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Tesis Doctoral

Marcadores de angiogénesis, inflamación y coagulación en pacientes con glioblastoma

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CERTIFICAN:
Que la presente Tesis Doctoral titulada: Marcadores de angiogénesis, inflamación y coagulación en pacientes con glioblastoma, ha sido realizada favorablemente por D. Gaspar Reynés Muntaner, Licenciado en Medicina por la Universidad de Valencia, bajo nuestra dirección en el Servicio de Oncología del Hospital Universitario y Politécnico La Fe y en el Instituto de Investigación Sanitaria La Fe.

Para que conste, en cumplimiento de la legislación, firmamos el presente certificado.

Valencia, a 24 de julio de 2017

Fdo. Ana Lluch Hernández Fdo. Jaime Font de Mora Saínz Fdo. Vicenta Martínez Sales

A Inma

Mi agradecimiento sincero a las muchas personas que, de manera directa o indirecta, han hecho posible este trabajo.

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A mi querida familia, por todo.

INDICE

ABREVIATURAS

1CCL2/MCP−1	Proteína quimioatrayente de monocitos
7-AAD	7-aminoactinomicina D
ADN	Ácido desoxirribonucleico
ANXA3	Anexina A3
Arg-1	Arginasa 1
ATRX	Síndrome de alfa-talasemia/retraso mental, de tipo sin deleción, ligado al cromosoma X
bFGF	Factor de crecimiento para fibroblastos básico
CBTRUS	*Central Brain Tumor Registry of the United States*
CCL2/MCP−1	Ligando de quimiocinas−2/ Proteína quimioatrayente de monocitos 1
CCL5	Ligando de quimiocinas−5
CCL5/RANTES	Citocina expresada y secretada por el linfocito T normal en función de su grado de activación
CEC	Célula endotelialcirculante
CEP	Célula endotelial progenitora
Cox2	Ciclooxigenasa 2
CRP	Proteína C reactiva
CXCL12/SDF-1α	Ligando de quimiocinas−12/ Factor derivado del estroma-1α
CXCL7	Ligando de quimiocinas−7
CXCL8	Ligando de quimiocinas−8
CXCR4	Receptor de CXCL12
DMT	Dosis máxima tolerada
DRAQ5	Antraquinona 5 rojo oscuro
EGFR	Receptor del factor de crecimiento epidérmico
ERK	Cinasa regulada por señales extracelulares
FITC	Isotiocianato de fluoresceína
FT	Factor tisular
G−MDSC	Células supresoras de estirpe mieloide granulocíticas
GB	Glioblastoma
G-CSF	Factor estimulante de colonias de granulocitos
GM-CSF	Factor estimulante de colonias de granulocitos y monocitos
HIF1α	Factor inducible de hipoxia 1α
HUVEC	Células endoteliales de cordón umbilical
IDH	Isocitratodehidrogenasa
IDO	Indolamina 2,3-dioxigenasa

IL10	Interleucina 10
IL6	Interleucina 6
IL8/CXCL8	Interleucina 8
iNOS	Sintasa inducible del óxido nítrico
M–MDSC	Células supresoras de estirpe mieloide monocíticas
MCA	Membrana corioalantoidea
MDSC	Células supresoras de estirpe mieloide
MGMT	O6-alquilguanin-ADN-metiltransferasa
MMP2	Metaloproteasa de matriz–2
MMP9	Metaloproteasa de matriz–9
MP	Micropartícula
NF1	Gen de la neurofibromatosis 1
NFκB	NFκB, factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas
NPTX2	Pentraxina neuronal 2
PAR	Receptor activado por proteasas
PDGFRA	Factor de crecimiento derivado de plaquetas alfa
PGE2	Prostaglandina E–2
PKC	Proteína kinasa C
PlGF	Factor de crecimiento placentario
PTEN	Homólogo de fosfatasa y tensina
RT	Radioterapia
S100 A8	Proteína de enlace de calcio A8
S100 A9	Proteína de enlace de calcio A9
SG	Supervivencia global
SLP	Supervivencia libre de progresión
SNC	Sistema nervioso central
STAT3	Transductor de señal y activador de la transcripción 3
sVEGFR–1	Receptor 1 soluble del factor de crecimiento endotelial vascular
TAM	Macrófagos asociados al tumor
TERT	Telomerasa transcriptasa inversa
TGFβ	Transforming growth factor β
TIMP-2	Inhibidor tisular de la metaloproteinasa 2
TMZ	Temozolomida
TNF	Factor de necrosis tumoral

TNF-α	Factor de necrosis tumoral alfa
TPA	Activador tisular del plasminógeno
Treg	Linfocito T regulador
TSP-1	Trombospondina-1
UEA–1	*Ulex europaeus*
VEGF	Factor de crecimiento endotelial vascular
VEGFR	Receptor del factor de crecimiento endotelial vascular

1. INTRODUCCIÓN

1.1 Epidemiología del glioblastoma

El glioblastoma (GB) es el tumor cerebral maligno más frecuente y es, además, el de peor pronóstico. En España, la tasa estandarizada por edad de los tumores del sistema nervioso central (SNC) es de 7,7 casos por 100.000 habitantes y año [1]. No es sencillo extraer la incidencia del GB debido a diferencias en la inclusión de casos entre los diferentes registros. Según datos de *Central Brain Tumor Registry of the United States* (CBTRUS), un registro detallado de tumores del SNC en Estados Unidos, el GB representa el 15% de todos los tumores del SNC (benignos y malignos) con una incidencia de 3,2 casos por 100.000 habitantes y año, con una ratio hombre/mujer de 1,57 [2] (Figura 1). En Europa, algunos registros también han comunicado datos de incidencia del GB, como el de Zurich, que da una tasa de incidencia algo mayor (3,39) [3]. Extrapolando las cifras a nuestro país (46.438.442 habitantes a 1 de enero de 2016), la incidencia anual se puede situar en torno a los 1.500 casos.

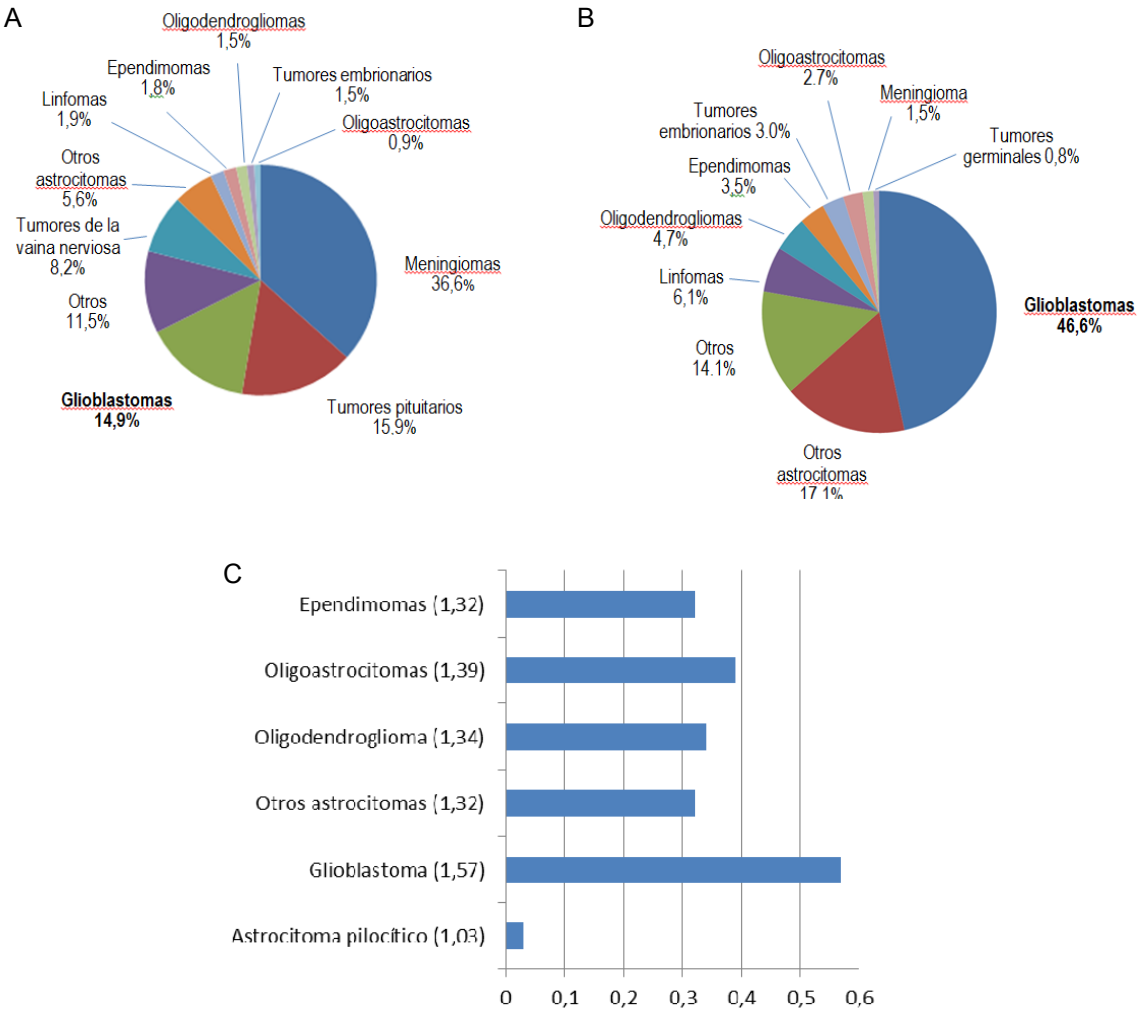

Figura 1. A: Porcentajes de todos los tumores del SNC. B: Porcentajes de los tumores malignos del SNC. CBTRUS, 2009–2013. C: Ratio hombre/mujer de los tumores gliales [2].

1.2 Situación general

Desde que se estableció, hace más de una década, la combinación de radioterapia (RT) y temozolomida (TMZ) como nuevo estándar terapéutico del GB tras la cirugía [4], se han hecho pocos avances en el tratamiento de la enfermedad, cuya mediana de supervivencia supera escasamente el año. La edad y el estado cognitivo influyen de forma importante en la supervivencia [5]. Entre los factores pronóstico biológicos destaca el estado de metilación del promotor del gen O6-alquilguanin-ADN-metiltransferasa (*MGMT)*, que condiciona la expresión de la proteína del mismo nombre en las células tumorales. MGMT es una enzima capaz de revertir la acción metiladora de los agentes alquilantes y la metilación del gen, que silencia su expresión, confiere mejor pronóstico [6]. La existencia de mutación del gen isocitrato deshidrogenasa (IDH) tipo I o II se asocia igualmente a mejor pronóstico. Clásicamente, los GB se han dividido en: a) GB secundarios, que aparecen por evolución de astrocitomas de menor grado, se dan en personas por debajo de los 55 años y representan alrededor del 10% de todos los GB. b) GB primarios, que aparecen *de novo*, se dan generalmente en personas de más de 55 años y representan alrededor del 90% de los GB. A la luz de los conocimientos sobre las características moleculares de los gliomas, la actual clasificación de la Organización Mundial de la Salud denomina *glioblastomas con mutación de IDH* a los GB secundarios y *glioblastomas con IDH nativo* a los GB primarios [7].

La mitad de los pacientes con GB han recaído a los 7 meses de la cirugía. Sin embargo, no existe un tratamiento estándar para el tratamiento de la recidiva. En los ensayos clínicos que se han llevado a cabo en esta situación, la supervivencia libre de progresión (SLP) se sitúa entre 1 y 5 meses y la supervivencia global (SG) entre 6 y 10 meses aproximadamente [8]. El tratamiento antiangiogénico ha proporcionado resultados modestos en recaída [9,10] y no ha aumentado la SG en primera línea [11,12]. La inmunoterapia, en sus diferentes modalidades, ha despertado expectativas en el tratamiento del GB; sin embargo, la respuesta inmunitaria antitumoral produce reacciones inflamatorias que, en el caso de los tumores cerebrales, pueden suponer una importante limitación. Por otra parte, aún no se dispone de resultados decisivos [13]. A todo ello se suma la dificultad de la evaluación de respuesta en estos tumores, que pueden presentar imágenes de pseudoprogresión o de pseudorrespuesta a determinados tratamientos [14]. En los últimos años se ha profundizado en el conocimiento de las características moleculares de los gliomas y del GB en particular, lo que ha permitido establecer subtipos con implicaciones pronósticas pero con escasas repercusiones terapéuticas [15–17]. Por todo ello es necesario buscar nuevas

estrategias terapéuticas y encontrar marcadores de respuesta, a fin de seleccionar con más precisión el tratamiento de cada paciente. La Tabla 1 muestra las alteraciones génicas más características de los subtipos moleculares definidos por Verhaak *et al*. [16]

Tabla 1. Alteraciones génicas características de los subtipos moleculares de glioblastoma.

Subtipo	Alteraciones génicas fundamentales
Proneural	Sobreexpresión de PDGFRA. Mutaciones de IDH1 o IDH2. Mutaciones de P53
Neural	Expresión de marcadores neuronales
Clásico	Amplificación de EGFR. Pérdida de PTEN
Mesenquimal	Pérdida de NF1. Sobreexpresión de genes correspondientes a vías de la superfamilia TNF y vías de NFκB

PDGFRA, factor de crecimiento derivado de plaquetas α; IDH, isocitratodehidrogenasa; EGFR, receptor del factor de crecimiento epidérmico; PTEN, homólogo de fosfatasa y tensina; NF1, gen de la neurofibromatosis 1; TNF, factor de necrosis tumoral; NFκB, factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas.

La Tabla 2 muestra la distribución de las alteraciones génicas más relevantes correspondientes a los GB con IDH nativo y a los GB con IDH mutado (GB primarios y GB secundarios, respectivamente, en la antigua denominación).

Tabla 2. Frecuencia de las alteraciones génicas características de los glioblastomas con IDH nativo y con IDH mutado.

Alteraciones génicas fundamentales	Glioblastoma IDH nativo	Glioblastoma IDH mutado
Mutaciones de P53	23%	74%
Amplificación de EGFR	42%	4%
Mutaciones de PTEN	24%	5%
Mutaciones de TERT	72%	26%
Mutaciones de ATRX	Excepcional	71%

EGFR, receptor del factor de crecimiento epidérmico; PTEN, homólogo de fosfatasa y tensina; TERT, telomerasa transcriptasa inversa; ATRX, síndrome de alfa-talasemia/retraso mental, de tipo sin deleción, ligado al cromosoma X [18].

1.3 Histopatología

El GB es un glioma, es decir, un tumor derivado de la glía, que está formada por astrocitos, oligodendrocitos y ependimocitos. Estudios recientes señalan como origen iniciador del glioma a variantes malignas de las células troncales neurales [19]. El GB se caracteriza por su alta densidad celular y la frecuencia de mitosis. Otra característica histológica propia del GB es la proliferación microvascular, que consiste en una superposición de células endoteliales con actividad mitótica junto con células de musculatura lisa y pericitos, adoptando una forma glomeruloide, expresión de la activa angiogénesis del GB. Estas formaciones se acompañan con frecuencia de trombosis vascular y de necrosis rodeada de células en pseudoempalizada, otra característica histológica del GB [20]. Por otra parte, como ocurre en otros tumores, el GB puede estar infiltrado por una variedad de células inflamatorias, incluyendo macrófagos, células progenitoras mieloides y diferentes tipos de linfocitos (Figura 2).

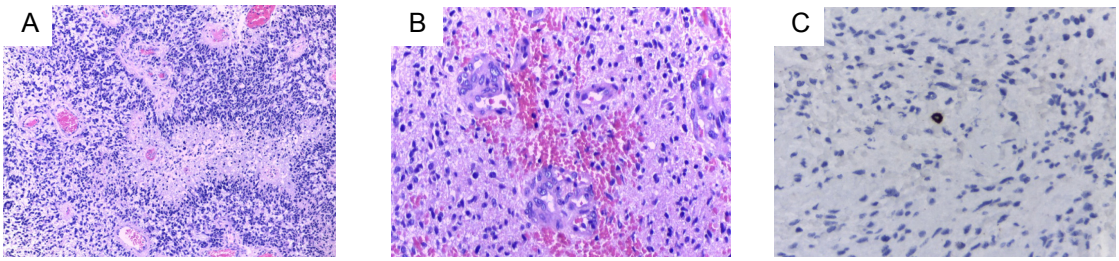

Figura 2. Histología. A: Necrosis con pseudoempalizada. B: Proliferación microvascular. C: Linfocito CD4+. Imágenes cedidas por el Dr. Jaime Ferrer, Servicio de Anatomía Patológica, Hospital Universitari i Politècnic La Fe, Valencia.

1.4 Biopatología

Hanahan y Weinberg, en un trabajo clásico, han señalado diez características distintivas del cáncer [21] (Figura 3). Entre ellas están la inducción de la angiogénesis, los fenómenos inflamatorios y la evitación de la destrucción del tumor por el sistema inmunitario. Los rasgos histológicos del GB que acabamos de describir tienen una relación directa con algunas de estas características.

Figura 3. Características distintivas del cáncer. Modificado de Hanahan D y Weinberg RA [21]

.

La angiogénesis y los fenómenos inflamatorios son rasgos especialmente destacados en el GB que pueden también correlacionarse con biomarcadores en la sangre periférica de los pacientes [22]. Ambos fenómenos se relacionan con la capacidad de evasión del GB a la acción del sistema inmunitario [23]. A su vez, el GB induce un estado protrombótico responsable de una alta incidencia de enfermedad tromboembólica en los pacientes [24], provocado en parte por la sobreexpresión de factor tisular (FT), que a su vez promueve la angiogénesis [25]. Inflamación, inmunosupresión y trombosis son por tanto fenómenos altamente relacionados. A continuación se resumen algunos aspectos de cada uno de ellos en el GB.

1.4.1 Angiogénesis

La hipoxia de las zonas de rápido crecimiento del tumor provoca áreas de necrosis e inhibe la degradación del factor inducible de hipoxia 1α (HIF1α), un factor de transcripción que también puede estar sobreexpresado por la activación oncogénica de las células tumorales. Las células tumorales hipóxicas migran alejándose de las zonas

necróticas centrales y forman las pseudoempalizadas, a la vez que secretan factores proangiogénicos que aumentan la proliferación microvascular observada en el GB [26]. HIF1α induce la expresión de una serie de proteínas relacionadas con la angiogénesis y la migración celular. Entre ellas figuran los factores de crecimiento endotelial vascular (VEGF) A, B, C y D, el factor de crecimiento placentario (PlGF) 1 y 2 y los receptores (VEGFR) 1 a 3, así como el factor derivado del estroma-*1α* (SDF-1α o CXCL12). VEGF-A juega un papel fundamental en el proceso de angiogénesis (proliferación de nuevos vasos a partir de los preexistentes) mediante la unión a sus receptores, VEGFR-1 y VEGFR-2. El VEGFR-1 tiene una función bivalente, actuando como proangiogénico si se homo- o hetero-dimeriza con otro receptor, pero su forma soluble (sVEGFR–1), detectable en el suero, actúa también como inhibidor de la angiogénesis al secuestrar a VEGF-A [27,28]. Además, VEGF–A, CXCL12 y otras moléculas, como el factor estimulante de colonias de granulocitos y monocitos (GM-CSF) y el factor estimulante de colonias de granulocitos (G-CSF) intervienen en la vasculogénesis, es decir, en la incorporación de células progenitoras de la médula ósea al entorno tumoral para la formación de nuevos vasos [29]. Tanto VEGF-A como VEGFR-1 se correlacionan con la densidad vascular y con el grado de malignidad de los gliomas [30]. HIF-1α regula negativamente al inhibidor tisular de la metaloproteinasa 2 (TIMP-2) y positivamente a la metaloproteasas de matriz MMP–2 y MMP–9, favoreciendo no sólo la expansión de los neovasos, sino también la migración de las células tumorales [31].

1.4.2 Inflamación

Se sabe que la inflamación crónica puede conducir al inicio del cáncer a través de un microentorno propicio en el que las células inflamatorias secretan un gran número de citocinas. Sin embargo, incluso en ausencia de inflamación previa, todo tumor desarrolla una reacción inflamatoria mediada en parte por las células tumorales apoptóticas y en parte por la activación en las células tumorales de factores de transcripción, como NFκB, HIF-1α y el transductor de la señal y activador de la transcripción 3 (STAT3). La activación de estas moléculas provoca la secreción de citocinas, las cuales reclutan monocitos y linfocitos y activan los mismos factores de transcripción en éstos y en los fibroblastos, macrófagos y mastocitos de su entorno [27,32,33]. Entre las citocinas secretadas por el tumor están el factor de necrosis tumoral alfa (TNF-α), la interleucina 6 (IL6) y la interleucina 8 (IL8/CXCL8) que, entre otras funciones, pueden dar señales proangiogénicas y tumorigénicas [34,35]. La IL6 producida por las células tumorales,

inflamatorias y endoteliales está sobreexpresada en los gliomas. La amplificación del gen *IL6,* presente en cerca del 50% de los GB se asocia a menor supervivencia [36].

1.4.3 Inmunosupresión

El reclutamiento de células derivadas de la médula ósea no sólo interviene en la vasculogénesis. Las células supresoras de estirpe mieloide (*myeloid–derived suppressor cells*, MDSC), aparte de su acción proangiogénica, ejercen un papel crucial en la evasión inmunitaria del cáncer. Existen dos tipos de MDSCs: monocíticas (M–MDSC) y granulocíticas (G–MDSC). La acumulación y diferenciación de las MDSCs es un proceso complejo que se desarrolla en dos fases: expansión y diferenciación, en las que están implicadas numerosas moléculas. Entre ellas destacan STAT3 (fase de expansión), NFκB, prostaglandina E–2 (PGE2) y ciclooxigenasa 2 (Cox2) (fase de diferenciación) [37,38]. Las MDSC son atraídas al tumor por quimiocinas producidas por las células tumorales, con pequeñas variaciones dependiendo del tipo de tumor. Las principales quimiocinas que intervienen en el reclutamiento de las M–MDSC son la proteína quimioatrayente de monocitos 1 (CCL2/MCP–1) y la citocina expresada y secretada por el linfocito T normal en función de su grado de activación (RANTES/ CCL5) pero también se han descrito otras, como CCL7, CXCL8 y CXCL12. En el reclutamiento de las G–MDSC intervienen varias quimiocinas del tipo CXC, incluyendo CXCL12 [39].

