UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
DEPARTAMENTO DE CIRURGIA VETERINÁRIA E REPRODUÇÃO ANIMAL

AVALIAÇÃO DOS BIOMARCADORES NO LÍQUIDO SINOVIAL DE OVINOS COM OSTEOARTRITE TRATADOS COM MEMBRANAS SINTÉTICAS E CÉLULAS-TRONCO MESENQUIMAIS

GUSTAVO DOS SANTOS ROSA

Botucatu, São Paulo
2022

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Tese apresentada junto ao Programa de Biotecnologia Animal para a obtenção do título de Doutor em Biotecnologia Animal. Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho".

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Data: 27 de outubro de 2022

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSANGELA APARECIDA LOBO-CRB 8/7500

Rosa, Gustavo dos Santos.
Avaliação dos biomarcadores no líquido sinovial de ovinos com osteoartrite tratados com membranas sintéticas e células-tronco mesenquimais / Gustavo dos Santos Rosa. - Botucatu, 2022

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina Veterinária e Zootecnia
Orientador: Ana Liz Garcia Alves
Capes: 50501003

1. Articulação. 2. Cartilagem. 3. Colágeno. 4. Matriz extracelular. 5. Polidioxanona.

Palavras-chave: Articulação; Cartilagem; Colágeno; Matriz extracelular; Polidioxanona.

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Agradecimentos

Agradeço à Profª Dra. Ana Liz Garcia Alves por me aceitar como orientado no doutorado; por compartilhar seus conhecimentos em terapias regenerativas e engenharia tecidual durante todos esses ano; pelos conselhos dados e principalmente por todo o suporte para a viabilização da conclusão do doutorado.

À minha família, por todo o suporte e amor nas maiores adversidades, apesar de nossa distância física.

Aos demais professores e funcionários do Serviço de Cirurgia de Grandes Animais da FMVZ UNESP Botucatu, por toda a ajuda.

Aos colegas de pós-graduação, em especial João Pfeifer, Fernanda Stievani, Emanuel Appolonio e Vittoria Altheman, pelo companheirismo e apoio em meio à pandemia e durante o projeto, e também pelos momentos de descontração ao longo destes anos.

À equipe do Hospital Israelita Albert Einstein, em especial aos doutores Camila Cohen, Pedro de Bieux e Moisés Cohen, pela viabilização desta pesquisa e pela colaboração.

À CAPES, CNPq e FAPESP, pela viabilização da realização de todo o doutorado. O presente trabalho foi realizado com apoio do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), processo n. 15915/2019-3 GM/GD. Processo FAPESP n. 2017/12815-0.

ROSA, G.S. Avaliação dos biomarcadores no líquido sinovial de ovinos com osteoartrite tratados com membranas sintéticas e células-tronco mesenquimais. Botucatu, 2022. Tese de Doutorado. 87 páginas. Faculdade de Medicina Veterinária e Zootecnia, campus Botucatu, Universidade Estadual Paulista “Júlio de Mesquita Filho”.

RESUMO

Devido ao baixo potencial de reparação apresentado pela cartilagem articular, as enfermidades articulares são causas frequentes de dor e diminuição de desempenho. Por seu caráter crônico, a osteoartrite representa um desafio aos tratamentos disponíveis. Assim, novas estratégias e abordagens são necessárias para aprimorar o reparo do tecido cartilaginoso. A engenharia tecidual se destaca neste sentido, valendo-se da associação entre biomateriais, células e fatores de crescimento, para promover um aporte biomecânico e celular potencialmente benéfico para a reparação condral. O objetivo do presente trabalho foi avaliar os biomarcadores articulares após tratamento da osteoartrite experimental em 32 ovinos, utilizando diferentes membranas sintéticas associadas ou não a células-tronco mesenquimais. As membranas constituídas por polidioxanona apresentaram os resultados mais relevantes, incluindo maior concentração de biomarcadores de síntese e menor concentração de biomarcadores de degradação de matriz extracelular. A comparação dos resultados nos diferentes grupos indica que a associação de diferentes princípios da engenharia tecidual, como a inclusão de uma fonte celular em um arcabouço, é a estratégia que gera melhores parâmetros nos biomarcadores do metabolismo da matriz extracelular condral

Palavras-chave: articulação; cartilagem; colágeno; matriz extracelular; polidioxanona.

ROSA, G.S. Evaluation of ovine synovial fluid biomarkers in experimental osteoarthritis treated with synthetic membranes and mesenchymal stem cells. Botucatu, 2022. PhD Thesis. 87 pages. School of Veterinary Medicine and Animal Science, Botucatu, Brazil. São Paulo State University - UNESP.

ABSTRACT

Due to the limited repair potential of articular cartilage, joint diseases are a common cause of pain and low performance. The chronic nature of osteoarthritis is a challenge for the current treatments. Thus, new strategies and approaches are required to improve cartilage repair. Tissue engineering rises as a potential treatment option, associating biomaterials, cells, and growth factors to provide biomechanical and cellular support for chondral repair. The aim of the present study was to evaluate joint biomarkers after treatment of experimental osteoarthritis in 32 sheep, using different synthetic membranes associated or not with mesenchymal stem cells. Membranes made of polydioxanone presented the most relevant results, including greater concentration of biomarkers of extracellular matrix synthesis and lower levels of biomarkers of extracellular matrix degradation. The comparison of the results in the different groups indicates that the association of different principles of tissue engineering, such as the inclusion of a cellular source in a scaffold, is the strategy that generates better parameters in the biomarkers of extracellular matrix metabolism.

Keywords: joint disease; cartilage; collagen; extracellular matrix; polydioxanone.

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CAPÍTULO 1

1. Introdução

As articulações apresentam características únicas de resistência e flexibilidade simultâneas, diferentemente de outros tecidos do organismo. Dentre os componentes das articulações, a cartilagem articular possui importantes peculiaridades. A ausência de vasos sanguíneos limita a perfusão de oxigênio e nutrientes para a cartilagem, tornando o acesso a essas moléculas mediado estritamente pelo contato com o líquido sinovial, produzido e absorvido pela membrana sinovial (SCANZELLO, 2022). Tal característica determina um ritmo metabólico reduzido, com baixa capacidade de reparação tecidual após lesões condrais (VAN WEEREN, 2016).

As lesões condrais podem decorrer de traumas físicos, estímulos químicos ou envelhecimento. O estresse biomecânico articular, aplicado de forma recorrente, pode predispor o desenvolvimento de microlesões no tecido articular, podendo gerar degradação da cartilagem articular (GOLDRING; GOLDRING, 2007).

Independente do fator promotor do desequilíbrio, a primeira estrutura articular afetada é a membrana sinovial. A inflamação tem início quando a membrana sinovial é exposta a moléculas sinalizadoras, que induzem a produção de mediadores inflamatórios que ativam o sistema imune e tornam a inflamação cíclica (ARLEEVSKAYA et al., 2019; LORENTE-SOROLLA et al., 2019), com destruição da cartilagem articular. Caso o estímulo persista, o quadro avançará para um estágio crônico de inflamação denominado osteoartrite, caracterizada pelo envolvimento não somente da cartilagem articular e da membrana sinovial, mas também das estruturas extra-articulares e do osso subcondral (MCILWRAITH, 2016). O diagnóstico das enfermidades articulares deve considerar

achados de exame clínico e complementares, como radiográficos, ultrassonográficos, tomografia computadorizada e ressonância magnética (KAWCAK, 2016). Análises moleculares revelam importantes dados sobre a articulação pela mensuração de biomarcadores capazes de indicar diferentes graus de síntese e degradação da cartilagem e a qualidade do líquido sinovial (GARNERO, 2004; AMEYE et al., 2007; PRINK et al., 2010).

A urina, o sangue e o líquido sinovial são os alvos mais comuns para a análise de biomarcadores. Destes, o líquido sinovial apresenta como vantagens a maior concentração e maior especificidade de biomarcadores locais (LAMBERT et al., 2021). Adicionalmente, a análise do líquido sinovial fornece informações sobre a real condição da articulação avaliada (MANDELL, 2022). Portanto, a mensuração de biomarcadores no líquido sinovial em situações experimentais é uma maneira objetiva de avaliar a resposta a diferentes tratamentos que visem promover a reparação da cartilagem articular.

1.1 Objetivo

O objetivo do presente trabalho foi avaliar os biomarcadores de síntese e degradação da matriz extracelular condral após tratamento da osteoartrite experimental em ovinos, utilizando diferentes membranas sintéticas associadas ou não a células-tronco mesenquimais.

2. Revisão de Literatura

A estrutura típica de uma articulação sinovial inclui tecidos que, embora tenham em comum sua origem mesodermal conjuntiva, são morfológica e funcionalmente distintos, agindo conjuntamente para garantir a movimentação entre dois ou mais ossos.

O perfeito funcionamento das estruturas articulares garante a congruência entre as extremidades ósseas, a absorção do impacto recebido, a diminuição do atrito entre as extremidades ósseas e a execução precisa dos diversos movimentos que o corpo realiza. As articulações devem ser resistentes, visto que recebem carga similar à carga exercida sobre os ossos durante o movimento. Contudo, ao mesmo tempo também precisam ser flexíveis e suaves, para absorver os impactos e garantir o menor atrito possível (VAN WEEREN, 2016).

Os tecidos responsáveis por manter o equilíbrio entre resistência e flexibilidade sinoviais são a membrana sinovial, a cápsula articular fibrosa, a cartilagem articular, o osso subcondral, a cavidade sinovial contendo líquido sinovial, e os ligamentos colaterais, os intra-articulares e os extra-articulares (JUNQUEIRA, 2018).

A cápsula articular une as extremidades ósseas, delimitando uma cavidade circunscrita denominada cavidade sinovial ou cavidade articular. Há duas camadas distintas na cápsula articular. A mais externa é a cápsula fibrosa, constituída de tecido conjuntivo fibroso muitas vezes conectado intimamente a estruturas extra-articulares como os ligamentos colaterais que, associados àquela, propiciam a sustentação da articulação (VAN WEEREN, 2016). Internamente, a cápsula articular apresenta a camada sinovial, também chamada de membrana sinovial ou sinóvia. A membrana sinovial, por sua vez, é subdividida em uma camada interna - a camada íntima - que contém 1 a 4 estratos de sinoviócitos, e uma camada externa, a sub-íntima ou lâmina

própria, composta por vasos sanguíneos que promovem o aporte de oxigênio e nutrientes, além de vasos linfáticos, terminações nervosas, tecido adiposo e tecido conjuntivo propriamente dito (BANKS, 1993; SMITH, 2011). O aspecto histológico da camada sub-íntima apresenta variações de acordo com a região, sendo classificado em três padrões: areolar, adiposo e fibroso (VEALE; FIRESTEIN, 2021). O padrão areolar possui grande quantidade de tecido conjuntivo frouxo e vasos sanguíneos, enquanto o padrão fibroso é pouco vascularizado e contém tecido conjuntivo fibroso, e o padrão adiposo é caracterizado por uma alta concentração de adipócitos (SCANZELLO, 2022).

A membrana sinovial é a estrutura responsável pela produção e absorção do líquido sinovial, cuja função primordial é lubrificar as superfícies articulares e reduzir o atrito e o estresse mecânico em sua superfície (KEENAN, 2022). O líquido sinovial é composto primariamente por ácido hialurônico, um biopolímero hidrofílico extremamente lubrificante que colabora com suas as propriedades reológicas (VAN WEEREN, 2016).

Os sinoviócitos, células presentes na membrana sinovial, produzem um transudato do plasma e secretam ácido hialurônico, lubricinas, proteases, colagenases e prostaglandinas para compor o líquido sinovial (BARTL; BARTL, 2019; FREEMONT , 1996). Além da lubrificação articular, o líquido sinovial também nutre estruturas intra-articulares como a cartilagem articular e os meniscos por difusão (FOX et al., 2009; SEIDMAN; LIMAIEM, 2022). Seu componente principal, o ácido hialurônico, possui múltiplas funções além da lubrificação da cartilagem, dentre elas a absorção de impactos, a analgesia por interagir direta ou indiretamente com nociceptores articulares, além de colaborar com a manutenção da homeostase pelo estímulo à síntese de matriz extracelular (TAMER, 2013). A lubricina, outro componente do líquido sinovial, é uma

glicoproteína semelhante à mucina, cuja função é reter uma camada protetora de água, lubrificar a articulação e prevenir a adesão de células e proteínas (JAY; WALLER, 2014).

A cartilagem presente na superfície das articulações é do tipo hialina, caracterizada pela ausência de vasos sanguíneos, linfáticos e terminações nervosas, e pela presença de células e matriz extracelular (MEC) abundante e rica em colágeno tipo II (VAN WEEREN, 2016). Em virtude de sua composição estrutural, a cartilagem hialina é capaz de facilitar a transmissão de cargas com baixo coeficiente friccional (FOX et al., 2009). Por não possuir pericôndrio, seu aporte nutricional é conferido pelo líquido sinovial (JUNQUEIRA et al., 2018).

A cartilagem hialina articular possui células especializadas - os condrócitos - envoltos por uma complexa estrutura de MEC. Os condrócitos compõem aproximadamente 5% da cartilagem articular madura, enquanto a MEC representa 95% de sua composição. Em nível estrutural, a MEC é composta primariamente por água, colágeno tipo II e glicosaminoglicanos (GAGs) associados a proteínas, formando os proteoglicanos (PGs). Em menor proporção são encontrados minerais, glicoproteínas e lipídeos (VAN WEEREN, 2016).

