TRASPLANTE AUTÓLOGO DE PROGENITORES HEMATOPOYÉTICOS COMO OPCIÓN TERAPÉUTICA EN LA ESCLEROSIS MÚLTIPLE

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CERTIFICAMOS que la memoria titulada "TRASPLANTE AUTÓLOGO DE PROGENITORES HEMATOPOYÉTICOS COMO OPCIÓN TERAPÉUTICA EN LA ESCLEROSIS MÚLTIPLE", presentada por Yolanda Blanco Morgado, se ha realizado bajo nuestra dirección y consideramos que reúne las condiciones necesarias para ser defendida ante el Tribunal correspondiente para optar al grado de Doctor en Medicina y Cirugía.

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GLOSARIO DE ABREVIATURAS

ATG..... Anti-thymocyte globulin / Globulina antitimocítica
BCNU..... Carmustina
BDNF..... Brain derived neurotrophic factor / Factor neurotrófico derivado del cerebro
BHE..... Barrera hematoencefálica
CD34…..... Antígeno de superficie marcador de célula progenitora
CMH..... Complejo mayor de histocompatibilidad
CPA..... Célula presentadora de antígeno
Cy..... Ciclofosfamida
G-CSF..... Granulocyte-colony estimulating factor / Factor estimulante de colonias granulocíticas
EAE..... Encefalomielitis autoinmune experimental
EDSS..... Expanded dissability severity score
EMPP..... Esclerosis múltiple primariamente progresiva
EMRR..... Esclerosis múltiple remitente-recidivante
EMSP..... Esclerosis múltiple secundariamente progresiva
IL..... Interleucina
INF-β..... Interferón beta
INF-γ..... Interferón gamma
LCR..... Líquido cefalorraquídeo
MMP-9..... Matrix metaloproteinase-9 / Metaloproteinasa-9 de matriz
MTP..... Metilprednisolona
mRNA..... RNA mensajero
PBMC..... Peripheral blood mononuclear cells / Células mononucleares de sangre periférica
PCR…… Polymerase chain reaction / Reacción en cadena de la polimerasa
RA..... Antígeno de superficie de célula *naïve*
RCT…… Receptor de la célula T
RM..... Resonancia magnética
RO….... Antígeno de superficie de célula memoria

SNC..... Sistema nervioso central

TAPH..... Trasplante autólogo de progenitores hematopoyéticos

TGF-β..... Transforming growth factor-beta / Factor de crecimiento transformante beta

Th1..... Célula T *helper* tipo 1

Th2..... Célula T *helper* tipo 2

TIMP-1..... Tissular inhibitor of metaloproteinases 1/ Inhibidor tisular de mataloproteinasas 1

TNF-α..... Tumoral necrosis factor-alfa / Factor de necrosis tumoral-alfa

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I. INTRODUCCIÓN

I.1. Introducción a la esclerosis múltiple

La esclerosis múltiple (EM) es el trastorno neurológico discapacitante no traumático más común en adultos jóvenes en el mundo occidental, con un pico de incidencia en la tercera década de la vida. Aproximadamente, un 70% de casos debuta entre los 20 y 40 años de edad, con un pico en la edad de inicio alrededor de los 23 o 24 años, por lo que resulta fácilmente comprensible el gran impacto que ocasiona sobre la vida profesional, familiar y social de los afectados, así como el enorme gasto económico y social que genera.[1] Si bien, la prevalencia global de EM es el doble en mujeres que en hombres, en las formas de inicio progresivo la prevalencia entre sexos es aproximadamente la misma.[2]

Los diferentes estudios de epidemiología descriptiva realizados en España, muestran tasas de prevalencia de alrededor de 50 casos /100.000 habitantes y de incidencia de unos 3 nuevos casos/100.000 habitantes-año, lo que sitúa a nuestro país en la franja de riesgo medio de desarrollo de la enfermedad.[3]

La EM es una enfermedad heterogénea en su presentación y evolución en la que la mayoría de los pacientes (80-85%) presentan un curso que evoluciona a brotes, o forma remitente-recidivante (EMRR), autolimitados, que a medida que se repiten van ocasionando un déficit residual funcional. Tras 10-15 años de evolución el 50% de ellos pasarán a presentar un curso secundariamente progresivo de incremento de la discapacidad no relacionado con los brotes (EMSP), y tras 25 años el porcentaje alcanza al 90% de los pacientes.[4] En un 10% de los casos el curso es progresivo desde el inicio, o forma primariamente progresiva. Un 10 a 20% de los pacientes se mantendrán sin secuelas importantes 15 años después del inicio de la enfermedad, y en un 1-3% de los casos, sin embargo, los pacientes evolucionarán

acumulando una gran discapacidad en pocos meses tras el debut de la enfermedad (EM maligna o fulminante). (Ver Figura 1)

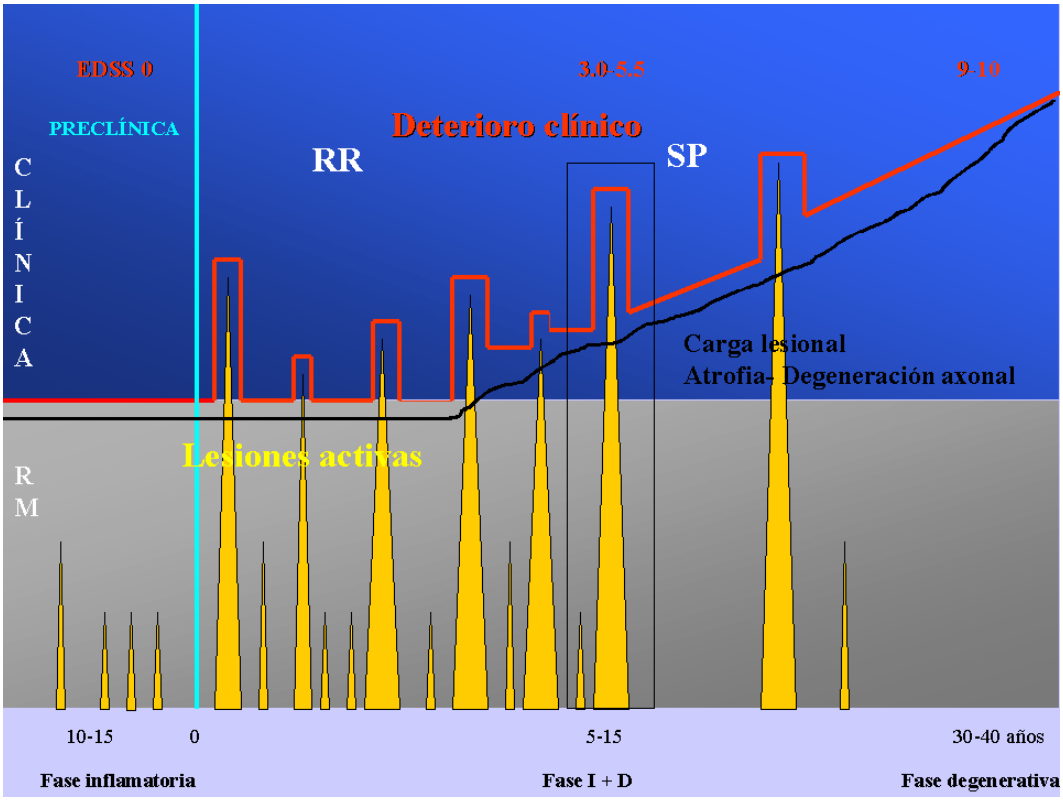

Figura 1

Si bien, la progresión de la discapacidad en la EM es altamente variable entre pacientes, un gran número de estudios con un seguimiento a largo plazo sugieren que la mediana de duración de la enfermedad para alcanzar una puntuación de 4.0 (dificultad para caminar ilimitadamente) en la escala de discapacidad modificada de Kurtzke[5] (EDSS) es de 10 años, 15-20 años para alcanzar un EDSS de 6.0 (necesidad de un apoyo para andar unos 100 metros) y aproximadamente de unos 30 años para llegar a un EDSS de 7.0 (incapacidad de andar más de 5 metros).[6,7]

Dada esta heterogeneidad característica de la EM muchos estudios han buscado factores predictivos que nos ayuden a seleccionar aquellos casos de peor pronóstico evolutivo a corto o largo plazo, y que podrían beneficiarse de un

tratamiento más intenso. Estudios de historia natural de la enfermedad han identificado como variables clínicas predictoras de un tiempo más largo hasta el inicio de la discapacidad irreversible a: sexo femenino, menor edad de inicio, curso inicial RR, recuperación completa tras el primer brote, inicio en forma de neuritis óptica, ausencia de síntomas de afectación de vías largas, un menor número de brotes durante los primeros años desde el inicio, y un mayor tiempo entre el primer y segundo brote. Así, el 50% de los pacientes con 5 o más brotes en los 2 primeros años de enfermedad precisarán de un apoyo para caminar tras 4-5 años de evolución, porcentaje que no se alcanza pasados incluso 15 años de evolución cuando el paciente presenta ≤ 2 brotes.[8]

Sin embargo, en el subgrupo de pacientes con un curso progresivo, o una vez se ha alcanzado un EDSS de 4.0 (dificultad para caminar ilimitadamente) estas variables ya no se mantienen como factores predictores de la discapacidad subsiguiente. Ello apoyaría la visión de la EM como una enfermedad con dos estadíos evolutivos como mostraba la Figura 1. Una fase inicial inflamatoria, de duración variable, influenciada por variables clínicas y una segunda fase, bastante independiente de las características clínicas basales, caracterizada por el descenso de la actividad inflamatoria de la enfermedad, y el acúmulo de discapacidad neurológica independiente de los brotes.[9]

Finalmente, los estudios de historia natural muestran que la esperanza de vida de los pacientes se reduce en unos 6-7 años cuando se compara con la población normal apareada por edad y sexo, y cerca del 50% de los pacientes fallecen por complicaciones derivadas de la enfermedad. La supervivencia se correlaciona también con el grado de discapacidad. Menos del 6% de los pacientes sin restricción

en las actividades de la vida diaria fallecen a los 10 años en comparación con el 70% de mortalidad para los pacientes confinados en una silla de ruedas.[10]

I.2. Patogenia de la esclerosis múltiple

La esclerosis múltiple (EM) se considera una enfermedad autoinmune desmielinizante crónica del sistema nervioso central (SNC), de etiología desconocida, mediada por linfocitos T autorreactivos frente a antígenos mielínicos. Así, se cree que un factor externo desconocido desencadenaría la respuesta autoinmune contra diferentes antígenos mielínicos en personas genéticamente predispuestas.[1]

La Figura 2 representa de forma esquemática los puntos más destacables de la patogenia de la EM.[11,12]

La lesión aguda de la EM se caracteriza por la infiltración multifocal perivenular de la sustancia blanca del SNC por linfocitos y monocito-macrófagos, y una destrucción de la mielina y oligodendrocitos formadores de mielina. En contra de la concepción clásica, estudios recientes sugieren que ya desde estadios iniciales de la enfermedad existiría un daño axonal progresivo.[13,14] El proceso inmunopatogénico de la EM se iniciaría con la existencia de células T autorreactivas que habrían escapado al proceso de tolerancia central tímica durante la ontogenia. Así, estas células circulantes en periferia, también presentes en individuos sanos, por una suma de factores desconocidos escaparían, años más tarde, de los mecanismos de tolerancia periférica y lograrían ser activadas tras la exposición a antígenos endógenos o exógenos.[15] A través del modelo animal de enfermedad desmielinizante autoinmune, la encefalomielitis autoinmune experimental (EAE), se

ha demostrado la trasferencia adoptiva de la enfermedad mediante clones de células T $CD4^+$, y más recientemente $CD8^+$, por lo que células T circulantes periféricas serían suficientes para desencadenar la desmielinización central.[16] Sin embargo, tales células autorreactivas precisan atravesar la barrera hematoencefálica (BHE) para iniciar la respuesta citotóxica local responsable de la destrucción del complejo oligodendrocito-mielina.

La migración transendotelial del linfocito T, y el resto de células inflamatorias implicadas, está mediada por moléculas de adhesión, cuya expresión en el endotelio vascular aumenta durante las fases de actividad de la enfermedad. A través de selectinas y sus ligandos, se establece una primera unión débil y reversible del linfocito T a la célula endotelial ("tethering"), lo que reduce la velocidad de paso del linfocito, facilitando la unión definitiva e irreversible al endotelio vascular, o adhesión, por medio de integrinas expresadas en la superficie linfocitaria.[11,12] La presencia de una BHE intacta es un factor limitante de la migración de células al SNC y para ello, las células T activadas, los monocitos y la propia célula endotelial, incrementan por efecto de citocinas inflamatorias la secreción de metaloproteinasa-9 de matriz (MMP-9), una endopeptidasa con capacidad proteolítica sobre la membrana basal subendotelial.[17] Así, la MMP-9, amplificaría el proceso de la migración celular mediante la agresión y lisis de la BHE, así como incrementando la expresión de integrinas. Se ha demostrado que la MMP-9 presenta actividad proteolítica sobre otras estructuras proteicas tales como la proteína básica de la mielina y el precursor el TNF-alfa, contribuyendo a la desmielinización, perpetuación de la respuesta inflamatoria por liberación de péptidos inmunogénicos, o amplificación antigénica, y al incremento de la respuesta inflamatoria.[17] Por ello,

es una enzima sujeta a una estrecha regulación, en especial a nivel de la trascripción génica, y una vez secretada, por la unión a su inhibidor tisular específico o TIMP-1.[18] Múltiples líneas de evidencia implican a la MMP-9 en la patogenia de la EM. Los niveles séricos y la expresión de MMP-9 en linfocitos de sangre periférica están elevados en pacientes con EMRR respecto a controles sanos, se incrementan durante fase de brote,[19,21] y además, se ha demostrado que un aumento de la MMP-9 junto al descenso de TIMP-1 precede a la aparición de nuevas lesiones captantes de gadolinio.[19-22] Estudios anatomopatológicos en lesiones de EM han demostrado un aumento de la expresión de MMP-9 en la pared vascular, microglia y macrófagos de la lesión activa, a diferencia de la crónica inactiva.[23]

Una vez los primeros linfocitos activados específicos de antígeno han accedido al SNC, la secreción de citocinas proinflamatorias incrementa la expresión de moléculas del CMH clase II en macrófagos y microglia, células que actuarán como presentadoras de antígeno. Ahora, la unión del receptor del linfocito T a antígenos mielínicos provocará su reactivación, así como la activación de células T *naïve* ($CD4^+CD45^+RA^+$) recién llegadas, con la aparición local de las subpoblaciones linfocitarias T *helper* efectora y T *helper* memoria central ($CD4^+CD45^+RO^+$). La comunicación bidireccional entre células del sistema inmune y residentes del SNC potenciará la red de citocinas que generarán ondas adicionales de reclutamiento celular manteniendo el proceso inflamatorio hasta desembocar en la lesión del objetivo, el oligodendrocito y la mielina. Tal destrucción comporta la exposición de múltiples antígenos nuevos, lo que conduce a la formación de clonas autorreactivas adicionales frente a determinantes antigénicos diferentes al original, conocido como amplificación antigénica.[11,12]

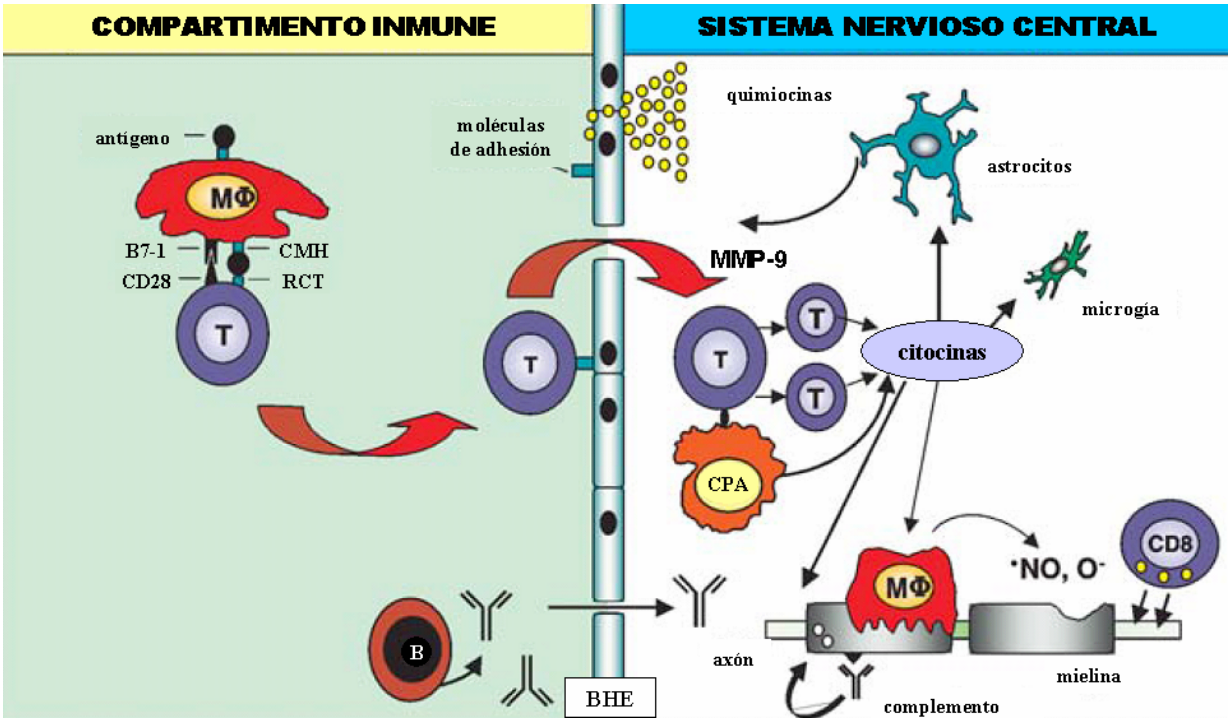

Figura 2

La producción de citocinas locales es un evento dinámico que varía dependiendo del estadio de activación celular, y a lo largo del curso de la enfermedad. El sistema inmune y el entramado de citocinas dispondría de sus propios mecanismos de contrarregulación negativa, moduladores de la respuesta inflamatoria, estableciéndose así un balance entre citocinas inflamatorias tales como el INF-gamma, TNF-α y β, IL-2 e IL-1 (patrón Th1) y antinflamatorias, como el TGF-β, IL-4 e IL-5 (patrón Th2) lo que nos conduce al concepto del paradigma Th1/Th2. El balance entre ambos fenotipos determina si la población celular T tiene una actividad global predominante proinflamatoria o antinflamatoria. Así, las células Th1CD4^{+} específicas de antígeno desencadenarían y amplificarían la respuesta inflamatoria, mientras que los niveles bajos y la deficiente producción de citocinas antinflamatorias en pacientes con EM activa, su papel protector en la EAE, y la evidencia experimental con terapias inmunomoduladoras sugieren que el perfil Th2

está fundamentalmente implicado en la inducción de remisión y en la supresión del proceso de la enfermedad.[11,12]

Tras los episodios de inflamación aguda, la resolución del proceso inflamatorio también estaría orquestada por la actividad de las células del sistema immune que infiltran la lesión. Dicha población celular es capaz de reconocer epítopos antigénicos y liberar un perfil de citocinas antinflamatorias junto a factores neurotróficos. Con ello se consigue, como se ha comentado, contrabalancear la respuesta Th1 y desviarla hacia un perfil Th2 con el objetivo, no sólo de limitar la lesión, sino de favorecer la reparación de la misma. Una de estas citocinas es el factor neurotrófico derivado del cerebro, o BDNF, una neurotrofina fundamental para el desarrollo neuronal y la sinaptogénesis.[24] Numerosos modelos experimentales de agresión neuronal han demostrado que el BDNF, además de incrementar la supervivencia neuronal, favorece la preservación axonal gracias a un transporte anterógrado, estimula la proliferación del oligodendrocito y la remielinización, y ejerce un papel inmunomodulador al disminuir la expresión de moléculas del CMH clase-II.[25-27] Todo ello es posible gracias a que tras la agresión del SNC se produce un aporte adicional de BDNF a través de las células inmunes.[28] Estudios inmunohistoquímicos en lesiones de EM han demostrado inmunorreactividad positiva para BDNF en linfocitos y macrófagos a nivel del infiltrado perivascular de la lesión activa, a diferencia de la inactiva.[29] Asimismo, se han demostrado que pacientes con EM presentan niveles superiores de BDNF en suero y LCR, y una mayor expresión linfocitaria que la población control sana, así como durante la fase de brote respecto a la fase de remisión.[30]

La existencia de un polimorfismo funcional, Val66Met, en el gen del BDNF ha puesto de manifiesto la implicación de esta proteína en procesos neuropatológicos. Este polimorfismo consiste en el cambio de una valina por una metionina en posición 66 del prodominio del BDNF, lo que condiciona una alteración en su transporte intracelular y posterior secreción, y finalmente un déficit neuronal de BDNF.[31,32] Varios estudios publicados demuestran que dicho polimorfismo se asocia a diferentes trastornos neuropsiquiátricos.[33-37] Sin embargo, se desconoce si el polimorfismo Val66Met influye en la susceptibilidad a padecer EM o en el curso evolutivo de la enfermedad. Tampoco se conoce si este polimorfismo funcional es deletéreo en la capacidad de secreción del BDNF por las células inmunes.

