Universidad de Santiago de Compostela

Facultad de Medicina y Odontología

Departamento de Medicina

Hospital Clínico Universitario de Santiago de Compostela

Laboratorio de Investigación 2

Factores genéticos implicados en el desarrollo de la artrosis y la artritis reumatoide. Estudio de polimorfismos funcionales en genes candidatos

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Tesis Doctoral

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2008

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CERTIFICAN QUE:

El presente trabajo que lleva por título: *Factores genéticos implicados en el desarrollo de la artrosis y la artritis reumatoide. Estudio de polimorfismos funcionales en genes candidatos,* realizado por Julio Rodríguez López en el Departamento de Medicina de la Universidad de Santiago de Compostela bajo nuestra dirección, ha sido revisado y está en disposición de ser presentado para optar al grado de Doctor en Biología.

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Fdo: Dr. Antonio González Martínez-Pedrayo Fdo: Dr. Juan J. Gómez-Reino Carnota

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A la gente que me ha hecho ser como soy; excluyéndome a mí mismo...

Hay que sufrir y trabajar mucho hasta dar con los colores, con el pincel y con el lienzo.
E incluso entonces estaremos muy lejos aún de dominar el arte de vivir,
aunque por lo menos, seremos dueños de nuestro propio taller.
Friedrich Nietzsche

Si un hombre comienza con certezas terminará dudando, pero si se
contenta con empezar dudando, terminará poseyendo certezas
Francis Bacon

Ex nihilo nihil fit

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Agradecimientos

Algunos dicen que la vida son las cosas que hacemos para algún día llegar a echarlas de menos. Supongo que eso es porque siempre nos gustaría estar haciendo aquello que nos hace felices, aquello que nos sale de dentro. Un día, cuando era niño, soñé con descubrir las reglas básicas que se encuentran por detrás de todo lo real, y ahora, mientras escribo esto, tomo conciencia de que estoy en el camino de hacerlo. Quizá nunca lo consiga, honestamente creo que la humanidad nunca lo conseguirá porque la vida es mucho más que números y fórmulas, y porque no nos va a dar tiempo antes de que destruyamos el planeta. Otros universos acaecerán, y quizás ellos sí lo llegarán a saber, pero no estaremos allí para comprobarlo, aunque igual alguno de nuestros inmortales átomos sí, pero lamentablemente, no seremos conscientes de ello. No me importa, porque la sensación de poner un granito de arena en el gran castillo del conocimiento es una de las más grandes del firmamento, y tan sólo por eso merece la pena estar vivo y viviendo. Y me siento satisfecho por ello, y NADIE puede cambiar mi opinión al respecto.
Siempre he querido que llegara este momento, el de la consecución de todo un proceso, el de llegar a una meta que lleva revoloteando durante mucho tiempo en tu cabeza. Ha costado mucho esfuerzo, pero la satisfacción de estar cumpliendo un sueño ha sido energía más que suficiente para mantener una sempiterna sonrisa (aunque fuera interiormente) cada uno de los días que he pasado entre probetas, poyatas, pipetas, reactivos de SNaP Shot, estadísticas y secuencias.

Primero, dar las gracias a TODO el mundo, por aguantarme.
En segundo lugar al doctor Antonio González, mi director de tesis, por haber confiado en mí y permitirme acceder a este apasionante mundo de la investigación científica y aportar mi pequeña contribución; por darme la posibilidad de conocer como es la ciencia en país que cree en ella y la respeta (U.K.), por la ciclópea paciencia mostrada durante estos tres meses de interminables correcciones de la tesis, y por compartir (en parte) conmigo una visión de *como se deben hacer las cosas*, que por desgracia para nosotros y para el mundo en general, no tiene muy buena acogida mediática.
Gracias también al doctor Juan J. Gómez-Reino codirector de mi tesis por ofrecerme la posibilidad de realizar mi labor en colaboración con el Servicio de Reumatología del Hospital Clínico Universitario de Santiago de Compostela.
Gracias, también, a todos aquellos participantes anónimos, pacientes y controles que por su labor altruista (¡existe el altruismo!) en favor de la ciencia me han permitido la posibilidad de hacer este estudio, así como a todos aquellos investigadores y clínicos que han colaborado directa o indirectamente en este trabajo. Mención especial al doctor John Loughlin de la Universidad de Oxford (ahora quizá Newcastle) y a todo el personal de su grupo de investigación por su hospitalidad, atención y docencia durante mi estancia en el Botnar Research Centre (Oxford University Institute of Musculoskeletal Sciences) durante los meses de junio y julio de 2007.

Luego, agradecer a mis padres porque, desde el punto de vista genético, este trabajo les corresponde en un 50% a cada uno de ellos (descontando el porcentaje debido a cambios ambientales y culturales, pero eso

lo dejo para un debate etólogo-político que ahora no viene al caso…), y desde el punto de vista espiritual (metafísico, trascendental, mental,…), por que son los mejores y nada más. A mi madre en especial, porque como dijo Saramago “A una madre se la quiere toda la vida con el mismo cariño, y cuando una madre se muere a cualquier edad se es niño”; y por el incontestable mérito de aguantar, con una paciencia que sólo las madres conocen, aquellas mis primeras parrafadas científicas por los ya lejanos años 90, cuando en primero de BUP descubrí por primera vez las leyes de Mendel y supe que mi destino había sido fijado para siempre. Por apoyarme siempre en mis controvertidas decisiones vitales y por no dejar de creer en mí por absurdas e ilusorias que sean mis aspiraciones (entre ellas la de cambiar, que no conquistar, el mundo). Es por las madres por las que había que levantar catedrales…
Al resto de mi familia por estar siempre ahí. (No voy a calcular el tanto por ciento desde el punto de vista genético que les corresponde de este trabajo…ya bastante tengo con escribir esto…). Mención especial para mis hermanos pequeños, no sé exactamente por qué, supongo que porque son geniales y porque me han hecho descubrir una faceta (oculta) de mi personalidad que, de una manera imposible de abordar desde un punto de vista científico, me ha aprendido un poco más lo que es la vida y el vivir, y me han hecho madurar, alcanzar otro estadio de Erikson más. También a mi primo Miguel (Beckham) porque su emoción al ver mi primer artículo publicado me hizo corroborar que lo que estaba haciendo merecía la pena y mucho más.

A todos mis compañeros de todos los laboratorios porque la compañía siempre es buena y el trabajo en conjunto es más ameno y más llevadero (¿siempre?) y, aunque no todos tengamos los mismos objetivos, en la medida de lo posible hemos sido capaces de compenetrarnos. No puedo mencionaros a todos y cada uno, pero siento que debo hacer algunas alusiones especiales por razones malamente disimuladas bajo una aparente estocástica, pero que espero entendáis y respetéis todos los demás. (Acordaos de que el orden de los factores no altera el resultado…) A Cristina (ex jefa del servicio técnico) por dejarme las pinzas para encender el coche y porque siempre lo da todo por los demás sin esperar recibir nada a cambio, y ese tipo de persona tiene que perdurar para bien de la humanidad. Desde el principio, cuando llegué aquí encerrado en un caparazón de hierro forjado, se acercó e intentó entenderme, comprenderme y tratar conmigo abiertamente, y eso tiene mucho mérito y, aunque parezca un arte sencillo, son muy contados aquellos que lo manejan con soltura y naturalidad. A Rebe porque en sí misma, mismamente es una gran persona y siempre ha sido ameno y divertido (y a veces peligroso) trabajar a su lado. Y también por los queiques, que no creo que nunca haya merecido ni la mitad de todos los que me ha regalado. A Marta (actual jefa del servicio técnico) por formar parte conmigo del frente revolucionario que combatió los envites del “lado oscuro de la fuerza”. A Marián porque creo que a todos nos ha contagiado un poco su serenidad y no nos ha venido mal. A Manuel por haber sido el contrapunto ideal a la hora de relajar el estrés del trabajo y por haberme ayudado desinteresadamente siempre que lo he necesitado. A Manolo por su original humor, por ese punto de vista distintamente calculado que siempre le aporta a todo y por las ayudas con las *chicuadrado*. A Rudi porque dudo mucho que pueda volver a encontrar a alguien con el que mantener esas apasionadas e interminables (no por interesantes si no por no existir final) discusiones sobre física cuántica, la no-existencia del tiempo, *end of evangelion* y demás frikadas manga, los gatos

envenenados de Heissenberg, los artículos del Reverte, la posibilidad de reinventar (que no deconstruir) nuestra tan bonita y pluricultural y socialdemocratacapitalista sociedad actual … y por estar siempre creando (tol rato genialidades, tol rato…). A Samu porque es del barsa… y porque nos conocemos desde la carrera y, a pesar de algún que otro plantón, hemos recorrido más o menos juntos el duro camino que lleva a la consecución de una tesis doctoral y siempre es bueno tener a alguien al lado que sufra los mismos tormentos. A Manuel (biologuer) por haberlo dejado "a cuadros" cuando al empezar juntos el camino del tercer ciclo decidí separarme para dejarme llevar por lo que me decía mi instinto (y que no eran las microalgas… con todos mis respetos por otra parte), y por haber compartido conmigo cinco años de carrera y aquel T.A.D. en fisio vegetal que lo pasamos genial trabajando con plumífero en la cámara de frío, y que nos permitió sentirnos *científicos* por primera vez. A Elisa porque, sin hacer ningún ruido, ha sabido ganarse perfectamente su sitio, una actitud admirable, que no estaría de más que fuese copiada por algunas (muchas) personas, y por que le gusta *Superstar* y *Zelda,* y eso es difícil de encontrar. A Bruno porque hemos pasado muy buenos ratos recordando viejas películas acompañados de mala comida y parrafando a las ocho de la mañana (de la noche). A Isabel (Barcelona) por su ayuda en el diseño del maravilloso "sujetatapas de centrífuga" y por tener que aguantar más de cinco borderías marca de la casa. A Servet por su "mala leche" y demás repertorio espanglish que le fuimos enseñando. A Isa por las inacabables discusiones sobre política y demás movimientos socioculturales, por servir de modelo e inspiración y por su ayuda y paciencia en el inicio y final de mi formación; la paciencia más bien al final que al principio, pero bueno, ha sido un placer trabajar codo con codo con un espíritu tan temperamental y decidido. A Cristina (estudiante) por ofrecerme la posibilidad de enseñar (un poco de) aquello que había aprendido y porque le gusta Mozart, uno de mis ídolos y uno de los personajes que más me ha influido. A Yolanda porque sin su trabajo de recogida de muestras nunca podría llevar a cabo mi proyecto de investigación, y por ser mi taxista durante una temporada, hace ya bastante tiempo, y eso de aguantarme a las siete y media de la mañana recién levantado merece un monumento por lo menos…A Ezequiel porque por fin alguien se decidió a organizar pachangas en horario champions, a Roberto por su gloriosa "olimpiada de calceta", a Ana, por compartir conmigo el esprín final de la tesis, a Miguel por ser socio del club de fans de Conchita Martínez (lo cual tiene mucho mérito) y a Sonia por la perseverancia que mostró para conseguir ver su sueño hecho realidad a pesar de todo y de todos, y eso es digno de admirar.
A Carmen, Fran y Oreste les quiero agradecer su atención y asesoramiento siempre que les he pedido algún tipo de ayuda, además de alguna que otra charla interesante (de cine y fútbol básicamente (grande Dino Zoff)) en las pocas ocasiones que tuvimos oportunidad…

A todos aquellos profesores (de colegio, instituto y universidad), personajes varios y medios audiovisuales y escritos (series de televisión, películas y libros) que me han hecho elegir ser como soy y amar el estudio y la investigación.
También le quiero dedicar este trabajo a mis amigos de Carballiño y alrededores, en especial a Alfredo, por haber mostrado siempre un interés desinteresado por todo aquello que hacía, y por prestarme su incondicional apoyo siempre que lo he necesitado; a Pako,… por ser un gran tipo, el tipo más auténtico que he conocido. A Serxo por los buenos momentos de ocio que hemos pasado juntos alrededor del

mundo, desde la *piazza navona* hasta los supermercados de Amsterdam, pasando por la *petit france* y el *manneken piss*. A Marcos Rúa por venirse conmigo a “buscar trabajo” a Irlanda y por ser un *crack* de la vida entre otras cosas más. A Montxo por ser la primera persona *extranjera* que conocí al llegar a Santiago con los inolvidables dieciocho años, y por acogerme en aquellos quince maravillosos días en Praga, un viaje que marcó un antes y un después en mi discurrir vital. Y, finalmente a Oskar Insua, teniente alcalde de Corcubión (quien lo diría…), por compartir conmigo los años de carrera en la residencia entre fiesta y fiesta y más fiesta (qué mal vivíamos…), y los primeros de doctorado en el antro de “Santiago de Chile” y en el congelador con camas de “Galeras”, por cantar juntos por las calles de Salamanca y por ganar aquel concurso amañado disfrazados de los cantantes de ABBA (o algo por el estilo).

Por supuesto no podía excluir de la lista de agradecimientos a la Muchachada (Mojamuto E. et al 2007), grandísimos personajes que han aderezado con buen rollo, consejos inolvidables (enorme Chimo Bayo y Marlo Brando) y formidable sentido del humor la última etapa de mi tesis.

Y ya finalmente (y ahora viene la parte sensiblera) quiero dedicarle este trabajo con todo mi cariño y corazón a Andrea V.P.. Porque sin ella nunca podría ser capaz de estar escribiendo esto de la manera en la que lo estoy haciendo, y por haberme permitido comprender (con harta paciencia por su parte) que son ciertas aquellas sabias palabras de José Agustín Goytisolo que decían “Un hombre solo, una mujer, así tomados, de uno en uno, son como polvo, no son nada”, y de esa manera ayudarme a encontrar ese minúsculo reducto interior de energía ilimitada no regida por las leyes de la física cuántica, que me ha concedido la capacidad de, una vez llegado a la cumbre de la montaña más alta, desplegar las alas y volar; volar mucho, pero mucho, mucho, mucho más allá...

Esta tesis doctoral fue realizada en el Laboratorio de Investigación 2 del Hospital Clínico Universitario de Santiago de Compostela en el periodo comprendido entre 2004-2007 con la ayuda de una beca FPU (Formación de Profesorado Universitario) financiada por el Ministerio de Educación y Ciencia de España.

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A continuación se exponen las abreviaturas más importantes utilizadas en el texto. Las que han sido omitidas en este apartado son de una menor relevancia para la comprensión global o se encuentran perfectamente especificadas en el texto.

ACR	Colegio Americano de reumatología
ADAM	Desintegrina y metaloproteasa
ADAMTS	Desintegrina y Metaloproteasa con motivos Trombospondina
ASPN	Asporina
BMP	Proteínas morfogénicas del hueso (del inglés *Bone mophogenic proteins*)
ECM	Matriz extracelular (del inglés *Extracellular matrix*)
EMSA	Ensayo de cambio de movilidad electroforética
FRZB	Proteína frizzled relacionada
GDF5	Factor de crecimiento y diferenciación 5
HOA	Artrosis de mano (del inglés *Hand Osteoarthritis*)
HWE	Equilibrio de Hardy-Weinberg
LD	Desequilibrio de ligamiento
MMP	Metaloproteasas de matriz extracelular
nsSNP	Polimorfismo no sinónimo de un sólo nucleótido
OA	Artrosis (del inglés *Osteoarthritis*)
OR	*Odds ratio*
PAI	Inhibidor del activador del plasminógeno
PAs	Sistema Plasminógeno-plasmina
PCR	Reacción en Cadena de la Polimerasa
PLAT	Activador tisular del plasminógeno
PLAU	Activador del plasminógeno tipo urokinasa
RA	Artritis Reumatoide (del inglés *Rheumatoid Arthritis*)
SNP	Polimorfismo de un sólo nucleótido (del inglés *Single nucleotid polymorphism*)
Ta	Temperatura de anillamiento
TGF-β	Factor transformador del crecimiento beta

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THR	Reemplazo total de la cadera (del inglés *Total Hip Replacement*)
TIMP	Inhibidor Tisular de Metaloproteasas
TKR	Reemplazo total de la rodilla (del inglés *Total Knee Replacement*)
Tm	Temperatura de disociación (del inglés *Melting Temperature*)
Wnt	"Sin alas" (del inglés *wingless*)

Introducción

Introducción

Artrosis

La Artrosis u osteoartrtosis (OA) (OMIM 165720), es un tipo de artritis causada por un deterioro progresivo e ininterrumpido del cartílago articular, modificación del hueso subcondral e inflamación del tejido sinovial. Su nombre deriva del griego "*osteo*", que significa "del hueso", "*arthro*", que significa "articulación", e "*itis*", que significa "inflamación", aunque en muchos casos se observa muy poca o ninguna. Su patofisiología es en gran medida una incógnita, aunque se sabe que influyen tanto efectos mecánicos como biológicos que provocan la desestabilización del equilibrio existente entre síntesis y degradación de los componentes de la matriz extracelular (ECM) y hueso subcondral (Kuettner KE, et al, 1995). La OA puede afectar a todas las articulaciones sinoviales del cuerpo aunque principalmente se encuentra en la rodilla, cadera, mano y la columna vertebral.

La OA tiene una prevalencia muy alta en la población adulta mundial y es la principal causa de incapacidad crónica, sobre todo debido a las afectaciones de la rodilla y la cadera. Además, los tratamientos actuales para la OA pueden mejorar la sintomatología pero no pueden frenar la progresión de la enfermedad, lo que hace que al llegar a altos niveles de severidad la única solución posible es el remplazo articular. Estas características provocan que la enfermedad tenga un impacto muy elevado en la salud pública, con el consiguiente coste substancial para el individuo y la sociedad (Van Saase JL et al, 1989) (Lawrence JS et al, 1966) (Global economics and health care burden of muscoloskeletal disease, 2001).

En la OA se producen cambios morfológicos, bioquímicos, moleculares y biomecánicos tanto en los condrocitos como en la ECM. Esto trae como consecuencia la degeneración cartilaginosa en áreas de tamaño irregular, fibrilación, ulceración, esclerosis del hueso adyacente, quistes óseos subcondrales y osteofitos marginales. Cuando la enfermedad se hace clínicamente evidente, la OA se caracteriza por la presencia de dolor articular, rigidez de las articulaciones afectadas con la consecuente limitación de la función, crepitación y derrame articular esporádico con grados variables de inflamación local sin repercusión sistémica. (Kuettner KE et al, 1995). **(Figura 1)**

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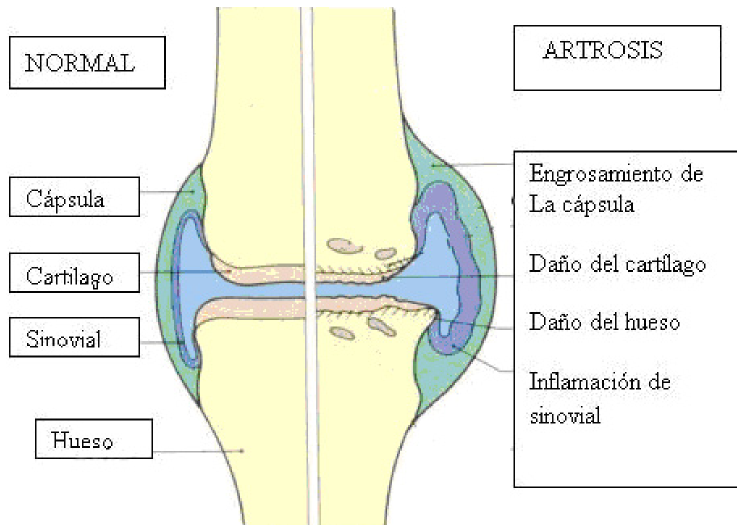

Figura 1: Esquema de los cambios observados en una articulación con artrosis (derecha) comparados con una articulación normal (izquierda)

En la OA se pueden distinguir dos subgrupos dependiendo del factor causal, la OA primaria o idiopática, cuya etiología es desconocida, y la OA secundaria que es debida a factores conocidos como alteraciones metabólicas, anormalidades anatómicas, traumatismos o artritis inflamatorias. Se ha propuesto que factores ambientales y antropométricos, como edad, sexo, obesidad o etnicidad puedan ser causas indirectas de la OA primaria, pero su papel no está muy bien definido y es algo confuso. En general, el cartílago de mucha edad tiene alteradas funciones básicas de los condrocitos y propiedades de la ECM, además de responder de manera menos eficiente a citoquinas y factores de crecimiento. Con la edad, factores neurológicos y mecánicos que pueden actuar como protectores de la articulación, como por ejemplo la propiocepción y la fuerza muscular, también pierden eficacia con lo que también pueden contribuir al desarrollo de la OA (Slemenda C et al, 1997). La OA es más frecuente en mujeres que en hombres aunque las diferencias dependen de la articulación. Por ejemplo, la OA de la rodilla es bastante más frecuente en mujeres que en hombres pero la diferencia se reduce mucho e incluso se iguala en el caso de la OA de la cadera (Danielsson L et al, 1997). En el caso de la OA de la mano, se ha visto que las mujeres tienen un riesgo 2.6 (95% CI 1.65-4.18) veces mayor de desarrollar OA que los hombres (Carman WJ et al, 1994). La manera en que el sexo puede influir en la susceptibilidad a la OA es a través de múltiples rutas que incluyen desde factores hormonales a

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probabilidades de prácticas de riesgo, pasando por la existencia de un distinto ambiente mecánico en la articulación (fuerza muscular relativa a la masa corporal). La masa corporal es uno de los factores de riesgo que más influyen en el desarrollo de la OA. Estudios longitudinales han demostrado que las mujeres con un índice de masa corporal (BMI) alto tienen 2.07 veces (95% CI 1.67-2.55) más riesgo de sufrir OA de la rodilla, mientras este valor es de 1.51 veces en hombres. En este mismo estudio se observó que una disminución de 2 unidades en el BMI producía un descenso del riesgo de sufrir OA (O.R. 0.46 (95% I.C. 0.24-0.86) (Felson DT et al, 1997). Por otra parte, en la OA de la cadera o la OA de la mano, la importancia del BMI como factor de susceptibilidad no está muy clara y en algunos estudios incluso se llega a demostrar que no existe ninguna correlación entre estos dos factores (Reijman M et al, 2007) (Sowers MF et al, 1996).

Últimamente se ha resaltado la importancia de los factores genéticos como en el riesgo de desarrollo y progresión de la OA. Varios estudios hechos con gemelos situaron la heredabilidad de la OA entre el 40 y un 79% dependiendo del fenotipo (Spector TD et al, 1996) (Zhang W et al, 2005). La evidencia del componente genético de la OA se ha hecho cada vez más manifiesta. Por otra parte en estos estudios también se ha resaltado la complejidad y heterogeneidad de la enfermedad.

Genética de la OA

La existencia de un componente genético como factor de riesgo en la OA es una evidencia desde que Stecher observara que los nódulos de Heberden eran tres veces más comunes de lo esperado en hermanas de pacientes (Stecher RM, 1955), y Kellgren mostrara que los parientes de primer grado de pacientes con OA generalizada tenían muchas más probabilidades de tener OA (Kellgren JH et al, 1963). Posteriormente, mediante estudios con gemelos y estudios de segregación se ha cuantificado este componente genético. El porcentaje de varianza atribuible a factores hereditarios ha variado de unos estudios a otros pero se sitúa entre el 39 y el 65% en la artrosis de la mano y la rodilla, sobre un 60% en la artrosis de la cadera y sobre un 70% en la artrosis de la columna. Estos datos en conjunto sitúan la heredabilidad de la OA en torno a un 50% (Spector TD, 2004) (Loughlin J, 2001). El valor de riesgo relativo entre hermanos (λs) (que expresa el riesgo que tiene el hermano de un afectado a sufrir la enfermedad comparado con el riesgo que tiene la población en general) es alrededor de dos veces mayor que la población control en el caso de la OA de la cadera y hasta 12 veces mayor que en la población control en el caso de la OA de la rodilla (Chitnavis J et al, 1997). En estos estudios también se

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ha mostrado que la OA es una enfermedad (1) compleja que no se hereda de una manera mendeliana simple, o sea que es multifactorial ya que depende de muchos genes con un efecto débil, y (2) de una gran heterogeneidad, porque los resultados dependen de la articulación afectada, siendo mayor en la mano, luego en la cadera y después en la columna (Riyazi, N et al, 2005); del sexo, siendo mayor en mujeres que en hombres (Carman WJ et al, 1994); y de la etnia, existiendo variabilidad entre los resultados obtenidos en distintas poblaciones.

Para abordar el estudio de la genética de la OA e identificar genes de susceptibilidad, se han seguido distintas estrategias. Estas se pueden agrupar en tres categorías: estudios de genes candidato, estudios de ligamiento de genoma completo y estudios de ligamiento de escaneado fino.

Estudios de genes candidato

Los estudios de genes candidato se basan en los conocimientos sobre la biología y la patofisiología de la OA así como en resultados derivados de estudios de niveles de expresión para establecer los genes diana sobre los cuales realizar el estudio de asociación. Los candidatos iniciales en este tipo de estudios se centraron en los genes que codifican para las proteínas estructurales de la ECM. Entre ellos el candidato principal fue el *COL2A1*, que codifica para la cadena polipeptídica α1 del colágeno tipo II, que es el principal componente en peso seco del cartílago articular. También se estudiaron otros colágenos como el colágeno tipo IX y tipo XI y genes que codifican para otros componentes de la ECM, entre los que destacan la proteína oligomérica de la matriz del cartílago (COMP) y el gen del agrecano *AGC1*. En general, estos estudios no obtuvieron resultados concluyentes que apoyen el papel de polimorfismos comunes no sinónimos en genes de proteínas estructurales de la ECM como factores de riesgo en la susceptibilidad a la OA (Loughlin J, 2001).

También se estudiaron genes candidato que codifican proteínas implicadas en la densidad ósea. Estas proteínas fueron estudiadas ya que se ha visto que la esclerosis subcondral es una característica de las primeras fases de la OA en algunas articulaciones, y la hipótesis de que esta esclerosis puede afectar al cartílago modificando su resistencia a la carga mecánica. Los genes más estudiados han sido los del receptor de la vitamina D, *VDR,* y el gen del receptor estrogénico, *ESR1* (Uitterlinden AG et al, 1997). Los primeros estudios mostraron que existía asociación con ambos genes y susceptibilidad a la OA, sin embargo estas asociaciones no fueron replicadas en estudios posteriores (Loughlin J et al, 2000). En el caso de *ESR1,* nuevos polimorfismos han sido estudiados obteniéndose resultados positivos, por lo que este gen sigue

siendo un buen candidato para explicar parte de la susceptibilidad a la OA (Bergink AP et al, 2003).

Otra aproximación para descubrir nuevos genes candidato ha sido realizando análisis de los niveles de expresión en cartílago articular de pacientes con OA comparados con controles sanos. Una vez identificados los genes que muestran diferentes niveles de expresión se buscan variantes genéticas en estos y se testan en estudios de asociación. De esta manera se han encontrado asociaciones de susceptibilidad a la OA con los genes *BMP2* (proteína morfogenéticas del hueso 2), *CD36* (receptor de trombospondina y colágeno), *COX2* (ciclooxigenasa) y *NCOR2* (receptor nuclear corepresor). También se encontraron genes que están asociados, no con el desarrollo de la enfermedad sino con su progresión, como por ejemplo *CILP* (proteína de la cap intermedia del cartílago), *OPG* (osteoprogeterina) *TNA* (tetranectina) *y SRI* (receptor estrogénico α). También se encontró un gen asociado con la prevalencia y la progresión de la enfermedad, el *ADAM-12* (meteloproteasa). Aún así estás asociaciones no se mantuvieron en su mayoría cuando los resultados fueron corregidos por el número de test utilizados, tan sólo en el caso de el *ADAM-12* la asociación continuó siendo significativa (Valdes AM et al, 2004).

El gen de la calmodulina-1, *CALM-1,* es otro gen que se encuentra aumentado en pacientes con OA (Mototani H et al, 2005) La calmodulina-1, *CALM-1,* es una proteína intracelular que se une al Ca^{2+} e interacciona con un gran número de proteínas. En un estudio llevado a cabo por el grupo del Dr. Ikagawa se encontraron cinco SNPs (un SNP en la región promotora, dos SNPS en el intrón-1, un SNP en la región 3' UTR y un SNP en el intrón-3) en fuerte desequilibrio de ligamiento (LD) asociados con susceptibilidad a la OA de la cadera ($P<0.0006$). De todos los SNPs asociados, el que estaba situado en el promotor del gen, fue considerado como el que tenía más posibilidades de ser funcional por lo que se realizaron estudios para saber qué efectos tenía sobre la expresión del gen. Los resultados obtenidos demostraron que el alelo asociado del SNP (alelo T) provocaba un descenso de la expresión de *CALM-1.* También demostraron que CALM-1 influía en la síntesis de colágeno tipo II y agrecano. De esta manera construyeron un modelo de actuación mediante el cual explicaron el papel de estos SNPs en la susceptibilidad a la OA. El alelo de susceptibilidad provocaría una menor expresión de la calmodulina lo que causaría una disminución en la síntesis de colágeno tipo II y agrecano y como consecuencia un cartílago más débil. Estudios posteriores sobre este gen en población asiática no han replicado estos resultados (no publicados), y hasta ahora no se han realizado más estudios al respecto.

En general, estos estudios han aportado buenos resultados sobre la genética de la OA pero tienen una serie de limitaciones debidas por una parte al conocimiento incompleto de la biología

de la OA y que en ocasiones el genotipado de un número limitado de variantes dentro de los genes investigados reduce el poder estadístico de estos estudios.

Dentro del estudio de genes candidato, Hay que destacar dos estudios llevados a cabo en población japonesa, estudios en los que se han encontrado dos nuevos genes que pueden tener mucha importancia en la explicación de la susceptibilidad a la OA primaria: el gen de asporina, *ASPN* (Kizawa H et al, 2005) y el gen del factor de crecimiento y diferenciación 5 *GDF5* (Miyamoto Y et al, 2007).

La asporina es una molécula de la ECM que pertenece a la familia de los proteoglicanos pequeños ricos en leucina (SLRP) a la que también pertenecen la decorina, el biglicano, la fibromodulina y la condroaderina (Henry SP et al, 2001). Los miembros de esta familia son capaces de interactuar con otros componentes de la ECM, como el colágeno, y con factores que residan temporalmente en la ECM, como el factor de crecimiento tumoral β (TGF-β). El gen de la *ASPN* se encuentra en la región cromosómica 9q22.31, una localización que no había sido previamente encontrada como ligada con la OA en los estudios de ligamiento de genoma completo. El grupo japonés que realizó el estudio de asociación, identificó un polimorfismo tipo microsatélite que estaba asociado con OA de la rodilla y de la cadera. Este microsatélite, situado en el exon 2 del gen, está formado por repeticiones de trinucleótidos que codifican residuos de ácido aspártico (D) y tiene diez alelos (10-19D). Los alelos asociados fueron el D14, aumentado en pacientes de la rodilla y la cadera, y el D13 aumentado en los controles, por lo que D14 sería un alelo de susceptibilidad y D13 de protección. Estos resultados los confirmaron en una cohorte japonesa independiente. Posteriormente, un estudio llevado a cabo en población china obtuvo resultados similares (Jiang Q et al, 2006). El grupo japonés también demostró que el alelo D14 de asporina, asociado con susceptibilidad a la OA, provocaba una mayor inhibición de la actividad de TGF-β, lo que provocaba una menor síntesis de colágeno tipo II y agrecano. Esta es una hipótesis plausible para explicar porqué este alelo se encontró asociado con susceptibilidad a la OA en esta población y le da credibilidad al resultado. Estudios posteriores realizados en población caucásica no confirmaron totalmente lo obtenido en asiáticos con lo que se concluye que asporina es un locus de susceptibilidad a la OA pero su efecto es mucho mayor en poblaciones asiáticas que en poblaciones occidentales (Nakamura T et al, 2007).

El factor de crecimiento y diferenciación 5, *GDF5,* también conocido como proteína morfogenética derivada de cartílago 1, ha sido el último gen que ha sido encontrado asociado con susceptibilidad a la OA por el grupo del Dr. Ikegawa (Miyamoto Y et al, 2007). *GDF5* es un miembro de la superfamilia del TGF-β y muy relacionado con la familia de las BMPs. Diversos estudios han demostrado que GDF5 está implicado en la formación de articulaciones y

es expresado en las regiones articulares muy precozmente en el desarrollo También se ha visto que estimula la síntesis de proteoglicanos en explantes de cartílago articular. En el estudio de asociación llevado a cabo por el grupo del Dr. Ikegawa, un SNP funcional situado en la región 5' UTR del gen de *GDF5,* mostraba una asociación muy fuerte ($P = 1.8 \times 10^{-13}$) con OA de la cadera en dos poblaciones japonesas independientes, y este resultado fue replicado en una población china. Los autores demostraron que el alelo asociado provocaba una disminución de la expresión del gen y de esta manera explicaban la susceptibilidad a la OA. Los resultados obtenidos del estudio de asociación así como los estudios funcionales fueron replicados en población europea aunque la magnitud del efecto fue menor (Southam L et al, 2007).

Estudios de ligamiento de genoma completo

Los estudios de ligamiento de genoma completo se basan en análisis de la cosegregación de loci genéticos en familias con miembros afectados por una determinada enfermedad. Se estudian una serie de marcadores repartidos por todo el genoma en pequeñas familias. Si un marcador está en ligamiento con un locus de susceptibilidad, los miembros afectados de la misma familia tendrán el mismo alelo del marcador. De esta manera se observa en qué región cromosómica se encuentran los marcadores que segregan de una manera compartida entre los miembros afectados y se buscan posibles genes candidato en la enfermedad.

Los estudios más importantes se realizaron sobre pequeñas familias con parientes afectados por OA recogidos en U.K. (OA de la cadera y la rodilla), Finlandia, EEUU e Islandia (OA de la mano).

En el estudio del Reino Unido, realizado en Oxford por el grupo del Dr. John Loughlin, se observó una región de ligamiento en el cromosoma 2 para la OA de la cadera, y la posterior estratificación por sexos mostró también ligamiento en los cromosomas 6, 4 y 11. (Chapman K et al, 1999) (Loughlin J et al, 1999). El estudio finlandés, fue realizado en familias con miembros que tenían OA en articulaciones interfalángicas distales (DIP), y se obtuvo ligamiento en las regiones 2q, 4q, 7p y Xcen. (Leppävuori J et al, 1999), aunque los ligamientos de los cromosomas 2 y 4 no se solapaban con los encontrados en el estudio de Oxford. El estudio Islandés fue realizado en un gran número de familias en las que existían pacientes con OA de la mano y parientes afectados, obteniendo los mejores resultados en los cromosomas 2 y 4, aunque tampoco se solaparon con los resultados obtenidos por los grupos anteriores (Stefánsson SE et al, 2003). El estudio estadounidense fue realizado utilizando la cohorte de Framingham y la OA de la mano fue caracterizada radiográficamente. En este estudio fueron encontrados varios loci,

muchos de los cuales estaban restringidos a articulaciones específicas de la mano, y uno de ellos se solapaba con la región 2p encontrada en el estudio islandés (Hunter DJ et al, 2004).
Los resultados obtenidos de estos estudios ponen de manifiesto la complejidad de la OA, puesto que son varios los loci encontrados y son diferentes dependiendo de la articulación afectada y el sexo. Esto se pone de manifiesto sobre todo en el estudio estadounidense donde las diferentes articulaciones de la mano se encuentran ligadas con loci distintos.

Identificación de genes candidato en loci de ligamiento.
A continuación, el siguiente paso que se siguió en la investigación genética de la OA fue el de estudiar en detalle las amplias zonas de asociación que se habían propuesto en los estudios de ligamiento de genoma completo en busca de genes candidato de susceptibilidad a la OA. Lo que se denomina escaneado fino de la región. Diversos genes fueron propuestos, realizándose estudios de asociación caso control utilizando polimorfismos existentes en los mismos.
En el estudio de ligamiento realizado en familias finlandesas, se encontró un pico de ligamiento en el cromosoma 2q en el que se encontraba un cluster de al menos 11 genes pertenecientes a la familia IL1 (región cromosómica 2q11-2q13). Aunque la OA no es una enfermedad inflamatoria hay casos en los que un tejido sinovial inflamado puede exacerbar la enfermedad. Además, las interleucinas son sintetizadas por los condrocitos para que actúen de manera auto y paracrina y de esta manera controlar la homeostasis del cartílago (Pelletier JP et al, 2001) (Goldring MB et al, 2000). Debido a esto, este cluster genético IL1 fue escogido para estudiar genes candidato para la OA. Varios estudios fueron realizados, siendo los llevados a cabo por un grupo holandés (Meulenbelt I et al, 2004) y otro grupo de Londres (Smith AJ et al, 2004) los más importantes. En estos estudios se ha encontrado asociación con haplotipos de susceptibilidad a la OA con unos valores de P del orden de 0.0000003.
En el estudio de ligamiento llevado a cabo por el grupo de Islandia se identificó un gen candidato en la región de máximo ligamiento (2p24.1), *MATN3*, que codifica la matrilina 3, una proteína estructural que forma parte de los componentes de la ECM. Se encontró un SNP no sinónimo asociado con susceptibilidad a OA de la mano en una primera cohorte, y este resultado fue confirmado en un segundo grupo de pacientes y controles (Stefánsson SE et al, 2003). Sin embargo este nsSNP no parece ser el SNP causal puesto que resultados de ligamiento obviando este SNP siguen dando LOD score elevados en la misma zona, por lo que nuevos estudios son necesarios para esclarecer estos resultados.
Otro gen candidato detectado a partir de los estudios de ligamiento es el de la cadena α del receptor IL-4, *IL4R*. Dicho gen se encuentra en la posición 16p12.1, región que fue detectada

por el grupo de Oxford (grupo del Dr. John Loughlin) en mujeres con OA de la cadera. Un estudio de asociación llevado a cabo por el mismo grupo encontró dos nsSNPs asociados ($P<0.05$) con susceptibilidad a la OA, resultado que fue confirmado en una cohorte independiente (Forster T et al, 2004), resaltando de esta manera su importancia en la susceptibilidad a la OA.

El grupo del Dr. John Loughlin también realizó la identificación de la región 2q24.3-q31.1, en la que fueron encontrados varios genes candidatos como *TNFAIP6* (TNF-α-proteína inducida 6), *ACVR1* (receptor A de la activita), *FAP* (proteína α de activación de fibroblastos), *ATF2* (activador de factor de transcripción 2), *ITGA4* (integrina α 4), *FRZB* (proteína de secreción frizzled-relacionada 3) y *ITGAV* (integrina α 5). A continuación llevaron a cabo estudios de asociación caso-control utilizando microsatélites y nsSNPs. Encontraron asociación de los nsSNPs *FRZB* y susceptibilidad a la OA de la cadera en mujeres. Estudios funcionales pusieron de manifiesto que estos dos nsSNPs reducían la capacidad de *FRZB* para antagonizar la señal de Wnt (Loughlin J et al, 2004). Estudios posteriores realizados en poblaciones caucásicas independientes confirmaron el papel de *FRZB* en la OA, aunque también se puso de manifiesto que este efecto es dependiente del sexo y de la articulación. (Min JL et al, 2005) (Lane NE et al, 2006) (Rodriguez-Lopez J et al, 2007).

Otros genes candidato estudiados por el grupo del Dr. John Loughlin, fueron los de *LRP5* (11q13.2), *BMP5* y *COL9A1* (6p12.1) los cuales están localizados en las regiones de ligamiento 11q13.2 y 6p12.1. Tanto en el estudio llevado a cabo en *LRP5* como en *BMP5* y *COL9A1* los resultados de asociación fueron negativos (Southam L et al, 2003). En el caso de *BMP5* y *COL9A1,* se genotiparon además una gran cantidad de marcadores genéticos dentro del intervalo de ligamiento 6p12.1, con una distancia promedio muy pequeña, del orden de 0.36 cM, confirmando el ligamiento encontrado con anterioridad (LOD score 4.8). Posteriormente se analizaron los mismos marcadores para testar la asociación, encontrándose que un marcador situado en el intrón 1 de *BMP5* se encontraba asociado ($P < 0.05$) al igual que otros dos situados en la región *downstream* (Southam L et al, 2004). Estudios en ratones demostraron que la regulación de este gen es compleja y probablemente implique numerosos elementos reguladores en *cis* que pueden estar situados a gran distancia del gen (DiLeone RJ et al, 2000). Esto parece indicar que quizá los SNPs de susceptibilidad se encuentren en estas regiones reguladoras y no en los nsSNPs estudiados, siendo los SNPs reguladores los que expliquen el ligamiento. Hasta el momento no se ha encontrado ningún polimorfismo susceptible de ser estudiado en estas zonas por lo que no se puede concluir nada todavía.

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En conclusión, es posible afirmar que en estos últimos años se ha hecho un considerable progreso en el estudio y conocimiento de las bases genéticas y moleculares de la OA primaria. Se puede decir que esta es una enfermedad compleja (poligénica) porque en su desarrollo parece que están envueltos muchos genes con un efecto débil y su herencia no sigue las reglas básicas de la genética mendeliana. También se ha demostrado que la OA posee una gran heterogeneidad, ya que se ha visto que las asociaciones genéticas encontradas son dependientes de la articulación afectada, del sexo y de la etnicidad (Nakamura T et al, 2007) (Loughlin J et al, 2004) (Min JL et al, 2005) (Lane NE et al, 2006) (Rodriguez-Lopez J et al, 2006) (Miyamoto Y et al, 2007) (Southam L et al, 2007). Para intentar evitar la posible confusión derivada de estas dos importantes características, los estudios genéticos de la OA deben estar constituidos por un gran número de muestras de ambos sexos con el objetivo de aumentar la potencia estadística, distintos fenotipos y que estos estén bien definidos, e incluir diferentes poblaciones en las que se confirmen los resultados obtenidos.

El cartílago articular

El cartílago articular es un tejido que posee dos componentes fundamentales: los condrocitos y la ECM. Los condrocitos representan el único tipo celular del tejido. Están muy especializados y son poco numerosos. Los condrocitos se encuentran embebidos en una abundante ECM muy hidratada que es sintetizada y mantenida por ellos mismos y que es responsable de las características especiales del tejido (Poole AR et al, 2001).
El cartílago de las articulaciones sinoviales, limita por una cara con la cavidad articular, y por la otra, con el hueso subcondral a través de una capa fina de cartílago calcificado. La organización de la ECM y de los condrocitos son ligeramente diferentes en el cartílago articular inmaduro y en el maduro. En los individuos jóvenes, el grosor del cartílago articular es menor y la distribución de las células en la ECM es más aleatoria. Cuando el tejido madura, se produce una gran anisotropía en el tejido, estando las células y la matriz organizadas en capas bastante bien definidas. Estos cambios vienen acompañados de una mejora en las propiedades biomecánicas del cartílago que se convierte en un tejido de una gran dureza y resistencia a las fuerzas compresivas y de cizalla. (Mow V et al, 1987). Las zonas en las que se divide el cartílago articular, de la cavidad articular al hueso subcondral, son: zona superficial (tangencial), zona media (transicional), zona profunda (radial) y zona de cartílago calcificado. **(Figura 2)** Estas zonas no son equivalentes ya que presentan diferencias en las propiedades biomecánicas derivadas de la distinta composición de la ECM y en el número y organización de los

condrocitos. Los condrocitos de las distintas zonas del cartílago poseen distintas características bioquímicas (metabolismo, estabilidad fenotípica y respuesta a interleuquina 1-α) lo que le confiere a cada zona su identidad (Aydelotte MB et al, 1992).

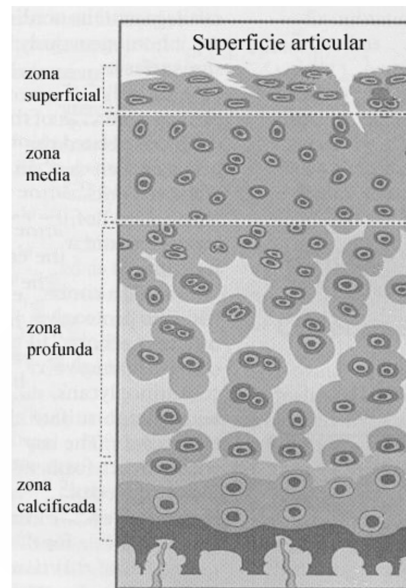

Figura 2: Representación esquemática de cartílago articular adulto mostrando las distintas zonas en las que se subdivide el mismo. En la superficie se representan erosiones típicas de un cartílago artrósico.

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Componentes del cartílago

La matriz extracelular

La ECM del cartílago es sintetizada por los condrocitos. Comparada con otros tejidos, la relación "volumen ECM/volumen células" es extremadamente alta.

El principal componente de la ECM es el agua que constituye alrededor del 60-80% del peso total. El 20-40% restante del peso húmedo del tejido está compuesto por dos componentes macromoleculares principales: el colágeno y los proteoglicanos. El colágeno constituye el 60% del peso seco del tejido y está formado básicamente por colágeno tipo II, con menos cantidad de otros tipos de colágeno como el colágeno tipo IX, XI, III, V, VI, X, XII y XIV. Los proteoglicanos constituyen aproximadamente el 40% del peso seco del tejido, siendo el agrecano el proteoglicano más abundante. El agrecano se encuentra formando largos agregados

con moléculas de ácido hialurónico (HA) en una organización típica en forma de escobilla (Aurich M et al, 2005). Los colágenos tipo II, IX y XI, se encuentran formando una red tridimensional que sirve de soporte estructural para la matriz, en la forma de una maraña no homogénea de fibras rodeada por una solución altamente concentrada del proteoglicano agrecano. El colágeno tipo VI, por otra parte, se encuentra formando una matriz microfibrilar en las proximidades de la superficie celular de los condrocitos (matriz pericelular) **(Figura 3)**

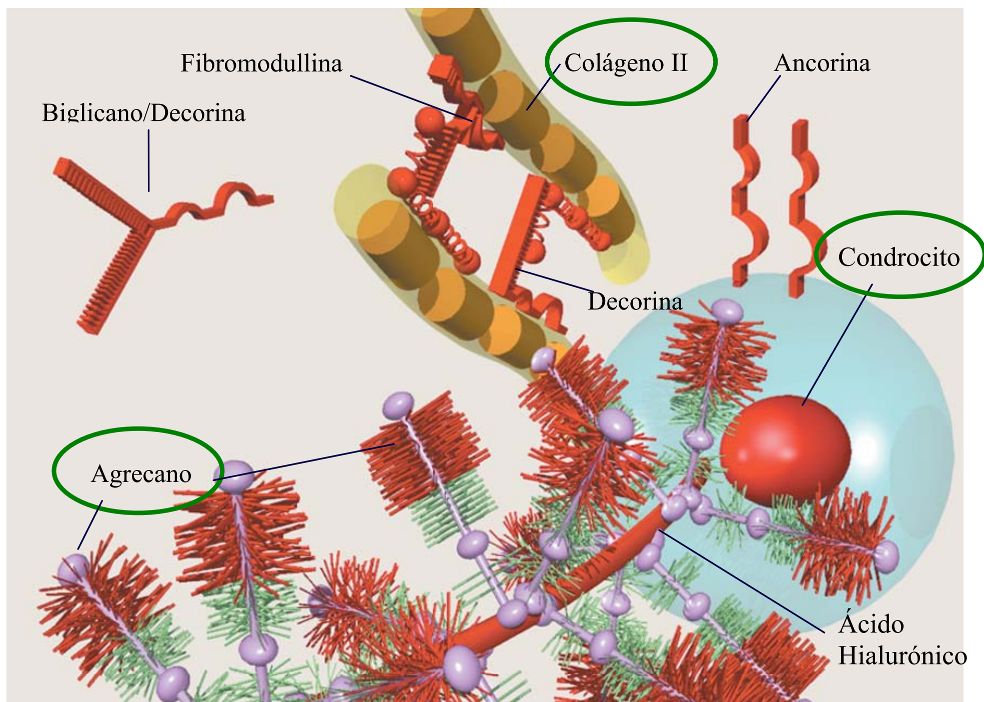

Figura 3: Representación esquemática de la matriz extracelular del cartílago

Los proteoglicanos son macromoléculas complejas constituidas por un núcleo proteico al que se encuentran unidas cadenas de glucosaminoglicanos, principalmente condroitín sulfato y keratán sulfato. **(Figura 4)** Casi la totalidad de los proteoglicanos que se encuentran en la ECM del cartílago articular está formada por agrecano, aunque pequeñas cantidades de otros proteoglicanos como los "proteoglicanos largos" (versicano), y los pequeños proteoglicanos pertenecientes a la familia de "proteoglicanos pequeños con repeticiones ricas en leucina" (asporina, biglicano y decorina), también pueden ser encontrados. Los proteoglicanos cumplen una función muy importante en la constitución fisiología del tejido ya que son las moléculas

responsables de la gran hidratación del mismo. Su carga neta negativa atrae a numerosos iones, con lo que se crea la presión osmótica necesaria para mantener las moléculas de agua en su interior. Esta característica acarrea importantes consecuencias en el comportamiento biomecánico del cartílago ya que es responsable de su gran resistencia a la compresión y su comportamiento viscoelástico (Hardingham TE et al, 1994) (Maroudas A et al, 1998).

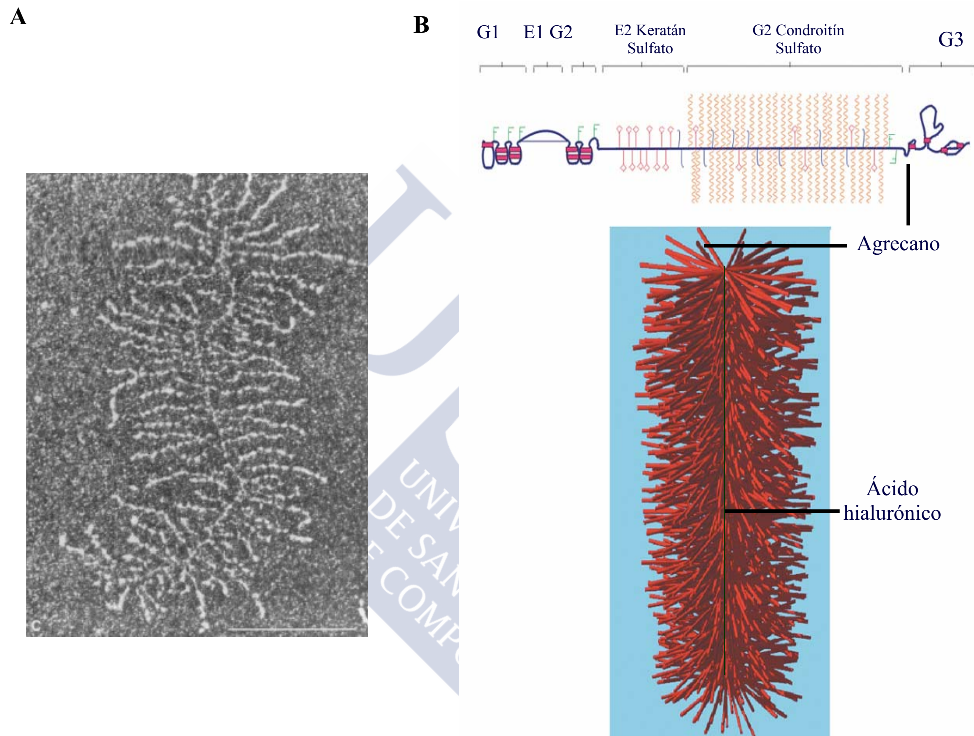

Figura 4: **(A)** Micrografía electrónica de un agregado macromolecular de ácido hialurónico y agrecano tal y como se encuentra en la matriz del cartílago. **(B)** Representación esquemática de la misma macromolécula. El agrecano se une al ácido hialurónico por su dominio G1 a través de una proteína de unión. Cadenas de polisacáridos cargados, como el Condroitín sulfato y Keratán sulfato, se encuentran unidas a los dominios E2 y G2 del agrecano.