A síntese e a degradação da MEC são realizadas pelos condrócitos, originados a partir da diferenciação de células multipotentes da mesoderme. Durante o desenvolvimento embrionário, as células progenitoras localizadas na mesoderme deixam sua morfologia fibroblastoide e adotam um aspecto menos alongado, mantendo ainda pequenos vilos, e recebem o nome de condroblastos. Os condroblastos contêm núcleo grande e retículo endoplasmático rugoso bastante desenvolvido, necessário à síntese dos constituintes da MEC (colágeno tipo II, proteoglicanos, glicosaminoglicanos,

condronectina, entre outros) nas etapas subsequentes da condrogênese (JUNQUEIRA et al., 2018). Embora a condrogênese seja regulada por diversos fatores de sinalização, a condensação celular é uma etapa crucial para o comprometimento das células progenitoras com a linhagem condrogênica. Assim, as células se multiplicam e se aglomeram, e a adesão célula-célula e célula-matriz inicia a sua condensação em nódulos condrogênicos (SHUM; NUCKOLLS, 2001). À medida que os condroblastos secretam a MEC, estes modificam sua estrutura, adotando morfologia globoide e núcleo ovoide, com poucas mitocôndrias. Uma vez envoltos pela MEC por eles produzida, os condroblastos passam a ser denominados condrócitos (JUNQUEIRA et al., 2018).

Após o nascimento, a cartilagem articular dos mamíferos é homogênea e a rede de fibrilas colágenas não apresenta disposição organizada de maneira zonal. Entretanto, durante o crescimento e maturação a cartilagem passa por uma série de modificações estruturais (VAN TURNHOUT et al., 2010). Em indivíduos adultos, apesar de parecer uma estrutura homogênea em seu aspecto macroscópico, a cartilagem articular apresenta zonas distintas morfo e fisiologicamente, de modo a suprir a demanda fisiológica que a biomecânica da locomoção exige.

A orientação e a organização das fibrilas possuem grande importância na função mecânica da cartilagem articular. O modelo de arranjo estrutural das fibrilas de colágeno foi inicialmente proposto por Alfred Benninghoff em 1925. Este modelo propõe uma organização em arcos, denominados “arcos de Benninghoff”, nos quais as fibrilas estariam dispostas tangencialmente na camada superficial, adotando orientação oblíqua na camada imediatamente inferior, para então assumirem disposição radial em relação ao osso subcondral (BENNINGHOFF, 1925).

O modelo proposto por Benninghoff foi confirmado em diversas espécies animais, respeitando-se ligeiras diferenças entre elas (KÄÄB; GWYNN; NÖTZLI, 1998). Assim, a porção hialina da cartilagem divide-se em 3 zonas: superficial ou tangencial, zona intermediária ou de transição e zona profunda ou radial. Logo abaixo encontra-se a zona de cartilagem calcificada, considerada a transição entre a cartilagem hialina e o osso subcondral, possuindo atividade metabólica extremamente baixa (JOHNSTON, 1997; JUNQUEIRA et al., 2018).

A zona superficial da cartilagem articular apresenta alta quantidade de fibrilas de colágeno compactadas e dispostas horizontalmente (paralelas à superfície), poucos proteoglicanos e alto teor de água. Nesta zona, os condrócitos apresentam-se mais planos em relação aos condrócitos localizados nas demais zonas da cartilagem hialina. A zona intermediária é mais rica em proteoglicanos, porém apresenta menor teor de água e menor densidade de colágeno, que possui orientação menos organizada, geralmente oblíqua. Nesta zona, os condrócitos adotam morfologia mais globoide em relação aos presentes na zona superficial. Por sua vez, a zona profunda é a que possui menor quantidade de colágeno e maior concentração de proteoglicanos. Os condrócitos e as fibrilas de colágeno desta zona estão dispostas de maneira perpendicular ao osso subcondral, adotando um arranjo em colunas. A zona profunda e a zona de cartilagem calcificada são separadas por uma linha mineralizada denominada '*tidemark*', que possui reentrâncias que atingem o osso subcondral e a medula óssea, realizando a comunicação entre estes e a cartilagem hialina. As fibrilas de colágeno da cartilagem hialina transpõem a zona de cartilagem calcificada e se ancoram no osso subcondral

(VAN TURNHOUT et al., 2008, 2010; RIEPPO et al., 2011; HOEMANN et al., 2011; VAN WEEREN, 2016) (Figura 1).

Apesar de sua função comum de absorção de impacto e diminuição do atrito, há variação tanto em sua espessura quanto no arranjo das fibrilas colágenas entre as diferentes espécies, principalmente nas zonas intermediária e profunda (KÄÄB; GWYNN; NÖTZLI, 1998). O arranjo das fibrilas também pode variar em diferentes regiões na mesma articulação, de acordo com a carga à qual cada região é submetida.

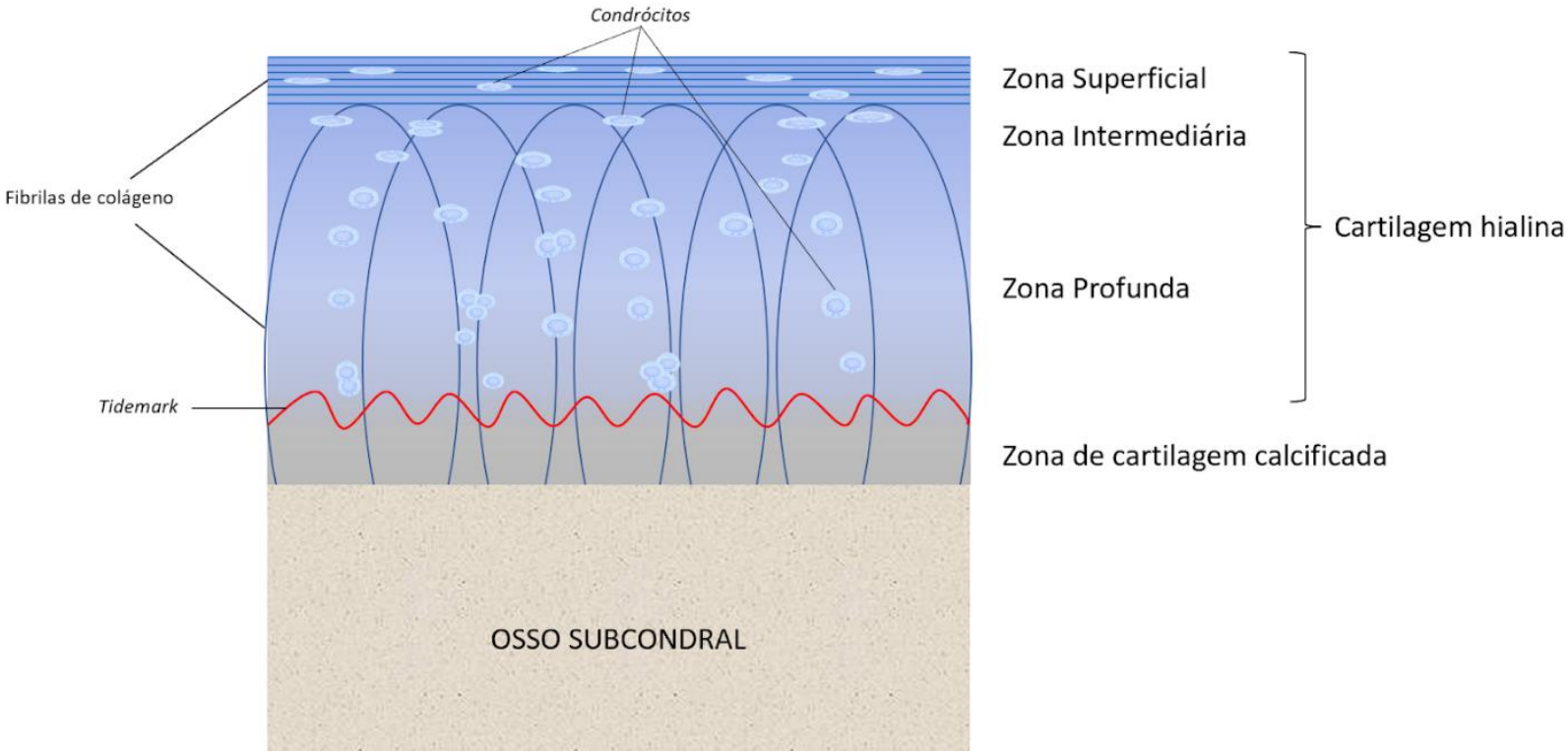

Figura 1. Organização estrutural das fibrilas colágenas, proposta por Benninghoff (1925).

A síntese e a degradação da cartilagem articular são mediadas pelos condrócitos. Diversas moléculas estimulam a síntese de MEC, como fatores de crescimento (FGF,

TGF-β, BMP), fatores de transcrição gênica (SOX5, SOX6 e SOX9), proteoglicanos (perlecan e versican), moléculas de sinalização (*indian hedgehog* e *sonic hedgehog*) e moléculas da matriz extracelular (matrilina e fibronectina) (WU et al., 2021).

O colágeno tipo II, principal colágeno da cartilagem hialina, é produzido exclusivamente pelos condrócitos. Os condrócitos produzem e secretam o colágeno em sua forma imatura - o procolágeno-, que é transformado em colágeno maduro no ambiente extracelular (VAN WEEREN, 2016). O procolágeno é formado por três cadeias de polipeptídeos (homotrímero), denominadas cadeias alfa (α). Cada cadeia é composta primariamente por glicina e prolina associadas à hidroxiprolina. Em sua terminação, cada cadeia α contém um grupamento amina ou um grupamento carboxila. As cadeias α compostas por uma terminação amina recebem o nome de pró-peptídeo N ou PIINP, enquanto as que apresentam um grupamento carboxila são denominadas pró-peptídeo C ou CP-II (figura 2). As terminações N e C são importantes para a formação da estrutura de tripla-hélice, mediada pela formação de pontes dissulfeto nessa região (GUDMANN et al., 2014). Uma vez liberado, o procolágeno tem suas terminações N e C clivadas por proteases específicas (N-protease e C-protease), levando à diminuição da solubilidade da molécula e formação do colágeno tipo II maduro. Ligações químicas adicionais criam um arranjo de fibrilas colágenas compactas e insolúveis, portanto capazes de suportar cargas mecânicas (SIEGEL, 1974; EYRE et al., 1984).

A remoção do pró-peptídeo C do procolágeno (CP-II) reflete diretamente a taxa de síntese de colágeno tipo II (NELSON et al., 1998), o que possibilita sua utilização como um biomarcador de síntese da porção colágena da MEC, uma vez que o CP-II

pode ser detectado em tecidos e fluidos corporais com a utilização de técnicas moleculares e imunoenzimáticas (LANE et al., 2019; JOHNSON et al., 2020).

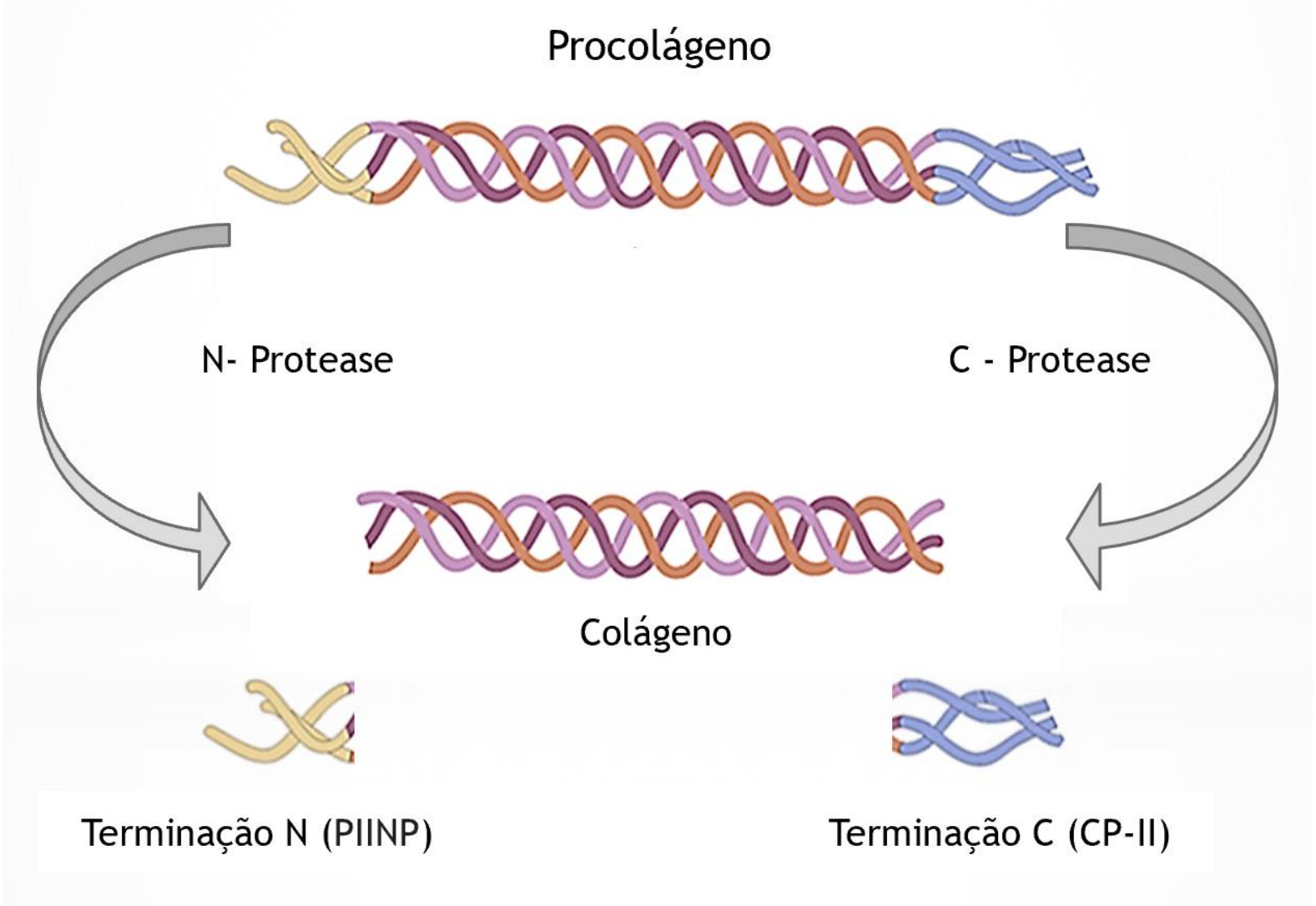

Figura 2. A molécula de procolágeno, produzida no citoplasma dos condrócitos, contém uma terminação amina (PIINP) e uma carboxila (CP-II). O procolágeno atinge o ambiente extracelular, onde é clivado pelas proteases N e C, dando origem à molécula de colágeno maduro. Adaptado de Peng et al. (2021).