Los principales mecanismos de inmunosupresión que utilizan las MDSC son la producción de arginasa-1 (a través de la vía Cox2→PGE2→E–prostanoide 4) y de óxido nítrico sintasa (M–MDSC) y la producción de especies reactivas de oxígeno (G–MDSC). Otros mecanismos incluyen la depleción de cisteína y la metabolización del triptófano a través de la producción de indolamina 2,3-dioxigenasa (IDO). También producen inmunosupresión a través de su influencia sobre otras células. Inducen la expansión de linfocitos T reguladores (Treg) a través de la secreción de factor de crecimiento transformante β (TGFβ), interleucina 10 (IL–10) e interferón γ. La producción de IL10 por las MDSC, induce la polarización de los macrófagos asociados al tumor (TAM) al tipo M2, con actividad inmunosupresora, angiogénica, antiapoptótica y facilitadora de la invasión tumoral. La producción de IL10 por las MDSC aumenta en un medio inflamatorio. Existe una compleja interrelación entre MDSC, TAM y células tumorales en la que IL6 e IL10 tienen un papel predominante [40,41]. Por otra parte, las M–MDSC pueden diferenciarse a macrófagos M2, por varios mecanismos, como la hipoxia, el HIF-1α, la IL6 o la trombina [39,42].

1.4.4 Coagulación

Las citocinas, la hipoxia y factores de crecimiento producidos por las células tumorales e inflamatorias inducen también la expresión de factor tisular (FT), la molécula iniciadora de la coagulación, y su nivel de expresión se correlaciona con el grado histológico del tumor [43]. La amplificación de EGFR o la expresión de su variante III (EGFRvIII), una mutación que activa constitucionalmente al receptor, inducen la expresión de FT, mientras que el homólogo de fosfatasa y tensina (PTEN) y p53 la inhiben [44]. El FT induce la angiogénesis mediante la activación de factores proangiogénicos como VEGF y la inhibición de trombospondina-1 (TSP-1) [45]. La unión del FT con el factor VII conduce a la generación de trombina. Ésta induce la angiogénesis a través de la escisión de los receptores activados por proteasas (PAR) [46] y provoca el aumento de la capacidad de adhesión y migración de las células endoteliales [47]. La trombina puede también desencadenar la conversión de fibrinógeno en fibrina al tiempo que activa las plaquetas que, al depositarse en la fibrina, forman el coágulo. Las plaquetas activadas liberan factores proangiogénicos favoreciendo la salida de proteínas y provocando la creación de una matriz de fibrina útil para la migración endotelial. La degradación de la fibrina libera factores de crecimiento secuestrados en el trombo [25,48]. La trombina, además, induce la activación del ciclo celular mediante la acción de su receptor PAR1 [49]. El complejo FT/factor VIIa puede también activar señales de migración e invasión a través de PAR2 y de las kinasas reguladas por señales extracelulares 1 y 2 (ERK1 y ERK2). En injertos murinos procedentes de una línea de glioma indolente, la expresión de FT provoca su paso a un estado activo precedido de infiltración por macrófagos y células mieloides y el inicio de angiogénesis. Estos fenómenos se acompañan de cambios en la expresión génica del tumor [50]. El mismo grupo ha estudiado el coaguloma de pacientes con GB, y observaron diferencias entre los subtipos moleculares, siendo el subtipo clásico el que presenta mayor expresión de FT y de PAR1 [51]. Todo ello subraya la importancia de la generación de trombina durante el desarrollo espontáneo del tumor y apoya la propuesta de su análisis como marcador de mal pronóstico. La Figura 4 esquematiza las complejas interacciones que tienen lugar entre células tumorales, coagulación, angiogénesis, inflamación e inmunosupresión.

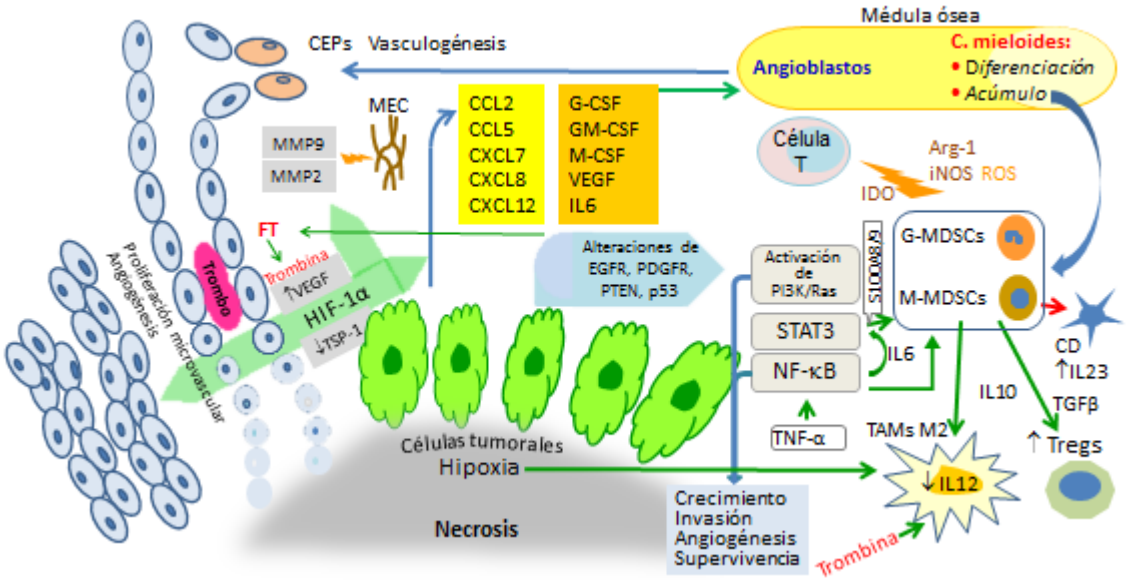

Figura 4. Esquema simplificado de las interrelaciones entre tumor, coagulación, angiogénesis, inflamación e inmunosupresión.

La hipoxia debida al rápido crecimiento del glioblastoma, así como las alteraciones génicas del tumor, provocan una acumulación de HIF1α que promueve la angiogénesis. Las células tumorales liberan quimiocinas que atraen CEP al entorno tumoral, contribuyendo a la vasculogénesis. Otras quimiocinas, con la intervención de STAT3, NFκB y otros factores de transcripción, provocan la diferenciación y acumulación de MDSC en el estroma tumoral. Estas células alteran la función de los linfocitos T citotóxicos mediante la depleción de L-arginina, entre otros mecanismos. También promueven la expansión de Tregs, así como la polarización de los macrófagos al fenotipo M2, en la que interviene también la trombina. La sobreexpresión de FT y la generación de trombina contribuyen a la aparición de trombosis vascular, que produce nuevas áreas de necrosis e hipoxia. El FT y la trombina contribuyen a la angiogénesis, a la proliferación tumoral y a la inmunosupresión.

CEP, células endoteliales progenitoras; MMP9, metaloproteasa de matriz–9; MMP2, metaloproteasa de matriz–2; FT, factor tisular; VEGF, factores de crecimiento endotelial vascular; TSP–1, trombospondina–1; CCL2, ligando de quimiocinas 2; CCL5, ligando de quimiocinas 5, CXCL7, ligando de quimiocinas 7; CXCL8, ligando de quimiocinas 8; CXCL12, ligando de quimiocinas 12; G–CSF, factor estimulante de colonias de granulocitos; GM–CSF, factor estimulante de colonias de granulocitos y macrófagos; M–CSF, factor estimulante de colonias de macrófagos; IL6, interleucina 6; HIF1α, factor inducible de hipoxia 1α; EGFR, receptor del factor de crecimiento epidérmico; PTEN, homólogo de fosfatasa y tensina. NF–kB, factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas. S100 A8 y A9, proteínas de enlace de calcio A8 y A9; Arg-1, arginasa 1; iNOS, sintasa inducible del óxido nítrico; IDO, indolamina 2,3-dioxigenasa; G–MDSC, células monocíticas supresoras de estirpe mieloide; M–MDSC, células granulocíticas supresoras de estirpe mieloide; CD, células dendríticas; IL6,

interleucina 6; IL23, interleucina 23; IL10, interleucina 10; IL12, interleucina 12; TGFβ, factor de crecimiento tumoral β; TAM, macrófagos asociados al tumor; STAT3, transductor de señal y activador de la transcripción 3.

1.5 Marcadores de disfunción endotelial: células endoteliales circulantes

Las células endoteliales circulantes son células maduras que se desprenden del endotelio vascular (CEC) o células progenitoras de endotelio que provienen de la médula ósea (CEP). Se han encontrado niveles aumentados de CEC en pacientes con diversos tipos de cáncer [52] y se ha establecido que los niveles altos de CEC viables son un indicador de progresión tumoral [53]. En un estudio en pacientes con cáncer de pulmón, el número de CEC fue mayor que en controles sanos y se asoció a respuesta al tratamiento y a mayor SLP [54]. En pacientes con cáncer de mama tratadas con quimioterapia metronómica (es decir, administrada de forma continua y a bajas dosis), un aumento significativo de CEC (la mayor parte apoptóticas) en el día 60 se asoció a mayor SLP y SG [55]. En otro ensayo en cáncer de mama, al mismo tipo de quimioterapia se añadió bevacizumab, un anticuerpo monoclonal anti VEGF; niveles basales altos de CEC viables se asociaron a mayor tiempo hasta la progresión y, cuando ésta se producía, se observaba una disminución en los niveles de CEC [56].

1.6 Micropartículas y cáncer

Las microvesículas o micropartículas (MP) son vesículas celulares formadas en los procesos de daño vascular, apoptosis y activación celular [57]. A diferencia de los exosomas, que son vesículas generadas por exocitosis desde el citoplasma, las MP son vesículas de 0,5 a 1 μm de diámetro formadas en la membrana celular, en las que la fosfatidilserina ha pasado de la cara interna a la externa de la membrana, favoreciendo así la formación de complejos enzimáticos de la cascada de coagulación [58]. Pueden tener diferentes orígenes, como plaquetas, eritrocitos, células endoteliales, linfocitos o monocitos [59]. Las MP de diferentes orígenes pueden identificarse usando anticuerpos adecuados. Por ejemplo, las MP de origen endotelial pueden seleccionarse como CD31+, CD41− y las de origen plaquetario, como CD31+, CD41+. Las MP protrombóticas que expresan fosfatidilserina en su superficie pueden seleccionarse como Anexina V+ [60] . Constituyen un reservorio de efectores celulares y ejercen diversas funciones biológicas: dependiendo de su origen pueden tener una acción anti o proangiogénica, proinflamatoria y procoagulante [61].

Las MP tienen un papel importante en el estado procoagulante inducido por muchos tumores, y concretamente por el GB [59,62,63]. Las plaquetas y diferentes tipos de células, entre ellas las tumorales y las endoteliales, liberan pequeñas vesículas como respuesta a estímulos o agresiones externas, o bien como parte del mecanismo de apoptosis [58]. La superficie de estas vesículas circulantes puede albergar moléculas con capacidad procoagulante, como FT y fosfatidilserina, previamente externalizada desde la capa interna de la membrana. Las MP promueven la angiogénesis y la migración de las células hematopoyéticas. Poseen también actividad proinflamatoria, tanto por su capacidad de favorecer la síntesis de mediadores de inflamación como por inducir la secreción de varias citocinas en las células endoteliales y monocitos [58]. El papel de las MP como una entidad procoagulante potencialmente importante en el cáncer está bien documentado [64–66]. En un estudio de nuestro grupo en pacientes con cáncer de pulmón se ha observado una asociación entre niveles elevados de MP y mayor supervivencia global [67]. Sin embargo, en pacientes con cáncer de próstata se asociaron a peor pronóstico [68]. Se ha observado que las MP derivadas de una línea celular de GB contienen enzimas proteolíticas, factores angiogénicos y CXCR4, receptor de CXCL12, que promueven la angiogénesis [69].

1.7 Marcadores en pacientes con glioma

En algunos ensayos en pacientes con GB que incluían diferentes tratamientos antiangiogénicos, varios marcadores han mostrado utilidad como factores pronóstico o como marcadores de respuesta o progresión; entre ellos, los receptores solubles 1 y 2 de VEGF (sVEGFR–1 y sVEGFR–2) y CXCL12/ SDF-1α [70,71].

Se han publicado varios estudios sobre CEC en pacientes con GB. Se ha visto que los niveles preoperatorios de CEP se correlacionan con la vascularización tumoral, medida por técnicas de perfusión [72]. Greenfield *et al.* encontraron un número significativamente mayor de CEP en pacientes con GB que en pacientes con gliomas de bajo grado. En los 26 pacientes con GB, el número de CEP se correlacionaba negativamente con la supervivencia. Además, los niveles plasmáticos de CXCL12/SDF1 estaban significativamente elevados en los pacientes con glioma de alto grado en comparación con controles sanos [73]. Rafat *et al.* estudiaron los niveles de CEP en 12 pacientes con GB, 10 con metástasis cerebrales y 10 controles sanos, encontrando niveles significativamente más altos en los pacientes con GB comparados con los

controles o los pacientes con metástasis [74]. El ensayo clínico BELOB comparó lomustina frente a bevacizumab o la combinación de ambos en GB recidivado. En los pacientes que recibieron lomustina en monoterapia, niveles altos de CEC a las 4 y 6 semanas de iniciar el tratamiento se asociaron a mayor SG [75]. Un estudio reciente encontró una asociación entre niveles altos de CEC CD109+ y mayor SLP y SG en pacientes con GB recidivado tratados con bevacizumab [76].

En dos pequeñas series de pacientes con GB, publicadas sucesivamente por el mismo grupo de trabajo, se analizaron las MP plasmáticas con diámetro ≥300 nm y positividad a anexina V. El número de MP fue menor en los pacientes con respuesta al tratamiento, enfermedad estable o pseudoprogresión que en los pacientes con progresión tumoral [77]. Además, su disminución o aumento durante la radio–quimioterapia con temozolomida se asoció a mejor o peor evolución, respectivamente, en términos de SLP y SG [78]. Se ha observado también que las MP derivadas de células de GB modifican el fenotipo de las células monocíticas en sentido protumoral [79] .

De todo lo expuesto se infiere que existe una estrecha correlación entre los fenómenos de angiogénesis, inflamación y coagulación en el contexto tumoral. Además, estas características son especialmente relevantes en el GB. Tomados conjuntamente, estos datos sugieren que los marcadores de angiogénesis, inflamación y coagulación, así como los niveles de CEC y MP, pueden tener utilidad como indicadores de pronóstico, respuesta o progresión en los pacientes con GB.

1.8 Acción de la quimioterapia metronómica

Varios estudios demuestran que la quimioterapia metronómica mejora el efecto antiangiogénico de diferentes fármacos citotóxicos a través de diferentes mecanismos [80–82]. Uno de estos mecanismos es la regulación negativa de la TSP–1 [83]. La exposición de células endoteliales a dosis bajas y continuas de diferentes fármacos quimioterápicos estimula la expresión de TSP–1, un inhibidor de la migración y la proliferación de células endoteliales [84]. En un estudio, la posología metronómica de ciclofosfamida provocó una caída prolongada de los niveles sanguíneos y la viabilidad de las CEP, mientras que la administración intermitente de las dosis máximas toleradas (DMT) fue seguida de una fuerte movilización de las CEP pocos días después de cada ciclo del fármaco [85].

La TMZ, fármaco fundamental en el tratamiento del GB, es un agente alquilante que introduce un radical metilo en el ADN celular, principalmente en tres localizaciones: N^7-guanina, N^3-adenina y O^6-guanina. La formación de O^6-metilguanina representa sólo el 5% del conjunto de nucleótidos metilados por el fármaco, pero es particularmente responsable de su actividad citotóxica [86]. Se ha demostrado un efecto sinérgico de TMZ con irinotecán cuando se administran en ese orden [87].

Kurzen *et al.* usaron membrana corioalantoidea *in vivo* (MCA) y ensayos de adhesión y proliferación de Matrigel *in vitro* basados en células endoteliales de cordón umbilical (HUVEC) para determinar los efectos antiangiogénicos de diferentes dosis de TMZ. En el ensayo MCA y en el ensayo de Matrigel *in vitro*, la angiogénesis se inhibió con 5 μM de TMZ, mientras que para la inhibición de la adhesión o proliferación dependientes del factor de crecimiento para fibroblastos básico (bFGF), VEGF o forbol-12-miristato-13-acetato (PMA), fueron necesarias concentraciones de TMZ de al menos 25 μM, lo que indica que las vías bFGF, VEGF o proteína kinasa C (PKC) pueden no estar implicadas inicialmente en el efecto antiangiogénico observado. La baja concentración de TMZ que se necesitaría para obtener un efecto antiangiogénico corresponde a las concentraciones plasmáticas logradas mediante una administración oral de 20 mg/m^2 cada 8 horas [88]. Experimentos in vitro han demostrado que la TMZ metronómica inhibe la angiogénesis y regula negativamente la expresión de MGMT en HUVEC [89]. En modelos murinos de glioma resistente a TMZ, el tratamiento metronómico con este fármaco inhibió la angiogénesis y el crecimiento tumoral [90]. En un modelo murino de cáncer de próstata tratado con ciclofosfamida metronómica, se observó que la aparición de resistencia al tratamiento se asociaba a la regulación positiva de genes de acción anticoagulante, como anexina A3 (*ANXA3*) y activador tisular del plasminógeno (*TPA*), y a la regulación negativa de la serpina clase A inhibidora de peptidasas*,* un inhibidor de *TPA*. Los autores conjeturan con que el tumor dificultaría los mecanismos de coagulación para vencer la escasez de nutrientes y de oxígeno provocada por la acción antiangiogénica del tratamiento [91].

Spiro *et al.* plantearon la hipótesis de que la TMZ continua podría agotar la MGMT de las células tumorales en los gliomas, mejorando así el efecto citotóxico del fármaco. Observaron que tras 5 días de tratamiento con TMZ, administrada bien 1 vez o 2 veces al día, la MGMT de los monocitos de sangre periférica disminuyó de forma consistente y con mayor rapidez en los pacientes tratados dos veces al día. Sin embargo, los cambios en MGMT del tumor fueron más variables [92].

Se han publicado varios ensayos clínicos de TMZ metronómica en GB recidivado. Brandes *et al*. utilizaron una dosis de 75 mg/m^2 días 1 a 21 cada 28 días, obteniendo

una SLP a 6 meses del 30% [93]. Perry *et al*. utilizaron TMZ continua a la dosis de 50 mg/m^2/día. La SLP a 6 meses fue del 23,9% en los pacientes con GB, pero con notables variaciones dependiendo del momento de la primera progresión con respecto al período de TMZ adyuvante [94].

1.9 Edema y necrosis tumoral

El edema peritumoral contribuye en gran medida a la sintomatología clínica de los pacientes con GB. El VEGF es un potente mediador de la permeabilidad vascular [95]. Dada la fuerte activación angiogénica del GB, es lógico que la vía VEGF juegue un papel importante en la aparición de edema peritumoral. Se ha demostrado la asociación entre la expresión de VEGF y el grado de edema en gliomas de alto grado, pero en tumores sin aumento significativo de la expresión de VEGF, el grado de edema se correlacionó con la expresión de otros genes proangiogénicos, como pentraxina neuronal 2 (*NPTX2*) [96]. Estudios más recientes han demostrado que las expresiones de VEGF y de STAT3 fosforilado (pSTAT3) en el GB están fuertemente correlacionadas y se asociaron positivamente al grado de extensión del edema peritumoral [97]. En otro estudio, la extensión del edema se asoció a peor SG en tumores con MGMT metilado, pero no en los no metilados [98]. Otro estudio reciente que incluyó 87 pacientes con GB, encontró que la mayor extensión del edema peritumoral y del área de necrosis se asociaba a peor SG [99].

2. HIPÓTESIS Y OBJETIVOS

2.1 HIPÓTESIS

Nuestra hipótesis es que la determinación de los niveles basales de una serie de marcadores circulantes de angiogénesis, inflamación y coagulación, así como los cambios producidos en dichos niveles por el tratamiento estándar de primera línea o por la quimioterapia metronómica en situación de progresión, pueden tener valor pronóstico y predictor de respuesta en los pacientes con glioblastoma.

2.2 OBJETIVOS

2.2.1 Objetivo general

El objetivo general de estos trabajos es la identificación de factores pronóstico relacionados con la angiogénesis, inflamación y coagulación en pacientes con glioblastoma.

2.2.2 Objetivos específicos

Artículo 1

Valoración de la necrosis tumoral, del edema periférico al tumor y de marcadores circulantes de angiogénesis, inflamación y coagulación, de las correlaciones entre ellos y de su posible valor pronóstico en el contexto del tratamiento de primera línea del glioblastoma.

Artículo 2

Valoración de los niveles circulantes de células endoteliales, de micropartículas circulantes y de la actividad procoagulante mediada por micropartículas en pacientes con glioblastoma, así como su valor pronóstico.

Artículo 3

Determinación de la dosis máxima tolerada de irinotecán quincenal en un esquema fijo de temozolomida metronómica administrada tres veces al día.

Artículo 4

Análisis de eficacia y seguridad de un esquema de temozolomida metronómica combinada con irinotecán. Análisis de marcadores circulantes de angiogénesis y coagulación en los pacientes tratados y de su valor pronóstico.

3. RESULTADOS

3.1 Artículo 1

Marcadores circulantes de angiogénesis, inflamación y coagulación en pacientes con glioblastoma.

3.1.1 Características de los pacientes

Se incluyeron 47 pacientes con diagnóstico histológico de glioblastoma y 60 voluntarios sanos. Quince pacientes no llegaron a recibir radioquimioterapia debido a deterioro clínico, hallazgo de otra neoplasia sincrónica o muerte por otras causas. El resto de pacientes se trató de acuerdo con el tratamiento estándar [4]: tras la cirugía, se administró radioterapia hasta un total de 60 Gy en 30 fracciones, 5 días por semana, con TMZ concomitante seguida, 4 semanas después, de TMZ adyuvante. Las características clínicas de los pacientes se describen en la Tabla 3.

Tabla 3. Características clínicas de los 47 pacientes con glioblastoma.

Característica	Valor
Edad (años)	61 ± 12
Sexo: hombre (%)	60
Indice de Karnofsky (%)	
>80	14,0
80-60	77,0
<60	4,5
ND	4,5
Extensión de la cirugía (%)	
Resección completa	42,5
Resección parcial >50%	31,9
Biopsia abierta <50%	8,5
Biopsia estereotáxica	17,0
Tumor residual (%)	
No	29,8
Sí, medible	53,2
Sí, no medible	17,0

La edad se expresa como media ± desviación estándar ; ND, No disponible.

3.1.2 Supervivencia global

La mediana de SG de toda la serie fue de 8,13 meses (IC 95%: 2,9–13,4. Rango: 0,5–30+ meses). La Figura 5 muestra la estimación de la función de supervivencia de Kaplan–Meyer.

