetero-dimer)
	IL-13 (Interleukin 13)
	IL-15 (Interleukin 15)
	IL-16 (Interleukin 16)
	IL-17A (Interleukin 17A)
	IL-17E (Interleukin 17E)
	IL-18 (Interleukin 18)
	IL-23 (Interleukin 23)
	M-CSF (Macrophage Colony-Stimulating Factor)
	MIF (Migration Inhibitory Factor)
	OPN (Osteopontin)
	RANKL (Receptor Activator of NF-kB Ligand)
	TNFα monomer (Tumor Necrosis Factor alpha)
	TNFα active trimer (Tumor Necrosis Factor alpha)
	TRAIL (TNF-Related Apoptosis-Inducing Ligand)
	TSLP (Thymic Stromal Lymphopoietin)
	TWEAK (TNF-relate and WEAK induder of apoptosis)
Cytokine Receptors	CD30/TNFRSF8 (Cluster of Differentiation 30)
	hSGP130
	IL-1RI (Interleukin 1 Receeptor I)
	IL-1RII (Interleukin 1 Receptor II)
	IL-2Rα (Interleukin 2 Receptor alpha)
	IL-2Rg (Interleukin 2 Receptor gamma)
	IL-4R (Interleukin 4 Receptor)
	IL-6R (Interleukin 6 Receptor)
	IL-13Rα1 (Interleukin 13 Receptor alpha 1)
	LIF (Leukemia Inhibitory Factor)

Supporting Information Table I (continued)

Cytokine Receptors	OPG (Osteoprotegerin)
	RANK (Receptor Activator of NF-kB)
	TNF-RI (Tumor Necrosis Factor alpha Receptor I)
	TNF-RII (Tumor Necrosis Factor alpha Receptor II)
Growth Factors	AR (Amphiregulin)
	EGF (Epidermal Growth Factor)
	EGFR (Epidermal Growth Factor Receptor)
	ErbB2/Her2 (Human Epidermal Growth Factor Receptor 2)
	HGH (Human Growth Hormone)
	IGFBP-1 (Insulin-like Growth Factor Binding Protein 1)
	IGFBP-2 (Insulin-like Growth Factor Binding Protein 2)
	IGFBP-3 (Insulin-like Growth Factor Binding Protein 3)
	SCF (Stem Cell Factor)
	TGFα (Transforming Growth Factor alpha)
	TGFβ1 (Transforming Growth Factor beta 1)
	TGFβ2 (Transforming Growth Factor beta 2)
Hormones	Acrp-30 (Adiponectin)
	CGα (Chorionic Gonadotropin alpha)
	C-peptide
	Insulin
	Leptin
	NT-proBNP (N-Terminal Prohormone of Brain Natriuretic Peptide)
	Prolactin
	Resistin
	SHBG (Sex Hormone Binding Globulin)
	Substance P
Immune Response	SAA (Serum Amyloid A)
	BD-2 (Beta Defensin 2)
	CD14 (Cluster of Differentiation 14)
	hsCRP (C-reactive protein)
	IgE (Immunoglobulin E)
	RAGE (Receptor for Advanced Glycation End Products)
Matrix Metalloproteinases	MMP-1 (Matrix Metalloproteinase 1)
	MMP-2 (Matrix Metalloproteinase 2)
	MMP-3 (Matrix Metalloproteinase 3)
	MMP-7 (Matrix Metalloproteinase 7)
	MMP-8 (Matrix Metalloproteinase 8)
	MMP-9 (Matrix Metalloproteinase 9)
	MMP-10 (Matrix Metalloproteinase 10)
	MMP-13 (Matrix Metalloproteinase 13)
	TIMP-1 (Tissue Inhibitor of Metalloproteinases 1)
	TIMP-2 (Tissue Inhibitor of Metalloproteinases 2)
Neurotrophic Factors	β-NGF (Beta Nerve Growth Factor)
	BDNF (Brain-Derived Neurotrophic Factor)
	CNTF (Ciliary Neurotrophic Factor)
	GDNF (Glial Cell-Derived Neurotrophic Factor)
	NT3 (Neurotrophin 3)
	PEDF (Pigment Epithelium-Derived Factor)
Other proteins	α2-Macroglobulin
	Apo A-1 (Apolipoprotein A-1)
	Apo B-100 (Apolipoprotein B-100)
	sAPPb (soluble Amyloid Precursor Protein beta)
	Cathepsin-D
	CC16 (Clara Cell Protein)
	Clusterin
	COX-2 (Cyclooxygenase 2)
	MPO (Myeloperoxidase)
	NGAL (Neutrophil Gelatinase-Assocciated Lipocalin)
	PAPP-A (Pregnancy-Associated Plasma Protein A)
	PD-1 (Programmed Death 1)
	RBP4 (Retinol Binding Protein 4)
	SP-C (Surfactant Protein C)
	SP-D (Surfactant Protein D)

List of 177 antibodies which were screened in the discovery phase classified in accordance to gene ontology biological processes.

Table II. Demographic and clinical factors from derivation and replication cohorts.

Factors	Derivation cohort (N=36)	Replication cohort (N=80)	p-value
Age, years median (IQR)	73.5 (62.5-81.5)	78 (71.2-82)	0.191
NIHSS at admission mean ± SD	15.6 ± 4.7	15.3 ± 6.6	0.745
Previous mRS median (IQR)	0 (0-0)	0 (0-0)	0.574
Gender (Male) % (n)	71.4 (25)	52.5 (42)	0.058
Smokers % (n)	18.8 (6)	14.5 (11)	0.577
Glucose, mg/dL median (IQR)	135.0 (97.0-177.0)	118 (95-151.8)	0.161
Arterial hypertension % (n)	64.7 (22)	67.5 (54)	0.772
Diabetes mellitus % (n)	32.4 (11)	26.3 (21)	0.507
Dyslipidemia % (n)	44.1 (15)	31.3 (25)	0.188
Atrial fibrillation % (n)	35.3 (12)	36.3 (29)	0.922
Ischemic cardiopathy % (n)	20.6 (7)	21.3 (17)	0.937
Early signs in CT scan % (n)	8.8 (3)	13.7 (10)	0.545
Previous stroke % (n)	20.6 (7)	21.3 (17)	0.937
TOAST			0.388
- Atherothrombotic % (n)	34.3 (12)	23.8 (19)	
- Cardioembolic % (n)	37.1 (13)	47.5 (38)	
- Undetermined % (n)	28.6 (10)	25 (20)	
Neurological worsening (24h) % (n)	25.7 (9)	21.5 (17)	0.622
Neurological improvement (24h) % (n)	40.0 (14)	32.9 (26)	0.464

NIHSS, National Institutes of Health Stroke Scale; mRS, modified Rankin scale; TOAST, etiology stroke subtype classification; IQR, interquartile range; CT, computerized tomography.
Missing data was not taken into account for percentages calculation of categorical variables.

