UNIVERSIDAD DE OVIEDO

PROGRAMA DE DOCTORADO

Investigación en Medicina

Caracterización de los componentes celulares y moleculares del microambiente inflamatorio y su implicación en el desarrollo de metástasis en el cáncer de mama

Noemí Eiró Díaz

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Abreviaturas

ADAM:	Desintegrina y metaloproteasa
ADAMTS:	Desintegrina y Metaloproteasa con motivos de la trombospondina
ADN	Ácido desoxirrubonucleico
AI:	Inhibidores de la aromatasa
AJCC:	American Joint Committee on Cancer
ASCOG:	American College of Surgeons Oncology Group
ATM:	Ataxia telangiectasia mutated
BCL-2	B-cell lymphoma 2
bcl-xl:	B-cell lymphoma-extra large
BRCA-1:	Breast Cancer-1
BRCA-2:	Breast Cancer-2
CCL3:	Quimioquina CCL3
CD:	Cluster Differentiation
c-erbB2:	Oncogen erbB2
c-FLIP:	Proteína inhibidora de FLICE
c-fos:	Proto-oncogén fos
CHEK2:	Cell-cycle-checkpoint kinase 2
c-jun:	Proto-oncogén jun
CMIs:	Células mononucleares inflamatorias
c-myc:	Proto-oncogén myc
COX-2:	Ciclooxigenasa-2
CSF:	Factor estimulante de colonia
cTNM:	Clasificación TNM basada en datos clínicos
EGFR:	Receptor del factor de crecimiento epidérmico
FISH:	Hibridación *in situ* fluorescente

GLAMs:	Ganglios linfáticos axilares metastáticos
HER2/neu:	Receptor 2 del factor de crecimiento epidermal humano
IAP-1:	Proteína inhibidora de la apoptosis 1
IAP-2:	Proteína inhibidora de la apoptosis 2
IFNβ:	Interferon β
IL:	Interleuquina
MATs:	Macrófagos asociados a tumores
MMP:	Metaloproteasa de la matriz
MoAb:	Anticuerpo monoclonal
Myd88:	Gen de respuesta primaria de diferenciación mieloide 88
NFκB:	Factor de transcripción nuclear κB
NIH:	National Institutes of Health
NPI:	Índice pronóstico de Nottingham
OSNA:	One-step-nucleic acid amplification
PAI-1:	Inhibidor del plasminógeno-1
PTEN:	Phosphatase and tensin homolog
pTNM:	Clasificación TNM basada en datos anatomo-patológicos
RE:	Receptores de estrógenos
RP:	Receptores de progesterona
SBR:	Scarf-Bloom-Richardson
STK11:	Serine-threonine kinase 11
Th17:	Células T helper 17
TIMPs:	Inhibidores tisulares de MMPs
UICC:	Unio Internationalis Contra Cancrum
uPA:	Uroquinase type plasminogen activator
VEGF:	Factor de crecimiento endotelial vascular
VEGFR:	Receptor factor de crecimiento del endotelio vascular
XIAP:	Inhibidor de la apoptosis ligado al cromosoma X

Introducción

I Historia del cáncer de mama

El papiro de Edwin Smith, así llamado por haber sido adquirido por este egiptólogo en 1862 en un mercado, es sin duda el documento médico más antiguo del que se tenía conocimiento en el mundo, pues data de unos 3000-2500 años antes de Cristo (a.C.) (Hajdu S.I., 2011). En ese documento aparece la primera descripción escrita de un cáncer, concretamente se describen ocho casos de cáncer de mama tratados con cauterización, pero con alusión a su incurabilidad cuando el tumor es sangrante, duro e infiltrante.

Quince siglos más tarde (1500 a.C.), en el papiro de Ebers se describe por primera vez el cáncer de mama con metástasis axilar, evocando además la posibilidad terapéutica mediante procedimientos quirúrgicos, medicamentosos o por ignición. Este autor insistió en no tratar el cáncer extendido y profundo, dado que las mujeres tratadas morían antes que las mujeres que no recibían tratamiento (Hajdu S.I., 2011).

El término "cáncer" se debe a Hipócrates (460–375 a.C.), quien nombró la enfermedad como *karkinos/karkinoma*, comparándola a la acción destructora que produce un cangrejo en los tejidos blandos de su víctima. Así, instauró la teoría de que dicha enfermedad era consecuencia del desorden entre la bilis negra, la bilis amarilla, y la flema (teoría humoral). Además, en sus escritos describió la enfermedad mamaria tumoral diseminada, y atribuyó el origen del cáncer mamario al cese de la menstruación. Siguiendo las ideas de Ebers, Hipócrates defiende no tratar los cánceres de mama ocultos (no ulcerados) porque se acelera la muerte de las enfermas (Lewison E.F., 1953; Hajdu S.I., 2011).

Posteriormente, el físico romano Aulus Cornelius Celsus (25 a.C.-50 d.C.), autor de *De re medica* y conocido por sus estudios sobre el origen de *tumor, calor y dolor*, ofrece la primera descripción clínica del cáncer de mama.

Además, llevó mas adelante la teoría de Hipócrates, estableciendo que sólo los tumores que ocupan menos de la mitad de la glándula mamaria, deben ser extirpados. En sus escritos, recopila los aspectos más importantes de la enfermedad tumoral mamaria desde el punto de vista quirúrgico en la época de Roma, dejando constancia que el cáncer de mama avanzado tiene una tendencia a reaparecer en la axila, con o sin hinchazón del brazo, y puede causar la muerte por la propagación a órganos distantes (Lewison E.F., 1953; Hajdu S.I., 2011).

En el siglo II d.C., Galeno (121-201), adopta la teoría humoral de Hipócrates y establece que el cáncer de mama escirro es causado por una acumulación del "humor espeso y lento". Así, aconseja tratamientos locales en los casos ulcerados y los casos ocultos. Sin embargo, si el tumor es voluminoso la única indicación es la cirugía, la cual se lleva a cabo de forma amplia, cauterizando los vasos. La mayor parte de los postulados de Galeno permanecieron en vigor hasta el siglo XVI, ya que la medicina antigua no generó ninguna contribución original posterior (Hajdu S.I., 2004; Hajdu S.I., 2005).

Con el renacimiento, el instrumento cortante fue tomando más protagonismo frente al cauterio. En el siglo XVI, Andrés Vesalio (1514-1564) realiza la extirpación del seno afecto y controla la hemorragia mediante ligaduras. Ambroise Paré (1510-1590) ofrece una detallada descripción clínica del cáncer de mama y utiliza también la ligadura vascular en cirugía. En el siglo XVII, el filósofo francés René Descartes (1596-1650) y el astrónomo italiano Galileo Galilei (1564-1642) retomaron de nuevo la teoría según la cual el cáncer es una "enfermedad general". Descartes señaló la linfa como el principal causante del *male oscuro*, en lugar de la bilis negra, considerándola como el fluido corporal donde el tumor tiene su origen, y la cual puede coagularse con relativa rapidez en cualquier lugar en el organismo y generar un cáncer localizado y claramente delimitado (Hajdu S.I., 2011).

Marco Aurelio Severino (1580-1634), fue el primer cirujano en comparar de forma rutinaria los hallazgos quirúrgicos y los patológicos. Este autor, distingue por primera vez, entre la evolución benigna y maligna de los tumores, postulando que cuando el tumor está fijado a la pared torácica es maligno, y cuando se puede mover libremente es benigno (fibroadenoma). Además, abogó por la escisión quirúrgica de los tumores benignos debido a la posibilidad de que puedan convertirse en cancerosos (Hajdu S.I., 2011).

En el comienzo de la Era de la Ilustración, a principios del siglo XVIII, los cirujanos se hacen más científicos, consiguen el título de médico y se separan de los barberos, quienes eran los que practicaban la cirugía de forma empírica hasta ese momento. En esta misma época, se desarrolla la idea de que el tumor mamario se puede diseminar por los vasos sanguíneos y linfáticos hacia los ganglios axilares, los pulmones, los huesos, y el cerebro.

El primer presidente de la Academia Francesa de Medicina, Jean-Louis Petit (1674-1760), fue el que más influyó en la evolución técnica de las intervenciones mamarias. Estableció el criterio de que el origen del cáncer de mama está en los ganglios axilares, por lo que la mama, el músculo pectoral mayor, y los ganglios axilares, deben ser extirpados en todos los casos. Esta teoría se convirtió en la base de la mastectomía radical clásica (Hajdu S.I., 2011).

Durante la segunda mitad del siglo XIX surgieron nuevos conocimientos sobre el cáncer de mama. En 1860, Rudolf Virchow (1821-1902), aplica los conceptos celulares al estudio de la histología tumoral, concluyendo que el crecimiento de los tejidos se realiza por división de células preexistentes, y que el origen de los tumores malignos es debido a cambios en el tejido conectivo (Hajdu S.I., 2012). En 1894, William Halsted (1852-1922) introdujo la mastectomía radical en Estados Unidos. Inicialmente, este procedimiento conllevaba la extirpación del músculo pectoral mayor, pero poco después se extirpaba ambos pectorales, vaciando la axila, la fosa supraclavicular y

subclavicular. Los resultados obtenidos con este procedimiento fueron muy superiores a los obtenidos hasta entonces, incluyendo una importante disminución de la mortalidad, por lo que la mastectomía radical se convirtió en la operación habitual para el tratamiento del cáncer de mama hasta bien entrado el siglo XX (Hajdu S.I., 2012).

La década de los años 1960 y 1970 marca una auténtica revolución en el conocimiento y tratamiento del cáncer de mama. La introducción de nuevas especialidades como la oncología y la radioterapia, y la realización de estudios de biología molecular dan un nuevo giro en el manejo de esta enfermedad. Se inicia la época de la cirugía conservadora, comienza el desarrollo de las terapias hormonales, y se aborda una alternativa a la disección axilar completa. De esta forma, el cáncer de mama es ya considerado una enfermedad sistémica.

II Fisiología de la mama

En los humanos la diferenciación embrionaria de la glándula mamaria es igual en el hombre que en la mujer. Sin embargo, a diferencia de en la glándula mamaria masculina, en la glándula mamaria femenina ocurren grandes modificaciones estructurales y funcionales relacionadas con el estado hormonal y la fisiología del sistema reproductor, que la convierten en un órgano complejo.

Las variaciones de la estructura histológica de la glándula se producen durante la pubertad, el ciclo menstrual, el embarazo y la menopausia. La mama de una mujer en edad fértil, no embarazada ni lactante, se denomina mama en reposo y es la que se describirá a continuación.

La mama en la mujer adulta está formada por un conjunto de 15 a 20 glándulas exocrinas túbulo-alveolares compuestas que forman los lóbulos. Estas

glándulas son independientes y están separadas por tejido conjuntivo denso y tejido adiposo. Estas estructuras, que tienen individualmente una parte secretora y un conducto excretor o galactóforo, se nombran unidad ducto-lobulillar terminal y constituyen la unidad estructural y funcional de la mama (Figura 1).

Cada lóbulo está integrado por los lobulillos, los conductos intralobulillares y el conducto terminal, que llega al conducto segmentario y finalmente se encuentran en el conducto galactóforo. Las ramificaciones van formando una estructura que se asemeja al árbol bronquial. El conducto galactóforo se dilata a nivel de la areola dando lugar al seno galactóforo donde se almacena la leche (Figura 2).

El epitelio de los conductos es diferente en cada tramo. Los interlobulares están revestidos por epitelio cilíndrico simple, el cual va disminuyendo de altura gradualmente hasta convertirse en cúbico simple en los conductos excretores intralobulillares más pequeños. Entre el epitelio y la membrana basal existe una capa de células mioepiteliales.

Los lóbulos están formados por los lobulillos, los cuales están rodeados por tejido conectivo laxo con capacidad de responder a las hormonas, y por tejido adiposo. Sin embargo, los lóbulos están separados por tejido conectivo denso. Los lóbulos no son estructuras completamente independientes, puesto que presentan cierto entrecruzamiento y están sujetos a la piel suprayacente por gruesas bandas de tejido conectivo, denominadas ligamento suspensorio o ligamentos de Cooper. Las porciones terminales secretoras de la glándula se componen de alvéolos revestidos por epitelio cúbico o cilíndrico con células mioepiteliales ramificadas ubicadas entre el epitelio glandular y la membrana basal (Stirling J.W. and Chandler J.A., 1976).

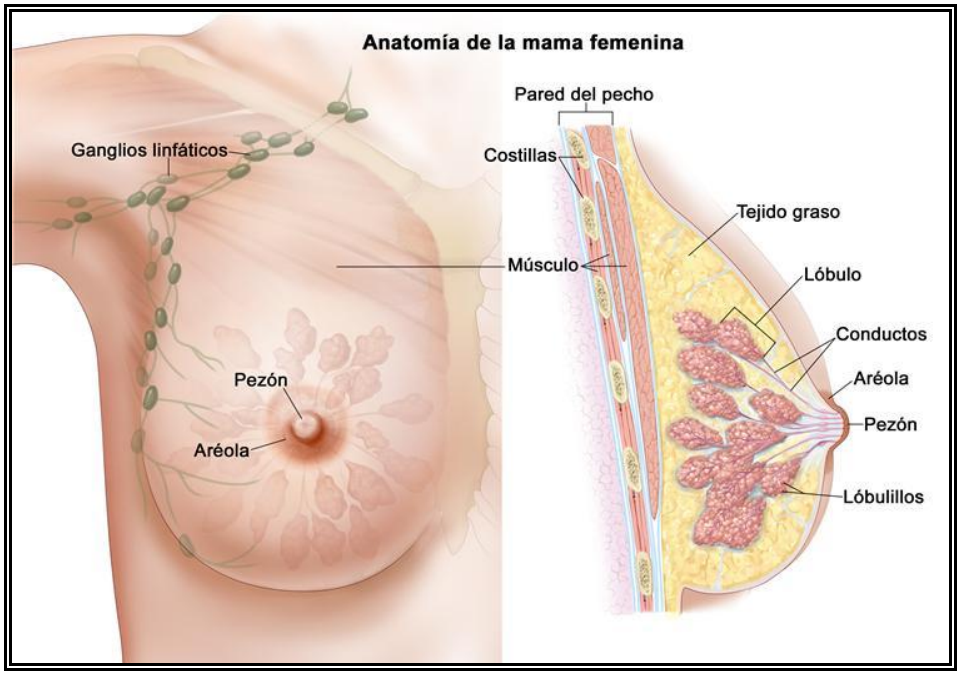

Figura 1: Estructura de la mama. (*Fuente: www.cancer.org*)

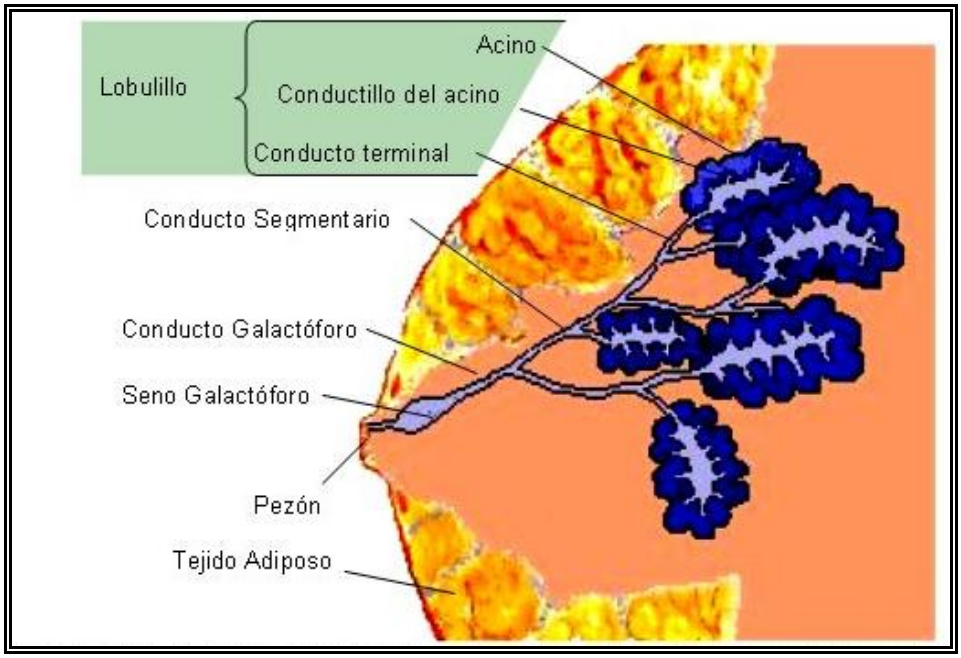

Figura 2: Anatomía del lobulillo y conductos mamarios. (*Fuente www.mastologia.net:*)

III Epidemiología del cáncer de mama

III.1 Incidencia

Según el estudio GLOBOCAN 2008, el cáncer de mama es el tumor más frecuente y la principal causa de muerte por cáncer en las mujeres de todo el mundo, representando el 23% (1.380.000) del total de nuevos casos de cáncer (Figura 3) (Jemal A. et al., 2011). Excluyendo el cáncer de piel, el cáncer de mama es la neoplasia maligna más frecuente entre las mujeres, representando casi 1 de cada 3 tumores diagnosticados en mujeres en Estados Unidos. En Europa, el cáncer de mama fue en 2004, el cáncer con mayor incidencia en las mujeres (29% de nuevos casos) (Althuis M.D. et al., 2005; Boyle P. and Ferlay J., 2005).

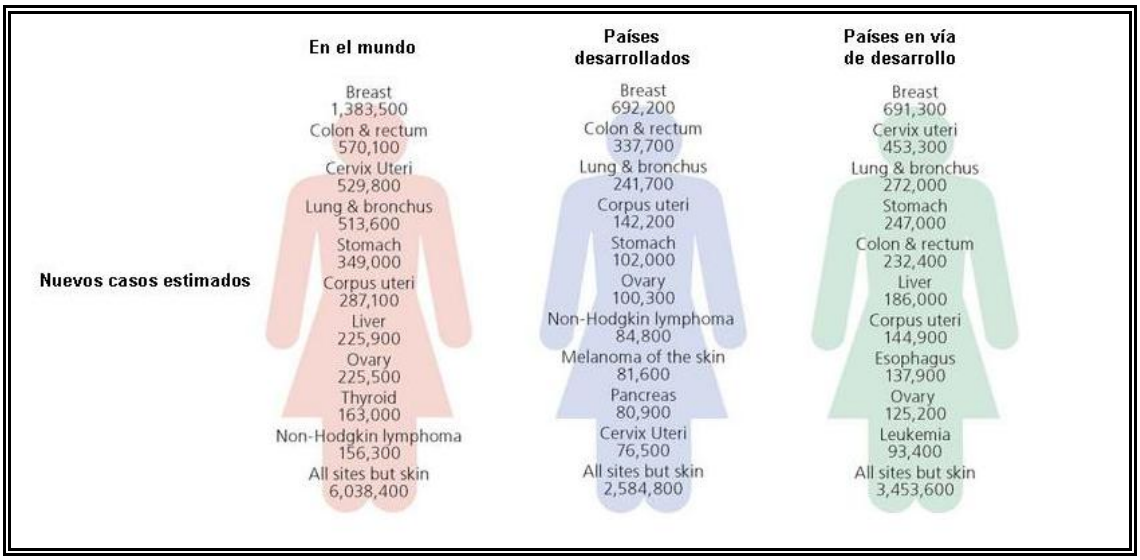

Figura 3: Estimación de nuevos casos de cáncer en todo el mundo, según el nivel de desarrollo económico. (*Fuente: GLOBOCAN 2008, Jemal A et al. Global Cancer Statistics, 2011*).

Se prevé que en 2013 en Estados Unidos, los tres tipos de tumores más comúnmente diagnosticado en las mujeres serán el cáncer de mama, el cáncer de pulmón, y el cáncer de colon y recto, llegando a representar el 51% del total de los nuevos casos de cáncer estimados en mujeres. Se espera que sólo el cáncer de mama represente el 29% (232.340) de todos los nuevos casos de cáncer entre las mujeres. La incidencia de los cuatro principales tipos de cáncer está disminuyendo, excepto en el caso del cáncer de mama, para el cual las tasas se mantuvieron relativamente estables desde 2005 hasta 2009, después de una reducción de un 2% por año entre 1999-2005 (Siegel R. et al., 2013).

III.2 Mortalidad

El cáncer de mama es la principal causa de muerte por cáncer en las mujeres en todo el mundo, representando el 14% (458.400) del total de muertes por cáncer en 2008 (Figura 4) (Jemal A. et al., 2011).

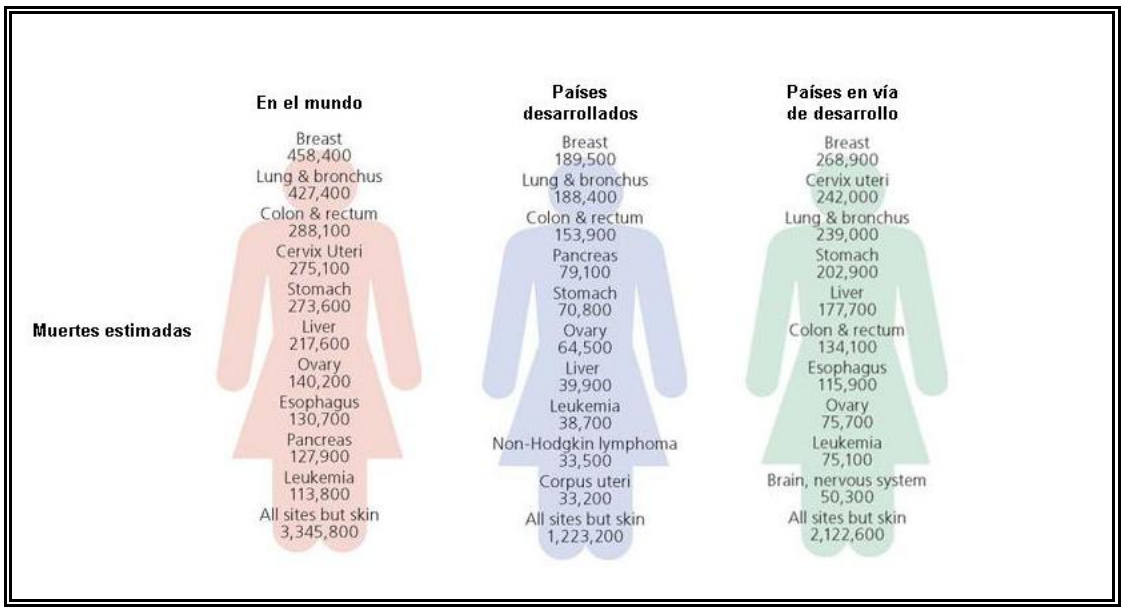

Figura 4: Mortalidad asociada a cáncer estimada en todo el mundo, según el nivel de desarrollo económico. (*Fuente: GLOBOCAN 2008, Jemal A et al. Global Cancer Statistics, 2011*).

Después de un ligero aumento durante varios años (0,4% anual entre 1975-1990), la tasa de mortalidad por cáncer de mama en mujeres en Estados Unidos disminuyó en un 2,2% por año desde 1990 hasta 2007. La disminución fue mayor para las mujeres menores de 50 años (3,2% anual) respecto a las mujeres mayores de 50 años (2% anual). La disminución de la mortalidad asociada al cáncer de mama se atribuye a la mejora en el tratamiento de la enfermedad y a su detección temprana (DeSantis C. et al., 2011). En España, entre 1980 y 2005, se produjeron 131.746 defunciones por cáncer de mama, con un cambio de tendencia descendente a partir de 1992, que persistió hasta 2005 (Vidal Lancis C. et al., 2010).

La supervivencia global del cáncer de mama asciende al 94% al año del diagnóstico, disminuyendo hasta el 84% a los 3 años y hasta el 78% a los 5 años del diagnóstico (Berrino F. et al., 2007).

IV Factores de riesgo

El 85% de las mujeres que padece un cáncer de mama no tiene ningún factor de riesgo identificable además de la edad, por tanto se considera que todas las mujeres corren riesgo de padecerlo.

La edad. El riesgo relativo de muerte por cáncer de mama aumenta con la edad, siendo así el factor de riesgo individualmente más importante. El 94% de todos los tumores de mama afectan a mujeres mayores de 40 años (Han W. et al., 2004). Sin embargo, los tumores diagnosticados en mujeres menores de 35 años suelen ser más proliferativos y por tanto tienen peor pronóstico tanto en lo que se refiere a tiempo libre de enfermedad como a supervivencia global (Bonnier P. et al., 1995; Park B.W. et al., 2002).

Historia ginecológica. La edad de la menarquia, del primer embarazo a término y de la menopausia, son tres etapas de la vida de una mujer que ejercen

un gran impacto sobre la incidencia del cáncer de mama. Los beneficios de la lactancia materna en la prevención del cáncer de mama es, en la actualidad un tema de debate (Yang L. and Jacobsen K.H., 2008).

Susceptibilidad genética. Se estima que entre el 5% y el 10% de casos de cáncer de mama son debidos a mutaciones en genes de alta penetrancia, de las cuales el 20- 25% ocurren en los genes BRCA-1 y -2 (Breast Cancer -1 y -2) (Chen S. and Parmigiani G., 2007). El riesgo de desarrollar un cáncer de mama a lo largo de la vida en portadoras de mutación en el gen BRCA-1 es superior al 60%, y supera al 50% en portadoras de la mutación en el gen BRCA-2 (Antoniou A.C. and Easton D.F., 2006). Otros genes de alta penetrancia son: p53, ATM (ataxia telangiectasia mutated), PTEN (phosphatase and tensin homolog), STK11 (serine-threonine kinase 11) (Wooster R. and Weber B.L., 2003). Algunos genes de menor penetrancia, como el recientemente identificado gen CHEK2 (cell-cycle-checkpoint kinase 2) (Meijers-Heijboer H. et al., 2002), podrían expresarse siguiendo un modelo poligénico (Pharoah P.D. et al., 2002).

Antecedentes familiares. Las mujeres con familiares de primer grado (madre o hermana) afectadas por cáncer de mama tienen de dos a tres veces más riesgo de padecer un cáncer mamario respecto a la población general. Los factores que intervienen en estos casos puedes ser genéticos o ambientales.