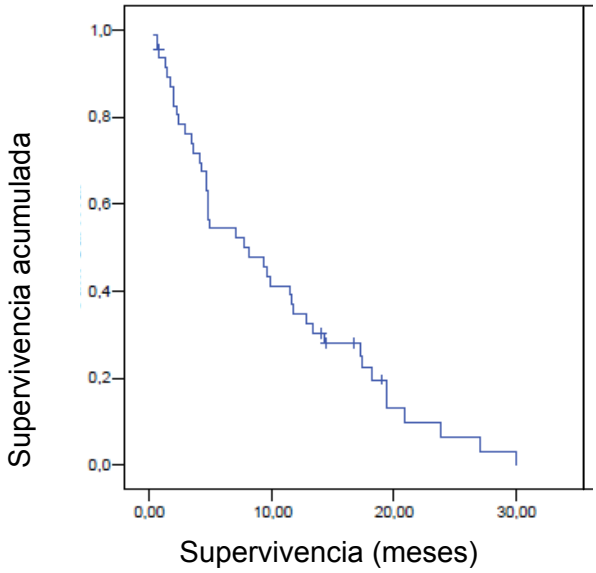

Figura 5. Curva de supervivencia de Kaplan–Meyer.

3.1.3. Supervivencia según las características de la neuroimagen

La presencia de necrosis y la extensión del edema peritumoral se valoró en 45 pacientes. Se observó una asociación estadísticamente significativa entre la existencia de necrosis central en las imágenes de resonancia magnética (RM), en secuencias T1 con gadolinio, y peor SG. La extensión del edema peritumoral se midió en secuencias T2. Se observó una tendencia a la asociación entre la extensión del edema a más de 1 cm desde el margen tumoral y peor SG, que no alcanzó significación estadística. La Tabla 4 muestra la SG de los pacientes de acuerdo con la presencia de edema y necrosis tumoral.

Tabla 4. Análisis de supervivencia de Kaplan–Meyer de acuerdo con las características de la imagen por resonancia magnética.

Variables	Número de pacientes (%)	Mediana de supervivencia (meses)		*Log rank*	*P*
		Sí	No		
Edema	41/ 45 (91)	7,03	20,8	3,05	0,08
Necrosis	31/45 (69)	4,87	13,4	6,53	<0,01

La Figura 6 muestra la estimación de la función de supervivencia de Kaplan–Meyer de acuerdo con la presencia o ausencia de necrosis

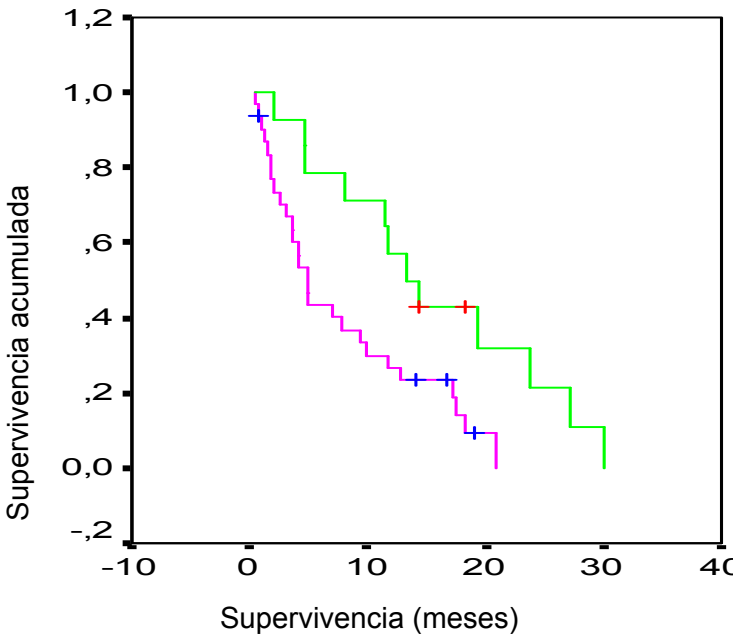

Figura 6. Curvas de supervivencia de los pacientes sin (color verde) y con (color magenta) necrosis tumoral, valorada en la RM en secuencias T1 con contraste.

3.1.4 Marcadores circulantes de coagulación, inflamación y angiogénesis

Se obtuvieron muestras de sangre de 40 pacientes, previas a la cirugía, y de 60 voluntarios sanos. Tras la obtención de plasma y suero se valoraron los niveles de marcadores de angiogénesis (VEGF–A, sVEGFR–1 y TSP-1), de inflamación (proteína C reactiva [CRP], IL6, TNF-α, fibrinógeno [Fg] y ácido siálico [AS]) y de coagulación (generación de trombina [GT], fragmentos 1+2 de la protrombina [F1+2] y FT).

3.1.4.1 Comparación entre pacientes y controles

Entre los marcadores de coagulación, los niveles de GT y F1+2 fueron más altos en los pacientes que en los controles.

Los niveles de todos los marcadores de inflamación estudiados fueron más altos en los pacientes que en los controles.

Entre los marcadores de angiogénesis, los niveles de VEGF–A y sVEGFR–1 fueron más altos en los pacientes que en los controles.

La Tabla 5 muestra los valores de los marcadores estudiados y la significación estadística de la diferencia entre pacientes y controles.

Tabla 5. Marcadores de coagulación, inflamación y angiogénesis en pacientes y controles.

	Pacientes (n = 40)	Controles (n = 60)	*P*
Coagulación			
F1+2 (nmol/L)	0.42 ± 0.50	0.20 ± 0.05	< 0.001
FT (pg/ml)	154 ± 93	153 ± 59	NS
GT (UI/ml)	10.9 ± 3.4	8.7 ± 2.4	< 0.01
Inflamación			
IL-6 (pg/ml)	3.5 ±7.1	0.7 ± 0.4	< 0.01
TNFα (pg/ml)	1.1 ± 0.9	0.6 ± 0.2	< 0.001
Fg (mg/dl)	300 ±156	232 ±31	< 0.01
AS (mg/dl)	71 ±22	55 ±10	< 0.001
CRP (mg/l)	17.0 ± 26.6	1.8 ± 2.7	< 0.001
Angiogénesis			
VEGF–A (pg/ml)	268 ±186	123 ± 64	< 0.001
sVEGFR–1 (pg/ml)	89 ± 29	77 ±17	< 0.05
TSP-1 (µ/ml)	47.2 ±14.1	45.9 ±10.3	NS

Los valores se expresan como media ± desviación estándar.

F1+2, factor de protrombina 1+2; FT, factor tisular; GT, generación endógena de trombina; IL-6, interleucina-6; TNFα, factor de necrosis tumoral alfa; Fg, fibrinógeno; AS, ácido siálico; CRP, proteína C reactiva; VEGF–A, factor de crecimiento endotelial vascular A; sVEGFR–1, receptor 1 soluble del factor de crecimiento endotelial vascular; TSP-1, trombospondina–1. NS, No significativo.

Las correlaciones entre marcadores se muestran en la Tabla 6. Cabe destacar la correlación negativa entre VEGF–A y sVEGFR–1.

Tabla 6. Correlaciones de Spearman de los marcadores de coagulación, inflamación y angiogénesis.

	GT	IL-6	TNFα	Fg	AS	CRP	sVEGFR-1
IL-6	NS	------					
TNFα rho *P*	NS	0.40 <0,02	------				
Fg rho *P*	0,44 <0,01	0,45 <0,01	NS	------			
AS rho *P*	NS	NS	NS	0,63 <0,001	------		
CRP rho *P*	NS	0,65 <0,001	0,56 <0,001	0,763 <0,001	0,60 <0,001	------	
VEGF–A rho *P*	NS	NS	NS	NS	NS	NS	**-0,35** **<0,02**

GT, generación de trombina; IL-6, interleucina-6; TNFα, factor de necrosis tumoral α; Fg, fibrinógeno; AS, ácido siálico; CRP, proteína C reactiva; VEGF–A, factor de crecimiento endotelial vascular A; sVEGFR–1, receptor 1 soluble del factor de crecimiento endotelial vascular; TSP-1, trombospondina 1. NS, no significativo.

No se encontraron asociaciones entre los niveles de los marcadores estudiados y la SLP o la SG de los pacientes. Tampoco se encontraron asociaciones entre los niveles de los marcadores y las características de las imágenes de RM.

3.2 Artículo 2

Células endoteliales y micropartículas circulantes en pacientes con glioblastoma: valor pronóstico.

3.2.1 Características de los pacientes

Se incluyeron 22 pacientes con diagnóstico histológico de GB y 40 controles sanos. Tras la cirugía, los pacientes recibieron el tratamiento estándar con radioterapia y TMZ concomitante y adyuvante. Las características de los pacientes se muestran en la Tabla 7.

Tabla 7. Características clínicas de los pacientes.

Características	Pacientes (n=22)
Edad en años	
Mediana (rango)	62 (41-83)
Sexo	
Hombre (%)	12 (54.5)
Mujer (%)	10 (45.5)
IK	
60-80 (%)	16 (72)
90-100 (%)	6 (27)
Extensión de la cirugía	
Biopsia E	5
Biopsia abierta	3
Resección parcial	12
Resección completa	2

IK: Indice de Karnofsky; Biopsia E, biopsia estereotáxica.

3.2.2 Valores de los marcadores estudiados y sus correlaciones

Las muestras de sangre de los pacientes se extrajeron dentro de las dos semanas previas al inicio de la radioquimioterapia y durante la última semana de dicho tratamiento. En ambos grupos de muestras se determinaron:

– Recuento de CEC
– Recuento de MP
– GT
– Tiempo de coagulación dependiente de fosfolípidos procoagulantes (TCDFP)

Además, se determinó el estado de metilación del promotor de *MGMT* en muestras parafinadas de los tumores.

Los niveles basales medios de CEC y de MP eran significativamente más altos en los pacientes que en los controles ($p<0.001$). En la segunda extracción, los niveles de CEC apenas se modificaron, permaneciendo más elevados que en los controles. Por el contrario, los niveles de MP y la GT disminuyeron de forma significativa, mientras que el TCDFP aumentaba. La Figura 7 muestra una célula endotelial correspondiente a un paciente del estudio.

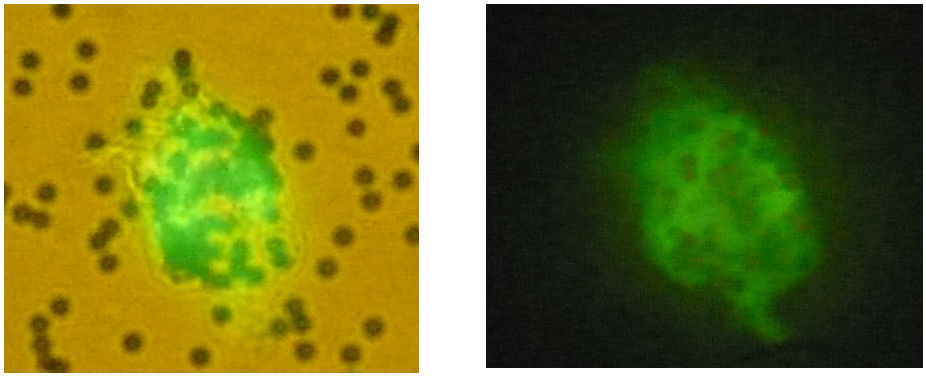

Figura 7. Célula endotelial circulante rodeada de las microesferas magnéticas conjugadas con CD 146 utilizadas para su aislamiento. Las microesferas tienen un diámetro de 4,5 µm.

La Figura 8 esquematiza los niveles de CEC, MP, GT y TCDFP obtenidos en pacientes y controles.

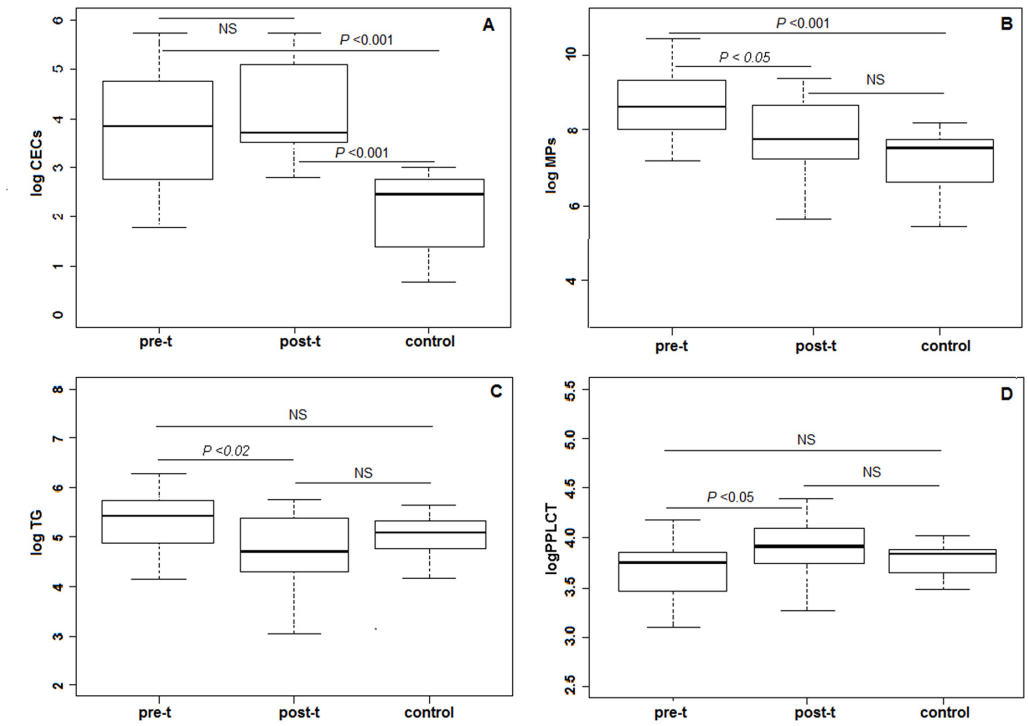

Figura 8. Niveles de biomarcadores pre y post tratamiento en pacientes y controles**.**

A: Células endoteliales circulantes (CEC), B: Micropartículas (MP), C: Generación de trombina (GT); D: Tiempo de coagulación dependiente de fosfolípidos procoagulantes (TCDFP). Se muestran los niveles de los marcadores y su desviación estándar pretratamiento, postratamiento y en controles sanos. Se ha hecho una transformación logarítmica de los datos para normalizar las distribuciones. NS: no significativo.

Se encontraron correlaciones significativas entre GT y MP, pre y postratamiento ($p<0.01$). El TCDFP se correlacionó inversamente con los niveles de MP y con la GT (Tabla 2). Los niveles de plaquetas y de leucocitos disminuyeron de forma significativa tras el tratamiento. El número de plaquetas se correlacionó con la GT, con las MP y con el TCDFP (Tabla 8).

Tabla 8. Correlación bivariante de Spearman entre MP y marcadores de coagulación

		MP	TCDFP
GT	Pretratamiento	0,732**	-0,627*
	Postratamiento	0,741**	
TCDFP	Pretratamiento	-0,858**	
	Postratamiento	NS	

MP, micropartículas circulantes; GT, generación de trombina; TCDFP: tiempo de coagulación dependiente de fosfolípidos procoagulantes. * P<0.05; ** P<0.01. NS, no significativo.

El promotor de *MGMT* estaba metilado en 6 muestras (27%).

3.2.3 Marcadores y resultados clínicos

Un recuento de CEC >20 células/mL (correspondiente al percentil 99 de los controles) se asoció a peor supervivencia (19 vs 72 semanas; Log rank 4,566; P=0,033) (Figura 9).

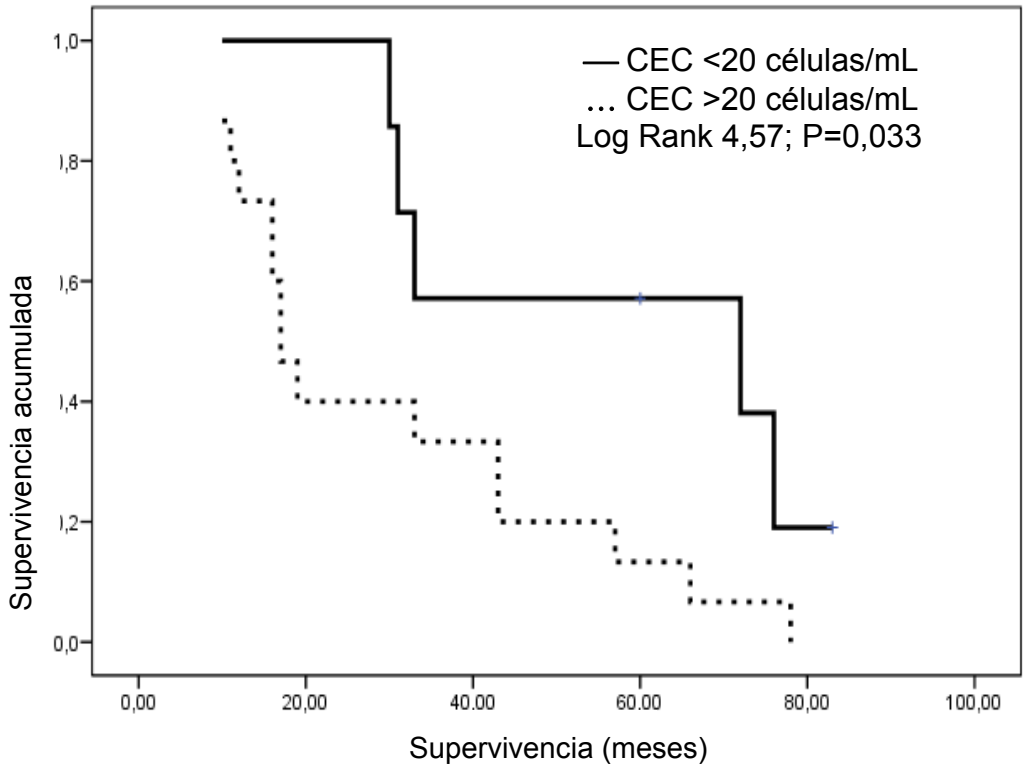

Figura 9. Análisis de supervivencia de Kaplan-Meyer según los niveles basales de CEC.

Curva de supervivencia de acuerdo con los valores de CEC pretratamiento. Los valores se dicotomizaron como mayores o menores del percentil 99% en los controles (CEC=20 células/mL) (Log rank=4,566; P = 0,033). CEC: células endoteliales circulantes.

Los niveles pre o postratamiento de MP, GT y TCDFP no se asociaron de forma significativa a la supervivencia de los pacientes.

La mediana de supervivencia de los pacientes cuyos tumores presentaban metilación del promotor de MGMT fue de 66 semanas (IC 95%: 44,6–87,4), y la de los no metilados de 30 semanas (IC 95%: 14,4–45,5), diferencia estadísticamente significativa (P<0,004). La Figura 10 muestra las curvas de SG de los pacientes con MGMT metilado y no metilado.

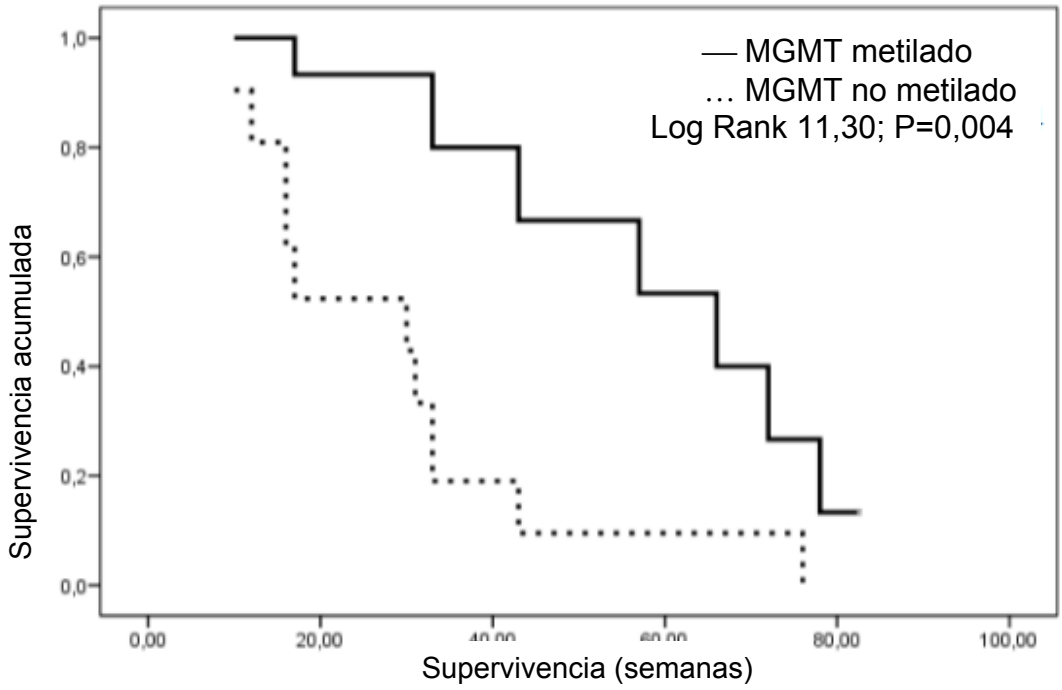

Figura 10. Supervivencia de acuerdo con el estado de metilación de MGMT

3.3 Artículo 3

Ensayo en fase I de irinotecán en combinación con temozolomida metronómica en pacientes con glioblastoma recidivado

3.3.1 Tratamiento

El objetivo de este estudio era determinar la dosis máxima tolerada (DMT) de irinotecán quincenal combinado con TMZ metronómica, de acuerdo con el siguiente esquema (Figura 11).

Días del ciclo de tratamiento													
1	2	3	4	5	6	7	8	9	10	11	12	13	14
○	○	○	○	○	○	○	■	○	○	○	○	○	○
○	○	○	○	○	○	○	▶	○	○	○	○	○	○
○	○	○	○	○	○	○		○	○	○	○	○	○
Días del ciclo de tratamiento													
15	16	17	18	19	20	21	22	23	24	25	26	27	28
○	○	○	○	○	○	○	■	○	○	○	○	○	○
○	○	○	○	○	○	○	▶	○	○	○	○	○	○
○	○	○	○	○	○	○		○	○	○	○	○	○

Figura 11. Esquema del tratamiento.
El tratamiento consistió en ciclos de 28 días repetidos sin pausa entre ellos. "○" indica TMZ, 50 mg/m^2/día, dividida en 3 dosis diarias. "■" indica TMZ, 100 mg/m^2 administrada en una sola dosis. "▶" indica irinotecán, administrado de 3 a 6 horas después de la TMZ, de acuerdo con el nivel de dosis apropiado (ver Tabla 9).

3.3.2 Metodología

Se partió de una dosis de 100 mg/m^2 de irinotecán (nivel 1), planificándose incrementos o decrementos de dicha dosis, de acuerdo con la cantidad de pacientes que presentaran toxicidad limitante de dosis (TLD) en cada nivel (ver Pacientes y Métodos). Los niveles de dosis planificados se muestran en la Tabla 9.

