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PhD Thesis

Novel evidence for the role of monomeric C reactive protein and WISP2 in the modulation of catabolic and inflammatory response in cartilage and intervertebral disc

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Doctoral Programme in Medicine Clinical Research

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NOVEL EVIDENCE FOR THE ROLE OF MONOMERIC C REACTIVE PROTEIN AND WISP2 IN THE MODULATION OF CATABOLIC AND INFLAMMATORY RESPONSE IN CARTILAGE AND INTERVERTEBRAL DISC

Clara Ruiz Fernández

INTERNATIONAL PHD SCHOOL OF THE UNIVERSITY OF SANTIAGO DE COMPOSTELA

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A mi madre, a mi padre y a mi hermana

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RESUMO

A dor lumbar (LBP) e a artrose (OA) son as dúas principais enfermidades musculoesqueléticas que causan discapacidade a nivel mundial. A etioloxía da dor lumbar é complexa e multifactorial; e a dexeneración do disco intervertebral (IVDD) considérase unha das súas principais causas. A dor, a disfunción e a rixidez que caracterizan aos pacientes con IVDD e OA son responsables dun grave deterioro da súa calidade de vida e dun gran impacto socioeconómico.

A artrose (OA) é unha enfermidade articular dexenerativa multifactorial caracterizada pola destrución progresiva da cartilaxe articular, cambios no óso subcondral, formación de osteofitos e inflamación sinovial. É o tipo de artrite máis frecuente, pero aínda non se aclarou a súa etioloxía. A inflamación crónica de baixo grao contribúe aos síntomas e á progresión da enfermidade. Na OA, un alto estrés mecánico, os produtos de degradación da matriz extracelular (ECM), as citocinas proinflamatorias e as adipocinas activan os condrocitos para estimular a produción de mediadores proinflamatorios e encimas que degradan a matriz, como a IL-1β e a MMP-13, que actúan sinérxicamente para promover e perpetuar estas respostas catabólicas.

No disco intervertebral adulto sans (IVD), a homeostase entre a síntese e a degradación da matriz extracelular está equilibrada por factores de crecemento e citocinas. Un desequilibrio dos procesos catabólicos contribúe á dexeneración estrutural do disco intervertebral, o que probablemente contribúa ao desenvolvemento da dor lumbar. A dexeneración do disco leva a múltiples cambios anatómicos, mecánicos e bioquímicos. Estas alteracións diminúen a estabilidade mecánica e as funcións de absorción de choques, o que contribúe á formación de osteofitos, fisuras anulares e á diminución do movemento dos segmentos da columna vertebral. En resposta á inflamación e á dexeneración, o crecemento vascular e nervioso prodúcese no disco, inicialmente avascular, dende as capas externas do anel fibroso, estendéndose ata o núcleo pulposo.

A dexeneración do disco intervertebral e a dexeneración da cartilaxe na artrose mostran marcadas semellanzas. Nas radiografías obsérvase: a perda da altura do disco ou do espazo articular, a esclerose do óso subcondral e o desenvolvemento de osteofitos. Ademais, tanto no disco intervertebral como no cartílago articular, a matriz extracelular está composta de

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compoñentes semellantes (aínda que en diferentes proporcións) e unha maquinaria de degradación da matriz extracelular similar está presente no proceso de dexeneración.

Hai un delicado equilibrio entre os procesos anabólicos e catabólicos na cartilaxe articular e no disco intervertebral. Cando se rompe o equilibrio, hai unha desigualdade entre a síntese e a degradación da matriz extracelular debido ao comportamento catabólico das células. Describíronse varios factores inflamatorios sistémicos que inclinan este equilibrio cara á dexeneración tanto en enfermidades musculoesqueléticas, como a diabetes, a obesidade, o tabaquismo e as infeccións sistémicas de baixo grao. A sobrecarga mecánica é outro factor que induce a inflamación local tanto no disco intervertebral como na cartilaxe. O comportamento celular catabólico caracterízase por un aumento da expresión de citocinas e encimas que degradan a matriz, e unha regulación á baixa dos seus inhibidores. As citocinas, como o factor de necrose tumoral alfa (TNFα) e a interleukina-1β (IL-1β), teñen un efecto prexudicial sobre a dexeneración do disco intervertebral e a OA. Estas citocinas xeran inflamación local nas células do disco e nos condrocitos ao aumentar as metaloproteinases da matriz de encimas que degradan a ECM (MMP) e as disintegrinas e metaloproteinases do motivo de trombospondina (ADAMTS)-4 e 5. Relacionáronse varias MMP coa dexeneración do disco, mostrando unha notable semellanza con aquelas. presentes na cartilaxe, aínda que con diferentes niveis de expresión. A degradación da ECM ten como resultado unha diminución da produción de proteoglicanos nas matrices de cartilaxe, o que leva a unha diminución da presión hidrostática. Isto provoca un estreitamento do espazo articular e un aumento da deformación do texido. Esta tensión é un sinal mecánico para que as células cambien a produción de coláxeno de tipo II a coláxeno de tipo I, dando como resultado un texido máis fibroso, que ten unha menor capacidade para soportar cargas de compresión.

En resumo, este estado proinflamatorio e catabólico relacionouse coa orixe e progresión tanto da fisiopatoloxía da OA como da IVDD. Para comprender mellor estes procesos moleculares, este traballo mostra a investigación realizada presentando o papel da proteína C reactiva monomérica (mCRP) na OA e IVDD e investigando o papel da adipocina WISP-2 na OA.

CAPÍTULO 1. PROTEÍNA C REACTIVA MONOMÉRICA EN CARTILAXE E DISCO INTERVERTEBRAL

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A proteína C reactiva (CRP), na súa forma pentamérica, é unha proteína sérica de fase aguda producida principalmente no fígado e un biomarcador ben establecido nunha gran cantidade de

enfermidades infecciosas e inflamatorias, incluíndo artrite reumatoide, OA e disco. hernia. Tamén se propuxo como mediador das respostas inflamatorias e inmunitarias no contexto do dano local do texido. As medicións seriadas de CRP no sangue son útiles non só para controlar as infeccións postoperatorias, senón tamén para controlar a eficacia do tratamento e para detectar recaídas en multitude de enfermidades inflamatorias. A CRP pertence á familia das pentraxinas e é o principal reactivo de fase aguda en humanos. Sintetízase nos hepatocitos e segrega ao torrente sanguíneo tras a estimulación con IL-6. Na súa forma circulante consta de cinco subunidades monoméricas unidas por enlaces disulfuro nun disco anular e a súa función biolóxica caracterízase por facilitar a eliminación dos produtos de destrución celular formados durante traumatismos, infeccións ou estados inflamatorios.

Existe outra forma de CRP, a CRP monomérica (mCRP), que se forma en sitios de inflamación local mediante a disociación das subunidades de CRP e, en menor medida, a través da síntese local. mCRP ten funcións completamente diferentes que a forma pentámera. Aínda que moito menos estudada, descubriuse que a forma monomérica ten un papel biolóxico diferente no proceso inflamatorio. Traballos científicos anteriores suxiren que a mCRP presenta diferentes propiedades antixénicas, biolóxicas e electroforéticas en comparación coa forma pentamérica e, en xeral, mostra marcadas propiedades proinflamatorias en varias liñas celulares, como células endoteliais vasculares, macrófagos e neutrófilos. Cando se disocia nas plaquetas e se adhire á parede dos vasos sanguíneos, a mCRP pode inducir a activación endotelial e o recrutamento de leucocitos. Tamén favorece a adhesión dos neutrófilos ás paredes endoteliais; a adhesión dos monocitos á matriz de coláxeno, fibrinóxeno e fibronectina; e extravasación de linfocitos T.
Algunhas publicacións anteriores hipotetizaron un posible papel da mCRP en enfermidades musculoesqueléticas, pero non había ningunha evidencia experimental da súa actividade en condrocitos humanos ou células de disco intervertebral humanos antes deste traballo.

En canto aos papeis fisiolóxicos e patolóxicos da mCRP e a súa relevancia na bioloxía da cartilaxe e na OA, algúns datos publicados avaliaron a relación entre o desenvolvemento e a progresión da artrose e os niveis de CRP. Os niveis de CRP foron significativamente elevados en pacientes con artrosis en comparación cos controis sans, e comprobouse que se correlacionan cos parámetros radiográficos e a gravidade clínica. Aínda que algúns estudos non demostraron ningunha asociación entre os niveis de CRP e OA radiográfica, atopouse unha correlación coa dor e a diminución da función física.

Estes resultados un tanto conflitivos, xunto coa falta de coñecemento sobre o papel fisiopatolóxico da mCRP na cartilaxe, eclipsaron a asociación entre os niveis de CRP e OA, facendo que o papel funcional da CRP na patoxénese da cartilaxe sexa incerto. De feito, antes do noso traballo, non se investigara o papel da mCRP na fisiopatoloxía da cartilaxe, nin a súa relevancia na etioloxía e progresión da OA.

Para abordar esta pregunta clave, primeiro probamos o efecto da mCRP no cultivo primario de condrocitos humanos illados de pacientes con OA da cadeira e do xeonllo ou de suxeitos sans. A liña celular condroxénica murina ATDC-5 tamén se utilizou como un valioso modelo de condrocitos para a comparación. Segundo o que sabemos, este é o primeiro traballo experimental que proporciona evidencia da actividade funcional da mCRP en condrocitos humanos e de rato. Para obter máis información sobre a acción da mCRP e verificar se esta estimulación pode producir efectos proinflamatorios sostidos, avaliamos a actividade dependente da dose de mCRP en marcadores inflamatorios e catabólicos clave na degradación da cartilaxe. A óxido nítrico sintasa tipo II (iNOS) e a COX-2 están implicadas clásicamente na amplificación das respostas inflamatorias. O óxido nítrico (NO) é citotóxico para os condrocitos, dana a cartilaxe e está implicado na regulación positiva das MMPs, entre as que a MMP13 é un dos principais mediadores da degradación da matriz extracelular. A COX-2 induce prostaglandinas que, a súa vez, aumentan a síntese doutros mediadores inflamatorios, como as citocinas, para perpetuar a destrución da cartilaxe. Polo tanto, centrámonos na regulación da produción de iNOS e NO, así como outros seis principais mediadores proinflamatorios con diferentes funcións na destrución da cartilaxe, como son: COX-2, MMP13, VCAM1, IL-6, IL-8 e LCN2. Todos estes mediadores son producidos por condrocitos e están elevados nas articulacións artríticas.

Estes resultados representan unha evidencia importante sobre a modulación do fenotipo articular e as funcións exercidas pola mCRP. En primeiro lugar, a exposición dos condrocitos á mCRP induciu múltiples xenes proinflamatorios, o que suxire que os seus efectos non se limitan a unha diana molecular específica, senón que a mCRP ten un espectro de acción máis amplo. O segundo punto importante é que o efecto da mCRP é persistente e sostido, independentemente do ambiente proinflamatorio. mCRP exerceu a súa acción sobre condrocitos humanos primarios non só de pacientes con OA, senón tamén de suxeitos sans. Este último descubrimento suxire que niveis elevados de mCRP producido localmente poden desencadear respostas inflamatorias multixénicas sostidas tamén en texidos normais non expostos previamente a un microambiente proinflamatorio. O terceiro punto importante é que

os efectos da mCRP nos condrocitos probablemente estean mediados pola vía de sinalización NF-κβ. En particular, os nosos resultados demostraron que o ditiocarbamato de pirrolidina (un inhibidor farmacolóxico coñecido de NF-κβ) anula parcialmente a expresión de todos os xenes proinflamatorios inducidos por mCRP. Aínda que son necesarios máis experimentos, tendo en conta a versatilidade da mCRP observada noutros traballos na activación de diferentes vías de sinalización, aquí describimos, por primeira vez, a participación de NF-κβ nos condrocitos tras a estimulación da mCRP.

En conxunto, os nosos datos sobre cartilaxe sentan as bases moleculares para unha acción sostida da mCRP en condrocitos humanos e murinos. Estes resultados suxiren que a mCRP exerce un efecto catabólico sostido ao aumentar a expresión de mediadores inflamatorios e encimas proteolíticos e, polo tanto, é capaz de promover a degradación da cartilaxe e desencadear respostas inflamatorias en cartilaxe san e OA. Finalmente, os nosos resultados indican que está implicada a vía desencadeada pola activación de NF-κβ. A pesar desta investigación, son necesarios máis estudos sobre a sinalización desencadeada pola mCRP para avaliar plenamente o seu papel na degradación da cartilaxe e na patoxénese e progresión da OA.

Despois de estudar o efecto inflamatorio da mCRP nos condrocitos, tentamos demostrar que esta actividade tamén pode ocorrer nas células do disco intervertebral.

Hai evidencias considerables de que o proceso inflamatorio xoga un papel importante no desenvolvemento da dexeneración do disco, e hai unha correlación significativa entre os niveis de marcadores inflamatorios e o grao de dexeneración. Na hernia de disco cervical, a dor prodúcese como resultado da liberación de citocinas inflamatorias locais. A concentración plasmática elevada de IL-6 producida polos macrófagos ao redor do texido do disco aumenta os niveis de CRP. Os niveis de CRP demostraron ser significativamente máis altos en pacientes con hernia de disco cervical e dor no pescozo, en comparación cos pacientes con dor pero cunha resonancia magnética cervical normal. Un estudo recente descubriu que os niveis de CRP no plasma sanguíneo periférico non diferían entre os controis sans e os pacientes con dexeneración do disco pero sen hernia.

Neste traballo, investigamos o efecto da mCRP no cultivo primario de células humanas do anel fibroso (AF) illadas de pacientes con dexeneración do disco ou de suxeitos sans. Para demostrar que estes efectos non son exclusivos das células do AF, utilizouse a liña celular HNPSV-1 inmortalizada a partir de células do núcleo pulposo (NP) humano como modelo de comparación

e aproximación ás células do núcleo, co obxectivo de poder estudar o efectos da mCRP sobre todo o disco, xa que as células NP adoitan presentar características similares ás dos condrocitos. Este traballo é tamén a primeira evidencia experimental da actividade funcional da mCRP en células de disco AF e NP. Para obter máis información sobre a acción da mCRP e verificar se esta estimulación pode producir efectos proinflamatorios sostidos, avaliamos a actividade dependente da dose da mCRP nalgúns dos principais marcadores inflamatorios e catabólicos na dexeneración do disco intervertebral. A IL-8 e a IL-6 son dúas das citocinas proinflamatorias altamente expresadas na dexeneración do disco. Tamén avaliamos LCN2, unha adipocina implicada no mecanismo catabólico da dexeneración do disco, MMP13, un encima catabólico, e os mediadores inflamatorios NOS2, COX2 e VCAM1. A COX-2 induce prostaglandinas que, á súa vez, aumentan a síntese doutros mediadores inflamatorios, incluídas as citocinas, para perpetuar a destrución do texido. Todos estes mediadores son producidos polas células do disco intervertebral e están elevados na dexeneración do disco.

Estes feitos proporcionan probas importantes para a modulación do metabolismo do disco intervertebral e as funcións exercidas pola mCRP, así como para a localización de mCRP nos texidos do disco intervertebral humano. En primeiro lugar, observamos que a exposición de células AF e NP a mCRP inducía varios xenes proinflamatorios, o que suxire que os seus efectos non se limitan a unha diana molecular específica, senón que a mCRP ten un espectro de acción máis amplo. O segundo punto importante é que o efecto da mCRP é persistente e sostido durante polo menos 24 horas, independentemente do ambiente proinflamatorio. A mCRP exerceu a súa acción sobre células AF humanas primarias derivadas non só de pacientes con dexeneración discal, senón tamén de suxeitos sans. De acordo con isto, demostramos que a mCRP pode localizarse fisioloxicamente tanto en texidos sans como dexenerados, e que está presente nos dous texidos principais do disco intervertebral, o AF e o NP. Ademais, a presenza de mCRP parece aumentar no texido dexenerativo de AF en comparación co texido san. O noso descubrimento suxire que niveis elevados de mCRP producido localmente poden desencadear respostas inflamatorias multixénicas sostidas tamén en texidos normais non expostos previamente a un microambiente proinflamatorio. Outro aspecto relevante é que os efectos da mCRP foron observados tanto nas células AF como nas NP, demostrando que a mCRP ten múltiples dianas celulares. Finalmente, a última idea importante deste traballo é que os efectos da mCRP nas células AF están mediados pola vía de sinalización NF-κβ. Probamos que tras o tratamento con mCRP, PI3K, ERK 1/2 e NF-κβ p65 foron fosforilados. Estes mediadores activáronse de forma similar tanto en células de AF de disco sas como dexenerativas, o que confirma que a acción da mCRP non depende dun estado inflamatorio previo.

En resumo, os nosos resultados proporcionan, por primeira vez, probas para a localización de mCRP en texidos de disco intervertebral sans e dexenerativos, e unha nova base molecular para a acción sostida da mCRP en células AF e NP humanas. Estes resultados suxiren que a mCRP exerce un efecto catabólico de longa duración ao aumentar a expresión de mediadores inflamatorios e encimas proteolíticos, promovendo potencialmente a degradación das células do disco e desencadeando respostas inflamatorias en células AF sas e dexenerativas e na liña celular NP inmortalizada. Ademais, a propia mCRP pode provocar un aumento severo da expresión de mediadores inflamatorios nas células do disco saudables, o que suxire un claro papel patóxeno da mCRP na enfermidade dexenerativa do disco. Os resultados indican que este efecto ocorre tanto nas células AF como nas NP, afectando así aos dous compartimentos celulares principais do disco. Ademais desta investigación, son necesarios máis estudos sobre a fisiopatoloxía da mCRP para determinar completamente o seu papel na patoxénese e progresión da dexeneración do disco intervertebral.

CAPÍTULO 2. WISP2 EN CARTILAXE

A adipocina WISP-2 identificouse previamente como unha proteína inducible pola vía de sinalización de Wnt. Na última década, varias probas suxeriron un papel clave para a sinalización de Wnt na fisiopatoloxía da OA. A vía canónica Wnt/β-catenina conduce a un catabolismo excesivo, degradación da cartilaxe e diferenciación hipertrófica dos condrocitos. Non obstante, a sinalización Wnt é necesaria para o mantemento da cartilaxe articular, pero a actividade excesiva é prexudicial.

Estudos recentes investigaron o papel das proteínas da familia CCN no desenvolvemento da OA. WISP-1/CCN4 e WISP-2/CCN5 exprésanse máis na cartilaxe do xeonllo OA e RA, e todos os xenes da familia CCN exprésanse en exemplares sinoviais que tamén presentan estas enfermidades. Ademais, o noso grupo identificou WISP-2 en cartilaxe, membrana sinovial e graxa infrapatelar, atopando un aumento significativo da súa expresión na graxa infrapatelar de pacientes con OA en comparación cos controis sans. Outros membros da superfamilia WISP exercen unha acción fisiopatolóxica nos texidos musculoesqueléticos. WISP-1 regula a expresión de agrecanasas e MMP nos condrocitos e é capaz de inducir danos na cartilaxe articular en modelos de OA. Pola contra, WISP-3/CCN6 reduce a expresión de ADAMTS-5 e provoca a regulación positiva de MMP-10 nos condrocitos humanos. En canto á participación de WISP-2 na OA, non houbo traballos previos que avaliasen a súa acción en condrocitos.

Neste traballo analizamos o efecto de WISP-2 no catabolismo da cartilaxe utilizando a súa proteína recombinante e experimentos de perda de función para aclarar o papel desta proteína na fisiopatoloxía dos condrocitos. Segundo o que sabemos, este é o primeiro traballo que mostra probas experimentales de que WISP-2 está modulado por mediadores inflamatorios e a súa implicación na degradación da cartilaxe.

En primeiro lugar, analizamos a regulación da expresión de WISP-2 ao longo da diferenciación dos condrocitos, observando un aumento da expresión de WISP-2 nas etapas iniciais da condroxénese, seguido dunha diminución ao comezo da etapa hipertrófica. Os nosos datos demostran que tanto o ARNm de WISP-2 como a expresión da proteína diminuíron significativamente. Cómpre sinalar que a marcada redución da expresión de WISP-2 na fase hipertrófica coincide cun aumento significativo da expresión de MMP-13, o principal marcador desta última etapa, o que suxire unha relación entre estes dous factores.

Varios estudos describiron a participación das citocinas na destrución da cartilaxe. A IL-1β é unha citocina proinflamatoria que actúa como un potente inductor da expresión de MMP-13. Así, estudamos o efecto de citocinas e adipocinas proinflamatorias relevantes implicadas en enfermidades reumáticas, como TNF-α, IL-1β ou leptina, sobre a expresión de WISP-2 nos condrocitos. WISP-2 está regulado por factores de transcrición que son inducidos pola inflamación, a activación da vía Wnt e a hipoxia. Ademais, a IL-1β induce a expresión das proteínas Wnt, dando como resultado a activación da vía de sinalización de Wnt. Por outra banda, a activación endóxena da sinalización de Wnt/β-catenina, como se observa en certas liñas celulares tumorais, caracterízase por unha expresión moi baixa de WISP-2. A diferenza doutros membros da familia CCN, como WISP-3, que os seus niveis aumentan nas condicións inflamatorias, observamos que TNF-α, IL-1β, LPS ou IL-6 reducen a expresión de WISP-2 nos condrocitos humanos. En conxunto, estes resultados suxiren que un ambiente proinflamatorio "canónico" caracterizado pola activación de citocinas ou TLR4 modula negativamente a expresión de WISP-2. Pola contra, as clásicas adipocinas proinflamatorias leptina e adiponectina non alteraron a expresión de WISP-2 nos condrocitos. Esperabamos que estas adipocinas, que son coñecidas como contribuíntes á creación dun ambiente proinflamatorio na obesidade e na OA, regulasen WISP-2, pero sorprendentemente non o fixeron.

Un aspecto que se desprende dos nosos resultados é que WISP-2 pode minimizar os efectos catabólicos da IL-1β sobre a cartilaxe. De feito, a proteína recombinante WISP-2 foi capaz de diminuír parcialmente as principais metaloproteasas e agrecanasas inducidas pola IL-1β. Nos nosos experimentos, o WISP-2 recombinante foi capaz de atenuar as vías de sinalización IL-1β/NF-κβ, así como Erk 1/2, JNK e p38. Polo tanto, estes datos, xunto coa observación de que

WISP-2 é capaz de aumentar a fosforilación de GSK-3, suxiren que WISP-2 podería actuar como un agonista de WNT con funcións anabólicas potenciais que son capaces de contrarrestar os efectos de WNT. IL-1β como un dos principais mediadores da resposta inflamatoria na cartilaxe. Confirmamos estas observacións silenciando a WISP-2 e verificamos o aumento da expresión de MMP-13 e doutras agrecanasas e citocinas proinflamatorias relevantes como IL-1β, IL-6 e IL-8. Finalmente, a activación da vía canónica WNT por BIO (un inhibidor clásico de GSK-3β) confirma os resultados mostrados anteriormente, mostrando que WISP-2 pode contribuír a contrarrestar os efectos nocivos da inflamación.

En resumo, demostramos por primeira vez que WISP-2 pode ter un papel relevante na modulación da expresión das encimas implicadas na renovación da matriz extracelular na cartilaxe e que a súa regulación negativa pode alterar negativamente o ambiente inflamatorio da cartilaxe. Tamén demostramos a participación da vía de sinalización Wnt/β-catenina nestes procesos. Polo tanto, WISP-2 pode representar un posible enfoque terapéutico para a OA, aínda que se necesitan máis investigacións para definir a contribución de WISP-2 na complexa rede metabólica de enfermidades dexenerativas e inflamatorias do sistema musculoesquelético.

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RESUMEN

El dolor lumbar (LBP) y la artrosis (OA) son las dos principales enfermedades musculoesqueléticas causa de discapacidad a nivel global. La etiología del dolor lumbar es compleja y multifactorial; y la degeneración del disco intervertebral (IVDD) se considera una de sus principales causas. El dolor, la disfunción y la rigidez que caracterizan a los pacientes con IVDD y OA son los responsables de un grave deterioro de su calidad de vida y un gran impacto socioeconómico.

La artrosis (OA) es una enfermedad degenerativa articular multifactorial caracterizada por la destrucción progresiva del cartílago articular, cambios en el hueso subcondral, formación de osteofitos e inflamación sinovial. Es el tipo de artritis más prevalente, pero su etiología todavía no ha sido clarificada. La inflamación crónica de bajo grado contribuye a los síntomas y a la progresión de la enfermedad. En la OA, el alto estrés mecánico, los productos de degradación de la matriz extracelular (ECM), las citoquinas proinflamatorias y las adipoquinas activan a los condrocitos para estimular la producción de mediadores proinflamatorios y enzimas que degradan la matriz, como IL-1β y MMP-13, que actúan sinérgicamente para promover y perpetuar estas respuestas catabólicas.

En el disco intervertebral adulto sano (IVD), la homeostasis entre la síntesis y la degradación de la matriz extracelular se equilibra mediante los factores de crecimiento y las citoquinas. Un desequilibrio hacia los procesos catabólicos contribuye a la degeneración estructural del disco intervertebral, lo que probablemente contribuya al desarrollo de dolor lumbar. La degeneración del disco conduce a múltiples cambios anatómicos, mecánicos y bioquímicos. Estas alteraciones disminuyen la estabilidad mecánica y las funciones de absorción de impactos, lo que contribuye a la formación de osteofitos, fisuras anulares y disminución del movimiento de los segmentos de la columna. En respuesta a la inflamación y a la degeneración, se produce el crecimiento vascular y nervioso en el disco, en principio avascular, desde las capas externas del anillo fibroso, extendiéndose hacia el núcleo pulposo.

La degeneración del disco intervertebral y la del cartílago en la artrosis muestran marcadas similitudes. En las radiografías se observa: la pérdida de la altura del disco o del espacio articular, la esclerosis del hueso subcondral y el desarrollo de osteofitos. Además, tanto en el disco intervertebral como en el cartílago articular, la matriz extracelular se compone de

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constituyentes similares (aunque en distinta proporción), y en el proceso de degeneración está presente una maquinaria de degradación de la matriz extracelular similar.

Existe un delicado equilibrio entre los procesos anabólicos y catabólicos en el cartílago articular y el disco intervertebral. Cuando se rompe el equilibrio, existe una desigualdad entre la síntesis y la degradación de la matriz extracelular debido al comportamiento catabólico de las células. Se han descrito varios factores inflamatorios sistémicos que inclinan este equilibrio hacia la degeneración en ambas enfermedades musculoesqueléticas, como la diabetes, la obesidad, el tabaquismo y la infección sistémica de bajo grado. La sobrecarga mecánica es otro factor que induce inflamación local tanto en el disco intervertebral como en el cartílago. El comportamiento celular catabólico se caracteriza por un aumento en la expresión de citoquinas y enzimas que degradan la matriz, y una regulación a la baja de sus inhibidores. Las citoquinas, como el factor de necrosis tumoral alfa (TNFα) y la interleukina-1β (IL-1β), tienen un efecto perjudicial en la degeneración del disco intervertebral y la OA. Estas citoquinas generan inflamación local en las células del disco y los condrocitos mediante una regulación al alza de las enzimas que degradan la ECM, las metaloproteinasas de matriz (MMP) y la desintegrina y metaloproteinasa con motivos de trombospondina (ADAMTS)-4 y 5. Varias MMPs se han relacionado con la degeneración en el disco, mostrando una notable similitud con las encontradas en el cartílago, aunque con diferentes niveles de expresión. La degradación de la ECM da como resultado una disminución en la producción de proteoglicanos en las matrices cartilaginosas, lo que conduce a una disminución de la presión hidrostática. Esto provoca una reducción del espacio articular y un aumento de la deformación del tejido. Esta tensión es una señal mecánica para que las células cambien la producción de colágeno tipo II a colágeno tipo I, lo que da como resultado un tejido más fibroso, que tiene una capacidad inferior para resistir cargas de compresión.

En resumen, este estado proinflamatorio y catabólico se ha relacionado con el origen y progresión tanto de la fisiopatología de la OA como de la IVDD. Con el objetivo de comprender mejor estos procesos moleculares, este trabajo muestra la investigación realizada presentando el papel de la proteína C reactiva monomérica (mCRP) en OA y IVDD e investigando el papel de la adipoquina WISP-2 en OA.

CAPÍTULO 1. LA PROTEÍNA C REACTIVA MONOMÉRICA EN EL CARTÍLAGO Y EL DISCO INTERVERTEBRAL

La proteína C reactiva (CRP), en su forma pentamérica, es una proteína sérica de fase aguda producida principalmente en el hígado, y un biomarcador muy establecido en un gran número de enfermedades infecciosas e inflamatorias, incluidas la artritis reumatoide, la OA y la hernia de disco. También se ha propuesto como mediador de respuestas inflamatorias e inmunitarias en el contexto de lesiones tisulares locales. Las medidas seriadas de CRP en sangre son útiles no solo para el seguimiento de infecciones postoperatorias, sino también para monitorear la eficacia del tratamiento y para la detección de recaídas en una multitud de enfermedades inflamatorias. La CRP pertenece a la familia de las pentraxinas y es el principal reactivo de fase aguda en humanos. Se sintetiza en los hepatocitos y se secreta al torrente sanguíneo tras la estimulación con IL-6. En su forma circulante consta de cinco subunidades monoméricas unidas con enlaces disulfuro en un disco en forma de anillo y su papel biológico se caracteriza por facilitar la eliminación de los productos de destrucción celular formados durante traumatismos, infecciones o estados inflamatorios.

Existe otra forma de CRP, la CRP monomérica (mCRP), que se forma en sitios de inflamación local a través de la disociación de las subunidades de CRP y, en menor medida, mediante síntesis local. La mCRP presenta unas funciones completamente diferentes a las de la forma pentamérica. Aunque mucho menos estudiada, se ha encontrado que la forma monomérica tiene un papel biológico diferente en el proceso inflamatorio. Previos trabajos científicos sugieren que la mCRP exhibe diferentes propiedades antigénicas, biológicas y electroforéticas en comparación con la forma pentamérica y, generalmente, muestra marcadas propiedades proinflamatorias en varias líneas celulares, como las células endoteliales vasculares, los macrófagos y los neutrófilos. Cuando se disocia en las plaquetas y se adhiere a la pared de los vasos sanguíneos, la mCRP puede inducir la activación endotelial y el reclutamiento de leucocitos. También promueve la adhesión de neutrófilos a las paredes endoteliales; la adhesión de monocitos a la matriz de colágeno, fibrinógeno y fibronectina; y la extravasación de linfocitos T.
Algunas publicaciones previas han hipotetizado sobre un posible papel de la mCRP en enfermedades musculoesqueléticas, pero no había ninguna prueba experimental de su actividad ni en condrocitos humanos ni en células de disco intervertebral humano anterior a este trabajo.

Con respecto a los roles fisiológicos y patológicos de la mCRP y su relevancia en la biología del cartílago y la OA, algunos datos publicados evaluaron la relación entre el desarrollo y la progresión de la artrosis y los niveles de CRP. Se ha visto que los niveles de CRP están

significativamente elevados en pacientes con OA en comparación con los controles sanos, y se observó que se correlacionan con los parámetros radiográficos y con la gravedad clínica. Aunque algunos estudios no mostraron asociación entre los niveles de CRP y la OA radiográfica, se ha encontrado una correlación con el dolor y la disminución de la función física. Estos resultados algo contradictorios, junto con la falta de conocimiento sobre el papel fisiopatológico de la mCRP en el cartílago, han eclipsado la asociación entre los niveles de CRP y la OA, lo que hace que la participación funcional de la CRP en la patogénesis de la OA sea incierta. De hecho, antes de nuestro trabajo, no se había investigado el papel de la mCRP en la fisiopatología del cartílago, ni su relevancia en la etiología y progresión de la OA.

Para abordar esta pregunta clave, primero probamos el efecto de la mCRP en el cultivo primario de condrocitos humanos aislados de pacientes con OA de cadera y rodilla, o de sujetos sanos. La línea celular condrogénica murina ATDC-5 también se usó como un modelo de condrocitos valioso para la comparación. Hasta donde sabemos, este es el primer trabajo experimental que proporciona evidencia de la actividad funcional de la mCRP en condrocitos humanos y de ratón. Para obtener más información sobre la acción de mCRP y para verificar si esta estimulación puede resultar en efectos proinflamatorios sostenidos, evaluamos la actividad dosis-dependiente de la mCRP en marcadores inflamatorios y catabólicos clave en la degradación del cartílago. La óxido nítrico sintasa tipo II (iNOS) y la COX-2 participan clásicamente en la amplificación de las respuestas inflamatorias. El óxido nítrico (NO) es citotóxico para los condrocitos, daña el cartílago y está involucrado en la regulación al alza de las MMP, entre las cuales la MMP13 es uno de los principales mediadores de la degradación de la matriz extracelular. La COX-2 induce prostaglandinas que, a su vez, aumentan la síntesis de otros mediadores inflamatorios, como las citoquinas, para perpetuar la destrucción del cartílago. Por lo tanto, nos hemos centrado en la regulación de la producción de iNOS y NO, así como de otros seis mediadores proinflamatorios principales con diferentes funciones en la destrucción del cartílago, tales como: COX-2, MMP13, VCAM1, IL-6, IL-8 y LCN2. Todos estos mediadores son producidos por los condrocitos y se encuentran elevados en las articulaciones artríticas.

Estos resultados suponen una evidencia importante con respecto a la modulación del fenotipo articular y las funciones ejercidas por la mCRP. En primer lugar, la exposición de los condrocitos a la mCRP indujo múltiples genes proinflamatorios, lo que sugiere que sus efectos no se limitan a un objetivo molecular específico, sino que la mCRP tiene un espectro de acción más amplio. El segundo punto importante es que el efecto de la mCRP es persistente y sostenido,

independientemente del entorno proinflamatorio. La mCRP ejerció su acción sobre los condrocitos primarios humanos provenientes no solo de pacientes con OA, sino también de sujetos sanos. Este último hallazgo sugiere que los niveles elevados de mCRP producidos localmente pueden desencadenar respuestas inflamatorias multigénicas sostenidas también en tejidos normales no expuestos previamente a un microambiente proinflamatorio. El tercer punto importante es que los efectos de la mCRP en los condrocitos probablemente estén mediados por la vía de señalización de NF-κβ. En particular, nuestros resultados demostraron que el ditiocarbamato de pirrolidina (un conocido inhibidor farmacológico de NF-κβ) anula parcialmente la expresión de todos los genes proinflamatorios inducidos por la mCRP. Aunque se necesitan más experimentos, teniendo en cuenta la versatilidad de la mCRP observada en otros trabajos en la activación de diferentes vías de señalización, aquí describimos, por primera vez, la participación de NF-κβ en condrocitos tras la estimulación de mCRP.

En conjunto, nuestros datos en cartílago sientan las bases moleculares para una acción sostenida de la mCRP en condrocitos humanos y murinos. Estos resultados sugieren que la mCRP ejerce un efecto catabólico sostenido al aumentar la expresión de mediadores inflamatorios y enzimas proteolíticas, y por lo tanto es capaz de promover la degradación del cartílago y desencadenar respuestas inflamatorias en cartílago sano y OA. Finalmente, nuestros resultados indican que la ruta desencadenada por la activación de NF-κβ está involucrada. A pesar de estos hallazgos, se necesitan más estudios sobre la señalización desencadenada por la mCRP para evaluar completamente su papel en la degradación del cartílago y la patogénesis y progresión de la OA.

Tras el estudio del efecto inflamatorio de la mCRP en condrocitos, intentamos demostrar que esta actividad se puede dar también en células de disco intervertebral.

Hay numerosas evidencias de que el proceso inflamatorio juega un papel importante en el desarrollo de la degeneración del disco y existe una correlación significativa entre los niveles de marcadores inflamatorios y el grado de degeneración. En la hernia de disco cervical, el dolor se produce como resultado de la liberación de citoquinas inflamatorias locales. La concentración plasmática elevada de IL-6 producida por los macrófagos alrededor del tejido del disco incrementa los niveles de CRP. Se ha demostrado que los niveles de CRP son significativamente más altos en pacientes con hernia de disco cervical y dolor de cuello, en comparación con aquellos pacientes con dolor, pero que presentan una resonancia magnética cervical normal. Un estudio reciente vio que los niveles de CRP en plasma en sangre periférica no diferían entre los controles sanos y los pacientes con degeneración de disco pero sin hernia.

En este trabajo, investigamos el efecto de la mCRP en el cultivo primario de células de anillo fibroso (AF) humanas aisladas de pacientes con degeneración de disco o de sujetos sanos. Para demostrar que estos efectos no son exclusivos de las células de AF, se utilizó la línea celular HNPSV-1 inmortalizada a partir de células de núcleo pulposo (NP) humanas, como modelo de comparación y aproximación a las células del núcleo, con el objetivo de poder estudiar los efectos de la mCRP en todo el disco, ya que las células de NP comúnmente exhiben características similares a las de los condrocitos.

Este trabajo es también la primera evidencia experimental de la actividad funcional de la mCRP en células de AF y NP de disco. Para obtener más información sobre la acción de mCRP y para verificar si esta estimulación puede resultar en efectos proinflamatorios sostenidos, evaluamos la actividad dependiente de la dosis de mCRP en alguno de los principales marcadores inflamatorios y catabólicos en la degeneración de disco intervertebral. IL-8 e IL-6 son dos de las citoquinas proinflamatorias fuertemente expresadas en la degeneración de disco. También evaluamos LCN2, una adipoquina implicada en el mecanismo catabólico de la degeneración del disco, MMP13, una enzima catabólica y los mediadores inflamatorios NOS2, COX2 y VCAM1. COX-2 induce prostaglandinas que, a su vez, aumentan la síntesis de otros mediadores inflamatorios, incluidas las citoquinas, para perpetuar la destrucción tisular. Todos estos mediadores son producidos por las células del disco intervertebral y están elevados en la degeneración de este.

Estos hallazgos aportan evidencias importantes sobre la modulación del metabolismo del disco intervertebral y las funciones ejercidas por la mCRP, así como de la localización de la mCRP en tejidos de disco intervertebral humanos. En primer lugar, observamos que la exposición de las células de AF y NP a mCRP indujo múltiples genes proinflamatorios, lo que sugiere que sus efectos no se limitan a un objetivo molecular específico, sino que mCRP tiene un espectro de acción más amplio. El segundo punto importante es que el efecto de mCRP es persistente y sostenido durante al menos 24 horas, independientemente del entorno proinflamatorio. La mCRP ejerció su acción sobre las células primarias de AF humanas provenientes no solo de pacientes con degeneración de disco, sino también de sujetos sanos. De acuerdo con esto, probamos que la mCRP puede localizarse fisiológicamente tanto en tejidos sanos como degenerados, y que está presente en los dos principales tejidos del disco intervertebral, el AF y el NP. Además, la presencia de mCRP parece aumentar en el tejido degenerativo de AF en comparación con el sano. Nuestro hallazgo sugiere que los niveles elevados de mCRP producidos localmente pueden desencadenar respuestas inflamatorias multigénicas sostenidas

también en tejidos normales no expuestos previamente a un microambiente proinflamatorio. Otro aspecto relevante es que los efectos de la mCRP se observaron tanto en células AF como NP, lo que demuestra que mCRP tiene múltiples dianas celulares. Finalmente, la última idea importante de este trabajo es que los efectos de mCRP en las células de AF están mediados por la vía de señalización de NF-κβ. Probamos que tras el tratamiento de mCRP, PI3K, ERK 1/2 y NF-κβ p65 se fosforilaron. Estos mediadores se activaron de manera similar tanto en células de AF de disco sanas como degenerativas, lo que confirma que la acción de mCRP no depende de un estado inflamatorio previo.

En resumen, nuestros resultados proporcionan, por primera vez, evidencia de la localización de mCRP en tejidos de disco intervertebral sanos y degenerativos, y una nueva base molecular para la acción sostenida de mCRP en células de AF y NP humanas. Estos resultados sugieren que la mCRP ejerce un efecto catabólico duradero al aumentar la expresión de mediadores inflamatorios y enzimas proteolíticas, pudiendo promover la descomposición de las células del disco y desencadenar respuestas inflamatorias en células de AF sanas y degenerativas y en la línea celular de NP inmortalizada. Además, la mCRP puede desencadenar por sí misma un aumento severo en la expresión de mediadores inflamatorios en las células sanas del disco, lo que sugiere un claro papel patogénico de la mCRP en la enfermedad degenerativa del disco. Los resultados indican que este efecto ocurre tanto en las células de AF como en las de NP, por lo que afecta a los dos compartimentos celulares principales del disco. Además de estos hallazgos, se necesitan más estudios sobre la fisiopatología de mCRP para determinar completamente su papel en la patogénesis y progresión de la degeneración de disco intervertebral.

CAPÍTULO 2. WISP2 EN EL CARTÍLAGO

La adipoquina WISP-2, se ha identificado anteriormente como una proteína inducible por la vía de señalización de Wnt. En la última década, distintas evidencias han sugerido un papel clave de la señalización de Wnt en la fisiopatología de la OA. La vía canónica de Wnt/β-catenina conduce a un catabolismo excesivo, a la degradación del cartílago y a la diferenciación hipertrófica de los condrocitos. Sin embargo, la señalización de Wnt es necesaria para el mantenimiento del cartílago articular, pero una actividad excesiva es perjudicial.

Estudios recientes han investigado el papel de las proteínas de la familia CCN en el desarrollo de la OA. WISP-1/CCN4 y WISP-2/CCN5 se expresan en mayor medida en el cartílago de rodilla con OA y RA, y todos los genes de la familia CCN se expresan en muestras sinoviales

que también presentan estas enfermedades. Además, nuestro grupo ha identificado WISP-2 en cartílago, membrana sinovial y grasa infrapatelar, encontrando un incremento significativo en su expresión en la grasa infrapatelar de pacientes con OA en comparación con controles sanos. Otros miembros de la superfamilia WISP ejercen una acción fisiopatológica en los tejidos musculoesqueléticos. WISP-1 regula la expresión de agrecanasas y MMPs en condrocitos y es capaz de inducir daños en el cartílago articular en modelos de OA. Por el contrario, WISP-3/CCN6 reduce la expresión de ADAMTS-5 y provoca la regulación positiva de MMP-10 en condrocitos humanos. En cuanto a la participación de WISP-2 en OA, no existían trabajos previos que evaluaran su acción en condrocitos.

En este trabajo hemos analizado el efecto de WISP-2 en el catabolismo del cartílago utilizando su proteína recombinante y experimentos de pérdida de función para aclarar el papel de esta proteína en la fisiopatología de los condrocitos. Hasta donde sabemos, este es el primer trabajo que muestra evidencia experimental de que WISP-2 está modulado por mediadores de la inflamación y su participación en la degradación del cartílago.

Primero analizamos la regulación de la expresión de WISP-2 a lo largo de la diferenciación de los condrocitos, observando un aumento de la expresión de WISP-2 en las primeras etapas de la condrogénesis, seguido de una disminución al inicio de la etapa hipertrófica. Nuestros datos demuestran que tanto la expresión de mRNA como de proteína de WISP-2 disminuyó considerablemente. Cabe destacar que la marcada reducción en la expresión de WISP-2 en la fase hipertrófica coincide con un aumento significativo en la expresión de MMP-13, el principal marcador de esta última etapa, lo que sugiere una relación entre estos dos factores.

Varios estudios han descrito la participación de las citoquinas en la destrucción del cartílago. IL-1β es una citoquina proinflamatoria que actúa como un potente inductor de la expresión de MMP-13. Así, hemos estudiado el efecto de citoquinas y adipoquinas proinflamatorias relevantes implicadas en enfermedades reumáticas, como TNF-α, IL-1β o leptina, sobre la expresión de WISP-2 en condrocitos. WISP-2 está regulado por factores de transcripción que son inducidos por inflamación, activación de la vía Wnt e hipoxia. Además, la IL-1β induce la expresión de proteínas Wnt, lo que da como resultado la activación de la vía de señalización Wnt. Por otro lado, la activación endógena de la señalización de Wnt/β-catenina, tal y como se observa en determinadas líneas celulares tumorales, se caracteriza por una expresión muy baja de WISP-2. A diferencia de otros miembros de la familia CCN, como WISP-3, cuyos niveles aumentan en condiciones inflamatorias, hemos observado que TNF-α, IL-1β, LPS o IL-6 reducen la expresión de WISP-2 en condrocitos humanos. En conjunto, estos resultados sugieren que un entorno proinflamatorio "canónico" caracterizado por citoquinas o activación

de TLR4 modula negativamente la expresión de WISP-2. Por el contrario, las clásicas adipoquinas proinflamatorias, leptina y adiponectina, no alteraron la expresión de WISP-2 en condrocitos. Esperábamos que estas adipoquinas, que son contribuyentes conocidos a la creación de un entorno proinflamatorio en la obesidad y la OA, pudieran regular WISP-2, pero, sorprendentemente, no lo hicieron.

Un aspecto que surge de nuestros resultados es que WISP-2 puede minimizar los efectos catabólicos de IL-1β en el cartílago. De hecho, la proteína WISP-2 recombinante fue capaz de disminuir parcialmente las principales metaloproteasas y agrecanasas inducidas por IL-1β. En nuestros experimentos, WISP-2 recombinante pudo atenuar las vías de señalización IL-1β/NF-κβ, así como Erk 1/2, JNK y p38. Por lo tanto, estos datos, junto con la observación de que WISP-2 es capaz de aumentar la fosforilación de GSK-3, sugieren que WISP-2 podría actuar como un agonista de WNT con funciones anabólicas potenciales que son capaces de contrarrestar los efectos catabólicos de IL-1β como uno de los principales mediadores de la respuesta inflamatoria en el cartílago. Confirmamos estas observaciones silenciando WISP-2, y comprobamos el aumento de la expresión de MMP-13, y de otras agrecanasas y citoquinas proinflamatorias relevantes como IL-1β, IL-6 e IL-8. Finalmente, la activación de la vía canónica WNT por BIO (un inhibidor clásico de GSK-3β) confirma los resultados mostrados anteriormente, mostrando que WISP-2 puede contribuir a contrarrestar los efectos perjudiciales de la inflamación.

En resumen, hemos demostrado por primera vez que WISP-2 puede tener un papel relevante en la modulación de la expresión de enzimas involucradas en la renovación de la matriz extracelular en el cartílago y que su regulación a la baja puede alterar negativamente el entorno inflamatorio en el cartílago artrósico. También demostramos la participación de la vía de señalización Wnt/β-catenina en estos procesos. Por lo tanto, WISP-2 puede representar un posible enfoque terapéutico para la OA, aunque es necesaria más investigación para definir la contribución de WISP-2 en la compleja red metabólica de enfermedades degenerativas e inflamatorias del sistema musculoesquelético.

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PUBLICATIONS INDEX

Article 1. Monomeric C reactive protein (mCRP) regulates inflammatory responses in human and mouse chondrocytes.

Article 2. Monomeric CRP regulates inflammatory responses in human intervertebral disc cells.

Article 3. WISP-2 modulates the induction of inflammatory mediators and cartilage catabolism in chondrocytes.

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Article 1.

Monomeric C reactive protein (mCRP) regulates inflammatory responses in human and mouse chondrocytes.

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Article 2.

Monomeric CRP regulates inflammatory responses in human intervertebral disc cells.

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Article 3.

WISP-2 modulates the induction of inflammatory mediators and cartilage catabolism in chondrocytes.

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ABSTRACT

Low back pain (LBP) and osteoarthritis (OA) are the two main musculoskeletal causes for disability worldwide. Low back pain etiology is complex and multifactorial. However, intervertebral disc degeneration (IVDD) is considered one of its major causes. Pain, dysfunction, and stiffness, that characterizes patients with IVDD and OA, are the cause of a huge socioeconomic impact.

Osteoarthritis (OA) is a multifactorial joint degenerative disease characterized by progressive destruction of articular cartilage, changes in subchondral bone, osteophyte formation, and synovial inflammation. During OA, high mechanical stress, extracellular matrix (ECM) degradation products, pro-inflammatory cytokines, and adipokines activate chondrocytes to stimulate the production of pro-inflammatory mediators and matrix degrading enzymes such as IL-1β and MMP-13, which act synergistically to promote and perpetuate chondrocyte catabolic responses.

In a healthy adult intervertebral disc (IVD), homeostasis between extracellular matrix (ECM) synthesis and degradation is balanced by growth factors and cytokines. An imbalance towards catabolic processes contributes to structural degeneration of the IVD, likely contributing to the development of LBP. IVD degeneration leads to multiple anatomical, mechanical, and biochemical disc changes. In response to inflammation and degeneration, vascular and nerve ingrowth into the avascular IVD occur from the outer layers of the annulus fibrosus, extending into the nucleus pulposus.

IVD degeneration and the degeneration of joint cartilage, as seen in osteoarthritis, show marked similarities. Striking similarities are seen on plain radiographs: the loss of disc height or joint space, sclerosis of the subchondral bone, and the development of osteophytes. Furthermore, in both IVD and joint cartilage, the ECM is composed of similar constituents (although in another ratio), and similar ECM-degrading machinery is present in the process of degeneration. There are also differences such as the type of forces applied to the ECM, and the absence of synovial fluid in IVDs.

The pro-inflammatory and catabolic state has been related to the origin and progression of the pathophysiology of both the OA and the IVDD. To further understand these molecular

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processes, this work presents the research done introducing monomeric C reactive protein (mCRP) role in OA and IVDD and investigating the role of the adipokine WISP-2 in OA.

C reactive protein (CRP) is an acute-phase protein that is used as a biomarker to follow severity and progression in infectious and inflammatory diseases. Its pathophysiological mechanisms of action are still poorly defined. CRP in its pentameric form exhibits weak anti-inflammatory activity. However, monomeric isoform (mCRP) exerts potent proinflammatory properties in endothelial cells and leucocytes. This is the first data published regarding mCRP pro-inflammatory and catabolic effects in human intervertebral disc cells and chondrocytes. Taken together, these results suggest that mCRP exerts a lasting catabolic effect by increasing the expression of inflammatory mediators and proteolytic enzymes, being able to promote IVD and cartilage cells breakdown and trigger inflammatory responses both in healthy and degenerative environments. Altogether, these molecules can cooperate, resulting in the enhancement and perpetuation of the ECM-degrading processes at IVD and cartilage level. In addition, mCRP by itself can trigger a severe increase in the expression of inflammatory mediators in healthy disc cells and chondrocytes, suggesting that mCRP may have a clear pathogenic role in disc disease an OA. Finally, we proved that NF-κβ signaling pathway is implicated in the mCRP mechanism of action.

To give a wider vision of the potential pathways involved in OA, we focused our attention on WISP-2. Wnt-1 inducible signaling pathway protein 2 (WISP-2) is a recently identified adipokine that has been described as an important mediator of canonical Wnt activation in adipogenic precursor cells. Although recent evidence suggests a role for Wnt signaling in OA physiopathology, little is known about the involvement of WISP-2 in cartilage degradation. With this work we have shown for the first time that WISP-2 may have relevant roles in modulating the turnover of extracellular matrix in the cartilage and that its downregulation may detrimentally alter the inflammatory environment in OA cartilage. We also proved the participation of Wnt/β-catenin signaling pathway in these processes.

Keywords: intervertebral disc degeneration, osteoarthritis, inflammation, monomeric C reactive protein, WISP2.

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INTRODUCTION

1. MUSCULOSKELETAL DISORDERS

Low back pain (LBP) and osteoarthritis (OA) are the two major musculoskeletal causes of disability worldwide[1–3]. Intervertebral disc degeneration (IVDD) is, at the same time, the main cause of low back pain[4–6]. Pain, dysfunction, and stiffness affecting patients with IVDD and OA can cause significant rates of disability and lead to high socio-economic costs[7]. Both diseases are caused by degeneration of the intervertebral disc and articular joint, following a vicious circle towards joint deterioration[8,9].

