

Osteoarthritis and Cartilage



Review

Chondrocyte secretome: a source of novel insights and exploratory biomarkers of osteoarthritis

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ARTICLE INFO

Article history:

Received 30 November 2016

Accepted 14 February 2017

Keywords:

Cartilage
Chondrocyte
Osteoarthritis
Biomarker
Proteomics
Secretome

SUMMARY

The extracellular matrix (ECM) of articular cartilage is comprised of complex networks of proteins and glycoproteins, all of which are expressed by its resident cell, the chondrocyte. Cartilage is a unique tissue given its complexity and ability to resist repeated load and deformation. The mechanisms by which articular cartilage maintains its integrity throughout our lifetime is not fully understood, however there are numerous regulatory pathways known to govern ECM turnover in response to mechanical stimuli. To further our understanding of this field, we envision that proteomic analysis of the secretome will provide information on how the chondrocyte remodels the surrounding ECM in response to load, in addition to providing information on the metabolic state of the cell. In this review, we attempt to summarize the recent mass spectrometry-based proteomic discoveries in healthy and diseased cartilage and chondrocytes, to facilitate the discovery of novel biomarkers linked to degenerative pathologies, such as osteoarthritis (OA).

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Introduction

To gain a deeper understanding of the mechanisms that drive osteoarthritis (OA), it is important to appreciate the underlying biology of healthy and diseased joint tissues. Of interest is the pathophysiology of articular cartilage, and the processes that

govern synthesis and organisation of extracellular matrix (ECM) components secreted by chondrocytes into the pericellular milieu. The chondrocyte is the unique resident cell of articular cartilage and thus solely responsible for ECM composition and regulation. Chondrocyte metabolism is influenced by its micro-environment, and in return influences ECM composition, organization and ultimately the mechanical resilience of cartilage^{1–3}. As such, chondrocytes play a key role in ECM remodelling in physiological and pathological conditions⁴.

It is commonly established that healthy articular chondrocytes change into different phenotypes as OA develops and progresses:

- (i) A catabolic phenotype develops, associated with an increase in proteolytic enzymes and reactive oxygen/nitrogen species,

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in response to mechanical stress and inflammatory cytokines, tumour necrosis factor (TNF)- α and interleukin (IL)-1 β , leading to further ECM degradation.

- (ii) An anabolic phenotype emerges that is associated with regeneration of the ECM, including increased collagen type II and proteoglycan expression regulated by growth factors (transforming growth factor (TGF)- β , bone morphogenetic protein (BMP)s and insulin growth factor (IGF)-I), expressed either by the surrounding joint tissue or by the chondrocytes themselves.
- (iii) A hypertrophic phenotype develops, manifesting in expression of type X collagen and induction of apoptosis, ultimately resulting in osteophyte formation.
- (iv) A fibroblastic-like phenotype with an increased number of dedifferentiated chondrocyte and expression of type I collagen.
- (v) Lastly, a chondroblastic phenotype emerges with expression of foetal type IIA collagen, type III collagen and early/late differentiation markers⁵.

The specific phenotype that any individual chondrocyte exhibits is dependent on the zone in which the chondrocyte is situated and the stage of OA progression. In the upper zone, cellular proliferation and hypertrophy are observed, whereas the mid and deep zones display increased expression of type II collagen⁶. As OA progresses, cartilage is lost and chondrocytes undergo senescence, due to a combination of replicative exhaustion and oxidative stress⁷.

Eventually, chondrocytes will undergo apoptosis, and the articular cartilage will be destroyed. The ultimate goal of mass spectrometry-based proteomics strategies is the identification of a specific tissue-derived secretome that is unique to the diseased chondrocyte and surrounding ECM and is able to distinguish between healthy and diseased cartilage.

Because protein expression is dependent on environmental conditions, the secretome is highly dynamic in composition and turnover. In 2010, Agrawal *et al.* suggested defining the secretome as “the global group of secreted proteins into the extracellular space by a cell, tissue, organ, or organism at any given time and conditions through known and unknown secretory mechanisms involving constitutive and regulated secretory organelles”⁸. In this narrative review, we only considered the chondrocyte (or cartilage) as the source of secreted proteins, which have been uncovered by state-of-the-art mass spectrometry-based proteomics techniques.

Proteomic techniques include different methods, all relying on the separation of proteins and their further analysis using either gel-based (Two-dimensional electrophoresis) or gel-free methods. Protein separation methods are coupled to a mass spectrometer for identification of sequence by mass spectrometry^{9,10}. In differential analysis, the peptides may be marked with stable isotope at various stages of the analysis process, or Label-free methods could be performed. Several modes of analysis are available in mass spectrometry¹⁰. They differ markedly by the ionization source of the sample. The main sources used in proteomic analysis are matrix-assisted laser desorption/ionization (MALDI) and surface-enhancer laser desorption/ionization (SELDI)^{10,11}. These techniques allow a soft ionization of molecules without excessive fragmentation^{9,11}.

More than the total tissue protein extract, it is expected that well-defined protein fractions such as the secretome could be a source of novel OA biomarkers, with the potential to predict disease severity and monitor progression.

Materials and methods

A literature search was performed in Pubmed/Medline and Scopus, identifying articles published between January 2004 and March 2016. Keywords used in ‘Any fields’ were; (chondrocyte OR cartilage) AND secretome (19 relevant papers out of 38 found), or (chondrocyte OR cartilage) AND (proteomic OR mass spectrometry) (65 articles relevant papers out of 290 found). Only results from mass spectrometry-based studies were included in this article. The review has considered all species, even if the majority of studies have been performed on human source of chondrocytes or cartilage. Only research articles published in English were included. Supplementary files of all papers were analysed and included in this review.