Tabla 9. Niveles de dosis de irinotecán

Nivel	Dosis
-1	85 mg/m^2
-2	70 mg/m^2
1	100 mg/m^2
2	115 mg/m^2
3	130 mg/m^2
4	145 mg/m^2
5	160 mg/m^2

3.3.3 Resultados

3.3.3.1 Características de los pacientes

La Tabla 10 muestra las características de los pacientes incluidos

Tabla 10. Características de los pacientes

Características	Valor
Número total	12
Edad (años)	
Mediana (rango)	58 (44-68)
Sexo	
Hombre	7
Mujer	5
IK (número de pacientes)	
70	4
90	6
100	2
Ciclos previos de TMZ	
Mediana (rango)	8,5 (3-16)
Tiempo desde la cirugía hasta la progresión (meses)	
Mediana (rango)	9,3 (6,8-28,9)

3.3.3.2 Toxicidad

La Tabla 11 muestra las toxicidades observadas en los niveles de dosis de irinotecán 1 y 2.

Tabla 11. Toxicidades observadas durante el período de evaluación

	Nivel 1				Nivel 2				
	Número de pacientes evaluables				Número de pacientes evaluables (número total de pacientes[a])				
Toxicidad	G 1	G 2	G 3	G 4	G 1	G 2	G 3	G 4	G 5
Hematológica									
Anemia	1	-	-	-	2 (4)	-	-	-	-
Linfopenia	-	-	-	-	2 (4)	1	2 (3) 1 (2)	1[b]	-
Neutropenia	1	-	-	-	2 (3)	1	-	1[b]	-
Trombocitopenia	1	-	-	-	1 (2)	-	1[b]	-	-
No hematológica									
Astenia	1	2	-	-	2	- (1)	-	-	-
Estreñimiento	-	-	-	-	1	-	-	-	-
Diarrea	-	-	-	-	1	-	1	-	-
Estomatitis	-	1	-	-	-	-	-	-	-
Neutropenia febril	-	-	-	-	-	-	1	-	-
Vómitos	1	-	-	-	2	- (1)	-	-	-
Neumonía	-	-	-	-	-	-	-	-	1[b]

[a]Tres pacientes no fueron evaluables para toxicidad porque progresaron durante el período de evaluación. En estos casos, un nuevo paciente se incluyó en el mismo nivel de dosis.

[b]Toxicidades limitantes de dosis: paciente 7, neumonía fatal con neutropenia y linfopenia grado 4 y trombocitopenia grado 3.

G, grado de toxicidad (National Cancer Institute Common Toxicity Criteria, versión 3.0).

3.3.3.3 Evaluación de las respuestas objetivas

Al final del período de observación, 9 pacientes eran valorables para respuesta: un paciente alcanzó una respuesta parcial con una duración de 8 meses. Cuatro pacientes presentaron enfermedad estable con una duración de 4, 4, 6 y 6 meses. Cuatro pacientes progresaron en la primera evaluación.

3.3.3.4 Resultado de la escalada de dosis

En el nivel 2 de dosis, 3 de los 6 pacientes evaluables experimentaron TLD. En consecuencia, el nivel 1 (100 mg/m^2 de irinotecán) fue seleccionado para la fase II del estudio.

3.4 Artículo 4

Ensayo clínico en fase II de irinotecán y temozolomida metronómica en pacientes con glioblastoma recidivado

3.4.1 Tratamiento

El tratamiento se estructuró en ciclos de 28 días, sin solución de continuidad entre ellos. Los pacientes recibieron TMZ oral, 50 mg/m2/día dividida en tres dosis, salvo los días 8 y 22 de cada ciclo, en los que se administró una dosis única de 100 mg/m^2/día, entre 3 y 6 horas antes de recibir una infusión intravenosa de irinotecán de 100 mg/m^2. La dosis de irinotecán utilizada es la que se identificó como DMT en el ensayo previo de escalada de dosis.

3.4.2 Resultados

3.4.2.1 Pacientes

Se incluyeron inicialmente 30 pacientes de ocho centros. Tres de ellos eran los que en el estudio previo recibieron la dosis de irinotecán seleccionada para la fase II, es decir, 100 mg/m^2. Tres pacientes fueron excluidos por diferentes razones. La figura 12 muestra el flujo de pacientes en un diagrama CONSORT.

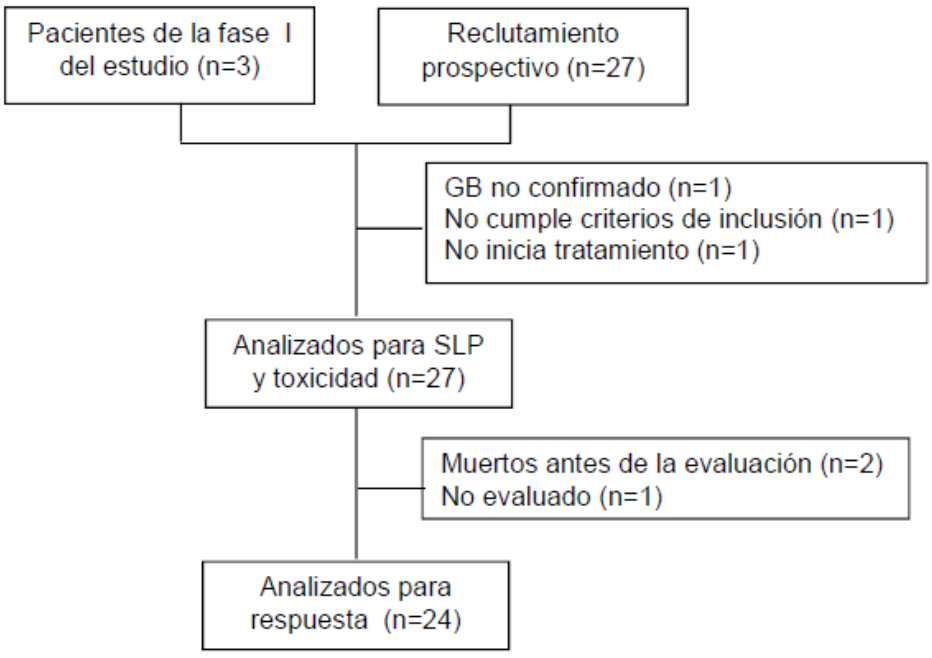

Figura 12. Flujo de pacientes. GB, glioblastoma; SLP, supervivencia libre de progresión.

La Tabla 12 muestra las características de los pacientes incluidos en el estudio.

Tabla 12. Características de los pacientes

Características	Valor
Número total de pacientes	27
Mediana de edad (rango)	56 (42-77)
Hombre	19
Mujer	8
Indice de Barthel	
50-70	4
80-90	9
95-100	10
ND	4
IK	
70	10
80	6
90	8
100	3
TMZ adyuvante: Mediana del número de ciclos (rango)	6 (3-15)
Tiempo hasta la recidiva:	
Mediana de tiempo desde la cirugía en meses (rango)	9,2 (5,5-28,6)
Durante la TMZ adyuvante	18
< 3 meses tras la TMZ adyuvante	2
3 – 6 meses tras la TMZ adyuvante	1
> 6 meses tras la TMZ adyuvante	6

IK, Indice de Karnofsky; TMZ, temozolomida; ND, no hay datos.

3.4.2.2 Eficacia

3.4.2.2.1 Tasa de respuestas objetivas

Un total de 24 pacientes fueron evaluables para respuesta. No se observaron respuestas completas ni parciales. En 9 pacientes se objetivó enfermedad estable, con una mediana de duración de 11,2 semanas (rango: 4,2–35,85 semanas).

3.4.2.2.2 Supervivencia libre de progresión

A los 6 meses, 5 pacientes de los 27 que iniciaron el tratamiento (20,8%) seguían libres de enfermedad. La mediana de la SLP fue de 11,6 semanas (IC 95%: 7,5 – 15,7). (Figura 13).

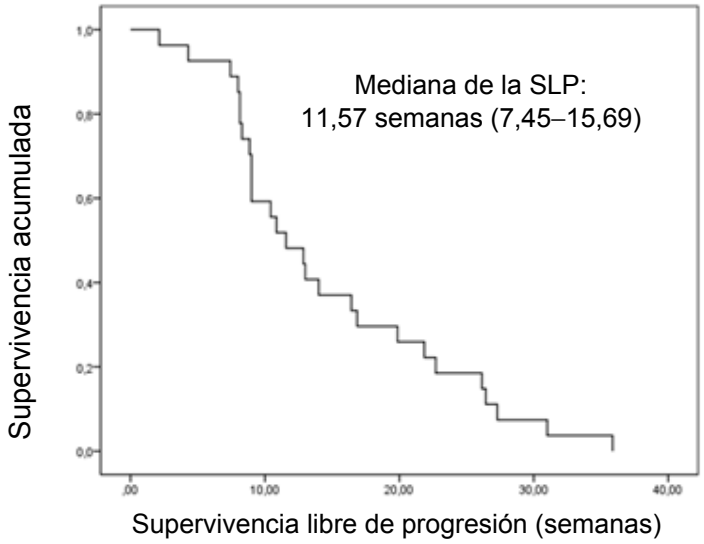

Figura 13. Curva de supervivencia libre de progresión.

No se observaron diferencias en la tasa de respuestas ni en la SLP entre los pacientes que progresaron durante la fase de TMZ adyuvante o después de haberla completado.

3.4.2.2.3 Toxicidad

No se observaron infecciones oportunistas por linfopenia. Un paciente falleció por neumonía el día 16 del primer ciclo. Otro paciente falleció durante el tercer ciclo por un cuadro clínico compatible con tromboembolismo pulmonar. La tabla 13 muestra las toxicidades registradas.

Tabla 13. Toxicidad

Toxicidad	Grado	Grado	Grado	Grado	Grado
	N (%)	N (%)	N (%)	-	N (%)
Hematológica					
Anemia	3 (11,1)	1 (3,7)	-	-	-
Linfopenia	-	3 (11,1)	4 (14,8)	1 (3,7)	-
Neutropenia	1 (3,7)	2 (7,4)	3 (11,1)	-	-
Trombocitopenia	1 (3,7)	1 (3,7)	-	-	-
No hematológica					
Astenia	6 (22,2)	7 (25,9)	1(3,7)	-	-
Estreñimiento	3 (11,1)	2 (7,4)	-	-	-
Diarrea	2 (7,4)	3 (11,1)	1 (3,7)	-	-
Estomatitis	2 (7,4)	-	-	-	-
Neutropenia febril	-	-	1 (3,7)	-	-
Náuseas/vómitos	6 (22,2)	1 (3,7)	3 (11,1)	-	-
Neumonía	-	-	-	-	1 (3,7)
Disnea[a]					1 (3,7)

[a]Un paciente presentó disnea aguda clínicamente compatible con tromboembolismo pulmonar y murió.

3.4.2.2.4 Biomarcadores

Con excepción de la TSP–1, todos los marcadores estudiados presentaban niveles más altos en los pacientes que en los controles (Tabla 14). Sin embargo, ninguno de ellos mostró asociación con los resultados clínicos.

Tabla 14. Niveles plasmáticos de marcadores de angiogénesis y de micropartículas

Marcadores	Pacientes	Controles	*P*
VEGF–A (pg/ml)	234±133	147±50	0,002
sVEGFR–1(pg/ml)	116± 38	72±17	<0,001
TSP-1 (µg/ml)	56±25	46±10	0,1
MP (sucesos/µl)			0,02
GT (nM)	243±125	158±58	<0,01

Los datos se presentan como medias ± SD. VEGF–A, factor de crecimiento endotelial vascular A; sVEGFR–1, receptor 1 soluble del factor de crecimiento endotelial vascular; TSP–1, trombospondina–1; MP, micropartículas; GT, generación de trombina.

4. DISCUSIÓN

Estos trabajos muestran una clara elevación de todos los marcadores de inflamación estudiados en los pacientes con GB frente a los controles sanos. El TNFα se correlacionó positivamente con IL6, lo que resulta lógico ya que TNFα induce la expresión de IL6 a través de la fosforilación de NFκB y de STAT3, entre otros mecanismos [100]. Esta correlación ha sido también encontrada por otros autores en suero de pacientes con GB [22]. La expresión de IL6 en el tejido tumoral no se observa en todos los GB y muestra una tendencia a menor SG [101], mientras que la amplificación del gen IL6 se asocia a menor SG [102]. Los niveles circulantes de IL6 se han asociado a toxicidad, particularmente a astenia, en pacientes con GB tratados con el quelante del VEGF aflibercept [103]. En un estudio reciente se estudiaron 120 proteínas y se identificó un panel de citosinas asociadas a buen y mal pronóstico en gliomas de alto grado, pero la IL6 no estaba entre ellas [104]. En otro estudio de marcadores de inflamación y angiogénesis en pacientes con GB, los niveles plasmáticos de IL6 y de TNFα, entre otros, no se asociaron al pronóstico [22]. Una posibilidad es que IL6 y TNFα tengan un papel más relevante en el entorno tumoral promoviendo, entre otros fenómenos, la inmunosupresión, pero siendo sus niveles circulantes poco significativos. La CRP es una proteína sintetizada por los hepatocitos en respuesta a niveles altos de citosinas [105]. En un estudio reciente, los niveles séricos altos de CRP en pacientes con glioma de alto grado (N=142) y de GB (N=111) se han asociado a peor SG; los autores mencionan nuestro estudio (artículo 1) y especulan que el motivo de que no encontremos dicha asociación puede deberse al menor número de pacientes [106]. En nuestro estudio cabe destacar la fuerte correlación de los niveles de CRP con los niveles de todos los demás marcadores de inflamación estudiados. Estos resultados apoyan la importancia del papel que juega la inflamación en el GB.

Los niveles circulantes de VEGF–A, factor clave en la angiogénesis, no han mostrado valor pronóstico. Tampoco se asoció con la SG en la pequeña serie de 14 pacientes estudiada por Chiorean *et al.* [22] . En nuestro estudio observamos una asociación negativa entre los niveles de VEGF–A y sVEGFR–1. Esta observación apoya el papel del sVEGFR–1 como quelante del VEGF–A, aunque la función de este receptor parece ser más compleja, actuando como inhibidor o activador de la señal angiogénica en función del tipo de dimerización que experimenta [107]. Los niveles de sVEGFR–1 y la razón VEGF–A/VEGFR–1 tampoco han mostrado valor pronóstico en nuestro estudio.

Los niveles circulantes de FT en pacientes y controles no mostraron diferencias significativas. En cáncer de mama se ha demostrado una asociación negativa entre los niveles plasmáticos del inhibidor de la vía del factor tisular (TFPI) y el pronóstico; sin embargo, los niveles de FT no influyeron en la SG de las pacientes [108]. La expresión

de FT se ha asociado al grado tumoral en los gliomas [109,110]. No conocemos datos que asocien niveles circulantes de FT y pronóstico en estos tumores.

En nuestro estudio se observó una asociación entre la existencia de necrosis central y peor supervivencia. El hecho de no encontrar una asociación similar entre edema y supervivencia, como han hecho otros autores [98,99], puede deberse al número de pacientes o a la metodología utilizada. En otros estudios se han encontrado asociaciones del edema con la expresión local de factores de transcripción, factores de crecimiento o citocinas relacionados con la angiogénesis y la inflamación [96,97]. No se han confirmado en nuestro estudio estas asociaciones del edema peritumoral con los niveles circulantes de marcadores como VEGF–A o IL6.

Nuestro trabajo (Artículo 1) mostró una elevación del VEGF–A circulante (y del sVEGFR–1 en menor medida) en pacientes con GB respecto a controles sanos, sin que se observara una asociación de sus niveles con la SG ni con la SLP. Una posible explicación es que, aunque la angiogénesis es una característica típica de los GB, los niveles circulantes de sus marcadores no representan la mayor o menor agresividad del tumor. De hecho, estudios clínicos con un anticuerpo anti VEGF como bevacizumab no ha demostrado una mejora significativa de la SG [11,12]. Por otra parte, es posible que tanto la asociación de los niveles de VEGF circulante con la SG como la eficacia del tratamiento antiangiogénico dependan del subtipo de GB [111]. El estímulo angiogénico y vasculogénico produce un crecimiento caótico de los vasos sanguíneos peritumorales; los niveles de CEC en los pacientes con cáncer pueden reflejar el rápido recambio del endotelio tumoral y correlacionarse con el volumen vascular [112]. El GB es, *a priori*, un tumor idóneo para valorar el significado pronóstico de las CEC dado su fuerte componente angiogénico, manifestado histológicamente por la aparición de vasos anómalos con capas superpuestas de células endoteliales (la llamada proliferación microvascular). Nuestro estudio ha demostrado que niveles altos de CEC antes de iniciar el tratamiento con quimiorradioterapia se asocian a peor SG, lo que sugiere que los GB con mayor actividad angiogénica comportan peor pronóstico. Este hallazgo, junto con la ausencia de asociación entre niveles séricos de VEGF y SG registrada en el artículo 1 de este compendio, parece indicar que las CEC reflejan mejor los efectos reales que el conjunto de la actividad angiogénica aberrante produce en los vasos peritumorales. Hay que recordar que, aunque VEGF y sVEGFR–1 juegan un papel fundamental en este proceso, son sólo parte de una compleja orquestación de factores que actúan en el microentorno tumoral y en el reclutamiento de CEC, CEP y células mieloides supresoras [113]. Otros trabajos en pacientes con GB han encontrado que los niveles basales altos de CEP se asociaban a peor pronóstico [73]. Los niveles de CEC

se han estudiado también en pacientes con GB: Cuppini *et al.* [76] estudiaron varias poblaciones de células endoteliales en pacientes con GB recidivado tratados con quimioterapia sola o con bevacizumab, asociado o no a irinotecán. Las CEC se identificaron como Syto 16 (marcador de ácido nucleico)+, CD45 (marcador leucocitario)–, CD31 (marcador de células hematopoyéticas)+, CD146 (marcador endotelial)+, con la adición de 7-aminoactinomicina D (7-AAD) para distinguir entre células viables y apoptóticas. Los niveles basales de CEC no se asociaron al pronóstico de los pacientes. Sin embargo, cuando se añadió el marcador CD109 +, niveles basales altos (>1er cuartil) de CEC se asociaron a mejor SLP y SG en los pacientes que habían recibido bevacizumab, pero no en los que fueron tratados con quimioterapia sola. Además, los pacientes que recibieron bevacizumab y estaban libres de progresión a los 2 meses, mostraron un descenso significativo de las CEC CD109+. El grupo de diferenciación CD109 corresponde a una proteína de superficie que actúa como correceptor TGFβ inhibiendo la señalización [114]. CD 109 Se expresa en varios tumores sólidos, incluyendo el GB [115]. Además, su expresión es mayor en el endotelio tumoral que en el endotelio normal [116]. Previamente, Calleri *et al.* [56] habían estudiado el valor pronóstico de las CEC DNA+, CD45-, CD31+, CD146+ (sin utilizar CD109) en pacientes con cáncer de mama tratadas con quimioterapia metronómica más bevacizumab, encontrando que niveles basales altos (> 1er cuartil) de CEC se asociaban a mayor SLP. En pacientes con cáncer de colon tratados con bevacizumab asociado a diversos regímenes de quimioterapia, los niveles basales bajos de CEC totales o apoptóticas mostraron una tendencia no significativa a mejor pronóstico en términos de respuesta objetiva. En este estudio las CEC totales se definieron con la combinación CD45–, CD146+, CD34+ y CD133–, y las CEC apoptóticas, con igual firma y Anexina V+ [117]. Batchelor *et al*. [118] estudiaron los niveles de CEC en pacientes con GB tratados con cediranib, un inhibidor de tirosina kinasa pan–VEGFR, y encontraron que niveles altos de CEC seleccionadas mediante los marcadores CD31, CD34, CD45 y CD133 se asociaban a peor pronóstico.

El estudio BELOB [75] comparó lomustina frente a bevacizumab o la combinación de ambos en GB recidivado. Los niveles basales de CEC no tuvieron valor pronóstico, pero niveles altos de CEC a las 4 y 6 semanas de iniciar tratamiento se asociaron a mayor SG en los pacientes que recibieron lomustina en monoterapia. Las CEC aumentaron de forma significativa durante el tratamiento únicamente en los pacientes que recibieron la combinación. En este estudio, las CEC se definieron como CD34+, CD146+, CD45– y DRAQ5+ (antraquinona 5 rojo oscuro, un marcador de ADN) [74]. Los autores mencionan nuestro estudio (artículo 2) para comentar que tampoco observaron

cambios en los niveles de CEC en los pacientes que recibieron quimioterapia. En nuestro estudio, sin embargo, los pacientes recibieron la combinación de quimioterapia con TMZ y radioterapia. Corsini *et al*. [119] encontraron una reducción de CEP en pacientes con gliomas tratados con radioquimioterapia, pero no tenemos datos sobre los cambios en los niveles de CEC en este contexto.

En nuestro estudio las CEC se seleccionaron como CD146+, un marcador endotelial que aparece también en una pequeña fracción de linfocitos T y B. Para descartar la unión de leucocitos a las esferas cargadas con CD146, las células se incubaron con lectina–1 de *Ulex europaeus* (UEA–1), un marcador de endotelio humano. Al no utilizar marcadores de ADN, es posible que se seleccionaran no sólo CEC sino también fragmentos de células endoteliales. En el estudio de Batchelor *et al.* [118] antes mencionado, en el que los niveles basales de CEC tuvieron también valor pronóstico, tampoco se utilizaron marcadores de ADN. Por el contrario, en el estudio BELOB, en el que se incluyó un marcador de ADN, los niveles basales de CEC no se asociaron a SG. Es posible que, al no utilizar este tipo de marcador, se detecte mejor el grado de alteración de la vasculatura tumoral como resultado de la activación angiogénica. Dado que el subtipo mesenquimal de GB es el que presenta mayor grado de angiogénesis [16], podría conjeturarse que los pacientes con niveles altos de CEC tendrían esta firma molecular. En cualquier caso, las múltiples técnicas utilizadas para definir las CEC hacen difícil la comparación entre estudios.

Por lo que se refiere a las MP, los estudios de Koch *et al.* [77] y de Evans *et al.* [78] (publicados sucesivamente por el mismo grupo de trabajo) seleccionaron MP ≥0,3 μm positivas para anexina V conjugada con isotiocianato de fluoresceína (FITC) y encontraron que el número de MP antes de la radioquimioterapia, y su aumento tras dicho tratamiento se asociaban a peor pronóstico. En nuestro trabajo se seleccionaron MP de 0,5–1 μm, igualmente positivas para FITC – anexina V, pero no encontramos asociación con la supervivencia. En el estudio de Evans *et al*. [78], la mitad de los pacientes presentaron un aumento de los niveles de MP y en la otra mitad disminuyeron o permanecieron estables a lo largo del tratamiento. En nuestro estudio se observó una disminución significativa en el recuento de MP tras la radioquimioterapia. No parece probable que la pequeña diferencia en el diámetro de las vesículas seleccionadas (≥0,3 μm frente a 0,5–1 μm) sea un factor relevante. Encontramos una esperable y muy importante correlación positiva entre niveles de MP y GT, y una correlación negativa de ambos parámetros con el TCDFP.