Table III. Biomarker levels in the replication cohort regarding demographic and clinical variables.

	BD-2 (ng/mL)			BCA-1 (pg/mL)			Exodus-2 (pg/mL)			IL-4R (pg/mL)		
Factor	Yes	No	p-value	Yes	No	p-value	Yes	No	p-value	Yes	No	p-value
Male	1.6 (1.0-3.2)	1.1 (0.5-2.8)	0.088*	53.0 (41.6-85.7)	73.8 (49.5-98.8)	0.094*	62.3 (57.6-81.8)	63.7 (55.1-77.6)	0.942	480.5 ± 137.8	471.9 ± 110.0	0.762
Smoker	2.7 (1.4-4.1)	1.2 (0.6-2.4)	**0.012**	66.6 (56.8-115.1)	68.1 (41.8-93.2)	0.281	91.6 (62.5-107.6)	63.2 (55.1-77.6)	**0.035**	522.6 ± 104.7	472.1 ± 129.7	0.226
Hypertension	1.2 (0.5-2.8)	1.4 (1.1-3.6)	0.172	71.4 (48.8-98.8)	61.3 (39.2-91.3)	0.353	63.8 (59.2-78.9)	62.7 (51.3-83.4)	0.491	479.6 ± 121.3	470.2 ± 134.1	0.755
Diabetes mellitus	1.3 (0.6-5.8)	1.2 (0.7-2.6)	0.301	71.4 (48.8-82.2)	65.8 (42.5-104.7)	0.978	67.6 (59.5-78.9)	63.4 (55.0-82.3)	0.454	508.7 ± 159.9	464.8 ± 108.8	0.169
Atrial fibrillation	1.1 (0.7-1.7)	1.3 (0.6-2.8)	0.438	84.5 (51.8-109.2)	55.8 (41.7-85.8)	0.066*	63.8 (59.7-81.8)	62.2 (55.0-79.1)	0.339	484.3 ± 109.1	472.2 ± 133.6	0.683
Ischemic cardiopathy	1.4 (0.6-2.2)	1.2 (0.7-2.9)	0.702	52.9 (41.8-89.4)	68.1 (47.1-96.0)	0.634	62.3 (57.6-77.2)	63.8 (55.5-82.2)	0.438	450.3 ± 127.0	483.7 ± 124.3	0.332
Dyslipidemia	1.3 (0.6-2.9)	1.3 (0.7-2.6)	0.954	65.8 (43.4-82.2)	68.1 (48.0-104.2)	0.445	62.5 (57.6-77.2)	63.8 (55.5-87.7)	0.278	465.2 ± 117.2	481.7 ± 129.0	0.586
Previous stroke	1.2 (0.5-2.7)	1.3 (0.7-2.8)	0.462	67.2 (52.6-119.8)	67.9 (42.5-90.3)	0.488	60.9 (57.6-66.4)	63.7 (55.5-82.3)	0.221	495.5 ± 127.4	471.3 ± 124.7	0.481
Atherothrombotic	1.3 (0.8-3.5)	1.2 (0.7-2.7)	0.53	66.6 (46.4-84.8)	67.9 (46.9-98.8)	0.782	67.6 (58.4-82.3)	63.4 (55.1-78.9)	0.769	474.6 ± 171.2	477.1 ± 108.0	0.941
Cardioembolic	1.1 (0.5-1.7)	1.7 (1.0-3.2)	0.063*	71.4 (48.8-108.1)	62.4 (41.8-91.3)	0.447	63.5 (55.9-85.6)	62.9 (55.1-77.6)	0.576	470.4 ± 102.3	481.8 ± 142.8	0.688
Neurological improvement at 24h	1.2 (0.7-2.4)	1.2 (0.7-3.2)	0.851	64.1 (40.3-112.4)	68.0 (48.8-88.7)	0.849	59.7 (50.3-63.8)	63.7 (55.9-81.8)	0.370	470.3 ± 88.1	476.6 ± 139.8	0.836
Neurological worsening at 24h	1.3 (1.2-3.9)	1.1 (0.6-2.1)	**0.046**	66.6 (41.6-79.3)	68.1 (48.7-99.5)	0.317	61.7 (52.9-73.0)	62.7 (55.5-80.3)	0.377	543.2 ± 175.0	455.3 ± 99.9	0.062*

Supporting Information Table III (continued).