Además del colágeno y los proteoglicanos existen un gran número de otros componentes de la ECM: Trombospondinas (TSPs), grupo al que pertenece la proteína de matriz oligomérica (COMP) (Adams JC et al, 2004); Matrilinas (Deak F et al, 1999); CILP (proteína de capa intermedia del cartílago), que fue identificada como un componente de la ECM del cartílago que se encuentra sobreexpresada en la OA (Lorenzo P et al, 2004), y se vio asociado con la enfermedad de disco lumbar (LDD) (Seki S et al, 2005). También se encuentran otras proteínas

como la proteína similar a la Chitinasa 3, la proteína de matriz Gla/MGP, la Cromomodulina, la Condrocalcina, y PARP. Estos otros componentes minoritarios de la matriz desempeñan distintas funciones como facilitar el ensamblaje de los componentes de la matriz, el mantenimiento de las propiedades mecánicas del tejido, el secuestro de de factores de crecimiento como TGF-β y proteasas de ECM en compartimentos específicos de la matriz (Kizawa H et al, 2005), e interacción con los condrocitos, por lo que se postula que puedan intervenir también en la regulación de actividades celulares.

El condrocito

El componente celular del cartílago está constituido únicamente por los condrocitos (Archer CW et al, 2003). La morfología del condrocito varía desde redondeada o poligonal en las zonas media y profunda del cartílago, hasta una forma discoidal más o menos aplanada que se puede encontrar en las zonas superficiales del cartílago. En el tejido humano adulto, los condrocitos ocupan tan solo el 2% del volumen total. Desde un punto de vista embriológico los condrocitos de los elementos articulares son derivados del mesodermo y los del esqueleto facial derivan directamente de la cresta neural. Los primeros condrocitos en diferenciarse surgen de una aglomeración de células mesenquimáticas en los sitios en los que se van a originar los elementos esqueléticos. En este proceso de diferenciación se produce la expresión de genes específicos del tejido como Sox9, L-Sox5, Sox6 y el gen del colágeno tipoIIA (Hall BK et al, 2000) (Hall BK et al, 1992). A continuación, los condrocitos que van a formar parte del cartílago articular persisten en ese estado de diferenciación mientras que los condrocitos de la zona de crecimiento epifisiario se transforman en condrocitos hipertróficos. Estos condrocitos hipertróficos situados dentro de la zona epifisiaria llevan a cabo el crecimiento del tejido mediante el incremento del número de células, síntesis de componentes de la ECM y el incremento del volumen celular en las últimas etapas de la diferenciación. El condrocito es una célula que posee los rasgos intracelulares característicos de una célula metabólicamente activa (gran retículo endoplásmico rugoso y aparato de Golgi), debido a que su papel esencialmente es el de síntesis y degradación de los componentes de la ECM en la cual se encuentran embebidos. Una muestra de esto es que el mapa proteómico de los condrocitos humanos muestra que el 26% de las proteínas sintetizadas están implicadas en la organización celular, el 16% en la producción de energía, el 14% en el tráfico proteico, el 12% en el metabolismo, y el 12% en respuesta a estrés (Ruiz-Romero C et al, 2005) **(Fig 5)**

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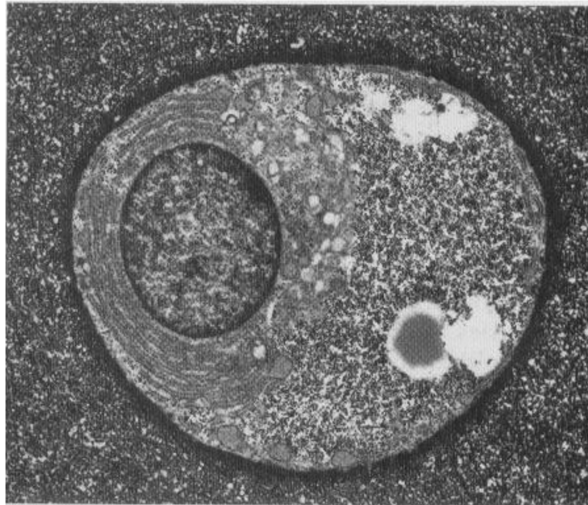

Figura 5: Micrografía electrónica de un condrocito. La forma de la célula puede variar de aplanada o discoide cerca de la zona superficial del cartílago articular, a una forma redondeada en las zonas media y profunda. (Tomado de Archer and Francis-West, Int. J. Biochem. Cell Biol 35:404-404, 2003)

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Función del cartílago articular

La función del cartílago articular es la de proporcionar una superficie de contacto que facilita el movimiento de los componentes de la articulación. Se caracteriza por ser un tejido que produce una muy baja fricción, sufre un mínimo desgaste y tiene una gran resistencia a las fuerzas de tensión y compresión. Estas propiedades se derivan de la particular composición y estructura de los constituyentes del cartílago y de su distribución espacial en el tejido.

Desde un punto de vista puramente biomecánico, el cartílago articular puede ser entendido como un material fibroso, poroso y permeable, el cual se encuentra saturado por un fluido intersticial, el agua (Mow VC et al, 1980). Esto hace que responda a las tensiones y la carga de una manera viscoelástica (dependiente del tiempo), anisótropa (dependiente de la dirección) y no lineal (dependiente de la magnitud de la tensión). El agua es el principal componente del cartílago. Esto le proporciona un gran apoyo mecánico al tejido en respuesta a una carga aplicada permitiéndole soportar grandes presiones mediante mecanismos de presurización (manteniendo la presión constante en el tejido) y disipación de la energía (Mow VC et al, 1980). De esta manera el agua absorbería la práctica totalidad de la carga (casi el 90%) y se reduciría el estrés mecánico sobre la fase sólida de la ECM (Park S et al, 2003). El segundo componente más abundante del cartílago, el colágeno, se encuentra en forma de fibras lo que le proporciona

al cartílago articular la propiedad de resistencia a la tensión. Las fibras de colágeno no se encuentran distribuidas de una manera homogénea en el tejido sino que su disposición varía dependiendo de la zona (en profundidad) del cartílago, lo que hace que las propiedades derivadas de las fibras de colágeno tampoco sean iguales en todas las zonas (Kempson GE et al, 1973) (Roth V et al, 1980). Las propiedades biomecánicas del cartílago van decayendo conforme aumenta la edad del individuo, especialmente una vez que se han sobrepasado los 30 años lo que conlleva un aumento de la susceptibilidad a sufrir OA (Kempson GE 1991). Los cambios en las propiedades biomecánicas del cartílago relacionados con la edad se cree que son debidos en parte a la acumulación de los denominados "productos finales avanzados de glicosilación no enzimática" (AGEs), que resultan de la reacción espontánea de azúcares reducidos con las proteínas de la ECM (Chen AC et al, 2002) (Reiser KM et al, 1998).

El fluido sinovial también juega un papel significativo en la biomecánica del tejido articular ya que proporciona lubricación a la articulación constituyendo una capa fina de gran viscosidad que minimiza el contacto cartílago-cartílago bajo condiciones de compresión (Hou JS et al, 1992). Además, el fluido sinovial sirve como una fuente de moléculas lubrificantes y de nutrientes para el cartílago. Dentro de las moléculas lubrificantes destacan la lubricina y la "proteína de zona superficial" (SZP), que son adsorbidas por la superficie del cartílago y ayudan a reducir la fricción en las situaciones en las que se produce un contacto real entre las dos superficies cartilaginosas (Swann DA et al, 1985). La importancia de este tipo de moléculas ha sido demostrada ya que, por ejemplo, una pérdida de la función de SZP está asociada con la enfermedad de "fallo articular juvenil". El aporte de nutrientes al cartílago es también una función muy importante realizada por la sinovia ya que el cartílago es un tejido avascular, aneural y alinfático, por lo que se encuentra aislado del torrente nutricional del organismo (Davies DV et al, 1962).

Regulación del metabolismo del cartílago

En el cartílago existe una gran regulación de la síntesis y degradación de los componentes de la ECM. Varios factores participan activamente en esta regulación, influyendo directamente en la bioquímica del condrocito. En condiciones fisiológicas, los componentes de la ECM del cartílago se encuentran en un estado de renovación lenta pero continua, mantenida por un equilibrio homeostático entre los procesos catabólicos y anabólicos del condrocito. Esta actividad se encuentra controlada por la interacción de factores genéticos y ambientales, entre los que se incluyen mediadores solubles como hormonas y citoquinas (IL-1, IL-6 o TNF α) y la carga mecánica a la que esté sometida la articulación (Helminen HJ et al, 1983). De hecho

varios estudios han mostrado que la carga mecánica a la que está sometida la articulación es un factor crítico en la regulación de la actividad del condrocito *in vivo* (Guilak F et al, 1994). Los mecanismos celulares a través de los cuales la carga mecánica es convertida en una señal bioquímica aún distan de ser totalmente entendidos. En general la evidencia que existe hasta el momento sugiere que los condrocitos pueden transducir las señales mecánicas en señales bioquímicas a través de varias rutas de señalización intracelulares e intercelulares, incluyendo las vías clásicas de segundos mensajeros como el AMP cíclico (cAMP), inositol trifosfato o iones de calcio (Stockwell RA 1978). Se han llevado a cabo numerosos experimentos para intentar comprender este proceso siendo los explantes de cartílago sometidos a diferentes cargas (comprensión, tensión, cizalla, presión osmótica o hidroestática,…), los modelos que han generado mejores resultados (Guilak F et al, 1994). El consenso general es que la carga mecánica es necesaria para el mantenimiento de la salud del tejido articular. Existen evidencias de que la compresión estática y continuada, así como la inmovilización de la articulación, inhiben la síntesis de los compuestos de la ECM, mientras que cargas cíclicas e intermitentes activan el metabolismo del condrocito (Gray ML et a 1988l) (Sah RL et al, 1991) (Guilak F et al, 1994) (Torzilli PA et al, 1997). Además estas respuestas se dan en un rango muy amplio de magnitudes de carga y exhiben una dependencia de dosis, ya que si la carga es excesiva (durante largo tiempo o de una gran magnitud) tiene un efecto deletéreo, provocando la muerte de los condrocitos, disrupción del tejido e hinchazón (Chen CT et al, 2001) (Farquhar T et al, 1996). Estos resultados demuestran que el metabolismo basal del condrocito *in vivo* necesita estar activado, y no funciona correctamente en un estado de no estimulación e independencia de señales externas.

Degradación de la matriz del cartílago: Proteasas de matriz extracelular

El papel que juegan en el proceso de degradación de la matriz las proteasas de ECM es muy relevante y ha sido objeto de estudio intensivo. Estas proteasas son sintetizadas por los condrocitos como parte del proceso normal de remodelado de la ECM. La alteración de la regulación de la expresión y activación de estas proteasas es uno de los mecanismos propuestos para explicar el desplazamiento del equilibrio entre síntesis y degradación de los componentes de la matriz que se produce en la OA y la AR. Conocer las causas, tanto genéticas como bioquímicas que producen este desequilibrio y las proteasas que se encuentran alteradas, ayudaría en gran medida a diseñar fármacos para inhibir su actividad.

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El grupo de las proteasas de ECM es muy heterogéneo. Aún así se pueden encontrar algunas características estructurales y funcionales comunes que nos permiten agrupar a las proteasas de ECM en seis tipos dependiendo de su mecanismo catalítico: Glutamil proteasas, Treonín proteasas, Cisteín proteasas, Aspartil proteasas, Serín proteasas y Metaloproteasas. Numerosas proteasas de los distintos grupos han sido asociadas a patología articular, pero las aspartil proteasas, las cisteín proteasas, las serín proteasas y, en especial, las metaloproteasas (ADAMTSs y MMPs), son las que parecen tener un protagonismo mayor (Brinckerhoff CE et al, 2002).

Metaloproteasas

Las metaloproteasas, también conocidas como metizincinas, son, con gran diferencia, los enzimas considerados más importantes en el cartílago artrósico. Su importancia se deriva del hecho de que los dos mayores componentes de la ECM, el colágeno tipo II y el agrecano, son degradados por componentes de este grupo. Las metaloproteasas son una superfamilia de enzimas caracterizados por poseer en su sitio activo un ion metálico (generalmente Zinc) que es fundamental para su función proteolítica, así como la existencia de una metionina altamente conservada que se encuentra situada muy próxima al sitio activo.

Las metaloproteasas se dividen a su vez en cuatro grupos bien diferenciados: Las astacinas (zinc-endopeptidasa del cangrejo de río *Astacus astacus*) (Stocker W et al, 1991), las adamlisinas (zinc endopeptidasa del veneno de serpientes de la familia Crotalidae, ADAMS y ADAMTSs) (Shannon JD et al, 1989), las serralisinas (zinc-endopeptidasas secretadas por algunas bacterias del género Serratia (Nakahama K et al, 1986) y las metaloproteasas de matriz extracelular (MMPs)(zinc endopeptidasas que degradan componentes de la ECM) (Nagase H et al, 1992). Las familias más importantes con actividad en el cartílago articular son dos, las metaloproteasas de matriz extracelular (MMPs) y las adamlisinas (ADAMs y ADAMTS)

Metaloproteasas de matriz extracelular, MMPs

Los numerosos miembros de la familia de las MMPs son capaces de degradar un gran número de componentes de la ECM del cartílago. Hasta el momento se han detectado 24 genes distintos codificadores de MMPs en el genoma humano. Las MMPs han sido relacionadas sobre todo con cáncer y enfermedades cardiovasculares (López-Otín C et al, 2007) (Phatharajaree W, et al, 2007), aunque su importancia en la degradación de la ECM les haya hecho que ocupen un lugar destacado como candidatos a explicar las lesiones que ocurren en la OA y la AR. Dentro de las

MMPs, podemos distinguir seis subfamilias distintas: colagenasas, estromalisinas, gelatinasas, matrilisinas, MMP de membrana (MT-MMP) y otras MMPs. **(Tabla1)**.
Con respecto al cartílago, la MMP-1 (colagenasa intersticial), MMP-2 (72 kDa gelatinasa), MMP-3 (estromalisina), MMP-7 (Matrilisina), MMP-8 (colagenasa de neutrófilos), MMP-9 (95-kDa gelatinasa) y MMP-13 (colagenasa-3), son considerados los más importantes pues participan en la degradación del agrecano y el colágeno tipo II (Lee ER et al, 1998) (Mitchell PG et al, 1996). Los sistemas encargados de la regulación de las MMPs actúan fundamentalmente a tres niveles: regulación de la expresión, activación intra o extracelular de zimógenos, e inhibición por TIMPs (inhibidores endógenos específicos). El nivel de regulación transcripcional es el más importante pero también su complejidad es muy alta debido a que existen muchas variables a tener en cuenta (Crawford H. C et al, 1996). Hay diversos trabajos en los que se ha puesto de manifiesto la existencia de una estructura común de los promotores de las MMPs con la presencia de elementos reguladores de la expresión como la caja TATA, el motivo AP-1 y elementos PEA3 además de otros (Jonat C et al, 1990) (Gaire M et al, 1994). Estos motivos son sitios consenso de unión tanto para factores de crecimiento (c-fos, c-jun, EGF y Ets) como para activadores tumorales (McDonnell S et al, 1994) (Crawford HC et al, 1994). Además se ha visto que la respuesta a estos elementos es distinta dependiendo de las secuencias promotoras que se encuentran flanqueándolas, lo que indica que existen otros elementos reguladores que determinan la expresión de éstas MMPs (Gaire M et al, 1994). Numerosos estudios han relacionado polimorfismos en estas regiones reguladoras con susceptibilidad a distintas enfermedades (Decock J et al, 2007) (Baig S et al, 2007) (Holla LI et al, 2006), destacando así la importancia de este nivel de regulación.
También influyen sobre los niveles de expresión de estas MMPs diversos factores como citoquinas proinflamatorias y los factores nucleares como NF-kappa-B (Westermarck J et al, 1999). Estos agentes pueden tener distintos efectos dependiendo de los tipos celulares en los que actúan, con lo que se añade un nivel más de complejidad adicional a la regulación transcripcional de las MMPs (Bond M et al, 1998).
Post-traduccionalmente, el primer nivel de regulación es el de la activación. Las MMPs son secretadas como enzimas inactivas o zimógenos. En esta forma su protodominio se encuentra plegado de manera que una cisteína situada en el mismo interacciona con el zinc del sitio activo formando un enlace tiol, lo que se denomina "*cysteine switch*". Al perder este protodominio el sitio activo queda libre y la MMP se transforma en un enzima activo. Este proceso se conoce con el nombre de activación y normalmente es un mecanismo autocatalítico (Nagase H et al, 1997).

Recientemente se han verificado nuevos mecanismos de activación de las MMPs en las que participan otras MMPs como la MT1-MMP (metaloproteasa de membrana 1) (Knauper V et al, 1996), proteínas como RECK y TFPI-2, y el sistema plasminógeno-plasmina (Oh J et al, 2001) (Herman MP et al, 2001).El último nivel de regulación de las MMPs es la inhibición por TIMPs (Inhibidores Tisulares de Metaloproteasas). Los TIMPs son inhibidores endógenos específicos que ejercen parte de su acción inhibidora sobre miembros de esta familia aunque también actúan sobre otras proteasas (Brew K et al, 2000).

Se han caracterizado 4 miembros de la familia llamados TIMP-1, TIMP-2 TIMP-3 y TIMP-4. TIMP-1 y TIMP-2 son capaces de inhibir la actividad de todas las MMPs conocidas en una unión estiquiométrica 1:1, por lo que se puede decir que su actividad no es muy específica (Sato H et al, 1992). Los TIMP modulan la actividad de las MMPs por unión directa al sitio activo o la forma precursora inactiva de la MMP. Con respecto a la regulación de los TIMP cabe destacar que factores activadores del catabolismo de la ECM como la interleucina-1 (IL-1) y el factor de necrosis tumoral-α (TNF-α) inhiben su expresión en condrocitos (Lotz M et al, 1991). Se ha propuesto que la regulación fina de la renovación de la ECM en el cartílago depende del balance entre la actividad de MMPs y TIMPs, y alteraciones en este sistema de equilibrio han sido relacionados con niveles excesivos de degradación de la ECM similares a los observados en la OA y la RA (Cawston TE et al, 1995).

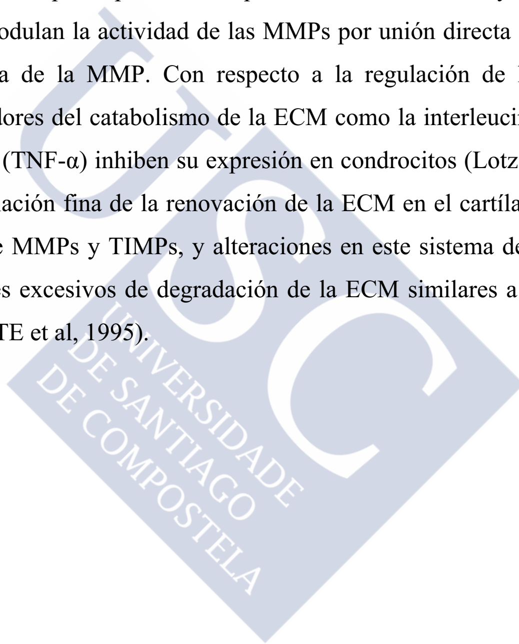
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Tabla 1: Miembros de la familia de las Metaloproteasas de matriz extracelular (MMPs), clasificadas en seis grupos de acuerdo con su organización en dominios

Grupo	Enzima	MMP	Sustrato in vivo
Colagenasas	Colagenasa-1	MMP-1	Colágeno (tipo I, II, III, VII, VIII, X y XI)
	Colagenasa-2	MMP-8	Colágeno (tipo I, II y III), agrecano
	Colagenasa-3	MMP-13	Colágeno (tipoI, II, III, IV, VI, IX, X y XI)
	Colagenasa-4	MMP-18	Colágeno tipo I (rata)
Estromalisinas	Estromalisina-1	MMP-3	Colágeno telopéptidos, componentes de la matriz (gelatina, elastina, fibronectina, vitronectina, laminina, estactina, perlecano, ...)
	Estromalisina-2	MMP-10	
	Estromalisina-3	MMP-11	
Matrilisinas	Matrilisinas –1	MMP-7	Colágeno (tipo I y IV), componentes de la matriz.
	Matrilisina-2	MMP-26	
Gelatinasas	Gelatinasa-A	MMP-2	Colágeno, componentes de la matriz, Citoquinas proinflamatorias
	Gelatinasa-B	MMP-9	Colágenos, componentes de la matriz
MT-MMPs	MT1-MMP	MMP-14	Colágenos componentes de la matriz, Otras proteasas (activación)
	MT2-MMP	MMP-15	
	MT3-MMP	MMP-16	
	MT5-MMP	MMP-24	
	MT4-MMP	MMP-17	
	MT6-MMP	MMP-25	
Otras MMPs	Metaloestatasa	MMP-12	Colágenos, componentes de la matriz
	-------	MMP-19	Colágenos componentes de la matriz
	Enamelisina	MMP-20	Agrecano, amelogenina.
	X-MMP(xenopus)	MMP-21	Gelatina
	CA-MMP	MMP-23	Gelatina
	CMMP (gallus)	MMP-27	Gelatina
	Epilisina	MMP-28	desconocido

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Familia ADAMTS

Las ADAMTS son enzimas con múltiples dominios que poseen una serie de características conservadas entre los distintos miembros de la familia como son la posesión de un péptido señal y un protodominio, un sitio catalítico con un átomo de zinc unido al sitio activo, una región similar a la desintegrina, una zona de repeticiónes de trombospondina-1 (TSR), un dominio rico en cisteína, y una región "*spacer"* libre de cisteína que en muchos miembros de la familia es seguida de nuevo por otra región TSR y un dominio C-terminal (Apte SS et al, 2004). Las ADAMTS-1,-4, -5, -9, y -15 poseen la capacidad de degradar el agrecano cortándolo en el sitio de corte exclusivo de agrecanasas situado entre los dominios G1 y G2 de la molécula (Glu^{373}–Ala^{374}) (**Figura 6**).

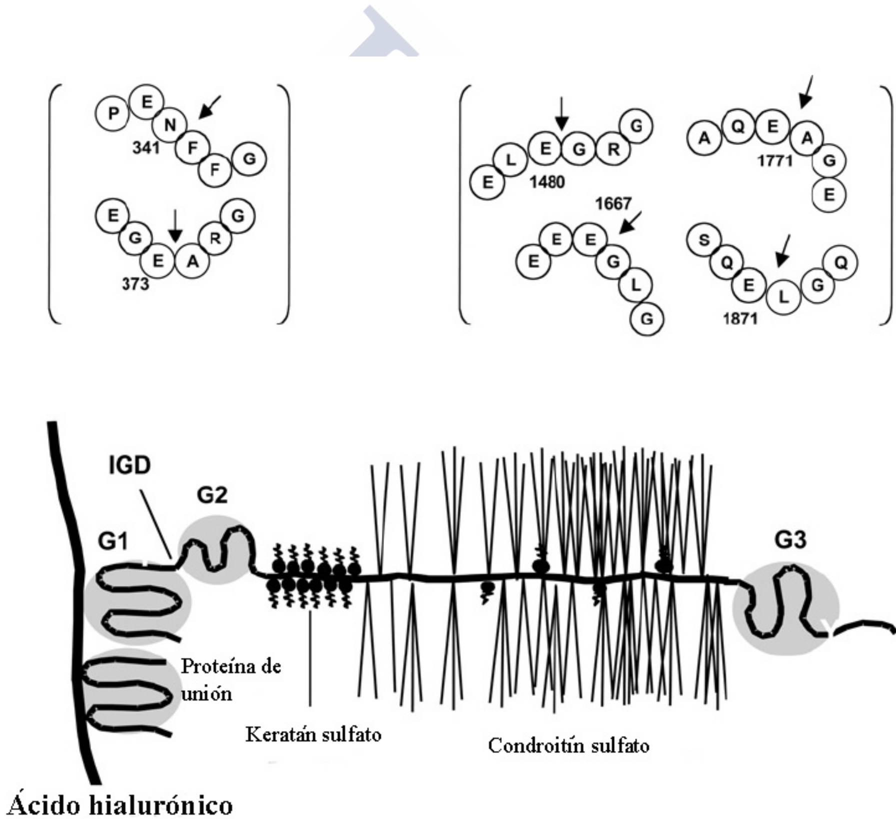

Figura 6: Representación esquemática de la molécula de agrecano y sus sitios de corte. El agrecano tiene dos sitios de corte situados en el dominio G1/G2 IGD. El sitio de corte Asn^{341}–Phe^{342} es el sitio de corte principal de las MMPs, mientras que el Glu^{373}–Ala^{374} es el principal sitio de corte de las agrecanasas. También existen otros cuatro sitios de corte más en la zona rica en condroitina sulfato en los que al menos se sabe que pueden cortar ADAMTS-4 y -5. Las flechas negras indican los sitios de corte. (Tomado de 169 Porter S et al,. The ADAMTS metalloproteinases. Biochem J. 2005 Feb 15; 386(Pt 1):15-27. Review

Diferentes experimentos han puesto de manifiesto que las principales agrecanasas *in vivo* son ADAMTS-4 y -5 (Porter S et al, 2005) (Nagase H, 2003). Estudios de expresión en cartílago de pacientes con OA, mostraron que ADAMTS-1, -4, -5, -9 y -15, las cuales son todas agrecanasas, se encontraban disminuidas, mientras que ADAMTS-2, -12, -14, y -16, las cuales participan principalmente en la ruta anabólica, estaban aumentadas en comparación con los niveles obtenidos en cartílago control (Kevorkian L et al, 2004). El cartílago examinado en este experimento se corresponde con fases tardías de la OA por lo que no es representativo de la regulación de esos genes en fases tempranas de la OA, que es precisamente donde se produce la mayor degradación del agrecano.

Experimentos con ratones knockout para ADAMTS-5 y ADAMTS-4, han puesto de manifiesto que la ADAMTS-5 es la principal agrecanasa *in vivo* (Glasson SS et al, 2005) (Stanton H et al, 2005). En estos experimentos, usando modelos murinos de OA y RA inflamatoria respectivamente,se demostró que en los ratones knockout para ADAMTS-5, pero no aquellos para ADAMTS-4, se producía una disminución de la degradación de agrecanos de la ECM. Como consecuencia, los ratones knockout de ADAMTS-5 desarrollaban una menor erosión articular y posterior lesión de la articulación que los ratones control. Estos resultados han sido muy importantes, pues ponen de manifiesto la importancia de la actividad de un sólo enzima en el mantenimiento de la integridad del tejido articular y abren las puertas a una posible diana terapéutica en la OA o la RA. En todo caso, la importancia de este enzima en humanos no es del todo clara y son necesarios más estudios para una correcta comprensión de su función.

Algunos genes de ADAMTS son inducidos por factores de crecimiento, citoquinas inflamatorias y hormonas, siendo una regulación muy específica: Así, por ejemplo, TGF-β induce la expresión de *ADAMTS-4* pero no de *ADAMTS-5* en sinoviocitos tipo fibroblasto (Kashiwagi M et al, 2004) y la hormona triiodothyronine (TE) produce la sobreexpresión de *ADAMTS-5* pero no de *ADAMTS-4* en la placa de crecimiento del cartílago durante la osificación endocondral. (Makihira S et al, 2003).

La regulación de las ADAMTS por TIMPs también es muy importante. Su labor es mucho más selectiva que la vista en las MMPs, donde un solo TIMP regulaba la actividad de un gran espectro de enzimas. En el caso de las ADAMTS, ADAMTS-4 y -5 son inhibidas por TIMP-3, pero no por TIMP-2, -1 o -4, y ADAMTS-1 es inhibida por TIMP-3 y TIMP-2, pero no por TIMP-1 o -4 (Kashiwagi M et al, 2001). Esta selectividad sugiere que variaciones específicas en los niveles de expresión y activación de los TIMPs pueden ser muy importantes a la hora de estudiar las ADAMTSs en relación con la OA.

Miembros de La familia de ADAMTS proteasas han sido relacionados con enfermedades cardiovasculares, articulares y con cáncer. Estudios en cáncer de mama y cáncer de próstata han demostrado que los niveles de expresión de 19 ADAMTS se encuentran alterados en estas situaciones patológicas (S. Porter, A.C.P. Riddick, K.K. Sethia, R.Y. Ball and D.R. Edwards, trabajo no publicado). Hasta la fecha, no han sido descritos polimorfismos en elementos genéticos reguladores asociados a ninguna enfermedad, ya que la mayoría de los experimentos han sido realizados con respecto a su activación e inactivación y a los mecanismos implicados en estos procesos (Malemud CJ 2006).

Familia ADAMs

Las ADAMs son una familia de proteínas transmembra que comparten una estructura de dominios característica que les confiere un comportamiento modular. En su región extracelular poseen actividad proteolítica y de unión a receptor, mientras que en su parte citoplasmática tienen capacidad de interacción con proteínas transductoras de señal. La ADAMs son similares a las MMPs por la posesión de un dominio metaloproteasa, pero se diferencian claramente de estas porque en su estructura existe además un dominio desintegrina específico de unión a receptor que permite adhesiones celulares mediadas por integrina (Seals DF et al, 2003). Además, no todas las ADAMs tienen actividad proteolítica ya que aproximadamente un 50% de las ADAMs conocidas no poseen los aminoácidos críticos para la actividad catalítica (White JM 2003). Las ADAMs han sido relacionadas con el control de la fusión de membranas, liberación de citoquinas y factores de crecimiento, y la migración celular, así como con diferentes procesos como el desarrollo muscular, la fertilización y la determinación del destino celular en el desarrollo. En determinados proceso patológicos como la inflamación y el cáncer también se ha visto que están implicados miembros de la familia ADAM (Kheradmand F et al, 2002). El posible papel de las ADAMs en patología articular se debe a su capacidad de degradar componentes de la ECM. Diferentes trabajos *in vitro* han mostrado la actividad catalítica de los miembros de esta familia frente a colágeno tipo IV (ADAM-10 y ADAM-15) (Millichip MI et al, 1998), gelatinas (ADAM-15) (Martin J et al, 2002) y fibronectina (ADAM-13) (Alfandari D et al, 2001). Además, se observó que los niveles de expresión del gen *ADAM-12,* también conocida como meltrina α, están aumentados en el cartílago de pacientes con OA (Valdes AM et al, 2004). En este mismo trabajo también se realizó un estudio de asociación encontrando que este gen *ADAM-12* está asociado con la prevalencia y la progresión de la OA. ADAM-12 es una metaloproteasa que media interacciones y respuestas celulares (Tian BL et al, 2002). Aparentemente, participa en la regulación de la formación de células gigantes derivadas de

macrófagos, posiblemente mediando los efectos en la fusión celular de la 1.25-hidroxivitamina D_3 la cual, a su vez, ha sido relacionada varias veces con patología articular (Felson DT et al, 2007). Además, se ha visto que la adición de mRNA antisentido de *ADAM-12* a células mononucleares precursoras de osteoclastos provoca una reducción de un 50% en la formación de células gigantes (Abe E et al, 1999).

La regulación de las ADAMs es similar a la que hemos descrito para las otras metaloproteasas. La expresión genética está regulada por factores extracelulares como citoquinas y factores de crecimiento (Seals DF et al, 2003). Por ejemplo, *ADAM-17* está sobreexpresada en procesos inflamatorios y en condiciones de deprivación de oxígeno y glucosa (Hurtado O et al, 2002). Las ADAMs, al igual que las MMPs, son sintetizadas como enzimas inactivos siendo necesaria para su activación la escisión de su protodominio por una convertasa (Seals DF et al, 2003). Como último nivel de regulación, la actividad proteolítica de algunas ADAMs también puede estar regulada por TIMPs. Experimentos realizados en ratones knockout para TIMP-3 han puesto de manifiesto un incremento de la actividad de ADAM-17 (Mohammed FF et al, 2004).

Serín proteasas

Estructuralmente relacionadas con la tripsina, las enzimas clasificadas en este grupo son activas a pH neutro y poseen un residuo de serina en su centro catalítico. Parte de sus componentes son miembros del sistema Plasminógeno-plasmina (PLAT, u-PA, PAI, Plasminógeno, Plasmina). La plasmina proviene de su precursor inactivo el plasminógeno y su función principal es la de degradar la fibrina la cual ha sido relacionada sucesivas veces con la patología articular (Cook AD et al, 2002) (Busso N et al, 1997). De una manera indirecta las serín proteasas pueden ser activadores importantes de las proMMPs a través de la acción propeptidasa de la plasmina, aunque también puede actuar degradando directamente componentes de la ECM como los proteoglicanos o el colágeno tipo IV y V. El plasminógeno es activado mediante corte por TPA o PLAT (tissue-type plasminogen activator) y uPA o PLAu (urokinase-type plasminogen activator). Inhibidores de estos enzimas, como PAI (inhibidor del activador del plasminógeno) son muy importantes a la hora de regular el funcionamiento de este sistema ya que por ejemplo se ha visto que la inactivación de serín proteasas, incluida la plasmina, provoca una inhibición de la activación de proMMP-1, proMMP-3 y proMMP9 en cultivos celulares de condrocitos de conejo (Hashimoto K et al, 1998). Otros miembros de las serín proteasas como la elastasa de PMN (elastasa de leucocitos polimorfonucleares) y la catepsina G, también pueden jugar un papel en la degradación de los componentes de la ECM del cartílago (Hashimoto K et al, 1996). La elastasa de PMN es capaz de degradar varios componentes de la ECM como la fibronectina,

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laminina, proteoglicanos y colágeno tipo IV, aunque su papel en la OA aún no ha sido muy estudiado.

Los integrantes de este grupo de proteasas han sido muy estudiados en relación con enfermedades cardiovasculares en donde han sido descubiertos varios polimorfismos genéticos que afectan a la regulación de la expresión de dichos genes (Ladenvall P et al, 2000) (Jern C et al, 1999). El estudio de estos polimorfismos en relación a la OA puede ser de gran importancia debido a que variaciones en la expresión de las serín proteasas pueden afectar del cartílago.

Cisteín proteasas

Caracterizadas por poseer un residuo de cisteína en su sitio catalítico, las cisteín proteasas participan en la degradación de la ECM en procesos de desarrollo, crecimiento y remodelación. También se ha visto que presentan una gran actividad degradativa en procesos patológicos y de envejecimiento.

Miembros de este grupo, como las catepsinas -B, -H, -K, -L y -S tienen niveles de expresión aumentados en hueso y cartílago, sobre todo en el caso de la catepsina-K (Morko JP et al, 2004). La catepsina-B puede degradar el agrecano en el sitio de corte descrito para las agrecanasas. Experimentos con conejos demostraron que la catepsina-B estaba sobreexpresada en el tejido sinovial en los primeros estadios del proceso degradativo del cartílago, y que la progresión de la OA estaba correlacionada con un aumento de su expresión en cartílago (Mehraban F et al, 1997). Las calpainas, otros miembros de las cisteín proteasas que se caracterizan por ser calcio-dependientes, también se han relacionado con patología articular. La m-calpaina se expresa en condrocitos articulares y sinoviocitos. Se ha visto que esta proteasas es la responsable de la producción de la mayor parte de las formas truncadas de agrecano encontradas en cartílago articular maduro in vivo (Oshita H et al, 2004).

Aspartil proteasas

En este grupo se engloban aquellas enzimas que se caracterizan por poseer al menos un residuo de ácido aspártico en su sitio catalítico y por ser activas a bajos pH. Su importancia en fenómenos relacionados con la OA no alcanza el nivel de los otros tres grupos, pero aún así miembros de esta familia como la catepsina-D pueden estar implicados en la degradación de la ECM del cartílago. Además sus niveles de expresión se han encontrado aumentados en el cartílago articular de pacientes con OA primaria con respecto a los niveles encontrados en controles sanos (Sapolsky AI et al, 1973).

Biosíntesis del cartílago

Diversos estudios han puesto de manifiesto que variaciones en los mecanismos que participan en el desarrollo y homeostasis del tejido articular pueden tener un papel muy importante en la etiología y patogénesis de la OA. Deficiencias en la síntesis de compuestos de la ECM o en la formación del tejido articular pueden tener el mismo efecto que un exceso de actividad catabólica. De hecho, una descompensación de efectos biomecánicos puede contribuir de manera indirecta a la activación de proteasas de ECM con la consecuente degradación de la matriz. Diversos trabajos han mostrado que varios genes como *FRZB, BMP-5, BMP-2* o *GDF-5,* que pertenecen a rutas de señalización celular que participan en la biosíntesis del cartílago, pueden ser posibles agentes implicados en la susceptibilidad a la OA. Desde un punto de vista anabólico hay que considerar dos aspectos en la biogénesis del tejido articular:

a- Síntesis de los compuestos de la ECM.
b- Condrogénesis y diseño de la arquitectura y organización de la articulación

Síntesis de los compuestos de la ECM
La ECM del cartílago es una vasta red interconectada de colágeno, proteoglicanos y glicoproteínas que es sintetizada, secretada, mantenida y degradada por los condrocitos. La OA puede surgir como consecuencia de cambios cualitativos en los colágenos o proteoglicanos de la matriz producto de una alteración de la biosíntesis. Se sabe que la ratio de síntesis de compuestos de la ECM decrece con la edad como efecto de una disminución de la actividad celular general, lo que contribuye al aumento de riesgo de padecer OA en la población adulta. Esta fase de síntesis es importante desde el punto de vista de la OA puesto que es la encargada de reponer las moléculas de la matriz que van siendo degradadas por la normal renovación de los compuestos de la ECM. Una disminución de la síntesis también provoca que la capacidad de recuperación de la ECM después de haber sufrido una lesión sea menor, con lo que se produce la debilitación del cartílago y el desarrollo de la OA. Existen múltiples factores que afectan a la síntesis de compuestos de la ECM. Así se ha encontrado que la inflamación o determinados medicamentos como la aspirina, esteroides y antiinflamatorios no esteroideos (NSAIDs) pueden alterar el metabolismo del cartílago (Brandt KD 1987) (Hugenberg ST et al, 1993). Por otra parte, moléculas como TGF-β y GDF5 también influyen sobre la síntesis de componentes de la ECM y diversos estudios han mostrado la importancia de estas moléculas en la susceptibilidad a la OA.

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Condrogénesis y diseño de la arquitectura y organización de la articulación

Los miembros esqueléticos de los vertebrados surgen por condensación de poblaciones celulares mesenquimáticas a partir de las cuales se diferenciarán las células condroprogenitoras que darán lugar posteriormente al cartílago y al hueso. El proceso de la condensación de células mesenquimáticas está dirigido por interacciones célula-célula y célula-matriz así como por la actuación de factores solubles. Existen evidencias tanto *in vitro* como *in vivo* de que este proceso de condensación celular es una etapa crítica en la diferenciación del tejido articular. Varios trabajos han mostrado que el grado de agregación celular está correlacionado con el nivel de condrogénesis (Evans MS et al, 1988) y se ha descrito que mutantes defectivos en la condensación de células mesenquimáticas poseen anormalidades esqueléticas en las extremidades (Hall BK et al, 1992). Posteriormente, las células condroprogenitoras sufren cambios en su morfología y en su metabolismo pasando a tener una forma esférica y a secretar colágeno tipo II y agrecano. Esto provoca la formación de una nueva ECM y la diferenciación progresiva de los condrocitos, lo que finalmente conducirá a la formación del cartílago maduro en los márgenes epifisiarios. Los condrocitos situados en el eje del miembro esquelético se organizan en placas de crecimiento y son finalmente remplazados por células óseas. Este proceso está regulado principalmente por tres factores interconectados: (1) moléculas condrogénicas (TGF-β, Wnt, BMPs y GDFs), (2) expresión de genes específicos de cartílago (genes E Sox), y (3) la ECM, la cual forma el substrato para la agregación de las células condrogénicas pero también tiene un papel importante en el secuestro y liberación de factores de diferenciación (Montero JA et al, 2007) (Church V et al, 2002). En este estadio también se crea el andamiaje en el que quedan establecidas la forma, posición y número de los elementos esqueléticos. El correcto diseño y organización de la articulación es un proceso de gran importancia ya que deficiencias en el mismo pueden afectar a la estabilidad de la misma lo que, pasado el tiempo, puede provocar el desarrollo de la OA. Todo este proceso está finamente regulado a nivel genético, a nivel de señalización celular y de interacciones célula-célula y célula-matriz. Además, también existe una modulación general que se da a nivel sistémico (Tuan RS 2004).

Una gran cantidad de factores específicos son expresados en estas primeras fases del desarrollo siendo los más importantes los miembros de la ruta Wnt (wingless), TGF-β y BMP/GDF (Enomoto-Iwamoto M et al, 2002) (Ameye L et al, 2002) (Merino R et al, 1999). Las proteínas Wnt son agentes señalizadores solubles que están implicados en una gran variedad de procesos del desarrollo. Ejercen su acción uniéndose a receptores Frizzled de la superficie celular (Hsieh JC et al, 1999). Varias proteínas Wnt, así como moléculas antagonistas como FRZB, se

expresan durante la esqueletogénesis. Wnt-3a, Wnt-5a, y Wnt-7a caracterizan el primer brote de la extremidad y se cree que regulan el patrón esquelético distal-proximal y dorsoventral (Kengaku M et al, 1998). La expresión de los Wnt está normalmente ausente en las condensaciones de células mesenquimáticas precondrogénicas, mientras que FRZB se expresa específicamente en ellas, lo que se correlaciona con el hallazgo que la supresión de de Wnt-1 y Wnt-7 inhibe la diferenciación de los condrocitos (Baranski M et al, 2000). Además, posteriormente se ha demostrado que FRZB es el modulador principal en la condrogénesis tanto *in vitro* e *in vivo.* Ya que se ha visto que una falta de expresión de FRZB mantiene a las células condrogénicas en cultivo en un estado no diferenciado y retarda gravemente la diferenciación de los condrocitos en modelos animales (Jones SE et al, 2002) (Enomoto-Iwamoto M et al, 2002).

Las BMPs forman un subgrupo dentro de la familia de moléculas señalizadotas TGF-β, representando casi un tercio de los miembros que componen esta familia. Las BMPs han sido consideradas como muy importantes en esqueletogénesis debido a su habilidad para inducir la formación de hueso subcondral cuando son implantadas subcutáneamente en ratas adultas (Rosen V et al, 1992). Se expresan de manera diferencial durante el desarrollo esquelético, y junto con las GDFs, poseen la habilidad de modular la condrogénesis y de controlar de manera secuencial diferentes aspectos del desarrollo esquelético (Roark EF et al, 1994). Cuatro miembros de las familias BMP/GDF, BMP-2, -4, GDF-5 y -6, se expresan durante el desarrollo temprano de las articulaciones (Zou H et al, 1997). Su papel es clave para el correcto desarrollo de los miembros esqueléticos. Diversos trabajos llevados a cabo con ratones knockout o ratones silenciados para estos genes han demostrado que la pérdida de su función trae graves consecuencias en el desarrollo de las extremidades como polidactilia, pérdida de falanges, defectos en el esqueleto axial, braquidactilia y braquipodismo (desarrollo anormalmente corto de los dedos y de los huesos largos de las extremidades respectivamente) (Selever J et al, 2004) (Kingsley DM et al, 1992) (King JA et al, 1994) (Storm EE et al, 1994). Para finalizar, indicar que otros miembros de la familia de TGF-β también participan en la condrogénesis mediando la inducción de moléculas requeridas para el proceso de agregación precondrogénica como fibronectina, N-CAM, N-Caderina o tenascina (Chimal-Monroy J et al, 1999). TGF-β2 se expresa se expresa en la agregación de células mesenquimáticas en etapas tempranas de la formación de los dedos (Merino R et al, 1999), y la aplicación de TGF-β1, TGF-β2 o TGF-β3 en embriones de pollo inhibe la apoptosis programada de las células de la articulación y promueve la condrogénesis ectópica (Ganan Y et al, 1996).

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Degradación del cartílago por las proteasas de matriz extracelular en artrosis

El estudio de la degradación de la ECM en situaciones normales y patológicas sugiere que es un proceso en etapas, en el que las MMPs y las agrecanasas desempeñan papeles específicos y secuenciales. El espectro de proteasas de matriz extracelular expresadas por el tejido articular es suficiente por sí mismo para destruir completamente el colágeno estructural y el resto de los componentes de la ECM del cartílago y de las membranas basales.

La proteolisis y la consecuente pérdida del agrecano ocurre en las primeras fases del proceso de degradación del cartílago, mientras que el catabolismo de la estructura fibrilar de colágeno ocurre tardíamente y representa el punto de no retorno en el daño articular (Jubb KW et al, 1980). El agrecano puede ser degradado tanto por agrecanasas como por las MMPs. Los resultados experimentales utilizando ensayos *in vitro* con explantes de cartílago tratados con IL-1, TNF-α o ácido retinoico, muestran que en la degradación del agrecano también podemos diferenciar dos etapas: en una primera etapa la degradación del agrecano sería llevada a cabo sólo por las agrecanasas, mientras que en etapas más tardías también pasarían a efectuar esta actividad degradativa las MMPs (Little CB et al, 2002) (Little CB et al, 1999). Se ha observado que los fragmentos de agrecano derivados de la actividad degradativa de las MMPs (neoepitopos VDIPEN) se encuentran abundantemente en el cartílago articular normal y su cantidad va aumentando con la edad, sin embargo estos neoepitopos no están significativamente incrementados en el cartílago de pacientes con OA o RA. Además, estudios inmunohistoquímicos llevados a cabo en cartílago de pacientes con OA o RA muestran que los neoepitopos VDIPEN no colocalizan con los derivados de la actividad degradativa de las agrecanasas (neoepitopos NITEGE) (Lark MW et al, 1997). En las primeras fases de la OA, lo neoepitopos NITEGE se encuentran abundantemente en el líquido sinovial, mientras que los derivados de las MMPs permanecen mayoritariamente en el cartílago (la ratio NITEGE/VDIPEN pasa de 2.5 en el líquido sinovial normal a 12 en el artrósico), lo que parece indicar que la actividad de las agrecanasas *in vivo* es destructiva para la matriz mientras que la actividad de las MMPs no lo es, reduciéndose al corte del extremo C-terminal de los monómeros de agrecano, dejando el resto de la molécula intacto (Sandy JD et al, 2001).

Por su parte, las MMPs tomadas en conjunto son capaces de degradar la totalidad de los componentes de la ECM. Las colagenasas (MMP-1, -8 y -13), las gelatinasas A y B (MMP-2 y -9 respectivamente), las estromalisinas (MMP-3, -10 y -11) y las matrilisinas (MMP-7 y -26) se expresan todas en niveles bajos en el tejido articular normal, sin embargo su expresión está muy incrementada en la OA y la RA (Yoshihara Y et al, 2000). La actuación de estos enzimas se

produce en cascada. Las MMPs son sintetizadas como zimógenos o enzimas inactivos y en su activación pueden participar diversos factores, entre ellos las MMPs de membrana. En la OA y la RA tan sólo MT1- y MT3-MMP juegan un papel importante. MT1- y MT3-MMP contribuye a la degradación de la matriz básicamente de una manera indirecta ya que activan a proMMP-2 y proMMP-13 mediante corte proteolítico, permitiendo que estos enzimas degraden colágeno, gelatinas y proteoglicanos (Knauper V et al, 1996) (Imai K et al, 1997).

El colágeno está en forma de triple hélice siendo esta una estructura muy resistente que sólo puede ser rota por las colagenasas. MMP-13 se expresa en condrocitos y degrada colágeno tipo II preferentemente, teniendo 10 veces más actividad que MMP-1 sobre este colágeno. MMP-1 y MMP-8 son sintetizadas por los sinoviocitos y neutrófilos respectivamente y presentan su mayor actividad enzimática sobre el colágeno tipo III (MMP-1) y el colágeno tipo I (MMP-8) (Burrage PS et al, 2006). Por ello, se piensa que MMP-13 tiene un efecto primordial como colagenasa. Idea reforzada por diversos experimentos realizados en animales (Neuhold LA et al, 2001). Una vez que el colágeno ha sido degradado por las colagenasas, los fragmentos del colágeno desnaturalizado pueden ser degradados aún más por el efecto de las gelatinasas (MMP-2 y MMP-9). A continuación la degradación del resto de los componentes de la ECM es llevada a cabo por las estromalisinas y las matrilisinas. Las estromalisinas (MMP-3, -10 y -11) pueden degradar fibronectina, laminina, elastina y agrecano. Además MMP-3 puede actuar en las primeras fases de la degradación activando por corte a la pro-MMP-1 (Unemori E. N. et al, 1991). Por su parte las matrilisinas (MMP-7 y -26) pueden degradar proteoglicanos y son inducidas por TNF-α e IL-1 beta (Ohta S et al, 1998). De esta manera, la actividad coordinada de las proteasas de ECM es por sí misma capaz de provocar la rotura de la integridad de la articulación por degradación de las fibras de colágeno y los demás constituyentes de la ECM trayendo como consecuencia la pérdida de su función, tal como se observa en la OA y la RA. Sin embargo, este esquema de actuación, está incompleto ya que todavía no se sabe si hay alguna proteasa extra en alguno de los pasos, o si este es exactamente el orden en que se producen las lesiones articulares en la OA.

Artritis Reumatoide y proteasas de matriz extracelular

La RA se trata en este trabajo sólo por la posible repercusión que las variantes genéticas en proteasas de ECM puedan tener en esta enfermedad y en la susceptibilidad a la RA, tanto desde el punto de vista de la degradación de la ECM como de su participación indirecta en los procesos de inflamación. La RA comparte con la OA la erosión del cartílago articular aunque

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los mecanismos de iniciación y la localización de las lesiones son diferentes. El efecto observado en la RA es secundario a un proceso de inflamación crónico de tipo sistémico y con implicación del sistema inmunológico que afecta fundamentalmente a las articulaciones periféricas con distribución simétrica. Este proceso está mediado por una red interdependiente de citoquinas, prostanoides y enzimas proteolíticos (Dinarello C et al, 2002). La RA se caracteriza por dolor, rigidez, hinchazón, pérdida de la movilidad, y puede llegar a provocar la destrucción de la arquitectura de la articulación causando graves deformidades (Bresnihan B et al, 1998). Su evolución es progresiva o con remisiones y exacerbaciones, acompañándose en ocasiones de alteraciones en diferentes órganos como vasculitis, pleuritis o pericarditis. La prevalencia de la RA es bastante elevada, afectando a un 1% de la población y siendo más frecuente en mujeres que en hombres en una relación de 3:1. La etiología de la RA es desconocida pero el componente genético se estima que explica alrededor del 50% de la varianza en la susceptibilidad a la enfermedad (Harney S et al, 2002).