Chondrocyte secretome

Recent mass spectrometry-based proteomic studies have identified several proteins which form the chondrocyte secretome. In this part of the review, we focused on proteins identified either directly in the secretome of cartilage explants^{12–20} or chondrocytes cultures^{18,21–31}, both of which are listed in Table I and illustrated in Fig. 1. We further complete this list with proteins recently identified by proteomic analysis performed directly on fresh chondrocytes or cartilage tissue^{32–36} (sometimes de-cellularized³⁷) whereby different locations in the joint were compared, or healthy joint tissues were compared with OA tissues. The characteristics of all these studies are summarized in Table II. Proteins have been classified in different sections: ECM proteins, cytokines and growth factors, enzymes and miscellaneous.

ECM proteins

As expected, the most abundant ECM proteins produced by chondrocytes, and detected by proteomic analysis, are collagens and proteoglycans, Table I. Thirteen collagens are found in the chondrocytes secretome, of which type II, VI and XII are the most abundant³⁵. Collagen type XII is also known to interact with other cartilage elements such as cartilage oligomeric matrix protein (COMP), decorin and fibromodulin³⁸. Collagen type II and VI levels are increased in the secretome of chondrocytes taken from the medial condyle of patients with early OA (Mankin score 0–3), compared with samples taken from patients with severe OA (Mankin score 5–10)²⁵.

Beside the collagens, other ECM proteins found in high abundance in the chondrocyte secretome include; aggrecan, HPLN-1 (proteoglycan link protein), biglycan, COMP, fibronectin, prolargin (Proline-arginine-rich end leucine-rich repeat protein (PRELP)), matrilin-3, cartilage acidic protein-1 (CRTAC1 or ASPIC), latent-transforming growth factor beta-binding protein-1 (LTBP1), extracellular matrix protein-1 (ECM-1), tenascin, lubricin and chitinase-3-like protein 1 (CHI3L1), also known as YKL-40). CHI3L1 was found at lower levels in cartilage explants treated with IL-1 β compared to controls¹². CHI3L1, is a biomarker of OA found in synovial fluid and serum³⁹, and plays a role in tissue remodelling and inflammation. The concentration of CHI3L1 in OA synovial fluid positively correlates with levels of matrix metalloproteinase (MMP)-1, MMP-3, IL-6 and IL-17.

IL-6 and IL-17 enhanced CHI3L1 production in human primary chondrocyte cultures⁴⁰ and CHI3L1 serum concentration positively correlated with osteophyte size in OA patients⁴¹. This protein is more abundant in knee compared to hip cartilage³⁷ and in OA

Table 1

Summary of recent mass spectrometry-based proteomic studies carried out on human chondrocytes and cartilage to identify secretome components

ECM proteins	Specificity	OA vs normal	With IL-1 β	References
Aggrecan core protein		\		13,14,17,18,20,22,27,31–37
Asporin				32,34,37
Basement membrane-specific heparin sulfate proteoglycan core protein (PGBM)		↗		13,18,20,30
Biglycan		↗		13,14,17,18,20–22,27,30,32–37
Cartilage acidic protein-1 (CRTAC1, ASPIC)	Specific chondrocyte marker	↗		13,16,22,26,33,35
Chitinase-3-like protein -1, -2	Tissue remodelling, inflammation	↗	\	13,16,18,20–22,24,25,27,30,31,33,35,37
Chondroitin sulfate proteoglycan 4 (CSPG4)				18
Collagens type I, II, III, V, VI, VIII, IX, X, XI, XII, XIV, XV, XVI		↗		13,14,16,18,20,21,24–27,30–35,37
Cartilage oligomeric matrix protein (COMP)		↗		13,14,17,18,20,22,26,27,30–35,37
Decorin				12–14,18–21,27,30,32,34,35,37
Extracellular matrix protein-1	Negative regulator of bone mineralization, promote angiogenesis, inhibit MMP-9 activity	↗		13,14,19,22,25,33
Fibrillin-1				18,22,25,27,30,35,37
Fibromodulin			↗	14,16–20,22,24,26,27,32–34,36
Fibronectin				14,16–18,20–22,26,27,30,32,34,35,37
Fibulin-1, -3, -4, -7		↗		13,18,20–22,24,25,27,30,33,34
HPLN-1 (proteoglycan link protein-1)	Bind hyaluronic acid and aggrecan	↗	↗	13,14,18,19,22,24,27,32–35,37
Latent-transforming growth factor beta-binding protein -1, -2 (LTBP1, 2)	Storage/activation TGF- β 1, structural role in ECM			13,20,26,27,30
Lubricin (proteoglycan-4)		↗		13,14,18,20–22,26,27,30,32–35,37
Lumican				13,14,16,18–22,24,25,27,30,32–35,37
Matrilin-2*, -3*				25,33–35,37
Matrix gla protein (MGP)		↗	↗	14,19,20,22,24,26,33–35,37
Mimiccan (osteoglycin)		↗		13,18,22,27,30,32–34,37
Osteomodulin				18,22,30–32,34,37
Periostin		↗		13,22,35
Perlecan				34,37
Prolargin (PRELP)	Bind type II collagen and perlecan			13,17,18,20,22,25,27,32–37
Syndecan-2, -4			↗	19,21,35
Tenascin C, X		↗		13,18,20–22,30–32,34,35,37
Versican		↗		13,32,34,37
Cytokines and growth factors	Specificity	OA vs normal	With IL-1 β	References
CCL2, -8, -14, -20			↗	14,21,22,25,33
CSF-1			↗	21,27
CTGF				16,18,20,22,24,30,31,33
CXCL1, -3, -5, -6			↗	27,33
Cytokine receptor like factor 1	Decrease aggrecan and type II collagen synthesis			20,26
Gremlin-1	BMP antagonist, inhibit bone mineralization	\		14,35
IGFBP-2, -3, -4, -5, -6, -7		IGFBP3 ↗		12,18,20–22,24–27,30
IL-6, -8, -17 β		IL-17 β \	IL-6, -8 ↗	14,19–21,25,27,33
Inhibin- β A/proinhibin- β A	dimerise to form Activin A, stimulating TIMP-1 production	↗	↗	12,16,20,27
Leukocyte cell-derived chemotaxin 2 (LECT2)	reduce IL-1 β , IL-6 and other chemokines reduce IL-1 β , IL-6 and other chemokines ⁷⁹	↗		18,33
Pleiotrophin (PTN)			↗	14,33
TGF- β 2				22
Enzymes	Specificity	OA vs normal	With IL-1 β	References
ADAMTS-1, -2, -4, -5				12,20,22,25
Angiogenin	Deep layer – stimulate angiogenesis	↗		12,18,32–34,37
Carboxypeptidase E				14
Cathepsins (B, D, F, K, L1, Z)		B ↗		12,14,16,18,20–22,25,30,35
ENPP2	NTP pyrophosphatase, mineralization			22
Extracellular sulfatase Sulf1 Sulf2				14
HTRA1 (serine protease)	Increase with TGF- β 1	\		12,14,16,18,20,22,24,26,30,32,34,35,37
Lysozyme C			↗	12,14–16,20,32,34
MMPs (-1, -2, -3, -7, -10, -13, -14, -16)		↗	↗	12–16,18,20–25,27,30,31,34,35,37
Pappalysin-1	Metalloproteinase, cleave IGFBP-4,-5			22
Peroxiredoxin-1, -2, -4	Protecting cells from free-radical damage	2 ↗		13,16,20,23,37
Phospholipase A2*		↗		12,13,32,34,35,37
Procollagen C-endopeptidase enhancer (PCOLCE)-1, -2	Enhances procollagen C-proteinase activity. C-terminal processed part of PCPE (CT-PCPE) may have an metalloproteinase inhibitory activity. Decrease with TGF- β 1	↗		12,14–16,18,20,22,24,26,30,33,34,37
Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1, 2	Collagen fibre glycosylation		↗	22,31,58
Putative trypsin 6 (serine protease)				21