	IL-12p40 (pg/mL)			TWEAK (pg/mL)			LIF (pg/mL)			MIP-1b (pg/mL)		
Factor	Yes	No	p-value	Yes	No	p-value	Yes	No	p-value	Yes	No	p-value
Male	4.0 (0.1-9.6)	10.3 (4.9-15.9)	**0.003**	261.2 (230.4-312.5)	274.6 (239.5-323.6)	0.749	0.5 (0.5-2.6)	0.5 (0.5-4.3)	0.133	54.9 (39.0-87.8)	70.4 (50.1-89.4)	0.245
Smoker	4.8 (0.1-9.1)	7.8 (1.1-14.7)	0.164	340.6 (294.0-398.4)	260.3 (229.1-309.5)	**<0.001**	0.5 (0.5-3.1)	0.5 (0.5-3.9)	0.631	52.3 (28.1-75.4)	61.2 (45.3-94.0)	0.284
Hypertension	8.2 (1.5-14.1)	5.3 (0.2-11.2)	0.268	262.1 (226.5-320.2)	278.2 (239.0-371.9)	0.212	0.5 (0.5-3.0)	0.5 (0.5-6.3)	0.504	67.0 (46.0-98.6)	57.4 (39.0-75.4)	0.244
Diabetes mellitus	9.6 (3.3-15.3)	7.2 (0.3-11.7)	0.354	309.7 (268.0-339.8)	259.2 (228.4-301.2)	**0.020**	0.5 (0.5-3.0)	0.5 (0.5-3.8)	0.937	62.9 (46.0-80.6)	60.7 (41.6-94.0)	0.930
Atrial fibrillation	10.4 (5.9-14.9)	4.4 (0.1-11.5)	**0.010**	262.0 (235.8-314.8)	267.3 (234.6-334.5)	0.786	0.5 (0.5-3.9)	0.5 (0.5-3.4)	0.532	70.6 (57.0-96.4)	52.4 (39.2-79.2)	0.070*
Ischemic cardiopathy	5.3 (0.2-11.5)	7.8 (0.9-14.4)	0.435	252.0 (213.9-276.3)	274.2 (239.0-334.6)	**0.028**	0.5 (0.5-0.5)	0.5 (0.5-4.1)	0.178	60.5 (57.0-87.2)	60.7 (39.8-91.6)	0.409
Dyslipidemia	2.8 (0.1-11.7)	8.5 (4.0-14.7)	**0.044**	273.9 (250.8-316.2)	262.6 (230.4-323.6)	0.920	0.5 (0.5-2.2)	0.5 (0.5-3.8)	0.297	57.3 (39.2-72.7)	66.7 (48.2-97.1)	0.139
Previous stroke	7.3 (4.1-11.7)	7.2 (0.3-13.3)	0.707	279.5 (250.8-323.2)	262.0 (226.4-323.6)	0.482	0.5 (0.5-0.5)	0.5 (0.5-4.1)	0.061*	55.8 (34.7-122.3)	61.7 (45.8-79.4)	0.926
Atherothrombotic	6.8 (3.6-13.5)	7.3 (0.3-12.6)	0.727	279.2 (244.3-334.5)	262.0 (229.1-321.7)	0.667	0.5 (0.5-2.5)	0.5 (0.5-3.7)	0.768	72.6 (45.8-126.5)	57.9 (41.6-79.2)	0.322
Cardioembolic	8.1 (2.4-13.3)	5.3 (0.3-11.8)	0.300	266.5 (242.2-323.2)	265.2 (230.4-332.3)	0.772	0.5 (0.5-2.4)	0.5 (0.5-5.4)	0.282	61.2 (49.8-79.3)	57.9 (40.7-110.0)	0.895
Neurological improvement at 24h	8.5 (0.1-11.7)	7.0 (1.1-13.6)	0.863	275.1 (232.1-316.2)	263.2 (238.8-323.4)	0.654	0.5 (0.5-3.8)	0.5 (0.5-2.6)	0.440	57.0 (41.6-104.7)	61.2 (46.0-79.3)	0.702
Neurological worsening at 24h	4.3 (1.7-12.7)	7.4 (0.3-13.3)	0.818	263.2 (238.8-320.2)	267.3 (232.1-323.6)	0.863	0.5 (0.5-2.9)	0.5 (0.5-3.8)	0.563	61.7 (54.8-70.7)	60.0 (40.7-94.0)	0.892

IL-4R is expressed as mean ± SD; all other biomarkers are expressed as median (IQR). Statistically significant differences between groups are expressed as bold p-value; * stands for statistical trend (p<0.1).

Table IV. Comparison between predictive models with only clinical variables and models including biomarkers for worsening at 48h.

		Model – Worsening 48h			
		Only Clinical	**Clinical + BD-2**	**Clinical + IL-4R**	**Clinical + Combination BD-2 & IL-4R**
Logistic regression (OR adj)	NIHSS admission	1.1 (1.0-1.2), 0.054*	1.1 (1.0-1.2), 0.059*	1.1 (1.0-1.2), **0.032**	1.1 (1.0-1.2), **0.033**
	DM	10.2 (2.5-41.6), **0.001**	14.5 (3.0-71.3), **0.001**	8.8 (2.1-37.2), **0.003**	10.8 (2.4-49.5), **0.002**
	Age	1.0 (0.9-1.0), 0.325	1.0 (0.9-1.0), 0.377	1.0 (0.9-1.0), 0.304	1.0 (0.9-1.0), 0.349
	Gender (female)	0.6 (0.2-2.1), 0.434	0.3 (0.1-1.5), 0.158	0.6 (0.1-2.2), 0.415	0.4 (0.1-1.7), 0.217
	BD-2	-	5.8 (1.3-26.1), **0.023**	-	-
	IL-4R	-	-	3.1 (0.9-11.1), 0.082*	-
	BD-2 + IL-4R	-	-	-	3.3 (1.3-8.4), **0.014**
Categorical NRI	NRI events	-	9.5%	0%	4.8%
	NRI non-events	-	13.3%	11.4%	22.7%
	NRI	-	22.8% (-5.3 – 50.9)	11.4% (-7.4 – 30.2)	27.5% (4.3 – 50.7)
	p-value	Ref.	0.111	0.236	**0.020**
IDI statistics	IDI events	-	0.053	0.034	0.071
	IDI non-events	-	0.025	0.009	0.026
	IDI	-	0.078 (0.014 - 0.141)	0.043 (-0.012 - 0.098)	0.097 (0.024 – 0.171)
	p-value	Ref.	**0.017**	0.130	**0.009**
ROC curves	AUC	0.775 (0.654-0.869)	0.823 (0.708-0.906)	0.811 (0.694-0.897)	0.840 (0.728 - 0.919)
	p-value	Ref.	0.187	0.228	0.074*

All logistic regression models were adjusted by NIHSS at admission, age and gender and OR_{adj} (95% CI) and p-value were given. Biomarkers were added to clinical logistic regression model using cut-off point: BD-2 > 1.15 ng/mL and IL-4R > 503.40 pg/mL. NRI: Net Reclassification Improvement index (risk categories used: ≤10%, 10-90% and >90%); percentage of reclassification given for both events (i.e. patients who worsened at 48h) and non-events and for the sum of both (with 95% CI). IDI: Integrated Discrimination Improvement index; index given for both events and non-events and for the sum of both (with 95% CI). AUC: Area Under the ROC Curve; area with 95%CI given for each model. Clinical model always used as reference model to compare. Statistically significant results expressed as bold p-values; * statistical trend.

VI. Blood gene expression profiling to predict ischemic stroke outcome.

(*Manuscript in preparation)*

Blood gene expression profiling to predict ischemic stroke outcome

Teresa García-Berrocoso[a], Israel Fernández-Cadenas[a], Joan Montaner[a,b,§]

[a]Neurovascular Research Laboratory, Vall d'Hebron University Hospital Research Institute (VHIR), Universitat Autònoma de Barcelona, Barcelona, Spain
[b]Neurovascular Unit, Department of Neurology, Vall d'Hebron University Hospital, Barcelona, Spain

[§]Corresponding author:
Joan Montaner
Neurovascular Research Laboratory
Vall d'Hebron Institute of Research
Vall d'Hebron University Hospital
Pg. Vall d'Hebron 119-129, 08035
Barcelona, Spain.
+34 93 489 40 73.
31862jmv@comb.es

Abbreviations

RNA: ribonucleic acid
rt-PA: recombinant tissular plasminogen activator
NIHSS: National Institutes of Health Stroke Scale
RT-PCR: reverse transcription polymerase chain reaction
DCAKD: dephospho-CoA kinase domain containing
PMS2: Mismatch repair endonuclease

ABSTRACT

Nowadays clinicians have no accurate tools to predict stroke outcome beside clinical and neuroimaging data. To study gene expression changes associated with neurological outcome after ischemic stroke, we pooled RNA from blood samples of 44 rt-PA-treated stroke patients depending on outcome group: worsening, stability or improvement (NIHSS score-based). Affymetrix GeneChip arrays were used and 4,581 genes were found to be differentially expressed after statistical analysis. 85 genes were associated to poor or good outcome, mainly involved in cell-death, cell-to-cell signaling and metabolism networks. Replication of 30 candidates by RT-PCR using microfluidic cards with individual samples from 60 new patients revealed the same trend in 10 genes. *DCAKD* and *PMS2* were significantly associated with neurological improvement at 24 and 48 hours after stroke and form part of a predictive panel that improved clinical information. Gene expression levels might contribute as markers for stroke prognosis.