A cartilagem articular também possui uma ampla gama de proteoglicanos como o biglicano, a fibromodulina, a decorina e os agrecanos, sendo os últimos os principais PGs encontrados na MEC condral (aproximadamente 90%). Os proteoglicanos são estruturas compostas por uma proteína central ligada a cadeias de glicosaminoglicanos sulfatados, como o sulfato de queratano e o sulfato de condroitina (WU et al., 2021). Juntos, os PGs e os GAGs representam aproximadamente 10% do peso seco da cartilagem (KIANI et al., 2002). Os proteoglicanos são sintetizados e secretados pelos condrócitos, a partir da sinalização por fatores de crescimento e peptídeos (FOX et al., 2011).

Estruturalmente, o agrecano contém três domínios globulares (G1, G2 e G3) e três domínios estendidos (domínio interglobular, *interglobular domain* ou IGD; sulfato de queratan, *keratan sulphate* ou KS; e sulfato de condroitina, *chondroitin sulphate* ou CS) (GARNERO, 2004). Destes, o domínio CS é o maior, contendo cerca de 100 cadeias de sulfato de condroitina (KIANI et al., 2002) (figura 3). Epítopos específicos, como o CS-3-B-3 e CS-846 possuem alta expressão durante a condrogênese da vida fetal, desaparecendo progressivamente, de modo a serem praticamente indetectáveis na cartilagem adulta. Entretanto, a expressão de CS-846 é aumentada em casos de artrite e fragmentação osteocondral (FRISBIE et al., 1999). Deste modo, a detecção dos epítopos dos agrecanos localizados em suas cadeias CS, como o CS-3-B-3, CS-7-D-4 e o CS-846 indica neogênese dos agrecanos, tornando estas moléculas importantes biomarcadores do metabolismo da MEC (BAKKER et al, 2011).

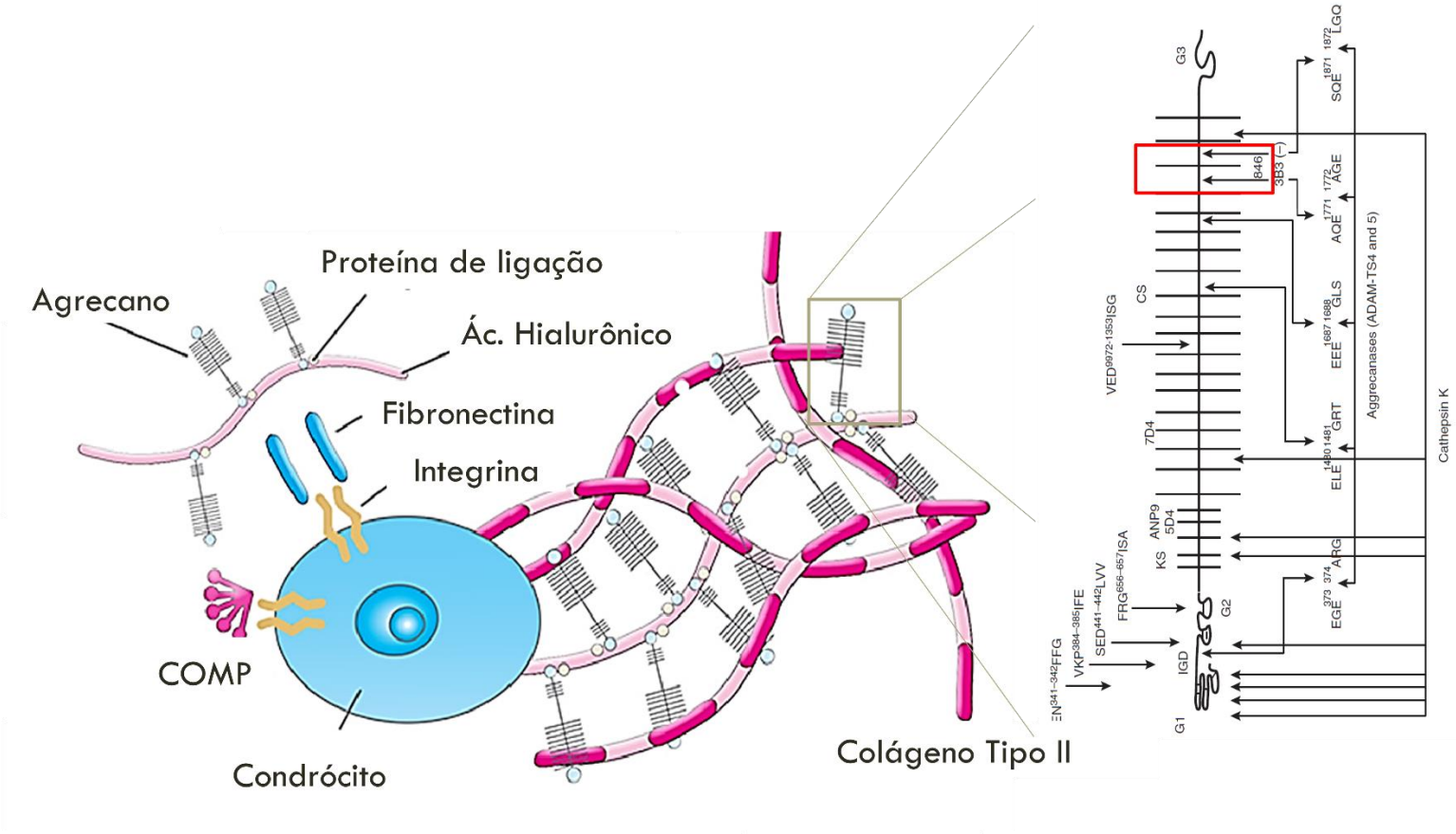

Figura 3. Estrutura do agrecano e ilustração de sua ligação com as fibrilas colágenas. O epítopo 846 da cadeia de sulfato de condroitina do agrecano (CS-846) encontra-se destacado em vermelho no quadrante superior direito da imagem. Adaptado de GARNERO (2004) e WU et al. (2021).

Em uma articulação hígida, os agrecanos se ligam a moléculas de ácido hialurônico, formando agregados de proteoglicanos que ocupam o espaço interfibrilar, ou seja, o espaço entre as fibras de colágeno tipo II (MÖRGELIN et al., 1994; HAN et al., 2011), fixando-se a elas por intermédio de proteínas ligadoras de colágeno, como a proteína da matriz oligomérica da cartilagem (*cartilage oligomeric matrix protein* ou COMP) (GARCÍA-CARVAJAL et al., 2013). O ácido hialurônico, além de constituinte fundamental do líquido sinovial, também possui um papel estrutural importante na matrix

extracelular da cartilagem hialina, e sua detecção por ensaios moleculares fornece importantes informações sobre o estado da cartilagem articular e do líquido sinovial. A COMP também é uma proteína presente na MEC, embora também seja encontrada nos tendões, ligamentos, músculo liso, membrana sinovial e enfermidades fibróticas (MÜLLER et al., 1998; RIESSEN et al., 2001; WYNN, 2008). Na cartilagem, sua função é catalisar a fibrilogênese, uma vez que as interações entre esta molécula e o colágeno tipo IX são essenciais para a síntese e a manutenção adequadas da MEC (GARCÍA-CARVAJAL et al., 2013), tornando-a um potencial biomarcador de progressão da osteoartrite (ZUO et al., 2015).

Os demais proteoglicanos, como o perlecan, o biglicano e a decorina, possuem alta similaridade estrutural protéica, mas diferem na composição de glicosaminoglicanos e na sua função. Enquanto a decorina e a fibromodulina interagem com as fibrilas de colágeno tipo II, compondo a malha da MEC, o biglicano é mais comumente encontrado nas imediações dos condrócitos, auxiliando em sua comunicação com o colágeno tipo VI e com a MEC adjacente (FOX et al., 2009).

A conexão entre todas estas moléculas cria uma complexa estrutura tridimensional de matriz extracelular extremamente organizada, capaz de manter as características de rigidez e elasticidade da MEC para promover simultaneamente a absorção de impacto e a resistência estrutural à aplicação de cargas compressivas. Isto se deve, por um lado, à organização estrutural das fibrilas de colágeno tipo II, que fornece o arcabouço de contenção que mantém a resistência da cartilagem durante a recepção de impactos (HAN et al., 2011). Em contrapartida, a carga negativa dos PGs produz alta

pressão osmótica na MEC, sendo responsável pela atração de moléculas de água, o componente mais abundante da cartilagem (que corresponde a até 80% de seu peso).

A capacidade de retenção de água é o que assegura não só a hidratação da MEC e dos condrócitos, mas também o pleno desempenho das propriedades mecânicas de pressurização de fluidos e a capacidade de absorção de cargas recorrentemente, reduzindo o estresse sobre o componente sólido da MEC (GUILAK et al., 2018). Em conjunto, os componentes da matriz extracelular sustentam e nutrem os condrócitos, formando um nicho para o metabolismo da cartilagem articular através do envio de sinais anabólicos e catabólicos (PENG et al., 2021).

Ainda que em ritmo metabólico reduzido, os eventos de catabolismo e anabolismo da cartilagem articular ocorrem simultânea e ininterruptamente em um ambiente de homeostase articular. A cartilagem articular sofre remodelamento constante, em uma combinação de produção de MEC, degradação de MEC mediada por proteases específicas, e inibição de sua atividade, realizada por inibidores teciduais de MMPs (TAKAHASHI et al., 2005). Deste modo, apesar da taxa metabólica reduzida observada fisiologicamente, a cartilagem articular apresenta um elevado potencial metabólico cuja ativação é observada em condições não-fisiológicas, como ocorre durante o desenvolvimento da osteoartrite (TCHETINA, 2011).

Duas famílias de proteases estão envolvidas no catabolismo da MEC, as metaloproteinases de matriz e as agrecanases. As metaloproteinases de matriz (MMP) são enzimas dependentes de zinco que regulam a degradação de MEC através da clivagem de ligações peptídicas específicas. De acordo com sua estrutura e seu substrato, as MMPs podem ser divididas em diversos grupos, como as

colagenases (MMP-1, MMP-13), gelatinases (MMP-2, MMP-9), estromelisinas (MMP-3), metaloelastases (MMP-12), matrilisinas (MMP-7) e metaloproteinases de matriz associadas à membrana (MT-MMPs).

O colágeno é clivado pelas MMP-1, MMP-8 e MMP-13, sendo a última a mais eficiente na clivagem do colágeno tipo II (VAN WEEREN, 2016). A clivagem ocorre no *sítio* ¾ do colágeno (figura 4), liberando dois produtos de clivagem do colágeno: o fragmento de tamanho ¾ e o fragmento de tamanho ¼ (DAHLBERG et al., 2000). A degradação do fragmento 3⁄4 do colágeno dá origem ao neoepítopo C2C, oriundo da primeira clivagem da tripla hélice do colágeno tipo II (AMEYE et al., 2007) (figura). Assim, os níveis de C2C podem ser utilizados como biomarcadores de degradação do componente colagênico da MEC (PRINK et al., 2010).

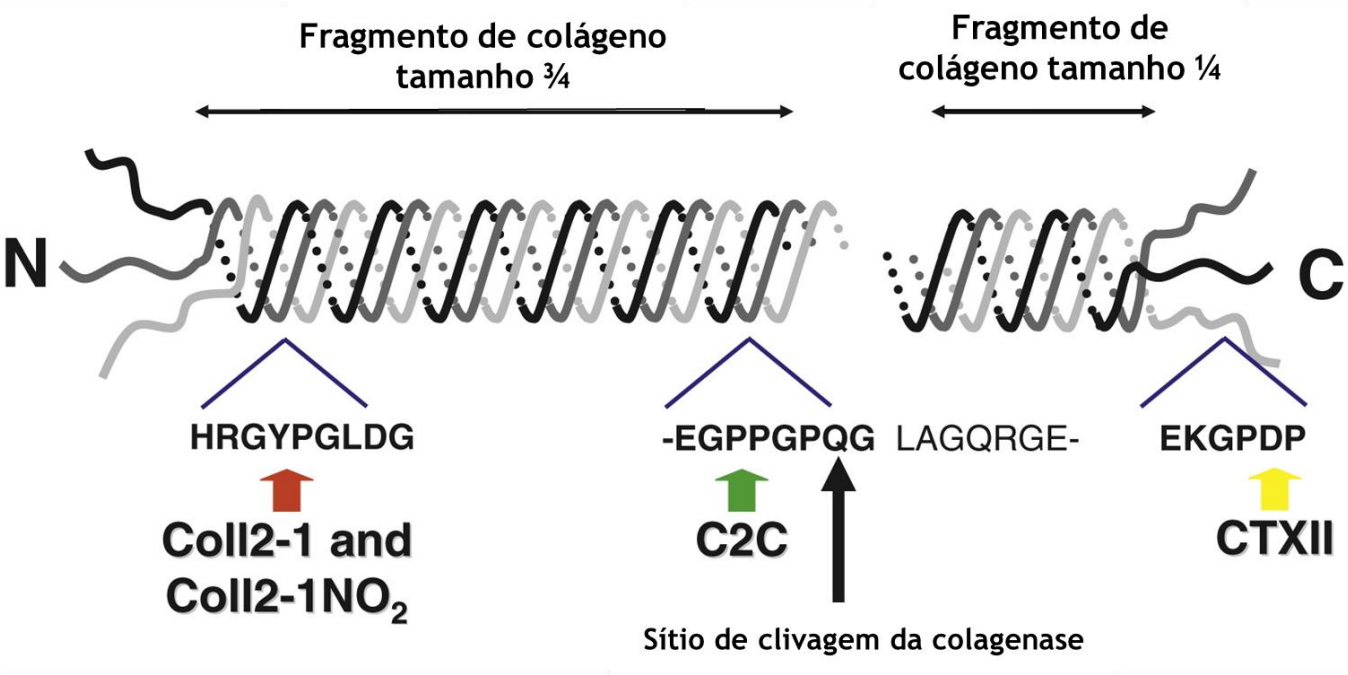

Figura 4. Clivagem do colágeno maduro. A clivagem ocorre no sítio ¾, dando origem aos fragmentos de colágeno ¾ e ¼ de colágeno. O C2C é um neoepítopo componente do fragmento de colágeno ¾. Adaptado de Huebner et al. (2009).