I.3. Tratamiento de la esclerosis múltiple

En los últimos años, la interacción de la neurología con la inmunología ha permitido mejorar la comprensión de la patogénesis de la EM y el desarrollo de los fármacos inmunomoduladores de los que disponemos actualmente para su tratamiento. La mayoría de ellos actúan en uno o en varios niveles de la cascada de acontecimientos ya citados, actuando así de forma preferencial en la vertiente inflamatoria de la enfermedad, pero sólo han mostrado un efecto discreto en el control de la EM en los casos más agresivos, o sobre la progresión de la discapacidad.[1]

En la actualidad disponemos de 4 tipos de fármacos cuya indicación aprobada es la esclerosis múltiple y que podemos agrupar en inmunomoduladores, como el interferón beta (INF-β; Betaferon®, Rebif®, Avonex®) y acetato de

glatiramero o copolímero (Copaxone®), e inmunodepresores, como la azatioprina y la mitoxantrona.

Según varios estudios de clase I, el INF-β ha demostrado reducir la tasa de brotes en pacientes con esclerosis múltiple (recomendación de tipo A), así como un efecto beneficioso en las medidas de actividad evaluada por RM, tal como la carga lesional en T2 (grado de recomendación A).[38-42] Sin embargo, su efecto en retrasar la progresión de la discapacidad no está claro ya que difiere entre estudios. Así, el INF-β 1b (Betaferon®) en un estudio con un seguimiento de tres años, fue capaz de reducir de forma significativa la proporción de pacientes con progresión confirmada de la discapacidad frente a placebo (39% frente a 50%), pero su efecto fue muy discreto ya que tan solo retrasó en nueve meses el tiempo para alcanzar la misma evolución.[40] Además, este mismo estudio no pudo replicarse en otro estudio con una población diferente.[41] El INF-β 1a (Rebif® 44 μg) probablemente sólo retrase la progresión de la discapacidad en aquellos pacientes que aún presentan brotes.[42]

El acetato de glatiramero (Copaxone®), una mezcla estandarizada de polipéptidos sintéticos, es eficaz en reducir la tasa de brotes (recomendación de tipo A), pero no ha demostrado que sea capaz de retrasar la discapacidad (grado de recomendación A). [43,44]

La azatioprina, un fármaco inmunosupresor análogo del nucleósido 6-mercaptopurina, es posible que reduzca la tasa de brotes (recomendación de tipo C), mientras que su efecto para retrasar la progresión de la discapacidad no ha sido demostrado (grado U), y por ello se considera una alternativa al tratamiento con INF- β o con acetato de glatiramero en la esclerosis múltiple RR.[45,46]

La mitoxantrona, derivado antraciclínico con efecto inmunosupresor, ha mostrado cierta eficacia en las formas severas RR y SP en un estudio clase III. El estudio MIMS que incluyó a 194 pacientes con esclerosis múltiple remitente-progresiva y esclerosis múltiple SP demostró un efecto favorable significativo a corto plazo en un análisis combinado de cinco variables clínicas (cambio en el EDSS, en el índice de deambulación, tiempo hasta el primer brote tratado, tiempo hasta el primer brote, y la escala neurológica SNS), pero que no se mantuvo pasados los 3 primeros años de seguimiento.[47] Una reciente revisión que analiza la eficacia de la mitoxantrona a partir de los datos de varios ensayos doble-ciego y controlados con placebo concluye que la mitoxantrona es sólo moderadamente eficaz en el control de la progresión de la discapacidad a corto plazo, sin poder estimar su eficacia a más largo plazo.[48] Sin embargo, su posible toxicidad cardiológica y el riesgo de desarrollo de leucemia limitan su uso, por lo que debe reservarse para pacientes con enfermedad rápidamente progresiva en los cuales han fallado otras terapias convencionales. En España está aprobado su uso en la esclerosis múltiple RR y en la SP con gran actividad clínica (brotes frecuentes, acúmulo progresivo de discapacidad) y de resonancia magnética, y falta de respuesta al tratamiento inmunomodulador estándar.

En resumen, la EM representa una enfermedad muy discapacitante, que afecta fundamentalmente a una población joven, y para la que no disponemos por el momento de ningún tratamiento capaz de modificar de forma satisfactoria el curso de la enfermedad. Por ello, parece necesario que los esfuerzos futuros se dirijan al desarrollo de nuevas terapias que optimicen el control de la actividad y, en especial, la progresión de la discapacidad de la enfermedad.

I.3.1. Trasplante y autoinmunidad

La aplicación del trasplante de médula ósea para el tratamiento de enfermedades autoinmunes se fundamenta en el hallazgo de la capacidad de transferencia adoptiva de enfermedades autoinmunes a través de la infusión de células hematopoyéticas de médula ósea de animales afectos.[49,50] Estudios posteriores demostraron que estas mismas enfermedades autoinmunes podían ser curadas a través del mismo principio, la infusión del injerto de médula ósea, pero en este caso del animal sano al enfermo.[51] Estos primeros trabajos utilizaron el trasplante **alogénico** en sus experimentos, es decir, donante y receptor presentan un HLA diferente, aunque compatible. La eficacia del trasplante alogénico en la curación de enfermedades autoinmunes en humanos se ha demostrado a través de los casos anecdóticos de pacientes sometidos a un trasplante alogénico de médula ósea por una enfermedad hematológica maligna coexistente.[52-56] Pero la mortalidad asociada al trasplante alogénico, pudiendo alcanzar hasta el 40%, y las complicaciones tóxicas e infecciosas del acondicionamiento, y las derivadas de la enfermedad del injerto contra el huésped, han limitado su uso como tratamiento opcional en enfermedades autoinmunes.[57] Es por ello que, cuando la evidencia experimental descubrió que la erradicación del sistema inmune seguida de la infusión de las propias células hematopoyéticas de médula ósea del individuo enfermo conseguía la remisión de la enfermedad autoinmune, el trasplante **autólogo** emergió como una terapia alternativa viable al trasplante alogénico.

No es descartable que los avances terapéuticos en el campo de la hematología permitan en un futuro reconsiderar el papel del trasplante alogénico. En los últimos años, se ha desarrollado una nueva opción de acondicionamiento para dicho

trasplante, el acondicionamiento no mieloablativo o de intensidad reducida, consiguiendo un evidente descenso en la toxicidad y mortalidad del procedimiento convencional.[58] Ello ha sido posible gracias al conocimiento de la capacidad del sistema inmune del injerto donante de erradicar células tumorales persistentes del receptor, el llamado efecto "injerto contra leucemia" ("*graft vs leukemia effect"*).[52] Estos protocolos, inicialmente utilizados en pacientes hematológicos de mayor edad y un peor status basal, están siendo actualmente aplicados de forma más generalizada para el tratamiento de pacientes hematológicos en remisión y tumores sólidos metastáticos, y han demostrado ser eficaces en modelos animales de enfermedades autoinmunes.[59-62] El mejor control de la enfermedad del injerto contra el huésped probablemente será el factor decisivo que permitirá extender su uso al campo de las enfermedades autoinmunes.[63-65]

I.3.2. Trasplante autólogo de progenitores hematopoyéticos de sangre periférica en la esclerosis múltiple.

La aplicación del trasplante autólogo en enfermedades autoinmunes surgió de la evidencia inesperada en el modelo animal de artritis adyuvante de que el trasplante de médula ósea de un donante HLA idéntico, usado como tratamiento control fue capaz, de forma sorprendente, de conseguir la remisión de la enfermedad al igual que el trasplante alogénico.[66] Estudios posteriores en el modelo animal de EAE demostraron que si bien, el trasplante autólogo lograba la remisión completa de la enfermedad, su eficacia en descencer la frecuencia de brotes subsiguientes era inferior al trasplante alogénico.[67-69] Por lo tanto, la evidencia experimental junto a su

menor toxicidad y mortalidad, inferior al 10%, han hecho que el trasplante autólogo haya emergido como un tratamiento potencial para la EM severa.[57]

Las dos premisas fundamentales en las que se basa el trasplante autólogo son la erradicación de las células autorreactivas del paciente, y la posterior reconstitución de un nuevo sistema inmune tolerante a los antígenos responsables de desencadenar y perpetuar la respuesta inmunitaria. La erradicación de los linfocitos T del paciente se consigue mediante el tratamiento inmunosupresor del acondicionamiento, y la depleción de células T del injerto mediante la selección de las células $CD34^+$ (o stem cell). Aunque el grado de éxito de la autotolerancia depende en gran medida de la eficacia de tal erradicación, es el resultado de un conjunto de hechos aún no bien conocidos en la actualidad. Sin embargo, habría que tener en cuenta tres puntos fundamentales para incrementar la eficacia y seguridad del procedimiento: la fuente de obtención de células progenitoras, la manipulación del injerto y por último, el régimen de acondicionamiento utilizado.

Respecto al primero de ellos, si bien las células progenitoras se encuentran en gran cantidad en la médula ósea se recomienda la extracción de las mismas de sangre periférica tras la movilización con G-CSF (granulocyte-colony stimulating factor), ya que ello acelera la recuperación hematológica del paciente.[70] Un punto debatido en lo referente a la manipulación del injerto es la intensidad de la depleción de las células T que debe hacerse. Si bien, una depleción insuficiente facilitará la reexpansión de las células autorreactivas del paciente, pudiendo repercutir en la eficacia del trasplante, y una depleción excesiva comportará un incremento en la frecuencia de infecciones oportunistas y trastornos linfoproliferativos, la experiencia clínica limitada en pacientes con EM impide llegar a conclusiones definitivas al

respecto. La selección de un régimen de acondicionamiento adecuado, en el caso particular de la EM, debe asociar la eliminación de las células autorreactivas del paciente en todo el cuerpo, incluido el SNC, sopesando la posible toxicidad aguda y el riesgo de cáncer asociado a largo plazo. Aunque pueden utilizarse diferentes combinaciones, incluyendo alguna de ellas la irradiación corporal total, no se ha encontrado que ello condicione la eficacia clínica. Sin embargo, alguno de ellos sí parece asociarse a una mayor toxicidad con un incremento en la mortalidad por lo que se desaconseja su uso.[71]

El fundamento de la aplicación del trasplante autólogo de progenitores hematopoyéticos (TAPH) en la EM se presenta en forma de un artículo de revisión publicado en la revista Lancet Neurology en el año 2005.[72] En este artículo se revisan los modelos animales experimentales y las bases racionales que llevaron a la indicación del TAPH en la EM, así como algunos de los aspectos prácticos más debatidos del propio procedimiento del trasplante. Asimismo, se detallan los resultados sobre la evolución clínica y de resonancia de los diferentes estudios clínicos publicados hasta el momento, apartado en el que incluimos nuestra experiencia personal, con 14 pacientes afectos de EM agresiva sometidos a un TAPH desde el inicio de un protocolo terapéutico en el año 1998. Hay que tener en cuenta que la experiencia acumulada al respecto se basa en ensayos clínicos fase I y II que incluyen un número pequeño de pacientes, heterogéneos en el protocolo de tratamiento y evaluación, y con un corto periodo de seguimiento lo que dificulta alcanzar conclusiones firmes sobre la seguridad y eficacia del procedimiento. La optimización de los criterios de selección del paciente, especialmente en lo referente a edad y discapacidad neurológica en el momento de la inclusión, el evitar el uso de

determinados protocolos de acondicionamiento y su aplicación en centros acreditados para el trasplante alogénico parecen ser los puntos clave para la reducción de la tasa de mortalidad a valores aceptables para su indicación en la EM.

Si bien, queda por aclarar el verdadero efecto que el TAPH tendría sobre la progresión de la enfermedad, su efecto predominante sobre la actividad inflamatoria justificaría su evaluación futura en ensayos multicéntricos aleatorizados que comparen su eficacia con otras terapias.

Y. Blanco, A. Saiz, E. Carreras, F. Graus. Autologous hematopoietic stem cell transplantation for multiple sclerosis. Lancet Neurology 2005;4:54-63

Autologous haematopoietic-stem-cell transplantation for multiple sclerosis

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Intense immunosupression followed by autologous haematopoietic-stem-cell transplantation (HSCT) is being assessed as a potential treatment for patients with severe multiple sclerosis (MS). The treatment was developed from research that showed autologous HSCT was as effective as allogeneic HSCT in the treatment of experimental autoimmune encephalomyelitis. The treatment is thought to eradicate the defective immune system, and the infused haematopoietic stem cells reconstitute an immune system that is more tolerant to the nervous system. About 250 patients with MS have been treated with autologous HSCT as part of phase I and phase II open trials. Autologous HSCT seems feasible in MS and assessment with clinical and MRI measures suggests it induces a profound and long-lasting suppression of inflammation. The course of MS seems to be stabilised after autologous HSCT, especially in ambulatory patients with evidence of active disease. Autologous HSCT deserves further study in randomised controlled trials.

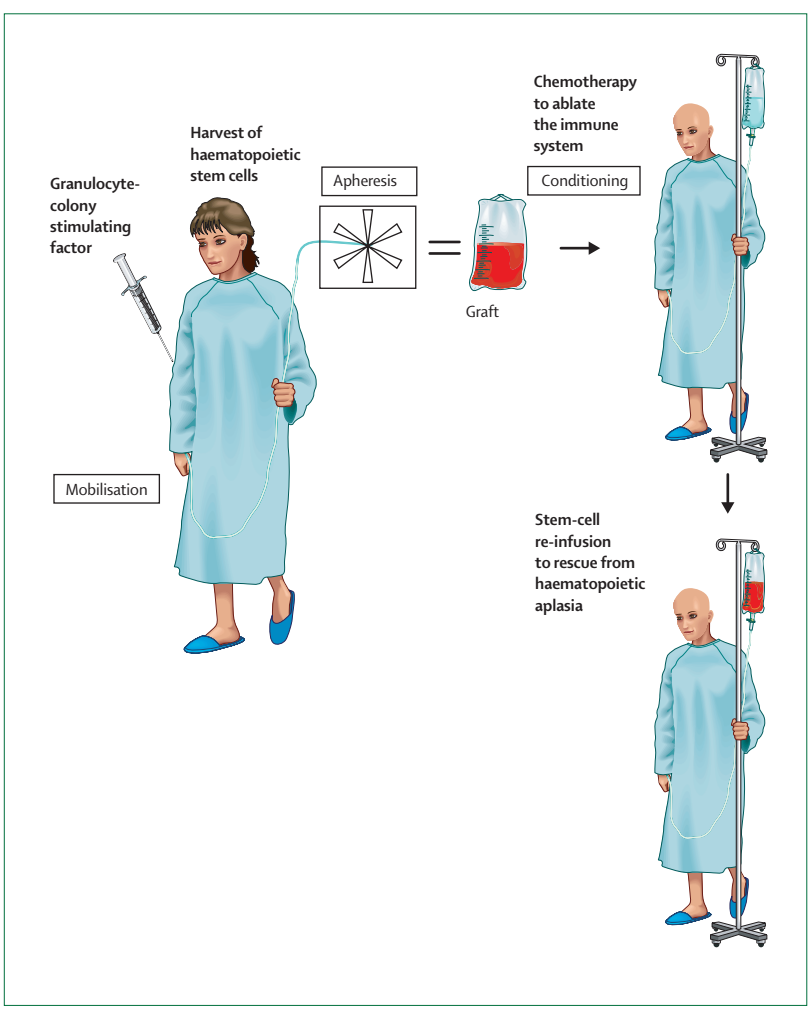

Figure 1: **Autologous HSCT**

The mobilisation of haematopoietic stem cells from bone marrow to peripheral blood is done by subcutaneous injection of granulocyte-colony stimulating factor. Serial peripheral-blood analysis establishes the optimal day (1–2 weeks later) for apheresis (extraction of peripheral blood haematopoietic stem cells with an apheresis machine that uses an immunomagnetic method). The apheresis product is the graft, which will be stored until used. Next, the patient receives the conditioning regimen (chemotherapy with or without total body irradiation) to ablate the immune system. Subsequently, the graft is re-infused to the patient.

Multiple sclerosis (MS) is thought to be an autoimmune disorder in which aberrant immune responses lead to T-cell mediated focal myelin destruction and secondary oligodendrocyte and axonal damage. Although the disease course is highly variable, 50% of patients will not be able to walk independently within 15 years of onset.[1,2] Current treatments for MS include immunomodulatory and immunosuppressive drugs. Interferon beta and glatiramer acetate reduce the number of relapses, but if these therapies are not successful or the disease develops into a progressive phase there are no effective treatments for modification of the course of the disease.[3] Mitoxantrone showed the clinical progression of secondary progressive MS in a randomised clinical trial, although its long-term clinical effect is unknown.[4] The limited effectiveness of these treatments justifies the assessment of alternative therapeutic strategies in patients with MS with aggressive clinical course.

Bone-marrow transplantation is the standard treatment for several haematological malignant disorders and is being assessed for the treatment of severe forms of several autoimmune disorders including MS.[5] Haematopoietic progenitor cells might re-establish the defective immune system in patients with autoimmune disorders. The cells can be obtained from a sibling or an unrelated donor who is closely matched on HLA (allogeneic transplantation), an identical twin (syngeneic transplantation), or the patient before chemotherapy (autologous transplantation). The haematopoietic progenitor cells can be directly harvested from the bone marrow or collected from peripheral blood; the term haematopoietic-stem-cell transplantation (HSCT) includes both sources (figure 1).

In allogeneic HSCT chronic immunosuppression is needed to prevent graft rejection and graft-versus-host disease. Allogeneic HSCT is also associated with higher mortality rates than autologous HSCT—up to 40% if the donor is not a sibling. By contrast, the mortality from autologous HSCT typically is less than 10%.[5]

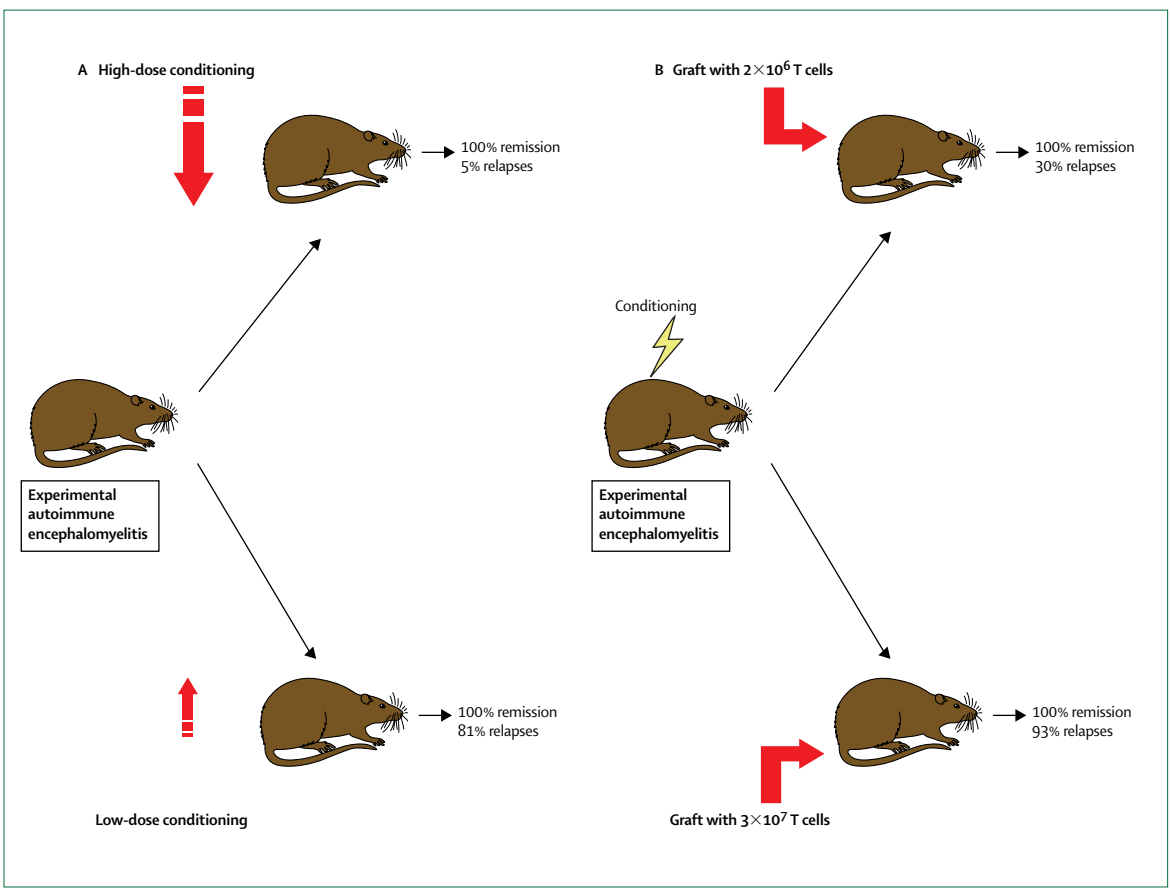

Figure 2: **Effect of conditioning and T-cell number on allogeneic and autologous bone marrow transplantation**
(A) Effect of the intensity of conditioning on the efficacy of allogeneic bone marrow transplantation in rats with chronic relapsing-remitting experimental autoimmune encephalomyelitis. The use of a suboptimal or low-dose conditioning (in this experiment 7 Gy of total body irradiation) induces complete remission, but significantly more spontaneous relapses than high-dose conditioning (10 Gy total body irradiation) treatment.[13] These results reflect the importance of the eradication of host T cells to achieve optimal results.(B) Effect of the number of T cells in the graft on the efficacy of autologous bone marrow transplantation in rats with chronic relapsing-remitting experimental autoimmune encephalomyelitis. The use of a T-cell enriched graft (T cells added to those typically present in bone marrow) after the same type of conditioning led to a significantly higher incidence of relapse.[15] These results support that the use of a T-cell depleted graft for autologous HSCT in MS should be recommended.