Historia personal de cáncer de mama. Una mujer que padece cáncer de mama tiene entre tres y cinco veces más riesgo de desarrollar un cáncer en la otra mama, sobre todo si es joven y posee antecedente familiares de cáncer de mama. Además, las mujeres que han padecido un tumor *in situ* tienen un riesgo más elevado de desarrollar un cáncer de mama invasivo (Silverstein M.J. et al., 1998; Li C.I. et al., 2006).

Factores hormonales: La exposición a elevadas concentraciones de estrógenos endógenos aumenta el riesgo de padecer cáncer de mama. El embarazo parece tener un doble efecto: a corto plazo se asocia con un incremento del riesgo inicial debido al aumento de hormonas circulantes, pero a largo plazo constituye un factor protector como consecuencia de la maduración del tejido mamario, que se traduce en una menor tasa de proliferación del mismo (Pike M.C. et al., 2004). El riesgo de padecer un cáncer de mama parece aumentar durante la época de ingesta de anticonceptivos orales, sobre todo cuando el tratamiento supera los 10 años.

Otros factores. Determinadas lesiones benignas de la mama, como las lesiones proliferativas con atipia, pueden aumentar el riesgo relativo de cáncer de mama. Los factores ambientales, el estatus socioeconómico alto (que refleja distintos patrones reproductivos) y el empleo de programas de cribado poblacional influyen en el riesgo de desarrollar cáncer de mama. La influencia de hábitos de vida como la actividad física y los factores dietéticos, como la ingesta de grasas, alcohol o fitoestrógenos, son también considerados factores de riesgo (Friedenreich C.M. and Cust A.E., 2008). Además, la exposición a radiaciones ionizantes, principalmente en edades jóvenes, incrementa el riesgo (Boyd N.F. et al., 2003).

V Factores pronósticos clásicos

V.1 Concepto de factores pronósticos y predictivos de respuesta.

Un factor pronóstico nos aporta información sobre la evolución clínica de la enfermedad en el momento del diagnóstico independientemente del tratamiento aplicado. En oncología, suelen ser variables relacionadas con el crecimiento, invasión tumoral o el potencial metastático del tumor. Un buen factor pronóstico ha de cumplir, según la conferencia consenso del National

Institutes of Health (NIH) (1991), una serie de mínimos como son: aportar un valor predictivo independiente y significativo validado en la clínica; que su determinación sea factible, reproducible y disponible de manera más o menos generalizada con un adecuado control de calidad; que los resultados de su determinación sean interpretables por el clínico y tengan trascendencia terapéutica y que la medida del marcador no emplee tejido necesario para la realización de otros estudios principalmente la caracterización histológica. Un factor predictivo en cambio, aporta información relacionada con la probabilidad de respuesta a un tratamiento determinado, son variables relacionadas en distinta medida con las dianas de los tratamientos. Algunos factores, como la expresión del oncogen HER2/neu, son tanto factores pronósticos como predictivos de respuesta al tratamiento, pero no necesariamente los factores pronósticos de supervivencia han de ser también predictivos ni viceversa (Subramaniam D.S. and Isaacs C., 2005).

Parece haber un claro acuerdo, en que el tamaño del tumor, la afectación ganglionar y el grado histológico son clásicos factores pronósticos que permiten diferenciar categorías de riesgo (Goldhirsch A. et al., 2009).

V.2 Tamaño tumoral.

El tamaño macroscópico (diámetro máximo que se obtiene de la medición del tumor en al menos dos dimensiones) se considera uno de los más importantes factores pronósticos independientemente de la afectación ganglionar (Rosen P.P. et al., 1991).

Se ha demostrado que los tumores de mayor tamaño se acompañan de intervalos más breves hasta la recurrencia de la enfermedad y conllevan una menor supervivencia. Así, en base a los numerosos estudios realizados, se puede afirmar que el tamaño tumoral constituye el marcador pronóstico más

importante en pacientes con cáncer de mama y ganglios axilares negativos, correlacionándose con la supervivencia libre de enfermedad y con la supervivencia total (Carter C.L. et al., 1989).

V.3 Afectación ganglionar

La afectación ganglionar es, aún en la actualidad, el factor pronóstico independiente más importante en el cáncer de mama. El 30% de las pacientes con ganglios negativos recidivan a los 10 años, mientras que la tasa de recurrencia de pacientes con ganglios positivos supera el 70% (Lonning P.E. et al., 2007).

La técnica de la biopsia selectiva del ganglio centinela, mediante la cual se detecta y extirpa el primer ganglio de la axila que recibe la linfa de la mama, permite realizar estudios anatomopatológicos cada vez más precisos. El concepto de biopsia del ganglio centinela se basa en dos principios básicos: la existencia de un patrón ordenado y predecible de drenaje linfático a una cadena de ganglios linfáticos regionales, y el funcionamiento de un primer ganglio linfático como un filtro efectivo para las células tumorales. Los métodos actuales para evaluar la afectación del ganglio centinela durante la cirugía emplean secciones congeladas, citología por impronta o raspado, inmunocitoquímica rápida y combinaciones de los mismos; así como nuevas técnica moleculares, como el OSNA (One-step-nucleic acid amplification).

La disección de los ganglios axilares en el cáncer de mama es un procedimiento originalmente diseñado para maximizar la supervivencia y el control regional, y para determinar la clasificación de los ganglios. La disección de los ganglios linfáticos axilares con eliminación y examen histopatológico de al menos 10 ganglios, ha sido una parte inherente del tratamiento quirúrgico del cáncer de mama, durante un largo periodo. En la pasada década, la disección de los ganglios linfáticos axilares fue sustituida por la biopsia del ganglio linfático centinela. La resección del ganglio centinela fue diseñada para

minimizar los efectos secundarios de la cirugía de los ganglios linfáticos, pero aún ofrece resultado equivalente a la disección de los ganglios axilares, cuando el ganglio centinela está afectado. En el caso de que el ganglio linfático centinela sea positivo, se completa la disección de los ganglios linfáticos axilares de forma rutinaria debido a la posibilidad de que estén afectados por tumor los ganglios no centinelas. Sin embargo, en entre un 50 y un 68% de las pacientes con ganglio centinela positivo, este es el único ganglio de la axila afectado por células tumorales (Hung W.K. et al., 2005; Domenech A. et al., 2009). Para evitar las disecciones de los ganglios linfáticos axilares innecesarias, se han desarrollado diferentes herramientas, llamadas nomogramas, para predecir la probabilidad de metástasis en ganglios no centinelas en pacientes con cáncer de mama con ganglio centinela positivo. Sin embargo, los nomogramas disponibles actualmente aún no son lo suficientemente fiables para permitir una estratificación terapéutica durante la cirugía para evitar las disecciones de los ganglios linfáticos axilares extensas en pacientes con un bajo riesgo de recurrencia axilar.

V.4 Grado histológico

El método de estadificación histológica utilizado en la actualidad, es el llamado "grado de diferenciación histológico de Scarf-Bloom-Richardson" o grado SBR (Simpson J.F. and Page D.L., 1994) que consta de los siguientes parámetros:

- el grado de formación de túbulos,
- la regularidad en el tamaño, la forma y el carácter de tinción del núcleo, y
- la hipercromasia nuclear y la actividad mitótica.

A cada uno de los tres factores anteriormente señalados se les asigna una puntuación de 1 a 3, siguiendo los criterios descritos a continuación:

- Formación de túbulos:
  - > 75% del tumor constituido por túbulos 1
  - 10-75% del tumor constituido por túbulos 2
  - < 10% del tumor constituido por túbulos 3

- Pleomorfismo nuclear:
  - núcleos pequeños y uniformes 1
  - moderada variabilidad en forma y tamaño 2
  - marcado aumento de tamaño y variabilidad 3

- Índice mitótico : 1
  2
  3

La calificación total alcanzada puede variar de 3 a 9. Una calificación de 3 a 5 etiqueta a los carcinomas como un tumor de grado I (bien diferenciado), 6 ó 7 como grado II (moderadamente diferenciado), y 8 ó 9 como grado III (poco diferenciado).

V.5 Índice pronóstico de Nottingham

El índice pronóstico de Nottingham (NPI) tiene como pilares las características pronósticas más importantes de un tumor: el tamaño, el estado de los ganglios linfáticos y el grado histológico.

La fórmula para obtenerlo es la siguiente:

NPI= 0,2 x tamaño tumoral + estadio de los ganglios + grado histológico.

El tamaño tumoral se expresa en centímetros, y tanto el estadio de los ganglios linfáticos como el grado histológico cuentan con una puntuación de 1 a 3. El valor más pequeño del NPI, tiene el mejor pronóstico.

V.6 Sistema TNM

El sistema de clasificación TNM es el método de estadiaje de neoplasias, desarrollado por la AJCC (American Joint Committee on Cancer) en colaboración con la UICC (Unio Internationalis Contra Cancrum), basado en la extensión del tumor primario (T), la presencia o ausencia del tumor en los ganglios regionales (N) y la presencia o ausencia de metástasis distantes (M). El sistema TNM de los tumores malignos fue desarrollado entre los años 1943 y 1952 por Pierre Denoix. Desde su primera edición en 1954, se ha actualizado en diferentes ocasiones el sistema TNM para el cáncer de mama, hasta la actual séptima edición, publicada en 2010 (Edge SB B.D., Compton CC, et al, 2010). Dicha clasificación incluye un estadiaje basado en datos clínicos cTNM y patológicos pTNM.

Tumor Primario (T)	
TX	El tumor primario no se puede evaluar.
T0	No evidencia de tumor primario.
Tis	Carcinoma in situ.
Tis (DCIS)	Carcinoma ductal "in situ".
Tis (LCIS)	Carcinoma Lobulillar "in situ".
Tis (Paget)	Enfermedad de Paget del pezón sin tumor. La enfermedad de Paget asociada a tumor se clasifica de acuerdo al tamaño del tumor.
T1	Tumor < 2 cm. en su mayor dimensión.
T1mic	Microinvasión < 0.1 cm. en su mayor dimensión.
T1a	Tumor > 0.1 cm. pero no > 0.5 cm. en su mayor dimensión.
T1b	Tumor > 0.5 cm. pero no > 1 cm. en su mayor dimensión.
T1c	Tumor > 1 cm. pero no > 2 cm. en su mayor dimensión.
T2	Tumor > 2 cm. pero no > 5 cm. en su mayor dimensión.
T3	Tumor > 5 cm. en su mayor dimensión.
T4*	Tumor de cualquier tamaño con extensión directa a pared torácica y/o piel.
T4a	Extensión a pared torácica sin incluir el músculo pectoral mayor.
T4b	Edema (incluyendo piel de naranja) o ulceración de la piel de la mama, o nódulos cutáneos satélites confinados a la misma mama.
T4c	Ambos T4a y T4b.
T4d	Carcinoma inflamatorio.**

* La invasión de la dermis sólo no clasifica al tumor como T4.

** La definición de Carcinoma Inflamatorio es fundamentalmente clínica. Implica la presencia de eritema y edema (piel de naranja) difuso de la mama asociado o no a una masa palpable subyacente. Estos cambios deben afectar a la mayor parte de la piel de la mama (la presencia de estos cambios asociados de forma limitada a un tumor localmente avanzado no implica el diagnóstico de carcinoma inflamatorio). La presencia de linfáticos dérmicos infiltrados sin los cambios clínicos descritos no implica por si solo el diagnóstico de carcinoma inflamatorio.

La detección clínica de los ganglios es realizada por estudios de imagen (excluyendo la linfogammagrafía) o por exploración física con características de alta sospecha o posible macrometástasis basado en PAAF.

Ganglios linfáticos regionales (cN)	
NX	Cuando no se pueden evaluar (p.e. Cirugía previa).
N0	No metástasis en los ganglios linfáticos regionales.
N1	Metástasis en ganglios homolaterales móviles.
N2	Metástasis en ganglios axilares homolaterales fijos o agrupados, o en ganglios de la cadena mamaria interna clínicamente aparentes* en ausencia de afectación clínica axilar.
N2a	Metástasis en ganglios axilares homolaterales fijos entre si o a otras estructuras.
N2b	Metástasis sólo en ganglios homolaterales de la cadena mamaria interna clínicamente aparentes y en ausencia de afectación clínica evidente axilar.
N3	Metástasis en ganglios infraclaviculares homolaterales, o en cadena mamaria interna homolateral clínicamente aparentes en presencia de afectación axilar clínicamente evidente; o metástasis en ganglios supraclaviculares homolaterales con o sin afectación axilar o de la mamaria interna.
N3a	Metástasis en ganglios infraclaviculares y axilares homolaterales.
N3b	Metástasis en cadena mamaria interna y axilar homolateral.
N3c	Metástasis en ganglios supraclaviculares homolaterales.

La clasificación pN está basada en la disección axilar con o sin estudio del ganglio centinela y se usa en conjunción con una valoración patológica de la T.

Ganglios linfáticos regionales (pN)	
pNX	Cuando no son evaluables (no extirpados o por cirugía previa).
pN0	No afectación histológica, sin estudios adicionales para células tumorales aisladas.
pN0(i-)	No afectación ganglionar histológica, hematoxilina e inmunohistoquímica negativas.
pN0(i+)	Presencia de células tumorales aisladas por hematoxilina o inmunohistoquímica, ninguna agrupación> 0.2 mm.
pN0(mol-)	No afectación ganglionar histológica, estudio molecular negativo (RT-PCR).
pN0(mol+)	No afectación ganglionar histológica, estudio molecular positivo (RT-PCR).
pN1mi	Micrometástasis (> 0.2 mm, < 2.0 mm).
pN1	Metástasis en 1 a 3 ganglios axilares linfáticos y/o en la mamaria interna con afectación microscópica detectada por biopsia de ganglio centinela pero no clínicamente aparente.
pN1a	Metástasis en 1 a 3 ganglios axilares linfáticos.
pN1b	Metástasis en la mamaria interna con afectación microscópica detectada por biopsia de ganglio centinela pero no clínicamente aparente.
pN1c	Metástasis en 1 a 3 ganglios axilares linfáticos y en la mamaria interna con afectación microscópica detectada por biopsia de ganglio centinela pero no clínicamente aparente.
pN2	Metástasis en 4 a 9 ganglios axilares linfáticos, o afectación clínicamente aparente de la mamaria interna en ausencia de afectación axilar.
pN2a	Metástasis en 4 a 9 ganglios axilares linfáticos (al menos un depósito tumoral > 2.0 mm).
pN2b	Metástasis clínicamente aparente en ganglios de la mamaria interna en ausencia de afectación axilar.
pN3	Metástasis en > 10 ganglios axilares, o en ganglios infraclaviculares, o en ganglios de la mamaria interna ipsilaterales clínicamente aparentes en presencia de 1 o mas ganglios axilares positivos; o en mas de 3 ganglios axilares con metástasis microscópicas clínicamente negativas de la mamaria interna; o en ganglios supraclaviculares ipsitaleterales.
pN3a	Metástasis en más de 10 ganglios axilares (al menos un depósito tumoral > 2,0mm), o metástasis en ganglios linfáticos infraclaviculares.
pN3b	Metástasis en ganglios de la mamaria interna ipsilaterales clínicamente aparentes en presencia de 1 o más ganglios axilares positivos; o en más de 3 ganglios axilares con metástasis microscópicas de la mamaria interna detectadas por biopsia de ganglio centinela pero no clínicamente aparentes.
pN3c	Metástasis en ganglios supraclaviculares ipsitaleterales.

Metástasis a distancia (M)	
MX	No se pueden evaluar.
M0	No metástasis a distancia.
cM0(i+)	No evidencia clínica ni radiológica de metástasis pero presencia de depósitos de células tumorales detectadas en sangre circulante, médula ósea u otro tejido ganglionar no regional que es < 0,2mm en un paciente sin signos ni síntomas de metástasis.
M1	Metástasis a distancia presentes.

V.7 Estadío tumoral

Las diversas combinaciones de las subcategorías de T, N, y M definen los siguientes cuatro estadios designados como I, II, III, y IV en orden ascendente de gravedad (Tabla 1).

Tabla 1: Definición del estadío tumoral en función del TNM.

Estadío	T	N	M
0	Tis	N0	M0
IA	T1b	N0	M0
IB	T0	N1mi	M0
	T1b	N1mi	M0
IIA	T0	N1c	M0
	T1b	N1c	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	T1b	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Cualquier T	N3	M0
IV	Cualquier T	Cualquier N	M1

V.8 Tipos de cáncer de mama

Los tumores mamarios pueden clasificarse en dos grupos:

- Cáncer no invasivo o in situ. Este tipo de tumor tiene como origen el epitelio lobulillar o ductal; existiendo entonces dos grupos:
    - Carcinoma lobulillar *in situ*.
    - Carcinoma ductal *in situ*.

En estos tipos de carcinomas se inicia una proliferación atípica del epitelio mamario hasta llegar a ocupar los conductos, pero sin invadir el tejido adiposo circundante. Los tumores no invasivos tienen un crecimiento localizado y lento.

- Cáncer invasivo o infiltrante. El origen de estos tumores es el mismo que el de los carcinomas in situ, sin embargo en estos casos las células cancerosas invaden el tejido circundante, pudiendo llegar a otros órganos. Los tumores invasivos más frecuentes son:

    - Carcinoma lobulillar infiltrante, se origina en las glándulas productoras de leche (lóbulos) y es capaz de extenderse hasta otros tejidos circundantes y distantes. Este tipo de tumores representa el 10-15% de los tumores invasivos.

    - Carcinoma ductal infiltrante, se origina en los ductos o conductos galactóferos de la mama e invade el tejido adiposo de la mama, llegando a producir metástasis. Este tipo de tumor representa el 80% de los tumores de mama.

Otros tipos de tumores invasivos, de menor incidencia, son el carcinoma medular, carcinoma mucinoso o coloide, carcinoma tubular o el carcinoma inflamatorio.

V.9 Invasión vascular linfática

Los vasos linfáticos que rodean a un carcinoma ductal infiltrante pueden estar invadidos por células tumorales. En los pacientes sin afectación ganglionar, la invasión vascular linfática es un factor predictivo de mal pronóstico, de recurrencia precoz y metástasis a distancia (Mirza A.N. et al., 2002; Schoppmann S.F. et al., 2004; Lee A.H. et al., 2006). Este factor indica un pronóstico desfavorable en términos de recaída local y reducción de la supervivencia global, por lo que se recomienda su evaluación en el estudio anátomo-patológico de la pieza tumoral (Hoda S.A. et al., 2006).

V.10 Invasión vascular venosa

La diseminación tumoral a distancia puede realizarse por vía hematógena, afectando principalmente a pulmones, hígado y hueso. Nuevos marcadores específicos del endotelio, como D2-40, hacen que sea posible distinguir entre la invasión vascular venosa y la invasión vascular linfática. Aunque se siga determinado, la presencia de invasión vascular venosa perdió significación estadística en un estudio al compararse con la invasión vascular linfática (Van den Eynden G.G. et al., 2006).

VI Factores pronósticos biológicos y moleculares

VI.1 Receptores hormonales

Los receptores de estrógenos (RE) y de progesterona (RP) han sido de los primeros factores moleculares en usarse en la práctica clínica (Maass H. et al., 1975; Wittliff J.L., 1984), pues contribuyen en la regulación de la proliferación y diferenciación celular mamaria. Se recomienda la determinación de estos

receptores en el tumor primario, recurrencias y en las lesiones metastásicas (Allred D.C. et al., 2009; Hammond M.E. et al., 2010), ya que es imprescindible para la selección de pacientes que se beneficiarán de un tratamiento hormonal, mejorando así el pronóstico y la supervivencia de estas pacientes. La expresión de estos factores se asocia con una menor agresividad tumoral. Entre el 55-65% de los cánceres de mama y el 45-55% de sus metástasis expresan los receptores estrógenos y entre el 55-60% responden a la administración de terapia hormonal (Cui X. et al., 2005). Se considera que la presencia conjunta de receptores de estrógeno y progesterona en un mismo tumor, aumenta la probabilidad de respuesta a la hormonoterapia, desde un 55% en pacientes con única expresión del receptor de estrógeno, a un 75-80% (Wittliff J.L., 1984). La expresión del receptor de progesterona es considerada al menos tan importante como la de los receptores de estrógeno para predecir la respuesta del cáncer de mama, así la pérdida de expresión del receptor de progesterona por las células tumorales está asociada con un peor pronóstico (McGuire W.L. and Clark G.M., 1986).

Una disminución en la expresión de RE, la baja o no expresión de RP y la positividad del receptor Her-2 se asocian con una menor probabilidad de respuesta a cualquier tipo de terapia hormonal (Rastelli F. and Crispino S., 2008). Los tumores que sobreexpresan Her2-neu/c-erbB2 son resistentes al tratamiento hormonal y por tanto requieren el bloqueo de la vía de Her-2, además de la deprivación estrogénica (Shou J. et al., 2004; Osborne C.K. et al., 2005). La respuesta a hormonoterapia puede estar influenciada por la relación entre diferentes receptores y las vías intracelulares del RE (Jansen M.P. et al., 2005; Osborne C.K. et al., 2005; Massarweh S. and Schiff R., 2006) (Figura 3).

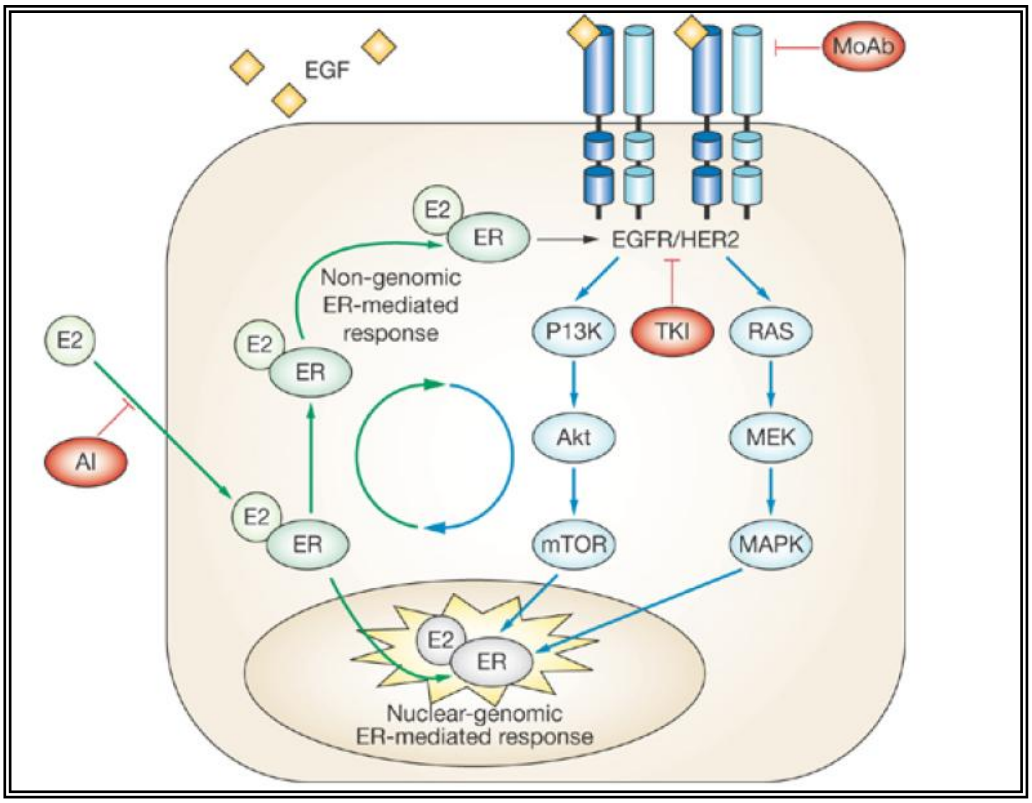

Figura 3: Relación del receptor de estrógenos con diferentes vías de señalización celulares. (*Fuente: Massarweh S et al. 2006*)

La interacción entre el RE y el receptor Her-2 puede explicar la resistencia a Tamoxifeno y el beneficio de los inhibidores de la aromatasa (AI) o el complemento de la terapia hormonal con el Trastuzumab (MoAb) a en algunas pacientes hormonosensibles.

VI.2 Oncogen c-ErbB2

El protooncogen c-erbB2 se encuentra en el cromosoma 17, pertenece a la familia del receptor del factor de crecimiento epidérmico (EGFR) y codifica una proteína-receptor tirosina-quinasa transmembrana de 185 kD (Her2/neu o receptor Her2 o p185). Se considera que dicho receptor está sobreexpresado si se observa positividad de tres cruces (3+) en la determinación por inmunohistoquímica de la expresión de la proteína Her2/neu en las células

tumorales o, en casos de menor positividad (2+), si la hibridación *in situ* fluorescente (FISH) es positiva, es decir, si se observan amplificación de las copias del gen c-erbB2 en las células tumorales.

La sobreexpresión de Her2/neu se correlaciona con un peor pronóstico, un menor tiempo libre de enfermedad y supervivencia global, la presencia de metástasis ganglionares, hormono-independencia, una mayor capacidad proliferativa y una resistencia al tratamiento con tamoxifeno. Sin embargo, este factor no ha sido relacionado con la edad, el tamaño tumoral ni el grado histológico (Dandachi N. et al., 2004; Viani G.A. et al., 2007).

Desde los años 80 hasta la actualidad, se ha acreditado el valor pronóstico y predictivo de Her2/neu (Ferretti G. et al., 2007; Shah S. and Chen B., 2011) y, su determinación está universalmente aceptada en la práctica clínica, reconociéndose imprescindible para la estrategia terapéutica.

VI.3 Ki-67

El antígeno Ki-67, identificado por Gerdes y cols. en 1991, fue descrito como una proteína nuclear tipo no histona. La localización de la proteína Ki-67 es variable y heterogénea durante las diferentes fases del ciclo celular. Durante el inicio de la fase G1 se observa una débil tinción que progresivamente aumenta por condensación en gránulos situados alrededor del nucleolo. Durante las fases S y G2 se encuentra principalmente en la región del nucleolo y alrededor de la heterocromatina. Cuando la membrana nuclear se rompe durante la mitosis temprana, Ki-67 muestra una intensa expresión asociada a la condensación de los cromosomas en el citoplasma. Dicha intensidad desaparece en la anafase y la telofase. Su medición puede realizarse por técnicas inmunohistoquímicas con el anticuerpo monoclonal MIB-1. Se considera un tumor con baja actividad proliferativa cuando existe menos de un 10% de células tumorales positivas; si es superior al 20%, el tumor es de alta actividad proliferativa.