Dado el papel primordial de la angiogénesis en la aparición y mantenimiento del GB, se han ensayado diversos tratamientos antiangiogénicos en situación de

enfermedad progresiva que hasta el momento han tenido resultados modestos [9,10, 120]. En el ensayo clínico GENOM 007 nos planteamos utilizar un esquema terapéutico con las siguientes características: a) una tolerancia aceptable; b) un aumento de la densidad de dosis de TMZ sobre el esquema estándar de 150–200 mg/m^2/día, 5 días cada 4 semanas; c) una administración continua para aprovechar el efecto antiangiogénico de la quimioterapia metronómica [80–82] y su acción depletiva sobre la MGMT [92], y d) la combinación de la TMZ con irinotecán para aprovechar el efecto sinérgico descrito previamente [91]. Se decidió que el irinotecán se administrara cada 2 semanas, como en otros esquemas con este fármaco [9,10]. La dosis de 50 mg/m^2 en una sola toma al día de TMZ de forma continua se había demostrado factible [94], pero los datos disponibles sobre la farmacocinética de la TMZ [121] y sobre la concentración plasmática necesaria para lograr un efecto antiangiogénico [90] nos llevaron a dividir la dosis en 3 tomas diarias. Por otra parte, se decidió dar una dosis única y mayor de TMZ (100 mg/m^2) previa a cada administración de irinotecán para aprovechar mejor la sinergia con dicho fármaco. Este esquema supone una dosis total de 1.500 mg/m^2 cada 4 semanas, ligeramente superior a la obtenida con el esquema clásico (1.250 mg).

Dada la ausencia de precedentes con esta pauta terapéutica, planificamos y llevamos a cabo un ensayo clínico multicéntrico de identificación de la DMT de irinotecán en combinación con la pauta prefijada de TMZ. Dicho ensayo mostró que la combinación era factible y que podía tener actividad en pacientes con GB recidivado. Cabe resaltar que un paciente falleció de neumonía por legionella, probablemente relacionada con la linfopenia que presentó, un efecto adverso a tener en cuenta en los pacientes tratados con TMZ en dosis extendidas [122]. La DMT de irinotecán fue de 100 mg/m^2, lo que coincidió con nuestros cálculos (100 mg/m^2 correspondía al nivel 1 de dosis).

La fase II del estudio incluyó 30 pacientes, de los cuales 3 tuvieron que descartarse por diversas razones (Figura 11). Si bien la SLP a 6 meses fue superior a la de algunos estudios de TMZ extendida en GB recidivado [123,124], excedió a la de otros regímenes utilizados en esta situación. La SLP a 6 meses, objetivo principal del estudio, fue del 20,8%, y la mediana de SLP fue 10,4 semanas. Perry *et al.* [94] utilizaron una dosis única diaria de 50 mg/m^2 de forma continua. La SLP a 6 meses varió de forma notable dependiendo del momento de la progresión, siendo del 27,3% en los pacientes que progresaron antes de completar 6 ciclos de TMZ adyuvante, del 7,4% en los pacientes que progresaron durante la TMZ adyuvante después del 6º ciclo y del 35,7% en los pacientes que progresaron tras completar la TMZ adyuvante con un intervalo libre de tratamiento mayor de 2 meses. Los autores sugieren que el resultado

del segundo grupo puede deberse a que los pacientes desarrollaron más resistencias a la TMZ por el prolongado tiempo de exposición al fármaco. Un estudio retrospectivo analizó los resultados de la TMZ utilizada de nuevo a la progresión en tres instituciones. El análisis se efectuó en dos situaciones: en pacientes que progresaron durante la TMZ adyuvante y fueron tratados con esquemas alternativos del mismo fármaco, y en pacientes con un intervalo libre de tratamiento con TMZ de al menos 8 semanas. En ambos casos, el tratamiento de la recaída proporcionó una SLP a 6 meses en torno al 27% [125]. Al igual que en esta revisión, en nuestro estudio no se apreciaron diferencias en la SLP entre los pacientes que progresaron durante o después de la TMZ adyuvante. En cualquier caso, el número de pacientes incluidos es insuficiente para sacar conclusiones fiables al respecto, especialmente si se tienen en cuenta los conocimientos actuales sobre la complejidad del GB. Por una parte, los criterios de inclusión de los ensayos clínicos deberán tener más en cuenta las características moleculares de los GB; además, la heterogeneidad de estos tumores añade incertidumbre a la hora de valorar los resultados [126].

Por lo que respecta a la toxicidad, la linfopenia, un riesgo conocido en los esquemas de TMZ extendida, alcanzó un 18,5% en nuestro estudio y un 15,8% en el estudio de Perry *et al.* Aunque no hubo infecciones oportunistas por este motivo, se registraron dos muertes durante el tratamiento, una por neumonía y otra por un probable tromboembolismo pulmonar. Aunque estos eventos podrían plantear dudas sobre la seguridad del tratamiento ensayado, se trata de complicaciones relativamente frecuentes en pacientes con GB avanzado y no es posible asociarlas con certeza a este régimen terapéutico en particular.

Al igual que en nuestros estudios anteriores, los niveles de VEGF–A, sVEGFR–1 y MP, así como la GT, fueron más altos en los pacientes que en los controles sanos, pero sin asociación con los resultados clínicos. En este ensayo clínico decidimos no medir los niveles de CEC por tratarse de un estudio multicéntrico que obligaba a manejar muestras de sangre congeladas. Las muestras congeladas no permiten un buen contaje de CEC y, además, el coeficiente de variación intra e interlector aumenta [127], lo que lleva a un grado de incertidumbre importante en una muestra relativamente pequeña de pacientes.

En resumen, los estudios presentados muestran, de forma conjunta, una elevación de marcadores de angiogénesis, inflamación y coagulación en pacientes con GB, así como importantes correlaciones entre ellos. Además, hemos observado que niveles altos de CEC previos a la radioquimioterapia concomitante se asocian a peor pronóstico. También hemos comprobado que los niveles de MP procoagulantes plasmáticas están elevadas en los pacientes con GB con respecto a los controles sanos,

al igual que la GT asociada a las MP. Además, hemos observado una disminución significativa de los niveles de MP y de GT tras el tratamiento con radioterapia y TMZ concomitante, lo que sugiere que la radioquimioterapia inhibe la liberación de MP por sus células de origen. Ni los niveles basales de MP y GT ni su disminución tras el tratamiento se han asociado al pronóstico.

Actualmente, nuestro grupo de trabajo se centra en el estudio de factores asociados a la inmunotolerancia generada por el GB a través de varios mecanismos, algunos de ellos relacionados con las alteraciones de la angiogénesis, la inflamación y la coagulación que se muestran y discuten aquí.

5. CONCLUSIONES

1. Los pacientes con GB muestran una elevación significativa de marcadores de angiogénesis, inflamación y coagulación con respecto a controles sanos.

2. Existen correlaciones significativas entre varios marcadores de inflamación, especialmente con la CRP.

3. Existe una correlación negativa entre los niveles séricos de VEGF y de sVEGFR−1 que apoya el papel de quelante de este último sobre el VEGF.

4. Los niveles de MP están elevados en los pacientes con respecto a los controles sanos y disminuyen tras la radioquimioterapia.

5. La combinación de TMZ metronómica en combinación con irinotecán quincenal es un esquema de tratamiento factible, pero no ha superado los resultados de otros esquemas terapéuticos en pacientes con GB recidivado.

6. Los niveles basales altos de CEC (>percentil 99 en los controles sanos) en pacientes con GB, medidos tras la cirugía y antes de iniciar el tratamiento con temozolomida y radioterapia concomitante, se asocian a peor SG.

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7. ARTÍCULOS PUBLICADOS

7.1 Artículo 1

Circulating markers of angiogenesis, inflammation, and coagulation in patients with glioblastoma.

Gaspar Reynés, Virtudes Vila, María Martín, Antonio Parada, Tania Fleitas, Edelmiro Reganon, Vicenta Martínez-Sales.

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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Circulating markers of angiogenesis, inflammation, and coagulation in patients with glioblastoma

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Abstract Inflammation, angiogenesis, and coagulation are linked to the development of cancer. In glioblastoma, microvascular proliferation is a hallmark, and lymphocytic infiltration is a common finding. Thromboses are frequent in patients with glioblastoma. The objective of this study was to assess presurgical levels of circulating markers of inflammation, angiogenesis, and coagulation in a prospective series of patients with glioblastoma, and to explore their correlations and possible associations with clinical findings. Angiogenesis markers included were vascular endothelial growth factor (VEGF), soluble vascular endothelial growth factor-receptor 1 (sVEGFR-1), and thrombospondin-1 (TSP-1). Inflammatory markers included were C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and sialic acid (SA). Coagulation markers included were fibrinogen (Fg), endogen thrombin generation (ETG), prothrombin fragments 1 + 2 (F1 + 2), and tissue factor (TF). Forty-seven patients and 60 healthy subjects were included in the study. Signs of tumor necrosis in presurgical MRI were associated with shorter survival ($P < 0.01$). All inflammation markers, F1 + 2, ETG, VEGF and sVEGFR-1, were significantly elevated in glioblastoma patients. Correlations were found between ETG and Fg ($r = 0.44$, $P < 0.01$). Sialic acid correlated with Fg ($r = 0.63$, $P < 0{,}001$); CPR correlated with SA ($r = 0.60$, $P < 0.001$), Fg ($r = 0.76$, $P < 0.001$), TNFα ($r = 0.56$, $P < 0.001$), and IL-6 ($r = 0.65$, $P < 0.001$); and IL-6 also correlated positively with TNFα ($r = 0.40$, $P < 0.02$) and Fg ($r = 0.45$, $P < 0.01$). Vascular endothelial growth factor inversely correlated with sVEGFR-1 ($r = -0.35$, $P < 0.02$). No associations were found between marker levels and survival or progression-free survival.

Keywords Glioblastoma · Inflammation · Angiogenesis · Coagulation · Circulating markers

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Introduction

Glioblastoma, one of the most malignant neoplasms in humans, is a highly vascularized tumor. Microvascular proliferation and necrosis are key histological characteristics of glioblastomas, and lymphocytic infiltration is also a common finding [1]. Furthermore, high-grade gliomas are among the tumors with the highest association with thrombotic events [2]. Angiogenesis, inflammation and coagulation are complex and highly interrelated processes which are linked to the initiation and development of cancer. Epidemiological studies have revealed that chronic inflammation caused by infections, autoimmune diseases, or other conditions increases the risk of developing some types of cancer. Apart from other mechanisms, the inflammatory cell-driven microenvironment, composed of a variety of cytokines, chemokines, and enzymes, might lead to tumor initiation and promotion [3]. However, signs of chronic inflammation are present in virtually all tumors, irrespective of the existence of a previous infectious or inflammatory disease. Oncogene-induced activation of transcription factors unleashes the production of tumor-promoting cytokines

and chemokines that in turn recruit and activate inflammatory cells. The activation of transcription factors in stromal, inflammatory and tumor cells induces the release of mediators that stimulate cell proliferation, migration, survival, and angiogenesis [4].

In glioblastoma, angiogenesis is triggered by a number of proteins secreted by tumor cells, and by stromal and inflammatory cells [5]. Interleukin-6 (IL-6), produced by tumor, inflammatory, and endothelial cells of newly formed vessels, is often overexpressed in these tumors. Interleukin-6 amplification is associated with shorter survival [6] and it is involved in angiogenesis and tumor progression. Plasma levels of the acute-phase inflammatory markers sialic acid (SA) and C reactive protein (CRP) have prognostic and predictive value in some tumors [7–10].

Vascular endothelial growth factor (VEGF) plays a central role in angiogenesis, and upregulation of VEGF levels in tumor tissue was found to be associated with the grade of malignancy and with microvascular density in a series of gliomas [11]. Vascular endothelial growth factor binds to two endothelial cell membrane receptors, vascular endothelial growth receptor-1 (VEGFR-1) and vascular endothelial growth receptor-2 (VEGFR-2). The soluble form of VEGFR-1 (sVEGFR-1) is a truncated receptor which lacks the transmembrane and intracellular domains. Similarly to VEGF, the tissue concentration of sVEGFR-1 correlates with malignancy grade and with microvascular density in gliomas [12]. Tumor angiogenesis is inhibited by thrombospondin-1 (TSP-1), a matricellular glycoprotein which in turn is downregulated by hypoxia [13]. Thrombospondin-1 is overexpressed in high-grade astrocytomas compared with low-grade astrocytomas and normal brain [14].

Tissue factor (TF), the primary initiator of coagulation, is expressed in gliomas, and the level of expression correlates with the histologic grade of the tumor [15]. Tissue factor expression is induced by cytokines and growth factors produced by tumor and inflammatory cells. Overexpression of TF has a key role in the increased risk of thrombosis in these patients. Together with thrombin, it also promotes angiogenesis by clotting-independent and clotting-dependent mechanisms. Tissue factor, IL-6, and VEGF have been proposed as possible cancer therapeutic targets [16, 17].

A substantial number of studies has focused on different aspects of inflammation, angiogenesis, or coagulation in gliomas, partially unveiling complex links among them. We have therefore assessed levels of circulating markers of inflammation, angiogenesis, and coagulation in a series of patients with glioblastoma, with the objective of establishing their correlations and their possible association with clinical features and outcome.

Materials and methods

Subjects

The study population consisted of a prospective series of patients with histologically proven glioblastoma, according to the World Health Organization Classification [1], admitted at the La Fe University Hospital. Before surgery, all patients were studied by magnetic resonance imaging (MRI), with complementary spectroscopy or perfusion measurements when indicated. After tumor resection or stereotaxic biopsy, an MRI or TC scan was performed within 72 h to assess the extent of resection and the appearance of postsurgical complications. After surgery, patients were treated with concomitant radiotherapy and temozolomide, followed by five-day cycles of temozolomide, in accordance with the current standard adjuvant treatment for glioblastoma [18]. The control group was composed of healthy subjects from the same demographic area, whose age and sex matched those of the patients.

The study was approved by the Clinical Research and Ethics Committee of La Fe University Hospital. Patients and controls were informed of the study objectives and gave written informed consent to participate in the study. All study procedures comply with the Declaration of Helsinki.

Neuroimaging assessment

The presence of peritumoral edema was evaluated as described by Schoenegger et al. [19]. Edema was defined as a region of increased T2 signal intensity. The maximum extent of edema from the tumor margin was measured on both axial and coronal sequences. Edema extending less than 1 cm from the tumor margin was regarded as minor or absent, and edema extending more than 1 cm from the tumor margin was regarded as major. Necrosis was defined as a region within the tumor that does not enhance or shows markedly diminished enhancement, surrounded by a ring enhancement area [20]. To evaluate the degree of tumor resection, we observed the presence of remaining enhancing tumor on the postoperative MRI.

Sample collection

Venous blood samples were obtained from patients before surgery. Blood was collected in a BD Vacutainer tube containing sodium citrate (129 mM) at a sodium citrate to blood ratio of 1:9 (*v*/*v*) and a dry BD Vacutainer tube. Samples were centrifuged at 1,500×*g* for 30 min at 4°C to obtain plasma or serum and then stored at −80°C for later batch analysis.

Markers included in the study and biochemical determinations

Angiogenesis markers included were VEGF, VEGF-R1, and TSP-1. Total serum levels of VEGF and sVEGFR-1 were determined by ELISA according to manufacturers' instructions (VEGF Biosource International and Quantikine Human sVEGFR1 R&D Systems, respectively). Thrombospondin-1 levels were quantified by an indirect ELISA, as described elsewhere [21].

Inflammatory markers included were CRP, IL-6, tumor necrosis factor alpha (TNFα), and SA. C-reactive protein plasma levels were measured by nephelometry using a commercial method (Dade-Behring, Germany). Interleukin-6 serum levels were determined by a commercial ELISA method (High Sensitivity Human IL-6 ELISA kit, Diaclone). Tumor necrosis factor alpha plasma levels were measured by ELISA using a commercial kit (Quantikine HS Human TNF-alpha, R&D Systems). Total SA plasma levels were measured using a commercial enzymatic-colorimetric method (Sialic acid Farbtest, Boehringer Mannheim, Germany).

Coagulation markers included were fibrinogen (Fg), endogen thrombin generation (ETG), prothrombin fragments 1 + 2 (F1 + 2), and TF. Fibrinogen level was determined by measuring the plasma fibrin formation rate by a turbidity assay [22]. Endogen thrombin generation was evaluated in fresh whole blood after adding 12.5 mM $CaCl_2$ (final concentration), and stopped after 20 min by adding 20 mM EDTA (final concentration). Free thrombin activity was determined using the chromogenic substrate S-2238 (1 mM final concentration) (Chromogenix-Instrumentation Laboratory). Plasma F1 + 2 levels were measured using a commercial ELISA method (Enzygnost F1 + 2 kit, Dade-Behring). Plasma TF levels were measured by ELISA using a commercial method (Immubind Tissue Factor, America Diagnostica).

Statistical analysis

Results are given as mean values ± standard deviations (SD) for continuous variables, and as percentages for categorical variables. The Kolmogorov–Smirnov test was used to evaluate whether each value follows a normal distribution. Analysis of variance (ANOVA) with the Bonferroni post-hoc test was used to assess differences among patients and healthy controls. Bivariate correlation was performed by use of Spearman′s correlation test. Survival time was analyzed by means of the Kaplan–Meyer method, and survival curves of subgroups were compared by use of the log-rank test. The upper reference limit levels of the markers were calculated as values higher than the 95th percentile of the control group (healthy subjects) and were set at: F1 + 2 = 0.23 nmol/l; ETG = 13.25 IU thrombin/ml; SA = 68 mg/dl; CRP = 2.4 mg/l; IL-6 = 3.6 pg/ml; TNFα = 1.1 pg/ml, and VEGF = 238.5 pg/ml.

All statistical analyses were performed with the SPSS computer software, version 12.0 for Windows (SPSS, Chicago, Ill, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Between April 2005 and June 2008, 47 patients and 60 healthy subjects were included in the study. Healthy control group has age 65 ± 15 years, sex 62% male. Clinical characteristics of patients are summarized in Table 1. All patients were on dexamethasone, with a dose range between 3 and 24 mg/day (one patient with > 24 mg/day). In this series, an unusual proportion of patients (15 patients, 32% of the total population) did not receive chemo-radiotherapy. Reasons included synchronous neoplasia (1), rapid clinical deterioration (10), and death from other causes (4). Median survival time from surgery was 8.13 months (95% IC: 2.9–13.4. Range: 0.5–30+ months).

Neuroimaging characteristics and survival

Peritumoral edema and necrosis on presurgical MRI were assessed in 45 patients. Edema was present in 41 patients, and necrosis in 31. Global survival was shorter for patients with peritumoral edema than for those without, although this difference was not statistically significant. Signs of

Table 1 Clinical characteristics of 47 glioblastoma patients

Age (years)	61 ± 12
Sex: male (%)	60
Karnofsky index (%)	
>80	14.0
80–60	77.0
<60	4.5
NA	4.5
Extent of surgery (%)	
Complete resection	42.5
Partial resection > 50%	31.9
Open biopsy < 50%	8.5
Stereotaxic biopsy	17.0
Residual tumor (%)	
No	29.8
Yes, measurable	53.2
Yes, non measurable	17.0

Age is expressed as mean ± standard deviation

NA, not available

Table 2 Kaplan–Meyer analysis of survival according to magnetic resonance imaging characteristics

Variables	Number of patients (%)	Median survival (months)		Log rank	*P*
		Yes	No		
Edema	41/45 (91)	7.03	20.8	3.05	0.08
Necrosis	31/45 (69)	4.87	13.4	6.53	<0.01

tumor necrosis (i.e., ring enhancement) were present in 31 patients, and was significantly ($P < 0.01$) associated with shorter survival (Table 2).

Coagulation, inflammation and angiogenesis marker levels, and their correlations

Levels of circulating markers of coagulation, inflammation and angiogenesis were assessed in 40 patients. Table 3 summarizes the marker levels in patients and controls. Among the coagulation markers, F1 + 2 and ETG levels were significantly higher in patients than in controls. All inflammation markers were significantly elevated in gliobastoma patients compared with controls. Among the angiogenesis markers, VEGF ($P < 0.001$) and sVEGFR-1 ($P < 0.05$) levels were significantly higher in patients than in controls.

Correlations among circulating markers in patients are shown in Table 4. A strong correlation was found between ETG and Fg ($r = 0.44, P < 0.01$). Among the inflammatory markers, SA directly correlated with Fg ($r = 0.63$, $P <$ 0,001); CPR correlated with SA ($r = 0.60$, $P < 0.001$), Fg ($r = 0.76$, $P < 0.001$), TNFα ($r = 0.56$, $P < 0.001$) and IL-6 ($r = 0.65$, $P < 0.001$); and IL-6 also correlated positively with TNFα ($r = 0.40$, $P < 0.02$) and Fg ($r = 0.45$, $P < 0.01$). Among the angiogenesis markers, VEGF inversely correlated with sVEGFR-1 ($r = -0.35$, $P < 0.02$).

Associations between circulating coagulation, inflammation, and angiogenesis markers and clinical outcome

Survival time and log-rank test for patients with different coagulation, inflammation, and angiogenesis marker levels higher or lower than the cut-off points were not statistically significantly different. No associations were found between marker levels and survival or progression-free survival. Levels of circulating IL-6 were not associated with the presence of peritumoral edema.