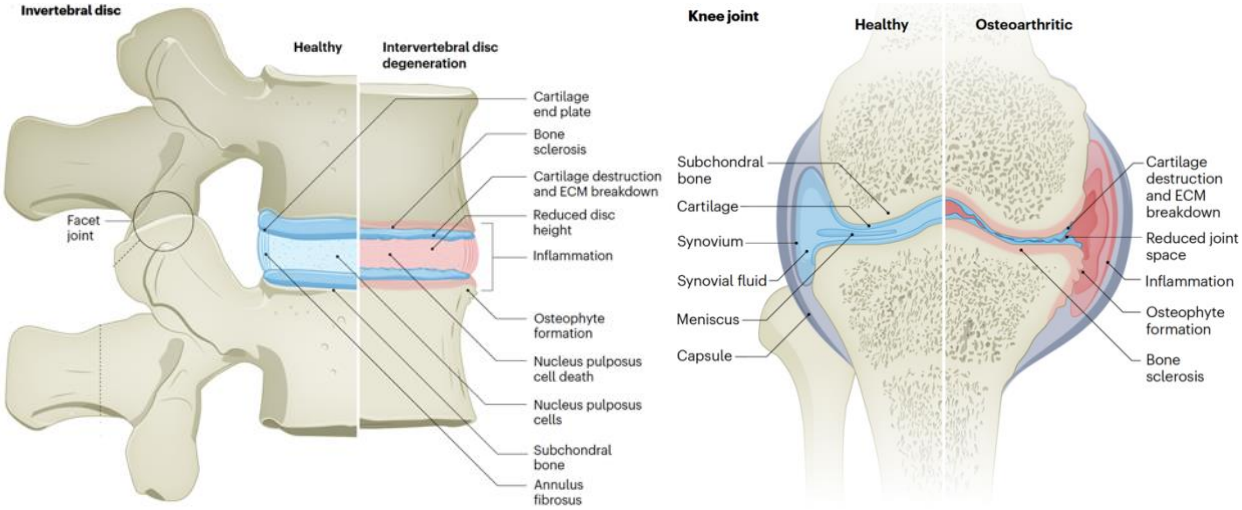

Figure 1. Comparison of normal and degenerated vertebral and knee joints. The figure represents the healthy and degenerating intervertebral disc and knee joint. Overlapping phenotypes of the degenerating joints include reduced disc or joint space, cartilage, and extracellular matrix (ECM) breakdown, inflammation, oxidative stress, suppression of autophagy, apoptosis, senescence, and subchondral bone changes, including osteophyte formation. In the knee, degeneration of the meniscus also occurs. *Fine, Noah et al. "Intervertebral disc degeneration and osteoarthritis: a common molecular disease spectrum." Nature reviews. Rheumatology vol. 19,3 (2023): 136-152. doi:10.1038/s41584-022-00888-z*[10]

1.1. SYNOVIAL JOINTS. PATHOPHYSIOLOGY AND OSTEOARTHRITIS

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1.1.1. STRUCTURE AND FUNCTION

Synovial joints are the most numerous in the human body. They are mainly composed by the articular cavity, surrounded by the joint cartilage and the joint capsule, that is the protective element that covers the entire articular cavity linking the bones of the joint. It has a fibrous outer layer and a synovial inner layer. The fibrous membrane is very resistant and not very elastic. The synovial membrane is a thin, transparent, vascularized membrane attached to the deep surface of the fibrous membrane. It contains the synoviocytes and is responsible for secreting synovial fluid, a transparent and viscous fluid formed by a filtrate plasma and very rich in mucin responsible for lubricating the joint, in addition to nourish and oxygenate the cartilage; and the infrapatellar fat pad, also called Hoffa's fat, that is a region of fatty tissue located below the patellar tendon with lubricating and shock absorbing functions.

1.1.1.1. Cartilage

Cartilage is a type of specialized, elastic connective tissue, lacking blood vessels, and made up mainly of extracellular matrix and scattered cells called chondrocytes. The outer part of the cartilage, called perichondrium, is responsible for providing life support to the chondrocytes. Cartilage is found lining joints, at the junctions between the ribs and the sternum, as reinforcement in the trachea and bronchi, in the external ear and in the nasal septum. It is also found in embryos of vertebrates and cartilaginous fish. There are 3 types of cartilage tissue:

Fibrous cartilage or fibrocartilage: It is a transitional form between regular dense connective tissue and hyaline cartilage. It is composed of chondrocytes and fibroblasts, surrounded by type I collagen fibers. It lacks a perichondrium. It is usually avascular. It is found in the intervertebral discs, articular edges, articular discs and menisci, sternoclavicular joints, jaw, pubic symphysis, as well as in the insertion sites of ligaments and tendons.

Elastic cartilage: Formed by chondrocytes surrounded by territorial and interterritorial matrices, which contain type II collagen, which interacts with proteoglycans and elastic fibers. It is surrounded by perichondrium. It is avascular. It forms the epiglottis, corniculate or Santorini cartilage, cuneiform or Wrisberg cartilage, in the larynx, the external ear (acoustic meatus) and in the walls of the external auditory canal and the Eustachian tube. It forms the pinna of the ear. It is yellowish and has greater elasticity and flexibility than hyaline. Its main difference with

the latter is that the matrix has a dense interweaving of fine elastic fibers that are basophilic and stain with hematoxylin and eosin, as well as orcein. It has more axial and poriferous isogenic groups.

Hyaline cartilage: It has chondrocytes arranged in groups (isogenic groups), each group surrounded by the territorial matrix, and between them there is an interterritorial matrix. These matrices mainly contain type II collagen fibrils, which interacts with proteoglycans. It is surrounded by perichondrium (except for articular cartilage). It is the most abundant type of cartilage in the body. It has a bluish-white appearance. It is found in the cartilage of the respiratory system: the nasal skeleton, the larynx, the trachea, the bronchi; and in the costal arches (ribs), the articular ends of the bones and in the temporal skeleton of the embryo. It is avascular, nourished by diffusion from the synovial fluid. It has few fibers.

Articular cartilage is the highly specialized connective tissue of diarthrodial joints. Its principal function is to provide a smooth, lubricated surface for articulation and to facilitate the transmission of loads with a low frictional coefficient. Articular cartilage is devoid of blood and lymphatics vessels, and nerves and is bound to a harsh biomechanical environment. Most important, articular cartilage has a limited capacity for intrinsic healing and repair. In this regard, the preservation and health of articular cartilage are of paramount relevance to joint health. It presents a glossy whitish appearance and, with a thickness between 1 and 5 mm, is capable of resisting compression forces. Its physical properties of resistance and elasticity are due to the integrity and structure of their extracellular matrix (ECM). It is a tissue with a low metabolic rate. However, it presents an elaborate and highly organized structure, with complex interactions between the chondrocytes and the matrix, responsible for tissue maintenance.
Articular cartilage contains only 2-5% cellular component (chondrocytes), while the remaining 95-98% of the tissue volume is extracellular matrix (ECM).

1.1.1.2. Chondrocytes

The chondrocyte is the only cell type present in articular cartilage. Each chondrocyte is isolated within the pericellular matrix, where it creates a specific microenvironment responsible for the synthesis and maintenance of the ECM. However, there are cytoplasmic projections that physically communicate chondrocytes located in distant lacunae[11]. Chondrocytes vary in size,

shape, and number depending on the region of the cartilage. Chondrocytes in the outer zone are smaller, flattened, and they present a larger density than in the inner areas of the cartilage. Chondrocytes come from a cell precursor of mesenchymal origin. As the cell differentiates, its phenotype changes and expresses different types of matrix formation marker genes. Thus, chondroprogenitor cells express type II collagen, proliferative chondrocytes express type II, IX and XI collagens in addition to aggrecan, and hypertrophic chondrocytes express type X collagen, osteocalcin, alkaline phosphatase, and MMP-13. Regarding their metabolic activity, chondrocytes present a high anabolic activity during growth phases, synthesizing different enzymes, growth factors, cytokines, and components of the ECM. However, this activity turns limited in mature cartilage, as their capacity of cell division.

1.1.1.3. Extracellular matrix (ECM)

ECM is made up of a liquid fraction (70-80%) and a solid scaffold (20-30%) of macromolecules that give cartilage its specific biomechanics. Water constitutes the liquid fraction of the matrix. Its distribution is heterogeneous within the tissue, in the outer layer it represents 80%, while in the inner only 60%. Its presence is crucial to maintain the characteristics of elasticity, flexibility, ability to reduce friction, and distribution of forces when interacting with the ECM macromolecules. The scaffold of structural macromolecules is composed of collagens (50%), proteoglycans (30-35%) and other matrix proteins representing the remaining 15-20%. Although different types of collagens coexist in cartilage (II, VI, IX, XI, XII, XIV), 90-95% is collagen type II. This type of collagen is organized according to a three-dimensional network, oriented to unload the forces of traction and thus provide tensile strength. Proteoglycans are macromolecules made up of a backbone protein to which glycosaminoglycan (GAG) chains are attached. Aggrecan, composed mainly by the GAG chondroitin sulfate, is the most common proteoglycan. Another GAG, the hyaluronic acid, forms aggregates by linking several aggrecan molecules through a protein bond.

1.1.1.4. Water

Water represents the predominant constituent of articular cartilage, contributing significantly to its wet weight, accounting for up to 80% of its composition. Among this aqueous content, roughly 30% is localized within the intrafibrillar space within the collagen structure, while a

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smaller fraction resides within intracellular compartments. The remaining water is distributed within the interstitial spaces of the extracellular matrix. Inorganic ions, such as sodium, calcium, chloride, and potassium, are solubilized within this tissue water. Notably, there is a gradual decline in the relative water concentration from approximately 80% in the superficial zone to about 65% in the deep zone. The movement of water through cartilage, extending across the articular surface, plays a critical role in facilitating the transportation and distribution of nutrients to chondrocytes, serving also as an essential lubricant. A substantial portion of the interfibrillar water appears to assume a gel-like state, with most of it being mobilized through the extracellular matrix either via the application of a pressure gradient across the tissue or as a consequence of compressing the solid matrix. It is worth noting that the frictional resistance to water flow within the matrix is considerably high, resulting in a notably low permeability of the tissue[12]. The collective effect of this frictional resistance to water flow and the pressurization of water within the matrix constitutes the foundational mechanisms by which articular cartilage derives its remarkable capacity to withstand substantial loads, often exceeding an individual's body weight several times over.

1.1.1.5. Collagens

Collagen stands as the predominant structural macromolecule within the extracellular matrix (ECM), constituting approximately 60% of the dry weight of cartilage. Within the ECM, type II collagen represents 90% to 95% of the collagen content. It forms fibrils and fibers that intricately intertwine with proteoglycan aggregates. While other collagen types such as I, IV, V, VI, IX, and XI are also present, they contribute only a minor fraction to the composition. These minor collagen types play a supplementary role in constructing and reinforcing the network of type II collagen fibrils. The collagen family comprises at least 15 distinct types, each comprised of no fewer than 29 polypeptide chains. All members of the collagen family feature a region composed of three polypeptide chains (α-chains) that are coiled into a triple helix structure. The predominant amino acids within these polypeptide chains are glycine and proline, with hydroxyproline contributing to stability via the formation of hydrogen bonds along the molecule's length. This triple helix configuration of polypeptide chains imparts significant shear and tensile properties to articular cartilage, thereby enhancing matrix stability[12].

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1.1.1.6. Proteoglycans

Proteoglycans are protein monomers extensively glycosylated. Within articular cartilage, they constitute the second-largest category of macromolecules within the extracellular matrix (ECM), making up approximately 10% to 15% of its wet weight. Proteoglycans are composed of a protein core to which one or more linear glycosaminoglycan chains are covalently attached. These chains can consist of over 100 monosaccharide units and project outward from the protein core, maintaining separation due to repulsive electrostatic charges. Articular cartilage harbors a diverse repertoire of proteoglycans that play essential roles in normal physiological function. These include aggrecan, decorin, biglycan, and fibromodulin. Among these, aggrecan stands out as the largest in size and the most abundant by weight. Aggrecan possesses more than 100 chondroitin sulfate and keratin sulfate chains. Notably, aggrecan exhibits the capacity to interact with hyaluronan (HA), forming substantial proteoglycan aggregates through the participation of link proteins. Aggrecan predominantly occupies the interfibrillar space within the cartilage ECM and provides cartilage with crucial osmotic properties, which are indispensable for its ability to withstand compressive loads[12].

1.1.1.7. No collagenous proteins and glycoproteins

While several non-collagenous proteins and glycoproteins have been identified within articular cartilage, their precise functions remain incompletely characterized. Some of these molecules, such as fibronectin and CII, a chondrocytes surface protein, are likely involved in orchestrating and preserving the macromolecular architecture of the extracellular matrix.

1.1.1.8. Zones of articular cartilage

Mature articular cartilage is a highly heterogeneous tissue. Four different zones are distinguished from the surface to the depth, depending on the size, shape, and number of chondrocytes; as well as the content of the ECM, which gives it different mechanical properties.

Zone I. The superficial zone: The surface or superficial tangential zone covers the articular surface. It has a smooth contour, allows the ends of the bones to slide, forms about 10% to 20% of the thickness of the articular cartilage. It has the highest collagen content of all areas. The

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collagen fibers of this zone (primarily, type II and IX collagen) are packed tightly and aligned parallel to the articular surface. The superficial layer contains a relatively high number of flattened chondrocytes, and the integrity of this layer is imperative in the protection and maintenance of deeper layers. This zone is in contact with synovial fluid and is responsible for most of the tensile properties of cartilage, which enable it to resist the sheer, tensile, and compressive forces imposed by articulation. The chondrocytes in this area have an elongated shape.

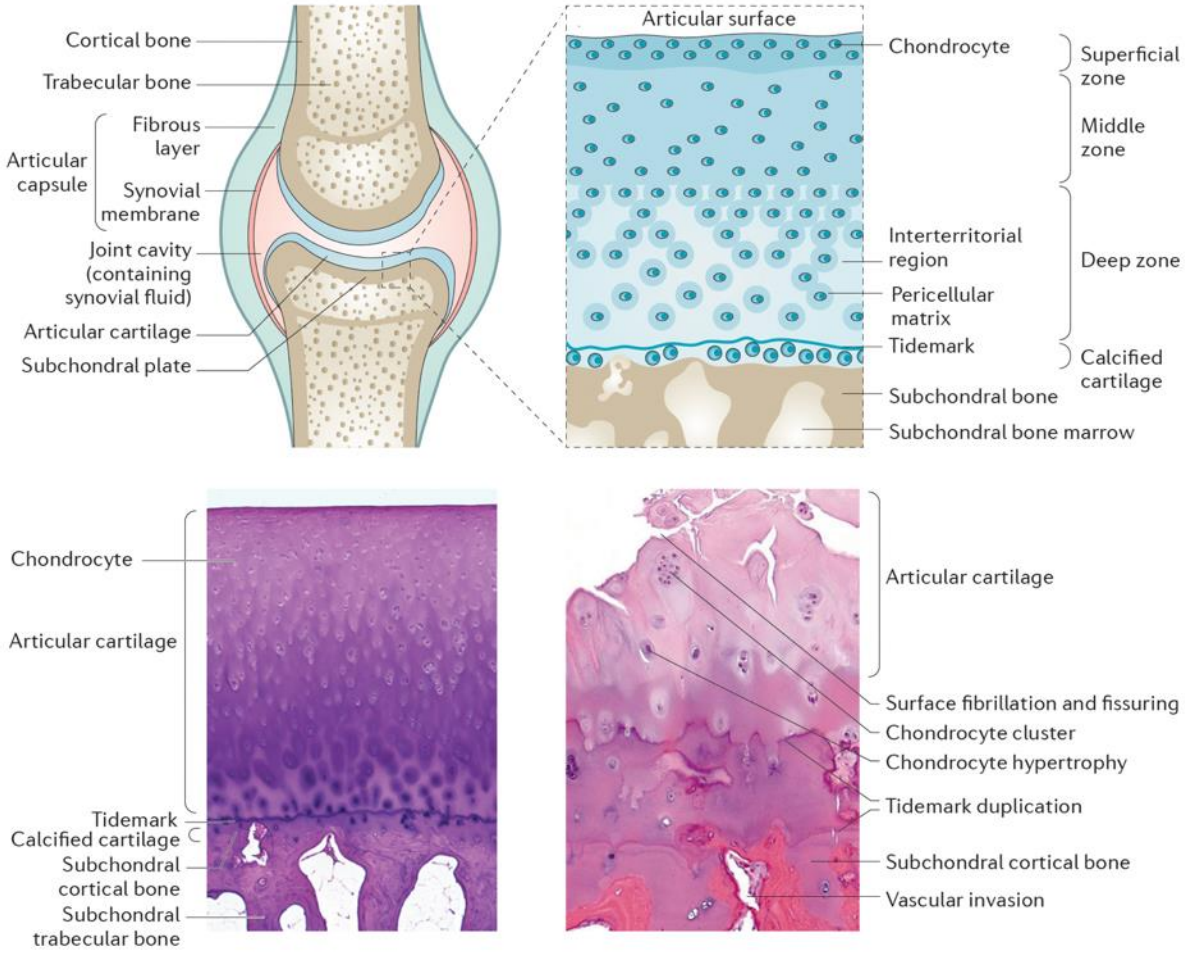

Figure 2. Diarthrodial joints in health and OA. Cross-section of the articular surface of a diarthrodial joint illustrating schematically and histologically the main structural elements, including the articular cartilage (with chondrocytes), tidemark (separating the calcified and articular cartilage), calcified cartilage, and subchondral cortical and trabecular bone. Histopathological cross-section of the articular surface showing advanced osteoarthritic changes characterized by fissuring and fragmentation of the articular cartilage, chondrocyte proliferation and hypertrophy, duplication and advancement of the tidemark, expansion of the zone of calcified cartilage, thickening of the subchondral cortical plate and vascular invasion of the bone and

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calcified cartilage. *Martel-Pelletier, Johanne et al. "Osteoarthritis." Nature reviews. Disease primers vol. 2 16072. 13 Oct. 2016, doi:10.1038/nrdp.2016.72*[13]

Zone II. The middle (or transition) zone: It represents 40% to 60% of the volume of articular cartilage, which provides an anatomic and functional bridge between the superficial and deep zones. There is a greater presence of proteoglycans than in the superficial zone, and the collagen fibrils are less numerous, thicker, and are loosely aligned and not parallel to the surface. Chondrocytes in this layer are more rounded. Functionally, the middle zone is the first line of resistance to compressive forces.

Zone III. The deep zone: It makes up to the 30% of cartilage. It is responsible for providing the greatest resistance to compressive forces, given that collagen fibrils are arranged perpendicular to the articular surface and have a large diameter. It has the highest proportion of proteoglycans and the lowest concentration of water. Chondrocytes are arranged in a column, parallel to the collagen fibers. The tide mark distinguishes the deep zone from the calcified cartilage. The deep zone is responsible for providing the greatest amount of resistance to compressive forces, given the high proteoglycan content. The calcified layer plays an integral role in securing the cartilage to bone, by anchoring the collagen fibrils of the deep zone to subchondral bone. In this zone, the cell population is scarce and chondrocytes are hypertrophic and present a more elliptical shape.

Zone IV. The calcified zone: It presents hypertrophic chondrocytes and type X collagen and acts as mechanical protection between the noncalcified articular cartilage and subchondral bone.

The main function of articular cartilage is to provide a smooth and low friction coefficient surface that allows articulation movement without pain. To facilitate mobility, cartilage surfaces are protected with a layer of lubricin and hyaluronic acid, both produced by chondrocytes and synoviocytes, allowing the sliding of the articular surfaces. The interaction between collagen, water and the different proteoglycans that form the ECM confers the articular cartilage its functions and physical and mechanical properties, being a highly resistant to deformation by compressive forces tissue. This resistance to deformation is mainly associated

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with its ability to remove water hydrating proteoglycans under stress pressures to subsequently recapture it by osmosis and recover its original shape.

1.1.1.9. Regions in articular cartilage

Beyond the zonal variations in structure and composition, the extracellular matrix (ECM) exhibits distinct regions that are delineated by their proximity to chondrocytes, composition, and the diameter and organization of collagen fibrils. The ECM can be categorized into three primary regions: the pericellular matrix, the territorial matrix, and the interterritorial region.

The pericellular matrix is a thin layer located adjacent to the cell membrane, encompassing the chondrocyte entirely. It primarily comprises proteoglycans, alongside glycoproteins and other noncollagenous proteins. This matrix region is believed to play a functional role in initiating signal transduction within cartilage, particularly under load-bearing conditions.

Surrounding the pericellular matrix there is the territorial matrix, mainly consisting of fine collagen fibrils, forming a basket-like network around the cells. Compared to the pericellular matrix, this region is thicker. It has been hypothesized that the territorial matrix serves to shield cartilage cells from mechanical stresses and contributes to the overall resilience of the articular cartilage structure, enabling it to withstand substantial loads.

The interterritorial region, the largest among the three matrix regions, significantly influences the biomechanical properties of articular cartilage. Characterized by randomly oriented bundles of large collagen fibrils, this region varies in alignment, being parallel to the surface in the superficial zone, oblique in the middle zone, and perpendicular to the joint surface in the deep zone. The interterritorial zone is rich in proteoglycans and plays a pivotal role in defining the mechanical properties of articular cartilage.

1.1.1.10. Metabolism

In adults, the articular cartilage matrix is separated from subchondral vascular spaces by the subchondral plate. Nutrient supply to articular cartilage relies on diffusion from the synovial fluid. The cartilage matrix regulates material passage based on factors like size, charge, and

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molecular configuration, with an estimated average pore size within the ECM of approximately 6.0 nm. In the absence of direct nutrient supply from blood vessels or lymphatics, chondrocytes predominantly rely on anaerobic metabolism.

Chondrocytes play a pivotal role in the development, maintenance, and repair of the ECM through a group of enzymes responsible for degradation. They also synthesize matrix components, including proteins and glycosaminoglycan side chains. Various factors within their chemical and mechanical environment can modify the metabolic activity of chondrocytes. Proinflammatory cytokines, such as interleukin-1 and tumor necrosis factor-alpha, exhibit both catabolic and anabolic effects that influence the degradation and synthesis of matrix macromolecules.

Proteoglycans are synthesized, sustained, and secreted into the ECM by chondrocytes. The regulation of proteoglycan metabolism involves several growth factors and regulatory peptides, including insulin-like growth factors, transforming growth factor-beta, interleukin-1, and tumor necrosis factor-alpha. However, the precise molecular mechanisms by which these growth factors and peptides exert their effects on proteoglycan metabolism remain poorly understood.

The ECM serves to shield chondrocytes from potentially damaging biomechanical forces. Homeostasis in ECM metabolism is maintained by balancing the degradation of various macromolecules with their replacement by newly synthesized products. Proteoglycan turnover can extend up to 25 years, while collagen has a half-life ranging from several decades to as much as 400 years.

The primary proteinases involved in cartilage turnover include metalloproteinases (collagenase, gelatinase, and stromelysin) and cathepsins (cathepsin B and D). Collagenase targets native helical collagen fibrils at a specific site, while gelatinase acts on denatured type II and type IV collagen, along with fibronectin, elastin, and collagen types V, VII, X, and XI. Stromelysin's role is to degrade the protein core of aggrecan. All metalloproteinases are initially secreted as latent proenzymes and require extracellular activation. Cathepsins play a role in aggrecan degradation.

Maintaining normal articular cartilage structure and function depends on joint motion and loading. Conversely, joint inactivity has been associated with cartilage degradation. Regular

joint movement and dynamic loading are crucial for preserving healthy articular cartilage metabolism[12]. Diseases like osteoarthritis emerge when there is a physiological imbalance between chondrocyte-driven degradation and synthesis processes.

1.1.1.11. Biomechanical Function

Articular cartilage, a specialized connective tissue, serves as a thin layer with unique viscoelastic properties. Its primary role is to offer a smooth, lubricated surface for articulation with minimal friction and to facilitate the transmission of loads to the underlying subchondral bone. Articular cartilage possesses remarkable resilience, demonstrating minimal evidence of damage or degeneration under high cyclic loads.

The biomechanical characteristics of articular cartilage are best comprehended when considering it as a biphasic medium, comprising a fluid phase and a solid phase. The fluid phase primarily consists of water, contributing up to 80% of the tissue's wet weight, along with inorganic ions like sodium, calcium, chloride, and potassium. The solid phase, characterized by the extracellular matrix (ECM), is porous and permeable. The relationship between proteoglycan aggregates and interstitial fluid confers compressive resilience to cartilage via negative electrostatic repulsion forces.

The initial application of contact forces during joint loading leads to an immediate increase in interstitial fluid pressure, causing fluid to flow out of the ECM, resulting in substantial frictional drag on the matrix. Upon the removal of compressive load, interstitial fluid returns to the tissue, aided by the low permeability of articular cartilage. The cartilage is confined beneath the contact surface by the opposing bones and surrounding cartilage boundaries, designed to restrict mechanical deformation.

Articular cartilage exhibits viscoelastic properties, displaying time-dependent behavior when subjected to a constant load or deformation. Two mechanisms contribute to viscoelasticity: flow-dependent and flow-independent. The flow-dependent mechanism depends on interstitial fluid and the associated frictional drag, known as biphasic viscoelastic behavior. The flow-independent component arises from macromolecular motion, specifically the intrinsic viscoelastic behavior of the collagen-proteoglycan matrix. Consequently, fluid pressure significantly contributes to total load support, reducing stress on the solid matrix.

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Articular cartilage also displays creep and stress-relaxation responses. Under constant compressive stress, the tissue gradually deforms until reaching equilibrium. Similarly, when deformed and held at a constant strain, the stress initially peaks and then undergoes slow stress-relaxation until reaching equilibrium. Articular cartilage does not conform to a single Young's modulus due to its stiffening behavior with increased strain. Instead, the modulus varies with the time at which force measurements are taken during stress-relaxation tests. The current approach involves applying known strains, immediately measuring the peak force, and recording the force/stress value upon reaching equilibrium.

The complex composition and organization of cartilage in the middle zones contribute significantly to its shear-resistant properties. The stretching of randomly distributed collagen fibrils enables cartilage to withstand shear stress. Precise molecular arrangements of collagen fibrils underlie tensile force-resisting properties, with intra- and intermolecular cross-links contributing to collagen fiber stabilization and ultimate tensile strength[12].

1.1.2. OSTEOARTHRITIS

Osteoarthritis (OA) is the most common degenerative joint disease characterized by the destruction of the hyaline cartilage that covers bone surfaces, and the simultaneous appearance of regenerative bone changes.

Osteoarthritis is the second cause of permanent disability, after cardiovascular diseases. More than a disease, it is a syndrome; a heterogeneous group of processes with varied etiopathogenic mechanisms, sometimes interrelated, end up causing the failure of the joint, weakening the cartilage, which then cannot withstand normal forces or gives way to abnormally intense forces. The importance of the disease in terms of economic and social cost is enormous in the Western world: it is the most important cause of functional disability in terms of processes related to the musculoskeletal system, in addition to promoting a high consumption of drugs (mainly NSAIDs and simple analgesics) and other health resources.

It is estimated that up to 70% of the population over 50 years of age has radiological signs of osteoarthritis in some joint of the body such as the spine, knees, hips, or hands. By the age of 75, up to 80% of the population has some radiological sign of osteoarthritis in different joints. However, many times these findings are not associated with the presence of symptoms or problems in daily life[12,13].

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Regarding its pathophysiology, OA is one of the diseases associated with cartilage destruction. It is a disorder that affects mobile joints characterized by cellular stress and degradation of the extracellular matrix initiated by microlesions. This activates repair responses that include pro-inflammatory pathways of the innate immunity. The disease first signs consist of molecular alteration (abnormal metabolism in joint tissue), followed by anatomical changes and/or physiological (characterized by cartilage degradation, subchondral bone remodeling, osteophyte formation, joint inflammation and loss of function joints), which can culminate in the appearance of disease symptoms[14].

The symptoms of the disease are usually accompanied by mechanical pain, joint stiffness, and loss of joint function in the more advanced stages. Even today, the causes of this disease are not clear. It is known that there is a mechanical component of wear of the articular cartilage when the balance between anabolic processes (matrix synthesis extracellular) and catabolic (degradation of extracellular matrix) that maintains the ECM integrity is lost in chondrocytes[14]. In addition, different studies have indicated a reduction in the number of chondrocytes, due in part to apoptotic processes[15]. As a result of the degradation of the cartilage matrix, fragments of ECM molecules are released into the synovial fluid. They are capable of inducing, both in the chondrocyte and in the synovial membrane, the production of cytokines and nitric oxide (NO), in addition to the synthesis of metalloproteases. Also, it has been described that the chondrocytes can react to the release of damage-associated molecular patterns (DAMPs), which have been described as Toll-like receptor (TLRs) ligands and receptors for glycosylation end products (RAGE)[13]. In this way, there is a intercommunication between the different tissues that make up the joint that causes and perpetuates an inflammatory response, through a large number of mediators, which end up irreversibly damaging the joint structure as a whole[14]. Among the mechanisms involved in the destruction of articular cartilage mechanical factors and biochemical factors are the most relevant.

Mechanical factors: mechanical stress in a physiological range induces the anabolic activity, producing the renewal of the matrix; while a range higher than the physiological catabolic activity increases, generating the ECM progressive lost. How the mechanical factor regulates the metabolic activity of the chondrocyte is not fully known; however, some mechanisms of mechano-transduction have been investigated in cartilage[16].

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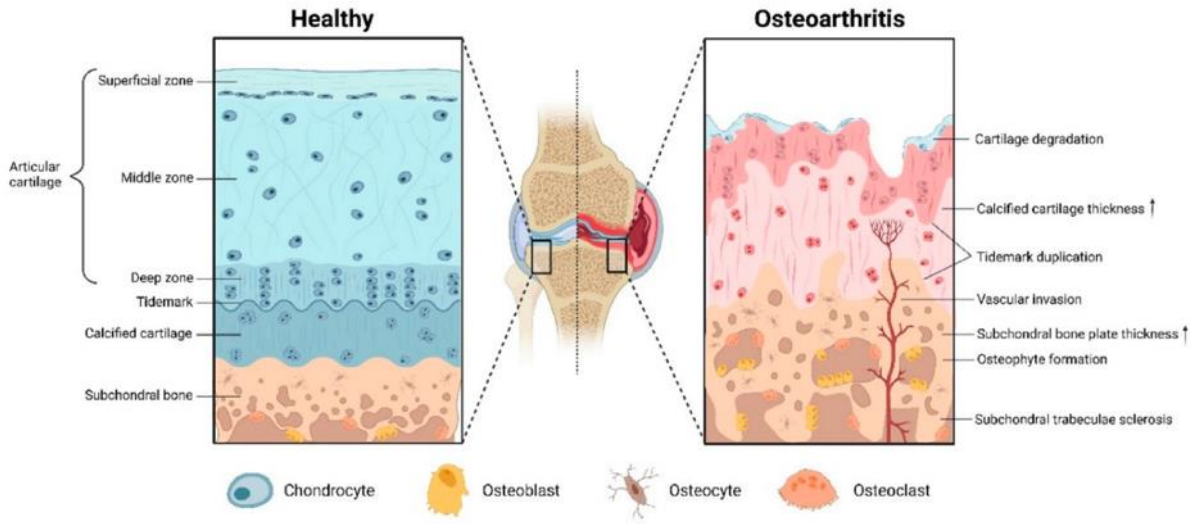

Figure 3. The physiological structure of articular cartilage and pathological changes of OA. *Li, Jiadong et al. "Targeted and responsive biomaterials in osteoarthritis." Theranostics vol. 13,3 931-954. 16 Jan. 2023, doi:10.7150/thno.78639*[17]

Biochemical factors: the most important element in the degradation of cartilage is the synthesis and activity of different types of proteases. The most studied and related to the development of OA proteases are matrix metalloproteases (MMPs) and ADAMTS proteases. The most important MMPs in the arthritic process are collagenases (MMP-1, MMP-8 and MMP-13) and the stromelyses (MMP-3 and MMP-10). The expression of the MMP-13 in arthritic cartilage and its ability to degrade type II collagen, make it one of the most important enzymes in the degradation of cartilage in OA patients. The proteolytic activity of MMPs is regulated at three levels: at the regulation of gene transcription, at the proenzyme activation, and at the inactivation by TIMPs (tissue inhibitors of metalloproteinases), also capable of inhibiting ADAMTS-5. ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) comprise the main aggrecanases (ADAMTS-1, ADAMTS-4, ADAMTS-5 and ADAMTS-9) responsible for the degradation of the ECM aggrecan. ADAMTS-5 is the predominant one in the development of OA[18].

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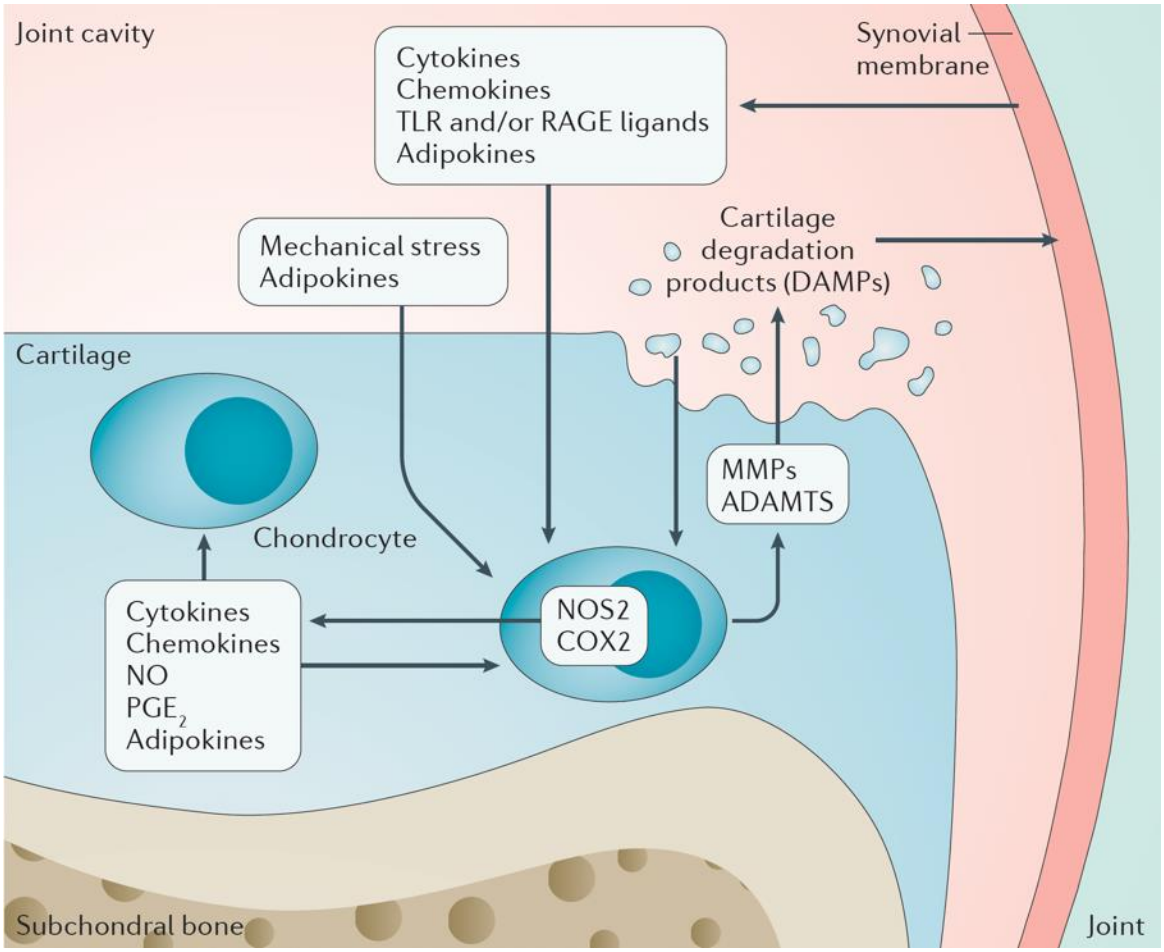

Figure 4. Crosstalk between cartilage and the synovium in the pathogenesis of OA. Products that are released from the cartilage matrix and/or the chondrocytes in response to adverse mechanical forces and other factors induce the release of products that deregulate chondrocyte function via paracrine and autocrine mechanisms. Catabolic enzymes, such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), released by chondrocytes degrade the cartilage matrix, releasing cartilage degradation products that, along with the other pro-inflammatory chondrocyte derived-products, act on the synovium to induce inflammation and the release of pro-inflammatory products that feedback on chondrocytes to further deregulate their function. *Martel-Pelletier, Johanne et al. "Osteoarthritis." Nature reviews. Disease primers vol. 2 16072. 13 Oct. 2016, doi:10.1038/nrdp.2016.72*[13]

Although OA was considered for decades as a not inflammatory arthropathy, it should be noted that pro-inflammatory mediators are produced by synoviocytes and chondrocytes during the development of the disease. In fact, inflammatory factors have been detected in the synovial fluid of patients with OA, although the levels are lower than those found in RA patients[19]. It is currently believed that the development and progression of OA also involve inflammation even in the early stages of the disease. The histological changes observed in the synovial membrane,

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both in the early phase as well as in the late phase of OA, include evidence of synovitis, such as membrane hyperplasia, macrophage infiltration and fibrosis. Although the causes of synovial inflammation in OA are unknown, the most accredited hypothesis describes that fragments of cartilage that precipitate into the joint encounter the synovial membrane, which recognizes them as foreign agents to the body. Consequently, synovial cells react by producing inflammatory mediators, that accumulate in the synovial fluid[20]. These mediators can activate chondrocytes present in the superficial layer of cartilage, inducing the production of MMPs and perpetuating cartilage destruction. Synovitis is associated with worsening of OA symptoms, such as joint pain, stiffness, and swelling, as well as a faster cartilage degeneration[21]. The degenerative processes of the joints are, to a great extent, modulated by the activity of pro-inflammatory cytokines and other factors that induce profound changes in the physiology of the chondrocyte, which result in dedifferentiation processes, apoptotic processes, and an increase in the production of MMPs.

Table 1. Local and general factors influencing osteoarthritis setup

Local	General
Congenital anomalies	Metabolic alterations
Joint overload	Joint laxity
Trauma	Previous inflammatory processes
Alignment defects	Endocrine disorders
Continued compression of the articular cartilage	Hematological, vascular, or neurological alterations
Acquired morphological conditions	Obesity and diet
	Age and sex
	Inheritance

1.1.2.1. Clinical manifestations

Pain, a symptom of the beginning of osteoarthritis, has an insidious onset, is deep, and poorly localized. Its intensity increases over the years, although in many patients, especially in the earliest stages, it shows an intermittent course and patients are symptom-free for years. Pain can occur as a survival mechanism, is triggered by exercise and improves until it disappears with rest. As the disease worsens, the pain becomes more continuous, persists for a longer time,

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and is induced by any movement of the affected joint. In the last evolutionary stages, it is almost continuous, even at rest, and does not respect the patients' night rest.

The causes of pain in this process are diverse. In the initial stages it is due to inflammation of periarticular structures, such as the capsule, tendons, and bursae, and to synovial inflammatory outbreaks induced by multiple factors: microtrauma, crystal deposits and synovial irritation due to cartilaginous debris. In no case do they come from cartilage, since it is a tissue that lacks nerve endings.

The origin of pain in the most advanced stages is not well known and has been attributed to venous congestion produced in the subchondral bone. The pain is accompanied by progressive functional disability, which consequences for the patient depend on the affected joint.

In advanced stages, large deformations and even subluxations of the joints appear caused by joint incongruity, osteophyte proliferation and capsular thickening. Stiffness also occurs after rest, which differs clinically from that observed in inflammatory processes due to its shorter duration; In general, it does not last more than half an hour and is limited to the affected joint.

Examination of the osteoarthritic joint under pressure shows mobility limitation with pain, that will be greater during outbreaks of inflammation of the synovial membrane or some periarticular structure. Clicking and harsh crepitation are common when the joint is mobilized, due to the irregularity of the surface. The characteristics of joint swelling depend on the degree of joint deformity and the coexistence of inflammatory foci, synovitis, or synovial effusion. Although the pathophysiological alterations usually continue once the symptoms and signs have appeared, and there are variable degrees of disability and functional compromise, sometimes the process stops or even normalizes.

1.1.2.2. Treatment

Treatment of osteoarthritis includes rehabilitation, which means preventing dysfunction, attempting to initiate care before disability develops, and reducing the intensity or duration of disability. The main considerations to take into account are the stage and magnitude of the tissue alterations in each particular patient, the number of joints affected, the pain cycle (taking into account whether this is due to biochemical alterations or inflammation) and the patient lifestyle.

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Treatment also includes patient education about the nature of the problem (physiology and biomechanics), the prognosis (usually benign), the need for cooperation, and the achievement and maintenance of an optimal level of overall fitness. Exercise (degree of mobility, isometric, isotonic, isokinetic, postural, strengthening) helps maintain the normality of the cartilage and the degree of mobility and development of the tendons and muscles that absorb tension. There must be a balance between rest (every 4-6 hours during the day in order to rehydrate the cartilage) and exercise and use of the joint. General advice to the patients includes paying attention to activities of daily living, instructing the patient to avoid soft chairs and cushions below the knees and to sit in straight-backed chairs that are not low, using a hard bed with a wood under the mattress, having a seat with a comfortable design in the car, performing postural exercises and maintaining their physical, work and intellectual activity.

Table 2. Basis for the treatment of osteoarthritis
Treatment individualization
Guidance and psychological support
Identification and modification of etiological and postural factors
Permanent functional and physical re-education
Indications for joint rest
Pharmacological treatment
Surgical treatment

Table 3. Objectives of osteoarthritis treatment
Easing the pain
Educating the patient
Restoring functionality
Preventing disability
Improving deformity
Slowing disease progression

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Table 4. General advice for the treatment of osteoarthritis
Correctly inform the patient about the illness
Prevent or treat the patient about the illness
Avoid joint overload (use canes if necessary)
Joint rest when there is pain and exacerbation of symptoms
Active and passive kinesitherapy techniques
Hydrotherapy
Cryotherapy in acute painful exacerbations
Thermotherapy that relieves chronic pain, stiffness, and muscle contracture
Massages

1.1.2.3. Pharmacotherapy

The pharmacological measures available are aimed at reducing pain: analgesics, NSAIDs, corticosteroids. It has been suggested that some drugs, such as glucosamine sulfate and others, can promote cartilage repair, and prevent the progression of the disease.

Analgesics: The most used is paracetamol. They present a low cost, fewer side effects than NSAIDs and are easy to use. Recent studies show that using paracetamol at a sufficient dose (equal to or greater than 2,600 mg/day) we obtain similar results than using NSAIDs in terms of response to pain and disability. For this reason, more and more voices are advocating for its use as the first choice in the usual treatment of osteoarthritis.

Opioid analgesics have not been shown to be more effective than paracetamol or acetylsalicylic acid in relieving pain and have a high risk of addiction and side effects, so their use is not recommended.

NSAIDs: They are a heterogeneous group of substances with anti-inflammatory, analgesic, and antipyretic activity. They inhibit the synthesis of prostaglandins, leukotrienes, and the release of other inflammatory mediators by polymorphonuclear cells in acute and chronic inflammation. All of them have a similar pharmacological action, clinical efficacy, and adverse effect profile. However, there are differences in effectiveness, depending on the individual, of an idiosyncratic nature.

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They have classically been widely used in the treatment of diseases of the musculoskeletal system. Its use in osteoarthritis should be reserved for the symptomatic treatment of pain as a second choice after the use of paracetamol or associated with it (in low doses) and in inflammatory outbreaks in short periods of time. The choice of the type of NSAID must be individualized for each patient considering their side effect profile, drug interactions, patient comorbidity (especially liver disease, kidney failure and gastropathy), the use of other drugs (oral anticoagulants), previous response to NSAIDs and dosage (compliance). They are used above all for their analgesic effects, so lower doses are generally required to obtain anti-inflammatory effects and, therefore, with fewer adverse effects.

Its route of administration should preferably be oral and on rare occasions rectal or parenteral (variable absorption and bioavailability).

Chondroprotective properties have been proposed in prolonged use but have not been demonstrated. Furthermore, some studies suggest that certain NSAIDs promote the destruction of articular cartilage, although there are no conclusive results at the moment.

Topical NSAIDs have controversial efficacy. They seem to produce a temporary moderate analgesia in the area where they are applied, derived from inhibition of the stimulation of dermal and subdermal nociceptive nerve terminals, lymphatic, and venous drainage of pro-inflammatory substances through massage, and thanks to the placebo effect. However, they are widely accepted among the population that uses them massively.

1.2. INTERVERTEBRAL DISC PATHOPHYSIOLOGY AND INTERVERTEBRAL DISC DEGENERATION

1.2.1. STRUCTURE AND FUNCTION

The vertebral column is composed of the vertebrae and the soft tissue in between the vertebral bodies, the intervertebral discs. They make up an amphiarthrosis joint since it allows very limited mobility. Its function is to provide rigidity to the axial skeleton while allowing limited rotation and bending including flexion-extension, axial rotation, lateral bending, and translatory motions of the cervical, thoracic, and lumbar vertebrae.

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Since vertebrae are relatively inelastic, the intervertebral disc main roles are to absorb biomechanical forces, allowing the movement of the spinal column, and to some extent, to protect the spinal cord and nerve roots.

It is composed of three distinct tissues: the nucleus pulposus, a central proteoglycan-rich core, enclosed by an outer circumferential ring of fibrocartilage, the annulus fibrosus, and the cartilage end plates, a thin cartilage layers that separate them caudally and cephalically from the vertebral body. Each of these tissues has a characteristic composition and structure which provide them with specific mechanical properties to perform their function. Their interaction enables the intervertebral disc to transmit loads while allowing a constrained flexibility between vertebral bodies[8].

To better understand the biomechanics of the intervertebral discs, it is necessary to know the characteristics of each one of these tissues.

1.2.1.1. Nucleus Pulposus

In a healthy intervertebral disc, the nucleus pulposus serves as a highly hydrated, gel-like core held in place by the annulus fibrosus and the cartilaginous endplates. Its primary role is to bear mechanical loads through hydraulic and osmotic pressure mechanisms.

The nucleus pulposus constitutes the soft, gelatinous, and markedly hydrophilic core of the intervertebral disc. It comprises water (making up 70–85% of its total weight), proteoglycans (constituting 30–50% of its dry weight), collagen (comprising 20% of its dry weight), and a few other minor proteins. This region of the disc possesses a notably higher concentration of proteoglycans, with aggrecan being the most abundant, and water compared to other disc regions. Conversely, the outer annulus exhibits the highest collagen levels, while the nucleus contains the lowest collagen concentration. Furthermore, the collagen content in the nucleus pulposus is highest in cervical discs and lowest in lumbar discs. In contrast, the proteoglycan content peaks in the nucleus pulposus of lumbar discs and is lowest in cervical discs.

The nucleus pulposus originates from the notochord, and notochordal cells can remain in the tissue after birth and in adulthood. During development, the nucleus is notably cellular, but cell numbers decrease after birth, resulting in a low cell density in adults. Histologically, nucleus pulposus cells are distinctive and complex, featuring large cells primarily organized in clusters

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and separated by an abundant extracellular matrix. These cells secrete aggrecan, as well as collagens I and II into the matrix. Additionally, the matrix includes collagens IX and XI, with collagen X presence being reported during degeneration. Due to the presence of aggrecan, the disc maintains high osmotic pressure. Furthermore, because it lacks a blood supply, the oxygen tension within the disc is quite low.

1.2.1.2. Annulus Fibrosus

The annulus fibrosus constitutes the outer part of the disc's structure and is comprised of lamellae—regular concentric bundles of parallel collagen fibers arranged around the central gelatinous nucleus pulposus. Each lamella contains collagen fibers running in one direction, while adjacent lamellae have fibers running in the opposite direction. Lamellar thickness increases from the inside to the outside. This alternating pattern is designed to withstand torsional stresses. The collagen fibers in the outer lamellae directly attach to the vertebral bodies, while the inner lamellae attach to the cartilaginous endplates. The annulus fibrosus serves to laterally confine the nucleus pulposus, support vertical loads, and restrict motion between the vertebral bodies.

Similar to the nucleus pulposus, the annulus fibrosus primarily comprises proteoglycans and collagen, but their relative content and arrangement differ significantly. In a healthy human annulus fibrosus, water content is 50%, collagen accounts for approximately 70% of the dry weight, and proteoglycans make up to 10% of the dry weight. The annulus fibrosus experiences both tensile and compressive stresses during physiological loading, which necessitates a high collagen content similar to other tension-bearing tissues like tendons and ligaments.

The annulus can be divided into an inner fibrocartilaginous region and an outer fibrous zone. The outer annulus fibrosus predominantly comprises well-defined collagen I fibers that bundle to form long, parallel concentric lamellae. Lamellar thickness varies circumferentially and radially, increasing significantly with age, location, and vertebral type. Central annulus fibers attach to the endplate cartilage, while peripheral ones anchor to the vertebral bone. In terms of collagen organization and cell composition, this region resembles tendon or ligament. The inner annulus fibrosus constitutes about 50% of the total radial thickness and differs notably from the outer region. Unlike the elongated, fusiform cells in the outer annulus, inner annulus cells are

spherical and many resemble chondrocytes, with few cells having short processes. There is also a chemical composition difference between the inner and outer annulus: the inner region contains both collagens I and II, while aggrecan is present in both annulus regions. Another significant protein is elastin, comprising 2% of the dry tissue weight. The change in the ratio of collagens I to II and the increase in proteoglycan content from the outer to the inner annulus reflect a shift in the loading environment from tension in the outer annulus fibrosus to compression toward the nucleus pulposus.

1.2.1.3. Cartilaginous Endplate

The human intervertebral disc includes the cartilaginous endplates, which are thin horizontal layers of hyaline cartilage typically less than 1 mm thick. These endplates separate the nucleus pulposus and annulus fibrosus from the adjacent vertebral bone. In adulthood, the cartilage endplate becomes narrow and often calcified, potentially disrupting nutrient supply to the nucleus pulposus. Collagen fibers within the endplates run parallel to the vertebral bodies and extend into the disc.

Cartilaginous endplates play a vital role in facilitating the exchange of nutrients, waste products, and metabolites between the nucleus and the blood vessels within the vertebral bodies. They also act as biomechanical barriers preventing direct pressure from the disc on the bone, imparting joint-like characteristics to the motion segment, and potentially preserving the viability of nucleus pulposus cells. Structurally, the endplate resembles articular hyaline cartilage, containing chondrocytes embedded in an extracellular matrix rich in aggrecan and collagen II. Although these cells do not undergo terminal differentiation, collagen X may be present in the central endplate region. The endplate transitions into bone through a region of calcified cartilage. The water content of human endplates is 58% of the wet weight, sulfated glycosaminoglycan (s-GAG) content is 17% of the dry weight, and the total collagen content ranges from 60% to 80% of dry weight. Vascular channels can penetrate the cartilage, but they narrow, constrict, or even obliterate with maturity, likely impacting nutrient supply to both cartilage and the disc. Notably, biomechanical studies of the cartilaginous endplate have been less explored compared to other disc tissues.

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While extensive research has contributed to understanding the structure-function relationships of intervertebral disc tissues, ongoing investigations aim to describe the mechanical behavior of healthy tissues, the effects of degeneration, and the implications of disc mechanics on cell biology. Recent findings on these structure-function relationships are presented in subsequent sections.

As previously mentioned, intervertebral disc tissues are primarily composed of water, proteoglycans, and collagen, each with varying relative content and organization. These differences in composition and organization confer distinct mechanical properties to disc tissues. For instance, the annulus fibrosus, due to its higher collagen content and fiber arrangement, exhibits superior tensile loading capacity, while the nucleus pulposus, with its high proteoglycan content, demonstrates remarkable compressive properties[22].

However, because these disc tissues share similar components, they also exhibit common mechanical behaviors, notably osmotic effects arising from the high negative charge density of proteoglycans. These osmotic effects have significant implications for disc mechanics. Osmotic pressure can induce tissue deformation, known as osmotic swelling, leading to tensile stresses and increased tissue stiffness. Osmotic swelling also draws water into these tissues, maintaining disc hydration. These osmotic effects are mediated by the tissue's proteoglycan content[23]. Proteoglycans are large molecules comprising multiple glycosaminoglycan units linked to a core protein. Glycosaminoglycans consist of polysaccharide chains that exhibit excess negative charge density at physiological pH. Due to their large size, proteoglycans are trapped within the collagen fiber network. Consequently, collagen and proteoglycans form a charged, porous, deformable solid material embedded in a solution of water and ions. These osmotic effects play a pivotal role in the mechanics of all disc tissues.

1.2.2. INTERVERTEBRAL DISC DEGENERATION

The intervertebral disc undergoes a series of biochemical and structural changes attributed to both aging and degeneration. Biochemical alterations involve a reduction in proteoglycan content, an increase in protein cross-linking, and modifications in collagen type and distribution. Notably, the biochemical changes seen in degeneration mirror those occurring during aging,

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but they manifest at an accelerated rate and are accompanied by structural changes that compromise disc function. These structural changes encompass a reduction in disc height, inward and outward bulging of the annulus fibrosus, and the loss of its lamellar organization[24].