Rationale and experimental models

The hypothetical basis for the use of allogeneic HSCT to treat MS is that the procedure will eradicate the abnormal immune system and establish a new one that is more tolerant to the nervous system. Since Morton and Siegel described the development of antinuclear antibodies in normal mice after allogeneic HSCT from NZB mice—a strain of mice that spontaneously develop a systemic-lupus-erythematosus-like disease[6]—experimental and clinical reports have confirmed the possibility of patients developing an autoimmune disorder from the haematopoietic stem cells of affected donors.[7–9] Laboratory animals with autoimmune disorders can be cured or improved by allogeneic HSCT from healthy donors.[7] The complete remissions obtained with allogeneic HSCT in animal models of several autoimmune disorders, including experimental allergic encephalomyelitis, were shown in human beings by anecdotal reports of patients who had malignancies and a coincidental autoimmune disorder and were treated with allogeneic HSCT.[8–12]

Despite promising reports, allogeneic HSCT cannot be used as a treatment for MS because high transplantation-associated toxicity precludes its application in diseases that are not life threatening. In addition, because we do not understand the mechanisms that lead to failure of tolerance in MS and other autoimmune disorders, we cannot be certain that tolerance will develop in cells grafted into the same or similar environment to that implicated in its loss.

Could autologous HSCT be a logical therapeutic approach for MS? The toxicity of the procedure may be acceptable for the treatment of patients with very aggressive forms of MS. However, the infused haematopoietic stem cells reproduce an immune system with potentially the same genetic defects. If MS is the result of an interaction between environmental factors and genetic predisposition, the rationale for the possible

***Panel 1:* Guidelines on autologous HSCT**

Consensus

Centre

Should be done in accredited bone-marrow transplant unit with experience in allogeneic HSCT

Source of haematopoietic stem cells

Peripheral blood

Mobilisation

Cells mobilised with GCSF plus cyclophosphamide or GCSF given alone*

Target cell dose for re-infusion

$>2\times10^6$ $CD34^+$ cell/kg

Antithymocyte globulin

Given if depletion not done†

No consensus

Ex vivo T-cell depletion

3 Log (1000 times fewer T cells) to 4 Log (10 000 times fewer T cells) depletion of T cells

Conditioning regimen

Several used

GCSF=granulocyte-colony stimulating factor. *If GCSF is given alone, MS flares prevented by steroids. CD34 is the antigen that identifies haematopoietic-stem cells. †Concomitant use of high-dose steroids (500 mg) to prevent neurological deterioration related to drug-induced fever.

beneficial effect of autologous HSCT would be that self tolerance will not be broken during the development of the new immune system because external factors that were present during the initial development of the immune system are absent.

Studies in animals with experimental allergic encephalomyelitis have shown that autologous HSCT can induce remissions of the disease. However, the occurrence of spontaneous and induced relapses was higher after autologous than after allogeneic HSCT.[13–15] To induce long-lasting remissions in MS and other autoimmune diseases autologous HSCT must eradicate all of the autoreactive lymphocyte population of the patient, including those in the target organ, and ensure minimum number of autoreactive T cells in the graft that is re-infused (figure 2).

Practical issues in autologous HSCT

As in other medical procedures, the immediate toxicity of the autologous HSCT depends on the expertise of the transplant team and the measures taken to prevent infectious complications. To guarantee the lowest rate of complications in patients with MS who have autologous HSCT, they must be treated by transplant teams with approved protocols for allogeneic HSCT and in isolated rooms with laminar airflow.[16]

Autologous HSCT is a complicated procedure with several steps that have not been done uniformly among different centres with approved protocols.[16,17] These steps may be important for outcome, including the success of improving the MS clinical course. A general consensus has not been achieved for all the issues (panel 1).[18]

Source of haematopoietic stem cells

Haematopoietic stem cells are mainly found in the bone marrow but they can be mobilised to the peripheral blood, in large numbers, by the administration of recombinant granulocyte colony-stimulating factor. Haematological (neutrophil, red cell, and platelet) and immune recovery is faster with peripheral blood cells than with bone marrow cells, owing to more rapid engrafment.[19]

Flares of MS, rheumatoid arthritis, and exacerbation of neurological symptoms in systemic lupus erythematosus have occurred while patients were taking granulocyte colony-stimulating factor.[20–22] Although the mechanism is unclear, it seems to be related to lymphocyte activation by release of cytokines. The concomitant use of steroids or cyclophosphamide with the granulocyte colony-stimulating factor is probably effective to suppress or at least reduce the risk of this complication.[20,23]

Graft manipulation

Experimental studies suggest the use of an unmanipulated graft increases the risk of disease recurrence because more autoreactive T cells are infused in the graft.[15,24] Furthermore, the number of T cells in the peripheral-blood transplant are much higher than in transplants where the haematopoietic stem cells are obtained from the bone marrow.[25] The number of re-infused T cells can be reduced ex vivo by T-cell depletion of the graft or in vivo by use of antithymocyte globulin, a potent lymphocytotoxic drug.[18,26] The convenience of T-cell depletion of the graft and the intensity of the depletion is debated. Data from allogeneic HSCT suggests that depletion should achieve a T-cell dose less than 10^5/kg to prevent re-expansion of autoreactive T cells (panel 1).[16] Although haematological recovery is not different between T-cell depleted and unmanipulated grafts,[27] the former slow down quantitative T-cell reconstitution, especially the CD4 T-cell counts.[28–31] In addition, aggressive T-cell depletion may result in increased opportunistic infections and lymphoproliferative disorders.[32–34]

The effect of T-cell depletion of the graft in the development of MS is unknown. In a pilot randomised trial comparing T-cell depleted with unmanipulated autologous HSCT for severe rheumatoid arthritis, a similar outcome was observed in the two arms after a follow-up of 1 year.[35] This result is not unexpected given that, in contrast to what happens in experimental allergic encephalomyelitis,[15] the addition of T cells to the graft does not increase the relapse rate in an adjuvant arthritis experimental model.[36] An alternative explanation is that

the conditioning was insufficient to eradicate the host's autoreactive T cells. The absence of randomised studies to address this issue in patients with MS prevents any statement on the advantage of T-cell depleted grafts in autologous HSCT.

Conditioning regimens

Conditioning regimens use a combination of cytotoxic drugs, and sometimes radiotherapy, to eliminate the immune cells, including those in the target organ. In MS, the conditioning regimen must destroy the autoreactive T cells in the nervous system, which are probably protected by the blood–brain barrier. There is no consensus on the ideal conditioning regimen to use in autologous HSCT for MS (table 1).[17,37–46]

Some protocols include total body irradiation as part of the conditioning regimen. In experimental allergic encephalomyelitis, total body irradiation as a single treatment has proved effective in the control of the disease and destroys the immune cells in the brain.[13–15,47,48] However, the use of radiotherapy in MS is controversial because it has been associated with worsening of disease activity in experimental allergic encephalomyelitis and may induce MS relapses in patients who receive cranial radiotherapy.[13,49–51] Use of total body irradiation at a dose of 800–1200 cGy as conditioning for MS was not associated with severe neurological toxicity.[42,43] Total body irradiation has been associated with high risk of solid tumours.[52]

The most common conditioning regimen used in autologous HSCT to treat patients with MS is BEAM (carmustine, etoposide, cytarabine, and melphalan), a standard treatment of patients with lymphomas who receive autotransplants (table 1).[37] This regimen, without T-cell depletion of the graft, and treatment with antithymocyte globulin after re-infusion of the graft has been selected for use in the open, randomised, phase III trial that will compare the efficacy of autologous HSCT with that of mitoxantrone to treat severe forms of MS.[53]

Besides the effectiveness of the conditioning regimen in T-cell depletion, there are other considerations in the selection of a regimen: the potential mortality risk of the treatment to patients in whom the expected risk of death from the disease is only slightly increased[54] and the long-term risk of cancer associated with the treatment.[52]

Clinical studies

Worldwide about 250 patients with MS have been treated with autologous HSCT. The Autoimmune Disease Working Party registry of the European Group for Blood and Marrow Transplantation[53] collected 168 cases up to June 23, 2004 (Dr A Tyndall for the European Group for Blood and Marrow Transplantation, personal communication). Data on toxicity and clinical outcome for the first 85 patients are available as result of a multicentre retrospective observational analysis.[17] In addition, several single-centre series from Europe and North America have been reported since 1997. Several patients described in these series were also included in the European Group for Blood and Marrow Transplantation report (table 1).

Study	Regimen	Number of patients	T-cell depletion of the graft
Fassas et al[17]	BEAM ± ATG	54	In 40 patients
Fassas et al[37,38]		25	In some patients*
Kozak et al[39,40]		10	In 7 patients
Mancardi et al[41]		10	No
Nash et al[42]	Cy + TBI + ATG	26	Yes
Burt et al[43]	Cy + TBI	21	Yes
Carreras et al[44,45]	Carm + Cy + ATG	14	Yes
Fassas et al[37,38]	Busulfan + ATG	10	In some patients*
Openshaw et al[46]	Busulfan + Cy + ATG	5	Yes
Fassas et al[17]	Fludarabine + ATG	1	Unknown*

BEAM=BCNU, etoposide, cytosine arabinoside, melphalan; ATG=antithymocyte globulin; Cy=cyclophosphamide; TBI=total body irradiation; Carm=carmustine; *Not stated in the paper

Table 1: **Conditioning regimens used in autologous HSCT for MS**

All the reported studies were phase I and phase II clinical trials assessing the feasibility and toxicity of the procedure rather than the efficacy. The series were heterogeneous for eligibility criteria and the procedures of stem-cell mobilisation, graft manipulation, and transplantation. The mean follow-up in some series was too short. Despite these shortcomings, the studies helped to improve the clinical criteria for the selection of patients with MS for the procedure (panel 2) and provided information on the morbidity of the autotransplant, the effect of this treatment on the immune reconstitution, and on the outcome of MRI and CSF variables.

Panel 2: **Proposed selection criteria in protocols of autologous HSCT for MS**

Inclusion criteria*

Relapsing-remitting MS with cumulative deficits

Secondary-progressive MS with or without relapses

Age 18–50 years

Current EDSS 3·5–6·5

Increase of the EDSS in the last year:

- at least 1·5 points if EDSS is 3·5–5·0, or at least 1·0 point if EDSS is ⩾5·5
- or
- at least 1·0 point if EDSS is 3·5–5·0, or at least 0·5 point if EDSS is ⩾5·5 with at least one enhancing lesion in brain MRI

Progression despite immunomodulating treatment

*Inclusion criteria of the ASTIMS trial (phase III study to compare efficacy and safety of autologous HSCT *vs* mitoxantrone therapy). These criteria are from the guidelines of the Milan consensus conference[18] and from the cumulative experience on autologous HSCT for MS.

Study	Number of patients	MS type	EDSS median	Conditioning regimen	Number and cause of deaths			
					Disease	Treatment-related progression		Overall mortality
						Infection	Other	
Fassas et al[17]	85	3 RR 60 SP 22 PP	6·5 (4·5–8·5)	Several	2 (at 63 and 81 days)	4	1	8·2%
Fassas et al[37,38]	35	2 RR 19 SP 14 PP	6·0 (4·5–8·0)	BEAM+ATG or ± T-cell depletion	Busulfan+ATG	1	1	5·7%
Nash et al[42]	26	1 RR 17 SP 8 PP	7·0 (5·0–8·0)	Cy+TBI+ATG + T-cell depletion	1 (at 23 months)	1		7·7%
Burt at al[43]	21	1 RR 14 SP 6 PR	6·5 (3·0–8·5)	Cy+TBI + T-cell depletion	2 (at 13 and 23 months)			9·5%
Saiz at al[45]	14	9 SP 5 RR	6·0 (4·5–6·5)	Carm+Cy+ATG + T-cell depletion				0%
Kozak et al[39,40]	10	SP	6·5 (5·5–7·5)	BEAM+ATG or BEAM+ T-cell depletion				0%
Mancardi et al[41]	10	SP	6·5 (5·0–6·5)	BEAM+ATG unmanipulated				0%

RR=relapsing-remitting; SP=secondary-progressive; PP=primary-progressive; PR= progressive-relapsing; BEAM=carmustine+etoposide+cytarabine+melphalan; ATG=antithymocyte globulin; Cy=cyclophosphamide; TBI=total body irradiation; Carm=carmustine

Table 2: **Mortality of patients with MS after HSCT**

Toxicity

The mortality in the European Group for Blood and Marrow Transplantation report, defined as death by any cause, was 8·2%.[17] There were five toxic deaths—four patients died from infection and one patient died from cardiac failure—and two patients died 3 months after the transplant from disease progression. Similar data were found in two North American series of patients treated with a combination of total body irradiation and cyclophosphamide with extensive T-cell depletion. Four of 47 patients (8·5%) treated with this regimen died. Three patients died from disease progression and one died from Epstein-Barr virus-related post-transplant lymphoproliferative disorder (table 2).[42,43]

When interpreting the European Group for Blood and Marrow Transplantation report, some limitations should be noted.[17] The report states that five of the seven dead patients did not fulfil the Milan consensus on old age and high expanded disability status scale (EDSS) score inclusion criteria.[18] Furthermore, three of seven patients were treated with a busulfan-based regimen (mortality in this group of patients was 20%) and another patient was the only one (of the 85 patients in this study) that was treated with a fludarabine-based regimen. All patients were not treated in institutions with the same expertise in HSCT. In our opinion, mortality risks might be lowered by better selection of patients and treatment in centres accredited for allogeneic transplantation.

Short-term toxicity after autologous HSCT in patients with MS is related to an occurrence of infections and engraftment syndrome—a non-infectious episode of fever and sometimes skin rash at the time of haematological recovery—compared with autologous HSCT for other indications.[37,55] Up to 27% of patients given HSCT for MS have a neurological deterioration that is associated with fever and infections and is commonly transient but can be irreversible (7%).[17] The use of higher doses of prednisone to prevent the fever induced by ATG or the flares associated with G-CSF, if cyclophosphamide is not included in the mobilisation, probably will reduce the incidence of these complications.[42,44,46]

Although autologous HSCT causes a profound immunosuppression, few long-term opportunistic infections have been reported.[23,38] The occurrence of permanent amenorrhea was observed in 30% of women older than 37 years at the time of the transplant,[45] a percentage that is not different from that reported in patients treated with mitoxantrone.[4] After the autologous HSCT, some patients with MS develop other autoimmune disorders such as autoimmune thyroiditis,[17,38,42] coagulopathy due to factor VIII-inhibitor,[38] uveitis,[44] lymphocitic gastritis, and brachial neuritis.[42] The transient presence of organ-specific autoantibodies is a well-known event but clinically evident autoimmune disorders are rarely observed after autologous HSCT.[56] Possible explanations include inhibition of the thymus-dependent clonal deletion of autoreactive T lymphocytes and an increased threshold of peripheral autoregulation.[57]

Clinical outcome

At the Milan consensus conference on the role of autologous HSCT in MS,[18] the procedure was suggested to be effective only if the rate of treatment failure at 3 ears is less than 20%. This means that 80% of the

Study	Number of patients	MS type	Median baseline EDSS	Median follow-up	Definition of progression	3 year progression-free survival	Definition of activity	3-year activity-free survival
Fassas et al[17]	85	3 RR	6·5 (4·5–8·5)	16 months (3–59)	Increase of 1·0 point	SP+RR=78%	Progression	55%
						if basal EDSS ⩽5·0		
		60 SP				or 0·5 points if basal	PP=66%	Relapse
		22 PP				EDSS ⩾5·5	Progression after improvement	
Nash et al[42]	26	1 RR	7·0 (5·0–8·0)	24 months (3–36)	1·0 point basal EDSS increase	73%		Not analysed
		17 SP						
		8 PP						
Fassas et al[37]	24	3 PR	6·0 (4·5–8·0)	40 months (21–55)	Increase of 1·0 point	RR=100%	Progression	PR: 0%
					if basal EDSS ⩽5·0			
		13 SP			or 0·5 points	SP=92%	Relapse	SP: 12%
					if basal EDSS ⩾5·5			
		8 PP			PP=39%			PP: 0%
Saiz et al[45]	14	9 SP	6·0 (4·5–6·5)	36 months (19–55)	Any basal EDSS increase	85·7%	Progression	46·4%
		5 RR					Relapse	
							Progression after improvement	
							AI worsening of 1 point	

RR=relapsing-remitting; SP=secondary-progressive; PP=primary-progressive; PR= progressive-relapsing; AI=ambulation index

Table 3: **Clinical outcome of patients with MS after autologous HSCT**

patients who had a transplant should not have confirmed progression as measured by the EDSS score. This proportion was derived from a meta-analysis of the progression-free survival in placebo groups included in treatment trials of progressive MS.

Only four series have reported their clinical outcome in comparable terms: probability of confirmed progression-free survival by Kaplan-Meier estimator (table 3). In all of them, the results were close to the established objective of efficacy of the Milan consensus conference. Another method to assess the clinical efficacy of the autologous HSCT is by recording any event after transplantation that indicates disease activity. This secondary outcome, disease-activity-free survival, defined as the probability of being alive without progression of any type, including no increase of the EDSS score, no increase of the EDSS score after initial improvement (even when the final EDSS is lower than the baseline EDSS score), and absence of relapses, was analysed in three studies. These series showed that less than 50% of patients achieved disease activity-free survival (table 3). These data suggest that autologous HSCT cannot be deemed a curative treatment for MS but support the view that it may cause prolonged stabilisation or change the aggressive course of the disease in the patients who are treated.

The studies of autologous HSCT in MS have not been blinded or have not randomly assigned patients to an alternative therapy, for example mitoxantrone. Therefore they have not answered the important question of whether the procedure is effective in modifying the progressive course of the disease. However, they provided important information on which patients may benefit from the autotransplant and when the treatment should be indicated in future prospective randomised protocols to test the efficacy of the procedure. Primary-progressive MS probably does not benefit from autologous HSCT. Less than 70% of patients with primary progressive MS had a stabilisation of the neurological deficit measured by the EDSS (table 3) and no patients were disease-activity free at 3 years.[17,37] Autologous HSCT probably will not help patients with high EDSS at entry. In one study, none of the nine patients with pre-transplant EDSS scores of 6·0 or lower progressed at least 1 point in the baseline EDSS score after a median follow-up of 18 months, in contrast with four out of 12 (33·3%) patients with pre-transplant EDSS scores of at least 6·5.[43] Lastly, the treatment has been very effective in reducing the number of relapses in those patients with MS who did not benefit from other immunomodulatory therapies.[45] These data suggest that the efficacy of autologous HSCT should be tested in patients with evidence of active MS, defined as patients with frequent relapses that are not controlled by accepted treatment and who have neurological dysfunction that has not exceeded an EDSS score of 6·0.