(continued on next page)

Table 1 (continued)

Enzymes	Specificity	OA vs normal	With IL-1 β	References
Pyruvate kinase		↑		12,13,22
Serine protease 23				22
Sulfhydryl oxidase				12,22
Superoxide dismutase (SOD1, SOD2, SOD3)	Protecting cells from free-radical damage		SOD2 ↓	12,14–16,22,23,25,31,32,34,35
Triosephosphate isomerase:	Carbohydrate degradation		↗	12,14,16,20,22
Enzymatic inhibitors	Specificity	OA vs normal	With IL-1 β	References
Antileukoprotease	Serine proteinase inhibitor			12,25
Cystatin C	Cysteine proteinase inhibitor			18,21,22,26
Inter α Globulin inhibitor H2	HA processing			12
Inter α trypsin inhibitor	HA processing,			
Heavy chain H1		↗		13
Heavy chain H4				18
SERPIN	Serine protease inhibitors			
A1: α 1-antitrypsin				16,21,22,25,32,34
A2: anti-trypsin related protein				22
A3: α 1-antichymotrypsin		↓		12,16,18,22,25,30,32–35
A5: Protein C inhibitor				12,22
E1: Plasminogen activator inhibitor 1				18,22,30,35
E2: Glia-derived nexin				14,20–22,32,35
F1: Pigment epithelium derived factor		↗		12,13,16,18,30
G1: Plasma protease C1 inhibitor				18,22,30
I2 α 1 antiproteinase 2				14,15
TIMPs (-1, -2, -3, -4)	TIMP-1 increase with TGF- β 1		TIMP-1 ↓	12,14–16,18,20–22,24–26,30,34,35,37
Tissue factor pathway inhibitor 1, 2 (TFPI)	Serine protease inhibitors			26
Miscellaneous	Specificity	OA vs normal	With IL-1 β	References
78 kDa glucose regulated protein				22,33–35
A1 acid glycoprotein 1, 2	Binding and modulation of cytokines and growth factor, like IL-6 and TNF- α	↗		13,34
ADAMTS-like 2	Bind to LTBP – no enzymatic function			25
Annexin A1, A2, A5,		A1, A2 ↗		14,18,20,22,30,35
Apolipoprotein AI, AII, D, E	Bind lipids – proteoglycans and collagens interaction	AI, AII ↗		13,18,19,30,34,35
		D ↓		
CILP (1-1, 1-2, 2-1, 2-2)	No intrinsic NTP pyrophosphatase activity but IGF-1/TGF- β antagonists, increase with TGF- β 1	↗	↗	12,14,16,18,20,24,25,32,34,36,37
Chondroadherin	Bind the cell to the type II collagen			13,14,16–18,20,25,32–35,37
Clusterin	Chaperon – inhibit protein aggregation and apoptosis	↓		14–20,22,24,26,30,34,35,37
Complement C1q, C1r, C1s, C3, C8, C9, factor B, factor D, factor H		B, C1r, C3 ↗		12–14,16,18,20–22,30,32,33,37
Emilin-1	Cell-ECM interaction			25,35,37
Ezrin-Radexin-Moesin-Transgelin	Cytoskeletal related			13,20,22,27,32,35,80
Gelsolin	Cytoskeletal related	↗		16,18,20,22,26,30–32,34,35,37,80
Lactadherin				15,18,20–22,30
Osteonectin (SPARC)	ECM-cytokine interactions			14–16,18,22,24,25,30,33–35
Osteopontin	Bind to mineral and inhibit mineralization	↗		13,20,34,37
Profilin-1	Cytoskeletal related			18,20,27,30,80
S100A1	Calcium binding proteins, role in inflammation	A1 ↓		13,19,21,22,25,27,32–35
		A8, A10 ↗		
Semaphorin 3A, 3C				22
Spondin -1 and -2	Cell adhesion-bind GAGs-Wnt agonist			12
Stanniocalcin 1, -2				18,26,30
Tetranectin	Mineralization – bind to GAGs	↗		13,18,21,30
TGFBI (Transforming Growth Factor, Beta-Induced)	Cell to type II collagen interactions, endochondral bone formation	↗		13,14,18,20–22,27,30,32–35,80
Thrombospondin-1, -3, -4	Cell-ECM interaction - angiogenesis	-3 ↗	-1 ↗	13,14,17–22,24,30,32,34,35,37
Vasorin	may act as an inhibitor of TGF- β signalling			22