Os agrecanos, por sua vez, são clivados em sua proteína central pela MMP-3 (também chamada estromelisina) e pelas gelatinases MMP-2 e MMP-9 (CLEGG et al., 1997; FOX et al., 2009). A MMP-2 também é capaz de clivar o colágeno tipo II, enquanto a MMP-9 cliva os colágenos tipo I e III (PATTERSON et al., 2001). A porção dos proteoglicanos constituída por glicosaminoglicanos, por sua vez, é clivada por outra classe de enzimas, denominadas agrecanases ou ADAMTS (do inglês '*a disintegrin and metalloproteinase with thrombospondin motifs'*) (TORTORELLA et al., 1999; BERTRAND et al., 2010; VAN WEEREN, 2016). As ADAMTS envolvidas na clivagem do agrecano são as ADAMTS-1, ADAMTS-4 e ADAMTS-5 (SCHNELLMANN, 2022). Embora outras ADAMTS sejam capazes de clivar demais componentes da MEC, os componentes formados por colágeno, a fibronectina e a trombospondina não são clivados pelas ADAMTS-1, ADAMTS-4 ou ADAMTS-5 (TORTORELLA et al., 2002; SCHNELLMANN, 2022).

Para que a homeostase articular seja mantida, as células precisam dispor de um mecanismo de inibição da degradação da MEC. As metaloproteinases são inibidas por moléculas como a α2-macroglobulina e os TIMPs (inibidores teciduais de metaloproteinases ou *tissue inhibitors of metalloproteinases*), especialmente o TIMP-1, TIMP-2 e TIMP-3 (SCHNELLMANN, 2022). As ADAMTS também são inibidas pela α2-macroglobulina e pelos TIMPS, especialmente o TIMP-3 (KASHIWAGI et al., 2001, SCHNELLMANN, 2022). Os TIMPs são encontrados em diversos tecidos conjuntivos e são considerados os principais inibidores do catabolismo da MEC, e a homeostase

articular é resultado do equilíbrio entre a concentração de TIMPs, ADAMTs e MMPs (MCILWRAITH, 2016).

Entretanto, a atividade metabólica dos condrócitos pode ser alterada por diversos fatores, sejam eles mecânicos ou químicos. Por exemplo, a simples movimentação regular da articulação é extremamente importante para a manutenção da estrutura da cartilagem articular, visto que a inatividade da articulação pode alterar o metabolismo da cartilagem, levando à sua degradação (BUCKWALTER; MANKIN, 1998). O estresse biomecânico e os sinais inflamatórios também são capazes de alterar o metabolismo da cartilagem articular. Assim, quaisquer estímulos não-fisiológicos sofridos pela articulação podem culminar em desequilíbrio entre catabolismo e anabolismo da cartilagem, o que inicia o processo inflamatório. O estresse biomecânico, por menor que seja, pode levar à inflamação de tecidos moles articulares, ocasionando a liberação de mediadores inflamatórios, o que gera desequilíbrio na homeostase articular. Por exemplo, sinoviócitos e condrócitos produzem MMP-3 na presença de interleucina-1 (MAY et al. 1992). Assim, o colágeno, os proteoglicanos e os glicosaminoglicanos da MEC são degradados pelas MMPs e ADAMTs, e seus produtos de degradação são liberados no líquido sinovial, dando continuidade ao processo inflamatório (HARDINGHAM et al., 1992).

Após a destruição da complexa rede de componentes da MEC, a capacidade de retenção de água é prejudicada devido à perda dos proteoglicanos, levando à diminuição da capacidade de resistência biomecânica da cartilagem (GRIMMER et al., 2006).

Devido à natureza avascular da cartilagem e ao seu ritmo metabólico reduzido, as lesões condrais tendem a permanecer ativas ao longo de anos, podendo levar à

degeneração articular adicional devido à constante imposição de cargas. Salienta-se que, embora muitas vezes fisiológicas, as cargas impostas sobre uma cartilagem que não possui a resistência e as propriedades biomecânicas de uma cartilagem hígida podem levar ao catabolismo articular (WU et al., 2010).

Diversas terapias celulares são utilizadas com o intuito de impedir o desenvolvimento e a progressão das enfermidades articulares. A terapia medicamentosa inclui fármacos anti-inflamatórios não-esteroidais (AINEs) e esteroidais (AIEs), glicosaminoglicanos polissulfatados (PSGAGs), ácido hialurônico (AH), N-acetilglicosamina (NAG, um componente do ácido hialurônico), ou ainda uma combinação entre alguns destes princípios (FRISBIE et al., 2009a; FRISBIE et al., 2009b; KEENAN, 2022). Terapias utilizando derivados biológicos, como células-tronco mesenquimais (CTMs), plasma rico em plaquetas (PRP) e soro autólogo condicionado (SAC/ IRAP®), também possuem utilização frequente por aumentarem a concentração de citocinas anti-inflamatórias e fatores de crescimento (HRAHA et al., 2011).

As CTMs são utilizadas para auxiliar o limitado potencial de reparação cartilaginosa, reduzir a formação de fibrocartilagem e bloquear o ciclo inflamatório articular, diminuindo a sinovite e consequentemente atuando como adjuvante no tratamento da OA (CAPLAN, 2007). As interações célula-célula e célula-ambiente possuem elevada importância neste contexto. Estes eventos possibilitam a ação parácrina das CTMs, que consiste na síntese e liberação de diversos peptídeos capazes de modificar o ambiente inflamatório, direcionando o processo de reparação articular para a resolução. As CTMs podem, ainda, colaborar diretamente com o restabelecimento da estrutura histológica articular devido à sua capacidade de diferenciação em diversos

tipos celulares especializados. Por fim, a presença de CTMs na articulação leva ao recrutamento quimiotático de CTMs endógenas contidas nos tecidos adjacentes que, uma vez presentes no ambiente, atuam de maneira sinérgica na reparação tecidual.

Nos casos mais avançados, onde a OA já se encontra estabelecida, o objetivo da utilização de CTMs é bloquear o ciclo inflamatório e melhorar a qualidade do tecido de reparação articular. De forma geral, estudos apontam melhora do tecido de reparação após administração intra-articular de CTMs livres (GE et al., 2021; YANG et al., 2021; RUSSO et al., 2022). Um fato importante que ocorre após a administração intra-articular é a dispersão das CTMs para a membrana sinovial, cartilagem articular e tecidos periarticulares (AGUNG et al., 2006; SANTOS et al., 2019). Assim, casos com comprometimento geral da articulação, como na OA acompanhada por sinovite ativa, onde a ação das CTMs é desejável em todos os componentes intra-articulares, podem se beneficiar da utilização de CTMs de maneira livre. Em contrapartida, a dispersão celular pode prejudicar a ação das CTMs nos casos em que a concentração destas no foco da lesão é desejada (SANTOS et al., 2019).

Neste sentido, o campo da terapia celular avançada e da engenharia tecidual também apresenta avanços notáveis nas possibilidades terapêuticas de enfermidades articulares mais avançadas. Terapias gênicas, especialmente o desenvolvimento de terapias que tenham como alvo microRNAs envolvidos na síntese e degradação de colágeno, são objetos de estudos frequentes e são referidas como potenciais ferramentas no tratamento da OA. Alguns exemplos citados são o microRNA-140, envolvido na proliferação e diferenciação de condrócitos e na diminuição da atividade da MMP13; o microRNA-27b, que diminui a expressão de MMP13 nos condrócitos; o

microRNA-146a, que diminui a expressão de MMP-13 em sinoviócitos e condrócitos, além de reduzir a ativação da via TLR-4 (LI; WU, 2021). A engenharia tecidual também é bastante empregada no tratamento de lesões condrais. Este é um ramo da terapia celular bastante promissor, que visa beneficiar o paciente através da ação sinérgica da combinação de uma fonte celular, um arcabouço e biocomponentes como fatores de crescimento. A associação de uma fonte celular com um arcabouço torna-se especialmente interessante nos casos de erosão da cartilagem articular, pois o fenótipo condrocítico de células pré-diferenciadas e diferenciadas é mantido e a condrogênese de células progenitoras é favorecida por sua inserção em um ambiente tridimensional, uma vez que os condrócitos naturalmente encontram-se imersos em um ambiente tridimensional complexo (YAMAGATA, NAKAYAMADA, TANAKA, 2018; SOUZA; ROSA et al., 2022)

Uma extensa variedade de arcabouços encontra-se disponível atualmente, cada um com suas vantagens e desvantagens. Estes são frequentemente utilizados em técnicas de impressão tridimensional com bioimpressoras, mas outras técnicas de polimerização podem ser utilizadas. Como exemplos podemos citar desde hidrogéis (compostos de polímeros hidrofílicos) de ácido hialurônico, alginato sódico, quitosana, PLGA (ácido lático-co-ácido glicólico), passando por polímeros termoplásticos, como a policaprolactona, até a própria cartilagem hialina descelularizada e arcabouços de colágeno (ARMIENTO et al., 2018; NIE et al., 2020; SOUZA; ROSA et at., 2021). Como cada arcabouço possui propriedades físico-químicas e biomecânicas distintas, a produção de um arcabouço híbrido, que combine diferentes materiais e suas propriedades, pode ser realizada para melhorar as características do produto final.

Por fim, os fatores de crescimento são proteínas naturalmente secretadas por células, que interagem com as células presentes no ambiente, estimulando a multiplicação e a diferenciação celular (WERNER; GROSE, 2003). Além destas características, a adição de fatores de crescimento também previne a hipertrofia dos condrócitos, a desdiferenciação e a transdiferenciação das células condrogênicas (TAN; HUNG, 2017). Os fatores de crescimento podem ser oriundos da própria fonte celular, quando estes são produzidos pelas células-tronco, ou podem provir de fontes externas, como a adição de fatores de crescimento isolados ou associados. As classes de fatores de crescimento mais utilizadas fazem parte dos TGF-βs, das BMPs (*bone morphogenetic proteins* ou proteínas morfogenéticas ósseas), dos FGFs (*fibroblast growth-factors* ou fatores de crescimento derivados de fibroblastos) e dos IGFs (*insulin-like growth-factors* ou fatores de crescimento semelhantes à insulina) (AUGUSTYNIAK et al., 2015). O plasma rico em plaquetas (PRP) e o concentrado plaquetário (CP) contêm uma ampla gama de fatores de crescimento em diferentes concentrações, estabelecendo uma alternativa que também gera resultados igualmente satisfatórios (XIE et al., 2012; ANITUA et al., 2021; ZHU et al., 2022).

O desenvolvimento da engenharia tecidual possibilitou notáveis avanços no campo da reparação articular, especialmente em enfermidades com extensa destruição da cartilagem articular. Em essência, a combinação de fatores de crescimento associados a um arcabouço é potencialmente capaz de direcionar a maquinaria celular, suficientemente complexa e especializada, a vias pró-resolutivas que favorecem a melhor qualidade final do tecido de reparação articular.

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CAPÍTULO 2

Artigo científico a ser submetido para o periódico **Frontiers in Bioengineering and Biotechnology**.

As normas para a publicação estão dispostas no ANEXO I, localizado após as referências bibliográficas ao final deste capítulo, e podem ser encontradas no endereço eletrônico:

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Title:

POLYDIOXANONE-BASED SCAFFOLDS PROVIDE BETTER RESULTS OF ARTICULAR METABOLISM BIOMARKERS IN AN OVINE *IN VIVO* OSTEOARTHRITIS MODEL

ABSTRACT

Due to the limited repair potential of articular cartilage, joint diseases tend to become chronic and cause pain and decrease in athletic performance. The chronic nature of osteoarthritis makes it a difficult injury to treat. In this sense, new strategies and approaches are highly required to improve cartilage repair. Tissue engineering rises as a potential treatment option by associating biomaterials, cells, and growth factors to provide biomechanical and cellular support for chondral repair. The aim of the present study was to evaluate joint biomarkers after treatment of experimental osteoarthritis in 32 ewes, using different synthetic membranes associated or no with mesenchymal stem cells. Membranes made of polydioxanone presented the most relevant results, including higher concentration in biomarkers of type-II collagen synthesis and lower levels of biomarkers of extracellular matrix catabolism. Polydioxanone properties associated with the paracrine effect of MSCs stimulated mechanisms of chondral repair, rising as a potentially new approach for the treatment of osteoarthritis. The association of different principles of tissue engineering, such as the inclusion of a cellular source in a scaffold, is the strategy that generates better parameters in the biomarkers of extracellular matrix metabolism.