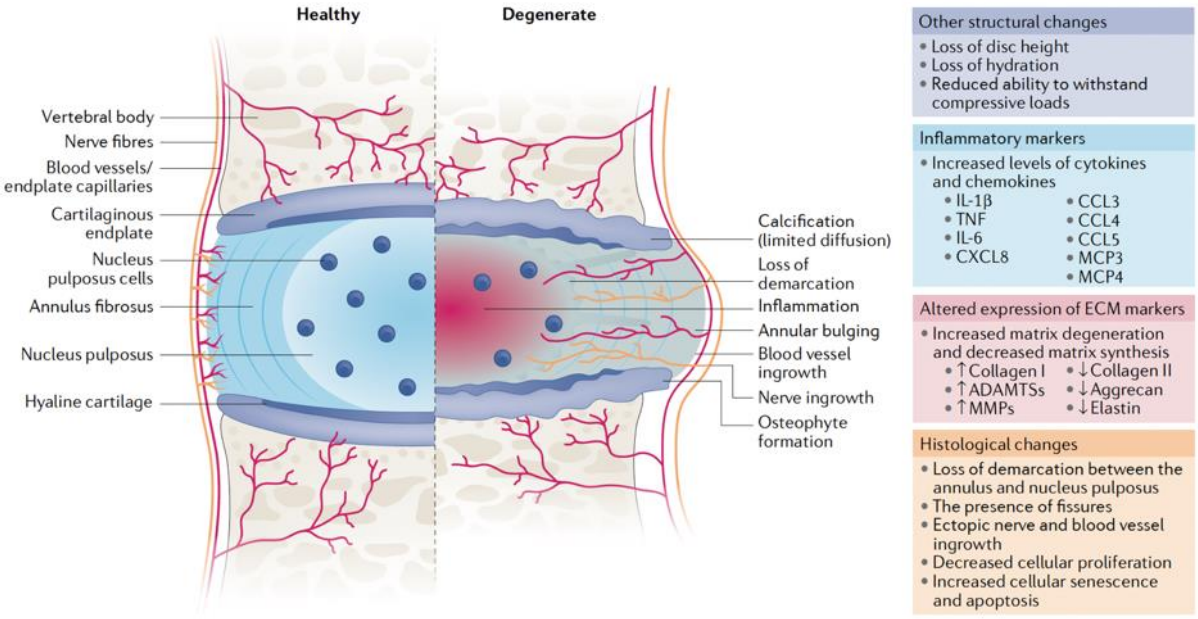

Figure 5. The intervertebral disc in health and disease. The healthy intervertebral (IVD) is characterized by three distinct anatomical regions: the cartilaginous endplates, annulus fibrosus and the gelatinous nucleus pulposus. Diffusion of nutrients occurs through capillary networks via the vertebral bodies. The degenerate disc is characterized by various structural and histological changes as well as an altered expression of extracellular matrix (ECM) markers and inflammatory markers. Degenerative changes are driven by inflammatory cytokines within the nucleus pulposus region, leading to altered matrix synthesis and reduced tissue integrity. Causes of low back pain in IVD degeneration are multifactorial; nevertheless, the occurrence of annular fissures provides an environment for the development of ectopic sensory nerve fibers and blood vessels, which are a major source of discogenic pain. *Binch, Abbie L A et al. "Cell-based strategies for IVD repair: clinical progress and translational obstacles." Nature reviews. Rheumatology vol. 17,3 (2021): 158-175. doi:10.1038/s41584-020-00568-w*[25]

Intervertebral disc degeneration can be described as a sequence of biochemical, mechanical, and structural transformations that impact disc functionality. Compositional changes in disc degeneration primarily involve the loss of proteoglycans, heightened cross-linking, and an increased prevalence of collagen I over collagen II. These alterations initially manifest in the nucleus pulposus and subsequently extend outward to affect the annulus fibrosus. Although many of these features are also observable during the normal aging process, they accelerate in

degeneration and are associated with discogenic pain, which arises due to spinal instability and the impingement of nerve roots within the spine[26].

The causes of disc degeneration remain largely unclear, but various factors have been linked to its onset and progression, including structural injury, genetic predisposition, age, inadequate metabolite transport, and an individual's history of spinal loading. Disc degeneration results primarily from genetic influences, although environmental factors also hold significance; it is not solely the result of wear and tear. Age and age-related factors play a crucial role in its development. Notably, disc degeneration exhibits a considerably higher prevalence and severity in the lower lumbar region compared to the upper lumbar region. The presence and severity of disc degeneration are typically assessed via MRI based on factors such as reduced signal intensity, disc bulging, narrowing of disc height, annular fissures, and the presence of osteophytes, either in isolation or in combination[27].

1.2.2.1. Low back pain

Usually, the process of vertebral pain has its origin in the degeneration of the intervertebral disc, approximately in 60% of cases, but also due to spondylolisthesis, scoliosis and canal stenosis. Although, in many cases, these pathologies are interrelated, and they will end up leading to a mechanical imbalance of the segment, today better known as "segmental instability". The vertebro-disc segment supports compression forces transmitted through the vertebral bodies, and the intervertebral discs, thanks to its capacity for elastic deformation, cushions and distributes these loads. Once these overloads cease, the disc recovers its original shape and the vertebrae return to their primitive physiological position. Segmental instability can then be defined as the mechanical failure of the functional unit of the spinal column.

When the intervertebral disc degenerates, occurs the onset of a process that is induced by the loss of its ability to return to an equilibrium position after the cessation of the load. The most obvious consequences of this functional loss of the intervertebral disc will secondarily favor the development of a series of alterations in the structures that make up the aforementioned functional unit.

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In 1978, Kirkaldy-Willis described the degenerative cascade, to name the succession of events that take place in the degeneration of the functional unit. It consists of three phases: dysfunction, instability and stabilization. It is necessary to differentiate mechanical instability from clinical or functional instability. Mechanical instability is the inability of the spine to support load. It is based on measurements obtained in flexion and extension radiographs and its approach can be surgical (using various fusion systems) or physiotherapeutic. Clinical instability includes clinical consequences, such as pain or neurological deficit. Its diagnosis and treatment is based on the neuromuscular control necessary to keep the spine stable during movement.

In the so-called dysfunction phase, the intervertebral disc begins its degenerative process, which is affected by a series of mechanical, biological, inflammatory, and metabolic factors, with overweight and cellular senescence also playing an important role. One of the most important factors in the onset of disc degeneration are alterations in the end plate which allows the passage of nutrients to the disc given that the disc is an avascular structure. The disc, whose nutrition depends on osmotic mechanisms coming from this vascular network, begins to suffer nutritional depletion that affects its cells and subsequently the extracellular matrix, which destabilizes its elastic properties and favors the formation of small fissures and tears in the discs components. The application of load to a damaged disc promotes rupture of the annulus fibrosus and herniation of the nucleus pulposus. This represents a threat to the integrity of the neural structures, as well as a complex inflammatory reaction, caused by mediators from the damaged disc (interleukins) responsible of low back pain intensity that is often discordant with the radiological degree of neural compression. This phenomenon is also favored by the appearance of a neovascularization process.

Another factor to take into account is that the degenerative processes of the intervertebral disc secondarily affect posterior structures of the spinal column, due to the spine functional unit is formed by the upper vertebral body, the disc, the lower vertebral body and posterior elements. such as the facet joints, which will always be affected by any malfunction of the intervertebral disc.

In this phase of instability, when the pattern of load transmission falls on a damaged disc, it motivates an increase in the load towards posterior vertebral elements (articular facets, laminae and ligamentum flavum); causing its hypertrophy. This initiates the process of spinal canal

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stenosis, in addition to generating the alteration in the transmission of loads, and both trabecular density and cortical mineralization of the anterior portion decrease. The vertebral body is thus more vulnerable to compressive fractures during ventral flexion of the trunk.

Likewise, in the instability phase, when the intervertebral disc is damaged, spinal mechanical hypermobility originates. In a normal disc the neutral zone is within the range of pain-free mobility. However, when the disc loses its biomechanical properties due to extracellular matrix depletion, the displacement caused by minimal loads is exaggerated. Secondarily, this alteration in the disc will cause mechanical changes that affect the articular processes, both with regard to the load and their ability to adapt to flexion-extension movements. These articular facets, when they receive a load that is abnormal, progressively deteriorate their articular cartilage and secondarily cause hypertrophic arthritic deformations and in this same process, these mechanical alterations also give rise to hypertrophy of the yellow ligament. These two circumstances in turn cause a stenosis of the spinal canal and the intervertebral foramina that can cause root compressions. Also the decrease in disc space, secondary to disc dehydration, will cause the ligaments to become more lax by reducing the disc space which causes abnormal intervertebral mobility, instability and olisthesis.

Also when the degenerated disc narrows diffusely, epiphyseal changes that occur in the subchondral bone begin, causing its deformation and the appearance of osteophytes that narrow the spinal canal even more.

In turn, the approach between the vertebral bodies causes functional elongation and laxity of the ligaments, losing their tension and their ability to stabilize intervertebral movement. This abnormal mobility facilitates greater deterioration of the functional unit of the vertebral column as a whole, which can cause a loss of spinal alignment in both the coronal and sagittal planes. In this situation, the so-called spinal stabilization system reacts by trying to immobilize the segment, initially contracting the paravertebral musculature, ultimately producing osteophytes. Although these osteophytes manage to contain the segmental instability, they are predictors of instability in the adjacent segments, which no longer manage to naturally transmit the load to the extremities. These functional alterations in the biomechanics of the vertebral segment are difficult to observe at the beginning of the degenerative process. Only in its most advanced phases is it possible to notice changes in its functioning and structure through imaging tests.

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Therefore, degeneration of the disc and facet joints can lead to this stenosis and can cause general instability of the spine.

An advanced process of intervertebral disc degeneration frequently leads to low back pain. It is estimated that as much as 84 % of the population will suffer from low back pain (LBP) at some point in their lifetime, with around 10 % of sufferers being chronically disabled. As such, LBP is one of the most prevalent musculoskeletal conditions affecting Western society, and its prevalence has increased over recent decades[28]. The socio-economic cost of LBP is also huge, with associated costs, in terms of lost productivity, disability benefits and direct and indirect health-care costs. Importantly, increases in both the size and average age of the population suggest that the prevalence and costs associated with LBP will continue to rise over future decades. While it is acknowledged that LBP is a multifactorial condition, a strong correlation with degeneration of the intervertebral disc has been shown in 40 % of cases. As degeneration proceeds, there are elevated levels of inflammatory cytokines, enhanced aggrecan and collagen degradation, and changes in disc cell phenotype[29]. The loss of hydrophilic matrix molecules leads to structural changes and spinal instability and is the main cause of herniation, sciatica and possibly stenosis. However, although a vast majority of adults over the age of 30 have some form of structural degeneration of one or more discs, this is not always accompanied by pain and may be a manifestation of ageing process. It is therefore likely that an event secondary to a structural deficit, such as injury or leakage of NP material through annular fissures results in recruitment of immune cells to the disc which then triggers pain generation. [27]
The origin of disc pain is still not understood. Pain most likely originates from receptors on nerve fibers on the surface of the posterior aspect of the intervertebral disc[30]. Pain activation may be due to leakage of disc-derived biologically active molecules which diffuse through annular tears of the annulus fibrosus[31].

2. INFLAMMATION AND CATABOLISM IN MUSCULOSKELETAL DISEASES

Although they are barely discussed simultaneously, IVD degeneration and the degeneration of joint cartilage in OA show marked similarities. On plain X-rays both, intervertebral disc degeneration and OA, present loss of joint space, osteophytes formation, subchondral cysts and sclerosis[32,33]. Furthermore, in both IVD and joint cartilage, the extracellular matrix (ECM) is

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composed of similar constituents (although in different ratio), and similar ECM-degrading machinery is present in the process of degeneration. Comparing the structure-function relationships of both the intervertebral disc and synovial joints, the separate tissues of the intervertebral disc are very similar to that of the diarthrodial joint: both types of joints are lined by cartilage, they are limited by an external ligament, and the joint space contains molecules that promote lubrication (lubricin and hyaluronan) and elevate the osmotic pressure (aggrecan). Indeed, even the presence of a band of nucleus pulposus tissue across the joint is not out of line with what is known of complex diarthrodial joints that contain cartilage and fibrocartilage discs and menisci.[34] Related to the function of the nucleus pulposus and the inner annulus, like the cells of the synovium, the resident disc cells do have the ability to mount a robust defense against bacterial attack[35,36] .

There are also some differences, such as the type of forces applied to the ECM and the absence of synovial fluid in IVDs[37–40]. No definitive treatment options are available to halt or reverse these conditions, in end-stage of IVDD and OA, spinal fusion and joint arthroplasty, respectively, are frequently the recommended option[41]. Taking into account these similarities, we investigated novel evidence in the modulation of the inflammatory and catabolic responses in both diseases.

There is a delicate balance between anabolic and catabolic processes in the articular joint and the intervertebral disc. When the balance breaks, there is an inequality between the synthesis and degradation of the ECM due to catabolic cell behavior[42–45]. Several systemic inflammatory factors have been described to incline this balance toward degeneration in both diseases, such as diabetes, obesity, smoking, and low-grade systemic infection[46–50]. Mechanical overloading is another established factor that induces local inflammation both in intervertebral disc and in cartilage[51,52]. Catabolic cell behavior is characterized by an increase in the expression of cytokines and matrix-degrading enzymes, and a downregulation of their inhibitors[53,54].

Cytokines, like tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1β), have a detrimental effect in intervertebral disc degeneration and OA[27,55,56]. These cytokines generate local inflammation in disc cells and chondrocytes by an upregulation of enzymes that degrade the ECM, matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and 5[41,57]. Simultaneously, their inhibitors are downregulated[58], pushing the ECM in a vicious cycle of degradation[8]. Several MMPs have been

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related to degeneration in the NP[58], showing remarkable similarity to those found in cartilage, although with different expression levels.

The degradation of the ECM results in a decrease in the production of proteoglycans in the cartilaginous matrices[59]. Consequently, less fluid is attracted, leading to a decrease in hydrostatic pressure[60]. This causes reduced joint space and an increase in tissue deformation. This shear stress is a mechanical cue for the cells to shift from collagen type II to collagen type I production, resulting in a more fibrous tissue[61], which has an inferior capacity to resist compressive loads[41].

Summing up, this pro-inflammatory and catabolic state has been related to the origin and progression of the pathophysiology of both the OA and the IVDD. Willing to further understand these molecular processes, this work presents the research done introducing monomeric C reactive protein (mCRP) role in OA and IVDD and investigating the role of the adipokine WISP-2 in OA.

3. CHAPTER 1. MONOMERIC C REACTIVE PROTEIN IN CARTILAGE AND INTERVERTEBRAL DISC

C-reactive protein (CRP) is an acute-phase inflammatory protein whose plasma levels sharply increase in response to injury, infection, and inflammation. It is a member of the pentraxin family, and it circulates prevalently in blood in its homopentameric form[62]. CRP plasma levels increase from around 1µg/mL to over 500 µg/mL within 24-72h upon severe tissue damage[63], thus CRP levels are a well-established biomarker for monitoring the inflammatory status in infections and other multiple diseases, including IVD herniation[64] and rheumatic diseases such as osteoarthritis (OA)[65].

The CRP molecule has a ligand-binding face that has two calcium ions per promoter. The calcium ions are important for the stability and binding of ligands. The opposite face interacts with C1q, from the complement pathway and with Fc receptors[66]. In the presence of calcium, CRP binds to polysaccharides such as phosphocholine (PCh) on microorganisms, triggering the classical complement pathway of innate immunity by activating C1q[67]. Transcriptional

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induction of the *CRP* gene mainly occurs in hepatocytes in response to increased levels of inflammatory cytokines, especially interleukin-6 (IL-6)[68].

The pentameric protein, also termed native CRP, is characterized by a discoid configuration of five identical non-covalently bound subunits of 206 amino acids long and about 23 kDa each one. It is synthesized primarily in liver hepatocytes but it has also been reported to be produced in other cell types such as smooth muscle cells[69], macrophages[70], endothelial cells[71], lymphocytes, and adipocytes[72].

Pentameric CRP can be irreversibly dissociated, with the resultant free subunits termed monomeric CRP (mCRP). The dissociation of CRP has been observed at either high concentrations of urea[73] or high temperatures in the absence of calcium[74]. The monomeric CRP (mCRP) differs from the pentameric CRP (pCRP) in its different antigenic, biological, and electrophoretic mobility[75] and in its expression of different neoepitopes[76]. These two distinct forms of CRP showed distinct biological functions in the inflammatory process. It has been proved that pCRP suppresses the adherence of platelets to neutrophils, whereas mCRP enhances these interactions[76]. This difference in function can be explained by the two isoforms binding to differing types of Fcγ-receptors involved in the signaling process. The mCRP isoform utilizes the low-affinity immune complex binding immunoglobulin G (IgG) receptor called FcγRIIIb (CD16b) on neutrophils and FcγRIIIa (CD16a) on monocytes, while pCRP binds to the low-affinity IgG receptor FcγRIIa (CD32)[77]. Evidence shows that pCRP tends to exhibit weak anti-inflammatory activities. In contrast, mCRP has marked pro-inflammatory properties both *in vitro* and *in vivo*[78] by promoting monocyte chemotaxis and the recruitment of circulating leukocytes to areas of inflammation via Fcγ-RI and Fcγ-RIIa signaling[79]. However, defining the pathophysiological role of CRP as a regulator of inflammatory processes remains still obscure. Elaborating on its role requires careful attention to which CRP conformation contributes to any described response.

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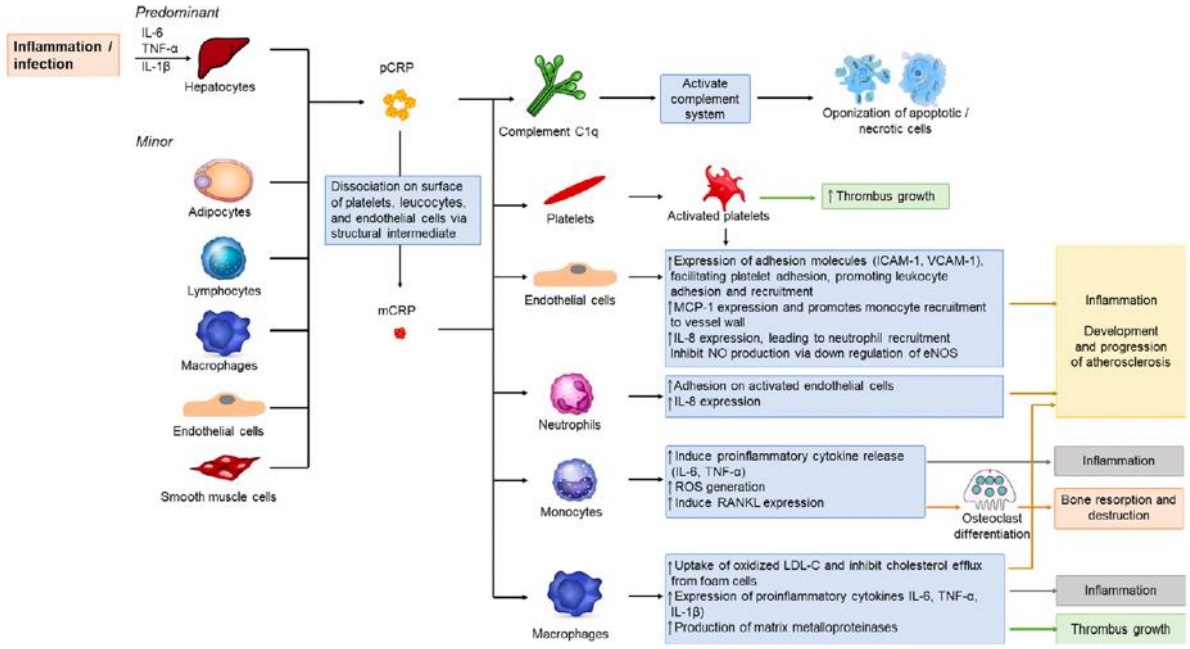

Figure 6. The biological role of C-reactive protein (CRP). *Pope, Janet E, and Ernest H Choy. "C-reactive protein and implications in rheumatoid arthritis and associated comorbidities." Seminars in arthritis and rheumatism vol. 51,1 (2021): 219-229. doi: 10.1016/j.semarthrit.2020.11.005*[80]

Several of the published OA and IVD disease literature assess CRP as a reference biomarker of an ongoing inflammatory process[64,81,82]. However, the role of mCRP in disc and joint pathophysiology has not been clarified yet.

Therefore, in this study, we evaluated first the functional role of mCRP as a modulator of the inflammatory response in chondrocytes. We evaluated the modulation of nitric oxide synthase type II (NOS2), an early inflammatory molecular mediator, as well as other relevant factors involved in the inflammatory response in human primary and immortalized mouse chondrocytes upon mCRP stimulation.

After, we tested these outcomes in intervertebral disc, evaluating the functional role of mCRP as a modulator of the inflammatory response in NP and AF human cells, assessing some pro-inflammatory and catabolic factors and the pathways involved in the signal transduction upon mCRP stimulation.

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4. CHAPTER 2. WISP2 IN CARTILAGE

Recent evidence from animal experiments and clinical studies highlights a role for Wnt signaling in OA pathology and other rheumatic diseases[83]. Progressive joint destruction in OA has been associated with overactivation of Wnt signaling[84]. The canonical Wnt pathway is initiated by the binding of Wnt ligands to frizzled receptors and co-receptors (LRP5/6), causing the inactivation of glycogen synthase kinase-3β (GSK-3β), which leads to intracellular β-catenin accumulation, since it is no longer marked for its degradation in the proteasome by phosphorylation of GSK3β, and nuclear translocation. Once in the nucleus, β-catenin interacts with the LEF/TCF family of transcription factors and activates Wnt target genes[85]. GSK-3β phosphorylation is a key step, and inhibition of this enzyme can lead to β-catenin stabilization and initiation of target gene expression independent of Wnt binding. Wnt ligands as Wnt-7b and Wnt target genes including Wnt-1 inducible signaling pathway protein 1 (WISP-1)[83] were found to be upregulated in OA cartilage, as well as nuclear β-catenin and the co-receptor LRP5[86]. Pro-catabolic factors such as IL-1β induced the expression of various Wnt proteins, resulting in the activation of β-catenin[87], and activation of Wnt/β-catenin signaling stimulated the expression of cartilage ECM-degrading MMPs[88]. The Wnt pathway has also been implicated in the development of RA, specifically, in processes of bone resorption and cartilage destruction; proteins like Wnt-1 are constitutively active in RA. Wnt activation produces inflammation, proliferation of the synovial membrane and degradation of the matrix of cartilage like seen in OA. This shows that the activation of the Wnt canonical pathway leads to an excess of catabolism and, therefore, cartilage degradation. However, the path Wnt is also necessary for the maintenance of articular cartilage, so it is an imbalance and excessive activation of the pathway what is deleterious.

Wnt-1 inducible signaling pathway protein 2 (WISP-2), also named CCN5, is a 27 kDa and 250 amino acids matricellular secreted protein that belongs to the CCN family. WISP-2 is considered a novel adipokine, as it was recently identified in a proteomics analysis of the secretome of human adipose tissue[89]. As a member of the CCN proteins family, WISP-2 shares the characteristic structure of this family. Most CCN proteins present an N-terminal secretion signal, or signal peptide, followed by four domains. All six CCN proteins have conserved domains with sequence homology. However, WISP-2 lacks the carboxy-terminal (CT) domain.

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Between two of the preserved domains, there is a highly variable region found between the different members of the CCN family, the hinge region, susceptible to proteolytic degradation by MMPs.

Although the WISP-2 receptor is unknown, as a secretion protein and member of the CCN family, one would expect that its activity would be mediated through membrane receptors. In fact, CCN proteins bind to receptors of surface, such as intergrins or co-receptors LRPs (low density lipoprotein receptor-related proteins). CCN proteins can modulate the Wnt pathway, in part, by binding to the LRP6 co-receptor (118), and, despite the lack of the CT domain, has recently been has demonstrated the phosphorylation of LRP6 by WISP-2 in murine adipocytes (115).

WISP-2 is highly expressed in mesenchymal stem cells (MSCs) and preadipocytes[90]. It was also found in smooth muscle cells (112), osteoblasts and chondrocytes (113). Several studies have described the involvement of WISP-2 in a wide variety of pathophysiological processes, such as embryonic development, cell differentiation and proliferation, tissue repair, motility and cancer (114). Grünberg *et al.* described WISP-2 as an important mediator of canonical WNT activation in adipogenic precursor cells, keeping the adipocytes in an undifferentiated state. They suggest that this adipokine may be involved in the development of obesity-related metabolic complications[91]. Frequently, WISP-2 is used as an indicator of canonical Wnt activation[92]. The identity of the WISP-2 receptor is currently unknown, although a Frizzled receptor would seem a likely possibility since the Frizzled co-receptor LRP5/6 is phosphorylated by WISP-2 in 3T3-L1 adipocytes[91].

To date, little is known about the role of this adipokine in cartilage pathophysiology. It has been demonstrated that WISP-2 expression was downregulated in human OA chondrocytes following sear stress[93]. WISP-2 has been found to be closely related to the pathogenesis of inflammatory arthritis[94] and the modulation of bone turnover[95]. In a previous study, we identified WISP-2 in synovium, infrapatellar fat pad, and chondrocytes obtained from OA patients[96], being its expression in infrapatellar fat pad adipocytes higher in OA patients than in healthy subjects. Therefore, the aim of this study was to analyze WISP-2 expression in OA chondrocytes, its effect on cartilage catabolism using recombinant WISP-2 protein and loss-of-function experiments and to elucidate the role of WNT/b-catenin pathway.

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OBJECTIVES

1. To verify the pathophysiological relevance of monomeric CRP (mCRP) in the etiology and progression of osteoarthritis.
   Addressed in ***Article 1. Monomeric C reactive protein (mCRP) regulates inflammatory responses in human and mouse chondrocytes.***

   1.1. To study the effect of mCRP in the inflammatory state of human primary healthy and osteoarthritic chondrocytes.
   1.2. To study the effect of mCRP in the inflammatory state on the murine chondrocyte cell line ATDC5.
   1.3. To study the NF-κβ role in mCRP signaling on the murine chondrocyte cell line ATDC5.

2. To verify the pathophysiological relevance and mechanism of action of monomeric CRP (mCRP) in healthy and degenerative intervertebral disc cells and in the etiology and progression of IVD degeneration.
   Addressed in ***Article 2. Monomeric CRP regulates inflammatory responses in human intervertebral disc cells.***

   2.1. To study the effect of mCRP in the inflammatory state of human primary healthy and degenerative intervertebral disc annulus fibrosus cells.
   2.2. To study the effect of mCRP in the inflammatory state of human nucleus pulposus cell line HNPSV-1.
   2.3. To study the mediators involved in mCRP intracellular signal transduction in human primary healthy and degenerative intervertebral disc annulus fibrosus cells.
   2.4. To investigate if mCRP can be located in human healthy and degenerative disc tissues.

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3. To study the Wnt signaling role in OA physiopathology, verifying the involvement of WISP2 in cartilage degradation.
   Addressed in ***Article 3. WISP-2 modulates the induction of inflammatory mediators and cartilage catabolism in chondrocytes.***

   3.1. To study WISP2 constitutive expression along the murine chondrocyte cell line ATDC5 differentiation.
   3.2. To study WISP2 mRNA and protein expression in healthy and OA human chondrocytes.
   3.3. To study the effects of recombinant human WISP2 protein on chondrocyte catabolism.
   3.4. To study the involvement of IL-1β and WNT/β-catenin downstream signaling pathways in the recombinant human WISP2 protein mechanism of action.
   3.5. To study the effects of WISP2 gene knockdown on chondrocyte catabolic markers and on WNT/β-catenin pathway.
   3.6. To study the effect of WISP2 siRNA knockdown on IL-1β-stimulated chondrocytes.
   3.7. To study the effect of the canonical WNT/β-catenin signaling on WISP2 siRNA knockdown mediated chondrocyte catabolism.

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METHODOLOGY

1. REAGENTS

Fetal bovine serum (FBS), lipopolysaccharide (LPS), human recombinant interleukin (IL)-1β, human recombinant tumor necrosis factor alpha (TNF-α), human recombinant IL-6, human recombinant leptin, and glycogen synthase kinase 3 (GSK-3) inhibitor 6-bromoindirubin-3'-oxime (BIO), were obtained from Sigma-Aldrich (St Louis, MO, USA). Dulbecco´s modified Eagle's medium (DMEM)/Ham's F12 medium, trypsin-ethylenediaminetetraacetic acid, L-glutamine, and penicillin-streptomycin mixture were obtained from Lonza Group (Basel, Switzerland). Pronase and collagenase P were obtained from Roche Applied Science (Penzberg, Germany). Recombinant mCRP (Roosevelt University, Schaumburg, IL, USA) was produced in *E. coli*, it had both cysteines replaced with alanine residues and was solubilized by an acylation technique (C-rmCRP) in 25mM NaPBS (pH7.4), purified and tested in several bioassays to confirm its equivalence to biological mCRP from the pentameric form and free of contaminating endotoxin. The final endotoxin level of all protein solutions was below the detection limit 0.06 EU/mL of the assay [97]. Human recombinant adiponectin and visfatin were obtained from BioVendor (Karasek, Brno, Czech Republic). Human recombinant WISP-2 protein (hrWISP-2) was purchased from PeproTech (Rocky Hill, NJ, USA).

2. CELL CULTURE CONDITIONS AND TREATMENTS

For the mCRP work, the murine chondrogenic cell line ATDC5 (RIKEN Cell Bank, Tsukuba, Japan) was cultured in DMEM/Ham's F12 supplemented with 5% FBS, 10 µg/ml human transferrin, 3 × 10M sodium selenite, 4mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin.

For the WISP2 work, the murine chondrogenic cell line ATDC-5 (purchased from RIKEN Cell Bank, Tsukuba, Japan) was cultured in DMEM–Ham's F-12 medium supplemented with 5% FBS, 10 µg/mL human transferrin, $3x10^{-8}$ M sodium selenite, 4mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin. Chondrogenic ATDC-5 cells were differentiated into mature chondrocytes as previously described[98]. Briefly, cells were seeded at a density of $6x10^4$ cells per well in 6-well plates with ATDC-5 standard medium supplemented with insulin (10 µg/mL). The differentiation medium was replaced every two days for 21 days. Differentiation

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was qualitatively characterized by increased formation of cell nodules. In other experiments (data not shown), differentiation was further analyzed by a sequential increase in the levels of type II collagen, aggrecan and type X collagen mRNA expression, as previously published[99].

The immortalized human juvenile costal chondrocyte cell line T/C-28a2 (a kind gift from Dr. M.B. Goldring, Hospital for Special Surgery, NYC, USA) was cultured in DMEM–Ham's F-12 medium supplemented with 10% FBS, 4mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin.

Human primary chondrocytes were isolated from healthy, or OA articular cartilage samples obtained from knee and hip joints of patients undergoing total joint replacement surgery. All the procedures were made under informed patient consent and approved by the local ethics committee, according to the declaration of Helsinki. Human primary chondrocytes were isolated and cultured as previously described[100]. They were cultured in DMEM/Ham's F-12 medium supplemented with 10% of FBS, 4mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin. Cells were seeded in monolayer up to the high density and used in the first passage of culture to avoid dedifferentiation.

Chondrocytes were seeded in six-well plates ($2.5x10^5$ cells per well) and treated in serum free conditions as indicated in each case, after overnight serum starvation. All the treatments concentrations were selected based on previous studies and in the absence of chondrocyte cell toxicity.

Human NP immortalized cell line HNPSV-1 [101] (Tokai University School of Medicine, Isehara, Japan) was cultured in DMEM/Ham's F12 supplemented with 10% FBS, 4mM L-glutamine, 50 units/ml penicillin and 50 µg/mL streptomycin. In these experiments we used a cell passage range of 13-22.

Healthy IVD samples were obtained from deceased multi-organ donors. Degenerative IVD samples were obtained, under written patient consent, from patients undergoing discectomy. Inclusion criteria: Male or female patients undergoing discectomy diagnosed with spinal stenosis, adult degenerative scoliosis, disc herniation and disc degeneration (Pfirrmann grade > 3-4). Exclusion criteria: Previous spinal malignancies or infections, previous spinal surgery and patients diagnosed with metabolic diseases. All IVD samples were obtained under permission from the local ethics committee, according to the declaration of Helsinki. AF cells were isolated and cultured as previously described [102]. Briefly, AF tissue aseptically dissected was diced and properly rinsed with phosphate-buffered saline (PBS). After removing PBS, tissue was

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incubated for 20 min with pronase at a final concentration of 1 mg/mL in DMEM/Ham's F12 at 37°C. Then pronase was removed and the tissue was rinsed 2-3 times with PBS. After removing PBS, digestion continued with collagenase P at a final concentration of 1 mg/ml in DMEM/Ham's F12 with 10% FBS. Tissue was incubated for 4–6 hours in agitation at 37°C. The resulting cell suspension was filtered with a 40μm nylon cell strainer (BD Biosciences Europe, Erembodegem, Belgium) to remove debris. Cells were centrifuged, washed with PBS, resuspended in DMEM/Ham's F12 supplemented with 10% FBS, 4mM L-glutamine, 50 units/ml penicillin and 50 μg/mL streptomycin, counted, and plated in 12-well culture plates. Cells were maintained in culture for a maximum of 10 days for expansion. All AF cells used in these experiments were at passage number 2.

AF cells and HNPSV-1 cells were seeded in 6-well plates ($2.5x10^5$ cells/well) and treated with 10, 25 or 50 μg/mL mCRP, 100 ng/mL LPS or 0.1ng/mL IL-1β, after serum starvation for 4 hours. All the concentrations were selected based on previous studies [78] and in the absence of IVD cell toxicity. All treatments were done in serum-free conditions after 4 hours of serum starvation and lasted 24 hours for the pro-inflammatory response experiments or 15 minutes for the intracellular signal transduction experiments.

3. NITRITE ASSAY

Nitrite accumulation was measured in the culture medium by the Griess reaction, as previously described[100,103,104]. Cell culture supernatant was incubated at room temperature with Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl), and the absorbance was measured at 550 nm in a microplate reader (Titertek Multiscan; Labsystems, Helsinki, Finland). Fresh culture medium was used as a blank, and nitrite concentration (μM) was calculated from a sodium nitrite standard curve.

4. IMMUNOHISTOCHEMICAL ASSAYS

Sections 4 μm thick obtained from human AF or NP tissue were mounted on silanized coated slides (Dako-Agilent, Santa Clara, CA, USA). Epitope retrieval was performed in a PT-Link (Dako-Agilent) at high pH for 20 minutes. Then the slides were automatically immunostained in an Autostainer-Link 48 (Dako-Agilent), employing the monoclonal anti-C reactive protein antibody, clone CRP-8, source mouse (Sigma-Aldrich, St. Louis, MO, USA), diluted 1:100 and 30-minute incubation time. As detection system, we used EnVision FLEX/HRP (Dako-Agilent),

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for 30 minutes, and 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen, for 5 minutes.

5. RNA ISOLATION AND REAL-TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT^2-qPCR)

Total RNA was isolated from cell culture with NZYol (NZYTech, Lisbon, Portugal) and E.Z.N.A.® Total RNA Kit I (Omega Bio-tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions; and reverse-transcribed using NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). Then, SYBR-green-based quantitative real-time PCR (RT-qPCR) was performed in Stratagene MX3005P thermal cycler as previously described [105] using a standard protocol with RT^2 SYBR Green qPCR Mastermix and specific PCR primers (Qiagen, Hilden, Germany) No-template controls were included to eliminate any non-specific amplification, and melting curves were generated to ensure a single gene-specific peak. Gene expression changes were determined by the comparative $\Delta\Delta C$t method in MxPro qPCR Software version 4.10 (Stratagene, La Jolla, CA, USA), and expressed as relative fold changes compared to control (C-) and normalized to GAPDH housekeeping gene.

6. PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

After treatment, cells were rapidly washed with ice-cold phosphate-buffered saline and scraped in lysis buffer (10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5% Triton X-100, 1 mM PMSF), freshly supplemented with protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA), then the total cell lysates were centrifuged at 14,000g for 20 min. SDS-PAGE and blotting procedures were performed as previously described[106]. Immunoblots were incubated with the specific antibody. The immune complexes were detected using anti-rabbit, anti-mouse (both from GE Healthcare, Chicago, IL, USA) or anti-goat (Santa Cruz Biotechnology, Dallas, TX, USA) horseradish-peroxidase-labeled secondary antibodies diluted 1:5000 and visualized with Immobilon Western Detection kit (Millipore, MA, USA). To verify equivalent protein loading, the membranes were stripped 15 minutes in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7), blocked and incubated with anti-GAPDH antibody diluted 1:2000 (Sigma-Aldrich, MO, USA). In all figures showing images of gels, the bands for each picture were obtained from the same gel, although they may have been

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spliced for clarity. The images were captured with ChemiDoc MP Imaging System and analyzed with Image Lab 6.0.1 Software, both from Bio-Rad Laboratories, Inc. (Hercules, CA, USA)

7. WISP-2 GENE KNOCKDOWN

For siRNA transfection experiments, T/C-28a2 cells and human primary OA chondrocytes were seeded at $2x10^5$ cells per well in 6-well plates and incubated overnight with DMEM/Ham's F-12 with 10% FBS. Before transfection, the medium was changed to serum and antibiotics free medium. Transfections were performed using TriFECTa RNAi Kit following manufacturer's instructions (Integrated DNA Technologies, Coralville, IA, USA). Gene silencing was made with 10 nM of three DsiRNAs that specifically target WISP-2 and 10 nM of nontargeting universal negative control RNA duplex that does not interact with any known sequence. Transfection with siRNA duplexes was performed using the cationic lipid siLentFect Lipid Reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's recommendations. Incubation was continued for 48 hours after siRNA transfection. WISP-2 gene knockdown was verified at mRNA and protein levels and supported with two control sequences provided by the manufacturer: a TYE 563-labeled transfection control and a positive control DsiRNA that targets the *HPRT1* gene and is prevalidated to give more than 90% knockdown of HPRT (presented as Supplementary data). At 48 hours after transfection, cells were treated with recombinant human IL-1β (0.5 ng/mL) for 24 hours. A specific pharmacological inhibitor (BIO 1μM) was added 1 hour before stimulation.

8. STATISTICAL ANALYSIS

Data are reported as mean ± standard error of the mean (SEM) of at least three independent experiments. Statistical analyses were performed with GraphPad Prism 9.3.1 software (GraphPad Software, La Jolla, CA, USA). When assuming a normal distribution, we used two-sided unpaired t test with Welch's correction or one-way ANOVA test corrected with Bonferroni's multiple comparison test or followed by Fisher´s LSD test, only when we performed few planned comparisons, *p*-values are not corrected for multiple comparisons, they apply individually to each value reported and not to the entire family of comparisons. When normal distribution was not assumed, we used Mann-Whitney test. *P*-values less than 0.05 were considered significant.

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RESULTS

1. Article 1. Monomeric C reactive protein (mCRP) regulates inflammatory responses in human and mouse chondrocytes.

As shown in Fig. 1A, mCRP at both 10 and 50 μg/ml induces a significant accumulation of nitrite levels in the supernatant of cultured primary human chondrocytes from OA patients. Accordingly, mCRP increases NOS-2 mRNA expression and protein levels, as confirmed RT-qPCR and western blot analysis' densitometry, respectively (1B). In a similar manner, mCRP also increased both mRNA and protein expression of COX-2 (C), MMP13 (D), VCAM1 (E), IL-6 (F), IL-8 (G) and LCN2 (H).

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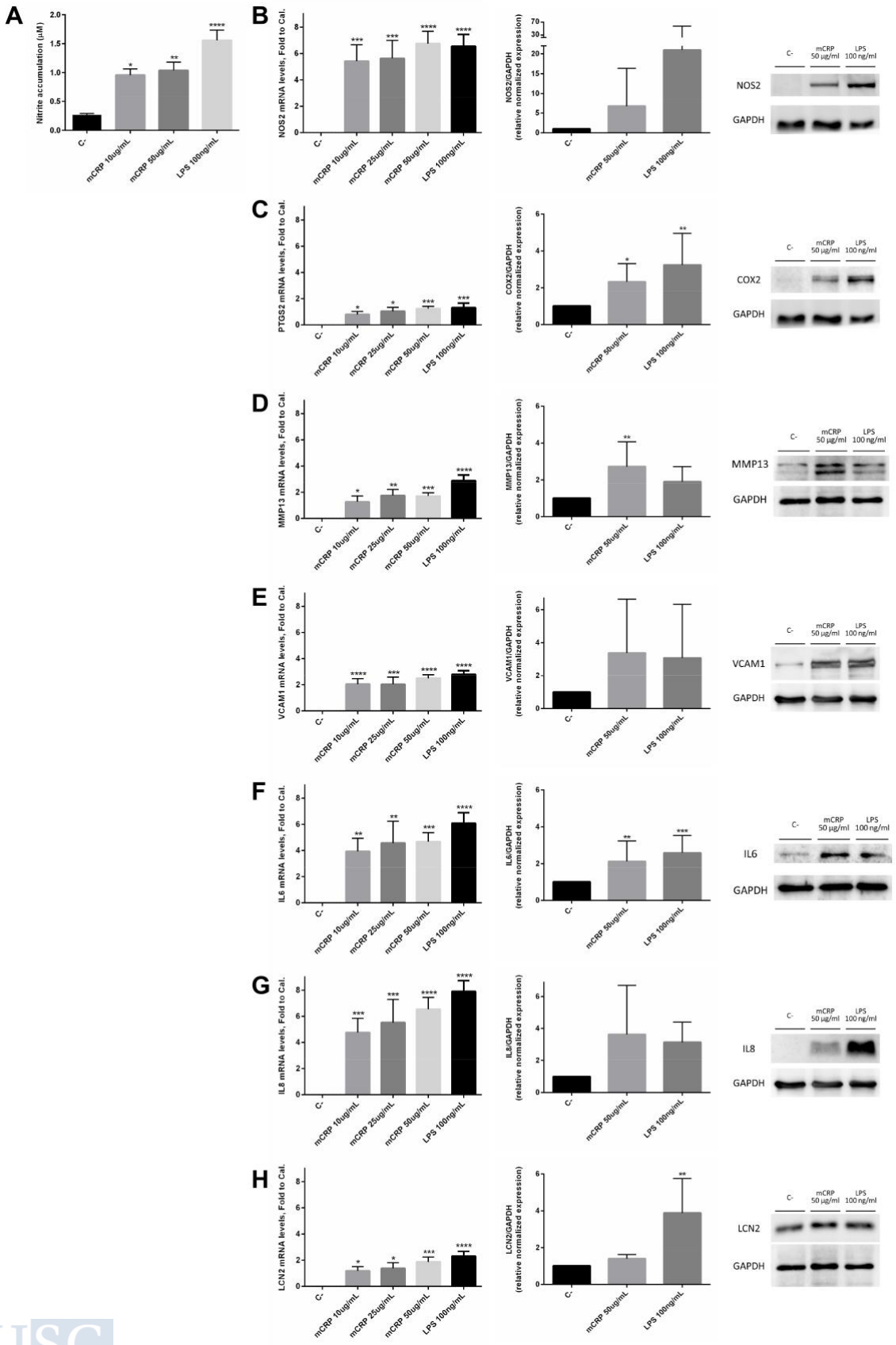
A
Nitrite accumulation (µM)
C-
mCRP 10ug/mL
mCRP 50ug/mL
LPS 100ng/mL
B
NOS2 mRNA levels, Fold to Cal.
mCRP 25ug/mL
NOS2/GAPDH (relative normalized expression)
mCRP 50ug/mL
NOS2
GAPDH
C
PTGS2 mRNA levels, Fold to Cal.
COX2/GAPDH (relative normalized expression)
COX2
D
MMP13 mRNA levels, Fold to Cal.
MMP13/GAPDH (relative normalized expression)
MMP13
E
VCAM1 mRNA levels, Fold to Cal.
VCAM1/GAPDH (relative normalized expression)
VCAM1
F
IL6 mRNA levels, Fold to Cal.
IL6/GAPDH (relative normalized expression)
IL6
G
IL8 mRNA levels, Fold to Cal.
IL8/GAPDH (relative normalized expression)
IL8
H
LCN2 mRNA levels, Fold to Cal.
LCN2/GAPDH (relative normalized expression)
LCN2
50 µg/ml
100 ng/ml

Fig. 1. mCRP pro-inflammatory effect on human primary osteoarthritis chondrocytes. Human primary OA chondrocytes in culture were challenged with 10, 25, or 50 μg/ml mCRP, or with 100 ng/ml LPS as positive control for 24 h, after 4 h serum starvation. A Nitric oxide production was evaluated by determining nitrite concentration (μM) in culture medium after 24 h treatment with 10, or 50 μg/ml mCRP, or with 100 ng/ml LPS as positive control using Greiss reaction. B–H (left panels) mRNA expression was determined by RT-qPCR. mRNA levels are presented as fold change relative to control (C−). B–H (middle and right panels) Protein expression was measured by western blot. Data from densitometric analyses normalized to GAPDH are shown as relative-to-control (C−) values. Data expressed as mean ± SEM of at least three independent experiments. Comparisons referred to control (C−): *P < 0.05, **P < 0.01, ***P < 0.001. mCRP monomeric C-reactive protein, LPS lipopolysaccharide, NOS2 nitric oxide synthase 2, PTGS2 prostaglandin-endoperoxide synthase 2, COX2 cyclooxygenase 2, MMP13 matrix metalloproteinase 13, VCAM1 vascular cell adhesion molecule 1, IL6 interleukin 6, IL8 interleukin 8, LCN2 lipocalin 2, GAPDH glyceraldehyde 3-phosphate dehydrogenase.

The induction of pro-inflammatory mediators by mCRP is also evident in human primary chondrocytes from healthy patients, suggesting that mCRP can modify the cell status by promoting the development of a pro-inflammatory environment in the cartilage (Fig. 2 from A to G). In all the experiments with human cells, LPS was used as positive control of inflammatory induction. Of note, the chondrocyte response to 50 μg/ml mCRP was comparable to that of 100 ng/ml LPS for the analyzed parameters.

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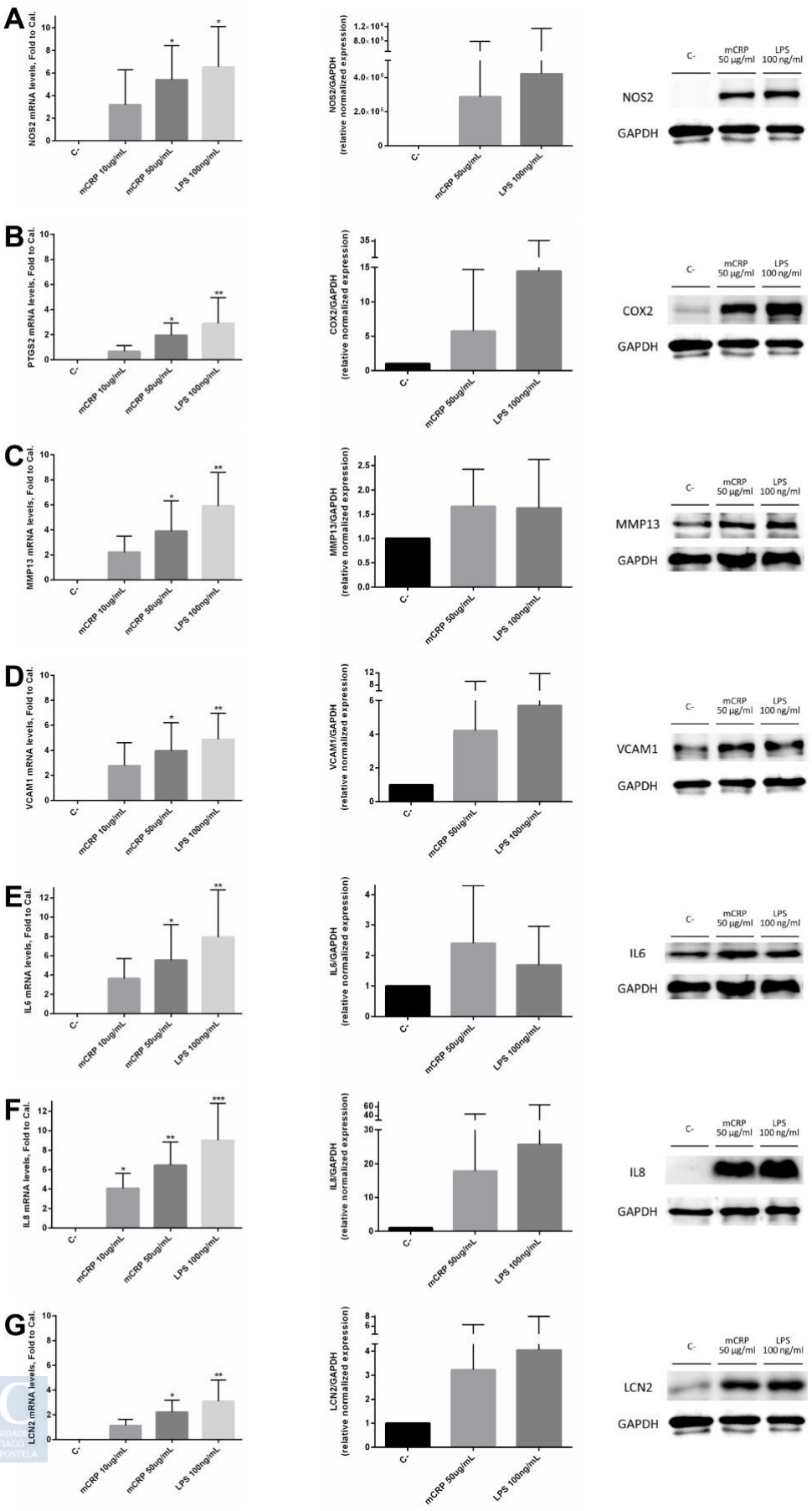

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Fig. 2. mCRP proinflammatory effect on human primary healthy chondrocytes. Human primary healthy chondrocytes in culture were challenged with 10 or 50 μg/ml mCRP, or with 100 ng/ml LPS as positive control for 24 h, after 4 h of serum starvation. A–G (left panels) mRNA expression was determined by RT-qPCR. mRNA levels are presented as fold change relative to control (C−). A–G (middle and right panels) Protein expression was measured by western blot. Data from densitometric analyses, normalized to GAPDH, are shown as relative-to-control (C−) values. Data expressed as mean ± SEM of at least three independent experiments. Comparisons referred to control (C−): *P < 0.05, **P < 0.01, ***P < 0.001. mCRP monomeric C-reactive protein, LPS lipopolysaccharide, NOS2 nitric oxide synthase 2, PTGS2 prostaglandin-endoperoxide synthase 2, MMP13 matrix metalloproteinase 13, VCAM1 vascular cell adhesion molecule 1, IL6 interleukin 6, IL8 interleukin 8, LCN2 lipocalin 2.

To further confirm our results, we tested mCRP activity in the ATDC5 murine chondrocyte cell line, a very useful tool for studying molecular and cellular mechanisms of cartilage inflammation in vitro[100].

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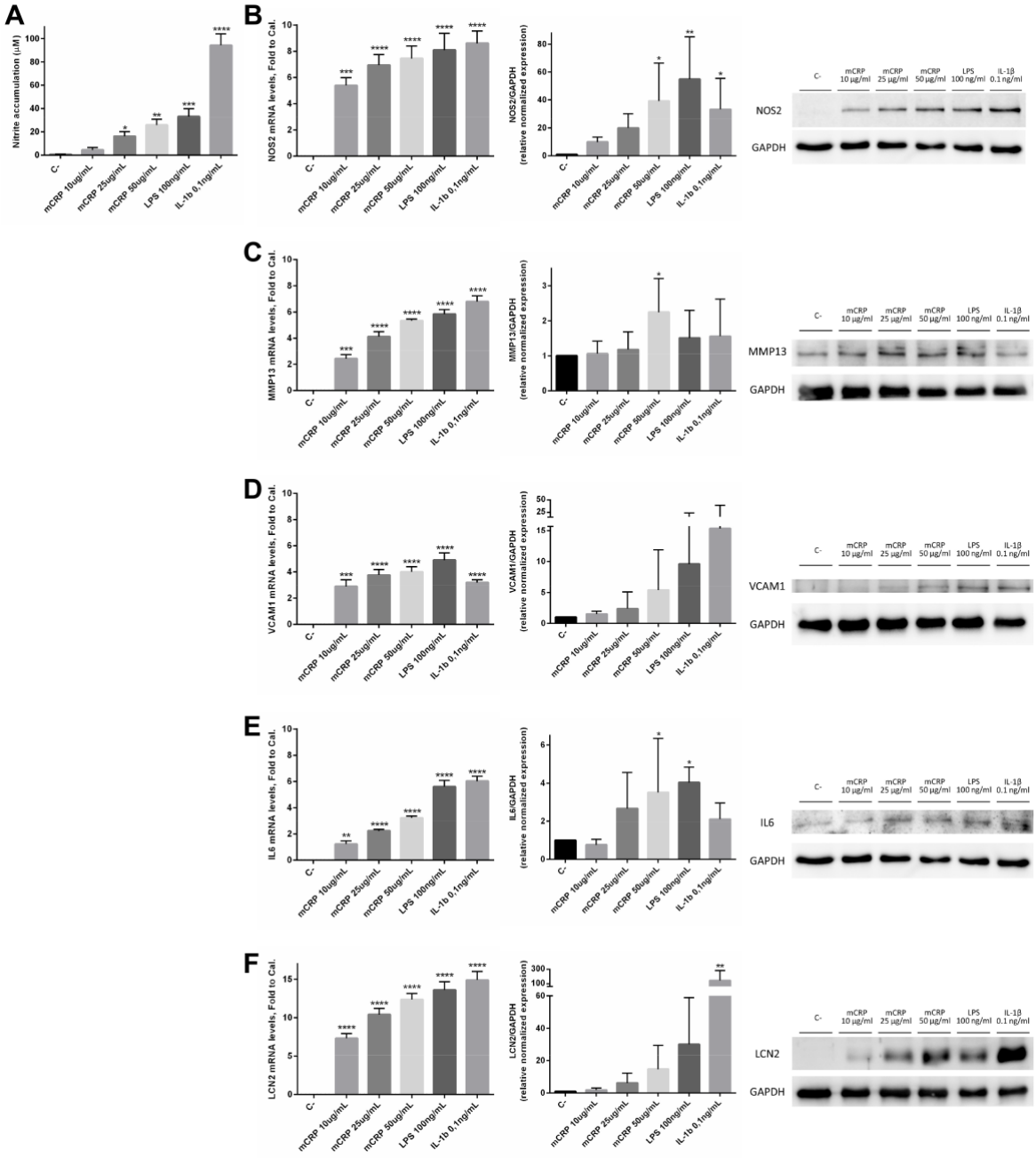

Fig. 3. mCRP pro-inflammatory effect on murine chondrocyte cell line. Murine chondrocyte cells were challenged with 10, 25, or 50 µg/ml mCRP, or with 100 ng/ml LPS or 0.1 ng/ml IL-1b as positive controls for 24 h, after overnight serum starvation. A Nitric oxide production was evaluated by determining nitrite concentration (µM) in culture medium after 24 h treatment using Greiss reaction. Data expressed as mean ± SEM of at least six independent experiments. B–F (left panels) mRNA expression was determined by RT-qPCR. mRNA levels are presented as fold change relative to control (C−) Data expressed as mean ± SEM of at least three independent experiments. B–F (middle and right panels) Protein expression was measured by western blot. Data from densitometric analyses normalized

to GAPDH are shown as relative-to-control (C−) values. Data expressed as mean ± SEM of at least three independent experiments. Comparisons referred to control (C−): *P < 0.05, **P < 0.01, ***P < 0.001. mCRP monomeric C-reactive protein, LPS lipopolysaccharide, IL-1b interleukin 1 beta, NOS2 nitric oxide synthase 2, MMP13 matrix metalloproteinase 13, VCAM1 vascular cell adhesion molecule 1, IL6 interleukin 6, LCN2 lipocalin 2, GAPDH glyceraldehyde 3-phosphate dehydrogenase.