INTRODUCTION

Ischemic stroke is the second cause of death and the major cause of disability worldwide[1].Despite of that the prognosis of stroke patients is still based only on clinical and neuroimaging data. To know which patients are going to develop medical complications would optimize care and monitoring of the patients, reducing in-hospital lethality and improving functional outcome[2,3]. Several blood biomarkers have been associated with stroke prognosis[4,5] and they might add information to improve the prediction of stroke outcome. Gene expression profiling techniques offer the opportunity to discover new markers. Our aim was to study gene expression changes associated with neurological outcome of stroke patients, using a blood samples-pooling strategy to reduce biological variability.

METHODS

Patients and samples

We included patients who were admitted to the emergency department of the Vall d'Hebron University Hospital (Barcelona, Spain) with an acute ischemic stroke within the first 4.5 hours after symptoms onset. Stroke diagnosis was performed based on a standardized protocol of clinical and neuroradiological assessments. All patients received intravenous recombinant tissue-

plasminogen activator (rt-PA) in a standard 0.9 mg/Kg dose (10% bolus, 90% continuous infusion during 1h). Stroke severity was assessed by using NIHSS[6]. We defined neurological improvement as a decrease in NIHSS score by 4 or more points, neurological stability as changes in NIHSS score of 3 or less points and neurological deterioration as death or an increase in NIHSS score by 4 or more points at 24 h or 48 h[7].

Before administration of any treatment, peripheral blood samples were drawn from each patient. White blood cells were obtained by centrifugation at 1500 *g* for 15 min at 4ºC, mixed with RNA*later*® solution (Ambion, USA) as RNA stabilizer and stored at -80ºC until use.

The local ethical committee approved the study and written consent was obtained from all patients or relatives in accordance with the Helsinki declaration.

RNA purification, pooling and hybridization

Total RNA was isolated with RiboPure™-Blood kit (Ambion) and globin mRNA was depleted with GLOBINclear™ kit (Ambion). The integrity of RNA was determined using the Bioanalyzer 2100 platform (Agilent, UK), with RNA integrity numbers of 5-7. RNA concentration was better determined by RediPlate™ 96 RiboGreen® RNA Quantitation Kit (Invitrogen, USA) and all RNA samples were diluted to 4 ± 0.5 ng/µL. Thus, equally amount of 2 to 4 RNA individual samples was pooled depending on neurological outcome:

1. Worsening (WOR): patients with neurological deterioration during in-hospital stay.
2. Stability (STA): patients without changes in neurological state during in-hospital stay.
3. Improvement (IMP): patients with neurological improvement during in-hospital stay.

In total, 16 pools were prepared: 4 pools for the worsening group and 6 pools for both stability and improvement groups. All three outcome groups were balanced regarding age and initial severity (Table 1).

RNA integrity was maintained after pooling. cDNAs obtained with Ovation Pico WTA system (Nugen, USA) were hybridized to GeneChip Human Exon 1.0 ST arrays (Affymetrix, USA).

Pool	number of samples	Mean NIHSS admission	Mean age	Mean NIHSS diff. (adm. - disch.)
WOR 1	3	14	70.7	-13.7
WOR 2	2	8	71	-15
WOR 3	3	21.3	74.3	-8.7
WOR 4	2	20	78	-10
STA 1	3	19.3	70	1.7
STA 2	2	16	80.5	1
STA 3	2	15	81	0.5
STA 4	3	12.7	59.3	1.7
STA 5	2	19	80	1.5
STA 6	3	19	69.7	2
IMP 1	4	16	79.8	11
IMP 2	2	16	82	14
IMP 3	2	16	61	14
IMP 4	2	14.5	76.5	11
IMP 5	3	10.3	66.3	9
IMP 6	3	16.3	79	14.3

Table 1. Clinical characteristics of pooled samples. NIHSS diff. = NIHSS at admission – NIHSS at discharge. Age given in years.
WOR: worsening; STA: stability; IMP: improvement; NIHSS: National Institutes of Health Stroke Scale.

Microarrays analysis

The images were processed with the Expression Console software (Affymetrix). The quality of the arrays was acceptable after the assessment of 6 quality control metrics and thus all 16 arrays were subjected to subsequent analysis. Raw expression values obtained directly from .CEL files were preprocessed using the Robust Multi-Array method, a three-step process which integrates

background correction, normalization and summarization of probe values[8]. After non-specific filtering to remove low signal and low variability genes, differentially expressed genes between outcome conditions were selected, based on a linear model analysis with empirical Bayes moderation of the variance estimates[9]. Age and initial severity were used as covariates. Spearman correlations between gene expression and NIHSS score difference as a continuous variable were also performed. All the statistical analyses were done using the free statistical language R and the libraries developed for microarray data analysis by the Bioconductor Project (www.bioconductor.org). Unadjusted p-values < 0.01 and Spearman correlation with R > 0.5 and p < 0.05 were used to consider differentially expressed genes associated with outcome.

Network analysis

Genes associated with outcome were analyzed through the use of Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com). Gene identifiers were mapped to its corresponding gene object in the IPA database in order to identify potential networks and associated canonical pathways. A score, derived from a p-value, was generated for each network according to the fit of the set of genes.