La expresión de Ki-67 se correlaciona directamente con el grado histológico (grado de diferenciación), la invasión vascular venosa, y las metástasis ganglionares. Sin embargo, se correlaciona inversamente con la presencia de receptores hormonales, y otros indicadores de proliferación como la proporción de células en la fase S del ciclo celular (Brown R.W. et al., 1996).

Por lo tanto, Ki-67 puede ser considerado un factor pronóstico en cáncer de mama puesto que una expresión elevada se asocia a un peor pronóstico y a un menor tiempo libre de enfermedad; además de un factor predictivo de respuesta a tratamiento hormonal adyuvante (Viale G. et al., 2008).

VI.4 p53

El gen TP53 o antioncogen p53, localizado en el cromosoma 17p13, es un estabilizador genómico que actúa como inhibidor de la progresión del ciclo celular y facilita la muerte celular programada. Concretamente, la proteína interviene en el control del ciclo celular en el paso G0 (en reposo) a G1 (en proliferación activa), como mediadora de la diferenciación, de la reparación del ADN y de la apoptosis. Las mutaciones de la proteína p53 llevan a una pérdida en la regulación del ciclo celular, encontrándose en el 20% de los cánceres de mama.

Existe una asociación significativa entre la sobre-expresión de la proteína p53 mutada y el tiempo libre de enfermedad, pudiendo además predecir la respuesta al tratamiento con tamoxifenos (Erdem O. et al., 2005; Dookeran K.A. et al., 2010); sin embargo si se considera el estatus hormonal de la paciente, p53 no parece tener impacto sobre el tiempo libre de enfermedad ni la supervivencia (Rossner P., Jr. et al., 2009).

VI.5 Bcl-2

El gen BCL-2 codifica una proteína intramitocondrial (Bcl-2) implicada en la supresión de la muerte celular programada o apoptosis. Este gen se expresa en el tejido normal mamario pero su expresión disminuye a medida que los tumores progresan desde un carcinoma *in situ* hacía un carcinoma invasivo. Un metanálisis reciente, apoya el valor pronóstico de la expresión de Bcl-2, medida por inmunohistoquímica, mostrando su indenpendencia del tamaño tumoral, del estado de afectación de los ganglios linfáticos axilares y del grado tumoral (Callagy G.M. et al., 2008). La utilidad clínica de Bcl-2 como marcador pronóstico independiente, necesita aún ser confirmada en estudios prospectivos

VI.6 Otros factores

Otros factores moleculares como otros miembros de la familia de EFGR o de la familia del receptor factor de crecimiento del endotelio vascular (VEGFR), como los marcadores de la capacidad invasiva uPA/PAI-1 (inhibidor del plasminógeno-1) y catepsina D o marcadores de la angiogénesis han sido estudiados como posible factores pronósticos. Pero a día de hoy, no está establecido rutinariamente su análisis y significado.

VII Inflamación y cáncer

La inflamación es un mecanismo que se desarrolla frente a un daño mecánico en los tejidos, o como respuesta a la presencia de un agente externo. Su fin es contener esos procesos en una zona determinada, impidiendo, por ejemplo, que los microorganismos se dispersen por el sistema. El proceso inflamatorio destruye, diluye o contiene al agente lesivo, y promueve la reparación del tejido. A principios del siglo XX, Rodolf Virchow postuló que la micro-inflamación resultante de la irritación lleva al desarrollo de la mayoría de

las enfermedades crónicas, incluyendo el cáncer (Balkwill F. and Mantovani A., 2001). Sin embargo, sólo recientemente los estudios experimentales y clínicos han confirmado esta hipótesis, que es un ahora un paradigma generalmente aceptado (Eiro N. and Vizoso F.J., 2012).

VII.1 La inflamación crónica como predisposición al cáncer

Actualmente, existen evidencias que apoyan la hipótesis de que la inflamación participa en la proporción de las condiciones que conducen al cáncer. Se estima que las infecciones subyacentes y las reacciones inflamatorias están vinculadas al 25% de los casos de cáncer.

Existe una asociación establecida entre el proceso inflamatorio y el cáncer, tal como la enfermedad inflamatoria intestinal (enfermedad de Crohn y especialmente la colitis ulcerosa) y el cáncer colorrectal (Eaden J. et al., 2000; van der Woude C.J. et al., 2004); la hepatitis viral B y C o la cirrosis hepática y el hepatocarcinoma (Matsuzaki K. et al., 2007); la esofagitis por reflujo que resulta en el esófago de Barrett y en el cáncer de esófago (van der Woude C.J. et al., 2004); la infección cervical por el virus del papiloma humano y el cáncer de cuello uterino; la prostatitis y el cáncer de próstata; la pancreatitis y el cáncer de páncreas; o la infección gástrica por *helicobacter pylori* que aumenta el riesgo de cáncer gástrico en un 75% (Hussain S.P. et al., 2000; Kuper H. et al., 2000).

Por tanto, una inflamación sin resolver debido a cualquier fracaso en el control de la respuesta inmune, puede perturbar el microambiente celular provocando alteraciones que favorecen el desarrollo de un tumor.

VII.2 El microambiente tumoral y su contribución a la progresión tumoral hacía las metástasis

Los tumores no sólo están compuestos de células tumorales, sino también de otros tipos de células que constituyen el estroma. Estas células estromales incluyen los fibroblastos asociados a tumor, las células endoteliales,

y una representación variable de leucocitos. Inicialmente, las células tumorales y las células estromales responden a la hipoxia tumoral y la necrosis; derivada de la excesiva proliferación de células tumorales; mediante la liberación de factores de crecimiento y citoquinas quimioatrayentes de monocitos y macrófagos (Balkwill F., 2004; Robinson S.C. and Coussens L.M., 2005).

Históricamente, los leucocitos infiltrantes asociados al tumor se han considerado como la manifestación de un mecanismo de defensa intrínseco contra el desarrollo tumoral (Lin E.Y. and Pollard J.W., 2004). Sin embargo, existe la evidencia creciente de que la infiltración de leucocitos puede promover cambios que conducen a un fenotipo tumoral más agresivo en el ámbito de la angiogénesis, crecimiento tumoral, invasión y metástasis. En condiciones fisiológicas, el estroma actúa como barrera que limita la transformación de las células epiteliales (Bhowmick N.A. and Moses H.L., 2005). Sin embargo, en respuesta a lesiones epiteliales, las células estromales se pueden alterar promoviendo el reclutamiento de nuevas células estromales que estimulan el crecimiento y la remodelación de la matriz extracelular (Bhowmick N.A. and Moses H.L., 2005; Tlsty T.D. and Coussens L.M., 2006). En este sentido, se ha descrito que la expresión génica del estroma predice el curso clínico en el cáncer de mama mejor que la expresión génica de las células epiteliales del tumor (Finak G. et al., 2008).

VII.3 Las metaloproteasas de la matriz y el cáncer de mama

La familia de las metaloproteasas de la matriz (MMPs), enzimas proteolíticas que poseen dos átomos de zinc en el sitio activo de la molécula, cuentan de 26 miembros. La familia de las MMPs se ha clasificado atendiendo a sus características funcionales y estructurales en 6 grupos diferentes (Brinckerhoff C.E. et al., 2000; Overall C.M. and Lopez-Otin C., 2002; Demers M. et al., 2005) (Tabla 2). Todas ellas presentan características muy similares e incluso se sobreponen en la especificidad por sustratos.

Tabla 2: Metaloproteasas de la matriz de origen humano.

Tipo de MMP	MMP	Nombre de la enzima	Peso molecular (kDa)	Substrato
Colagenasas	MMP-1	Colagenasa - I	57* 47 A	Colágenos (I, II, III, VII, and X), proteoglicanos, entactina, ovostatina, MMP-2, MMP-9.
	MMP-8	Colagenasa-2/ colagenasa de neutrófilo	85* 64 A	Colágenos (I, II, III, VII, VIII and X), fibronectina, proteoglicanos.
	MMP-13	Colagenasa-3	60* 48 A	Colágenos (I, II, III, VII, VIII and X), tenascina, plasminogeno, aggrecano, fibronectina, osteonectina, MMP-9
	MMP-18	Colagenasa-4	53* 51 A	Colágeno tipo I
Gelatinasas	MMP-2	Gelatinasa-A	72* 66 A	Gelatina, colágeno (IV, V, VII VI, IX and X), elastina, fibronectina.
	MMP-9	Gelatinasa-B	92* 86 A	Colágenos (IV, V, VII, X, and XIV), gelatina, entactina, elastina, fibronectina, osteonectina, plasminógeno, proteoglicanos.
Stromelisinas	MMP-3	Stromelisina-I	60* 52 A	Colágenos (IV, V, and IX), gelatina, aggrecano, laminina, elastina, caseína, osteonectina, fibronectina, ovostatina, entactina, plasminógeno.
	MMP-10	Stromelisina2	53* 47 A	Colágenos (I, II, IV and V), gelatina, caseína, elastina, fibronectina.
	MMP-11	Stromelysin3	60* 47 A	Colágenos (IV, V, IX and X), laminina, elastina, fibronectina, caseína, proteoglicanos.
Matrisilinas	MMP-7	Matrisilina	28* 19 A	Colágeno IV, gelatina, fibronectina, laminina, elastina, caseína, transferrina.
	MMP-26	Matrisilina -2	29	Colágeno IV, fibronectina, fibrinógeno, gelatina, pro-MMP9.
MT-MMP (MMP de membrana)	MMP-14	MT1-MMP	66* 54 A	Colágenos (I, II, III), gelatina, fibronectina, laminina, vitronectina, entactina, pro-MMP2.
	MMP-15	MT2-MMP	76	Fibronectina, gelatina, vitronectina, entactina, laminina, pro-MMP-2
	MMP-16	MT3-MMP	65* 63 A	Colágeno III, gelatina, caseína, fibronectina, pro-MMP-2.
	MMP-17	MT4-MMP	65* 63 A	Pro-MMP2, fibrinógeno, gelatina.
	MMP-24	MT5-MMP	73	Fibronectina, pro-MMP2, proteoglicanos, gelatina.
	MMP-25	MT6-MMP	62	Pro-MMP2, pro-MMP9, colágeno IV, gelatina, fibronectina, Proteínasa A.
Otras enzimas	MMP-12	Metaloelastasa de macrófago	54* 45 A	Colágeno IV, gelatina, elastina, caseína, fibronectina, vitronectina, laminina, entactina, fibrina/fibrinógeno.
	MMP-19	RASI I	59	Colágenos (I, IV) gelatina, fibronectina, laminina.
	MMP-20	Enamelisina	56	Amelogenina, aggrecano.
	MMP-21		65	
	MMP-22		58* 53 A	
	MMP-23		44	gelatina
	MMP-27		59	
	MMP-28	Epilisina	59	

La expresión de las MMPs es inducida por una variedad de estímulos externos tales como las citoquinas y los factores de crecimiento, incluyendo interferones, interleuquinas, el factor de crecimiento de fibroblastos, el factor de crecimiento endotelial vascular, el factor de necrosis tumoral alfa o beta, el factor de crecimiento epidermal y el inductor de metaloproteasas de la matriz extracelular (EMMPRIN) (Sternlicht M.D. and Werb Z., 2001). Las MMPs son sintetizadas como zimógenos; precursores inactivos; para su activación enzimática, es necesaria la ruptura del enlace cisteína-zinc, mediante la proteolisis del pro-péptido por otras MMPs o serine proteasas o un cambio conformacional de la proteína (inducido por compuestos organomercuriados, urea, detergentes o especies reactivas de oxígeno). La actividad de las MMPs es regulada por sus inhibidores endógenos, llamados inhibidores tisulares de MMPs (TIMPs, por sus siglas en inglés). Sus 4 miembros (TIMP-1 a -4) son proteínas capaces de inactivar las MMPs a través de su unión directa y reversible al dominio catalítico de las MMPs (Brew K. et al., 2000).

Las MMPs desempeñan un papel esencial en la degradación del tejido conectivo estromal y los componentes de la membrana basal, que son elementos clave en la invasión tumoral y las metástasis. Así, ha sido establecido la implicación de las MMPs en el desarrollo del cáncer (Shuman Moss L.A. et al., 2012), como el de mama (Vizoso F.J. et al., 2007), el colorrectal (Gonzalez L. et al., 2012; Herszenyi L. et al., 2012), de próstata (Fernandez-Gomez J. et al., 2011) y el hepatocarcinoma (Altadill A. et al., 2009), entre otros. Durante la invasión tumoral, las MMPs parecen tener diversas funciones, probablemente debido a su preferencia de sustrato. Existen datos que desafían el clásico dogma que establece que las MMPs promueven la metástasis exclusivamente mediante la modulación de la remodelación de la matriz extracelular; se describe, así, la influencia de las MMPs en el comportamiento de la célula tumoral *in vivo* como consecuencia de su capacidad para escindir los factores de crecimiento, los receptores de superficie de la célula, las moléculas de adhesión celular, así como las quimioquinas o citoquinas (Manes S. et al., 1999; Stetler-Stevenson W.G., 1999; Fingleton B. et al., 2001).

Nuestro grupo de investigación ha estudiado ampliamente el papel de la MMPs en la progresión y metástasis del cáncer de mama, además de en otros tumores como el cáncer de próstata. Hemos descrito que un 32% de los carcinomas mamarios presentan en el estroma tumoral células mononucleares inflamatorias (CMIs) mostrando un perfil molecular elevado de MMPs y TIMPs (matrilisina -MMP7-, gelatinasa B -MMP9-, estromelisina-3 -MMP3-, colagenasa-3 -MMP13-, metaloproteasa de membrana tipo I -MMP14-, TIMP-1 y TIMP-2) (Figura 4) (Gonzalez L.O. et al., 2007). Además, demostramos que el 97,6% de esos casos, caracterizados por la expresión de MMP11 por las CMIs, desarrollaron metástasis a distancia, lo que representó el factor independiente más potentemente asociado con la supervivencia libre de enfermedad (Gonzalez L.O. et al., 2007; Vizoso F.J. et al., 2007; Gonzalez L.O. et al., 2010). También cabe destacar que esa asociación fue detectada cuando consideramos separadamente tanto los tumores con fenotipo luminal A como los de tipo basal-like (Gonzalez L.O. et al., 2009). Asimismo, pudimos comprobar la existencia de diferentes fenotipos de las CMIs ya en las fases más iniciales de la transformación maligna, cuando esas células inflamatorias rodean los ductos neoplásicos de los carcinomas "in situ" de mama (Gonzalez L.O. et al., 2008). Además, cuando hemos comparado los valores de inmunotinción de las MMPs/TIMPs entre diferentes localizaciones tumorales (centro tumoral, frente invasivo o ganglios linfáticos axilares metastáticos (GLAMs)), altas correlaciones positivas han sido encontradas entre los GLAMs (Garcia M.F. et al., 2010).

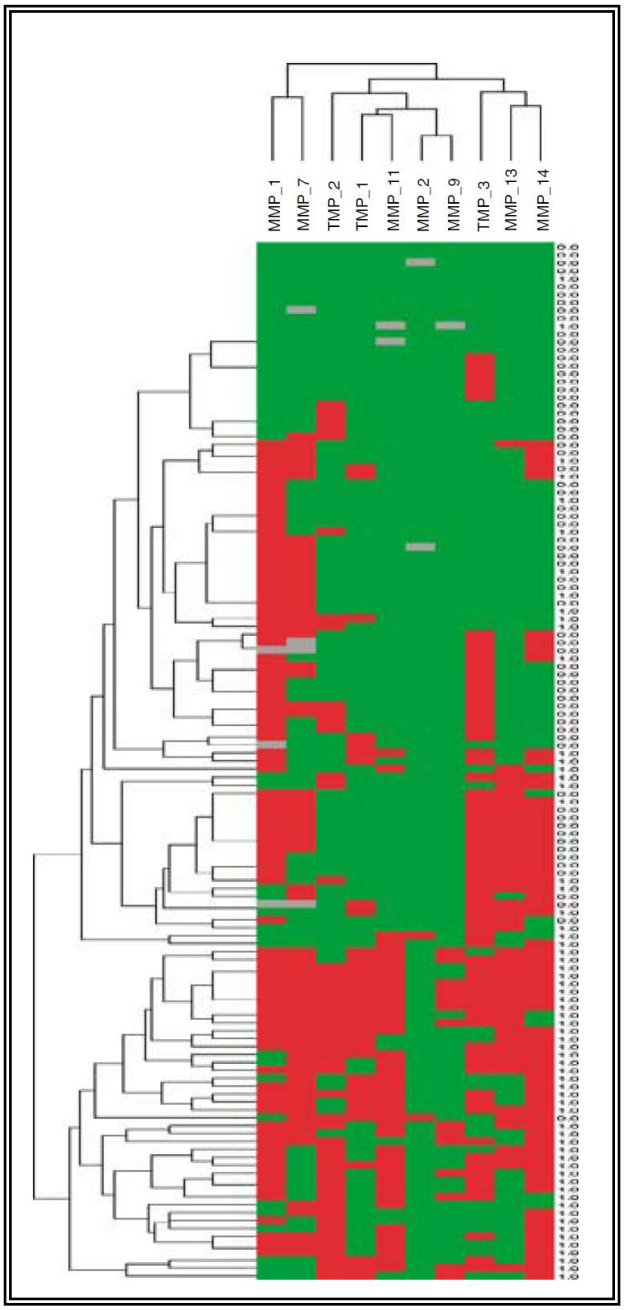

Figura 4: Cluster jerárquico no supervisado de la expresión de MMPs/TIMPs en las CMIs intratumorales de carcinomas mamarios. Columnas: MMPs/TIMPs; lineas: tumores. Rojo: expresión positiva; verde: expresión negativa: gris: falta de dato.

Objetivos

Las evidencias científicas indican que el microambiente inflamatorio de los carcinomas mamarios puede influir positivamente en una mayor agresividad biológica de los mismos. Así pues, el objetivo principal del presente estudio es caracterizar los componentes celulares y moleculares del microambiente inflamatorio implicados en el desarrollo de metástasis en el cáncer de mama así como evaluar su potencial pronóstico.

Los objetivos específicos son:

1. Evaluar la relación entre la expresión de MMP-11 por las células mononucleares inflamatorias intratumorales, la ocurrencia de metástasis a distancia y la expresión de un panel de factores relacionados con la progresión tumoral y la inflamación en el cáncer de mama.

2. Investigar la relevancia clínica de la cantidad relativa de macrófagos, linfocitos T y linfocitos B en la frontera tumoral de carcinomas mamarios.

3. Determinar la relación entre la cantidad relativa de macrófagos, linfocitos T y linfocitos B en la frontera tumoral y la expresión de metaloproteasas de la matriz y sus inhibidores en la frontera tumoral y el centro del tumor.

4. Identificar un factor molecular asociado al desarrollo de metástasis ganglionares capaz de predecir la afectación de los ganglios linfáticos axilares no centinela.

Aportaciones

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Relationship between the Inflammatory Molecular Profile of Breast Carcinomas and Distant Metastasis Development

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Abstract

Inflammatory conditions may promote tumor progression and aggressiveness. In previous reports, we found a group of breast cancer tumors characterized by metalloprotease-11 (MMP-11) expression by intratumoral mononuclear inflammatory cells (MICs), which was associated with distant metastasis development. Thus, in the present study we evaluated the relationship between MMP-11 expression by MICs, distant metastasis development, and a wide panel of inflammatory factors in breast carcinoma. In an initial approach, we analyzed 65 factors associated with tumor progression and inflammation, in a tumor population classified in good or bad prognosis, based on MMP-11 expression by intratumoral MICs. The most differentially expressed factors were then analyzed in a wider tumor population classified according to MMP-11 expression by MICs and also according to metastasis development. These analyses were carried out by Real-time PCR. The results showed that of the 65 starting factors analyzed, those related with MMP-11 expression by MICs were: IL-1, −5, −6, −8, −17, −18, MMP-1, TIMP-1, ADAM-8, −10, −15, −23, ADAMTS-1, −2, −15, Annexin A2, IFNβ, Claudin-3, CCL-3, MyD88, IRAK-4 and NFκB. Of them, factors more differentially expressed between both groups of tumors were IL-1, IL-5, IL-6, IL-17, IFNβ and NFκB. Thereafter, we confirmed in the wider tumor population, that there is a higher expression of those factors in tumors infiltrated by MMP-11 positive MICs. Altogether these results indicate that tumors developing worse prognosis and identified by MMP-11 expression by intratumoral MICs, shows an up-regulation of inflammatory-related genes.

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Introduction

Inflammatory cells and immune mediators in tumor microenvironment influence tumor progression and metastasis in cancers, such as breast cancer [1]. Historically, tumor-infiltrating leukocytes have been considered as an intrinsic defense mechanism against tumor development. However, increasing evidences indicate that leukocyte infiltration may favor tumor development by promoting angiogenesis, growth, and invasion. This may happen because inflammatory cells influence cancer promotion by secreting cytokines, growth factors, chemokines and proteases, which stimulate proliferation and invasiveness of cancer cells. Consequently, events and molecules implicated in this cross-talk between tumor and inflammatory microenvironment may emerge as attractive targets in anticancer intervention with significant clinical impact.

In previous reports, we found that 32% of breast carcinomas analyzed contained mononuclear inflammatory cells (MICs) in the intratumoral stroma with a high metalloproteases and tissue inhibitor metalloproteases (MMPs/TIMPs) expression profile, which is associated with a higher rate of distant metastasis development (97.6%), as compared with patients whose MICs had a low MMPs/TIMPs expression profile and are associated with a lower rate of distant metastasis (26.9%). Those prometastatic-related MICs were characterized by overexpression of MMP-7, 9, 11, 13 and 14, and TIMP-1 and 2 [2,3]. Of them, stromelysin-3 (MMP-11) was the most frequently expressed in this MICs population (85.7% *vs.* 4.6% in the low MMPs/TIMPs profile group), and therefore the expression of this factor is considered as a useful biological marker of these prometastatic-related MICs.

The high occurrence of distant metastasis in patients whose tumors are infiltrated by MICs overexpressing MMPs and TIMPs may be, in part, because MMPs play an essential role in the degradation of the stromal connective tissue and basement membrane components, which are key elements in tumor invasion and metastasis. MMPs are also able to impact on tumor cell behavior *in vivo* because of their capacity to cleave growth factors, cell surface receptors, cell adhesion molecules, and chemokines/

cytokines [4,5,6]. Furthermore, by cleaving proapoptotic factors, MMPs induce a more aggressive phenotype as a consequence of generation of apoptotic resistant cells [4]. MMPs also regulate cancer-related angiogenesis, both positively through their ability to mobilize or activate proangiogenic factors [7], or negatively via generation of angiogenesis inhibitors, such as angiostatin and endostatin, cleaved from large protein precursors [8]. Moreover, it is now accepted that TIMPs are multifactorial proteins involved in the induction of proliferation and the inhibition of apoptosis [9,10].

Nevertheless, we consider that these tumors containing MMPs/TIMPs-overexpressing MICs, and with a higher rate of distant metastasis, may also express other inflammatory factors, which may be potential biological markers of tumor aggressiveness and/or therapeutic targets in breast cancer. Therefore, the aim of the present study was to evaluate the relationship between MMP-11 expression by intratumoral MICs, distant metastasis development, and a wide panel of biological parameters related to tumor progression and inflammation in breast carcinoma.

Materials and Methods

Ethics Statement

Women were treated according to the guidelines used in our Institution. Written informed consent, approved by our Institution's Ethics and Investigation Committee, was obtained from all patients before the evaluation of tumor samples. The study adhered to National regulations and was approved by our Institution's Ethics and Investigation Committee.

Patient Selection and Study Design

We selected women with the following inclusion criteria: early invasive breast cancer (without distant metastasis at initial diagnosis), at least 6 histopathologically assessed axillary lymph nodes, T1 or T2 size tumors and a minimum of 10 years of follow-up for women without tumor recurrence. The exclusion criteria were the following: metastatic disease at presentation, prior history of any kind of malignant tumor, bilateral breast cancer at presentation, having received any type of neoadjuvant therapy, development of locoregional recurrence during the follow-up period and development of a second primary cancer (distant recurrence cases were not excluded).

From 320 patients fulfilling these criteria, diagnosed and treated between 1990 and 2005, we selected 6 patients whose tumors had MMP-11 negative MICs (Group A) and 6 patients whose tumors had MMP-11 positive MICs (Group B). In both groups of tumors we analyzed the expression of 65 factors (Table 1) by PCR. Proteins showing more significant differences between groups were then analyzed in a wider tumor population corresponding to 3 groups of patients, selected from the remainder patients who fulfilled the inclusion criteria, and stratified as follows: Group A1, 15 patients with MMP-11 negative intratumoral MICs and without distant metastasis during the follow-up period; Group A2, 15 patients with MMP-11 negative intratumoral MICs and with distant metastasis during the follow-up period; and Group B1, 15 patients with MMP-11 positive intratumoral MICs and with distant metastasis during the follow-up period. Given the number of breast cancers that meet the inclusion criteria, 15 patients per group is a representative sample of the population. It is important to emphasize that we did not find a critical number of patients to gather a group B2 (MMP-11 positive intratumoral MICs and without distant metastasis). Protein expression of the factors analyzed in the last three groups was confirmed by immunohistochemistry.

Patient characteristics are listed in Table 2. *Menopausal status was defined* as "postmenopausal" if 1 year was elapsed since the last menstrual period. For reporting the Histological Grade we used the Nottingham combined histologic grade (Elston-Ellis modification of Scarff-Bloom-Richardson grading system) [11]. For estrogen (ER) and progesterone (PgR) receptors evaluation we used mouse anti-ER (clone 1D5) diluted 1/50, and anti-PgR (clone PgR 636) diluted 1/50 (Dako, Glostrup, Denmark). Staining for ERs and PgRs was scored according to the method described by Allred et al. [12].

The endpoint of the study was distant metastatic relapse. The median follow-up period in patients without metastases was 85 months, and 46 months in patients who developed metastases.