As shown in Fig. 3, mCRP significantly induces nitrite accumulation in ATDC5 cells (3A), and highly augments NOS2 (B), MMP13 (C), VCAM1 (D), IL-6 (E) and LCN2 (F), either at mRNA or protein level, as previously observed in human primary chondrocytes.

To gain further insights into the action mechanisms responsible by mCRP-induced pro-inflammatory effects in chondrocytes, we analyzed the involvement of NF-kB signaling pathway. NF-κB is a transcription factor that has been widely demonstrated to be involved in cellular responses to different signals of stress, such as cytokines, free radicals, heavy metals, and bacterial or viral antigens. Over-expression or inappropriate activation of NF-κB is implicated in many pathological mechanisms of diseases, ranging from inflammation to cancer[107]. PDTC is a thiol compound, which has been considered an effective NF-kB inhibitor[108]. Thus, in order to investigate the potential protective effects of NF-κB inhibition on mCRP-induced pro-inflammatory mediators' expression, PDTC was used in combination with mCRP in chondrocytes.

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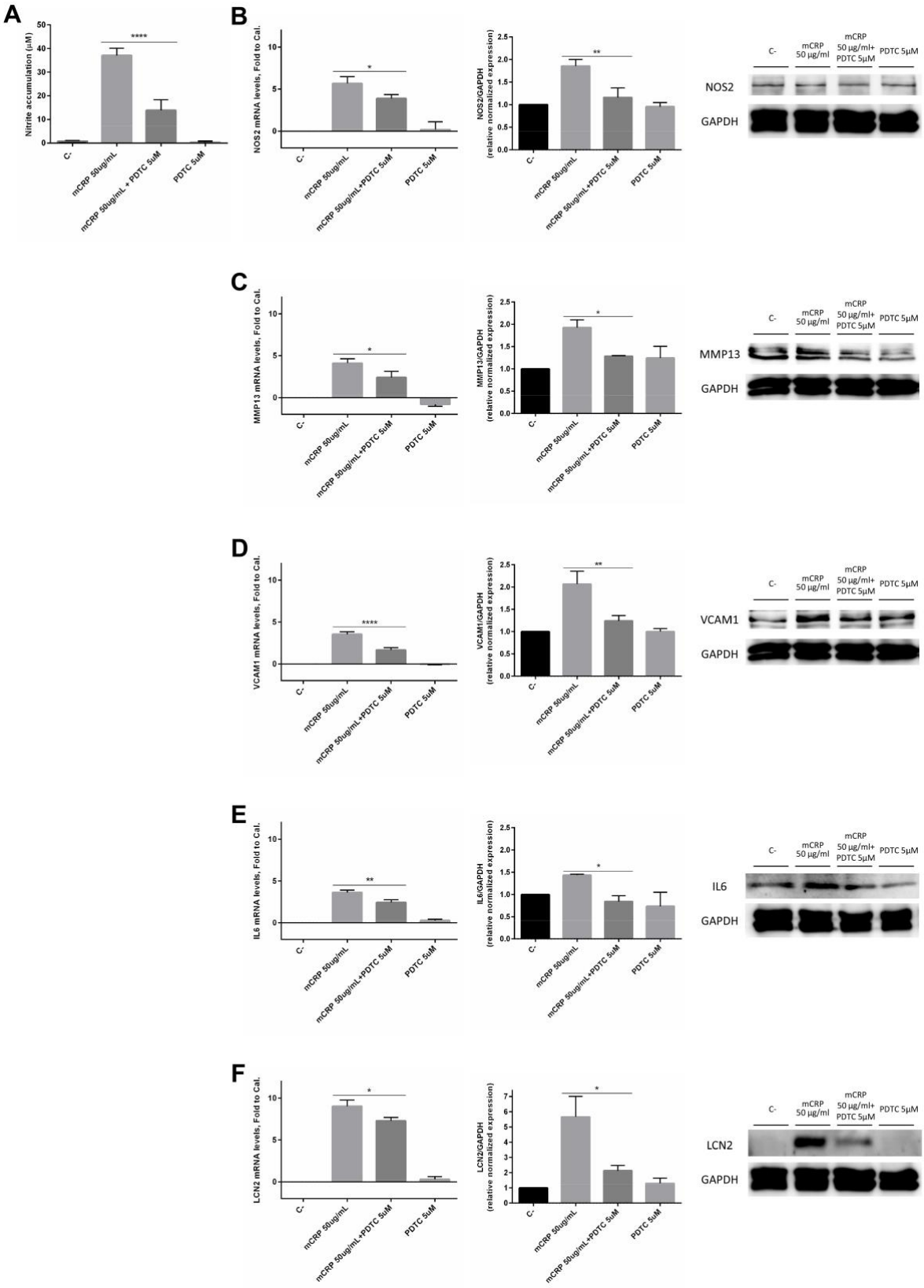

Fig. 4. Nf-κβ role in mCRP signaling: Nf-κβ inhibition with PDTC in murine chondrocyte cell line. Cells starved for serum overnight were pre-treated for 1 h with PDTC 5 µM. After keeping the culture

medium, were challenged with 50 μg/ml mCRP for 24 h. A Nitric oxide production was evaluated by determining nitrite concentration (μM) in culture medium after 24 h treatment using Griess reaction. Data expressed as mean ± SEM of six independent experiments. Comparisons mCRP 50 μg/mL vs. mCRP 50 μg/mL + PDTC 5 μM. B–F (left panels) mRNA expression was determined by RT-qPCR. mRNA levels are presented as fold change relative to control (C−). Data are expressed as mean ± SEM of at least five independent experiments. B–F (middle and right panels) Protein expression was measured by western blot. Data from densitometric analyses, normalized to GAPDH, are shown as relative-to-control (C−) values. Data expressed as mean ± SEM of at least three independent experiments. Comparisons mCRP 50 μg/mL vs. mCRP 50 μg/mL + PDTC 5 μM. *P < 0.05, **P < 0.01, ***P < 0.001. mCRP monomeric C-reactive protein, NF-kB nuclear factor kappa B, PDTC pyrrolidine dithiocarbamate, NOS2 nitric oxide synthase 2, MMP13 matrix metalloproteinase 13, VCAM1 vascular cell adhesion molecule 1, IL6 interleukin 6, LCN2 lipocalin 2.

As shown in Fig. 4 (panels A and B), PDTC significantly reduce both nitrite accumulation and NOS2 expression (at mRNA and protein levels) in mCRP-stimulated ATDC5 cells. In addition, PDTC also significantly reduced the expression of MMP13, VCAM1, IL-6, and LCN2 induced by 50 μg/ml mCRP (Fig. 4, panels C to F)

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2. Article 2. Monomeric CRP regulates inflammatory responses in human intervertebral disc cells.

As shown in Figure 1, mCRP increases IL-6, IL-8, LCN2, VCAM1, NOS2, COX2, and MMP13 mRNA expression and protein levels in cultured human primary AF cells from patients with IVD degeneration, as confirmed by RT-qPCR and western blot analysis.

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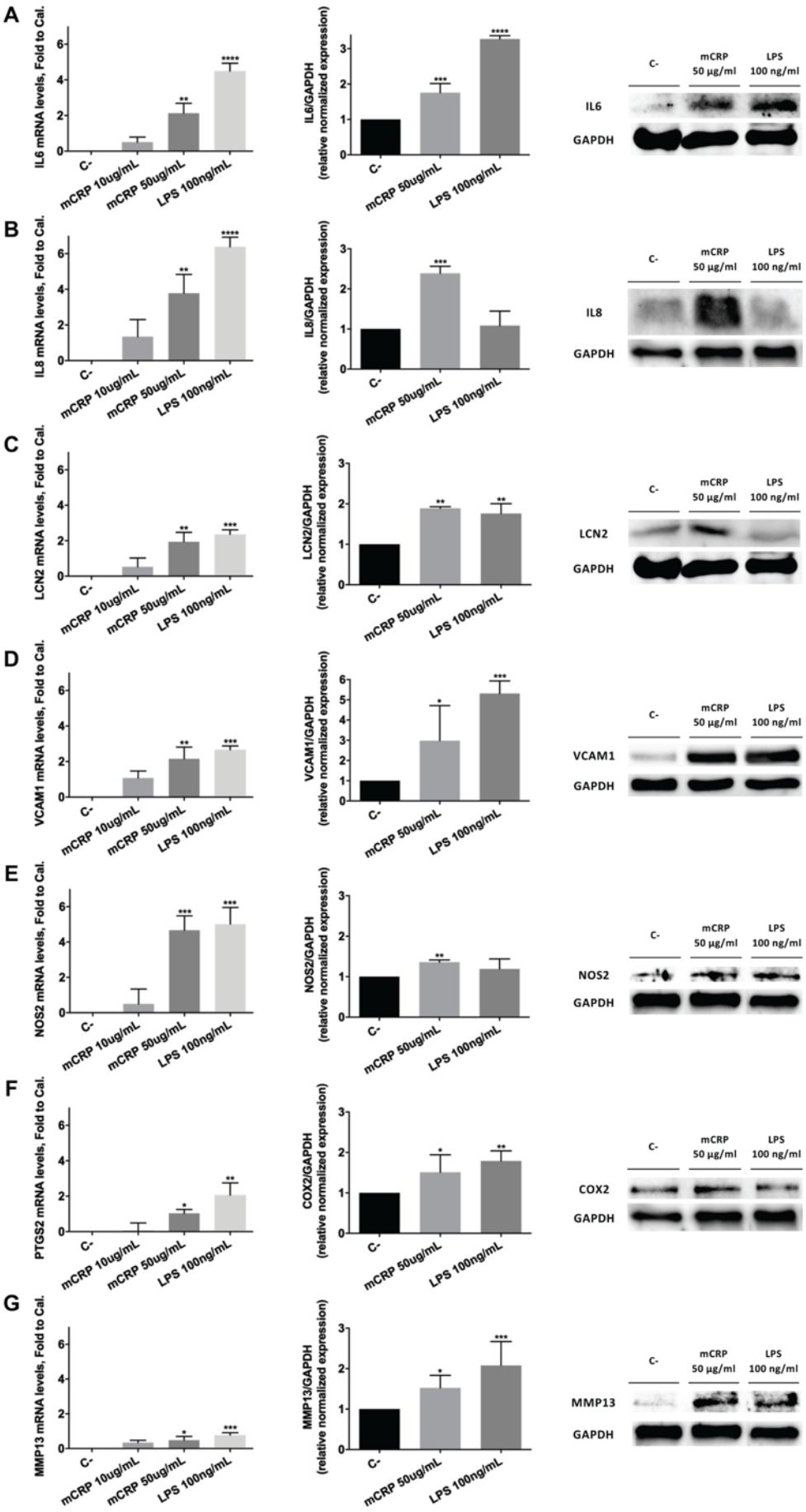

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Fig. 1. Monomeric CRP (mCRP) proinflammatory effect on human primary degenerative intervertebral disc (IVD) annulus fibrosus (AF) cells. Human primary AF cells derived from degenerative IVD in culture were challenged with 10 or 50 μg/ml mCRP, or with 100 ng/ml lipopolysaccharide (LPS) as positive control for 24 hours, after four hours of serum starvation. a) to g) Left panels. Messenger RNA (mRNA) expression was determined by quantitative real-time polymerase chain reaction. mRNA levels are presented as fold change relative to control (C-). a) to g) Middle and right panels. Protein expression was measured by western blot. Data from densitometric analyses normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown as relative-to-control (C-) values. Data are expressed as mean (standard error of the mean) of at least three independent experiments. Comparisons referred to control (C-): *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Cal., calibrator (refers to control (C-) condition); COX2, cyclooxygenase 2; IL, interleukin; LCN2, lipocalin 2; MMP13, matrix metalloproteinase 13; NOS2, nitric oxide synthase 2; PTGS2, prostaglandin-endoperoxide synthase 2; VCAM1, vascular cell adhesion molecule 1.

The induction of proinflammatory mediators occured in a dose-dependent manner after 24 hours.

The upregulation of these inflammatory and catabolic factors by mCRP was also determined in human primary AF cells from healthy patients, suggesting that mCRP can modify the healthy cell status by promoting the development of a proinflammatory environment in the IVD (Figures 2a to 2g). In all the experiments with human cells, LPS inflammatory stimulus was used as control of inflammatory induction.

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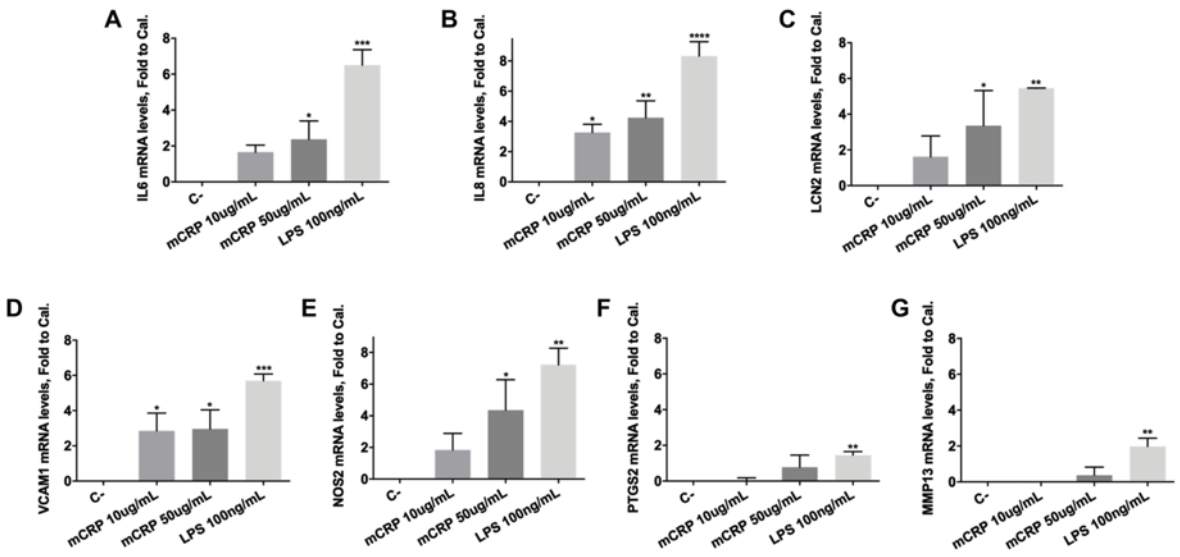

Fig. 2. Monomeric CRP (mCRP) proinflammatory effect on human primary healthy intervertebral disc (IVD) annulus fibrosus (AF) cells. Human primary AF cells from healthy IVD in culture were challenged with 10 or 50 µg/ml mCRP, or with 100 ng/ml lipopolysaccharide (LPS) as positive control for 24 hours, after four hours of serum starvation. a) to g) Messenger RNA (mRNA) expression was determined by quantitative real-time polymerase chain reaction. mRNA levels are presented as fold change relative to control (C-). Data expressed as mean (standard error of the mean) of at least three independent experiments. Comparisons referred to control (C-): *p < 0.05, **p < 0.01, ***p < 0.001. Cal., calibrator (refers to control (C-) condition); IL, interleukin; LCN2, lipocalin 2; MMP13, matrix metalloproteinase 13; NOS2, nitric oxide synthase 2; PTGS2, prostaglandin-endoperoxide synthase 2; VCAM1, vascular cell adhesion molecule 1.

To broaden the scope of our results, we also tested mCRP activity in HNPSV-1, a cell line derived from human NP cells that maintains the original architecture of the cells and their gene expression profile[101]. Figure 3 shows that mCRP at 10 µg/ml, 25 µg/ml, and 50 µg/ml significantly increases IL-6, IL-8, LCN2, VCAM1, PTGS2, and MMP13 mRNA expression levels, as previously observed in human primary AF cells. LPS and IL-1β, as well-known inflammatory stimuli, were used as controls of inflammatory induction.

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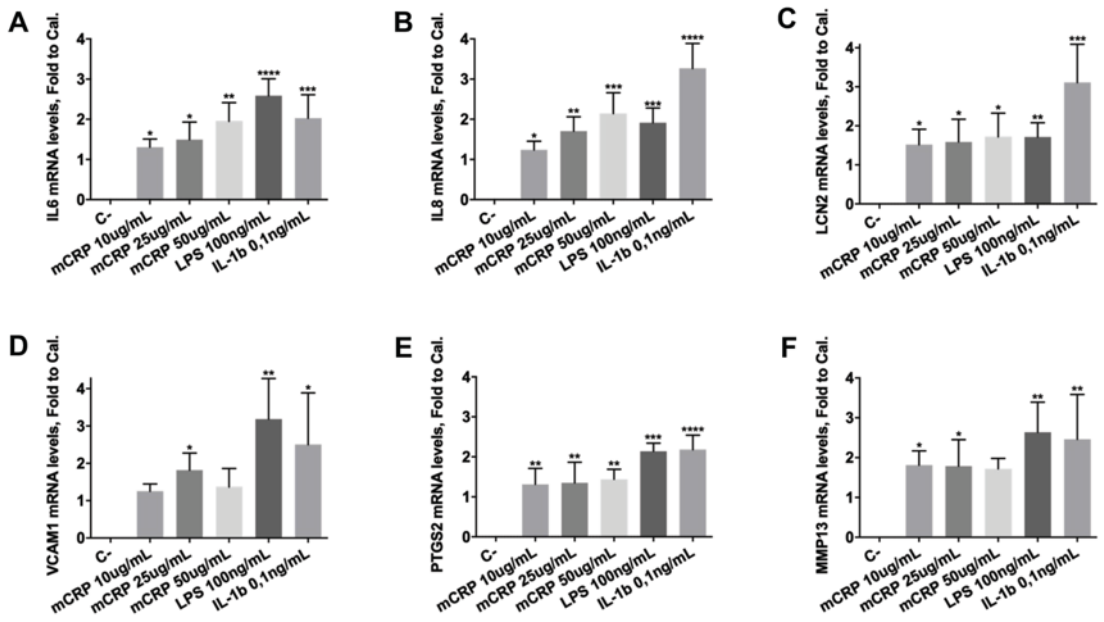

Fig. 3. Monomeric CRP (mCRP) proinflammatory effect on human intervertebral disc (IVD) nucleus pulposus (NP) cell line. HNPSV-1 human NP cells were challenged with 10, 25, or 50 µg/ml mCRP, or with 100 ng/ml lipopolysaccharide (LPS) or 0.1 ng/ml IL-1b as positive controls for 24 hours, after four hours of serum starvation. a) to f) Messenger RNA (mRNA) expression was determined by quantitative real-time polymerase chain reaction. mRNA levels are presented as fold change relative to control (C-). Data expressed as mean (standard error of the mean) of at least three independent experiments. Comparisons referred to control (C-): *p < 0.05, **p < 0.01, ***p < 0.001. Cal., calibrator (refers to control (C-) condition; IL, interleukin; LCN2, lipocalin 2; MMP13, matrix metalloproteinase 13; PTGS2, prostaglandin-endoperoxide synthase 2; VCAM1, vascular cell adhesion molecule 1.

For completeness, to elucidate which mediators might be implicated in mCRP intracellular signal transduction in both healthy and degenerative human disc cells, we analyzed NF-κβ signalling pathway upon mCRP challenge. Healthy or degenerative human primary AF cells were treated with 50 µg/ml mCRP or 100 ng/ml LPS as a positive control, for 15 minutes. The results obtained showed that mCRP induces PI3K phosphorylation, ERK1/2 phosphorylation, and NF-κβ p65 phosphorylation in healthy AF cells (Figures 4a to 4c) and degenerative AF cells (Figures 4d to 4f).

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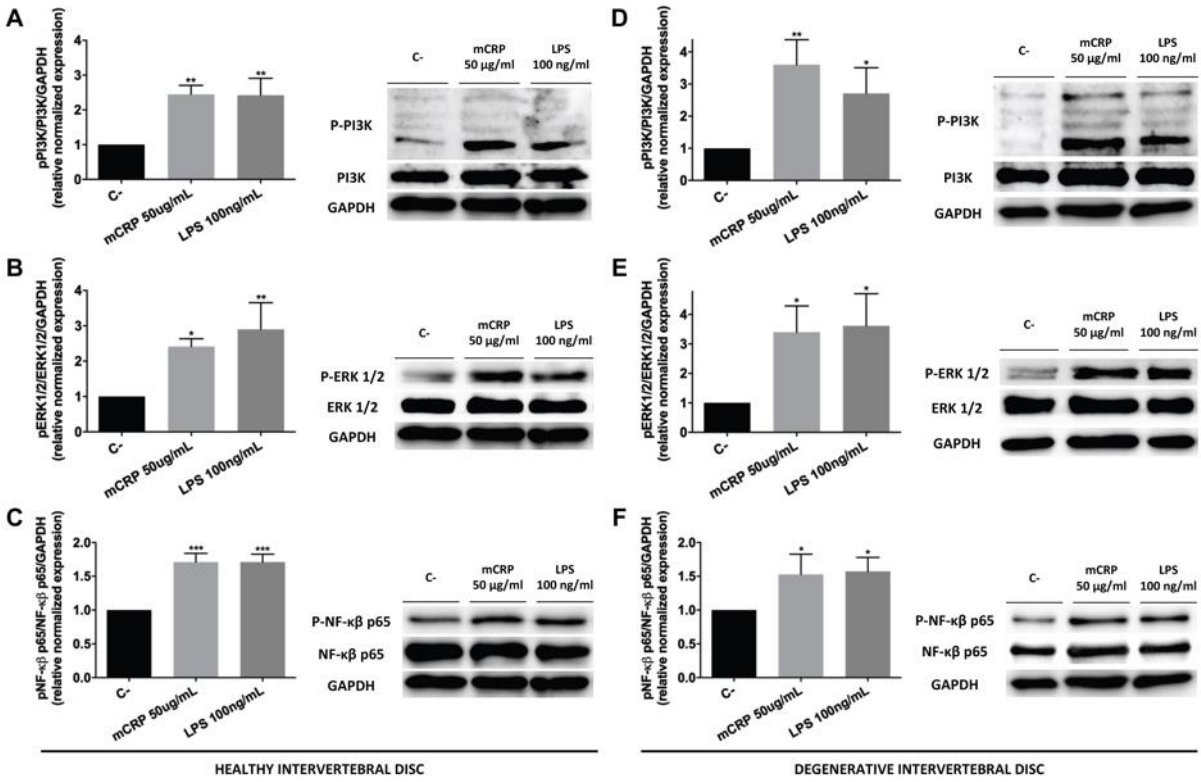

Fig. 4. Mediators involved in monomeric CRP (mCRP) intracellular signal transduction in human primary healthy and degenerative intervertebral disc (IVD) annulus fibrosus (AF) cells. Human primary AF cells from healthy or degenerative IVD in culture were challenged with 50 µg/ml mCRP or with 100 ng/ml lipopolysaccharide (LPS) as positive control for 15 minutes, after four hours of serum starvation. a) to f) Protein expression was measured by western blot. Determination of the phosphorylated protein normalized to total protein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data from densitometric analyses are shown as relative-to-control (C-) values. Data expressed as mean (standard error of the mean) of at least two or three independent experiments. Comparisons referred to control (C-): *p < 0.05, **p < 0.01, ***p < 0.001. NF-κβ p65, nuclear factor-κβ p65; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; PI3K, phosphoinositide 3-kinase; pPI3K, phosphorylated PI3-kinase; pNF-κβ p65, phosphorylated nuclear factor-κβ p65.

Finally, we performed immunohistochemical assays to investigate whether mCRP is located in IVD tissues. We assessed mCRP presence in human degenerative and healthy AF and healthy NP tissues, confirming its presence in physiological state in all of them.

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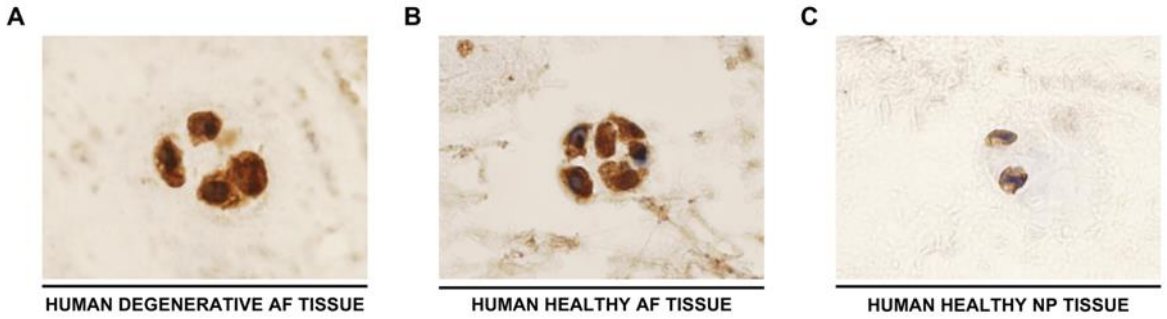

Fig. 5. Monomeric CRP (mCRP) localization in human degenerative and healthy intervertebral disc tissues. Immunohistochemical images showing mCRP protein presence (brown staining) in: a) human degenerative annulus fibrosus (AF) tissue; b) human healthy AF tissue; and c) human healthy nucleus pulposus (NP) tissue. Images taken at 60× magnification.

As we can observe in Figures 5a to 5c, mCRP location is predominantly cytoplasmic and perinuclear, being the more intense stain in the AF, especially in the degenerative tissue, than in the NP tissue.

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3. Article 3. WISP-2 modulates the induction of inflammatory mediators and cartilage catabolism in chondrocytes.

WISP-2 mRNA and protein basal expression along ATDC-5 differentiation

To evaluate whether WISP-2 mRNA and protein levels change along chondrocyte differentiation, we differentiated murine ATDC-5 cells into mature and hypertrophic chondrocytes.

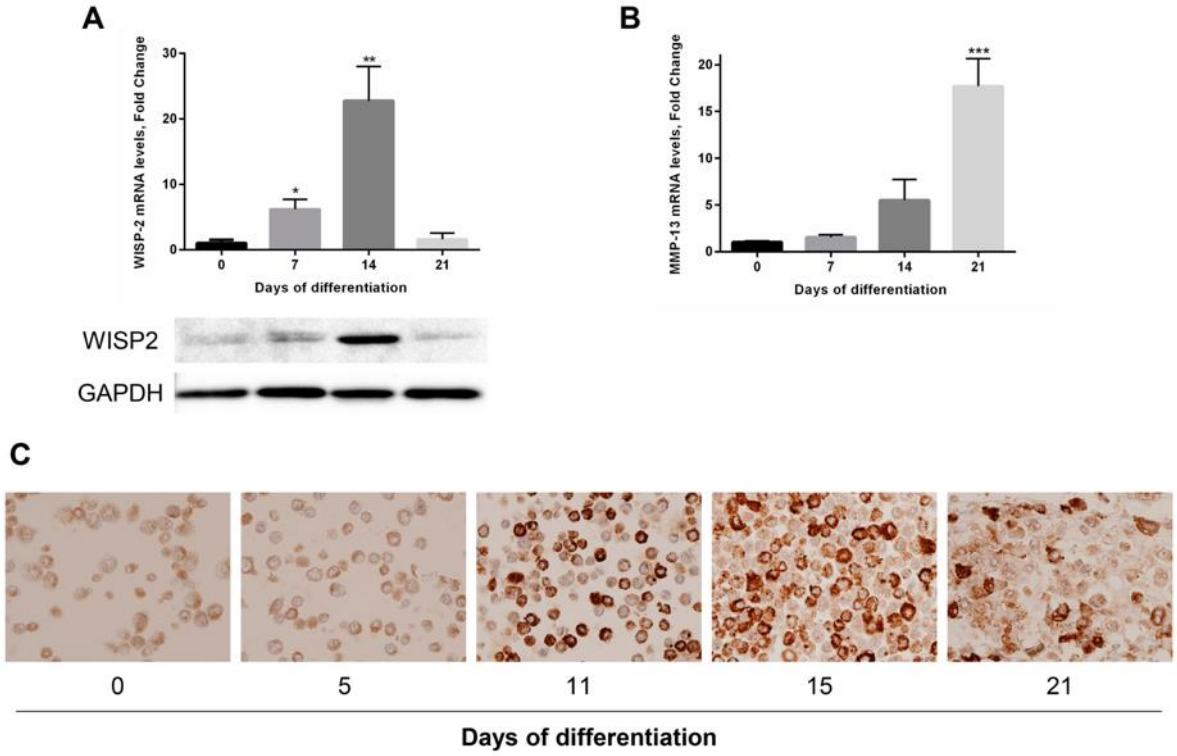

Fig. 1. WISP-2 mRNA and protein constitutive expression along ATDC-5 differentiation. A WISP-2 mRNA and protein expression along ATDC-5 differentiation after 7, 14, and 21 days. Cell lysates underwent western blot analysis using WISP-2 antibody. GAPDH was used as a loading control. Values are the mean ± SEM of at least four independent experiments (*p < 0.05, **p < 0.01 vs. control). B mRNA levels of MMP-13 along ATDC-5 differentiation. Values are the mean ± SEM of at least four independent experiments (***p < 0.001 vs. control). C Immunocytochemical images showing WISP-2 protein expression in ATDC-5 cells along cell differentiation at 0, 5, 11, 15, and 21 days.

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As shown in Fig. 1A, WISP-2 mRNA expression increased during the process of differentiation of ATDC-5 cells. This increase is significant after 7 and 14 days of differentiation in comparison to undifferentiated cells (day 0) (Fig. 1A, upper panel). However, WISP-2 mRNA expression dramatically declined at 21 days in hypertrophic chondrocytes. This effect was also evaluated in terms of protein expression as reported in Fig. 1A (lower panel). This decrease matches with a significant increment in MMP-13 expression at the same time point in ATDC-5 hypertrophic cells (Fig. 1B). The WISP2 protein expression along ATDC-5 differentiation was also assayed by immunocytochemistry (Fig. 1C)

Effect of pro-inflammatory cytokines and adipokines on WISP- 2 mRNA expression in human chondrocytes

To further elucidate the pattern of WISP-2 expression under inflammatory conditions, we treated T/C-28a2 cells with proinflammatory cytokines TNF-α and IL-1β and with representative adipokines leptin, adiponectin, and visfatin.

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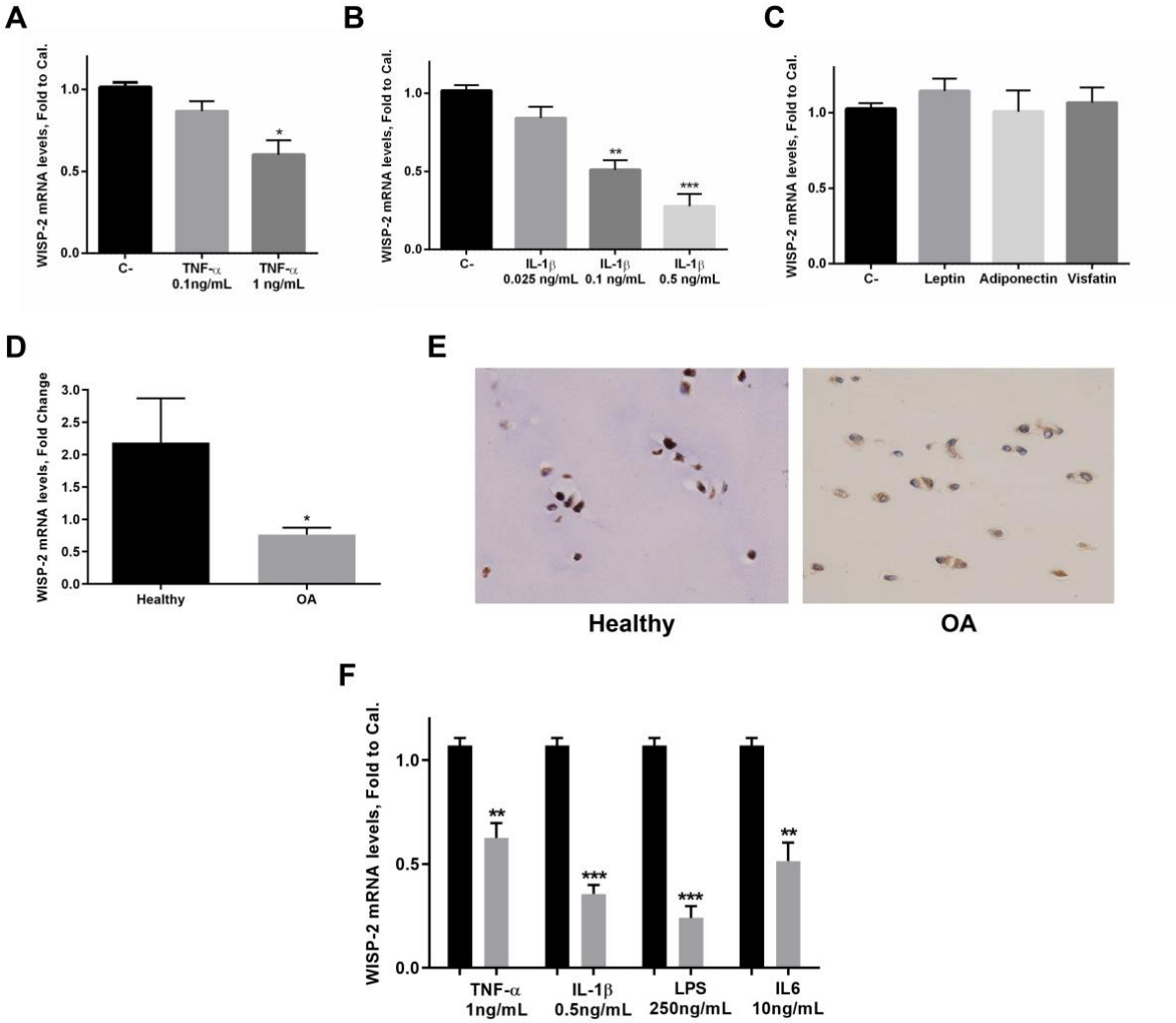

Fig. 2. WISP-2 mRNA and protein expression in human chondrocytes. A Human T/C-28a2 cells were treated with TNF-α 0.1 and 1 ng/mL, B IL-1β 0.025, 0.1 and 0.5 ng/mL, and C with leptin (800 nM), adiponectin (10 µg/mL) and visfatin (500 ng/mL) for 24 h. D WISP-2 mRNA basal expression in healthy and OA human primary chondrocytes. Values are the mean ± SEM of 4 independent human samples for the healthy cartilage, and 8 independent human samples for OA cartilage (*$p < 0.05$). E Immunocytochemical images showing WISP-2 protein expression in healthy and OA human cartilage. F Human primary OA chondrocytes were treated with TNF-α 1 ng/mL, IL-1β 0.1 ng/mL, LPS 250 ng/mL and IL-6 10 ng/mL for 24 h. WISP-2 mRNA expression was evaluated by RT-qPCR. Values are the mean ± SEM of at least three independent experiments (**$p < 0.01$, ***$p < 0.001$ vs. control).

As shown in Fig. 2A, cells stimulated with TNF-α 1 ng/mL for 24 h showed a significant inhibition of WISP-2 mRNA expression. A more pronounced effect was observed when cells were stimulated with IL-1β 0.5 ng/mL (Fig. 2B) for 24 h. Nonetheless, neither leptin (800 nM)

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nor adiponectin (10 μg/mL) or visfatin (500 ng/mL) had significant effects on WISP-2 expression (Fig. 2C).

We also examined the constitutive expression of WISP-2 in human chondrocytes derived from healthy and OA joints. As shown in Fig. 2D, WISP-2 mRNA levels in OA chondrocytes are lower than those observed in chondrocytes obtained from healthy subjects. We further confirmed these results by immunocytochemistry in human healthy and OA cartilage. As shown in Fig. 2E, immunocytochemical techniques showed cytoplasmic positivity for WISP-2 in human chondrocytes. In osteoarthritis cartilage samples the intensity of immunostaining was weak, and the WISP-2 immunoreactivity rate (WISP-2-immunostaine-positive cells divided by total cells) was 59.92 ± 0.76% [Fig. 2 panel E (OA)] In healthy cartilage controls the intensity of immunostaining was stronger, with a WISP-2 immunoreactivity rate of 81.02 ± 0.98% [Fig. 2 panel E (Healthy)].

OA chondrocytes were also stimulated with cytokines TNF-α, IL-1β, IL-6, and an agonist of TLR4, the bacterial lipopolysaccharide (LPS). As shown in Fig. 2F, TNF-α as well as IL-1β, LPS, and IL-6 were able to decrease WISP-2 mRNA expression.

Effect of recombinant human WISP-2 protein and IL-1β stimulation in human chondrocyte catabolism

To elucidate if WISP-2 can interfere in IL-1β-mediated MMPs and aggrecanase production, T/C-28a2 chondrocytes were pre-treated with human WISP2 recombinant protein (500 ng/mL) one hour before being stimulated with IL-1β 0.5 ng/mL for 24 h. Compared to IL-1β-stimulated cells, chondrocytes pre-treated with rhWISP-2 protein showed a significant reduction in MMP-13 and ADAMTS5 mRNA expression (Fig. 3A) These results were also confirmed at protein level (Fig. 3B)

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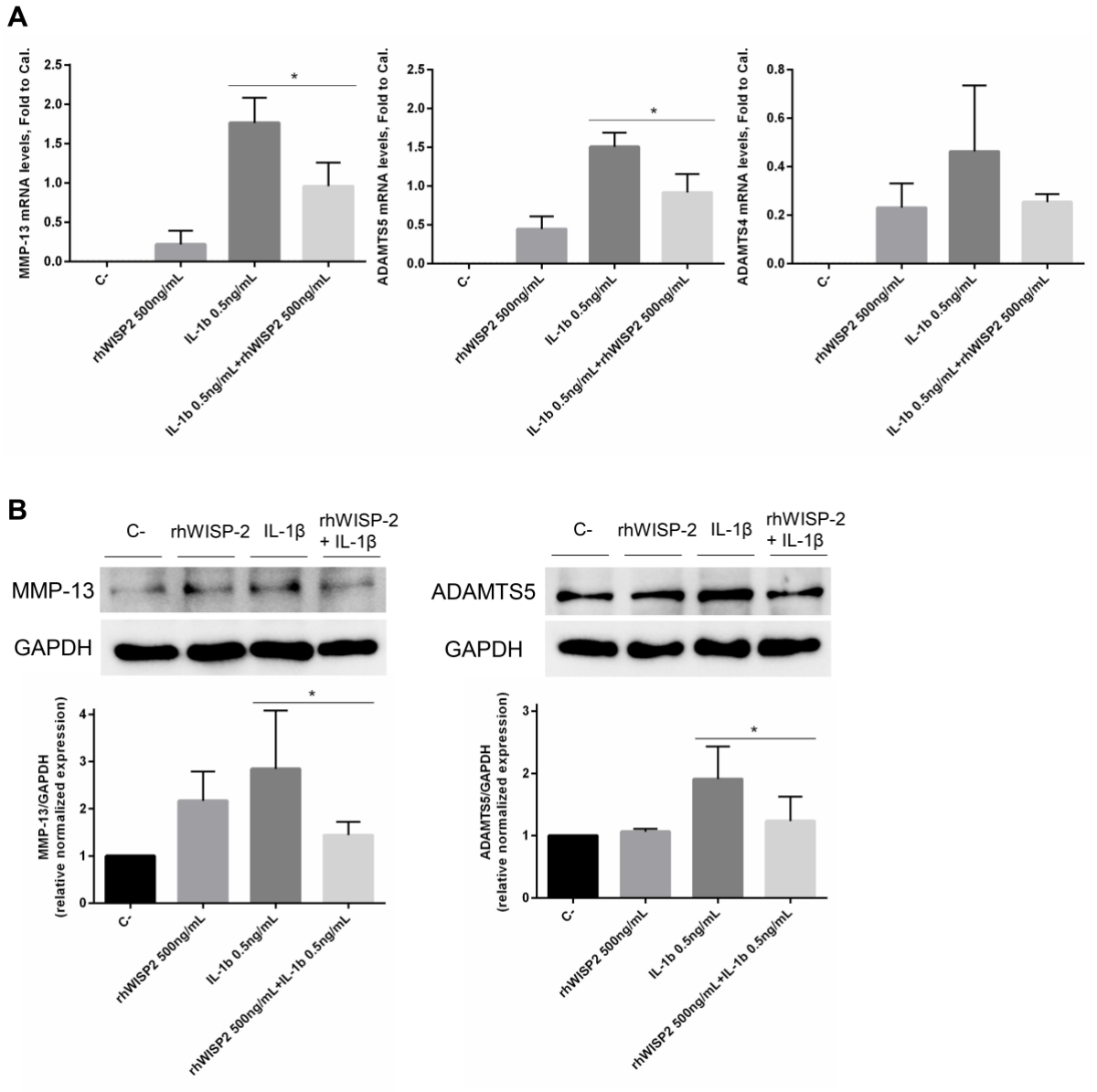

Fig. 3. Recombinant human WISP-2 protein reduces IL-1β-mediated chondrocyte catabolism. Human T/C-28a2 cells were pre-treated with rhWISP-2 (500 ng/mL) for one hour before being challenged with IL-1β 0.5 ng/mL for 24 h. Catabolism markers were evaluated in pre-treated and not pre-treated human chondrocytes. A Relative mRNA levels of MMP-13, ADAMTS-5, and ADAMTS-4 in human T/C-28a2 chondrocytes were determined by RT-qPCR. B Determination of MMP-13 and ADAMTS-5 protein expression by western blot in human T/C-28a2 chondrocytes. GAPDH was used as a loading control. Densitometric analysis is also shown (lower panels). Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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For completeness, ADAMTS4 was also examined since it is upregulated by inflammatory cytokines in chondrocytes. Interestingly, we observed that its mRNA expression is fairly lower than ADAMTS5. However, the pattern of response to rhWISP2 treatment is similar in both aggrecanases (Fig. 3A, right panel).

Downstream IL-1β and WNT/b-catenin signaling pathways involved in recombinant human WISP-2 protein and IL-1β stimulation action in human chondrocytes

In order to understand the molecular mechanisms involving WISP-2 we analyzed the downstream IL-1β signaling upon WISP-2 treatment. Human chondrocytes were pre-treated with human WISP2 recombinant protein (500 ng/mL) 1 h before being stimulated with IL-1β 0.5 ng/mL for 20 min. The obtained results clearly showed that rhWISP-2 significantly reduces IL-1β-induced NF-kβ p65 phosphorylation, Erk 1/2 phosphorylation, JNK phosphorylation and p38 phosphorylation (Fig. 4A–D) After confirming the WISP-2 inhibitory effect in the IL-1β signaling in human chondrocytes, we addressed Wnt/β-catenin signaling to elucidate if WISP-2 can act as a WNT agonist. For this purpose, we measured GSK-3 phosphorylation under the same experimental conditions and observed that rhWISP2 significantly activates Wnt signaling. Our results also showed that rhWISP2, in combination with IL-1β, has a cooperative effect, and that the Wnt pathway activation is significantly increased compared to single treatments (Fig. 4E).

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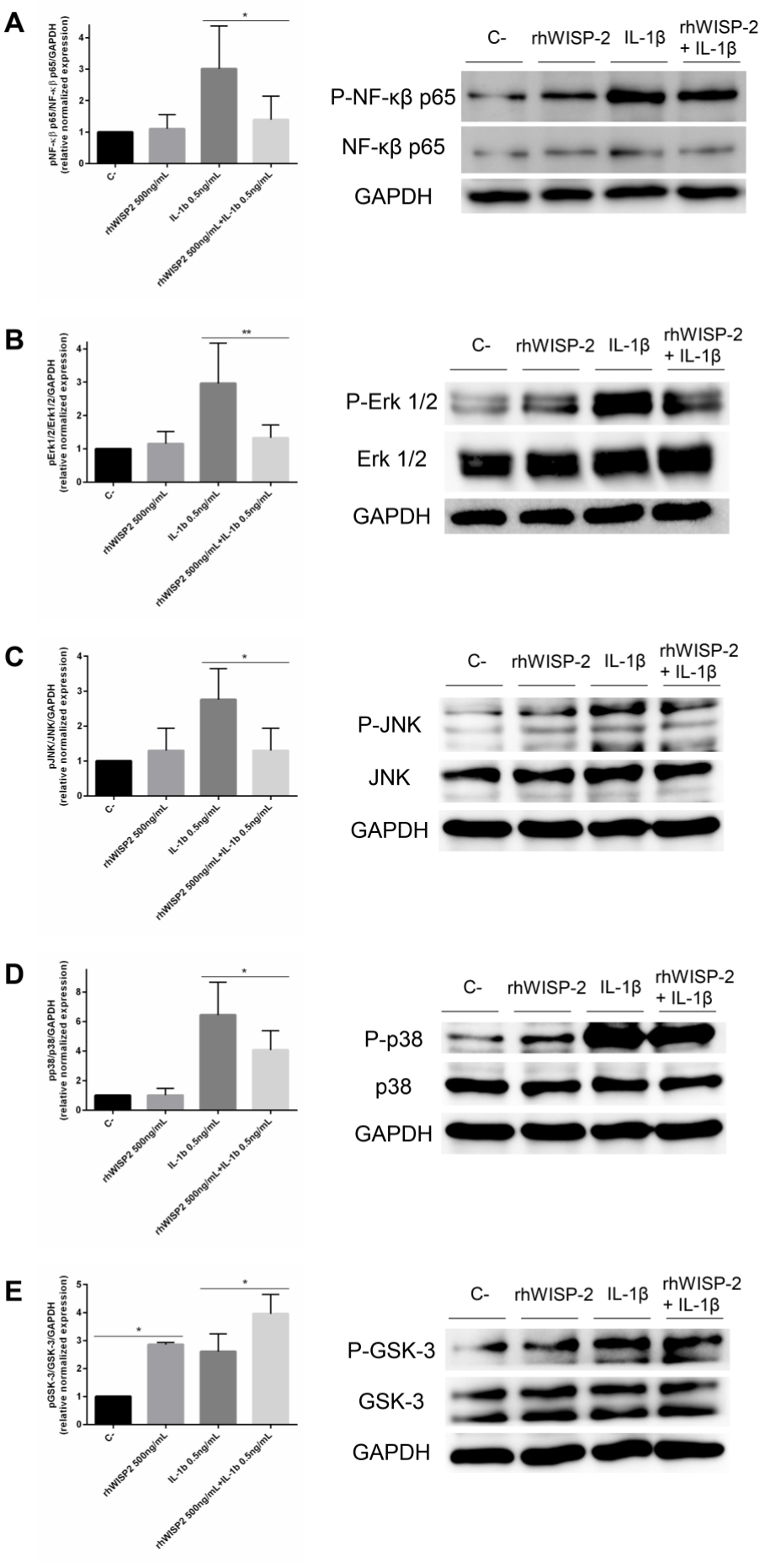

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Fig. 4. Downstream IL-1β and WNT/b-catenin signaling pathways involvement in recombinant human WISP-2 protein mechanism of action. Human T/C-28a2 cells were pre-treated with rhWISP-2 (500 ng/mL) for 1 h before being challenged with IL-1β 0.5 ng/mL for 20 min. IL-1β cascade and Wnt pathway activation were evaluated in pre-treated and not pre-treated human chondrocytes. A Determination of phosphorylated NF-kβ p65 and total NF-kβ p65 expression by western blot in human T/C-28a2 chondrocytes. B Determination of phosphorylated Erk 1/2 and total Erk 1/2 protein expression by western blot in human T/C-28a2 chondrocytes. C Determination of phosphorylated JNK and total JNK protein expression by western blot in human T/C-28a2 chondrocytes. D Determination of phosphorylated p38 and total p38 protein expression by western blot in human T/C-28a2 chondrocytes. E Determination of phosphorylated GSK-3 and total GSK-3 protein expression by western blot in human T/C-28a2 chondrocytes. GAPDH was used as a loading control. Densitometric analysis is also shown. Values are the mean ± SEM of at least three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001).

WISP-2 siRNA knockdown stimulates chondrocyte catabolism and downregulates WNT/b-catenin signaling

Based on the data above showing WISP-2 downregulation under pro-inflammatory stimulation, we investigated the effect of WISP-2 gene silencing on cartilage ECM breakdown mediators. As shown in Fig. 5A, WISP-2 gene knockdown led to increased MMP-13 and ADAMTS-5 expression in comparison to T/C-28a2 cells transfected with a non-targeting control siRNA. Apart from cartilage degrading enzymes, the catabolic effects of chondrocytes are mediated through several cytokines and chemokines. WISP-2 silencing increased IL-6 and IL-1β mRNA levels. The chemokine IL-8 was also significantly upregulated upon WISP-2 gene knockdown. In addition, as shown in Fig. 5B, MMP-13 mRNA expression is increased also in OA human primary chondrocytes after WISP-2 gene silencing. This effect was also evaluated in terms of protein expression as reported in Fig. 5C.

To gain further insights into the signaling pathways at play, we tested the hypothesis that WISP-2 might be an activator of canonical Wnt signaling[91]. To investigate Wnt/β-catenin signaling activation we addressed GSK-3 phosphorylation in WISP-2- silenced T/C-28a2 chondrocytes. As shown in Fig. 5D, upon WISP-2 silencing there is a significant decrease of phosphorylation of GSK-3, suggesting that the process of accumulation and translocation of β-catenin to the nucleus, as well as the expression of downstream target genes are compromised.

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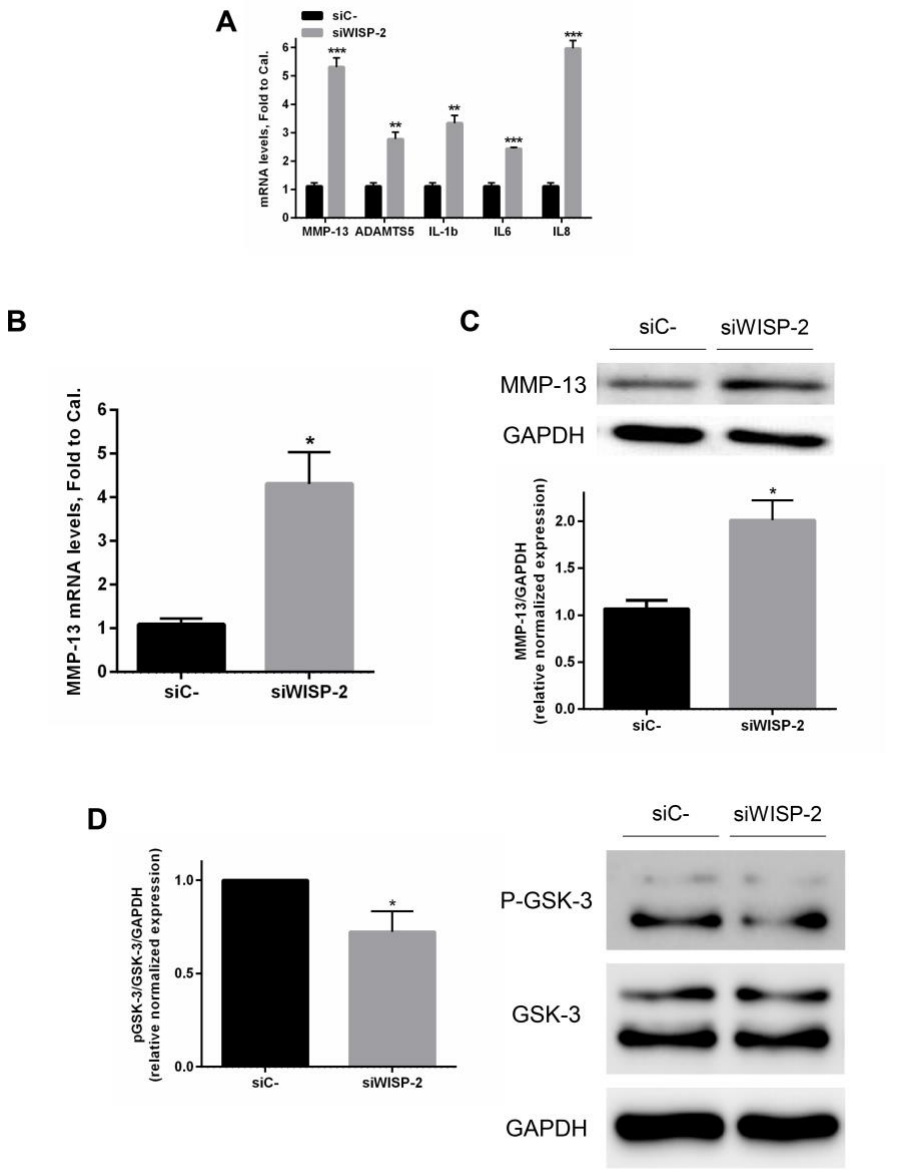

Fig. 5. Effect of WISP-2 gene knockdown on chondrocytes catabolic markers and in WNT/b-catenin pathway. Chondrocytes were transfected with negative control (siC-, 10 nM) or siRNA against WISP-2 (siWISP-2, 10 nM) for 48 h. A Relative mRNA levels of MMP-13, ADAMTS-5, IL-6, IL-8, and IL-1β were determined in human T/C-28a2 chondrocytes by RT-qPCR. B Determination of human MMP-13 mRNA expression by RT-qPCR in human primary OA chondrocytes. C Determination of human MMP-13 protein expression by western blot in human OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis was also shown. D Determination of phosphorylated GSK-3 protein expression by western blot in human OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis is also shown. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. siC-).