Quantitative RT-PCR

Total RNA was isolated with RiboPure™-Blood kit from individual samples from 60 new stroke patients and RNA quantity and quality (*A*260/*A*280 ratio) were determined by NanoDrop® ND-1000 (Nucliber, Spain). cDNAs were generated per High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA). qRT-PCR reaction were performed using TaqMan® Custom Arrays (Life Technologies), preconfigured in a 384-well format and spotted on a microfluidic card, for *GSTM1, FBXO32, KIAA0232, FUNDC1, MED20, SERHL2, MPZL3, OASL, CCL4, IFIT5, CCDC146, IER3, CEP19, ZNF384, CCDC93, S100A6, GPN1, YPEL4, DCAKD, PMS2, PFKFB2, MAP2K6, CYP2F1, SPRYD4, SH2D1B, NUTF2, QTRTD1, FAM134C, SLC22A15, HSPB11* and *GAPDH* & *PPIA* as endogenous controls. qRT-PCR amplifications were run in triplicates on an 7900HT Sequence Detection System (Life Technologies) and analyzed using the SDS 2.4 software (Life Technologies). Relative quantification (RQ) values were calculated by use of the Livak equation: $RQ = 2^{-\Delta\Delta Ct}$, using the geometric mean of both endogenous controls and a calibrator sample in all runs.

Statistical analyses

Analyses were conducted using SPSS 15.0 and R. Normality was assessed by Kolmogorov-Smirnov test. Intergroup differences between stroke outcome groups were assessed by ANOVA and Student's t tests and p-values <0.05 were considered significant. Mean and SEM values were displayed in bar graphs.

To know which genes might confer robustness on a potential biomarkers' panel we have used PanelomiX, a web-based tool that allows the iterative combination of biomarkers and clinical variables by selecting thresholds for an optimal classification performance, which is analyzed with cross-validation and receiver operator characteristic (ROC) curves[10]. Clinical variables considered were age, gender and NIHSS at admission.

The best panel was reproduced in a logistic regression analysis to obtain the model probabilities for each patient in order to calculate the integrated discrimination improvement (IDI) and the categorical net reclassification improvement (NRI) indexes[11]. Thus, the added value of biomarkers to clinical data could be addressed and, in the case of NRI, pre-specified clinically relevant thresholds of predicted risk (≤10% and >90%) allowed to calculate reclassification of patients into risk outcome groups[12].

RESULTS

Gene expression profiling using microarrays

After normalizing and filtering raw results, 4,581 genes were found to be differentially expressed (Figure 1A). When comparison by pairs was done, 33 genes were altered in the WOR vs. IMP analysis and 26 genes in both WOR vs. STA and IMP vs. STA analyses (Figure 1B). In total, 76 different genes were altered regarding early neurological outcome (p-value < 0.01). Five of these genes were exclusively associated with WOR, 2 over-expressed and 3 under-expressed genes; 3 genes were exclusively associated with IMP, 2 of them being over-expressed.

Correlations between gene expression and NIHSS score change between admission and discharge revealed 14 genes with a positive correlation (the more expression, the more reduction in NIHSS score, that is improvement) and 24 genes with a negative correlation (the more expression, the more increase in NIHSS score, that is worsening). These correlations suggested a direct relation with NIHSS score changes and not only with categorization of outcome groups.

Both analyses built a final list of 85 differentially expressed genes depending on early neurological outcome.

Network analysis

IPA analysis was performed with the list of genes from each comparison by pairs, in order to identify which biological processes lead to worsening and which ones lead to improvement.

The analyses WOR vs. STA and WOR vs. IMP revealed gene interactions in cell death and cell-to-cell signaling associated with worsening (Figure 2). On the other hand, the analysis IMP vs. STA found gene interactions in lipid and protein metabolism network (Figure 3).

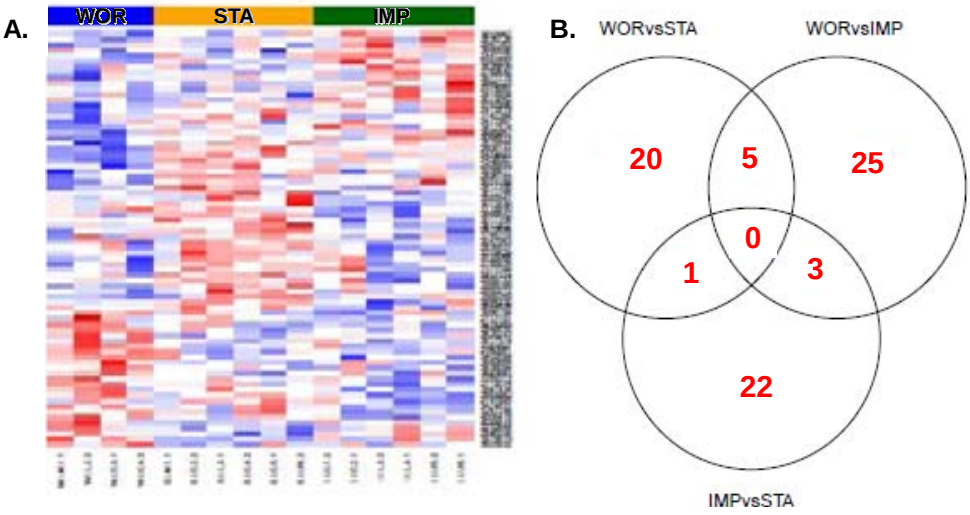


Figure 1. **Differential gene expression between outcome groups.** Heat map (1A; red for over-expression and blue for under-expression) and gene expression distribution (1B).
WOR: worsening; STA: stability; IMP: improvement.

