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# Caracterisations structurales et fonctionnelles des glycosaminoglycannes matriciels dans le cartilage humain : pour une utilisation spécifique de GAG et de cellules souches pour la réparation du cartilage dans l'Osteoarthrose.

Sara Shamdani

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# UNIVERSITÉ — — PARIS-EST

Thèse de doctorat  
Université Paris Est  
Spécialité: Biologie Cellulaire  
Ecole Doctorale Sciences de la Vie et de la Santé

**Caracterisations structurales et fonctionnelles des  
glycosaminoglycannes matriciels dans le cartilage humain au cours  
de l'Osteoarthrose.**

**Structural and functional characterization of matrix  
glycosaminoglycans of the human cartilage in Osteoarthritis.**

**Sara SHAMDANI**

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## Abbreviations

6-OST2: 6-O-sulfotransferase 2	CRP: Complement Regulatory Protein
AChRs: Acetylcholine Receptors	CS: Chondroitin Sulfate
ACI: Autologous chondrocyte implantation	CSPGs: Chondroitin Sulfate Proteoglycans
ACL: anterior cruciate ligament ACL	CTD: C-terminal TSR domain
AD: Alzheimer's disease	Cys-SOH: cysteine sulfenic acid
ADAMTS: A Disintegrin And Metalloproteinase with Thrombospondin motifs	DAG1: $\alpha$ -Dystroglycan
AD-MSCs: Adipose-derived MSCs	DAMPs: Damage-Associated Molecular Patterns
ALK: Activin Like Kinase	DDR2: Discoidin Domain Receptor 2
ALK: Anaplastic Lymphoma Kinase	DKK2: Dickkopf-related protein 2
AMP: Adenosine monophosphate	DMD: Duchenne Muscular Dystrophy
AMPK: 5'-AMP Activated Protein Kinase	DNA: Deoxyribonucleic Acid
AT: Adipose Tissue	DS: Dermatan Sulfate
ATP: Adenosine triphosphate	ECD: Extracellular Domain
BDNF: Brain-derived Neurotrophic Factor	ECM: Extracellular Matrix
BM: Bone Marrow	ELISA: Enzyme-linked immunosorbent assay
BMI: Body Mass Index	EMEA: European Medicine Agency
BM-MSCs: Bone Marrow-Derived-Mesenchymal Stem Cells	ERK1/2: Extracellular-Signal-Regulated Kinase 1/2
BMP: Bone morphogenetic proteins	EULAR: European Association of Rheumatology
C6ST: Chondroitin 6-sulfotransferase	EXT: Exostosin
CCL2: CC chemokine ligand 2	FGF: Fibroblast Growth Factors
CD: Cluster of Differentiation	FGFR: Fibroblast Growth Factors Receptor
CDKN2A: Cyclin-Dependent Kinase Inhibitor 2A	FLS: Fibroblast-Like Synoviocytes
CDMPs: Cartilage-Derived Morphogenetic Proteins	FN: Fibronectin
ChM-1: Chondromodulin-1	Foxp3: Forkhead box P3
CHPF: Chondroitin Polymerization Factor	FTDP: Frontotemporal dementia and parkinsonism
CNS: Central Nervous System	FZD: Frizzled receptor
COL10A1: Collagen type 10 alpha 1	GAG: Glycosaminoglycan
COX-2: Cyclooxygenase-2	Gal: Galactose
	GalTs: Galactosyltransferases