Tumor Tissue Handling and Immunohistochemistry

Breast carcinoma tissue samples were obtained at the time of surgery, routinely fixed (in 10% buffered formalin), paraffin-embedded and stored in our pathology laboratory. Serial 3 µm sections of these tumor samples were cut using a microtome (Leica Microsystems) and transferred to an adhesive-coated slide.

Immunohistochemistry was performed on tissue sections using a TechMate TM50 autostainer (Dako). To enhance antigen retrieval, tissue sections were treated in a PT-Link® (Dako) at 97°C for 20 min, in citrate buffer pH 6.1 for IL-1, −5, −6, −17, and in Tris-EDTA buffer pH 9 for IFNβ and NFκB, and then washed in phosphate buffered saline (PBS). Antibody for MMP-11 did not require antigen. The dilution for each antibody was as follows: 1/50 for IL-5; 1/200 for MMP-11; 1/300 for IL-17; 1/400 for IL-1, -6 and IFNβ; and 1/600 for NFκB. The negative control was DakoCytomation mouse or rabbit serum diluted at the same concentration as the primary antibody. Dilutions were made in antibody diluent (Dako) and incubated for 30 min to 2 h at room temperature. Breast tumor samples in which MMP-11 expression was confirmed by Western-blot analysis, were used as positive controls, as shown previously [2,13]. Endogenous peroxidase activity was blocked by incubating the slides in peroxidase blocking solution (Dako) for 5 min. The EnVision Detection kit (Dako) was used as the detection system. Sections were counterstained with haematoxylin, dehydrated with ethanol and permanently coverslipped.

In the present work we evaluated the immunoreactivity on stromal MICs exclusively. Each evaluated field (400× power objective) contained at least 10 stromal MICs. We considered a positive immunostaining, for MMP-11 by MICs, when at least 10% of MICs showed a positive immunostaining at each evaluated field in every case, as established previously [2]. We used several markers to distinguish mononuclear inflammatory cells: CD3 for T-cells, CD20 for B-cells and CD68 for macrophages, all from Dako. Ten fields per case, corresponding to areas with higher immunostaining and without necrosis, were evaluated for CD3, CD20 and CD68 cell counting in a 1 mm^2 final area.

We also evaluated the immunohistochemical staining for IL-1, −5, −6, −17, IFNβ and NFκB by each main cellular type: tumor cells, fibroblast and MICs. Stromal cells were distinguished from cancer cells because the latter are larger in size, and fibroblasts since they are spindle-shaped whereas mononuclear inflammatory cells are small round cells. Moreover, while cancer cells are arranged forming either acinar or trabecular patterns, stromal cells are spread, and also we used several markers to distinguish mononuclear inflammatory cells as describe above. We considered a positive immunostaining, for these inflammatory factors by any of these cell types, when at least 10% of cells showed a positive immunostaining at each evaluated field in every case.

Table 1. Factors analyzed by real-time PCR.

Symbol	Official name	Symbol	Official name
ADAM8	A desintegrin and metalloprotease 8	**IL8**	Interleukin 8
ADAM9	A desintegrin and metalloprotease 9	**IL10**	Interleukin 10
ADAM10	A desintegrin and metalloprotease 10	**IL12**	Interleukin 12
ADAM12	A desintegrin and metalloprotease 12	**IL17**	Interleukin 17
ADAM15	A desintegrin and metalloprotease 15	**IL18**	Interleukin 18
ADAM17	A desintegrin and metalloprotease 17	**IRAK4**	Interleukin-1 receptor-associated kinase 4
ADAM23	A desintegrin and metalloprotease 23	**IRF3**	Interferon regulatory factor 3
ADAM33	A desintegrin and metalloprotease 33	**MMP1**	Matrix metalloprotease 1(interstitial collagenase)
ADAMTS1	ADAM metalloprotease with thrombospondin type 1 motif, 1	**MMP2**	Matrix metalloprotease 2 (gelatinase A)
ADAMTS2	ADAM metalloprotease with thrombospondin type 1 motif, 2	**MMP3**	Matrix metalloprotease 3 (stromelysin 1)
ADAMTS4	ADAM metalloprotease with thrombospondin type 1 motif, 4	**MMP7**	Matrix metalloprotease 7 (matrilysin)
ADAMTS5	ADAM metalloprotease with thrombospondin type 1 motif, 5	**MMP9**	Matrix metalloprotease 9 (gelatinase B)
ADAMTS15	ADAM metalloprotease with thrombospondin type 1 motif, 15	**MMP11**	Matrix metalloprotease 11 (stromelysin 3)
ANGPT1	Angiopoietin 1	**MMP13**	Matrix metalloprotease 13 (collagenase 3)
ANGPT2	Angiopoietin 2	**MMP14**	Matrix metalloprotease 14 (membrane-inserted)
ANXA2	Annexin A2	**MyD88**	Myeloid differentiation primary response gene 88
CCL3	Chemokine (C-C motif) ligand 3	**NFκB**	Nuclear factor kappa B
CLDN3	Claudin-3	**TEK**	TEK tyrosine kinase, endothelial
COX2	Cyclooxygenase 2	**TGFβ1**	Transforming growth factor beta 1
CXCL16	Chemokine (C-X-C motif) ligand 16	**TIMP1**	TIMP metalloprotease inhibitor 1
CXCR4	Chemokine (C-X-C motif) receptor 4	**TIMP2**	TIMP metalloprotease inhibitor 2
EGF	Epidermal growth factor	**TIMP3**	TIMP metalloprotease inhibitor 3
FGF-2	Fibroblast growth factor 2	**TLR2**	Toll-like receptor 2
FN	Fibronectin	**TLR3**	Toll-like receptor 3
ICAM1	Intercellular adhesion molecule 1	**TLR4**	Toll-like receptor 4
IFNα	Interferon alpha	**TLR5**	Toll-like receptor 5
IFNβ	Interferon beta	**TLR7**	Toll-like receptor 7
IGF-1	Insulin-like growth factor 1 (Somatomedin C)	**TLR9**	Toll-like receptor 9
IGFBP-2	Insulin-like growth factor binding protein 2	**TNF**	Tumor necrosis factor
IL1	Interleukin 1	**VEGFA**	Vascular endothelial growth factor A
IL4	Interleukin 4	**VEGFC**	Vascular endothelial growth factor C
IL5	Interleukin 5	**VEGFD**	Vascular endothelial growth factor D
IL6	Interleukin 6		

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Real-time PCR

Total RNA was isolated from formalin-fixed paraffin-embedded tissue blocks using the Nucleospin® FFPE RNA Kit (Macherey-Nagel), including DNase treatment. We assessed the quality and quantity of extracted RNA using agarose gel electrophoresis and optical density measurements (NanoDrop Technologies, Wilmington, US). First strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the manufacturer's instructions. Reverse transcription step was carried out using the following program: 25°C for 10 min, 37°C for 120 min and 85°C for 5 sec. Expression of the different factors and β-actin (internal control) were assessed by real-time PCR using Fast SYBR Green Master Mix (Applied Biosystems) in an ABI Prism 7900 HT thermocycler (Applied Biosystems) with the following cycling conditions: 95°C for 20 sec, 40 cycles of 95°C for 1 sec and 60°C for 20 sec. Primers used are listed in Table 3.

Results

MMP-11 Immunostaining

We analyzed MMP-11 expression by MICs in breast carcinomas samples, to distinguish the two principal groups of tumors (Figure 1). Immunostaining for this protein has a cytoplasmic location in all positive cases. It was very easy to distinguish "positive" from "negative" cases, because all MMP-11 positive cases showed at least as 70% positive MICs; whereas in MMP-11 negative cases, no more than 10% of MICs were stained. We did not find cases with MMP-11 positive MICs and negative tumor cells; however, we found cases with MMP-11 positive tumor cells and negative or positive expression by MICs. T-cells ($CD3^+$), B-cells ($CD20^+$) and macrophages ($CD68^+$) counts showed no significant differences between each group of tumors studied (data not shown).

Table 2. Patient and tumor characteristics.

Characteristics	Group A1 N° (%)	Group A2 N° (%)	Group B1 N° (%)
Total cases	**15 (100)**	**15 (100)**	**15 (100)**
Menopausal status			
Premenopausal	4 (26.7)	5 (33.3)	5 (33.3)
Postmenopausal	11 (73.3)	10 (66.7)	10 (66.7)
Tumoral size			
T1	7 (46.7)	6 (40)	6 (40)
T2	8 (53.3)	9 (60)	9 (60)
Nodal status			
N(−)	5 (33.3)	6 (40)	7 (46.7)
N(+)	10 (66.7)	9 (60)	8 (53.3)
Histological grade			
Well Dif. (I)	5 (33.3)	4 (26.7)	5 (33.3)
Mod. Dif. (II)	7 (46.7)	7 (46.7)	5 (33.3)
Poorly Dif. (III)	3 (20)	4 (26.7)	5 (33.3)
Estrogen receptors			
Negative	6 (40)	9 (60)	8 (53.3)
Positive	9 (60)	6 (40)	7 (46.7)
Progesterone receptors			
Negative	5 (33.3)	11 (73.3)	9 (60)
Positive	10 (66.7)	4 (26.7)	6 (40)
Adjuvant radiotherapy			
No	6 (40)	8 (53.3)	9 (60)
Yes	9 (60)	7 (46.7)	6 (40)
Adjuvant systemic therapy			
Chemotherapy	6 (40)	8 (53.3)	7 (46.7)
Adjuvant Tamoxifen	2 (13.3)	2 (13.3)	4 (26.7)
Chemotherapy *plus* sequential Tamoxifen	5 (33.3)	3 (20)	2 (13.3)
No treatment	2 (13.3)	2 (13.3)	2 (13.3)

We evaluated 30 patients with tumors showing MMP-11 negative expression by MICs, without (Group A1) or with (Group A2) distant metastasis, and 15 patients with tumors showing MMP-11 positive expression by MICs and with distant metastasis (Group B1).
doi:10.1371/journal.pone.0049047.t002

Preliminary Screening of Factors Related to MMP11-expression

In an initial approach, we analyzed a sample size of 6 patients whose tumors have MMP-11 negative MICs (Group A) and 6 patients whose tumors have MMP-11 positive MICs (Group B). In these groups we analyzed by real-time PCR the differential expression of 65 factors related with tumor progression and inflammation (Table 1 and 3) and found differences in the RNA expression of 26 factors (Figure 2), that were therefore related with MMP-11 expression by MICs. All factors show raised levels in tumors with MMP-11 positive MICs. However, carcinoma samples with MMP-11 positive MICs showed a more important increase in the mRNA level of 19 factors: IL-1, −5, −6, −8, −17 and −18, ADAM-8, −10, −15, and −23, ADAMTS-1, −2, and −15, IFNβ, MMP-1, as well as mediators related to inflammation (CCL-3, IRAK-4, MyD88 and NFκB). It was remarkable that expression of several factors such as IL-1, −5, −6 and −17, NFκB and IFNβ was increased at least 8 to 15 fold in Group A compared with Group B samples. Initially, we considered factors increased at least 10 fold but with this threshold we discarded an important cytokine as IFNβ, therefore, we decided to include IFNβ and thus decrease the threshold to 8 fold. Thus, these six factors more differentially expressed between both groups of tumors are the factors selected to study differences between groups later on.

Relationship Between IL-1, −5, −6 and −17, IFNβ and NFκB, and Distant Metastasis Development

We analyzed by real-time PCR the expression of the selected factors (IL-1, −5, −6 and −17, IFNβ and NFκB) more differentially expressed in the preliminary screening, in a wider tumor population consisting of three differentiated groups according to MMP-11 expression by MICs and to distant metastasis development (n = 15 in each group) (Figure 3). The results indicate that the expression levels of these inflammatory factors were significantly higher in tumors with MMP-11 positive MICs and that develop distant metastasis during the follow-up period (Group B1), compared with tumors with MMP-11 negative MICs and distant metastasis (Group A2), or compared with tumors with MMP-11 negative MICs and without distant metastasis (Group A1), which showed the lower levels of these factors.

Table 3. Primers sequences used for real-time PCR analysis (listed 5′- to -3′ end).

Symbol	Primers sequences	Symbol	Primers sequences
ADAM8	F- GTGAATCACGTGGACAAGCTAT	**IL8**	F- TCTCAGCCCTCTTCAAAAACTTCTC
	R- TTCTTGCTGTGGTCCTGGTTCA		R- ATGACTTCCAAGCTGGCCGTGGCT
ADAM9	F- TTAGTGAAGATAGTGGATTGAGTACAGCTT	**IL10**	F- ATGCAGGACTTTAAGGGTTACTTGGGTT
	R- TGTTGGAGCCATGACATGCT		R- ATTTCGGAGAGAGGTACAAACGAGGTTT
ADAM10	F- TTTGGATCCCCACATGATTCTG	**IL12**	F- TCGCGTTCACAAGCTCAAGT
	R- GGTTGGCCAGATTCAACAAAAC		R- CAAACCTGACCCACCCAAGA
ADAM12	F- GGAATTGTCATGGACCATTCAG	**IL17**	F- GTCTGGGCGCAGGTATGTGG
	R- TTCCTGCTGCAACTGCTGAACA		R- CACCGTGGAGACCCTGGAGGC
ADAM15	F- AACATGGACCACTCCACCAGCA	**IL18**	F- CAGACAACTTTGGCCGACTTCA
	R- TTCGAAGAGGCAGCTGCCCATT		R- ACACAAACCCTCCCCACCTAACT
ADAM17	F- TACAAAGGAAGCTGACCTGGTT	**IRAK4**	F- CAGACTCTCTTGCTTGGATGGT
	R- TTCATCCACCCTCGAGTTCCCA		R- AGCTGACCCTGAGCAATCTT
ADAM23	F- TAGGGATCCCAAAGCTATTTGAGCCCA	**IRF3**	F- GTTCTGTGTGGGGGAGTCAT
	R- ATGAAGATTCGGTGGGCA		R- CTGTTGGAAATGTGCAGGTC
ADAM33	F- TGGTTCAAGTTTCGGTGCCGAG	**MMP1**	F- CAGTGGTGATGTTCAGCTAGCTCA
	R- GAGTGGCCTGATCACCCTCA		R- GCCGATGGGCTGGACA
ADAMTS1	F- CAGCCCAAGGTTGTAGATGGTA	**MMP2**	F- GAGGACTACGACCGCGACAA
	R- TTCACTTCGATGTTGGTGGCTC		R- CTTCACTTTCCTGGGCAACAA
ADAMTS2	F- GAACCATGAGGACGGCTTCTCCT	**MMP3**	F- GCCAGGGATTAATGGAGATG
	R- GGCTGCAGCGGGACCAGTGGAA		R- ATTTCATGAGCAGCAACGAG
ADAMTS4	F- GCAACGTCAAGGCTCCTCTT	**MMP7**	F- GTGGGAACAGGCTCAGGACTATCTCAA
	R- CTCCACAAATCTACTCAGTGAAGCA		R- CACATTGGGCTTCTGCATTATTACTA
ADAMTS5	F- AGGAGCACTACGATGCAGCTATC	**MMP9**	F- CCTGGAGACCTGAGAACCAATC
	R- CCCAGGGTGTCACATGAATG		R- GATTTCGACTCTCCACGCATCT
ADAMTS15	F- GCCTGGCAGAAGAAGCTGAAC	**MMP11**	F- GAGCAGGTGCGGCAGACGA
	R- GCTGTCCAGGAAGTCGGTGAT		R- CGAAAGGTGTAGAAGGCGGACA
ANGPT1	F- TTCTCTTCCCAGAAACTTCA	**MMP13**	F- ATCCCTTGATGCCATTACCA
	R- ACTGAACCTGACCGTACACATCTCCGACTT		R- AAGAGCTCAGCCTCAACCTG
ANGPT2	F- TATGGAAAACAACACTCAG	**MMP14**	F- GCCCAATGGGAAGACCTACT
	R- ACTGAACCTGACCGTACATTCTGTACTGCATTCTGCTG		R- AGGGTACTCGCTGTCCACTG
ANXA2	F- CTCTACACCCCCAAGTGCAT	**MyD88**	F- TGGCACCTGTGTCTGGTCTA
	R- TCAGTGCTGATGCAAGTTCC		R- ACATTCCTTGCTCTGCAGGT
CCL3	F- CTTGCTGTCCTCCTCTGCAC	**NFκB**	F- TCTCCCTGGTCACCAAGGAC
	R- TCACTGGGGTCAGCACAGAC		R- TCATAGAAGCCATCCCGGC
CLDN3	F- CTGCTCTGCTGCTCGTGTCC	**TEK**	F- TGTTCCTGTGCCACAGGCTG
	R- TTAGACGTAGTCCTTGCGGTCGTAG		R- CACTGTCCCATCCGGCTTCA
COX2	F- ATCATAAGCAGGGCCAGCT	**TGFβ1**	F- GCAACAATTCCTGGCGATAC
	R- AAGGCGCAGTTTACGCTGTC		R- AAGGCGAAAGCCCTCAAT
CXCL16	F- TCTCAAAGAATGTGGACATGC	**TIMP-1**	F- CCGCAGCGAGGAGTTTCTC
	R- CAGGGGTGTGGATATCTGAA		R- GAGCTAAGCTCAGGCTGTTCCA
CXCR4	F- AGCTGTTGGCTGAAAAGCTGGTCTATG	**TIMP-2**	F- CGACATTTATGGCAACCCTATCA
	R- GCGCTTCTGGTGGCCCTTGGAGTGTG		R- GCCGTGTAGATAAACTCTATATCC
EGF	F- ACATCAAATATCCTCAATGG	**TIMP-3**	F- GCGTCGGAGGTTAAGGTTGTT
	R- GTGGCATCAAGACCGGGCTGC		R- CTCTCCAAAATTACCGTACGCG
FGF-2	F- ACCCCGACGGCCGA	**TLR2**	F- CAGGGCTCACAGAAGCTGTAA
	R- TCTTCTGCCTTGAAGTTGTAGCTTGA		R- GCCCAGGGAAGAAAAAGAATC
FN	F- CCACAGTCGGGTCAGGAG	**TLR3**	F- TAGCAGTCATCCAACAGAATCAT
	R- CTGGCCGAAAATACATTGTAAA		R- AATCTTCTGAGTTGATTATGGGTAA
ICAM1	F- AGGCCACCCCAGAGGACAAC	**TLR4**	F- ACTCCCTCCAGGTTCTTGATTAC

Table 3. Cont.

Symbol	Primers sequences	Symbol	Primers sequences
	R- CCCATTATGACTGCGGCTGCTA		R- CGGGAATAAAGTCTCTGTAGTGA
IFNα	F- TGGCTGTGAAGAAATACTTCCG	TLR5	F- GATTCTTGCCCACCACAT
	R- TGTTTTCATGTTGGACCAGATG		R- GGTTCGCTGTAAGGTTGAT
IFNβ	F- TCTCCACGACAGCTCTTTCCA	TLR7	F- CGGCTTGATTTACTCTCCAT
	R- ACACTGACAATTGCTGCTTCTTTG		R- CAGTGGTCAGTTGGTTGTGG
IGF-1	F- TTGTGATTTCTTGAAGGTGAAGATG	TLR9	F- CTTCCCTGTAGCTGCTGTGTCC
	R- CGTGGCAGAGCTGGTGAAG		R- CCTGCACCAGGAGAGACAG
IGFBP-2	F- GCAGGTTGCAGACAATGGCG	TNF	F- CCAGGGACCTCTCTCTAATCAGC
	R- GTGGTCCAGCTTCTTGGGC		R- CTCAGCTTGAGGGTTTGCTACAA
IL1	F- TAGTAGCAACCAACGGGAAG	VEGFA	F- GCAGAATCATCACGAAGTGG
	R- CTCTGAGTCATTGGCGATG		R- GCAACGCGAGTCTGTGTTTTTG
IL4	F- CCACGGACACAAGTGCGATAT	VEGFC	F- GCCACGGCTTATGCAAGCAAAGAT
	R- CGTAACAGACATCTTTGCTGCC		R- AGTTGAGGTTGGCCTGTTCTCTGT
IL5	F- AAAGGCAAACGCAGAACGTGT	VEGFD	F- CGATGTGGTGGCTGTTGCAATGAA
	R- CTCTTGGAGGTGCCTAGTGT		R- GCTGTTGGCAAGCAAGCACTTGACAACCT
IL6	F- GAACTCCTTCTCCACAAGCGCCTT		
	R- CAAAAGACCAGRGATGATTTTCACCAGG		

F: forward primer; R: reverse primer.
doi:10.1371/journal.pone.0049047.t003

Thus, these results contribute to identify a tumor group with up-regulated inflammatory-related genes, which present worse prognosis. The classification of these tumor populations in good or bad prognosis was based on the expression of MMP11 by MICs, as described previously by our group [3]. Our study emphasizes the importance of IL-1, −5, −6 and −17, IFNβ and NFkB in promoting distant metastasis and recurrence, as demonstrated by their high expression in tumors from patients with a higher rate of distant metastasis development (97.6%) [3]. Some of these molecules implicated in the cross-talk between the tumor and the inflammatory microenvironment may emerge as attractive targets in breast cancer.

Expression of IL-1, −5, −6 and −17, IFNβ and NFκB by Tumor and Stromal Cells

We analyzed by immunohistochemistry the expression of IL-1, −5, −6 and −17, IFNβ and NFκB in three differentiated groups according to MMP-11 expression by MICs and to distant metastasis development (n = 15 in each group). Table 4 indicates that all cell type contribute to the overall expression of these factors, except NFκB which was expressed only by tumor cells. In addition, IL-6 was differentially expressed by fibroblasts between each group ($p = 0.006$), showing fewer positive cases in Group A2. In the same way, Group A2 show less positive cases for IL-17 by tumor cell ($p = 0.008$). In addition, IFNβ expression by tumor cells was similar in Group A1 and B1. Generally, differential expression of these factors is not dependent on one cell type.

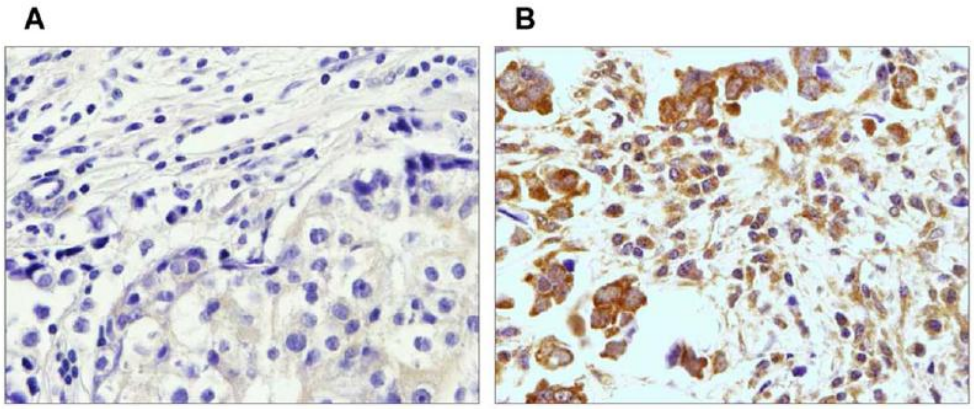

Figure 1. Representative examples of MMP-11 immunostaining in MICs (x400). (a) MMP-11 negative staining in MICs. (b) MMP-11 positive staining in MICs. The red arrow represents tumor cells and the green arrow represents MICs.
doi:10.1371/journal.pone.0049047.g001

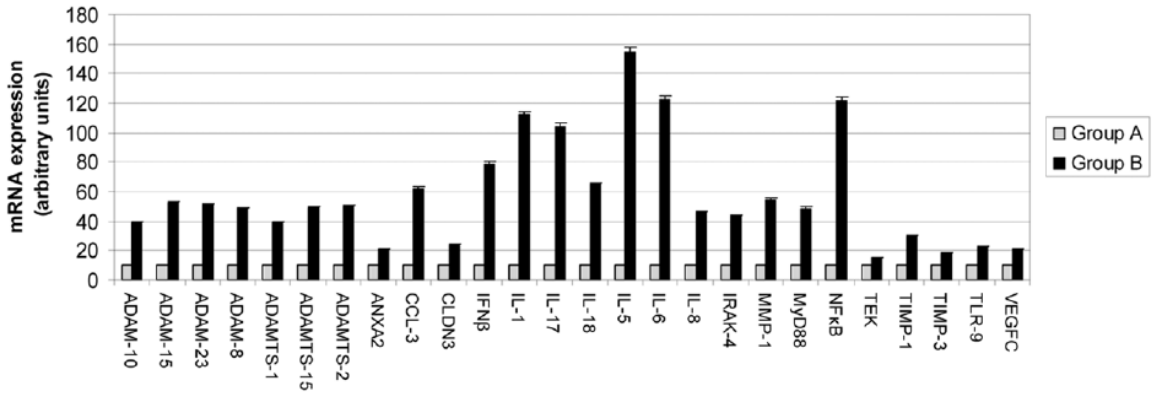

Figure 2. Real-time PCR analysis of factors differentially expressed between the two main tumor groups. Group A: tumors with MMP-11 negative MICs. Group B: tumors with MMP-11 positive MICs. Data represent the mean ± SD of three independent experiments.
doi:10.1371/journal.pone.0049047.g002

Discussion

The present study consists in a molecular characterization of the inflammatory process implicated in the tumor progression of breast cancer. In this study we used a classification of tumors in good or bad prognosis based on the expression of MMP11 by MICs, as described previously by our group [3]. The accomplishment of a pilot study has allowed us to perform a preliminary screening of molecules associated with tumor progression and inflammation, and to analyze the relationship between specific inflammatory factors and distant metastasis development in breast carcinomas based on MMP-11 expression by MICs.

Inflammatory cells can represent up to 50% of the total tumor mass in an invasive mammary carcinoma, and include macrophages, plasma cells, mast cells, T and B-lymphocytes [14,15]. Historically, the infiltrating tumor-associated leukocytes have been considered as an intrinsic mechanism of defense against tumor development [15,16]. Our results agree with increasing evidences indicating that leukocyte infiltration can promote changes leading to a more aggressive tumor phenotype in the scope of angiogenesis, tumor growth, invasion and metastasis [14,17]. Inflammatory cells probably influence tumor progression by secreting factors like cytokines, growth factors, chemokines and proteases, which stimulate the proliferation and invasiveness of tumor cells. According to this, recently Tan et al. have characterized a type of tumor-infiltrating lymphocytes that stimulate the development of breast cancer metastasis through signals related to the transcription factor NFκB [18].