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Effect of WISP-2 siRNA knockdown on IL-1β-stimulated chondrocytes

Since IL-1β has been implicated in cartilage degradation[109], we determined the effect of WISP-2 silencing on IL-1β-stimulated T/C-28a2 chondrocytes. As expected, IL-1β induced mRNA expression of MMP-13, ADAMTS-5, IL-6, and IL-8, while WISP-2 knockdown further increased IL-1β-induced expression of those genes (Fig. 6A–D). These results were confirmed also in human primary OA chondrocytes in terms of MMP-13 protein expression (Fig. 6E).

Effect of canonical Wnt/β-catenin signaling on WISP-2 siRNA knockdown mediated chondrocyte catabolism

To investigate the involvement of Wnt/β-catenin signaling, we transfected T/C-28a2 chondrocytes with siWISP-2 and then treated them with IL-1β in presence or not of selective GSK-3 inhibitor BIO. As shown in Fig. 7A–D, activation of canonical Wnt signaling by BIO significantly decreased the expression of MMP-13, as well as ADAMTS-5, IL-6, and IL-8. Moreover, BIO treatment blocked IL-1β-induced expression of those catabolic markers' mRNA. To note, blocking GSK-3 with BIO almost completely suppressed the IL-1β-mediated upregulation of MMP-13, ADAMTS-5, IL-6, and IL-8 in siWISP-2-transfected cells. This effect was confirmed in human primary OA chondrocytes at MMP-13 protein level (Fig. 7E).

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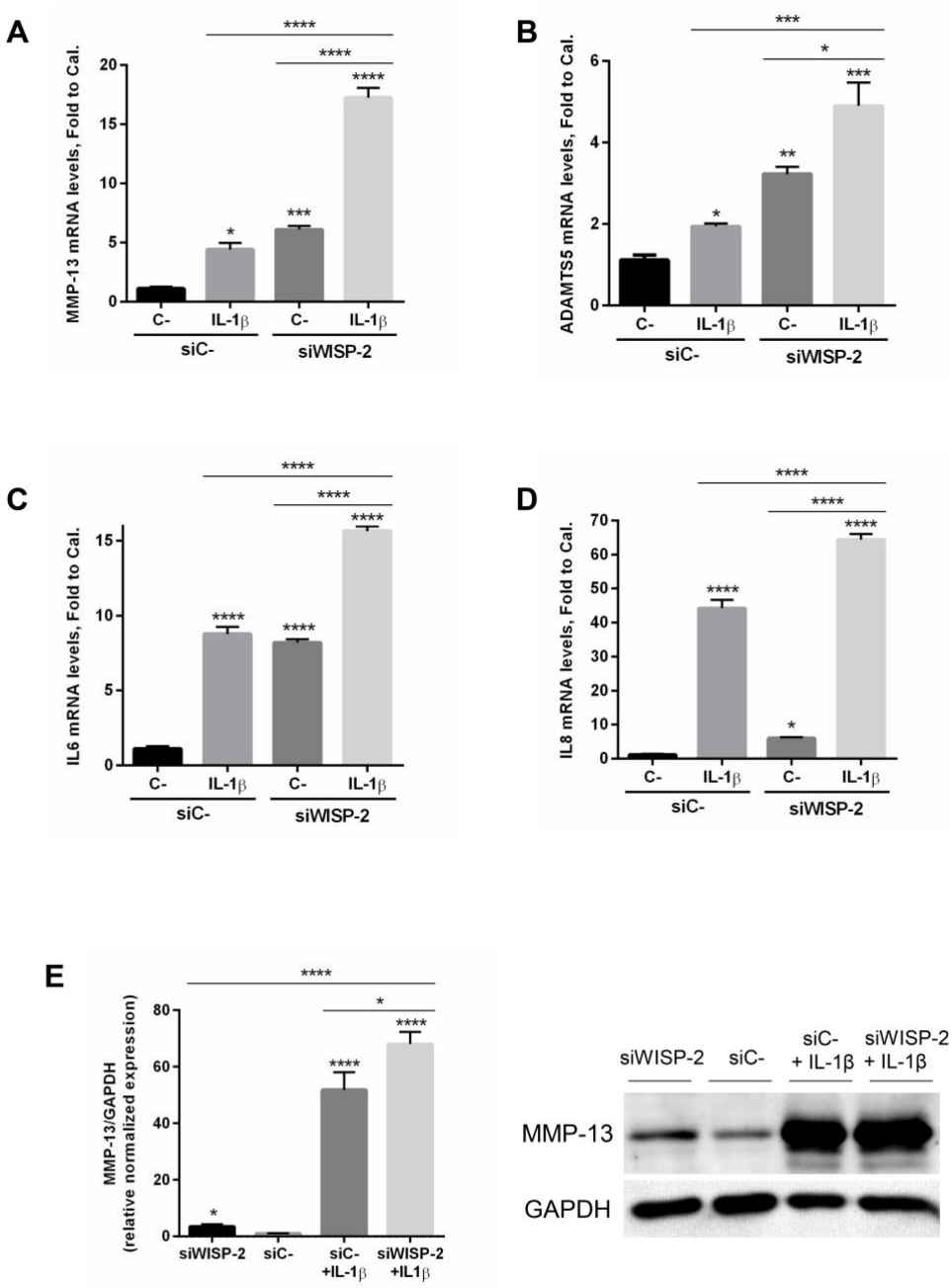

Fig. 6. Effect of WISP-2 siRNA knockdown on IL-1β-stimulated chondrocytes. Chondrocytes were transfected with negative control (siC-, 10 nM) or siRNA against WISP-2 (siWISP-2, 10 nM) in presence or not of IL-1β (0.5 ng/mL) for 24 h. A–D Relative mRNA levels of MMP-13, ADAMTS-5, IL-6, and IL-8 in human T/C-28a2 chondrocytes were determined by RT-qPCR. E Determination of human MMP-13 protein expression by western blot in human primary OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis was also shown. Values are the mean ± SEM of at least three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001 vs. siC-).

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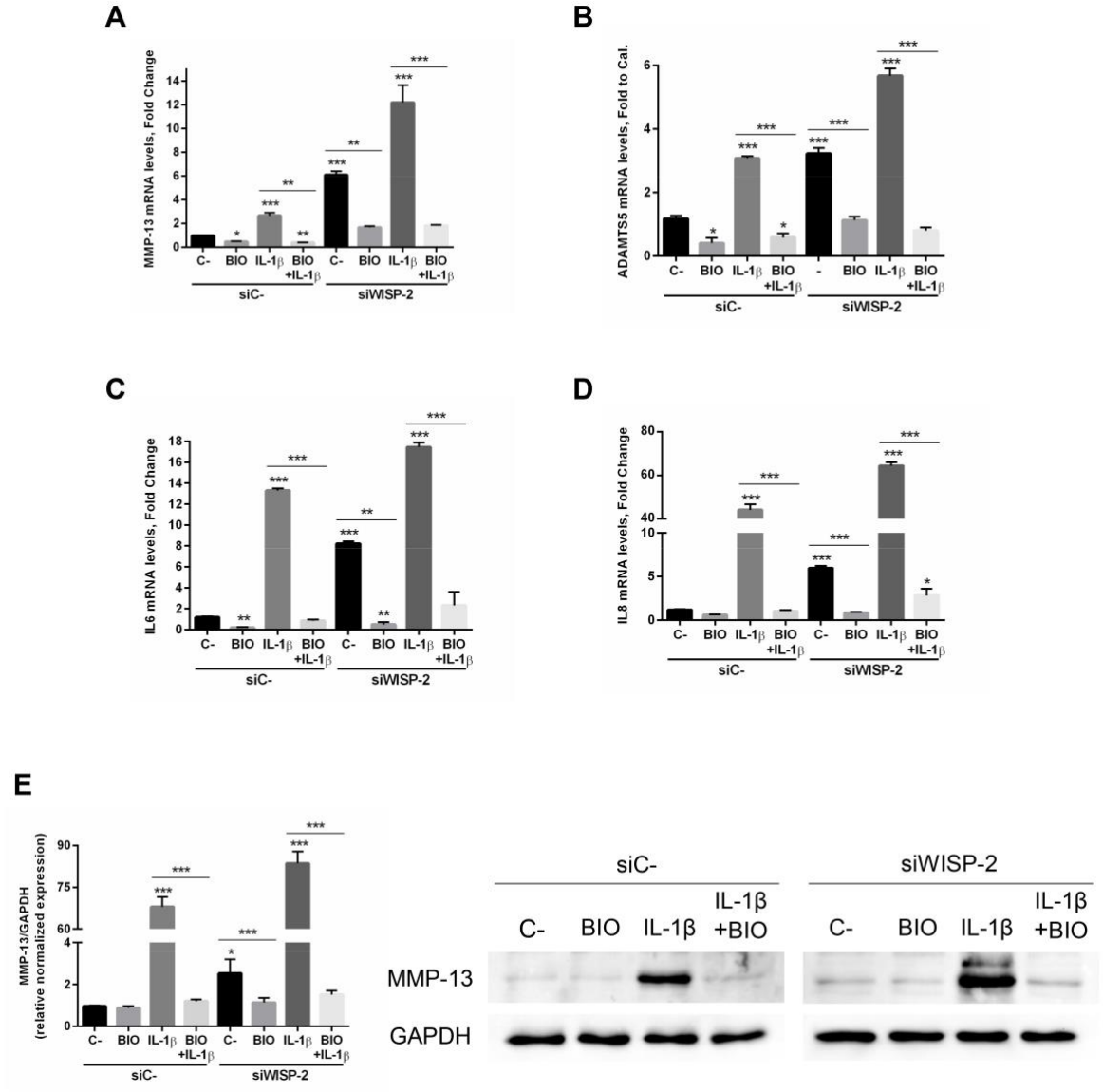

Fig. 7. Effect of canonical Wnt/β-catenin signaling on WISP-2 siRNA knockdown mediated chondrocyte catabolism. Chondrocytes were transfected with negative control (siC-, 10 nM) or siRNA against WISP-2 (siWISP-2, 10 nM) in presence or not of IL-1β (0.5 ng/mL) for 24 h in combination with 1 μM BIO. A–D Relative mRNA levels of MMP-13, ADAMTS-5, IL-6, and IL-8 in human T/C-28a2 chondrocytes. E Determination of human MMP-13 protein expression by western blot in human primary OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis was also shown. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. siC-).

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DISCUSSION

1. CHAPTER 1. MONOMERIC C REACTIVE PROTEIN IN CARTILAGE AND IN INTERVERTEBRAL DISC.

C reactive protein, in its pentameric form, is an acute-phase serum protein produced mainly in the liver upon IL-6 stimulation[110] and also a well-established biomarker in a great number of infectious and inflammatory diseases, including RA, OA and IVD herniation [111]. It has also been proposed as a mediator of inflammatory and immune responses in the context of local tissue injury [112,113]. Serial blood CRP measures are useful not only for the follow-up of postoperative infections but also for monitoring treatment's efficacy and for the detection of relapses [82,114] in a multitude of inflammatory diseases.

CRP belongs to the pentraxin family of acute-phase proteins. It is the primary acute-phase reactant in humans. CRP is synthesized in hepatocytes and secreted into the bloodstream upon stimulation with IL-6. Circulating CRP consists of five monomeric subunits bound with disulfide bonds in a ring-shaped disc[115]. Each subunit of the pentameric disc has a calcium-dependent binding site for lysophosphatidylcholine on one side and the complement component C1q on the other[116,117]. Phosphatidylcholine is a major structural component of cell and extracellular vesicle membranes. It is mainly present on the outer leaflet of the membrane phospholipid bilayer[118]. Secretory phospholipase A2 (PLA2) hydrolyzes it to lysophosphatidylcholine. Usually, phosphatidylcholine does not interact with PLA2. However, during apoptosis and cell injury, phospholipids of the inner part translocate to the outer layer of the cell membrane. These phospholipids include phosphatidylserine and phosphatidylethanolamine, which are PLA2 ligands. In the presence of these two phospholipids, PLA2 hydrolyzes phosphatidylcholine to biologically active lysophosphatidylcholine[118]. Lysophosphatidylcholine stimulates the endothelial synthesis of several chemokines, impairs, increases oxidative stress, suppresses endotheliocyte migration and proliferation, and facilitates macrophage activation and polarization to the inflammatory M1 phenotype[119].

Circulating CRP acts as an opsonin that binds to lysophosphatidylcholine on the surface of cell membranes and oxidized lipoproteins. The sites on the reverse side of the CRP disc interact with the complement component C1q. This results in activation of the classical complement

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cascade up to the C4 component. Thus, CRP-induced complement activation facilitates phagocytosis of damaged cells and oxidized lipoproteins but does not initiate the formation of the membrane-attack complex C5b–C9[120]. CRP can also interact with factor H and activate the complement cascade up to the C4 component via the alternative pathway[121]. Furthermore, CRP can opsonize nuclear antigens released by apoptotic and necrotic cells[122]. Therefore, the biological role of circulating CRP is characterized by the facilitation of the clearance of cell-destruction products formed during trauma, infection, or sterile inflammation[123].

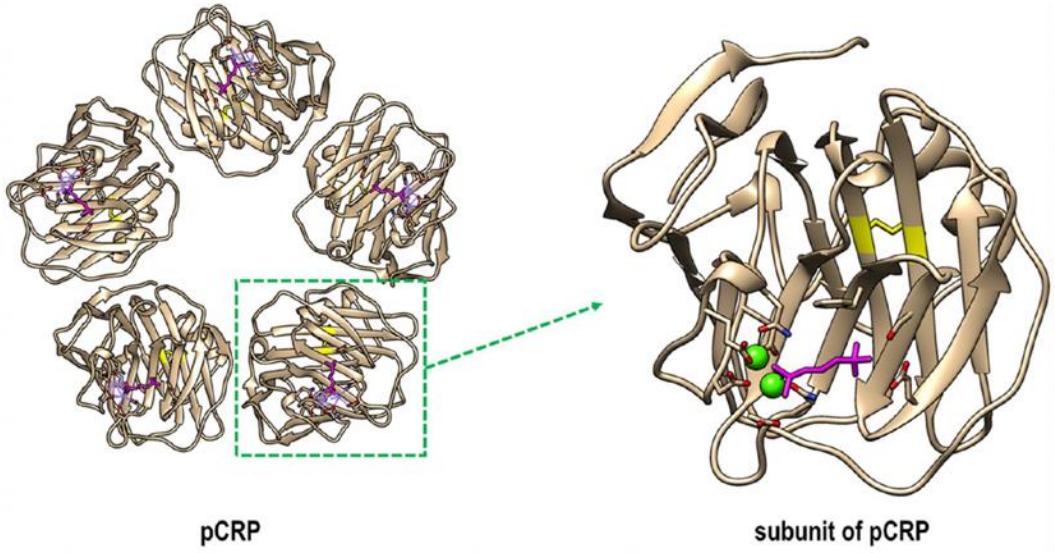

Figure 7. Structure of pCRP (PDB:1B09). pCRP consists of five identical subunits. Each subunit contains a hydrophobic core stabilized by the only intrachain disulfide bond (yellow). On the opposite side of the disulfide bond, the subunit can bind two calcium ions (green), which participate in the binding of PC (purple). *Yao, ZhenYu et al. "Regulation of C-reactive protein conformation in inflammation." Inflammation research: official journal of the European Histamine Research Society ... [et al.] vol. 68,10 (2019): 815-823. doi:10.1007/s00011-019-01269-1*[124]

There is another form of CRP, monomeric CRP (mCRP), which is formed at sites of local inflammation through the dissociation of CRP subunits and, to some extent, local synthesis. mCRP is essentially different from CRP in its functions[110,125]. Upon binding to lysophosphatidylcholine, the pentameric disc of CRP undergoes dissociation through intermediate forms into the final product, mCRP[126,127]. Dissociation occurs through the disintegration of disulfide bonds between the CRP subunits[128]. This process involves lysophosphatidylcholine but also requires other cell-membrane components and calcium. Soluble lysophosphatidylcholine does not dissociate CRP in the absence of cell membranes[127].

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Dissociation opens a neoepitope (octapeptide Phe-Thr-Lys- Pro-Gly-Leu-Trp-Pro) on the C-terminal end of monomeric subunits, which is concealed in the pentameric disc[127]. This dramatically changes the antigenic specificity and biological functions of CRP[129].

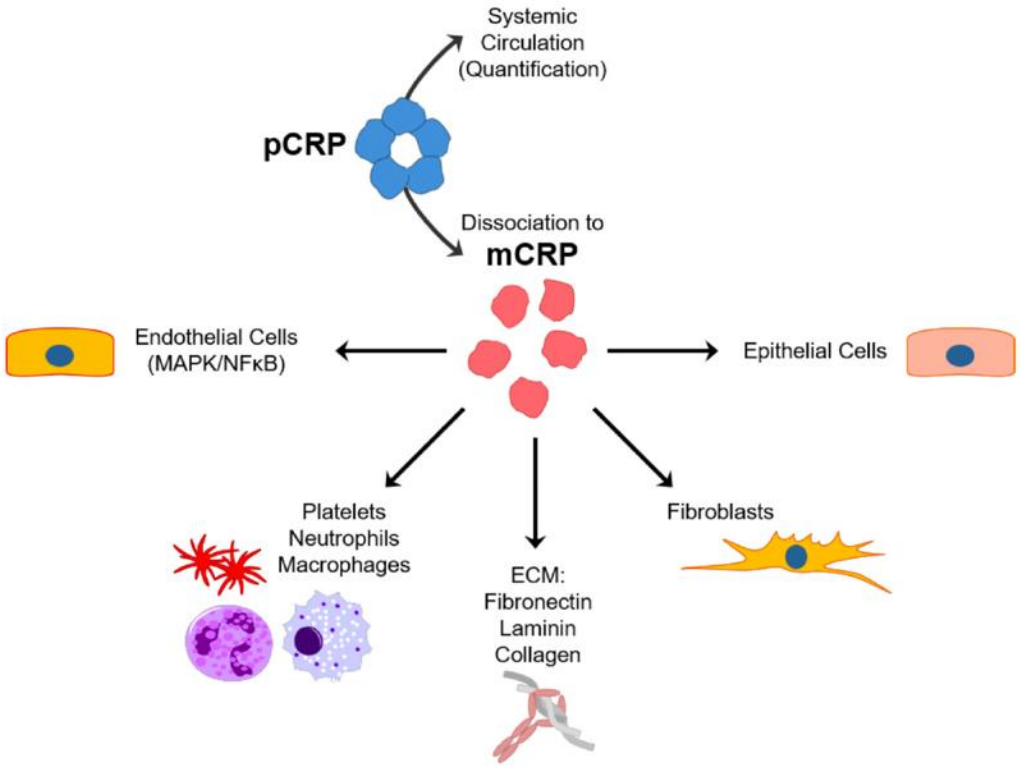

Figure 8. Schematic representation of the predominant interactions of pCRP and mCRP isoforms. Pentameric CRP (pCRP) released from hepatocytes due to inflammation circulates through the systemic vasculature and serves as the pool of quantifiable CRP that is used in diagnostic testing. pCRP, however, once dissociated to monomeric CRP (mCRP) at lipid rafts of cells involved in inflammatory responses instead is highly biologically active. mCRP in turn interacts with several different cell types at the sites of inflammation, including endothelial cells, epithelial cells, fibroblasts and immune cells (platelets, neutrophils, macrophages) as well as components of the extracellular matrix (ECM) such as fibronectin, laminin and collagen. *Hart, Peter C et al. "C-Reactive Protein and Cancer-Diagnostic and Therapeutic Insights." Frontiers in immunology vol. 11 595835. 19 Nov. 2020, doi:10.3389/fimmu.2020.595835*[130]

mCRP has reduced aqueous solubility and remains predominantly bound to the cell membranes[113]. It has been detected in extracellular vesicles circulating in the blood- stream. Although much less studied, the monomeric form has been found to have a different biological role in the inflammatory process. While clarifying its pathophysiological function needs further investigation, new insights could be extremely helpful for a better understanding of the inflammatory mechanism and how it works in the pathogenesis and disease progression.

Evidence suggests that mCRP exhibits different antigenic, biological, and electrophoretic properties compared to the pentameric form and it generally displays marked pro-inflammatory properties in several cell lineages, including vascular endothelial cells, macrophages, and neutrophils[131–133]. When dissociated on platelets and adhered to the vessel wall, mCRP can induce endothelial activation and leukocyte recruitment. mCRP enhanced endothelial activation and neutrophil attachment to the endothelium[134,135]; monocyte adhesion to the collagen[136], fibrinogen[79], and fibronectin matrix[137]; and T-lymphocyte extravasation[138]. Of note, it was recently described that mCRP directly binds to fibronectin without depending on calcium presence and pH (acid in the microenvironment of inflamed tissue, which promotes dissociation into the monomeric form). It was also shown that mCRP, deposited in the ECM, may regulate the recruitment and adhesion of monocytes thus triggering the inflammatory process[137]. mCRP strongly activates angiogenesis both *in vitro* and *in vivo* through a mechanism involving MAP kinase signaling and Notch-3, but this vasculogenic process appears to be flawed often originating leaky blood vessels[68]. In addition, over the last decade mCRP form has been highlighted as the major biologically active form, and tissue-associated mCRP within the brain has shown to be associated with development of dementia[139]. Furthermore, there is evidence of a powerful *in vivo* pro-inflammatory effect of mCRP in striated muscle, atherosclerotic plaque, and infarcted myocardium in human and rat tissues[79]. mCRP binds to macrophages via integrin α5β3 and subsequent AKT signaling inducing a pro-inflammatory status and chemotaxis. In endothelial cells, mCRP is known to bind to membrane-associated enriched lipid rafts and stimulate cellular activation through phospholipase C, MAP kinase, and NF-κB[140,141]. It was demonstrated that only mCRP originated from local pCRP in the joint compartment, may bind to RANKL, neutralizing its activity and downregulating osteoclast differentiation[142]. Nevertheless, it is unclear how mCRP accumulates in tissues. It could cross the endothelial barrier after dissociation[79] or be synthesized locally.

There are some recent works that show mCRP location in cortical vessels, macrophages, glia, cortical gray matter, neuron cells, hypothalamus[139], and in the kidney tubules cytoplasm[143,144], always upon an inflammatory condition. Also, related to tissue damage, it has been detected in skin, knee joint synovium tissues, colon, and thyroid gland tissues[144]. Of interest, mCRP has also been detected without being associated to an inflammatory or pathological state, in monocytes[145], in the vessels wall and in the extracellular space of the pulmonary endothelium,

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the ovarian and testicular tissues, and the cardiac tissue[146]. Although less investigated, some publications report that mCRP can be synthetized locally in monocytes, macrophages[145], lymphocytes[147,148], pancreas islet cells[149] and epithelial cells of the respiratory tract[150]. Nonetheless, the contribution of local synthesis to the total concentration of mCRP in the tissues and bloodstream is unknown.

Although several lines of evidence have postulated a role for mCRP in musculoskeletal diseases, there were no experimental evidence of its activity neither in human chondrocytes nor in human intervertebral disc cells.

1.1. MONOMERIC C REACTIVE PROTEIN IN CARTILAGE

Regarding the physiological and pathological roles of mCRP and its biological relevance in cartilage biology and OA, some published data assessed the relationship between OA development and progression and pentameric CRP levels. CRP levels have been reported to be significantly elevated in patients with OA compared to healthy controls, and they were observed to be correlated with radiographic parameters as well as clinical severity[151–153]. Although some studies showed no association between CRP levels and radiographic OA, a correlation with pain and decreased physical function has been found[154]. Of interest, most studies in OA patients[65,151–153] only quantified CRP levels in the high sensitivity range (i.e. hsCRP) describing values below 10 μg/ml. According to several studies[155–161] and to the guidance of US-FDA[162], the clinical significance of such values is undefined and considered controversial.

A recent publication demonstrated that CRP aggravates OA development in mice on a high-fat diet[163], although the mechanism of action for CRP involvement is not clarified yet. A hypothetical mechanism through which CRP could be involved in local cartilage disarrangements in OA might be via the pathogenic monomeric subunit of CRP (mCRP), originated by phospholipase-A2 (PLA_2) enzymatic action in an inflammatory environment[79]. Increased PLA_2 activity has been observed in synovial fluid from OA patients and in animal models of OA[164,165]. However, it is also hypothetically possible that pCRP increases locally where it works to dampen the acute inflammatory response leading to prolonged, chronic tissue damage associated with arthritis. Whereas the PC groups on PLA_2 binding to the PC face will make the pCRP cholesterol binding ligand accessible so that pCRP can bind to a membrane, and, consequently, it is juxtaposed to a lipid zone that will supply the biochemical energy to relax pCRP into an orientation that then dissociates to mCRP[166].

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These contradictory results, together with the lack of knowledge about the pathophysiological role of the mCRP in cartilage, have overshadowed the association between CRP levels and OA pathology, making the functional involvement of CRP in OA pathogenesis and/or progression uncertain. In fact, before our research, the role of mCRP in cartilage pathophysiology, as well as its relevance in OA etiology and progression, had barely been investigated.

To address this key question, we first tested the effect of mCRP in primary culture of human chondrocytes isolated from patients with hip and knee OA, or from healthy subjects. For completeness, the ATDC5 murine chondrogenic cell line was also used as a valuable chondrocyte model for comparison[78]. As far as we are aware, this is the first experimental article that provides evidence for the functional activity of mCRP in human and mouse chondrocytes. To gain further insights into the action of mCRP and to verify whether this stimulation can result in sustained pro-inflammatory effects, we evaluated the dose-dependent activity of mCRP in master inflammatory and catabolic players in cartilage degradation. Nitric oxide synthase type II (iNOS) and COX-2 are classically involved in the amplification of inflammatory responses. NO is cytotoxic for chondrocytes, it damages cartilage, and it is involved in the upregulation of MMPs, among which MMP13 is one of the major mediators of ECM degradation. COX-2 induces prostaglandins that, in turn, increase the synthesis of other inflammatory mediators, including cytokines, to perpetuate cartilage destruction[167]. Thus, we have focused on the regulation of iNOS and NO production, as well as other six major pro-inflammatory mediators with different functions in cartilage destruction, namely COX-2, MMP13, VCAM1, IL-6, IL-8, and LCN2. All these mediators are produced by chondrocytes and are elevated in arthritic joints[14,134,168–170].

Addressing these findings in cartilage, we brought out important evidence regarding the modulation of articular phenotype and functions exerted by mCRP. First, exposure of chondrocytes to mCRP induced multiple pro-inflammatory genes suggesting that its effects are not limited to a specific molecular target, rather mCRP has a broader spectrum of action. The second important point is that the effect of mCRP is persistent and sustained, regardless of the pro-inflammatory environment. Actually, mCRP exerted its action on human primary chondrocytes coming not only from OA patients, but also from healthy subjects. This latter finding suggests that elevated locally produced levels of mCRP may trigger sustained multigenic inflammatory responses also in normal tissues not previously exposed to a pro-

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inflammatory micro-environment, as in the case of OA chondrocytes. Of note, it was recently described that mCRP directly binds to fibronectin without depending on calcium presence and pH (acid in the micro-environment of inflamed tissue, which promotes dissociation into the monomeric form). It was also shown that mCRP deposited in the ECM may regulate the recruitment and adhesion of monocytes thus triggering the inflammatory process[137]. This could help to understand the similar response to mCRP in both healthy and OA chondrocytes. The third important point is that the effects of mCRP in chondrocytes are likely to be mediated by the NF-kB signaling pathway. In particular, our results demonstrated that pyrrolidine dithiocarbamate (a well-known pharmacological inhibitor of NF-kB) partially abrogates the expression of all pro-inflammatory genes induced by mCRP. Accordingly, in osteoclasts, mCRP has revealed to act primarily through NF-kB and phospholipase C pathways[68]. Furthermore, in endothelial cells, p38 MAPK was implicated in the mCRP-induced cytokine promotion[142,171]. Although further experiments are needed, considering the versatility of mCRP in activating different signaling pathways, we describe here, for the first time, the involvement of NF-kB in chondrocytes upon mCRP stimulation.

Taken together, our data in cartilage set the molecular basis for a sustained action of mCRP in both human and murine chondrocytes. These results suggest that mCRP exerts a sustained catabolic effect by increasing the expression of inflammatory mediators and proteolytic enzymes, such as MMP13, and is thus able to promote cartilage breakdown and to trigger inflammatory responses in healthy and OA cartilage. Altogether, these molecules can cooperate resulting in the enhancement and perpetuation of the ECM-degrading processes at cartilage level. Finally, our results indicate that the route triggered by the release of NF-kB is involved. Despite these findings, further studies on the signaling elicited by mCRP are needed to completely assess its role in cartilage degradation and the pathogenesis and progression of OA.

1.2. MONOMERIC C REACTIVE PROTEIN IN INTERVERTEBRAL DISC

After we had reported the inflammatory effect of mCRP in chondrocytes, demonstrating that mCRP triggers a sustained pro-inflammatory and catabolic state in OA and healthy cartilage, and that NF-κβ pathway is involved in its signal transduction, we tried to demonstrate that this activity can be exerted also in IVD cells. In both OA and IVDD, wear and tear or traumatic injury can lead to low-grade inflammation, and consequently to tissue damage, ECM degradation and altered homeostasis, resulting in compromised functionality and pain[10].

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There is evidence that the inflammatory process plays an important role in the development of disc degeneration and there is a significant correlation between the levels of inflammatory markers and the degree of degeneration[172]. An association between high-sensitivity CRP (hs-CRP) levels and severe pain has been reported [173]. Sugimori et al. showed significantly higher mean hs-CRP levels among patients with lumbar disc herniation (0.056 ± 0.076 mg/dl) than in the control group (0.017 ± 0.021 mg/dl), no significant correlation between hs-CRP levels and herniation level was reported, but those patients with a higher concentration of hs-CRP before operation showed a poorer recovery [174]. Of interest, some papers only quantified CRP levels in the high sensitivity range (i.e., hs-CRP) describing values below 10 μg/mL. According to several studies [155–161] and to the guidance of the US Food and Drug Administration (FDA) [162], the clinical significance of such values is questionable.
In cervical disc herniation, pain occurs as a result of the release of local inflammatory cytokines. The elevated plasma concentration of IL-6 produced by macrophages around the IVD tissue increments CRP levels [27,175]. It was showed that CRP levels were significantly higher in patients with cervical disc hernia and neck pain compared to those patients with neck pain but a normal cervical magnetic resonance [111]. A recent study found that plasma CRP levels in peripheral blood did not differ between healthy controls and non-herniated IVD degeneration patients [176].

In our work, we tested the effect of mCRP in primary culture of human AF cells isolated from patients with IVD degeneration or from healthy subjects. In order to show that these effects are not exclusive from AF cells, the human NP immortalized cell line HNPSV-1 was used as a NP cell model for comparison in order to study mCRP effects in the whole disc, since NP cells commonly exhibit chondrocyte-like characteristics [177].

As far as we know, this is also the first experimental evidence for the functional activity of mCRP in AF and NP disc cells. To gain further insights into the action of mCRP and to verify whether this stimulation can result in sustained pro-inflammatory effects, we first evaluated the dose-dependent activity of mCRP on master inflammatory and catabolic players in IVD degeneration. IL-8 and IL-6 are two of the pro-inflammatory cytokines strongly expressed in IVD degeneration [178]. In this work we explored the ability of mCRP to modulate some of the most important factors involved in the disc degeneration inflammatory and catabolic processes. We also assessed LCN2, an adipokine implicated in the catabolic mechanism of IVD

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degeneration, MMP13, a catabolic enzyme, and the inflammatory mediators NOS2, COX2 and VCAM1. Nitric oxide synthase type II (iNOS) and COX-2 are classically involved in the amplification of inflammatory responses. NO has also cytotoxic effects, damages the IVD, and is involved in the upregulation of MMPs, among which MMP13 is one of the major mediators of ECM degradation. COX-2 induces prostaglandins that in turn increase the synthesis of other inflammatory mediators, including cytokines, to perpetuate tissue destruction. All these mediators are produced by IVD cells and are elevated in IVD degeneration [14,134,168–170]

At molecular level, degenerative processes in the disc are characterized by a shift in the collagen synthesis profile and increased expression of catabolic enzymes, such as matrix metalloproteinases, with a subsequent loss of proteoglycans. The proteolytic disintegration of aggrecan, which is promoted by cytokine-regulated aggrecanases (ADAMTS-4 and -5), reduces the disc's barrier function to nerve ingrowth[178]. The importance of IVD inflammation is further supported by research describing the nociceptive role of abnormal levels of pro-inflammatory molecules secreted by NP and AF cells as well macrophages, T cells and neutrophils. Secreted pro-inflammatory cytokines irritate invading nerve endings, promote the expression of nerve growth factor from IVD cells, and worsen disc pathology through initiation of autophagy, senescence, apoptosis and induction of catabolic processes[27]. Abnormal apoptosis of NP cells is an important cause of IVDD. Excessive apoptosis of NP cells leads to decreased cell viability, which results in decreased synthesis of ECM[179], thereby suggesting a potential role of this process in the acceleration of IVDD development. These findings explain the potential impact of inflammatory cytokines on IVDD and its symptoms[180]. In the disc, oxidative stress and lesions due to mechanical loading result in higher levels of IL-8[181]. Interestingly, not only local IL-8 expression, but also serum and cerebrospinal fluid concentrations are affected by spinal pathologies[182,183]. More in detail, patients with higher lumbar radicular pain and/or more pronounced disc herniation possess higher IL-8 levels in cerebrospinal fluid[182] and serum[183], respectively. IL-6 is an inflammatory cytokine with multiple biological effects, it is believed to play a major role in the pathogenesis of spinal neuropathic pain, specifically in symptomatic radiculopathy (by inducing PGE2-mediated allodynia in experimental rat models) and peripheral nerve injury. The functional role of IL-6 in spinal pain has also been supported by studies demonstrating that lumbar radicular pain induced by disc herniation is associated with elevated IL-6 serum levels. It has been demonstrated that the expression of IL-6 was dependent

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on the disc degeneration grade (significative positive correlation) and the zonal region of the IVD (higher in AF than NP). LCN2 is an adipokine that has been described as a sensor of mechanical load and inflammatory status of the joint, contributing to deregulation of cartilage and subchondral bone homeostasis, as well as bone-cartilage crosstalk, thus being implicated in OA and RA pathophysiology[24]. However, the role of LCN2 in disc degeneration remains largely unknown. Kao et al. demonstrated that nerve growth factor, an IVDD inducer, up-regulates the expression of LCN2, which forms covalent complexes with MMP9, blocking its auto-degradation and hence, increasing its activity, in rat AF cells[184]. These results could indicate that LCN2 may be implicated in disc degeneration, although further studies are needed. Most MMPs in discs are produced by NP cells and inner AF cells, and they are usually considered inactive zymogens in normal discs[185]. Cascade amplification effects occur when MMPs are activated, which leads to the degradation of the ECM[186]. Overexpression of MMPs and ECM degradation are clearly associated with the pathological processes that lead to both osteoarthritis and IVDD, and it is known that MMP13 cleavages type II collagen[185].

Regarding intervertebral disc, our present findings bring out important evidence about the modulation of IVD metabolism and functions exerted by mCRP, as well as the localization of mCRP in human IVD tissues. First, exposure of AF and NP cells to mCRP induced multiple pro-inflammatory genes suggesting that its effects are not limited to one specific molecular target, rather mCRP has a broader spectrum of action. The second important point is that the effect of mCRP is persistent and sustained for at least 24 hours, regardless of the pro-inflammatory environment. Actually, mCRP exerted its action on human primary AF cells coming not only from patients with IVD degeneration, but also from healthy subjects. In accordance with this, we found that mCRP can be localized physiologically both in healthy NP and AF tissues, and in addition, mCRP presence seems to increase in the degenerative AF tissue compared to the healthy one. Our finding suggests that elevated locally produced levels of mCRP may trigger sustained multigenic inflammatory responses also in normal tissues not previously exposed to a pro-inflammatory micro-environment, as in the case of AF cells from degenerated IVD. Another relevant aspect is that the effects of mCRP were observed both in AF and NP cells, showing that mCRP has multiple cell targets. Finally, the last important insight of this work is that mCRP effects in AF cells are mediated by NF-κβ signaling pathway. We proved that upon mCRP challenge, PI3K, ERK 1/2 and NF-κβ p65 were phosphorylated. These

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mediators were activated in a similar manner both in healthy and degenerative AF disc cells, confirming that mCRP action does not depend on a previous inflammatory state.

Our results are in agreement with those obtained by other groups working with other cell types. For instance, it has been observed in osteoclasts that mCRP acts through PI3K signaling pathway [68]. Wang and collaborators demonstrated that CRP increases IL-8 gene expression in HUVEC cells [187]. In osteoclasts, mCRP has revealed to act primarily through NF-kB and phospholipase C pathways[68]. Furthermore, in endothelial cells, p38 MAPK was implicated in the mCRP-induced cytokine promotion[142,171]. Our results also proved that NF-κβ has a role in mCRP signaling in cartilage [78]. This experimental design and the results observed were similar to those obtained in IVD cells. Regarding the potential role of mCRP in inflammatory and autoimmune disorders, very recently it has been demonstrated that extracellular vesicles opsonized by mCRP, but not by pCRP, are significantly increased in patients with active systemic lupus erythematosus (SLE), as well as in anti-CRP antibody positive patients. This suggests that mCRP on extracellular vesicles may contribute to anti-CRP autoantibody generation serving as adjuvant and/or autoantigen and thus contributing to SLE disease progression[188].

It is important to keep in mind that mCRP has long remained the subject of basic research; however, it is only in recent years that the levels of mCRP in the blood of patients and healthy individuals have been assessed. To date, nine studies on the measurement of mCRP levels in human serum or plasma have been published[123]. In summary, these studies showed the possibility of measuring mCRP levels. Most of them reported mCRP values in a comparable range. Some of these studies have reported an association between mCRP levels and disease severity and outcome. Nevertheless, large prospective studies are required to provide sufficient evidence to support this association or even establish mCRP levels as a diagnostic or prognostic tool.[123]

To sum up, our present findings provide, for the first time, evidence of the mCRP localization in healthy and degenerative IVD tissues, and a novel molecular basis for the sustained action of mCRP in human AF and NP cells. Taken together, these results suggest that mCRP exerts a lasting catabolic effect by increasing the expression of inflammatory mediators and proteolytic

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enzymes, being able to promote IVD cells breakdown and to trigger inflammatory responses in healthy and degenerative AF cells and in the immortalized NP cell line. Altogether, these molecules can cooperate resulting in the enhancement and perpetuation of the ECM-degrading processes at IVD level. In addition, mCRP can trigger by itself a severe increase in the expression of inflammatory mediators in healthy disc cells, suggesting a clear pathogenic role of mCRP in disc disease. Our results indicate that this effect occurs both in AF and NP cells, so affects the two main disc cellular compartments. Besides these findings, further studies on the pathophysiology of mCRP are needed to completely determine mCRP role in the pathogenesis and progression of IVD degeneration.

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2. CHAPTER 2. WISP2 IN CARTILAGE

The recently discovered adipokine WISP-2 has been identified as a Wnt-inducible protein[189]. In the last decade, evidence has suggested a key role for Wnt signalling in the pathophysiology of OA. Canonical Wnt/β-catenin pathway leads to excessive catabolism and cartilage degradation and the hypertrophic differentiation of chondrocytes. Wnt ligands as Wnt-7b and Wnt target genes including Wnt-1 inducible signaling pathway protein 1 (WISP-1)[83] were found to be upregulated in OA cartilage, as well as nuclear β-catenin and the co-receptor LRP5[86]. Pro-catabolic factors such as IL-1β induced the expression of various Wnt proteins, resulting in the activation of β-catenin[87], and activation of Wnt/β-catenin signaling stimulated the expression of cartilage ECM-degrading MMPs[88]. However, Wnt signalling is necessary for maintenance of the articular cartilage, but an excessive activity is deleterious.

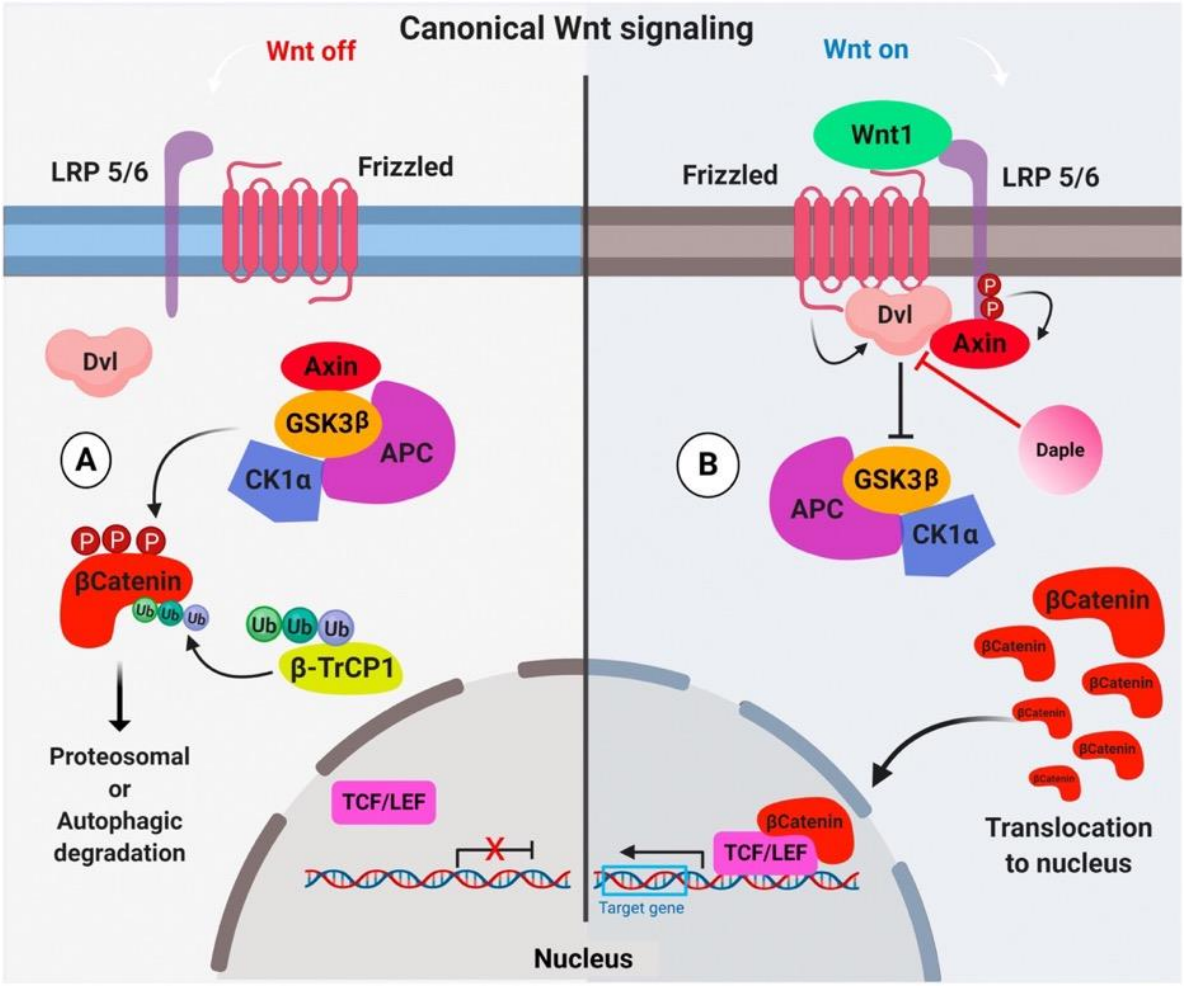

Figure 9. Canonical Wnt signaling. (A) In the absence of Wnt proteins, β-catenin is constantly phosphorylated by a multimeric protein complex consisting of Axin, APC, GSK-3β and CK1α. This labels

β-catenin for initial ubiquitination by β-TrCP1, and further degradation by proteasomes or autophagy. (B) When Wnt proteins bind to Frizzled receptors and a family of LRP 5/6 co-receptors, the cytoplasmic region of LRP 5/6 is phosphorylated by GSK3β. This leads to recruitment of Dvl and axin proteins to the cytoplasmic regions of Frizzled and LRP5/6 receptors, respectively. This process can be inhibited by Daple protein. Activated Dvl disrupts the destructive complex and inhibits GSK3β-mediated phosphorylation of β-catenin, leading to inhibition of degradation. Consequently, β-catenin accumulates in the cytoplasm, translocates into the nucleus, and binds to TCF/LEF transcription factors, acting as a co-activator for expression of Wnt target genes. *Lorzadeh, Shahrokh et al. "Autophagy and the Wnt signaling pathway: A focus on Wnt/β-catenin signaling." Biochimica et biophysica acta. Molecular cell research vol. 1868,3 (2021): 118926. doi:10.1016/j.bbamcr.2020.118926*[190]

Recent studies have investigated the role of CCN family proteins in the development of OA. WISP-1/CCN4 and WISP-2/CCN5 were found expressed to a greater extent in OA and RA knee cartilage and all CCN genes were expressed in OA and RA synovial samples[191]. Moreover, our group has identified WISP-2 in cartilage, synovium, and infrapatellar fat pad, finding a significative increment in WISP-2 expression in OA infrapatellar fat pad in comparison to healthy controls[96]. In developmental dysplasia of the hips, it was found during chondrogenesis that WISP-2 overexpression in primary chondrocytes reduces cell viability and PPARγ activation[192]. WISP-2 expression slightly increases during osteogenesis in vitro; exogenous WISP-2 has no effect on osteoblast proliferation, but induces osteoblast maturation by upregulating Osterix, ALP and Bsp via Smad 1/5/8, β-catenin and p38 mediated pathways[193] Interestingly, a previous work about the biological role of WISP-2 showed that it is not required for normal bone formation, although it is not crucial in bone biology, since Ccn3/Nov knockout mice exhibit accelerated bone healing after injury despite the lack of an overt skeletal phenotype, this might also be the case for WISP-2[194]. Other members of the WISP superfamily have been reported to exert pathophysiological action in musculoskeletal tissues. WISP-1 regulates chondrocyte MMP and aggrecanase expression and is capable of inducing articular cartilage damage in models of OA[83]. By contrast, WISP-3/CCN6-induced repression of ADAMTS-5 expression and up-regulation of MMP-10 in human chondrocytes[195]. Regarding the participation of WISP-2 in OA, there are currently no papers in the literature evaluating its action in chondrocytes. Thus, we have analyzed the effect of WISP-2 on cartilage catabolism using the recombinant protein and loss-of-function experiments to enlighten the role of this protein in chondrocyte pathophysiology. To the best of our knowledge, this is the first work

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showing experimental evidence that WISP-2 is modulated by mediators of inflammation and its involvement in cartilage degradation.

We first analyzed the regulation of WISP-2 expression along chondrocyte differentiation observing an increase of WISP-2 expression in the first stages of chondrogenesis, followed by a dramatic decrease at the onset of hypertrophic stage. Our data in ATDC-5 cells, a well-established cell line to study chondrogenesis[196], are in agreement with those obtained by Schutze *et al*.[197]. Although these authors used bone marrow-derived MSC, that in their undifferentiated stage do not express none of the chondrogenic markers, the ATDC-5 chondrogenic cell line expressed them also in the chondrogenic stage. Our experimental set provides novel data since we analyzed WISP-2 expression up to the onset of hypertrophy, showing that both mRNA and protein expression of WISP-2 strongly decreased. Noteworthy, the marked reduction in WISP-2 expression at hypertrophic phase was coincident with a significant increase in MMP-13 expression, the main marker of this last stage[198], suggesting a relationship between these two factors. Of note, CCN5 overexpression in primary chondrocytes reduces cell viability and PPARγ activation as observed in developmental dysplasia of the hips[192].

Plenty of studies have described the involvement of cytokines in cartilage destruction. IL-1β is a well-known pro-inflammatory cytokine that is a potent inductor of MMP-13 expression[199]. Thus, we have studied the effect of relevant pro-inflammatory cytokines and adipokines involved in rheumatic diseases, such as TNF-α, IL-1β or leptin, on WISP-2 expression in chondrocytes. WISP-2 is regulated by transcription factors which are induced by inflammation, activation of the Wnt pathway, and hypoxia, processes increased in obesity[90]. Furthermore, IL-1β induces expression of Wnt proteins, resulting in activation of Wnt pathway[87]. On the other hand, the endogenous activation of Wnt/β-catenin signalling, as observed in certain tumour cell lines, is characterized by a very low expression of WISP-2[91]. In contrast to other members of the CCN family, whose levels are increased under inflammatory conditions (i.e. WISP-3[200]), we have observed that either TNF-α, IL-1β, LPS, or IL-6 were able to reduce the expression of WISP-2 in human chondrocytes. Together, these results suggest that a "canonical" pro-inflammatory environment characterized by cytokines or TLR4 activation negatively modulates the expression of WISP-2. By contrast, the classic pro-inflammatory adipokines, leptin and adiponectin did not alter the expression of WISP-2 in T/C-28a2 chondrocytes. We expected that these adipokines, that are well known contributors to create a pro-inflammatory

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environment in obesity and OA, might be able to regulate WISP-2. Remarkably and surprisingly, they did not.

One aspect that arises from our current investigations is that WISP-2 can minimize the catabolic effects of IL-1β in cartilage. As a matter of fact, recombinant WISP-2 was able to partially decrease the main metalloproteases and aggrecanases induced by IL-1β. In our experiments, recombinant WISP-2 was able to attenuate the IL-1β/NF-κβ as well as the Erk 1/2, JNK and p38 signaling pathways. Therefore, these data, together with the observation that WISP-2 is able to increase the phosphorylation of GSK-3, suggested that WISP-2 may act as a WNT agonist with potential anabolic functions that are partially able to counteract the catabolic effects of IL-1β as one of the main mediators of inflammatory response in cartilage.

We confirmed these observations by silencing WISP-2. Indeed, the silencing of WISP-2 increased the expression of MMP-13, but also was able to upregulate the expression of other aggrecanases and relevant pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8. Finally, the activation of canonical WNT pathway by BIO (a classic GSK-3β inhibitor) confirms the previously showed results and agree with previous published literature. In concrete terms, WISP-2 may contribute to counteract the detrimental effects of inflammation. To this regard, CCN5/WISP-2 has been reported to have also protective effects counteracting the fibrosis in heart failure by inhibiting the TGF-β pathway[201]. In rheumatoid arthritis, Tanaka et al. reported that WISP-2 is prevalently expressed in arthritic synovial tissues[94] and specifically in fibroblast of the fibrotic area.

In conclusion, there are potentially significant physiologic and pathophysiologic aspects glanced from this investigation. To summarise, we have shown for the first time that WISP-2 may have relevant roles in modulating the expression of enzymes involved in the turnover of extracellular matrix in the cartilage and that its downregulation may negatively alter the inflammatory environment in OA cartilage. We also proved the participation of Wnt/β-catenin signalling pathway in these processes. Thus, targeting WISP-2 may represent a possible therapeutical approach to OA. Further research is needed to define the contribution of WISP-2 in the complex metabolic network of degenerative/inflammatory diseases of musculoskeletal system such as osteoarthritis.

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CONCLUSIONS

In ***Article 1. Monomeric C reactive protein (mCRP) regulates inflammatory responses in human and mouse chondrocytes.***

1. mCRP can modulate the expression of nitric oxide synthase type II (NOS2), an early inflammation molecular biomarker, raising its expression levels in human healthy and degenerative chondrocytes and in the murine chondrocyte cell line ATDC5.