MRI variables

MRI assessment has been included in most series as an additional outcome measure after autologous HSCT.[17,40–43,45,46] All studies included the number of gadolinium-enhancing T1 lesions and new T2-weighted lesions as a measure of disease activity, but only a few studies provided additional information on measures of disease burden or brain atrophy.[42,45,58]

The most impressive finding in all these studies is that autologous HSCT induces a profound and long-

lasting suppression of gadolinium-enhancing lesions. New gadolinium-enhanced lesions were rarely seen in the serial MRIs done after autologous HSCT.[17,40–43,45,46] In a series of ten patients, serial monthly MRI during the 3 month pretreatment period detected a total of 341 gadolinium-enhancing lesions. Eight of the ten patients did not present with gadolinium-enhancing lesions in the serial MRIs done after transplantation. The other two patients presented with five gadolinium-enhancing lesions during the first 3 months post-transplantation that disappeared over the next 24 months.[41] This effect seems to persist more than 3 years after the transplantation.[45] The positive effect on gadolinium-enhanced lesions was also observed in other inflammatory variables as the T2 lesion load that had a median decrease higher[45] than that reported in trials of immunomodulatory therapies in MS.[59]

The issue of long-term progression of brain atrophy after autologous HSCT has been analysed in only two studies, with conflicting results.[45,58] We[45] reported a mean decrease of 12·71% in the corpus callosum area at 3 years post-transplant compared with baseline. The atrophy was highest during the first year, the time of the greatest reduction of T2 lesion load. Over the ensuing years, the percentage of reduction of the corpus callosum area was lower than that reported in patients with MS treated with interferon or placebo.[60,61] The association between the reduction of the T2 lesion load and brain atrophy suggested that the resolution of oedema and inflammation due to the autologous HSCT could explain part of the increase in the measures of brain atrophy.[45] By contrast, in another study[58] there was a mean yearly decrease of about 1·9% in brain volume over the 2 year follow-up. A percentage that is about two-times higher than those reported in studies of brain atrophy in MS.[62] These data would suggest that the degenerative process is not halted by autologous HSCT despite the effect on improving the inflammatory MRI variables. None of these studies assessed the rate of brain atrophy in the year before the autotransplant and the direct effect of autologous HSCT on brain atrophy is not well known.[63] These limitations prevent any unambiguous statement on the role of autologous HSCT in the development of axonal loss and other non-inflammatory mechanisms implicated in MS. Future protocols should be designed to specifically address this issue and measure the clinical effect with serial neuropsychological assessments.

Oligoclonal IgG bands

The persistence of the CSF oligoclonal IgG bands after autologous HSCT has been reported in most studies.[42,46,64] In the single longitudinal study[64] on the development of oligoclonal bands in the CSF and serum after the autotransplant, CSF oligoclonal IgG bands persisted at 3 months post-transplant. In addition, there were multiple bands in the serum and the albumin index was high suggesting a disruption of the blood–brain barrier. Some of the observed oligoclonal bands in the baseline CSF were also identified in the serum, commonly with a lower intensity; this suggests the B cells that synthesise IgG were in the CNS and the bands diffused to the serum owing to the disruption of the blood–brain barrier. At 12 months, all patients had the same pattern of oligoclonal bands identified in the baseline CSF and most of the serum bands had disappeared.

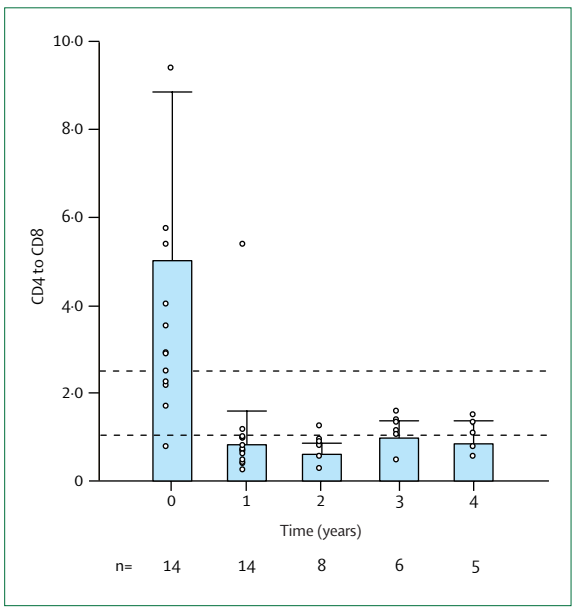

Figure 3: **Change in CD4/CD8 ratio after autologous HSCT in 14 patients with MS[45]**
Mean results are presented and error bars represent 95% CI of the mean. Dotted lines show the 25th and 75th percentiles in the control population; n=number of patients analysed at each point in time.

The development of serum bands after autologous and allogeneic HSCT is transient and seems to be related to the recovery of normal B-cell function.[65] The longitudinal analysis of the CSF oligoclonal IgG bands supports the idea that the B cells and presumably also the T cells located in the CNS at the time of the treatment, survived the conditioning regimen.[64] The persistence of the CSF bands after autologous HSCT with different protocols[42,46] suggests that the conditioning regimens used cannot completely eradicate the T-cells in the nervous system. The clinical significance of this observation is unclear.

Immune reconstitution after transplantation

Information on immune reconstitution after autologous HSCT for MS is scarce and mostly limited to the first year post-transplant.[17,39,42,44,66] The results of the general immune reconstitution are similar to those observed after autologous HSCT for other

indications.[67,68] Thus, the number of B cells, natural killer cells, and CD3 T cells reach normal values in 3 months. However, the subset of CD4 T cells is decreased and that of CD8 T cells increased resulting in an inverted CD4/CD8 ratio that lasts during the first year post-transplant[44,66] and beyond the third year (figure 3). In the early (3–6 month) period post-transplant most of the CD4 T cells are exclusively CD45RO (memory) T cells consistent with selective T-cell expansion from pre-existing T cells that survived the transplantation.[44,66] The number of myelin-basic-protein-reactive T cells greatly diminishes in this early period.[69] CD45RA (naive) T cells (thymus-dependent regeneration) gradually increase after 6 months.[44,66] In this second phase of thymus-dependent pathway recovery there is an expansion of myelin-basic-protein-reactive T cells that recognised a broad repertoire of epitopes including those recognised before the transplant. If confirmed, these findings, obtained from a few patients,[69] support the hypothesis that clonal composition of the reconstituted immune system is not substantially different from the original immune system before transplantation. These data coupled with the profound and long-lasting immunosuppresion of the treatment, suggest that immunosuppression may justify the effect of autologous HSCT on the improvement of the MRI inflammatory variables and, if proven in the future, the clinical efficacy.

Conclusion

Phase I and phase II clinical trials on autologous HSCT for MS have provided important insights on the morbidity and the outcome of MRI and CSF variables. The findings suggest that the treatment is feasible in severe forms of MS provided that strict eligibility criteria are applied to patients and centres. Although the treatment induces a profound and long-lasting suppression of MRI activity associated with inflammation, whether the procedure is really effective in modifying the progressive course of the disease deserves further assessment in the setting of randomised controlled trials. Preliminary studies suggest that autologous HSCT causes an important and persistent immunosuppression rather than a change in the reconstituted immune system.

Search strategy and selection criteria

Studies were identified by searches of PubMed from 1996 to June 2004 with the terms "multiple sclerosis", "magnetic resonance imaging", and "oligoclonal bands" and combining these terms with "haematopoietic-stem-cell transplantation" or "bone-marrow transplantation". Studies were also identified from the personal files of the authors. Abstracts and reports from meetings were not included.

Authors' contributions

YB and AS did the reference search, selected the references, and wrote the first draft of the review. EC gave his expert opinion and reviewed the haematological parts of the review. FG critically read and revised the review.

Conflicts of interest

We have no conflicts of interest.

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II. HIPÓTESIS Y OBJETIVOS

II. 1. HIPÓTESIS

H.1. La ablación del sistema inmune del paciente mediante el acondicionamiento conllevará la erradicación de las células autorreactivas frente a los antígenos diana responsables de la lesión desmielinizante, y la reconstitución inmune de los progenitores hematopoyéticos infundidos generará un nuevo sistema inmunológico tolerante con los autoantígenos mielínicos, con el subsiguiente descenso de la actividad inflamatoria inmunomediada de la enfermedad. Por lo tanto, tras el TAPH esperamos una mejoría en el curso clínico de la enfermedad caracterizada por un descenso en la tasa anual de brotes y el enlentecimiento en la progresión de la discapacidad, así como una reducción paralela de los parámetros radiológicos de actividad inflamatoria tales como el número de lesiones captantes de gadolinio y la carga lesional en resonancia cerebral.

H.2. El cambio en el curso clínico y radiológico de la enfermedad podría explicarse a través del efecto del TAPH sobre marcadores de actividad inflamatoria que participan en la patogenia de la enfermedad:

H.2.a. La eliminación de las células autorreactivas y la instauración de la tolerancia inmune conducirán a una limitación de la activación linfocitaria específica de antígeno, y de la posterior migración de dichas células hacia el SNC. Por lo tanto, esperamos encontrar un descenso en las células inmunes de la síntesis y secreción de MMP-9, enzima que amplifica el acceso de células inflamatorias hacia el SNC, así como un incremento de su inhibidor tisular, o TIMP-1, lo que se traducirá en un descenso de la actividad proteolítica expresada a través de la ratio MMP-9/TIMP-1.

Todo ello favorecerá el restablecimiento de la BHE y la supresión de la captación de contraste medida en resonancia.

H.2.b. Durante el proceso inflamatorio en el SNC se produce un aporte adicional de factores tróficos, en especial BDNF, por parte de las células del sistema inmune para garantizar los mecanismos de reparación que siguen a la agresión. Por lo tanto, el descenso de la respuesta inflamatoria tras el TAPH tendría que asociarse a una reducción en los requerimientos de BDNF, si bien esto podría tener una repercusión negativa sobre la vertiente beneficiosa de la inflamación. Así, el descenso del BDNF a niveles asociados con menor inflamación podría comportar un incremento de la atrofia cerebral ligada a la enfermedad.

H.2.c. La reconstitución inmune en un individuo adulto se caracteriza por una generación lenta de células T $CD4^+$ *naïve*, timodependientes, a diferencia de lo que ocurre con la población T $CD8^+$. Ello conduciría a un cambio fenotípico linfocitario de intensidad y duración variables, que podría condicionar un efecto inmunodepresor a largo plazo. Además, hipotetizamos que asociado a dicho efecto inmunodepresor la tolerancia inmune tras el TAPH podría explicarse también a través de un efecto inmunomodulador caracterizado por un desplazamiento del perfil de producción de citocinas hacia un patrón tipo Th2 (o antinflamatorio), a diferencia de la situación pretransplante donde predominaría el patrón estimulador de la inmunidad celular Th1.

H.3. El polimorfismo Val66Met (sustitución de una valina por metionina en la posición 66 de la proteína) del gen del BDNF es un polimorfismo funcional que condiciona a nivel neuronal una menor producción de BDNF por alteración de su

transporte intracelular y secreción. La presencia del alelo met podría determinar también una menor producción de BDNF a nivel de las células inmunes favoreciendo la neurodegeneración y la atrofia cerebral asociadas a la enfermedad.

II.2. OBJETIVOS

O1. Evaluar el efecto del TAPH sobre el curso de la enfermedad en un grupo de pacientes con EM agresiva resistente a terapia convencional, a través de parámetros de actividad clínicos y de resonancia cerebral en el marco de un protocolo terapéutico.

O2. Analizar la respuesta al TAPH a través de diferentes marcadores subrogados de actividad inflamatoria implicados en la patogenia de la esclerosis múltiple, y su correlación con la evolución clínica y radiológica tras el TAPH:

O2.a. Analizar la evolución de los niveles séricos y la expresión de mRNA en células mononucleares de sangre periférica de la MMP-9 y de su inhibidor tisular, el TIMP-1, y su correlación, en especial, con las medidas de la carga lesional y las lesiones captantes de gadolinio en resonancia.

O2.b. Analizar la evolución de los niveles de BDNF en suero, en líquido cefalorraquídeo y en sobrenadante de células mononucleares de sangre periférica, y su correlación con las medidas de atrofia cerebral en resonancia.

O2.c. Estudiar las características a largo plazo de la reconstitución inmune tras el TAPH a través del fenotipaje de las subpoblaciones linfocitarias de sangre periférica, y evaluar el posible efecto inmunomodulador del trasplante mediante el análisis del perfil de secreción de citocinas Th1 y Th2 por las células mononucleares de sangre periférica.

O3. Analizar si el polimorfismo Val66Met condiciona susceptibilidad a padecer EM o el curso evolutivo de la enfermedad, y analizar el impacto de dicho polimorfismo en la capacidad de secreción de BDNF por las células inmunes en el subgrupo de pacientes sometidos al TAPH.

III. PACIENTES Y MÉTODOS

III. 1. PACIENTES

Entre Abril de 1998 y Abril de 2001 un total de 15 pacientes con el diagnóstico de EM fueron inicialmente incluidos en el protocolo según los siguientes **criterios de inclusión:**

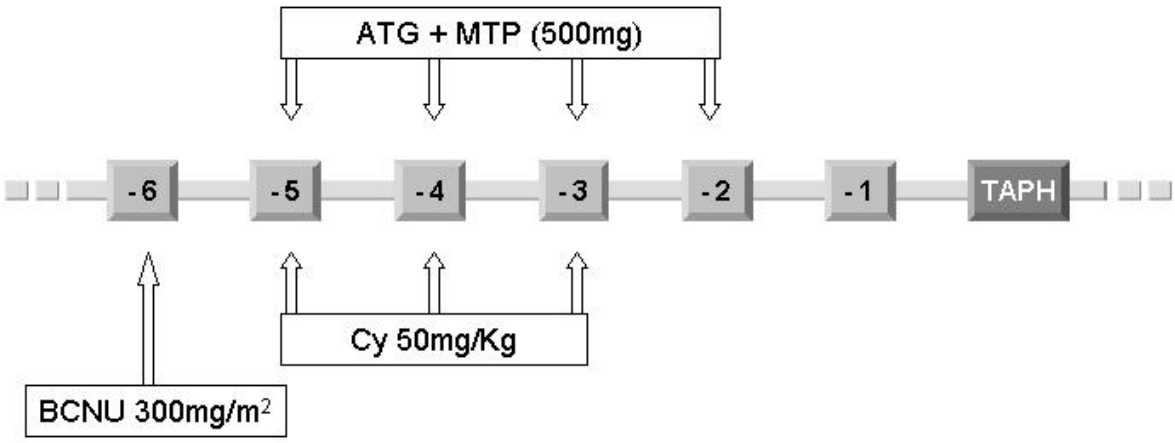
1) Edad entre 18 y 60 años
2) Esclerosis múltiple RR clínicamente definida o en forma SP, con una puntuación en la EDSS de 4.0 a 6.5
3) Incremento de 1 punto en la EDSS durante el año previo a la inclusión si la EDSS basal ≤ 5.5, o de 0.5 puntos si la EDSS basal >5.5 a pesar de tratamiento con interferón u otras inmunoterapias, retiradas al menos 1 mes antes del TAPH
4) Los pacientes con la forma RR debieron presentar al menos 2 brotes en el último año.

III. 2. MÉTODOS

III.2.1. Procedimiento del trasplante

Brevemente, el procedimiento del trasplante consistió en:

1) Obtención de células progenitoras hematopoyéticas mediante la administración de G-CSF asociado a ciclofosfamida (Cy).
2) Depleción de células T del injerto a través de la selección de células $CD34^+$ (progenitores hematopoyéticos) por un método inmunomagnético.
3) Régimen de acondicionamiento que incluía carmustina (BCNU), ciclofosfamida y globulina antitimocítica (ATG) asociada a metilprednisolona (MTP), según el siguiente esquema representado en días previos al día de la infusión del injerto (TAPH) (ver figura):



III.2.2. Evaluación clínico-radiológica

Para cumplir con el **objetivo 1** los pacientes seleccionados fueron sometidos antes del trasplante (basal), al mes, 3, 6, 9 y 12 meses postrasplante, y luego cada 6 meses (anualmente en el caso de la RM cerebral) a:

1) Exploración neurológica que incluía la puntuación en la EDSS y el índice de deambulación.
2) RM cerebral evaluada por un único neurorradiólogo que recogió de forma ciega en cada exploración las siguientes variables:
   - número de lesiones hipointensas en secuencias potenciadas en T1
   - número de lesiones captantes de contraste en T1
   - número de nuevas lesiones en T2
   - volumen lesional en T2
   - área del cuerpo calloso
   - volumen cerebral

La eficacia clínica del TAPH fue evaluada a través de 2 variables:

- ✓ Probabilidad actuarial acumulada de permanecer **libre de progresión** confirmada de la enfermedad. Se consideró progresión a cualquier incremento de la EDSS independientemente de la puntuación de la EDSS basal.
- ✓ Probabilidad actuarial acumulada de permanecer **libre de actividad** de la enfermedad. Se consideró actividad de la enfermedad cualquiera de los siguientes: incremento en la EDSS, incremento en la EDSS tras una mejoría inicial (aún cuando la EDSS final fuese menor a la EDSS basal), incremento

de al menos 1 punto en el índice de deambulación, o presencia de algún brote. El brote fue definido como la aparición de nueva sintomatología neurológica de más de 24h de duración.

III.2.3. Recogida de muestras

Para cumplir con los **objetivos** restantes se realizó la extracción de las siguientes muestras:

1) Obtención de suero antes del TAPH y a los 3, 6 y 12 meses, y luego anualmente tras el trasplante a partir de 10ml de sangre sin anticoagulante
2) Extracción de forma apareada con el suero de LCR basal, y a los 12 meses tras el trasplante.
3) Obtención de células mononucleares de sangre periférica (90% linfocitos y 10% monocitos) basal y anualmente tras el trasplante, que fueron posteriormente almacenadas en nitrógeno líquido.
4) Extracción basal, a los 3, 6, 9 y 12 meses tras el trasplante de 30-40ml de sangre en heparina Li estéril para la caracterización de las subpoblaciones celulares en sangre periférica.
5) Obtención de DNA a partir de sangre periférica de 12 de los 14 pacientes trasplantados, en el contexto de un estudio de casos y controles para el que se usaron además muestras de DNA de la genoteca de esclerosis múltiple.

III.2.4. Técnicas de laboratorio

Las diferentes técnicas de laboratorio utilizadas en los 4 trabajos que componen esta tesis fueron:

- Trabajo 2:

1) Técnica de **ELISA** para la cuantificación de los niveles séricos de MMP-9 y TIMP-1. El procedimiento se realizó en el laboratorio de Neurología.
2) **PCR cuantitativa**, o a tiempo real, para la determinación de la expresión de mRNA de MMP-9 y TIMP-1 en células mononucleares de sangre periférica. Para ello, se extrajo previamente el RNA de las muestras de células mononucleares de sangre periférica que se encontraban almacenadas en N líquido. El aprendizaje de la técnica se realizó gracias a la colaboración del Servicio de Pneumología.

- Trabajo 3:

1) Técnica de **ELISA** para la determinación de los niveles de BDNF en suero, LCR y en el sobrenadante de cultivos de células mononucleares de sangre periférica. Para ello se contó con la colaboración del departamento de Biología Celular de la Universidad de Barcelona.
2) La reconstitución inmunológica se monitorizó a través del análisis por **citometría de flujo** (FAC-Scalibur) de diferentes marcadores de superficie celular sobre muestras en fresco de células mononucleares de sangre periférica. Todo ello fue realizado por el Servicio de Hematología.
3) Estudio del **perfil de secreción de citocinas** en el sobrenadante de las células mononucleares de sangre periférica a través un kit comercial, *Human Th1/Th2 Cytometric Bead Array Kit,* y su posterior análisis por citometría de

flujo. Todo ello se realizó en colaboración con la Unidad de Inmunología de la UAB.

- Trabajo 4:

1) Determinación de las variantes alélicas en el codon 66 del gen del BDNF mediante el **genotipado** del DNA genómico a través de la técnica comercial TaqMan® SNP Genotyping Assay, en colaboración con el Servicio de Inmunología del Hospital Clínic.

IV. RESULTADOS

TRABAJO 1.

Saiz A., Blanco Y., Carreras E., Berenguer J., Rovira M., Pujol T., Marin P., Arbizu T., Graus F. Clinical and MRI outcome after autologous hematopoietic stem cell transplantation in MS. Neurology 2004; 62(2):282-4.

En este primer trabajo se evaluó la evolución clínica y radiológica de 14 pacientes con EM agresiva resistente a terapia convencional sometidos a un TAPH en el contexto de un ensayo clínico fase II de viabilidad y seguridad.

Mi participación en este trabajo fue colaborar con el Dr. Saiz y el Dr. Graus en la exploración clínica de los pacientes en las fechas correspondientes, creación de la base de datos y la explotación de los mismos. Asimismo, participé en la redacción del artículo.

Este trabajo fue presentado como comunicación oral en la XIII European Neurological Society, en Estambul en el año 2003.

Clinical and MRI outcome after autologous hematopoietic stem cell transplantation in MS

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Abstract—The authors report the outcome of 14 patients with severe multiple sclerosis treated with autologous hematopoietic stem cell transplantation (AHSCT) after a median follow-up period of 3 years. The 3-year actuarial probability of progression-free survival was 85.7% and that of disease activity-free survival was 46.4%. On MRI, no T1-enhanced lesions were detected after AHSCT. The mean change in T2 lesion volume from baseline to the third year was −20.2% and that of the corpus callosum area was −12.7%; 50% of this reduction was seen during the first year.