Table 3 Coagulation, inflammation, and angiogenesis markers in patients and controls

	Patients ($n = 40$)	Controls ($n = 60$)	*P*
Coagulation			
F1 + 2 (nmol/L)	0.42 ± 0.50	0.20 ± 0.05	<0.001
TF (pg/ml)	154 ± 93	153 ± 59	NS
ETG (UI/ml)	10.9 ± 3.4	8.7 ± 2.4	<0.01
Inflammation			
IL-6 (pg/ml)	3.5 ± 7.1	0.7 ± 0.4	<0.01
TNFα (pg/ml)	1.1 ± 0.9	0.6 ± 0.2	<0.001
Fg (mg/dl)	300 ± 156	232 ± 31	<0.01
SA (mg/dl)	71 ± 22	55 ± 10	<0.001
CRP (mg/l)	17.0 ± 26.6	1.8 ± 2.7	<0.001
Angiogenesis			
VEGF (pg/ml)	268 ± 186	123 ± 64	<0.001
sVEGF-R1 (pg/ml)	89 ± 29	77 ± 17	<0.05
TSP-1 (μg/ml)	47.2 ± 14.1	45.9 ± 10.3	NS

Values are expressed as mean ± standard deviation

F1 + 2, prothrombin factors 1 + 2; TF, tissue factor; ETG, endogenous thrombin generation; IL-6, interleukin-6; TNFα, tumor necrosis factor alpha; Fg, fibrinogen; SA, sialic acid; CRP, C-reactive protein; VEGF, vascular endothelial growth factor; sVEGF-R1, soluble vascular endothelial growth factor receptor-1; TSP-1, thrombospondin 1; NS, Not significant

Table 4 Spearman's correlations of coagulation, inflammation and angiogenesis markers

	ETG	IL-6	TNFα	Fg	SA	CRP	sVEGFR-1
IL-6	NS	–					
TNFα							
rho	NS	0.40	–				
P		<0.02					
Fg							
rho	0.44	0.45	NS	–			
P	<0.01	<0.01					
SA							
rho	NS	NS	NS	0.63	–		
P				<0.001			
CRP							
rho	NS	0.65	0.56	0.763	0.60	–	
P		<0.001	<0.001	<0.001	<0.001		
VEGF							
rho	NS	NS	NS	NS	NS	NS	−0.35
P							<0.02

ETG, endogenous thrombin generation; IL-6, interleukin-6; TNFα, tumor necrosis factor alpha; Fg, fibrinogen; SA, sialic acid; CRP, C-reactive protein; VEGF, vascular endothelial growth factor; sVEGF-R1, soluble vascular endothelial growth factor receptor-1; TSP-1, thrombospondin 1; NS, Not significant

Discussion

All circulating markers of inflammation included in our study were elevated in patients compared with healthy controls. Elevated levels of serum IL-6 have been found in many neoplastic diseases, for example gastrointestinal tumors, lung, prostate, and renal cancers, among others. In most of these studies, serum levels of IL-6 correlate with clinical extension of the tumor and with outcome [23]. Synthesis of CRP is induced by IL-6, interleukin-1, and TNFα. Serum levels of IL-6, TNFα, and CRP were significantly higher in patients with colorectal cancer than in normal controls, and they correlated with tumor size. High levels of IL-6 and CRP were associated with reduced survival [24]. In advanced non-small cell lung cancer, serum levels of TNFα and CRP, but not of IL-6, were elevated compared with normal controls [25]. In our study, strong correlations were found between IL-6 and CRP and between TNFα and CRP. Nevertheless, none of these markers were significantly associated with survival. Sialic acid has been linked to tumor potential for differentiation and progression, in part through modulation of the glycosylation status of integrins [26]. In one study, SA plasma levels were increased in patients with glioma [27]. Sialic acid is the terminal sugar of the carbohydrate chains bound to fibrinogen, so both inflammatory markers are closely related [28]. In our study a correlation of SA with Fg and CRP was found.

Among the coagulation markers, plasma levels of fibrinogen, F1 + 2, and ETG, as expression of the systemic hypercoagulability of patients with high-grade glioma, were higher in patients than in controls.

Vascular endothelial growth factor is overexpressed in glioblastoma [29]. However, studies measuring VEGF concentration in the serum of patients with glioblastoma have obtained conflicting results. Takano et al. found no difference between VEGF serum concentration in 19 patients with brain tumors and in healthy controls, and no correlation between serum and tissue extracts in any of the patients, but they included only six glioblastomas and VEGF serum concentration was assessed in two of them only [30]. Stockhammer et al. [31] found no difference between serum VEGF concentration in 14 patients with brain tumors, including six glioblastomas, and in healthy controls, but VEGF levels were 2 to >2000 times higher in fluids of tumor cysts than in the corresponding sera. In contrast, Salven et al. found serum levels of VEGF to be elevated in high-grade brain tumors (nine cases), but not in low-grade tumors (six cases) [32]. The small number of patients with glioblastoma included in these studies may explain their conflicting results. In our study, circulating markers were measured for 40 patients with proven glioblastoma, and serum levels of VEGF were twofold higher in patients compared with healthy controls.

Soluble VEGFR-1 is thought to have an inhibitory role in the VEGF signaling system, by sequestering VEGF and by forming non-functional heterodimers with membrane VEGFR monomers [33–35]. Lamszus et al. analyzed tissue levels of VEGF and sVEGFR-1 in gliomas of different grades of malignancy, including 46 glioblastomas. The markers correlated with each other. Elevated levels of both proteins were associated with high-grade glioma and with high microvessel density. The sVEGFR-1/VEGF ratio was 2.6-fold higher in low-grade astrocytomas than in glioblastomas. In 26 glioblastoma patients for whom survival data were available, sVEGFR-1 correlated with survival [12]. In a previous study of breast cancer patients, a sVEGFR-1/VEGF ratio > 10 was associated with favorable prognosis [36]. Other studies have analyzed the prognostic value of circulating sVEGFR-1 in different tumors. In a series of 38 lung cancer patients, pretreatment serum levels of sVEGFR-1 were not elevated compared with controls, and there were no significant differences in levels of sVEGFR-1 before and after treatment. However, there was a significant difference in sVEGFR-1 levels depending on treatment response [37]. In pancreatic cancer, circulating VEGF and sVEGFR-1 levels were elevated compared with healthy controls. A reverse correlation was observed between sVEGFR-1 level and advance of tumor stage. Higher VEGF/sVEGFR-1 ratio was significantly correlated with poor outcome [38]. Interestingly, an inverse correlation between VEGF and sVEGFR-1 was found in our study, suggesting an effective role of sVEGFR-1 as a VEGF trap. However, no associations with outcome were found for circulating VEGF, sVEGFR-1, or their ratio.

In our study, peritumoral edema and ring enhancement were associated with shorter survival, although only for ring enhancement did this reach statistical significance. In the study by Pope et al. [20] edema, but not necrosis or ring enhancement, had prognostic value in patients with glioblastoma, and in the study by Schoenegger et al. [19], peritumoral edema was confirmed as an independent negative prognostic factor. All patients received dexamethasone at different doses. Steroids can affect both extension of the peritumoral edema and several of the markers studied, thus limiting interpretation of the results. Nevertheless, the wide range of dosing and its inconsistency with the extension of edema made difficult to stratify the patients on the basis of this variable. Biological imaging is an interesting approach for non-invasive assessment of tumor characteristics and response to treatment. In our study, spectroscopy and blood perfusion tests were performed before surgery for eight and two patients, respectively. Assessment of potential correlations between such imaging studies and circulating markers, before and after treatment, could be of great interest, but exceeded the objectives of this study, because of the limited number of

patients and the lack of follow-up blood sampling. Note, in this series a high proportion of patients were unable to receive the complete scheduled treatment for different reasons, hence, this could have hindered the associations between clinical or experimental findings and survival.

In conclusion, our study revealed significant activation of inflammation, angiogenesis, and coagulation processes in patients with glioblastoma. In patients with extensive peritumoral edema survival was shorter than in those with moderate edema. Nevertheless, none of the circulating markers measured in this study could be associated with clinical outcome.

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7.2 Artículo 2

Circulating endothelial cells and procoagulant microparticles in patients with glioblastoma: prognostic value.

Gaspar Reynés, Virtudes Vila, Tania Fleitas, Edelmiro Reganon, Jaime Font de Mora, María Jordá, Vicenta Martínez-Sales.

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Circulating Endothelial Cells and Procoagulant Microparticles in Patients with Glioblastoma: Prognostic Value

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Abstract

Aim: Circulating endothelial cells and microparticles are prognostic factors in cancer. However, their prognostic and predictive value in patients with glioblastoma is unclear. The objective of this study was to investigate the potential prognostic value of circulating endothelial cells and microparticles in patients with newly diagnosed glioblastoma treated with standard radiotherapy and concomitant temozolomide. In addition, we have analyzed the methylation status of the *MGMT* promoter.

Methods: Peripheral blood samples were obtained before and at the end of the concomitant treatment. Blood samples from healthy volunteers were also obtained as controls. Endothelial cells were measured by an immunomagnetic technique and immunofluorescence microscopy. Microparticles were quantified by flow cytometry. Microparticle-mediated procoagulant activity was measured by endogen thrombin generation and by phospholipid-dependent clotting time. Methylation status of *MGMT* promoter was determined by multiplex ligation-dependent probe amplification.

Results: Pretreatment levels of circulating endothelial cells and microparticles were higher in patients than in controls ($p<0.001$). After treatment, levels of microparticles and thrombin generation decreased, and phospholipid-dependent clotting time increased significantly. A high pretreatment endothelial cell count, corresponding to the 99th percentile in controls, was associated with poor overall survival. *MGMT* promoter methylation was present in 27% of tumor samples and was associated to a higher overall survival (66 weeks vs 30 weeks, $p<0.004$).

Conclusion: Levels of circulating endothelial cells may have prognostic value in patients with glioblastoma.

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Introduction

The current standard of care for newly diagnosed glioblastoma is surgery, radiotherapy, and concomitant daily temozolomide, followed by cycles of this drug given for five days every four weeks. Despite treatment, most patients die within two years of surgery [1]. The proportion of patients who benefit from this therapy is determined only partially by the methylation status of the O6-alkylguanine-DNA-methyltransferase (*MGMT*) gene promoter, which is considered a prognostic factor rather than a predictor of response [2]. Assessment of response in glioblastoma patients is difficult because radiochemotherapy modifies vascular permeability in the tumor area. This alteration can lead to pseudoprogression, an increase in contrast tumor enhancement that mimics the true progression [3]. Therefore, it is crucial to identify biomarkers that may help establish the prognosis of patients with glioblastoma and predict their response to treatment.

Glioblastoma is a highly vascularized tumor that displays active angiogenesis [4]; thus, drugs with antiangiogenic properties, such as bevacizumab and cilengitide, are being tested for use with radiotherapy and temozolomide [5,6]. Nevertheless, continuous temozolomide, as described for other metronomic chemotherapy regimens, might have antiangiogenic activity by itself, mediated in part by a direct effect on tumor vessel endothelium [7].

Circulating endothelial cells (CECs) consist of at least endothelial progenitor cells (EPCs) that originate in the bone marrow, mature endothelial cells shed from vessel walls, apoptic endothelial cells and some cells with endothelial function from cancerous cells [8]. Recently, circulating endothelial cells (CECs) have been established as markers of endothelial damage or dysfunction [9]. CEC levels increase in many kinds of disorders such as cardiovascular [10,11], autoimmune [12], and infectious diseases [13], as well as in cancer [14]. In cancer patients, CEC number

correlates with tumor progression [15] and constitutes a promising tool for monitoring disease activity, with potential for the assessment of prognosis and response to treatment. In patients with non-small cell lung cancer (NSCLC), we observed an association between elevated CEC numbers and decreased overall survival (OS) [16], although in a study by Kawaishi et al. [17], high CEC numbers were associated with longer progression-free survival (PFS). In patients with breast cancer treated with metronomic chemotherapy, CEC levels after two months treatment were associated with prolonged PFS [18]; in another trial with metronomic chemotherapy and bevacizumab, baseline CEC levels were also associated with PFS [19]. It has been suggested that quantification of CECs is useful to identify patients who might benefit from antiangiogenic treatments [20]. Batchelor et al., in a series of patients with glioblastoma treated with AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, found that viable CEC number increased when tumors escaped treatment [21].

Microparticles (MPs) are small vesicles (100 nm–1 μm) which directly bud from the plasma membrane of different cells, including blood, endothelial and tumor cells [22,23]. During MP formation, phosphatidylserine (PS) is transferred from the inner to the outer leaflet of the membrane; this externalization of PS facilitates the assembly of components of the clotting cascade, thus increasing the procoagulant activity of MPs [24]. The procoagulant MP levels increase in cancer patients [25]. In patients with castration-resistant prostate cancer, high platelet-derived MP number is associated with shorter survival [26]. However, in patients with NSCLC we observed an association between elevated total MP count and increased OS [16]. Nevertheless, the potential prognostic value of MPs in glioblastoma patients remains unclear.

The aim of this study was to evaluate the potential prognostic value of CECs, MPs and MP-mediated procoagulant activity in patients with newly diagnosed glioblastoma. In addition, we have analyzed the methylation status of *MGMT* promoter in tumor tissue.

Materials and Methods

Study Design and Patients

This prospective study included consecutive patients with newly diagnosed, histologically proven glioblastoma who received standard treatment [1] at La Fe University Hospital. The control group comprised healthy subjects matched for sex and age with the patients. The study was conducted in accordance with the principles outlined in the Declaration of Helsinki. All participants gave written informed consent. The study was approved by the institutional Biomedical Research Ethics Committee.

After surgery, patients received radiotherapy to a total dose of 60 Gy in 30 fractions given five days per week, plus concomitant temozolomide at a daily dose of 75 mg/m^2. After a four-week rest, adjuvant temozolomide was administered at a dose of 150 to 200 mg/m^2 for five days every four weeks until progression, unaccepable toxicity or other reasons that hinder treatment. Patients were assessed by magnetic resonance imaging (MRI) at baseline. Subsequent imaging assessments were performed within 72 hours after surgery, to check the extent of tumor resection and to rule out postsurgical complications, and every three cycles of temozolomide thereafter. Perfusion, diffusion, and spectroscopy MRI procedures were performed when indicated. At progression, patients amenable for second-line treatment received bevacizumab plus irinotecan, fotemustine or rechallenge with temozolomide.

Biomarker Evaluation

Blood sampling. Venous blood samples were obtained from patients within two weeks before the start of radiochemotherapy and during the last week of this treatment. The initial 3 mL of blood was discarded to avoid contamination with endothelial cells from the puncture wound of the vein. Blood for quantification of CECs was collected in a tube containing ethylenediaminetetraacetic acid (1.8 mg/mL). For the determination of MP levels and MP-mediated procoagulant activity, blood was collected in a tube containing sodium citrate (129 mM) at a ratio of 1:9 (v/v, sodium citrate/blood). We have previously studied pre-analytical conditions to analyze MP count and pro-coagulant activity, and centrifugation at 1500×g, for 30 min, at 4°C and analysis on frozen plasma samples have been applied. Plasma was stored at –80°C to allow later batch analysis.

Quantification of circulating endothelial cells. The isolation and quantification of CECs was performed by immunomagnetic technique following a consensus protocol [27]. In brief, cells were isolated from whole blood at 4°C by means of an endothelial cell specific monoclonal antibody sEndo1 (BioCytex, Marseille, France) raised against the endothelial antigen CD146, coupled to micromagnetic beadsPan-Mouse M450 Dynabeads.-Dynal, Oslo, Norway. To avoid nonspecific binding of leukocytes to CD146-coated beads, cells were incubated after immunomagnetic isolation of CECs with fluorescein isotiocyanate-conjugated (FITC)-*Ulex europaeus* lectin-1 (UEA1). UEA-1 lectin (Sigma-Aldrich, Inc., Saint Louis, MO, USA) is a good histologic marker for endothelium in human, and constitutes a specific and sensitive additional tool in demonstrating endothelial cells and endothelial derivation of human tumors. After incubation, samples were washed, suspended in buffer, and counted with fluorescence microscopy using a Nageotte chamber. The size of the CEC population often exceeds 10 μm, which is not compatible with the typical size of endothelial progenitor cells. In addition, the morphology of our cells indicates considerable damage or even necrosis. Nucleated cells >10 μm in length with more than eight immunomagnetic beads attached and positive UEA1 staining were regarded as CECs. Conglomerates were counted as one cell. The number of CECs was expressed as cells/mL of blood. Reproducibility was tested by performing six replicates of 10 different samples; the coefficient of variation was 12%.

Quantification of total microparticles. Plasma MPs were quantified by flow cytometry in an EPICS XL-cytometer (Beckman Coulter, Brea, CA, USA) at high flow rate. Plasma was incubated with FITC–Annexin V conjugate (TACS Annexin V; Trevigen Inc. Gaithersburg, MD, USA) to detect accessible phosphatidylserine on MP membranes. Standard fluorescent beads of different diameters were used for size calibration (0.5–3.0 μm, Megamix; BioCytex, Marseille, France) and to set the gate for MP detection at a diameter of 0.5–1 μm following a consensus guideline on MP measurement [28]. The number of FITC–Annexin V-positive MPs was calculated and expressed as events/μL of plasma.

Assessment of MP-mediated Procoagulant Activity

The MP-mediated procoagulant activity of plasma was analyzed by thrombin generation (TG) assay without added exogenous tissue factor or phospholipids (Calibrated automated thrombogram, CAT; Thrombinoscope BV, Paris, FranceG). Under these conditions, the assay was critically dependent on MPs present in plasma. Curves were calculated using the Thrombinoscope software and the results were expressed as the thrombin peak (nM). MP activity depends on the exposure of anionic phospholipids that provide a surface for the assembly of the tenase and

prothrombinase complexes. To measure this activity the procoagulant phospholipid-dependent clotting time (PPLCT) assay was also analyzed (STA-Procoag-PPL; Diagnostica Stago, Paris, France).

MGMT methylation analysis. Formalin-fixed, paraffin-embedded tumor samples were subjected to careful histological assessment in order to select tumor areas. Three non-tumor areas of brain tissue were further isolated and independently mounted in paraffin blocks. DNA from 10 μm sections of each tumor and from the three controls was extracted with the deparaffination solution (Qiagen, Venlo, The Netherlands), followed by its purification with the DNA Investigator kit (Qiagen). Methylation status of *MGMT* promoter was determined by multiplex ligation-dependent probe amplification (MLPA) following the manufacturer's protocol (ME011-B1 MLPA, MRC-Holland). A methylation-sensitive restriction enzyme, HhaI (New England BioLabs), which cuts unmethylated GCGC sites, was applied to each set of samples. Reaction products were resolved on ABI3700 automated DNA sequencer and quantification of the methylation status of *MGMT* promoter was performed by Coffalyser software (MRC-Holland).

Statistical Analysis

The Kolmogorov-Smirnov test was used to evaluate whether each parameter came from a normal distribution. Statistical analyses were performed using a non-parametric test among sample types (control, pre-treatment and post-treatment) (Krusal-Wallis test) and for the independent relationship of the control samples with respect to patient groups (Chi-Square test with Yate's correction). Bivariate correlation was performed using Spearman's correlation test. OS and PFS were analyzed by Kaplan-Meyer method and survival curves of subgroups were compared using the log-rank test. CEC values were dichotomized as greater than 99% confidence interval in healthy controls (CEC = 20 cells/ml). All statistical calculations were performed using SPSS software (v. 15.0; SPSS Inc., Chicago, IL, USA).

Results

Patient Characteristics

Twenty-two patients and forty healthy subjects were included in the study during a period of 18 months. Patients' characteristics are shown in Table 1. All patients had histologically confirmed glioblastoma. Median PFS was 30 weeks (9–135) and median OS was 33 weeks (10–146).

Circulating Marker and Correlation Analysis

Levels of CECs, MPs, TG and PPLCT in patients and controls are shown in Figure 1. Compared with the healthy control group, mean pre-treatment levels of CECs and MPs were significantly higher ($p<0.001$). Post-treatment levels of CECs remained significantly higher in patients than in controls, while levels of MPs and TG decreased, and PPLCT increased significantly after treatment. Significant correlations were found between both pre- and post-treatment levels of TG and MPs ($p<0.01$), while PPLCT inversely correlated with pre-treatment levels of MPs ($p<0.01$) and TG ($p<0.05$) (Table 2). Platelet and leukocyte count significantly decreased after treatment (platelet: 283 vs 166×10^3, $p<0.0001$; leukocyte: 8.6 vs 6.2×10^3, $p = 0.021$). Platelet count significantly correlated with TG ($r = 0.52$, $p<0.001$), MPs ($r = 0.39$, $p = 0.013$) and PPLCT ($r = -0.49$, $p<0.01$).

Table 1. Clinical characteristics of patients.

Characteristics	Patients (n = 22)
Age in years	
Median (range)	62 (41–83)
Sex	
Male (%)	12 (54.5)
Female (%)	10 (45.5)
KPS score	
60–80 (%)	16 (72)
90–100 (%)	6 (27)
Extent of surgery	
S. Biopsy	5
Open biopsy	3
Partial resection	12
Complete resection	2

KPS: Karnofsky performance scale; S. Biopsy: stereotactic biopsy.
doi:10.1371/journal.pone.0069034.t001

Circulating Markers, *MGMT* Status and Clinical Outcome

The analysis of the associations between circulating markers and clinical outcome showed that pre-treatment CEC levels >20 cells/mL (corresponding to the 99th percentile in controls) were associated with poor OS (19 vs. 72 weeks; Log rank 4.566; $p = 0.033$) (Figure 2). No such association was found for pre- or post-treatment levels of MPs, TG and PPLCT.

MGMT promoter was methylated in 27% of tumor samples. *MGMT* status significantly influenced median OS, which was 66 weeks (95% CI, 44.6 to 87.4 weeks) in *MGMT* promoter methylated patients and 30 weeks (95% CI, 14.4 to 45.5 weeks) in *MGMT* promoter unmethylated patients ($P<0.004$), although its influence on PFS was not statistically significant. The difference in OS observed according CEC pre-treatment levels and TG post-treatment levels did not reach statistical significance when analyzed separately in methylated and unmethylated patients.