Keywords: cartilage; extracellular matrix; joint disease; mesenchymal stem cells; microfracture

1. Introduction

The hyaline cartilage found in the joint surfaces mainly is composed by chondrocytes and extracellular matrix (ECM) made of type II collagen, proteoglycans, and water (van Weeren, 2016). The special organization of ECM elements is responsible for the unique mechanical properties of articular cartilage, in a combination of flexibility and stiffness (FOX et al., 2009). Once damaged, articular cartilage has a limited capacity for self-renewal, mostly due to the absence of blood supply in its structure (McIlwraith, 2016). Long term joint injuries usually lead to osteoarthritis, a common pathway that represents the final stage of unbalanced joint metabolism (Lambert et al., 2021).

Different strategies have been employed intending to promote articular repair (McIlwraith, 2016). However, research often present widely different results among different techniques (Tengove et al., 2022), which brings scientific interest to this field. Tissue engineering is a promising approach in current osteoarthritis research, especially with the use of cartilage-like constructs made of a cell source integrated with tridimensional scaffolds (Zhou et al., 2019). Several scaffolds and cell sources are available commercially, that can be used either alone or in combination with different cell sources (Statham et al., 2022). Commercially available scaffolds are made of different biomaterials, including types I and III collagen, polydioxanone alone or in combination with other materials, making their use available in several chondral repair techniques.

Cartilage metabolism biomarkers are common targets to determine cartilage condition. Although biomarkers can be assessed in body fluids such as serum, urine, and synovial fluid, the last presents the higher concentration and specificity of local

biomarkers (Lambert et al., 2021), providing estimative about the actual articular metabolism (Mandell, 2022). Type-II collagen synthesis can be evaluated by the detection of procollagen carboxy propeptide (CP-II), whereas its degradation can be estimated by the measurement of epitopes of collagen cleavage, such as C2C. The synthesis of proteoglycan can be estimated by the presence of glycosaminoglycan epitopes such as CS-846.

Thus, synovial evaluation of chondral biomarkers in experimental conditions is an objective analysis, providing an estimative of the articular condition and being helpful in the evaluation of the treatment response. The aim of this study was to compare synovial biomarkers of cartilage metabolism (Hyaluronan, CP-II, C2C and CS846) in an induced osteoarthritis model treated with different commercial membranes, with or without mesenchymal stem cells and subchondral bone microfracture.

2. Material and Methods

This study was conducted in accordance with the principles of ethics and well-being in animal experimentation and was approved by the Ethics Committee on Animal Use (CEUA) of the School of Veterinary Medicine and Animal Science of UNESP, Brazil, in consonance with the international conventions of animal welfare (protocol n. 0068/2021).

2.1 Animals

Thirty-two Ile de France ewes were used in this study, with age ranging between 1 and 2 years and weight between 45 and 60 kg. Physical exams, complete blood count, lameness scores and radiographic examination of both femorotibial joints were performed in all animals. Individuals presenting any signs of systemic or articular abnormalities were excluded from this study. Animals were divided in eight groups, treated according to each tested therapy.

2.2 Mesenchymal stem cells

Subcutaneous adipose tissue was obtained from the supra-gluteal region of one donor that was not used in the experiment. The adipose tissue was maintained in PBS (Thermo-Fisher Scientific, Grand Island, NY, USA). Sample processing was performed according to the technique described by Carvalho et al. (2009). Briefly, sample was washed in PBS several times to remove blood excess, dried in sterile absorbent paper and fragmented with a scalpel. Tissue dissociation was made using 2 mg/ml of type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) diluted in DMEM F12 Glutamax™ (DMEM, Invitrogen, Grand Island, New York, USA). Each gram of adipose tissue was dissociated with 2 ml of the collagenase solution (for example: 1g of adipose tissue received 2 ml of DMEM containing 4 mg of type I collagenase). The content was homogenized and transferred to an incubator for 3 h at 37ºC and 5% of CO_2.

Collagenase was inactivated with DMEM F12 Glutamax™ supplemented with 10% fetal bovine serum (FBS - Thermo Fisher Scientific, Grand Island, New York, USA) and

the sample was centrifuged for 10 minutes at 760 x g. After disposing the supernatant, the sample was washed and centrifuged once again, and the pellet was diluted in 2 ml of culture medium (DMEM F12 Glutamax + 10% FBS + 1% penicillin, streptomycin and amphotericin B solution (Antibiotic-Antimycotic, Thermo Fisher Scientific, Grand Island, New York, USA) and seeded in 25 cm^2 culture bottles, changing the culture medium every 48h. When confluence reached 80% or more, cell adhesions were disrupted with 0.25% EDTA trypsin for 3 minutes. Trypsin was inactivated with culture medium. The bottle content was centrifuged and the pellet was resuspended in 2 ml of culture medium and seeded in 75cm^2 culture bottles (first passage or P1) and 175cm^2 bottles (second passage or P2 and third passage or P3). Cell viability was assessed after trypsinization of cultured cells by trypan blue exclusion method using an automatic counter (Countess II FL Automated Cell Counter™, Thermo-Fisher Scientific, Grand Island, NY, USA).

Cell characterization was performed by morphologic evaluation and trilineage (chondrogenic, osteogenic and adipogenic) differentiation assays. For adipogenic and osteogenic differentiation, cell cultures received medium StemPro™ Adipogenesis Differentiation Kit (Gibco, Grand Island, NY, USA), whereas osteogenic differentiation was performed using StemPro™ Osteogenesis Differentiation Kit (Gibco, Grand Island, NY, USA). Culture medium was changed every 48 h for 14 days. Cells were fixed in formaldehyde and the osteogenic/control groups were stained with Alizarin Red, whereas the adipogenic/control groups were stained with Oil Red O. Chondrogenic differentiation assay was made in biomass culture in a 15ml conical centrifuge tube using StemPro™ Chondrogenesis Differentiation Kit (Gibco, Grand Island, NY, USA) for 21 days. Culture

was fixed and submitted to histopathologic evaluation using Toluidine Blue and Alcian Blue staining.

Immunophenotyping by flow cytometry was not feasible due to the lack of antibody cross-reactivity of the available equine antibodies with the ovine species. Thus, gene expression of PCR for OCT-4, SOX-2, NANOG, CD73, CD90, CD105, CD34, CD45 and MHC class-II is set to be performed as a next step in this study, following techniques validated by previous authors (table 1).

Table 1. PCR sequencing primers for MSCs immunophenotyping.

N.	Gene	Primer sequence F: 5' – 3' R: 3'-5'	Access n.	Amplicon lenght	Ref
1	GAPDH	F – CTTCATTGACCTTCACTACATGG R – TGCAGGAGGCATTGCTGACAA	NM_001190390.1	365	Zhao et al., 2020
2	OCT-4	F - GATCGGGCCGGGGGTTGTGC R –TCGGCTCCAGCTTCTCCTTGTCCA	XM_004018968.1	235	Zhao et al., 2020
3	SOX-2	F- CATGAACGGCTCGCCCACCTACAG R -TCTCCCCCGCCCCCTCCAGTTCAC	XM_004003838.1	267	Zhao et al., 2020
4	NANOG	F – TTCCTTCCTCCATGGATCTG R - ACCACTGGTTGCTCCAAGAC	FJ970651.1	315	Zhao et al., 2020
5	$CD73^{+}$	F - TGGTCCAGGCCTATGCTTTTG R – GGGATGCTGCTGTTGAGAAGAA	BC114093	115	Lyahyai et al., 2012
6	$CD\ 90^{+}$	F - CAGAATACAGCTCCCGAACCAA R – CACGTGTAGATCCCCTCATCCTT	BC104530	96	Lyahyai et al., 2012
7	$CD105^{+}$	F - CGGACAGTGACCGTGAAGTTG R -TGTTGTGGTTGGCCTCGATTA	NM_001076397	115	Lyahyai et al., 2012
8	$CD34^{-}$	F – TGGGCATCGAGGACATCTCT R - GATCAAGATGGCCAGCAGGAT	AB021662	107	Lyahyai et al., 2012
9	$CD45^{-}$	F – CCTGGACACCACCTCAAAGCT R-TCCGTCCTGGGTTTTATCCTG	NM_001206523	101	Lyahyai et al., 2012
10	MHC-II HLA DRA	Primer sequence non available	qHsaCED 0037296	119	Bio-Rad Laboratories

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2.3 *Study design*

Thirty-two ewes were submitted to surgical induction of unilateral femorotibial osteoarthritis. Animas were divides in eight groups and treated with microfracture or three different synthetic membranes associated or no with mesenchymal stem cells. Synovial fluid was collected before and 6 months after the induction. Samples were submitted to ELISA to determine synovial concentration of cartilage biomarkers CP-II, C2C, CS-846 and Hyaluronan.

2.4 *Surgical induction of osteoarthritis and treatments*

Ewes were submitted to a 24h fasting before the surgical procedures. Sedation was performed with intramuscular injection of 2% xylazine hydrochloride (0.3 mg/kg bw) and intravenous general anesthesia was induced with diazepam 0.5% (0.5 mg/kg bw) and 10% ketamine (5 mg/kg bw). Animals were intubated and maintained under inhalation anesthesia using isoflurane in 100% oxygen.

Both femorotibial joints of all animals were clipped and submitted to surgical antisepsis. Samples of approximately 1 mL of synovial fluid were obtained immediately before the surgery, being used for the measurement of the biomarkers at the initial time point (group named “basal”).

Surgical access of the femorotibial joint was performed through arthrotomy on the dorsomedial aspect of the joint, following the previously described technique (Schinhan et al., 2012). In brief, a 5 cm skin incision was made to access the parapatellar aspect of joint capsule and exposition of the medial femoral condyle. After positioning the joint in semiflexion to expose articular surface, a 10 mm diameter chondral lesion was performed on the loading area using a Keyes cutaneous punch. The cartilage was debrided with a curette until only the calcified cartilage layer over the bone was left, without reaching or exposing the vascularized subchondral bone.

Tested membranes were ChondroGide™ (Geistlich Pharma AG, Switzerland), PlenunGide V1™ and PlenunGide V2™ (Plenum Biotechnology, São Paulo, Brazil). Besides the initial time point synovial fluid collection, considered as the “Basal” group, animals were randomly divided in 8 groups according to each treatment, as it follows:

Basal: samples obtained before the OA induction
+

Control: Chondral lesion

MF: Chondral lesion + Microfracture

CGide: Chondral lesion + Microfracture + ChondroGide™ membrane

V1: Chondral lesion + Microfracture + PlenumGide™ V1 membrane

V1MSC: Chondral lesion + Microfracture + PlenumGide™ V1 membrane + MSCs

V2: Chondral lesion + Microfracture + PlenumGide™ V2 membrane

V2MSC: Chondral lesion + Microfracture + PlenumGide™ V2 membrane + MSCs

CGideMSC: Chondral lesion + ChondroGide™ + MSCs

All treatments were implemented immediately after creating the lesion. Control group was submitted to chondral lesion without treatment. Groups MF, CGide, V1 and V2 were all treated with microfractures, made by subchondral bone perforation in 5 distinct points within the chondral lesion. While MF group received only microfractures, chondral defects of CGide group were covered with biological membrane Chondro-Gide™ (Geistlich Pharma AG, Switzerland). Group V1 was treated with PlenumGide V1™ membrane (Plenum Biotecnologia, São Paulo, Brazil), whereas animals in the V2 group received PlenumGide V2™ membrane (Plenum Biotecnologia, São Paulo, Brazil). Animals from the groups V1MSC, V2MSC and CGideMSC received Plenum Gide V1™, Plenum Gide V2™ and Chondro-Gide™, respectively, all of them seeded with $3X10^6$ mesenchymal stem cells for 24 h prior to the surgical procedure, to ensure proper cell adaptation to the tridimensional structure of the scaffolds. Membrane seeding was performed according to previously described methodology (Saska et al., 2021). All membranes were held on the lesion using Fibrin Sealant Tisseel ™ (Tisseel, Baxter Hospitalar, São Paulo, Brazil). Closure of the joint capsule was performed using 2-0 PDS-II (Ethicon, United States of America), followed by closure of subcutaneous and skin.

Post-operative protocol included Ceftiofur (4 mg/kg bw) for 5 days and Meloxicam (0.5 mg/kg bw) for 7 days.

2.5 Sample collection and molecular analyses

Synovial fluid was collected via arthrocentesis immediately before the surgery (group named “before surgery”) and after 6 months. Samples were centrifuged at 720 x g for 10 minutes at 4˚C, aliquoted in 1.5 ml polypropylene tubes and stored at -80˚C. Synovial levels of biomarkers CP-II, C2C, CS846 (IBEX Pharmaceuticals, Inc. Quebéc, CA) and Hyaluronan (R&D Systems, Minneapolis, MN, USA) were measured by multispecies enzyme-linked immunosorbent assay (ELISA) at initial time point (considered as initial control) and after 6 months (at this time point each group was evaluated individually and compared to the initial control), following the manufacturers’ instructions. Optic densities were read at 450 nm wavelength and the regression curve was performed and evaluated by the software Instat GraphPad™.

2.6 Statistical Analysis

Normality test was performed and parametric data were submitted to ANOVA and pairwise multiple comparisons using Tukey Test, whereas Kruskal-Wallis Test and Duncan’s Method were applied to nonparametric data. Differences were considered statistically significant when P-value was <0.05. The software used for all analyses was Sigmastat 3.5 (Systat Software, Inc., Point Richmond, CA, USA).