GDF: Growth/Differentiation Factor	IPAH: Idiopathic Pulmonary Arterial Hypertension
GF: Growth Factor	iPS: Induced Pluripotent Stem Cells
GI: Gastrointestinal	JAK: Janus Kinase
GlcA: Glucuronic Acid	KD: Kilo Dalton
GlcNAc: N-acetyl-glucosamine	KL: Kellgren-Lawrence
GlcNS: N-sulfated –Glucosamine namido	KS: Keratan Sulfate
GPI: glycosyl phosphatidylinositol	KSPGs: Keratin Sulfate Proteoglycans
GS: Glucosamine sulfate	LAMC1: Laminin- $\gamma$ 1
HA: Hyaluronic Acid	LKB1: Liver Kinase B1
HARP: Heparin affinity regulatory peptide	LPs: link proteins
HAS: Hyaluronan synthase	LRP: LDL Receptor-Related Protein
HB-GAM: Heparin- binding growth-associated molecule	LRP: Low-density lipoprotein receptor-related protein
HGBFs: Heparin Binding Growth Factors	MACI: Matrix-induced autologous chondrocyte implantation
HBP: Heparin Binding Proteins	MAPK: Mitogen-activated protein kinase
HCA: Hydrocortisone Acetate	MCP-1: Monocyte Chemoattractant Protein 1
hESC: Human Embryonic Stem Cells	M-CSF: Macrophage Colony-Stimulating Factor
HIF: Hypoxia-inducible factor	MEKK: mitogen-activated protein Kinase/ERK kinase kinase 3
HS: Heparan Sulfate	MHC II: Major Histocompatibility Complex class II
HS2ST: HS 2-O-sulfotransferase	MIP-1a: Macrophage Inflammatory Protein 1a
HS3ST2: Heparan Sulfate-Glucosamine 3-Sulfotransferase 2	MK: Midkine
HSPGs: Heparan Sulfate Proteoglycans	MMP: Matrix Metalloproteinase
hTNFtg: human TNF transgenic	MPA: Methylprednisolone Acetate
IA: Intra-articular	MRI: Magnetic Resonance Imaging
IDO: Indoleamine 2, 3-Dioxygenase	mRNA: messenger Ribonucleic Acid
IdoA: Iduronic Acid	MSCs: Mesenchymal stem cells
IGF: Insulin-like Growth Factor	mTOR: Mechanistic target of Rapamycin
IGFBPs: IGF-Binding Proteins	MuSK: Muscle-Specific Kinase
IHH: Indian Hedgehog	
IL: Interlukine	

MW: Molecular Weight	Ras: Rat sarcoma small GTP-ase
NDST: N-deacetylase/N-sulfotransferase	RNA : Ribonucleic Acid
NFκB: Nuclear factor-kappa B	ROS: Reactive Oxygen Species
NLRs: Nod-Like Receptors	RTKs: Receptor Tyrosine Kinases
NMJ: Neuromuscular Junction	RUNX2: Runt-related Transcription Factor 2
NOS: Nitric Oxide Synthases	SASP: Senescence-Associated Secretory Phenotype
NSAIDs: Nonsteroidal Anti-Inflammatory Drugs	SDCs: Syndecans
NTD: N-terminal TSR domain	SF: Synovial Fluid
OA: Osteoarthritis	SHAP: Serum-derived Hyaluronan-Associated Protein
OP-1: Osteogenic Protein-1	sIL-6R: soluble IL-6 receptor
OPG: Osteoprotegerin	SLRPs: Small leucine-rich proteoglycans
OSF-1: Osteoblast-specific factor 1	SM: Synovial Membrane
PCP: Planar Cell Polarity	SM-MSCs: Synovial Membrane-Derived MSCs
PDGFs: Platelet-Derived Growth Factors	SOD2: Superoxide Dismutase 2
PF: Pulmonary Fibrosis	SOX: Sex determining region Y box
PGs: Proteoglycans	STAT: Signal Transducer and Activator of Transcription
PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase	STs: Sulfotransferases
PIGF: Placental Growth Factor	TCA: Triamcinolone Acetonide
PKC: Protein kinase C	TGFβ: Transforming Growth Factor beta
PLA2: Phospholipase A2	TIMP: Tissue inhibitors of metalloproteinases
PRR: Particle Recognition Receptors	TLR: Toll-Like Receptors
PTB: Phosphotyrosine binding domain	TM: Transmembrane
PTHrP: Parathyroid Hormone-related Peptide	TNFα: Tumor Necrosis Factor-alpha
PTK: Protein tyrosine kinase	TSG6: TNF-Stimulated Gene 6
PTN: Pleiotrophin	TSP-1: Thrombospondin-1
PTPRZ: Protein Tyrosine Phosphatase Zeta	VEGF: Vascular Endothelial Growth Factor
RANKL: Receptor Activator of NFκB Ligand	VEGFR: Vascular Endothelial Growth Factor Receptor
RANTES: Regulated and Normal T cell Expressed and Secreted	WHO: World Health Organization
	XylT: Xylosyltransferase