2. mCRP increases the mRNA and protein expression levels of some relevant factors involved in inflammatory and catabolic responses: NOS2, COX2, MMP13, VCAM1, IL-6, IL-8, and LCN2; in human healthy and degenerative chondrocytes and in murine chondrocyte cell line ATDC5. This shows that mCRP exerts a sustained catabolic effect on chondrocytes, increasing the expression of inflammatory mediators and proteolytic enzymes, which can promote ECM breakdown both in healthy and OA cartilage.

3. NF-kβ is a key factor in the intracellular signaling of mCRP-driven induction of pro-inflammatory and catabolic mediators in chondrocytes.

In ***Article 2. Monomeric CRP regulates inflammatory responses in human intervertebral disc cells.***

4. mCRP can be present in healthy and degenerative IVD tissues, both in annulus fibrosus and nucleus pulposus tissues. Its location is predominantly cytoplasmic and perinuclear, and its presence seems higher in AF, especially in the degenerative tissue, than in the NP.

5. mCRP induces the expression of multiple proinflammatory and catabolic factors in both healthy and degenerative human AF cells and in the immortalized NP cell line. The effect of mCRP is persistent and sustained, being able to promote IVD cell breakdown and to trigger inflammatory responses regardless of the proinflammatory environment. In addition, mCRP by

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itself can trigger a severe increase in the expression of inflammatory mediators in healthy disc cells, suggesting a pathogenic role in disc disease.

6. mCRP effects in healthy and degenerative AF cells are mediated by phosphoinositide 3-kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2), and nuclear factor-κβ (NF-κβ) p65 signaling pathways.

7. This is the first experimental work that provides evidence for the functional activity of mCRP in healthy and degenerative human AF and NP disc, and that demonstrates the localization of mCRP in intravertebral disc cells of the AF and NP.

In ***Article 3. WISP-2 modulates the induction of inflammatory mediators and cartilage catabolism in chondrocytes.***

8. WISP2 expression increases along chondrocyte differentiation and is downregulated at the onset of hypertrophy, matching the time point in which MMP-13 expression is significantly upregulated. This suggests that WISP2 may have relevant roles in modulating the turnover of extracellular matrix in cartilage.

9. Under inflammatory conditions originated by TNF-α, IL-1β, IL-6 or LPS treatments, WISP2 expression is significantly downregulated in chondrocytes.

10. WISP2 expression is significantly downregulated in OA chondrocytes compared to chondrocytes from healthy subjects.

11. Recombinant human WISP2 protein significantly reduces IL-1β-mediated chondrocyte catabolism.

12. Recombinant human WISP2 protein significantly reduces IL-1β-induced NF-κβ p65 phosphorylation, Erk 1/2 phosphorylation, JNK phosphorylation, and p38 phosphorylation. This confirms the WISP2 inhibitory effect in the IL-1β signaling pathway in human chondrocytes.

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13. Recombinant human WISP2 protein significantly activates Wnt signaling. Also, rhWISP2 in combination with IL-1β presents a cooperative effect, showing an increased activation of Wnt pathway comparing to the single treatments.

14. WISP2 gene knockdown increases the expression of ECM breakdown mediators in human OA chondrocytes.

15. WISP2 gene knockdown causes a significant decrease of GSK-3 phosphorylation, suggesting that the process of accumulation and translocation of β-catenin to the nucleus, and the expression of downstream target genes are compromised.

16. WISP2 gene knockdown on IL-1β-stimulated chondrocytes originates a significantly higher expression of the catabolic markers MMP-13 and ADAMTS-5, and the inflammatory mediators IL-6 and IL-8, than in the non-silenced cells. This shows that WISP2 downregulation may detrimentally alter the inflammatory environment in OA cartilage, in a Wnt/β-catenin dependent manner.

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ARTICLE

Monomeric C reactive protein (mCRP) regulates inflammatory responses in human and mouse chondrocytes

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Abstract

C-reactive protein (CRP) is an acute-phase protein that is used as an established biomarker to follow disease severity and progression in a plethora of inflammatory diseases. However, its pathophysiologic mechanisms of action are still poorly defined and remain elusive. CRP, in its pentameric form, exhibits weak anti-inflammatory activity. On the contrary, the monomeric isoform (mCRP) exhibits potent pro-inflammatory properties in endothelial cells, leukocytes, and platelets. So far, no data exists regarding mCRP effects in human or mouse chondrocytes. This work aimed to verify the pathophysiological relevance of mCRP in the etiology and/or progression of osteoarthritis (OA). We investigated the effects of mCRP in cultured human primary chondrocytes and in the chondrogenic ATDC5 mouse cell line. We determined mRNA and protein levels of relevant factors involved in inflammatory responses and the modulation of nitric oxide synthase type II (NOS2), an early inflammatory molecular target. We demonstrate, for the first time, that monomeric C reactive protein increases NOS2, COX2, MMP13, VCAM1, IL-6, IL-8, and LCN2 expression in human and murine chondrocytes. We also demonstrated that NF-kB is a key factor in the intracellular signaling of mCRP-driven induction of pro-inflammatory and catabolic mediators in chondrocytes. We concluded that mCRP exerts a sustained catabolic effect on human and murine chondrocytes, increasing the expression of inflammatory mediators and proteolytic enzymes, which can promote extracellular matrix (ECM) breakdown in healthy and OA cartilage. In addition, our results implicate the NF-kB signaling pathway in catabolic effects mediated by mCRP.

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Introduction

C-reactive protein (CRP) is an acute-phase reactant and a member of the pentraxin family [1]. It circulates prevalently in the blood as a pentamer of identical monomeric subunits. For many decades, CRP levels have been used as a circulating biomarker to monitor the inflammatory status in infections and other multiple diseases, including rheumatic diseases such as rheumatoid arthritis (RA) and osteoarthritis (OA) [2]. CRP has at least two naturally occurring and conformationally distinct isoforms described as pentameric CRP (pCRP) and monomeric CRP (mCRP) [3]. Defining the pathophysiological role of CRP as a regulator of inflammatory processes remains obscure and poorly described; elaborating on its role now requires careful attention to which CRP conformation contributes to any described response. OA is a degenerative disease mainly characterized by articular cartilage degradation. A combination of multiple factors, including genetic, mechanical, inflammatory, and metabolic, is likely to be involved in both pathogenesis and progression of the disease [4], but most of them still remain elusive.

CRP levels have been reported to be significantly elevated in patients with OA compared to healthy controls, and they were observed to be correlated with radiographic parameters as well as clinical severity [5–7]. Although some studies showed no association between CRP levels and radiographic OA, a correlation with pain and decreased physical function has been found [8]. Of interest, most studies in OA patients (2, 5–7) only quantified CRP levels in the high sensitivity range (i.e. hsCRP) describing values below 10 µg/ml. According to several studies [9–15] and to the guidance of US-FDA [16], the clinical significance of such values is undefined.

A recent paper by Kozijn et al. [17] demonstrated that CRP aggravates OA development in mice on a high-fat diet, although the mechanism of action for CRP involvement is not clarified yet. A hypothetical mechanism through which CRP could be involved in local cartilage disarrangements in OA might be via the pathogenic monomeric subunit of CRP (mCRP), originated by phospholipase-A2 (PLA_2) enzymatic action in an inflammatory environment [18]. Increased PLA_2 activity has been observed in synovial fluid from OA patients and in animal models of OA [19, 20]. However, it is also hypothetically possible that pCRP increases locally where it works to dampen the acute inflammatory response leading to prolonged, chronic tissue damage associated with arthritis. Whereas the phosphatidyl choline (PC) groups on PLA_2 binding to the PC face will make the pCRP cholesterol binding ligand accessible so that pCRP can bind to a membrane, and, consequently, it is juxtaposed to a lipid zone that will supply the biochemical energy to relax pCRP into pCRP* orientation then dissociates to mCRP [21].

To date, the role of mCRP in cartilage pathophysiology, as well as its relevance in OA etiology and progression, has not been investigated. Therefore, in this study, we evaluated the functional role of mCRP as a modulator of the inflammatory response in chondrocytes. We evaluated the modulation of nitric oxide synthase type II (NOS2), an early inflammatory molecular mediator, as well as other relevant factors involved in the inflammatory response in human primary and immortalized mouse chondrocytes upon mCRP stimulation.

Materials and methods

Reagents

Fetal bovine serum (FBS), human transferrin, sodium selenite, lipopolysaccharide (LPS), mouse recombinant interleukin (IL)1β, and nuclear factor kappa B (NF-kB) inhibitor pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium, trypsin-ethylenediaminetetraacetic acid, L-glutamine, and penicillin-streptomycin mixture were obtained from Lonza Group (Basel, Switzerland). Pronase and collagenase P were obtained from Roche Applied Science (Penzberg, Germany).

Recombinant mCRP produced in *Escherichia coli* had both cysteines replaced with alanine residues and was solubilized by an acylation technique (C-rmCRP) in 25 mM NaPBS (pH7.4), purified and tested in several bioassays to confirm its equivalence to biological mCRP from the pentameric form and free of contaminating endotoxin. The final endotoxin level of all protein solutions was below the detection limit 0.06 EU/ml of the assay [22].

Cell culture conditions and treatments

The murine chondrogenic cell line ATDC5 (RIKEN Cell Bank, Tsukuba, Japan) was cultured in DMEM/Ham's F12 supplemented with 5% FBS, 10 µg/ml human transferrin, 3×10 M sodium selenite, 4 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin. Articular cartilage samples were obtained from knee and hip joints of patients undergoing total joint replacement, under written patient consent, and permission from the local ethics committee, according to the declaration of Helsinki. Human primary chondrocytes were isolated and cultured as previously described [23].

Chondrocytes were seeded in six-well plates (2.5×10^5 cells/well) and treated with 10, 25, or 50 µg/ml mCRP, 100 ng/ml LPS or 0.1 ng/ml IL-1β for 24 h, after serum starvation of human primary chondrocytes or ATDC5 cell

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line for 4 h or overnight, respectively. All the concentrations were selected based on previous studies and in the absence of chondrocyte cell toxicity. All treatments lasted 24 h, after 4 h of starvation for FBS in human primary chondrocytes and overnight starvation in ATDC5 cell line.

Nitrite assay

Nitrite accumulation was measured in the culture medium by the Griess reaction, as previously described [24–26]. Cell culture supernatant was incubated at room temperature with Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl), and the absorbance was measured at 550 nm in a microplate reader (Titertek Multiscan; Labsystems, Helsinki, Finland). Fresh culture medium was used as a blank, and nitrite concentration (μM) was calculated from a sodium nitrite standard curve.

RNA isolation and real-time reverse transcription-polymerase chain reaction

Total RNA was isolated from cell culture with NZYol (NZYTech, Lisbon, Portugal) and E.Z.N.A.® Total RNA Kit I (Omega Bio-tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions; and reverse-transcribed using NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). Then, SYBR-green-based quantitative real-time PCR (RT-qPCR) was performed in Stratagene MX3005P thermal cycler as previously described [27] using a standard protocol with RT^2 SYBR Green qPCR Mastermix and specific PCR primers (Qiagen, Hilden, Germany) (human GAPDH, 175 bp, PPH00150E, reference position 1287, Gen-Bank accession no. NM_002046.3; mouse GAPDH, 140 bp, PPM02946E, reference position 309, Gen-Bank accession no. NM_008084.2; human NOS2, 132 bp, PPH00173E, reference position 3962, Gen-Bank accession no. NM_000625.4; mouse NOS2, 122 bp, PPM02928B, reference position 2728-2748, Gen-Bank accession no. NM_010927.3; human MMP13, 61 bp, PPH00121B, reference position 1380, Gen-Bank accession no. NM_002427.3; mouse MMP13, 88 bp, PPM03675A, reference position 1145, Gen-Bank accession no. NM_008607; human VCAM1, 141 bp, PPH00623E, reference position 2980, Gen-Bank accession no. NM_001078; mouse VCAM1, 78 bp, PPM03208C, reference position 2307, Gen-Bank accession no. NM_011693; human LCN2, 87 bp, PPH00446E, reference position 626-644, Gen-Bank accession no. NM_005564.3; mouse LCN2, 81 bp, PPM03770A, reference position 364-383, Gen-Bank accession no. NM_008491.1; human IL6, 98 bp, PPH00560C, reference position 816, Gen-Bank accession no. NM_000600.3; mouse IL6, 178 bp, PPM03015A, reference position 120, Gen-Bank accession no. NM_031168; human IL8, 126 bp, PPH00568A, reference position 326, Gen-Bank accession no. NM_000584.3; human PTGS2, 63 bp, PPH01136F, reference position 1502, Gen-Bank accession no. NM_000963.2) No-template controls were included to eliminate any nonspecific amplification and melting curves were generated to ensure a single gene-specific peak. Gene expression changes were determined by the comparative ($\Delta\Delta C$t) method in MxPro qPCR Software version 4.10 (Stratagene, La Jolla, CA, USA), and expressed as relative fold changes compared to control and normalized to GAPDH housekeeping gene.

Protein extraction and western blot analysis

After treatments, total cell lysates were obtained using lysis buffer (10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5% Triton X-100, 1 mM PMSF), freshly supplemented with protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA), and then centrifuged at 14,000 *g* for 20 min. SDS-PAGE and blotting procedures were performed as previously described [28]. Immunoblots were incubated with the specific antibody against (NOS-2 or COX-2 (Cell Signaling, Danvers, MA, USA), IL-6 or IL-8 (Santa Cruz Biotechnology, Dallas, TX, USA), MMP13 (Abcam, Cambridge, UK), mouse LCN2, or human LCN2 (R&D Systems, Minneapolis, MN, USA)).

The immune complexes were detected using anti-rabbit (GE Healthcare, Chicago, IL, USA), anti-mouse (both from GE Healthcare, Chicago, IL, USA) or anti-goat (Santa Cruz Biotechnology, Dallas, TX, USA) horseradish-peroxidase-labeled secondary antibodies and Immobilon ECL. To verify equivalent protein loading, the membranes were incubated with anti-GAPDH antibody (Sigma-Aldrich).

The signals generated were detected in ChemiDoc MP Imaging System (Bio-Rad Laboratories, Inc.) and analyzed with Image Lab 6.0.1 Software (Bio-Rad Laboratories, Inc., Hercules CA, USA).

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM) of at least three independent experiments. Statistical tests were applied using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). Assuming a normal distribution, one-way ANOVA followed by Fisher´s LSD test was used. As we performed few planned comparisons, *p* values are not corrected for multiple comparisons, they apply individually to each value reported and not to the entire family of comparisons. *P* values less than 0.05 were considered significant.

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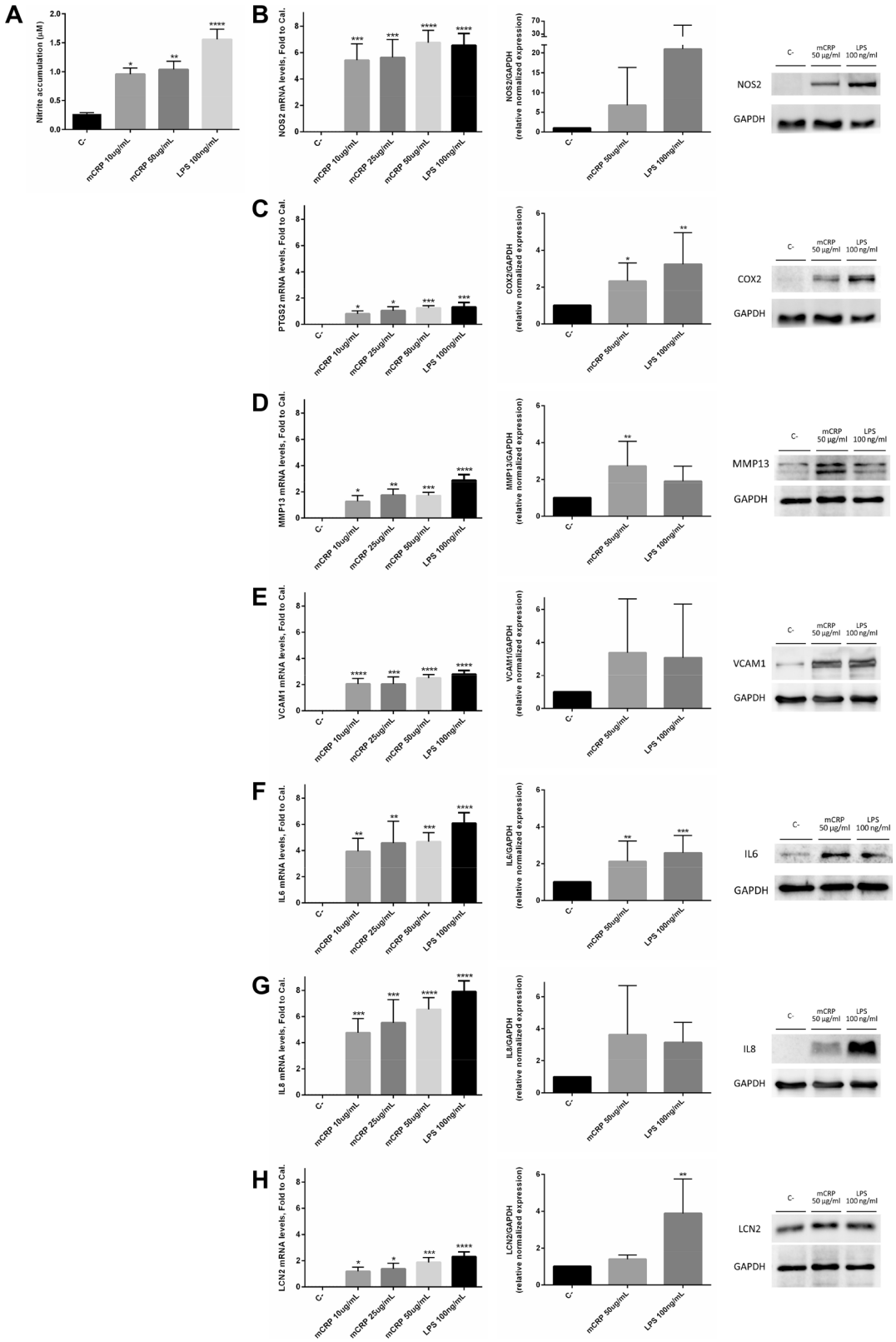

Results

As shown in Fig. 1A, mCRP at both 10 and 50 μg/ml induces a significant accumulation of nitrite levels in the supernatant of cultured primary human chondrocytes from OA patients. Accordingly, mCRP increases NOS-2 mRNA expression and protein levels, as confirmed RT-qPCR and western blot analysis' densitometry, respectively (1B). In a

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◀ **Fig. 1 mCRP pro-inflammatory effect on human primary osteoarthritis chondrocytes.** Human primary OA chondrocytes in culture were challenged with 10, 25, or 50 µg/ml mCRP, or with 100 ng/ml LPS as positive control for 24 h, after 4 h serum starvation. **A** Nitric oxide production was evaluated by determining nitrite concentration (µM) in culture medium after 24 h treatment with 10, or 50 µg/ml mCRP, or with 100 ng/ml LPS as positive control using Greiss reaction. **B–H** (left panels) mRNA expression was determined by RT-qPCR. mRNA levels are presented as fold change relative to control (C−). **B–H** (middle and right panels) Protein expression was measured by western blot. Data from densitometric analyses normalized to GAPDH are shown as relative-to-control (C−) values. Data expressed as mean ± SEM of at least three independent experiments. Comparisons referred to control (C−): $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. mCRP monomeric C-reactive protein, LPS lipopolysaccharide, NOS2 nitric oxide synthase 2, PTGS2 prostaglandin-endoperoxide synthase 2, COX2 cyclooxygenase 2, MMP13 matrix metalloproteinase 13, VCAM1 vascular cell adhesion molecule 1, IL6 interleukin 6, IL8 interleukin 8, LCN2 lipocalin 2, GAPDH glyceraldehyde 3-phosphate dehydrogenase.

similar manner, mCRP also increased both mRNA and protein expression of COX-2 (C), MMP13 (D), VCAM1 (E), IL-6 (F), IL-8 (G) and LCN2 (H).

The induction of pro-inflammatory mediators by mCRP is also evident in human primary chondrocytes from healthy patients, suggesting that mCRP can modify the cell status by promoting the development of a pro-inflammatory environment in the cartilage (Fig. 2 from A to G). In all the experiments with human cells, LPS was used as positive control of inflammatory induction. Of note, the chondrocyte response to 50 µg/ml mCRP was comparable to that of 100 ng/ml LPS for the analyzed parameters.

To further confirm our results, we tested mCRP activity in the ATDC5 murine chondrocyte cell line, a very useful tool for studying molecular and cellular mechanisms of cartilage inflammation in vitro [26]. As shown in Fig. 3, mCRP significantly induces nitrite accumulation in ATDC5 cells (3A), and highly augments NOS2 (B), MMP13 (C), VCAM1 (D), IL-6 (E) and LCN2 (F), either at mRNA or protein level, as previously observed in human primary chondrocytes.

To gain further insights into the action mechanisms responsible by mCRP-induced pro-inflammatory effects in chondrocytes, we analyzed the involvement of NF-kB signaling pathway. NF-κB is a transcription factor that has been widely demonstrated to be involved in cellular responses to different signals of stress, such as cytokines, free radicals, heavy metals, and bacterial or viral antigens. Over-expression or inappropriate activation of NF-κB is implicated in many pathological mechanisms of diseases, ranging from inflammation to cancer [29]. PDTC is a thiol compound, which has been considered an effective NF-kB inhibitor [30]. Thus, in order to investigate the potential protective effects of NF-κB inhibition on mCRP-induced pro-inflammatory mediators' expression, PDTC was used in combination with mCRP in chondrocytes. As shown in Fig. 4 (panels A and B), PDTC significantly reduce both nitrite accumulation and NOS2 expression (at mRNA and protein levels) in mCRP-stimulated ATDC5 cells. In addition, PDTC also significantly reduced the expression of MMP13, VCAM1, IL-6, and LCN2 induced by 50 µg/ml mCRP (Fig. 4, panels C to F)

Discussion

CRP is a well-established biomarker in a plethora of infectious and inflammatory diseases, including RA and OA. Furthermore, CRP levels are closely associated with disease severity and/or progression [31], however little is known about its pathophysiological role. It is still matter of debate whether CRP plays any specific role in the elementary pathological processes.

CRP is an acute-phase protein that circulates in the blood prevalently as an annular (ring-shaped) pentamer of five identical monomer subunits (pCRP), which has been evidenced as a mediator of inflammatory and immune responses in the context of local tissue injury [32, 33]. At bone level, CRP has been reported to have controversial biological effects on osteoclast differentiation. However, recently, it has been demonstrated that only mCRP originated from local pCRP in the joint compartment, may bind to RANKL, thus, neutralizing its activity and downregulating osteoclast differentiation [34].

The physiological and pathological roles of mCRP and its biological relevance in cartilage biology and in OA is practically unknown.

Several published data regarding the relationship between OA development and progression and the levels of CRP are contradictory since CRP levels are in the range of hsCRP (below 10 µg/ml) whose clinical significance is matter of debate. These contradictory results, together with the lack of knowledge about the pathophysiological role of the mCRP in cartilage, have overshadowed the association between CRP levels and OA pathology, making the functional involvement of CRP in OA pathogenesis and/or progression uncertain. Evidence suggests that mCRP exhibits different antigenic, biological, and electrophoretic properties compared to the pentameric form and it generally displays marked pro-inflammatory properties in several cell lineages, including vascular endothelial cells, macrophages, and neutrophils [35–37].

Although several lines of evidence have postulated a role for mCRP in cartilage, there is no experimental evidence of its activity in human or mouse chondrocytes. To address this key question, we tested the effect of mCRP in primary culture of human chondrocytes isolated from patients with hip and knee OA, or from healthy subjects. For the sake of

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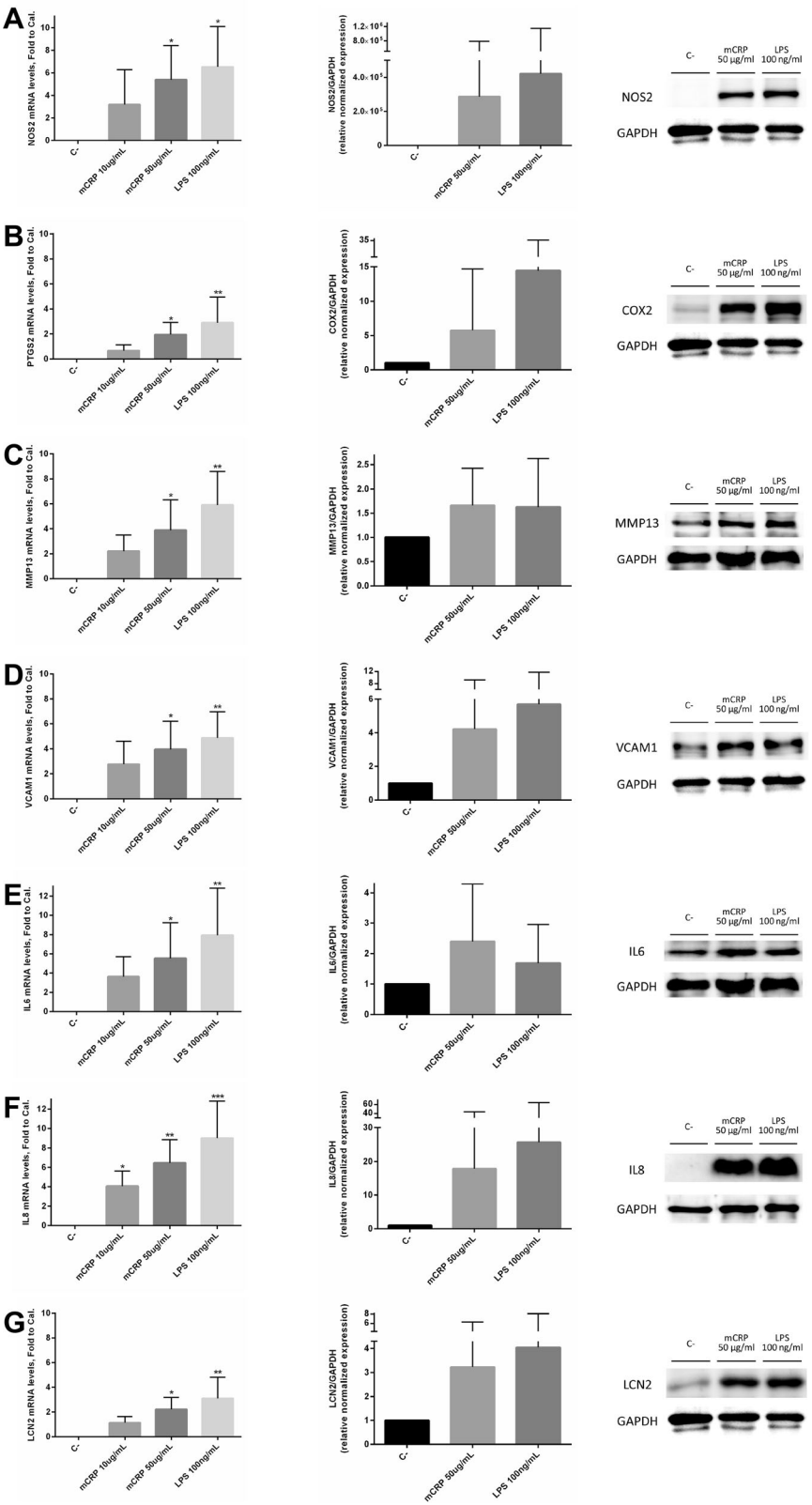

Fig. 2 mCRP pro-inflammatory effect on human primary healthy chondrocytes. Human primary healthy chondrocytes in culture were challenged with 10 or 50 µg/ml mCRP, or with 100 ng/ml LPS as positive control for 24 h, after 4 h of serum starvation. **A–G** (left panels) mRNA expression was determined by RT-qPCR. mRNA levels are presented as fold change relative to control (C−). **A–G** (middle and right panels) Protein expression was measured by western blot. Data from densitometric analyses, normalized to GAPDH, are shown as relative-to-control (C−) values. Data expressed as mean ± SEM of at least three independent experiments. Comparisons referred to control (C−): $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. mCRP monomeric C-reactive protein, LPS lipopolysaccharide, NOS2 nitric oxide synthase 2, PTGS2 prostaglandin-endoperoxide synthase 2, MMP13 matrix metalloproteinase 13, VCAM1 vascular cell adhesion molecule 1, IL6 interleukin 6, IL8 interleukin 8, LCN2 lipocalin 2.

completeness, the ATDC5 murine chondrogenic cell line was also used as another chondrocyte model for comparison. As far as we are aware, this is the first experimental article that provides evidence for the functional activity of mCRP in human and mouse chondrocytes. To gain further insights into the action of mCRP and to verify whether this

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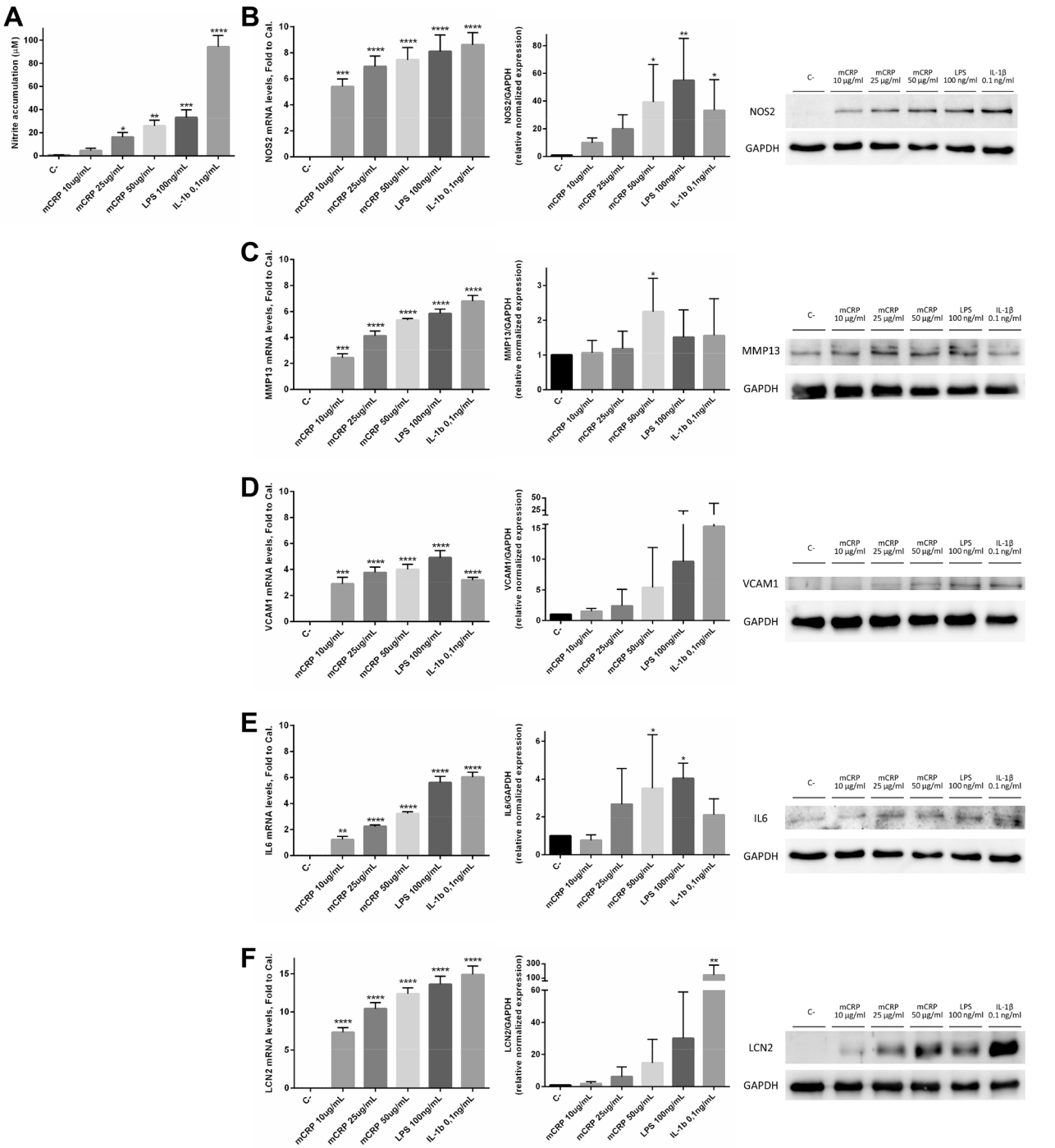

Fig. 3 mCRP pro-inflammatory effect on murine chondrocyte cell line. Murine chondrocyte cells were challenged with 10, 25, or 50 μg/ml mCRP, or with 100 ng/ml LPS or 0.1 ng/ml IL-1b as positive controls for 24 h, after overnight serum starvation. **A** Nitric oxide production was evaluated by determining nitrite concentration (μM) in culture medium after 24 h treatment using Greiss reaction. Data expressed as mean ± SEM of at least six independent experiments. **B**–**F** (left panels) mRNA expression was determined by RT-qPCR. mRNA levels are presented as fold change relative to control (C−) Data expressed as mean ± SEM of at least three independent experiments. **B**–**F** (middle and right panels) Protein expression was measured by western blot. Data from densitometric analyses normalized to GAPDH are shown as relative-to-control (C−) values. Data expressed as mean ± SEM of at least three independent experiments. Comparisons referred to control (C−): $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. mCRP monomeric C-reactive protein, LPS lipopolysaccharide, IL-1b interleukin 1 beta, NOS2 nitric oxide synthase 2, MMP13 matrix metalloproteinase 13, VCAM1 vascular cell adhesion molecule 1, IL6 interleukin 6, LCN2 lipocalin 2, GAPDH glyceraldehyde 3-phosphate dehydrogenase.

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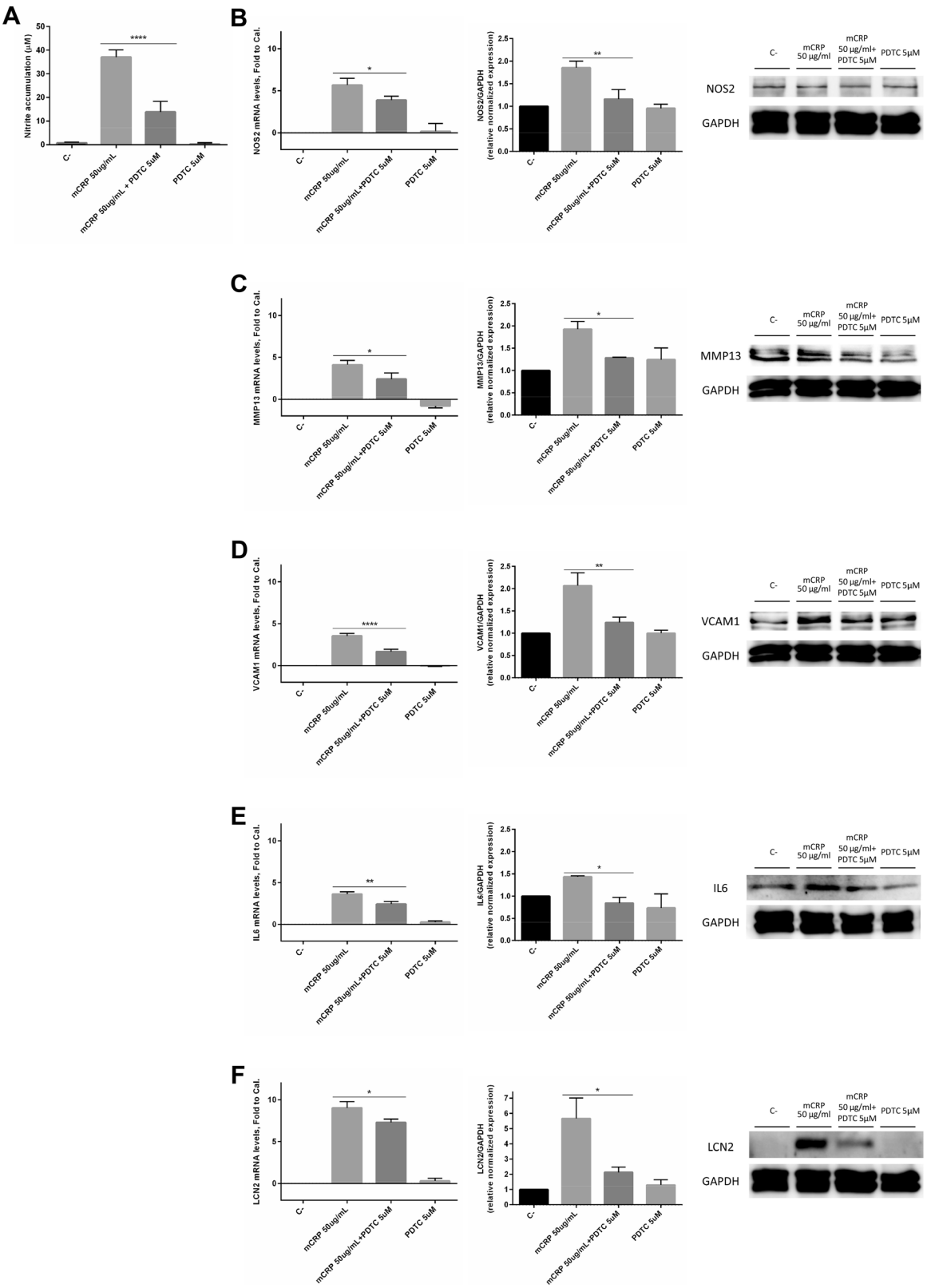

stimulation can result in sustained pro-inflammatory effects, we evaluated the dose-dependent activity of mCRP in master inflammatory and catabolic players in cartilage degradation. Nitric oxide synthase type II (iNOS) and COX-2 are classically involved in the amplification of inflammatory responses. NO is cytotoxic to chondrocytes,

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◀ **Fig. 4 Nf-κβ role in mCRP signaling: Nf-κβ inhibition with PDTC in murine chondrocyte cell line.** Cells starved for serum overnight were pre-treated for 1 h with PDTC 5 μM. After keeping the culture medium, were challenged with 50 μg/ml mCRP for 24 h. **A** Nitric oxide production was evaluated by determining nitrite concentration (μM) in culture medium after 24 h treatment using Griess reaction. Data expressed as mean ± SEM of six independent experiments. Comparisons mCRP 50 μg/mL vs. mCRP 50 μg/mL + PDTC 5 μM. **B–F** (left panels) mRNA expression was determined by RT-qPCR. mRNA levels are presented as fold change relative to control (C−). Data are expressed as mean ± SEM of at least five independent experiments. **B–F** (middle and right panels) Protein expression was measured by western blot. Data from densitometric analyses, normalized to GAPDH, are shown as relative-to-control (C−) values. Data expressed as mean ± SEM of at least three independent experiments. Comparisons mCRP 50 μg/mL vs. mCRP 50 μg/mL + PDTC 5 μM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. mCRP monomeric C-reactive protein, NF-kB nuclear factor kappa B, PDTC pyrrolidine dithiocarbamate, NOS2 nitric oxide synthase 2, MMP13 matrix metalloproteinase 13, VCAM1 vascular cell adhesion molecule 1, IL6 interleukin 6, LCN2 lipocalin 2.

damages cartilage, and is involved in the upregulation of MMPs, among which MMP13 is one of the major mediators of ECM degradation. COX-2 induces prostaglandins that in turn increase the synthesis of other inflammatory mediators, including cytokines, to perpetuate cartilage destruction [38]. Thus, we have focused on the regulation of iNOS and NO production, as well as other six major pro-inflammatory mediators with different functions in cartilage destruction, namely COX-2, MMP13, VCAM1, IL-6, IL-8, and LCN2. All these mediators are produced by chondrocytes and are elevated in arthritic joints [4, 39–42] Our results agree with those obtained by other authors in other cell types. For instance, Wang and collaborators demonstrated that CRP increases IL-8 gene expression in HUVEC cells [43]. Of note in Wang's report, the effect of CRP on gene expression was measured after a 24-h cell culture incubation of CRP with vascular endothelial cells. It has been established that incubating CRP in cell culture or with liposomes for just 30 min, and notably within the first 24 h, can cause pCRP to change conformation in mCRP isomeric forms [30, 44–47]. As Wang's report appeared prior to widespread awareness of distinct CRP structural isoforms with distinctive bioactivities, and in reflecting on the results on direct comparison of mCRP-specific bioactivities presented herein, Wang's results may more precisely reflect on mCRP effects rather than pCRP.

Our present findings bring out important evidence regarding the modulation of articular phenotype and functions exerted by mCRP. First, exposure of chondrocytes to mCRP-induced multiple pro-inflammatory genes suggesting that its effects are not limited to a specific molecular target, rather mCRP has a broader spectrum of action. The second important point is that the effect of mCRP is persistent and sustained, regardless of the pro-inflammatory environment. Actually, mCRP exerted its action on human primary chondrocytes coming not only from OA patients, but also from healthy subjects. This latter finding suggests that elevated locally produced levels of mCRP may trigger sustained multigenic inflammatory responses also in normal tissues not previously exposed to a pro-inflammatory micro-environment, as in the case of OA chondrocytes. Of note, it was recently described that mCRP directly binds to fibronectin without depending on calcium presence and pH (acidic in the micro-environment of inflamed tissue, which promotes dissociation into the monomeric form). It was also shown that mCRP deposited in the ECM may regulate the recruitment and adhesion of monocytes thus triggering the inflammatory process [48]. This could help to understand the similar response to mCRP in both healthy and OA chondrocytes. The third important point is that the effects of mCRP in chondrocytes are likely to be mediated by NF-kB signaling pathway. In particular, our results demonstrate that pyrrolidine dithiocarbamate (a well-known pharmacological inhibitor of NF-kB) partially abrogates the expression of all pro-inflammatory genes induced by mCRP. Accordingly, in osteoclasts, mCRP has revealed to act primarily through NF-kB and phospholipase C pathways [49]. Furthermore, in endothelial cells, p38 MAPK was implicated in the mCRP-induced cytokine promotion [34, 50]. Although further experiments are needed due to the versatility of mCRP in activating different signaling pathways, we describe here, for the first time, the involvement of NF-kB in chondrocytes upon mCRP stimulation.

In conclusion, our present findings provide, for the first time, a molecular basis for the sustained action of mCRP in human and murine chondrocytes. Taken together, these results suggest that mCRP exerts a sustained catabolic effect by increasing the expression of inflammatory mediators and proteolytic enzymes, such as MMP13, and is thus able to promote cartilage breakdown and to trigger inflammatory responses in healthy and OA cartilage. Altogether, these molecules can cooperate resulting in the enhancement and perpetuation of the ECM-degrading processes at cartilage level. Finally, our results indicate that the route triggered by the release of NF-kB is involved. Despite these findings, further studies on the signaling elicited by mCRP are needed to completely assess its role in cartilage degradation and the pathogenesis and progression of OA.

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Author contributions CRF, MG, and VF participated in acquisition of data, analysis, and interpretation of data and critical revision of the manuscript. AM, RG, JC, FL, JP, MAGG, and AM participated in the acquisition of data and samples, drafting of the manuscript, and statistical analysis. IMR participated in C-rmCRP preparation and editing the manuscript. LAP participated in the analysis and interpretation of data and drafting/editing the manuscript. OG participated in the conception and design of the study, in the analysis and interpretation of data, critical revision of the manuscript, and scientific supervision of experiments.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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Monomeric CRP regulates inflammatory responses in human intervertebral disc cells

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Aims

CRP is an acute-phase protein that is used as a biomarker to follow severity and progression in infectious and inflammatory diseases. Its pathophysiological mechanisms of action are still poorly defined. CRP in its pentameric form exhibits weak anti-inflammatory activity. The monomeric isoform (mCRP) exerts potent proinflammatory properties in chondrocytes, endothelial cells, and leucocytes. No data exist regarding mCRP effects in human intervertebral disc (IVD) cells. This work aimed to verify the pathophysiological relevance of mCRP in the aetiology and/or progression of IVD degeneration.

Methods

We investigated the effects of mCRP and the signalling pathways that are involved in cultured human primary annulus fibrosus (AF) cells and in the human nucleus pulposus (NP) immortalized cell line HNPSV-1. We determined messenger RNA (mRNA) and protein levels of relevant factors involved in inflammatory responses, by quantitative real-time polymerase chain reaction (RT-qPCR) and western blot. We also studied the presence of mCRP in human AF and NP tissues by immunohistochemistry.

Results

We demonstrated that mCRP increases nitric oxide synthase 2 (NOS2), cyclooxygenase 2 (COX2), matrix metalloproteinase 13 (MMP13), vascular cell adhesion molecule 1 (VCAM1), interleukin (IL)-6, IL-8, and Lipocalin 2 (LCN2) expression in human AF and NP cells. We also showed that nuclear factor-κβ (NF-κβ), extracellular signal-regulated kinase 1/2 (ERK1/2), and phosphoinositide 3-kinase (PI3K) are at play in the intracellular signalling of mCRP. Finally, we demonstrated the presence of mCRP in human AF and NP tissues.

Conclusion

Our results indicate, for the first time, that mCRP can be localized in IVD tissues, where it triggers a proinflammatory and catabolic state in degenerative and healthy IVD cells, and that NF-κβ signalling may be implicated in the mediation of this mCRP-induced state.

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Keywords: Monomeric CRP, Inflammation, Intervertebral disc, Annulus fibrosus, Nucleus pulposus, Low back pain

Article focus

- This study aimed to verify the pathophysiological relevance and mechanism of action of monomeric CRP (mCRP) in healthy and degenerative intervertebral disc (IVD) cells.

Key messages

- mCRP can be present in IVD tissues and induces the expression of multiple proinflammatory and catabolic factors in human annulus fibrosus (AF) and nucleus pulposus (NP) cells.
- The effect of mCRP is persistent and sustained, regardless of the proinflammatory environment, as it was similar in healthy and degenerative human primary AF cells.
- mCRP effects in healthy and degenerative AF cells are mediated by phosphoinositide

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3-kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2), and nuclear factor-κβ (NF-κβ) p65 signalling pathways.

Strengths and limitations

- This is the first experimental article that provides evidence for the functional activity of mCRP in healthy and degenerative human AF and NP disc cells.
- This is the first article demonstrating the localization of mCRP in intravertebral disc cells of the AF and NP.
- Further studies are needed to confirm the role of mCRP in IVD degeneration. A larger number of subjects studied would give more strength to our results; the limited access to primary human disc cells is a limitation of this study.

Introduction

Low back pain (LBP) is the main cause of disability worldwide, affecting around 700 million people.[1] It has been estimated that 70% to 85% of the global population has LBP at some time in their lives, and it can limit the activity of people under 45 years of age, generating a huge socioeconomic impact.[2] The aetiology of LBP is complex, multifactorial, and still unclear. However, histological and MRI data associate chronic LBP with lumbar intervertebral disc (IVD) degeneration.[3] IVDs are embedded between the vertebrae and provide flexibility to the spine. They consist of three anatomical parts: an inner cell-sparse gelatinous nucleus pulposus (NP), an outer fibrous region, the annulus fibrosus (AF), and, limiting above and below the AF, the hyaline cartilaginous end plates (CEPs).[4] In a healthy adult IVD, homeostasis between extracellular matrix (ECM) synthesis and degradation is balanced by growth factors and cytokines. An imbalance towards catabolic processes contributes to structural degeneration of the IVD, likely contributing to the development of LBP.[3,5]

IVD degeneration leads to multiple anatomical, mechanical, and biochemical disc changes. These alterations decrease mechanical stability and shock absorber functions, contributing to osteophyte formation, annular fissures, and decreased motion of spinal segments.[3] Genetic risk has an important influence on IVD degeneration, however mechanical trauma, injuries, smoking, obesity, and ageing also play a role.[6] In response to inflammation and degeneration, vascular and nerve ingrowth into the avascular IVD occur from the outer layers of the AF, extending into the NP.[7,8]

IVD degeneration and the degeneration of joint cartilage as seen in osteoarthritis (OA) show marked similarities, although they are barely discussed simultaneously. Striking similarities are seen on plain radiographs: the loss of disc height or joint space, sclerosis of the subchondral bone, and the development of osteophytes. Furthermore, in both IVD and joint cartilage, the ECM is composed of similar constituents (although in another ratio), and similar ECM-degrading machinery is present in the process of degeneration. There are also differences such as the type of forces applied to the ECM, and the absence of synovial fluid in IVDs.[9-12]

CRP is an acute-phase inflammatory protein whose plasma levels sharply increase in response to injury, infection, and inflammation. It is a member of the pentraxin family, and circulates prevalently in blood in its homopentameric form.[13] CRP plasma levels increase from around 1 μg/ml to over 500 μg/ml within 24 to 72 hours upon severe tissue damage.[14] Thus CRP levels are a well-established biomarker for monitoring the inflammatory status in infections and other multiple diseases, including IVD herniation[15] and rheumatic diseases such as OA.[16]

The pentameric protein is characterized by a discoid configuration of five identical non-covalently bound subunits of about 23 kDa each. It is synthesized primarily in liver hepatocytes but has also been reported to be produced in other cell types such as smooth muscle cells,[17] macrophages,[18] endothelial cells,[19] lymphocytes, and adipocytes.[20] The monomeric CRP (mCRP) differs from the pentameric CRP (pCRP) in its different antigenic, biological, and electrophoretic mobility,[21] and in its expression of different neoepitopes.[22] These two distinct forms of CRP showed distinct biological functions in the inflammatory process. It has been proved that pCRP suppresses the adherence of platelets to neutrophils, whereas mCRP enhances these interactions.[22] This difference in function can be explained by the two isoforms binding to differing types of Fcγ-receptors involved in the signalling process. The mCRP isoform uses the low-affinity immune complex binding immunoglobulin G (IgG) receptor called FcγRIIIb (CD16b) on neutrophils and FcγRIIIa (CD16a) on monocytes, while pCRP binds to the low-affinity IgG receptor FcγRIIa (CD32).[23] Evidence shows that pCRP tends to exhibit weak anti-inflammatory activities. In contrast, mCRP has marked proinflammatory properties both in vitro and in vivo by promoting monocyte chemotaxis and the recruitment of circulating leucocytes to areas of inflammation via Fcγ-RI and Fcγ-RIIa signalling.[24,25] However, the pathophysiological role of CRP as a regulator of inflammatory processes remains obscure.

Some of the published IVD disease literature assesses CRP as a reference biomarker of an ongoing inflammatory process.[15,26,27] Ruiz-Fernández et al[24] recently investigated the inflammatory effect of mCRP in chondrocytes, proving that mCRP triggers a sustained proinflammatory and catabolic state in healthy and OA cartilage. However, the role of mCRP in disc pathophysiology has not yet been clarified. In this current study, we evaluated the functional role of mCRP as a modulator of the inflammatory response in NP and AF human cells, assessing some proinflammatory and catabolic factors and the pathways involved in the signal transduction upon mCRP stimulation.