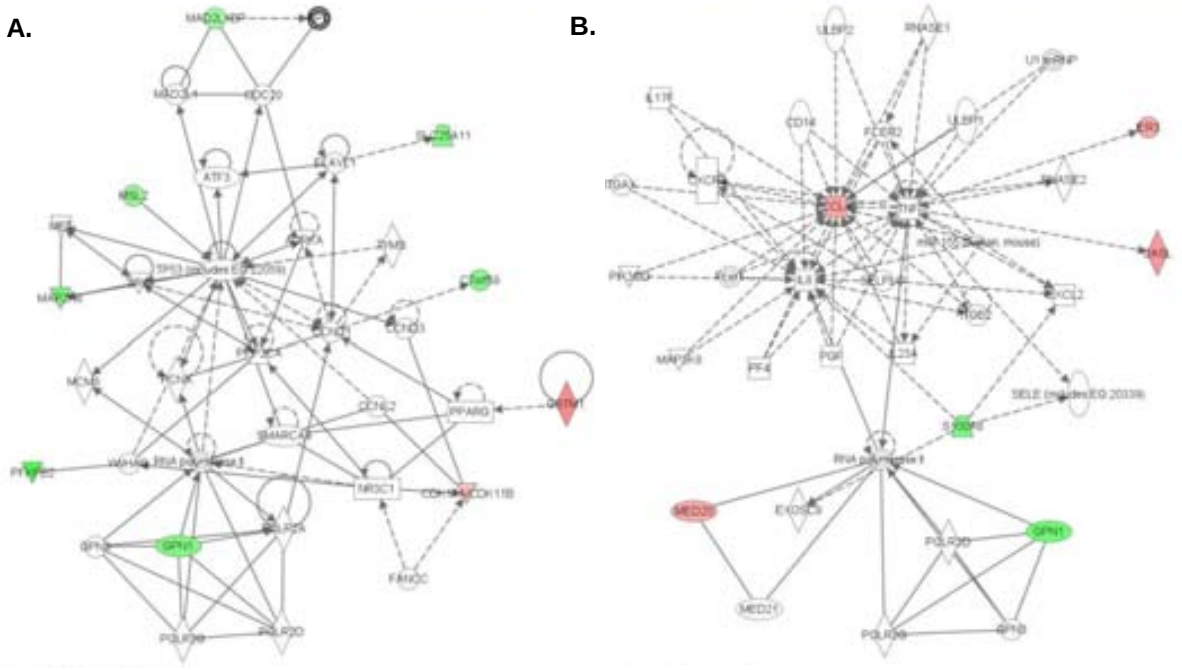


Figure 2. **Biological pathways associated with worsening. 2A.** Cell cycle and cell death pathway. **2B.** Cell-to-cell signaling and interaction and cell movement pathway. In both cases red is used for over-expressed genes in patients who worsened their neurological status and green for under-expression. Continuous line indicated direct interaction and

Replication of microarray data

From the 85 altered genes list we selected 30 candidates for replication, based on association with outcome ($p<0.01$), correlation with NIHSS difference ($p<0.02$) and availability of commercial probe.

CYP2F1 was not amplified by qRT-PCR in our replication cohort. From the other genes *QTRTD1, FAM134C, HSPB11, SH2D1B, NUTF2, SPRYD4, OASL* and *CCL4* showed the same trend in association with outcome than in the microarrays analysis; *DCAKD* and *PMS2* were significantly associated with neurological improvement at 24 h (Figure 4; Table 2) and at 48 h after stroke.

Biomarkers' panel for improvement

As an 80% of the replicated genes were associated with improvement, we built a biomarkers' panel to predict this outcome. A panel including *DCAKD* >0.92, *PMS2* >1.01

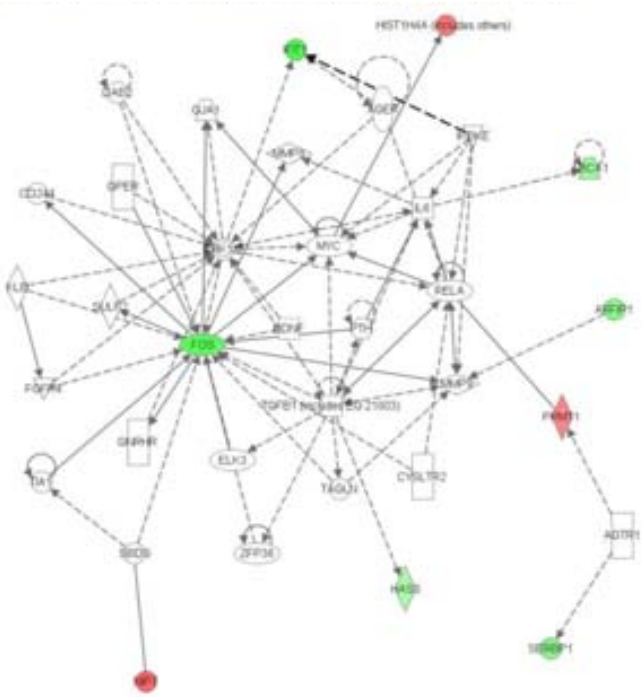


Figure 3. **Biological pathway associated with improvement.** Lipid metabolism and protein synthesis pathway. Red is used for over-expressed genes in patients who improved their neurological status and green for under-expression.

and *CCL4* <0.93 together with NIHSS at admission <15.5, age <74.5 years and male gender, showed a good discrimination of patients who improved at 24h after stroke,

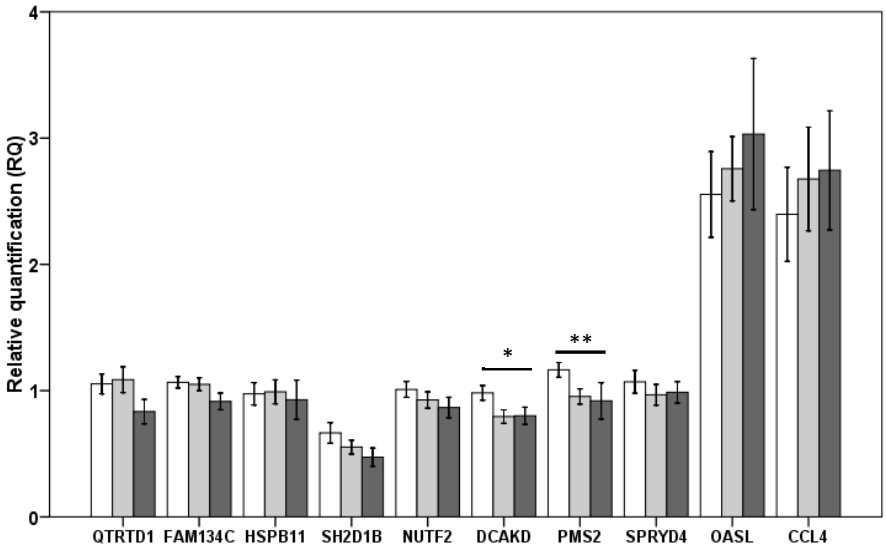


Figure 4. **Gene expression depending on neurological outcome at 24h after stroke.** Relative quantification of gene expression by real-time RT-PCR regarding housekeeping expression of *GAPDH* and *PPIA*. Bars display mean ± SEM. White bars represent patients who improved, light grey bars represent patients who remained stable and dark grey bars represent patients who worsened. * p<0.05; ** p<0.01.

with an AUC of 79.3% after cross-validation (with a 77% specificity and a 83% of sensitivity). When only clinical variables were used the AUC fell to 69.6% (63% specificity, 67% sensitivity). This three-marker panel enhanced the discrimination of patients who improved after stroke in a 10% using IDI statistics, increasing the detection of both true positives and false positives (Figure 5). Regarding reclassification, the three-marker panel classified better a 21% of the patients into predefined categories of <10%, 10-90% and >90% probability of improving at 24h (p=0.03).