NEUROLOGY 2004;62:282–284

We assessed immune ablation with autologous hematopoietic stem cell transplantation (AHSCT) as potential treatment for patients with severe multiple sclerosis (MS).[1,2] Previous series have emphasized the tolerance of the procedure with limited information on the neurologic or MRI outcome beyond the first year after AHSCT.[3-6] We reported the short-term MRI evolution of the first five MS patients included in our AHSCT protocol[7] and the toxicity results of the entire series of 14 patients who were treated.[8] Here, we describe the clinical outcome and MRI evolution after a median follow-up period of 3 years.

Methods. Fifteen patients (13 women, 2 men; median age, 30 years; range, 22 to 45 years) were initially included in a prospective protocol to evaluate the safety of T-cell–depleted AHSCT. Eligibility criteria followed the recommendations of the Milan consensus conference.[2] Briefly, the criteria were 1) aged 18 to 60 years; 2) clinically definite secondary progressive MS (SPMS) or relapsing-remitting MS (RRMS) with a Kurtzke's Expanded Disability Status Scale (EDSS) score of 4.0 to 6.5; and 3) an increase in the EDSS by 1.0 point with an EDSS of ≤5.5 or 0.5 with an EDSS >5.5 over the previous year despite treatment with interferon or other immunotherapies that are stopped at least 1 month before the AHSCT. Six patients had RRMS with cumulative residual deficits on recovery, and nine patients had SPMS. The median number of relapses in the year before transplantation was three (range, one to seven) in the entire series and six (range, three to seven) in the RRMS group. The median EDSS at entry was 6.0 (range, 4.5 to 6.5). The median EDSS increase in the year before transplantation was 1.0 (range, 0.5 to 2.0) in the SPMS group and 1.5 (range, 1.0 to 4.5) in the RRMS group.

The transplantation procedure was previously described in detail.[7,8] Hematopoietic stem cells were obtained with cyclophosphamide (Cy) and granulocyte colony-stimulating factor. The grafts were depleted of T cells by $CD34^+$ immunomagnetic selection. Conditioning regimen included carmustine, Cy, and antithymocyte globulin (ATG). Patients were evaluated (neurologic examination, ambulation index [AI], EDSS, brain MRI) before AHSTC, at 1, 3, 6, 9, and 12 months after AHSCT, and then every 6 months (MRI done yearly after the first year). The MRI protocol was previously described in detail.[7]

The 3-year progression-free survival was defined as the probability to be alive without increase in the EDSS score (confirmed over 6 months) after AHSCT as compared with baseline measurement.[2,9] The 3-year disease activity-free survival was defined as the probability of being alive without progression of any type, which included no increase of the EDSS or AI at last assessment, no increase of the EDSS after initial improvement even if the worsening did not reach the baseline EDSS, and absence of objective relapses. The Kaplan–Meier estimator was used to assess progression-free and disease activity-free survival.

Results. Mobilization of hematopoietic stem cells from peripheral blood was unsuccessful in one RRMS patient. The tolerance to the AHSCT of the remaining 14 patients was previously reported.[8] Briefly, no patient died or had grade III/IV (severe) systemic complications as result of the procedure. Neurologic deterioration was observed in three patients. It was transient in two (one during mobili-

See also page 168

From the Services of Neurology (Drs. Saiz, Blanco, and Graus), Bone Marrow Transplantation Unit, Hematology (Drs. Carreras and Rovira), Radiology (Drs. Berenguer and Pujol), and Blood Bank and Cryopreservation Unit (Dr. Marin), Hospital Clínic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, Spain; and Service of Neurology (Dr. Arbizu), Unidad de Esclerosis Múltiple, C. S. U. de Bellvitge, L'Hospitalet, Barcelona, Spain.

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Table *Baseline and follow-up of clinical and MRI characteristics after autologous hematopoietic stem cell transplantation*

Patient no.	Age, y/sex	MS type	Disease duration, y	Baseline EDSS	Last EDSS (month post AHSCT)	Relapses pre AHSCT	Relapses post AHSCT*	Baseline AI	Last AI*	MRI Gad+ pre	MRI Gad+ post	% change T2 lesion load compared with baseline			% change corpus callosum area compared with baseline		
												1 y	2 y	3 y	1 y	2 y	3 y
1	30/F	SP	8	6.0	6.0 (55)	3	0	4	6	+	−	−19.77	−51.97	−47.64	−7.62	−9.43	−12.41
2	43/F	SP	7	6.5	8.0 (54)	2	0	6	8	−	−	−26.62	−31.89	N.D.	−19.69	−20.27	N.D.
3	24/F	RR	9	5.0	5.0 (48)	6	2†	2	2	+	−	−10.33	−43.78	−39.95	−13.65	−15.29	−15.52
4	44/M	SP	14	6.5	5.0 (46)	2	1†	4	2	−	−	−5.94	2.86	3.28	−11.06	−10.78	−7.99
5	27/F	RR	8	6.5	6.5 (45)	6	0	4	6	+	−	−11.79	−9.09	−11.40	−17.83	−21.64	−21.3
6	45/F	SP	19	6.5	7.5 (39)	3	0	6	8	−	−	−11.66	−24.31	−22.69	−13.89	−12.8	−12.16
7	23/F	RR	6	6.5	6.5 (37)	6	0	5	6	−	−	1.01	1.45	−1.11	−9.15	−7.3	−10.46
8	37/F	SP	3	5.5	5.5 (36)	1	0	3	3	+	−	−26.79	−23.02	−21.64	−8.21	−10.24	−9.11
9	31/F	SP	10	6.0	5.0 (33)	1	0	4	2	−	−	−2.69	−6.05		−2.2	0.31	
10	28/F	RR	1	5.5	4.5 (30)	6	4	2	2	−	−	−5.03	−3.96		−4.09	−3.35	
11	28/F	SP	9	6.5	6.5 (25)	1	0	5	5	−	−	−7.04	−5.39		−1.15	−0.94	
12	33/M	SP	8	6.5	6.5 (22)	3	3	5	5	−	−	−23.97	−23.05		−0.82	−1.01	
13	37/F	SP	10	5.5	5.5 (20)	1	0	5	5	−	−	5.32			−0.3		
14	22/F	RR	6	4.5	4.0 (19)	7	0	2	2	+	−	1.77			−8.65		

AHSCT = autologous hematopoietic stem cell transplantation; MS = multiple sclerosis; SP = secondary progressive MS; RR = relapsing-remitting MS; EDSS = Expanded Disability Status Scale score; AI = ambulation index.

*See follow-up in the column of last EDSS.

†Subjective sensory symptoms without change in the neurological examination; N.D. = Not done at 3 years, but at 4 years the % change of the T2 lesion load compared with baseline was −36.2%, and the reduction of the corpus callosum area of −19.9%.

zation and another with high fever related to ATG administration) and persistent in the third patient (related to ATG-induced fever).[7] Four of the 12 female patients (all aged >37 years) had secondary amenorrhea.

After a median follow-up period of 36 months (range, 19 to 55 months), the EDSS remained stable in eight patients, improved in four (median, 1.0; range, 0.5 to 1.5), and worsened in two (1.0 and 1.5) (table). In one patient, the deterioration was related to the procedure. The 3-year actuarial probability of progression-free survival was 85.7 (95% CI, 60 to 96%) and that of disease activity-free survival was 46.4 (95% CI, 24 to 76%) (figure 1). An increase of one point in the AI was observed in 3 of 12 patients considered progression-free by the EDSS criteria (see table). In one patient, after an initial improvement that lasted for 12 months, the EDSS score progressed to baseline and then remained stable for 31 months.

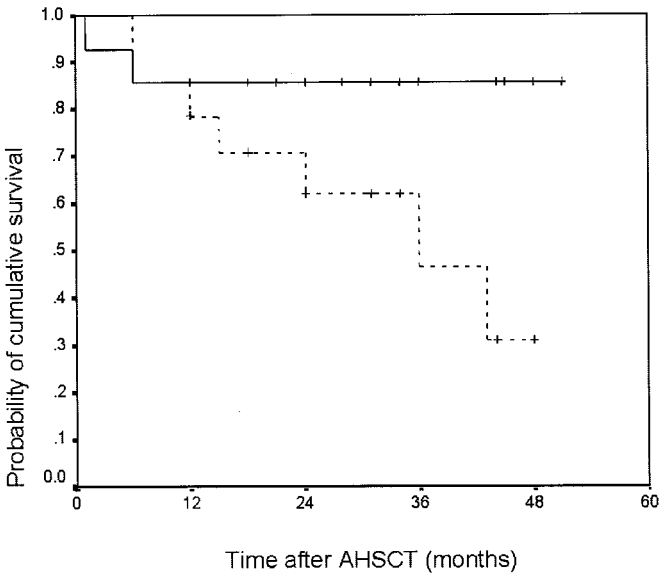

Figure 1. Actuarial probability of progression-free survival (Expanded Disability Status Scale stable or better) (continuous line) and disease activity-free survival (see text for definition) after autologous hematopoietic stem cell transplantation in 14 patients with multiple sclerosis.

Two patients had three episodes of transient subjective sensory symptoms that did not require treatment. The number of objective relapses decreased from 48 in the year before AHSCT to 7 (in two patients) during the follow-up period (see table). No patient received any additional immunotherapy during the entire follow-up period after the AHSCT.

Five patients had gadolinium-enhancing lesions on basal MRI. No T1-enhanced lesions were detected since the first month after AHSCT in any of the follow-up MRI studies. The mean percentage reduction of the T2 lesion volume at 3 years was 20.2% compared with baseline (figure 2); 50% of this reduction seen in the first year (see table).

A decrease in the corpus callosum area was observed throughout the study with a mean reduction of 12.71% at 3 years compared with baseline (see figure 2). The main reduction occurred during the first year. The decrease of the area of the corpus callosum was only 0.37% between the first- and second-year MRI (12 patients evaluated) and 0.20% between the second- and third-year MRI (7 patients) (see table).

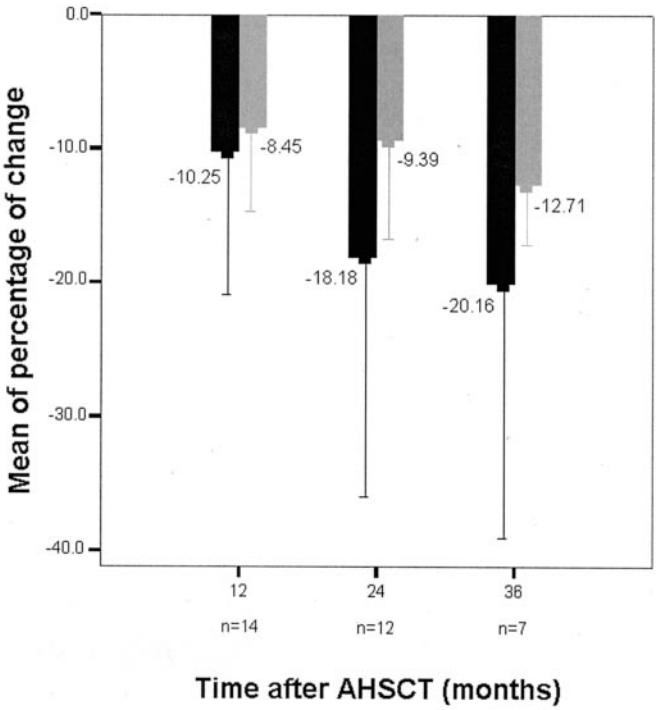

Figure 2. Mean percentage change of the T2 lesion volume (black columns) and reduction of the corpus callosum area (gray columns) compared with baseline. Error bars represent the SD of the mean.

Discussion. AHSCT remains an experimental treatment option for patients with severe forms of MS that should be considered only in the setting of approved protocols. Our 3-year results in this pilot trial showing an actuarial probability of progression-free survival of 85.7% with 46.4% of patients free of clinical disease activity support the view that AHSCT deserves further evaluation in the setting of multicenter controlled trials.

A main concern of AHSCT is the morbidity of the treatment. In a recent retrospective multicenter study of 85 MS patients who underwent AHSCT, 7 (8%) died and 27% had transient or, less frequent, long-lasting neurologic deterioration.[9] These figures are higher than those observed in our protocol and probably are explained by selection criteria (age >40 years and EDSS >6.5 probably predispose to higher morbidity) and use of more aggressive conditioning regimens.[9]

The probability of progression-free and disease activity-free survival at 3 years of our study should be taken with caution because of the small number of patients. A previous study with a similar follow-up period of AHSCT in 16 patients with progressive-relapsing MS (three patients) or SPMS showed a 3-year progression-free survival of 92%.[3] However, the 3-year disease activity-free survival was <20%.[3] The absence of a plateau in the disease activity-free survival curves suggests that AHSCT will not be a definitive cure of MS but may cause prolonged stabilization or perhaps change the aggressive course of the disease. Our results in the EDSS stabilization after AHSCT are supported with a dramatic decrease in the number of objective relapses that changed from 48 in the year before AHSCT to 7 (in two patients) after the procedure. Whether the same goal may be achieved with less-intensive immunosuppressor treatments is open to debate.

Unlike previous series of AHSCT in patients with MS, our study provided information on the long-term evolution of MRI measures of inflammation and brain atrophy. The procedure completely abolished gadolinium-enhancing lesions during the 3-year period, confirming the experience of a previous study with a shorter median follow-up period of 15 months.[6] Accordingly, we observed an important decrease in T2 lesion load. The mean decrease of 20.2% at 3 years was higher than that reported in trials of RRMS and SPMS patients treated with different types of beta interferon or glatiramer acetate.[10] It is important to note that the greatest reduction of T2 lesion load occurred during the first year after AHSCT, when we also observed an important decrease in the corpus callosum area. The trend toward an association between the reduction of the T2 lesion load and the increase of brain atrophy measures suggests that the resolution of edema and inflammation resulting from the AHSCT could explain part of this finding and masks any potential effect of the transplant in the rate of real degenerative changes. Alternatively, the atrophy of the corpus callosum could be a reflection of the damaging inflammatory events that were present before the transplant.

Acknowledgment

The authors thank the neurologists who referred their patients.

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TRABAJO 2.

Blanco Y., Saiz A., Carreras E., Graus F. Changes of matrix metalloproteinase-9 and its tissue inhibitor (TIMP-1) after autologous hematopoietic stem cell transplantation in multiple sclerosis. J Neuroimmunol. 2004;153(1-2):190-4.

En el segundo trabajo se analizó la evolución tras el trasplante de los niveles de MMP-9 y de su inhibidor, TIMP-1, y la expresión de ambos en células inmunes.

Mi participación en este trabajo fue el aprendizaje y la realización de las técnicas de ELISA y PCR cuantitativa, y la explotación estadística de los datos que fueron añadidos a la base ya existente. Asimismo, participé en la redacción del artículo.

Este trabajo fue presentado como póster en el 19th European Committee for Treatment and Research in Multiple Sclerosis y en la LV Reunión Anual de la Sociedad Española de Neurología, ambos durante el año 2003.

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Changes of matrix metalloproteinase-9 and its tissue inhibitor (TIMP-1) after autologous hematopoietic stem cell transplantation in multiple sclerosis

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Abstract

Elevated levels of matrix metalloproteinase-9 (MMP-9) associates with predictors of multiple sclerosis (MS) activity. We analysed serum levels and mRNA expression of MMP-9 and its inhibitor TIMP-1 in peripheral mononuclear cells of 14 MS patients after autologous hematopoietic stem cell transplantation (AHSCT). All but 2 patients stabilized after AHSCT. A significant decrease of MMP-9 levels was seen up to 36 months after AHSCT. TIMP-1 levels did not change. MMP-9 mRNA levels correlated with the CD4+ T cell count ($p < 0.0001$). The significant and persistent change in MMP-9 activity after AHSCT may be caused in part by the effect of AHSCT in the CD4+ T cell count.

Keywords: Multiple sclerosis; Transplantation; Matrix metalloproteinase; Real time polymerase chain reaction

1. Introduction

Matrix metalloproteinase-9 (MMP-9) expression is a crucial pathogenic feature in multiple sclerosis (MS) (Leppert et al., 1995). The expression and activity of MMP-9 is tightly regulated and includes inhibition of MMP-9 activation and proteolytic activity through binding to an endogenous tissue inhibitor of metalloproteinase or TIMP-1 (Kleiner and Stetler-Stevenson, 1993).

Serum and mRNA levels of MMP-9 or the MMP-9/TIMP-1 ratio are increased in MS patients compared to healthy controls, are higher during clinical relapses (Waubant et al., 1999; Lee et al., 1999; Lichtinghagen et al., 1999) and predict new gadolinium-enhanced MRI lesions (Waubant et al., 1999, 2003). Part of the beneficial effect of interferon beta (INFβ) may result from altering the balance between MMP-9 and TIMP-1 (Trojano et al., 1999; Galboiz et al., 2001; Waubant et al., 2003).

The current study analysed the changes of serum and mRNA levels of MMP-9 and TIMP-1 in 14 MS patients treated with autologous hematopoietic stem cell transplantation (AHSCT), an experimental treatment proposed for severe, refractory autoimmune diseases (Marmont, 1994).

2. Material and methods

2.1. Patients

Blood samples were taken at baseline, 12, 24 and 36 months from 14 MS patients treated with AHSCT. The protocol has been previously reported in detail (Carreras et al., 2003; Saiz et al., 2004). Briefly, 14 patients, 5 with relapsing–remitting MS (RRMS) and 9 with secondary progressive MS (SPMS), were included. The median EDSS increase in the year before AHSCT was 3.0 (range, 0.5 to 2.0), and the median number of relapses in the year before AHSCT was 3 (range, 1 to7). After a median follow-up of 36 months, the EDSS remained stable in nine patients and improved in three patients. No enhanced lesions were

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identified after AHSCT in any of the follow-up MRI studies. Mean number of T lymphocytes was normal at 1 year after AHSCT but the CD4+ T cells remained lower during the 3 years of follow-up (mean 0.55 cells × 109/l at 36 months; Carreras et al., 2003).

2.2. Determination of MMP-9 and TIMP-1 serum levels

A commercially available ELISA kit was used to deterine the serum levels of MMP-9 and TIMP-1. Assays were performed following manufacturer's instructions (Amersham-Pharmacia, Little Chalfont, UK). In addition, serum samples were obtained from 32 healthy controls.

2.3. Determination of MMP-9 and TIMP-1 mRNA expression

PBMCs were isolated by Ficoll (Pharmacia, Uppsala, Sweden) density centrifugation and subjected to RNA extraction according to the manufacturer's instructions (Qiagen, USA). Total RNA was reverse transcribed to cDNA in 20 μl of reaction final volume using 250 ng of random hexamers, 0.5 mM dNTPs, 200U Superscript II reverse transcriptase, 50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl^2$, 10 mM dTT and 40 U Rnase OUT (Invitrogen, Paisley, UK). Quantitative PCR analysis was done on a LightCycler using a Fast Start DNA Master SYBR Green I kit according to the manufacturer's instructions (Roche, Mannheim, Germany). The primers for MMP-9 and glyceraldehyde 3-phosphate dehydrogenase (GPDH) were those previously described (Galboiz et al., 2001; Pujols et al., 2001, respectively). For TIMP-1, we used a commercial primer set (LC-Search, Heidelberg, Germany). PCR was performed in a 20 μl reaction volume containing 2 μl of 1:5 diluted cDNA, 3 mM MgCl2, 5 μM specific sense and antisense primers, 1 unit of uracil-DNA glycosylase and 2 μl dye SYBR green I 1:50,000 (Roche, Indianapolis, USA).

The following real time PCR protocol was used:

1. denaturation program (95 °C for 10 min);
2. amplification program repeated for 35 (TIMP-1) or 45 (MMP-9, GPDH) cycles, each cycle containing a denaturation step (10 s, 95 °C), annealing step (MMP-9, 57 °C; GPDH, 58 °C; TIMP-1, 68 to 58 °C using touchdown technique) and elongation step at 72 °C (MMP-9 15 s; GPDH, 40 s; TIMP-1, 10 s) with a single fluorescence measurement at each cycle;
3. melting curve program (65–95 °C), with a heating rate of 0.05 °C/s and continuous fluorescence measurements; and
4. cooling program down to 40 °C. For MMP-9 analysis, serial dilutions of a cDNA PBMCs sample with the same amplification efficiency were used as external standard. For TIMP-1 analysis, an external standard was provided by the commercial kit.