Nevertheless, the prognostic significance of the lymphoid infiltrate at the tumor site remains controversial perhaps because the evaluation criteria for these tumor infiltrates are not sufficiently standardized to yield reliable and reproducible results in different institutions. Therefore, our results may contribute to a better characterization of the inflammatory phenotype of mammary carcinomas associated with unfavorable prognosis. Altogether, these results suggest that identification of these cases turns out to be an important key in the molecular biology of mammary carcinomas associated with tumor progression, and depending not only on the tumor cells themselves but also on the surrounding inflammatory cell infiltrate.

Our results demonstrate that, of the 65 factors analyzed and related to the inflammatory process and tumor progression, those related to MMP-11 expression by MICs in the intratumoral stroma were IL-1, −5, −6, −8, −17, −18, MMP-1, TIMP-1, ADAM-8, −10, −15, −23, ADAMTS-1, −2, −15, CCL-3,

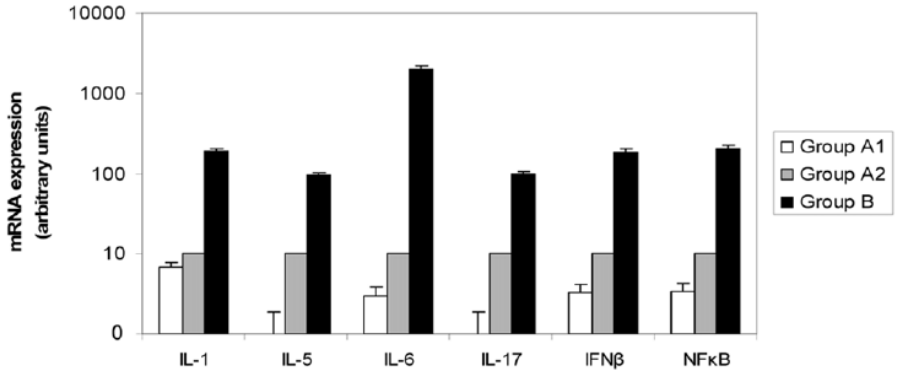

Figure 3. Real-time PCR analysis of the 6 most differentially expressed factors between the three tumors groups. Group A1: tumors with MMP-11 negative MICs and without distant metastasis. Group A2: tumors with MMP-11 negative MICs and distant metastasis. Group B: tumors with MMP-11 positive MICs and distant metastasis. Data represent the mean ± SD of three independent experiments.
doi:10.1371/journal.pone.0049047.g003

Table 4. Percentage of cases positive for IL-1, −5, −6, −17, IFNβ and NFκB by each cell type as function of group.

	Group A1			Group A2			Group B1		
	Tumor cell	MIC	Fibroblast	Tumor cell	MIC	Fibroblast	Tumor cell	MIC	Fibroblast
IL-1	90.9	90.9	90.9	100	100	100	100	90	100
IL-5	95	50	25	89	58	22	100	62.5	12.5
IL-6	89.5	52.6	36.8 *	66.7	66.7	66.7	100	88.9	100
IL-17	100 *	78.9	89.5	66.7	66.7	66.7	100	100	88.9
IFNβ	90.9 *	100	100	33.3	100	100	90	90	100
NFκB	81.8	0	0	66.7	0	0	80	0	0

MIC: mononuclear inflammatory cells; *$p<0.05$.
doi:10.1371/journal.pone.0049047.t004

Annexin A2, IFNβ, Claudin3, IRAK-4, MyD88 and NFκB. Of them, factors more differentially expressed between both main types of tumors were IL-1, −5, −6, −17, IFNβ and NFκB. These latter factors were analyzed in a wider tumor sample, in which we confirmed the higher expression of those factors in tumors infiltrated by MMP-11 positive MICs and that the expression not depend of only one cell type. Thus, our study contributes to a better biological characterization of mammary carcinomas, especially with regard to the molecular profile of its inflammatory component.

These factors showing an increased expression level in tumors infiltrated by MMP-11 positive MICs, like IL-1, −5, −6, −17, IFNβ and NFκB, have a great biological interest because of their relation with tumor progression. IL-1 is essentially produced by activated macrophages, and induces a great variety of genes like IL-5, IL-6, oncogenes (c-fos, c-myc, c-jun), IFN-β and collagenases. Different experimental models have shown that local production of IL-1 influences tumor growth and metastasis development, either through direct proliferative effects or through the activation of the inflammation and angiogenesis signaling [19,20]. The production of IL-1 by tumor or stromal cells has been associated with an aggressive tumor phenotype in several types of mouse and human cancers [21]. These data support our results, in which patients with a higher frequency of metastasis (97.6%) present a higher expression of IL-1.

IL-5 is essentially produced by T-helper type-2 lymphocytes and mast cells, stimulates B cells growth and increases the production of immunoglobulins. This interleukin has not yet been described as an important factor in the development of breast cancer metastasis, but our results indicate that could be an important target to analyze in these cases.

IL-6 seems to play an important role in the resistance to the apoptotic process. IL-6 is produced by stromal cells like T-cells, fibroblasts or monocytes and also by tumor cells. Some studies show the role of IL-6 in tumor cells growth *in vitro*, but its exact role is still unclear. Also, studies evaluating IL-6 expression in mammary carcinomas, show contradictory results. Marrogi et al. analyzed the expression profile of IL-6 in 19 mammary carcinomas and detected no mRNA expression [22]. Nevertheless, other studies have detected and quantified IL6 expression in breast cancer [23,24]. Bachelot et al. studied the clinical meaning of vascular endothelial growth factor (VEGF) and IL-6 expression in hormone-refractory mammary carcinomas and observed that presence of IL-6 in patient's serum (but not VEGF), was correlated with a shorter survival [25]. In our case, we found that intratumor expression of IL-6 correlates with a higher risk of metastasis.

In the last few years, IL-17 has been considered as a key link between adaptive and innate immunity, and also plays a critical role in inflammation and autoimmune diseases. In spite of the role of IL-17 in autoimmunity, it is relatively little known about its function in cancer, and the published data are still contradictory. Some studies support its role in tumor progression, probably due to the stimulation of angiogenic factors [26,27]. On the contrary, other studies suggest that IL-17 promotes tumor rejection through a T-cell-dependent mechanism [28]. $CD8^+$ T-cells and non-T-cells have been reported to produce Th17 cytokines [29], including IL-17, but the role of non-T-cell-derived IL-17 remains to be further defined. Our data suggest that IL-17 contributes to tumor progression and aggressiveness, showing an expression decrease in at least 100 fold in tumors that do not develop metastasis compared to tumors with unfavorable prognosis.

The production of IFNβ by T and B-cells, macrophages, fibroblasts, or endothelial cells among others, is induced by other cytokines like IL-1, IL-2, TNF and CSF. The function of IFNβ in breast cancer progression has already not been described. Nevertheless, this cytokine well-known because of its role in antiviral immunity, can be related with the recent association between human papilloma virus and breast cancer [30,31,32].

Nuclear factor kappa (NFkB) has a specific role in tumor progression, and also has been associated to cancer stem cells survival [33]. NFkB regulates the expression of numerous antiapoptotic proteins associated with tumor survival (bcl-xl, bcl-2, XIAP, c-FLIP, IAP-1, IAP-2, and survivin), as well as genes associated with tumor progression (cyclin D1, c-myc and COX-2). In addition, numerous data support the role of NFkB in the regulation of tumor inflammation and progression [34].

The result of the present study was that tumors developing worse prognosis and identified by MMP-11 expression by intratumoral MICs, showed an up-regulation of inflammatory-related genes. The classification of these tumor groups in good or bad prognosis was based on the expression of MMP-11 by MICs, as described previously by our group [3]. Our study emphasizes the importance of IL-1, −5, −6 and −17, IFNβ and NFkB in promoting disease metastasis and recurrence, as demonstrated by their high expression in tumors from patients with a higher rate of distant metastasis development (97.6%) [3]. Some of these molecules implicated in the cross-talk between the tumor and inflammatory microenvironment may emerge as attractive targets in breast cancer. Therefore, these data contribute to a better biological characterization of tumors and open up the possibility of undergoing new studies to determine which cell type specifically express those factors, and their biological signification.

Author Contributions

Conceived and designed the experiments: NE LG LOG BFG MLL LM SGR JMdC FJV. Performed the experiments: NE LG LOG BFG LM SGR FJV. Analyzed the data: NE LG LOG BFG MLL LM SGR JMdC FJV. Contributed reagents/materials/analysis tools: NE LG LOG BFG MLL LM SGR JMdC FJV. Wrote the paper: NE LG LOG BFG MLL LM SGR JMdC FJV.

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Impact of CD68/(CD3+CD20) Ratio at the Invasive Front of Primary Tumors on Distant Metastasis Development in Breast Cancer

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Abstract

Tumors are infiltrated by macrophages, T and B-lymphocytes, which may favor tumor development by promoting angiogenesis, growth and invasion. The aim of this study was to investigate the clinical relevance of the relative amount of macrophages ($CD68^+$), T-cells ($CD3^+$) and B-cells ($CD20^+$) at the invasive front of breast carcinomas, and the expression of matrix metalloproteases (MMPs) and their inhibitors (TIMPs) either at the invasive front or at the tumor center. We performed an immunohistochemical study counting CD3, CD20 and CD68 positive cells at the invasive front, in 102 breast carcinomas. Also, tissue sections were stained with MMP-2, -9, -11, -14 and TIMP-2 antibodies, and immunoreactivity location, percentage of reactive area and intensity were determined at the invasive front and at the tumor center. The results showed that an increased CD68 count and CD68/(CD3+CD20) ratio were directly associated with both MMP-11 and TIMP-2 expression by mononuclear inflammatory cells at the tumor center ($p = 0.041$ and $p = 0.025$ for CD68 count and $p = 0.001$ and $p = 0.045$ for ratio, respectively for MMP-11 and TIMP-2). In addition, a high CD68/(CD3+CD20) ratio (>0.05) was directly associated with a higher probability of shortened relapse-free survival. Multivariate analysis revealed that CD68/(CD3+CD20) ratio was an independent factor associated with distant relapse-free survival (RR: 2.54, CI: (1.23–5.24), $p<0.01$). Therefore, CD68/(CD3+CD20) ratio at the invasive front could be used as an important prognostic marker.

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Introduction

Development of an invasive cancer is not only the result of genetic changes in tumor cells but also the result of the interplay between tumor and stromal cells [1]. Tumors are infiltrated by a large number of immune cells that constitute the main cell population of tumor microenvironment, where they can account for up to 50% of the total tumor mass in invasive breast carcinomas. Historically, tumor-infiltrating leukocytes have been considered as an intrinsic defensive mechanism against developing tumors [2–3]. However, increasing evidence indicates that leukocyte infiltration may favor tumor development by promoting angiogenesis, growth, and invasion [4–5]. This may be due to inflammatory cells that probably influence cancer promotion by secreting cytokines, growth factors, chemokines and proteases, which stimulate proliferation and invasiveness of cancer cells [6–8].

Inflammatory cells have gained a renewed interest in breast cancer research due to our increased understanding of their role in tumor development, and also due to our increased ability to identify each cell type. Leukocyte infiltrate includes a variable representation of leukocytes, including macrophages, neutrophils, mast cells, and T and B-lymphocytes [4,9]. There are evidences indicating that different types of breast carcinomas may have different types of leukocyte infiltrate with distinct abilities to control tumor growth according to their tumor dissemination. Thus, whereas macrophages are known to have several pro-tumor functions and macrophage infiltration has also been associated with worse prognosis [4,10–11], it has been reported that both T- and B-lymphocytes perform an important immunological response by inhibiting cancer development and progression [12–20].

Metastasis development is regulated not only by intrinsic genetic changes in malignant cells, but also by the tumor microenvironment. Matrix metalloproteases (MMPs) play an essential role in the degradation of the stromal connective tissue and basement membrane components, which are key elements in tumor invasion and metastasis. In fact, in the metastatic process across the axillary lymph node chain in breast cancer, MMP-1 expression by mononuclear inflammatory cells (MICs) from the sentinel lymph node (SLN) was significantly associated with metastatic spread to non-SLNs [21]. MMPs cleave proapoptotic factors and induce a more aggressive phenotype generating apoptotic resistant cells [22], and also regulate cancer-related angiogenesis, both positively

Table 1. Basal characteristics of 102 patients with invasive ductal carcinoma of the breast.

CHARACTERISTICS	Without recurrence No. (%)	With recurrence No. (%)
Total cases	59 (100)	43 (100)
Menopausal status		
Premenopausal	18 (30.5)	12 (27.9)
Postmenopausal	41 (69.5)	31 (72.1)
Tumoral size		
T1	31 (52.5)	19 (44.2)
T2	28 (47.5)	24 (55.8)
Nodal status		
N (−)	28 (47.5)	12 (27.9)
N (+)	31 (52.5)	31 (72.1)
Histological grade		
Well Dif. (I)	20 (33.9)	7 (16.3)
Mod. Dif. (II)	31 (52.5)	16 (37.2)
Poorly Dif. (III)	8 (13.6)	20 (46.5)
Nottingham prognostic index		
<3.4	25 (42.4)	8 (18.6)
3.4–5.4	25 (42.4)	22 (51.2)
>5.4	9 (15.3)	13 (30.2)
Estrogen Receptor		
Negative	16 (27.1)	23 (53.5)
Positive	31 (52.5)	18 (41.9)
Progesterone Receptor		
Negative	20 (33.9)	27 (62.8)
Positive	27 (45.8)	14 (32.6)
Adjuvant radiotherapy		
No	44 (74.6)	21 (48.8)
Yes	15 (25.4)	22 (51.2)
Adjuvant systemic therapy		
Chemotherapy	18 (30.5)	18 (41.9)
Tamoxifen	24 (40.7)	9 (20.9)
Chemotherapy plus sequential Tamoxifen	10 (16.9)	7 (16.3)
No treatment	7 (11.9)	9 (20.9)
HER2 Status		
Negative	49 (83.1)	36 (83.7)
Positive	8 (13.6)	7 (16.3)
Basal like phenotype		
Non basal like	30 (50.8)	23 (53.5)
Basal like	15 (25.4)	18 (41.9)

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through their ability to mobilize or activate proangiogenic factors [23], or negatively through the generation of angiogenesis inhibitors, such as angiostatin and endostatin [24]. The activity of MMPs is specifically inhibited by the so-called tissue inhibitors of metalloproteases (TIMPs). In previous reports we analyzed the expression of several MMPs and TIMPs (MMP-1, 2, 7, 9, 11, 13 and 14, and TIMP-1, 2 and 3), either at the invasive front or at the tumor center of breast carcinomas, in many of the women included in the present study [25–28]. Thus, we identified a phenotype of MICs characterized by the expression of specific MMPs and TIMPs (MMP-2, 9 11 and 14, and with TIMP-2) in the tumor center, associated with distant metastasis development [25–26], suggesting that inflammatory cells at the invasive front can polarize their phenotype impacting on tumor progression [27]. These tumors also showed an up-regulation of inflammatory-related genes (IL-1, -5, -6 and -17, IFNβ and NFkB), which emphasize their importance in promoting disease metastasis and recurrence [29].

Considering that the invasive front is the area where some of the most important interactions between cancer cells and tumor supporting stroma take place [30], we investigate the relevance of the relative amount of macrophages (CD68), T-cells (CD3) and B-

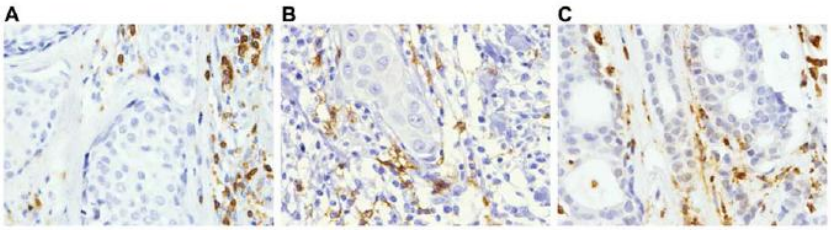

Figure 1. Representative examples of immunohistochemical stainings at the invasive front from breast carcinomas (×200 magnification). (A) Membranous staining of CD3 indicating T-lymphocytes. (B) Membranous staining of CD20 indicating B-lymphocytes. (C) Cytoplasmic staining of CD68 indicating macrophages.
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cells (CD20) in this tumor location from breast carcinomas. Also, we study their relationship with MMPs and TIMPs expression, either at the invasive front or at the tumor center. Thus, we found that a high CD68/(CD3+CD20) ratio (>0.5) at the invasive front is associated with tumor aggressiveness and poor prognosis in patients.

Materials and Methods

Ethics Statement

Women were treated according to the guidelines used in our Institution (Hospital de Jove). Written informed consent, approved by "Hospital de Jove Ethics and Investigation Committee", was obtained from all patients before the evaluation of tumor samples.

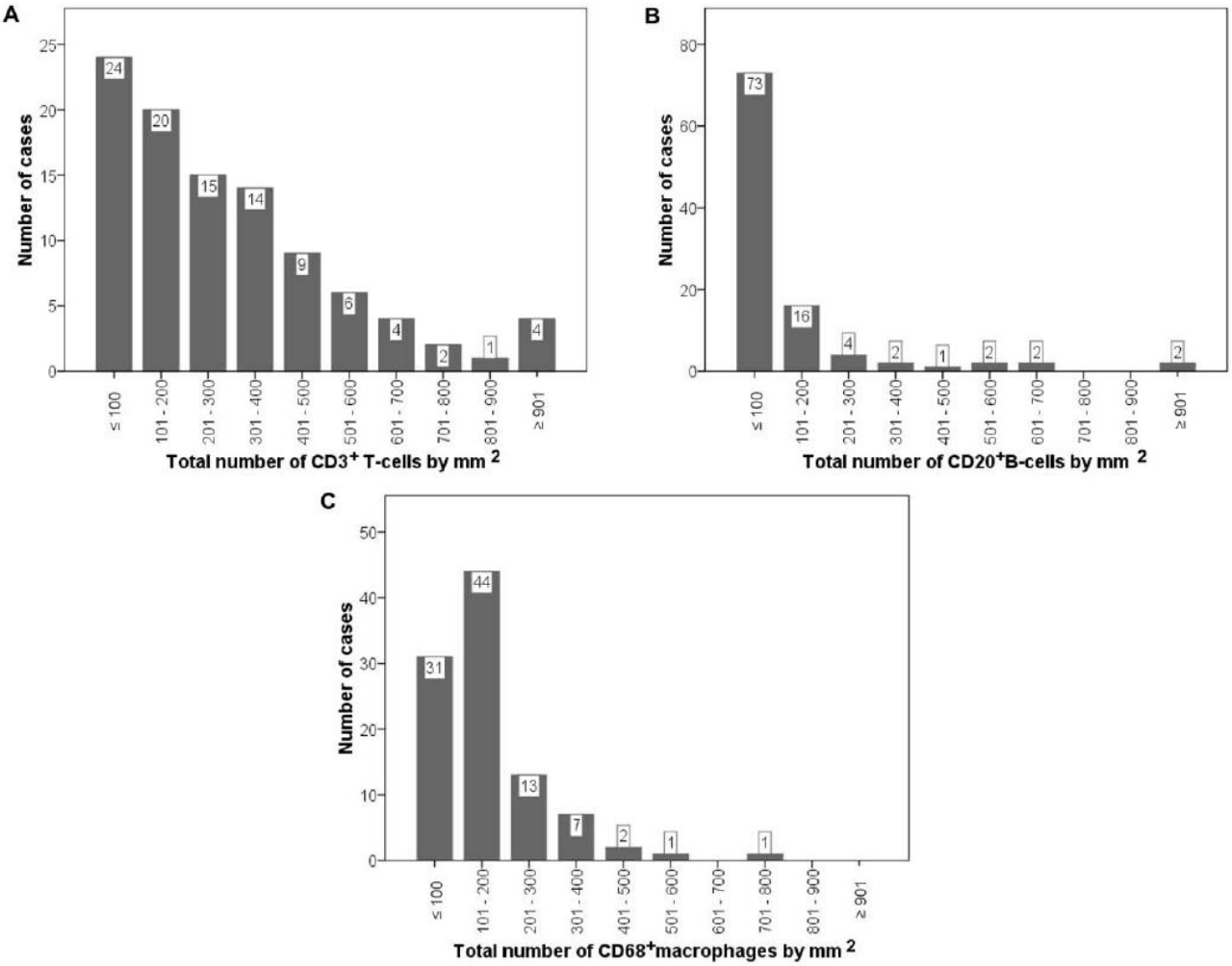

Figure 2. Distribution of the total number of CD markers by mm^2 at the invasive front, in 102 breast carcinomas. CD3 (A), CD20 (B) and CD68 (C).
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Table 2. Relationship between inflammatory cells count or ratio and clinico- pathological characteristics in 102 patients with invasive ductal carcinoma of the breast.

CHARACTERISTICS	No.	CD3	CD20	CD68	CD68/(CD3+CD20)
		median (range)	median (range)	median (range)	median (range)
Total cases	102	214 (0–999)	29 (0–1152)	141 (14–727)	0.5 (0–6.6)
Menopausal status		$p=0.009$			
Premenopausal	30	322 (9–999)	50 (0–1121)	158 (31–404)	0.3 (0.1–5.4)
Postmenopausal	72	167 (0–987)	18 (0–1152)	128 (14–727)	0.5 (0–6.6)
Tumoral size					
T1	50	207 (0–987)	22 (0–1152)	128 (15–727)	0.5 (0–5.4)
T2	52	242 (12–999)	34 (0–1121)	154 (14–577)	0.6 (0.1–6.6)
Nodal status					
N (−)	40	201 (9–987)	27 (0–1152)	136 (15–727)	0.5 (0.1–6.3)
N (+)	62	250 (0–999)	32 (0–1121)	142 (14–577)	0.6 (0–6.6)
Histological grade					
Well Dif. (I)	27	197 (9–987)	25 (0–1152)	140 (15–727)	0.5 (0.1–5.4)
Mod. Dif. (II)	47	228 (12–999)	30 (0–1121)	142 (49–577)	0.6 (0.1–6.6)
Poorly Dif. (III)	28	252 (0–542)	35 (0–156)	139 (14–416)	0.6 (0–5.4)
Nottingham prognostic index					
<3.4	33	172 (9–954)	7 (0–655)	122 (15–727)	0.5 (0.1–5.4)
3.4–5.4	47	267 (0–999)	41 (0–1152)	143 (21–577)	0.5 (0–6.6)
>5.4	22	250 (14–756)	40 (0–252)	170 (14–416)	0.5 (0.1–4.0)
Estrogen Receptor		$p=0.040$			
Negative	39	298 (0–987)	41 (0–1152)	181 (14–727)	0.6 (0–4)
Positive	49	151 (9–895)	10 (0–1121)	122 (34–362)	0.6 (0.1–6.3)
Progesterone Receptor		$p=0.003$		$p=0.002$	
Negative	47	267 (27–987)	40 (0–1152)	182 (14–727)	0.6 (0.1–6.1)
Positive	41	144 (0–895)	10 (0–1121)	105 (35–314)	0.6 (0–6.3)
HER2 Status			$p=0.009$	$p=0.027$	
Negative	85	209 (0–999)	16 (0–1152)	137 (14–727)	0.5 (0–6.6)
Positive	15	359 (36–917)	101 (0–576)	186 (54–577)	0.6 (0.1–1.1)
Basal like phenotype					
Non basal like	53	197 (9–895)	14 (0–1121)	137 (34–577)	0.7 (0.1–6.3)
Basal like	33	251 (0–987)	40 (0–1152)	142 (14–727)	0.4 (0–4.0)

Mann-Whithney or Kruskall-Wallis tests.
doi:10.1371/journal.pone.0052796.t002

The study adhered to National regulations and was approved by our Institution's Ethics and Investigation Committee.

Patient selection, characteristics and tissue specimen handling

This study comprises 102 women with a histological confirmed diagnosis of early invasive breast cancer and treated between 1990 and 2003. Many of these women have been included in previous studies of our group [25–28]. We selected women with the following inclusion criteria: invasive ductal carcinoma and a minimum of 5 years of follow-up for those women without tumor recurrence. The exclusion criteria were the following: metastatic disease at diagnosis, prior history of any kind of malignant tumor, bilateral breast cancer at diagnosis, have been treated with any type of neoadjuvant therapy, development of loco-regional recurrence during the follow-up period or development of a second primary cancer. From patients fulfilling these criteria, we randomly selected a sample size of 102 patients in accordance to 4 different groups stratified with regard to nodal status and to the development of metastatic disease, which were the key measure variables of the study. Thus, we included an important number of cases in both node-positive and node-negative patient subgroups in order to guarantee the statistical power of the survival analysis. Patient characteristics included in the two main groups, with or without distant metastases, are listed in Table 1. Menopausal status was defined as "postmenopausal" if 1 year was elapsed since the last menstrual period. For reporting the Histological Grade we used the Nottingham combined histologic grade (Elston-Ellis modification of Scarff-Bloom-Richardson grading system) [31].

The end-point of our study was distant metastatic relapse. The median follow-up period in patients without metastases was 85 months, and 52 months in patients with metastases.