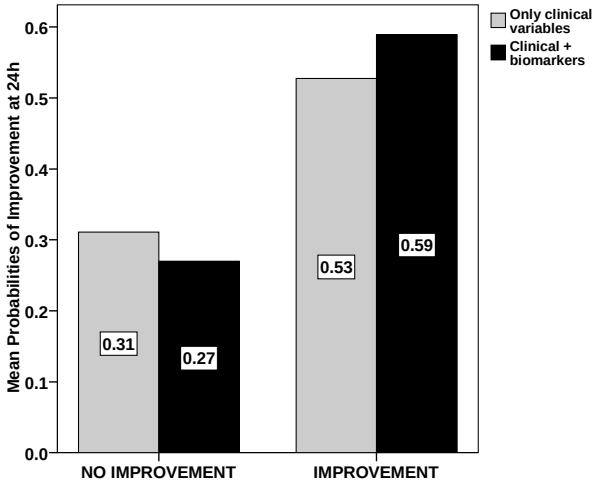


Figure 5. **Graphical representation of the comparison of predictive models by IDI index.**

Table 2. Gene expression of genes associated with neurological outcome at 24h after stroke.

Genes	Microarrays				Replication (qRT-PCR)			
	WORvsSTA (FC, p)	**WORvsIMP (FC, p)**	**IMPvsSTA (FC, p)**	**Correlation (R, p)**	**WOR (RQ)**	**STA (RQ)**	**IMP (RQ)**	**Correlation (R, p)**
QTRTD1	n.a.	-0.72, 0.008	n.a.	0.64, 0.008	0.83	1.09	1.05	n.a.
FAM134C	-0.60, 0.032	-0.77, 0.008	n.a.	0.64, 0.008	0.91	1.05	1.07	n.a.
HSPB11	-0.65, 0.066	-1.03, 0.005	n.a.	0.61, 0.011	0.93	0.99	0.97	n.a.
SH2D1B	n.a.	-0.94, 0.007	0.54, 0.079	0.69, 0.003	0.47	0.55	0.66	n.a.
NUTF2	-0.79, 0.016	-1.07, 0.002	n.a.	0.68, 0.004	0.86	0.93	1.01	n.a.
DCAKD	n.a.	-0.75, 0.007	0.70, 0.007	0.62, 0.010	0.80	0.79	0.98	0.25, 0.053
PMS2	-1.97, 0.001	-1.18, 0.003	n.a.	0.56, 0.024	0.92	0.95	1.16	0.25, 0.060
SPRYD4	n.a.	-0.9, 0.005	n.a.	0.69, 0.003	0.99	0.97	1.07	0.22, 0.098
OASL	n.a.	0.98, 0.007	n.a.	-0.64, 0.007	3.03	2.76	2.55	n.a.
CCL4	n.a.	0.88, 0.007	-0.69, 0.020	-0.61, 0.013	2.74	2.68	2.40	n.a.

Fold changes (FC) and p-values from each of the statistical comparisons performed in the microarrays analysis together with R and p-values from Spearman's correlation with NIHSS difference. Relative quantification (RQ) values of gene expression by qRT-PCR in the replication phase for worsening (WOR), stability (STA) and improvement (IMP) together with correlation with NIHSS difference.

DISCUSSION

Our strategy has allowed us to describe general changes in the expression of genes in the circulating leukocytes of ischemic stroke patients associated with early neurological outcome.

The mechanisms of cell death and cell-to-cell signaling seem relevant in those patients who worsened after stroke, maybe related to the inflammatory process that takes place in the acute phase of stroke. However, a pathway involving metabolism and protein synthesis was associated with improvement of baseline neurological deficit, maybe reflecting the generation of energy and products needed in the cells of the immune system to act in order to reestablish the normal status after stroke. Moreover, one of the genes associated with improvement, *PMS2*, has a role in damaged DNA repair. Thus, it might be that patients who had more capability of maintaining immune system active and in an appropriate state had more chance to overcome stroke.

The replication phase in individual samples is desirable after a pooling-based discovery phase to demonstrate that the general changes found are individually applicable[13,14]. In order to increase the number of genes to be analyzed, we used microfluidic cards, what allowed us to analyze 30 genes plus 2 housekeeping genes in a rapid and reproducible manner[15]. *DCAKD* and *PMS2* maintained their association with improvement in the replication cohort. Other 8 genes showed a trend in the same direction than in microarrays analysis and *CCL4* expression level enter together with *DCAKD* and *PMS2* into a panel that showed some added value to clinical predictive data. If a panel including more markers can increase that amelioration in predicting a good early outcome will be further explore.

The exploration of gene expression in different subtypes of leukocytes (monocytes, neutrophils, limphocytes) might contribute to the understanding of the physiopathology of stroke at the immunological level[16]. If the expression of the candidates shown up in this study is different among subtypes of leukocytes could be addressed to know if the different cell contribution makes slighter the differences between outcome groups.

REFERENCES

1. Roger VL, Go AS, Lloyd-Jones DM, *et al*. Heart disease and stroke statistics--2012 update: A report from the american heart association. *Circulation* 2012; 125: e2-e220.

2. Álvarez-Sabín J, Molina C, Montaner J, *et al*. Beneficios clínicos de la implantación de un sistema de atención especializada y urgente del ictus. *Med Clin (Barc)* 2004; 122: 528-531.

3. Ruiz V, Ramón N, Juan Ó, *et al*. Unidades de ictus: más supervivencia. Una revisión sistemática. *Med Clin (Barc)* 2005; 124: 22-29.

4. Whiteley W, Chong WL, Sengupta A, *et al*. Blood markers for the prognosis of ischemic stroke: a systematic review. *Stroke* 2009; 40: e380-e389.

5. Katan M, Elkind MSV. Inflammatory and neuroendocrine biomarkers of prognosis after ischemic stroke. *Expert Rev Neurother* 2011; 11: 225-239.

6. Brott T, Adams HP Jr, Olinger CP, *et al*. Measurements of acute cerebral infarction: a clinical examination scale. *Stroke* 1989; 20: 864-870.

7. Brott TG, Haley EC Jr, Levy DE, *et al*. Urgent therapy for stroke. Part I. Pilot study of tissue plasminogen activator administered within 90 minutes. *Stroke* 1992; 23: 632-640.

8. Irizarry RA, Hobbs B, Collin F, *et al*. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* 2003; 4: 249-264.

9. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004; 3: Article 3.

10. Robin X, Turck N, Hainard A, *et al*. PanelomiX: A threshold-based algorithm to create panels of biomarkers. *Transl Proteomics* 2013; 1: 57-64.

11. Pencina MJ, D'Agostino RB Sr, D'Agostino RB Jr, *et al*. Evaluating the added predictive ability of a new marker: From area under the ROC curve to reclassification and beyond. *Statist Med* 2008; 27: 157-172.

12. Whiteley W, Wardlaw J, Dennis M, *et al*. The use of blood biomarkers to predict poor outcome after acute transient ischemic attack or ischemic stroke. *Stroke* 2012; 43: 86-91.