Methods

Reagents. Fetal bovine serum (FBS), lipopolysaccharide (LPS), and human recombinant interleukin (IL)-1β

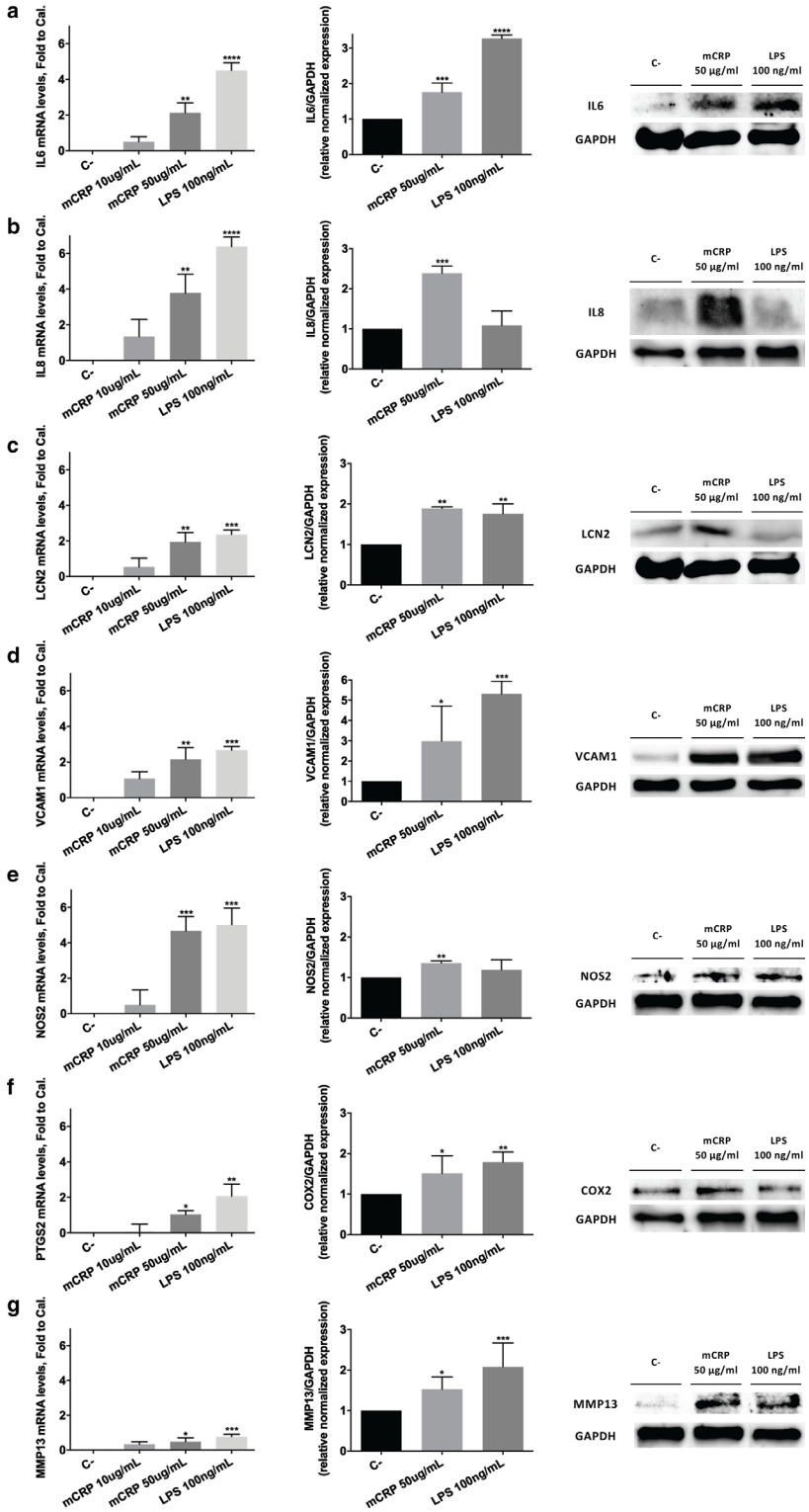

Fig. 1

Monomeric CRP (mCRP) proinflammatory effect on human primary degenerative intervertebral disc (IVD) annulus fibrosus (AF) cells. Human primary AF cells derived from degenerative IVD in culture were challenged with 10 or 50 µg/ml mCRP, or with 100 ng/ml lipopolysaccharide (LPS) as positive control for 24 hours, after four hours of serum starvation. a) to g) Left panels. Messenger RNA (mRNA) expression was determined by quantitative real-time polymerase chain reaction. mRNA levels are presented as fold change relative to control (C-). a) to g) Middle and right panels. Protein expression was measured by western blot. Data from densitometric analyses normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are shown as relative-to-control (C-) values. Data are expressed as mean (standard error of the mean) of at least three independent experiments. Comparisons referred to control (C-): *p < 0.05, **p < 0.01, ***p < 0.001. Cal., calibrator (refers to control (C-) condition); COX2, cyclooxygenase 2; IL, interleukin; LCN2, lipocalin 2; MMP13, matrix metalloproteinase 13; NOS2, nitric oxide synthase 2; PTGS2, prostaglandin-endoperoxide synthase 2; VCAM1, vascular cell adhesion molecule 1.

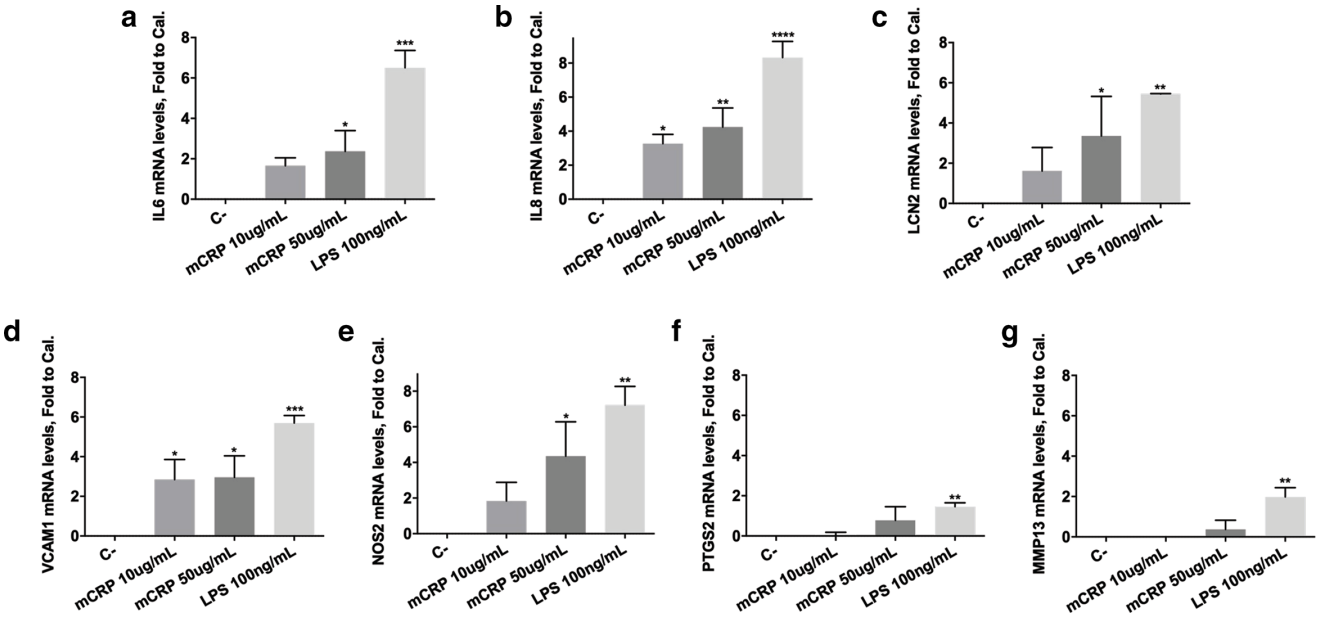

Fig. 2

Monomeric CRP (mCRP) proinflammatory effect on human primary healthy intervertebral disc (IVD) annulus fibrosus (AF) cells. Human primary AF cells from healthy IVD in culture were challenged with 10 or 50 µg/ml mCRP, or with 100 ng/ml lipopolysaccharide (LPS) as positive control for 24 hours, after four hours of serum starvation. a) to g) Messenger RNA (mRNA) expression was determined by quantitative real-time polymerase chain reaction. mRNA levels are presented as fold change relative to control (C-). Data expressed as mean (standard error of the mean) of at least three independent experiments. Comparisons referred to control (C-): *p < 0.05, **p < 0.01, ***p < 0.001. Cal., calibrator (refers to control (C-) condition); IL, interleukin; LCN2, lipocalin 2; MMP13, matrix metalloproteinase 13; NOS2, nitric oxide synthase 2; PTGS2, prostaglandin-endoperoxide synthase 2; VCAM1, vascular cell adhesion molecule 1.

were obtained from MilliporeSigma (USA). Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 medium, trypsin-ethylenediaminetetraacetic acid, L-glutamine, and penicillin-streptomycin mixture were obtained from Lonza Group (Switzerland). Pronase and collagenase P were obtained from Roche Diagnostics (Germany).

Recombinant mCRP (Roosevelt University, College of Pharmacy, USA) was produced in *Escherichia coli*, had both cysteines replaced with alanine residues and solubilized by an acylation technique (C-rmCRP) in 25 mM NaPBS (pH7.4), and was purified and tested in several bioassays to confirm its equivalence to biological mCRP from the pentameric form and free of contaminating endotoxin. The final endotoxin level of all protein solutions was below the detection limit 0.06 endotoxin units (EU)/ml of the assay.[28]

Cell culture conditions and treatments. Human NP immortalized cell line HNPSV-1[29] (Tokai University School of Medicine; Japan) was cultured in DMEM/Ham's F12 supplemented with 10% FBS, 4 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. In these experiments we used a cell passage range of 13 to 22.

Healthy IVD samples were obtained from deceased multi-organ donors. Degenerative IVD samples were obtained, under written patient consent, from patients undergoing discectomy. Inclusion criteria were: male or female patients undergoing discectomy diagnosed with spinal stenosis; adult degenerative scoliosis; disc herniation; and disc degeneration (Pfirrmann grade > 3 to 4). Exclusion criteria were: previous spinal malignancies or infections; previous spinal surgery; and patients diagnosed with metabolic diseases. All IVD samples were obtained under permission from the local ethics committee, according to the Declaration of Helsinki.[30] AF cells were isolated and cultured as previously described.[31] Briefly, aseptically dissected AF tissue was diced and properly rinsed with phosphate-buffered saline (PBS). After removing PBS, tissue was incubated for 20 minutes with pronase at a final concentration of 1 mg/ml in DMEM/Ham's F12 at 37°C. Then, pronase was removed and the tissue was rinsed two to three times with PBS. After removing PBS, digestion continued with collagenase P at a final concentration of 1 mg/ml in DMEM/Ham's F12 with 10% FBS. Tissue was incubated for four to six hours in agitation at 37°C. The resulting cell suspension was filtered with a 40 µm nylon cell strainer (BD Biosciences Europe, Belgium) to remove debris. Cells were centrifuged, washed with PBS, resuspended in DMEM/Ham's F12 supplemented with 10% FBS, 4 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin, counted, and plated in 12-well culture plates. Cells were maintained in culture for a maximum of ten days for expansion. All AF cells used in these experiments were at passage number 2.

AF cells and HNPSV-1 cells were seeded in six-well plates (2.5×10^5 cells/well) and treated with 10, 25, or 50 µg/ml mCRP, 100 ng/ml LPS, or 0.1 ng/ml IL-1β, after serum starvation for four hours. All the concentrations

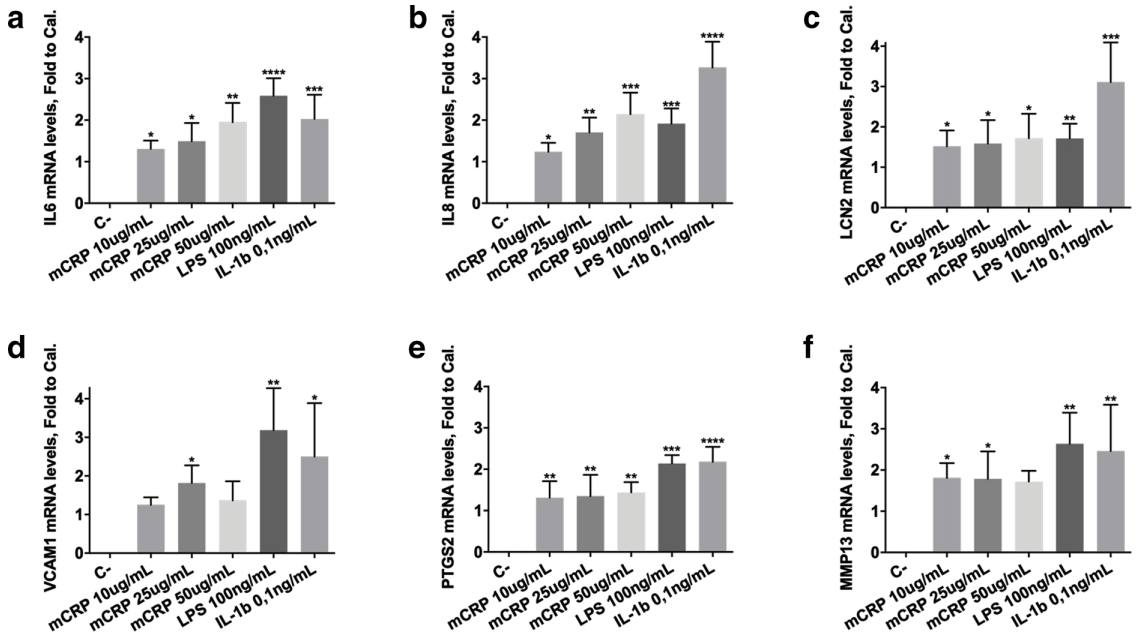

Fig. 3

Monomeric CRP (mCRP) proinflammatory effect on human intervertebral disc (IVD) nucleus pulposus (NP) cell line. HNPSV-1 human NP cells were challenged with 10, 25, or 50 µg/ml mCRP, or with 100 ng/ml lipopolysaccharide (LPS) or 0.1 ng/ml IL-1b as positive controls for 24 hours, after four hours of serum starvation. a) to f) Messenger RNA (mRNA) expression was determined by quantitative real-time polymerase chain reaction. mRNA levels are presented as fold change relative to control (C-). Data expressed as mean (standard error of the mean) of at least three independent experiments. Comparisons referred to control (C-): *p < 0.05, **p < 0.01, ***p < 0.001. Cal., calibrator (refers to control (C-) condition; IL, interleukin; LCN2, lipocalin 2; MMP13, matrix metalloproteinase 13; PTGS2, prostaglandin-endoperoxide synthase 2; VCAM1, vascular cell adhesion molecule 1.

were selected based on a previous study[24] and in the absence of IVD cell toxicity. All treatments were done in serum-free conditions after four hours of serum starvation and lasted 24 hours for the proinflammatory response experiments (Figures 1 to 3), or 15 minutes for the intracellular signal transduction experiments (Figure 4).

Immunohistochemical assays. Sections 4 µm thick obtained from human AF or NP tissue were mounted on silanized coated slides (Dako-Agilent; USA). Epitope retrieval was performed in a PT-Link (Dako-Agilent) at high pH for 20 minutes. Then, the slides were automatically immunostained in an Autostainer-Link 48 (Dako-Agilent), employing the monoclonal anti-CRP antibody, clone CRP-8 (MilliporeSigma), diluted 1:100, and 30-minute incubation time. As a detection system, we used EnVision FLEX/HRP (Dako-Agilent), for 30 minutes, and 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen, for five minutes.

RNA isolation and real-time reverse transcription-polymerase chain reaction. Total RNA was isolated from cell culture with NZYol (NZYTech, Portugal) and E.Z.N.A. Total RNA Kit I (Omega Bio-tek, USA) according to the manufacturer's instructions, and reverse-transcribed using NZY First-Strand cDNA Synthesis Kit (NZYTech). Then, SYBR-green-based quantitative real-time polymerase chain reaction (RT-qPCR) was performed in Stratagene MX3005P thermal cycler as previously described,[32] using a standard protocol with RT² SYBR Green qPCR Mastermix and specific PCR primers (Qiagen, Germany) (human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 175 bp, PPH00150E, reference position 1287, Gen-Bank accession no. NM_002046.3; human nitric oxide synthase 2 (NOS2), 132 bp, PPH00173E, reference position 3962, Gen-Bank accession no. NM_000625.4; human matrix metalloproteinase 13 (MMP13), 61 bp, PPH00121B, reference position 1380, Gen-Bank accession no. NM_002427.3; human vascular cell adhesion molecule 1 (VCAM1), 141 bp, PPH00623E, reference position 2980, Gen-Bank accession no. NM_001078; human Lipocalin 2 (LCN2), 87 bp, PPH00446E, reference position 626 to 644, Gen-Bank accession no. NM_005564.3; human IL-6, 98 bp, PPH00560C, reference position 816, Gen-Bank accession no. NM_000600.3; human IL-8, 126 bp, PPH00568A, reference position 326, Gen-Bank accession no. NM_000584.3; human prostaglandin-endoperoxide synthase 2 (PTGS2), 63 bp, PPH01136F, reference position 1502, Gen-Bank accession no. NM_000963.2). No-template controls were included to eliminate any non-specific amplification, and melting curves were generated to ensure a single gene-specific peak. Gene expression changes were determined by the comparative ΔΔCt method in MxPro qPCR Software version 4.10 (Stratagene, USA), and expressed as relative fold changes compared to control (C-) and normalized to GAPDH housekeeping gene.

Protein extraction and western blot analysis. After treatment, cells were rapidly washed with ice-cold PBS and scraped in lysis buffer (10 mM Tris–HCl, pH 7.5, 5 mM

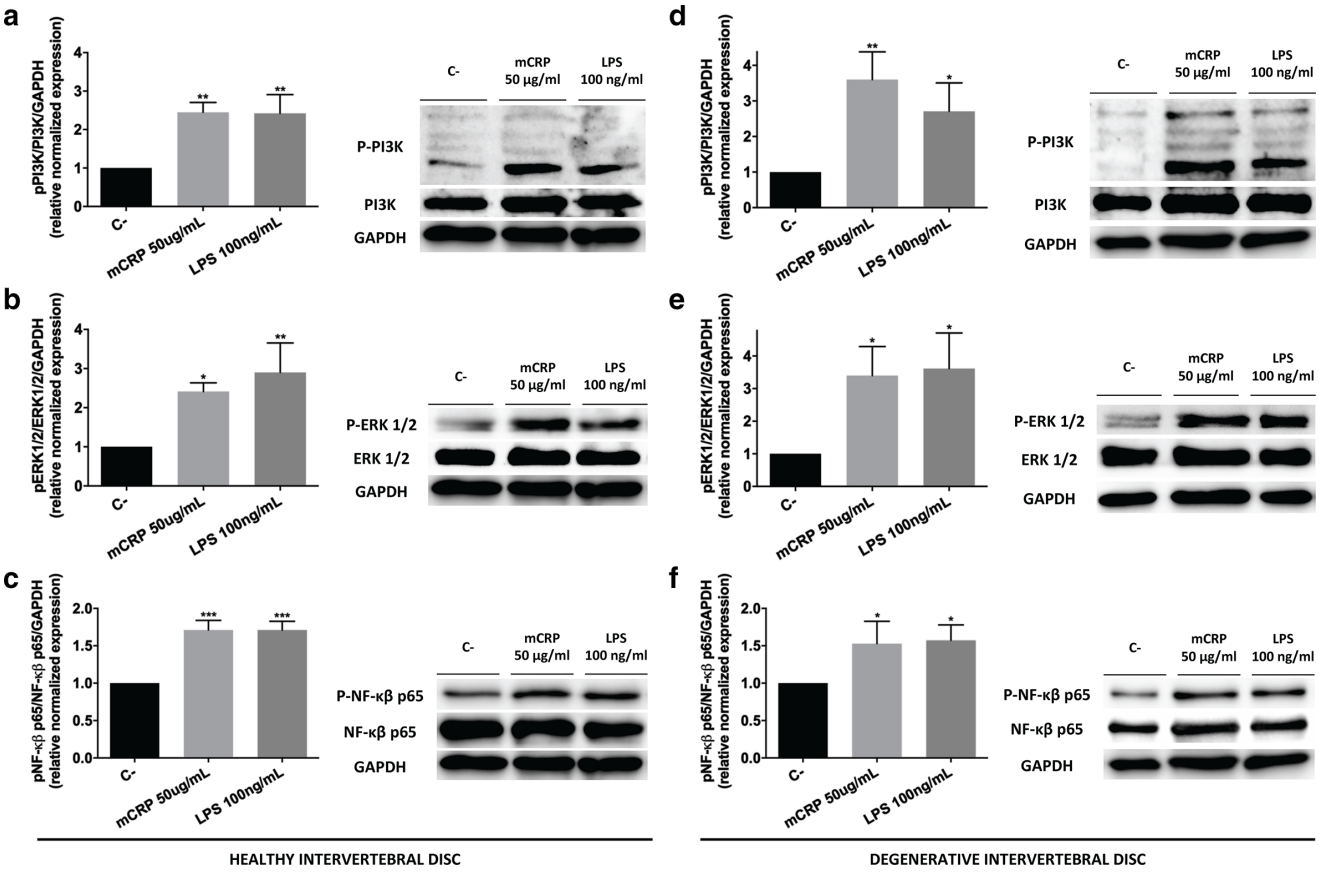

Fig. 4

Mediators involved in monomeric CRP (mCRP) intracellular signal transduction in human primary healthy and degenerative intervertebral disc (IVD) annulus fibrosus (AF) cells. Human primary AF cells from healthy or degenerative IVD in culture were challenged with 50 µg/ml mCRP or with 100 ng/ml lipopolysaccharide (LPS) as positive control for 15 minutes, after four hours of serum starvation. a) to f) Protein expression was measured by western blot. Determination of the phosphorylated protein normalized to total protein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data from densitometric analyses are shown as relative-to-control (C-) values. Data expressed as mean (standard error of the mean) of at least two or three independent experiments. Comparisons referred to control (C-): *p < 0.05, **p < 0.01, ***p < 0.001. NF-κβ p65, nuclear factor-κβ p65; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; PI3K, phosphoinositide 3-kinase; pPI3K, phosphorylated PI3-kinase; pNF-κβ p65, phosphorylated nuclear factor-κβ p65.

ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF)), freshly supplemented with protease inhibitor cocktail (Thermo Fisher Scientific, USA), then the total cell lysates were centrifuged at 14,000 g for 20 minutes. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotting procedures were performed as previously described.[33] Immunoblots were incubated with the specific antibody against NOS2, cyclooxygenase 2 (COX2), phospho-phosphoinositide 3-kinase (PI3K) p85/p55, phospho-p44/p42 MAPK (extracellular signal-regulated kinase 1/2 (ERK1/2)), phospho-nuclear factor-κβ (NF-κβ) p65, or NF-κβ p65 diluted 1:1000 (Cell Signaling Technology, USA), PI3K p85 or MAPK 1/2 (ERK1/2) diluted 1:1000 (MilliporeSigma), VCAM1 diluted 1:500 (EnoGene Biotech, USA), IL-6 or IL-8 diluted 1:100 (Santa Cruz Biotechnology, USA), MMP13 diluted 1:500 (Abcam, UK), and human LCN2 diluted 1:1,000 (R&D Systems, USA).

The immune complexes were detected using anti-rabbit, anti-mouse (both from GE Healthcare, USA), or anti-goat (Santa Cruz Biotechnology) horseradish-peroxidase-labelled secondary antibodies diluted 1:5,000 and visualized with Immobilon Western Detection kit (Millipore, USA). To verify equivalent protein loading, the membranes were stripped 15 minutes in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7), blocked and incubated with anti-GAPDH antibody diluted 1:2,000 (MilliporeSigma). In all figures showing images of gels, the bands for each picture were obtained from the same gel, although they may have been spliced for clarity. The images were captured with ChemiDoc MP Imaging System and analyzed with Image Lab 6.0.1 Software, both from Bio-Rad Laboratories (USA)

Statistical analysis. Data are reported as mean (standard error of the mean (SEM)) of at least three independent experiments. Statistical analyses were performed with GraphPad Prism 9.3.1 software (GraphPad Software, USA). Assuming a normal distribution, one-way analysis

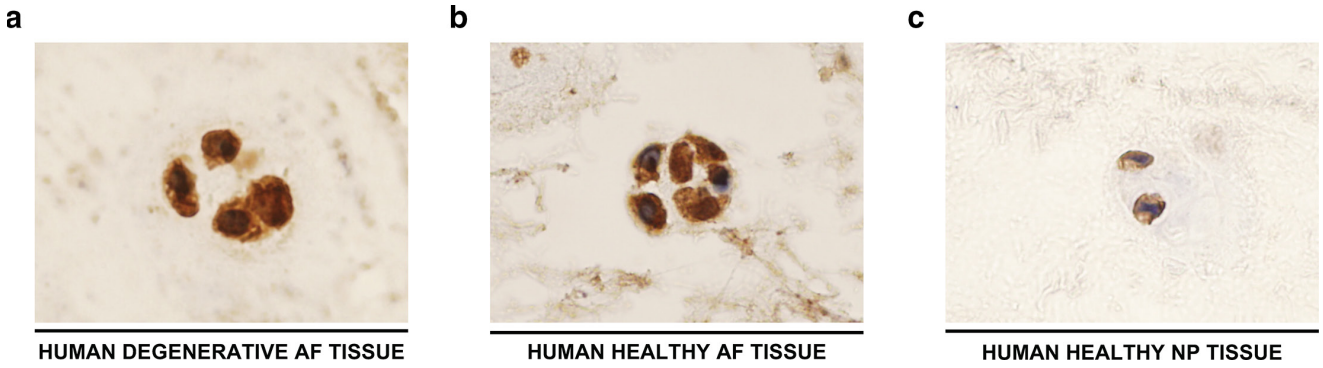

Fig. 5

Monomeric CRP (mCRP) localization in human degenerative and healthy intervertebral disc tissues. Immunohistochemical images showing mCRP protein presence (brown staining) in: a) human degenerative annulus fibrosus (AF) tissue; b) human healthy AF tissue; and c) human healthy nucleus pulposus (NP) tissue. Images taken at 60× magnification.

of variance (ANOVA) followed by Fisher's least significant difference (LSD) test were employed. As we performed few planned comparisons, p-values have not been corrected for multiple comparisons; they apply individually to each value reported and not to the entire family of comparisons. A p-value less than 0.05 was considered to be statistically significant.

Results

As shown in Figure 1, mCRP increases IL-6, IL-8, LCN2, VCAM1, NOS2, COX2, and MMP13 mRNA expression and protein levels in cultured human primary AF cells from patients with IVD degeneration, as confirmed by RT-qPCR and western blot analysis. The induction of proinflammatory mediators occured in a dose-dependent manner after 24 hours.

The upregulation of these inflammatory and catabolic factors by mCRP was also determined in human primary AF cells from healthy patients, suggesting that mCRP can modify the healthy cell status by promoting the development of a proinflammatory environment in the IVD (Figures 2a to 2g). In all the experiments with human cells, LPS inflammatory stimulus was used as control of inflammatory induction.

To broaden the scope of our results, we also tested mCRP activity in HNPSV-1, a cell line derived from human NP cells that maintains the original architecture of the cells and their gene expression profile.[29] Figure 3 shows that mCRP at 10 µg/ml, 25 µg/ml, and 50 µg/ml significantly increases IL-6, IL-8, LCN2, VCAM1, PTGS2, and MMP13 mRNA expression levels, as previously observed in human primary AF cells. LPS and IL-1β, as well-known inflammatory stimuli, were used as controls of inflammatory induction.

For completeness, to elucidate which mediators might be implicated in mCRP intracellular signal transduction in both healthy and degenerative human disc cells, we analyzed NF-κβ signalling pathway upon mCRP challenge. Healthy or degenerative human primary AF cells were treated with 50 µg/ml mCRP or 100 ng/ml LPS as a positive control, for 15 minutes. The results obtained showed that mCRP induces PI3K phosphorylation, ERK1/2 phosphorylation, and NF-κβ p65 phosphorylation in healthy AF cells (Figures 4a to 4c) and degenerative AF cells (Figures 4d to 4f).

Finally, we performed immunohistochemical assays to investigate whether mCRP is located in IVD tissues. We assessed mCRP presence in human degenerative and healthy AF and healthy NP tissues, confirming its presence in physiological state in all of them. As we can observe in Figures 5a to 5c, mCRP location is predominantly cytoplasmic and perinuclear, being the more intense stain in the AF, especially in the degenerative tissue, than in the NP tissue.

Discussion

CRP in its pentameric form is an acute-phase serum protein and a well-established biomarker in a great number of infectious and inflammatory diseases, including IVD herniation.[34] It has also been evidenced as a mediator of inflammatory and immune responses in the context of local tissue injury.[35,36] Serial CRP measures are useful not only for the follow-up of postoperative infection, but also for monitoring treatment efficacy and for the detection of relapses.[27,37]

CRP monomeric isoform, although much less studied, has been found to have different biological functions in the inflammatory process. While clarifying its pathophysiological function needs further investigation, new insights could be extremely helpful in better understanding the inflammatory mechanism and how it works in the pathogenesis and disease progression.

There is evidence that the inflammatory process plays an important role in the development of disc degeneration, and there is a marked correlation between the levels of inflammatory markers and the degree of degeneration.[38] An association has been previously reported between high-sensitivity CRP (hs-CRP) levels and severe pain (VAS of > 40).[39] Sugimori et al[40] found markedly higher mean hs-CRP levels among patients with lumbar disc herniation (0.056 mg/dl (standard deviation (SD) 0.076)) than in the control group (0.017 mg/dl (SD

0.021)); no significant correlation between hs-CRP levels and herniation level was reported, but those patients with a higher concentration of hs-CRP before operation showed a poorer recovery. Of interest, some papers only quantified CRP levels in the high sensitivity range (i.e. hs-CRP), describing values below 10 µg/ml. According to several studies[41–47] and to the guidance of the USA Food and Drug Administration (FDA),[48] the clinical significance of such values is questionable.

In cervical disc herniation, pain occurs as a result of the release of local inflammatory cytokines. The elevated plasma concentration of IL-6 produced by macrophages around the IVD tissue increments CRP levels.[49,50] It has been shown that CRP levels were considerably higher in patients with cervical disc hernia and neck pain compared with those patients with neck pain but a normal cervical magnetic resonance.[34] A recent study found that plasma CRP levels in peripheral blood did not differ between healthy controls and non-herniated IVD degeneration patients.[51]

Recently, it has been demonstrated that only mCRP originated from local pCRP in the joint compartment may bind to receptor activator of nuclear factor-κB ligand (RANKL), neutralizing its activity and downregulating osteoclast differentiation.[52] Ruiz-Fernández et al[24] recently reported the inflammatory effect of mCRP in chondrocytes, demonstrating that mCRP triggers a sustained proinflammatory and catabolic state in OA and healthy cartilage, and that the NF-κβ pathway is involved in its signal transduction. To demonstrate that this activity can also be exerted in IVD cells, we tested the effect of mCRP in primary culture of human AF cells isolated from patients with IVD degeneration or from healthy subjects. In order to show that these effects are not exclusive to AF cells, the human NP immortalized cell line HNPSV-1 was used as a NP cells model for comparison in order to study mCRP effects in the whole disc, since NP cells commonly exhibit chondrocyte-like characteristics.[53]

As far as we know, this is the first experimental article that provides evidence for the functional activity of mCRP in AF and NP disc cells. To gain further insights into the action of mCRP and to verify whether this stimulation can result in sustained proinflammatory effects, we evaluated the dose-dependent activity of mCRP in master inflammatory and catabolic players in IVD degeneration. IL-8 and IL-6 are two of the proinflammatory cytokines strongly expressed in IVD degeneration.[54] In this work, we explored the ability of mCRP to modulate some of the most important factors involved in the disc degeneration inflammatory and catabolic processes. We also assessed LCN2, an adipokine implicated in the catabolic mechanism of IVD degeneration, MMP13, a catabolic enzyme, and the inflammatory mediators NOS2, cyclooxygenase 2 (COX2), and VCAM1. Nitric oxide synthase type II (iNOS) and COX2 are classically involved in the amplification of inflammatory responses. NO has cytotoxic effects, damages the IVD, and is involved in the upregulation of MMPs, among which MMP13 is one of the major mediators of ECM degradation. COX2 induces prostaglandins that in turn increase the synthesis of other inflammatory mediators, including cytokines, to perpetuate tissue destruction. All these mediators are produced by IVD cells and are elevated in IVD degeneration.[55–59]

Our present findings bring out important evidence regarding the modulation of IVD metabolism and functions exerted by mCRP, as well as the localization of mCRP in human IVD tissues. First, exposure of AF and NP cells to mCRP induced multiple proinflammatory genes, suggesting that its effects are not limited to one specific molecular target, rather, mCRP has a broader spectrum of action. The second important point is that the effect of mCRP is persistent and sustained for at least 24 hours, regardless of the proinflammatory environment. In fact, mCRP exerted its action on human primary AF cells coming not only from patients with IVD degeneration, but also from healthy subjects. In accordance with this, we found that mCRP can be localized physiologically both in healthy NP and AF tissues and, in addition, mCRP presence seems to increase in the degenerative AF tissue compared to the healthy one. Our finding suggests that elevated locally produced levels of mCRP may also trigger sustained multigenic inflammatory responses in normal tissues not previously exposed to a proinflammatory micro-environment, as in the case of AF cells from degenerated IVD. Another relevant aspect is that the effects of mCRP were observed both in AF and NP cells, showing that mCRP has multiple cell targets. The last important insight of this work is that mCRP effects in AF cells are mediated by the NF-κβ signalling pathway. We proved that upon mCRP challenge, PI3K, ERK1/2, and NF-κβ p65 were phosphorylated. These mediators were activated in a similar manner both in healthy and degenerative AF disc cells, confirming that mCRP action does not depend on a previous inflammatory state.

Our results are in agreement with those obtained by other groups working with other cell types. For instance, it has been observed in osteoclasts that mCRP acts through the PI3K signalling pathway.[60] Wang et al[61] demonstrated that CRP increases IL-8 gene expression in human umbilical vein endothelial cells (HUVECs). Previous published results in cartilage showed the proinflammatory effect of mCRP in chondrocytes, clearly demonstrating that mCRP triggers a sustained proinflammatory and catabolic state in OA and healthy cartilage. These results also proved that NF-κβ has a role in mCRP signalling in cartilage.[24] This experimental design and the results observed were similar to those obtained in IVD cells.

The limited access to primary degenerative and healthy human disc cells is a limitation of this study. Further studies involving a larger number of subjects would be needed to fully understand the role of mCRP in IVD degeneration pathophysiology.

In conclusion, our present findings provide, for the first time, evidence of the mCRP localization in healthy and degenerative IVD tissues, and a novel molecular basis for the sustained action of mCRP in human AF and NP cells.

Taken together, these results suggest that mCRP exerts a lasting catabolic effect by increasing the expression of inflammatory mediators and proteolytic enzymes, being able to promote IVD cell breakdown and trigger inflammatory responses in healthy and degenerative AF cells and in the immortalized NP cell line. Altogether, these molecules can cooperate, resulting in the enhancement and perpetuation of the ECM-degrading processes at IVD level. In addition, mCRP by itself can trigger a severe increase in the expression of inflammatory mediators in healthy disc cells, suggesting that mCRP has a clear pathogenic role in disc disease. Our results indicate that this effect occurs both in AF and NP cells, and so affects the two main disc cellular compartments. Besides these findings, further studies on the pathophysiology of mCRP are needed to completely determine the role of mCRP in the pathogenesis and progression of IVD degeneration.

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ARTICLE

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WISP-2 modulates the induction of inflammatory mediators and cartilage catabolism in chondrocytes

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Wnt-1 inducible signaling pathway protein 2 (WISP-2/CCN5) is a recently identified adipokine that has been described as an important mediator of canonical Wnt activation in adipogenic precursor cells. In osteoarthritis (OA), the most common form of arthritis, chondrocytes exhibit aberrant and increased production of pro-inflammatory mediators and matrix degrading enzymes such as IL-1β and MMP-13. Although recent evidence suggests a role for Wnt signaling in OA physiopathology, little is known about the involvement of WISP-2 in cartilage degradation. In the present study, we determined the expression of WISP-2 in healthy and OA human chondrocytes. WISP-2 expression is modulated along chondrocyte differentiation and downregulated at the onset of hypertrophy by inflammatory mediators. We also investigated the effect of WISP-2 on cartilage catabolism and performed WISP-2 loss-of-function experiments using RNA interference technology in human T/C-28a2 immortalized chondrocytes. We demonstrated that recombinant human WISP-2 protein reduced IL-1β-mediated chondrocyte catabolism, that IL-1β and WNT/b-catenin signaling pathways are involved in rhWISP-2 protein and IL-1β effects in human chondrocytes, and that WISP-2 has a regulatory role in attenuating the catabolic effects of IL-1β in chondrocytes. Gene silencing of WISP-2 increased the induction of the catabolic markers MMP-13 and ADAMTS-5 and the inflammatory mediators IL-6 and IL-8 triggered by IL-1β in human primary OA chondrocytes in a Wnt/β-catenin dependent manner. In conclusion, here we have shown for the first time that WISP-2 may have relevant roles in modulating the turnover of extracellular matrix in the cartilage and that its downregulation may detrimentally alter the inflammatory environment in OA cartilage. We also proved the participation of Wnt/β-catenin signaling pathway in these processes. Thus, targeting WISP-2 might represent a potential therapeutical approach for degenerative and/or inflammatory diseases of musculoskeletal system, such as osteoarthritis.

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INTRODUCTION

Osteoarthritis (OA) is a multifactorial joint degenerative disease characterized by progressive destruction of articular cartilage, changes in subchondral bone, osteophyte formation, and synovial inflammation[1]. It is the most prevalent type of arthritis, but its etiology is still incomplete[2,3]. Recently, inflammation has been recognized as contributing to the symptoms and progression of OA[4]. Chondrocytes, as the only resident cells in articular cartilage, preserve the integrity of the cartilage itself. However, during OA, high mechanical stress, extracellular matrix (ECM) degradation products, pro-inflammatory cytokines, and adipokines activate chondrocytes to stimulate the production of inflammatory mediators, such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α and degradative enzymes, such as matrix metalloproteinase (MMP)-13 and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-5[2]. In addition, IL-1β and TNF-α also induce other pro-inflammatory cytokines, such as IL-6, and chemokines, like IL-8.

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These mediators act synergistically to promote and perpetuate chondrocyte catabolic responses[2].

Recent evidence from animal experiments and clinical studies highlights a role for Wnt signaling in OA pathology[5]. Progressive joint destruction in OA has been associated with overactivation of Wnt signaling[6]. The canonical Wnt pathway is initiated by the binding of Wnt ligands to frizzled receptors and co-receptors (LRP5/6), causing the inactivation of glycogen synthase kinase-3β (GSK-3β), which leads to intracellular β-catenin accumulation and nuclear translocation. Once in the nucleus, β-catenin interacts with the LEF/TCF family of transcription factors and activates Wnt target genes[7]. GSK-3β phosphorylation is a key step, and inhibition of this enzyme can lead to β-catenin stabilization and initiation of target gene expression independent of Wnt binding. Wnt ligands as Wnt-7b and Wnt target genes including Wnt-1 inducible signaling pathway protein 1 (WISP-1)[5] were found to be upregulated in OA cartilage, as well as nuclear β-catenin and the co-receptor LRP5[8]. Pro-catabolic factors such as IL-1β induced the expression of various Wnt proteins, resulting in the activation of β-catenin[9], and activation of Wnt/β-catenin signaling stimulated the expression of cartilage ECM-degrading MMPs[10].

Wnt-1 inducible signaling pathway protein 2 (WISP-2), also named CCN5, is a 27 kDa matricellular protein that belongs to the CCN family. WISP-2 is considered a novel adipokine, as it was recently identified in a proteomics analysis of the secretome of human adipose tissue[11]. It is a secreted protein, highly expressed in mesenchymal stem cells (MSCs) and preadipocytes[12]. Grünberg et al. described WISP-2 as an important mediator of canonical WNT activation in adipogenic precursor cells, keeping the adipocytes in an undifferentiated state. They suggest that this adipokine may be involved in the development of obesity-related metabolic complications[13]. Frequently, WISP-2 is used as an indicator of canonical Wnt activation[14]. The identity of the WISP-2 receptor is currently unknown, although a Frizzled receptor would seem a likely possibility since the Frizzled co-receptor LRP5/6 is phosphorylated by WISP-2 in 3T3-L1 adipocytes[13].

To date, little is known about the role of this adipokine in cartilage pathophysiology. It has been demonstrated that WISP-2 expression was downregulated in human OA chondrocytes following sear stress[15]. WISP-2 has been found to be closely related to the pathogenesis of inflammatory arthritis[16] and the modulation of bone turnover[17]. In a previous study, we identified WISP-2 in synovium, infrapatellar fat pad, and chondrocytes obtained from OA patients[18], being its expression in infrapatellar fat pad adipocytes higher in OA patients than in healthy subjects. Therefore, the aim of this study was to analyze WISP-2 expression in OA chondrocytes, its effect on cartilage catabolism using recombinant WISP-2 protein, and loss-of-function experiments and to elucidate the role of WNT/b-catenin pathway.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (FBS), human recombinant interleukin (IL)-1β, human recombinant tumor necrosis factor alpha (TNF-α), lipopolysaccharide (LPS), human recombinant IL-6, human recombinant leptin, and glycogen synthase kinase 3 (GSK-3) inhibitor 6-bromoindirubin-3′-oxime (BIO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant adiponectin and visfatin were obtained from BioVendor (Karasek, Brno, Czech Republic). Human recombinant WISP-2 protein (hrWISP-2) was purchased from PeproTech (Rocky Hill, NJ, USA). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium, L-glutamine, penicillin-streptomycin mixture, and trypsin-EDTA were purchased from Lonza Group (Basel, Switzerland). Pronase and collagenase P were obtained from Roche Applied Science (Penzberg, Germany).

Cell culture and treatments

The murine chondrogenic cell line ATDC-5 (purchased from RIKEN Cell Bank, Tsukuba, Japan) was cultured in DMEM–Ham's F-12 medium supplemented with 5% FBS, 10 µg/mL human transferrin, 3×10^{-8} M sodium selenite, 4mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin. Chondrogenic ATDC-5 cells were differentiated into mature chondrocytes as previously described[19]. Briefly, cells were seeded at a density of 6×10^4 cells per well in six-well plates with ATDC-5 standard medium supplemented with insulin (10 µg/mL). The differentiation medium was replaced every two days for 21 days. Differentiation was qualitatively characterized by increased formation of cell nodules. In other experiments (data not shown), differentiation was further analyzed by a sequential increase in the levels of type II collagen, aggrecan and type X collagen mRNA expression, as previously published[20]. The immortalized human juvenile costal chondrocyte cell line T/C-28a2 (a kind gift from Dr. M.B. Goldring, Hospital for Special Surgery, NYC, USA) was cultured in DMEM–Ham's F-12 medium supplemented with 10% FBS, 4mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin. Human primary chondrocytes were isolated from healthy, or OA articular cartilage samples obtained from knee and hip joints of patients undergoing total joint replacement surgery. All the procedures were made under informed patient consent and approved by the local ethics committee, according to the declaration of Helsinki. Human primary chondrocytes were isolated and cultured as previously described[21]. They were cultured in DMEM/Ham's F-12 medium supplemented with 10% of FBS, 4 mM L-glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin. Cells were seeded in monolayer up to the high density and used in the first passage of culture to avoid dedifferentiation.

Chondrocytes were seeded in six-well plates (2.5×10^5 cells per well) and treated in serum-free conditions as indicated in each case, after overnight serum starvation. All the treatments concentrations were selected based on previous studies and in the absence of chondrocyte cell toxicity.

Immunocytochemical assays

Immunohistochemistry was automatically performed using an Autostainer-Link 48 immunostainer from Dako-Agilent (Santa Clara, CA, USA). Briefly, the slides were incubated at room temperature in: (1) heat-induced epitope retrieval solution at high pH (Dako-Agilent) for 20 min at 97 °C; (2) human polyclonal antibody to WISP-2 (ABK1-A4795) from Abyntek Biopharma (Derio, Biscay, Spain) at 1:100 for 30 min; (3) EnVision®+ Dual Link System-HRP (dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-mouse and goat anti-rabbit immunoglobulins) (Dako-Agilent, K4065) for 20 min; (4) DAB + substrate-chromogen solution (1 mL of substrate buffer solution containing hydrogen peroxide and 20 µL of 3,3′-diaminobenzidine tetrahydrochloride chromogen solution) for 10 min; and (5) EnVision FLEX hematoxylin for 15 min.

RNA isolation and real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cell culture with TRIzol LS Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and NucleoSpin RNA/Protein Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions; and reverse-transcribed using Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) at 42 °C for 30 min, followed by 2 min incubation at 95 °C. Then, SYBR-green-based quantitative real-time PCR (RT-qPCR) was performed in a Stratagene MX3005P thermal cycler using a standard protocol (10 min at 95 °C followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 1 min at 60 °C) with RT^2 SYBR Green qPCR Mastermix and specific PCR primers (Qiagen, Hilden, Germany) (mouse GAPDH, 140 bp, PPM02946E, reference position 309, Gen-Bank accession no. NM_008084.2; human GAPDH, 175 bp, PPH00150E, reference position 1287, Gen-Bank accession no. NM_002046.3; mouse WISP-2, 135 bp, PPM02952F, reference position 1044, Gen-Bank accession no. NM_016873.2; human WISP-2, 123 bp, PPH00981B, reference position 1257, Gen-Bank accession no. NM_003881.2; mouse MMP-13, 88 bp, PPM03675A, reference position 1145, Gen-Bank accession no. NM_008607; human MMP-13, 61 bp, PPH00121B, reference position 1380, Gen-Bank accession no. NM_002427.3; human ADAMTS-5, 170 bp, PPH30803E, reference position 7506-7527, Gen-Bank accession no. NM_011782.2; human ADAMTS-4, 4410 bp, PPH14490A, reference position 2675, Gen-Bank accession no. NM_005099; human IL-6, 98 bp, PPH00560C, reference position 816, Gen-Bank accession no. NM_000600.3; human IL-8, 126 bp, PPH00568A, reference position 326, Gen-Bank accession no. NM_000584.3; human IL-1β, 126 bp, PPH00171C, reference position 574, Gen-Bank accession no. NM_000576.2). No-template controls were included to eliminate any non-specific amplification, and melting curves were generated to ensure a single gene-specific peak. Gene expression changes were determined by the comparative ΔΔCt method in MxPro qPCR Software version 4.10 (Stratagene, La Jolla, CA, USA), expressed

as relative fold change compared to control (C-), and normalized to GAPDH housekeeping gene.

Protein extraction and western blot analysis

After treatment, cells were rapidly washed with ice-cold phosphate-buffered saline and scraped in lysis buffer for protein extraction (10 mM Tris/HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5% Triton X-100, 1 mM PMSF, and protease inhibitor cocktail from Thermo Fisher Scientific (Waltham, MA, USA)). Lysed cells were centrifuged at 14.000 × *g* for 20 min. SDS-PAGE and blotting procedure were performed as previously described[22]. Immunoblots were incubated with the pertinent antibody against (WISP-2 diluted 1:400 (Abyntek Biopharma, Derio, Biscay, Spain); MMP-13, Phospho-GSK-3α/β, GSK-3α/β, Phospho-NF-κβ p65, NF-κβ p65, Phospho-p44/p42 MAPK (Erk1/2), and Phospho-p38 MAPK diluted 1:1000 form Cell Signaling (Danvers, MA, USA); MAPK 1/2 (Erk1/2), p38/SAPK2, Phospho-JNK 1/2, and JNK/SAPK1 diluted 1:1000 from Upstate (Syracuse, NY, USA); ADAMTS-5 diluted 1:500 (Abcam, Cambridge, UK) The immune complexes were detected using anti-rabbit or anti-mouse horseradish-peroxidase-labeled secondary antibodies diluted 1:5000 (both from GE Healthcare, Chicago, IL, USA) and visualized with Immobilon Western Detection kit (Millipore, MA, USA) To confirm equal loading in each sample, the membranes were stripped in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and re-blotted with anti-GAPDH antibody diluted 1:2000 (Sigma Aldrich, MO, USA). In all figures showing images of gels, the bands for each picture were obtained from the same gel, although they may have been spliced for clarity. The images were captured with ChemiDoc MP Imaging System and analyzed with Image Lab 6.0.1 Software, both from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

WISP-2 gene knockdown

For siRNA transfection experiments, T/C-28a2 cells and human primary OA chondrocytes were seeded at 2×10^5 cells per well in six-well plates and incubated overnight with DMEM/Ham's F-12 with 10% FBS. Before transfection, the medium was changed to serum and antibiotics free medium. Transfections were performed using TriFECTa RNAi Kit following the manufacturer's instructions (Integrated DNA Technologies, Coralville, IA, USA). Gene silencing was made with 10 nM of three DsiRNAs that specifically target WISP-2 and 10 nM of nontargeting universal negative control RNA duplex that does not interact with any known sequence. Transfection with siRNA duplexes was performed using the cationic lipid siLentFect Lipid Reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's recommendations. Incubation was continued for 48 h after siRNA transfection. WISP-2 gene knockdown was verified at mRNA and protein levels and supported with two control sequences provided by the manufacturer: a TYE 563-labeled transfection control and a positive control DsiRNA that targets the *HPRT1* gene and is prevalidated to give more than 90% knockdown of HPRT (presented as Supplementary Data). At 48 h after transfection, cells were treated with recombinant human IL-1β (0.5 ng/mL) for 24 h. A specific pharmacological inhibitor (BIO 1 μM) was added 1 h before stimulation.

Statistical analysis

Data are reported as the mean ± standard error of the mean (SEM) of at least three independent experiments. Statistical analyses were performed with GraphPad Prism 9.3.1 software (GraphPad Software, La Jolla, CA, USA). When assuming a normal distribution, we used two-sided unpaired t test with Welch's correction or one-way ANOVA test corrected with Bonferroni's multiple comparison test. When normal distribution was not assumed, we used Mann–Whitney test. *P*-values less than 0.05 were considered significant.

RESULTS

WISP-2 mRNA and protein basal expression along ATDC-5 differentiation

To evaluate whether WISP-2 mRNA and protein levels change along chondrocyte differentiation, we differentiated murine ATDC-5 cells into mature and hypertrophic chondrocytes. As shown in Fig. 1A, WISP-2 mRNA expression increased during the process of differentiation of ATDC-5 cells. This increase is significant after 7 and 14 days of differentiation in comparison to undifferentiated cells (day 0) (Fig. 1A, upper panel). However, WISP-2 mRNA expression dramatically declined at 21 days in hypertrophic

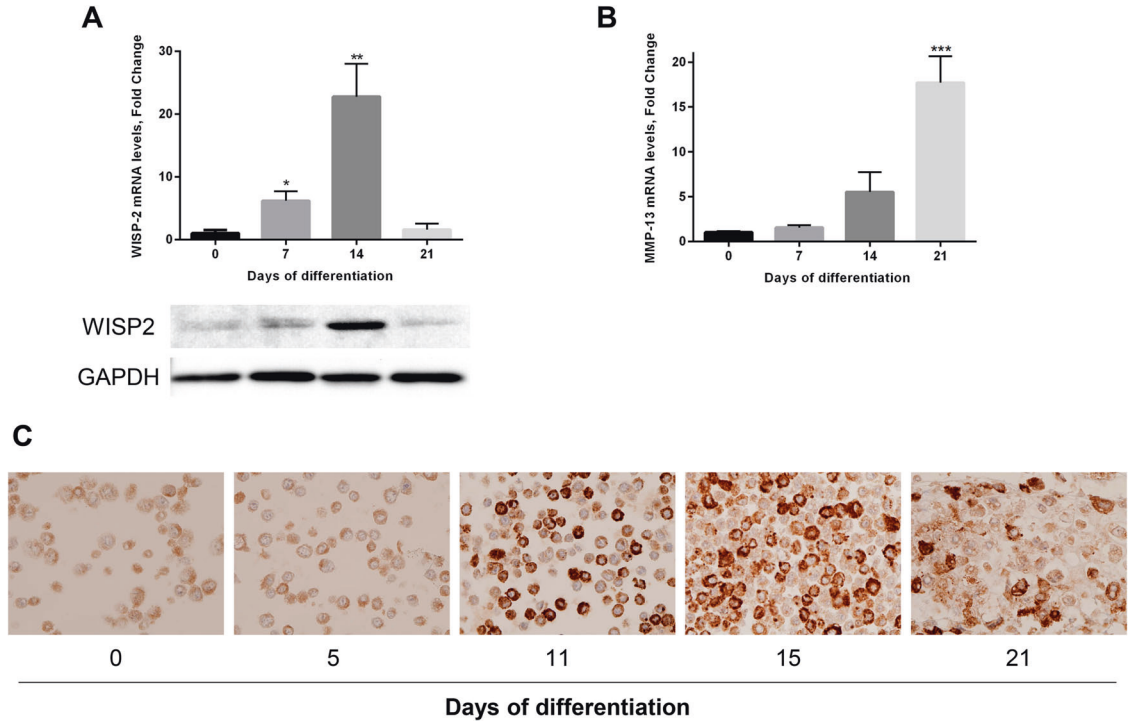


Fig. 1 WISP-2 mRNA and protein constitutive expression along ATDC-5 differentiation. A WISP-2 mRNA and protein expression along ATDC-5 differentiation after 7, 14, and 21 days. Cell lysates underwent western blot analysis using WISP-2 antibody. GAPDH was used as a loading control. Values are the mean ± SEM of at least four independent experiments (*$p < 0.05$, **$p < 0.01$ vs. control). **B** mRNA levels of MMP-13 along ATDC-5 differentiation. Values are the mean ± SEM of at least four independent experiments (***$p < 0.001$ vs. control). **C** Immunocytochemical images showing WISP-2 protein expression in ATDC-5 cells along cell differentiation at 0, 5, 11, 15, and 21 days.