Tissue arrays and immunohistochemistry

Breast carcinoma tissue samples were obtained at the time of surgery. Samples were removed from the tumors, avoiding grossly

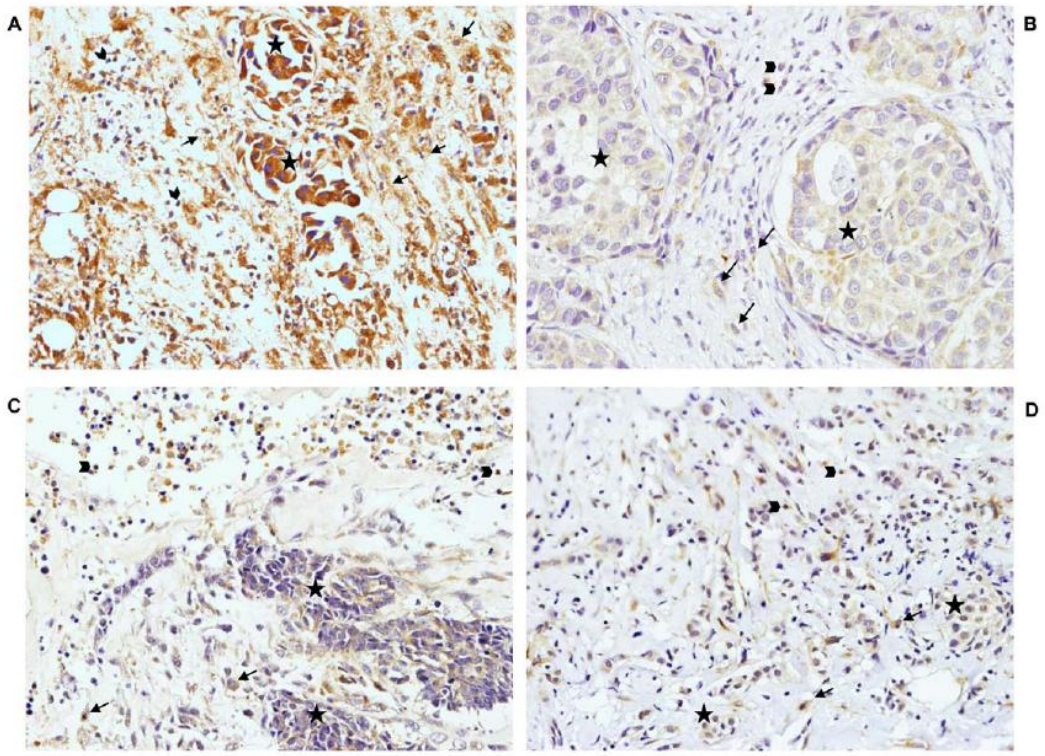

Figure 3. Representative example of immunostaining. MMP11 (A) and TIMP2 (B) immunostaining at the tumor center and MMP9 (C) and MMP14 (D) at the invasive front (×200 magnification), indicating the different cell types. Tumor cells (★), lymphocytes (◗) and macrophages (⟶). doi:10.1371/journal.pone.0052796.g003

necrotic tissues, routinely fixed, paraffin-embedded and stored. Histopathological representative tumor areas of invasive front and tumor center were defined in hematoxylin and eosin-stained sections and marked on the slide. The invasive front was defined as the tumor advancing edge, which corresponds to a 2 mm margin surrounding the tumor and containing cancerous cells, and the tumor center was defined as the tumor area inside the invasive front. Tumor tissue microarray (TMA) blocks containing primary tumor samples were performed as described previously [25]. We analyzed 2 cores of the invasive front and 2 cores of the tumor center in each case (double redundancy) as it has been demonstrated to correlate properly with conventional immunohistochemical staining methods [25,27].

Four composite high-density TMA blocks were performed, consecutively cut in 5 µm sections with a microtome (Leica Microsystems GmbH, Wetzlar, Germany) and transferred to adhesive-coated slides. One section from each TMA block was stained with hematoxylin and eosin, and these slides were then reviewed to confirm that the sample was representative of the invasive front and tumor center of the original tumor. Immunohistochemistry was performed using a TechMate TM50 autostainer (Dako, Glostrup, Denmark), where sections were incubated with the following antibodies (ready to use): CD3 (T-lymphocytes), CD20 (B-lymphocytes) and CD68 (macrophages) all purchased from Dako (Glostrup, Denmark).

In previous reports from our group, we found a specific MICs phenotype characterized by high MMP-2, 9, 11, 14, and TIMP-2 expression, which correlated significantly with distant metastasis development [25–28]. Consequently, in the present study we performed a new staining set using antibodies against these specific proteins, in the tissue arrays from the invasive front and those from the tumor center. Antibodies for MMPs and TIMPs were purchased from Neomarker (Lab Vision Corporation, Fremont, CA, USA), and the dilution used was: 1/50 for MMP-2, -14 and TIMP-2; 1/100 for MMP-9; and 1/200 for MMP-11. To enhance antigen retrieval, tissue sections were treated in a PT-Link® (Dako) at 97°C for 20 min, in citrate buffer of pH 6.1 for MMP-14, in EDTA buffer of pH 9 for TIMP-2. Antibodies for MMP-2, -9 and -11 do not require antigen retrieval. The negative control was DakoCytomation mouse or rabbit serum diluted at the same concentration as the primary antibody. All the dilutions were made in Antibody Diluent, (Dako, Glostrup, Denmark) and incubated 30 min at room temperature.

Endogenous peroxidase activity was blocked by incubating the slides in peroxidase-blocking solution (Dako) for 5 min. The EnVision Detection Kit (Dako) was used as the staining detection system. Sections were counterstained with hematoxylin, dehydrated with ethanol, and permanently coverslipped.

Immunohistochemistry analysis

Five fields per core, corresponding to areas of higher immunostaining and without necrosis, were evaluated with a 400× power objective, counting CD3, CD20 and CD68 positive cells, in 1 mm^2 final area, at the invasive front. If there was no tumor sample in a particular core, 10 fields were then evaluated in another one in order to obtain the same final area. We obtain a total score and this is the value of CD3, CD20 or CD68 for each tumor.

For each MMP or TIMP antibody studied, we determined the immunoreactivity location, percentage of reactive area and intensity, at the invasive front and at the tumor center. An image analysis system composed of the Olympus BX51 microscope,

Table 3. Relationship between inflammatory cells count or ratio at invasive front and MMPs/TIMPs expression by mononuclear inflammatory cells at invasive front or tumor center.

	MICs at invasive front											
	MMP-9			MMP-11			MMP-14			TIMP-2		
	−	+	*p*	−	+	*p*	−	+	*p*	−	+	*p*
CD3	185	327	**0.006**	209	266.5	N.S	210	261	N.S	154	317.5	**0.001**
	(0–987)	(76–999)		(9–954)	(0–999)		(0–999)	(12–542)		(0–895)	(27–999)	
CD20	15.5	85	**0.029**	25	38.5	N.S	23	55.5	N.S	8	74.5	**0.002**
	(0–1152)	(0–576)		(0–655)	(0–1152)		(0–1152)	(0–302)		(0–1121)	(0–1152)	
CD68	130.5	184	**0.036**	128	166	N.S	132.5	186.5	**0.015**	118	178.5	**0.002**
	(14–727)	(15–416)		(21–727)	(14–577)		(15–727)	(14–577)		(21–416)	(14–727)	
CD68/(CD3+CD20)	0.6	0.43	N.S	0.5	0.48	N.S	0.45	0.7	N.S	0.6	0.43	N.S
	(0–6.6)	(0.1–0.9)		(0.1–5.4)	(0–6.6)		(0–6.1)	(0.2–6.6)		(0–6.6)	(0.1–6.1)	
	MICs at tumor center											
	MMP-9			**MMP-11**			**MMP-14**			**TIMP-2**		
	−	+	*p*	−	+	*p*	−	+	*p*	−	+	*p*
CD3	209	256	N.S	222.5	199	N.S	209	234	N.S	210	252.5	N.S.
	(0–999)	(36–451)		(0–999)	(9–542)		(12–999)	(0–917)		(0–999)	(9–954)	
CD20	25	75	N.S	29.5	29	N.S	30	21	N.S	22.5	35.5	N.S.
	(0–1152)	(0–211)		(0–1152)	(0–211)		(0–1152)	(0–576)		(0–1152)	(0–664)	
CD68	140	167	N.S	130.5	166.5	**0.041**	143	137	N.S	128	184.5	**0.025**
	(14–727)	(62–577)		(14–727)	(40–577)		(15.727)	(14–577)		(14–577)	(40–727)	
CD68/(CD3+CD20)	0.5	0.6	N.S	0.44	0.9	**0.001**	0.43	0.6	N.S	0.4	0.7	**0.045**
	(0–6.6)	(0.2–1.1)		(0–6.61)	(0.2–6.3)		(0.1–6.6)	(0–6.3)		(0–5.37)	(0.1–6.6)	

Mann-Whithney test. MICs: mononuclear inflammatory cells. Data are expressed as median (range). N.S: not significant.

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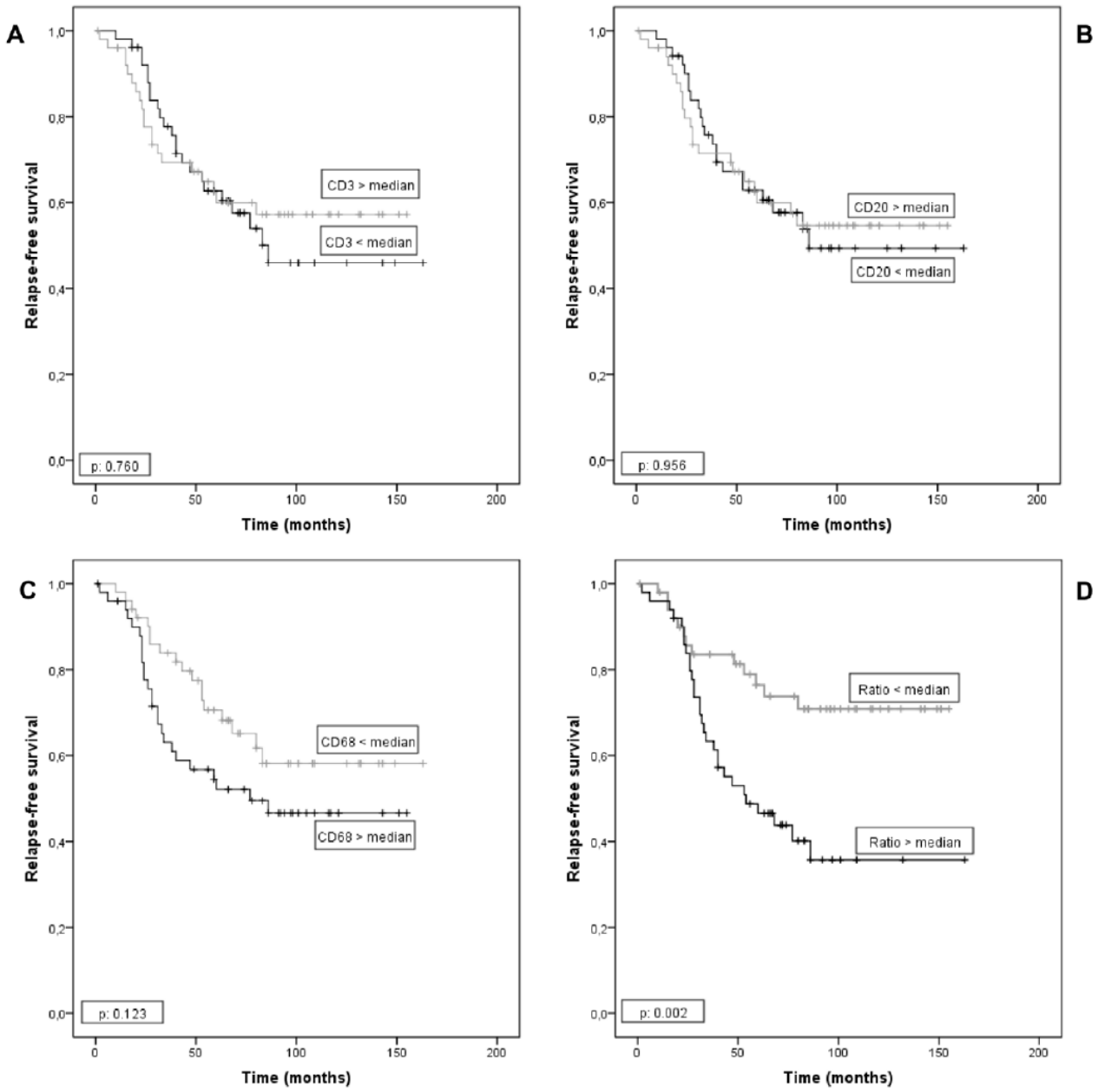

Figure 4. Probability of relapse-free survival as a function of CD markers count for 102 patients with invasive ductal carcinoma. CD3 count (A), CD20 count (B), CD68 count (C) and CD68/(CD3+CD20) ratio (D).
doi:10.1371/journal.pone.0052796.g004

digital camera system DP12 and soft analysis (analySIS®, Soft Imaging System, Münster, Germany) was used in the tumor sections (stained with antibodies and counterstained with hematoxylin), as described before [32]. To evaluate immunostaining intensity we used a numeric score ranging from 0 to 3, reflecting the intensity as follows: 0, no reactivity; 1, weak reactivity; 2, moderate reactivity; and 3, intense reactivity. Using an Excel spreadsheet, the mean score was obtained by multiplying the intensity score (I) by the percentage of reactivity area (PA) and the results were added together (total score: I×PA). This overall score was then averaged with the number of cores performed for each patient. If there was no tumor in a particular core, then no score was given. In addition, the mean score of two core biopsy samples was calculated for each tumor. This scoring evaluation was based on a global evaluation of staining areas corresponding to tumor cells as well as to stromal cells. Nevertheless, in the present work we also evaluated the immunohistochemical staining exclusively for mononuclear inflammatory cells (MICs).

Statistical analysis

Differences in percentages were calculated with the chi-square test. Immunostaining score values for each protein were expressed as a median (range). Correlation between score values was

Table 4. Cox's univariate (HR) and multivariate (RR) analysis of the significant relationships between MMPs, TIMPs expression or CD68/(CD3+CD20) ratio at the tumor center or at the invasive front, and relapse-free survival.

Tumor location	Factor	No. of patients	Event frequency	HR (95% CI)	RR (95% CI)
TUMOR CENTER	**TIMP2**				
	Score < median vs. >median	51/51	9/34	4.62 (2.21–9.65)****	3.23 (1.51–6.92)***
	MIC (−) vs. (+)	72/30	20/23	3.77 (2.06–6.89)****	4.37 (2.31–8.25)****
	MMP11				
	MIC (−) vs. (+)	76/26	18/25	9.19 (4.73–17.85)****	8.80 (4.40–17.61)****
INVASIVE FRONT	**MMP9**				
	Score < median vs. >median	50/49	16/25	2.03 (1.08–3.80)*	2.22 (1.15–4.29)*
	MMP14				
	MIC (−) vs. (+)	74/24	24/17	3.38 (1.81–6.31)****	3.41 (1.75–6.63)****
	TIMP2				
	MIC (−) vs. (+)	49/50	15/26	1.89 (1.01–3.58)*	2.51 (1.28–4.92)**
	CD68/(CD3+CD20) Ratio	51/50	13/29	2.68 (1.39–5.17)***	2.54 (1.23–5.24)**

Abbreviations: MIC: mononuclear inflammatory cells; HR: hazard ratio; RR: relative risk; CI: confidence interval.
*p<0.05;
**p<0.01;
***p<0.005;
****p<0.001.
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calculated by using the Spearman correlation test. Comparison of immunostaining values between groups was made with the Mann-Whitney or Kruskall-Wallis tests. Statistical results were corrected applying Bonferroni's correction. For relapse-free survival analysis we used the Cox's univariate method. Cox's regression model was used to examine interactions between different prognostic factors in multivariate analysis. Only parameters that achieve statistical significance for distant relapse-free survival in the univariate analysis were included in the multivariate analysis. The PASW Statistics 18.0 software (SPSS Inc, Chicago, IL, USA) was used for all calculations. $p<0.05$ was considered as significant.

Results

Immunostainings for CD3, CD20 and CD68 were performed in TMA blocks from invasive ductal carcinoma of the breast (Figure 1), showing a membranous staining for CD3 and CD20, whereas CD68 staining is found in the cytoplasm. Our results demonstrate a wide variability among tumors in the number of $CD3^+$ T-cells (median: 214.00 (0–999), $CD20^+$ B-cells (29.50 (0–1152) or $CD68^+$ macrophages (141.00 (14–727), by 1 mm^2 at the invasive front (Figure 2). We found direct correlations between the number of $CD68^+$ macrophages and the number of $CD3^+$ T-cells (r sub S = 0.57; p = 0.0001) or the number of $CD20^+$ B-cells (r sub S = 0.51; p = 0.0001), and specially between the number of $CD3^+$ T-cells and the number of $CD20^+$ B-cells (r sub S = 0.71; p = 0.0001).

We examined the possible relationship between the overall number of intratumoral MICs at the invasive front, or the relative ratio of these cells [number of $CD68^+$ macrophages/number of lymphocytes (number of $CD3^+$ T-cells + number of $CD20^+$ B-cells), further named as CD68/(CD3+CD20) ratio], and the clinico-pathological characteristics of patients and tumors (Table 2). Our results demonstrated a direct relationship between the number of $CD3^+$ T-cells and premenopausal status (p = 0.009); whereas this same cell count was inversely associated with both ER^+ and PgR^+ status (p = 0.04 and p = 0.003, respectively). The number of $CD20^+$ B-cells was directly associated with $HER2^+$ status (p = 0.009). The number of $CD68^+$ macrophages was inversely associated with PgR^+ status and directly associated with $HER2^+$ status (p = 0.027). However, our results showed no significant association between the CD68/(CD3+CD20) ratio and any clinico-pathological characteristics (Table 2).

We had previously identified a significant percentage of tumors with a MICs phenotype characterized by a molecular profile with specific MMPs and TIMPs increased expression, and associated with a high metastatic rate [25–28]. Thus, in the present work we determined the expression of these significant proteins (MMP-2, 9, 11, 14, and TIMP-2) in the tumor samples, and analyzed the possible relationship between the presence of different MICs phenotypes at the invasive front, and MMPs and TIMPs expressions by tumors both in the invasive front and in the tumor center.

With regard to global expression (score values) of MMPs and TIMPs, our result showed a direct correlation between MMP-2 score values and CD3 (r = 0.21, p = 0.038), CD20 (r = 0.25, p = 0.011) or CD68 (r = 0.32, p = 0.001) counts at the invasive front; whereas MMP-9 score values correlated with CD68 count (r = 0.21, p = 0.041) in this same tumor location. On the other hand, TIMP-2 score values at the tumor center correlated inversely with CD3 (r = −0.23, p = 0.021) or with CD20 (r = −0.21, p = 0.036) count in the invasive front, but correlated directly with CD68/(CD3+CD20) ratio in this same tumor location (r = 0.24, p = 0.014).

Figure 3 shows examples of immunostaining for different MMPs and TIMPs, at tumor center and at the invasive front. We found several significant associations between the different MICs counts at the invasive front and the expression of MMPs and TIMPs by MICs from the invasive front or from the tumor center (Table 3). Thus, high CD3, CD20 or CD68 counts were significantly associated with MMP-9 expression, at the invasive front; whereas high CD68 count was significantly associated with MMP-14 and TIMP2 in this same tumor location. Also, we found that high CD68 count and CD68/(CD3+CD20) ratio were associated with both MMP-11 and TIMP-2 expressions by MICs at the tumor

center. In addition, it is interesting our finding indicating that if there is a high CD68/(CD3+CD20) ratio at the invasive front, most of MICs with a positive MMP-11 or TIMP-2 phenotype at the tumor center are macrophages (Figure 3A and B, respectively). In this figure, MMP-11 staining demonstrates that apart from tumor cells with large nucleus and an intense cytoplasmic staining, there are a small number of lymphocytes with rounded nucleus surrounded by a small positive cytoplasm, but the most abundant cells type in the tumor center are macrophages, which are the large, round cells that contain a central round nucleus and an abundant clear positive cytoplasm.

The possible influence of the number of the different inflammatory cell types on relapse-free survival was evaluated in all patients included in the present study. For this purpose, we took the corresponding median value of the total number of each cell type by 1 mm^2 at the invasive front as cut-off point. Univariate analysis indicates that CD3, CD20, or CD68 count showed no significant associations with relapse-free survival (Figure 4). Nevertheless, our results showed that a high CD68/(CD3+CD20) ratio was significantly associated with a higher probability of shortened relapse-free survival ($p = 0.002$) (Table 4 and Figure 4D). Multivariate analysis according to Cox's model demonstrated that tumor stage (II: (relative risk (RR) (confidence interval (CI) = 1.8(0.7–4.5); III: 4.6(1.8–12.0); $p = 0.003$) and PgR status (positive: 0.4(0.2–0.8), $p = 0.011$) were significant and independently associated with distant relapse-free survival. Nevertheless, this same analysis also demonstrated that CD68/(CD3+CD20) ratio was significant and independently associated with distant relapse-free survival (Table 4).

Discussion

Inflammation is now considered a hallmark of cancer and can play a role in all aspects of tumor biology, including initiation, promotion, angiogenesis, and metastasis [4,27,33–34]. It is known that the activation of oncogenes can trigger the production of inflammatory molecules and the recruitment of inflammatory cells. But the potential effects of the inflammatory cell infiltrate in breast cancer seem to be diverse and complex. Therefore, in this study we investigate the impact of different inflammatory cell types at the invasive front from breast carcinomas on distant metastasis development. We consider that this is of special interest because the invasive front is the area where some of the most important interactions between cancer cells and the tumor supporting stroma take place [30]. Our results showed a biological heterogeneity among breast tumors with regard to these cellular infiltrates at the invasive front. In addition, we found that a high CD68/(CD3+CD20) ratio at the invasive front is significant and independently associated with the occurrence of distant metastasis.

There are data indicating that, depending on the cell type present and their functional profile, inflammatory cells can either suppress or promote tumor growth. We analyzed the expression profile of the individual inflammatory cell types, and our results are in accordance with other studies indicating that tumor-infiltrating lymphocytes correlate with hormone receptor-negative or $HER2^+$ status, or with high grade/highly proliferative tumors, although we did not find correlation with favorable long-term prognosis [12–19]. In addition, it has been reported that activated B cells can mediate tumor regression by itself and confers host T-cell antitumor immunity. Likewise, it was suggested that effector B cells can serve as a useful adjunct in adoptive T-cell therapy [35].

Tumor-associated macrophages arise from circulating monocytes that migrate into tissues in response to chemical signals and differentiate into macrophages. In breast cancer, macrophages have been found to comprise up to 50% of the breast tumor mass [36]. Tumor-associated macrophages produce a variety of cytokines and chemokines, as well as growth factors for both epithelial and endothelial cells, which play a key role in tumor growth and metastasis [4,10–11]. Our results are in accordance with previous studies reporting an association between macrophages density and PgR^- or $HER\text{-}2^+$ status [37]. However, also in accordance with Mahmoud *et al.*, we found that overall macrophage numbers are not related to prognosis in breast cancer in a multivariate analysis [37]. This may be due the density of macrophages was correlated with higher tumor grade in the present study as well as in previous studies [37–39]. Hence, multivariate analysis is thus essential when examining the relation between macrophage infiltration and survival. Nevertheless, this latter analysis led us to identify a high CD68/(CD3+CD20) ratio was a potent independent factor for predicting distant metastasis relapse-free survival in our patient population. Therefore, we describe here, for the first time, a study evaluating the relative amount of different MICs at the invasive front in breast carcinomas, using a new ratio that correlates with patient survival and could be useful in predicting patient outcome. We consider this is a relevant finding since the role of inflammatory cells in cancer seems to be complex, and this ratio can reflect a more objective result of the interactions between both anti-tumor and pro-tumor effects of the different inflammatory cells.

The end point of the present study was the occurrence of distant metastasis, which is regulated not only by intrinsic genetic changes in malignant cells, but also by the microenvironment. MMPs play an essential role in tumor invasion and metastasis via degradation of the stromal connective tissue and basement membrane components, and are inhibited by TIMPs. In previous reports we identify a phenotype of MICs characterized by the expression of specific MMPs and TIMPs at the tumor center, and associated with distant metastasis development [25–28], which also showed an up-regulation of inflammatory-related genes [29]. According to this, in the present study we determined the expression of these significant proteins (MMP-2, 9, 11, 14, and TIMP-2) in those breast cancer samples and analyzed the possible relationship between the different inflammatory cells counts at the invasive front and the expression of MMPs and TIMPs, either at the invasive front or at the tumor center. Then, we found several associations between the inflammatory cell types and some of these factors. Nevertheless, the most relevant finding was the association between high CD68/(CD3+CD20) ratio and the expression of MMP-11 (stromalysin-3) or TIMP-2 by the MICs at the tumor center. This is a relevant finding considering that both MMP-11 and TIMP-2 are the two principal factors defining the pro-metastatic phenotype of MICs in our previous studies [25–28]. Therefore, these results may indicate that a high CD68/(CD3+CD20) ratio at the invasive front contributes to polarize macrophages to achieve a high metastatic phenotype at the tumor center. In addition, it is remarkable our finding indicating that if there is a high CD68/(CD3+CD20) ratio at the invasive front, most of MICs with a positive MMP-11 or TIMP-2 phenotype at the tumor center are macrophages.

A limitation of the present study was the lack of a complete study of the count for the different MICs at the tumor center. It was due to the absence of enough tissue sample in many cases, because of their utilization in our previous expression studies on MMPs, TIMPs and other factors in breast carcinomas. Nevertheless, we observed that most of MICs in tumor center have macrophage-like morphology, indicating an important contribution of these stromal cells to tumor biology in this tumor location.

In summary, our results contribute to characterize the inflammatory cell infiltrate in breast cancer, and their relationship with prognostic evaluation and MMPs/TIMPs expression. Further studies will be necessary to assess if this CD68/(CD3+CD20) ratio at the invasive front can contribute to identify patients with breast cancer candidates to different therapeutic strategies based on immuno-modulation. In fact, several strategies against tumor-associated macrophages have already been published [40–42], and several reports indicate the effectiveness of activated B-cells in cellular immunotherapy of malignancies [43–46]. Hence, to design breast tumor immunotherapy and vaccine strategies hereafter, it will be necessary to consider humoral immunity in addition to the cell mediated immunity, as a potential therapeutic tool.

Author Contributions

Conceived and designed the experiments: NE IP LOG ALM FJV. Performed the experiments: NE IP BFG SJ MLL JMDC LOG ALM FJV. Analyzed the data: NE IP BFG SJ MLL JMDC LOG ALM FJV. Contributed reagents/materials/analysis tools: NE IP BFG SJ MLL JMDC LOG ALM FJV. Wrote the paper: NE IP BFG SJ MLL JMDC LOG ALM FJV.