Las proteasas de ECM en AR

Debido a su función, las proteasas de ECM se han señalado como posibles efectores a la hora de explicar parte de la susceptibilidad a la RA (Mengshol JA et al, 2002). Se puede señalar un doble papel, por una parte participan en la degradación de la ECM del cartílago y el hueso de una manera similar a la ya revisada en el caso de la OA, y por otra, juegan un papel importante en el proceso inflamatorio (Firestein GS et al, 2003) (Martel-Pelletier J et al, 2001) (Parks WC et al, 2004). Estudios recientes han mostrado que MMP-3 es la proteasa más abundante en la RA tanto en las primeras etapas de la enfermedad como en estados más avanzados. En este estudio también se observó que la concentración sérica de MMP-3 estaba fuertemente correlacionada con la severidad de la RA y que los niveles en suero de esta metaloproteasa podían ser utilizados como marcadores para predecir el daño articular (Yamanaka H et al, 2000). Sin embargo, estudios de asociación con polimorfismos reguladores en la región promotora de esta proteasa no han encontrado asociación con susceptibilidad a la RA (Tsukahara S et al, 2008) aunque sí se ha visto asociación con progresión radiográfica (Nemec P et al, 2007). Otros estudios muestran que los niveles de MMP-1, MMP-9 y MMP-13 también se encuentran elevados en el suero de pacientes con RA (Fiedorczyk M et al, 2006). Con respecto a las otras proteasas de ECM, destacan algunos trabajos que resaltan el papel de ADAMTS-12 y ADAMTS-7 por su participación en la degradación de la proteína oligomérica de la matriz (COMP) (Liu CJ et al, 2006) (Liu CJ et al, 2006(2)).

En lo que respecta al papel de las proteasas de ECM en la inflamación, varios trabajos señalan el papel de MMP-2 y MMP-9, y también de la ADAM-15. Se ha visto que los niveles de expresión de MMP-2 y MMP-9 se encuentran aumentados en pacientes con RA, aunque sus efectos parecen antagónicos (Burrage PS et al, 2006). En el caso de MMP-2 se ha visto que tiene efectos anti-inflamatorios degradando moléculas biológicamente activas como citoquinas, quimiocinas o receptores de factor de crecimiento (Xue M et al, 2007). Por el contrario, MMP-9 puede actuar como enzima proinflamatorio facilitando la infiltración leucocitaria y activando a otras proteasas (Nemec P et al, 2007). ADAM-15 por su parte participa en los fenómenos de inflamación mediante mecanismos de interacción célula-célula, interacción célula-ECM y actividad de podado celular (Charrier-Hisamuddin L et al, 2007).

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Hipótesis

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Hipótesis

1. Las proteasas de ECM son importantes en múltiples procesos en OA y RA. La degradación del cartílago es el más evidente, pero también juegan un papel relevante en la activación de otras proteasas, liberación de factores solubles secuestrados en la matriz, desactivación de mediadores inflamatorios y regulación de la interacción entre los condrocitos y la matriz.

2. Es muy difícil determinar qué funciones de las proteasas dependen específicamente de alguna de ellas ya que su actividad *in vitro* es bastante redundante.

3. La investigación genética permite identificar proteasas críticas como aquellas que muestran frecuencias alélicas diferentes en pacientes con OA y RA en relación con cambios modestos en los niveles de expresión o en la función.

4. La solidez de las conclusiones de la investigación genética depende de su potencia estadística, por lo tanto, la obtención de grandes tamaños muestrales y la replicación en estudios independientes son requisitos imprescindibles para el progreso de esta área de investigación biomédica.

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Objetivos

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Objetivos

1. Análisis de los polimorfismos conocidos que afectan a la función o la expresión de las proteasas de ECM en la susceptibilidad a OA.

2. Análisis de algunos polimorfismos que afectan a la expresión de las proteasas de EMC en la susceptibilidad a RA.

3. Replicación de hallazgos significativos de estudios independientes que han demostrado asociación con susceptibilidad a OA.

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Resultados, discusión y procedimiento experimental

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Resultados, procedimiento experimental y discusión. Esquema general

1. Proteasas de matriz extracelular

a. Artrosis

a.1 ADAMTSs y Adamlisinas (ADAMs)

Publicación 1:

- Rodriguez-Lopez J 1, Mustafa Z, Pombo-Suarez M, Malizos K, Rego I, Blanco FJ, Tsezou A, Loughlin J, Gomez-Reino JJ, Gonzalez A. Nonsynonymous Polymorphisms of a Major Aggrecanase, ADAMTS5, in Susceptibility to Osteoarthritis. Arthritis Rheum. 2008 Jan 31; 58(2):435-441

Publicación 2:

- Rodriguez-Lopez J, Pombo-Suarez M, Loughlin J, Tsezou A, Malizos KN, Rego I, Blanco FJ, Meulenbelt I, Valdes AM, Spector TD, Gomez-Reino JJ, Gonzalez A. A change of Glutamic acid for Glicine in the ADAMTS14 protease associated with susceptibility to primary osteoarthritis

a.2 MMPs

Material y métodos

Resultados

Discusión

a.3. Serín proteasas (sistema plasminógeno-plasmina)

Material y métodos

Resultados

Discusión

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b. Artritis reumatoide

Publicación 3

- Rodriguez-Lopez J, Perez-Pampin E, Gomez-Reino JJ, Gonzalez A Regulatory polymorphisms in extracellular matrix protease genes and susceptibility to rheumatoid arthritis: a case-control study Arthritis Research & Therapy 2006, 8:R1 (1 November 2005)

2. Estudios de replicación

a FRZB

Publicación 4

- Rodriguez-Lopez J, Pombo-Suarez M, Liz M, Gomez-Reino JJ, Gonzalez A. Further evidence of the role of frizzled-related protein gene polymorphisms in osteoarthritis. Ann Rheum Dis. 2007 Aug; 66(8):1052-5. Epub 2007 Jan 19

b. ASPN

Publicación 5

- Rodriguez-Lopez J, Pombo-Suarez M, Liz M, Gomez-Reino JJ, Gonzalez A Lack of association of a variable number of aspartic acid residues in the asporin gene with osteoarthritis susceptibility: case-control studies in Spanish Caucasians Arthritis Research & Therapy 2006, 8:R55 (10 March 2006).

Publicación 6:

- Nakamura T, Shi D, Tzetis M, Rodriguez-Lopez J, Miyamoto Y, Tsezou A, Gonzalez A, Jiang Q, Kamatani N, Loughlin J, Ikegawa S. Meta-analysis of association between the ASPN D-repeat and osteoarthritis. Hum Mol Genet. 2007 Jul 15; 16(14):1676-81. Epub 2007 May 20. PMID: 17517696.

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c. GDF5

Publicación 7:

a. Southam L & Rodriguez-Lopez J, Wilkins JM, Pombo-Suarez M, Snelling S, Gomez-Reino JJ, Chapman K, Gonzalez A, Loughlin J. An SNP in the 5'-UTR of GDF5 is associated with osteoarthritis susceptibility in Europeans and with in vivo differences in allelic expression in articular cartilage. Hum Mol Genet. 2007 Sep 15; 16(18):2226-32. Epub 2007 Jul 6. PMID: 17616513

Publicación 8:

- Kay Chapman1, Atsushi Takahashi2, Ingrid Meulenbelt3, Chris Watson, Julio Rodriguez-Lopez, Rainer Egli1, Aspasia Tsezou, Konstantinos N.Malizos, Margreet Kloppenburg, Dongquan Shi, Lorraine Southam, Ruud van der Breggen, Rachelle Donn, Jianghui Qin, Michael Doherty, P Eline Slagboom, Gillian Wallis, Naoyuki Kamatani, Qing Jiang, Antonio Gonzalez, John Loughlin and Shiro Ikegawa. A meta-analysis of European and Asian cohorts reveals a global role of a functional SNP in the 5’ UTR of GDF5 with osteoarthritis susceptibility. Hum Mol Genet. 2008 Feb 24

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1. Proteasas de matriz extracelular

a. Artrosis

a.1 ADAMTs y Adamlisinas (ADAMs)

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ARTHRITIS & RHEUMATISM
Vol. 58, No. 2, February 2008, pp 435–441
DOI 10.1002/art.23201
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Genetic Variation Including Nonsynonymous Polymorphisms of a Major Aggrecanase, ADAMTS-5, in Susceptibility to Osteoarthritis

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Objective. **Given the recent characterization of ADAMTS-5 as the main aggrecanase of cartilage destruction in mouse models, we explored whether genetic variation and, in particular, putative damaging polymorphisms in the ADAMTS-5 gene modify susceptibility to osteoarthritis (OA).**

Methods. **Two likely deleterious nonsynonymous single-nucleotide polymorphisms (SNPs) were identified in ADAMTS-5 by bioinformatics analysis, rs2830585 in exon 5 affecting a thrombospondin 1 motif, and rs226794 in exon 7. Exploration of their role was carried out in 3 steps, discovery, extension, and replication, on samples obtained from 4 European Caucasian collections, comprising a total of 2,715 patients with knee, hip, or hand OA and 1,185 OA-free controls. In addition, 6 tagSNPs were studied to fully evaluate genetic variation in the ADAMTS-5 locus.**

Results. **Initial analyses of 2 sample collections (n = 277 and n = 159) showed a trend toward decreased frequency of the putative deleterious allele of rs226794 among patients with severe knee OA (*P* = 0.047 versus controls). However, results in patients with knee OA from 2 additional sample collections (n = 360 and n = 265) did not confirm this trend. No association was found with hip OA or hand OA. None of the other SNPs or haplotypes constructed with these SNPs showed a significant association with OA susceptibility.**

Conclusion. **Use of several collections of OA samples allowed us to obtain sound evidence against the participation of genetic variation in ADAMTS-5 in OA susceptibility. These results indicate the need to further explore the function of this aggrecanase in human OA to determine whether it is as critical as has been observed in mouse models.**

Supported by the Instituto de Salud Carlos III (Spain), with participation from the European Union FEDER Fund, and by the Fundacion MMA (Spain). Mr. Rodriguez-Lopez is recipient of a Spanish Ministry of Education scholarship. Dr. Pombo-Suarez is recipient of a Fundacion Española de Reumatologia bursary. Dr. Loughlin's work was supported by Research into Ageing (UK) and the Arthritis Research Campaign (UK).

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Submitted for publication April 24, 2007; accepted in revised form November 5, 2007.

Primary osteoarthritis (OA) is a complex disease without obvious causes, in which a major genetic component has been demonstrated (1,2). This etiologic component accounts for a large fraction of disease variability, with heritability estimates of ~50%. Candidate gene approaches have already allowed identification of some of the genetic variants that modify OA susceptibility. The genetic effects of each of these polymorphisms are small. Nevertheless, they provide exciting starting points for new hypotheses and for the exploration of disease mechanisms in OA.

In the present study we explored genetic variation in a critical protease in the initial stages of OA, ADAMTS-5, and, in particular, in 2 nonsynonymous single-nucleotide polymorphisms (nsSNPs) that are likely to affect the function of ADAMTS-5 (by altering the amino acid sequence of the protein). This protease

seems critical in the catabolic component of OA because it is involved in degradation of aggrecan, which is one of the major components of cartilage extracellular matrix. Loss of aggrecan precedes collagen damage at the beginning of the OA lesion (3). Recent evidence indicates that, in vivo, the only critical aggrecanase, at least in mice, is ADAMTS-5 (4,5). Its absence was observed to prevent lesions in animal models of OA and inflammatory arthritis.

Herein we studied 8 SNPs that cover genetic variation in ADAMTS-5 and its neighboring sequences. Two of these SNPs were nsSNPs that were shown, by bioinformatics analysis, to be deleterious in ADAMTS-5 function. The remaining 6 were tagSNPs. The results of our study, however, did not reveal any significant effect on OA susceptibility.

PATIENTS AND METHODS

Patients and controls. Three of the 4 sample collections that were used for our study have been described previously (6–8). Collections were obtained from the Hospital Clinico de Santiago in Spain, from the University of Thessaly in Greece, from the Institute of Musculoskeletal Sciences at Oxford in the UK, and from the Hospital Juan Canalejo in Corunna, Spain. The collection from Santiago included samples from patients with severe primary OA who were undergoing total hip replacement (THR) (307 patients, of whom 185 were women and 122 were men) or total knee replacement (TKR) (277 patients, of whom 223 were women and 54 were men) and patients with hand OA (242 patients, of whom 213 were women and 27 were men, with unrecorded data for 2 patients). Controls comprised 294 subjects (115 women and 179 men) older than age 55 years who did not show clinical manifestations compatible with OA; only 31.6% of the recruited control subjects older than age 55 years passed this selection. The median age of the control subjects was 68 years (interquartile range 61–75 years) at the time of recruitment.

The collection from Thessaly included samples from 159 patients with OA who had undergone TKR (139 women and 20 men) and 193 control subjects (137 women and 56 men). The control subjects were older than age 45 years and were free of clinical OA. The collection from Oxford comprised 360 samples from patients with OA who had undergone TKR (196 women and 164 men), 1,105 samples from patients with OA who had undergone THR (629 women and 476 men), and samples from 698 control subjects (356 women and 342 men). The control subjects had no signs or symptoms of arthritis or joint disease (pain, swelling, tenderness, or restriction of movement). The median age of the control subjects at the time of recruitment was 69 years (range 55–89 years). Due to ethical and financial constraints, the hip and knee joints of the control subjects were not subjected to radiographic analysis. The collection of samples from Corunna was obtained from patients with clinically symptomatic and radiographically confirmed knee OA (265 patients, of whom 222 were women and 43 were men).

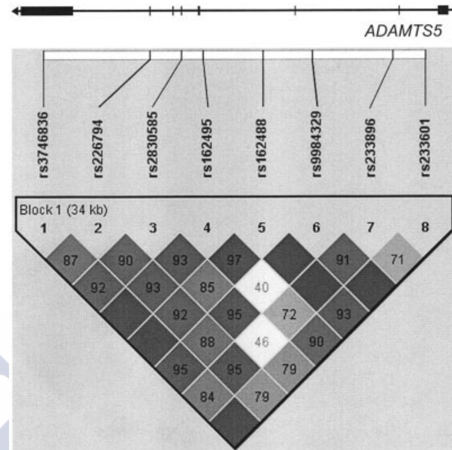

Figure 1. Map of linkage disequilibrium (D′) of the 8 tag single-nucleotide polymorphisms in the ADAMTS-5 gene.

All patients and controls gave their informed consent for the study. Each collection of samples was obtained with the approval of the respective ethics committees.

SNP selection. Seven nsSNPs in ADAMTS-5 were found in the dbSNP database, of which 2, rs2830585 G/A (R614H in exon 5) and rs226794 C/T (P692L in exon 7), were predicted to be both frequent in and deleterious for ADAMTS-5 protein function. Functional prediction was done using SIFT software (9). TagSNPs were selected from the HapMap CEPH (Utah residents with ancestry from northern and western Europe) database and included the aforementioned nsSNPs of the ADAMTS-5 gene as well as 10 kb of flanking sequence to each side (Figure 1). The tagSNPs were selected using Haploview software, in which an r^2 threshold of 0.8 was applied in the pairwise correlation, and SNPs with a minor allele frequency $<10\%$ were excluded.

Genotyping. The rs226794 nsSNP and the 6 tagSNPs (rs3746836, rs162495, rs162488, rs9984329, rs233896, and rs233601) were genotyped by single-base extension with the SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA) in 2 independent assays. A fluorescence 5′ exonuclease assay that was designed as a TaqMan Genotyping Assay (Applied Biosystems) was used to genotype the rs2830585 nsSNP. Several samples with different genotypes were sequenced to check the accuracy of the results (details on the oligonucleotide sequences are available online at http://bioinformatics.cesga.es/supmat/). The genotype call rate was 0.98 for both rs226794 and rs2830585 across sample collections and across patients and controls. For the tagSNPs, the genotype call rate was 0.85 in UK samples, 0.93 in Greek samples, and 0.97 in Spanish samples. Genotype frequencies were in Hardy-Weinberg equilibrium.

Table 1. Frequencies of alleles of the 2 putative damaging nonsynonymous single-nucleotide polymorphisms in ADAMTS-5 in different groups of patients with osteoarthritis (OA) and OA-free controls*

	rs226794 C/T P692L			rs2830585 G/A R614H		
Collection, group	T allele, %	*P*	OR (95% CI)	A allele, %	*P*	OR (95% CI)
Santiago						
Control (n = 588)	11.5	–	–	18.5	–	–
OA						
TKR (n = 554)	8.2	0.06	0.68 (0.5–1.0)	21.0	0.3	1.16 (0.9–1.6)
THR (n = 614)	11.3	0.9	0.98 (0.7–1.4)	20.5	0.4	1.13 (1.0–1.8)
Hand OA (n = 484)	9.6	0.3	0.81 (0.5–1.2)	23.0	0.08	1.31 (1.0–1.8)
Thessaly						
Control (n = 386)	9.8	–	–			
OA TKR (n = 318)	8.0	0.4	0.80 (0.5–1.4)			
Oxford						
Control (n = 1,396)	11.1	–	–			
OA						
TKR (n = 720)	11.2	0.9	1.01 (0.8–1.4)			
THR (n = 2,210)	9.9	0.3	0.88 (0.7–1.1)			
Corunna						
Knee OA (n = 530)	11.1	0.8†	0.96 (0.7–1.4)†			

* OR = odds ratio; 95% CI = 95% confidence interval; n = number of alleles; TKR = total knee replacement; THR = total hip replacement.
† Compared with Santiago controls.

Statistical analysis. Statistica software (Statsoft, Tulsa, OK) and Haploview software were used for the data analyses. Allele frequencies as well as odds ratios and their 95% confidence intervals were calculated. Comparison of allele frequencies was performed with a chi-square test. Evidence of a gene-dose effect was evaluated with univariate logistic regression, applying an additive genetic model. Estimation of the linkage disequilibrium of haplotype frequencies and comparison of those frequencies between patients and controls were carried out with Haploview software.

Data from the populations of Spain, Greece, and the UK were combined for assessment by Mantel-Haenszel test, using 2 × 2 contingency tables that were stratified by sample collection. Homogeneity of the effect size across collections was assessed with the Breslow-Day test. Correlation analysis, Student's *t*-test, and chi-square analysis were used to explore possible associations of OA clinical features with the genotypes at the 2 nsSNPs. Available information on the demographic and clinical characteristics of the patients in the Santiago collection comprised sex, age at disease onset, age at surgery, time from disease onset to surgery, body mass index, assessment of pain and disability on a visual analog scale, family medical history, presence of Heberden's or Bouchard's nodes, and OA in other joints. Control subjects were also analyzed for age at recruitment.

RESULTS

Discovery step. Our initial analysis involved genotyping 2 nsSNPs of ADAMTS-5, rs2830585 G/A, which provokes an R614H substitution in exon 5, and rs226794 C/T, which codes for a P692L change in exon 7, in the 3-branched OA sample collection from Santiago. No significant differences were detected between any of these 3 OA groups and the controls (Table 1). However, there was a trend toward significance in the decreased frequency of the minor allele, T, of rs226794 among the OA patients who underwent TKR as compared with the control subjects (frequency of T allele 8.2% in OA TKR patients versus 11.5% in control subjects; *P* = 0.06).

Analysis of the genotype frequencies showed a dose effect of the T allele of rs226794, with the differences between OA TKR patients and controls approaching significance (frequencies of CC, CT, and TT 83.9%, 15.7%, and 0.4%, respectively, in OA TKR patients versus 79.4%, 18.2%, and 2.4%, respectively, in control subjects; *P* for trend = 0.06). No differences between female and male subjects were observed in these analyses of the OA TKR and control sample sets.

Furthermore, there was a lack of effect of the rs226794 nsSNP among patients with hand OA and among OA patients who underwent THR, as determined in samples from the Santiago collection. The latter finding was confirmed in a large number of samples from the Oxford collection (Table 1).

The other nsSNP, rs2830585, showed a trend that was contrary to our hypothesis. Frequencies of the putative damaging allele (the A allele) in rs2830585 were seemingly larger in the OA patients compared with the control subjects (Table 1), especially in the group of patients with hand OA (23.0% versus 18.5% in controls; *P* = 0.08).

Genotypes at the 2 nsSNPs did not correlate with

Table 2. Effect of the rs226794 nonsynonymous single-nucleotide polymorphism in different combined collections of samples from OA TKR patients and controls*

Collections, group	Mantel-Haenszel test			Breslow-Day test, *P*†
	T allele, %	OR (95% CI)	*P*	
Santiago and Thessaly				
Control (n = 974)	11.1	–	–	–
OA TKR (n = 872)	7.9	0.73 (0.5–1.0)	0.047	0.7
Santiago, Thessaly, and Oxford				
Control (n = 2,370)	11.0	–	–	–
OA TKR (n = 1,592)	9.5	0.87 (0.7–1.1)	0.2	0.3
Female				
Control (n = 1,216)	10.9	–	–	–
OA TKR (n = 1,116)	9.0	0.88 (0.7–1.2)	0.4	0.8
Male				
Control (n = 1,154)	11.1	–	–	–
OA TKR (n = 476)	10.6	0.97 (0.7–1.4)	0.9	0.8

* See Table 1 for definitions.
† Test of heterogeneity.

the clinical features of OA in any of the patient groups, nor did the genotypes correlate with the age at recruitment in the OA-free controls. Specifically, the putative protective alleles were not more frequent in older control subjects; among the 40 control subjects older than age 79 years, the frequency of the rs226794 T allele was 11.5%, and the frequency of the rs2830585 A allele was 21.2%.

The 2 nsSNPs showed linkage disequilibrium, as evaluated with the D′ parameter (D′ = 0.67), but there was a very weak correlation in their genotypes (r^2 = 0.012). Haplotype estimation from the genotypes of the 2 nsSNPs in the Santiago sample collection revealed only 3 of the 4 possible haplotypes (GC, AC, and GT) that accounted for >99% of all chromosomes. The unique haplotype containing the minor allele of the rs226794 SNP (haplotype GT in the rs2830585, rs226794 order) was slightly less frequent in the OA patients who underwent TKR than in the control subjects (frequency of GT 7.5% in OA TKR patients versus 10.9% in controls; P = 0.048). Since this was the only haplotype with the minor rs226794 allele, this result was equivalent to the findings with regard to rs226794 allele frequencies.

Multivariate logistic regression analysis showed a lack of detectable interaction between the 2 nsSNPs (results not shown). None of the analyses showed a significant effect in the OA patients who underwent THR or in the patients with hand OA from the Santiago collection.

Extension step. Given the suggestive evidence for an effect of the rs226794 SNP in OA TKR patients from the Santiago collection, we analyzed additional OA TKR samples from Thessaly. In this extension step (Table 1), the frequency of the T allele of the rs226794 SNP was not significantly different between OA TKR patients and controls, although the nominal percentage was smaller among the OA TKR patients. Combined analysis of the Santiago and Thessaly OA TKR samples (Table 2) showed a slightly significant difference compared with controls, indicating that the T allele of this nsSNP could be less frequent among OA TKR patients. Genotype analysis of these data yielded results similar to those for the allele frequencies (P = 0.024 for OA patients who underwent TKR versus control subjects). As a result, we decided to replicate this analysis in other sample collections.

Replication steps. The rs226794 SNP was genotyped in the 360 OA TKR samples and 698 control samples from the Oxford collection. The frequency of the minor allele of rs226794 was not different in the OA TKR patients compared with the control subjects (Table 1). Combined analysis of the Santiago, Thessaly, and Oxford OA TKR sets, using the Mantel-Haenszel approach, showed a lack of association of this nsSNP with TKR in these patients (Table 2), indicating that this polymorphism does not modify susceptibility to severe knee OA requiring joint replacement. Results from each collection and the combined results from the 3 collections were similar between female and male subjects (Table 2).

We also obtained an additional sample collection from Corunna, Spain, a town near Santiago, that contained samples from patients with symptomatic and radiographic knee OA (without the need for TKR). Comparison of the frequency of the T allele of the rs226794 nsSNP in these patients with that in the control

Table 3. Associations of alleles of the ADAMTS-5 tag single-nucleotide polymorphisms (tagSNPs) in OA TKR patients and controls from the combined Spanish, Greek, and UK sample collections*

	Minor allele, %		Mantel-Haenszel test		
TagSNP	Controls (n = 1,710)	OA TKR (n = 1,562)	OR (95% CI)	*P*	Breslow-Day test, *P*†
rs233601 (T/C)	25.1	24.9	1.01 (0.86–1.19)	0.91	0.023
rs233896 (A/C)	42.3	43.2	0.96 (0.83–1.11)	0.61	0.86
rs9984329 (A/G)	37.8	37.7	1.01 (0.87–1.67)	0.91	0.28
rs162488 (G/A)	32.3	31.6	1.03 (0.89–1.21)	0.62	0.25
rs162495 (A/G)	37.5	35.0	1.11 (0.76–1.05)	0.15	0.04
rs3746836 (C/T)	25.8	28.0	1.14 (0.96–1.35)	0.12	0.22

* TagSNPs are listed in 5′–3′ order. See Table 1 for other definitions.
† Test of heterogeneity.

subjects from Santiago showed that the frequency of this allele was not decreased in the OA TKR patients (Table 1).

TagSNP and haplotype analyses. To further investigate the possible involvement of ADAMTS-5 genetic variation, we selected tagSNPs that, together with the 2 nsSNPs, cover the gene and its neighboring sequences. We studied them in all of the OA TKR and control samples. None of the tagSNPs was found to be associated with susceptibility to OA, when analyzed with a Mantel-Haenszel test that accounted for the different sample collections (Tables 3 and 4).

Two of the tagSNPs showed significant effect heterogeneity between the collections. The frequency of the minor allele rs233601 was significantly greater in Spanish OA TKR patients compared with Spanish controls and UK or Greek OA TKR patients or controls. The frequency of the minor allele rs162495 was significantly lower in Greek OA TKR patients compared with Greek controls and Spanish or UK OA TKR patients or controls. These differences were most likely due to chance fluctuations and were not related to OA susceptibility.

In addition, haplotypes of the tagSNPs were estimated, because they could be used to uncover genetic effects of polymorphisms that have not been genotyped. However, the frequencies of the haplotypes of the tagSNPs were not different between the OA TKR patients and controls (Table 5).

DISCUSSION

Study of genetic susceptibility factors in OA requires large sample collections to differentiate between genuine effects and random variation in genotype frequencies (1,2). In the present study we used a sufficiently large and varied amount of DNA samples to obtain sound conclusions. One of the explored nsSNPs, rs2830585, showed unpromising results in our initial analyses, and was therefore not studied further. The other nsSNP, rs226794, appeared promising in patients with severe knee OA in the first 2 collections analyzed. However, the results from 2 additional sample collections did not confirm this trend. These observations led us to conclude that our initial results were most likely due to chance variation. In addition, we were able to

Table 4. Frequencies of alleles of the 6 tag single-nucleotide polymorphisms in OA TKR patients and controls from each sample collection*

	rs233601 (T/C)		rs233896 (A/C)		rs9984329 (A/G)		rs162488 (G/A)		rs162495 (A/G)		rs3746836 (C/T)	
Collection, group	C allele, %	*P*	C allele, %	*P*	G allele, %	*P*	A allele, %	*P*	G allele, %	*P*	T allele, %	*P*
Santiago												
Control (n = 588)	25.8	–	40.1	–	37.7	–	30.2	–	37.4	–	26.5	–
OA TKR (n = 524)	31.5	0.04	41.1	0.72	33.7	0.17	29.5	0.81	39.6	0.46	25.1	0.61
Thessaly												
Control (n = 386)	24.0	–	43.4	–	37.3	–	30.7	–	38.4	–	26.5	–
OA TKR (n = 318)	21.3	0.40	45.9	0.51	39.2	0.62	34.8	0.26	28.7	0.01	28.7	0.53
Oxford												
Control (n = 736)	25.2	–	43.6	–	38.3	–	35.0	–	37.2	–	24.8	–
OA TKR (n = 720)	21.6	0.11	43.6	1.00	40.1	0.50	31.7	0.19	34.4	0.29	29.8	0.04

* See Table 1 for definitions.

Table 5. Frequencies of ADAMTS-5 haplotypes, including those of the 6 tag single-nucleotide polymorphisms (SNPs) and the rs226794 nonsynonymous SNP, in OA TKR patients and controls from the combined Santiago, Thessaly, and Oxford sample collections*

		Haplotype frequency		
Haplotype†	Sequence	OA TKR (n = 1,562)	Controls (n = 1,710)	*P*
I	TCAAACC	30.3	30.8	0.75
II	TAGGACT	23.8	22.4	0.35
III	CAAGGCC	20.6	21.8	0.44
IV	TAGGGTC	7.9	9.9	0.06

* n = number of chromosomes (see Table 1 for other definitions).
† SNP order: rs233601, rs233896, rs9984329, rs162488, rs162495, rs226794, and rs3746836. The rs2830585 SNP was not included because it was not genotyped in all samples; however, in the Santiago collection, its rare allele split haplotype III into 2 haplotypes of similar frequencies, both in patients and in controls.

exclude a role of rs226794 in hip OA, according to the concordant results obtained in 2 of the sample collections. A similar result was observed in the unique collection of samples from patients with hand OA. Therefore, neither of the 2 putative damaging nsSNPs in ADAMTS-5 seems to have a significant effect on OA susceptibility.

We believe our study was well suited to detect a protective effect from damaging nsSNPs, because controls were selected for the absence of clinical OA and for old age. An alternative could have been to use even older subjects than were included in our study as OA-free controls. However, there is no evidence that this would increase the sensitivity of the study. In fact, we have shown that the age of the OA-free controls was not correlated with the allele frequencies in the nsSNPs. In addition, OA prevalence is near its maximum in the age range of our controls (10).

Although nsSNPs are more likely than other SNPs to be functional, only a fraction of them will significantly affect protein function. Several software programs have been developed to estimate their deleterious potential. We used SIFT software (9) to perform analyses of amino acid conservation for screening. Analysis by different software, PolyPhen (11), which is based on slightly different criteria, confirmed the possible damaging effect of rs226794 but not that of rs2830585. In contrast, application of SNPs3D software (12) confirmed the possible deleterious effects of both nsSNPs, attributing a larger likelihood of being deleterious to rs2830585. The high degree of conservation of the sequences of the 2 nsSNPs is due to their position in functional domains; rs2830585 maps to the first of the 2 thrombospondin type 1 domains of ADAMTS-5, whereas rs226794 maps to a putative functional domain of unknown function. Therefore, both SNPs seem likely to affect ADAMTS-5 function.

We are unaware of other ADAMTS-5 polymorphisms that could affect its function. However, bioinformatic prediction of sequences with regulatory function is very poor, and therefore it remains possible that other polymorphisms could be identified. Our exploration of genetic variation along the locus with tagSNPs and haplotypes showed that even if other ADAMTS-5 functional polymorphisms are present, they do not have a relevant role in OA susceptibility.

The place of the different extracellular matrix proteases in OA is not completely clear. Two recent reports describing the findings in murine models of OA and rheumatoid arthritis attributed a unique role to ADAMTS-5 (13,14). However, it is unclear whether ADAMTS-5 is as necessary for OA in humans as it is in mice. Aggrecan is lost as a consequence of its cleavage at specific sites flanked proximally by glutamic acid. This activity has been characterized as "aggrecanase" activity, which is in contrast to cleavage of aggrecan at other sites by matrix metalloproteinases that do not cause aggrecan loss from cartilage (15). Only ADAMTS-1, -4, -5, -8, -9, and -15 show aggrecanase activity in vitro (3). However, most of these ADAMTS family members are unlikely to play a significant role in OA, since they are absent from joint cartilage or have low activity. Only ADAMTS-4 and ADAMTS-5 are considered likely candidates for this function, although there is no clear evidence of a dominant role of any of these ADAMTS family members in humans (13,14,16). Results from our present study suggest that ADAMTS-5 could be less critical in human OA than has been observed in the murine models.

Our study is the first to explore genetic variation in ADAMTS-5 in OA susceptibility. The number of samples analyzed and the variety of studied phenotypes led us to conclude that it is unlikely that the 2 studied nsSNPs modify OA susceptibility. The nsSNPs are likely to affect enzyme function, and consequently, their lack of effect and lack of association in terms of genetic variation in ADAMTS-5 emphasize the need to further determine whether ADAMTS-5 has an exclusive and necessary role in human OA as has been described in experimental murine models.

ACKNOWLEDGMENTS

We thank Elisa Alonso-Perez, Manuel Calaza, and Cristina Fernandez for their help in the study of the tagSNPs.

AUTHOR CONTRIBUTIONS

Dr. Gonzalez had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Rodriguez-Lopez, Gonzalez.

Acquisition of data. Rodriguez-Lopez, Mustafa, Pombo-Suarez, Malizos, Rego, Blanco, Tsezou, Loughlin, Gomez-Reino, Gonzalez.

Analysis and interpretation of data. Rodriguez-Lopez, Mustafa, Pombo-Suarez, Malizos, Rego, Blanco, Tsezou, Loughlin, Gonzalez.

Manuscript preparation. Rodriguez-Lopez, Gomez-Reino, Gonzalez.

Statistical analysis. Rodriguez-Lopez, Gonzalez.

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A Change of Glutamic Acid for Glycine in ADAMTS14 Associated to Primary Osteoarthritis Susceptibility

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Running title: ADAMTS14 aminoacid change in OA susceptibility

Keywords: Genetic susceptibility, Osteoarthritis, Matrix metalloproteases, Non-synonymous polymorphisms

ABSTRACT

Objective: We aimed to investigate the effect of putative damaging changes in the aminoacid sequence of the ADAMTS and ADAM matrix proteases (except ADAMTS5 that was already studied) in OA susceptibility.

Material and methods: Non-synonymous single nucleotide polymorphisms (nsSNP) in 18 ADAMTS and 31 ADAM genes were analyzed with two softwares for prediction of functional damage, SIFT and PolyPhen. Four damaging nsSNP were found in ADAMTS2, ADAMTS14, ADAMTS16 and ADAM12, respectively. Their genotypes were analyzed in case-control sample collections totalling 3217 OA patients with different phenotypes and 2214 healthy controls, all of them European Caucasians.

Results: No statistically significant differences were found in the ADAMTS2, ADAMTS16 and ADAM12 nsSNPs. Conversely, the rare allele of the rs4747096 nsSNP in exon 21 of ADAMTS14 was overrepresented in women with severe knee OA ($O.R._{M-H}$ = 1.39, 95% C.I. = 1.1-1.7; p = 0.001) and patients with symptomatic hand OA (O.R. = 1.37, 95% C.I. = 1.0-1.9; p = 0.047). A near significant increase was also found in patients with severe hip OA ($O.R._{M-H}$ = 1.14, 95% C.I. = 1.0-1.3; p = 0.08).

Conclusion: Our findings implicate a new player, ADAMTS14, in OA because a putative damaging change of glutamic acid for glycine was associated with an increased risk of OA, specifically to severe knee OA in women and to hand OA. Independent confirmation of a role in female knee OA has been communicated. ADAMTS14 involvement highlights a new area of interest in OA pathogenesis by its role in the maturation of collagen fibers.

INTRODUCTION

Primary osteoarthritis (OA) is the most prevalent form of arthritis and a cause of important handicap among the elderly. Available treatments are of low efficacy and mostly symptomatic. This situation is compounded by our incomplete knowledge of OA molecular mechanisms and etiological factors. The latter are complex, including a multiplicity of weak genetic factors that jointly set the threshold of susceptibility for each individual (1-3); and a wide array of non-genetic factors such as age, obesity, profession, joint malalignment, trauma and gender. The genetic component can explain around 50 % of disease variance in twin concordance studies and has been shown to increase OA risk among patient siblings. Some of the specific genetic variants have been identified. Notable examples are asporin (4), FRZB (5) and GDF5 (6). Other factors are less certain like matrilin 3, the IL-1 gene cluster and the IL-4R gene. Many other

genes have been implicated in OA by some studies but not generally confirmed. The three best studied genetic factors are involved in growth and differentiation regulatory pathways, asporin by regulating the availability of free TGF-β in the peri-chondrocyte matrix (7); FRZB as a soluble inhibitor competing with Wnt receptors for their ligands; and GDF5 by its action as cartilage growth and differentiation factor. However, we do not know yet the details of how the susceptibility alleles impinge in OA. For example, it is unclear if the FRZB effect is related with cartilage repair or with subchondral bone stiffness (8); or at what level the change in GDF5 expression is important, during joint formation, in joint homeostasis or during cartilage repair. Also, we do not know why these factors show heterogeneity of effects depending on joint, gender and ethnicity. For example, the OA associated alleles in two non-synonymous SNPs of FRZB increase risk to OA only in females (5, 9); or the asporin length polymorphism has a more marked effect in Asians than in Europeans (10). Further research is needed to solve these questions and to identify other factors contributing to OA. In this effort, there is still room for studies addressing genes of interest in OA, the so-called candidate genes. In brief, whole genome association studies (WGAS) will be the main tool of genetic research. The strength of the WGAS is the combination of very large number of SNPs, typically more than 300 000, and large collections of samples.

An analysis of OA pathogenesis shows many genes of potential interest for genetic analysis. Two large families of proteases, ADAM and ADAMTS, are among them (11). They share two functional domains, a metalloprotease domain, the active site common to all metalloproteases, and a disintegrin domain that can interact with integrins and mediate adhesion. In addition, the ADAMTS include also several thrombospondin motifs. Most ADAMs are membrane-bound proteases and their better known functions are ectodomain shedding and regulation of cell adhesion, although they can have also a role in matrix degradation (12, 13). ADAMs are the main mediators of ectodomain shedding, the proteolytic cleavage of extracellular protein domains. This affects growth factors, cytokines and cell adhesion molecules. In this way, ADAMs eliminate some adhesion molecules from the cell surface, but they also increase cell adhesion to immunoglobulin family integrins and to elements of the extracellular matrix trough their disintegrin domain. Some members of the ADAM family have a function that could be related with OA. For example, ADAM-8 and -12 are involved in osteoclast differentiation, ADAM-10 is implicated in cell fate determination of osteoblasts, ADAM-15 enhances chondrocyte adhesion to types II and VI collagen, promotes chondrocyte survival and protects from experimental OA (14); and ADAM-17 or TNF-α-converting enzyme (TACE) that releases TNF-α form its membrane-bound precursor.

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The ADAMTS family is also of great interest in OA because it contains all the known aggrecanases (ADAMTS-1, -4, -5, -8, -9, -15); enzymes that cut the aggrecan core protein in a particular site that seems critical for cartilage damage in arthritis (15). One of the aggrecanases, ADAMTS-5 has been shown to be necessary for cartilage damage in murine models of arthritis (16, 17). However, it is unknown if it is as crucial in humans as in these models (18). We have recently shown that two putative damaging nsSNPs in the ADAMTS5 gene are not associated to OA protection and, more generally, that genetic variation in this gene seems irrelevant for OA predisposition (19). Other three ADAMTS, ADAMTS-2, -3 and -14, are involved in processing of collagen precursors, type I, II or III procollagen, and receive the name of procollagen-N-propeptidases because they cleave the N-terminal propeptide. In addition, some of the ADAMTS are able to degrade other matrix components apart from aggrecan and, in this way, they could be involved in OA. None is known to interact with integrins in spite of their disintegrin-like domain (15).

Systematic analyses of gene expression in OA cartilage by microarray hybridization have shown a few significant changes among ADAM and ADAMTS proteases, down-regulation of ADAM8 in early OA (20), and increase in ADAM12 in damaged regions versus preserved cartilage (21). In contrast, studies addressing specifically the genes of these proteases have shown a wide array of changes in OA (22, 23)::: up-regulation of ADAMTS-2, -10 and -16 in OA cartilage and synovium; up-regulation of ADAMTS-3, -7, -12, -14, -15, -18 and -20 only in OA cartilage with no changes in synovium; up-regulation of ADAMTS-8, -13 and -17 only in synovium; and down-regulation of ADAMTS-1, -4, 5 and -9 in OA cartilage and synovium. Also indicative of the possible relevance of some ADAM or ADAMTS proteases are studies showing genetic association to OA of a nsSNP in ADAM12 (24).

Here, we have examined genomic sequences of all the ADAMTS and ADAM members looking for nsSNPs. Those predicted to be damaging for protein function were studied in several sets of samples including a variety of OA phenotypes and one came out as associated with OA in ADAMTS14.

MATERIAL AND METHODS

Patients and controls

Six already described sample collections of European Caucasians were used (19, 25-29). Details of patient and control selection are omitted here. They were divided in two sets, a first set included samples with a severe OA phenotype in which all SNPs were analyzed in the Santiago

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laboratory. The second set, included other OA phenotypes in which only the rs4747096 SNP was studied in three different laboratories. The first set included samples from three collections, the Hospital Clinico de Santiago in Spain; the Institute of Musculoskeletal Sciences, University of Oxford in the UK; and the Departments of Biology and Genetics and of Orthopaedics, University of Thessaly in Greece. The Santiago collection included patients with severe primary OA that were undergoing total hip replacement (THR; 307 subjects, 185 women and 122 men) or total knee replacement (TKR; 262 patients, 211 women and 51 men) and patients complaining of hand OA (HOA; 242 subjects, 213 women, 27 men and 2 unrecorded). Controls were 294 subjects (115 women and 179 men) older than 55 years that did not show clinical manifestations of OA. The Oxford's collection was of 1105 THR samples (629 females and 476 males), 360 TKR samples (196 women, 164 men), and 698 controls (356 women, 342 men). The Thessaly's collection included 159 TKR patients (139 women, 20 men) and 193 controls (137 women, 56 men). Therefore, the first set amounted to 1412 THR, 781 TKR, 242 HOA and 1185 control samples. The second set of sample collections was made of 265 clinically symptomatic and radiographically confirmed knee OA (KOA; 222 women and 43 men) from the Hospital Juan Canalejo, A Corunna in Spain (this collection did not include controls); 358 generalized OA samples (292 women and 66 men of Dutch ancestry sib pairs) from the Genetics osteoARthritis and Progression (GARP) study and 712 controls (409 women, 303 men) of the Leiden University Medical Centre in the Netherlands; and 317 controls and 159 OA samples (all women) selected randomly (a subject from each twin pair) from the UK Adult Twin Registry of the Twin Research and Genetic Epidemiology Unit in London. OA in this later collection was defined as radiographic OA of the hand (n =72), knee (n = 71) or hip (n =71; 5 of them also with OA in the knee). Patients and controls from every collection gave their informed consent. Each collection of samples has obtained the approval of the relevant ethics committee.

SNP prediction

The gene sequences of the 18 ADAM and the 18 ADAMTS (except the previously studied ADAMTS5 (19)) proteases whose genome sequence is available were searched for non-synonymous SNPs (nsSNPs) in the dbSNP database of the NCBI. The 152 nsSNPs that were found were filtered for validation status considering information in the same database. The likely functional consequences of the nsSNPs were assessed with two prediction softwares: SIFT (Sorting Intolerant From Tolerant) (30) (available at http://blocks.fhcrc.org/sift/SIFT.html) that uses alignment to orthologous and homologue protein sequences to determine if the specific

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aminoacid is conserved, and PolyPhen (*Poly*morphism *Pheno*typing) (31) (available at http://genetics.bwh.harvard.edu/pph/index.html) that is based on empirical rules derived from phylogenetic and structural information that are applied to the sequence. Four nsSNPs were predicted as likely deleterious: rs1054480 C/T or P1177S in exon 22 of ADAMTS2, rs4747096 A/G or E1049G in exon 21 of ADAMTS14, rs1019747 T/C or S104P in exon 3 of ADAMTS16, and rs3740199 G/C or G48R in exon 2 of ADAM12. We also searched the PubMED bibliographic database for functional evidence of SNPs modifying ADAM or ADAMTS expression, but none was found.

nsSNP Genotyping

The three sample collections of the first set and the Corunna collection were genotyped by multiplex single-base extension in Santiago. Primers and probes for the four nsSNPs were designed with the FastPCR (obtained from Dr Ruslan Kalendar, University of Helsinki) and Primer3 softwares (Steve Rozen et al) (available at http://frodo.wi.mit.edu/). PCR fragments were amplified in a multiplex PCR reaction (Qiagen Multiplex PCR, Valencia, CA, USA). Oligonucleotide sequences and PCR conditions are detailed in Supplementary Table 1. PCR products were purified by digestion with Exonuclease I (Epicentre, Madison, WI, USA) and shrimp alkaline phosphatase (Amersham Biosciences, Barcelona, Spain). Single-base extension reactions were done with the SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA). Samples were analyzed in the ABI Prism 3130XL Genetic Analyzer (Applied Biosystems). Several samples with different genotypes were sequenced to check for accuracy of results with the Big Dye Ready Reaction Kit (Applied Biosystems). The rs4747096 nsSNP was studied also in samples from the Leiden (GARP) and London (UK Adult Twin Registry) collections. The Leiden samples were genotyped by mass spectrometry (the homogeneous MassARRAY system; Sequenom, San Diego, CA), using standard conditions. The London samples were genotyped at Kbioscience Ltd (Hertfordshire UK) using the KASPar chemistry, which is a competitive allele-specific PCR genotyping system using FRET quencher cassette oligos (http://www.kbioscience.co.uk/genotyping/genotyping-chemistry.htm).

Statistical analysis

A customized version of the Statistica (Statsoft, Tulsa OK) software was used thoroughly. Allele frequencies, odds ratios (O.R.) and their 95% confidence intervals (95% CI) were calculated. Comparison of allele frequencies was done using two by two contingency tables with chi-square tests. This type of analysis was used also for carrier analysis. Evidence of a gene dose effect was evaluated with univariate logistic regression applying an additive genetic model

(codes were: 0 for AA, 1 for Aa and 2 for aa genotypes). For stratification analysis, female patients were compared with female controls and male patients with male controls. Data from populations of Santiago, Thessaly and Oxford were combined using the Mantel-Haenszel test for the 2 x 2 contingency tables stratifying by collection of samples. Homogeneity of effect size across collections was assessed with the Breslow-Day test. Power and sample size calculations were done with the Power and Sample Size software for a value of $\alpha = 0.05$ (32). The Leiden collection required specific assays to compensate for familial relationships: strength of association was assessed with logistic regression analysis performed in STATA and robust standard errors were estimated from the variance between sib pairs.

RESULTS

Search for SNPs likely to affect function of any of the ADAM or ADAMTS proteases (except ADAMTS5) lead to the selection of four nsSNPs that were likely to have deleterious effects according to prediction softwares. These four nsSNPs were studied in three sample collections that included 1412 THR, 781 TKR, 242 HOA and 1185 control samples from Santiago (Spain), Thessaly (Greece) and Oxford (United Kingdom). Genotyping was successful in most samples with a uniform and high calling rate (mean = 0.98) and with results that were in Hardy-Weinberg equilibrium in each of the sample collections. Allele frequencies (Table 1) showed that the nsSNPs rs1054480 in ADAMTS2 and rs1019747 in ADAMTS16 were unrelated with any of the OA phenotypes analyzed, TKR, THR or HOA. Analysis of combined data across the three sample collections confirmed the lack of association (Table 2). The ADAMTS2 and ADAMTS16 nsSNP showed pooled Mantel-Haenszel odds ratios that were indistinguishable from 1 in analyses of TKR and THR patients. These results were not affected by stratifying by gender (Table 2). Analogously, the allele frequencies of the rs3740199 nsSNP in ADAM12 were similar in cases and controls (Table 1), but there were two particularities with this nsSNP. First, it showed significant association in THR males from the Santiago collection (60.8 % of allele C vs. 52.2 % in controls, p = 0.04). However, this difference was not confirmed in THR males from the Oxford collection (46.2 % vs. 46.4 % in controls) and was not present in the joint analysis (Table 2). Second, this ADAM12 SNP showed different allele frequencies depending on the population, with the lowest MAF in the Oxford controls and the highest in the samples from Thessaly (Table 1; Oxford vs. Thessaly $p = 2.2 \times 10^{-7}$; Oxford vs. Santiago p = 0.0003).

The rs4747096 A/G nsSNP in ADAMTS14 was significantly associated to OA in two groups of patient samples (Table 1). The clearest difference was between Oxford TKR patients and controls (allele G frequencies 21.6 vs. 16.0 %, respectively). The significant increase in the allele G frequency was restricted to TKR women of this collection (24.6 % vs. 15.7 % in female controls) with an O.R. of 1.75 (95 % C.I = 1.3-2.4). The TKR men from Oxford showed a G allele percentage that was not significantly larger than in men controls (18.0 % vs. 16.4 %, respectively). There was some indication of a dominant effect of the G allele in the Oxford's TKR women because the association was stronger in carrier analysis (O.R. = 2.05, 95% C.I. 1.4-3.0; p = 0.0001) than in allelic analysis (above) or with an additive genotype model (O.R. = 1.78, 95% C.I. 1.3-2.4; p = 0.0003). TKR samples from the Santiago collection showed also a modest increase in the G allele frequency (Table 1), but it was not significant. The Thessaly samples were neutral, or slightly in the same direction than the Oxford samples (O.R. for women = 1.10, 95% C.I. = 0.7-1.7). Pooled analysis of the three groups of TKR patients showed significant association that was specific of women (Table 2; $O.R._{M-H}$ for women = 1.41, 95 % C.I = 1.1-1.8). TKR men showed a non-significant increase in G allele frequency. Pooled carrier analysis across the three TKR collections showed a slightly stronger association than allelic analysis ($O.R._{M-H}$ of carrier females = 1.47, 95% C.I. 1.1-1.9; p = 0.0036).
The second group of patients that showed significant association to the ADAMTS14 nsSNP was the HOA group from Santiago, which was the unique available with this phenotype. In this instance, the rare G allele was also more frequent in patients than in controls (Table 1; O.R. = 1.37, 95 % C.I = 1.0-1.9), but the increase was similar in women (O.R. = 1.43, 95 % C.I = 0.9-2.2) and men (O.R. = 1.47, 95 % C.I = 0.7-3.1). Carrier analysis showed a similar strength of association (O.R. = 1.44, 95 % C.I = 1.00-2.1) as well as the additive genotype analysis (O.R. = 1.37, 95 % C.I = 1.0-1.9).
In addition to the TKR and HOA significant associations, it was notable that the same allele of the ADAMTS14 nsSNP showed a trend to be more frequent also in the two available groups of THR patients (Table 1). These changes were very modest but they became near significant (p = 0.08) in the combined analysis (Table 2). Carrier analysis of the two sample collections together also showed a near significant association (p = 0.07; $O.R._{M-H}$ = 1.18, 95 % C.I = 0.9-1.5).
Given the interesting results obtained with the rs4747096 ADAMTS14 nsSNP we extended our analysis to other collections of OA samples (Table 3). The Corunna samples were of symptomatic knee OA and were compared with the Santiago controls, as Santiago and Corunna share the same population and are only 60 Km apart. This group showed also a nominal increase in G allele frequency, but only among women (17.9 % *vs.* 15.2 % in controls) and this change

was not significant (O.R. = 1.21, 95 % C.I. = 0.8-1.9). The samples from the GARP study have been selected for sib pair concordance in a generalized OA phenotype. They showed no change in the frequency of the rs4747096 alleles. Finally, we obtained rs4747096 genotypes from some samples with radiographic OA of the UK Adult Twin Registry. They did not show significant frequency changes (Table 3), but this collection was the single one in which OA patients showed a nominal decrease in the frequency of the minor allele of the rs4747096 nsSNPs.