compared to healthy cartilage²⁷ and is considered to be an early OA biomarker in the Stenberg' proteomic study, which compared early and severe OA secretomes²⁵.

In comparison with normal cartilage explants, CRTAC1 is found elevated in the OA cartilage secretome¹³. This protein is considered a cartilage specific protein, appearing early during chondrocyte differentiation of mesenchymal stem cells (MSCs)²⁶. Expression of CRTAC1 allows discrimination between human chondrocytes and osteoblasts, or MSCs, in monolayer cell culture^{26,42}. This protein is therefore considered a good marker of chondrocytic differentiation of MSCs.

LTBP1 also seems to be an important ECM protein secreted early in the differentiation of MSCs to chondrocytes²⁶. Beside its structural role in the ECM, this protein is involved in the storage and the

activation of TGF- β 1, and therefore may play an important role in facilitating cartilage homeostasis.

ECM-1 is a protein involved in endochondral bone formation serving as a negative regulator of bone mineralization⁴³. It is able to enhance the proliferation of endothelial cells during angiogenesis⁴⁴ and inhibit MMP-9 proteolytic activity⁴⁵. Louridos *et al.* observed that the level of ECM-1 secreted by cells originating from damaged OA cartilage is 3-fold higher than that of healthy cartilage¹³.

Periostin secretion from explants increases with cartilage degradation¹³, and was only found in cultured OA chondrocytes³⁵. Expression of periostin was previously shown to be elevated in OA cartilage compared to normal, and located in the pericellular ECM close to damaged areas of cartilage^{46,47}. Periostin is able to increase