Discussion

Our results show that pre-treatment CEC levels were significantly elevated in patients with glioblastoma compared with controls, a finding that is consistent with those of previous studies in different types of cancer [14–16]. We have found an association between higher basal CEC count (>99th percentile of the CEC count in controls) and poor survival. In other tumors, the association between baseline CEC count and clinical outcome is conflicting [15–17,29]. Specific tumor characteristics and the variety of methods being used to identify CECs may explain these discrepancies. The standard treatment received by all patients in the present study includes low-dose, daily temozolomide along with radiation therapy. A study using a murine model reported that low-dose; continuous (metronomic) chemotherapy leads to apoptosis of endothelial cells within the tumor bed, resulting in increased apoptosis of tumor cells [30]. In patients with breast cancer receiving metronomic chemotherapy with methotrexate and cyclophosphamide with or without thalidomide, an increase in CEC count after two months was associated with a better PFS [18]. Similar results (i.e., an association between good outcome and an increase in CEC count after several treatment cycles) have been found in patients with cancer receiving antiangiogenic drugs [31,32]. Nevertheless, a recent study in patients with colorectal

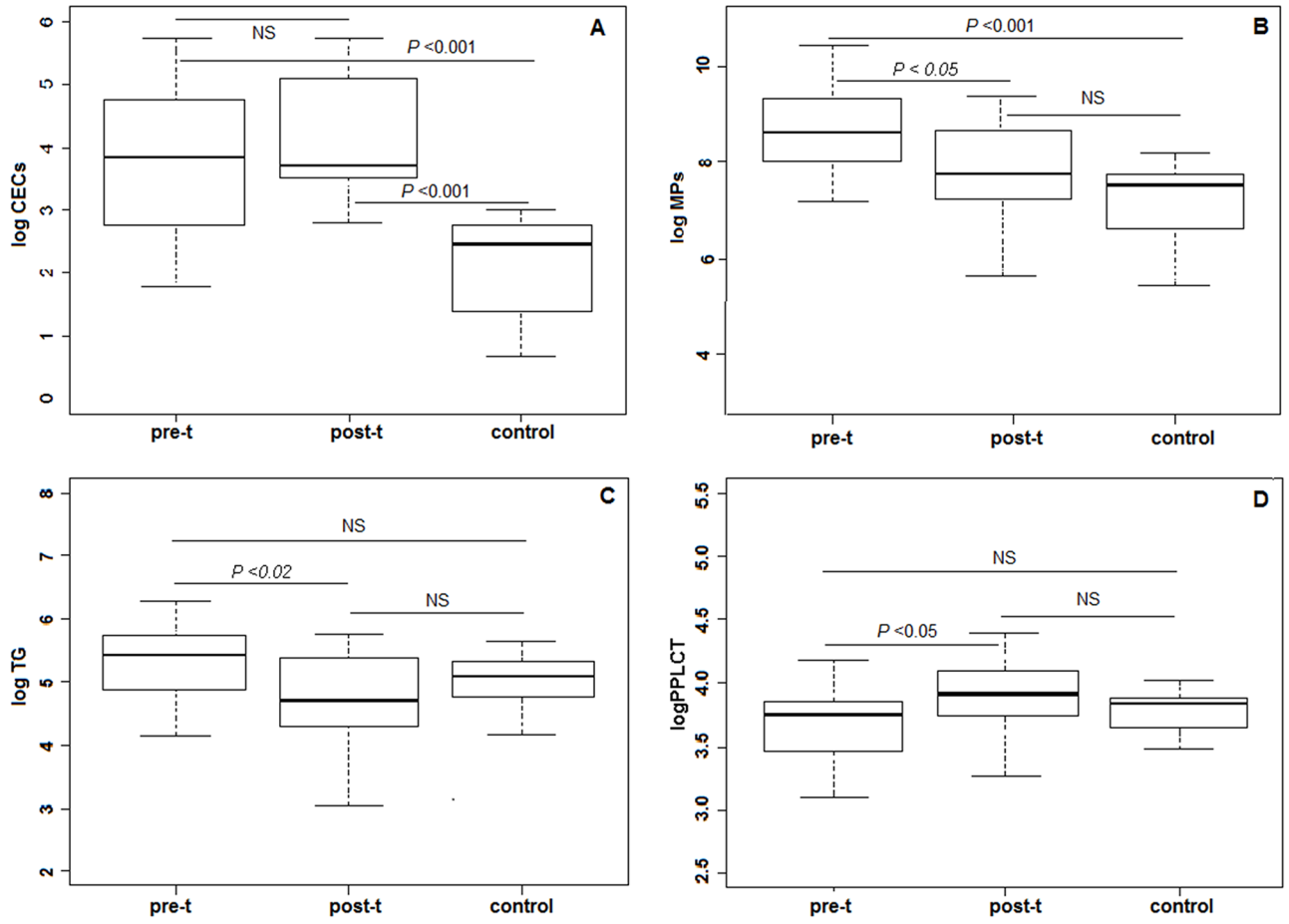

Figure 1. Pre- and posttreatment levels of biomarkers in patients and in controls. A: Circulating endothelial cells (CECs), B: Microparticles (MPs), C: Endogen thrombin generation (TG); D: Procoagulant phospholipid-dependent clotting time (PPLCT). Marker levels and their standard deviations are shown for pre-t: pretreatment; post-t: posttreatment; c: controls. Logarithmic transformation of data was made to normalize the distributions. NS: no significant.
doi:10.1371/journal.pone.0069034.g001

cancer concluded that high viable CEC count both at baseline and after the first cycle of chemotherapy plus bevacizumab, was associated with a worse outcome [33]. In the present study, the CEC levels were higher than in controls; however, they did not increase significantly after radiochemotherapy. The impact of radiation therapy on CEC number has not been studied fully, and any effect could have influenced the post-treatment CEC count in our patients.

Table 2. Spearman's bivariate correlation between MP and coagulation markers.

		MPs	PPLCT
TG	Pretreatment	0.732**	−0.627*
	Posttreatment	0.741**	
PPLCT	Pretreatment	−0.858**	
	Posttreatment		

MPs: circulating microparticles; TG: endogenous thrombin generation; PPLCT: procoagulant phospholipid-dependent clotting time.
*p<0.05;
**p<0.01.
doi:10.1371/journal.pone.0069034.t002

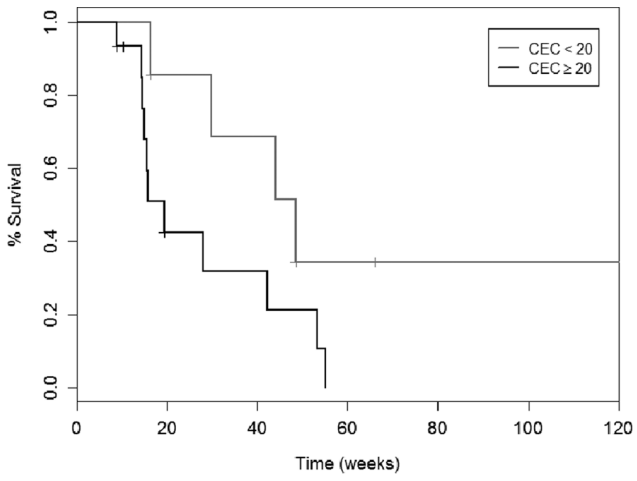

Figure 2. Kaplan-Meyer analysis of survival. Overall survival curve according to pretreatment CECs values were dichotomized as greater than 99% confidence interval in controls (CEC = 20 cells/ml) (Log rank = 4.566; p = 0.033). CECs: circulating endothelial cells.
doi:10.1371/journal.pone.0069034.g002

We found that the pretreatment MP count was significantly elevated in glioblastoma patients compared with controls. In agreement with our findings, microvesicles together with exosomes have also been reported to be elevated in glioblastoma patients and decrease upon temozolomide treatment [34].

Another finding of the present study is the decrease of MP and TG levels, and the increased PPLCT observed after treatment, an effect that could be explained by a decrease in the levels of their parent cells or by an inhibition of MP release induced by radiochemotherapy; although leukocytes and platelets decreased after treatment, only platelet count significantly correlated with TG, MPs and PPLCT. In a recent work by Sartori et al. [35], the procoagulant activity of annexin V-positive MPs was analyzed in 61 patients with glioblastoma at different times in their evolution; in accordance with the present study, they found that MP activity became significantly lower 1 and 4 months after surgery, though only in patients achieving complete surgical resection. As expected, in the present study OS was higher in patients with methylated *MGMT* promoter.

In summary, this exploratory study suggests an association between postsurgical higher CEC count and shorter survival in patients with glioblastoma. We believe that these findings warrant further investigation with a larger number of patients.

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Author Contributions

Conceived and designed the experiments: GR VV VM-S. Performed the experiments: VV JFdM MJ VM-S. Analyzed the data: VV TF ER JFdM VM-S. Wrote the paper: GR VV TF JFdM VM-S.

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7.3. Artículo 3

A phase I study of irinotecan in combination with metronomic temozolomide in patients with recurrent glioblastoma.

Gaspar Reynés, Carmen Balañá, Oscar Gallego, Luis Iglesias,
Pedro Pérez, José L. García.

Anticancer Drugs. 2014;25(6):717-22.

A phase I study of irinotecan in combination with metronomic temozolomide in patients with recurrent glioblastoma

Gaspar Reynés[a], Carmen Balañá[b], Oscar Gallego[c], Luis Iglesias[d], Pedro Pérez[e] and José L. García[f]

To determine the maximum tolerated dose of irinotecan administered every 2 weeks, in combination with a fixed and continuous administration of temozolomide, in patients with glioblastoma at first relapse. Patients received oral temozolomide at a fixed and continuous dose of 50 mg/m^2 divided into three daily doses, except for a single 100 mg/m^2 dose, administered before every irinotecan infusion. Irinotecan was given intravenously on days 8 and 22 of 28-day cycles. The starting dose of irinotecan was 100 mg/m^2, and this was escalated by increments of 15 mg/m^2 in cohorts of 3–6 evaluable patients. Determination of the dose-limiting toxicity was based on toxicities recorded from day 1 of the first cycle to day 8 of the third cycle. Enzyme-inducing antiepileptic drugs were not allowed. Tumor response was assessed by MRI every 8 weeks. Twelve patients were enrolled in this phase I study. The three patients enrolled at dose level 1 and six of nine patients enrolled at dose level 2 were evaluable for toxicity. The maximum tolerated dose of irinotecan was 100 mg/m^2. The dose-limiting toxicities were hematologic and gastrointestinal. Nine patients were evaluable for response: one patient achieved a partial response, four patients remained stable, and four patients had disease progression. The combination of metronomic temozolomide and irinotecan every 2 weeks can be safely administered at the recommended doses; a phase II study with this combination was started and has completed accrual. *Anti-Cancer Drugs* **25:717–722 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: irinotecan, metronomic chemotherapy, phase I, recurrent glioblastoma, temozolomide

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Introduction

Despite progress made in the treatment of glioblastoma, most patients relapse within 2 years after surgery [1]. Although targeted therapies have provided encouraging results [2], the search for new therapeutic strategies remains an important goal.

Temozolomide (TMZ), an oral alkylating agent, given at a dose of 150–200 mg/m^2 for 5 days every 4 weeks, demonstrated a modest activity in recurrent glioblastoma not previously treated with this drug [3,4]. It has been argued that extended-dose TMZ allows the administration of a higher dose density. Moreover, it could lead to depletion of O^6-methylguanine-DNA methyltransferase from tumor cells that express this DNA repair enzyme, thus increasing their sensibility to TMZ [5].

Many cytotoxic agents, administered at low and continuous doses (metronomic chemotherapy), inhibit angiogenesis through different mechanisms [6–10]. Kurzen *et al.* [11] observed that angiogenesis was significantly inhibited by 5 μmol/l TMZ, a concentration that could be achieved with the administration of 20 mg/m^2 of body surface every 8 h. In a pharmacokinetic study, after the oral administration of 200 mg of ^{14}C-TMZ, the maximum plasma concentration was 5.8 μg/ml, and was reached between 0.33 and 2.0 h (mean, 1.2 h) after drug intake. The drug was undetectable after 8 h of treatment in most patients [12]. These data support the feasibility of giving TMZ three times a day.

Irinotecan, a derivative of camptothecin that inhibits DNA-topoisomerase I, has shown variable activity in high-grade gliomas, reaching up to 15% objective response [13]. The metabolism of irinotecan is increased by concomitant use of enzyme-inducing antiepileptic drugs (EIADs) [13–15]. Preclinical studies have demonstrated a synergistic interaction between TMZ and irinotecan; this effect is schedule-dependent, and was observed when TMZ was administered before irinotecan [16]. Synergy between the two drugs occurs because DNA methylation produced by TMZ results in an increment of DNA-topoisomerase I complexes, thereby increasing the effectiveness of irinotecan [17]. Moreover, the cytotoxic activity of the combination TMZ–irinotecan appears to be partly independent on the status of O^6-methylguanine-DNA methyltransferase and the phenotype of mismatch repair mechanisms [18]. Finally, TMZ and irinotecan have different toxicity profiles, with myelosuppression and diarrhea being their limiting toxicities, respectively [19,20].

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We present the results of a multicentre, open label phase I trial conducted by the Grupo Español de Investigación en Neurooncología (GEINO). The aim of the present study was to determine the maximum tolerated dose (MTD) of irinotecan administered every 2 weeks, in combination with a fixed and continuous administration of TMZ.

Patients and methods

Eligibility

Patients aged 18 years or older, with a histologic diagnosis of glioblastoma at first recurrence, a Karnofsky performance status score of at least 70, not receiving corticosteroids, or with a stable or decreasing dose at least 72 h before basal MRI and until starting the study treatment, were eligible to participate in this protocol. Prior treatment with radiotherapy and concomitant TMZ followed by adjuvant TMZ, according to the protocol described by Stupp *et al*. [1], was also required. To rule out the possibility of pseudoprogression, a minimum of three cycles of adjuvant TMZ was essential before establishing the diagnosis of recurrence, by means of an MRI performed within 3 weeks before starting treatment. To enter the study, a measurable tumor progression or recurrence, assessed by the Macdonald criteria, was required. Additional requirements included adequate bone marrow function, with hemoglobin level > 10 g/dl, absolute neutrophil count (ANC) ≥ 1500/μl, and platelet count $> 100\,000$/μl; renal function, with serum creatinine $\leq 1.5\times$ institutional upper normal limit (UNL), and hepatic function, with total serum bilirubin $< 1.5\times$ institutional UNL; and alanine aminotransferase and aspartate aminotrasnferase $< 3\times$ institutional UNL.

Patients were ineligible if they were pregnant or breast-feeding, if they had a neurological dysfunction that hampered the understanding of treatment, and in case of vomiting or other conditions that impede oral treatment. Patients with other malignancies, excluding basal cell carcinoma or cervical carcinoma *in situ*, or other medical conditions that, in the opinion of the investigator, would make the patient unsuitable to participate in the study, were also ineligible. Simultaneous treatment with drugs having a potent inhibitory or inducing activity on cytochrome P450 enzymes (CYP450) was prohibited. Patients taking EIADs had to replace them, if indicated, by non-EIADs, at least 1 week before starting protocol treatment. The protocol was approved by the ethics committees of the participating centers. Informed consent was obtained from all patients.

Treatment plan

TMZ was supplied in capsules of 5, 20, 100, and 250 mg. All patients received oral TMZ at a fixed and continuous dose of 50 mg/m^2 divided into three daily doses, except for a single 100 mg/m^2 dose, administered between 3 and 6 h before every irinotecan infusion (Fig. 1). The calculated dose was rounded to the nearest 5 mg. To facilitate dose adjustments, a table with TMZ doses corresponding to frequent body surface values was provided. Irinotecan was given intravenously on days 8 and 22 of 28-day cycles. TMZ was given at least 2 h after and 1 h before a meal. Antiemetics, preferably metoclopramide, were allowed in case of nausea or vomiting. An oral serotonin antagonist could be administered when necessary. Before every irinotecan infusion, 0.25 mg subcutaneous atropine and an intravenous serotonin antagonist were administered. Prophylactic administration of granulocyte colony-stimulating factors was not allowed, but erythropoietin and red blood cell or platelet transfusions could be administered at the discretion of the investigator.

Five irinotecan dose levels were planned. The starting dose of irinotecan was 100 mg/m^2 and subsequent dose levels were established in increments of 15 mg/m^2 up to a maximum of 160 mg/m^2. For the case that dose-limiting toxicity (DLT) was reached at dose level 1, two additional dose levels below the starting dose were planned: dose level -1, at 85 mg/m^2; and dose level -2, at 70 mg/m^2 (Table 1).

Dose escalation and definition of dose-limiting toxicity

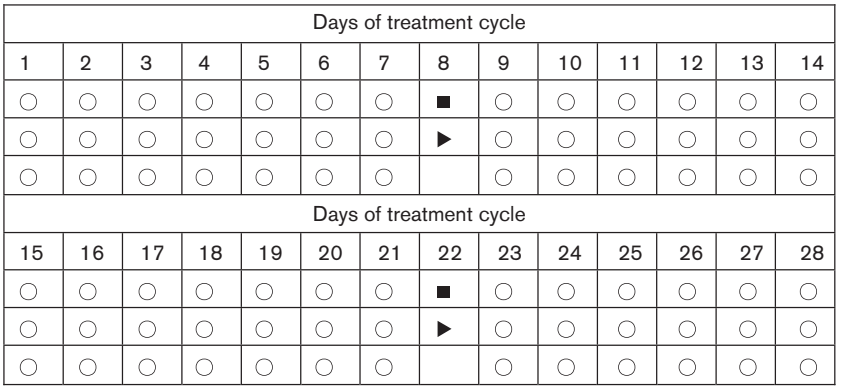
The dose escalation plan was as follows. Three patients were included at dose level 1. If no cases of DLT were observed, the dose level was escalated in successive cohorts of three patients. If one or two patients experienced DLT at a given dose level, three additional patients were treated at the same dose level. If no more than two of the six patients experienced DLT, dose escalation continued. If more than two of a six-patient cohort, or all three initial patients included in a dose level, experienced DLT, the MTD was surpassed and the previous dose level was considered as the MTD. Once the MTD was established, all patients included in the higher dose level were rescheduled to receive the MTD.

Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria, version 3.0. Determination of the DLT was on the basis of the toxicities recorded from day 1 of the first cycle to day 8 of the third cycle. DLT was defined as any of the following toxicities: ANC < 500/μl lasting > 7 days; platelet count $< 25\,000$/μl; a delay > 7 days in the beginning of the second or the third cycle to allow recovery of hematologic toxicity (ANC ≥ 1500/μl and platelet count $\geq 100\,000$/μl); febrile neutropenia grade ≥ 3; and nonhematologic toxicity grade ≥ 3, except alopecia and nausea or vomiting without prophylaxis or appropriate treatment.

Patient monitoring and dose modifications

During the first two cycles, a physical examination and a complete blood count were performed weekly. A serum biochemistry profile including electrolyte levels and renal

Fig. 1



Treatment schedule. Treatment consisted of 28-day cycles, repeated without break. "○" denotes TMZ, 50 mg/m^2/day, divided in three daily doses. "■" denotes TMZ, 100 mg/m^2 given in a single dose. "▶" denotes irinotecan, administered 3–6 h after TMZ, according to appropriate dose level (Table 2). TMZ, temozolomide.

Table 1 Dose levels of irinotecan

Level	Dose (mg/m^2)
−1	85
−2	70
1	100
2	115
3	130
4	145
5	160

The starting dose of irinotecan was 100 mg/m^2 (dose level 1).

and liver tests was obtained on day 8 of each treatment cycle. Treatment with TMZ was maintained if ANC was at least 1000/μl and platelet count at least 75 000/μl. Otherwise, TMZ was discontinued for 7 days and was resumed when ANC was at least 1500/μl and platelet count at least 75 000/μl. Patients left the study if ANC and platelet count were not recovered after 4 weeks. TMZ dose was decreased by 20% (always in three equal fractions per day, except on days 8 and 22) if ANC was less than 1000/μl in two successive weekly determinations, or febrile neutropenia with ANC was less than 500/μl, or a platelet count was less than 75 000/μl in two successive weekly determinations, or a platelet count was less than 25 000/μl in any determination. If nonhematologic toxicity grade greater than 2 occurred (except for alopecia and nausea, vomiting, and diarrhea not properly treated), TMZ was discontinued for 7 days; it was resumed if toxicity recovered to a grade of up to 1 or biochemical values did not exceed those specified in the inclusion criteria. Otherwise, the process was repeated up to 4 weeks; if any of the mentioned toxicities persisted within that period, the patient left the study. TMZ dose was decreased by 20% in case of delay lasting 2 weeks due to nonhematologic toxicity, or if nonhematologic toxicity reached grade of at least 3. TMZ dose reduction was maintained until the end of the study. No further dose reductions were allowed.

Irinotecan was given as scheduled if ANC was $\geq$ 1500/μl and platelet count $\geq$ 100 000/μl. If ANC was >999/μl and <1500/μl, or platelet count $\geq$ 75 000/μl and <100 000/μl, irinotecan was not administered and the next infusion was given at the same dose in case of blood count recovery (ANC $\geq$ 1500/μl and platelet count $\geq$ 100 000/μl). If a new suspension was required, the following irinotecan infusions were reduced to 75% of the initial dose. No further dose reductions were allowed. If ANC was <1000/μl or platelet count <75 000/μl, irinotecan was not administered and the following infusions were reduced to 75% of the initial dose, once blood count values were recovered. No further dose reductions were allowed. If more than two successive dose suspensions were required, the patient left the study.

Irinotecan was given on the scheduled date and at full dose if nonhematologic toxicity was of grade 2 or lower. In the case of nonhematologic toxicity greater than 2 (except for alopecia and nausea, vomiting, or diarrhea not properly treated) registered at the date scheduled for irinotecan administration, irinotecan infusion was suspended and the following dose was given if nonhematologic toxicity was of grade 1 or lower or biochemical values did not exceed those specified in the inclusion criteria. If more than two successive dose suspensions were required, the patient left the study.

Response evaluation

All patients who completed two cycles of treatment were evaluable for response. Determination of response was assessed by MRI in accordance with Macdonald's criteria [21], which takes into account changes in the size of the enhancing tumor, along with corticosteroid requirements and neurologic clinical changes.

Results

Patient characteristics

A total of 12 patients from six hospitals were enrolled in this phase I study, over 12 months. Nine patients were evaluable for response at the end of the observation period. There were seven men and five women, with a median age of 58 years. Most patients had a Karnofsky performance status score of at least 90. Patient characteristics are detailed in Table 2.

Toxicity: three patients were included at dose level 1. None of them developed DLT; therefore, subsequent patients were enrolled at dose level 2. Three patients enrolled at dose level 2 progressed before reaching the eighth day of the third treatment cycle, and were thus not evaluable for toxicity, and were replaced by three new patients. Only one patient, enrolled at dose level 1, had a treatment delay within the period established to determine the DLT; in this patient, the third cycle was delayed by 1 week due to ANC of 800/μl.

Three patients experienced DLT during the toxicity evaluation period. One of the first three evaluable patients included at dose level 2 experienced dose-limiting grade 3 diarrhea after the first infusion of irinotecan. Although the patient recovered in less than 1 week with loperamide and dietary measures, diarrhea reappeared after the next two infusions of irinotecan and the patient left the study. Therefore, this cohort was expanded to six evaluable patients. Among the three new evaluable patients treated at dose level 2, one patient developed grade 4 lymphopenia and neutropenia, grade 3 thrombocytopenia, and grade 1 anemia on day 16 of the first cycle; he died the day after from a grade 5 pneumonia with positive serology for *Legionella*. Another patient developed grade 3 febrile neutropenia during the second cycle, with negative blood and urine cultures, and he recovered with standard broad-spectrum antibiotics. With three of six evaluable patients having experienced DLT at dose level 2, we considered 100 mg/m^2 (corresponding to dose level 1) as the MTD of irinotecan. Hence, we selected this dose for a subsequent phase II study with this treatment schedule. Table 3 summarizes the toxicities of all patients included in this phase I study.

Table 2 Patient characteristics

Characteristics	
Total number	12
Age (years)	
Median (range)	58 (44–68)
Sex	
Male	7
Female	5
KPS (number of patients)	
70	4
90	6
100	2
Prior cycles of TMZ	
Median (range)	8.5 (3–16)
Time from surgery to progression (months)	
Median (range)	9.3 (6.8–28.9)

KPS, Karnofsky performance status score; TMZ, temozolomide.

Tumor response

At the end of the observation period, nine patients were evaluable for response: one patient achieved a partial response with a duration of 8 months, and four patients remained stable at 4, 4, 6, and 6 months, respectively. Four patients had disease progression.

Discussion

This trial showed that the combination of daily TMZ and irinotecan every 2 weeks is feasible and well tolerated. At the recommended dose (100 mg/m^2) of irinotecan, toxicity was manageable. At 115 mg/m^2 irinotecan, three evaluable patients experienced DLT; one of them developed severe hematologic toxicity and died of *Legionella*-related pneumonia.