DISCUSSION

This study has examined genetic association with some SNPs likely to have functional effects on a group of genes of potential importance in OA, the ADAMTS and ADAM proteases (11, 15, 22-24). It was not intended as a comprehensive assessment of these two protease families because genetic variation in these genes is much wider than the four nsSNPs studied here. It will not be even a comprehensive analysis of nsSNPs in these genes as new SNPs have continued to be described specially in some of the less studied proteases. Selection of putative deleterious nsSNPs increases the chances of finding an association in a genetic study (33, 34). However, even in this subgroup of nsSNPs the most likely outcome is lack of association. In agreement with this expectation, we did not find association with three of the four nsSNPs studied, in ADAMTS-2, -16 and ADAM-12, respectively. Only the ADAM12 rs3710199 nsSNP deserves a specific commentary because this nsSNP has previously been studied in relation with knee OA susceptibility. The rare allele of this nsSNP has been associated to the presence of osteophytes and change in osteophyte grade in a prospective study of UK women with radiographic knee OA (24). A subsequent study in UK patients with symptomatic knee OA showed no association with this nsSNP, although a haplotype of the ADAM12 gene was associated (35). Our results confirm the lack of association of this SNP with severe knee OA (TKR) and indicate that it is not associated to THR or HOA. However, it should be noted that osteophyte formation and other polymorphisms in the ADAM12 gene were not addressed in our study.

The rs4747096 nsSNP in ADAMTS14 showed significant association to severe OA. Association was clear with severe knee OA, the TKR group, and especially in women from the UK. In addition, association was present in clinical hand OA, HOA. The strength of association was similar in HOA and in TKR females as evaluated by the O.R. (1.37 vs. 1.41, respectively). However, the limited number of available HOA samples made this result more uncertain than the obtained in TKR females. We also noted a near significant association to severe hip OA, the

THR group. In this case, the effect size was lower (O.R. = 1.14), but the large sample size of our THR group (1412 patients) allowed for better detection sensitivity. These are promising but no definitive results. The association to TKR and HOA will require additional studies for confirmation (but one already communicated, see below). Also additional studies will be needed to delineate the effects that this ADAMTS14 nsSNP could have in other OA forms.

We already tried to obtain more information by analyzing the ADAMTS14 nsSNP in other three sample collections. Unfortunately, the results were inconclusive. The symptomatic KOA collection from Corunna showed a trend to increased frequency of the G allele in the women's group, consistent with the observed in TKR women. No change was observed in the generalized OA patients from the GARP study or in the radiographic OA samples from the UK Twin registry. These three collections were of different OA phenotypes and each of them provided limited statistical power. These two aspects make the results in these three collections of uncertain interpretation. Available evidence shows that each OA phenotype is dependent on different genetic factors or with different strength (1-3). Our data in the first set of samples seem to indicate that the ADAMTS14 nsSNP shows also a variable effect depending on joint and gender. In addition, effects of the ADAMTS14 nsSNP are small and their demonstration requires large studies. Sample sizes large enough to detect with 80 % power a difference with O.R. = 1.3 were only available in the pooled analyses of TKR and of THR patients because over 700 cases and 700 controls are needed.

Confidence on the association between symptomatic knee OA in women and ADAMTS14 genetic variation has been reinforced by results of a WGAS done in the UK and communicated in a recent meeting (36). This study has analyzed more than 400 000 SNPs spread over the genome in female knee OA patients and the SNPs showing the largest differences were genotyped in additional collections of samples totalling more than 3000 women. One of the few SNPs that remained associated after replication was a SNP in the forth intron of ADAMTS14 that showed a pooled $O.R._{M-H}$ = 1.2 (95 % C.I. = 1.02-1.41). This study did not overlap with the samples included in our TKR collections. Therefore, two independent studies with a similar OA phenotype and large sample sizes have found association with SNPs in the ADAMTS14 gene. Further analyses will be necessary to show conclusively if the E1049G change determined by the rs4747096 nsSNP is a functional polymorphism or if other functional polymorphisms are present in the ADAMTS14 gene.

The 22 exons of the ADAMTS14 gene are spread over 89.6 Kb in chromosome 10q22.1. This gene was not cloned until 2001 (37) and little is still known about its functions (38, 39). The rs4747096 nsSNP determines a change of glutamic acid for glycine in exon 21 in a segment of

the protein of unclear functional significance. This aminoacid change was predicted as damaging by PoylPhen, a software application that evaluates several characteristics including hydrophobicity, stability, binding, proximity to active sites, homology…(31) Its predictions of damaging changes have specificity over 90 % (40). A glutamic acid in this position is exclusive of the human ADAMTS14 gene. Glycine is likely the ancestral allele as this is the aminoacid in ADAMTS14 orthologues in chimpanzee and rhesus macaque. However, the glutamic acid allele is more common than the glycine allele in Europeans, Africans and Asians, raising the possibility of some advantage associated to it that will support its positive selection in humans. However, little can be said about this or the possible consequences of the aminoacid change until more is known about the function of ADAMTS14. Most available information has been inferred by its similarity to two other ADAMTS, ADAMTS-2 and -3. It has 63 % homology to ADAMTS-3 and 56 % to ADAMTS-2 (37). The three are required for cleaving the N-telopeptides of procollagen before the collagen monomers can be incorporated into fibers. Expression of the three propeptidases has been found to be up-regulated in OA cartilage (22, 23). This over-expression can be related with cartilage repair and production of matrix components as part of the anabolic compensation to cartilage degradation. ADAMTS-14 is produced in a latent form and, after activation, it processes procollagen I in an in vitro system. However, no other substrates have been examined (39). The relevance of ADAMTS-14 activity on procollagen I is unclear as it is not able to replace ADAMTS-2 deficiency in Ehlers-Danlos syndrome type VIIC.

In conclusion, we have found that a change of glutamic acid for glycine in ADAMTS14 was associated with severe knee OA in females and with clinical hand OA. The first association was more robust because of the larger samples size and because of evidence from other study of association between ADAMTS14 genetic variation and knee OA in females. Our results suggested also that the same nsSNP could have association, but weaker, with severe hip OA. These results require confirmation in well powered studies. Also, a systematic study of the gene variation should be done to uncover possible additional polymorphisms. Other OA phenotypes we have examined showed lack of association. The incomplete knowledge of ADAMTS14 functions hampers prediction of its possible role in OA, but it is likely that deficient ADAMTS14 could lead to collagen fibers with incompletely processed collagen that will provoke detrimental effects on cartilage matrix structure and function.

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ACKNOWLEDGEMENTS

We thank Carlos Lopez Otin from the University of Oviedo for advice on ADAMTS14, Manuel Calaza for his help in customizing Statistica software, and Cristina Fernandez for her outstanding technical work. This project was financed by the Fundacion MMA (Madrid, Spain). JR-L is the recipient of a scholarship of the Spanish Ministry of Education. MP-S received a bursary from the Fundacion Española de Reumatologia (Madrid) and a Post-MIR contract from the Instituto de Salud Carlos III (Madrid). The Oxford study was supported by Research into Ageing and by The Arthritis Research Campaign. The Leiden University Medical Centre, the Dutch Arthritis Association and Pfizer Inc., Groton, CT, USA supported the GARP study and controls (Leiden samples). The genotyping of Leiden samples was supported by the Netherlands Organization of Scientific Research (MW 904-61-095, 911-03-016, 917 66344 and 911-03-012), Leiden University Medical Centre and the Centre of Medical System Biology (CMSB).

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Table 1: Allele frequencies of four putative damaging nsSNPs in sample collections with severe OA

		ADAMTS2			ADAMTS16			ADAM12			ADAMTS14		
Collection	Group[a]	T %	P	O.R. (95% C.I.)	C %	P	O.R. (95% C.I.)	C %	P	O.R. (95% C.I.)	G %	P	O.R. (95% C.I.)
Santiago	Control (n = 588)	29.7			36.0			55.6			16.2		
	TKR (n = 524)	31.3	ns	1.07 (0.8-1.4)	36.8	ns	1.03 (0.8-1.3)	55.3	ns	0.98 (0.8-1.2)	17.4	ns	1.09 (0.8-1.5)
	THR (n = 614)	26.5	ns	0.85 (0.7-1.1)	36.3	ns	1.01 (0.8-1.3)	58.8	ns	1.14 (0.9-1.4)	18.4	ns	1.17 (0.9-1.6)
	HOA (n = 484)	28.1	ns	0.92 (0.7-1.2)	33.5	ns	0.89 (0.7-1.15)	57.3	ns	1.06 (0.8-1.4)	20.9	0.047	1.37 (1.0-1.9)
Thessaly	Control (n = 386)	31.9			33.3			62.0			11.5		
	TKR (n = 318)	35.9	ns	1.19 (0.9-1.6)	29.6	ns	0.84 (0.6-1.2)	56.7	ns	0.80 (0.6-1.1)	11.4	ns	0.99 (0.6-1.6)
Oxford	Control (n= 1396)	46.6			38.5			46.6			16.0		
	TKR (n = 720)	48.6	ns	1.07 (0.9-1.3)	36.1	ns	0.90 (0.7-1.1)	48.6	ns	1.07 (0.9-1.3)	21.6	0.001	1.44 (1.1-1.8)
	THR (n= 2210)	46.4	ns	0.99 (0.8-1.2)	42.3	0.06	1.21 (0.98-1.5)	46.4	ns	0.99 (0.8-1.2)	19.0	ns	1.19 (0.9-1.55)

[a] THR = total hip replacement, TKR = total knee replacement, HOA = hand OA, (n = number of alleles).

Table 2: Mantel-Haenszel pooled analyses of the four putative damaging ADAMTS and ADAM nsSNPs in three sample collections with severe OA

Group[a]	ADAMTS2			ADAMTS16			ADAM12			ADAMTS14		
	$O.R._{M-H}$[b] (95 % C.I.)	P	P_{B-D}[c]	$O.R._{M-H}$ (95 % C.I.)	P	P_{B-D}	$O.R._{M-H}$ (95 % C.I.)	P	P_{B-D}	$O.R._{M-H}$ (95 % C.I.)	P	P_{B-D}
TKR (n=1562) (n ctrl=2370)	1.06 (0.9-1.2)	ns	ns	0.9 (0.8-1.1)	ns	ns	1.09 (0.96-1.2)	ns	ns	1.25 (1.05-1.5)	0.009	ns
TKR women (n= 1092) (n ctrl=1216)	1.01 (0.9-1.3)	ns	ns	0.9 (0.8-1.1)	ns	ns	1.12 (0.9-1.3)	ns	ns	1.41 (1.1-1.8)	0.002	ns
TKR men (n = 470) (n ctrl=1154)	0.99 (0.8-1.3)	ns	ns	0.9 (0.7-1.2)	ns	ns	1.17 (0.9-1.45)	ns	ns	1.12 (0.8-1.45)	ns	ns
THR (n=2824)[d] (n ctrl=2370)	1.00 (0.9-1.1)	ns	ns	1.08 (0.96-1.2)	ns	ns	0.92 (0.8-1.04)	ns	ns	1.14 (0.98-1.3)	0.08	ns
THR women (n=1628) (n ctrl=1216)	1.01 (0.8-1.2)	ns	ns	1.05 (0.9-1.2)	ns	ns	0.95 (0.8-1.1)	ns	ns	1.14 (0.9-1.4)	ns	ns
THR men (n = 1194) (n ctrl=1154)	1.01 (0.8-1.2)	ns	ns	1.13 (0.95-1.3)	ns	ns	0.90 (0.8-1.1)	ns	0.08	1.16 (0.9-1.5)	ns	ns

[a] n = total number of alleles of Santiago, Thessalay and Oxford.
[b] Mantel-Haenszel odds ratio
[c] Breslow-Day heterogeneity test
[d] One sample (two alleles) haven't got defined sex

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Table 3. Minor allele frequencies of the ADAMTS14 nsSNPs in other three sample collections with varied OA phenotypes

Collection	Group[a]	ADAMTS14		
		G %	p	O.R. (95% C.I.)
Corunna	Controls[b] (n=588)	16.2		
	KOA (n=530)	16.7	ns	1.04 (0.75-1.4)
Leiden	Controls (n=1424)	16.9		
	GARP (n=716)	17.5	ns	1.04 (0.8-1.3)
London[c]	Controls (n=634)	18.6		
	ROA (n=318)	15.4	ns	0.79 (0.55-1.15)

[a] n = Number of alleles; KOA = sympthomatic and radiographically confirmed knee OA; GARP = Genetics, Arthrosis and Progression, sib pairs concordant for generalized OA; ROA = radiographic OA
[b] Controls from the Santiago collection
[c] All samples of this collection are from women

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Supplementary table1. Primers and probes used in the study

Gene	SNP	PCR primers (5'-3')	SNaP Shot probes (5'-3')	
ADAMTS2	rs 1054480 C/T	tcataggggctcggtcgtcg	aatccatggcctggaagatgaagtccagcca	*R*[a]
		ctccagcaccaatgccacag		
ADAMTS14	rs 4747096 A/G	cacaggaaatcaccagaactcc	gaggggcagtgggtgccacaatctg	*F*
		ctgcaacaccagggccactg		
ADAMTS16	rs 1019747 T/C	tgtcccatgaaatcatgcacc	agtctcttcaccttcggctgaaaggc	*F*
		gcacagacttagtgcctgtc		
ADAM12	rs3740199 G/C	tccctcatcagcactgtcac	cttatggaaccaaggaagagctgatgaagttgtcagtgcctctgtt	*R*
		ccttttgattcaggggtgagc		

[a] Orientation of the probes: *R* = reverse; *F* = forward

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a.2 MMPs

Material y métodos

Pacientes y controles

La colección de muestras del Hospital Clínico Universitario de Santiago de Compostela ha sido descrita con anterioridad (Rodriguez-Lopez J et al, 2007). Está formada por pacientes con OA primaria severa de la cadera que han sufrido remplazo articular total (THR; 307 sujetos, 185 mujeres y 122 hombres) o remplazo articular total de la rodilla (TKR; 277 pacientes, 223 mujeres y 54 hombres) y pacientes aquejados de OA de la mano (HOA; 242 sujetos, 213 mujeres, 27 hombres y 2 no catalogados). El grupo control estaba formado por 294 sujetos (115 mujeres y 179 hombres) mayores de 55 años, los cuales no presentaban ninguna manifestación clínica compatible con OA (tan sólo el 31.6% de los controles reclutados mayores de 55 años pasaron esta selección) (**Tabla R.1**). Los controles tenían una media de edad de 68 años (rango intercuartílico: 61-75 años) en el momento de la recogida. Debido a restricciones éticas y financieras, las articulaciones de la cadera y la rodilla de los controles no fueron sometidas a análisis radiográfico. Todos los pacientes y controles dieron su consentimiento informado para la participación en el estudio y la colección de muestras y el diseño del estudio obtuvieron la aprobación del Comité Ético de Investigación Clínica de Galicia.

Tabla R[a].1: Relación de pacientes y controles de la colección de Santiago. Los datos se muestran también estratificados por sexo y articulación. THR: remplazo articular de la cadera. TKR: remplazo articular de la rodilla. HOA: OA de mano

	Hombres	Mujeres	Total
Controles	179	115	294
THR	122	185	307
TKR	54	223	277
HOA	27	213	242[b]

[a] Se refiere al apartado: resultados, procedimiento experimental y discusión (R)

[b] Dos muestras en este grupo sin dato de sexo

Selección de SNPs

En este estudio se realizó una selección de SNPs reguladores y SNPs codificadores no sinónimos (nsSNPs). Los nsSNPs se obtuvieron de la base de datos de SNPs del NCBI-Entrez. Fueron encontrados varios nsSNPs para cada locus y sólo uno de ellos, rs2250889 C/G (R574P en el exon 4 de *MMP-9*), fue escogido para el estudio ya que presentaba las características de

estar validado y de afectar probablemente al correcto funcionamiento de la proteína. Dicha predicción fue hecha con el software SIFT (Sorting Intolerant From Tolerant) (Ng PC et al, 2006) (disponible en http://blocks.fhcrc.org/sift/SIFT.html), el cual utiliza alineamientos de secuencias de proteínas ortólogas y homólogas para determinar la conservación de aminoácidos específicos. Otro software también utilizado fue PolyPhen (*Poly*morphism *Pheno*typing) (Sunyaev S, et al, 2000) (disponible en http://genetics.bwh.harvard.edu/pph/index.html), el cual está basado en reglas empíricas sencillas que se aplican a la información de la secuencia, de la filogenia y de la estructura de la proteína para caracterizar la sustitución de los aminoácidos, pero con este software no se identificó ningún nsSNP de MMPs como probablemente dañino.
Los polimorfismos reguladores fueron escogidos usando la base de datos de PubMed. Se buscaron SNPs de los que existía evidencia experimental de un efecto regulador y que afectasen a genes de MMPs posiblemente implicadas en el desarrollo y progresión de la OA. Se escogieron 16 SNPs y un microsatélite. Estos polimorfismos se encuentran localizados en 7 MMPs: tres en MMP-1; cuatro en MMP-2; uno en MMP-3; dos en MMP-7; tres en MMP-8; dos SNPs y un microsatélite en MMP-9 y uno en MMP-13 (**Tabla R.2**)

Genotipado

Todos los SNPs, excepto el microsatélite de MMP-9, fueron genotipados utilizando *single-base extensión* con el SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA). Los primers fueron diseñados con los software FastPCR (obtained from Dr Ruslan Kalendar, University of Helsinki) y Primer3 (Rozen S et al, 2000) (disponible en http://frodo.wi.mit.edu/). Los fragmentos de PCR fueron amplificados en reacciones de PCR multiplex (Qiagen Multiplex PCR, Valencia, CA, USA). Los productos de PCR fueron purificados por digestión con *Exonuclease I* (Epicentre, Madison, WI, USA) y *Shrimp Alkaline Phosphatase* (Amersham Biosciences, Barcelona, Spain). Las reacciones de SNaPshot fueron hechas siguiendo las especificaciones del kit. Las muestras fueron posteriormente analizadas en un *ABI Prism 3130XL* Genetic Analyzer Applied Biosystems, Foster City, CA, USA). Varias muestras con diferentes genotipos fueron secuenciadas para chequear la exactitud de los resultados con el *Big Dye Ready Reaction K*it (Applied Biosystems).
El microsatellite de MMP-9 fue amplificado con primers de PCR marcados con FAM. Posteriormente el tamaño de los productos de PCR (indicativo de los distintos alelos del polimorfismo) fue determinado por electroforesis capilar en un *ABI prism 3100 Avant Genetic Analyzer* (Applied Biosystems, Foster City, CA, USA).

Tabla R.2: Polimorfismos estudiados en MMPs. Se señala la posición en el cromosoma y en relación con el inicio de la transcripción. Nótese que 5 MMPs están en el cluster de MMps situado en el cromosoma 11q. También se indican los alelos común y raro y el efecto que el alelo raro produce sobre la expresión o actividad de la proteína. Up: eleva el nivel de expresión. Down: baja el nivel de expresión. – no se conoce. Del: afecta negativamente a la actividad de la proteína.

MMP	Cromosoma	Posición en Cromosoma[a]	Posición inicio transcripción[a]	Número de identificación	Alelos	Efecto alelo raro
MMP-9	20q	44069383	-1562	rs3918242	C/T	Up
		44070820	(~)-80	rs3222264	microsatélite	-
		44075813	+574	rs2250889	C/G	Del
MMP-2	16q	54069038	-1575	rs243866	G/A	Down
		54069307	-1306	rs243865	C/T	Down
		54069823	-790	rs243864	T/G	-
		54069879	-735	rs2285053	C/T	Down
MMP-7	11q	101906843	-155	rs11568819	C/T	Up
		101906871	-183	rs11568818	A/G	Up
MMP-8	11q	102100876	+17	rs2155052	C/G	Up
		102101273	-381	rs1320632	T/C	Up
		102101690	-799	rs11225395	C/T	Up
MMP-1	11q	102174440	-340	rs514921	A/G	Down
		102174619	-519	rs1144393	A/G	Down
		102175707	-1607	rs11292517	A/G	Up
MMP-3	11q	102221162	-1171	rs3025058	T/C	Up
MMP-13	11q	102331749	-77	rs2252070	T/C	Up

[a] En pares de bases

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Las secuencias de oligonucleótidos utilizados para el genotipado se muestran en la **tabla R.3**. El porcentaje de genotipos válidos fue alto (0.98 para todos los SNPs). Las frecuencias genotípicas estaban en equilibrio Hardy-Weinberg (HWE) para todos los SNPs.

Análisis estadístico

El software STATISTICA (Statsoft, Tulsa OK) fue utilizado para el análisis de los datos. Se calcularon frecuencias alélicas, odds ratios (O.R.) y los intervalos de confianza (95% CI). La comparación de las frecuencias alélicas fue hecha utilizando una tabla de contingencia dos por dos con un test de chi-cuadrado. Evidencia de un efecto de dosis génica fue evaluada con regresión logística univariable usando un modelo genético aditivo (Los códigos fueron: 0 para el genotipo AA, 1 para el genotipo Aa y 2 para el genotipo aa). Para el análisis estratificado, los pacientes mujeres fueron comparados con los controles mujeres y los pacientes hombres con los controles hombres. El estudio de "desequilibrio de ligamiento" (LD), la estimación de las frecuencias haplotípicas y la comparación de las mismas entre pacientes y controles fue realizada con el software Haploview. Para el estudio del microsatélite de MMP9, la comparación de frecuencias alélicas fue llevada a cabo usando tablas de contingencia 2 x n después de colapsar columnas de baja frecuencia utilizando la opción T2 del software Clump (Sham PC et al, 1996). El análisis del poder del estudio fue determinado con el software Power and Sample Size para un valor de $\alpha = 0.1$ (Dupont WD et al, 1990).

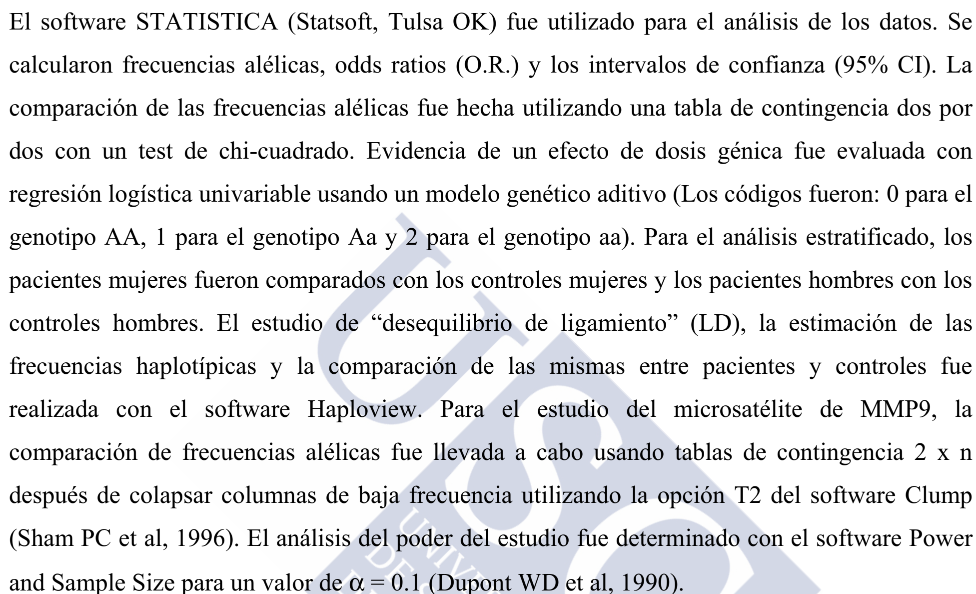

Tabla R.3: Primers de PCR y sondas utilizadas en el estudio

MMP	Polimorfismo		Primer de PCR	Sonda de SNaP Shot[a]
MMP-9	-1562 C/T	Izquierdo	agagccgggatggaaatc	acagagagagtctggacacgtgggg (R)
		Derecho	agcctccctcactcctttct	
	(~)-80	Izquierdo	FAMgacttggcagtggagactgcgggca	
		Derecho	caccсctccttgacaggcaa	
	+574 C/G	Izquierdo	tggcgattctctgagggcag	gagggaaagtaggtaactaaccagagaagaagaaaagcttcttggagagc (F)
		Derecho	cttgactgatggggattgacc	
MMP-2	-1575 G/A	Izquierdo	acacccaccagacaagcctg	GCTAATccgcagagacttttctagctgtgatgatcaagacataatc[b] (F)
		Derecho	acagggtgaggggatggagc	
	-1306 C/T	Izquierdo	caagccgcagagacttttcta	gtgacttctgagctgagacctgaagagctaaagaggt (R)
		Derecho	tcaggtcgaatcccaatttt	
	-790 T/G	Izquierdo	tcgctttctttgccatcttgg	tgcatactgttttatcagtggggtctttgtgacctctatc (F)
		Derecho	gttggaaccagagcgactcc	
	-735 C/T	Izquierdo	tcgctttctttgccatcttgg	GACGATGCTAATatttctatctcatcctgtgaccgagaatgcggac (F)
		Derecho	gttggaaccagagcgactcc	
MMP-7	-155 C/T	Izquierdo	ccatctttcccctgtatgga	gaactttgaaagtatgtgttatttttcattaactaaaacgaggaagtattacatc (R)
		Derecho	ttctcagcctcgaatgtgg	
	-183 A/G	Izquierdo	ccatctttcccctgtatgga	catggagtcaatttatgcagcagacagaaaaaaaaatcctttgaaagac (F)
		Derecho	ttctcagcctcgaatgtgg	

[a] Orientación de la sonda: R = reverse; F = forward
[b] En mayúsculas oligonucleótido de secuencia no humana

Tabla R.3 continuación

MMP	Polimorfismo		Primer de PCR	Sonda de SNaP Shot[a]
MMP-8	+17 C/G	Izquierdo	tgcttcatattcctgatgcagc	GCTGTGACGATGCTAATaagagagctgtgagtgacacatgatgctgtgaac (F)
		Derecho	tttctgtccctctgggtagg	
	-381 T/C	Izquierdo	acatcctccagaagcagctc	TATCGCGCTGTGACGATGCTAATggaccaaggagcccaactagagaatctctgtactta (R)
		Derecho	ttgggaccaaggagcccaac	
	-799 C/T	Izquierdo	tctcccttgtcttctgcctg	GATGCTAATcaagtgggagactaccatgcagagc (F)
		Derecho	catcttcacatagccttgggag	
MMP-1	-340 A/G	Izquierdo	gcttgagccaccatgcctgg	GTGACGATGCTAATGccatggtctttttgaaaaagactggttctgatggtcataa[b] (R)
		Derecho	gcgaaggagacacactctgc	
	-519 A/G	Izquierdo	gcttgagccaccatgcctgg	attaagggaagccatggtgctatcgcaatagggt (F)
		Derecho	gcgaaggagacacactctgc	
	-1607 A/G	Izquierdo	tcacttcagtggcaagtgttct	cctcttgaactcacatgttatgccacttagatgaggaaattgtagttaaataattagaaag (F)
		Derecho	tggtgctaggcagcatttat	
MMP-3	-1171 T/C	Izquierdo	tggccaaatattttccctgta	aaatattttccctgtatttcaatcaggacaagacatggttttt (F)
		Derecho	gagctctgggctcaagtgat	
MMP-13	-77 T/C	Izquierdo	tgcttcttcccacagtatcca	ttatggataggtttccacttcc (R)
		Deerecho	tcgtcaagtttgcgagtcac	

[a] Orientación de la sonda: R = reverse; F = forward
[b] En mayúsculas oligonucleótido de secuencia no humana

Resultados

Estudio de Desequilibrio de ligamiento

Se realizó un estudio de desequilibrio de ligamiento (LD) entre los SNPs situados en un mismo gen y entre los situados en las MMPs del cluster de MMPs del cromosoma 11q. El estudio de desequilibrio de ligamiento realizado con los SNPs que se encontraban en el cluster de MMPs del cromosoma 11q reveló que, en general, el LD era muy débil entre SNPs que no se encontraban en el mismo gen. Tan sólo el caso del SNP -340 de MMP-1 mostró un LD relativamente alto con los SNPs -381 y +17 de MMP-8 (D' = 0.72). También se observó un cierto LD entre el SNP -1171 de MMP-3 y el SNP -1607 de MMP-1 (D' = 0.59) (**Figura R.1**).

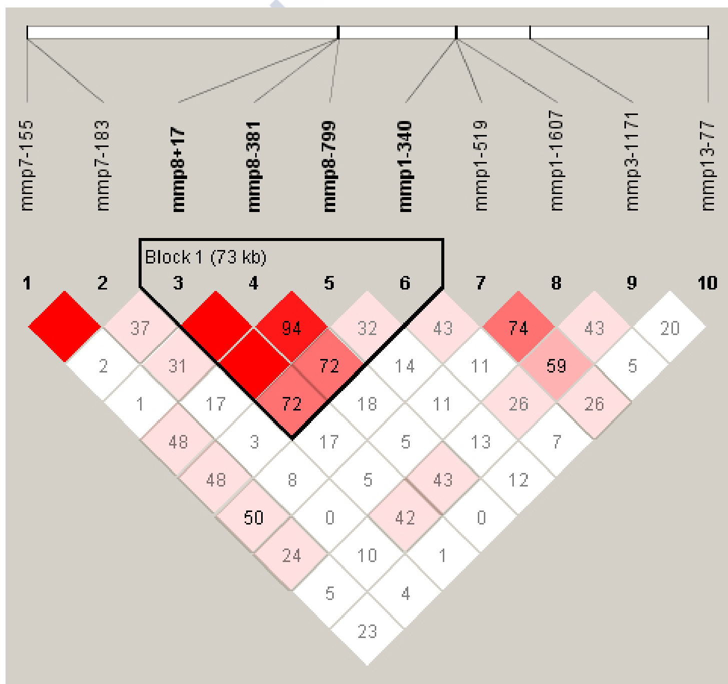

Figura R.1: Desequilibrio de ligamiento (LD) según el parámetro D' entre los 10 polimorfismos existentes en las MMPs encontradas en el cluster de MMPs del cromosoma 11q. El color rojo indica D' = 1 y LOD ≥ 2. En el bloque se muestran los tres SNPs de MMP-8 con un alto LD junto con el SNP -340 de MMP-1, que muestra una D' bastante alta con +17 y -381 (D' = 0.72).

El análisis de la r^2 demostró que la correlación entre genotipos era muy baja entre los SNPs de distintos genes. Tan sólo en el caso del SNP MMP-3 -1171 se muestra un valor ligeramente elevado de r^2 con los SNPs MMP-1 -1607 y MMP-1 -519 (0.17 y 0.19 respectivamente). Los

SNPs que se encuentran en un mismo gen tampoco muestran valores de r^2 muy significativos excepto en el caso de los SNPs MMP-8 +17 y MMP-8 -381, que presentan una r^2 de 0.98 (**Figura R.2**).

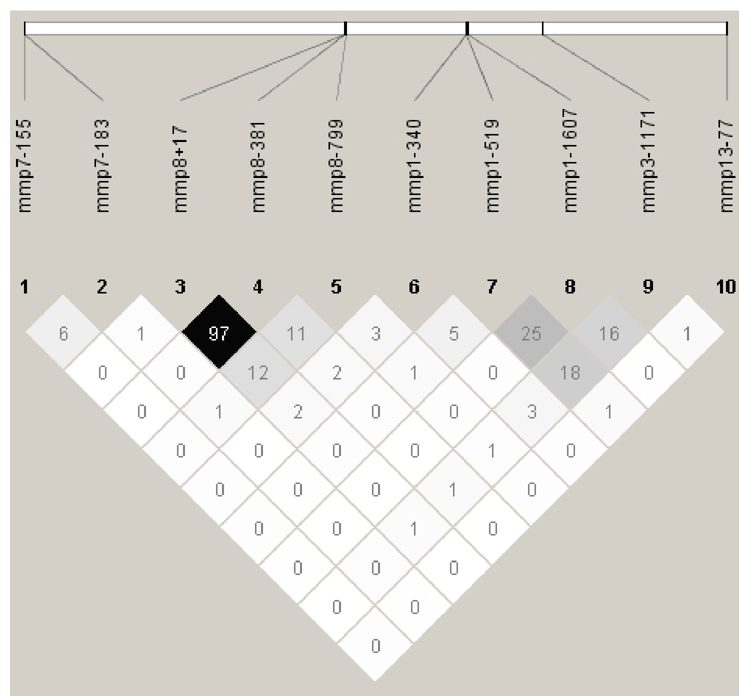

Figura R.2: Desequilibrio de ligamiento (LD) según el parámetro r^2 entre los 10 polimorfismos existentes en las MMPs encontradas en el cluster de MMPs del cromosoma 11q.

Entre los SNPs situados dentro de un mismo gen, los situados en MMP-2 mostraron un gran desequilibrio de ligamiento con altos valores de D’ y r^2 (**Figura R.3**). En la figura se puede observar como los SNPs -1575, -1306 y -790 están correlacionados con valores de r^2 muy altos mientras el SNP -735 es independiente. En el caso de los SNPs -1562 y +574 del gen *MMP-9,* se observó un valor de D’ elevado (0.92) pero sin embargo el valor de r^2 fue de cero (datos no mostrados). El resto de MMPs estudiadas se encuentran en el cluster de MMPs del cromosoma 11q por lo que sus valores de D’ y r^2 se pueden ver en las figuras R.1 y R.2.

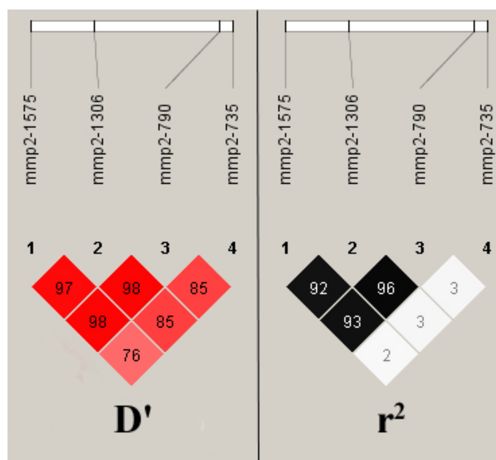

Figura R.3: Desequilibrio de ligamiento (LD) según el parámetro D' y r^2 entre los 4 polimorfismos estudiados en el gen *MMP-2.*

Frecuencias alélicas pacientes y controles

Para analizar si SNPs en proteasas de ECM eran factores de riesgo para OA, comparamos frecuencias alélicas de pacientes y controles, estratificando por la articulación afectada (THR, TKR o HOA). El estudio se realizó en primer lugar sobre la población en general y posteriormente se realizó una estratificación por sexos. En el caso de los pacientes de THR se encontró que de los dieciséis SNPs estudiados, catorce de ellos tenían frecuencias similares en pacientes y controles. Tan sólo dos SNPs en el gen de *MMP-2,* presentaron diferencias significativas entre pacientes THR y controles (-790 T/G; -1575 G/A) (**Tabla R.4**). Para discernir si los SNPs de *MMP-2* asociados eran independientes entre sí o si los efectos podrían ser atribuirse a desequilibrio de ligamiento entre ellos, se hizo un análisis de los genotipos mediante regresión logística condicional. En este análisis se tomaba como base el SNP asociado de manera más significativa (SNP principal) y se testaba la asociación de los otros SNPs utilizando un modelo condicionado de dos variables (el SNP que se testaba más el SNP principal). De esta manera, si el SNP testado dejaba de estar asociado y el principal seguía estándolo, se concluía que su efecto no era independiente del SNP principal. Si los dos SNPs dejaban de estar asociados, entonces era que el efecto podía ser explicado por los dos en conjunto o por separado. En MMP-2, los dos SNPs asociados tienen una r^2 muy elevada y, por lo tanto, cualquiera de ellos o los dos pueden explicar la asociación.

Tabla R.4: Frecuencias alélicas de los SNPs estudiados en controles y pacientes THR.

MMP	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
MMP-9	-1562 C/T	14.85	17.61	ns	1.22 (0.89-1.67)
	+574 C/G	3.57	3.85	ns	1.10 (0.59-1.97)
MMP-2	-1575 G/A	19.90	25.08	0.03	1.35 (1.02-1.77)
	-1306 C/T	19.45	24.08	0.05	1.31 (0.99-1.73)
	-790 T/G	19.22	25.41	0.01	1.43 (1.08-1.88)
	-735 C/T	15.48	13.70	ns	0.87 (0.63-1.19)
MMP-7	-155 C/T	4.93	5.33	ns	1.10 (0.65-1.82)
	-183 A/G	45.75	44.00	ns	0.93 (0.74-1.17)
MMP-8	+17 C/G	7.65	7.26	ns	0.94 (0.61-1.45)
	-381 T/C	7.85	7.26	ns	0.92 (0.59-1.41)
	-799 C/T	38.44	36.59	ns	0.93 (0.72-1.18)
MMP-1	-340 A/G	33.39	31.71	ns	0.93 (0.72-1.18)
	-519 A/G	34.71	37.17	ns	1.11 (0.88-1.41)
	-1607 A/G	46.76	48.83	ns	1.08 (0.86-1.36)
MMP-3	-1171 T/C	50.17	46.33	ns	0.85 (0.68-1.07)
MMP-13	-77 T/C	32.42	32.50	ns	1.00 (0.79-1.28)

[a] Se muestra la frecuencia del alelo raro en %

A continuación, se realizó una estratificación por sexos de las poblaciones de pacientes y controles y se compararon las frecuencias alélicas. En el caso de las mujeres se obtuvieron resultados significativos con los SNPs de *MMP-2* (-790 T/G; -1575 G/A; -1306 C/T) y *MMP-1*(-519 A/G; -340 A/G) (**Tabla R.5)**. En los hombres, tan sólo uno de los dieciséis SNPs estudiados, el SNP -1562 C/T de *MMP-9,* mostró una diferencia nominalmente significativa de frecuencias alélicas entre pacientes y controles. Otros dos SNPs mostraron una tendencia. MMP-2 -790 T/G, que ya se había observado en el análisis global y en mujeres, y el SNP MMP-3 -1171 T/C, que mostró una tendencia que no se había visto en los análisis anteriores (**Tabla R.6**).

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Tabla R.5: Frecuencias alélicas de los SNPs estudiados en controles y pacientes THR mujeres.

MMP	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
MMP-9	-1562 C/T	17.83	16.76	ns	0.93 (0.60-1.43)
	+574 C/G	2.61	3.93	ns	1.53 (0.58-4.03)
MMP-2	-1575 G/A	16.96	24.86	0.02	1.62 (1.06-2.46)
	-1306 C/T	16.96	24.59	0.02	1.59 (1.05-2.43)
	-790 T/G	16.52	24.59	0.01	1.64 (1.08-2.51)
	-735 C/T	17.83	15.03	ns	0.81 (0.52-1.27)
MMP-7	-155 C/T	5.65	5.80	ns	1.03 (0.50-2.09)
	-183 A/G	48.70	45.30	ns	0.87 (0.63-1.21)
MMP-8	+17 C/G	4.78	7.92	ns	1.71 (0.83-1.21)
	-381 T/C	4.78	7.92	ns	1.71 (0.83-3.50)
	-799 C/T	30.43	35.99	ns	1.28 (0.90-1.83)
MMP-1	-340 A/G	40.27	32.04	0.04	0.69 (0.49-0.98)
	-519 A/G	28.51	39.01	0.008	1.60 (1.12-2.29)
	-1607 A/G	50.88	48.07	ns	0.89 (0.64-1.25)
MMP-3	-1171 T/C	47.37	47.51	ns	1.00 (0.72-1.40)
MMP-13	-77 T/C	31.30	33.79	ns	1.12 (0.79-1.60)

[a] Se muestra la frecuencia del alelo raro en %

En las mujeres THR, se realizó el análisis de genotipos mediante regresión logística condicionada para discernir qué SNPs de *MMP-2* y *MMP-1* eran los responsables del efecto observado. Los resultados mostraron que los efectos de los SNPs de *MMP-2* no eran independientes, pudiendo ser el SNP causal cualquiera de los dos por separado o los dos en conjunto. El análisis de los SNPs de *MMP-1,* mostró resultados análogos a los obtenidos para MMP-2.

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Tabla R.6: Frecuencias alélicas de los SNPs estudiados en controles y pacientes THR hombres.

MMP	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
MMP-9	-1562 C/T	12.92	18.91	0.048	1.57 (1.00-2.46)
	+574 C/G	4.19	3.75	ns	0.89 (0.38-2.07)
MMP-2	-1575 G/A	21.79	25.63	ns	1.24 (0.84-1.82)
	-1306 C/T	21.07	23.31	ns	1.14 (0.77-1.69)
	-790 T/G	20.95	26.89	0.09	1.39 (0.95-2.04)
	-735 C/T	13.97	11.34	ns	0.79 (0.48-1.30)
MMP-7	-155 C/T	4.47	4.62	ns	1.03 (0.47-2.27)
	-183 A/G	43.85	42.02	ns	0.93 (0.67-1.29)
MMP-8	+17 C/G	9.50	6.30	ns	0.64 (0.64-1.20)
	-381 T/C	9.83	6.30	ns	0.62 (0.33-1.16)
	-799 C/T	43.58	37.82	ns	0.79 (0.56-1.10)
MMP-1	-340 A/G	28.90	31.03	ns	1.11 (0.77-1.59)
	-519 A/G	38.70	34.62	ns	0.84 (0.59-1.18)
	-1607 A/G	44.13	50.00	ns	1.27 (0.91-1.76)
MMP-3	-1171 T/C	51.99	44.54	0.07	0.74 (0.53-1.03)
MMP-13	-77 T/C	33.15	30.51	ns	0.88 (0.62-1.26)

[a] Se muestra la frecuencia del alelo raro en %

La comparación de las frecuencias alélicas entre el grupo de pacientes con OA de la rodilla (TKR) y el grupo control, mostró que sólo el SNP -340 A/G de *MMP-1* presentaba diferencias significativas (**Tabla R.7**). Esta diferencia era en el mismo sentido que la encontrada en el grupo de pacientes de THR y HOA.

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Tabla R.7: Frecuencias alélicas de los SNPs estudiados en controles y pacientes TKR.

MMP	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
MMP-9	-1562 C/T	14.85	17.12	ns	1.18 (0.86-1.63)
	+574 C/G	3.57	3.21	ns	0.89 (0.47-1.71)
MMP-2	-1575 G/A	19.90	23.85	ns	1.26 (0.85-1.68)
	-1306 C/T	19.45	23.18	ns	1.25 (0.94-1.67)
	-790 T/G	19.22	23.08	ns	1.26 (0.94-1.68)
	-735 C/T	15.48	14.81	ns	0.95 (0.68-1.32)
MMP-7	-155 C/T	4.93	4.41	ms	0.89 (0.51-1.56)
	-183 A/G	45.75	44.83	ns	0.96 (0.76-1.22)
MMP-8	+17 C/G	7.65	10.19	ns	1.37 (0.90-2.07)
	-381 T/C	7.85	10.62	ns	1.39 (0.92-2.10)
	-799 C/T	38.44	36.92	ns	0.94 (0.73-1.19)
MMP-1	-340 A/G	33.39	27.45	0.03	0.75 (0.58-0.98)
	-519 A/G	34.71	35.33	ns	1.03 (0.80-1.32)
	-1607 A/G	46.76	48.27	ns	1.06 (0.84-1.34)
MMP-3	-1171 T/C	50.17	46.55	ns	0.86 (0.68-1.09)
MMP-13	-77 T/C	32.42	32.76	ns	1.01 (0.79-1.30)

[a] Se muestra la frecuencia del alelo raro en %

El estudio realizado estratificando por sexos mostró que, en las mujeres, seis de los dieciséis SNPs analizados presentaban diferencias significativas (**Tabla R.8**). Los SNPs asociados se encontraban en los genes *MMP-2* (-1575 G/A; -790 T/G) y *MMP-1* (-340 A/G; -519 A/G), con cambios en el mismo sentido que los observados en las mujeres con THR. Además, fueron significativamente distintas las frecuencias alélicas de los SNPs de *MMP-8,* -381 T/C y +17 C/G, con cambios en el mismo sentido que las tendencias observadas en mujeres con THR. En el estudio llevado a cabo en los hombres no se obtuvo ninguna diferencia significativa, aunque la frecuencia del alelo raro del SNP -1562 C/T de *MMP-9* mostró una tendencia a ser más frecuente en pacientes (**Tabla R.9**). No parece que el menor número de muestras de hombres pueda explicar completamente las distintas variaciones encontradas entre hombres y mujeres.

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Tabla R.8: Frecuencias alélicas de los SNPs estudiados en controles y pacientes TKR mujeres.

MMP	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
MMP-9	-1562 C/T	17.83	16.51	ns	0.91 (0.59-1.39)
	+574 C/G	2.61	3.27	ns	1.27 (0.48-3.33)
MMP-2	-1575 G/A	16.96	24.05	0.03	1.55 (1.03-2.34)
	-1306 C/T	16.96	23.33	0.05	1.49 (0.98-2.25)
	-790 T/G	16.52	23.33	0.04	1.53 (1.01-2.33)
	-735 C/T	17.83	14.52	ns	0.78 (0.51-1.21)
MMP-7	-155 C/T	5.65	3.81	ns	0.66 (0.31-1.40)
	-183 A/G	48.70	44.76	ns	0.85 (0.62-1.18)
MMP-8	+17 C/G	4.78	9.76	0.02	2.15 (1.08-4.27)
	-381 T/C	4.78	10.29	0.01	2.28 (1.15-4.52)
	-799 C/T	30.43	36.43	ns	1.31 (0.93-1.85)
MMP-1	-340 A/G	40.27	27.07	0.0007	0.55 (0.39-0.77)
	-519 A/G	28.51	36.12	0.049	1.42 (0.99-2.01)
	-1607 A/G	50.88	43.37	ns	0.87 (0.63-1.20)
MMP-3	-1171 T/C	47.37	44.76	ns	0.90 (0.65-1.24)
MMP-13	-77 T/C	31.30	34.52	ns	1.16 (0.82-1.63)

[a] Se muestra la frecuencia del alelo raro en %

A continuación se realizó el estudio de los genotipos mediante regresión logística condicionada para los SNPs de *MMP-2, MMP-1 y MMP-8* asociados con TKR en el subgrupo de las mujeres. Este análisis puso de manifiesto que el SNP -340 A/G en el caso de *MMP-1* es el SNP que explica el efecto observado, siendo el otro SNP asociado, -519 A/G, dependiente de él ya que su valor de “p” pasa a ser no significativo mientras que el valor de -340 A/G, aunque disminuye, sigue siendo significativo (Datos no mostrados). En el caso de las asociaciones encontradas en MMP-2 y MMP-8, los análisis realizados mostraron que los SNPs asociados no eran independientes entre ellos por lo que el efecto observado puede estar explicado por cualquiera de ellos. Por otra parte, *MMP-1* y *MMP-8* son dos genes que codifican para la colagenasa 1 y la colagenasa 2 respectivamente que están situados en el cluster de metaloproteasas de matriz extracelular del cromosoma 11q. Su distancia en el cromosoma es de 65 Kb y la D’ entre los SNPs -381 y -340 es de 0.72 (**Figura R.1**). El análisis de los genotipos con regresión logística condicionada mostró que el efecto observado para MMP-1 -340 A/G y MMP-8 -381T/C es principalmente debido a MMP-1 -340 A/G ya que los dos SNPs no son independientes. El

efecto de MMP-8 -381T/C pasa a ser no significativo al condicionarlo a MMP-1 -340 A/G mientras que MMP-1 A/G mantiene su significación al condicionarlo al MMP-8 -381T/C. De esta manera podríamos decir que el efecto del SNP MMP-8 -381 T/C puede estar explicado por MMP-1 -340 A/G, pero este resultado hay que tomarlo con cautela porque son SNPs de genes distintos bastante separados físicamente en el cromosoma y que poseen una r^2 muy pequeña ($r^2 = 0.022$) (**Figura R.2**).

Tabla R.9: Frecuencias alélicas de los SNPs estudiados en controles y pacientes TKR hombes.

MMP	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
MMP-9	-1562 C/T	12.92	19.61	0.09	1.64 (0.92-2.93)
	+574 C/G	4.19	2.94	ns	0.69 (0.20-2.44)
MMP-2	-1575 G/A	21.79	23.00	ns	1.07 (0.63-1.82)
	-1306 C/T	21.07	22.55	ns	1.09 (0.64-1.85)
	-790 T/G	20.95	22.00	ns	1.06 (0.62-1.82)
	-735 C/T	13.97	16.00	ns	1.17 (0.63-2.16)
MMP-7	-155 C/T	4.47	6.86	ns	1.57 (0.63-3.94)
	-183 A/G	43.85	45.10	ns	1.05 (0.67-1.64)
MMP-8	+17 C/G	9.50	12.00	ns	1.29 (0.65-2.61)
	-381 T/C	9.83	12.00	ns	1.25 (0.62-2.51)
	-799 C/T	43.58	39.00	ns	0.83 (0.53-1.30)
MMP-1	-340 A/G	28.90	29.00	ns	1.00 (0.61-1.64)
	-519 A/G	38.70	32.00	ns	0.74 (0.46-1.19)
	-1607 A/G	44.13	51.96	ns	1.37 (0.88-2.13)
MMP-3	-1171 T/C	51.99	53.92	ns	1.08 (0.69-1.68)
MMP-13	-77 T/C	33.15	25.49	ns	0.69 (0.42-1.13)

[a] Se muestra la frecuencia del alelo raro en %

El estudio de asociación caso control en pacientes con OA de la mano reveló que las frecuencias fueron muy similares entre pacientes y controles en todos los SNPs estudiados. Tan sólo se observó una tendencia en el caso de los SNPs -381 T/C y +17 C/G de *MMP-8* pero las diferencias no fueron significativas (**Tabla R.10**).

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Tabla R.10: Frecuencias alélicas de los SNPs estudiados en controles y pacientes HOA.

MMP	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
MMP-9	-1562 C/T	14.85	13.79	ns	0.92 (0.65-1.30)
	+574 C/G	3.57	4.18	ns	1.18 (0.63-2.20)
MMP-2	-1575 G/A	19.90	21.46	ns	1.10 (0.81-1.48)
	-1306 C/T	19.45	21.04	ns	1.10 (0.82-1.49)
	-790 T/G	19.22	21.67	ns	1.16 (0.86-1.57)
	-735 C/T	15.48	13.09	ns	0.82 (0.58-1.67)
MMP-7	-155 C/T	4.93	4.34	ns	0.87 (0.49-1.55)
	-183 A/G	45.75	44.01	ns	0.93 (0.73-1.19)
MMP-8	+17 C/G	7.65	10.94	0.06	1.48 (0.97-2.26)
	-381 T/C	7.85	10.94	0.08	1.44 (0.95-2.19)
	-799 C/T	38.44	40.49	ns	1.10 (0.85-1.40)
MMP-1	-340 A/G	33.39	30.93	ns	0.89 (0.68-1.17)
	-519 A/G	34.71	37.80	ns	1.14 (0.88-1.48)
	-1607 A/G	46.76	48.54	ns	1.07 (0.84-1.37)
MMP-3	-1171 T/C	50.17	49.59	ns	0.98 (0.77-1.24)
MMP-13	-77 T/C	32.42	31.77	ns	0.97 (0.74-1.28)

[a] Se muestra la frecuencia del alelo raro en %

La estratificación por sexos mostró que existían SNPs con diferencias significativas entre pacientes y controles en mujeres y en hombres. En las mujeres se encontraron diferencias en los tres SNPs de *MMP-8* estudiados (+17 C/G; -381 T/C y -799 C/T). Estos cambios fueron en el mismo sentido que los encontrados en mujeres con THR y mujeres con TKR. En el SNP -340 A/G de *MMP-1* se observó también una disminución del alelo raro en mujeres similar a la observada en mujeres con THR y mujeres con TKR. A su vez también se observaron cambios no significativos en los SNPs -790 T/G y -735 C/T de *MMP-2* y -519 A/G de *MMP-1* (**Tabla R.11**).