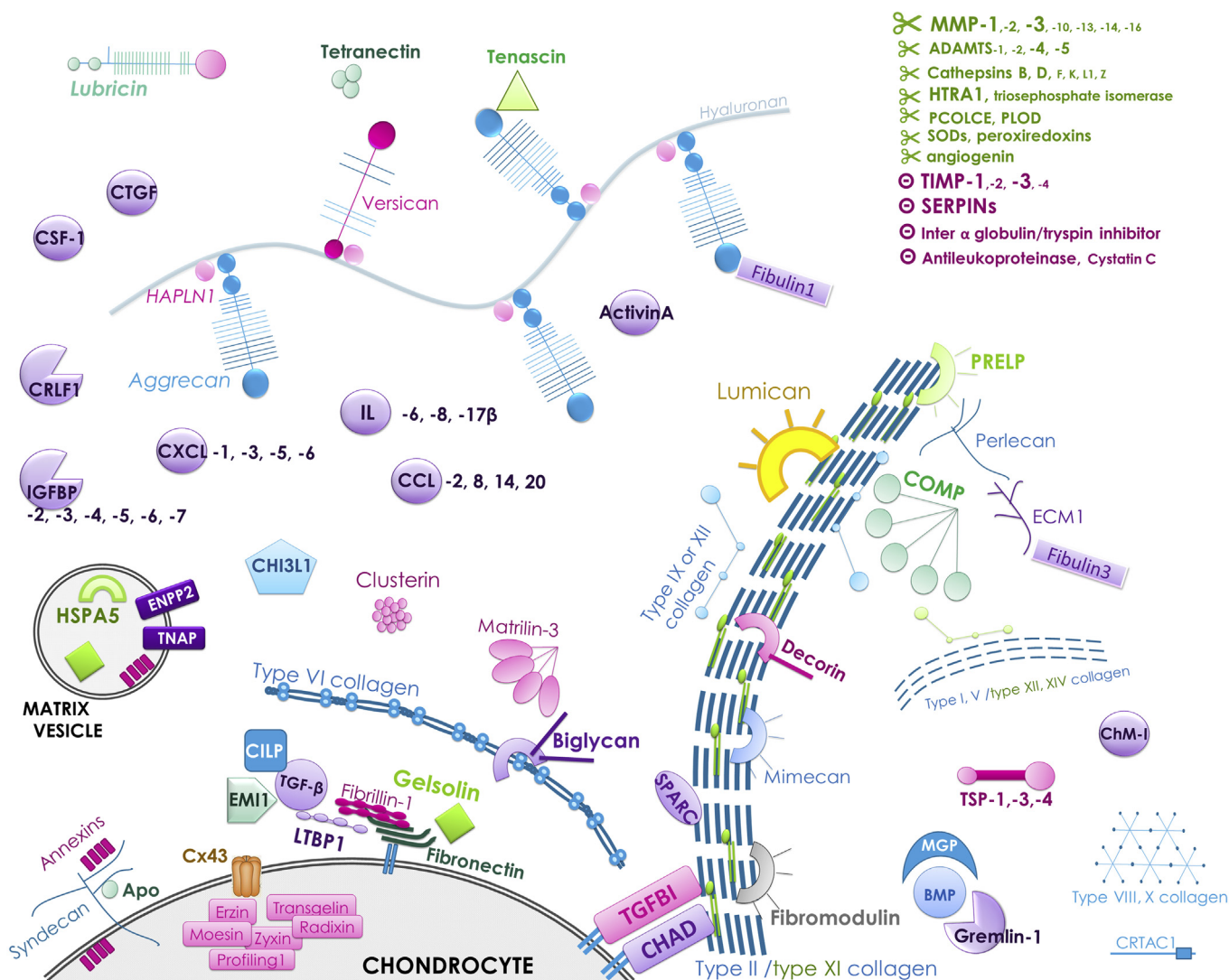


Fig. 1. Schematic representation of the chondrocyte secretome, based on data from mass-spectrometry based proteomic studies. ADAMTS: A Disintegrin And Metalloproteinase with Thrombospondin Motifs, Apo: apolipoprotein, BMP: Bone morphogenetic protein, CCL: Chemokine ligand, CHAD: Chondroadherin, ChM: chondromodulin, CILP: Cartilage intermediate layer protein, CHI3L1: Chitinase-3-like protein 1, COMP: Cartilage oligomeric matrix protein, CRLF: Cytokine receptor-like factor, CRTAC: Cartilage acidic protein, CSF: Macrophage colony-stimulating factor, CTGF: Connective tissue growth factor, Cx: connexin, CXCL: C-X-C motif chemokine ligand, ECM: extracellular matrix protein, ENPP: Ectonucleotide pyrophosphatase/phosphodiesterase family member, EMI: emilin, HSPA5: 78 kDa glucose-regulated protein, HTRA: High-temperature requirement A serine peptidase, IL: Interleukin, LTBP: Latent-transforming growth factor beta-binding protein, MGP: matrix gla protein, MMP: matrix metalloproteinase, PCOLCE: Procollagen C-endopeptidase enhancer, PLOD: Procollagen-lysine,2-oxoglutarate 5-dioxygenase, PRELP: proline-arginine-rich end leucine-rich repeat protein, SERPIN: serine protease inhibitors, SOD: superoxide dismutase, SPARC: Secreted protein acidic and rich in cysteine, TGFBI: Transforming growth factor-beta-induced protein ig-h3, TGF: transforming growth factor, TIMP: tissue inhibitor of metalloproteinase, TNAP: tissue non-specific alkaline phosphatase, TSP: thrombospondin.

expression of IL-6, IL-8, MMP-1,-3,-13 and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 by chondrocytes *in vitro*^{46,47}. Fibronectin and fibromodulin are secreted at comparable levels by OA and normal chondrocytes, but fragmentation of these ECM proteins is found to differ depending on whether the cell is healthy or diseased^{36,48}.

Cytokines and growth factors

Classical cytokines and chemokines like IL-6, IL-8, CCL8-20, CXCL1-3-5-8, CSF-1 are detected by tandem mass spectrometry only after an IL-1 β stimulation of chondrocytes, cultured either in explant or in monolayer^{12,14,19,22,27}.

The IGFBP family is widely represented in chondrocyte secretome by IGFBP-2,-3,-4,-5,-6 and -7^{12,21,22,24-27}. Expression of IGFBP-3,-4,-6 and -7 is increased in early OA²⁵. These proteins serve as a

carrier protein for IGF-1 and modulate the availability of IGF-1 to bind its receptor. Furthermore, IGFBP3 is known to induce chondrocyte apoptosis during OA⁴⁹. In contrast IGFBP5 that has a positive role in cartilage anabolism, and inhibits enzymatic degradation; in fact IGFBP5 promotes cartilage extracellular matrix formation *in vivo* in the DMM OA rat model⁵⁰.

Among growth factors detected in the chondrocyte secretome, gremlin-1, chondromodulin and pleiotrophin are known to be involved in hypertrophic differentiation regulation. Gremlin-1 is a BMP antagonist presenting reduced secretion from OA chondrocytes compared to normal^{14,35}, and plays an important role in bone development by inhibiting bone formation⁵¹. Gremlin-1 is therefore, potentially, a potent inhibitor of chondrocyte hypertrophy in cartilage. Chondromodulin is also important for chondrocytes stabilization, acting by inhibiting hypertrophic differentiation, and is shown to be secreted only by superficially

Table II
Characteristics of the reviewed studies

Study	Method	Secretome	Chondrocyte culture	Explant culture
Peffer et al. 2013 ¹²	OA ± IL-1β	X		X
Lourido et al. 2014 ¹³	Normal vs wound or unwound OA	X		X
Svala et al. 2015 ¹⁴	Equin ± IL-1β	X		X
Williams et al. 2013 ¹⁵	Equin ± IL-1β	X		X
Hermansson et al. 2004 ¹⁶	OA only	X		X
Clutterbuck et al. 2011 ¹⁷	Equin ± IL-1β	X		X
Peffer et al. 2016 ²⁰	Equin ± IL-1β	X		X
Polacek et al. 2010 ¹⁸	OA monolayer vs explant culture	X	X	X
Swan et al. 2013 ¹⁹	Canine ± IL-1β	X		X
Calamia et al. 2012 ²¹	Normal + IL-1β	X	X	
Calamia et al. 2014 ²²	OA ± chondroitine or glucosamine sulfate	X	X	
Catterall et al. 2006 ²³	OA ± IL-1β	X	X	
Riffault et al. 2015 ²⁴	OA ± TGF-β1	X	X	
Stenberg et al. 2013 ²⁵	Early OA vs late OA	X	X	
Rocha et al. 2014 ²⁶	bMSCs chondrogenic differentiation	X	X	
Lourido et al. 2015 ²⁷	OA or normal + IL-1β	X	X	
Taylor et al. 2015 ²⁸	Bovine P0 vs P2 chondrocytes	X	X	
Haglund et al. 2008 ²⁹	Rat, +/- LPS	X	X	
Polacek et al., 2011a ³⁰	Chondrocytes vs MSCs	X	X	
Polacek et al., 2011b ³¹	Monolayer vs aggregate culture	X	X	
Onnerfjord et al. 2012 ³²	Type of cartilage			X
Ikedo et al. 2013 ³³	Normal vs OA hip			X
Muller et al. 2014 ³⁴	Normal at superficial vs intermediate vs deep layer			X
Tsolis et al. 2015 ³⁵	Normal vs OA		X	
Cillero-Pastor et al. 2013 ³⁶	Normal vs OA from superficial to deep layer with MALDI-IMS			X
Gago-Fuentes et al. 2015 ⁸⁰	Cx43 complexes from normal vs OA chondrocytes		X	
Hsueh et al. 2015 ³⁷	Decellularized cartilage from normal vs OA, knee vs hip and superficial vs intermediate vs deep layer			X