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Prediction of metastatic breast cancer in non-sentinel lymph nodes based on metalloprotease-1 expression by the sentinel lymph node

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KEYWORDS
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Abstract ***Aims:*** Sentinel lymph node (SLN) biopsy is the current standard axillary approach for patients with clinically node-negative breast cancer. If the SLN is positive, is still also standard in most centres to proceed with a complete axillary dissection to predict the remaining nodes affectation, despite of SLN is the only positive lymph node in 50–68% of cases. If we could identify them, these patients could be spared a complete axillary dissection.
Methods: Elevated expression of specific matrix metalloproteases (MMPs) and their inhibitors (TIMPs) by stromal mononuclear inflammatory cells (MICs) of primary tumours is significantly associated with distant metastasis development in breast cancer. In the present study we first identified candidate MMPs/TIMPs associated with axillary metastasis in a preliminary group ($n = 50$), and subsequently examined the potential of their expression in the SLN for predicting the status of the remaining nodes, in 105 patients with intraoperative SLN-assessment.
Results: MMP-1 expression by MICs from SLNs was significantly associated with metastatic spread to non-SLNs. Moreover, in all cases with negative MMP-1 expression by MICs from

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SLNs, the remaining non-SLNs were not affected ($p < 0.0001$). Therefore, we demonstrate that MMP-1 expression by MICs from SLNs had 100% sensitivity, 100% negative-predictive value and 61.5% specificity to predict non-SLNs status.

Conclusion: This is the first time that tumour spread to the remaining axillary nodes has been predicted from molecular features of the SLN(s). If confirmed in larger studies, patients could be spared the morbidity associated with an unnecessary complete lymphadenectomy.

1. Introduction

Sentinel lymph node (SLN) biopsy is the current standard axillary approach for patients with clinically node-negative breast cancer.[1,2]

When the SLN is involved with tumour, it is also standard in most centres to proceed with a complete axillary dissection to ascertain the status of the remaining nodes (non-sentinel lymph nodes, non-SLNs), although a recent report from a randomised prospective trial claims that patients obtain no objective benefit from it.[3] In many other centres, radiotherapy is used as an alternative to surgery for the treatment of SLN biopsy positive patients. Furthermore, the SLN is the only positive lymph node in 50% to 68% of cases.[4,5] Thus, in retrospect, these cases could have been spared a complete axillary dissection and its accompanying morbidity.

Several studies have investigated clinical parameters to estimate the probability of non-sentinel node metastasis in SLN-positive patients. These reports found that the incidence of non-SLNs metastasis increased as the metastases in the SLN increased in size,[4,6–10] and also with increasing number of positive SLN,[4,11,12] extracapsular extension of the SLN-metastasis[13] or lymphovascular invasion of the primary tumour.[8,9,14–16] In addition, several nomogram scores based on these factors have been defined to determine the risk of finding additional positive axillary nodes if axillary node dissection is performed.[13,17–20] However, the results obtained using the cited nomogram scores are still unsatisfactory for clinical use.[21,22]

One still scarcely explored strategy is the study of candidate molecular markers involved in metastatic progression through the axillary lymph node chain in breast cancer. One family of promising markers for this purpose is human matrix metalloproteases (MMPs). In fact, in previous reports, we were able to show that an elevated expression of several MMPs and their specific inhibitors, tissue inhibitors of MMPs (TIMPs), by mononuclear inflammatory cells (MICs) from the stroma of primary tumours is significantly associated with the occurrence of distant metastasis in breast cancer.[23–27] Based on these findings, we devised the present study, in order to explore the possible role of MMPs/TIMPs expression in the metastatic process across the axillary lymph node chain in breast cancer.

2. Patients and methods

We studied axillary node samples from patients with breast cancer operated upon at "Fundación Hospital de Jove", Gijón (Spain). As a first step, we studied archival axillary samples from the "pre-sentinel-node" era with known metastatic involvement, in order to identify possible MMPs or TIMPs related with the metastatic process. For this purpose, we considered a population of 50 women having undergone classical upfront axillary lymph node dissection (Group 1) between January 1990 and January 2003, with the following inclusion criteria: invasive ductal carcinoma, lymph node involvement. The exclusion criteria were as follows: distant metastatic disease at presentation, prior history of any kind of malignant tumour, bilateral breast cancer at presentation, having received any type of neoadjuvant therapy. Table 1 shows the clinical and pathological features of the population. In addition, the number of histologically assessed axillary lymph nodes ranged from 11 to 23.

Once having identified candidate MMPs and TIMPs as described above, in a subsequent step we studied a second patient population having undergone intraoperative sentinel lymph node assessment (Group 2) in order to explore the possible role of MMPs or TIMPs expression by the positive SLNs to predict the involvement or not of the remaining nodes. For this purpose, we revised the databases from "Fundación Hospital de Jove", Gijón (Spain) and "Hospital Clinico Universitario de Santiago de Compostela" (Spain) including patients having undergone SLN biopsy for invasive breast cancer with a clinically negative axilla between January 2007 and January 2012. We selected SLN-positive patients having undergone subsequent axillary dissection, using the following inclusion criteria: invasive ductal carcinoma, metastasis (tumour cell clusters) in the SLN identified by means of haematoxylin/eosin (H&E)-staining, and not merely by means of immunohistochemistry, and only one or two positive SLNs. The exclusion criteria were as follows: prior history of any kind of malignant tumour, bilateral breast cancer at presentation, multifocality and having received any type of neoadjuvant therapy. A total of 105 patients fulfilling these criteria were included in the present study. Table 2 shows the clinical and pathological features of the population. In addition, the number of histologically assessed axillary lymph nodes ranged from 10 to 29.

Table 1
Basal characteristics of Group 1 of patients with invasive ductal carcinoma of the breast.

Characteristics	Group 1 *N* (%)
Total cases	50 (100)
Age (years)	
<62	29 (58)
>62	21 (42)
Menopausal status	
Premenopausal	9 (18)
Postmenopausal	41 (82)
Tumour size	
T1	18 (36)
T2	32 (64)
Number of affected lymph node	
1	12 (24)
> 1	38 (76)
Histological grade	
Well Dif.	13 (26)
Mod. Dif.	29 (58)
Poorly Dif.	8 (16)
Oestrogen receptors	
Negative	20 (40)
Positive	30 (60)
Progesterone receptors	
Negative	19 (38)
Positive	31 (62)
HER-2/neu status	
Negative	43 (86)
Positive	7 (14)
Lymphovascular invasion	
No	30 (58)
Yes	21 (42)

The patients signed an informed consent for the secondary use of their tissue samples for research. The study adhered to national regulations and was approved by our institution Ethics and Research Committee.

2.1. Surgical procedures

Our method of sentinel node identification uses both radioisotope and blue dye injection (detailed in Supplemental Methods). We defined sentinel nodes as nodes that contained ^{99m}TC-labelled sulphur colloid and/or isosulfan blue or that were suspicious on palpation. While the nodal tissue was being processed by frozen section, the primary tumour was resected.

2.2. Pathological examination of the SLN

Once excised, the SLN was sent to the laboratory for frozen section examination (detailed in Supplemental Methods). Patients who have tumour cells in their SLN biopsy on frozen section analysis underwent immediate level I–II axillary lymph node dissection with all resected tissue being sent for standard pathological processing.

2.3. Tissue handling and tissue arrays

Lymph node tissue samples formalin-fixed and paraffin-embedded (FFPE) were stored in our pathology laboratory prior to their use in the present investigation. Histopathologically representative tumour areas of metastatic axillary lymph nodes (MALNs) were defined on H&E-stained sections and marked on the slide. Tumour tissue microarray (TMA) blocks were obtained as described elsewhere[5] from Group 1 of patients tissue samples. We performed one core with a diameter of 1.5 mm per each MALN. In patients with more than one MALN, one lymph node was randomly chosen among them and analysed as described. We analysed one core histologically representative of the SLN involvement from tissue block as it has been shown, by our group, to correlate properly with conventional immunohistochemical staining methods.[28] Samples from Group 2 of patients were evaluated from FFPE tissue block, without conducting tissue microarray. Although in this case samples were previously frozen, SLN can be correctly evaluated, as this is an accepted routine process for subsequent evaluation by pathologists.[29]

2.4. Immunohistochemistry

Serial 3-μm sections of the high-density blocks were consecutively cut with a microtome (Leica Microsystems GmbH, Wetzlar, Germany) and transferred to adhesive-coated slides. Immunohistochemistry was carried out on these sections using a TechMate TM50 autostainer (Dako, Glostrup, Denmark) (detailed in Supplemental Methods).

2.5. Immunohistochemical analysis

For each antibody studied, the location of immunoreactivity, percentage of reactive area and intensity were determined. All cases were semiquantified for each protein-stained area. An image analysis system using an Olympus BX51 microscope and software analysis (analySIS®, Soft Imaging System, Münster, Germany) was used as described previously.[27] For TMAs samples from Group 1 of patients, each core was scanned with a 400X power objective in two fields per core. Fields were selected around tumour areas searching for protein-reactive areas.

In Group 2 of patients, SLN samples were scanned with a 400× power objective in two fields. Fields were selected on the basis of the most representative area of lymph node metastasis in each individual biopsy section.

In the present work, we also evaluated individually the immunohistochemical staining by each main cellular

Table 2
Basal characteristics of Group 2 of patients with invasive ductal carcinoma of the breast, based on non-SLNs involvement.

Characteristics	Negative non-SLNs cases N° (%)	Positive non-SLNs cases N° (%)	p-Value
Total cases	**65 (100)**	**40 (100)**	
Age (years)			
⩽58	38 (58.5)	14 (35.0)	**0.016**
>58	27 (41.5)	26 (65.0)	
Menopausal status			
Premenopausal	17 (26.6)	6 (15.0)	0.167
Postmenopausal	48 (73.4)	34 (85.0)	
Characteristics of primary tumours			
Tumour size			
T1	42 (64.6)	20 (50.0)	**0.010**
T2	23 (35.4)	20 (50.0)	
Histological grade			
Well Dif.	20 (30.8)	6 (15.0)	0.191
Mod. Dif.	30 (46.2)	24 (60.0)	
Poorly Dif.	15 (23)	10 (25.0)	
Oestrogen receptors			
Negative	12 (18.5)	4 (10.0)	0.318
Positive	53 (81.5)	36 (90.0)	
Progesterone receptors			
Negative	16 (24.6)	8 (20.0)	0.699
Positive	49 (75.4)	32 (80.0)	
HER-2/neu status			
Negative	58 (89.2)	33 (82.5)	0.223
Positive	7 (10.8)	7 (17.5)	
Lymphovascular invasion			
No	37 (56.9)	16 (40.0)	0.107
Yes	28 (43.1)	24 (60.0)	
Characteristics of SLN			
Number of positive SLN			
1 positive SLN	38 (58.5)	25 (62.5)	0.837
2 positive SLN	27 (41.5)	15 (37.5)	
Size of largest SLN metastasis (mm)			
⩽2 mm	22 (33.8)	8 (20.0)	**0.0001**
2–6 mm	33 (50.8)	11 (27.5)	
>6 mm	10 (15.4)	21 (52.5)	
Characteristics of non-SLNs			
Positive non-SLNs versus total non-SLNs	0/800	185/583	

Abbreviations: SLN, sentinel lymph node; non-SLNs, non-sentinel lymph nodes. Significative values ($p < 0.05$) are shown in bold.

type: tumour cells and peritumoural MICs. MICs were distinguished from cancer cells because these latter are larger in size, whereas MICs are small round cells. On the other hand, while cancer cells are arranged forming either acinar or trabecular patterns, MICs show a sheet pattern. We considered a positive immunostaining, for a given MMP or TIMP by any of these cell types, when at least 10% of cells showed a positive immunostaining at each evaluated field in every case.

Staining for oestrogen receptors (ER) and progesterone receptors (PgR) was scored according to the method described by Allred DC et al.,[30] and human epidermal growth factor receptor 2 (HER-2) staining according to the criteria used for the Herceptest.[31] Controls included breast cancer tissue with known immunoreactivity for each antibody used in the study. For negative controls, the primary antibody was omitted and replaced by Antibody Diluent (Dako, Glostrup, Denmark). The following antibodies, from Dako, were used for these latter determinations: mouse anti-ER clone 1D5 (ready-to-use); anti-PR clone PgR 636, at a dilution of 1/50; rabbit polyclonal anti-HER-2/neu oncoprotein at a dilution of 1/250.

The evaluation of immunostaining was blinded to outcome. The two pathologists have evaluated each immunostaining not knowing the outcome. Also, we confirmed that they obtained the same results.

2.6. Statistical analysis

Differences between percentages were calculated using the chi-squared test. The PASW Statistics 18 programme was used for all calculations. p-Values < 0.05 were considered as significant.

3. Results

3.1. Group 1 of patients

Table 3 shows the global expression (median score values) of the different MMPs and TIMPs, as well as by the different cell types from MALNs, depending on the number of affected MALNs. Cases with more than one affected MALN had a higher ratio of positive cases for MMP-1 and TIMP-1, by MICs, than cases with just one affected MALN. Likewise, cases with more than one affected MALN had a higher ratio of expression for MMP-13 by MICs, than cases with one single affected MALN. Also, cases with more than one affected MALN had a higher ratio of positive cases for MMP-7 by MICs, than cases with just one affected MALN.

3.2. Group 2 of patients

Statistical analysis to determine the relationship between clinicopathological variables and positive SLNs (Table 2) demonstrated that age of patients, tumour size and size of SLN metastasis were significantly associated with non-SLNs status.

Proteins more significantly associated with the metastatic process across the axillary lymphatic system in Group 1 of patients, were investigated in Group 2 of patients, where less tissue was available after SLN biopsy had been performed. The median expression score values for the different MMPs and TIMPs tested in SLNs, together with their range of variation, were as follows: MMP-1: 68.79 (0–198.30); MMP-7: 114.58 (0–268.68); MMP-13: 47.82 (0–244.19); and TIMP-1: 39.17 (0–286.29).

Fig. 1 shows representative examples of MMP-1 expression by MICs in positive SLNs of breast carcinomas. Table 4 shows the global expression (median score values) of MMPs and TIMP-1 by the different cell types depending on the degree of tumoural involvement of the remaining axillary nodes (non-SLNs). A high MMP-1 score value was significantly associated with positive non-SLNs ($p < 0.0001$). We also found that all of cases with negative MMP-1 expression by MICs (<10% of stained MICs), from the SLNs ($n = 40$) showed negative non-SLNs status ($p < 0.0001$). It was very straightforward to distinguish "positive" from "negative" cases, because all MMP-1 positive cases in SLNs showed at least as 70% positive MICs; whereas in MMP-1 negative cases in SLNs, no more than 10% of MICs were stained.

Our results demonstrated that MMP-1 expression by MICs in SLNs had a sensitivity of 100%, a negative-predictive value of 100% and a specificity of 61.5%, to predict non-SLNs status.

4. Discussion

Complete axillary dissection in patients with positive SLNs is currently under discussion, since approximately only 32–50% of such patients carry metastasis in the remaining lymph nodes.[4,5] The next step of SLN biopsy in breast cancer is to determine which patients need axillary lymph node dissection following a positive SLN biopsy. In the present study we found a new molecular marker candidate for identifying these patients.

Our results are in agreement with several previous studies indicating that the size of the primary tumour[32] and SLN metastasis diameter[4,6–10] are significant predictors of non-SLNs involvement. Our results also show a trend for lymphatic invasion[8,9,14–16] to predict non-SLN involvement. However, today several experts consider that nomogram models based on such individual factors are not adequate to identify patients in whom axillary dissection can be omitted.[17,21,22,33]

Our results showed that global expression (score values) of MMP-1 at the SLNs correlates positively and significantly with non-SLN status. In addition, none of the SLNs negative for MMP-1 expression by MICs ($n = 0$) was associated with positive non-SLNs, whereas all of the positive non-SLN cases (40 out of 40, 100%) showed MMP-1 expression by MICs. Therefore, our finding that MMP-1 (interstitial collagenase, also named collagenase-1) was very significantly associated with tumour involvement of non-SLNs, is especially relevant. MMP-1 is the most ubiquitously expressed interstitial collagenase. MMP-1 cleaves several components of the extracellular matrix, including collagen of types I (the principal component of connective tissue), II, III, VII, VIII and IX, aggrecan, as well as serin proteinase inhibitors, and α2 macroglobulin.[34,35] The degradation capacity of MMP-1 may be the responsible for promoting tumour spread via the lymph nodes.

Our present results may seem in contradiction with those previously reported by us regarding the lack of association between high MMP-1 expression by MICs from primary tumours and distant metastasis in breast carcinomas.[27] However, the data of the present study seem to indicate that MMP-1 expression by MICs at the SLN is implicated in tumour progression through the axillary lymph system, which is a process entirely different from hematogenous spread, which is the one responsible for distant metastases.[36,37]

Table 3
Relationship between MMPs/TIMPs expression in metastatic lymph nodes and the number of affected nodes in Group 1 of patients.

Factor	1 Positive lymph node N° patients	>1 Positive lymph node N° patients	p-Value
MMP-1			
Score <median versus >median	**11/1**	**14/24**	**0.001**
Tumour cell (−) versus (+)	**8/4**	**3/35**	**0.0001**
MIC (−) versus (+)	**12/0**	**8/30**	**0.0001**
MMP-2			
Score <median versus >median	6/6	20/18	1.000
Tumour cell (−) versus (+)	4/8	19/19	0.476
MIC (−) versus (+)	10/2	33/5	0.862
MMP-7			
Score <median versus >median	7/5	18/20	0 754
Tumour cell (−) versus (+)	**3/9**	**7/31**	**0.042**
MIC (−) versus (+)	11/1	24/14	0.052
MMP-9			
Score <median versus >median	7/5	20/18	0.722
Tumour cell (−) versus (+)	2/10	5/33	0.973
MIC (−) versus (+)	10/2	28/10	0.148
MMP-11			
Score <median versus >median	6/6	19/19	1.000
Tumour cell (−) versus (+)	2/10	3/35	0.629
MIC (−) versus (+)	7/5	18/10	0.476
MMP-13			
Score <median versus >median	4/8	19/19	0.933
Tumour cell (−) versus (+)	2/10	4/34	0.791
MIC (−) versus (+)	**8/4**	**14/24**	**0.028**
MMP-14			
Score <median versus >median	7/5	19/19	1.000
Tumour cell (−) versus (+)	4/8	5/33	0.204
MIC (−) versus (+)	8/4	25/13	0.288
TIMP-1			
Score <median versus >median	8/4	16/22	0.441
Tumour cell (−) versus (+)	3/9	2/36	0.151
MIC (−) versus (+)	**11/1**	**19/19**	**0.039**
TIMP-2			
Score <median versus >median	7/5	12/16	0.150
Tumour cell (−) versus (+)	3/9	2/36	0.151
MIC (−) versus (+)	10/2	24/14	0.571
TIMP-3			
Score <median versus >median	8/4	17/21	0.291
Tumour cell (−) versus (+)	4/8	7/31	0.327
MIC (−) versus (+)	8/4	25/13	0.288

Abbreviations: MIC, mononuclear inflammatory cell; MMP, matrix metalloprotease; TIMP, tissue inhibitors of MMP. Significative values ($p < 0.05$) are shown in bold.

Our results are potentially important because, to our knowledge, it is the first time that tumour spread to the remaining axillary nodes has been predicted from molecular features of the sentinel node(s). Classically, biological factors in breast cancer predicting axillary involvement have been investigated in primary tumours.[38–41] However, draining lymph nodes, and especially SLNs, are of great interest as a study target because of their exposure to all soluble factors coming from the tumour; also, they may be colonised by aggressive clones deriving from primary tumour cells. In a prior report, we found that when we compared the immunostaining values of MMPs and TIMPs between the different tumour localisations (tumour centre, invasive front and MALNs), the higher positive correlations were found between MALNs themselves.[42] This finding suggests that primary tumour cells clones which colonise regional lymph nodes show a tendency to have a similar phenotype of MMPs/TIMPs. In addition, in the present study we also found that MMP-1 expression by the peritumoural stromal MICs of SLNs was associated with the number of invaded nodes. This seems to indicate that metastatic cancer cells have the ability to induce the production of these proteins in host cells within

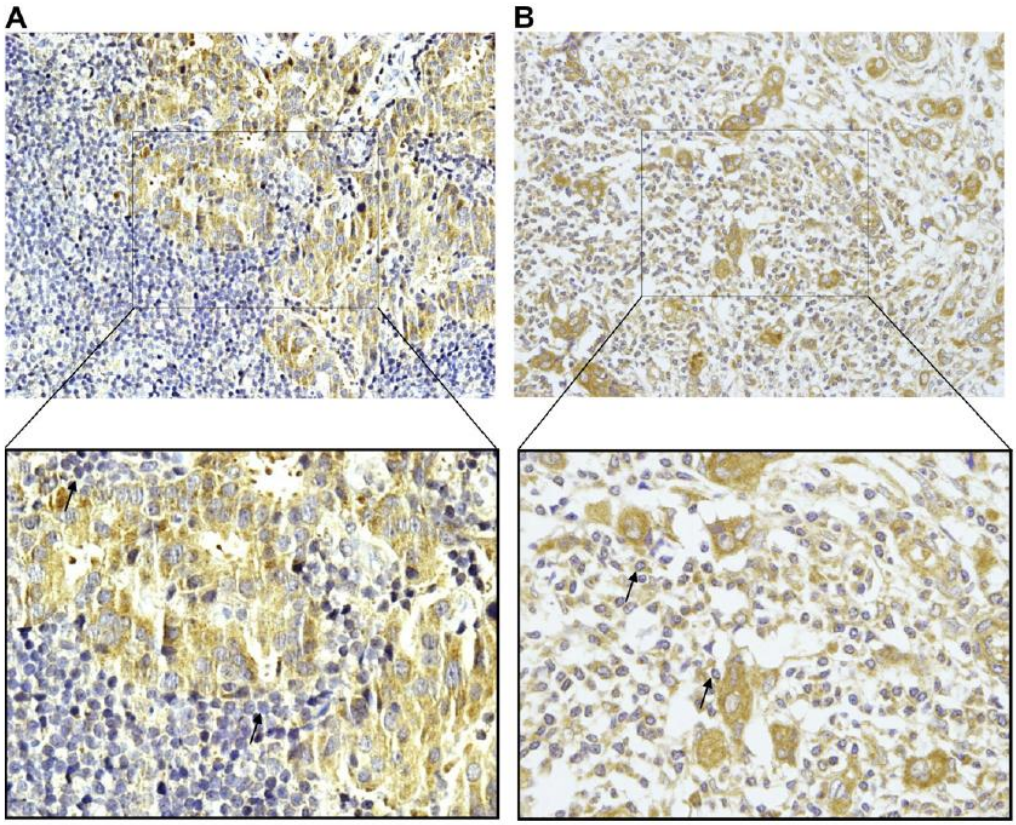

Fig. 1. Example of immunohistochemical staining for matrix metalloprotease (MMP)-1 in positive sentinel lymph node. (A) MMP-1 immunostaining (left 200×) and a 400× magnification (right) of the selected area showing mononuclear inflammatory cells (MICs) with negative staining (arrows) and positive tumour cells. (B) MMP-1 immunostaining (left 200×) and a 400× magnification (right) of the selected area showing positive stained MICs (arrows) and positive tumour cells.

Table 4
Relationship between MMPs/TIMPs expression in positive SLN, and non-SLNs status in 105 patients of Group 2.

Factors	Negative non-SLNs cases *N* (%)	Positive non-SLNs cases *N* (%)	*p*-Value
Total cases	**65 (100)**	**40 (100)**	
MMP-1			
Score <median versus >median	**42 (64.6)/23 (35.4)**	**11 (27.5)/29 (72.5)**	**0.0001**
Tumour cell (−) versus (+)	19 (29.2)/46 (70.8)	7 (17.5)/33 (82.5)	0.263
MIC (−) versus (+)	**40 (61.5)/25 (38.5)**	**0 (0)/40 (100)**	**0.0001**
MMP-7			
Score <median versus >median	33 (50.8)/31 (47.7)	19 (47.5)/20 (50.0)	0.939
Tumour cell (−) versus (+)	12 (18.5)/52 (80.0)	5 (12.5)/34 (85.0)	0.608
MIC (−) versus (+)	23 (35.4)/41 (63.1)	19 (47.5)/20 (50.0)	0.283
MMP-13			
Score <median versus >median	36 (55.4)/28 (43.1)	16 (40.0)/24 (60.0)	0.158
Tumour cell (−) versus (+)	20 (30.8)/44 (67.7)	13 (32.5)/27 (67.5)	1.000
MIC (−) versus (+)	36 (55.4)/28 (43.1)	21 (52.5)/19 (47.5)	0.864
TIMP-1			
Score <median versus >median	33 (50.8)/30 (46.2)	19 (47.5)/21 (52.5)	0.779
Tumour cell (−) versus (+)	24 (36.9)/39 (60.0)	18 (45.0)/22 (55.0)	0.625
MIC (−) versus (+)	50 (76.9)/13 (20.0)	27 (67.5)/13 (32.5)	0.263

Abbreviations: SLN, sentinel lymph node; non-SLNs, non-sentinel lymph nodes; MIC, mononuclear inflammatory cell; MMP, matrix metalloprotease; TIMP, tissue inhibitors of MMP. Significative values ($p < 0.05$) are shown in bold.

the lymph nodes, which emphasises the importance of the stromal-epithelial interactions in tumour progression among MALNs.

In summary, our present results suggest that MMP-1 expression by MICs at SLNs showed a 100% sensitivity, a negative-predictive value of 100% and 61.5% specificity for predicting the status of the remaining axillary nodes. Therefore, if these results are confirmed in larger studies, they could help to avoid unnecessary axillary node dissection in a significant percentage of cases (50–68%) after the identification of a metastatic SLN, at least as long as axillary dissection is not abandoned as standard practise when metastatic spread to other axillary nodes, different from the SLN, is suspected.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejca.2012.09.019.

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Discusión

La presente tesis doctoral consiste en la caracterización celular y molecular del proceso inflamatorio implicado en la progresión tumoral del cáncer de mama. La realización de un estudio piloto de caracterización molecular nos ha permitido realizar un "*screening*" de moléculas asociadas a la progresión tumoral y la inflamación según una clasificación de los tumores de buen y mal pronóstico que hemos descrito anteriormente, y basada en el fenotipo de las CMIs en cuanto a la expresión de MMPs y TIMPS, proteínas involucradas en diferentes aspectos básicos de la progresión tumoral (Vizoso F.J. et al., 2007). De esos factores, la MMP11 es el que muestra una mayor expresión diferencial entre esos dos tipos de tumores (Vizoso F.J. et al., 2007) y, por tanto, es en el que nos hemos basado en el presente estudio para la caracterización inicial de los tumores.