Al igual que en los anteriores análisis, se hizo un estudio de las frecuencia genotípicas mediante regresión logística condicionada en los casos en los que se encontró más de un SNP asociado dentro del mismo gen para discernir a cuál de los SNPs era debido el efecto observado. En el caso de *MMP-8,* los SNPs +17 C/G y -381 T/C tenían una distribución de frecuencias exactamente iguales por lo que ya se demuestra que no son independientes. Se tomó como SNP

Tabla R.11: Frecuencias alélicas de los SNPs estudiados en controles y pacientes HOA mujeres.

MMP	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
MMP-9	-1562 C/T	17.83	13.24	ns	0.70 (0.45-1.09)
	+574 C/G	2.61	4.29	ns	1.67 (0.65-4.27)
MMP-2	-1575 G/A	16.96	22.33	ns	1.41 (0.93-2.13)
	-1306 C/T	16.96	22.17	ns	1.39 (0.92-2.11)
	-790 T/G	16.52	22.82	0.05	1.49 (0.98-2.27)
	-735 C/T	17.83	12.62	0.07	0.67 (0.43-1.04)
MMP-7	-155 C/T	5.65	4.69	ns	0.82 (0.40-1.68)
	-183 A/G	48.70	45.77	ns	0.89 (0.64-1.23)
MMP-8	+17 C/G	4.78	11.17	0.004	2.50 (1.27-4.93)
	-381 T/C	4.78	11.17	0.004	2.50 (1.27-4.93)
	-799 C/T	30.43	39.80	0.018	1.51 (1.07-2.13)
MMP-1	-340 A/G	40.27	31.68	0.03	0.69 (0.49-0.97)
	-519 A/G	28.51	35.22	0.09	1.36 (0.95-1.95)
	-1607 A/G	50.88	49.29	ns	0.93 (0.68-1.29)
MMP-3	-1171 T/C	47.37	48.59	ns	1.05 (0.76-1.45)
MMP-13	-77 T/C	31.30	31.18	ns	0.99 (0.69-1.43)

[a] Se muestra la frecuencia del alelo raro en %

de referencia el -381 T/C por los resultados observados en TKR mujeres. El análisis del SNP -799 C/T, demostró que este SNP no tenía un efecto independiente ya que su p valor pasaba de de 0.02 a 0.31, sin embargo el SNP -381 T/C sí seguía estando significativamente asociado. En *MMP-1,* el análisis demostró que los dos SNPs asociados, el SNP -519 A/G y el -340 A/G, no eran independientes por lo que el efecto puede deberse a cualquiera de los dos o a los dos conjuntamente. También en este caso se hizo el análisis de regresión logística condicionada de los SNPs -381 T/C de *MMP-8* y -340 A/G de *MMP-1.* El resultado mostró que en este caso, a diferencia de lo observado en mujeres con TKR, el SNP causante del efecto era el -381 T/C ya que seguía manteniendo su nivel de significación. Al igual que lo dicho anteriormente, este resultado debe tomarse con cautela debido a que ambos SNPs están bastante separados y la r^2 entre ellos es pequeña (r^2 = 0.022).

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El estudio llevado a cabo en los hombres mostró que existían diferencias significativas en uno de los SNPs estudiados de MMP-1, concretamente el -519 A/G. También se encontró que había un cambio entre pacientes y controles en el caso del SNP -183 A/G de *MMP-7*, pero este no llegaba a ser significativo (**Tabla R.12**).

Tabla R.12: Frecuencias alélicas de los SNPs estudiados en controles y pacientes HOA hombres.

MMP	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
MMP-9	-1562 C/T	12.92	19.23	ns	1.60 (0.75-3.42)
	+574 C/G	4.19	3.70	ns	0.88 (0.19-3.96)
MMP-2	-1575 G/A	21.79	15.38	ns	0.65 (0.29-1.44)
	-1306 C/T	21.07	12.96	ns	0.56 (0.24-1.28)
	-790 T/G	20.95	13.46	ns	0.59 (0.25-1.35)
	-735 C/T	13.97	15.38	ns	1.12 (0.49-2.52)
MMP-7	-155 C/T	4.47	1.85	ns	0.40 (0.05-3.10)
	-183 A/G	43.85	31.48	0.08	0.58 (0.32-1.08)
MMP-8	+17 C/G	9.50	9.62	ns	1.01 (0.37-2.72)
	-381 T/C	9.83	9.62	ns	0.97 (0.36-2.61)
	-799 C/T	43.58	46.00	ns	1.10 (0.61-1.99)
MMP-1	-340 A/G	28.90	23.91	ns	0.77 (0.38-1.58)
	-519 A/G	38.70	58.70	0.01	2.25 (1.20-4.20)
	-1607 A/G	50.88	49.29	ns	0.94 (0.68-1.29)
MMP-3	-1171 T/C	51.99	57.41	ns	1.24 (0.69-2.22)
MMP-13	-77 T/C	33.15	36.36	ns	1.15 (0.60-2.21)

[a] Se muestra la frecuencia del alelo raro en %

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Frecuencias haplotípicas pacientes y controles

El análisis de haplotipos apenas aportó nuevas diferencias significativas con respecto al estudio de frecuencias alélicas. El único resultado distinto fue el de los haplotipos de MMP-8 en hombres con THR, ya que no se había encontrado nada parecido en el análisis de frecuencias alélicas (**Tabla R.13**). El haplotipo asociado en MMP-8, C-T-C, está formado por los alelos que provocan una disminución de la expresión de *MMP-8.* Este haplotipo se encontró aumentado en los casos que es el mismo efecto que se observó para +17 C/G y -381 T/C en el estudio de frecuencias alélicas en hombres con THR. De todos modos, este efecto sólo se vio en ese subgrupo de pacientes siendo el contrario que el observado en todos los demás, por lo que puede haberse debido a fluctuaciones al azar de las frecuencias alélicas provocadas por el pequeño

tamaño muestral de los varones con THR (n = 122). Además, Se encontraron asociaciones con los haplotipos definidos en MMP-1 y MMP-2 en pacientes con THR (**Tabla R.13**). Los resultados obtenidos en MMP-2 representan la idea de que los tres SNPs que mostraron asociación, -1575 G/A, -1306 C/T y -790 T/G, son indistinguibles pues los alelos asociados con THR se encuentran exclusivamente en el único haplotipo asociado: A-T-G-C.

Tabla R.13: Distribución de las frecuencias de los haplotipos de MMPs que han mostrado asociación con THR. Se muestran los haplotipos con una frecuencia superior al 5%. Los resultados pertenecen al estudio global así como los derivados de la estratificación por sexos.

	Haplotipo	Controles	Casos	P valor
Total				
MMP2				
-1575/-1306/-790/-735	G-C-T-C	64.9	60.7	ns
	A-T-G-C	18.7	24.3	0.019
	G-C-T-T	15.0	13.3	ns
Mujeres				
MMP2				
-1575/-1306/-790/-735	G-C-T-C	65.7	59.7	ns
	A-T-G-C	15.6	24.7	0.008
	G-C-T-C	16.9	13.5	ns
MMP1				
-340/-519/-1607	A-A-G	26.3	27.4	ns
	A-G-A	20.1	29.4	0.01
	G-AG	21.5	17.0	ns
	A-A-A	12.0	10.0	ns
	G-A-A	11.8	7.4	ns
	G-G-A	5.1	5.0	ns
Hombres				
MMP8				
+17/-381/-799	C-T-C	55.6	65.3	0.02
	C-T-T	34.6	28.1	ns
	G-C-T	9.5	6.6	ns

Los haplotipos de MMP-1 fueron muy informativos. Por una parte, el análisis con los tres SNPs mostró que son totalmente independientes (ya sugerido por el análisis de LD, **Figura R.2**). Se observaron 6 haplotipos con frecuencia mayor del cinco por ciento y alguno de ellos se encontró asociado en el subgrupo de mujeres con THR (**Tabla R.13**). Estos resultados parecen contradictorios con los encontrados en los SNPs por separado (**Tabla R.4 a R.12**), por lo que se realizó un nuevo análisis de haplotipos de MMP-1 tomando únicamente los SNPs -340 A/G y -519 A/G, que fueron los que se encontraron asociados de manera individual. Como se vio que este tipo de análisis era más coherente con los resultados de los SNPs individuales, y como los SNPs mostraban un escaso LD, se aplicó a todos los fenotipos (**Tabla R.14**).

Tabla R.14: Distribución de las frecuencias de los haplotipos de MMP-1 con los SNPs -340 A/G y -519 A/G que han mostrado asociación con THR, TKR y HOA. Se muestran los haplotipos con una frecuencia superior al 5%.

	Haplotipo	Controles	Casos	P valor
THR mujeres				
-340/-519	A-A	37.7	37.0	ns
	G-A	33.8	24.7	0.017
	A-G	22.0	31.4	0.013
	G-G	6.5	7.0	ns
TKR mujeres				
-340/-519	A-A	36.9	39.3	ns
	G-A	34.6	25.1	0.009
	A-G	22.8	32.3	0.01
	G-G	5.7	3.4	ns
HOA mujeres				
-340/-519	A-A	38.2	40.4	ns
	G-A	33.3	24.4	0.01
	A-G	21.5	27.9	ns
	G-G	7.0	7.4	ns
HOA hombres				
-340/-519	A-A	38.7	25.3	ns
	G-A	22.6	18.4	ns
	A-G	32.3	49.7	0.02
	G-G	6.4	6.6	ns

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Este análisis muestra cuatro haplotipos frecuentes, dos de ellos son neutros en todas las comparaciones, A-A y G-G. Los otros dos son diferentes entre casos y controles en una parte de los análisis. Este patrón nos muestra claramente que el alelo A de -340 A/G sólo predispone a OA en el haplotipo con el alelo G del SNP -519 A/G, ya que el haplotipo A-A es neutro. De forma similar, el alelo G del SNP -340 A/G sólo protege de OA en el haplotipo G-A y no en el haplotipo G-G. Por lo tanto, el efecto de estos dos SNPs es interdependiente y sólo se observa en combinaciones específicas de los alelos, lo que sugiere una relación funcional entre ambos (**Tabla R.14**).

El estudio de los haplotipos en relación con el grupo de pacientes con TKR, mostró asociación sólo en el subgrupo de las mujeres, tanto en MMP-2 como en MMP-1. El haplotipo de MMP-2 fue el mismo asociado con THR, y, por lo tanto, los efectos de estas variantes parecen similares en estos dos grupos de OA (**Tabla R.15**).

Tabla R.15: Distribución de las frecuencias de los haplotipos de MMPs que han mostrado asociación con TKR (sólo en mujeres). Se muestran los haplotipos con una frecuencia superior al 5%.

	Haplotipo	Controles	Casos	P valor
MMP2				
-1575/-1306/-790/-735	G-C-T-C	60.2	65.4	ns
	A-T-G-C	15.9	23.8	0.02
	G-C-T-T	14.1	17.2	ns
MMP1				
-340/-519/-1607	A-A-G	25.4	27.8	ns
	A-G-A	20.3	29.2	0.01
	G-A-G	22.2	15.4	0.03
	A-A-A	11.6	11.5	ns
	G-A-A	12.3	9.6	ns
	G-G-A	5.3	3.4	ns

La asociación con MMP-1 se debe a dos haplotipos incompatibles en los dos SNPs y de efectos contrarios, A-G asociado con susceptibilidad, y G-A asociado con protección. Este es un resultado similar al obtenido en el grupo de THR y ya comentado (**Tabla R.14**).

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En cuanto a la OA de la mano, se encontraron diferencias significativas en los haplotipos de MMP-1, ya comentados (**Tabla R.14**), y dos efectos significativos en la distribución de haplotipos de MMP-8 en mujeres (**Tabla R.16**). Los dos haplotipos que mostraron asociación, de los tres observados, tuvieron efectos opuestos, luego parece que los SNPs +17 C/G y -381 T/C, que están en casi completo LD, tienen un efecto independiente y opuesto al del SNP -799 T/C. Sin embargo, estos resultados son muy sorprendentes pues la asociación de estos dos haplotipos es la opuesta a la observada en los varones con THR, en los que el haplotipo C-T-C estuvo aumentado en pacientes y el haplotipo G-C-T mostró una tendencia a estar disminuido (**Tabla R.12**).

Tabla R.16: Distribución de las frecuencias de los haplotipos de MMPs que han mostrado asociación con HOA (sólo en mujeres). Se muestran los haplotipos con una frecuencia superior al 5%

	Haplotipo	Controles	Casos	P valor
MMP8				
+17/-381/-799	C-T-C	68.6	60.1	0.03
	C-T-T	26.6	28.7	ns
	G-C-T	4.7	10.8	0.0083

También se realizó un análisis de haplotipos con los SNPs de las MMPs del cluster de MMPs del cromosoma 11q. El objetivo fue el de estudiar la posibilidad de que algún haplotipo pudiese incluir SNPs no analizados en el estudio pero que se encontrase en LD con los SNPs estudiados. Los resultados no mostraron ninguna diferencia significativa entre pacientes y controles (Datos no mostrados)

Microsatélite de MMP-9

Pacientes y controles fueron genotipados para un polimorfismo funcional tipo microsatélite en la región promotora del gen *MMP-9*. Este microsatélite consiste en repeticiones del dinucleótido CA. En población humana existen nueve alelos distintos de este microsatélite (aunque en algunas ocasiones se encontraron diez) repartidos entre trece y veinticuatro repeticiones. Previamente, se había demostrado la funcionalidad de este microsatélite con ensayos reporter y EMSA. Se observó que se producía un aumento de la expresión en las construcciones que portaban por encima de veintitrés repeticiones y que esta variación de la expresión era debida a

diferencias en la unión de factores de transcripción (Peters DG et al, 1999). En nuestro estudio, los datos de genotipado en pacientes de OA y controles fueron analizados con el software Clump (Sham PC et al, 1996) (ver métodos). No se encontró ningún resultado significativo en ninguno de los fenotipos de OA estudiados (**Tabla R.17**). La estratificación por sexos tampoco reveló ninguna asociación (**Tabla R.18 y R.19**).

Tabla R.17: Frecuencias alélicas del microsatélite (CA) situado en la región promotora del gen *MMP-9* en los grupos de pacientes y controles estudiados. Los datos son dados como % en la población general.

	Alelos[a]									
	<14	14	15	19	20	21	22	23	24	P valor[b]
Controles	0.00	54.45	2.57	0.68	1.03	19.86	14.90	5.65	0.86	
THR	0.32	50.98	2.46	1.80	1.48	22.13	15.25	4.92	0.66	0.64
TKR	0.18	53.64	2.73	1.14	2.55	19.64	14.18	4.00	1.64	0.24
HOA	0.21	56.02	2.07	1.45	2.07	16.60	15.98	4.77	0.83	0.47

[a] número de repeticiones
[b] Obtenido de tablas contingencia 2 x n después de colapsar columnas de baja frecuencia

Tabla R.18: Frecuencias alélicas del microsatélite (CA) situado en la región promotora del gen *MMP-9* en los grupos de mujeres pacientes y controles estudiados. Los datos son dados como %.

	Alelos[a]									
	<14	14	15	19	20	21	22	23	24	P valor[b]
Controles	0.00	53.10	2.21	0.44	1.33	20.35	14.16	7.52	0.00	
THR	0.54	49.46	2.17	1.90	2.45	23.37	14.95	4.62	0.54	0.39
TKR	0.23	52.70	2.70	1.35	2.93	19.82	14.86	3.60	0.23	0.18
HOA	0.23	52.70	2.70	1.35	2.93	16.67	16.90	3.99	0.23	0.22

[a] número de repeticiones
[b] Obtenido de tablas de contingencia 2 x n después de colapsar columnas de baja frecuencia

Tabla R.19: Frecuencias alélicas del microsatélite (CA) situado en la región promotora del gen *MMP-9* en los grupos de hombres pacientes y controles estudiados. Los datos son dados como %.

	Alelos[a]								
	14	15	19	20	21	22	23	24	P valor[b]
Controles	55.31	2.79	0.84	0.84	19.55	15.36	4.47	0.84	
THR	53.31	2.89	1.65	0.00	20.25	15.70	5.37	0.83	0.99
TKR	57.55	2.83	1.89	0.94	18.87	11.32	5.66	0.94	0.48
HOA	55.77	0.00	5.77	0.00	17.31	9.62	11.54	0.00	0.32

[a] número de repeticiones
[b] Obtenido de tablas de contingencia 2 x n después de colapsar columnas de baja frecuencia

Discusión

Se encontraron diversas asociaciones significativas entre polimorfismos presentes en MMPs y susceptibilidad a OA en los tres fenotipos de la enfermedad estudiados. El elevado número de asociaciones puede reflejar la importancia de este grupo de proteasas en la OA. También es posible que este número sea debido a que esta familia de proteasas es la más estudiada y, por lo tanto, hay más SNPs funcionales definidos. Además, las diferencias encontradas en MMP-8 y MMP-1 pueden no ser independientes pues estos dos genes se encuentran en LD. Por otra parte, contribuye a mitigar el entusiasmo con estos resultados el análisis de la potencia estadística post-hoc. Sólo fue de magnitud suficiente en los SNPs de MMP-2 asociados en THR (0.8 para detectar un efecto de *odds ratio* de 1.4). Por el contrario, fue marcadamente débil en los análisis estratificados por sexos o en los SNPs +17 C/G y -381 T/C de MMP-8 debido a su baja frecuencia. Por todo esto, los resultados obtenidos deben ser tomados con relativa prudencia.

MMP-1 es una colagenasa (colagenasa-1) que degrada el colágeno nativo tipo-II y -III. Se ha visto que la expresión de *MMP-1* se encuentra influenciada por el efecto de citoquinas proinflamatorias como IL1-β (Klatt AR et al, 2006), aunque no existen diferencias en los niveles de expresión entre pacientes de OA y controles (Kevorkian L et al, 2004). Los SNPs que se vieron asociados con OA en este trabajo son SNPs funcionales que afectan al nivel de transcripción, pero los estudios funcionales que se han realizado no guardan correlación con los efectos observados (Pearce E et al, 2005). Así, por ejemplo, en ensayos tipos EMSA se vio que

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el alelo G del SNP -340 A/G une proteínas nucleares, mientras que no lo hace el alelo A. Sin embargo, los estudios reporter muestran que la expresión depende del haplotipo -519/-340 y no del alelo -340 A/G aislado. Por lo tanto, no tiene sentido explorar sus efectos por separado. Así, se ha visto que de los cuatro haplotipos posibles con estos dos SNPs, dos producen niveles elevados de transcripción en ensayos reporter, A-A y G-G, y dos producen niveles bajos, G-A y A-G. Estas diferencias se han demostrado también en los mRNAs extraídos de tejidos (Pearce E et al, 2005). Por lo tanto, es sorprendente que los dos haplotipos estén asociados con un nivel disminuido de expresión y, sin embargo, nosotros hayamos encontrado que uno de ellos es de susceptibilidad y otro es de protección. Esta paradoja nos lleva a desconfiar de nuestros resultados, como también las diferencias notables entre las frecuencias alélicas en controles mujeres y hombres (especialmente en el SNP -340 A/G que pasa de 40.3% a 28.9%). Sin embargo, nuestros resultados son muy similares a los obtenidos en dos colecciones demuestras en relación con el infarto de miocardio (Pearce E et al, 2005). También en este caso, el haplotipo G-A fue protector y el haplotipo A-G fue de susceptibilidad. Estos datos, de replicarse, indican que en estos haplotipos de MMP-1 hay interacciones funcionales entre los dos SNPs que se nos escapan y que deben ser investigadas.

También hemos encontrado asociación con otra colagenasa, MMP-8 (colagenasa-2). MMP-8 es la colagenasa de neutrófilos, que también degrada colágeno nativo tipo-II y -I. Los SNPs analizados en este estudio son funcionales (Wang H et al, 2004) y los alelos de los SNPs individuales aumentados en pacientes con OA, +17 G en el grupo de mujeres con TKR y con HOA, y -381 C en mujeres con TKR, provocan un aumento de la expresión de MMP-8. Un haplotipo formado por los alelos G y C de estos dos SNPs más el alelo T del SNP -799 C/T se ha visto que provoca un aumento de hasta tres veces en el nivel de transcripción de *MMP-8* en células de trofoblasto, y se ha visto asociado con la ruptura prematura de membranas (Wang H et al, 2004). En nuestro análisis de haplotipos los resultados positivos que hemos obtenido no son muy fiables puesto que el haplotipo C-T-T se encuentra aumentado en los hombres con THR, y por el contrario, está disminuido en mujeres con HOA (**Tabla R.13 y R.15**). Esto puede deberse al marcado contraste en las frecuencias del alelo raro del SNP -799 C/T entre las mujeres y varones de nuestra población control (30.4% vs 43.6%, respectivamente). Esta variación, probablemente debida al azar, condiciona todos los análisis que incluyen este SNP. El mismo tipo de diferencia se observó en los SNPs -381 T/C y +17G/C que mostraron frecuencias notablemente menores en las mujeres controles que las observadas en los varones (4.8 vs >9.5 respectivamente). Por lo tanto, los resultados obtenidos en MMP-8 necesitan, todavía con más claridad que el resto, replicación en nuevos estudios.

MMP-2, también conocida como gelatinasa-A, participa en la degradación de los fragmentos de colágeno tipo-I generados por las colagenasas y que han perdido la estructura típica en triple hélice. De esta manera, la gelatinasa MMP-2 actúa sinérgicamente con las colagenasas en la degradación de los colágenos fibrilares. Además, ejerce su función también sobre el colágeno tipo IV y tiene efecto proteolítico contra proteoglicanos y elastina, los cuales son resistentes a otras MMPs. MMP-2 también degrada MCP3 (proteína quimiotáctica de monocitos 3) y SDF1 (factor quimiotáctico de linfocitos), por lo que controla el influjo de células proinflamatorias (McQuibban, G. A et a l 2000). No se han detectado diferencias en los niveles de expresión de *MMP-2* entre pacientes de OA y controles sanos (Kevorkian L et al, 2004). Los SNPs que resultaron asociados con OA son todos funcionales (Price SJ et al, 2001) (Harendza S et al, 1995). Diversos trabajos muestran que los alelos raros A y T de los SNPs MMP-2 -1575 G/A y -1306 C/T provocan una disminución de la expresión de MMP-2. Del SNP MMP-2 -790 T/G, no se conoce su repercusión funcional en los niveles de transcripción de *MMP-2.* Tanto el análisis de LD como el estudio de los haplotipos han mostrado que los tres SNPs están asociados con la OA de forma imposible de diferenciar ya que la asociación se produce con el único haplotipo que contiene el alelo raro de cada uno de estos tres SNPs (A-T-G). Por el contrario, el SNP -735 C/T, que también parece afectar a los niveles de transcripción de *MMP-2* (Yu C et al, 2004), no mostró ningún efecto. El haplotipo asociado con la OA mostró diferencias significativas sólo en las mujeres, tanto con THR como con TKR. Hubo una tendencia en el mismo sentido en mujeres con HOA, pero la diferencia no fue significativa. Por el contrario, en los hombres no se encontró ninguna tendencia clara.

Teniendo en cuenta estos resultados, la posible participación de MMP-2 en la susceptibilidad a OA sería difícil de explicar por su efecto proteolítico sobre los componentes de la ECM, ya que los SNPs asociados estarían disminuyendo la expresión de la proteína. Por otra parte su efecto sobre factores quimiotácticos como MCP-3 y SDF-1, sí podrían explicar parte de la patología asociada a la OA (McQuibban GA et al, 2000) (McQuibban GA et al, 2001). MMP-2 degrada estos sustratos frenando así la inflamación por infiltración de células inflamatorias y atenuando de manera indirecta los efectos inflamatorios de estas células. En la OA, se produce una inflamación secundaria al proceso de degradación de la ECM. Esta inflamación actúa acelerando la activación de enzimas proteolíticos por citoquinas proinflamatorias, lo que contribuye a una mayor degradación de la ECM. De esta manera, todo el proceso se repite e incrementa continuamente por retroalimentación. El papel de MMP-2 sería el de actuar como factor protector mitigando este proceso. Al estar disminuida su expresión, la inflamación no se atenúa, o se atenúa más lentamente, con lo que se produce un aumento progresivo de enzimas

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proteolíticos activos con el consecuente incremento de la degradación de los componentes de la ECM y la aceleración de la progresión de la OA.
Otra de las asociaciones encontradas fue en el gen de la gelatinasa-B (MMP-9). Al igual que MMP-2 (gelatinasa-A), MMP-9 también participa en la degradación de los fragmentos de colágeno generados por las colagenasas. Además, MMP-9 tiene actividad catalítica sobre otros componentes de la ECM. Su expresión está aumentada en el cartílago y la sinovia de pacientes con OA (Davidson RK et al, 2006). La asociación con el SNP -1562 C/T se encontró en el subgrupo de hombres con THR y fue sólo nominalmente significativa ($p = 0.048$, O.R. 1.57, 95% I.C. 1.00-2.46). El alelo asociado, T, provoca un aumento de la expresión de *MMP-9*. Este alelo T se encontró aumentado en todos los grupos de pacientes de OA hombres, aunque no de forma significativa. Su distribución en los grupos de mujeres fue variable (**Tablas R.4 a R.12**). Un aumento de la expresión de *MMP-9* provocado por el alelo T del SNP -1562 C/T, aumentaría el ritmo de degradación de la ECM en el cartílago, lo que podría correlacionarse lógicamente con un aumento de la susceptibilidad a OA. Sin embargo, este resultado requiere confirmación antes de afirmar un papel del SNP -1562 C/T en la susceptibilidad a OA.

La falta de asociación encontrada en los otros SNPs estudiados en las proteasas de ECM fue algo, en cierto modo, inesperado porque afectaban a proteasas con un papel reconocido en OA (Martel-Pelletier J et al, 2001). Especialmente llamativo es el caso de MMP-13 y MMP-3, colagenasa-3 y estromalisina-1 respectivamente, ya que son activas frente a los principales componentes de la ECM y sus niveles de expresión se encuentran alterados en OA. MMP-13 (colagenasa-3), es la más activa de las colagenasas frente a colágeno tipo-II nativo llegando a ser cinco a diez veces más activa que la colagenasa-1 (MMP-1). MMP-13 es expresada por los condrocitos articulares y se encuentra aumentada en el cartílago y tejido sinovial de pacientes con OA (Davidson RK et al, 2006). Además, experimentos *in vitro* han mostrado que los condrocitos articulares aumentan la expresión de MMP-13 en respuesta a IL-7 (Long DL et al, 2008). Sin embargo, otros autores han sugerido que el papel de MMP-13 en la OA es de remodelado de las estructuras dañadas y no estaría implicada en la destrucción del tejido, basándose en que se encuentra en las zonas profundas del cartílago artrósico y sus niveles permanecen constantes durante la progresión de la enfermedad (Fernandes JC et al, 1998). Los niveles de MMP-3 (estromalisina-1) se encuentran aumentados en OA (Martel-Pelletier J et al, 2001) aunque esto no se ha observado en todos los estudios (Davidson RK et al, 2006). MMP-3 posee actividad proteolítica frente a una gran cantidad de moléculas de la matriz como fibronectina, elastina, laminina y agrecano. Estudios histoquímicos han relacionado los niveles

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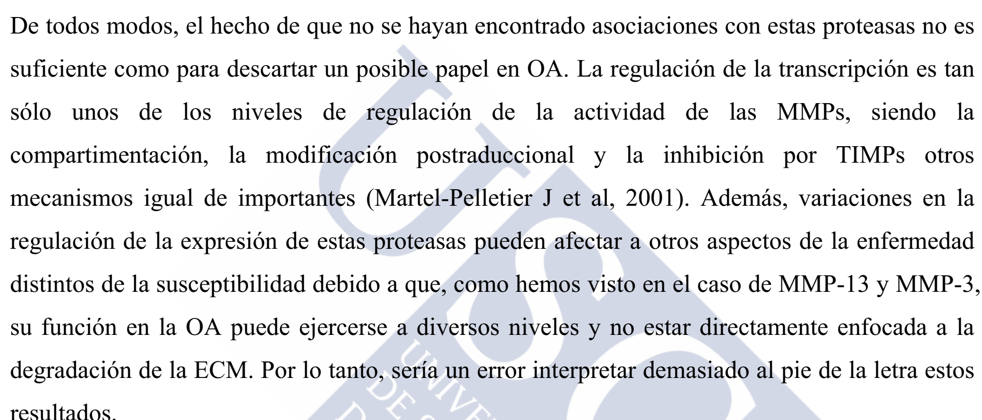
de MMP-3 y el grado de degradación de los proteoglicanos (Bonassar L. J et al, 1995). Además, MMP-3 contribuye a la activación de la procolagenasa-1 (MMP-1) (Unemori E.N et al, 1991), lo que da a MMP-3 un doble papel en la destrucción de la ECM. Por otra parte, MMP-7 (Matrilisina-1), es otra de las proteasas que no se ha visto asociada en ninguno de nuestros análisis y que, teóricamente, puede tener un papel importante en la OA. Los niveles de expresión de *MMP-7* también se encuentran aumentados en el tejido sinovial y el cartílago de pacientes con OA (Davidson RK et al, 2006). Además, este enzima también puede degradar proteoglicanos y su expresión se ve aumentada por la acción de citoquinas proinflamatorias como IL1-β y TNF-α (Ohta S et al, 1998).

De todos modos, el hecho de que no se hayan encontrado asociaciones con estas proteasas no es suficiente como para descartar un posible papel en OA. La regulación de la transcripción es tan sólo unos de los niveles de regulación de la actividad de las MMPs, siendo la compartimentación, la modificación postraduccional y la inhibición por TIMPs otros mecanismos igual de importantes (Martel-Pelletier J et al, 2001). Además, variaciones en la regulación de la expresión de estas proteasas pueden afectar a otros aspectos de la enfermedad distintos de la susceptibilidad debido a que, como hemos visto en el caso de MMP-13 y MMP-3, su función en la OA puede ejercerse a diversos niveles y no estar directamente enfocada a la degradación de la ECM. Por lo tanto, sería un error interpretar demasiado al pie de la letra estos resultados.



a.3 Serín proteasas (sistema plasminógeno-plasmina)

Material y Métodos
Véase material y métodos del capítulo dedicado a las MMPs más las siguientes peculiaridades

Pacientes y controles
Se estudiaron las muestras de la colección de Santiago excepto en el análisis del polimorfismo repetición Alu en PLAT, en el que se han utilizado otras dos colecciones de muestras además de la de Santiago. Ambas han sido previamente descritas (Fytili et al, 2005) (Mustafa Z et al, 2005) y pertenecen al Departamento de Biología y Genética y de Ortopedia, Universidad de Tesalia (Grecia), "colección de Tesalia"; y al Instituto de Ciencias Musculoesqueléticas, Universidad de Oxford (Reino Unido, U.K.), "colección de Oxford". La "colección de Tesalia" está formada por 159 pacientes del grupo TKR (139 mujeres y 20 hombres), y 193 controles (137 mujeres y 56 hombres), mayores de 45 años. La "colección de Oxford" comprende 360 muestras del grupo TKR (196 mujeres y 164 hombres), 1105 muestras del grupo THR (629 mujeres y 476 hombres) y 698 controles (356 mujeres y 342 hombres), mayores de 55 años (**Tabla R.20**)

Tabla R.20: Relación de pacientes y controles de las colecciones de Tesalia y Oxford. Los datos se muestran también estratificados por sexo y articulación. THR: remplazo articular de la cadera. TKR: remplazo articular de la rodilla.

	Hombres	Mujeres	Total
Oxford			
Controles	342	356	698
THR	476	629	1105
TKR	164	196	360
Tesalia			
Controles	56	137	193
TKR	20	139	159

Selección de SNPs
Se siguieron los criterios de selección similares a los utilizados en los estudios de MMPs y de ADAMs y ADAMTS. Así, se seleccionaron SNPs no-sinónimos (nsSNP) de la base de datos de SNPs del NCBI-Entrez. Sólo dos de ellos, rs2020921 C/T (R164W en el exon 6 del gen *PLAT*) y rs2227564 C/T (L141P en el exon 6 del gen de *PLAU*), fueron escogidos para el estudio ya que presentaba las características de estar validados y afectar probablemente al funcionamiento correcto de la proteína.

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También se escogieron polimorfismos reguladores a partir de la base de datos de PubMed. Se seleccionaron aquellos polimorfismos en los que estuviese demostrada su funcionalidad, además de provocar un efecto clínico derivado de su acción. Fueron escogidos cinco polimorfismos: dos SNPs en PAI-1, un SNP en PLAU y un SNP y una repetición Alu en el gen de PLAT (**Tabla R.21**)

Tabla R.21: Polimorfismos estudiados en Proteasas de Serina. Se señala la posición en el cromosoma y en relación con el inicio de la transcripción. También se muestran los alelos común y raro así como el efecto que el alelo raro produce sobre la expresión o actividad de la proteína. Up: eleva el nivel de expresión. Down: baja el nivel de expresión. – no se conoce. Del: disminuye la actividad de la proteína.

Serín proteasa	Cromosoma	Posición en cromosoma[a]	Posición inicio transcripción[a]	Número de identificación	Polimorfismo	Efecto alelo raro
PLAT	8p	42159057	(Alu) Intron 8		I/D	Down
		42164122	+164	rs2020921	C/T	Del
		42191595	-7351	rs2020918	C/T	Down
PLAU	10q	75343107	+141	rs2227564	C/T	Del
		75346470	+4065	rs4065	G/A	-
PAI-1	7q	100362973	-844	rs2227631	T/C	Down
		100363141	-675	rs1799768	G/A	Up

[a] En pares de bases

Genotipado

Los SNPs incluídos en este estudio se genotiparon como en el caso de los SNPs de MMPs. Para genotipar la repetición Alu de PLAT se amplificó un fragmento de PCR con un primer marcado con FAM. Se utilizó una PCR con un set de tres primers, añadiendo un primer reverse extra que reconociese una secuencia específica de la inserción (Primer interno). De esta manera se evitó la posible clasificación errónea de heterocigotos ID como homocigotos DD provocada por una diferencia en la eficiencia de la PCR al amplificar los fragmentos conteniendo o no conteniendo la inserción derivada de su gran diferencia de tamaño (440 y 167 pb respectivamente) (Jern C et al, 1999). La presencia de la repetición se determinó mediante electroforesis capilar de los productos de esta PCR en un ABI prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Las secuencias de oligonucleótidos utilizados para el genotipado se muestran en la **tabla R.22**. El porcentaje de genotipos válidos fue alto (0.98 para todos los SNPs). Las frecuencias genotípicas estaban en equilibrio Hardy-Weinberg (HWE) para todos los SNPs y para la repetición Alu.

Análisis estadístico

El análisis estadístico se realizó básicamente siguiendo lo detallado para el estudio de las MMPs, salvo que los datos de las poblaciones de Santiago, Tesalia y Oxford. fueron combinados utilizando el test de Mantel-Haenszel para tablas de contingencia 2 x 2 estratificando por colección de muestras. La homogeneidad del efecto entre las colecciones fue valorada con el test de Breslow-Day.

Tabla R.22.: Primers de PCR y sondas utilizadas en el estudio

Serín proteasa	Polimorfismo		Primer de PCR	Sonda de SNaP Shot[a]
PLAT	Alu I/D	Izquierdo	tccgtaacaggacagctcacFAM	
		Derecho	tcttgaccttgcagcacacc	
		Interno	gtgctgggattacaggcgtg[c]	
	+164 C/T	Izquierdo	agaaccctgacggatctgac	cctgatggcgtctggcctcc (R)
		Derecho	acgaggaccagggcatcagc	
	-7351 C/T	Izquierdo	agcggcgagtcctgtgatgc	catggctgtgtctggggc (R)
		Derecho	tgcactccagcctgggagac	
PLAU	+141 C/T	Izquierdo	tggttgagtcttccctgagg	catgcaccatgcactcttggacaagc (F)
		Derecho	tcccctccaccccactgtc	
	+4065 G/A	Izquierdo	actgggaagataggctctgc	atgcctgagggtaaagctattgtcgttc (R)
		Derecho	tctgggcagccagcacccag	
PAI-1	-844 T/C	Izquierdo	agcacaccctgcaaacctgc	caggagaccaacgtgtaagtttcacttc (R)
		Derecho	ccttgtctggctgaggttgg	
	-675 G/A	Izquierdo	gggcacagagagagtctgga	acagagagagtctggacacgtgggg (F)
		Derecho	cccctagtgttcagcttgga	

[a] Orientación de la sonda: R = reverse; F = forward
[b] En mayúsculas oligonucleótidode secuencia no humana
[c] Se utilizó un primer interno que hibridase en la repetición Alu debido a que el tamaño de esta era de 316pb

Resultados

Frecuencias alélicas pacientes y controles

Para analizar si SNPs en proteasas de Serina de ECM eran factores de riesgo para OA, comparamos frecuencias alélicas de pacientes y controles, estratificando por la articulación afectada (THR, TKR o HOA). Al igual que en las MMPs, el estudio se realizó en primer lugar sobre la población en general y posteriormente se realizó una estratificación por sexos. En el caso de los pacientes de THR se encontró que de los siete polimorfismos estudiados, cinco de ellos tenían frecuencias similares en pacientes y controles. Tan sólo dos polimorfismos, el PLAT Alu I/D y el SNP PAI-1 -844 T/C, presentaron diferencias significativas entre pacientes THR y controles (**Tabla R.23**). Los resultados que se obtuvieron después de la estratificación por sexos no mostraron ningún resultado significativo y tan sólo mostraron cambios reseñables en el caso de los SNPs -844 G/A de *PAI-1* en mujeres y +164 C/T de *PLAT* en hombres. (**Tablas R.24 y R.25**). Este resultado del SNP +164 C/T de *PLAT* es muy cuestionable dado la baja frecuencia del alelo raro de este SNP.

Tabla R.23: Frecuencias alélicas de los polimorfismos estudiados en controles y pacientes THR.

Serín proteasa	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
PLAT	(Alu) Intron 8 I/D	37.76	43.77	0.03	1.28 (1.02-1.62)
	+164 C/T	1.36	2.51	ns	1.86 (0.78-4.43)
	-7351 C/T	31.40	35.52	ns	1.20 (0.95-1.53)
PLAU	+141 C/T	16.33	17.73	ns	1.10 (0.81-1.19)
	+4065 G/A	36.73	34.49	ns	0.91 (0.71-1.15)
PAI-1	-844 T/C	46.26	40.26	0.04	0.78 (0.62-0.98)
	-675 G/A	47.28	51.83	ns	1.20 (0.91-1.57)

[a] Se muestra la frecuencia del alelo raro en %

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Tabla R.24: Frecuencias alélicas de los polimorfismos estudiados en controles y pacientes THR mujeres.

Serín proteasa	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
PLAT	(Alu) Intron 8 I/D	39.57	45.38	ns	1.27 (0.91-1.77)
	+164 C/T	2.17	2.25	ns	1.03 (0.33-3.20)
	-7351 C/T	34.35	37.57	ns	1.15 (0.81-1.62)
PLAU	+141 C/T	16.96	17.13	ns	1.01 (0.65-1.57)
	+4065 G/A	37.39	33.61	ns	0.85 (0.60-1.19)
PAI-1	-844 T/C	46.09	38.52	0.07	0.73 (0.52-1.02)
	-675 G/A	45.65	50.00	ns	1.19 (0.82-1.73)

[a] Se muestra la frecuencia del alelo raro en %

Tabla R.25: Frecuencias alélicas de los polimorfismos estudiados en controles y pacientes THR hombres.

Serín proteasa	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
PLAT	(Alu) Intron 8 I/D	36.59	41.32	ns	1.22 (0.87-1.70)
	+164 C/T	0.84	2.92	0.05	3.55 (0.91-13.89)
	-7351 C/T	29.49	32.33	ns	1.14 (0.80-1.63)
PLAU	+141 C/T	15.92	18.75	ns	1.22 (0.79-1.87)
	+4065 G/A	36.31	36.13	ns	1.00 (0.72-1.42)
PAI-1	-844 T/C	46.37	42.44	ns	0.85 (0.61-1.19)
	-675 G/A	48.32	54.92	ns	1.30 (0.86-1.97)

[a] Se muestra la frecuencia del alelo raro en %

En el grupo de pacientes con OA de la rodilla, tan sólo se encontró un resultado significativo (PAI -1 -675 G/A). También se encontraron cambios importantes en las frecuencias entre pacientes y controles en el caso del SNP +4065 G/A de PLAU aunque estas no llegaron a ser significativas. (**Tabla R.26**). La estratificación por sexos mostró que las frecuencias en pacientes y controles eran bastante similares en todos los polimorfismos estudiados en las mujeres (**Tabla R.27**), mientras que en los hombres, se encontró un resultado significativo (PAI-1 -675 G/A), aunque hay que tomarlo con cierta cautela debido a que el número de hombres TKR es pequeño (**Tabla R.28**).

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Tabla R.26: Frecuencias alélicas de los polimorfismos estudiados en controles y pacientes TKR.

Serín proteasa	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
PLAT	(Alu) Intron 8 I/D	37.76	41.27	ns	1.16 (0.91-1.47)
	+164 C/T	1.36	0.94	ns	0.69 (0.22-2.12)
	-7351 C/T	31.40	33.91	ns	1.12 (0.87-1.44)
PLAU	+141 C/T	16.33	15.09	ns	0.91 (0.66-1.26)
	+4065 G/A	36.73	31.73	0.08	0.80 (0.62-1.02)
PAI-1	-844 T/C	46.26	44.62	ns	0.93 (0.74-1.19)
	-675 G/A	47.28	40.23	0.02	0.75 (0.59-0.95)

[a] Se muestra la frecuencia del alelo raro en %

Tabla R.27: Frecuencias alélicas de los polimorfismos estudiados en controles y pacientes TKR mujeres.

Serín proteasa	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
PLAT	(Alu) Intron 8 I/D	39.57	43.24	ns	1.16 (0.84-1.61)
	+164 C/T	2.17	0.93	ns	0.42 (0.11-1.59)
	-7351 C/T	34.35	33.57	ns	0.97 (0.69-1.36)
PLAU	+141 C/T	16.96	15.19	ns	0.88 (0.57-1.35)
	+4065 G/A	37.39	31.90	ns	0.78 (0.56-1.10)
PAI-1	-844 T/C	46.09	43.81	ns	0.91 (0.66-1.26)
	-675 G/A	45.65	42.38	ns	0.87 (0.63-1.21)

[a] Se muestra la frecuencia del alelo raro en %

Tabla R.28: Frecuencias alélicas de los polimorfismos estudiados en controles y pacientes TKR hombres.

Serín proteasa	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
PLAT	(Alu) Intron 8 I/D	36.59	33.02	ns	0.85 (0.54-1.35)
	+164 C/T	0.84	0.98	ns	1.17 (0.12-11.38)
	-7351 C/T	24.49	35.29	ns	1.30 (0.82-2.10)
PLAU	+141 C/T	15.92	14.71	ns	0.91 (0.49-1.69)
	+4065 G/A	36.31	31.00	ns	0.79 (0.49-1.27)
PAI-1	-844 T/C	46.37	48.00	ns	1.07 (0.68-1.66)
	-675 G/A	48.32	31.37	0.002	0.49 (0.31-0.78)

[a] Se muestra la frecuencia del alelo raro en %

El estudio de asociación en pacientes con OA de la mano mostró que las frecuencias de los siete polimorfismos estudiados fueron similares entre pacientes y controles tanto en la población general como en las subpoblaciones de hombres y mujeres no encontrándose ninguna diferencia significativa. Tan sólo en el caso del SNP PAI-1 -675 G/A se observó una cambio destacable en las frecuencias, pero este no llegó a ser significativo (**Tablas R.29, R.30 y R.31**)

Tabla R.29: Frecuencias alélicas de los polimorfismos estudiados en controles y pacientes HOA.

Serín proteasa	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
PLAT	(Alu) Intron 8 I/D	37.76	38.84	ns	1.05 (0.82-1.34)
	+164 C/T	1.36	2.29	ns	1.70 (0.68-4.26)
	-7351 C/T	31.40	29.87	ns	0.93 (0.71-1.21)
PLAU	+141 C/T	16.33	17.29	ns	1.07 (0.78-1.48)
	+4065 G/A	36.73	37.12	ns	1.02 (0.79-1.31)
PAI-1	-844 T/C	46.26	47.16	ns	1.03 (0.82-1.32)
	-675 G/A	47.28	41.21	0.05	0.78 (0.61-0.99)

[a] Se muestra la frecuencia del alelo raro en %

Tabla R.30: Frecuencias alélicas de los polimorfismos estudiados en controles y pacientes HOA mujeres.

Serín proteasa	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
PLAT	(Alu) Intron 8 I/D	39.57	38.97	ns	0.97 (0.70-1.35)
	+164 C/T	2.17	2.61	ns	1.20 (0.41-3.51)
	-7351 C/T	34.35	28.96	ns	0.78 (0.55-1.10)
PLAU	+141 C/T	16.96	18.25	ns	1.10 (0.71-1.67)
	+4065 G/A	37.39	38.35	ns	1.04 (0.75-1.45)
PAI-1	-844 T/C	46.09	46.32	ns	1.01 (0.73-1.39)
	-675 G/A	45.65	41.90	ns	0.86 (0.62-1.19)

[a] Se muestra la frecuencia del alelo raro en %

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Tabla R.31: Frecuencias alélicas de los polimorfismos estudiados en controles y pacientes HOA hombres.

Serín proteasa	Polimorfismo	Controles[a]	Casos[a]	P valor	O.R. (95% C.I.)
PLAT	(Alu) Intron 8 I/D	36.59	37.04	ns	1.02 (0.56-1.84)
	+164 C/T	0.84	0.00	ns	-
	-7351 C/T	29.49	37.50	ns	1.43 (0.77-2.68)
PLAU	+141 C/T	15.92	11.11	ns	0.66 (0.29-1.61)
	+4065 G/A	36.31	26.92	ns	0.64 (0.34-1.24)
PAI-1	-844 T/C	46.37	54.00	ns	1.36 (0.75-2.46)
	-675 G/A	48.32	38.89	ns	0.68 (0.38-1.22)

[a] Se muestra la frecuencia del alelo raro en %

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Extensión del estudio de la repetición Alu de PLAT en OA

Con el objetivo de confirmar los resultados obtenidos en la colección de Santiago, el polimorfismo repetición Alu del gen *PLAT,* fue también estudiado en otras dos poblaciones europeas independientes: la colección de Tesalia (Grecia) y la colección de Oxford (Reino Unido). Los resultados obtenidos en la población española no se confirmaron en ninguna de las otras dos poblaciones, no encontrándose asociación ni en el análisis global ni en la estratificación por sexos (**Tabla R.32**).

Tabla R.32: Frecuencias alélicas de la repetición Alu de PLAT en pacientes y controles de las dos poblaciones estudiadas. N se refiere al número de alelos, I = presencia de la repetición Alu; D = ausencia de la repetición Alu.

Colección	Grupo	PLAT Alu (I/D)		
		D %	p	O.R. (95% C.I.)
Tesalia	Control (n = 386)	37.77		
	Control mujeres (n = 274)	39.31		
	Control hombres (n = 112)	33.96		
	TKR (n = 318)	43.06	0.17	1.24 (0.91-1.70)
	TKR mujeres (n = 278)	42.00	0.54	1.12 (0.78-1.59)
	TKR hombres (n = 40)	50.00	0.08	1.94 (0.92-4.13)
Oxford	Control (n= 1396)	42.12		
	Control mujeres (n = 712)	42.77		
	Control hombres (n = 684)	41.45		
	TKR (n = 720)	40.66	0.52	0.94 (0.78-1.13)
	TKR mujeres (n = 392)	41.44	0.67	0.95 (0.73-1.22)
	TKR hombres (n = 328)	39.75	0.61	0.93 (0.71-1.22)
	THR (n= 2210)	41.58	0.75	0.98 (0.85-1.12)
	THR mujeres (n = 1258)	42.31	0.84	0.98 (0.81-1.18)
	THR hombres (n = 952)	40.60	0.73	0.97 (0.79-1.18)

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El análisis conjunto de las tres colecciones puso de manifiesto la ausencia de asociación con OA en los dos subgrupos de pacientes estudiados. La estratificación por sexos tampoco mostró ningún resultado positivo (**Tabla R.33**)

Tabla R.33: Análisis de asociación de la repetición Alu de PLAT en controles y pacientes de THR y TKR en población caucásica (Santiago, Tesalia y Oxford.)

Grupo	Individuos	Mantel-Haenszel Test		
		P valor	OR M-H	Interacción M-H
Controles combinados (Santiago, Tesalia, Oxford)	1185			
Controles mujeres combinados (Santiago, Tesalia, Oxford)	608			
Controles hombres combinados (Santiago, Tesalia, Oxford)	577			
TKR combinados (Santiago, Tesalia, Oxford)	782	0.49	1.05 (0.92-1.20)	0.30
TKR mujeres combinados (Santiago, Tesalia, Oxford)	546	0.79	1.02 (0.86-1.22)	0.60
TKR hombres combinados (Santiago, Tesalia, Oxford)	236	0.99	0.99 (0.80-1.25)	0.17
Controles combinados (Santiago, Oxford)	992			
Controles mujeres combinados (Santiago, Oxford)	471			
Controles hombres combinados (Santiago, Oxford)	521			
THR combinados (Santiago, Oxford)	1412	0.34	1.06 (0.94-1.19)	0.05
THR mujeres combinados (Santiago, Oxford)	814	0.64	1.04 (0.90-1.22)	0.18
THR hombres combinados (Santiago, Oxford)	598	0.58	1.05 (0.88-1.25)	0.26

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Análisis de las frecuencias haplotípicas

El estudio de LD en las proteasas de serina se realizó en los tres genes analizados (*PLAT, PAI-1* y *PLAU*). El LD existente entre los polimorfismos de un mismo gen fue lo suficientemente alto como para justificar un análisis de haplotipos tanto en valores de D' como en valores de r^2 (datos no mostrados). El análisis de haplotipos no aportó ningún resultado adicional con respecto al estudio de frecuencias alélicas. El único resultado positivo fue encontrado con el haplotipo T-G de PAI-1 en OA de la mano (**Tabla R.34**). El haplotipo T-A refleja el efecto protector observado con el alelo raro del SNP -675 G/A. Sin embargo, el haplotipo asociado, T-G, muestra un efecto contrario de susceptibilidad que no se apreciaba en el estudio de los SNPs por separado y que sugiere algún tipo de interacción entre los SNPs, o la presencia de otros SNPs con repercusión en la OA de la mano. Estos dos SNPs muestran una D' de 0.93 y una r^2 de 0.62 (Datos no mostrados). En el caso de PLAT se encontró un haplotipo que presentaba diferencias en su distribución de frecuencias entre pacientes con THR y controles, pero estas no llegaban a ser significativas (p = 0.07) (Datos no mostrados).