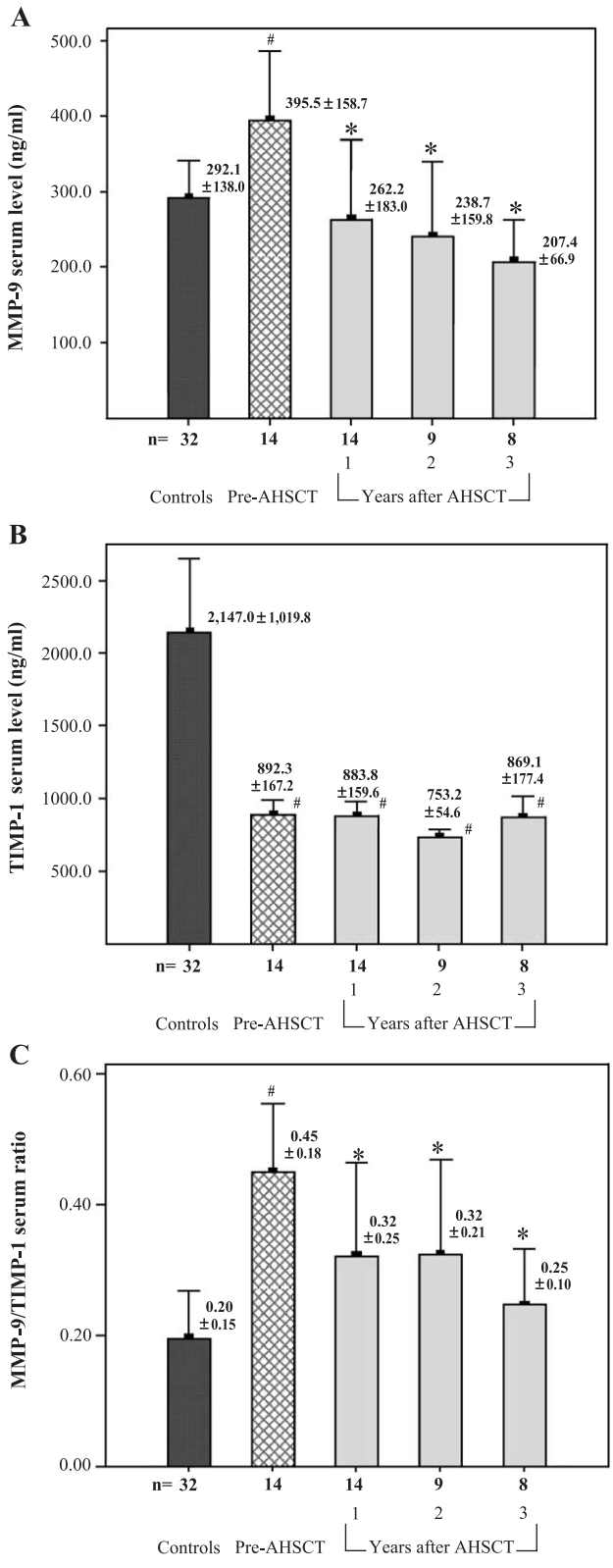

Fig. 1. Serum levels of MMP-9 (A), TIMP-1 (B) and MMP-9/TIMP-1 ratio (C) in 32 healthy controls (dark bars) and 14 MS patients before (striped bars) and after AHSCT (grey bars). Results are expressed as mean ± standard deviation. Error bars represent CI 95% of the mean. n = number of individuals analysed at each point. $^{\#}p < 0.05$ compared to controls. $^{*}p < 0.05$ compared to pre-AHSCT.

Standard curves were calculated referring the threshold cycle to the log of each cDNA dilution step. In each standard curve, we demanded a slope between −5.7 and −2.9, r between −0.98 and −1, and error <0.1. The quantification analysis was made by the Fit Points method using LightCycler software provided with the instrument (version 3.5; Roche). Specific amplicon was confirmed by the presence of one single peak in the melting curve plots and also by agarose gel electrophoresis of the PCR products. Results were expressed as pg equivalents—pg cDNA/capillar adjusted by amount of reverse-transcribed RNA for MMP-9 and G3PH, and the number of copies/capillar for TIMP-1 (commercial kit). The housekeeping gene glyceraldehyde phosphate dehydrogenase (GPDH) was amplified from all samples to normalize expression of MMP-9 and TIMP-1.

2.4. Statistical methods

Nonparametric tests were used for all mean comparisons: Friedman and Wilcoxon signed ranks tests for paired patients groups (baseline, 12, 24 and 36 months) and Mann–Whitney U test for patients and controls. Pearson's coefficients were used for correlations.

3. Results

3.1. Baseline MMP-9 and TIMP-1 serum levels in MS patients compared with controls

Baseline mean MMP-9 serum levels in MS patients were significantly higher and the TIMP-1 serum levels were lower than in controls ($p=0.023$ and $p<0.001$, respectively). The baseline MMP-9/TIMP-1 ratio in patients was significantly higher than in controls ($p<0.0001$; Fig. 1).

3.2. Evolution of the MMP-9 and TIMP-1 serum levels after AHSCT

A significant decrease in MMP-9 levels was seen at 12, 24 and 36 months after AHSCT ($p=0.009$, $p=0.025$ and $p=0.017$, respectively). These MMP-9 serum levels dropped to those seen in controls. In contrast, TIMP-1 levels remained unchanged. After AHSCT, the MMP-9/TIMP-1 ratio decreased during the follow-up ($p=0.05$, $p=0.025$ and $p=0.017$, respectively) and reached similar values as in controls (Fig. 1).

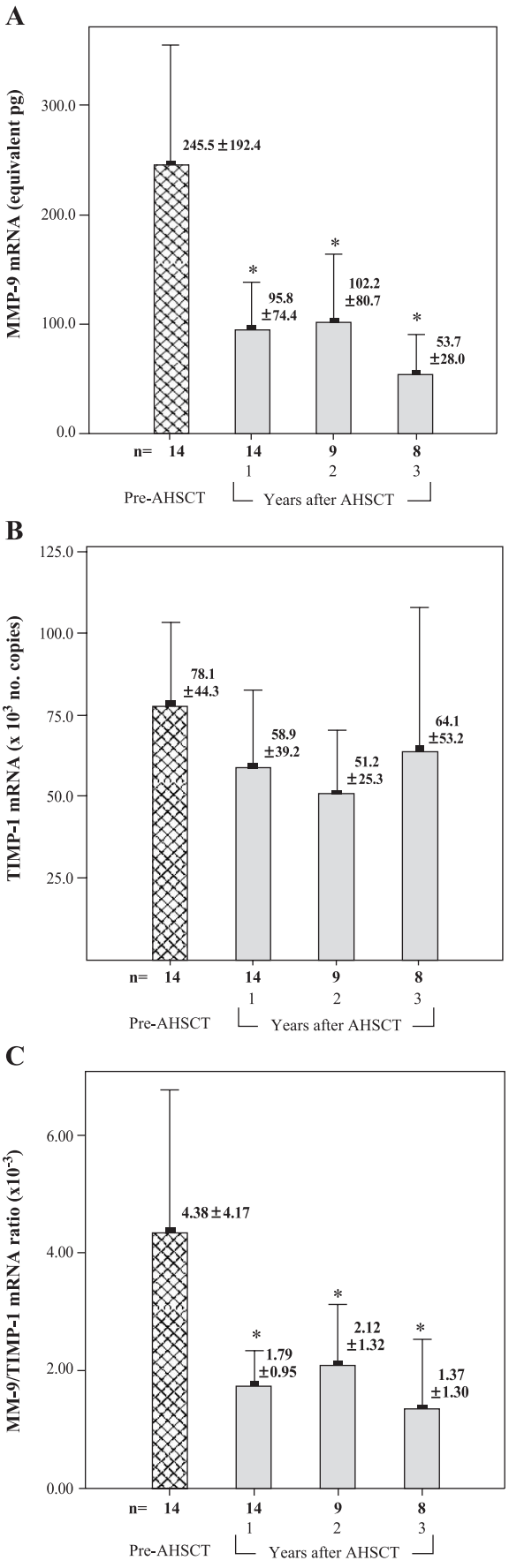

Fig. 2. Evolution of MMP-9 (A), TIMP-1 (B) and MMP-9/TIMP-1 mRNA expression levels at 1, 2 and 3 years after AHSCT (grey bars). Baseline levels are shown in striped bars. Results are expressed as mean ± standard deviation. Error bars represent CI 95% of the mean. n = number of patients analysed at each point. $^{\#}p<0.05$ compared to controls, $^{*}p<0.05$ compared to pre-AHSCT.

3.3. Evolution of the MMP-9 and TIMP-1 mRNA expression after ahsct

A significant decrease of MMP-9 mRNA expression was found at 12, 24 and 36 months after AHSCT compared with that found before treatment ($p=0.001$, $p=0.01$ and $p=0.018$, respectively; Fig. 2). In contrast, the TIMP-1 mRNA expression did not change significantly post-AHSCT. However, the mRNA MMP-9/TIMP-1 expression ratio showed and maintained a significant decrease during the follow-up compared with the baseline ratio ($p=0.039$, $p=0.021$ and $p=0.043$, respectively; Fig. 2). MMP-9 mRNA expression levels correlated with the number of CD4+ T cells ($r=0.6$, $p<0.0001$).

4. Discussion

There is evidence that the levels of serum or mRNA of MMP-9 and TIMP-1 may be useful surrogate markers of disease activity and effect of treatment in MS (Rosenberg, 2001). After AHSCT, serum MMP-9 levels and MMP-9/TIMP-1 ratio dropped to levels of healthy controls during the 3 years of the study. These results coupled with a reduced mRNA expression, suggesting that AHSCT induces a shift towards inhibition of MMP-9 activity—a finding that is in agreement with the clinical and MRI reduction of disease activity observed in these patients. After a median follow-up of 3 years, the EDSS only worsened in two patients and the number of relapses decreased from 48 in the year before AHSCT to 7 (in two patients) after the procedure (Saiz et al., 2004).

The present study showed some differences between our results and those described with INFβ therapy. Thus, in one study, serum MMP-9 activity in RRMS patients decreased during the first year of INFβ treatment, but later, they returned to baseline (Trojano et al., 1999). In SPMS patients, serum MMP-9 levels or MMP-9/TIMP-1 ratio decreased after 3 years on INFβ compared with those treated with placebo. However MMP-9 changes after INFβ compared to pretherapy were not significant (Waubant et al., 2003). High serum MMP-9/TIMP-1 ratios predict new enhanced lesions on brain MRI (Lee et al., 1999; Waubant et al., 2003), suggesting that an imbalance between proteolytic/inhibitory activity might be implicated in the formation of MS lesions. Our results support this view, none of our 14 MS patients presented new enhanced lesions after AHSCT in the 3-year period of follow-up (Saiz et al., 2004).

MMP-9 facilitates the migration of activated peripheral cells into the central nervous system, but the exact contribution of the different PBMCs to the serum MMP-9 is unknown (Opdenakker et al., 2001). The number of CD4+ T cells remained low in the years after AHSCT. The observed correlation between MMP-9 mRNA levels and the CD4+ T cell counts suggests that the low MMP-9 mRNA expression by PBMCs after AHSCT could be related to the underepresentation of the CD4+ population. Further studies on MMP-9 mRNA expression by specific T cells are needed to clarify this issue.

A downregulation of MMP-9 mRNA expression by INFβ has been reported in RRMS patients (Gilli et al., 2004; Galboiz et al., 2001) but not in those with SPMS (Galboiz et al., 2001). Although we have not shown the results of our study by MS subtypes, baseline and follow-up MMP-9 and TIMP-1 serum levels and their mRNA expression were not different between patients with RRMS and SPMS. The small series prevents further statistical analysis to ascertain if the mRNA levels of MMP-9 change differently in RRMS and SPMS patients after AHSCT.

In conclusion, our study suggests that AHSCT in patients with severe MS induces a shift in the protein and mRNA levels or the MMP-9/TIMP-1 ratio toward a reduced proteolytic load. Whether these changes contribute to the reduction of disease activity observed in these patients or merely express the effect of AHSCT remains to be elucidated.

Acknowledgements

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TRABAJO 3.

Evolution of brain-derived neurotrophic factor levels after autologous hematopietic stem cell transplantation in multiple sclerosis. Y. Blanco, A. Saiz, M. Costa, J.F. Torres-Peraza, E. Carreras, J. Alberch, D. Jaraquemada, F. Graus. Neurosc Lett, 380 (2005), 122-126.

En el tercer trabajo se analizó la evolución de los niveles de BDNF, y se estudió la reconstitución inmune y el perfil de secreción de citocinas tras el trasplante.

Mi participación en este trabajo fue la coordinación con el Servicio de Biología Celular de la Universidad de Barcelona y con la Unidad de Inmunología de la UAB del análisis de BDNF y de la determinación del perfil de secreción de citocinas de las muestras, respectivamente. Asimismo, me encargué de recopilar los datos del inmunofenotipaje de las subpoblaciones celulares de sangre periférica realizada de forma asistencial por el Servicio de Hematología. Todos estos datos fueron añadidos a la base de datos de los pacientes trasplantados para su explotación estadística conjunta. Asimismo, participé en la redacción del artículo.

Este trabajo fue presentado como póster en el 20th European Committee for Treatment and Research in Multiple Sclerosis, y como comunicación oral en la LVI Reunión anual de la Sociedad Española de Neurología, ambos durante el año 2004.

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Evolution of brain-derived neurotrophic factor levels after autologous hematopietic stem cell transplantation in multiple sclerosis

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Abstract

A neuroprotective role of inflammation has been suggested based on that immune cells are the main source of brain-derived neurotrophic factor (BDNF). We investigated the 3-year evolution of BDNF levels in serum, CSF and culture supernatant of peripheral blood mononuclear cells (PBMC), unstimulated and stimulated with anti-CD3 and soluble anti-CD28 antibodies, in 14 multiple sclerosis patients who underwent an autologous hematopoietic stem cell transplantation (AHSCT). BDNF levels were correlated with previously reported MRI measures that showed a reduction of T2 lesion load and increased brain atrophy, mainly at first year post-transplant. A significant decrease of serum BDNF levels was seen at 12 months post-transplant. BDNF values were found significantly lower in stimulated but not in unstimulated PBMC supernatants during the follow-up, supporting that AHSCT may induce a down-regulation of BDNF production. The only significant correlation was found between CSF BDNF levels and T2 lesion load before and 1 year after AHSCT, suggesting that BDNF reflects the past and ongoing inflammatory activity and demyelination of these highly active patients. Our study suggests that AHSCT can reduce BDNF levels to values associated with lower activity. This decrease does not seem to correlate with the brain atrophy measures observed in the MRI.

Keywords: Multiple sclerosis; Hematopoietic stem cell transplantation; BDNF; Cytokines; Lymphocyte immunophenotyping

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that have the capability to promote neuronal survival and induction of oligodendrocyte proliferation and myelination [1,9]. Several lines of evidence show that inflammation may have, besides the detrimental effect on the nervous system, a neuroprotective role based on that immune cells are the mayor source of BDNF in neuroinflammatory diseases such as multiple sclerosis (MS) [5–7]. Hence, it has been suggested that the limited efficacy of immunosuppressive therapies in MS may be related to the suppression of the beneficial component of inflammation [6].

Autologous hematopietic stem cell transplantation (AHSCT) is currently evaluated as a potential treatment for severe cases of MS. We previously reported that AHSCT in 14 rapidly evolving MS patients induced in most of them a stabilization of the disease along with a persistent suppression of magnetic resonance imaging (MRI) variables associated with inflammation. Despite the improvement on gadolinium-enhanced lesions and T2 lesion load, increased brain atrophy was observed mainly during the first year post-transplant [14]. The aim of the current study was to assess the effect of AHSCT on BDNF levels and to correlate it with previously reported MRI measures of lesion load and brain atrophy [14]. We also analyzed the immune reconstitution and the evolution of the cytokine profile,

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as an indicative measure of Th1/Th2 balance, after the transplant.

Serum samples and peripheral blood mononuclear cells (PBMC) were taken at baseline, 12, 24 and 36 months, and cerebrospinal fluid (CSF) samples at baseline and 12 months, from 14 MS patients treated with AHSCT. The protocol and clinical and MRI outcomes has been previously reported in detail [3,14]. Briefly, 14 patients, 5 with relapsing-remitting and 9 with secondary progressive MS were included. The median Kurtzke's Expanded Disability Status Scale (EDSS) score increase in the year before AHSCT was 1.0 (range, 0.5–2.0), and the median number of relapses in the year before AHSCT was 3 (range, 1–7). The toxicity results of the protocol (no patient died or had severe systemic complications) have been previously reported in detail [3,14]. After a median follow-up of 36 months, the EDSS remained stable in 9 patients and improved in 3 patients. Two patients presented relapses but none had enhancing lesions in any of the follow-up MRI studies. The mean change in T2 lesion load from baseline to the third year was −20.2% and that of the corpus callosum area was −12.7%; 50% of this reduction was seen in the first year. The decrease of the corpus callosum area was only 0.37% between the first- and second-year MRI and 0.20% between the second- and third-year MRI [14].

Non-parametric tests were used for all comparisons: Friedman and Wilcoxon signed ranks tests for paired patients groups (baseline, 12, 24 and 36 months), and Mann–Whitney *U* test for patients and controls. Pearson's correlation coefficient was used for the correlation analysis. Significance levels were set at 5% ($p < 0.05$).

BDNF levels in serum, CSF, and culture supernatants from peripheral blood mononuclear cells were determined using an ELISA kit following manufacturer's instructions (Promega, Madison, USA) as previously described [11]. Serum samples were diluted 1:50, supernatants 1:5 and CSF undiluted. Serum samples from 17 healthy controls were included in the analysis. BDNF release levels were measured in the supernatants from PBMC stimulated with anti-CD3 and soluble anti-CD28 antibodies for 72 h (stimulation that involve T-cell receptor-mediated activation) as used to evaluate the cytokine profile (see the following). Before AHSCT, mean BDNF serum levels were higher than in controls ($p = 0.024$). At 12 months after AHSCT a significant decrease was observed ($p = 0.048$), reaching values similar to controls throughout the follow-up (Fig. 1). In the same way, an almost significant decrease of the mean CSF BDNF levels was observed at 12 months after AHCST as compared with basal values (22.9 ± 17.9 versus 9.3 ± 4.9 pg/ml, $p = 0.066$) in the nine patients with available CSF. Of note, at 12 months all patients had the same oligoclonal IgG bands pattern identified in the baseline CSF [3], and only in one out of four the IgG index persisted elevated (>0.7).

Because the source of BDNF in serum includes other non-immune cells [13,17], we analyzed the BDNF production by PBMC upon stimulation that mimics a physiologic mode of T-cell activation [12]. A significant decrease of mean levels of

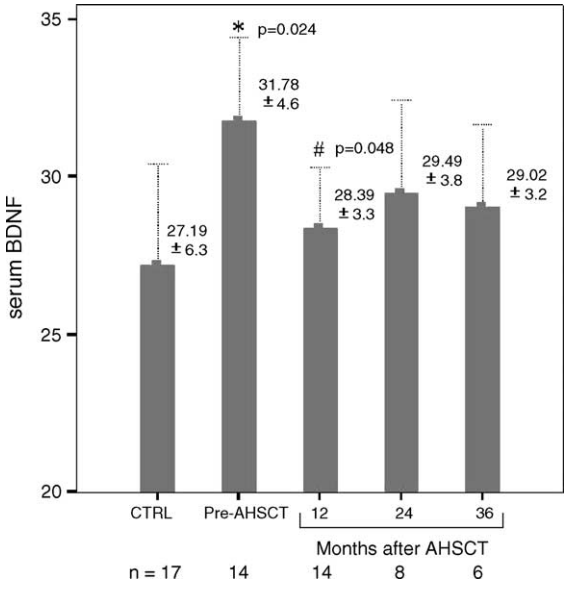

Fig. 1. Evolution of BDNF serum levels (ng/ml) along the 3-year follow-up after autologous haematopoietic stem cell transplantation. Results are expressed as mean ± S.D. Error bars represents 95% CI of the mean. (*) Significant *p*-value compared with controls. (#) Significant *p*-value compared with baseline. CTRL: controls. AHSCT: autologous hematopoietic stem cell transplantation; *n*: number of individuals analyzed at each point.

BDNF in the supernatants of stimulated PBMC was seen at 12 months (238.9 ± 194.6 pg/ml, $p = 0.021$) and 24 months (246.4 ± 207.4 pg/ml, $p = 0.028$) and a trend at 36 months (179.9 ± 176.7 pg/ml, $p = 0.057$) after AHSCT as compared with baseline (411.5 ± 366.1 pg/ml). However, no significant differences were found in the supernatants of unstimulated PBMC (data not shown) suggesting that AHSCT induces a down-regulation of BDNF production.

According to the view that BDNF is produced by immune cells [7], the decrease in BDNF levels found after transplant is not an unexpected finding considering that AHSCT involves high-dose immunosuppression. To our knowledge, this is the first report showing longitudinal data of BDNF evolution after an immunosuppressive treatment. Our patients had a highly active disease profile before transplant despite being under standard therapies for MS. In this setting, an increased baseline levels is expected according to a previous study in relapsing-remitting MS patients that showed higher BDNF levels in serum, CSF and supernatants of PBMC in active periods of the disease (relapses or with enhancing lesions on MRI) compared with those detected in periods without disease activity [15].