13. Sham P., Bader J.S., Craig I., *et al.* DNA pooling: a tool for large-scale association studies. *Nat Rev Genet* 2002; 3: 862-871.

14. Walker L.C., Thompson B.A., Waddell N, *et al*. Use of DNA-Damaging agents and RNA pooling to assess expression profiles associated with *BRCA1* and *BRCA2* mutation status in familial breast cancer patients. *PLoS Genet* 2010; 6: e1000850.

15. Abruzzo LV, Lee KY, Fuller A, *et al*. Validation of oligonucleotide microarray data using microfluidic low-density arrays: a new statistical method to normalize real-time RT-PCR data. *BioTechniques* 2005; 38: 785-792.

16. Tang Y, Xu H, Du XL *et al*. Gene expression in blood changes rapidly in neutrophils and monocytes after ischemic stroke in humans: a microarray study. *J Cereb Blood Flow Metab* 2006; 26: 1089-1102.

VII. Abreviaciones

2D-DIGE: electroforesis bidimensional diferencial en gel.

A^2DS^2: *Age¸ Atrial fibrillation, Dysphagia, Sex, stroke Severity score.*

AIS-APS: *acute ischemic stroke-associated pneumonia score*.

AIT: ataque isquémico transitorio.

ARN: ácido ribonucleico.

ASPECTS: *Alberta stroke program early CT score*.

ASTRAL: *the acute stroke registry and analysis of Lausanne*.

ATP: adenosín trifosfato.

AUC-ROC: área bajo la curva de característica operativa del receptor.

BD-2: beta-defensina 2.

BI: índice de Barthel.

BNP: péptido natriurético tipo B.

BOAS: *the Bologna outcome algorithm for stroke*.

CADASIL: arteriopatía cerebral autosómica dominante con infartos subcorticales y leucoencefalopatía.

CNS: *Canadian neurological scale*.

CNTN1: contactina 1.

CRP: proteína C reactiva.

CYBP: proteína de unión a calciclina.

CYTA: cistatina A.

DCAKD: proteína con dominio defosfo-coA quinasa.

DM: diabetes mellitus.

DRAGON: *hyperDense cerebral artery sign, prestroke mRS, Age, Glucose, Onset-to-treatment time, NIHSS score.*

DRP-2: proteína relacionada con la dihidropirimidinasa 2.

DTI: imagen de tensor de difusión.

DWI: *diffusion weighted imaging*.

ECASS: *European cooperative acute stroke study*.

ELISA: ensayo por inmunoabsorción ligado a enzimas.

ESS: *European stroke scale*.

FA: fibrilación auricular.

fMRI: resonancia magnética funcional.

GAPDH: gliceraldehído-3-fosfato deshidrogenasa.

GCS: *Glasgow coma scale.*

GDIR1: inhibidor de disociación de GDP Rho alfa.

GELS: gelsolina.

GFAP: proteína gliofibrilar ácida.

GODS: *genetic contribution to functional outcome and disability after stroke*.

GOS: *Glasgow outcome scale*.

GRASPS: *Glucose Race Age Sex Pressure stroke Severity score.*

GTPase: guanosina trifosfatasa.

GWAS: estudio de asociación del genoma completo.

GWTG: *get with the guidelines*.

HAT: *Hemorrhage After Thrombolysis score*.

HI: infarto hemorrágico.

HSPB11: proteína de choque térmico 11.

IDI: índice de mejoría de la discriminación.

IL-4R: receptor de la interleucina 4.

IL-6: interleucina 6.

IM: infarto de miocardio.

IPA: *Ingenuity Pathways Analysis*.

IPD: metaanálisis de pacientes individuales.

iScore: *ischemic stroke predictive risk score*.

ISSS: *ischemic stroke survival score*.

JCS: *Japan coma scale*.

LC-MS/MS: cromatografía líquida acoplada a espectrometría de masas en tándem.

Lp-PLA2: fosfolipasa A2 asociada a lipoproteína.

MAC: molécula de adhesión celular.

MALDI: desorción/ionización láser asistida por matriz.

miR: microARN.

MMP: metaloproteinasa de matriz.

MRM: proteómica cuantitativa dirigida (*multiple reaction monitoring*).

mRS: escala modificada de Rankin.

NIHSS: *National Institutes of Health stroke scale*.

NRI: índice de mejoría de la reclasificación.

NT-proBNP: fragmento aminoterminal del pro-péptido natriurético tipo B.

NUTF2: factor de transporte nuclear 2.

OASL: proteína análoga a la 2'-5' oligoadenilato sintasa.

OCSP: *Oxfordshire community stroke project classification*.

OHS: *Oxford handicap scale*.

OR: cociente de probabilidades u *odds ratio*.

PAIS: *Paracetamol (Acetaminophen) In Stroke.*

PASS: *Preventive Antibiotics in Stroke Study*

pAUC: área bajo la curva parcial.

PET: tomografía por emisión de positrones.

PH: hematoma parenquimatoso.

PLAN: *Preadmission comorbidities, Level of consciousness, Age, Neurologic deficit score.*

PMS2: endonucleasa de reparación de desapareamiento de bases.

POC: *point-of-care devices*.

PPIA: ciclofilina.

PWI: *perfusion weighted imaging*.

QTRTD1: t-ARN guanine transglicosilasa.

RDRS: *rapid disability rating scale*.

RM: resonancia magnética.

RQ: cuantificación relativa.

rt-PA: forma recombinante del activador tisular del plasminógeno.

RT-PCR: Reacción en cadena de la polimerasa con transcriptasa inversa.

SEDAN: *Sugar, Early signs on CT, hyperDense artery sign, Age, NIHSS score.*

SEM: error estándar de la media.

SH2D1B: proteína con dominio SH2 1B.

SID: dilución de isótopo estable.

SOAR: *systolic blood pressure, oxygenation, age and respiratory rate score*.

SPECT: tomografía computerizada de emisión monofotónica.

SPRYD4: proteína con dominio SPRY 4.

sRAGE: receptor soluble de productos de glicación avanzada.

SSS: *Scandinavian stroke scale*.

SSV: *six simple variable model*.

STAIR: *stroke therapy academic industry roundtable*.

s-TPI: *stroke thrombolytic predictive instrument*.

STRAWINSKI: *STRoke Adverse outcome is associated WIth NoSocomial Infections.*

TABASCO: *Tel Aviv Brain Acute Stroke Cohort.*

TC: tomografía computerizada.

Th: linfocito T cooperador.

TOAST: *trial of Org 10172 in acute stroke treatment*.

TOF: tiempo de vuelo.