Tabla R.34: Frecuencias de los haplotipos estimados a partir de los SNPs estudiados en PAI-1 en controles y pacientes con OA de la mano.

	Haplotipo	Controles	Casos	P valor
Total				
PAI-1 -844/-675	C-G	44.9	46.3	ns
	T-A	45.9	40.3	ns
	T-G	7.8	11.8	0.03

Discusión

En el grupo de Serín proteasas estudiadas, se encontraron diversas asociaciones con susceptibilidad a la OA en los tres fenotipos estudiados. Concretamente, con la repetición Alu en el intrón 8 de *PLAT* y el SNP -844 T/C de PAI-1 en el grupo THR, aunque la asociación de la repetición Alu no fue confirmada, el SNP -675 G/A de PAI-1 en el grupo TKR y con un haplotipo de PAI-1 en el grupo HOA.

PLAT codifica para el activador tisular del plasminógeno. Su papel en enfermedades articulares ha sido estudiado sobre todo en relación a la artritis reumatoide (RA), encontrándose que en los pacientes con RA se observa una baja expresión de *PLAT* (Busso N et al, 1997) (Sanchez-

Pernaute O et al, 2003) (So AK et al, 2003). En la OA, también se observó un descenso de la expresión de *PLAT* en pacientes comparado con controles (Busso N et al, 1997) y, aunque, esta diferencia de expresión no llegó a ser significativa, sugiere que PLAT puede estar implicado en la patogénesis de la OA. El papel de PLAT en la OA sería indirecto, agravando la inflamación secundaria a la enfermedad por acumulación de fibrina. De esta manera contribuiría a la activación de MMPs por parte de citoquinas proinflamatorias, lo que traería como consecuencia una aceleración de la degradación del cartílago. (Busso N et al, 2002). La repetición Alu que nosotros encontramos asociada en pacientes con THR españoles altera los niveles de expresión de *PLAT*. En concreto, el alelo D (ausencia de la repetición Alu), que se encontró aumentado en los pacientes con THR, se correlaciona con bajos niveles de PLAT en plasma (Jern C et al, 1999). Este dato ha sido corroborado por nuestro laboratorio en colaboración con el laboratorio del Dr John Loughlin de la Universidad de Oxford mediante análisis de expresión alélicas diferencial en tejido sinovial de pacientes con OA (Datos no mostrados). Sin embargo, el intento por confirmar la asociación de la repetición Alu en pacientes con THR en muestras independientes produjo resultados negativos. Además, el análisis conjunto de las poblaciones estudiadas mostró que no había diferencias ni en THR ni en TKR. Estos resultados indican que no existe asociación entre la repetición Alu de *PLAT* y susceptibilidad a la OA. A su vez, esto indica que posiblemente nuestros primeros resultados se debieran a variaciones al azar de las frecuencias alélicas.

Otro de los resultados positivos encontrados en Serín proteasas se corresponde con los SNP de PAI-1. PAI-1 (inhibidor del activador del plasminógeno-1) es otro miembro del sistema plasminógeno-plasmina (PAs). Su función, como su nombre indica, es la de inhibir los activadores del plasminógeno, PLAT y PLAU (Andreasen PA et al, 1990). Los estudios que se han realizado sobre PAI-1 y su posible papel en la OA están basados en que sus niveles se encuentran aumentados en pacientes con OA (Busso N et al, 1997). Su posible acción en la degradación del cartílago es a través de la actividad proteolítica mediada directamente por la plasmina, o indirectamente a través de la activación de proteasas de matriz extracelular por la propia plasmina o por citoquinas proinflamatorias.

En nuestro estudio, los dos SNPs analizados en PAI-1 se encuentran asociados con alguna forma de OA. Como los efectos descritos para los alelos raros de estos dos SNPs son opuestos, cabría esperar que los cambios en la distribución de las frecuencias también lo fueran. Sin embargo, el alelo C del SNP -844 T/C está disminuido en el grupo de pacientes con THR y el alelo A del SNP -675 G/A está disminuido en el grupo de pacientes con TKR y muestra una tendencia a la disminución en los pacientes con OA de mano. No está claro como se puede

explicar esta contradicción. Por un lado el alelo C del SNP -844 T/C determina una menor expresión de *PAI-1* y, por lo tanto, sería esperable que estuviese aumentado entre los pacientes, debido a la actividad inhibidora de PAI-1 sobre la activación del plasminógeno. La disminución en frecuencia del alelo A del SNP -675 G/A en los pacientes con TKR, por el contrario, es compatible con su efecto aumentando la expresión de *PAI-1*.

En el estudio de haplotipos de PAI-1 se encontró una asociación con el haplotipo T-G en pacientes con HOA (**Tabla R.34**), estando este haplotipo significativamente aumentado en los casos con respecto a los controles. Al mismo tiempo el haplotipo T-A es menos frecuente en los pacientes, lo que concuerda con los datos del SNP -675 G/A. El efecto del haplotipo T-A es compatible con el efecto atribuido a los alelos de los dos SNPs. Esto es, un aumento relativo de la expresión de *PAI-1* sería un obstáculo a la predisposición a la OA. Una alta expresión de PAI-1 prevendría la activación del plasminógeno moderada por PLAT y PLAU. Al haber menos plasmina activa no podría ejercer su actividad proteolítica sobre los componentes de la ECM ni activar a las MMPs. De esta manera, se produciría una reducción de la actividad catalítica en el cartílago. La asociación del haplotipo T-G con susceptibilidad a la OA es más difícil de entender ya que combina un alelo de aumento de expresión con uno de disminución. Es posible que los efectos funcionales de estos SNPs no sean independientes y que los resultados obtenidos con los haplotipos se deban a interacción entre los dos SNPs. De todas formas, estos resultados tienen que ser confirmados por estudios independientes y con un mayor tamaño muestral para demostrar su veracidad.

En conclusión, los resultados obtenidos en este estudio muestran que PLAT y PLAU no tienen una actividad determinante como factores de susceptibilidad a la OA. En el caso de PAI-1, los resultados observados parecen indicar que algunos de los SNPs tengan influencia en la OA. Sin embargo, es difícil concluir con firmeza y debemos esperar a estudios de replicación para estar seguros. Al igual que en las MMPs, las serín proteasas pueden estar sujetas a distintos niveles de regulación además del transcripcional y afectar a otros aspectos de la enfermedad como la progresión o la inflamación (Gyetko M et al, 1994).

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Research article

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Regulatory polymorphisms in extracellular matrix protease genes and susceptibility to rheumatoid arthritis: a case-control study

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Received: 16 Aug 2005 Revisions requested: 26 Sep 2005 Revisions received: 3 Oct 2005 Accepted: 10 Oct 2005 Published: 1 Nov 2005

Arthritis Research & Therapy 2006, **8**:R1 (doi:10.1186/ar1849)
This article is online at: http://arthritis-research.com/content/8/1/R1
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Abstract

Many extracellular matrix (ECM) proteases seem to be important in rheumatoid arthritis (RA) and regulation of their transcription levels is a critical mechanism for controlling their activity. We have investigated, therefore, whether the best-characterized single nucleotide polymorphisms (SNPs) affecting transcription of the ECM proteases that have been related with joint pathology are associated with RA susceptibility. Nine SNPs in eight genes were selected by bibliographic search, including SNPs in the genes encoding matrix metalloproteinase (MMP)1, MMP2, MMP3, MMP7, MMP9, MMP13, plasminogen activator, tissue type (PLAT) and PAI-1. They were studied in a case-control setting that included 550 RA patients and 652 controls of Spanish ancestry from a single center. Genotyping was performed by single-base extension. Only two of the nine SNPs showed significant association with RA susceptibility. RA patients showed increased frequencies of the -7351 T allele of the gene encoding PLAT (36.4% versus 32.1% in controls, $p = 0.026$) and the -1306 T allele of the gene encoding MMP2 (24.5% versus 20.3% in controls, $p = 0.013$). These two alleles seemed to cooperate according to an additive model with respect to increased RA susceptibility ($p = 0.004$), and they were the low-expression alleles of the respective SNPs in a *PLAT* enhancer and the *MMP2* promoter. These findings are in agreement with previous data suggesting that these two ECM proteases have a protective role in RA pathology. Confirmation of these associations will be needed to support these hypotheses. The remaining SNPs did not show association, either individually or collectively. Therefore, although regulatory SNPs in ECM proteases did not show any major effect on RA susceptibility, it was possible to find modest associations that, if replicated, will have interesting implications in the understanding of RA pathology.

Introduction

Many studies support an important role for genetic factors in rheumatoid arthritis (RA) susceptibility and progression [1]. Overall, the genetic component has been estimated to account for about 50% of the variance in disease susceptibility, the remainder being environmental and stochastic components. The best known RA genetic factor is the human leukocyte antigen (*HLA*) gene, where multiple alleles of the DRbeta1 chain that share a common epitope in the third hypervariable region determine disease susceptibility and severity. Other HLA molecules and several non-*HLA* genes have also been related with RA susceptibility. Among the many genes that have been studied, only two that encode extracellular matrix (ECM) proteases have been explored [2-6], despite the unequivocal involvement of this family of proteins in RA.

The ECM proteases comprise a large family of proteins grouped in several subfamilies, including the matrix metalloproteinases (MMPs), the most extensively studied in RA [7-9]. Many MMPs are expressed at increased levels in RA tissues and in synoviocyte cultures in response to inflammatory cytokines, show specificity for joint tissue components and affect the evolution of experimental models of arthritis. Drugs able to inhibit a wide array of MMPs have been tried for the treatment of RA and, although effective in experimental models, human clinical trials had to be discontinued due to intolerable side effects. It is expected that more specific protease

CI = confidence interval; ECM = extracellular matrix; IQR = interquartile range; LD = linkage disequilibrium; MMP = matrix metalloproteinase; OR = odds ratio; PAI = plasminogen activator inhibitor; PCR = polymerase chain reaction; PLAT = plasminogen activator, PLAU: plasminogen activator, urokinase tissue type; RA = rheumatoid arthritis; SAP = shrimp alkaline phosphatase; SNP = single nucleotide polymorphism.

inhibitors will retain therapeutic potential without the associated side effects. It is unclear what ECM proteases to target with these drugs, however, because it has been difficult to ascertain the specific participation of each of them in RA. As a group, they are the major actors in the degradation of ECM in RA cartilage and bone. In addition, they increase and perpetuate joint inflammation through the activation of cytokines, chemokines and other proteases by cleavage of their precursors at specific sites [7-10]. They can also contribute to inflammation by exposing cryptic epitopes in ECM components that have biological actions in angiogenesis, cell migration and proliferation [10]. The difficulty in discerning the specific role of each of the ECM proteases found in the joints stems from the apparent redundancy of their effects and the wide variety of targets that each could degrade. We expect that genetic studies will provide clues to the identities of proteases that are critical for the RA process.

We have searched bibliographic databases for ECM proteases and their specific inhibitor proteins that have been described as involved in cartilage homeostasis and joint pathology. About 35 were identified with very varied supporting evidence, including some with a putative protective effect. We looked for evidence of single nucleotide polymorphisms (SNPs) in the genes encoding these ECM proteases that have shown a regulatory effect on their transcription level, most often from reporter gene assays but also from electrophoretic mobility-shift assays and in some cases from *ex vivo* studies. Nine SNPs in eight genes that fulfilled these criteria were found. In addition, each of these SNPs has been associated with susceptibility to at least one from a wide list of diseases, including cardiovascular diseases, aneurysms, preterm rupture of amniotic membranes, and tumor metastasis, indicating that their effects in gene transcription have a significant *in vivo* repercussion. The nine SNPs were found in the promoters or enhancers of the genes encoding MMP1 [11], MMP2 [12], MMP3 [13], MMP7 (with two SNPs) [14], MMP9 [15], MMP13 [16], plasminogen activator, tissue type (PLAT) [17] and plasminogen activator inhibitor-1 (PAI-1) [18]. They were selected as appropriate candidates to participate in RA susceptibility and studied in a large case-control setting. Two of the SNPs, those in *PLAT* and *MMP2*, showed moderate association with RA. The alleles of these two SNPs found with increased frequency in RA patients are low-expression alleles, which is in agreement with previous data suggesting that these ECM proteases have a protective effect in RA.

Materials and methods

Patients and controls

We sought to include all the 980 RA patients followed in the Rheumatology Unit of the University Clinical Hospital of Santiago de Compostela. Of these, 91 patients were not retrievable, 98 had died or were too sick to participate and 85 were unwilling to collaborate. Of the remaining 706 patients, 156 were excluded because they had non-Spanish ancestry or because of discrepancies with the American College of Rheumatology revised classification criteria for RA; 550 RA patients were available for the study. The control samples were from 652 subjects older than 55 years of age undergoing preoperative work-up for elective surgery excluding orthopedics. All were of Spanish ancestry and resided in the reference area of the Hospital. The Ethical Committee for Clinical Research of Galicia approved this study and all participants gave their written informed consent.

Genotyping

Peripheral blood DNA was used to genotype the following nine SNPs: MMP1 -1607 1G/2G (rs1799750); MMP2 -1306 C/T (rs243865); MMP3 -1171 6A/5A (rs3025058); MMP7 -181 A/G and -153 C/T; MMP9 -1562 C/T (rs3918242); MMP13 -77 A/G (rs2252070); PLAT -7351 C/T (rs2020918); and PAI-1 -675 5G/4G (rs1799768).

PCR was performed in two multiplex reactions with the QIAGEN Multiplex PCR Kit (QIAGEN, Valencia, CA, USA), each containing 30 ng of genomic DNA. One multiplex reaction was carried out for PLAT, MMP13, PAI, MMP9 and MMP2, and the other included MMP3, MMP7 and MMP1. PCR conditions were: initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s, and extension at 72°C for 90 s. Final extension was performed for 10 minutes at 72°C. Primers were designed with the FastPCR software (obtained from Dr Ruslan Kalendar, University of Helsinki). PCR products were purified by Exo-SAP digestion with Exonuclease I (Epicentre, Madison, WI, USA) and shrimp alkaline phosphatase (SAP; Amersham Biosciences, Barcelona, Spain) for 1 h at 37°C, and 15 minutes at 75°C to inactivate the enzymes. Single-base extension reactions with the SNaPshot Multiplex Kit (Applied Biosystems, Foster City, CA, USA) were done. Reaction conditions were: 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and single-base extension at 60°C for 30 s. Post-extension treatment with SAP was done for 1 h at 37°C. Samples were analyzed in the ABI prism 3100 Avant Genetic Analyzer (Applied Biosystems). Sequences of the PCR primers and of the single base extension oligonucleotides are available from the authors upon request.

Sequencing

Several samples with each of the observed genotypes were sequenced to test the accuracy of genotyping. The system used for sequencing was the Big Dye Ready Reaction Kit (Applied Biosystems) on an ABI prism 3100 Avant Genetic Analyzer (Applied Biosystems). Cycling conditions were: initial denaturation at 96°C for 4 minutes, followed by 30 cycles of denaturation at 96°C for 15 s, annealing at 50°C for 10 s, and extension at 60°C for 3 minutes. Final elongation was done at 60°C for 10 minutes.

Table 1

Clinical characteristics of the rheumatoid arthritis patients included in the study

Characteristic	Value
Females (%)	421 (76.5)
Age of disease onset (median, IQR)	49 (37–57)
Follow-up (median, IQR)	13 (7–21)
Rheumatoid factor (%)[a]	280 (62.5)
Bone erosions (%)[a]	284 (64.8)
Extra-articular manifestations (%)[a]	48 (11.2)
DMARDS use (%)[a]	465 (94.9)
Tobacco use (%)[a]	84 (18.4)

[a]This information was not available for a variable number of patients (from 10.9 for disease-modifying anti-rheumatic drugs (DMARDS) use to 21.8% for extra-articular manifestations). IQR, interquartile range.

Statistical and genetic analysis

Statistical analysis was done with the Statistica software (Statsoft, Tulsa, OK, USA). Allele frequencies, their interquartile ranges (IQR), odds ratios (ORs) and their 95% confidence intervals (95% CI) were calculated. Comparison of allele frequencies was done using a two by two contingency table with a chi-square test. Evidence of a gene dose effect was evaluated with univariate logistic regression applying an additive genetic model (codes were: 0 for AA, 1 for Aa and 2 for aa genotypes). Multivariate comparison of the coordinate effect of the protease genotypes, as well as analysis of the effect of clinical features as covariants, was done with stepwise backward logistic regression analysis. Analysis of the gene-gene interaction between the *PLAT* and *MMP2* SNPs was done with the LRASSOC software [19], which implements well-defined genetic models for gene interactions. Model selection was based in the lowest Akaike's information criterion. *Post hoc* power of the study was estimated for alfa = 0.05 with the Gpower software [20]. Relationships between clinical features and genotypes were analyzed with Student's *t* test for quantitative variables and chi-squared test for the contingency tables of qualitative features.

Linkage disequilibrium (LD) between the SNPs in chromosome 11 was analyzed with the *ldmax* software [21]. Haplotype frequencies were estimated with the PL-EM software [22], which uses an implementation of the expectation-maximization algorithm. Comparison of haplotype frequencies was done with a nonparametric homogeneity test and with a permutation test performed with the Clump software [23].

Results

Study characteristics

The characteristics of the RA patients are shown in Table 1. Women were more abundant in the RA group (421 of 550, 76.5%, 95% CI = 73–80) than in the control group (344 of the 642, 52.8%, 95% CI = 49–56). This difference did not affect the results, however, as associations were independent of sex as shown below. The median age at disease onset was 49 years (IQR 37–57) and the median follow-up was 13 years (IQR 7–21 years). Controls (median age = 69 years, IQR 62–76 years) were selected over 55 years of age that corresponded to percentile 70 of the age at disease onset in our series of RA patients. Genotypes for the nine SNPs analyzed were determined unambiguously in 99.7% of the samples, and confirmed by sequencing a fraction of them. The genotype distributions of all SNPs were in concordance with the Hardy-Weinberg equilibrium.

Genetic susceptibility to RA

Allelic frequencies of seven of the nine SNPs were similar in RA patients and controls (Table 2). Only the *PLAT* -7351 C/T SNP and the *MMP2* -1306 C/T SNP were significantly different. In the case of *PLAT*, the T allele was significantly (p = 0.026) more frequent in the RA patients (36.4%, 95% CI = 33–39) than in controls (32.1%, 95% CI = 29–34). In the case of *MMP2*, the T allele was significantly (p = 0.013) more frequent in RA patients (24.5%, 95% CI = 22–27) than in controls (20.3%, 95% CI = 18–22). Analysis of genotype frequencies by univariate logistic regression produced similar results: the effect of the T allele of *PLAT* -7351 C/T was dose-dependent according to an additive genetic model (p = 0.026; OR = 1.21, 95% CI 1.02–1.43) as shown in Figure 1a; similarly, the effect of the T allele of *MMP2* -1306 C/T was in agreement with an additive genetic model (p = 0.013; OR = 1.27, 95% CI 1.05–1.55) as shown in Figure 1b. These results were not significantly modified by the inclusion of sex as a covariant (Table 2): the OR for *PLAT* genotypes was 1.20 after adjusting for sex and the OR for *MMP2* was unchanged after the inclusion of this covariant (OR = 1.27). Similarly, the sex-adjusted ORs of the other ECM protease SNPs were not significantly different from the unadjusted ORs (Table 2).

Gene-gene interactions that could involve any of the nine ECM protease SNPs were ascertained with multivariate logistic regression analysis. Only an additive genetic model was tested. The inclusion of the different SNPs was based on a backwards stepwise approach. The best model included only the two previously mentioned SNPs (*PLAT* -7351 C/T and *MMP2* -1306 C/T) and it showed a slightly better fit to data than any of the two SNPs separately (p for the model with the two SNPs = 0.004). There was no evidence of significant gene-gene interactions with any of the other seven SNPs, those without an effect in the individual analyses. We also explored whether there were any specific relationships between the clinical features of the RA patients (Table 1) and the nine SNPs, but none was found.

The statistical characteristics of the interaction between the *PLAT* and *MMP2* SNPs were analyzed with the LRASSOC

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Table 2

Allele frequencies of the nine regulatory single nucleotide polymorphisms in extracellular matrix proteases

Gene	SNP	Allele	RA patients	Controls	OR	*p*	Sex-adjusted OR	Change in transcription[a]
MMP1	-1607 1G/2G	2G	47.8	48.2	0.98	0.8	0.96	Up
MMP2	-1306 C/T	T	24.5	20.3	1.27	0.01	1.27	Down
MMP3	-1171 6A/5A	5A	50.5	51.7	0.95	0.5	0.96	Up
MMP7	-181 A/G	G	43.8	45.3	0.94	0.4	0.95	Up
	-153 C/T	T	5.0	5.1	0.99	0.9	0.98	Up
MMP9	-1562 C/T	T	14.3	13.6	1.05	0.6	1.02	Up
MMP13	-77 A/G	A	68.0	65.6	1.13	0.2	1.09	Up
PLAT	-7351 C/T	T	36.4	32.1	1.21	0.03	1.20	Down
PAI-1	-675 5G/4G	4G	47.6	46.8	1.03	0.7	1.05	Up

Frequency values for rheumatoid arthritis (RA) and controls are percentages.
[a]Reported up- or downregulation of gene expression determined by the mentioned allele. MMP, matrix metalloproteinase; OR, odds ratio; PAI, plasminogen activator inhibitor; PLAT, plasminogen activator, tissue type; SNP, single nucleotide polymorphism.

software. This software checks the relative fitting to data of a series of genetic models that include parameters for additive, dominance and epistatic interactions between two genes. The model that best accounted for the data was the model assuming additive effects of both SNPs, without dominance or interactive components.

Five of the studied ECM protease genes (*MMP7*, *MMP1*, *MMP3* and *MMP13*) are in a MMP cluster that covers 500 kb in chromosome 11q and includes at least another five MMP-encoding genes. Therefore, we checked if they were in LD and if the haplotypes defined by them were associated with RA as a way to explore possible effects in the region not accounted for by the studied SNPs. There was significant LD between the two SNPs in *MMP7* (-181 A/G and -153 C/T), between the *MMP1* -1607 1G/2G and *MMP3* -1171 6A/5A SNPs, and between *MMP13* -77 A/G and two other SNPs (*MMP3* -1171 6A/5A and *MMP7* -153 C/T). Comparison of the frequencies of the haplotypes defined by the pairs of SNPs in LD did not disclose significant differences between RA patients and controls (not shown).

Discussion

The lack of association with RA of seven of the regulatory SNPs in ECM proteases was somehow unexpected because they affect proteases with a recognized role in RA [7-9] and because the size of the study allowed for detection of modest effects (for example, the *post hoc* power to detect an excess of the T allele of the *MMP9* SNP was 87% for a risk ratio of 1.2). In fact, there is much more published evidence supporting the involvement of some of the ECM proteases that did not show association in our study than the two that were associated with RA. This is especially clear for the metalloproteinases MMP13, MMP1, MMP3 and MMP9. We also analyzed the possibility of cooperation or of cumulative effects between these SNPs with regard to their association with RA. It would be a mistake, however, to interpret too strongly these results as questioning the importance of these six ECM proteases in RA. At least two factors moderate a conclusion of this type. The multiplicity of control mechanisms of ECM protease activity, of which transcription regulation is only one of importance, compartmentalization by pericellular accumulation, activation by cleavage of latent pro-enzymes and inhibition by specific proteins being the others [7,8]. Also, variation in these proteases could impinge on other aspects of disease progression different from disease susceptibility, although we did not find significant association with any of the clinical features available for study. They did not include disease activity indexes or quantitative assessment of bone erosions, however, which could be more informative of the possible involvement of these SNPs. This seems the case for the SNP in *MMP3* found in previous studies to be associated with quantitatively evaluated RA erosions [3,5], but not to RA susceptibility [3,6]. It is also possible that the SNP in *MMP1* predisposes to some RA features because there are reports of association with RA inflammatory activity [4] or with RA erosions [6] although these associations were not found in other studies [2].

The two regulatory SNPs that showed moderate association with RA susceptibility cause lower transcription of their respective genes, *PLAT* and *MMP2*. Both of them seem genuine associations because they have been found in a hypothesis driven case-control study and have shown modest effects, in the range that is expected in complex diseases and specifically in RA [1]. The size of the study, 550 cases and 642 controls, effectively prevents interference from random variation of allelic frequencies, which is the major cause of false positive results in genetic association studies [24]. In this regard, it is reassuring that about 90% of the associations shown in studies involving more than 150 cases and controls

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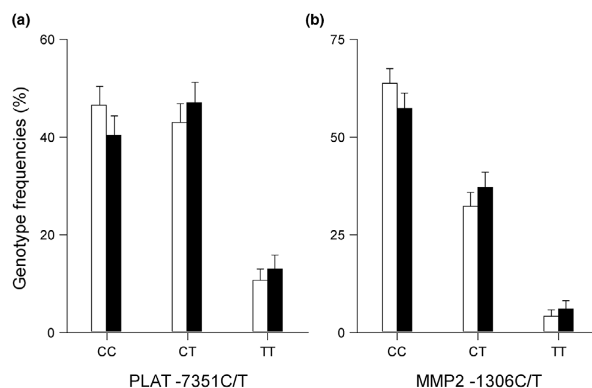
Figure 1



Genotype frequencies (%) of single nucleotide polymorphisms (SNPs) associated with rheumatoid arthritis (RA). **(a)** The *PLAT* -7351 C/T and **(b)** the *MMP2* -1306 C/T SNP in controls (blank columns) and RA patients (filled columns). Error bars represent the 95% confidence interval.

have subsequently been replicated, as pointed out in a recent meta-analysis [24]. Finally, population stratification, another widely claimed cause of spurious association results, is not a significant concern in this study as all cases and controls comprised a very homogenous population. They resided in a largely rural area where immigration has been very restricted. Specifically, 71.8% of the RA patients and 74.0% of the controls had all known ancestors from the same province (Corunna), and 95.4% of the RA patients and 95.3% of the controls were from the same historic region (Galicia, composed of four of the 52 Spanish provinces). In addition, analysis of data restricted to study participants with all known ancestors from Galicia or from Corunna gave similar results to those from the whole study. Nevertheless, circumspection should be exercised in interpreting these associations until the results can be replicated.

PLAT participation in RA has been related to fibrin accumulation in RA synovial cavities [25-27] and the association found here could be involved in this process. A contributing factor in the increase of fibrin could be the lower or unchanged expression of PLAT in RA synovium compared to healthy synovium [28,29], which would cause the availability of plasmin, the major fibrinolytic enzyme, to be restricted. A putative protective role for PLAT in arthritis has also been shown in experimental models of RA [30,31]. As the T allele of *PLAT* -7351 disrupts a GC box in the *PLAT* enhancer [17,32] it could contribute to the insufficient fibrinolysis and, thus, to RA. Other processes in which hypofibrinolysis is a contributing disease mechanism, such as myocardial infarction [33] and lacunar stroke [34], have also been associated with the T allele of the *PLAT* -7351 C/T SNP and these two diseases are observed at increased rate in RA.

Very few studies have addressed the role of MMP2 in RA. It has been assumed that MMP2 promotes RA by participating in cartilage degradation and by activating pro-inflammatory mediators based on its *in vitro* reactivity [8,9]. However, MMP2 levels and activity seem to be unaltered in human RA [35-37] and in experimental models of RA [38]. In addition, MMP2 plays a suppressive role in the pathogenesis of antibody-induced arthritis, as shown by an exacerbated disease in MMP2 deficient mice [39]. A likely mechanism for MMP2-mediated protection against RA involves the inactivation of chemokines (CCL7 and SDF1), thereby limiting inflammatory infiltration [40,41]. Consistent with a protective role for MMP2 in RA, the allele associated with increased RA susceptibility in our study is the low-expression allele [42,43]. The *in vivo* relevance of this change has been demonstrated by the association of the *MMP2* -1306 C/T SNP with several types of cancer [43-48], although a recent report showed no association with chronic periodontitis [49], a disease with many similarities to RA with respect to inflammation and ECM proteases [50].

Our study indicates that both SNPs act independently in their contribution to RA liability. This conclusion is consistent with the independent roles proposed for the two proteases for their putative protective effect in RA. Interaction with other gene polymorphisms should be explored as it is likely that variants of cytokine genes that are important in RA and that trigger ECM protease gene expression, especially tumor necrosis factor and interleukin-1 but also interleukin-6, epidermal growth fac-

tor, platelet-derived growth factor, basic fibroblast growth factor and transforming growth factor-beta, potentiate the effect of the SNPs studied here. In the same way, it is possible that variants of the genes encoding chemokines that are cleaved by MMP2 could interact with the -1306 C/T SNP in determining increased RA susceptibility.

Conclusion

It seems that genetic variants affecting transcription of ECM proteases are not major contributors to RA susceptibility. It is possible, however, that some play a minor role as shown here for the *PLAT* and *MMP2* SNPs. These associations need to be confirmed, although it is already possible to see that the likely effects of these SNPs are consistent with previous evidence supporting a protective effect of the two proteases in arthritis. Confirmation of these associations will lend support to this hypothesis and will show how important it is to define the participation of each ECM protease in joint pathology before trying to manipulate them therapeutically.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to the final manuscript. In addition, JR-L did the genotyping and participated in the design of the study and the analysis of results, EP-P reviewed all the clinical data from the patients and selected them according to the ACR classification criteria, JG-R participated in the selection of patients, and AG coordinated the study and participated in its design, experiments, analysis and in the writing of this manuscript.

Acknowledgements

We thank sample donors for their collaboration and Drs Antonio Mera, Manuel Caamaño, Jorge Blanco and Santos Insua for providing access to their patients. Lorena Fernandez-Blanco collaborated in the selection of the functional SNPs. We thank also Yolanda Lopez-Golan and Fina Meijide for their help in recruiting study participants. This project was supported by grant PI02/0713 from the Instituto de Salud Carlos III (Spain) with participation of funds from FEDER (European Union). JR-L is the recipient of a scholarship of the National Program for the Training of University Professors of the Spanish Ministry of Education. AG was supported by the Instituto de Salud Carlos III (Spain).

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Further evidence of the role of frizzled-related protein gene polymorphisms in osteoarthritis

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Ann Rheum Dis 2007;**66**:1052–1055. doi: 10.1136/ard.2006.065938

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Accepted 13 January 2007
Published Online First 19 January 2007

Objective: To replicate the association of frizzled-related protein (FRZB) non-synonymous polymorphisms with osteoarthritis (OA) susceptibility.
Methods: Three groups of Spanish patients with OA were included: with total joint replacement due to primary OA in the hip (n = 310), or the knee (n = 277), or with hand OA (n = 242). Controls were more than 55 years old and did not show OA (n = 294). SNPs rs288326 (R200W) and rs7775 (R324G) were genotyped.
Results: There were no significant differences in allele frequencies between controls and each of the three groups of OA patients. However, allele G of the R324G SNP showed a trend to be more frequent in patients with a clinical OA syndrome at multiple joints (p = 0.07), specifically in women of the total hip replacement group (8.3% in patients without other affected joints, 13.1% with one, 15.9% with two and 24.1% with more than two additional joints, p for trend = 0.008).
Conclusions: No direct replication of previous OA association findings was obtained but the results suggest that the R324G SNP of the FRZB gene may have an effect in OA development in multiple joints, with a specific severe involvement of the hip in women. This phenotype could reconcile previous studies that showed association either with generalised OA or with hip OA in women.

It is well known that osteoarthritis (OA) is the commonest form of joint disease and a leading cause of disability in older people,[1] but while some people develop OA changes in their 40s and 50s, others enjoy long lives without joint problems. Several factors play a role in the variable OA risk, including age, sex, genetics, ethnicity, behavioural influences, obesity and occupation.[2] Genetic factors have been shown to account for between 40% and 85%, depending on the phenotype considered, of the variance in liability to OA and they contribute to a twofold to fivefold increase in OA risk in patient siblings.[3] However, each genetic factor has a modest effect and, consequently, their identification and study are difficult.[2 4] A further complication of research in OA derives from a variety of disease phenotypes that seem to depend on different susceptibility factors[2 4] and could be related to different pathogenic mechanisms.[5] Therefore genetic variants predisposing to OA in one joint do not have, in general, the same effect in other joints and may even differ between men and women.[6] Our current knowledge of the disease is too crude to explain these differential effects, or to identify differences between these joint-specific factors and others that seem to predispose to OA more broadly. In spite of these difficulties, several genetic factors have been shown reproducibly to participate in OA predisposition, such as polymorphisms in the oestrogen receptor a gene (ESR1) or the interleukin 1 (IL1) cluster and a repeat polymorphism in the asporin gene.[2 7]

A series of recent studies show some evidence implicating non-synonymous single nucleotide polymorphism (nsSNPs) of the frizzled-related protein (FRZB) gene, coding for the secreted frizzled-related protein 3 (SFRP3), in a couple of OA phenotypes. The first evidence was obtained by linkage analysis of UK sibling pairs concordant for total hip replacement (THR) for primary OA.[8] FRZB is in the linked locus, in chromosome 2q, and is a likely candidate because, as in its role of inhibitor of WNT signalling, it is involved in bone and joint development.[9–11] Association to a nsSNP (rs7775 causing a R324G substitution) in FRZB was found in female probands. Replication of the association in the THR female subset of a case-controlled UK cohort in the same report strengthened the case for a direct role of the R324G SNP of FRZB in severe hip OA in women.[8] There was also evidence of a possible effect of another FRZB nsSNP (rs288326 causing a R200W substitution) only in women bearing both the G allele of R324G and the W allele of R200W. Functional experiments showed that the 324G allele of SFRP3 is less effective in blocking WNT signalling, an effect that will be related to a decreased sequestration of WNT ligands in the extracellular space. Additional experiments showed that other SNPs in the FRZB promoter do not significantly affect transcription levels and are unlikely to be alternative causal variants.[12] A subsequent study has obtained confirmatory evidence for the role of the FRZB nsSNPs in radiographic hip OA characterised by severe joint space narrowing in women.[13] In this case the effect was mainly associated with the R200W SNP, although carriage of both the R200W and R324G rare alleles was a risk factor. Another study showed an effect of the R200W SNP, but not of the R324G SNP, in the difference between women with THR and women with osteoporosis but not in the comparison with healthy controls.[14] Neither of these two replication studies included patients with OA in other joints or men with hip OA. A broader range of OA phenotypes was explored in a third replication study that included both male and female subjects with radiographic OA in the hip or in multiple joint regions in a population-based cohort.[15] They also included sibling pairs selected for the presence of symptomatic OA at multiple sites. In this study the OA predisposition effect of the FRZB nsSNPs was only evident for the R324G SNP and in patients with polyarticular OA (no sex effect was mentioned) but not in those with hip OA. Therefore, it seems that there is already convincing evidence, though not completely conclusive, of the involvement in OA of nsSNPs in the FRZB gene. However, there are still many unresolved questions including whether they play a role only in women, only in hip OA, or in polyarticular OA, or if each of the two non-synonymous SNPs

Abbreviations: FRZB, frizzled-related protein; HOA, hand osteoarthritis; OA, osteoarthritis; THR, total hip replacement; TKR, total knee replacement

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has an effect on their own or whether both rare alleles need to be present in the same subject. We have tried to address some of these outstanding questions.

MATERIAL AND METHODS

Patients and controls

Selection criteria and patient characteristics have been described.[16] In brief, subjects were recruited from patients undergoing total hip replacement (THR) or total knee replacement (TKR) only if a rheumatologist considered them to suffer from severe primary OA (a directed questionnaire, the clinical history and radiographs previous to surgery were reviewed). A third group included patients attending the rheumatology unit because of hand OA (HOA) complaints who fulfilled American College of Rheumatology classification criteria.[17] The presence of chronic pain and incapacity in other joint regions different from the one motivating recruitment of the patients, as well as hand deformities in THR and TKR patients, were recorded. These manifestations were considered as evidence of clinical OA by a rheumatologist after excluding other causes by means of anamnesis following a directed questionnaire and review of the clinical history. Radiographs of the additional joint regions were not a requisite.

Controls were older than 55 years. Only the 31.6% who were free of clinical manifestations compatible with OA (radiographic examination was not performed) were included. This selection introduced a bias in the controls: of the 929 voluntary donors 58.2% were women, but after exclusion of those with features compatible with OA only 39.1% were women. Accordingly, comparisons were stratified by sex. The final numbers in each group are shown in table 1. This study was approved by the Ethical Committee for Clinical Research of Galicia and all participants gave their written informed consent. All were of Spanish origin and resided in the reference area of the hospital.

Genotyping

Peripheral blood samples were used to obtain DNA. TaqMan (Applied Biosystems, Foster City, CA) 5′ nuclease assays were used to genotype two non-synonymous FRZB SNPs: rs288326 C/T that causes the R200W substitution in exon 4 and rs7775 C/G producing the R324G substitution in exon 6. Primers and fluorescence-labelled probes were designed and synthesised by Applied Biosystems and their sequences are available from the authors. TaqMan reactions were performed in a total volume of 10 µl containing 24 ng genomic DNA following the Applied Biosystems protocol. A Chromo4 real-time PCR system (MJ Research, Waltham, MA) was used to run these assays. Several samples with different genotypes were sequenced with the Big Dye Ready Reaction Kit (Applied Biosystem) to test the accuracy of the genotypes.

Statistical analysis

Statistical analysis was carried out with the Statistica software (Statsoft, Tulsa OK). Allele frequencies, odds ratios (OR) and their 95% confidence intervals were calculated. Comparison of allele frequencies was done using a two-by-two contingency table with a X^2 test. Evidence of a gene dose effect was evaluated with univariate logistic regression applying an additive genetic model (codes were: 0 for AA, 1 for Aa and 2 for aa genotypes). Multivariate logistic regression was used to evaluate the effect of the two nsSNPs together. The effect of genotypes in number of OA localisations was determined via logistic regression. Linkage disequilibrium, estimation of haplotype frequencies and comparison of those frequencies between patients and controls were made with the Haploview software.

RESULTS

Three groups of patients have been included and their characteristics are shown in table 1. We have included in this table the percentages of patients showing clinical manifestations compatible with OA affectation in multiple joint regions. Controls were older than 55 years and selected to be free of symptomatic OA. Numbers of men in the TKR and HOA groups were small and results relative to them should be taken with caution. Power to detect an effect with OR = 2.0 in the THR patients was over 0.98, and for an OR = 1.5 it was 0.65 for the R324G SNP and 0.75 for the R200W SNP. Valid genotypes for the two FRZB nsSNPs were obtained in 98.7% of the subjects. Their frequencies were in accordance with the Hardy–Weinberg equilibrium.

In contrast with what could be expected based on some previous reports,[8 13] the frequencies of the rare alleles of the R324G SNP and of the R200W SNP were not significantly elevated in women with hip OA compared with healthy controls (table 2). In addition, women with the other two OA phenotypes, TKR and HOA that were studied for the first time

Table 1 Characteristics of patients and controls included in the different groups

Characteristics	OA patients THR	TKR	HOA	Controls
Number of subjects	310	277	242	294
Sex (% women, women/total)	60.6 188/310	80.5 223/277	88.4 214/242	39.1 115/294
Age of disease onset (median IQR*)	61 55–67	56 50–63	50 44–56	
Age at recruitment (median IQR)	69 64–73	69 64–72	61 54–68	68 61–75
BMI (median IQR)	30.1 27.7–32.9	32.9 29.9–36.0	28.3 26.0–31.3	
OA in other joint regions % (number)				
No other region	42.6 (132)	35.0 (97)	44.2 (107)	
1 region	31.3 (97)	31.8 (88)	30.2 (73)	
2 regions	16.1 (50)	22.4 (62)	14.5 (35)	
>2 regions	10.0 (31)	10.8 (30)	11.1 (27)	

THR, total hip replacement; TKR, total knee replacement; HOA, hand osteoarthritis.
*IQR, Interquartile range.

Table 2 Minor allele frequencies* of the two nsSNPs of FRZB

	Women	Men	Total
R324G			
Controls	11.6 (26/224)	10.7 (38/354)	11.1 (64/578)
THR	14.1 (52/368)	9.7 (23/236)	12.4 (75/604)
TKR	11.0 (49/444)	12 (13/104)	11.3 (62/548)
HOA	12.1 (51/420)	9 (5/54)	11.8 (58/474)
R200W			
Controls	11.3 (26/230)	17.6† (63/358)	15.1 (89/588)
THR	9.9 (37/372)	14.0 (34/242)	11.6 (71/614)
TKR	13.5 (59/436)	16 (17/106)	14.0 (76/542)
HOA	11.8 (50/422)	9 (5/54)	11.5 (55/476)

*Frequencies expressed as % and number of minor alleles/total number of alleles.
†This frequency was significantly larger than in female controls (p = 0.03). However, 126 additional male controls showed a W allele frequency (11.5%) very similar to the female frequency.
THR, total hip replacement; TKR, total knee replacement; HOA, hand osteoarthritis.

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in relation with these SNPs, did not show differences from controls. In the same way, there were not differences between male patients from any of the three patient groups and male controls (as mentioned male numbers in the TKR and, specially, in the HOA groups were small). Similar negative results were observed in the genotype analysis of the two nsSNPs using an additive genetic model (not shown).

A previous report has indicated that the G allele of the R324G SNP is associated with polyarticular OA.[15] We have found some supportive evidence by comparing the frequencies of this SNP in patients with manifestations compatible with OA in additional joint regions. Notably, the strength of the association increased with the number of joint regions involved. There was a near significant trend when men and women from the three patient groups were considered together, but the relationship became clear after stratifying by groups of patients (table 3). The effect was restricted to patients of the THR group, with no differences in the other two groups: TKR and HOA. Further stratification by sex showed that the association was exclusively observed in women (table 4). Women in the TKR and the HOA groups did not show this association. Frequencies of the R200W SNP, on the other hand, did not show any relationship with the number of additional joint regions with OA-compatible affectation (data not shown).

Finally, there was some previous evidence of a collective effect of the rare alleles of the two nsSNPs in OA susceptibility.[8 13] The two FRZB SNPs were not in linkage disequilibrium ($D' = 0.07$, $r^2 = 0.003$) in our samples as in all the previously studied populations. The haplotype frequency distribution was not different between THR women and controls, or between the other groups of patients and controls (not shown). In addition, there was not any evidence of a collective effect of the two SNPs in the number of additional joint regions involved. However, it should be noted that the rare alleles of the two nsSNPs appear together in very few subjects, given their low frequency and lack of linkage disequilibrium, and this study lacks power to detect a moderate effect of such a rare event.

Table 3 Minor allele frequencies* of the two nsSNPs of FRZB in patients with OA stratified by number of joint regions in addition to the one that had determined patient recruitment showing OA compatible features

	Number of additionally affected joint regions				
	0	1	2	>2	p for trend
R324G					
All groups	10.1 67/662	12.2 62/506	13.4 39/290	14.5 25/172	0.05
THR	9.7 25/258	12.2 23/188	14.3 14/98	22 13/60	0.01
TKR	10.3 20/194	13.4 23/172	11.3 14/124	9 5/58	0.9
HOA	10.5 22/210	11.0 16/146	16 11/68	13 7/54	0.3
R200W					
All groups	12.9 86/666	11.8 60/508	11.1 32/288	13.8 24/174	0.9
THR	13.0 34/262	8.8 17/192	10.0 10/100	17 10/60	0.9
TKR	16.7 32/192	12.9 22/170	12.5 15/120	12 7/60	0.2
HOA	9.4 20/212	14.4 21/145	10 7/68	13 7/54	0.5

*Frequencies were expressed as % and number of minor alleles/total number of alleles.
THR, total hip replacement; TKR, total knee replacement; HOA, hand osteoarthritis.

Table 4 Minor allele frequencies* of the R324G SNP in patients of the THR group stratified by sex

	Number of additionally affected joint regions				
	0	1	2	>2	p for trend
Women	8.3 8/96	13.1 17/130	16 14/88	24 13/54	0.008
Men	10.5 17/162	10 6/58	0 0/10	0 0	0.3

*Frequencies were expressed as % and number of minor alleles/total number of alleles.

DISCUSSION

Progressively, new genetic factors that seem implicated in OA susceptibility have been reported.[2 4] Evidence involving several of them starts to be compelling though it is not yet clear if any has reached a level of confirmation that could be taken as definitive. Experience in the study of other complex diseases, where rarely a genetic variant is consistently found to have the same effect in different studies, has led some authors[18] to think that only very significant and straightforward results, not involving analysis of subphenotypes or subgroups of patients, should be considered as evidence of a true genetic effect. However, research into OA has been providing examples of variants that show association in successive studies in spite of modest p values and of being restricted to a limited phenotype in the wide spectrum of the disease. In the case of the FRZB gene, some evidence of an effect of two nsSNPs in OA susceptibility has already been found in the four studies that have been conducted.[8 13–15] In each case the effect has been modest at best, with p values barely below 0.05, and always restricted to a subgroup of patients. In addition, the evidence of association has not been identical but with particular aspects in each case. Our results should be considered within this framework.

The nsSNP that we have found associated, R324G, has been the susceptibility SNP in two of the previous studies,[8 15] and is also the SNP that has been shown to have a functional effect.[8] This makes it likely that it will be a genuine OA susceptibility variant. In contrast, the R200W SNP was associated with OA susceptibility only in two studies[13 14] and no functional consequences have been found for this nsSNP.[8] This makes it less likely that it will have an independent effect by itself. Some of the previous reports have obtained evidence of an increased susceptibility due to the concurrent presence of the rare alleles of these two nsSNPs.[8 13] We did not find any evidence of this, but due to the rarity of the presence of the two rare alleles in the same subject (expected frequency of about 1%) it will be necessary to use very large sample sizes to address this point adequately.

The most interesting aspect of our study was the suggestion of a particular subgroup of patients where the effect of FRZB seems more likely: women who were recruited because of THR by primary OA and had referred clinical complaints compatible with OA in other joint regions. The strength of the association increased with the number of additional joint regions affected. This phenotype is interesting because it can be regarded as compatible with that found in previous studies and provides a way to conciliate evidence that previously seemed contradictory. In effect, FRZB association with OA has been restricted to women, as in our results, in the only study where sex has been analysed.[8] Also, only hip OA, apart from polyarticular forms of OA, has been found to be associated with nsSNPs in FRZB. We have expanded the range of OA forms studied to TKR and HOA, but they did not show evidence of association. Finally, there was also a study showing association to polyarticular OA but not to hip OA.[15] We interpret our results as a possible bridge between the association with hip OA and

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with polyarticular OA. Against this interpretation, it can be argued that there was not any mention of polyarticular involvement in the reports showing association with hip OA[8 13 14] or of a particular involvement of the hip joint in the study reporting association with polyarticular OA.[15] Also, a limitation in the comparison of the different studies is that patients and controls have been selected according to different criteria. This is a universal problem in OA genetic epidemiology derived from the variety of clinical presentations of OA, lack of knowledge of the relation between the different forms and differences in emphasis on the different disease features.[2 3] It is certain that each of the approaches has limitations but they have also advantages over their alternatives. In any case, the association we have found, though related to previous findings, has new aspects and should be considered in this regard as a new hypothesis requiring further confirmation.

ACKNOWLEDGEMENTS

The authors would like to thank sample donors for their collaboration. They would also like to thank Yolanda Lopez-Golan and Fina Meijide for their help in recruiting study participants. Marta Picado and Cristina Fernandez provided outstanding technical assistance.

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Funding: This project was supported by grant PI02/0713 from the Instituto de Salud Carlos III (Spain) with participation of funds from FEDER (European Union), and by the Fundacion MMA (Madrid, Spain). JR-L is the recipient of a scholarship of the Spanish Ministry of Education. MP-S received a bursary from the Fundacion Española de Reumatologia. AG was supported by the Instituto de Salud Carlos III, Spain.

Competing interests: None declared

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Research article

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Lack of association of a variable number of aspartic acid residues in the asporin gene with osteoarthritis susceptibility: case-control studies in Spanish Caucasians

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Received: 21 Dec 2005 Revisions requested: 18 Jan 2006 Revisions received: 3 Feb 2006 Accepted: 14 Feb 2006 Published: 10 Mar 2006

Arthritis Research & Therapy 2006, **8**:R55 (doi:10.1186/ar1920)
This article is online at: http://arthritis-research.com/content/8/3/R55
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Abstract

A recent genetic association study has identified a microsatellite in the coding sequence of the asporin gene as a susceptibility factor for osteoarthritis (OA). Alleles of this microsatellite determine the variable number of aspartic acid residues in the amino-terminal end of the asporin protein. Asporin binds directly to the growth factor transforming growth factor beta and inhibits its anabolic effects in cartilage, which include stimulation of collagen and aggrecan synthesis. The OA-associated allele, with 14 aspartic acid residues, inhibits the anabolic effects of transforming growth factor beta more strongly than other asporin alleles, leading to increased OA liability. We have explored whether the association found in several cohorts of Japanese hip OA and knee OA patients was also present in Spanish Caucasians. We studied patients that had undergone total joint replacement for primary OA in the hip ($n = 303$) or the knee ($n = 188$) and patients with hand OA ($n = 233$), and we compared their results with controls ($n = 294$) lacking overt OA clinical symptoms. No significant differences were observed in any of the multiple comparisons performed, which included global tests of allele frequency distributions and specific comparisons as well as stratification by affected joint and by sex. Our results, together with reports from the United Kingdom and Greece, indicate that the stretch of aspartic acid residues in asporin is not an important factor in OA susceptibility among European Caucasians. It remains possible that lifestyle, environmental or genetic differences allow for an important effect of asporin variants in other ethnic groups as has been reported in the Japanese, but this should be supported by additional studies.

Introduction

Current concepts in osteoarthritis (OA) imply an imbalance of cartilage anabolic and catabolic processes in response to mechanical stress with the participation of inflammatory mediators [1]. Recent investigation has also shown that genetic factors account for a substantial portion of OA etiology [2,3]. These two considerations contribute to the interest of a recent report showing that a variant of asporin (ASPN) is a susceptibility factor for OA [4]. This association points to a defect in the anabolic side of cartilage homeostasis, identifies ASPN and transforming growth factor beta as important molecular players in this process, and suggests that regulatory genetic variants are important in OA susceptibility [5,6].

ASPN is a new member of the small leucin-rich proteoglycan subfamily of proteins [7,8] that is expressed at low levels in normal cartilage but that is notably increased in OA cartilage [4]. Variant ASPN proteins are due to a microsatellite in the ASPN coding sequence that determines a variable number of aspartic acid (D) residues in the amino terminal region. The D14 allele is associated with increased OA susceptibility in the Japanese (odds ratio = 1.66–2.49, depending on the cohort) [4] due to its strong inhibition of the cartilage anabolic effects induced by transforming growth factor beta [4]. Given the significance of these results, we explored the ASPN effect in Spanish patients with severe knee or hip OA or with hand OA, but we did not find association. This result is similar to

ASPN = asporin; D = aspartic acid; OA = osteoarthritis; PCR = polymerase chain reaction; THR = total hip replacement; TKR = total knee replacement.

findings in UK Caucasians [9] and in the Greek population [10], and together the observations indicate that the ASPN microsatellite does not have a significant effect on OA susceptibility in European Caucasians.