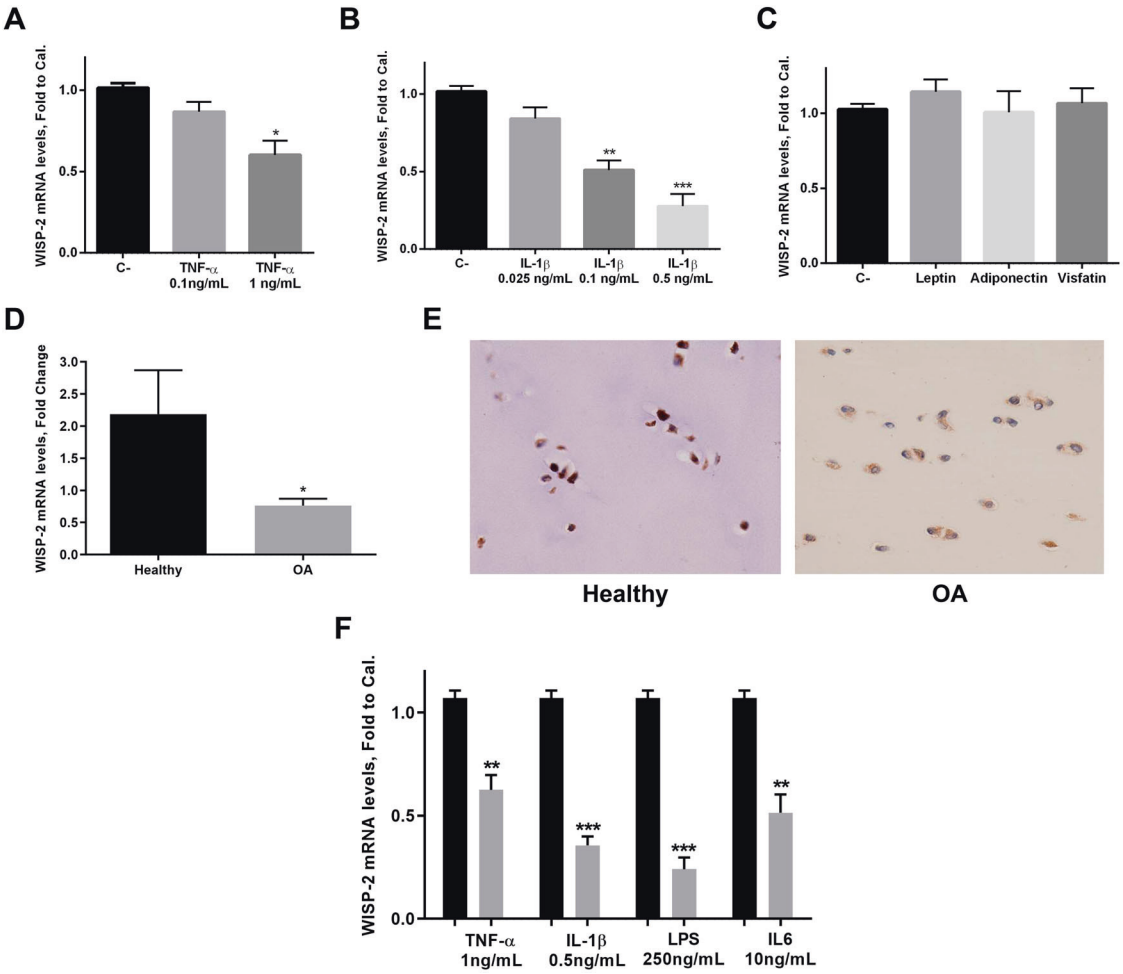


Fig. 2 WISP-2 mRNA and protein expression in human chondrocytes. A Human T/C-28a2 cells were treated with TNF-α 0.1 and 1 ng/mL, **B** IL-1β 0.025, 0.1 and 0.5 ng/mL, and **C** with leptin (800 nM), adiponectin (10 µg/mL) and visfatin (500 ng/mL) for 24 h. **D** WISP-2 mRNA basal expression in healthy and OA human primary chondrocytes. Values are the mean ± SEM of 4 independent human samples for the healthy cartilage, and 8 independent human samples for OA cartilage (*$p < 0.05$). **E** Immunocytochemical images showing WISP-2 protein expression in healthy and OA human cartilage. **F** Human primary OA chondrocytes were treated with TNF-α 1 ng/mL, IL-1β 0.1 ng/mL, LPS 250 ng/mL and IL-6 10 ng/mL for 24 h. WISP-2 mRNA expression was evaluated by RT-qPCR. Values are the mean ± SEM of at least three independent experiments (**$p < 0.01$, ***$p < 0.001$ vs. control).

chondrocytes. This effect was also evaluated in terms of protein expression as reported in Fig. 1A (lower panel). This decrease matches with a significant increment in MMP-13 expression at the same time point in ATDC-5 hypertrophic cells (Fig. 1B). The WISP2 protein expression along ATDC-5 differentiation was also assayed by immunocytochemistry (Fig. 1C)

Effect of pro-inflammatory cytokines and adipokines on WISP-2 mRNA expression in human chondrocytes

To further elucidate the pattern of WISP-2 expression under inflammatory conditions, we treated T/C-28a2 cells with pro-inflammatory cytokines TNF-α and IL-1β and with representative adipokines leptin, adiponectin, and visfatin. As shown in Fig. 2A, cells stimulated with TNF-α 1 ng/mL for 24 h showed a significant inhibition of WISP-2 mRNA expression. A more pronounced effect was observed when cells were stimulated with IL-1β 0.5 ng/mL (Fig. 2B) for 24 h. Nonetheless, neither leptin (800 nM) nor adiponectin (10 µg/mL) or visfatin (500 ng/mL) had significant effects on WISP-2 expression (Fig. 2C).

We also examined the constitutive expression of WISP-2 in human chondrocytes derived from healthy and OA joints. As shown in Fig. 2D, WISP-2 mRNA levels in OA chondrocytes are lower than those observed in chondrocytes obtained from healthy subjects. We further confirmed these results by immunocytochemistry in human healthy and OA cartilage. As shown in Fig. 2E, immunocytochemical techniques showed cytoplasmic positivity for WISP-2 in human chondrocytes. In osteoarthritis cartilage samples the intensity of immunostaining was weak, and the WISP-2 immunoreactivity rate (WISP-2-immunostaine-positive cells divided by total cells) was 59.92 ± 0.76% [Fig. 2 panel E (OA)] In healthy cartilage controls the intensity of immunostaining was stronger, with a WISP-2 immunoreactivity rate of 81.02 ± 0.98% [Fig. 2 panel E (Healthy)].

OA chondrocytes were also stimulated with cytokines TNF-α, IL-1β, IL-6, and an agonist of TLR4, the bacterial lipopolysaccharide (LPS). As shown in Fig. 2F, TNF-α as well as IL-1β, LPS, and IL-6 were able to decrease WISP-2 mRNA expression.

Effect of recombinant human WISP-2 protein and IL-1β stimulation in human chondrocyte catabolism

To elucidate if WISP-2 can interfere in IL-1β-mediated MMPs and aggrecanase production, T/C-28a2 chondrocytes were pre-treated with human WISP2 recombinant protein (500 ng/mL) one hour before being stimulated with IL-1β 0.5 ng/mL for 24 h. Compared to IL-1β-stimulated cells, chondrocytes pre-treated with rhWISP-2

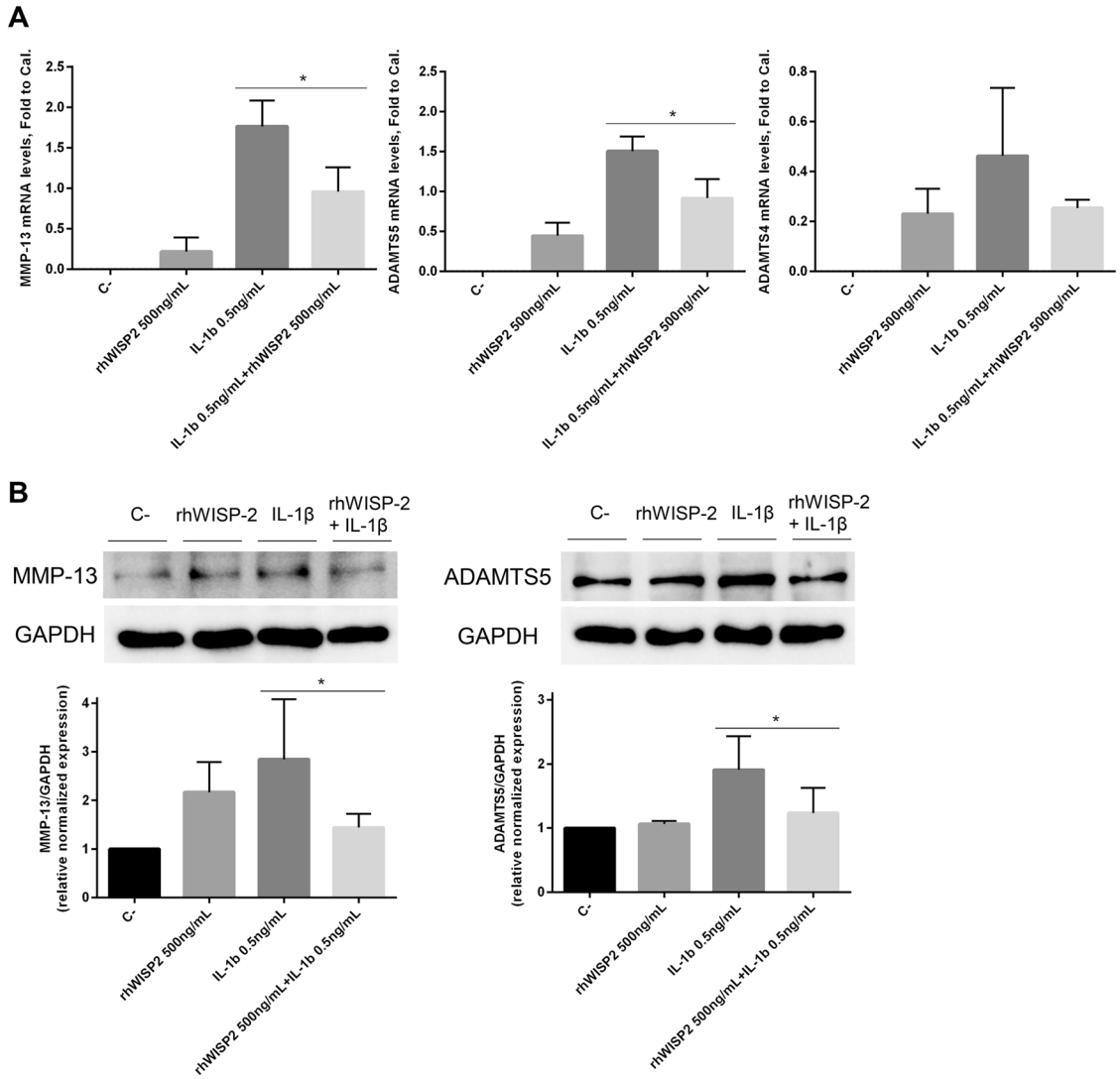


Fig. 3 Recombinant human WISP-2 protein reduces IL-1β-mediated chondrocyte catabolism. Human T/C-28a2 cells were pre-treated with rhWISP-2 (500 ng/mL) for one hour before being challenged with IL-1β 0.5 ng/mL for 24 h. Catabolism markers were evaluated in pre-treated and not pre-treated human chondrocytes. **A** Relative mRNA levels of MMP-13, ADAMTS-5, and ADAMTS-4 in human T/C-28a2 chondrocytes were determined by RT-qPCR. **B** Determination of MMP-13 and ADAMTS-5 protein expression by western blot in human T/C-28a2 chondrocytes. GAPDH was used as a loading control. Densitometric analysis is also shown (lower panels). Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

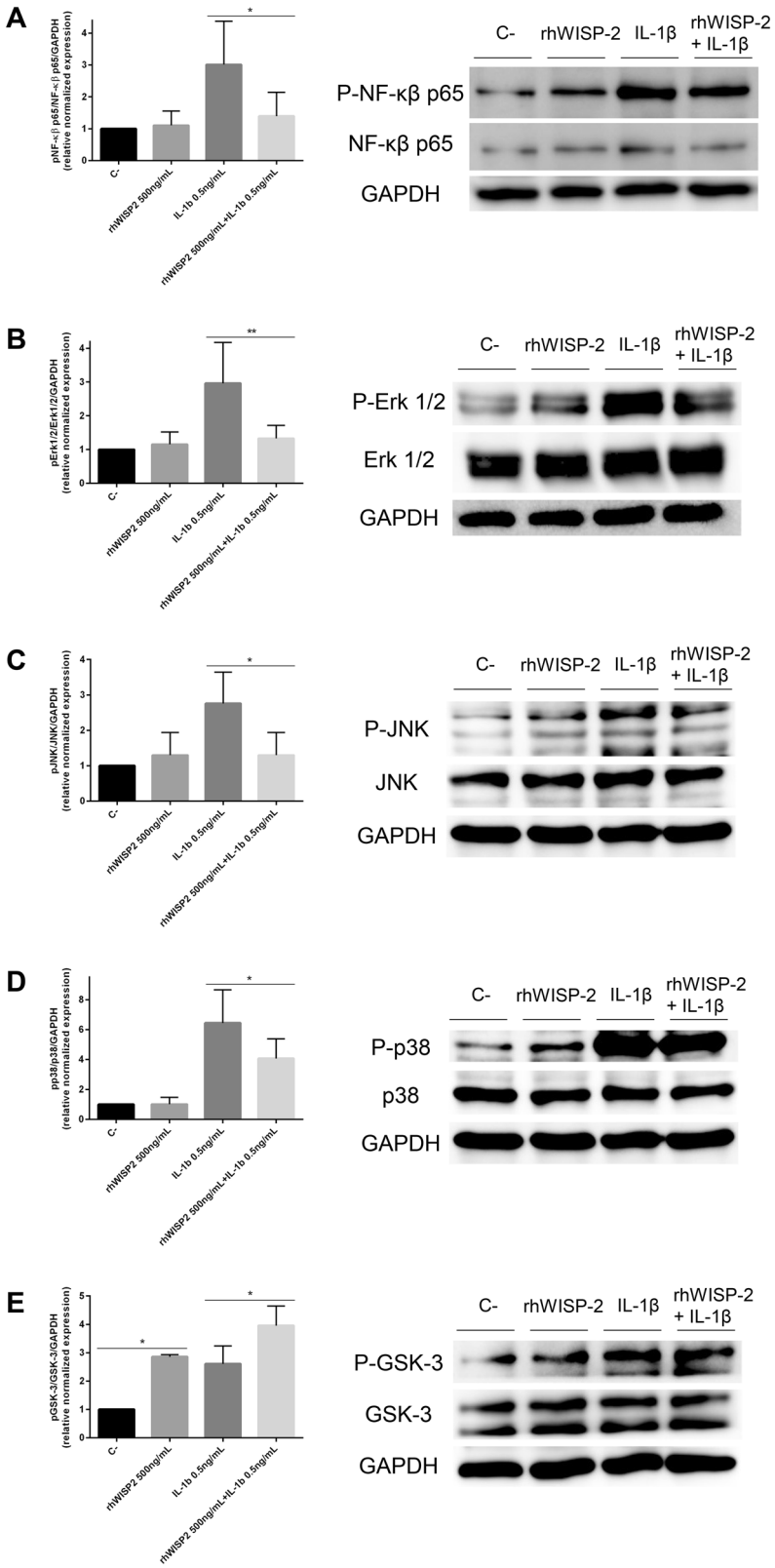
protein showed a significant reduction in MMP-13 and ADAMTS5 mRNA expression (Fig. 3A) These results were also confirmed at protein level (Fig. 3B) For completeness, ADAMTS4 was also examined since it is upregulated by inflammatory cytokines in chondrocytes. Interestingly, we observed that its mRNA expression is fairly lower than ADAMTS5. However, the pattern of response to rhWISP2 treatment is similar in both aggrecanases (Fig. 3A, right panel).

Downstream IL-1β and WNT/b-catenin signaling pathways involved in recombinant human WISP-2 protein and IL-1β stimulation action in human chondrocytes

In order to understand the molecular mechanisms involving WISP-2 we analyzed the downstream IL-1β signaling upon WISP-2 treatment. Human chondrocytes were pre-treated with human WISP2 recombinant protein (500 ng/mL) 1 h before being stimulated with IL-1β 0.5 ng/mL for 20 min. The obtained results clearly showed that rhWISP-2 significantly reduces IL-1β-induced NF-kβ p65 phosphorylation, Erk 1/2 phosphorylation, JNK phosphorylation and p38 phosphorylation (Fig. 4A–D) After confirming the WISP-2 inhibitory effect in the IL-1β signaling in human chondrocytes, we addressed Wnt/β-catenin signaling to elucidate if WISP-2 can act as a WNT agonist. For this purpose, we measured GSK-3 phosphorylation under the same experimental conditions and observed that rhWISP2 significantly activates Wnt signaling. Our results also showed that rhWISP2, in combination with IL-1β, has a cooperative effect, and that the Wnt pathway activation is significantly increased compared to single treatments (Fig. 4E).

WISP-2 siRNA knockdown stimulates chondrocyte catabolism and downregulates WNT/b-catenin signaling

Based on the data above showing WISP-2 downregulation under pro-inflammatory stimulation, we investigated the effect of WISP-2 gene silencing on cartilage ECM breakdown mediators. As shown in Fig. 5A, WISP-2 gene knockdown led to increased MMP-13 and ADAMTS-5 expression in comparison to T/C-28a2 cells transfected with a non-targeting control siRNA. Apart from cartilage-degrading enzymes, the catabolic effects of chondrocytes are mediated through several cytokines and chemokines. WISP-2 silencing increased IL-6 and IL-1β mRNA levels. The chemokine IL-



8 was also significantly upregulated upon WISP-2 gene knockdown. In addition, as shown in Fig. 5B, MMP-13 mRNA expression is increased also in OA human primary chondrocytes after WISP-2 gene silencing. This effect was also evaluated in terms of protein expression as reported in Fig. 5C.

To gain further insights into the signaling pathways at play, we tested the hypothesis that WISP-2 might be an activator of canonical Wnt signaling[13]. To investigate Wnt/β-catenin signaling activation we addressed GSK-3 phosphorylation in WISP-2-silenced T/C-28a2 chondrocytes. As shown in Fig. 5D, upon

Fig. 4 Downstream IL-1β and WNT/b-catenin signaling pathways involvement in recombinant human WISP-2 protein mechanism of action. Human T/C-28a2 cells were pre-treated with rhWISP-2 (500 ng/mL) for 1 h before being challenged with IL-1β 0.5 ng/mL for 20 min. IL-1β cascade and Wnt pathway activation were evaluated in pre-treated and not pre-treated human chondrocytes. **A** Determination of phosphorylated NF-kβ p65 and total NF-kβ p65 expression by western blot in human T/C-28a2 chondrocytes. **B** Determination of phosphorylated Erk 1/2 and total Erk 1/2 protein expression by western blot in human T/C-28a2 chondrocytes. **C** Determination of phosphorylated JNK and total JNK protein expression by western blot in human T/C-28a2 chondrocytes. **D** Determination of phosphorylated p38 and total p38 protein expression by western blot in human T/C-28a2 chondrocytes. **E** Determination of phosphorylated GSK-3 and total GSK-3 protein expression by western blot in human T/C-28a2 chondrocytes. GAPDH was used as a loading control. Densitometric analysis is also shown. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

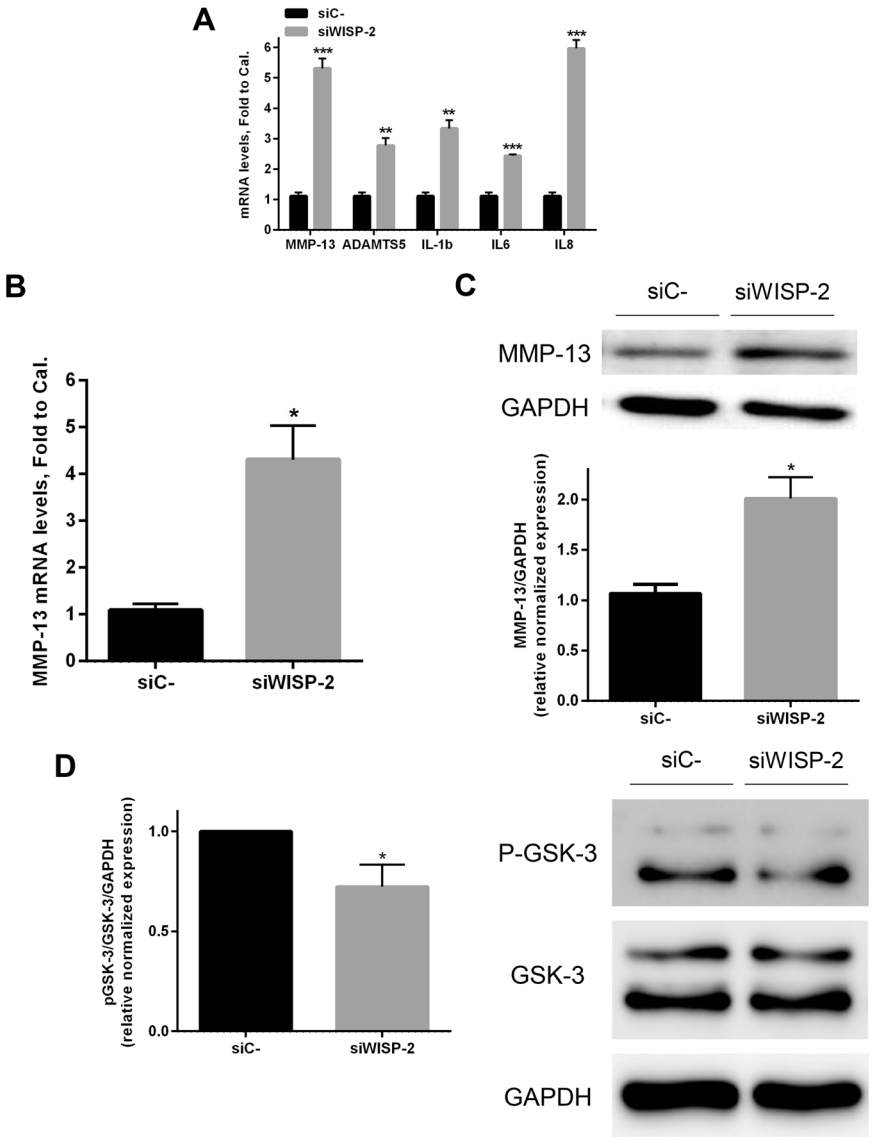


Fig. 5 Effect of WISP-2 gene knockdown on chondrocytes catabolic markers and in WNT/b-catenin pathway. Chondrocytes were transfected with negative control (siC-, 10 nM) or siRNA against WISP-2 (siWISP-2, 10 nM) for 48 h. **A** Relative mRNA levels of MMP-13, ADAMTS-5, IL-6, IL-8, and IL-1β were determined in human T/C-28a2 chondrocytes by RT-qPCR. **B** Determination of human MMP-13 mRNA expression by RT-qPCR in human primary OA chondrocytes. **C** Determination of human MMP-13 protein expression by western blot in human OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis was also shown. **D** Determination of phosphorylated GSK-3 protein expression by western blot in human OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis is also shown. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. siC-).

WISP-2 silencing there is a significant decrease of phosphorylation of GSK-3, suggesting that the process of accumulation and translocation of β-catenin to the nucleus, as well as the expression of downstream target genes are compromised.

Effect of WISP-2 siRNA knockdown on IL-1β-stimulated chondrocytes

Since IL-1β has been implicated in cartilage degradation[23], we determined the effect of WISP-2 silencing on IL-1β-stimulated T/C-

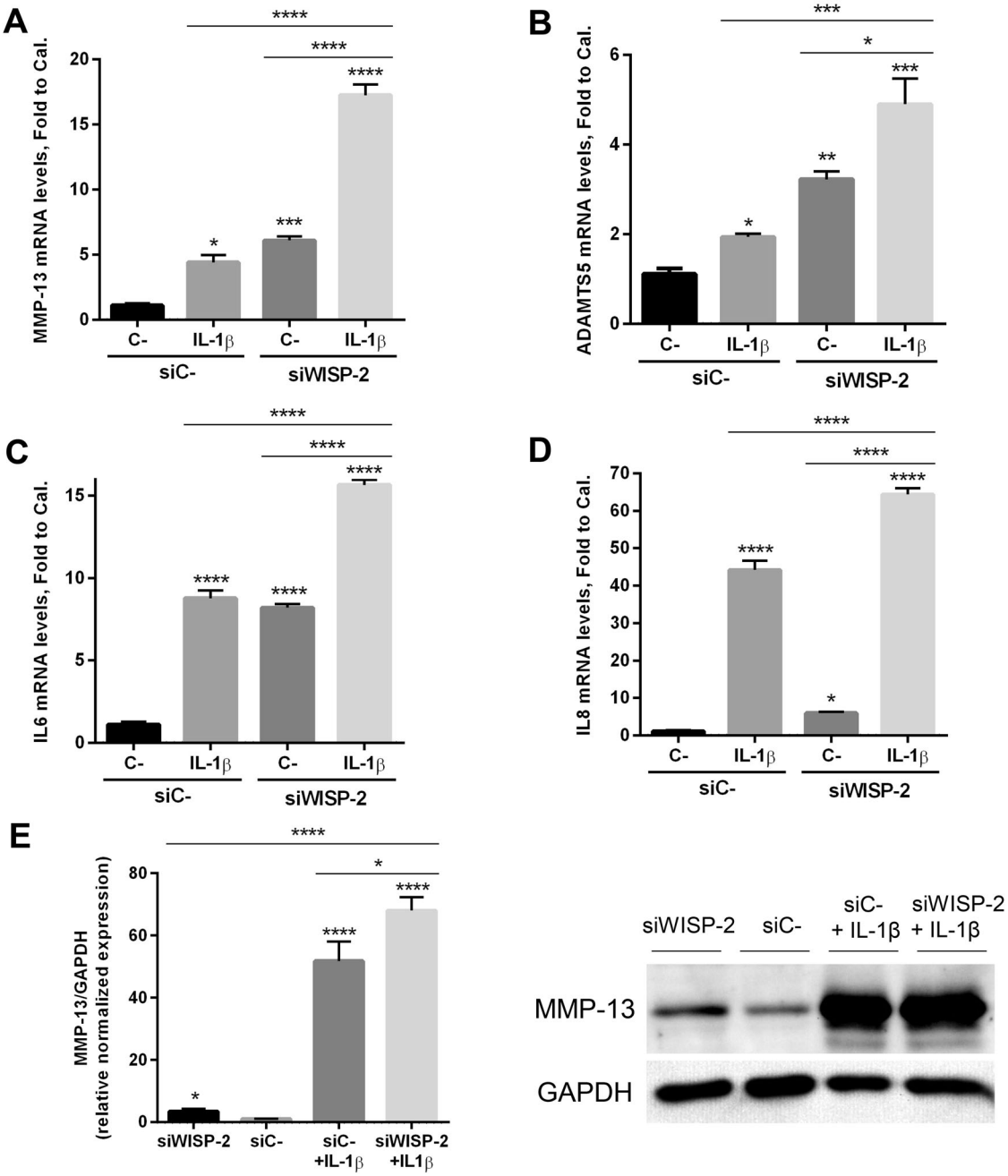


Fig. 6 Effect of WISP-2 siRNA knockdown on IL-1β-stimulated chondrocytes. Chondrocytes were transfected with negative control (siC-, 10 nM) or siRNA against WISP-2 (siWISP-2, 10 nM) in presence or not of IL-1β (0.5 ng/mL) for 24 h. **A**–**D** Relative mRNA levels of MMP-13, ADAMTS-5, IL-6, and IL-8 in human T/C-28a2 chondrocytes were determined by RT-qPCR. **E** Determination of human MMP-13 protein expression by western blot in human primary OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis was also shown. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. siC-).

28a2 chondrocytes. As expected, IL-1β induced mRNA expression of MMP-13, ADAMTS-5, IL-6, and IL-8, while WISP-2 knockdown further increased IL-1β-induced expression of those genes (Fig. 6A–D). These results were confirmed also in human primary OA chondrocytes in terms of MMP-13 protein expression (Fig. 6E).

Effect of canonical Wnt/β-catenin signaling on WISP-2 siRNA knockdown mediated chondrocyte catabolism

To investigate the involvement of Wnt/β-catenin signaling, we transfected T/C-28a2 chondrocytes with siWISP-2 and then treated them with IL-1β in presence or not of selective GSK-3 inhibitor BIO. As shown in Fig. 7A–D, activation of canonical Wnt signaling by BIO significantly decreased the expression of MMP-13, as well as ADAMTS-5, IL-6, and IL-8. Moreover, BIO treatment blocked IL-1β-induced expression of those catabolic markers' mRNA. To note, blocking GSK-3 with BIO almost completely suppressed the IL-1β-mediated upregulation of MMP-13, ADAMTS-5, IL-6, and IL-8 in siWISP-2-transfected cells. This effect was confirmed in human primary OA chondrocytes at MMP-13 protein level (Fig. 7E).

DISCUSSION

The recently discovered adipokine WISP-2 was former identified as a Wnt-inducible protein[24]. In the last decade, evidence has

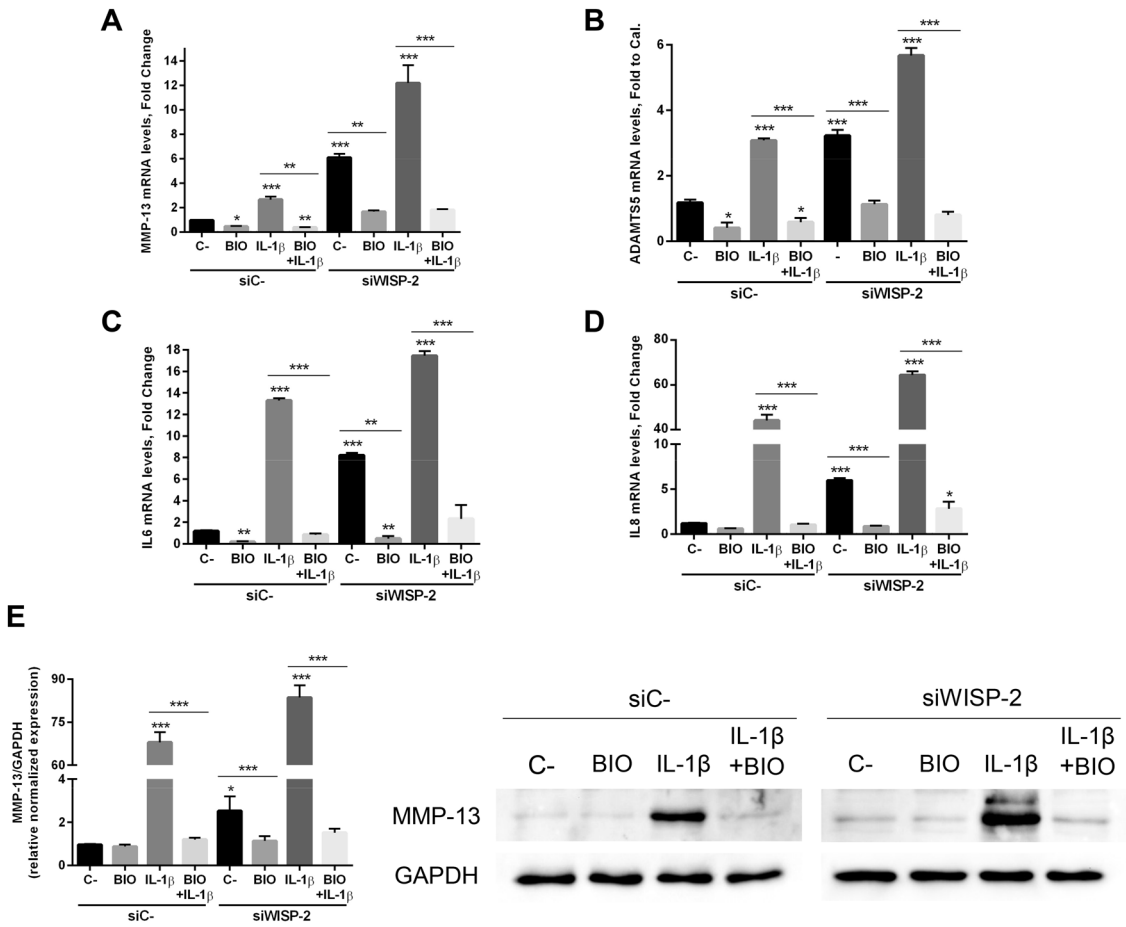


Fig. 7 Effect of canonical Wnt/β-catenin signaling on WISP-2 siRNA knockdown mediated chondrocyte catabolism. Chondrocytes were transfected with negative control (siC-, 10 nM) or siRNA against WISP-2 (siWISP-2, 10 nM) in presence or not of IL-1β (0.5 ng/mL) for 24 h in combination with 1 μM BIO. **A**–**D** Relative mRNA levels of MMP-13, ADAMTS-5, IL-6, and IL-8 in human T/C-28a2 chondrocytes. **E** Determination of human MMP-13 protein expression by western blot in human primary OA chondrocytes. GAPDH was used as a loading control. Densitometric analysis was also shown. Values are the mean ± SEM of at least three independent experiments (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. siC-).

suggested a key role for Wnt signaling in the pathophysiology of OA. Canonical Wnt/β-catenin pathway leads to excessive catabolism and cartilage degradation and the hypertrophic differentiation of chondrocytes. However, Wnt signaling is necessary for maintenance of the articular cartilage, but an excessive activity is deleterious.

Recent studies have investigated the role of CCN family proteins in the development of OA. WISP-1/CCN4 and WISP-2/CCN5 were found expressed to a greater extent in OA and RA knee cartilage and all CCN genes were expressed in OA and RA synovial samples[25]. Moreover, our group has identified WISP-2 in cartilage, synovium, and infrapatellar fat pad, finding a significative increment in WISP-2 expression in OA infrapatellar fat pad in comparison to healthy controls[18]. WISP-1 regulates chondrocyte MMP and aggrecanase expression and is capable of inducing articular cartilage damage in models of OA[5]. By contrast, WISP-3/CCN6-induced repression of ADAMTS-5 expression and upregulation of MMP-10 in human chondrocytes[26]. Regarding the participation of WISP-2 in OA, there are currently no papers in the literature evaluating its action in chondrocytes. Thus, we have analyzed the effect of WISP-2 on cartilage catabolism using the recombinant protein and loss-of-function experiments in order to enlighten the role of this protein in chondrocyte pathophysiology. To the best of our knowledge, this is the first paper showing experimental evidence that WISP-2 is modulated by mediators of inflammation and its involvement in cartilage degradation.

We first analyzed the regulation of WISP-2 expression along chondrocyte differentiation observing an increment of WISP-2 expression in the first stages of chondrogenesis, followed by a dramatic decrease at the onset of hypertrophic stage. Our data in ATDC-5 cells, a well-established cell line to study chondrogenesis[27], are in agreement with those obtained by Schutze et al.[28]. Although these authors used bone marrow-derived MSC, that in their undifferentiated stage do not express none of the chondrogenic markers, the ATDC-5 chondrogenic cell line expressed them also in the chondrogenic stage. Our experimental set provides novel data since we analyzed WISP-2 expression up to the onset of hypertrophy, showing that both mRNA and protein expression of WISP-2 strongly decreased. Noteworthy, the marked reduction in WISP-2 expression at hypertrophic phase was coincident with a significant increase in MMP-13 expression, the main marker of this last stage[29], suggesting a relationship between these two factors.

Plenty of studies have described the involvement of cytokines in cartilage destruction. IL-1β is a well-known pro-inflammatory cytokine that is a potent inductor of MMP-13 expression[30]. Thus, we have studied the effect of pro-inflammatory cytokines and adipokines involved in rheumatic diseases, such as TNF-α, IL-1β, or leptin, on WISP-2 expression in chondrocytes. WISP-2 is regulated by transcription factors which are induced by inflammation, activation of the Wnt pathway, and hypoxia, processes increased in obesity[12]. Furthermore, IL-1β induces expression of Wnt

proteins, resulting in activation of Wnt pathway[9]. On the other hand, the endogenous activation of Wnt/β-catenin signaling, as observed in certain tumor cell lines, is characterized by a very low expression of WISP-2[13]. In contrast to other members of the CCN family, whose levels are increased under inflammatory conditions (i.e., WISP-3[31]), we have observed that either TNF-α, IL-1β, LPS, or IL-6 were able to reduce the expression of WISP-2 in human chondrocytes. Taking together, these results suggest that a "canonical" pro-inflammatory environment characterized by cytokines or TLR4 activation negatively modulates the expression of WISP-2. By contrast, the classic pro-inflammatory adipokines, leptin, and adiponectin did not alter the expression of WISP-2 in T/C-28a2 chondrocytes. We expected that these adipokines, which are well known contributors to create a pro-inflammatory environment in obesity and OA, might be able to regulate WISP-2. Remarkably, they did not.

One aspect that arises from our current investigations is that WISP-2 can minimize the catabolic effects of IL-1β in cartilage. As a matter of fact, recombinant WISP-2 was able to partially decrease the main metalloproteases and aggrecanases induced by IL-1β. In our experiments, recombinant WISP-2 was able to attenuate the IL-1β/NF-κβ as well as the Erk 1/2, JNK, and p38 signaling pathways. Therefore, these data, together with the observation that WISP-2 is able to increase the phosphorylation of GSK-3, suggested that WISP-2 may act as a WNT agonist with potential anabolic functions that are partially able to counteract the catabolic effects of IL-1β as one of the main mediators of inflammatory response in cartilage.

We confirmed these observations by silencing *WISP-2*. Indeed, the silencing of *WISP-2* increased the expression of MMP-13, but also was able to upregulate the expression of other aggrecanases and relevant pro-inflammatory cytokines such as IL-1β, IL-6, and IL-8. Finally, the activation of canonical WNT pathway by BIO (a classic GSK-3β inhibitor) confirms the previously showed results and are in agreement with previous published literature. In concrete terms, WISP-2 may contribute to counteract the detrimental effects of inflammation. To this regard, CCN5/WISP-2 has been reported to have also protective effects counteracting the fibrosis in heart failure by inhibiting the TGF-β pathway[32]. In rheumatoid arthritis, Tanaka et al. reported that WISP-2 is prevalently expressed in arthritic synovial tissues[16] and specifically in fibroblast of the fibrotic area. In conclusion, there are potentially significant physiologic and pathophysiologic aspects glanced from this investigation.

In conclusion, we have shown for the first time that WISP-2 may have relevant roles in modulating the expression of enzymes involved in the turnover of extracellular matrix in the cartilage and that its downregulation may negatively alter the inflammatory environment in OA cartilage. We also proved the participation of Wnt/β-catenin signaling pathway in these processes. Thus, targeting WISP-2 may represent a possible therapeutical approach to OA. Further research is needed to define the contribution of WISP-2 in the complex metabolic network of degenerative/inflammatory diseases of musculoskeletal system such as osteoarthritis.

DATA AVAILABILITY

All the data are available in the manuscript. Data sets are available to readers promptly upon request.

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AUTHOR CONTRIBUTIONS

C.R.-F., M.G.-R., V.A., V.F., A.C.-B., J.P., F.L., J.C.-A., M.A.G.-G., A.M., D.A.E., Y.F., L.G.-C., and M.G.-C., participated in acquisition of data and samples, drafting the manuscript, analysis and interpretation of data, and statistical analysis. A.M. participated in scientific discussions, provided feedback, and critically revised the manuscript. O.G. and M.S. participated in conception and design of the study, in analysis and interpretation of data, critical revision of the manuscript, and scientific supervision of the experiments.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

This study was conducted with the approval of the Santiago University Clinical Hospital Ethics Committee (CAEIG 2014/310).

ADDITIONAL INFORMATION

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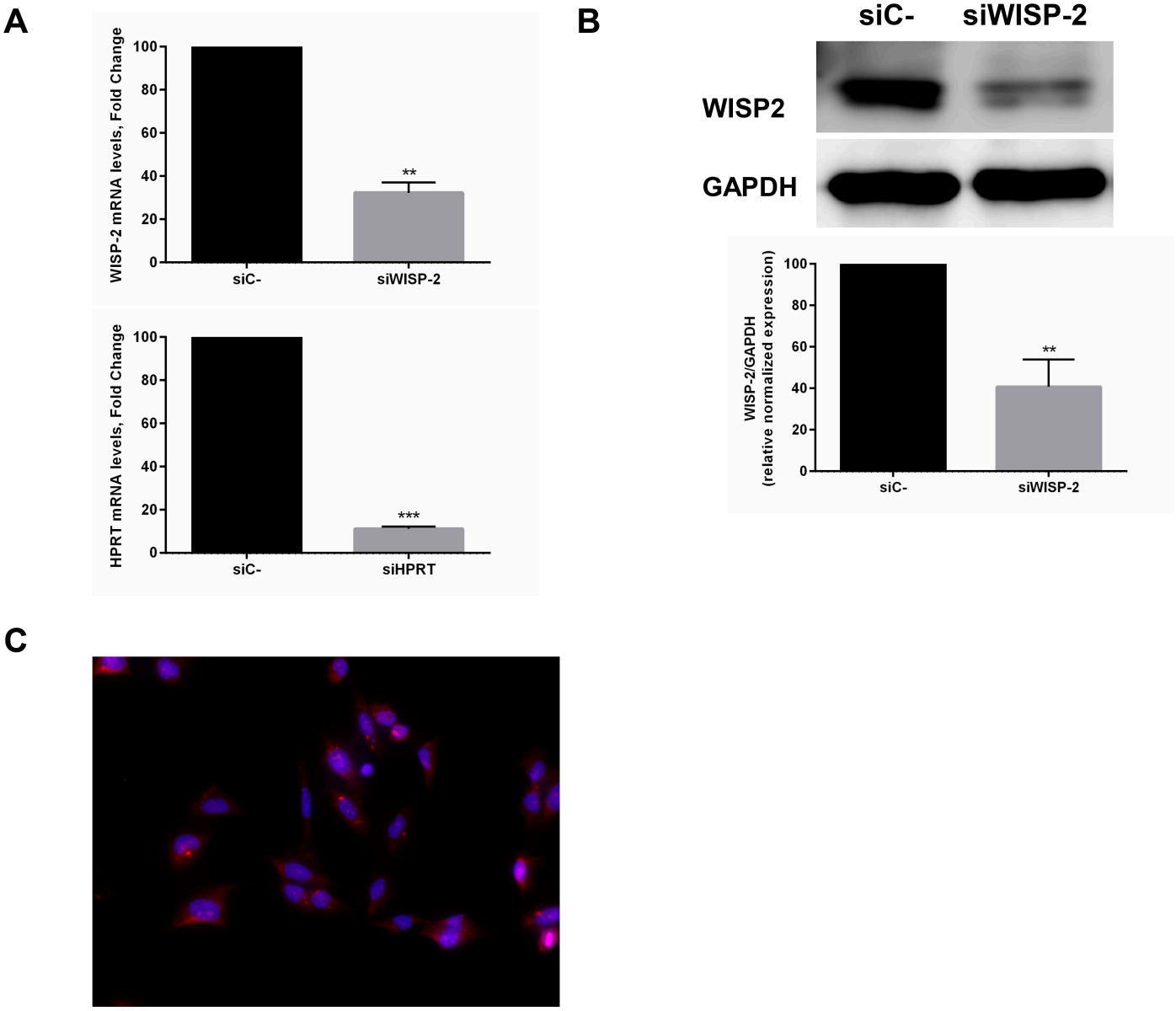

Supplementary data. WISP-2 transfection efficiency. Human T/C-28a2 cells were transfected with TriFECTa RNAi Kit following manufacturer's recommendations. *WISP2* gene knockdown was verified at mRNA and protein levels using three different control DsiRNAs provided by the manufacturer. **(A)** Relative mRNA levels of WISP-2 and HPRT were determined by RT-qPCR after 48 hours of transfection with 10nM of the three DsiRNAs that specifically target WISP-2 or with 10nM of the positive control DsiRNA that targets the *HPRT1* gene, respectively. Values were referred to siC- (T/C-28a2 cells after 48h transfection with 10nM of nontargeting universal negative control RNA duplex that does not interact with any known sequence) **(B)** Determination of WISP-2 protein expression by western blot after 48 hours of transfection with 10nM of the three DsiRNAs that specifically target WISP-2 or with 10nM of negative control DsiRNA (siC-). GAPDH was used as a loading control. Densitometric analysis are also shown. **(C)** Fluorescence microscopy image of labeled T/C-28a2 cells after 48h pf transfection with 10nM of TYE 563 Transfection Control DsiRNA. DAPI was used as a control of cell viability. Values are the mean ± SEM of at least three independent experiments (*p<0.05, **p<0.01, ***p<0.001)

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Fine, Noah et al. "Intervertebral disc degeneration and osteoarthritis: a common molecular disease spectrum." Nature reviews. Rheumatology vol. 19,3 (2023): 136-152. doi:10.1038/s41584-022-00888-z

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Figure 2. Diarthrodial joints in health and OA.

Martel-Pelletier, Johanne et al. "Osteoarthritis." Nature reviews. Disease primers vol. 2 16072. 13 Oct. 2016, doi:10.1038/nrdp.2016.72

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Figure 3. The physiological structure of articular cartilage and pathological changes of OA.

Li, Jiadong et al. "Targeted and responsive biomaterials in osteoarthritis." Theranostics vol. 13,3 931-954. 16 Jan. 2023, doi:10.7150/thno.78639

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Figure 4. Crosstalk between cartilage and the synovium in the pathogenesis of OA.

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Figure 5. The intervertebral disc in health and disease.

Binch, Abbie L A et al. "Cell-based strategies for IVD repair: clinical progress and translational obstacles." Nature reviews. Rheumatology vol. 17,3 (2021): 158-175. doi:10.1038/s41584-020-00568-w

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Figure 6. The biological role of C-reactive protein (CRP).

Pope, Janet E, and Ernest H Choy. "C-reactive protein and implications in rheumatoid arthritis and associated comorbidities." Seminars in arthritis and rheumatism vol. 51,1 (2021): 219-229. doi: 10.1016/j.semarthrit.2020.11.005

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Figure 7. Structure of pCRP (PDB:1B09).

Yao, ZhenYu et al. "Regulation of C-reactive protein conformation in inflammation." Inflammation research: official journal of the European Histamine Research Society ... [et al.] vol. 68,10 (2019): 815-823. doi:10.1007/s00011-019-01269-1

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Figure 8. Schematic representation of the predominant interactions of pCRP and mCRP isoforms.

Hart, Peter C et al. "C-Reactive Protein and Cancer-Diagnostic and Therapeutic Insights." Frontiers in immunology vol. 11 595835. 19 Nov. 2020, doi:10.3389/fimmu.2020.595835

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Figure 9. Canonical Wnt signaling.

Lorzadeh, Shahrokh et al. "Autophagy and the Wnt signaling pathway: A focus on Wnt/β-catenin signaling." Biochimica et biophysica acta. Molecular cell research vol. 1868,3 (2021): 118926. doi: 10.1016/j.bbamcr.2020.118926

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Tel: 881546425. Correo-e: ceic@sergas.es

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DICTAMEN DEL COMITÉ DE ÉTICA DE LA INVESTIGACIÓN DE SANTIAGO-LUGO

Guillermo José Prada Ramallal, Secretario del Comité de Ética de la Investigación de Santiago-Lugo,

CERTIFICA:

Que este Comité evaluó en su reunión del día 22 de junio de 2017 el estudio**:**

Título: Papel de las adipokinas, de los factores neuroendocrinos y de las nuevas citokinas en la degeneración del disco intervertebral (DDIV)
Promotor*:* Jesús Pino Mínguez, Oreste Gualillo
Tipo de estudio: Outros
Versión:
Código del Promotor:
Código de Registro: 2017/279

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humana, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Postautorización de Tipo Observacional para medicamentos de uso humano, y la Circular nº 07/2004, de investigaciones clínicas con productos sanitarios.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos da Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del Comité.

Emite un dictamen **FAVORABLE** para la realización del estudio **por el/la investigador/a del centro**:

Centros	**Investigadores Principales**
IDIS-CHUS	Jesús Pino Mínguez, Oreste Gualillo

En Santiago de Compostela, a 30 de junio 2017.

El Secretario del Comité Territorial de Ética de la Investigación de Santiago Lugo,

XUNTA DE GALICIA SECRETARÍA XERAL TÉCNICA SECRETARÍA DE COMITÉS DE ÉTICA DA INVESTIGACIÓN CONSELLERÍA DE SANIDADE

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Guillermo José Prada Ramallal, Secretario del Comité de Ética de la Investigación de Santiago-Lugo,

HACE CONSTAR QUE:

1.- El Comité Territorial de Ética de la Investigación de Santiago-Lugo cumple tanto en su composición como en sus PNTs los requisitos legales vigentes (RD 1090/2015 de ensayos clínicos, y la Ley 14/2007 de Investigación Biomédica).

2.- La composición actual del Comité Territorial de Ética de la Investigación de Santiago-Lugo es:

- **Juan Manuel Vázquez Lago (Presidente).** Médico especialista en Medicina Preventiva y Salud Pública. Área de Gestión Integrada de Santiago**.**
- **Pilar Rodríguez Ledo (Vicepresidenta).** Médico especialista en Medicina Familiar y Comunitaria. Área de Gestión Integrada de Lugo.
- **Guillermo José Prada Ramallal (Secretario).** Médico especialista en Farmacología Clínica. Área de Gestión Integrada de Santiago. Fundación Ramón Domínguez.
- **Lorenzo Armenteros del Olmo (Vicesecretario).** Médico especialista en Medicina Familiar y Comunitaria. Área de Gestión Integrada de Lugo.
- **Francisco Campos Pérez.** Biólogo. Instituto de Investigación Sanitaria de Santiago de Compostela.
- **Rosana Castelo Domínguez.** Farmacéutica de Atención Primaria. Área de Gestión Integrada de Santiago.
- **Ricardo García Martínez.** Licenciado en Derecho. Área de Gestión Integrada de Lugo.
- **Jaime Gulín Dávila**. Farmacéutico especialista en Farmacia Hospitalaria. Área de Gestión Integrada de Lugo.
- **Victor Herrán Carreira.** Paciente. ADIL-Asociación de Diabéticos Lucense.
- **María Jesús Lamas Díaz.** Farmacéutica especialista en Farmacia Hospitalaria. Área de Gestión Integrada de Santiago.
- **Carlos Rodríguez Moreno.** Médico especialista en Farmacología Clínica. Área de Gestión Integrada de Santiago.
- **Rafael Carlos Vidal Pérez.** Médico especialista en Cardiología. Área de Gestión Integrada de Lugo.
- **María Jesús Wandosell Picatoste.** Enfermera. Área de Gestión Integrada de Santiago.

Para que conste donde proceda, y a petición del promotor/investigador, en Santiago de Compostela, a 30 de junio de 2017.

El Secretario del Comité Territorial de Ética de la Investigación de Santiago Lugo,

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DICTAMEN DEL COMITÉ DE ÉTICA DE LA INVESTIGACIÓN DE SANTIAGO-LUGO

Ana Estany Gestal, Secretaria del Comité de Ética de la Investigación de Santiago-Lugo,

CERTIFICA:

Que este Comité evaluó en su reunión del día 25 de mayo de 2021 la modificación del estudio**:**

> **Título:** Papel de las adipokinas, de los factores neuroendocrinos y de las nuevas citokinas en la degeneración del disco intervertebral (DDIV)
> **Versión modificación**: modificación Abril 2021
> **Promotor/a**: Jesús Pino Mínguez, Oreste Gualillo
> **Investigador/a**: Jesús Pino Mínguez, Oreste Gualillo
> **Código de Registro**: 2017/279

Y que este Comité acepta de conformidad con sus procedimientos normalizados de trabajo y tomando en cuenta los requisitos éticos, metodológicos y legales exigibles a los estudios de investigación con seres humanos, sus muestras o registros, que dicha modificación sea incorporada al estudio de investigación mencionado.

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NOTA: Se le recuerda que en el caso de que en este estudio se recluten pacientes, el equipo investigador debe tener disponible el Documento de Consentimiento Informado (Hojas de Información y Hojas de Firma) tanto en *galego* como en castellano en el momento de comenzar el reclutamiento.

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Y HACE CONSTAR QUE:

1.- El Comité Territorial de Ética de la Investigación de Santiago-Lugo cumple tanto en su composición como en sus PNTs los requisitos legales vigentes.

2.- La composición actual del Comité Territorial de Ética de la Investigación de Santiago-Lugo es:

Vicepresidenta
Pilar Rodríguez Ledo. Médico especialista en Medicina Familiar y Comunitaria.

Secretaria
Ana Estany Gestal. Licenciada en Farmacia.

Vicesecretaria
María Mercedes Rodicio García. Médico especialista en Pediatría.

Vocales
Lorenzo Armenteros del Olmo. Médico especialista en Medicina Familiar y Comunitaria.
Beatriz Bernardez Ferrán. Farmacéutica especialista en Farmacia Hospitalaria
Cristina Blanco Freire. Enfermera.
Francisco Campos Pérez. Licenciado en Biología.
Jesús Fernández Álvarez. Miembro lego.
Ricardo García Martínez. Licenciado en Derecho.
Eva Marcos Doldán. Analista-programadora.
Jesús Prego Domínguez. Enfermero.
Carlos Rodríguez Moreno. Médico especialista en Farmacología Clínica.
Juan Manuel Vázquez Lago. Médico especialista en Medicina Preventiva y Salud Pública.

Para que conste donde proceda, y a petición de quien proceda, en Santiago de Compostela,
La Secretaria del Comité Territorial de Ética de la Investigación de Santiago Lugo,

Low back pain and osteoarthritis (OA) are the two main musculoskeletal diseases cause of disability worldwide, and intervertebral disc degeneration (IVDD) is considered the main cause of low back pain. A sustained inflammatory and catabolic state has been related to the origin and progression of both, OA and IVDD.

This research work introduces monomeric C reactive protein (mCRP) pro-inflammatory and catabolic effects in healthy and degenerative human intervertebral disc cells and chondrocytes, suggesting its possible role in the pathophysiology of OA and IVDD. Furthermore, this thesis describes the adipokine WISP2 relevant roles in modulating the turnover of extracellular matrix in cartilage and how its downregulation may detrimentally alter the inflammatory environment in OA joints.