located chondrocytes³⁴. Pleiotrophin is a secreted heparin-binding peptide expressed in mesodermal and neuroectodermal cells during development, but rarely in adult tissues. Pleiotrophin is abundant in foetal and juvenile cartilage, but not in mature. Furthermore, pleiotrophin is re-expressed in chondrocytes in early OA, and is involved in the clustering and proliferation of chondrocytes observed in the early stages of OA⁵². Pleiotrophin is an inducer of hypertrophy during chondrogenic differentiation of MSCs⁵³ and is also a potent pro-angiogenesis factor.

Proteolytic enzymes and their regulators

Proteomic analysis of the chondrocyte secretome shows that the most abundant family of enzymes secreted is the MMPs, along with their endogenous inhibitors, the TIMPs. MMP-1 and 3, and TIMP-1, are particularly abundant. TIMP-3 appears to be more abundant in normal hip cartilage than knee, but MMP-1 is contrarily abundant in normal knee cartilage³⁷. According to these studies, MMP-13 was only identified following IL-1β-stimulation¹⁵, or is at very low levels without stimulation¹⁴. With the exception of MMP-2¹³, MMP protein levels do not vary substantially between normal and OA chondrocytes, but all increase with IL-1β treatment. TIMP-1 and -2 levels are decreased in OA medial condyles²⁵, and TIMP-1 is increased with TGF-β1 stimulation²⁴.

The other family of metalloproteinases expected to be found in cartilage is the ADAMTSs. Although ADAMTSs were found in some proteomic secretome studies^{12,22,25}, they were not identified in all studies; most likely due to varying extraction methods which can eliminate ADAMTSs, along with the glycosaminoglycan (GAG) attachments of proteoglycans¹⁵. ADAMTS's may not be easily identified in mass spectrometry-based proteomic studies also due their relatively low abundance. Indeed, saturation of detectors with high abundance ions, type of protease used in mass spectrometry based studies (mainly trypsin) which may lead to non-identification of ionized peptides as they are too small, too large or contain amino acid sequences less likely to be 'seen' following ion identification. Positive

studies, which identified ADAMTSs in the secretome, were all performed using OA chondrocytes or OA cartilage explant culture^{12,22,25}. A recent study published by Svala, showed that peptides cleaved from aggrecan, following IL-1β stimulation in chondrocytes, were generated by MMPs, but not by ADAMTSs¹⁴. This finding confirms the importance of MMPs in cartilage ECM breakdown mediated by IL-1β. However, the fragmentation patterns, and differential distribution between cartilage and synovial fluid, are consistent with the existence of at least two proteolytic pathways for aggrecan degradation in human OA, generating both ³⁴²FFGV- and ³⁷⁴ARGS-fragments⁵⁴. Both the MMP-generated N-terminus of ³⁴²FFGV-fragment and the aggrecanase ³⁷⁴ARGS-fragment are found in the OA synovial fluid, but only the ³⁴²FFGV is found in cartilage tissue itself⁵⁴.

A further intriguing proteomic study was conducted to elucidate the target of MMPs and ADAMTSs in articular cartilage^{20,55}. Human articular cartilage⁵⁵ or crude equine cartilage proteoglycan extract²⁰ was digested by the addition of exogenous metalloproteases, including MMP-2, -3, -8, -9, -12, and -13 and the aggrecanases ADAMTS-4 and ADAMTS-5. Digestion products were identified by proteomic methods, and complete sequences of generated peptides were determined. A wide variety of peptides, originating from collagen types I, II, and III, biglycan, prolargin, fibromodulin, fibronectin, decorin, COMP, cartilage intermediate-layer protein, megakaryocyte-stimulating factor, clusterin, mimican, aggrecan, and lumican, were obtained^{20,55}. MMP-2 was the most active protease used in the study, and the aggrecanases were the least active in generating peptides from cartilage digestion. The aggrecanases showed a preference for cleaving proteoglycan-containing proteins. However, all of the proteases cleaved many types of cartilage ECM proteins⁵⁵. Interestingly, IL-1β treatment generated many COMP neopeptides^{14,20}. Some biglycan and COMP neopeptides were identified as being generated by ADAMTS-4 or MMP-3 and even increased by IL-1β concerning COMP²⁰ are found increased in the horse OA synovial fluid compared to normal one, i.e., ¹⁹¹CIEMGGNPL for biglycan and ¹⁴⁹CEACPPGYSGPTHEGVGM¹⁶⁶ and ⁸⁷AQCAPGSCFPVACTQ¹⁰² for COMP⁵⁶.