Materials and methods

Patients and controls

Patients were selected from consecutive patients undergoing total hip replacement (THR) or total knee replacement (TKR) and patients complaining of hand OA that were followed in the Rheumatology Unit. THR and TKR patients were included only if surgery has been performed at ages ranging from 55 to 75 years and if a rheumatologist considered them to suffer from severe primary OA after compatible evaluation and exclusion of the following confounding factors: inflammatory, infectious, traumatic or congenital joint pathology and lesions due to crystal deposition or osteonecrosis. Evaluation of patients included an interview with each patient specifically for this study and a review of the clinical history and a review of radiographs. Analysis of synovial fluid was not a requisite. Obesity and occupational strain were not considered exclusion causes. Patients with hand OA were required to fulfill American College of Rheumatology classification criteria [11].

The final numbers of patients in each group were 303 THR patients (183 women, 120 men), 188 TKR patients (153 women, 35 men) and 232 hand OA patients (205 women, 27 men). We recruited controls among subjects older than 55 years undergoing preoperative workup for elective surgeries other than joint surgery. We restricted analysis to the 35.5% of controls (294/828 donors, 115 women and 179 men) that did not show clinical manifestations of OA (absence of chronic pain or restriction of mobility in the two years before recruitment, no hand enlargements or deformities, and no previous medical evaluation as OA). Radiographic exploration was not performed in controls.

This study was approved by the Ethical Committee for Clinical Research of Galicia and all cases and controls gave their written informed consent to participate. All participants were of Spanish ancestry and resided in the reference area of the hospital.

Genotyping

Peripheral blood DNA was used to genotype the D-repeat microsatellite by PCR with the primers 5'-FAM-TGGCTTTGT-GCTCTGCCAAACC-3' and 5'-TCTGAGCAATGTA-CAACTCGTG-3'. The size of the fluorescence-labeled products was determined by capillary electrophoresis on an ABI prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Several samples with different geno-

Table 1

Frequencies of the asporin microsatellite alleles in Spanish controls and each of the patient groups

	Allele							
Group	D12	D13	D14	D15	D16	D17	D18	D19
Controls								
All controls ($n = 294$)	31(5.3)	248 (42.2)	74 (12.6)	150 (25.5)	55 (9.3)	12 (2.0)	10 (1.7)	8 (1.3)
Males ($n = 179$)	22 (6.15)	154 (43.0)	48 (13.4)	90 (25.1)	29 (8.1)	5 (1.4)	6 (1.7)	4 (1.1)
Females ($n = 115$)	9 (3.9)	94 (40.9)	26 (11.3)	60 (26.1)	26 (11.3)	7 (3.0)	4 (1.7)	4 (1.7)
Total hip replacement								
All patients ($n = 303$)	22 (3.6)	262 (43.3)	59 (9.7)	156 (25.7)	68 (11.2)	13 (2.1)	13 (2.1)	13(2.1)
Males ($n = 120$)	7 (2.9)	105 (43.7)	25 (10.4)	64 (26.7)	20 (8.3)	6 (2.5)	9 (3.7)	4 (1.7)
Females ($n = 183$)	15 (4.1)	157 (42.9)	34 (9.3)	92 (25.1)	48 (13.1)	7 (1.9)	4 (1.1)	9 (2.5)
Total knee replacement								
All patients ($n = 188$)	14 (3.7)	156 (41.5)	56 (14.9)	93 (24.7)	30 (7.9)	14 (3.7)	7 (1.8)	6 (1.6)
Males ($n = 35$)	3 (4.3)	36 (51.4)	11 (15.7)	14 (20.0)	4 (5.7)	0 (0.0)	2 (2.8)	0 (0.0)
Females ($n = 153$)	11 (3.6)	120 (39.2)	45 (14.7)	79 (25.8)	26 (8.5)	14 (4.6)	5 (1.6)	6 (2.0)
Hand osteoarthritis								
All patients ($n = 232$)	16 (3.4)	209 (44.8)	57 (12.2)	113 (24.2)	46 (9.9)	13 (2.8)	8 (1.7)	4 (0.8)
Males ($n = 27$)	2 (3.7)	18 (33.3)	6 (11.1)	19 (35.2)	6 (11.1)	0 (0.0)	2 (3.7)	1(1.8)
Females ($n = 205$)	14 (3.4)	189 (46.1)	51 (12.4)	94 (22.9)	40 (9.8)	13 (3.2)	6 (1.5)	3 (0.7)

Values presented the number of alleles (%).

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Table 2

Comparisons of the D14 and D13 allelic frequencies of the asporin microsatellite between Spanish osteoarthritis patients and controls represented as *P* values

	Comparison		
Group	D14 versus others	D13 versus others	D14 versus D13
Total hip replacement (*n* = 303)	0.12	0.71	0.15
Total knee replacement (*n* = 188)	0.31	0.83	0.37
Total hip replacement + total knee replacement (*n* = 597)	0.61	0.88	0.63
Hand osteoarthritis (*n* = 233)	0.86	0.38	0.65

types were sequenced to test the accuracy of genotyping with the Big Dye Ready Reaction Kit (Applied Biosystems).

Statistical analysis

Allele frequencies and their 95% confidence intervals were calculated. Comparison of allele frequencies was carried out using the 2 × *n* contingency table after collapsing columns with low frequency, as implemented in the T2 option of the Clump software that follows a Monte Carlo approach [12]. Post-hoc power was determined with the GPower software [13] for each comparison of the D14 and D13 allelic frequencies between patients and controls.

Results and discussion

This study explored whether the sound association between the ASPN microsatellite and OA in the Japanese population [4] was also present in the Spanish population. We therefore searched for evidence of association in multiple ways. First, the allelic frequency distribution of the microsatellite was compared between the three groups of patients separated by the affected joint and the controls (Table 1). None of these three comparisons showed differences. All *P* values were far larger than the threshold for significance: TKR patients versus controls, $P = 0.6$; THR patients versus controls, $P = 0.5$; and hand OA patients versus controls, $P = 0.9$.

Similar results were obtained after stratifying by sex (Table 1). Female patients and controls had similar allele frequencies (TKR patients versus controls, $P = 0.9$; THR patients versus controls, $P = 0.9$; and hand OA patients versus controls, $P = 0.8$), as well as male patients and controls (TKR patients versus controls, $P = 0.6$; THR patients versus controls, $P = 0.3$; hand OA patients versus controls, $P = 0.4$). We also pooled data from TKR and THR patients as both patient groups were associated with ASPN in the Japanese population, but even for this larger group there was no difference with the controls ($P = 0.8$). A similar result was observed if all the OA patients were taken together (THR + TKR + hand OA = 723 patients, $P = 0.8$).

These results are in clear contrast with the association described in several cohorts of Japanese subjects, where the frequency distributions of this microsatellite were consistently different between patients with knee OA or hip OA and controls [4], but they were similar to recent results in the UK population [9] and the Greek population [10].

The differences in the ASPN microsatellite frequency distributions reported in the Japanese study were due mainly to an increased frequency of the D14 allele and to a lesser extent due to a decreased frequency of the D13 allele in OA patients [4]. The Greek study also found a significant decrease of the D13 allele in TKR patients but there was no change in the D14 frequency. The UK study did not find significant differences but observed a tendency in the same sense as the Japanese study: an increase of the D14 allele and a decrease of the D13 allele in THR and TKR patients [9]. We therefore specifically tested these two alleles for differences in our subjects (Table 2).

No differences were detected in the D14 allele frequencies between controls and TKR patients, THR patients, both TKR and THR patients together, or hand OA patients (this later group had not been included in previous studies). We did not even observe a tendency to a higher frequency of the D14 allele in patients. The same pattern was found when this analysis was repeated after stratifying by sex. In the same way, the frequency of the D13 allele was similar in all patient groups and in controls (Table 2) and no difference was observed after stratifying by sex. Finally, as the most sensitive test, we considered only the D13 and the D14 alleles and compared their relative frequencies in each patient group with their frequencies in the controls, but again no differences were found (Table 2).

Even with this later comparison that gave the largest difference in the Japanese population, we did not find evidence of association of OA with the ASPN microsatellite in our patients. The present study has enough post-hoc power (>0.8), however, to detect a D14 effect of the size found in the Japanese [4], with $P < 0.05$. The power for the comparisons of THR

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patients versus controls and TKR patients versus controls is 0.83 and 0.94 for the D14 allele frequencies and is 0.90 and 0.98 for the D14 versus D13 frequencies, respectively. The comparison lacks power to detect a decrease of the D13 allele in OA patients, similar to the Japanese study (power is 0.16 for the comparison of THR patients versus controls and is 0.29 for the comparison of TKR patients versus controls), probably due to the weak effect of this allele (odds ratio = 0.84 both for hip OA and knee OA in the Japanese population).

Our results were similar to the UK study [9] except for a significant increase of the D14 allele in UK THR males that did not persist after correction for the multiple tests performed. In contrast, our patients showed a nonsignificant decrease of this allele in THR males (10.4%, 95% confidence interval 3.4–17.4% in THR males versus 13.4%, 95% confidence interval 5.6–21.2% in male controls). The D14 allele was also similar in Greek TKR patients and controls [10]. The Greek TKR patients, however, showed significantly increased frequencies of the D15 and D18 alleles, which were not found in our study (Table 1) or in the other two larger studies, and which did not remain significant after accounting for the multiple comparisons performed. The overall results therefore indicate that the ASPN microsatellite is not associated with OA susceptibility in European Caucasians. Discrepancies between the Japanese and Caucasians could be multifactorial, involving differences in disease phenotype, in lifestyle, and in environmental and genetic factors.

Conclusion

There was no detectable effect of variation in the number of D residues in ASPN in hip OA, knee OA or hand OA susceptibility in Spanish Caucasians. This result reinforces the overall lack of association reported in other studies in Caucasians. The association between variation in the number of D residues and OA described in the Japanese study is robust, however, and is not refuted by these studies, but it will require confirmation in the same ethnic population. Therefore, although it remains possible that ASPN could be a crucial modulator in OA by fine-tuning transforming growth factor beta in the repair of damaged cartilage, this effect will be limited to some ethnic groups or environmental settings.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to the final manuscript. In addition, JR-L genotyped the samples and participated in the design and analysis of the study, MP-S, ML and JJG-R evaluated the patients, and AG coordinated the study and participated in its design and analysis.

Acknowledgements

The authors thank sample donors for their collaboration. They thank also Yolanda Lopez-Golan and Fina Meijide for their help in recruiting study participants. Marta Picado and Cristina Fernandez provided outstanding technical assistance. This project was supported by grant PI02/0713 form the Instituto de Salud Carlos III (Spain) with participation of funds from FEDER (European Union), and by a grant of the Fundacion Pedro Barrie de la Maza (A Corunna, Spain). JR-L is the recipient of a scholarship of the National Program for the Training of University Professors of the Spanish Ministry of Education. AG was supported by the Instituto de Salud Carlos III (Spain).

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Human Molecular Genetics, 2007, Vol. 16, No. 14 ***1676–1681***
doi:10.1093/hmg/ddm115
Advance access published May 20, 2007

Meta-analysis of association between the *ASPN* D-repeat and osteoarthritis

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Received March 26, 2007; Revised and Accepted April 26, 2007

Osteoarthritis (OA) is the most common form of human arthritis. Genetic factors have been implicated in OA. It was reported that an aspartic acid (D)-repeat polymorphism in the gene encoding asporin (*ASPN*) was associated with OA of knee and hip joints in Japanese; in the three independent studies performed, the D14 allele of the *ASPN* polymorphism was over-represented and the D13 allele was under-represented. Subsequently, four replication studies, three in Europeans and one in Chinese populations, have been reported; however, they showed inconsistent results. To evaluate between-study heterogeneity and to estimate the common genetic effect of the D-repeat polymorphism on OA, we performed a meta-analysis of the five reports that include seven association studies, using the DerSimonian–Laird procedure. We detected association between knee OA and the susceptible D14 allele [*P* = 0.003, summary odds ratio (OR) = 1.46] with significant heterogeneity (*P* = 0.047) among the studies. We also detected positive association between knee OA and the protective D13 allele (*P* = 0.026, summary OR = 0.84) with significant heterogeneity (*P* = 0.040) among the studies. Because of significant heterogeneity, we stratified the studies by ethnicity. We detected positive association between knee OA and the D14 allele (*P* = 0.0000013, summary OR = 1.95) with non-significant heterogeneity (*P* = 0.535) in Asian populations. In hip OA, significant heterogeneity was identified and there was no positive association for any allele in any comparison. The present results suggest that the association of the ASPN D14 allele and knee OA has global relevance, but that its effect has ethnic differences.

INTRODUCTION

Osteoarthritis (OA) is one of the most common joint diseases. Epidemiological studies suggest that genetic factors strongly affect the onset and development of OA (1–3). Several susceptibility genes have been reported, which is consistent with the polygenic nature of the disease (4–10). Kizawa *et al.* (11) reported an association between OA and the gene encoding asporin (*ASPN*) in Japanese: using a cohort sample, they found a positive association between knee OA and the D14 allele of the aspartic acid (D)-repeat polymorphism in *ASPN* [$P = 0.0013$, odds ratio (OR) = 2.49]. The association was replicated in a separate case–control sample of knee OA ($P = 0.018$, OR = 1.66). Furthermore, they

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Table 1. Allele counts for the D-repeat polymorphisms in *ASPN* in the six studies from five articles

Study	Country	Case					Control				
		Count			Frequency		Count			Frequency	
		D14	D13	Others	D14	D13	D14	D13	Others	D14	D13
Knee OA											
Kizawa/cohort	Japan	30	163	81	0.109	0.595	22	314	132	0.047	0.671
Kizawa/case–control	Japan	61	459	266	0.078	0.584	36	479	233	0.048	0.640
Mustafa	UK	76	258	222	0.137	0.464	190	752	554	0.127	0.503
Kaliakatsos	Greece	47	119	146	0.151	0.381	51	192	141	0.133	0.500
Rodriguez	Spain	56	156	164	0.149	0.415	74	248	266	0.126	0.422
Jiang	China	41	300	95	0.094	0.689	45	603	260	0.050	0.664
Hip OA											
Kizawa/case–control	Japan	93	731	362	0.078	0.616	36	479	233	0.048	0.640
Mustafa	UK	258	858	692	0.143	0.475	190	752	554	0.127	0.503
Rodriguez	Spain	59	262	285	0.097	0.432	74	248	266	0.126	0.422

replicated the association of the polymorphism in a case–control study of hip OA ($P = 0.0078$, OR $= 1.70$). Subsequent functional analyses presented compelling evidence for causality of asporin in OA (11).

Despite the strong evidence indicating association between OA and *ASPN* in Japanese populations, subsequent studies of other populations showed inconsistent results. Mustafa *et al*. (12) assessed the association in UK cases (ascertained by hip or knee total joint replacement for primary OA) and controls, but did not detect significant association. They found only marginal evidence of association to the D14 allele in male patients with hip OA ($P = 0.025$, OR $= 1.41$). A Greek case–control study (13), comprising OA cases ascertained by total knee joint replacement, indicated that the D13 allele decreased the risk of knee OA ($P = 0.002$, OR $= 0.62$), but that the D14 allele did not increase risk ($P = 0.65$, OR $= 1.1$). In a Spanish case–control study (14), comprising cases with hand OA or with hip or knee OA ascertained by total joint replacement, no significant associations were detected between the *ASPN* D-repeat and OA (all $P > 0.05$). In a Chinese case–control study (15), comprising cases with symptomatic and radiographically defined knee OA, significant association was detected between the D14 allele and OA ($P = 0.0013$, OR $= 2.04$). However, the association with the D13 allele was not replicated ($P = 0.4$, OR $= 1.11$). The discrepant results may be due to differences in inclusion criteria. The Japanese and Chinese studies used patients with symptomatic hip or knee OA ascertained radiographically, whereas the three European studies recruited hip or knee OA patients who had undergone joint replacement. Alternatively, the discrepancy may be due to ethnic differences.

Meta-analysis is a widely accepted tool for summarizing different studies beyond the between-study variance (16,17). Its main objective is to assess heterogeneity and association. Meta-analysis can assess the significance of observed heterogeneity. It can exclude differences in allele frequencies and affection rates between studies that can be confounding factors in genetic association studies. Exclusion of confounding factors increases the quality of the evidence. For example, Iwamoto *et al*. (18) used meta-analysis to summarize several studies of association between *PADI4* and rheumatoid arthritis. They found that the discrepancies between the studies were not significant, and that overall association was significant.

The purpose of the present study is to evaluate between-study heterogeneity of the association studies on the *ASPN* D-repeat polymorphism and OA and to estimate the common genetic effect of the polymorphism. Using meta-analysis of the genotyping data, we integrated the previous association studies and evaluated the global significance of the association. We found strong association between the D14 allele of the *ASPN* D-repeat and knee OA in Asian populations.

RESULTS

Genotyping data and patient clinical data of a total of 5446 subjects from the five participating groups (Japan, China, UK, Spain and Greece) were obtained from the original researchers. There were 1370 cases and 2296 controls in the knee OA study and 1800 cases and 1416 controls in the hip OA study.

Allelic and genotypic counts were evaluated for the D14 and D13 alleles, respectively (Table 1 and Supplementary Material, Table S1). The frequencies of the D14 allele and of the D13 allele differed between the Asian and European studies (Table 1). The differences of the allele frequencies between the Asian and European studies were tested by chi-square test using 2×3 tables, Asian/European and D13/D14/others, in case and control samples separately. The P-values for the tests were significant ($P < 0.0001$). The variation of the frequencies of the D13 allele between studies was larger than those of allele D14, particularly in the case groups. The frequency of the D14 allele in the control groups was very similar between Japanese and Chinese (Asian) studies and between the European studies. The frequency of the D14 allele was higher in OA cases than in control individuals in all studies, except for the Spanish hip OA cases; the frequency of the D13 allele was lower in OA cases than in control individuals in all studies, except for the Chinese study.

Because differences of the clinical covariates related to OA between the different genotype groups might cause a spurious association, we compared the age, sex and body mass index

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Table 2. Summary OR of six studies and summary studies regarding the association of knee OA and the asporin D-repeat polymorphisms

Study (country)	OR (95%CI)		
	D14 versus others	D14 versus D13	D13 versus others
Original study			
Kizawa/cohort (Japan)	2.49 (1.41–4.42)	2.63 (1.47–4.70)	0.72 (0.53–0.98)
Kizawa/case–control (Japan)	1.66 (1.09–2.54)	1.77 (1.15–2.72)	0.79 (0.64–0.97)
Mustafa (UK)	1.09 (0.82–1.45)	1.17 (0.86–1.58)	0.86 (0.70–1.04)
Kaliakatsos (Greece)	1.16 (0.75–1.78)	1.49 (0.94–2.35)	0.62 (0.46–0.84)
Rodriguez (Spain)	1.22 (0.84–1.77)	1.20 (0.81–1.80)	0.97 (0.75–1.26)
Jiang (China)	1.99 (1.28–3.09)	1.83 (1.17–2.86)	1.12 (0.87–1.43)
Summary study			
All	1.46 (1.14–1.87)	1.53 (1.22–1.92)	0.84 (0.72–0.98)
Replication	1.34 (1.07–1.68)	1.41 (1.17–1.70)	0.86 (0.72–1.02)
Asian	1.95 (1.49–2.55)	1.96 (1.49–2.57)	0.86 (0.67–1.12)
European	1.14 (0.93–1.39)	1.24 (1.00–1.53)	0.81 (0.64–1.03)
	P-value for the test of summary OR		
All	0.0030	0.00020	0.026
Replication	0.0090	0.00040	0.085
Asian	0.0000013	0.0000015	0.26
European	0.20	0.058	0.081
	P-value for the test of heterogeneity		
All	0.047	0.12	0.040
Replication	0.15	0.34	0.033
Asian	0.54	0.53	0.043
European	0.90	0.67	0.075

Replication: all studies except for the Kizawa's cohort study. Asian: Japanese and Chinese studies. European: UK, Greece and Spain studies.

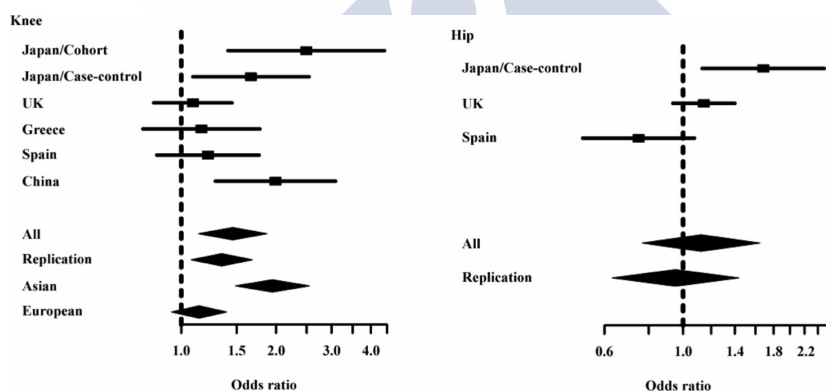

Figure 1. A meta-analysis for association between OA and the *ASPN* D-repeat in the comparison of the D14 allele versus other alleles combined. Bar, 95% CI of OR; diamond, OR and its 95% CI for the summary study. In knee OA (left panel), the summary ORs except for the European studies do not include 1, indicating the positive association between D14 and knee OA. In hip OA (right panel), the summary ORs include 1.

(BMI) status between the D13 and the D14 genotypes for each study (Supplementary Material, Tables S2–S4). There were no differences (all nominal $P > 0.05$) in these analyses except for the age and the D14 genotype in the Japanese cohort study ($P = 0.002$ in a dominant model) and sex and the D13 genotype in the Japanese case–control study ($P = 0.035$). Neither difference would remain significant if the results were subjected to Bonferroni correction for multiple testing [corrected $P = 0.0007$ (0.05/72)].

Knee OA

We analyzed six studies of association between knee OA and the *ASPN* D-repeat alleles. The summary OR for the contingency table of the D14 allele versus other alleles combined and its 95% CI exceeded 1 (Table 2; Fig. 1), indicating a significant association. The same result was obtained in the analysis for the contingency table of the D13 allele versus other alleles combined. D14 was over-represented, and D13

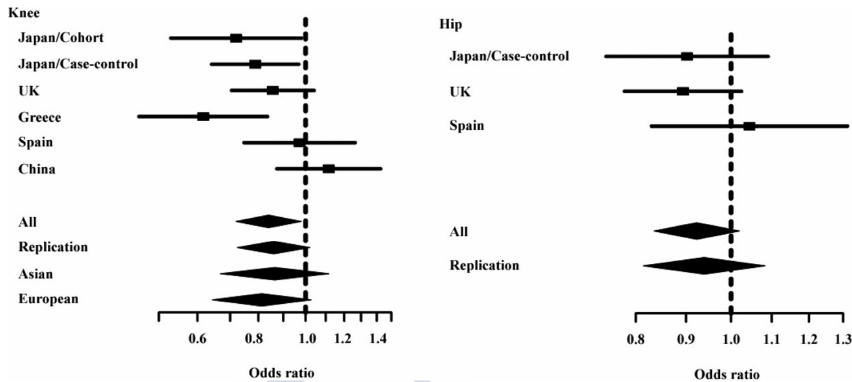

Figure 2. A meta-analysis for association between OA and the *ASPN* D-repeat in the comparison of the D13 allele versus other alleles combined. ORs for the Jiang's study (China) for knee OA and for the Rodrigues's study (Spain) for hip OA are above 1. Summary ORs except for all studies of knee OA include 1.

was under-represented; the *P*-values for the summary OR of D14 and D13 were 0.003 and 0.026, respectively (Table 2). Furthermore, the ORs of each of the six studies all exceeded 1 in the contingency table of D14 (Fig. 1). Under the null hypothesis, the probability that the OR exceeds 1 in a single study is 1/2. Therefore, the *P*-value for the sign test (two-sided test) is 1/32 (<0.05), also supporting a positive association between the D14 allele and knee OA.

To exclude the possible publication and selection bias of the first study (the Japanese knee cohort study), we excluded this study and analyzed the remaining five knee case–control studies (the 'replication' studies). The summary OR of D14 versus other alleles combined was lower for the replication studies than for all six studies, but still exceeded 1, as did its 95% CI (Table 2). The *P*-value for D14 for the replication studies was significant ($P = 0.009$). The summary OR of D13 versus other alleles combined exceeded 1 in the replication studies, but its 95% CI also intercepted 1, generating a non-significant *P*-value of 0.085 (Fig. 2; Table 2).

Significant heterogeneity was detected among the studies in the analysis of association of the D14 allele versus other alleles combined ($P = 0.047$, Table 2). Because of this, we stratified the studies by ethnicity, into Asian and European groups (Table 2). We detected association between knee OA and the D14 allele ($P = 0.0000013$, $OR = 1.95$) with non-significant heterogeneity ($P = 0.54$) in the Asian group. In contrast, the D14 allele was not associated in the European group ($P = 0.2$, $OR = 1.14$) with non-significant heterogeneity ($P = 0.90$). D13 was not associated in the Asian group ($P = 0.26$) or in the European group ($P = 0.081$).

We analyzed genotype data with the assumption of the inheritance modes of dominant or recessive. A stronger association was not found with the assumption of mode of inheritance. The tendency found in the analysis of allele count data was maintained in the analysis with the assumption of dominance of the D14 allele for all studies ($P = 0.006$), the replication studies ($P = 0.02$) and for Asians ($P = 0.0000014$) but not for Europeans ($P = 0.32$) (Supplementary Material, Table S5; Fig. 3). An association with the D13 allele was detected in the recessive mode for all studies ($P = 0.009$), for the replication study ($P = 0.038$) and for Europeans ($P = 0.049$) but not for Asians ($P = 0.14$). (Supplementary Material, Table S6; Fig. 3).

Hip OA

We analyzed three case–control studies of association between hip OA and *ASPN* D-repeat alleles: one in Japanese and two in European subjects (UK and Spain). The tests for the D14 allele versus other alleles combined and for the D14 allele versus the D13 allele showed significant heterogeneity (Supplementary Material, Table S7). The 95% CI for the summary OR included 1 when all studies were analyzed together (Japanese, UK and Spain) and when the replication studies (UK and Spain) were analyzed together (Fig. 1), indicating that there was no positive association between *ASPN* and hip OA. The same tendency was observed in the analysis of genotype data assuming inheritance modes of dominant or recessive except for the assumption of a recessive mode for the D13 allele in all three hip studies ($P = 0.026$, summary $OR = 1.20$) (Supplementary Material, Table S8).

Other alleles

Kaliakatsos *et al*. (13). reported that the D15 allele of the D-repeat also associated with knee OA ($P = 0.018$, $OR = 1.54$). We performed a meta-analysis for this allele as well as for additional common alleles of the repeat other than D13 and D14. No significant association was detected for any allele (Supplementary Material, Table S9).

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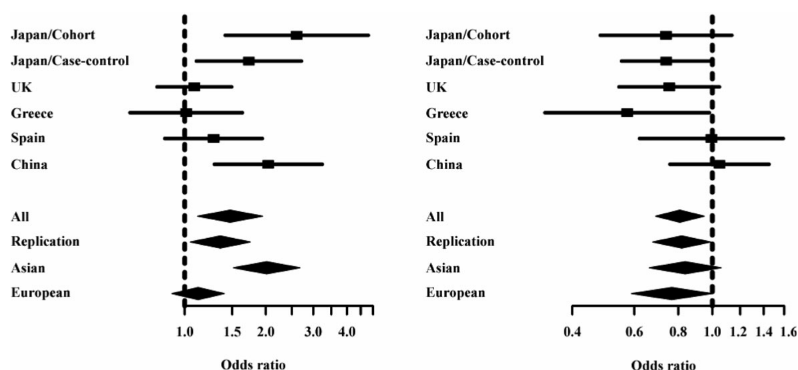

Figure 3. A meta-analysis of knee OA under the assumption of the inheritance mode. *Left*: knee OA in the dominant mode of the D14 allele. Summary ORs do not include 1 except for the European studies. *Right*: knee OA in the recessive mode of the D13 allele. Summary ORs do not include 1 except for the Asian studies.

DISCUSSION

This report represents the first comprehensive meta-analysis of OA-associated genes. We enrolled a large number of subjects for the *ASPN* D-repeat association from Europe and Asia. There were two objectives of the study. The first was to assess heterogeneity among the association studies so far published. The OR of association between OA and *ASPN* D-repeat alleles in the studies examined showed significant heterogeneity. These results suggest that there are ethnic differences in the effect of the *ASPN* on OA susceptibility.

The second objective was to test association by summarizing allele and genotype information. The summary OR of the D14 allele for knee OA showed a significant association and was greater than 1. The sensitivity analysis also indicated significance, despite use of the conservative DerSimonian–Laird (DSL) procedure. These results, together with evidence from functional analyses (11), enhance the evidence for the association of the D14 allele of the *ASPN* D-repeat with knee OA. However, the effect is heterogeneous. The lower effect of this allele in Europeans may be caused by the influence of other genes or by environmental effects that vary between Europeans and Asians. We detected strong association between knee OA and the D14 allele with non-significant heterogeneity in Asian populations. These results suggest that the genetic effect of the *ASPN* D-repeat in the Asian population is stronger than in the European population.

In contrast, the summary OR for hip OA did not indicate a significant association. Allelic frequencies differed greatly between the two European studies (UK and Spain). These results may indicate either lack of association, population differences or a lack of power to detect minor associations. The OR for association of hip OA was substantially lower than that of knee OA, even in the Japanese studies.

The recessive protective effects was observed for the D13 allele in knee and hip European populations (Supplementary Material, Tables S6 and S8), which is inconsistent with the previous *in vitro* functional study where the inhibitory effect of the D13 allele on TGF-β induction on cartilage marker gene is similar to those of the D16 and D17 alleles (11). The effect of the D13 allele may just be a reflection of the absence of the D14 allele that has a dominant positive effect.

Our conclusion is that the D-repeat of *ASPN* is globally associated with knee OA, but its effect size has ethnic difference: strong in Eastern Asians and weaker in Europeans. The heterogeneity of genetic effects between Asian and European suggests the existence of gene–gene interaction or gene–environmental interaction. The summary ORs presented here are marginal effects of *ASPN* polymorphism for knee OA. The combination of *ASPN* D-repeat and the other polymorphisms that are common in Asian and uncommon in European may increase the risk of knee OA. Otherwise, the polymorphism of *ASPN* may increase the risk of knee OA with the other environmental factors. The meta-analysis using ORs corrected by the other information, clinical parameters and the other polymorphisms that are associated with knee OA, is efficient. Further replication studies are required in order to detect the true effects of the *ASPN*.

MATERIALS AND METHODS

Data collection

To find articles about association between *ASPN* and OA, the first author (Takahiro Nakamura) searched the PubMed database for articles containing the terms 'asporin' and 'osteoarthritis'. Five relevant articles were published between February 2005 and October 2006 and are described in Introduction (11–15). Under the initiative of the corresponding author (Shiro Ikegawa), the authors of the five articles joined together to form a collaborative group. The genotype data and clinical covariates, age, sex and BMI of each study were collected together.

Statistical analysis

We used the software R (http://www.R-project.org) (19) for all the calculations of statistical analysis. We compared the difference of the clinical covariates between the genotypes of the *ASPN* D-repeat polymorphisms using Mann–Whitney and Kruskal–Wallis tests. We used OR as a measure of the effect of the genetic factor. There are two major models for meta-analysis: the fixed-effect model and the random-effect model (16,17). The fixed-effect model interprets variations in genetic effects between studies as just errors, whereas the random-effect model interprets variations as the sum of errors and heterogeneity. We principally used the DSL procedure (20) for the analysis. The DSL procedure is based on the random-effect model and contains two tests. One is a test of heterogeneity for testing the null hypothesis that all the populations in the studies have a common OR; the other is a test of significance for testing the null hypothesis that the common OR is equal to 1.

We performed the tests for three comparisons of the allele count data: the D14 allele versus other alleles combined, the D13 allele versus the D14 allele and the D13 allele versus the other alleles combined. If the onset of OA depends on the mode of inheritance then a test assuming the mode of inheritance (dominant or recessive) would be more powerful. Therefore, we also performed the tests using the genotype data. There was a tendency for the Japanese knee cohort study to have a high OR (21), which may have been caused by the selection of major significant genes from among multiple candidates, as well as by the publishing bias. Therefore, in addition to the analyses using all seven studies (Japanese knee cohort, Japanese knee case–control, Japanese hip case–control, Chinese, UK, Spanish and Greek), we performed a sensitivity analysis in which the Japanese knee cohort study was excluded. Furthermore, we examined the data following stratification by ethnicity (Asian or European) and by joint site (hip or knee) for the meta-analysis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We would like to thank Atsushi Takahashi (SNP Research Center, RIKEN). This work was supported by The Arthritis Research Campaign (to J.L.), Research into Ageing (to J.L.), Grant PI02/0713 form the Instituto de Salud Carlos III (Spain) with participation of funds from FEDER (European Union) (to J.R.-L. and A.G.), National Nature Science Foundation of China (3057874) (to D.S. and Q.J.), Programme of Technology Development of Nanjing (200603001) (to D.S. and Q.J.) and The Ministry of Education, Science, Sports and Culture Grant-in-Aid for Young Scientists (B) 18700283 (2006) (to T.N.).

Conflict of Interest statement. The authors declare that they have no competing financial interests.

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UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Human Molecular Genetics, 2007, Vol. 16, No. 18 ***2226–2232***
doi:10.1093/hmg/ddm174
Advance Access published on July 6, 2007

An SNP in the 5′-UTR of *GDF5* is associated with osteoarthritis susceptibility in Europeans and with *in vivo* differences in allelic expression in articular cartilage

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Received June 8, 2007; Revised and Accepted July 2, 2007

A compelling genetic association with osteoarthritis (OA) of a functional SNP (rs143383, T/C) in the 5′-UTR of the *GDF5* gene was recently reported in case–control cohorts from Japan and China. GDF5 is a pro-chondrogenic growth factor. The T-allele frequency of the gene was elevated in cases, with an odds ratio (OR) of 1.79, and *in vitro* functional studies demonstrated that this allele mediated a moderate but significant reduction in the activity of the *GDF5* promoter in several cell lines. Our initial objective was to assess whether the SNP was also associated with OA in a broad European population by genotyping the SNP in 2487 cases and 2018 age-matched controls from the UK and Spain. The T-allele was associated with OA ($P = 0.03$, OR = 1.10) as was carrier status for this allele ($P = 0.004$, OR = 1.28), demonstrating that the SNP is associated with OA in two diverse ethnic groups, Asians and Europeans. We subsequently assessed the functional effect of the SNP on *GDF5* allelic expression using RNA extracted from the cartilage of OA patients who had undergone joint-replacement surgery. The associated T-allele showed up to a 27% reduction in expression relative to the C-allele ($P = 0.00007$), revealing that the functional effect mediated by SNP rs143383 on *GDF5* expression is active in patients who have severe disease up to the point at which they require surgery. A small but persistent imbalance of *GDF5* expression throughout life therefore appears to render an individual more susceptible to OA.

INTRODUCTION

Osteoarthritis (OA) is a common multifactorial disease characterized by the degeneration of the protective cartilage layer in articulating synovial joints. It is largely a disease associated with ageing, being rare in individuals below the age of 45 years with an increase in incidence and prevalence up to 80 years of age. A common treatment for patients who have severe disease of the hip or the knee is total joint replacement surgery (THR and TKR, respectively). Growth and differentiation factor 5 (also known as cartilage-derived morphogenetic protein 1) is a member of the transforming growth factor-β (TGF-β) superfamily and participates in the development, maintenance and repair of bone, cartilage and other soft tissues of the synovial joint (1–5). An association with hip and knee OA of a single

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nucleotide polymorphism (SNP) (rs143383, T/C) located in the 5′-UTR of the growth and differentiation factor 5 gene, *GDF5*, was recently reported in Japanese and in Chinese case–control cohorts (6). The major, T-allele, of the SNP was common in the Asian populations, with frequencies >70% in controls, and was at an elevated frequency in OA cases, with odds ratios (ORs) of 1.79 in the Japanese hip cases ($n = 998$), 1.30 in the Japanese knee cases ($n = 718$) and 1.54 in the Chinese knee cases ($n = 313$). *In vitro* cell transfection studies using luciferase reporter assays revealed that the T-allele-mediated a moderate but significant reduction in the activity of the *GDF5* promoter in chondrogenic and non-chondrogenic cell lines. Our initial objective was to assess the role of this SNP on OA development in Europeans by case–control association analysis. Since the SNP was associated in both Japanese and Chinese, representing a relatively broad group of ethnic Asians, we hypothesized that if the SNP were associated in Europeans then it would be associated in a broad rather than a narrow European group. We therefore chose to study North European (UK) and South European (Spanish) populations in a combined analysis of 4505 cases and controls. We subsequently assessed whether the SNP influenced allelic expression of *GDF5 in vivo* by extracting the RNA from the cartilage of nine OA patients who had undergone a THR or a TKR. This RNA was then used to measure *GDF5* allelic expression by a single base-pair extension assay that can discriminate and quantify the mRNA synthesized from each allele.

RESULTS

Power of the association study

We determined that the combined cohort ($n = 4505$), the UK cohort alone ($n = 2491$) and the Spanish cohort alone ($n = 2014$) were all adequately powered, at a significance level of 5%, to detect an OR comparable to those reported in the Asian study (data not shown). There was no significant difference in genotype or allele frequencies when the UK controls were compared with the Spanish controls, with a genotype P of 0.46 and an allele P of 0.25, implying that for this SNP the two populations are equivalent and can therefore be combined.

Single nucleotide polymorphism rs143383 is associated with osteoarthritis in Europeans

In the combined analysis, the SNP demonstrated association at both the genotypic ($P = 0.01$) and the allelic [$P = 0.03$, OR = 1.10 (95% CI 1.01–1.20)] level (Table 1). This association involved an elevation of the T-allele, in line with the Asian study. The significances of these associations did not increase following stratification by sex or by site of disease (hip OA, knee OA or hand OA), implying that a particular stratum does not drive them. Carriers of the T-allele [(TT) + (TC)] were also significantly more common in cases than in controls (Table 2), with a P of 0.004 and an OR of 1.28 (95% CI 1.08–1.51). In the T-allele carrier analysis, the knee stratum, the hip stratum and the hip and knee stratum (cases who had undergone joint replacement at both a hip and a knee) were all significant ($P \leq 0.05$) and all had positive ORs whose 95% CI did not include 1, whereas the hand stratum was not significant ($P = 0.86$) and had an OR whose 95% CI did encompass 1 (Fig. 1). This implies that the rs143383 T-allele carrier association is restricted in Europeans to larger, weight-bearing synovial joints such as those of the hip and knee. It is noteworthy that the highest frequency of T-allele carriage is in the hip and knee stratum, with a carrier frequency of 91.9% in hip and knee cases versus 84.1% in controls [Table 2; $P = 0.04$; OR = 2.14 (95% CI 1.03–4.46)], implying that SNP rs143383 is a particular risk factor for OA development at multiple large joints in an individual.

When we studied the UK case–control cohort alone, we did not observe a significant difference in genotype or allele frequencies for the un-stratified analysis between cases and controls, although the UK sample did approach significance for the genotype comparison, with a P of 0.07, and was significant for the stratum of cases who had undergone both a hip and a knee joint replacement, with a genotype P of 0.04 (Supplementary Material, Table S1). Carriers of the T-allele were also more common in the UK cases than in the UK controls [$P = 0.02$; OR = 1.32 (95% CI 1.04–1.68); Supplementary Material, Table S3). As mentioned above, in the combined analysis, the hip and knee cases showed the highest frequency of T-allele carriage. All of these cases were from the UK cohort. When these hip and knee cases were compared with only UK controls, the difference in T-allele carriage remained significant [$P = 0.05$; OR = 2.06 (95% CI 0.98–4.35)] implying that this association is not an artefact of examining two combined populations (UK and Spain). For the Spanish case–control cohort alone, there were no significant differences between the cases and controls (Supplementary Material, Table S2), although there was a non-significant increase in the frequency of carriers of the T-allele in the cases (Supplementary Material, Table S3).

Significantly less *GDF5* transcript is produced by the T-allele of SNP rs143383 in the cartilage of osteoarthritis patients

We next set out to assess whether the 5′-UTR SNP correlated with differences in allelic expression using RNA extracted from the articular cartilage of nine OA patients who were heterozygous for the SNP and who had undergone either a THR or a TKR. These nine individuals had been collected subsequent to our association analysis. All nine patients demonstrated a reduction in the expression of the T-allele relative to the C-allele, with allelic ratios below 1.0, and for six of the nine patients, the reductions were significant ($P < 0.005$, two-tailed Mann–Whitney exact test, Fig. 2A). The greatest allelic difference was for patient 7, a male who had undergone a TKR at the age of 66 years. His *GDF5* T-allele showed a 27% reduction in expression relative to his *GDF5* C-allele ($P = 0.00007$). When the data for all nine patients was studied together, the T-allele demonstrated an average 12% reduction in relative expression ($P = 0.006$, two-tailed t-test, Fig. 2B).

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Table 1. Genotype and allele association analysis of SNP rs143383 in cases with osteoarthritis and controls

Group		Genotype			*P*-value	Allele		*P*-value
		(CC)	(CT)	(TT)		(C)	(T)	
All cases	Count	319	1194	974	0.01	1832	3142	0.03
(*n* = 2487)	Frequency (%)	12.8	48.0	39.2		36.8	63.2	
All controls	Count	320	935	763		1575	2461	
(*n* = 2018)	Frequency (%)	15.9	46.3	37.8		39.0	61.0	
Female cases	Count	207	785	631	0.29	1199	2047	0.20
(*n* = 1623)	Frequency (%)	12.8	48.4	38.9		36.9	63.1	
Female controls	Count	154	493	388		801	1269	
(*n* = 1035)	Frequency (%)	14.9	47.6	37.5		38.7	61.3	
Male cases	Count	111	409	342	0.05	631	1093	0.08
(*n* = 862)	Frequency (%)	12.9	47.4	39.7		36.6	63.4	
Male controls	Count	166	442	375		774	1192	
(*n* = 983)	Frequency (%)	16.9	45.0	38.1		39.4	60.6	
All knees	Count	76	304	243	0.08	456	790	0.12
(*n* = 623)	Frequency (%)	12.2	48.8	39.0		36.6	63.4	
Female knees	Count	52	193	157	0.62	297	507	0.38
(*n* = 402)	Frequency (%)	12.9	48.0	39.1		36.9	63.1	
Male knees	Count	24	111	86	0.07	159	283	0.19
(*n* = 221)	Frequency (%)	10.9	50.2	38.9		36.0	64.0	
All hips	Count	198	728	599	0.06	1124	1926	0.06
(*n* = 1525)	Frequency (%)	13.0	47.7	39.3		36.9	63.1	
Female hips	Count	118	461	369	0.29	697	1199	0.21
(*n* = 948)	Frequency (%)	12.4	48.6	38.9		36.8	63.2	
Male hips	Count	80	267	230	0.28	427	727	0.19
(*n* = 577)	Frequency (%)	13.9	46.3	39.9		37.0	63.0	
All hips and knees[a]	Count	8	57	34	0.04	73	125	0.54
(*n* = 99)	Frequency (%)	8.1	57.6	34.3		36.9	63.1	
Female hips and knees	Count	4	37	21	0.09	45	79	0.59
(*n* = 62)	Frequency (%)	6.5	59.7	33.9		36.3	63.7	
Male hips and knees	Count	4	20	13	0.47	28	46	0.79
(*n* = 37)	Frequency (%)	10.8	54.1	35.1		37.8	62.2	
All hands[b]	Count	37	105	98	0.65	179	301	0.46
(*n* = 240)	Frequency (%)	15.4	43.7	40.8		37.3	62.7	
Female hands[b]	Count	33	94	84	0.72	160	262	0.76
(*n* = 211)	Frequency (%)	15.6	44.5	39.8		37.9	62.1	
Male hands[b]	Count	3	11	13	0.52	17	37	0.24
(*n* = 27)	Frequency (%)	11.1	40.7	48.1		31.5	68.5	

[a]Cases who had undergone both a hip and a knee joint replacement.
[b]Two hand osteoarthritis cases had unrecorded sex status.

DISCUSSION

Using a large cohort of 2487 OA cases and 2018 controls, we have replicated in Europeans the association of the *GDF5* 5′-UTR SNP rs143383 that is reported as associated to OA in Asians (6). We hypothesized that since rs143383 showed association in a relatively broad group of ethnic Asians, then any association in Europeans would also be ethnically broad, that is it would be unlikely to be associated in Asians and to only a specific ethnic subgroup of Europeans. We therefore studied Europeans from the North and South of the continent. There were no significant differences in genotype or allele frequencies between the UK controls and the Spanish controls, implying that for this SNP the two populations are equivalent and can therefore be combined. In our combined study we detected association at the genotype level, at the allele level and when carriers of the T-allele were combined [(TT) + (TC)]; this latter result implying that the allele has a dominant effect on OA susceptibility. Association was detected in the UK cohort alone but not in the Spanish cohort alone, although trends towards association were evident in the Spanish cohort. The UK cohort is larger than the Spanish cohort (2491 individuals versus 2014 individuals) and does therefore have more power to detect an association.

The unstratified ORs detected in our study are not as large as those reported in the Asian study. We must conclude therefore that the *GDF5* SNP rs143383 is not as major a susceptibility locus in Europeans as it is in Asians. This may reflect differences in the genetic background or in non-genetic, environmental influences between Europeans and Asians. Other loci that are risk factors for OA development in Asians have been studied in Europeans and their effects have not been replicated to the same degree as in the primary report (7–14). An example of this is the asporin gene *ASPN*, which harbours an aspartic-acid repeat polymorphism that has a highly significant effect upon OA susceptibility in Asians, but which shows only a moderate association in Europeans (9–11,14–16).

Growth and differentiation factor 5 is a member of the TGF-β superfamily of signalling molecules. Post-development,

Table 2. Association analysis of carriers of the T-allele of SNP rs143383 in cases with osteoarthritis and controls

Group		Carriers of the T-allele (TT) + (TC)	Non-carriers (CC)	*P*	OR (95% CI)
All cases	Count	2168	319	0.004	1.28
(*n* = 2487)	Frequency (%)	87.2	12.8		(1.08–1.51)
All controls	Count	1698	320		
(*n* = 2018)	Frequency (%)	84.1	15.9		
Female cases	Count	1416	207	0.12	1.20
(*n* = 1623)	Frequency (%)	87.2	12.8		(0.95–1.50)
Female controls	Count	881	154		
(*n* = 1035)	Frequency (%)	85.1	14.9		
Male cases	Count	751	111	0.02	1.37
(*n* = 862)	Frequency (%)	87.1	12.9		(1.06–1.78)
Male controls	Count	817	166		
(*n* = 983)	Frequency (%)	83.1	16.9		
All knees	Count	547	76	0.03	1.36
(*n* = 623)	Frequency (%)	87.8	12.2		(1.04–1.77)
Female knees	Count	350	52	0.35	1.18
(*n* = 402)	Frequency (%)	87.1	12.9		(0.84–1.65)
Male knees	Count	197	24	0.03	1.67
(*n* = 221)	Frequency (%)	89.1	10.9		(1.06–2.63)
All hips	Count	1327	198	0.02	1.26
(*n* = 1525)	Frequency (%)	87.0	13.0		(1.04–1.53)
Female hips	Count	830	118	0.12	1.23
(*n* = 948)	Frequency (%)	87.6	12.4		(0.95–1.59)
Male hips	Count	497	80	0.11	1.26
(*n* = 577)	Frequency (%)	86.1	13.9		(0.95–1.69)
All hips and knees[a]	Count	91	8	0.04	2.14
(*n* = 99)	Frequency (%)	91.9	8.1		(1.03–4.46)
Female hips and knees	Count	58	4	0.07	2.53
(*n* = 62)	Frequency (%)	93.5	6.5		(0.91–7.08)
Male hips and knees	Count	33	4	0.33	1.68
(*n* = 37)	Frequency (%)	89.2	10.8		(0.59–4.80)
All hands[b]	Count	203	37	0.86	1.03
(*n* = 240)	Frequency (%)	84.6	15.4		(0.71–1.48)
Female hands[b]	Count	178	33	0.78	0.94
(*n* = 211)	Frequency (%)	84.4	15.6		(0.63–1.42)
Male hands[b]	Count	24	3	0.43	1.63
(*n* = 27)	Frequency (%)	88.9	11.1		(0.48–5.46)

Data is presented un-stratified and following stratification by sex and by joint.
[a]Cases who had undergone both a hip and a knee joint replacement.
[b]Two hand osteoarthritis cases had unrecorded sex status.

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this superfamily facilitates synovial joint homeostasis in a pro-anabolic manner (2). Disruption to the anabolic–catabolic balance is often cited as a likely mechanism that influences OA development (17). Our *in vivo* allelic expression data demonstrates that the *GDF5* gene is also expressed in the cartilage of elderly adults and that the functional difference mediated by SNP rs143383 on *GDF5* expression that was intimated by the *in vitro* reporter experimemt carried out in the Asian study (6) is active in patients who have severe end-stage disease up to the point at which they require surgery. It could be therefore that a small but persistent imbalance of *GDF5* expression throughout life renders an individual more susceptible to OA. This is potentially a very important observation as it suggests that a therapy designed to counteract the functional susceptibility of *GDF5* may be beneficial even when administered to mature individuals.

A number of other genes encoding proteins mediating cell signalling and signal transduction have been implicated in OA susceptibility (18). *GDF5* joins this growing group. Manipulation of these genes and their encoded proteins, even in adult tissue, may be a fertile area for the development of novel OA therapeutics.

MATERIALS AND METHODS

UK case–control cohort

Cases were ascertained through the Nuffield Orthopaedic Centre in Oxford (*n* = 1669; 1006 females and 663 males). They had undergone total joint replacement of a hip (THR, *n* = 1221; 765 females and 456 males), of a knee (TKR, *n* = 349; 179 females and 170 males) or of a hip and a knee (*n* = 99; 62 females and 37 males) for primary OA. The cases were ascertained using the criteria of signs and symptoms of OA sufficiently severe to require joint replacement surgery. The radiological stage of the disease was a Kellgren and Lawrence grade of 2 or more in all cases with over 90% being grade 3 or 4. Inflammatory arthritis (rheumatoid, polyarthritic or autoimmune disease) was excluded, as was post-traumatic or post-septic arthritis. No cases suggestive of a skeletal dysplasia or developmental dysplasia were included. The average age of the cases at replacement surgery was 65 years with an age range of 56–85 years. The control group comprised 822 individuals (368 females and 454 males) with no signs or symptoms of arthritis or joint disease (pain, swelling, tenderness or restriction of movement). The

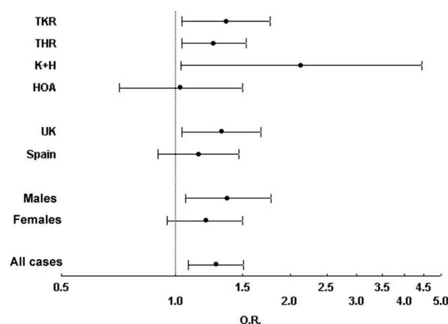

Figure 1. Odds ratios (OR) and 95% CI (bar) for carriers of the T-allele of *GDF5* SNP rs143383. TKR, knee cases (UK and Spanish data combined), THR, hip cases (UK and Spanish data combined), K + H, cases who have undergone a total knee and a total hip replacement (UK data only), HOA, cases with hand OA (Spanish data only). Also shown are the ORs for the UK cohort alone and for the Spanish cohort alone, for males and for females from the UK and Spanish data combined, and finally for the combined unstratified study.

average age of the controls at recruitment was 69 years with an age range of 55–89 years. Because of ethical and financial constraints, the hip and knee joints of the controls were not subjected to radiographic analysis. All cases and all controls were UK citizens of European ethnicity. Ethical approval for the study was obtained from appropriate ethics committees.