3. Results

3.1 Culture and characterization of MSCs

Enzymatic tissue dissociation was effective to recover adipose tissue stem cell population. Cultured cells presented capability to adhere to the plastic bottle, as well as fibroblastic morphology and clonal expansion potential. Cells presented mean viability of 93% after trypsinization. Differentiation assays revealed capability of chondrogenesis, adipogenesis and osteogenesis, confirmed by the specific staining of the cultures.

3.2 Synovial concentration of hyaluronan, C2C, CS846 and CP-II

Hyaluronan levels presented statistical differences between groups. Concentration of hyaluronan in V2 group was higher than the initial time point (P=0.022). There was also significantly higher hyaluronan concentration of V2 group synovial fluid compared to V1MSC (P=0.042) (figure 1).

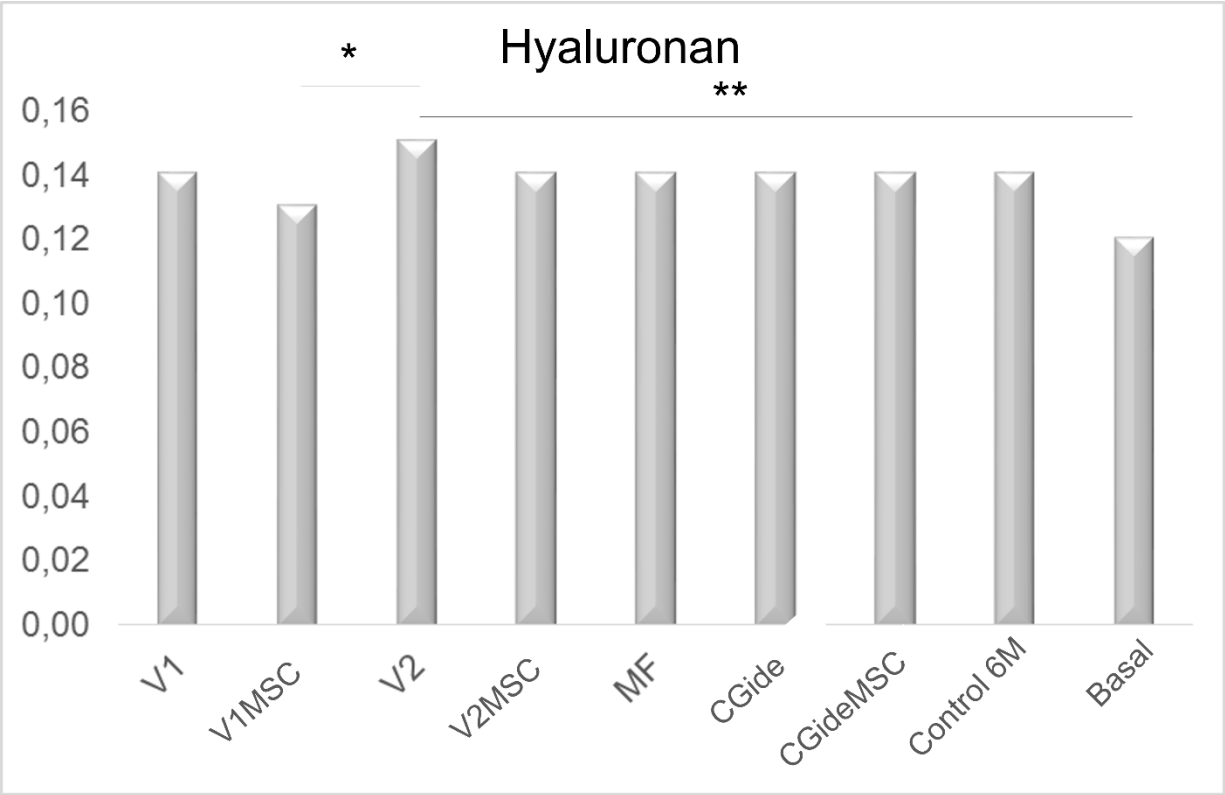
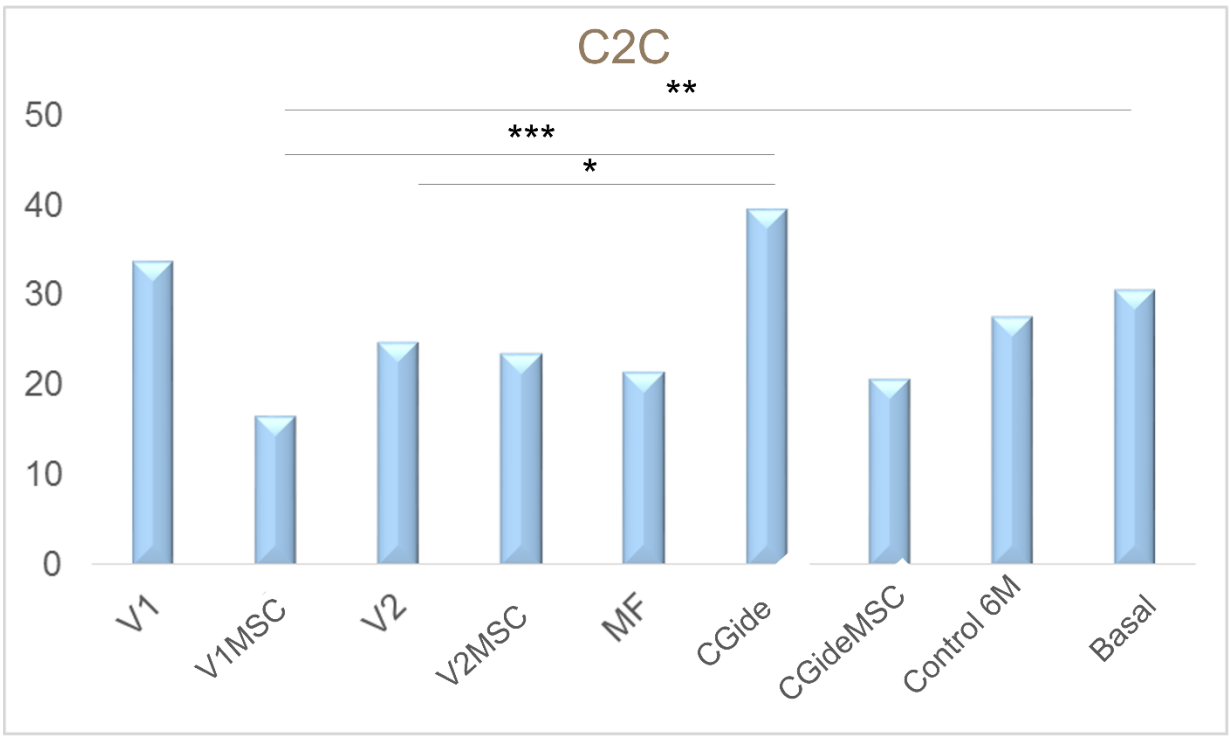


Figure 1. Synovial concentration of Hyaluronan at initial time point (Before Surgery) and 6 months after the surgery. ***** means P=0.022, whereas ****** means P=0.042.

The synovial concentration of C2C exhibited significantly higher degradation in the CG group compared to V2 (P=0.040) and to V1MSC (P=0.022). The V1MSC group also demonstrated lower degradation compared to the initial time point (P=0.036). None of the other groups presented significance in any comparison (Figure 2).

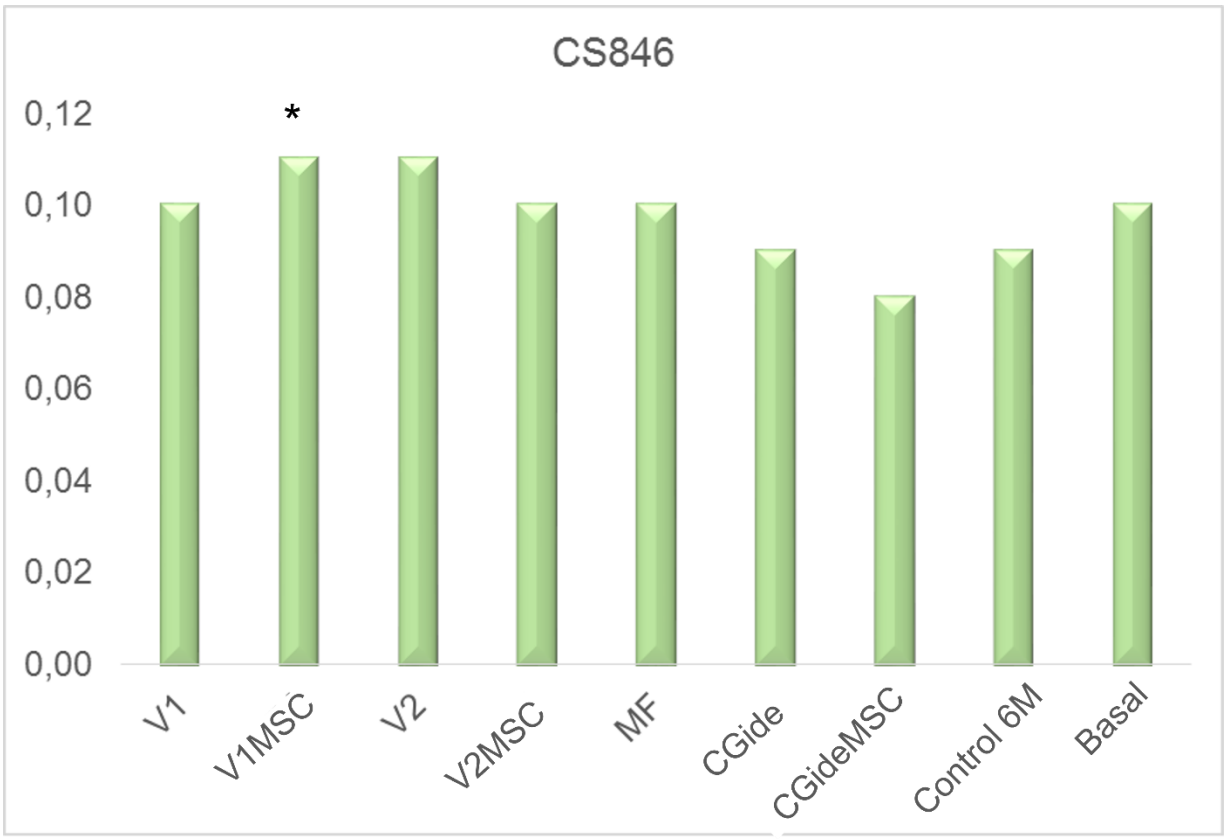


223

224 **Figure 2.** Synovial fluid concentrations of C2C before and 6 months after the OA
225 induction, demonstrating lower C2C levels in V1MSC group compared to initial time point
226 (** indicates P=0.036) and to the CG group (*** indicates P= 0.022), as well as lower C2C
227 levels in V2 group compared to CG group (* indicates P=0.040).

228

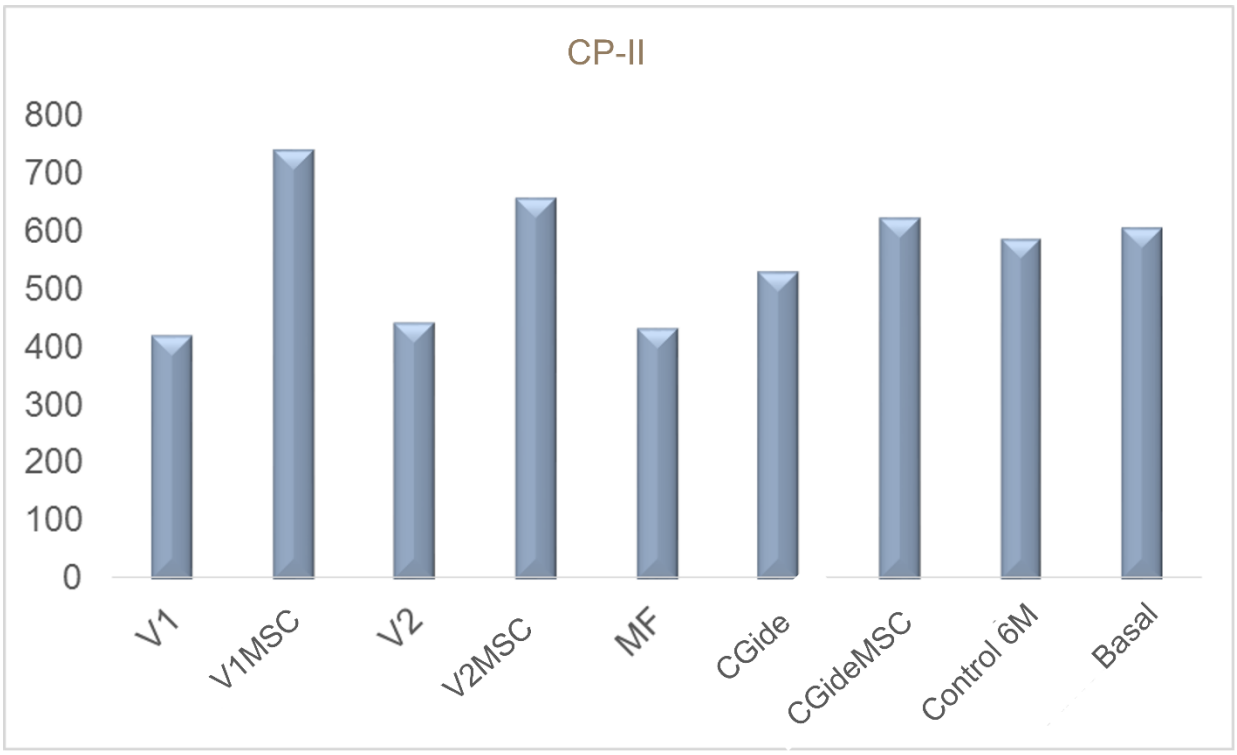
229 Evaluation of the biomarker CS846 revealed higher scores in animals treated with
230 the V1 membrane with MSCs (V1MSC), differing from all the other groups significantly
231 ($P<0.05$) (figure 3). Although there were noticeable differences among the groups,
232 synovial CP-II concentration did not present significant differences (P=0.091) between
233 initial timepoint and any treated or control group after 6 months (figure 4).