Correlation analysis between BDNF data and MRI variables were negative except for a positive correlation between CSF BDNF levels and T2 lesion load before ($r = 0.65$, $p = 0.015$) (Fig. 2) and one year after AHSCT ($r = 0.725$, $p = 0.027$).

The T2 lesion load is, in part, a measure of past and ongoing inflammatory activity and demyelination [10]. Active demyelination MS plaques harbor numerous BDNF positive

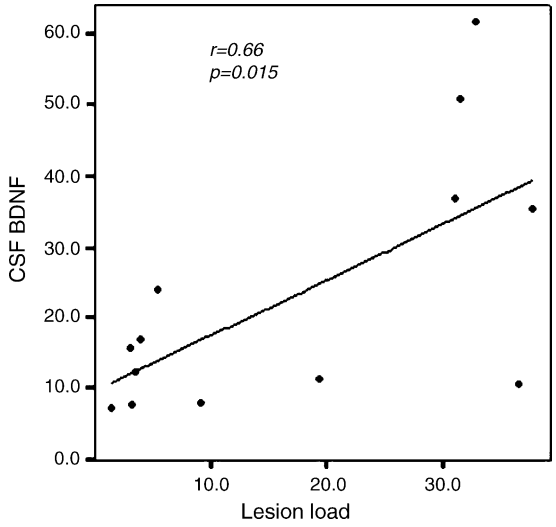

Fig. 2. Correlation between CSF BDNF levels (pg/ml) and MRI T2 lesion load (cm^3) before autologous hematopoietic stem cell transplantation; *r*: Pearson's correlation coefficient.

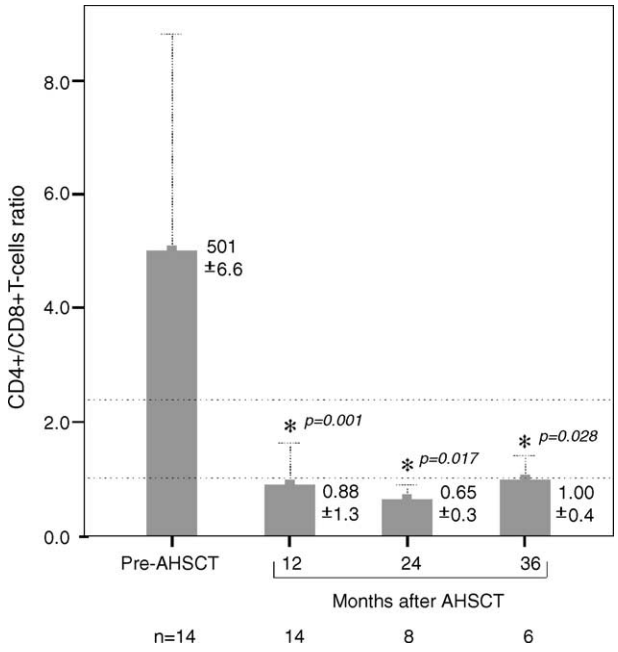

Fig. 3. Evolution of the $CD4^+/CD8^+$ lymphocytes ratio along the 3-year follow-up after autologous hematopoietic stem cell transplantation. Results are expressed as mean ± S.D. Error bars represents 95% CI of the mean. (*) Significant *p*-values compared with baseline. Discontinous lines refer to the 25 percentile and 75 percentile in healthy individuals; *n*: number of patients analyzed at each point.

inflammatory cells and astrocytes compared with chronic inactive plaques [7,16]. Hence, the positive correlation between BDNF levels and T2 lesion load in the MRI is not surprising. The absent correlation of the T2 lesion load with serum BDNF is probably explained by the fact that in CSF, but not in serum, BDNF levels are related to the brain parenchyma and reflect processes ongoing in this compartment, whereas in the peripheryl the source of BDNF levels is more widespread [7,13]. It is noteworthy that none of the CSF samples included showed disrupted blood–brain barrier (BBB) (albumin index <0.9), so a passive transfer of serum BDNF to the CSF should not be expected. Although we cannot ensure that CSF BDNF levels unambiguously reflect locally produced BDNF levels.

The question about the potential detrimental effect of decreasing BDNF levels on the clinical evolution is difficult to answer considering that the primary goal of our clinical trial was to evaluate the feasibility of the procedure. However, a neurological deterioration was not observed in the majority of the patients, and we did not find a correlation between any measure of BDNF and brain atrophy, measured by reduction in the area of the corpus callosum, either before or at 12 months after AHSCT.

BDNF can be secreted by almost cell types of the human immune system, including $CD4^+$ and $CD8^+$ T cells, B cells and monocytes [7]. To know the immune reconstitution following AHSCT is important for understanding the observed effect on BDNF production. Therefore, fresh peripheral blood was analyzed using a FAC-Scalibur [Becton Dickinson Immunocytometry System (BDSI), San José, CA, USA], as previously reported [3]. Monoclonal antibodies to the following surface markers were used: CD3, CD8, CD4, CD19, HLA-DR, CD62-L, CD45RA, CD45RO (all from BD Biosciences, San Jose, CA, USA). A significant decrease in $CD4^+$ T cells counts was seen at 12, 24, and 36 months after AHSCT ($p = 0.002$, $p = 0.036$, $p = 0.046$, respectively). The $CD4^+/CD8^+$ ratio decreased significantly after transplant ($p = 0.001$, $p = 0.017$, $p = 0.028$, respectively) and remained inverted throughout 3 years follow-up (Fig. 3). The same results were seen in the five patients analyzed at 4 years (data not shown). In the early (3–6 months) period post-transplant most of the $CD4^+$ T cells were exclusively $CD45RO^+$ (memory) T cells and after the first year and during the follow-up the predominant T cells were $CD45RA^+$ (naïve) T cells (data not shown). Finally, B-cells and NK-cells remained at normal levels after the third month [3]. Theses results provide evidence that AHSCT induce a profound and long-lasting immunosuppression. Whether the decrease of BDNF levels is related to the sustained depression of $CD4^+$ T cells, considering that BDNF mRNA levels in $CD4^+$ T cells is approximately two-fold increase compared to the other cell populations of PBMC [7], deserves more detailed studies on BDNF production by specific T cells. However, the observed reduction in BDNF secretion upon stimulation and the absence of changes when PBMC were not stimulated would favor a down-regulation of BDNF production after AHSCT.

We next determined whether AHSCT might have immunomodulatory properties, besides the well-established immunosuppressive effects. Thus, we analyzed the cytokine profile secreted in the supernatant of PBMC that had been isolated by Ficoll (Pharmacia, Uppsala, Sweden) density centrifugation and stored in liquid nitrogen in 90% FCS–10% DMSO. IL-2, TNF-α and INF-γ, proinflammatory cytokines as markers of Th1 phenotpye, and IL-4, IL-5 and IL-10,

Table 1
Secreted cytokines in the supernatant from peripheral blood mononuclear cells (PBMC) before the autologous haematopoietic stem cell transplantation (pre-AHSCT) and 12, 24 and 36 months after under two conditions: unstimulated and stimulated with anti-CD3 and soluble anti-CD28 antibodies (anti-CD3/-CD28)

PBMC cytokines		Pre-AHSCT	12 months post-AHSCT	24 months post-AHSCT	36 months post-AHSCT
TNF-α	Unstimulated	540 ± 748.8	415.8 ± 402.8	556.2 ± 603.5	732.2 ± 1436.4
	Anti-CD3/-CD28	597.5 ± 517.2	422.8 ± 468.4	591.4 ± 472.6	524.6 ± 473.8
INF-γ	Unstimulated	23.7 ± 23.2	15.5 ± 12.4	23.6 ± 28.6	30.8 ± 43.5
	Anti-CD3/-CD28	6685.9 ± 6976.6	5281.4 ± 5688.5	5024.8 ± 3599.9	5951.1 ± 7462.1
IL-2	Unstimulated	3.1 ± 2.3	3.7 ± 2.9	2.6 ± 2.0	2.5 ± 1.4
	Anti-CD3/-CD28	149.8 ± 174.1	37.1* ± 46.1	48.3* ± 44.1	54.2* ± 46.5
IL-4	Unstimulated	6.4 ± 5.5	5.6 ± 5.4	6.4 ± 6.6	1.8 ± 2.4
	Anti-CD3/-CD28	18.0 ± 16.4	18.5 ± 21.1	27.4 ± 25.9	21.5 ± 21.9
IL-5	Unstimulated	2.0 ± 0.9	1.9 ± 1.1	1.9 ± 1.2	1.3 ± 1.2
	Anti-CD3/-CD28	104.7 ± 158.7	325.8 ± 571.8	475.9 ± 971.1	90.7 ± 129.2
IL-10	Unstimulated	26.5 ± 37.6	19.4 ± 31.4	30.5 ± 41.1	29.5 ± 55.3
	Anti-CD3/-CD28	160.8 ± 164.3	112.7 ± 192.1	172.1 ± 215.1	157.8 ± 215.3
INF-γ/IL-4	Unstimulated	9.2 ± 1.8	2.7 ± 0.9	3.1 ± 2.3	10.7 ± 7.2
	Anti-CD3/-CD28	1137.9 ± 2723.3	424.9* ± 554.4	246.0* ± 280.3	219.5* ± 188.4
IL-2/IL-4	Unstimulated	0.8 ± 0.8	1.0 ± 0.8	0.7 ± 1.0	1.6 ± 0.9
	Anti-CD3/-CD28	8.0 ± 6.9	2.0* ± 1.5	2.7* ± 2.4	3.5* ± 3.2

Data are expressed as mean ± S.D. in pg/ml.
* p-value <0.05 compared with pre-AHSCT.

anti-inflammatory cytokines as markers of Th2 phenotype were measured. PBMC were cultured for 4 h either alone or for 48 h with plastic-bound anti-CD3 antibody (1:100 dilution of OKT3 hybridoma supernatant) and soluble anti-CD28 antibody (1:40 dilution of 152-2E10 hybridoma supernatant, a kind gift of Dr. R. Vilella) (anti-CD3/-CD28 Ab). Cytokine production was detected with the Human Th1/Th2 Cytokine Cytometric Bead Array Kit (CBA, BD Pharmingen) as previously reported [4]. Briefly, 25 μl of each sample supernatant or the provided Standard cytokines was mixed with 25 μl of mixed capture beads and 25 μl of human PE detection reagent, consisting of a mixture of PE-conjugated antibodies against the human cytokines. After incubation, samples were analysed in a FACS calibur flow cytometer (BD Biosciences) using CBA software (BD Pharmingen). No significant changes were seen after AHSCT compared with baseline for any of the cytokines analyzed, except for IL-2 production under stimulation (Table 1). However, a significant decrease in the IL-2/IL-4 ratio after anti-CD3/-CD28 Ab stimulation was seen at 12, 24 and 36 months after AHSCT compared with that found at baseline ($p = 0.006$, $p = 0.008$, and $p = 0.05$, respectively). The same results were found for INF-γ/IL-4 ratio ($p = 0.047$, $p = 0.039$, $p = 0.048$, respectively) (Table 1). Because both ratios reflect the Th1/Th2 ratio, AHSCT seems to modulate the Th1/Th2 balance with a relative shift toward the anti-inflammatory Th2 profile response. The observed correlation between pre-transplant $CD4^+/CD8^+$ ratio and the Th1/Th2 cytokine profile, either INF-γ/IL-4 or IL-2/IL-4 ratios ($r = 0.9$, $p = 0.0001$ and $r = 0.7$, $p = 0.007$, respectively; corrected p-values by absolute number of $CD4^+$ and $CD8^+$ T cells), support the view that the immunosuppressive effect of AHSCT might be amplified by an immunomodulatory effect on the immune system. Whether the influence of AHSCT on cytokine production is related to the reduction of disease activity observed in these patients or contributes to reduced tissue damage is presently unknown. In fact, we previously reported a significant decrease of the MMP-9 mRNA expression levels after transplant that correlated with the number of $CD4^+$ T cells [2], and MMP-9 activity has also been considered an important step involved in MS disease activity [8]. The aforementioned limitations of the present study prevent any unambiguous statement on the role of AHSCT in the evolution of non-inflammatory mechanisms implicated in MS.

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TRABAJO 4.

No association of the Val66Met polymorphism of brain derived neurotrophic factor (BDNF) to multiple sclerosis. Y. Blanco, M. Gómez-Choco, J.L. Arostegui, B. Casanova, J.E. Martínez-Rodríguez, I. Boscá, E. Munteis, J. Yagüe, F. Graus, A. Saiz. Neurosc Lett. PMID: 16356643

Por último, en el cuarto trabajo se estudió si el polimorfismo Val66Met del gen del BDNF confería susceptibilidad a padecer EM o influía en el curso evolutivo de la misma. También evaluamos la influencia del polimorfismo en la capacidad de secreción de BDNF por las células inmunes en los pacientes sometidos al TAPH.

Mi participación en este trabajo fue calcular el tamaño de muestra necesario y coordinar con los Servicios de Neurología del hospital La Fe de Valencia y el Hospital del Mar de Barcelona la colaboración el envío de las muestras de DNA. Una vez realizado el genotipado por el Servicio de Inmunología del Hospital Clínic fui la encargada del análisis de los resultados, de la creación de la base de datos clínicos de todos los pacientes analizados, y del estudio de la asociación entre ellos. Asimismo, participé en la redacción del artículo.

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No association of the Val66Met polymorphism of brain-derived neurotrophic factor (BDNF) to multiple sclerosis

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Abstract

Brain-derived neurotrophic factor (BDNF), a neurotrophin produced by neurons and immune cells, promotes neuronal survival and repair during development and after CNS injury. The BDNF-Val66Met polymorphism is functional and induces abnormal intracellular trafficking and decreased BDNF release. Therefore, we investigated the impact of the BDNF-Val66Met polymorphism on the susceptibility and clinical course in a case–control study of 224 multiple sclerosis (MS) Spanish patients and 177 healthy controls. We found no evidence for association to susceptibility or severity of the disease in our population. Moreover, we did not observe, in a subgroup of 12 MS patients, that the methionine substitution at position 66 in the prodomain had negative impact in the capacity to produce BDNF by peripheral blood mononuclear cells (PBMC).

Keywords: Brain-derived neurotrophic factor; BDNF-Val66Met polymorphism; Multiple sclerosis; Genetic susceptibility

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS), with both inflammatory and neurodegenerative components. Although the mechanisms underlying both processes remain unclear, genetic and environmental factors seem to contribute to the etiology and the course of the disease [5]. Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family, which promotes neuronal survival and repair during development and after CNS injury [1]. Thus, BDNF is immunolocalized in inflammatory cells in active MS lesions and immune cells are the main source of BDNF, supporting the concept that inflammation may have a neuroprotective role in MS [9,10,16,18].

A frequent single nucleotide polymorphism in the exon 5 of the human *BDNF* gene (GenBank accession no. NM170735) determines a valine to methionine substitution at amino acid position 66 of the prodomain of BDNF. This change, Val66Met, has been associated with abnormal intracellular trafficking and decreased BDNF release in an activity-dependent manner, and with deleterious influence in several neuropsychiatric diseases [6–8,12,17].

In the present study, we analyzed the effect of the BDNF-Val66Met polymorphism on the susceptibility and the clinical course of the disease in a Spanish MS population. Furthermore, we assessed the impact of these genotypes in the production of BDNF by immune cells.

In a cross-sectional manner, we collected clinical and genetic data of 224 unrelated MS patients of Spanish origin, diagnosed by standard criteria [3], recruited from the outpatient Service of Neurology of three centers, and followed according to standardised protocol. One hundred and forty of them were previously described in a study that analysed the influence of NOS2A polymorphism to MS [3]. The demographic and clinical data collected were: age, gender, type of MS, age at onset, disease duration, disease severity according to the expanded disability status scale (EDSS) score, annualized relapse rate, time between first and second relapse, time to reach an EDSS score of 4.0 and

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Table 1
Demographic and clinical characteristics of the MS patients included in the study

Type of MS, *n* (%):	
Relapsing–remitting	138 (61.6)
Secondary progressive	66 (29.5)
Primary progressive	20 (8.9)
Age mean (S.D.) (year)	42.8 (12.2)
Gender (W/M)	1.73
Age at onset mean (S.D.) (year)	31.7 (10.5)
Disease duration mean (S.D.) (year)	11.3 (8.9)
EDSS median (range)	3.2 (0–9.0)
Annualised relapse rate mean (S.D.)	0.8 (0.6)
Time between first and second relapse median, mo	15
Time to reach mean (S.D.) (year):	
EDSS of 4.0	8.6 (6.3)
EDSS of 6.0	13.5 (4.8)
Time to secondary progressive mean (S.D.) (year)	13.4 (5.8)
Progression index mean (S.D.)	0.45 (0.4)
MSSS mean (S.D.)	4.6 (2.7)

EDSS: Kurtzke's expanded disability severity score; MSSS: multiple sclerosis severity score; S.D.: standard deviation.

6.0, and time to develop secondary progressive MS. The rate of accumulation of neurological disability was expressed by the progression index (PI = EDSS/duration [years]) and the multiple sclerosis severity score (MSSS) [15]. The main clinical characteristics of the patients included are shown in Table 1.

One hundred and seventy-seven age- and sex-matched unrelated healthy blood donors from the same geographical area and ethnicity and of Spanish origin were included as controls. Informed consent was obtained from all patients and the Ethical Committee of the participant centers approved the study.

Genomic DNA from whole blood samples from patients and controls was isolated using QIAmp DNA Blood Mini Kit (QIAgen, Germany). Allelic variants at codon 66 (SNP# rs6265) of *BDNF* gene were genotyped using TaqMan® SNP Genotyping Assay (PE Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Control samples of each genotype were included in each run.

Patients and controls were in Hardy–Weinberg equilibrium (MS $\chi^2 = 0.14$, 2df, $p = 0.92$; and controls $\chi^2 = 2.23$, 2df, $p = 0.33$). As shown in Table 2, the alleles and genotypes distribution frequencies of the BDNF-Val66Met polymorphism of the MS patients were not different from those of the control population examined by using chi-square test and the Fisher's exact test. The heterozygosity was 0.37 in MS patients and 0.36 in controls. No differences were found in the allelic and genotypic frequencies among the three participant hospitals.

Neither allele nor genotype was associated with the demographic or clinical variables analysed. Thus, the genotype distribution frequencies were similar in the different clinical form of disease, and between genders. Kruskal–Wallis test did not identify differences in baseline EDSS or PI. By using ANOVA test, a difference among the three genotypes was not found for age, age at onset, disease duration, annualized relapse rate, time between first and second relapse, and MSSS. Moreover, the time to achieve an EDSS of 4.0 and 6.0, and the time to develop a secondary progressive MS was studied by using Kaplan–Meier curves, and after log rank test comparison between genotypes no differences were found. In a model of logistic regression, disease duration was associated with severe disease outcome, analysed by MSSS (OR = 1.1 per year, 95% CI 1.06–1.15, $p < 0.0001$). After correction for this variable, BDNF-Val66Met polymorphism remained without association with disability outcome measured by PI or MSSS.

We previously reported BDNF levels in serum and culture supernatant of peripheral blood mononuclear cells in 14 rapidly evolving MS patients who underwent autologous hematopoietic stem cell transplantation [2]. Here, in an exploratory study, we analyzed the genotype of this homogeneous group of patients to evaluate its influence in the BDNF production. Nine of the 12 analyzed patients were Val/Val, and 3 Val/Met heterozygous. Baseline serum levels were not different between both groups (31.46 ng/ml versus 30.5 ng/ml, respectively, $p = 0.64$). By using Mann–Whitney test, a non-significant increased level was found in the supernatant of unstimulated PBMC of the Val/Met genotype patients (905.13 pg/ml versus 623.45 pg/ml, $p = 0.2$). After PBMC stimulation with anti-CD3 and soluble anti-CD28 antibodies (stimulation that mimics a physiological mode of T-cell activation), the BDNF production of Val/Met patients was 4.43-fold higher than that of Val/Val homozygous patients ($p = 0.017$). Taken together, our data suggests that the methionine substitution at position 66 in the prodomain has no negative impact on the capacity to produce BDNF by immune cells, considering the

Table 2
Allele and genotype frequencies of the BDNF-Val66Met polymorphism in MS patients and healthy controls

Val66Met polymorphism	Controls, *n* (%)	MS, *n* (%)	OR[a]	CI (95%)	*P*-value
Alleles (2*n*)					
Val	282 (79.7)	344 (76.8)	0.84	0.60–1.18	0.33
Met	72 (20.3)	104 (23.2)	1.18	0.84–1.66	0.33
Total	354	448			
Genotypes (*n*)					
Val/Val	109 (61.6)	131 (58.5)	0.88	0.59–1.31	0.53
Val/Met	64 (36.2)	82 (36.6)	1.02	0.68–1.54	0.92
Met/Met	4 (2.2)	11 (4.9)	2.23	0.70–7.13	0.17
Total	177	224			

[a] Odds ratio (OR) and 95% (CI) confidence interval were calculated by logistic regression.

limitation of the small sample size and the absence of Met/Met homozygous cases in this cohort of MS patients.