Proteomics studies also highlight a serine protease, HTRA1, as one of the most abundant secreted protein by chondrocytes. This enzyme cleaves aggrecan, within the interglobular domain, in human cartilage⁵⁷. Levels of HTRA1-generated aggrecan fragments, containing the VQTV (356) neoepitope, were significantly elevated in OA cartilage compared with cartilage from healthy joints; implicating HTRA1 as a critical protease involved in proteoglycan turnover and cartilage degradation during degenerative joint disease⁵⁷. Cleavage of aggrecan by HTRA1 was strongly enhanced by HTRA1 agonists such as CPII, a C-terminal hexapeptide derived from the C-propeptide of procollagen II α 1⁵⁷. HTRA1 is preferentially localized in the deep layer of cartilage³⁴ and increased during chondrocyte differentiation of MSCs²⁶, and by TGF- β 1 stimulation²⁴. HTRA1 is reduced 4-fold in OA vs normal chondrocytes³⁵, with no difference detected between OA and normal decellularized cartilage³⁷.

Procollagen C-endopeptidase enhancer (PCOLCE)-1, and 2, are proteins which enhance procollagen C-proteinase activity, commonly identified in many proteomic studies. Furthermore, the C-terminal processed domain of PCPE (CT-PCPE) may have metalloproteinase inhibitory activity. PCOLCE1 is increased during chondrocyte differentiation of MSCs²⁶ and in OA cartilage³⁷, but is decreased with IL-1 β ^{14,15} and with TGF- β 1²⁴.

Other enzymes that could be important in the regulation of collagen metabolism are the procollagen-lysine, 2-oxoglutarate 5-dioxygenases 1 and 2. Expression of these enzymes is increased upon IL-1 β stimulation^{22,58}, and they could be responsible for an over glycosylation of collagen fibrils, observed in OA, which decreases fibril flexibility⁵⁸.

Another set of enzymes found to be greatly increased in the OA chondrocyte secretome are the lysosomal enzymes, including cathepsins, phospholipases, peroxiredoxins, ovochymase-1^{36,37}. In fact, cathepsin activity based probes have detected increased cathepsin B activity in blood and synovial fluid of early OA patients, while cathepsin S was associated with RA⁵⁹.

Other enzyme families detected in chondrocyte secretome comprise of SERPINs, superoxide dismutases (SODs), triosephosphate isomerase and lysozyme C enzymes. These are modulated by IL-1 β , but are not significantly modified with the disease, except SERPINA3. SERPINA3, also known as α 1-antichymotrypsin, is mainly located in the superficial layer of the articular cartilage³⁴ and is decreased during OA³⁵.

Miscellaneous secretome proteins

The chondrocyte secretome contains many proteins involved in cellular regulatory pathways, cell–cell or cell-ECM interactions, including chaperons, alarmins, apolipoproteins and chondrocalcin. Chondrocalcin is the C-terminal of type II collagen, associated with the calcification of cartilage ECM.

Gelsolin, clusterin and transforming growth factor-beta-induced protein ig-h3 (TGFB1) appeared in nearly all secretome studies and are one of the most highly secreted proteins by chondrocytes^{14–19,22,24,26,30,34,35,37}. Gelsolin is increased in OA compared to normal chondrocytes^{35,37}. Clusterin inhibits protein aggregation and apoptosis. This protein is detected more in healthy than osteoarthritic cartilage³⁷. TGFB1 is known to be involved in the interaction of the cell with type II collagen, and plays an important role in endochondral bone formation. Indeed, this factor is an inhibitor of mineralization. It was found to be highly secreted by OA chondrocytes, compared to normal chondrocytes¹³, and present at a greater abundance in hip OA than healthy cartilage³³.

Osteonectin is involved in ECM-cytokine interactions. Similar to osteopontin, osteonectin is highly secreted in deep layer

chondrocytes³⁴, and found to be increased in the secretome of medial condylar early OA cartilage^{16,25,27}. TGF- β 1 decreases the production of osteonectin²⁴.

Finally, thrombospondin-1,-3,-4 are all involved in cell-ECM interaction and angiogenesis, and are also secreted by chondrocytes^{13,14,17,19,21,24,32,34,35,37}. They are more abundant in the superficial layer of cartilage³⁴. Thrombospondin-3 is increased in OA compared to normal chondrocyte secretomes¹³, and thrombospondin-1 is increased with IL-1 β treatment¹⁹.

Discussion

The “omics” approach is a general exploratory approach used to investigate alterations in an enormous number of genes, transcripts, proteins, lipids and metabolites, in healthy vs diseased tissues. The challenge of using this approach is to identify those candidates that are specifically involved in the disease process. The most commonly used omics approaches include genomics, proteomics, lipidomics, metabolomics and transcriptomics. Such “omics” technologies applied to serum or urine samples have uncovered numerous new biomarkers, which are ubiquitous molecules in most cases. Therefore, it would be beneficial to refine “omics” technologies specifically to joint tissues (cartilage, bone, meniscus, synovial membrane) and compare “omics” profiles of the various articular tissues taken at different stages of evolution and correlate those amongst the various diseased tissues for the joint. In particular, the secretome of chondrocytes is of great interest because this approach provides a range of biomarkers reflecting the metabolic changes occurring in the main tissue affected by OA. Further, by investigating the chondrocyte secretome we increase the chance of identifying a circulating biomarker specific to disease, even specific to a particular joint. In this paper, we have reviewed and summarized data produced by proteomic analysis of cartilage or chondrocyte culture supernatants. Interestingly, some proteins have been found to increase in the secretome of OA explants or OA chondrocytes, while few were found decreased in comparison with normal secretome. Among these, some have been found in synovial fluid, serum or urine and proposed as potential biomarkers. For example, type II collagen^{60–64}, aggrecan^{63,65,66}, lumican^{67,68}, COMP^{64,66}, gelsolin^{67,69}, fibulin-3⁷⁰, mimecan⁶⁹, periostin^{13,71,72}, SERPINs^{67,70,73} are found in these fluids in their native form, but also post-transcriptionally modified and/or fragmented. Some of these proteins or protein fragments have demonstrated to be burden of disease, prognostic, and efficacy of intervention or diagnostic soluble biomarkers, according the Burden of Disease, Investigative, Prognostic, Efficacy of Intervention and Diagnostic (BIPEDS) criteria. Some peptides generated during type II collagen degradation have been particularly well investigated, because type II collagen is the most specific protein of cartilage. Further, their concentration appears to be modified in joint disease. As previously mentioned, many aggrecan neoepitopes are found in cartilage tissue, but those generated by aggrecanases are largely released from cartilage and found in blood circulation, while the majority of fragments generated by MMPs remain entrapped in the tissue⁵⁴.