234

235 **Figure 3.** Results of CS846 synovial measurements at the initial time point (Before
236 surgery) and after 6 months (all the other groups). There was a significantly higher CS846
237 concentration in V1MSC group compared to the other groups. ***** indicates P<0.05.

238



239

240 **Figure 4.** Concentrations of CP-II before and 6 months after the OA induction. No
241 significant differences were observed.

242

243 **4. Discussion**

244 This study assessed the synovial levels of chondral metabolism biomarkers in
245 response to different treatments, all employed with the intention of supporting chondral
246 healing after cartilage loss. The evaluation of biomarkers of ECM metabolism is well
247 described in the literature and can reveal relevant information regarding joint condition
248 (Garnero, 2004; McIlwraith, 2005).

Osteoarthritis remains one of the most challenging conditions to handle, mostly due to the limited potential of repair that cartilage presents (Vinatier et al., 2009). Hyaline cartilage ECM is mainly composed of collagen fibrils and proteoglycans, which are responsible for its important mechanical properties (van Weeren, 2016). Although no differences were observed in synthesis of collagenous ECM components, aggrecan synthesis was significantly elevated in the group treated with the membrane V1Gide™ and MSCs. The V1Gide™ is composed of 100% synthetic polydioxanone, an absorbable polymer widely used in tissue engineering due to its combined properties of cell adhesion and mechanical resistance (Goonoo et al., 2015). Scaffolds composed of polydioxanone can induce expression of cartilage-specific genes in vitro (Jeong et al., 2010) and are able to stimulate chondrogenesis with good production of proteoglycans and moderate production of type II collagen (Kaps et al., 2006). The results of this study corroborate previous findings about low collagen production and relevant proteoglycan production induced by polydioxanone, since CP-II levels were maintained, but lower C2C and higher CS846 levels were observed.

The lower collagen catabolism in V2 and in V1MSC groups points to the hypothesis that polydioxanone might also play a chondroprotective role, that favored aggrecan synthesis and led to less cartilage catabolism, since polydioxanone is the common component of both membranes. Additionally, ChondroGide™ is composed of types I and III collagen molecules, in opposition to the native type II collagen found in chondral ECM. Compared to type II collagen, type I collagen has less interactions with ECM

proteoglycans due to the minor content of hydroxy-lysine and galactosyl and glucosyl residues (Gelse et al., 2003).

Alongside with polydioxanone, the V2 membrane also has graphene in its composition. Graphene is a thin yet very resistant element, known as the strongest material ever measured in the universe (Geim, 2009). The higher hyaluronan expression in the group treated with the V2 membrane can be related to graphene's capability of stimulating glycosaminoglycan synthesis (Zhou et al., 2017). Graphene also presents important hydrophilic and mechanical features (Karbasi et al., 2017) that can be useful to preserve ECM architecture and water content, thus favoring cartilage function. Moreover, tissue engineering techniques using graphene have demonstrated good results in stimulating chondrogenesis (Yoon et al., 2014; Zhou et al., 2017; Zhou et al., 2019), as well as adipogenesis and osteogenesis (Kim et al., 2013), making graphene a promising candidate for new strategies of tissue engineering.

Besides its purpose of ensuring proper membrane attachment to the lesion site, fibrin also has important chondrogenic properties (Ahmed et al., 2008) that can potentially contribute to cartilage repair. However, although V1MSC group may have benefited from these properties, all the other groups treated with fibrin sealant did not demonstrate similar results. Previous studies that evaluated chondral repair showed inferior cartilage quality after using a type II collagen hydrogel associated with fibrin sealant, but relevant tissue formation after combining both with MSCs (Lazarini et al., 2017). Our results also demonstrated superior ECM production when MSCs were associated with the scaffold and the fibrin sealant, even though the microfracture technique also promotes migration

of the subchondral bone autologous MSCs population to the joint (Erggelet & Vavken, 2016). Moreover, microfracture alone leads to lower cartilage repair compared to microfracture associated with fibrin sealant and MSCs (Koh et al., 2016). Thus, the presence of a higher population of MSCs *in loco* in our study was considered more effective than the autologous migration of MSCs. This indicates a major role of MSCs in the coordination of cartilage repair process, corroborating previously published data that demonstrated their enhanced paracrine effect in a tridimensional organization (Lee & Lee, 2022). However, a limiting factor of this study was the absence of a group treated with MSCs alone, which impairs the proper observation of the isolated effect of MSCs in this specific experimental model.

Although the experimental model of osteoarthritis used in this study design does not induce a low-grade inflammatory process in all articular components, the results of a 6-month follow-up demonstrate that the cartilage metabolism is affected by the surgery, indicating the efficacy of the model.

Even though additional time points of synovial fluid collection and analysis would facilitate the interpretation of the results, repeated arthrocentesis in the ovine species is not a feasible option, mainly due to the scarce amount of synovial fluid and to the small anatomical window used for joint access. Given the feasibility in the detection of cartilage metabolism biomarkers in other body fluids such as urine (Deberg et al., 2005; van Tuyl et al., 2008) and serum (Billinghurst et al., 2003; Conrozier et al., 2007; Frisbie et al., 2008), the periodic measurement of articular biomarkers in serum or urine is a non-to-little invasive tool that can be useful to monitor joint disease progression and treatment

response. Thus, further studies might benefit from parallel collection of urine or serum samples to evaluate the sensitivity of biomarker detection, as well as to compare synovial and urine/serum levels and determine the urine/serum equivalent levels of biomarkers that correspond to specific synovial levels.

The results of this study substantiate additional exploration of cartilage-like constructs associated with MSCs to treat chondral lesions. Although important advances have been made in the treatment of articular injuries, further work is still needed to reach the excellence of a well-designed tissue-engineered construct capable of adequately improving mechanical properties of the articular cartilage.

5. Conclusion

Taken together, our results substantiate the use of synovial biomarkers for osteoarthritis research and suggest that polydioxanone-based scaffolds are preferable over type I and type III collagen scaffolds for chondral defects, showing better results of ECM synthesis and degradation biomarkers. Polydioxanone properties place it as a promising biomaterial for new therapeutic approaches in tissue engineering. The association of a higher population of purified MSCs with an appropriate scaffold provides better results in biomarkers of cartilage metabolism, reinforcing the importance of associating complementary techniques to enhance cartilage aspect and functionality through different mechanisms of repair.

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Abstract

As a primary goal, the abstract should render the general significance and conceptual advance of the work clearly accessible to a broad readership. In the abstract, minimize the use of abbreviations and do not cite references, figures or tables.

For Clinical Trial articles, please include the Unique Identifier and the URL of the publicly accessible website on which the trial is registered.

Keywords

All article types require a minimum of five and a maximum of eight keywords.

Text

The entire document should be single-spaced and must contain page and line numbers in order to facilitate the review process. The manuscript should be written using either Word or LaTeX. See above for templates.

Nomenclature

The use of abbreviations should be kept to a minimum. Non-standard abbreviations should be avoided unless they appear at least four times, and must be defined upon first use in the main text. Consider also giving a list of non-standard abbreviations at the end, immediately before the acknowledgments.

Equations should be inserted in editable format from the equation editor.

Italicize gene symbols and use the approved gene nomenclature where it is available. For human genes, please refer to the HUGO Gene Nomenclature Committee (HGNC). New symbols for human genes should be submitted to the HGNC here. Common alternative gene aliases may also be reported, but should not be used alone in place of the HGNC symbol. Nomenclature committees for other species are listed here. Protein products are not italicized.

We encourage the use of Standard International Units in all manuscripts.

Chemical compounds and biomolecules should be referred to using systematic nomenclature, preferably using the recommendations by the International Union of Pure and Applied Chemistry (IUPAC).

Astronomical objects should be referred to using the nomenclature given by the International Astronomical Union (IAU) provided here.

Life Science Identifiers (LSIDs) for ZOOBANK registered names or nomenclatural acts should be listed in the manuscript before the keywords. An LSID is represented as a uniform resource name (URN) with the following format: urn:lsid:<Authority>:<Namespace>:<ObjectID>[:<Version>]

For more information on LSIDs please see the 'Code' section of our polices and publication ethics.

Sections

The manuscript is organized by headings and subheadings. The section headings should be those appropriate for your field and the research itself. You may insert up to 5 heading levels into your manuscript (i.e.,: 3.2.2.1.2 Heading Title).

For Original Research articles, it is recommended to organize your manuscript in the following sections or their equivalents for your field.

Introduction
Succinct, with no subheadings.

Materials and methods
This section may be divided by subheadings and should contain sufficient detail so that when read in conjunction with cited references, all procedures can be repeated. For experiments reporting results on animal or human subject research, an ethics approval statement should be included in this section (for further information, see the 'Bioethics' section of our polices and publication ethics.)

Results
This section may be divided by subheadings. Footnotes should not be used and must be transferred to the main text.

Discussion
This section may be divided by subheadings. Discussions should cover the key findings of the study: discuss any prior research related to the subject to place the novelty of the discovery in the appropriate context, discuss the potential shortcomings and limitations on their interpretations, discuss their integration into the current understanding of the problem and how this advances the current views, speculate on the future direction of the research, and freely postulate theories that could be tested in the future.

For further information, please check the descriptions defined in the journal's 'Article types' page, in the 'For authors' menu on every journal page.

Acknowledgements

This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors. Should the content of the manuscript have previously appeared online, such as in a thesis or preprint, this should be mentioned here, in addition to listing the source within the reference list.

Contribution to the field statement

When you submit your manuscript, you will be required to briefly summarize in 200 words your manuscript's contribution to, and position in, the existing literature in your field. This should be written avoiding any technical language or non-standard acronyms. The aim should be to convey the meaning and importance of this research to a non-expert.

While Frontiers evaluates articles using objective criteria, rather than impact or novelty, your statement should frame the question(s) you have addressed in your work in the context of the current body of knowledge, providing evidence that the findings – whether positive or negative – contribute to progress in your research discipline. This will help the chief editors to determine whether your manuscript fits within the scope of a specialty as defined in its mission statement; a detailed statement will also facilitate the identification of the editors and reviewers most appropriate to evaluate your work, ultimately expediting your manuscript's initial consideration.

Example statement on: Markram K and Markram H (2010) The Intense World Theory – a unifying theory of the neurobiology of autism. Front. Hum. Neurosci. 4:224. doi: 10.3389/fnhum.2010.00224

Autism spectrum disorders are a group of neurodevelopmental disorders that affect up to 1 in 100 individuals. People with autism display an array of symptoms encompassing emotional processing, sociability, perception and memory, and present as uniquely as the individual. No theory has suggested a single underlying neuropathology to account for these diverse symptoms. The Intense World Theory, proposed here, describes a unifying pathology producing the wide spectrum of manifestations observed in autists. This theory focuses on the neocortex, fundamental for higher cognitive functions, and the limbic system, key for processing emotions and social signals. Drawing on discoveries in animal models and neuroimaging studies in individuals with autism, we propose how a combination of genetics, toxin exposure and/or environmental stress could produce hyper-reactivity and hyper-plasticity in the microcircuits involved with perception, attention, memory and emotionality. These hyper-functioning circuits will eventually come to dominate their neighbors, leading to hyper-sensitivity to incoming stimuli, over-specialization in tasks and a hyper-preference syndrome. We make the case that this theory of enhanced brain function in autism explains many of the varied past results and resolves conflicting findings and views and makes some testable experimental predictions.

Figure and table guidelines
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instructions requested by third-party rights holders, and cover any supplementary charges.

For additional information, please see the 'Image manipulation' section of our polices and publication ethics.

Figure requirements and style guidelines

Frontiers requires figures to be submitted individually, in the same order as they are referred to in the manuscript; the figures will then be automatically embedded at the end of the submitted manuscript. Kindly ensure that each figure is mentioned in the text and in numerical order.

For figures with more than one panel, panels should be clearly indicated using labels (A), (B), (C), (D), etc. However, do not embed the part labels over any part of the image, these labels will be replaced during typesetting according to Frontiers' journal style. For graphs, there must be a self-explanatory label (including units) along each axis.

For LaTeX files, figures should be included in the provided PDF. In case of acceptance, our production office might require high-resolution files of the figures included in the manuscript in EPS, JPEG or TIF/TIFF format.

To upload more than one figure at a time, save the figures (labeled in order of appearance in the manuscript) in a zip file and upload them as 'Supplementary Material Presentation.'

Please note that figures not in accordance with the guidelines will cause substantial delay during the production process.

Captions

Captions should be preceded by the appropriate label, for example 'Figure 1.' Figure captions should be placed at the end of the manuscript. Figure panels are referred to by bold capital letters in brackets: (A), (B), (C), (D), etc.

Image size and resolution requirements

Figures should be prepared with the PDF layout in mind. Individual figures should not be longer than one page and with a width that corresponds to 1 column (85 mm) or 2 columns (180 mm).

All images must have a resolution of 300 dpi at final size. Check the resolution of your figure by enlarging it to 150%. If the image appears blurry, jagged, or has a stair-stepped effect, the resolution is too low.

The text should be legible and of high quality. The smallest visible text should be no less than eight points in height when viewed at actual size.

Solid lines should not be broken up. Any lines in the graphic should be no smaller than two points wide.