Spanish case–control cohort

The selection criteria and case characteristics have been described previously (7). In brief, OA cases were recruited among patients undergoing total hip replacement (THR, $n = 304$; 183 females and 121 males) or total knee replacement (TKR, $n = 274$; 223 females and 51 males) or among patients attending the Rheumatology Unit because of hand OA [HOA, $n = 240$; 211 females and 27 males (two hand OA cases had unrecorded sex status)] complaints and fulfilling ACR classification criteria (19). Controls ($n = 1196$; 667 females and 529 males) were older than 55 years. This study was conducted following the Helsinki Declaration, was approved by the Ethical Committee for Clinical Research of Galicia and all participants gave their written informed consent. All subjects were of Spanish ancestry and resided in the reference area of the Hospital Clinico Universitario de Santiago, Spain.

Genotyping SNP rs143383 in the UK case–control cohort

The SNP alters a *Bsi*EI restriction enzyme site and was genotyped using a PCR-restriction enzyme analysis. The forward primer had the sequence 5′-AGCACACAGGCAGCATTACG-3′ and the reverse primer the sequence 5′-CCAGTCCCATAGTGGAAA TG-3′, creating a 197 bp PCR product. Following digestion with *Bsi*EI (New England Biolabs, Hitchin, UK) a T-allele remains uncut, while a C-allele generates two fragments of 106 bp and 91 bp. Digestion products were electrophoresed through 3% agarose and scored following ethidium bromide staining.

Genotyping SNP rs143383 in the spanish case–control cohort

A TaqMan (Applied Biosystems, Foster City, CA, USA) assay was used, with primers and fluorescence-labelled probes designed and synthesized by Applied Biosystems (sequences are available from the authors). TaqMan reactions were performed in a total volume of 10 μl containing 24 ng of genomic DNA following the Applied Biosystems protocol. A Chromo4 real-time PCR system (MJ Research, Waltham, MA, USA) was used to run the assays. Several samples with different genotypes were sequenced with the Big Dye Ready Reaction Kit (Applied Biosystems) to confirm the accuracy of genotypes.

Power calculations and statistical analysis

The minimum detectable OR under the log-additive model with power greater than or equal to 80% and significance level of 5% was calculated using Quanto version 1.1 (http://hydra.usc.edu/gxe) (20,21) and using the Power and Sample Size software (22). The T-allele frequency was set to 62% (the frequency for all 822 of the UK controls) or to 60.2% (the frequency for all 1196 of the Spanish controls) and the population risk of OA was set to 5%.

Genotype and allele distributions in cases and controls were compared using standard χ^2 analysis-of-contingency tables. ORs were calculated with 95% CIs. For stratification analysis, female cases were compared with female controls, and male cases were compared with male controls.

Allelic expression analysis

Using a protocol described previously (23), nucleic acid was extracted from the macroscopically normal articular cartilage of UK OA patients who had undergone hip or knee replacement surgery (THR and TKR, respectively). The cartilage genomic DNA was used to genotype the patients for SNP rs143383 using the restriction enzyme assay described above. Two THR (one female and one male) and seven TKR patients (five females and two males) were identified as heterozygous for SNP rs143383 (these nine patients were collected subsequent to the case–control cohort). The cartilage RNA from these nine patients was then taken forward for an allelic expression analysis using a single base extension (SBE) assay that we have described in detail previously (23). At least 800 ng of RNA was used for the cDNA synthesis using random hexamers and the SuperScript™ kit (Invitrogen, Paisley, UK). Two reverse transcription (RT) reactions were performed for each patient: with reverse transcriptase (+RT) and without reverse transcriptase (−RT). From each +RT reaction, ≥19 individual PCR amplifications were carried out using forward primer 5′-CTTCAAGCCCTCAGTCAGT TG-3′ and reverse primer 5′-CGGGTGTGTGTTTGTATCC AG-3′, both located in the 5′-UTR of *GDF5*. The (−RT)

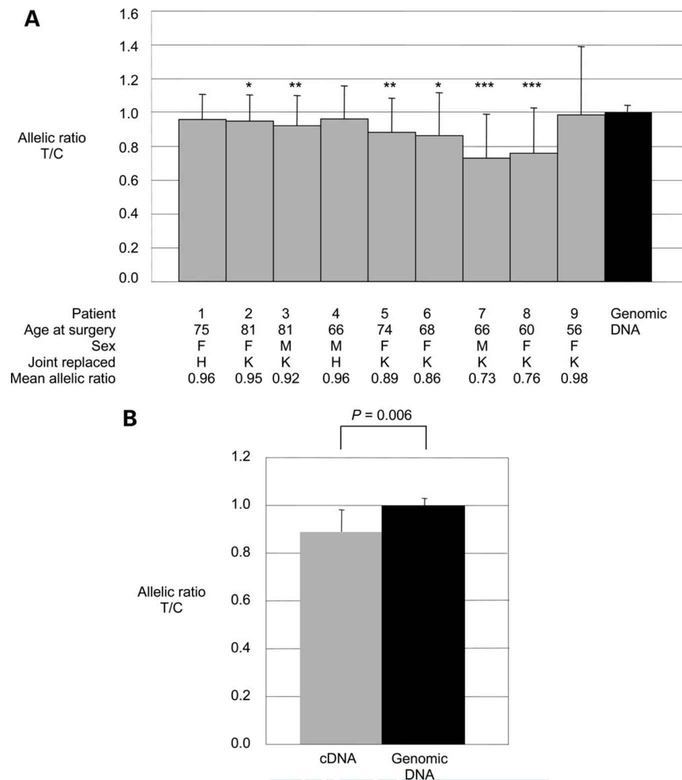

Figure 2. Allelic expression analysis at *GDF5* SNP rs143383 using RNA extracted from articular cartilage. (**A**) The expression analysis was performed on the cartilage of nine UK patients who had severe end-stage OA, seven of whom had undergone a TKR (K) and two of whom had undergone a THR (H). For each patient, 19 (patients 1, 4, 5 and 7) or 20 (patients 2, 3, 6, 8 and 9) individual cDNA PCR amplifications and SBE extension reactions were performed. Forty-five individual PCR and SBE reactions were performed on genomic DNA (five reactions per patient). The cDNA allelic ratios from each patient were then compared with the 45 genomic allelic ratios by a two-tailed Mann–Whitney exact test. Data shown are the mean + SD. $^{*}P < 0.005$; $^{**}P < 0.0005$; $^{***}P < 0.0001$. (**B**) The mean allelic ratios for the nine patient cDNAs were compared with the mean allelic ratios for the nine patient genomic DNAs using a two-tailed *t*-test. Data shown are the mean + SD.

controls did not yield detectable PCR products. The primer 5′-CTCGTTCTTGAAAGGAGAAAGCC-3′, which is located immediately adjacent to SNP rs143383, was used for the SBE assay. To ascertain the peak pattern for an assumed 1:1 ratio between alleles, we performed five individual PCR and SBE reactions on the cartilage genomic DNA of each of the nine patients to give a total of 45 individual genomic DNA measurements. The same PCR primers and SBE primer were used for the cDNA and genomic template. This use of the same analytic conditions for the cDNA and genomic DNA measurements enabled us to use the average of the 45 genomic DNA allelic ratio measurements (representing the assumed 1:1 ratio between alleles) to correct the allelic ratios obtained from the cDNA measurements and thus to account for differences in fluorescent yield and terminator dye incorporation specific to the assay. This correction allowed us to obtain exact values of the relative allelic expression of each cDNA measurement. To determine if there was a significant difference in allelic expression for each patient, we compared the cDNA allelic ratios for that patient to the pooled genomic allelic ratios using a two-tailed Mann–Whitney exact test. To determine if there was an overall difference in expression between the T-allele and the C-allele, we compared the mean allelic ratios for the nine patient cDNAs to the mean allelic ratios for the nine patient genomic DNAs using a two-tailed *t*-test.

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SUPPLEMENTARY MATERIAL

Supplementary material is available at HMG Online.

ACKNOWLEDGEMENTS

We thank Andrew Carr, Kim Clipsham, Bridget Watkins and Nick Athanasou, who helped organize the collection of samples from UK individuals evaluated in this report, and Yolanda Lopez-Golan and Fina Meijide for their help in recruiting Spanish study participants. Marta Picado and Cristina Fernandez provided outstanding technical assistance. This study was supported by Research into Ageing (J.L.), the Arthritis Research Campaign (J.L.), by grant PI02/0713 from the Instituto de Salud Carlos III, Spain (A.G.), with funds from FEDER of the European Union (A.G.) and by the Fundacion MMA of Madrid, Spain (A.G.). J.R.-L. is the recipient of a scholarship of the Spanish Ministry of Education. J.M.W. is the recipient of a Marshall Scholarship. M.P.-S. received a bursary from the Fundacion Española de Reumatologia.

Conflict of Interest statement. None declared.

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HMG Advance Access published February 24, 2008

A meta-analysis of European and Asian cohorts reveals a global role of a functional SNP in the 5' UTR of *GDF5* with osteoarthritis susceptibility

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Abstract

We have performed a meta-analysis combining data for over 11,000 individuals. It provides compelling evidence for a positive association between a functional SNP in the 5' UTR of *GDF5* (+104T/C; rs143383) and osteoarthritis (OA) in European and Asian populations. This SNP has recently been reported to be associated with OA in Japanese and Han Chinese populations. Attempts to replicate this association in European samples have been inconclusive as no association was found in the case-control cohorts from the UK, Spain and Greece when studied individually. However, the pooled data of UK and Spain found an association of the T-allele with an odds ratio (OR) 1.10. Whilst the European studies had adequate power to replicate the original findings from the Japanese cohort (OR = 1.79), these results suggest that the role of the *GDF5* polymorphism may not be as strong in Europeans. To clarify whether the European studies were hampered by insufficient power we combined new data from the UK and the Netherlands with the three published studies of Europe and Asia. The results provide strong evidence of a positive association of the *GDF5* SNP with knee OA for Europeans as well as for Asians. The combined association for both ethnic groups is highly significant for the allele frequency model (P = 0.0004, OR = 1.21) and the dominant model (P <0.0001, OR = 1.48). These findings represent the first highly significant evidence for a risk factor for the development of OA which affects two highly diverse ethnic groups.

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INTRODUCTION

The role of growth and differentiation factor 5 GDF5 in the development and maintenance of bone and cartilage has been recognised for some time [1-5]. Also known as cartilage-derived morphogenetic protein 1, it is a member of the transforming growth factor- (TGF-) superfamily and is closely related to the subfamily of bone morphogenetic proteins (BMPs). Mutations in *GDF5* have been implicated in several disorders of skeletal development, for example various forms of chondrodysplasia, synphalangism and type C brachydactyly. Thus its function in the etiology of OA, a progressive degeneration of articular cartilage, appears highly plausible.

OA is a common multifactorial disease affecting about 5% of the population mostly over 45 years of age. As such it has a large economic impact. Early changes most likely take place in the articular cartilage that result in bone remodelling, new bone and (over a period of time) loss of articular cartilage which appears radiographically as joint space narrowing. A later severe end-stage of the disease is usually treated by surgery involving total joint replacement of the hip and/or knee (THR and TKR). A number of studies in recent years have reported finding susceptibility genes for the disease [6-10], although replication of many of these findings has been mixed[11]. Possible reasons proposed for the noted inconsistencies in results include, ethnic differences between cohorts, the heterogeneity of OA and perhaps the inadequate statistical power of some of the studies. These inconsistencies can be overcome by a meta-analysis which provides a quantitative approach for the combination of different independent studies, thereby maximising the overall power.

It was to resolve some of these difficulties that a meta-analysis was conducted between an aspartic acid (D)-repeat polymorphism in the gene encoding asporin (ASPN) and OA[12]. The initial association was found in a Japanese cohort between knee OA and the D14 allele of the gene (P = 0.0013, OR = 2.49). This result was replicated in two independent samples of knee OA and hip OA with functional analyses providing strong evidence for the causality of the asporin variant [7]. Whilst three European studies from the UK, Greece and Spain failed to

replicate the findings [13-15]; a Chinese case-control study utilising a cohort of radiographically defined knee OA, did find significant association with the D14 allele (P = 0.0013, OR = 2.04) [16]. A meta-analysis of all the data revealed significant heterogeneity between ethnic groups of Europeans and Asians[12]. The study detected a strong association between the D14 allele and knee OA in Asian populations (P = 0.0000013, summary OR = 1.95) that was not replicated in the European samples. Thus it was concluded that there are ethnic differences in the effect of the ASPN D14 allele on OA susceptibility.

Recently, an association with hip and knee OA of the single nucleotide polymorphism (SNP) in the 5' UTR of *GDF5* (+104T/C; rs143383) was reported in Japanese and Chinese cohorts [17]. An elevated frequency of the T allele was found in two independent Japanese hip cohorts with a combined OR = 1.79 (P = 1.8×10^{-13}). This finding was replicated in two knee OA cohorts from Japan (P = 0.0021; OR = 1.30) and China (P = 0.00028; OR = 1.54). Furthermore, *in vitro* cell transfection studies employing luciferase reporter assays revealed that the T-allele mediated a moderate but significant reduction in the activity of the *GDF5* promoter in both chondrogenic and non-chondrogenic cell lines. Two European studies have attempted to reproduce these results [18, 19]. The first study employed two large independent populations from the UK (n = 2491) and Spain (n = 2014) with knee and hip OA as defined by having THR and/or TKR. No association was detected in the Spanish cohort either between allele or genotype frequencies. The UK population alone did reveal moderate association when carriers of the T-allele were combined (TT + TC) and compared with the CC genotypes (implying that the allele has a dominant effect on OA susceptibility). However, a trend towards an elevated T-allele in OA cases for both cohorts was observed. The data was pooled and this larger cohort of 4505 individuals revealed association at the allele level (P = 0.03), the genotype level (P = 0.01) and with a dominant effect model (P = 0.004). As with the original Asian study, over-representation of the T-allele in the OA individuals was identified as the risk allele. Stratification by both joint site and sex revealed no significant increase in the association. The second study utilised a Greek population (n = 519) of radiographically

assessed knee OA cases and controls where no association was identified with either allele frequency data or genotype data. There were also no differences detected when stratification for sex was considered. Interestingly, an increased frequency of the T-allele in the case individuals (62.9%) versus the controls (60.3%) was noted which is comparable to that observed in the pooled UK and Spanish sample (63.3% cases vs 61.0% controls). It is possible that the predisposing risk factor of *GDF5* has a modest effect in European populations and hence these studies were of inadequate sample size.

In this paper, meta-analyses were performed to assess the association of *GDF5* on OA. The analysis incorporates unpublished data from the Netherlands and UK (Manchester and Oxford) to add to previously published results from Japan, China, UK (Oxford), Spain and Greece [17-19].

RESULTS

Table 1 shows the allele counts and genotypes for the SNP rs143383 in cases and controls from Europe and Asia. It includes both the published and unpublished data. The total data for each study is presented along with the stratification into hip, knee and hand OA.

Allele frequency model

Initially, the analysis was conducted on the allele data following the initial finding from the Asian populations of association of the T-allele of *GDF5* and OA (Table 2). The forest plots for the allele model are shown in Figure 1. There was very significant heterogeneity when all the studies were combined for all the cases (I^2 = 84.8%). This heterogeneity was also present in the combined hip OA cases (I^2 = 88.3%), but was less apparent when only the knee studies were pooled (I^2 = 36.9%). The summary OR for all cases was significant (P = 0.006) but the exclusion of the European studies increased the significance considerably (OR = 1.55, P = 0.000001) and no association was detected when the pooled European data is considered alone. The most significant result and strongest summary OR is obtained when just the two Asian hip cohort are combined (OR = 1.78, P < 0.0000001), with the European studies showing no significant association in this strata. The four combined European studies for knee

OA, however, demonstrate significant association (P = 0.022) as well as the pooled two Asian studies (P = 0.00012). Interestingly, there is no significant heterogeneity in the knee strata either between or within the two ethnic groups and the combined OR for Asian and European populations is highly significant (OR = 1.21, P = 0.0004). These results indicate that the initial finding of a strong association in the Japanese and Chinese populations of knee OA with *GDF5* is independently replicated in European groups. This result is not seen with European hip OA and hand OA cases however.

Dominant model

We then examined the effects of genotype differences following a dominant model, in response to the previous findings from the UK and Spanish cohorts. Their results suggest that the T-allele has a dominant effect on OA in European populations. This analysis indicates very strong association for both ethnic groups (Table2, Figure 2). For all cases the association is highly significant for Europeans (P = 0.0045), Asians (P = 0.0004) and the combined group of Europeans plus Asians (P = 0.0007). Moderate but significant heterogeneity was seen when these studies were combined (I^2 = 51.9%, P = 0.042) but was not apparent when the groups were considered separately. The summary ORs for the Asian group is much stronger compared with the Europeans (1.87 and 1.24, respectively) indicating perhaps the more moderate role *GDF5* plays in populations of European ancestry. For hip OA the association is only significant when the two ethnic groups are combined (P = 0.035) but again significant heterogeneity is detected. The trend of the ORs for each of the European hip cohorts of UK, Spain and Netherlands is similar (Figure 2). It may be that the sample size is too small to detect the seemingly weak genetic effect of *GDF5* with hip OA. However Europeans and Asians do show significant association for *GDF5* with knee OA individually (Europeans, P = 0.001; Asians, P = 0.005). Combining all studies of Europeans and Asians together increases the significance of association (P < 0.0001) without any evidence for heterogeneity. The consistency of these results is under-lined by the fact that all the CIs across individual studies overlap and contain the summary OR estimate of the meta-analysis (Figure 2). The OR for

knee OA shows that the risk allele for *GDF5* has a moderate to strong effect in both ethnic groups (OR = 1.43 [Europeans]; 1.64 [Asians]; 1.48 [both]).

The combined OR for hand OA is also significant (P = 0.045, OR = 1.26). This was the smallest category (n = 955 cases) in the analysis where just three European studies were combined and so this result may well be more significant with an increase in sample size to increase statistical power. In addition, there were differences in the clinical definition of hand OA in the three cohorts and hence future studies may need to address whether association may be to a specific hand OA trait.

DISCUSSION

GDF5 plays a major role in joint formation and articular cartilage homeostasis [3]. The rs143383 T-allele, associated with increased risk for OA, has been demonstrated to show decreased transcriptional activity in chondrogenic cells [17]. In addition, *in vivo* allelic expression data has demonstrated that the *GDF5* gene is expressed in the cartilage of elderly adults with a small but statistically significant decrease in the expression of the T-allele relative to the C-allele [18]. Recently, the *GDF5* polymorphism has been reported to be associated with a decrease in human height in both males and females [20]. This interesting finding indicates that the reduced expression of *GDF5* which renders an individual susceptible to OA in later life may also result in a reduction in limb bone growth during the earlier stage of life.

We performed a meta-analysis because of the concern that the individual studies may have insufficient power to detect the small effect of the functional polymorphism of *GDF5* on OA susceptibility in populations of European ancestry. Combining eight studies from six countries across two continents, we were able to perform an analysis that included 11,213 individuals. The results confirm the strong effect that the rs143383 polymorphism of *GDF5* plays in OA susceptibility in Asians with very highly significant association for both the hip and knee OA strata as measured by allele frequency comparison. The effect appears to be strongest in the hip OA category where the OR = 1.78 (P < 0.0000001). This association was not detected in

the hip OA stratum for the European studies, as measured by allele frequency and when considering the dominant model. Significant heterogeneity was measured between the ethnic groups for this category however making conclusions difficult although the trend does indicate that there would be significant association for the dominant model in European (P = 0.069) and Asian (P = 0.070) groups alone if the sample size was increased further. It is interesting that there is heterogeneity between the hip and knee strata for the Asian group alone (P = 0.042). This may reflect a differential effect of the risk allele between the two joint strata. All centres defined their cases as having a KL grade • 2. The UK, Spanish and Greek centres cases also had a total knee or hip replacement. Whilst it is possible that the differences found between the populations for the risk association are the result of the OA phenotypes defined within each centre; it is also likely that it reflects differences in environmental influences or even in the genetic background of the two groups.

In contrast, the highly significant association, as measured by allele frequencies, in the Asian populations for knee OA cases was detected in the Europeans. The combined summary OR was also highly significant (P = 0.0004). This result is also seen when the dominant model for carriers of the T-allele [(TT) + (TC)] is considered. In contrast to the hip OA stratum, the knee stratum was found to have no significant heterogeneity (Table 2).

Of final interest is the association in the hand OA stratum. Association has been reported between the presence of hand OA (particularly Heberden's nodes) and radiographic knee OA [21, 22], it cannot be excluded at this stage that the association seen in the hand OA category may be the results of concordant knee OA which was not scored in the UK and Spanish study. In fact in the GARP study, where OA was assessed at 4 joint locations, the largest effect was found in subjects with OA at multiple joint locations involving at least one large joint stratum (knee or hip) and hand (results not shown). The effect in GARP among subjects without large joint involvement stayed behind as reflected in the "all cases" analyses of Figure 1 and 2.

Together, we found evidence that the functional polymorphism in the 5' UTR of *GDF5* plays a significant role in OA susceptibility in European as well as Asian populations. Overall

significance for the dominant model was observed for knee, hip and hand OA cases, which indicates that the *GDF5* variant contributes to OA susceptibility at different joint locations and across different ethnic groups.

MATERIALS AND METHODS

New European Data

The Netherlands data consists of 724 independent controls (ages 30 – 80 years) and 191 Dutch sibling pairs (ages 40 – 70 years) from the GARP study [23]. Subjects of the GARP study were included with symptomatic and radiographic OA in two or more joint sites of the hand, spine (cervical or lumbar), knee or hip. Patients with secondary OA and familial syndromes were excluded. Radiographic OA at knee hip hand and spine was assessed according to the Kellgren/Lawrence (KL) scale (0 – 4) [24], while symptomatic OA of those joints was defined according to the American College of Rheumatology (ACR) recommendations [25-27]. As such the GARP study is the only one in which OA was assessed in 4 different joint locations. In the current analyses, GARP knee and hip OA was defined as radiographic OA, whereas hand OA was defined as radiographic OA in 3 or more hand joints out of 20 scored irrespective of the OA at other joint locations. In the "all OA" analyses all sibling pairs of GARP (N=91 pairs) were included. The new UK (Manchester) data consisted of 515 unrelated cases with hand OA defined as clinical evidence of Heberden's nodes on at least two or more distal interphalangeal joints of each hand [28]. The new UK (Oxford) cases were ascertained through the Nuffield Orthopaedic Centre in Oxford and had undergone a total joint replacement of the hip (THR, n = 69) or of a knee (TKR, n = 160). The ascertainment criteria for the new hip and knee cases were as previously published for the Oxford, UK[18] cohort. The new cases increased the UK study from 1669 to 2413 individuals. These were compared against the published UK controls of 822 individuals.

Published data

A PubMed database search for osteoarthritis and *GDF5* revealed just three relevant articles. These were published between April and September 2007: a study from Greece[19], another which combined UK (Oxford) and Spanish populations[18], and a third from Asia that initially reported the finding in two independent Japanese cohorts with replication in a Chinese population[17]. The Japanese and Chinese cases were defined radiographically with a KL •2. The Greek cases had undergone a total knee replacement. Anteroposterior weight-bearing radiographs were obtained for all the Greek individuals (cases and controls). Cases were defined as having a KL score > 2. The UK (Oxford) cases were ascertained using the criteria of signs and symptoms of OA sufficiently severe to require joint replacement surgery. The radiological stage of the disease was a KL grade > 2 in all cases with over 90% being grade 3 or 4 [29]. The Spanish hip and knee cases were also defined by total hip or knee replacement. Radiographs were used to verify that the OA was primary. The hand OA was defined using clinical ACR criteria [30]. Collaboration was established between the authors of these papers and the groups responsible for the new data. The subsequent meta-analysis combined data from 8 independent populations from Europe and Asia to give a total of 5874 cases and 5339 controls (Table 1).

Statistical analysis

Odd ratios were estimated for all the studies in the standard way except for the Netherlands data. In this case, to adjust for the family relationship among sibling pairs, standard errors were estimated from the variance between the sibling pairs (robust standard errors) and used in all the comparisons between the GARP subjects and controls including the meta-anlayses[31, 32]. Meta-analyses were performed, using the random-effects model of DerSimonian and Laird (DSL) [33]. The random-effects model anticipates that the studies may have genuine differences in their results which are not due to chance alone and thus incorporates a between-study variance in its estimate. It was found that there was highly significant heterogeneity between the frequencies of the common T-allele between the control populations of the two ethnic

groups, as measured by Fisher's exact test (P = 0.0000001). Consequently, a DSL model was deemed to be the most appropriate method even though it generally gives wider confidence intervals (CIs) than a fixed-effect model and is thus considered to be more conservative [34]. In the absence of any between-dataset heterogeneity, fixed-effect model estimates are identical to random-effect model estimates.

Heterogeneity between studies was evaluated using the I^2 statistic for inconsistency [35] and the ^{2} distributed Cochran Q statistic [36]. I^2 is given by 100% x (Q-df)/Q), where df = degrees of freedom. It describes the proportion of variation that is unlikely to be due to chance and is considered large for values over 50%[37]. The Q test is the most widely used test for heterogeneity, but is recognised to have poor power when there are few studies. Hence Q is considered statistically significant for $p < 0.10$ [35, 36]. However I^2 is unaffected by the number of studies and consequently is useful when comparing subgroups within the overall study. The I^2 estimate and the Q statistic were computed for all study comparisons.

We estimated summary odds ratios and 95% CIs for each ethnic group as well as for the entire set. We also sub-divided for knee, hip and hand OA, following the findings of previous studies. Finally we examined the effects of allele frequency differences as well as genotype differences (following a dominant model) in response to the previous findings from the UK and Spanish cohort. All statistical analyses were performed using the R software (http,//www.R-project.org) [38] and StatsDirect version 2.6.3 (http,//www.statsdirect.com) [39].

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Acknowledgments

This study was supported by Research into Ageing, the Arthritis Research Campaign, the Leiden University Medical Centre, the Dutch Arthritis Association and Pfizer Inc., Groton, CT, USA support the GARP study. The genotypic work was supported by the Netherlands Organization of Scientific Research (MW 904-61-095, 911-03-016, 917 66344 and 911-03-012), Leiden University Medical Centre and the Centre of Medical System Biology. We thank

Jeanine Houwing Duistermaat (LUMC, Dept. Medical Statistics, Leiden, The Netherlands) for statistical support.

Conflict of Interest Statement. The authors declare that they have no competing financial interest.

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Figure 1. DerSimonian-Laird OR meta-analysis (random effects) of the association between *GDF5* SNP rs143383 and OA as measured by allele frequency data. Black squares (OR) and 95% CI (bar) are shown for each study. The pooled ORs and their 95% CIs are represented by the un-shaded diamonds. Summary ORs are given for each ethnic group as well as all groups combined. The left-hand column lists the individual studies, each one defined by country of origin. The three cohorts from Japan are defined as follows: Japan – Hip1 represents the first Japanese cohort of hip OA; Japan – Hip2 is the second (replicating) Japanese hip OA cohort; Japan – Knee represents the Japanese cohort of knee OA.

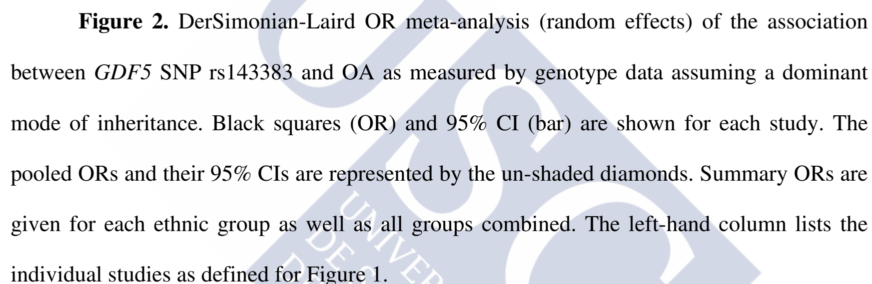

Figure 2. DerSimonian-Laird OR meta-analysis (random effects) of the association between *GDF5* SNP rs143383 and OA as measured by genotype data assuming a dominant mode of inheritance. Black squares (OR) and 95% CI (bar) are shown for each study. The pooled ORs and their 95% CIs are represented by the un-shaded diamonds. Summary ORs are given for each ethnic group as well as all groups combined. The left-hand column lists the individual studies as defined for Figure 1.

Table 1

[a] Some cases from the UK and Spain were excluded from the stratified groups because they were diagnosed as having OA at more than one major site.

[b] Analysis includes unpublished data in addition to data presented in Southam *et al.*

[c] Not previously published data. Cases are subjects of the GARP sibling pair study. To adjust for family relationships between subjects of the GARP study, standard errors were estimated from the variance between sibling pairs (robust standard errors) and used in each analyses [31, 32].

[d] In Spain hand OA was defined by ACR hand criteria. In the UK hand OA was defined as nodal OA. In The Netherlands hand OA was defined as radiographic OA at 3 or more joint sites out of 20 scored.

[e] Unpublished data compared against UK controls of Southam *et al.*

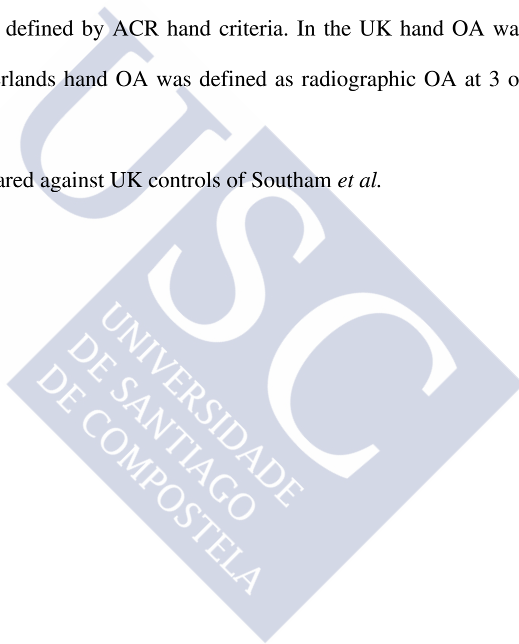
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Table 1 Allele counts and genotypes for the SNP rs143383 in cases and controls for eight studies from Europe and Asia. Data is presented un–stratified and then sub–divided by joint[a]. Study names are defined in Figure 1

Country (study)	Case		Control		Case				Control			
	Allele Count (%)		Allele Count (%)		Genotype				Genotype			Total
	T	C	T	C	TT	TC	CC	Total	TT	TC	CC	
All Cases												
UK (Southam *et al*)[b]	3138 (65.0)	1688 (35.0)	1020 (62.0)	624 (38.0)	1005	1128	280	2413	324	372	126	822
Spain (Southam *et al*)	1002 (61.20	634 (38.8)	1441 (60.2)	951 (39.8)	302	398	118	818	439	563	194	1196
Greece (Tsezou *et al*)	316 (62.9)	186 (37.1)	323 (60.3)	213 (39.7)	95	126	30	251	99	125	44	268
Netherlands[c]	431 (59.4)	295 (40.6)	909 (62.8)	539 (37.2)	121	189	53	363	289	331	104	724
Japan – Hip1 (Miyamoto *et al*)	386 (81.1)	90 (18.9)	375 (73.0)	139 (27.0)	160	66	12	238	135	105	17	257
Japan – Hip2 (Miyamoto *et al*)	1282 (84.3)	238 (15.7)	1080 (74.4)	372 (25.6)	541	200	19	760	407	266	53	726
Japan – Knee (Miyamoto *et al*)	1131 (78.8)	305 (21.2)	1276 (74.1)	446 (25.9)	444	243	31	718	473	330	58	861
China (Miyamoto *et al*)	491 (78.4)	135 (21.6)	681 (70.2)	289 (29.8)	197	97	19	313	244	193	48	485
Total								5874				5339
Hip OA												
UK (Southam *et al*)	1645 (63.8)	935 (36.2)	1020 (62.0)	624 (38.0)	519	607	164	1290	324	372	126	822

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Spain (Southam *et al*)	361 (59.4)	247 (40.6)	1441 (60.2)	951 (39.8)	102	157	45	304	439	563	194	1196
Netherlands[c]	136 (64.2)	76 (35.8)	909 (62.8)	539 (37.2)	43	50	13	106	289	331	104	724
Japan – Hip1 (Miyamoto *et al*)	386 (81.1)	90 (18.9)	375 (73.0)	139 (27.0)	160	66	12	238	135	105	17	257
Japan – Hip2 (Miyamoto *et al*)	1282 (84.3)	238 (15.7)	1080 (74.4)	372 (25.6)	541	200	19	760	407	266	53	726
Total								2698				3725
Knee OA												
UK (Southam *et al*)	676 (66.4)	342 (33.6)	1020 (62.0)	624 (38.0)	219	238	52	509	324	372	126	822
Spain (Southam *et al*)	340 (62.0)	208 (38.0)	1441 (60.2)	951 (39.8)	102	136	36	274	439	563	194	1196
Greece (Tsezou *et al*)	316 (62.9)	186 (37.1)	323 (60.3)	213 (39.7)	95	126	30	251	99	125	44	268
Netherlands[c]	180 (63.4)	104 (36.6)	909 (62.8)	539 (37.2)	54	72	16	142	289	331	104	724
Japan – Knee (Miyamoto *et al*)	1131 (78.8)	305 (21.2)	1276 (74.1)	446 (25.9)	444	243	31	718	473	330	58	861
China (Miyamoto *et al*)	491 (78.4)	135 (21.6)	681 (70.2)	289 (29.8)	197	97	19	313	244	193	48	485
Total								2207				4356
Hand OA[d]												
UK[e]	692 (67.2)	338 (32.8)	1020 (62.0)	624 (38.0)	233	226	56	515	324	372	126	822
Spain (Southam *et al*)	301 (62.7)	179 (37.3)	1441 (60.2)	951 (39.8)	98	105	37	240	439	563	194	1196
Netherlands[b]	239 (59.8)	161 (40.2)	909 (62.8)	539 (37.2)	64	111	25	200	289	331	104	724
Total								955				2742

Table 2. Random–effects meta–analysis of eight studies from Europe and Asia for the association of *GDF5* (allele frequency and dominant model) with Osteoarthritis. Heterogeneity was evaluated using the Cochran Q test. I^2 percentage values are also given (%).

Summary (number of studies)	Allele frequency model			Dominant Model		
	OR (95% CI)	P–value for the test of combined OR	P–value for the test of heterogeneity (I^2%)	OR (95% CI)	P–value for the test of combined OR	P–value for the test of heterogeneity (I^2%)
All OA studies combined (8)	1.26 (1.07 – 1.48)	0.006	< 0.0000001 (84.8)	1.43 (1.16 – 1.76)	0.0007	0.042 (51.9)
European (4)	1.05 (0.94 – 1.17)	0.400	0.144 (44.6)	1.24 (1.07 – 1.44)	0.0045	0.403 (0)
Asian (4)	1.55 (1.30 – 1.85)	0.000001	0.042 (63.5)	1.87 (1.33 – 2.65)	0.0004	0.199 (35.5)
All Hip studies combined (5)	1.26 (0.97 – 1.64)	0.083	< 0.0001 (88.3)	1.43 (1.03 – 2.00)	0.0352	0.029 (62.8)
European (3)	1.04 (0.94 – 1.15)	0.438	0.621 (0)	1.20 (0.99 – 1.46)	0.069	0.885 (0)
Asian Hip (2)	1.78 (1.52 – 2.08)	<0.0000001	0.389 (na)[a]	2.12 (0.94 – 4.78)	0.070	0.078 (na)
All Knee studies combined (6)	1.21 (1.09 – 1.34)	0.0004	0.160 (36.9)	1.48 (1.24 – 1.78)	<0.0001	0.941 (0)
European (4)	1.13 (1.02 – 1.25)	0.0222	0.714 (0)	1.43 (1.15 – 1.76)	0.001	0.860 (0)
Asian (2)	1.39 (1.17 – 1.64)	0.00012	0.234 (na)	1.64 (1.16 – 2.32)	0.005	0.869 (na)
All Hand OA studies combined (3)	1.08 (0.89 – 1.32)	0.4392	0.045 (67.8)	1.26 (1.01 – 1.58)	0.0446	0.417 (0)

Figure 1

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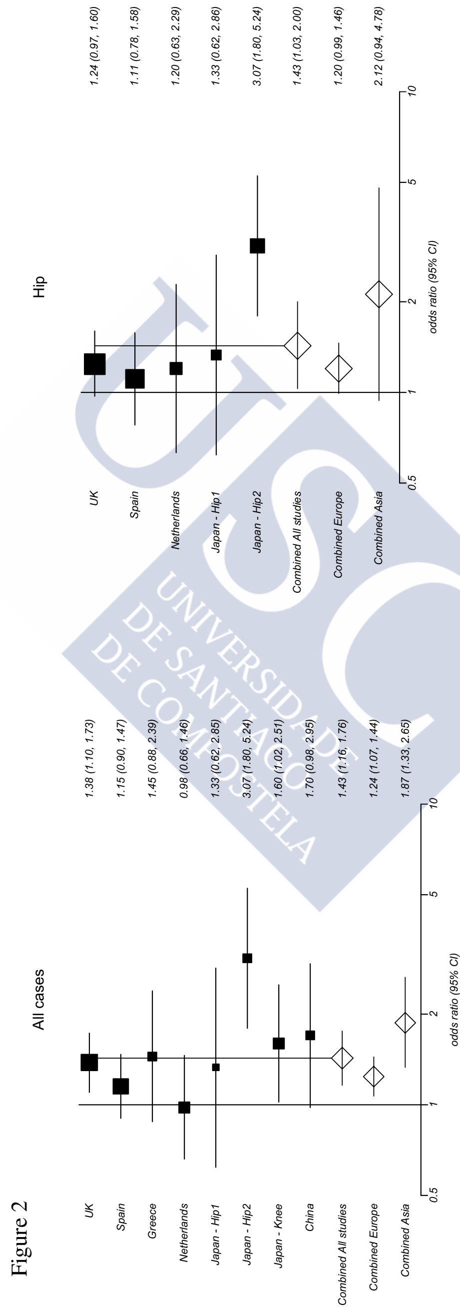
Figure 2



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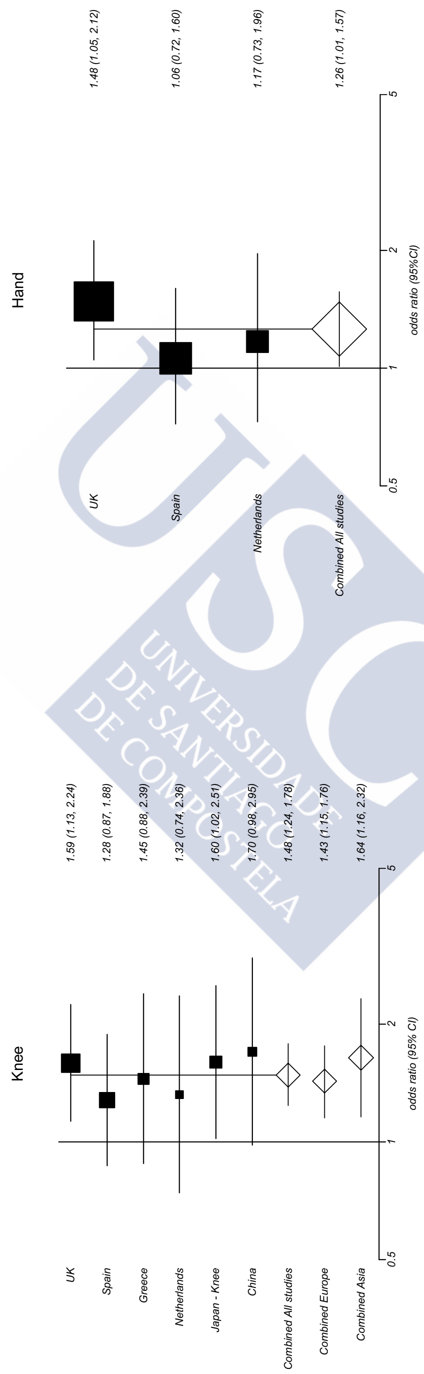

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Discusión general

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Discusión general

Nuestro trabajo ha abordado el estudio de las proteasas de ECM en la OA desde un punto de vista puramente genético. Además, ha analizado algunas de estas proteasas en relación con la RA y se han replicado estudios de asociación de tres genes reguladores del desarrollo, maduración y reparación del cartílago.
La OA es una enfermedad de alta prevalencia en la población que muestra una etiología multifactorial. Estudios epidemiológicos han demostrado que el componente genético de esta enfermedad es elevado situándose en torno a un 40-79% dependiendo del fenotipo. Los resultados obtenidos por los estudios genéticos muestran que la OA es una enfermedad compleja y poligénica y que además, en su etiología parecen influir factores pertenecientes tanto a la ruta catabólica como a la anabólica del cartílago.

Hemos encontrado algunas asociaciones nuevas en los genes *ADAMTS-14, MMP-8, MMP-2, MMP-1, MMP-9* y *PAI-1.* Todos ellas requieren confirmación en estudios independientes. Sin embargo, la asociación entre ADAMTS-14 y OA es la que parece más sólida, y no sería arriesgado plantear estudios enfocados a identificar el papel funcional de esta proteasas en la OA y en el desarrollo y reparación del cartílago. También hay que destacar que en este trabajo no se encontró asociación entre SNPs en la que era considerada la mayor agrecanasa *in vivo*, ADAMTS-5, y susceptibilidad a la OA. Nuestros resultados indican la posibilidad de que los sistemas catabólicos del cartílago, y la regulación de estos, no sean los mismos en el ratón y en el humano o, al menos, que el papel de ADAMTS-5 no sea tan crítico en humanos como en ratones.

Otros genes candidatos que también fueron tomados en consideración en este estudio fueron los genes de los inhibidores tisulares de metaloproteasas (TIMPs), y las catepsinas –K, -B y –D. Los TIMPs son importantes ya que ejercen una actividad inhibidora sobre las MMPs, ADAMTS y ADAMs, por lo que variantes genéticas que afectasen a su expresión o a su actividad podrían tener repercusión en la susceptibilidad a la OA. Experimentos con ratones knockout han demostrado que su desactivación aumenta la tasa de degradación de colágeno y agrecano en el cartílago articular (Sahebjam S et al, 2007). Por otro lado, las catepsinas también han sido relacionadas con la OA ya que tienen actividad proteolítica sobre muchos de los componentes de la ECM y sus niveles de expresión se encuentran aumentados en el cartílago, tejido sinovial y hueso subcondral de pacientes con OA (Söderström M et al, 1999) (Mehraban F et al, 1997)

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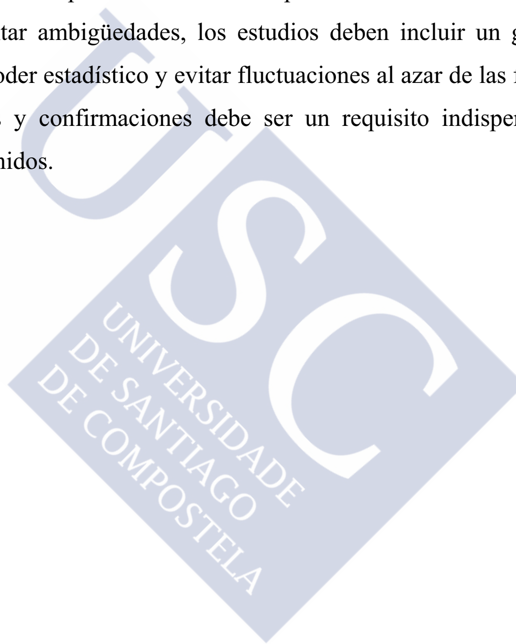
(Sapolsky AI et al, 1973). La búsqueda de polimorfismos reguladores y el análisis de nsSNPs para estos genes fueron realizados a la vez que para los otros genes candidatos de proteasas de matriz extracelular, pero no arrojó ningún resultado positivo. El papel de estos factores en la susceptibilidad a la OA no puede ser descartado sin embargo, ya que las variantes genéticas pueden tener repercusión que no es detectada con nuestros criterios de selección.
Además, hemos contribuido a establecer que polimorfismos en ASPN, FRZB y GDF5 tienen un efecto en la predisposición a OA. Gracias a la realización de estudios de este tipo se van identificando una lista de factores genéticos definitivamente implicados en la OA y se van definiendo sus efectos y los fenotipos que están afectados. En particular, los estudios de dos de estos factores genéticos se han seguido de estudios de metaanálisis que han clarificado algunos de estos aspectos.

En conclusión, estos resultados nos permiten vislumbrar un escenario de desarrollo de la OA. Esta comenzaría con deficiencias en la biogénesis del tejido articular, ejemplificados por GDF5, y posiblemente FRZB y ASPN, lo que pasados los años podría crear una situación de estrés tanto en las células como en el propio cartílago, que activaría los mecanismos catalíticos. Esto, unido a variaciones funcionales en los genes implicados en la degradación de la ECM, reflejados en las asociaciones con MMP-1, MMP-8 y MMP-9, posiblemente provocaría que se acentuara la situación de desequilibrio entre los procesos de síntesis y degradación de los componentes de la ECM. Las fases iniciales de degradación se pueden potenciar por una reacción inflamatoria frente a los fragmentos del cartílago que no esté adecuadamente controlada. Los polimorfismos en MMP-2 y PAI-1 podrían modificar la susceptibilidad a la OA a este nivel. Además, es posible que la respuesta regeneradora del cartílago dañado sea insuficiente, nivel en el que podrían incidir los polimorfismos de ADAMTS-14, ASPN y FRZB.
Todos estos factores, actuando de forma independiente contribuirían a aumentar la posibilidad de desarrollar OA a lo largo de la vida de un individuo. Una posibilidad atractiva es la de analizar la interacción de todas estos factores a través de un estudio genético. Sin embargo, es de una gran complejidad debido a las limitaciones estadísticas que este tipo de estudios suponen (Thornton-Wells TA et al, 2004).

En la RA, hemos encontrado dos SNPs moderadamente asociados con susceptibilidad. Los alelos de susceptibilidad en ambos casos provocan un descenso de la expresión de sus respectivos genes, *PLAT* y *MMP-2*. Una baja expresión de estos genes provocaría una facilitación de los fenómenos de inflamación por quimiotaxis de células inflamatorias (Dvorak

HN et al, 1985) (Forsyth CB et al, 2001) (Itoh T et al, 2002). (McQuibban GA et al, 2000), y limitaría la nutrición normal del tejido provocando hipoxia y acidosis en le líquido sinovial (Mapp PI et al, 1995). Es necesario completar este estudio con el análisis de otros SNPs en proteasas para completar el análisis del papel que los enzimas degradadores de cartílago tienen en la RA, y comparar su efecto con el que tienen en la OA.

Por último, en este trabajo se ha puesto de manifiesto la dificultad que atañe el estudio genético de enfermedades complejas. Se ha demostrado que la única manera de conseguir resultados fiables es realizar estos estudios genéticos eliminando factores de confusión. Para ello, el criterio fenotípico que utilicemos para seleccionar los pacientes de OA tiene que estar bien definido y no puede presentar ambigüedades, los estudios deben incluir un gran número de muestras para aumentar el poder estadístico y evitar fluctuaciones al azar de las frecuencias, y la realización de replicaciones y confirmaciones debe ser un requisito indispensable para dar validez a los resultados obtenidos.



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Conclusiones

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Conclusiones

1. La variación genética en *ADAMTS-14* está asociada con la OA, al menos con formas severas de la OA de rodilla en mujeres. Este efecto ha sido identificado en nuestro estudio y confirmado en un estudio independiente. Todo indica que la asociación es debida a un SNP no sinónimo con probable efecto deletéreo. Este hallazgo potenciará el estudio de ADAMTS-14 que es una proteasa casi desconocida. Es posible que su papel crítico esté relacionado con la formación de fibras de colágeno imperfectas.

2. Se han encontrado evidencias que indican que SNPs funcionales en varias proteasas contribuyen a la OA. Los detalles de esta relación todavía no están claros y sólo la replicación permitirá ser más concluyente, pero en una primera interpretación se puede sugerir que los SNPs en *MMP-1* y *MMP-8* muestran como estas proteasas contribuyen a la degradación del colágeno nativo, y que este paso es crítico en la OA. Por su parte un SNP en *MMP-9* mostraría la contribución de proteasas menos específicas pero muy activas. El haplotipo asociado con OA en *MMP-2* determina una disminución de esta proteasa y, probablemente, refleja la importancia de su efecto limitando la quimiotaxis de células inflamatorias que predominaría sobre la posible función colagenolítica. Por último, la asociación con PAI-1, puede interpretarse como reflejo de la importancia que esta molécula inhibidora tiene en la regulación del sistema plasminógeno-plasmina en la OA.

3. La variación genética en *ADAMTS-5*, incluyendo dos SNPs no sinónimos de probable efecto deletéreo, no modifica de forma detectable la predisposición a la OA. Por lo tanto, no está claro que ADAMTS-5 tenga un papel crítico en la OA como se había sugerido a partir de estudios en ratones. Algunos polimorfismos reguladores en otras proteasas no parecen tener un efecto en OA. Incluso en proteasas que según múltiples evidencias son importantes, como MMP-13 y MMP-3. Sin embargo, la complejidad de la OA y de la regulación de las proteasas no permite interpretar la ausencia de asociación como contraria a su relevancia.

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4. SNPs no sinónimos en FRZB contribuyen a la susceptibilidad a la OA de cadera en mujeres. Nuestro estudio ha corroborado este efecto indicando la posibilidad de que sea más específico de las pacientes con afectación en múltiples articulaciones. Este hallazgo indica la importancia que la ruta WNT tiene en la OA, aunque todavía no se ha identificado a qué nivel FRZB es crítico.

5. La longitud variable de la cadena de ácido aspártico de ASPN está asociada con la OA de rodilla, con un efecto más marcado en asiáticos que en europeos. Por este motivo, nuestro estudio no mostró asociación, pero contribuyó a identificar la asociación al participar en un metaanálisis. Esta asociación se ha interpretado como reflejo de la importancia de TGF-β en la reparación del cartílago dañado, y de la regulación de la acción del TGF-β por la ECM.

6. Un alelo que disminuye la transcripción de *GDF5* aumenta el riesgo de sufrir OA de forma global, pero de forma más clara en asiáticos que en europeos. Nuestro trabajo ha contribuido a confirmar y delimitar este efecto. De este modo, se ha puesto de manifiesto como un pequeño defecto en la expresión de un factor crítico en las fases iniciales del desarrollo de las articulaciones tiene consecuencias en la susceptibilidad a la OA.

7. Se ha encontrado asociación entre susceptibilidad a RA y alelos en SNPs reguladores de MMP-2 y de PLAT que determinan una menor expresión de estas dos proteasas. Esta asociación, de confirmarse, implicaría que estos dos genes tienen un efecto protector en RA. MMP-2, probablemente, por degradar factores quimiotácticos para células inflamatorias. PLAT, por su parte, podría proteger de RA porque la activación del plasminógeno contribuiría a impedir la acumulación intraarticular de fibrina, que tiene efectos proinflamatorios.

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