Las células inflamatorias pueden representar hasta el 50% de la masa tumoral total de los carcinomas mamarios infiltrantes. Las células inflamatorias que infiltran los carcinomas mamarios incluyen macrófagos, células plasmáticas, linfocitos T y B (Coussens L.M. and Werb Z., 2002; Lin E.Y. and Pollard J.W., 2004). Históricamente, los leucocitos infiltrantes asociados al tumor se han considerado como la manifestación de un mecanismo de defensa intrínseco contra el desarrollo de tumores (Lin E.Y. and Pollard J.W., 2004; Pollard J.W., 2004). Los resultados presentados coinciden con la evidencia creciente de que la infiltración de leucocitos puede promover cambios que conducen a un fenotipo tumoral más agresivo en el ámbito de la angiogénesis, crecimiento tumoral, invasión y metástasis (Coussens L.M. and Werb Z., 2002; Daniel D. et al., 2005). Esto puede ser debido a que las células inflamatorias probablemente influencian la progresión tumoral secretando moléculas como las citoquinas, los factores de crecimiento, las quimioquinas y las proteasas, que estimulan la proliferación e invasividad de las células tumorales. En este mismo sentido, recientemente Tan y cols. han caracterizado un tipo de linfocitos que infiltran los tumores y que estimulan el desarrollo de metástasis de cáncer de mama a través de señales relacionadas con el factor de transcripción NFκB (Tan W. et al., 2011).

Nuestros resultados demuestran que, de los 65 factores analizados y relacionados con el proceso inflamatorio y la progresión tumoral, los relacionados con la expresión de MMP11 por las CMIs del estroma intratumoral fueron IL-1, IL-5, IL-6, IL-8, IL-17, IL-18, MMP1, TIMP1, ADAM8, ADAM10, ADAM15, ADAM23, ADAMTS1, ADAMTS2, ADAMTS15, CCL3, Anexina A2, IFNβ, Claudina3, Myd88 y NFκB. De ellos, los más diferencialmente expresados por los dos principales tipos de tumores fueron IL-1, IL-5, IL-6, IL-17, IFNβ y NFκB. Esos últimos factores fueron analizados en un grupo más amplios de tumores, en los que confirmamos su elevada expresión por los tumores infiltrados por las CMIs con expresión positiva de MMP11. Nuestro estudio contribuye a una más óptima caracterización biológica de los carcinomas mamarios, en especial en cuanto al perfil molecular de su componente inflamatorio.

Los factores más expresados por los tumores con MMP11 positivos por las CMIs, como el IL-1, IL-5, IL-6, IL-17, IFNβ Y NFκB, tienen un gran interés biológico por su relación con la progresión tumoral.

La IL-1 producida fundamentalmente por los macrófagos activados estimula la función de una gran variedad de genes, entre otros la IL-5, IL-6, oncogenes (c-fos, c-myc, c-jun), IFN-β y colagenasas. Los modelos experimentales han mostrado que la producción local de IL-1 influencia el crecimiento del tumor y el desarrollo de metástasis con efectos proliferativos directos o promoviendo la activación de las vías de señalización de la inflamación y la angiogénesis (Saijo Y. et al., 2002; Salven P. et al., 2002). La producción de IL-1 por las células tumorales o estromales ha sido asociada a un fenotipo tumoral agresivo, en varios tipos de tumores murinos y humanos (Gemma A. et al., 2001). Estos datos apoyan nuestra observación, en el sentido de que las pacientes con una mayor frecuencia de metástasis (97,6%) presentan un perfil de alta expresión de IL-1.

La IL-5, esencialmente producida por los linfocitos T-helper 2 y los mastocitos, tiene como principal función estimular el crecimiento de las células B y aumentar la producción de inmunoglobulinas. Esta interleuquina no ha sido

relacionada como un factor importante en el desarrollo de metástasis en el cáncer de mama. Algunos estudios que han comparado el perfil inflamatorio de carcinomas mamarios y tejido normal no han encontrado transcripto de este factor (Green A.R. et al., 1997); sin embargo nuestros datos indican que podría ser una diana importante que debe ser estudiada en mayor profundidad en estos casos.

La IL-6 parece desempeñar un papel importante en la resistencia al proceso apoptótico. Algunos estudios muestran el papel de la IL-6 en el crecimiento de las células tumorales *in vitro*, pero su papel exacto sigue siendo confuso. La IL-6 es producida por las células estromales como los linfocitos T, los fibroblastos o monocitos y también por las células tumorales. Los estudios que evalúan la expresión de IL-6 en los carcinomas mamarios, muestran resultados contradictorios. Marrogi y cols. (Marrogi A.J. et al., 1997; Ueno T. et al., 2000; Knupfer H. et al., 2004) han analizado el perfil de expresión de la IL-6 en 19 carcinomas mamarios y no han detectado la expresión de ARNm. Sin embargo, otros estudios han mostrado y cuantificado su expresión (Ueno T. et al., 2000; Knupfer H. et al., 2004). Bachelot y cols. (Bachelot T. et al., 2003) estudiaron la significación clínica del factor de crecimiento endotelial vascular (VEGF) e IL-6 en carcinomas mamarios hormono-refractarios y observaron que la presencia IL-6 en el suero de las pacientes (pero no de VEGF), se correlaciona con una supervivencia más corta. En nuestro caso, relacionamos la expresión intratumoral de IL-6 con un mayor riesgo de desarrollo de metástasis.

Estos últimos años, se ha considerado la IL-17 como mediador clave de la relación entre la inmunidad adaptativa y adquirida. La IL-17 desempeña un papel crítico en la inflamación y enfermedades autoinmunes. A pesar del papel de la IL-17 en la autoinmunidad, se sabe relativamente poco sobre su papel en el cáncer, y los datos obtenidos hasta ahora son algo contradictorios. Algunos estudios apoyan su papel en la progresión tumoral, probablemente debido a una estimulación de factores angiogénicos (Kato T. et al., 2001; Numasaki M. et al., 2003). Por el contrario, otros estudios sugieren que la IL-17 promueve el rechazo del tumor por mediación de los linfocitos T. Los linfocitos T-$CD8^+$, y

otros tipos celulares, producen las citoquinas del perfil Th17 (Numasaki M. et al., 2003), incluyendo la IL-17; no obstante, el papel de la IL-17 producida por células no linfocitos T-CD8$^+$ queda por definir. Nuestros datos sugieren que la IL-17 contribuye a la progresión y agresividad tumoral, existiendo una expresión 100 veces menor en los tumores que no desarrollan metástasis respecto a los tumores de pronóstico desfavorable.

La producción de IFNβ, por los linfocitos T y B, los macrófagos, los fibroblastos, las células endoteliales entre otras, es inducida por otras citoquinas, como por ejemplo, la IL-1, IL-2, TNF y el factor estimulante de colonia (CSF). Además, los agentes quimioterápicos como el cisplatino o el paclitaxel inducen la expresión del IFNβ (Wan S. et al., 2012) que puede inhibir la angiogénesis, mediante la regulación de la expresión de genes pro-angiogénicos en el infiltrado inflamatorio (Jablonska J. et al., 2010). Sin embargo, esta citoquina es conocida por su papel en la respuesta viral, lo que puede permitir establecer una relación con la recién asociación entre el virus del papiloma humano y el cáncer de mama (Kan C.Y. et al., 2005; Heng B. et al., 2009; Lawson J.S. et al., 2009).

Existen evidencias que indican el papel del factor de transcripción nuclear kappa (NFκB) en la progresión tumoral. En efecto, NFκB está asociado a la supervivencia de las células madre del cáncer (Guzman M.L. et al., 2007). Asimismo, regula la expresión de numerosas proteínas anti-apoptóticas asociadas a la supervivencia tumoral (bcl-xl, bcl-2, XIAP, c-FLIP, IAP-1, IAP-2, y survivina), así como la expresión de genes asociados a la progresión tumoral (cyclin D1, c-myc and COX-2). Además, numerosos datos apoyan el papel de NF-κB en la regulación de las vías de inflamación y progresión tumoral (Aggarwal B.B. et al., 2006).

En definitiva, estos resultados muestran que la expresión de MMP11 por las CMIs del estroma intratumoral identifica un grupo de tumores con un perfil de expresión elevado de moléculas relacionadas con el proceso inflamatorio, que se asocia con un peor pronóstico.

Es sabido que la activación de oncogenes puede desencadenar la producción de moléculas inflamatorias y el reclutamiento de células inflamatorias. Sin embargo, los efectos potenciales del infiltrado inflamatorio en el cáncer de mama parecen ser diversos y complejos. Por lo tanto, hemos investigado el impacto de la presencia de diferentes tipos de células inflamatorias en la frontera tumoral sobre el desarrollo de metástasis a distancia. Hemos estudiado esa relación en la frontera invasiva pues es dónde tienen lugar importantes interacciones entre las células tumorales y el estroma es la zona en la que algunos de los más importantes las interacciones entre las células cancerosas y el estroma de soporte del tumor tener lugar (Giatromanolaki A. et al., 2004).

Nuestros resultados muestran que existe una heterogeneidad biológica entre los tumores de mama con respecto al infiltrado celular en el frente invasivo. Asimismo, hemos mostrado que un alto ratio de macrófagos / linfocitos T y B [CD68 / (CD3 + CD20)] en la frontera invasiva se asocia significativa e independientemente con el desarrollo de metástasis a distancia. Las células inflamatorias pueden suprimir o promover el crecimiento tumoral, dependiendo del tipo celular y su perfil funcional. Así, el análisis del perfil de expresión proteíca de los linfocitos que hemos realizado, coincide con otros estudios que sugieren que el infiltrado linfocitario se correlaciona con tumores con receptores hormonales negativos o $HER2^{+}$, o con tumores de alto grado; sin embargo no hemos podido establecer una relación con el pronóstico (Arnould L. et al., 2006; Bates G.J. et al., 2006; Alexe G. et al., 2007; Desmedt C. et al., 2008; Rody A. et al., 2009; Denkert C. et al., 2010; Mahmoud S.M. et al., 2011; Lofdahl B. et al., 2012). Asimismo, los linfocitos B activados pueden influir en la regresión tumoral promoviendo una inmunidad antitumoral a los linfocitos T; lo que sugiere que los linfocitos B pueden ser un complemento a la inmunoterapia adoptiva basada en células T (Li Q. et al., 2011).

Los macrófagos asociados a tumores (MATs) provienen de los monocitos circulantes que migran a los tejidos en respuesta a señales químicas y se

diferencian a macrófagos. En el cáncer de mama, los MATs pueden llegar a representar hasta el 50% de la masa del tumor. Los MATs producen una variedad de factores tales como citoquinas y quimioquinas, o factores de crecimiento dirigidos tanto a las células epiteliales como a las células endoteliales, las cuales juegan un papel clave en el crecimiento tumoral y la metástasis (Bingle L. et al., 2002; Coussens L.M. and Werb Z., 2002; Lewis C.E. and Pollard J.W., 2006). De acuerdo con Mahmud et al, hemos relacionado la densidad de macrófagos con tumores receptor de progesterona negativos o $HER2^{+}$; sin poder relacionarla con el pronóstico (Mahmoud S.M. et al., 2012).

El análisis multivariante es, pues, fundamental para establecer una relación entre la infiltración leucocitaria y el pronóstico las pacientes con cáncer de mama. La realización de este tipo de análisis nos ha permitido identificar que un alto ratio [CD68 / (CD3 + CD20)] es un potente factor independiente para la predicción de desarrollo de metástasis a distancia en nuestra población de pacientes. Así, se describe por primera vez, un estudio de la evaluación de la cantidad relativa de diferentes tipos de CMIs en la frontera tumoral de carcinomas mamarios, que puede permitir predecir la evolución de las pacientes. Este dato pone de manifiesto el complejo papel de las células inflamatorias en el cáncer de mama, reflejando de forma más objetiva las interacciones pro- y anti-tumorales de los diferentes tipos de células inflamatorias.

El desarrollo de metástasis a distancia no es sólo promovido por los cambios genéticos intrínsecos en las células malignas, sino también por el microambiente tumoral. En ese sentido, las MMPs juegan un papel esencial en la invasión tumoral y la metástasis a través de la degradación del tejido conectivo del estroma y los componentes de la membrana basal. En estudios previos, hemos identificado un fenotipo de CMIs caracterizado por la expresión de determinadas MMPs y TIMPs en el centro tumoral y asociado al desarrollo de metástasis a distancia en pacientes con cáncer de mama (Gonzalez L.O. et al., 2007; Vizoso F.J. et al., 2007; Del Casar J.M. et al., 2009; Gonzalez L.O. et al., 2010). Además, tal y como describimos en el presente estudio, ese fenotipo de

CMIs muestra una elevada expresión de factores relacionados con la inflamación (Eiro N. et al., 2012; Eiro N. et al., 2013). En base a esos datos, hemos analizado la posible relación entre el número de macrófagos y linfocitos (T y B) en la frontera tumoral y la expresión de MMP-2, 9, 11, 14, y TIMP-2, en la frontera tumoral como o en el centro del tumor. Hemos establecido diversas asociaciones entre los recuentos de los diferentes tipos de células inflamatorias y la expresión de MMPs/TIMP; sin embargo, el resultado más pertinente fue la asociación establecida entre el ratio [CD68 / (CD3 + CD20)] y la expresión de MMP11 y TIMP2 por las CMIs en el centro del tumor. La MMP11 y TIMP2 son los dos principales factores que definen el fenotipo pro-metastático de las CMIs, descrito en nuestros estudios previos (Gonzalez L.O. et al., 2007; Vizoso F.J. et al., 2007; Del Casar J.M. et al., 2009; Gonzalez L.O. et al., 2010); lo que indica que un alto ratio [CD68 / (CD3 + CD20)] en la frontera tumoral contribuye a polarizar los macrófagos hacia un fenotipo metastático en el centro del tumor.

Los presentes resultados contribuyen a caracterizar el infiltrado inflamatorio y su relación con el pronóstico y la expresión de MMPs/TIMPs en el cáncer de mama. La determinación de este ratio [CD68 / (CD3 + CD20)] podría contribuir a la identificación de pacientes con cáncer de mama candidatos a ser tratados con terapias basadas en la inmuno-modulación. En la actualidad, se han establecido diferentes estrategias contra los MATs (Griffiths L. et al., 2000; Luo Y. et al., 2006; Mukhtar R.A. et al., 2011), y se ha evaluado la eficacia de las células B activadas en inmunoterapia celular en tumores malignos (Schultze J.L. et al., 1997; Lapointe R. et al., 2003; Coughlin C.M. et al., 2004; DiLillo D.J. et al., 2010). Por tanto, el diseño de inmunoterapia en el cáncer de mama requiere tener en cuenta la inmunidad celular así como la inmunidad humoral.

Los factores moleculares y su implicación en el desarrollo de metástasis a distancia o metástasis hematógenas han sido ampliamente estudiados en el cáncer de mama. Sin embargo, el conocimiento de los determinantes implicados en la metástasis ganglionar y el valor pronóstico de los mismos es aún insuficiente. El descubrimiento de factores implicados en las metástasis ganglionares es biológicamente relevante en cuanto al conocimiento de un mecanismo distinto al desarrollo de metástasis hematógenas; pero sobre todo es clínicamente trascendental en cuanto a evitar el sobre tratamiento quirúrgico de las pacientes con cáncer de mama.

La disección axilar completa en pacientes con ganglio centinela positivo es en la actualidad objeto de debate, puesto que aproximadamente sólo el 32-50% de estas pacientes desarrolla metástasis en el resto de los ganglios axilares (Hung W.K. et al., 2005; Domenech A. et al., 2009). Ante toda esa perspectiva actual, se impone la búsqueda de nuevos factores predictivos de la afectación de los ganglios no centinela, de cara a optimizar la estrategia terapéutica en el cáncer de mama. Por tanto, el siguiente paso es determinar qué pacientes necesitan una disección axilar de ganglios linfáticos tras un resultado positivo de la biopsia de ganglio centinela; existiendo así la necesidad de identificar un método fiable para la predicción de la afectación tumoral de los ganglios linfáticos no centinela. Ello, con el fin de evitar una disección de ganglios linfáticos axilares innecesaria y así eludir los efectos adversos de la cirugía. Además, cabe destacar que se desconoce en la actualidad el efecto que puede tener la linfadenectomía axilar completa, y la disminución de la capacidad de vigilancia y defensa inmunológica que ello conlleva sobre el desarrollo de recurrencias tumorales y/o nuevos tumores en el cáncer de mama. Así pues, se considera muy importante la necesidad de investigar factores que predigan la afectación tumoral de los ganglios linfáticos no centinelas. Con el fin de identificar la posible relación de las MMPs o TIMPs con la afectación tumoral de los ganglios linfáticos axilares, hemos realizado un "screening" en una población de mujeres que se sometieron a una disección axilar clásica. Una vez identificadas las MMPs y TIMPs candidatas a predecir la afectación ganglionar,

se investigó dicha relación en una población de pacientes que se sometieron a la evaluación intraoperatoria del ganglio centinela.

Nuestros resultados coinciden con diversos estudios que indican que el tamaño tumoral (Fougo J.L. et al., 2009) y el diámetro de la metástasis en el ganglio centinela (Cserni G., 2001; Hwang R.F. et al., 2003; Fleming F.J. et al., 2004; Viale G. et al., 2005; Wada N. et al., 2006; Domenech A. et al., 2009) son factores predictivos significativos de la afectación de los ganglios linfáticos no centinela. Asimismo, nuestros datos muestran una tendencia de la invasión linfática para predecir la afectación de los ganglios no centinela (Turner R.R. et al., 2000; Abdessalam S.F. et al., 2001; Degnim A.C. et al., 2003; Hwang R.F. et al., 2003; Viale G. et al., 2005). Distintos scores de nomogramas basados en esos factores fueron definidos para determinar el riesgo de encontrar ganglios axilares adicionales positivos cuando la linfadenectomía axilar completa es realizada (Tousimis E. et al., 2003; Van Zee K.J. et al., 2003; Katz A. et al., 2008; Gur A.S. et al., 2009; Unal B. et al., 2009). Sin embargo, los resultados obtenidos utilizando los citados nomogramas resultan todavía insatisfactorios para la práctica clínica (Van Zee K.J. et al., 2003; Erb K.M. and Julian T.B., 2009; Gur A.S. et al., 2009; Scow J.S. et al., 2009). Así, se estima que las pacientes con la más favorable combinación de factores predictivos, no tienen menos de un 13% de riesgo de presentar metástasis en los ganglios linfáticos no centinela, por lo que debería aún serle ofrecida la práctica de una linfadenentomía axilar completa (Viale G. et al., 2005). Además, el valor predictivo positivo de los nomogramas se sitúa entre el 44% y 64%, con importantes variaciones entre las diferentes instituciones donde se aplique (Cserni G. et al., 2007; Pinero A. et al., 2013).

La situación de incertidumbre, en cuanto a la práctica de la disección axilar completa tras una biopsia positiva del ganglio centinela, se ha incrementado recientemente debido a la presentación de los resultados del ensayo Z011 del American College of Surgeons Oncology Group (ASCOG) (Giuliano A.E. et al., 2011). En ese ensayo se incluyeron mujeres diagnosticadas de un carcinoma infiltrante de mama con tumores menores de 5 cm (T1-T2), sin

afectación clínica axilar e intervenidas mediante un procedimiento quirúrgico conservador. Las pacientes en las que el ganglio centinela estaba afectado se aleatorizaron por observación y linfadenectomía axilar. Todas las mujeres incluidas en el estudio recibieron tratamiento radioterápico mediante dos campos tangenciales. Se excluyeron del estudio las pacientes con invasión extracapsular del ganglio centinela o aquellas con más de dos ganglios centinela afectados. Tras un seguimiento mínimo de seis años, no se observaron diferencias significativas en la tasa de recurrencias axilares entre los dos grupos. Sin embargo, se ha señalado que ese ensayo muestra defectos metodológicos relevantes, entre otros: (i) El estudio no concluyó el reclutamiento de pacientes previstas (se incluyeron 891 pacientes de las 1.900 previstas). Ello condicionó una baja representatividad de la muestra debido al escaso reclutamiento de pacientes en muchos hospitales. (ii) El 80% de las pacientes tenían receptores de estrógenos positivos, lo que supone un sesgo en la selección de pacientes hacia el buen pronóstico. (iii) La mayoría de las pacientes presentaron una mínima carga tumoral en la axila debido a que se excluyeron mujeres con más de 2 ganglios centinela afectados y con afectación extracapsular. (iv) En la mayoría de las pacientes no se realizó análisis inmunohistoquímico del ganglio centinela, por tanto no se tuvo en consideración la posible existencia de micrometástasis en este estudio. (v) No se realizaron estudios del HER-2, con lo cual no se pudo valorar convenientemente los resultados en los tumores HER-2-positivos o de tipo triple negativo. (vi) Se observó una tendencia a que los tumores incluidos en el grupo de mujeres que se sometieron a linfadenectomía fueron más frecuentemente de mayor tamaño, con invasión linfovascular y con un mayor número de ganglios axilares afectados (40,8% vs. 21,9%). Todo ello ha condicionado un cierto escepticismo en cuanto a la aplicabilidad clínica del resultado del ensayo ASCOG Z0011 (Knauer M. et al., 2012).

En el presente estudio, hemos establecido una correlación positiva y significativa entre la expresión global de MMP1, en el ganglio centinela positivo, y la afectación de los ganglios no centinela. Pero es aún más remarcable que ninguno de los casos de ganglio centinela con expresión

negativa de MMP1 en las CMIs presentaba ganglios no centinela afectados; mientras que todos los casos de ganglios no centinela afectados mostraron una expresión positiva de MMP1 por las CMIs del ganglio centinela. Por tanto, la expresión de MMP1 es significativamente asociada con la afectación de los ganglios linfáticos no centinela. La MMP1 o colagenasa intersticial es la más ubicua y es capaz de degradar diversos componentes de la matriz extracelular, como el colageno de tipo 1, que constituye el principal componente del tejido conectivo, los colagenos de tipo II, III, VII, VIII y IX, los agrecanos así como los inhibidores de serín-proteasas y la α2 macroglobulina (Brinckerhoff C.E. et al., 2000; Ala-aho R. and Kahari V.M., 2005). La capacidad de degradación de la MMP1 puede ser la responsable de la promoción de la extensión del tumor a través de los ganglios linfáticos.

El sistema linfático axilar puede ser considerado como el primer lugar donde se reciben antígenos tumorales específicos y otras señales bioquímicas procedentes del tumor primario de la mama. Esas señales moleculares procedentes de los tumores pueden ocasionar modificaciones en las poblaciones linfocíticas de los ganglios linfáticos. Sin embargo, la naturaleza inmunológica de los ganglios linfáticos axilares ha sido tradicionalmente poco investigada. Se conoce que las células inflamatorias de los ganglios linfáticos pueden diferir no solo en el tipo sino también en su fenotipo en cuanto a su capacidad defensiva (Cochran A.J. et al., 2006). Además, se ha señalado que el perfil de las poblaciones de células inflamatorias de los ganglios axilares puede aportar información pronóstica adicional sobre la evolución clínica del cáncer de mama. Así, se ha identificado que unas poblaciones más elevadas de células T "helper" CD4 y células dendríticas (CD1a) (células presentadoras de antígenos) en los ganglios axilares sin afectación neoplásica, se asocian con un mejor pronóstico en el cáncer de mama (Kohrt H.E. et al., 2005). Por tanto, las células inflamatorias de los ganglios linfáticos así como los factores que expresan parecen tener un papel activo y relevante en la progresión de las metástasis ganglionares en el cáncer de mama. En ese sentido, hemos observado que la expresión de MMP1 por las CMIs peritumorales en el ganglio centinela ha sido

asociada con el número de ganglios no centinela afectados. Esto parece indicar que las células tumorales tienen la capacidad de inducir la producción de dicha proteína en las células del huésped en los ganglios linfáticos; lo que destaca la importancia de la relación estroma-epitelio en la progresión tumoral a través de la cadena ganglionar.

En definitiva, la expresión de MMP1 por las CMIs de los ganglios centinela positivos muestra una sensibilidad del 100%, una valor predictivo negativo del 100% y una especificidad del 61,5% para predecir la afectación de los ganglios linfáticos axilares no centinela. Si este resultado es confirmado en estudios más amplios, podría ayudar a evitar la realización de disecciones axilares innecesaria en un porcentaje significativo de casos (50-68%) después de la identificación de un ganglio centinela metastático.

Los resultados del presente estudio ponen de manifiesto la participación del microambiente inflamatorio en la progresión del cáncer de mama y el desarrollo de metástasis a distancia y metástasis ganglionares así como su valor pronóstico. Además, las células inflamatorias y las moléculas implicadas en la interacción entre el tumor y su microambiente inflamatorio pueden ser una diana para el desarrollo de nuevas terapias contra el cáncer de mama.

Conclusiones

Los resultados obtenidos y la oportuna discusión de los mismos, nos han llevado a las siguientes conclusiones:

1. La expresión de MMP11 por las células mononucleares inflamatorias del estroma intratumoral identifica un grupo de tumores con un perfil de expresión elevado de moléculas relacionadas con el proceso inflamatorio y la carcinogénesis, que se asocia con un peor pronóstico

2. El ratio macrófagos/(suma de linfocitos T y B) [CD68 / (CD3 + CD20)] es un factor pronóstico independiente para la predicción de desarrollo de metástasis a distancia.

3. El ratio [CD68 / (CD3 + CD20)] se relaciona con la expresión de los dos principales factores que definen el fenotipo pro-metastático de las células mononucleares inflamatorias en el centro del tumor, la MMP11 y el TIMP2.

4. La expresión de MMP-1 por las células mononucleares inflamatorias del ganglio linfático centinela positivo predice la afectación de los ganglios linfáticos axilares no centinela. Cuando la MMP1 no es expresada por las células mononucleares inflamatorias del ganglio linfático centinela positivo, en ningún caso los ganglios linfáticos axilares no centinela están afectos.

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