At the time we were analyzing our data, a study has shown no evidence of association between susceptibility or clinical course of MS and BDNF-Val66Met polymorphism in a UK MS population [11]. Our study confirms this negative result in our population, but also provides a possible explanation based on the functional effect of this polymorphism in the BDNF production by peripheral blood mononuclear cells.

The genotype distribution in our population is similar to those reported in other Spanish samples and European and American populations [4,7,11,14]. Therefore, the lack of association we found does not seem to be related to a specific genetic background of our population.

Although it is well known the negative effect of BDNF-Met 66 in regulated BDNF secretion from neuronal cells, reducing the amount of BDNF released in an activity-dependent manner, this effect has not been demonstrated in other cell populations such as endothelial cells [6]. Moreover, in vivo analysis on effects of BDNF genotype by using proton magnetic resonance spectroscopic imaging showed lower hippocampal levels of *N*-acetyl-aspartate in Val/Met heterozygous compared to Val/Val subjects, but no effect of BDNF genotype in other brain regions [13]. In spite of the limitations of our study on the functional effect of BDNF genotype in immune cells, all these data would suggest that the BDNF-Val66Met polymorphism is related to a selective impairment. In any case, our study suggests that this functional polymorphism is not influencing the clinical course of the disease.

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V. DISCUSIÓN GENERAL

La EM es una enfermedad desmielinizante de base autoimmune mediada por células T activadas que penetran en el sistema nervioso y son capaces de iniciar una cascada de fenómenos inmunológicos que llevan a la lesión del complejo oligodendrocito-mielina. Si bien, el pronóstico individual de la enfermedad es difícil de predecir, de forma global un 50% de los pacientes pasan a una forma progresiva de la enfermedad tras 10-15 años de evolución, y además, en algunos pacientes la evolución clínica es muy agresiva causando un grado importante de discapacidad neurológica a corto plazo, a pesar de los tratamientos disponibles en la actualidad.

La ausencia de terapias eficaces ha llevado al ensayo de nuevas opciones terapéuticas como el TAPH en base a los resultados de eficacia derivados la experimentación animal, y de casos aislados de humanos con una enfermedad hematológica maligna coexistente. Además, los avances alcanzados en la última década en las medidas de soporte de los pacientes neutropénicos y el empleo de progenitores, y de factores de crecimiento hematopoyéticos, han disminuido la mortalidad relacionada con el TAPH a niveles aceptables para su indicación en pacientes con enfermedades de curso clínico muy invalidante. Todo ello ha facilitado que el TAPH esté siendo evaluado actualmente en diferentes centros como terapia experimental, si bien bajo criterios de selección y protocolos de actuación heterogéneos.

Así, en el año 1998 se inició en nuestro centro un ensayo clínico prospectivo fase I-II para la aplicación del TAPH en casos agresivos de EM resistente a la terapia convencional, con el objetivo principal de evaluar la viabilidad del procedimiento en una nueva indicación. Siguiendo las recomendaciones de la conferencia de consenso de Milán,[73] un total de 15 pacientes fueron inicialmente

incluidos entre Abril de 1998 y Abril de 2001, y 14 de ellos (9 en forma SP y 5 RR) fueron finalmente trasplantados. El protocolo incluía la evaluación clínica y radiológica basal y postrasplante, así como la recogida de muestras de sangre periférica para la evaluación futura de marcadores biológicos de actividad inflamatoria en un intento de profundizar en el mecanismo de acción del TAPH.

En el **trabajo 1** analizamos la evolución clínica y radiológica de 14 pacientes con EM sometidos a un TAPH en nuestro centro. Tras una mediana de seguimiento de 3 años se demuestra que el ***TAPH es un procedimiento seguro y viable, que provoca un notable descenso de la actividad inflamatoria de la enfermedad, y que parece enlentecer la progresión de la discapacidad en pacientes con un curso clínico agresivo.***

La mortalidad del procedimiento fue del 0% y ningún paciente presentó toxicidad sistémica grave. Encontramos a su vez una toxicidad neurológica del 25 %, en su mayor parte reversible, algo menor a lo descrito en otras series.[71] Estos datos son inferiores a los observados en otros protocolos que muestran una mortalidad relacionada con el procedimiento de alrededor del 6 %, probablemente debido a la aplicación de criterios de selección más estrictos en lo que se refiere a la edad de inclusión (<40 años) y la puntuación de la EDSS basal (<6.5), y al uso de regímenes de acondicionamiento menos agresivos en nuestro caso.[71,74-77] Nuestro estudio demuestra que el procedimiento del TAPH con una depleción intensa de células T es viable y presenta una toxicidad aceptable.

La eficacia del TAPH en la EM es difícil de evaluar teniendo en cuenta las características propias de la enfermedad y el número relativamente bajo de pacientes incluidos. En la reunión de consenso de Milán de 1998 sobre la aplicación del TAPH

en la EM se definió el trasplante como eficaz si el fracaso del tratamiento (progresión de la discapacidad) a 3 años no era superior al 20%.[73] Tras una mediana de seguimiento de nuestros pacientes de 36 meses la probabilidad actuarial de supervivencia libre de progresión de la enfermedad a 3 años fue 85,7%, dentro del límite marcado de eficacia, a pesar de la definición más estricta de progresión considerando como tal cualquier incremento en la EDSS independientemente del valor de la EDSS basal. El dato clínico más llamativo fue el gran descenso, un 80%, en el número de brotes durante el seguimiento, con un 85,7% de los pacientes libres de brotes tras el trasplante. Sin embargo, a través de un análisis secundario de eficacia se observó que la probabilidad actuarial de supervivencia libre de actividad de la enfermedad a 3 años fue tan solo del 46,4%, un resultado parecido al que describen el resto de series de la literatura. Por lo tanto, parece claro que si bien el trasplante tiene una alta probabilidad de frenar o cambiar el curso progresivo de la EM agresiva, y en especial, de disminuir la actividad inflamatoria medida clínicamente, no es capaz de curar definitivamente la enfermedad.

La evaluación de la eficacia radiológica del estudio incluía, a diferencia de otras series, información a largo plazo, no sólo de la evolución de parámetros inflamatorios sino de medidas de atrofia cerebral.[74,76,79] Tras una mediana de seguimiento de 3 años observamos, como dato más característico y homogéneo, la supresión mantenida de las lesiones captantes de gadolinio a partir del primer mes postrasplante, en línea con lo descrito por otros grupos con un seguimiento inferior. Este efecto antinflamatorio del TAPH se confirmó también en el análisis cuantitativo de la carga lesional tras observar un descenso medio de la misma a 3 años del 20,2%, claramente superior al descrito en ensayos clínicos con pacientes con EM RR

o SP tratados con INF-β o acetato de glatiramero.[80-82] Un 50 % de esta reducción se produjo durante el primer año post-trasplante, en especial en los 3 primeros meses, un 35% en el segundo año y se observó una tendencia a la estabilización a lo largo del tercer año.

El análisis de las medidas de atrofia cerebral evaluada través del área del cuerpo calloso evidenció una reducción media a 3 años del 12,7%. La mayor parte de la atrofia tuvo lugar a lo largo del primer año post-trasplante, al igual que sucedía con la carga lesional. Tras un análisis de correlación entre ambos parámetros observamos una tendencia hacia la asociación entre la reducción de la carga lesional y el incremento en las medidas de atrofia cerebral. Ello sugiere, que más que tratarse de una atrofia real, sería la resolución del edema y la inflamación la responsable de desenmascarar la atrofia previamente existente. Por lo tanto, resulta difícil el análisis tanto del efecto real degenerativo del TAPH como su efecto sobre la progresión de la atrofia cerebral asociada a la enfermedad, teniendo en cuenta además que no disponemos de datos de dicha progresión previa al procedimiento. Si bien, parece que esto sería así al menos durante el primer año, pues al comparar el área del cuerpo calloso del segundo año con la del primer año la reducción fue de un 0,37%, y de tan sólo un 0,2% a lo largo del tercer año. Se trata de unas cifras muy inferiores a las descritas en estudios de pacientes con EMRR muy poco discapacitados que formaban parte de grupos placebo o que fueron tratados con interferón-β, que es del 5% anual, lo que sugeriría que el TAPH podría tener algún efecto de enlentecimiento de la atrofia cerebral asociada a la enfermedad.[80,81,83]

En conclusión, los hallazgos en RM demuestran que el TAPH aplicado en pacientes con EM de curso agresivo produce un notable descenso de la actividad

inflamatoria, como demuestran la supresión de las lesiones captantes de gadolinio y el descenso de la carga lesional, apoyando la evolución clínica favorable descrita.

El **trabajo 2** muestra que el ***TAPH consigue normalizar los niveles séricos de MMP-9 y su actividad proteolítica a través de un efecto específico en las células inmunes.***

En este trabajo realizamos el análisis de la MMP-9 a la vista del claro efecto del TAPH sobre medidas inflamatorias en RM, en especial sobre la captación de gadolinio, y dada la implicación fisiopatológica de la MMP-9 en la disrupción de la BHE. Encontramos, que los pacientes tenían en comparación con el grupo control, niveles séricos basales de MMP-9 superiores, e inferiores de TIMP-1, y en consecuencia una actividad proteolítica, expresada en forma de cociente MMP-9/TIMP-1, superior. Un resultado esperable teniendo en cuenta el perfil intenso de actividad de la enfermedad de estos pacientes. Asimismo, en un análisis a posteriori encontramos una correlación (r=0,64) significativa entre los niveles de expresión de MMP-9 basal y la discapacidad neurológica medida por el Multiple Sclerosis Severity Score,[78] lo que reflejaría que la mayor parte de la discapacidad se ha adquirido como resultado de la gran actividad inflamatoria mantenida más que de un proceso lentamente degenerativo.

El TAPH descendió los niveles de MMP-9 y el cociente MMP-9/TIMP-1 a niveles similares a los de los controles sanos durante los 3 años postrasplante evaluados, todo ello asociado a la regulación a la baja en la expresión linfocitaria de MMP-9 y de la ratio de expresión MMP-9/TIMP-1, lo que confirma un efecto específico y mantenido a lo largo del seguimiento en las células inmunes. Además, la correlación entre los niveles de expresión de MMP-9 y el número de células

$CD4^+$, indicaría que posiblemente el efecto del TAPH medido a través de la MMP-9 traduciría sobretodo un efecto inmunomodulador sobre esta subpoblación celular inmune. Estos resultados apoyarían la implicación de la MMP-9 en la formación de las lesiones de EM, ya que éste descenso favorable en su actividad proteolítica se produce en el contexto de la supresión de la captación de gadolinio, la ausencia de nuevas lesiones captantes de gadolinio y de la reducción de la carga lesional en RM.

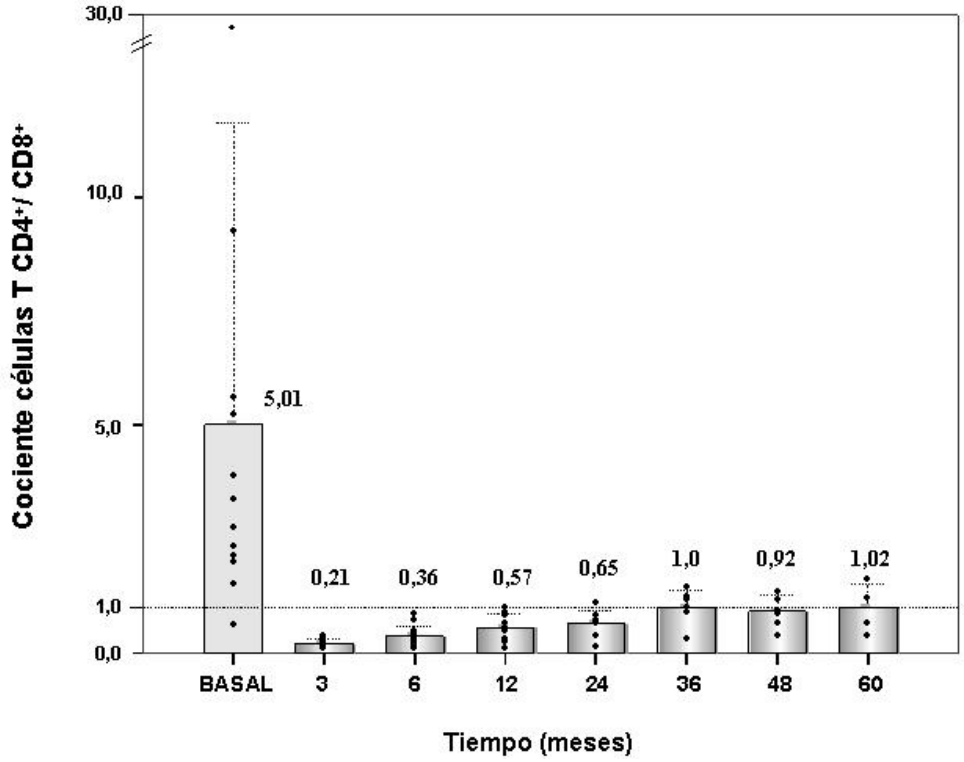
El **trabajo 3** muestra que el ***TAPH reduce los niveles de BDNF a valores asociados a una menor actividad inflamatoria en ausencia de un efecto desfavorable sobre la atrofia cerebral. Asimismo, el TAPH produce una profunda y duradera inmunosupresión con un efecto inmunomodulador añadido.***

Como era de esperar, dada la relación de BDNF con el proceso inflamatorio encontramos que en situación basal los pacientes, un grupo especialmente activo, presentaban niveles superiores al grupo control, y el TAPH fue capaz de descenderlos hasta cifras similares a las de la población sana control. Adicionalmente, en 9 de los 14 pacientes con LCR disponible se observó, en línea con lo anterior, un descenso significativo de los niveles de BDNF al año postrasplante. La regulación a la baja en la producción de BDNF observada en el análisis de secreción por las células inmunes circulantes demostró que el TAPH induce también un efecto específico inmunomodulador a este nivel. El análisis de correlación entre el BDNF y los parámetros de RM, no evidenció que el descenso del BDNF, en el contexto del efecto antinflamatorio del trasplante, tuviera una repercusión desfavorable en las medidas de atrofia cerebral. Sin embargo, sí mostró una correlación positiva entre el BDNF en LCR y las medidas de carga lesional, sugiriendo que el BDNF a este nivel reflejaría la actividad local acumulada

inflamatoria y de desmielinización de la enfermedad. Por lo tanto, es posible que la asociación preferente del BDNF con los parámetros inflamatorios enmascare algún posible efecto protector de esta neurotrofina sobre la atrofia cerebral.

Nuestro trabajo acerca de la reconstitución inmune postrasplante en pacientes con EM aporta una información a mayor plazo que lo descrito en la literatura.[71,76,84,85] El dato característico de dicha reconstitución es el descenso mantenido en la cifra de células $CD4^+$, en especial de la subpoblación *naïve*, debido a su lenta generación tímica en el adulto. Durante los 3 primeros años postrasplante la media de linfocitos $CD4^+$ fue significativamente inferior a la media pre-trasplante, y siempre situada por debajo del percentil 25 de los valores normales de referencia. Los datos disponibles en la actualidad de 5 pacientes seguidos a 5 años confirman el descenso significativo a largo plazo. Asimismo, la rápida recuperación de los linfocitos $CD8^+$, que se mantienen en cifras significativamente superiores a las basales durante los 3 primeros años postrasplante, conduce a una inversión significativa y mantenida de la ratio de células $CD4^+/CD8^+$ (análisis actual en el que 5 pacientes han llegado a 5 años de seguimiento inmunofenotípico) (Ver Figura).

Estos datos reflejarían la inmunodepresión profunda y duradera inducida por el trasplante, que justificaría la mejoría de las variables inflamatorias clínicas y en RM, y en especial ayudaría a comprender la supresión precoz de las lesiones captantes de gadolinio y el descenso preferencial de la carga lesional a lo largo del primer año de evolución.



También era interesante conocer si el efecto favorable del trasplante se debía, no sólo a un efecto inmunodepresor intenso, sino a propiedades inmunomoduladoras asociadas sobre la red de citocinas. Para ello, analizamos el perfil de secreción de citocinas por las células mononucleares de sangre periférica y encontramos un descenso significativo en los 3 primeros años postrasplante de la producción de IL-2, citocina Th1, y del cociente IL-2/IL-4 e INF-gamma/IL-4, como reflejo del balance Th1/Th2. Esto indicaría que el TAPH es capaz de modular la secreción de citocinas con un efecto neto global de desviación de la respuesta inmune hacia un perfil antinflamatorio. Además, la intensa correlación entre el cociente de células $CD4^+/C8^+$ y el perfil Th1/Th2 previo al trasplante sugiere que el efecto inmunodepresor del TAPH se ve potenciado por el efecto inmunomodulador sobre el sistema inmune.

El **trabajo 4** muestra que el ***polimorfismo Val66Met del gen de BDNF no incrementa el riesgo de padecer EM, ni condiciona su curso evolutivo***. Para ello analizamos el polimorfismo Val66Met en 224 pacientes de EM y en 177 controles sanos de nuestra población. No encontramos diferencias en las frecuencias alélicas ni genotípicas entre casos y controles, ni asociación con ninguna de las características clínicas analizadas, por lo que nuestro estudio confirmaría los resultados negativos sobre una población anglosajona recientemente publicados.[86]

Entre los casos analizados incluimos a los pacientes sometidos al TAPH (12 de ellos con DNA disponible) con el objetivo de evaluar la influencia del dicho polimorfismo en la producción linfocitaria de BDNF determinada en el trabajo 3. Así, no encontramos diferencia en los valores de BDNF sérico entre los 12 casos homocigotos Val/Val y los 3 heterocigotos Val/Met. Puesto que el BDNF deriva en gran parte de la secreción plaquetaria no es un buen marcador del efecto sobre las células inmunes. Por ello, analizamos la secreción por las células inmunes tras estimulación y observamos que en los pacientes Val/Met ésta fue 4,43 veces superior a la de los pacientes homocigotos Val/Val. Ello sugiere que la sustitución de una metionina por una valina en el prodominio del BDNF no compromete su secreción en las células mononucleares de sangre periférica, a diferencia de lo que se ha observado que ocurre a nivel neuronal.[32]

VI. CONCLUSIONES

C1. El TAPH aplicado a la EM provoca un descenso de la actividad inflamatoria de la enfermedad, tal y como muestran la franca reducción de la tasa anual de brotes, así como el descenso de la carga lesional y la práctica desaparición de las lesiones captantes de contraste en resonancia cerebral. Si bien, el TAPH no es capaz de curar la enfermedad, sí parece cambiar el curso agresivo de la misma retrasando la progresión de la discapacidad.

C2. De forma paralela a la respuesta clínica y radiológica, el TAPH modifica favorablemente los diferentes marcadores de actividad inflamatoria analizados, pudiendo explicar en parte su mecanismo de acción:

C2.a. El TAPH **c**omporta un descenso en los niveles séricos de la MMP-9 y en su actividad proteolítica medida a través de la ratio MMP-9/TIMP-1 (enzima/inhibidor tisular), así como una regulación a la baja de su expresión en los linfocitos de sangre periférica reflejando un efecto modulador específico en las células inmunes que podrían explicar la reducción de la actividad de la enfermedad observada en estos pacientes.

C2.b. El TAPH provoca un descenso en los niveles de BDNF a valores asociados con menor actividad inflamatoria, si bien, este descenso no parece influir negativamente en las medidas de atrofia cerebral observadas en resonancia. Los niveles de BDNF en LCR podrían reflejar la actividad inflamatoria y desmielinización acumuladas de la enfermedad.

C2.c. La reconstitución inmune postrasplante se caracteriza por una inmunodepresión prolongada, como reflejan el descenso mantenido en la cifra de células T $CD4^+$ y la inversión del cociente de células T $CD4^+/CD8^+$. El TAPH

comporta, además, un efecto inmunomodulador con una desviación de la respuesta inmune hacia un perfil Th2 antinflamatorio. Así el efecto inmunodepresor se ve potenciado por un efecto inmunomodulador como muestra la correlación entre el cociente de células T $CD4^+/CD8^+$ y el perfil Th1/Th2.

C3. El polimorfismo funcional Val66Met del gen del BDNF no confiere susceptibilidad a padecer EM en nuestra población, ni influye en el curso evolutivo de la enfermedad. Tampoco parece influir negativamente en la capacidad de secreción de BDNF en las células del sistema inmune, a diferencia de lo que ocurre en las neuronas.

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