Although the proteomic approach is a good approach to study secretome, there are some limitations associated with this method. Quantitative assessment of proteins secretome using these proteomic techniques remain hazardous, because the method promotes the quantification of some proteins relative to their solubility, binding to other components, their size, length, amino acid sequence and also their post-translational modifications. Small proteins, like cytokines, give few peptides after trypsinisation, and because at least 2 peptides are needed to assure the identification of the protein, they are probably excluded from the final observation.

In addition, there are some missing links in the secretome study. For example, there is to date few studies really dedicated and designed to investigate the dynamic kinetics of cartilage metabolism during OA disease. Most of the studies compare healthy and end-stage OA chondrocyte secretome, with the aim to discover new proteins secreted by the chondrocytes. For example, CRTAC1, HTRA1, PCOLCE, LTBP1, ECM-1, gremlin-1, clusterin, have been identified using this approach and now need more investigations to decipher their role in chondrocyte metabolism during OA pathogenesis.

The abundance of diverse models used in these proteomics studies also make them difficult to interpret. In monolayer, chondrocytes certainly have a different secretome than in their native 3D environment³¹. In explant culture, the majority of the newly synthesized proteins remains entrapped in the ECM and only degraded products are released into the supernatant¹⁸. Mechanical stimulation of the explant should be performed to mimic the physiological flow and help the protein to be released from tissue.

Posttranslational modifications like glycosylation, glycation, nitration are seldom investigated. A metabolomics approach would allow to investigate these changes and to complete knowledge coming from proteomic.

Many supposed cytoplasmic proteins are found in the chondrocyte secretome using proteomic technics. This finding seems surprising but can be explained by the secretion of ECM-derived vesicles by chondrocytes. Articular cartilage vesicles (ACVs) are 50–150 nm membrane-bound extracellular organelles found in normal articular cartilage. They were initially characterized in reference to their role in pathologic mineralization in cartilage in studies which mirrored those of matrix vesicles derived from growth plate cartilage and other normally mineralizing tissues. ACVs contain enzymes, ions and substrates necessary for mineral formation⁷⁴. The presence of these ACVs explain why cytoplasmic/membrane proteins, such as annexins, have been identified in the secretome of the chondrocytes. Transmembrane proteins can also be cleaved leading to the release in the extracellular space of extracellular part of the protein, which is the case of syndecans⁷⁵. Another explanation at the presence of membrane proteins in the secretome is the presence of apoptotic cells, mainly during OA^{76–78}. Loss of cell integrity during apoptosis further contribute to emerging proteins and pathway end-products in synovium and other bodily fluids, potentially giving rise to several biomarkers which may predict the susceptibility of an individual to develop OA.

Pro-inflammatory cytokines, prostaglandins and reactive oxygen species (ROS) activate the normally quiescent articular chondrocytes and induce them to undergo a phenotypic shift through a phenomenon recently described as “chondrosenescence”, leading to further disruption of homeostasis and metabolism in cartilage⁷. Effectively, chondrosenescence is the term that describes the age-dependent deterioration of chondrocyte function and how it undermines cartilage function in OA. Until now, this particular phenotype has not yet been investigated by proteomic technics. This should be added in the research agenda.

In conclusion, proteomic analysis of chondrocytes secretome is a promising approach for detecting changes in chondrocyte metabolism linked to OA diseases (Table 1). This review listed the advantages and disadvantages of secretome investigation using the proteomic methods. The main limitation is the lack of standardization of the culture protocols, while the main advantage is the possibility to compare different environmental condition on the secretome. We have also suggested some research perspectives, including comparison of secretome at different disease stages. Definitely, research on secretome using proteomic methods have to be encouraged with the objectives to identify new biomarkers reflecting chondrocyte metabolic changes in OA.

Author contribution

All author contributed to the collection, assembly, analysis and interpretation of data and critical revision of the article for important intellectual content.

All authors approved the final version of the manuscript.

Competing interest statement

ACBJ is a full-time employee and shareholder in Nordic Bioscience. YH is the founder and chairman of Artialis SA.

Role of the funding source

The authors' work is supported by the European Commission. A. Mobasher is the co-ordinator of the D-BOARD Consortium funded by European Commission Framework 7 programme (EU FP7; HEALTH.2012.2.4.5-2, project number 305815, Novel Diagnostics and Biomarkers for Early Identification of Chronic Inflammatory Joint Diseases). AM and YH are members of the Applied Public-Private Research enabling OsteoArthritis Clinical Headway (APPROACH) Consortium,^a a 5-year project funded by the European Commission's Innovative Medicines Initiative (IMI). APPROACH is a public-private partnership directed towards osteoarthritis biomarker development through the establishment of a heavily phenotyped and comprehensively analyzed longitudinal cohort. The research leading to these results has received partial support from the Innovative Medicines Initiative (IMI) Joint Undertaking under grant agreement no. 115770, resources of which are composed of financial contribution from the European Union's Seventh Framework programme (FP7/2007–2013) and EFPIA companies' in kind contribution. A.M. has also received funding from Arthritis Research UK (grant number 21076) The funding sources had no role in the writing of the manuscript or in the decision to submit the manuscript for publication.

Acknowledgments

The authors would like to thank the European Union's Seventh Framework Programme for research, technological development and demonstration for funding (grant agreement No. 305815).

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