Please note that saving a figure directly as an image file (JPEG, TIF) can greatly affect the resolution of your image. To avoid this, one option is to export the file as PDF, then convert into TIFF or EPS using a graphics software.

Format and color image mode

The following formats are accepted: TIF/TIFF (.tif/.tiff), JPEG (.jpg), and EPS (.eps) (upon acceptance). Images must be submitted in the color mode RGB.

Chemical structures

Chemical structures should be prepared using ChemDraw or a similar program. If working with ChemDraw please use our ChemDraw template. If working with another program please follow the guidelines below.

- Drawing settings: chain angle, 120° bond spacing, 18% width; fixed length, 14.4 pt; bold width, 2.0 pt; line width, 0.6 pt; margin width, 1.6 pt; hash spacing, 2.5 pt. Scale 100% Atom Label settings: font, Arial; size, 8 pt
- Assign all chemical compounds a bold, Arabic numeral in the order in which the compounds are presented in the manuscript text.

Table requirements and style guidelines

Tables should be inserted at the end of the manuscript in an editable format. If you use a word processor, build your table in Word. If you use a LaTeX processor, build your table in LaTeX. An empty line should be left before and after the table.

Table captions must be placed immediately before the table. Captions should be preceded by the appropriate label, for example 'Table 1.' Please use only a single paragraph for the caption.

Ensure that each table is mentioned in the text and in numerical order.

Large tables covering several pages cannot be included in the final PDF for formatting reasons. These tables will be published as supplementary material.

Tables which are not according to the above guidelines will cause substantial delay during the production process.

Accessibility

We encourage authors to make the figures and visual elements of their articles accessible for the visually impaired. An effective use of color can help people with low visual acuity, or color blindness, understand all the content of an article.

These guidelines are easy to implement and are in accordance with the W3C Web Content Accessibility Guidelines (WCAG 2.1), the standard for web accessibility best practices.

Ensure sufficient contrast between text and its background
People who have low visual acuity or color blindness could find it difficult to read text with low contrast background color. Try using colors that provide maximum contrast.

WC3 recommends the following contrast ratio levels:

- Level AA, contrast ratio of at least 4.5:1
- Level AAA, contrast ratio of at least 7:1

Avoid using red or green indicators
More than 99% of color-blind people have a red-green color vision deficiency.

Avoid using only color to communicate information
Elements with complex information like charts and graphs can be hard to read when only color is used to distinguish the data. Try to use other visual aspects to communicate information, such as shape, labels, and size. Incorporating patterns into the shape fills also make differences clearer; for an example please see below:

Supplementary material

Data that are not of primary importance to the text, or which cannot be included in the article because they are too large or the current format does not permit it (such as videos, raw data traces, and PowerPoint presentations), can be uploaded as supplementary material during the submission procedure and will be displayed along with the published article. All supplementary files are deposited to figshare for permanent storage and receive a DOI.

Supplementary material is not typeset, so please ensure that all information is clearly presented without tracked changes/highlighted text/line numbers, and the appropriate caption is included in the file. To avoid discrepancies between the published article and the supplementary material, please do not add the title, author list, affiliations or correspondence in the supplementary files.

The supplementary material can be uploaded as:

- data sheet (Word, Excel, CSV, CDX, FASTA, PDF or Zip files)
- presentation (PowerPoint, PDF or Zip files)

- image (CDX, EPS, JPEG, PDF, PNG or TIF/TIFF),
- table (Word, Excel, CSV or PDF)
- audio (MP3, WAV or WMA)
- video (AVI, DIVX, FLV, MOV, MP4, MPEG, MPG or WMV).

Technical requirements for supplementary images:

- 300 DPIs
- RGB color mode.

For supplementary material templates (LaTeX and Word), see our supplementary material templates.

References

Frontiers' journals use one of two reference styles, either Harvard (author-date) or Vancouver (numbered). Please check our help center to find the correct style for the journal to which you are submitting.

- All citations in the text, figures, or tables must be in the reference list and vice-versa
- The names of the first six authors followed by et al. and the DOI (when available) should be provided
- Given names of authors should be abbreviated to initials (e.g., Smith, J., Lewis, C.S., etc.)
- The reference list should only include articles that are published or accepted
- Unpublished data, submitted manuscripts, or personal communications should be cited within the text only, for article types that allow such inclusions
- For accepted but unpublished works use 'in press' instead of page numbers
- Data sets that have been deposited to an online repository should be included in the reference list. Include the version and unique identifier when available
- Personal communications should be documented by a letter of permission
- Website URLs should be included as footnotes
- Any inclusion of verbatim text must be contained in quotation marks and clearly reference the original source
- Preprints can be cited as long as a DOI or archive URL is available, and the citation clearly mentions that the contribution is a preprint. If a peer-reviewed journal publication for the same preprint exists, the official journal publication is the preferred source. See the preprints section for each reference style below for more information.

Harvard reference style (author-date)

Many Frontiers journals use the Harvard referencing system; to find the correct reference style and resources for the journal you are submitting to, please visit our help center. Reference examples are found below, for more examples of citing other documents and general questions regarding the Harvard reference style, please refer to the Chicago Manual of Style.

In-text citations

- For works by a single author, include the surname, followed by the year
- For works by two authors, include both surnames, followed by the year
- For works by more than two authors, include only the surname of the first author followed by et al., followed by the year
- For humanities and social sciences articles, include the page numbers.

Reference list examples

Article in a print journal
Sondheimer, N., and Lindquist, S. (2000). Rnq1: an epigenetic modifier of protein function in yeast. Mol. Cell. 5, 163-172.

Article in an online journal
Tahimic, C.G.T., Wang, Y., Bikle, D.D. (2013). Anabolic effects of IGF-1 signaling on the skeleton. Front. Endocrinol. 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book
Sorenson, P. W., and Caprio, J. C. (1998). "Chemoreception," in The Physiology of Fishes, ed. D. H. Evans (Boca Raton, FL: CRC Press), 375-405.

Book
Cowan, W. M., Jessell, T. M., and Zipursky, S. L. (1997). Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press.

Abstract
Hendricks, J., Applebaum, R., and Kunkel, S. (2010). A world apart? Bridging the gap between theory and applied social gerontology. Gerontologist 50, 284-293. Abstract retrieved from Abstracts in Social Gerontology database. (Accession No. 50360869)

Website
World Health Organization. (2018). E. coli. https://www.who.int/news-room/fact-sheets/detail/e-coli [Accessed March 15, 2018].

Patent
Marshall, S. P. (2000). Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity. U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

Data
Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and dissertations
Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the

deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

Preprint
Smith, J. (2008). Title of the document. Preprint repository name [Preprint]. Available at: https://persistent-url (Accessed March 15, 2018).

Vancouver reference style (numbered)

Many Frontiers journals use the numbered referencing system; to find the correct reference style and resources for the journal you are submitting to, please visit our help center.

Reference examples are found below, for more examples of citing other documents and general questions regarding the Vancouver reference style, please refer to Citing Medicine.

In-text citations

- Please apply the Vancouver system for in-text citations
- In-text citations should be numbered consecutively in order of appearance in the text – identified by Arabic numerals in the parenthesis (use square brackets for physics and mathematics articles).

Reference list examples

Article in a print journal
Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell (2000) 5:163-72.

Article in an online journal
Tahimic CGT, Wang Y, Bikle DD. Anabolic effects of IGF-1 signaling on the skeleton. Front Endocrinol (2013) 4:6. doi: 10.3389/fendo.2013.00006

Article or chapter in a book
Sorenson PW, Caprio JC. "Chemoreception". In: Evans DH, editor. The Physiology of Fishes. Boca Raton, FL: CRC Press (1998). p. 375-405.

Book
Cowan WM, Jessell TM, Zipursky SL. Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press (1997). 345 p.

Abstract
Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, editor. Genetic Programming. EuroGP 2002:

Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3–5; Kinsdale, Ireland. Berlin: Springer (2002). p. 182–91.

Website
World Health Organization. E. coli (2018). https://www.who.int/news-room/fact-sheets/detail/e-coli [Accessed March 15, 2018].

Patent
Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible Endoscopic Grasping and Cutting Device and Positioning Tool Assembly. United States patent US 20020103498 (2002).

Data
Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular tools for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

Theses and dissertations

Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

Preprint
Smith, J. Title of the document. Preprint repository name [Preprint] (2008). Available at: https://persistent-url (Accessed March 15, 2018).

ANEXO II

Dados brutos das análises de ELISA (C2C, CS846, CP-II e Hyaluronan)

Group	Time point	C2C conc	CS846	CP II conc	Hyaluronan
V1	Pós 6m	14,876	0,099	443,417	0,125
V1	Pós 6m	32,172	0,096	295,123	0,126
V1	Pós 6m	>1049,500	0,11	1458,506	0,141
V1	Pós 6m	29,707	0,106	119,508	0,151
V1	Pós 6m	23,854	0,228	386,086	0,131
V1	Pós 6m	739,351	0,093	204,653	0,143
V1	Pós 6m	34,623	0,096	464,8	0,141
V1	Pós 6m	>1049,500	0,101	464,8	0,121
V1 CTM	Pós 6m	9,461	0,683	789,887	0,13
V1 CTM	Pós 6m	24,961	0,068	721,859	0,126
V1 CTM	Pós 6m	<0,000	0,124	284,07	0,137
V1 CTM	Pós 6m	25,322	1,165	1227,895	0,12
V1 CTM	Pós 6m	<0,000	0,644	1219,856	0,161
V1 CTM	Pós 6m	14,123	0,085	765,364	0,123
V1 CTM	Pós 6m	18,581	0,077	546,801	0,119
V1 CTM	Pós 6m	27,461	0,098	736,014	0,173
V2	Pós 6m	329,841	0,102	460,747	0,197
V2	Pós 6m	24,582	0,094	437,313	0,136
V2	Pós 6m	10,654	0,109	514,237	0,165
V2	Pós 6m	22,081	0,115	116,714	0,16
V2	Pós 6m	619,137	0,12	424,679	0,129
V2	Pós 6m	17,687	0,098		0,122
V2	Pós 6m	>1049,500	0,193		
V2 CTM	Pós 6m	26,317	0,108	1045,006	0,158
V2 CTM	Pós 6m	23,153	0,11	628,057	0,131
V2 CTM	Pós 6m	24,06	0,103	<0,000	0,126
V2 CTM	Pós 6m	20,957	0,104	1375,097	0,126
V2 CTM	Pós 6m	<0,000	0,09	179,124	0,121
V2 CTM	Pós 6m	>1049,500	0,074	1056,107	0,143
V2 CTM	Pós 6m	23,012	0,08	388,697	0,153
V2 CTM	Pós 6m	25,724	0,141	652,185	0,143
V2 CTM	Pós 6m	<0,000	0,091	653,421	0,14

MF	Pós 6m	17,832	0,129	426,876	0,17
MF	Pós 6m	23,505	0,161	264,213	0,138
MF	Pós 6m	21,338	0,106	760,871	0,13
MF	Pós 6m	21,151	0,098	<0,000	0,132
MF	Pós 6m	37,183	0,077	57,362	0,141
MF	Pós 6m		0,718	743,22	0,146
MF	Pós 6m		0,075		0,139
MF	Pós 6m		0,101		
CG	Pós 6m	22,538	0,085	195,257	0,149
CG	Pós 6m	384,924	0,07	235,905	0,126
CG	Pós 6m	49,062	0,088	>2097,500	0,123
CG	Pós 6m	260,334	0,101	2077,934	0,145
CG	Pós 6m	39,122	0,096	526,4	0,141
CG	Pós 6m	21,778	0,102		0,13
CG	Pós 6m	39,338			
CG	Pós 6m	24,553			
CG CTM	Pós 6m	22,785	0,084	360,506	0,134
CG CTM	Pós 6m	20,989	0,099	307,677	0,142
CG CTM	Pós 6m	<0,000	0,095	683,968	0,154
CG CTM	Pós 6m	<0,000	0,074	1203,972	0,124
CG CTM	Pós 6m	24,553	0,085	843,593	0,123
CG CTM	Pós 6m	19,823	0,073	550,797	0,136
CG CTM	Pós 6m				0,141
Controle	Pós 6m	>1049,500	0,106	533,095	0,116
Controle	Pós 6m	18,272	0,091	260,52	0,123
Controle	Pós 6m	27,425	0,094	>2097,500	0,129
Controle	Pós 6m	227,624	0,081	581,931	0,153
Controle	Pós 6m	21,151	0,096	957,216	0,143
Controle	Pós 6m	24,41	0,082	>2097,500	0,14
Controle	Pós 6m	56,651	0,103		0,143
Controle	Antes Cir	25,897	0,096	455,145	0,121
Controle	Antes Cir	<0,000	0,094	194,372	0,124
Controle	Antes Cir	189,926	0,154	768,378	0,117
Controle	Antes Cir	15,658	0,094	600,534	0,126
Controle	Antes Cir	49,634	0,125	<0,000	0,127
Controle	Antes Cir	<0,000	0,096	432,056	0,15
Controle	Antes Cir	30,407	0,077	<0,000	0,14
Controle	Antes Cir	27,284	0,101	697,213	0,13
Controle	Antes Cir	151,553	0,108	730,312	0,155
Controle	Antes Cir	28,322	0,085	794,592	0,135
Controle	Antes Cir	286,687	0,093	330,941	0,149
Controle	Antes Cir	20,219	0,085	1341,583	0,135
Controle	Antes Cir	>1049,500	0,092		0,143
Controle	Antes Cir	754,059	0,101		0,161
Controle	Antes Cir	33,327			0,136
Controle	Antes Cir	29,894			0,125
Controle	Antes Cir	43,594			0,149
Controle	Antes Cir				0,144
					0,126
					0,117