Table 3 Toxicities during the evaluation period

	Level 1				Level 2				
	Number of evaluable patients				Number of evaluable patients (total number of patients[a])				
Toxicity	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Hematologic									
Anemia	1	–	–	–	2 (4)	–	–	–	–
Lymphopenia	–	–	–	–	2 (4)	1	2 (3) 1 (2)	1[b]	–
Neutropenia	1	–	–	–	2 (3)	1	–	1[b]	–
Thrombocytopenia	1	–	–	–	1 (2)	–	1[b]	–	–
Nonhematologic									
Fatigue	1	2	–	–	2	1	–	–	–
Constipation	–	–	–	–	1	–	–	–	–
Diarrhea	–	–	–	–	1	–	1	–	–
Stomatitis	–	1	–	–	–	–	–	–	–
Febrile neutropenia	–	–	–	–	–	–	1	–	–
Vomiting	1	–	–	–	2	1	–	–	–
Pneumonia	–	–	–	–	–	–	–	–	1[b]

[a]Three patients were not evaluable for toxicity because they progressed during the evaluation period. In such cases, a new patient was enrolled at the same dose level.
[b]Dose-limiting toxicities: patient 7: fatal pneumonia with grade 4 neutropenia and lymphopenia, and grade 3 thrombocytopenia.

Many studies have explored the activity of TMZ given in protracted schedules [22–28]. In a study by Perry *et al.* [23], TMZ was given daily at a dose of 50 mg/m^2, a schedule very similar to that used in the present study, though administered in a single daily dose. Although, in general, extended-dose TMZ regimens are well tolerated, they may result in prolonged lymphopenia with the risk of opportunistic infections, especially in patients receiving corticosteroids [27,28]. Nevertheless, the treatment combination proposed in the present study seems to be safe at the recommended doses.

The combination of TMZ administered at 150 or 200 mg/m^2/day for 5 days and irinotecan at varying doses has already been explored in two phase I studies in patients with relapsed gliomas. The DLTs of both studies taken together were hematologic, hepatic, nausea, vomiting, and diarrhea [29,30]. One of these treatment schedules was used in a phase II study, in which up to three 6-week cycles were given before radiotherapy in patients with newly diagnosed glioblastoma; the standard 5-day TMZ dosing was combined with irinotecan given on days 1, 8, 22, and 29. Of the 42 patients included, eight (19%) achieved a partial response. Grade 3–4 toxicity was observed in 36% of patients [31]. In another phase II trial in patients with recurrent high-grade glioma, most of the 22 patients enrolled were treated with the same TMZ 5-day schedule, followed by irinotecan 350 mg/m^2 on day 6 every 4 weeks; 28% of the patients with glioblastoma reached a partial response and toxicity was manageable [32].

As irinotecan plasma levels are strongly influenced by the concomitant administration of EIAEDs, efforts have been made to determine the adequate irinotecan dosing for patients receiving or not receiving these drugs [14]. When designing the present study, we considered that the non-EIAEDs currently available in the clinical practice allowed us to establish the treatment with enzyme-inducing drugs as an exclusion criterion. Irinotecan has been tested in patients with high-grade glioma, mainly using two dosing schedules: once every 3 weeks or once weekly for 4 weeks followed by a 2-week rest. In patients with recurrent glioblastoma, the first schedule resulted in a response rate of 4–6% and a maximum stabilization rate of 37% [15,33]. The relative high response rate (17% in patients with glioblastoma) obtained by Friedman *et al.* [13] with the weekly schedule was not confirmed in a subsequent study, in which only one partial response and five stabilizations were obtained in 18 patients with high-grade glioma [34]. To combine with metronomic TMZ, we have chosen a biweekly administration of irinotecan, a dosing schedule widely used in combination with bevacizumab at a dose of 125 mg/m^2 [2,35].

In summary, this phase I study shows that the combination of metronomic TMZ and irinotecan every 2 weeks can be safely administered at the recommended doses, and might be active in patients with recurrent glioblastoma. Hence, a phase II study with this combination was started; that trial has completed accrual, and results will soon be available and presented in a separate publication.

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Conflicts of interest

Consultant or Advisory Role: Gaspar Reynés (Pfizer Pharmaceuticals, Merck Sharp & Dohme Corp.). For the remaining authors there are no conflicts of interest.

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7.4. Artículo 4

Phase II trial of irinotecan and metronomic temozolomide in patients with recurrent glioblastoma.

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Phase II trial of irinotecan and metronomic temozolomide in patients with recurrent glioblastoma

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Objective **This phase II study was conducted to determine the efficacy and safety of metronomic temozolomide (TMZ) in combination with irinotecan in glioblastoma (GB) at first relapse.**

Patients and methods **Patients with GB at first relapse received TMZ 50 mg/m^2/day divided into three doses, except for a single 100 mg/m^2 dose, administered between 3 and 6 h before every irinotecan infusion. Irinotecan was given intravenously at the previously established dose of 100 mg/m^2 on days 8 and 22 of 28-day cycles. Treatment was given for a maximum of nine cycles or until progression or unacceptable toxicity occurred. Vascular endothelial growth factor and its soluble receptor 1, thrombospondin-1, microparticles, and microparticle-dependent procoagulant activity were measured in blood before treatment. The primary objective was 6-month progression-free survival (PFS).**

Results **Twenty-seven evaluable patients were enrolled. Six-month PFS was 20.8%. Median PFS was 11.6 weeks (95% confidence interval: 7.5–15.7). Stable disease was the best response for nine (37.5%) patients, with a median duration of 11.2 weeks (4.2–35.85 weeks). No differences in PFS or response were observed among patients who relapsed during or after completion of adjuvant TMZ. Grade 3/4 adverse events included lymphopenia (15%), fatigue, diarrhea and febrile neutropenia (3.7% each), lymphopenia, neutropenia, and nausea/vomiting (11.1% each). One patient died from pneumonia and one patient died from pulmonary thromboembolism. Pretreatment levels of angiogenesis biomarkers, microparticles, and microparticle-related procoagulant activity were elevated in patients compared with healthy volunteers.**

Conclusion **This regimen is feasible, but failed to improve the results obtained with other second-line therapies in recurrent GB.** ***Anti-Cancer Drugs*** **00:000–000**

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Keywords: irinotecan, metronomic chemotherapy, phase II, recurrent glioblastoma, temozolomide

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Introduction

The prognosis of glioblastoma (GB) remains poor. Standard therapy with surgery followed by radiation therapy and temozolomide (TMZ) provides a median progression-free survival (PFS) of around 7 months and a median overall survival of less than 15 months [1], although it varies according to the methylation status of the O6-alkylguanine-DNA-methyltransferase (*MGMT*) gene promoter [2]. Therefore, treatment of recurrent disease remains an important goal.

Treatment options for recurrent disease are limited [3], making obvious the need to explore new therapeutic strategies. Several studies have been carried out using dose-dense, extended TMZ, including treatment for 21 days every 28 days or every other week [4,5]. In 2008, Perry *et al.* [6] reported the preliminary results of TMZ at a continuous dose of 50 mg/m^2 in patients with recurrent GB. Patients were stratified into three groups, according to timing of recurrence related to the period of adjuvant TMZ. In the final report [7], 6-month PFS was 7.4% for patients who progressed while receiving adjuvant TMZ beyond six cycles, and 35.7% for patients who progressed after completion of adjuvant TMZ with a treatment-free interval more than 2 months.

Irinotecan has demonstrated clinical activity in recurrent GB, reaching 15% objective response in one study [8]. TMZ and irinotecan exert antitumoral activity through different mechanisms and have different toxicity profiles. Furthermore, preclinical data have demonstrated that the combination of TMZ and irinotecan has a synergistic and schedule-dependent antitumor activity [9]. Moreover, the cytotoxic effect of this combination seems to be

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independent of the methylation status of the *MGMT* gene promoter [10]. We recently carried out a phase I trial designed to find the maximum tolerated dose of irinotecan given every 2 weeks, combined with a fixed and continuous dose of TMZ, in patients with GB at first relapse [11]. We now report a phase II, multicenter clinical trial conducted by the Spanish Neuro-Oncology Research Group (Grupo Español de Investigación en Neurooncología, GEINO). The trial was designed to determine the efficacy and toxicity of this treatment schedule.

The primary objective of the study was to determine the PFS rate at 6 months. Secondary objectives included response rate and toxicity. Moreover, the study sought to evaluate the influence of plasma concentration of thrombospondin-1 (TSP-1), vascular endothelial growth factor (VEGF), soluble vascular endothelial growth factor receptor 1 (sVEGFR-1), and microparticles (MPs) on clinical outcome.

Patients and methods

Eligibility

To enter the study, patients were required to be at least 18 years old, have a Karnofsky Performance Status (KPS) score 70 or more, and a histologically confirmed GB at first recurrence. Initial standard treatment [1] with radiotherapy and concomitant TMZ, followed by a minimum of three cycles of adjuvant TMZ, was also mandatory. The diagnosis of recurrence was established by an MRI performed within 3 weeks before starting treatment, according to Macdonald's criteria [12]. Additional requirements included adequate bone marrow and renal and hepatic function, as previously described [11].

Patients were excluded if they were pregnant or breast-feeding, and if they had other malignancies, except basal cell carcinoma or cervical carcinoma *in situ*. Simultaneous treatment with drugs having a potent inhibitory or inducing activity on cytochrome P450 (CYP450) enzymes was not permitted. Enzyme-inducing antiepileptic drugs had to be replaced by non-enzyme-inducing antiepileptic drugs, at least 1 week before starting the protocol treatment. The study was approved by the ethics committees of the participating centers. To have a normal reference of circulating biomarkers, a control group was included. The control group was composed of healthy individuals from the same demographic area, whose age and sex matched those of the patients. Informed consent was obtained from all patients and healthy volunteers.

Study design and treatment plan

All patients received oral TMZ at a fixed and continuous dose of 50 mg/m^2 divided into three daily intakes, except for a single 100 mg/m^2 dose, administered between 3 and 6 h before every irinotecan infusion. The calculated dose was rounded to the nearest 5 mg. Irinotecan was given intravenously at the previously established dose of 100 mg/m^2 on days 8 and 22 of 28-day cycles. Antiemetic drugs, preferably metoclopramide, were allowed in case of nausea or vomiting. Oral serotonin antagonists were permitted when necessary. Before every irinotecan infusion 0.25 mg subcutaneous atropine and an intravenous serotonin antagonist were administered. Prophylactic administration of granulocyte-colony stimulating factors was not allowed. Erythropoietin and red blood cell or platelet transfusions could be administered. Treatment was given for a maximum of nine cycles or until progression or unacceptable toxicity occurred.

Surveillance and follow-up

Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria, Version 3.0. The baseline examinations were performed within 3 weeks before the start of treatment and included complete blood count, serum biochemistry profile (SBP), blood sampling for assessment of circulating biomarkers, brain MRI, physical examination, KPS, Barthel Index, and chest radiograph. Evaluation of complete blood count, SBP, and KPS, as well as assessment of toxicity, was repeated on days 8 and 22 of every treatment cycle. An SBP was repeated on day 8 of every cycle. A brain MRI and a Barthel Index were performed on day 8 of odd cycles, as from the third cycle. Dose delays, reductions, and discontinuations were applied according to the protocol, and have been detailed elsewhere [11]. Objective responses were assessed following Macdonald's criteria [12].

Blood sampling

For the determination of MP levels and MP-mediated procoagulant activity, blood was collected in a BD Vacutainer tube (BD, Franklin Lakes, New Jersey, USA) containing sodium citrate (129 mmol/l) at a ratio of 1 : 9 (v/v, sodium citrate/blood) and in dry BD Vacutainer tubes for the determination of angiogenic biomarkers. After 30–90 min, blood samples were centrifuged at 1500*g* for 15 min at room temperature to obtain plasma or serum, which was divided into 200 µl aliquots and stored at −80°C for later batch analysis.

Biomarker analysis

Total serum levels of VEGF and sVEGFR-1 were determined by ELISA according to the manufacturers' instructions [VEGF (Biosource International, Camarillo, California, USA) and Quantikine Human sVEGFR1 (R&D Systems, Minneapolis, Minnesota, USA)]; the coefficients of variation were 6.5 and 7.0%, respectively. TSP-1 levels were quantified by an indirect ELISA, as described elsewhere [13]; the coefficient of variation was 4.8%.

Plasma MPs were quantified by flow cytometry in an EPICS XL Cytometer (Beckman Coulter, Brea, California,

USA) at high flow rate. Plasma was incubated with FITC–Annexin V conjugate (TACS Annexin V; Trevigen Inc., Gaithersburg, Maryland, USA) to detect accessible phosphatidylserine on MP membranes, as previously described [14].

The MP-mediated procoagulant activity of plasma was analyzed by thrombin generation assay without added exogenous tissue factor or phospholipids (Calibrated Automated Thrombogram; Thrombinoscope BV, Paris, France), as previously described [15].

Statistical analysis

Among patients with recurrent GB treated with extended-dose TMZ, 6-month PFS varies widely, reaching 20% or more in most studies. The present study was initially designed to include 50 patients, assuming a 6-month PFS of 22% with a 95% confidence interval (CI) between 10.5 and 33.5%. Nevertheless, because of low recruitment, the protocol was modified to reduce the number of patients. On the basis of published data of dose-extended TMZ [4], a 6-month PFS of 30% was considered achievable; therefore, an amendment to the protocol was approved to reduce the number of patients to 30, resulting in a 95% CI between 13.6 and 46.4% for an estimated 6-month PFS of 30%.

PFS was analyzed by the Kaplan–Meier method. Results are given as mean ± SD for continuous variables. The Kolmogorov–Smirnov test was used to evaluate whether each parameter came from a normal distribution. The Mann–Whitney *U*-test was conducted to analyze the independent relationship of healthy individuals with patients. Bivariated correlation was performed using the Spearman correlation test. All statistical calculations were performed using SPSS software (v.15.0; SPSS Inc., Chicago, Illinois, USA). *P* values less than 0.05 were considered statistically significant.

Results

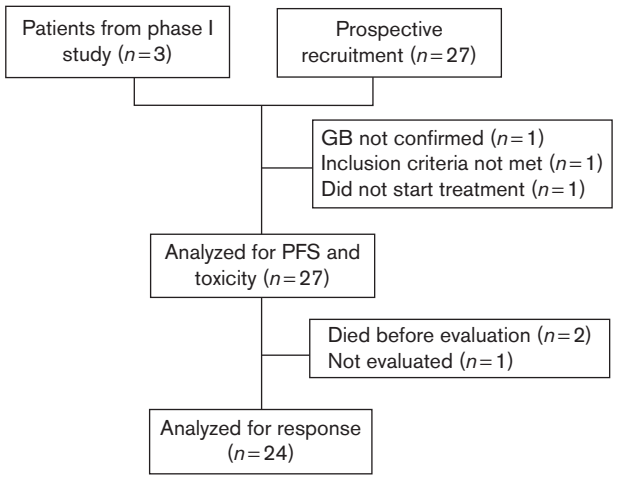
Patient recruitment and demographics

A total of 30 patients from eight centers were initially enrolled. Three patients had been included in the previous phase I trial and had received the dose of irinotecan selected for the phase II trial – that is, 100 mg/m^2. The remaining patients were recruited over 16 months. Patient flow is depicted in Fig. 1. Three patients were excluded from the study: in one the diagnosis of GB was not confirmed; another patient did not meet all inclusion criteria; and the other did not start treatment after signing the informed consent form. Clinical patient characteristics are shown in Table 1.

Progression-free survival

Twenty-seven patients started treatment. At 6 months, five (20.8%) patients remained progression free. Median PFS was 11.6 weeks (95% CI: 7.5–15.7) (Fig. 2).

Fig. 1



Patient flow. GB, glioblastoma; PFS, progression-free survival.

Table 1 Patient characteristics

Characteristics	Value
Total number of patients	27
Age [median (range)] (years)	56 (42–77)
Male	19
Female	8
Barthel Index	
50–70	4
80–90	9
95–100	10
ND	4
KPS score	
70	10
80	6
90	8
100	3
Adjuvant TMZ	
Median number of cycles (range)	6 (3–15)
Time of recurrence from surgery [median (range)] (months)	9.2 (5.5–28.6)
Number of patients at recurrence	
During adjuvant TMZ	18
< 3 months after adjuvant TMZ	2
3–6 months after adjuvant TMZ	1
> 6 months after adjuvant TMZ	6

KPS, Karnofsky Performance Status; ND, no data; TMZ, temozolomide.

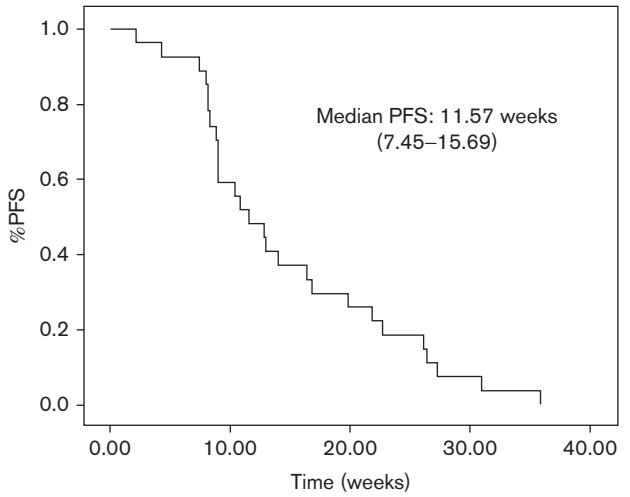
Response

Twenty-four patients were evaluable for response. No partial or complete responses were observed. Stable disease was the best response in nine (37.5%) patients, with a median duration of 11.2 weeks (4.2–35.85 weeks). No differences in PFS or response were observed among patients who relapsed during or after completion of adjuvant TMZ.

Toxicity

All 27 patients were assessable for toxicity. One patient died from pneumonia on day 16 of the first cycle. Another patient died on cycle 3 after an episode of acute dyspnea clinically compatible with pulmonary thromboembolism.

Fig. 2



Kaplan–Meier curve of progression-free survival (PFS).

No opportunistic infections were observed. Table 2 shows all grades of toxicity registered.

Circulating marker levels

Table 3 summarizes the levels of the angiogenesis markers (VEGF, VEGFR-1, TSP-1) and MPs in patients before the start of the study treatment, and in controls. Among the angiogenesis markers, VEGF levels ($P < 0.05$) and VEGFR-1 levels ($P < 0.001$) were significantly elevated in patients. Levels of MPs and their procoagulant activity were also higher in patients than in controls. No correlations have been found among the biomarkers studied. Moreover, no associations were found between marker levels and clinical outcome.

Table 2 Toxicity

	N (%)				
Toxicities	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Hematologic					
Anemia	3 (11.1)	1 (3.7)	–	–	–
Lymphopenia	–	3 (11.1)	4 (14.8)	1 (3.7)	–
Neutropenia	1 (3.7)	2 (7.4)	3 (11.1)	–	–
Thrombocytopenia	1 (3.7)	1 (3.7)	–	–	–
Nonhematologic					
Fatigue	6 (22.2)	7 (25.9)	1 (3.7)	–	–
Constipation	3 (11.1)	2 (7.4)	–	–	–
Diarrhea	2 (7.4)	3 (11.1)	1 (3.7)	–	–
Stomatitis	2 (7.4)	–	–	–	–
Febrile neutropenia	–	–	1 (3.7)	–	–
Nausea/vomiting	6 (22.2)	1 (3.7)	3 (11.1)	–	–
Pneumonia	–	–	–	–	1 (3.7)
Dyspnea[a]	–	–	–	–	1 (3.7)

[a]A patient presented with acute dyspnea clinically compatible with pulmonary thromboembolism, and died.

Table 3 Plasma levels of angiogenesis markers and microparticles

Markers	Patients	Controls	*P*
VEGF (pg/ml)	234 ± 133	147 ± 50	0.002
VEGFR-1 (pg/ml)	116 ± 38	72 ± 17	< 0.001
TSP-1 (μg/ml)	56 ± 25	46 ± 10	0.1
MP (events/μl)	4698 ± 5021	1840 ± 2060	0.02
TG (nmol/l)	243 ± 125	158 ± 58	<0.01

Data are presented as mean ± SD.
MP, microparticle; TG, endogen thrombin generation; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor 1.

Discussion

The rationale for this phase II study was the synergistic effect of TMZ and irinotecan that could result in improved results compared with other continuous-dose TMZ schedules. Nevertheless, although the 6-month PFS reached in the present study is superior to that reported in some phase II studies with dose-dense TMZ [16,17], it was not superior to other regimens used for the treatment of recurrent GB. The primary objective of the study, 6-month PFS, was 20.8%, and the median PFS was 10.4 weeks. In a review article [18], rechallenge with TMZ has been analyzed in two different settings: in patients experiencing progressive disease during TMZ therapy who were rechallenged with alternative TMZ regimens, and in patients rechallenged after a TMZ-free interval of at least 8 weeks. PFS-6 rates were 27.7 and 28.6%, respectively. Second-line treatment with the nitrosourea fotemustine has yielded 6-month PFS rates between 35 and 39%, even in elderly patients [19,20]. In a phase II study [21], bevacizumab has been used in monotherapy and in combination with irinotecan; reported 6-month PFS was 43 and 50%, respectively, although these results should be interpreted cautiously, given the difficulty of assessing response to antiangiogenic drugs. In another phase II study [22], monotherapy with bevacizumab resulted in a 6-month PFS of 29%.

Interestingly, grade 3 and 4 lymphopenia, a characteristic hematologic toxicity of metronomic TMZ regimens [23], has been registered in less than 20% of patients, and no opportunistic infections occurred, despite the lack of specific prophylaxis. However, two patients died because of adverse events (one from pneumonia and another one from a likely pulmonary thromboembolism), raising doubts about the safety of treatment.

Metronomic chemotherapy exerts antiangiogenic effect through different mechanisms [24]. Several studies *in vitro* and *in vivo* have shown that metronomic TMZ inhibited proliferation, adhesion, and migration of glioma-associated endothelial cells and human umbilical vein endothelial cells [25–27], and inhibited MGMT expression in human umbilical vein endothelial cell [26] compared with conventional TMZ dosing. Nevertheless, Virrey *et al.* [28], using different assays *in vitro* and *in vivo*,

observed no effect of TMZ on proliferation or migration of glioma-associated endothelial cells.

Consistent with previous results in newly diagnosed patients with GB [29], VEGF and VEGFR-1, but not TSP-1, were elevated in this series of patients at first relapse. MP levels and MP-related procoagulant activity were also elevated. We had previously observed increased numbers of MPs in patients with GB after surgery, before starting radiation therapy and TMZ [15]. Nevertheless, none of these markers showed associations with clinical outcome.

In summary, this regimen of irinotecan and metronomic TMZ was well tolerated, but failed to improve significantly the results obtained with other second-line therapies in recurrent GB.

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Conflicts of interest

Gaspar Reynés has an advisory role in Pfizer Pharmaceuticals and in Merck, Sharp & Dohme Corp. For the remaining authors there are no conflicts of interest.

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