## ABSTRACT IN ENGLISH

Osteoarthritis (OA) is the most prevalent joint disease with increasing socio-economic impact due to population aging, obesity epidemic, and finally absence of an efficient medical treatment that can repair cartilage. OA is characterized by degradation of articular cartilage, hypertrophy and apoptosis of chondrocytes, subchondral bone remodeling and joint synovial inflammation. Cartilage extracellular matrix (ECM) consists of collagens, glycoproteins and proteoglycans (PGs) that are composed of Glycosaminoglycans (GAGs) linked to core proteins, presents in the ECM or at the cell surface. GAGs are linear polysaccharidic sulfated chains including Heparine/Heparan Sulfate (Hep/HS), Chondroitin Sulfate (CS) and Keratan Sulfate (KS) families. Hyaluronic acid (HA) is a particular un-sulfated GAG not associated to core protein. In cartilage, one of the major component in ECM is aggrecan, a CS/KS PG that form aggregate through HA interaction. During the aging process, changes in PGs quality pave the way for OA and studies are focus on aggrecans and CS catabolism since 60 years. CS expression levels, chain size, sulfation patterns evolved during OA, affecting the mechanical properties of ECM. However, treatments based on visco-supplementation with CS local injections have demonstrated their limit since cartilage repair is not induced. Although they are rarely found in adult cartilage, HSPGs are expressed by chondrocytes also and their relevance was demonstrated mainly during bone development. HS chains are very important homeostatic regulators because they are able to bind and regulate the activity of several heparin binding proteins (HBP) (growth factors, cytokines, chemokines, morphogens), protecting them against proteolysis and potentiating their binding to their receptors. These interactions provide a stock of regulatory factors that can be release by selective degradation of the HS chains too. All these regulatory effects are mediated through the complex sulfation/acetylation pattern of HS chains but no data are available on this aspect during OA.

In this context, the goal of this Thesis was to characterize the evolution of HS chemical signature and functionality during OA, in cartilage and synovial fluids, that are two important compartments involved in the physiopathological evolution of this degenerative disease associated to aging. In collaboration with Rheumatology and Orthopedic clinical teams from Henri Mondor Hospital, a quantitative evaluation of HS and CS amount in control versus OA human cartilage and synovial samples was correlated to the structural damage severity. According to the tools of the CRRET's lab glycomic platform, structural changes on HS and CS sulfated disaccharides compositions was observed using HPLC. These structural features were correlated to functional changes on HBP affinities, such as FGF-2, VEGF and PTN, through ELISA based competition assay. Finally, GAGs from OA have different abilities to modulate properties (adhesion proliferation, phenotype...) of Mesenchymal Stem Cells, chondrocytes, fibroblast and endothelial cells. These results clearly make the proof that modifications of HS structures and functions could be involved in the evolution of cartilage homeostasis and pave the way for altered pathological processes during OA. In the discussion section of this thesis we try to propose some hypothesis on the type of properties that's HS modulate and the family of HSPG

that could bring such relevant HS chains in cartilage and synovium. This is important because it should permit to identify which kind of signaling pathways and molecular cascade of regulation could be involved. Moreover we try to highlight also a potential relevant role of these specific HS signatures in the chronic inflammatory process that take place during OA, between cartilage and synovium. This project is clearly positioned as a fundamental research that will permit to gain knowledge on the mechanisms regulating cartilage cells/matrix interactions during OA. However all the glycomic data that we will describe should permit also to develop more translational projects in the future: We propose to discuss that cellular therapeutic strategies currently used to treat OA, based on Mesenchymal stem cells or chondrocytes, could be optimized with glycanic products (mimetics or antagonists) based on a better understanding of the biological role of HS in cartilage.

Key words: Osteoarthritis, Cartilage, Chondrocyte, Glycosaminoglycans, Extracellular matrix, Heparine/Heparan Sulfate, Sulfation.

## ABSTRACT IN FRENCH

L'arthrose est la maladie articulaire la plus répandue avec un impact socio-économique croissant en raison du vieillissement de la population, de l'épidémie d'obésité et enfin de l'absence d'un traitement médical efficace capable de réparer le cartilage. L'arthrose est caractérisée par la dégradation du cartilage articulaire, l'hypertrophie et l'apoptose des chondrocytes, le remodelage osseux sous-chondral et l'inflammation synoviale articulaire. La matrice extracellulaire du cartilage (MEC) est constituée de collagènes, de glycoprotéines et de protéoglycanes (PG) composés de glycosaminoglycanes (GAG) liés à des protéines de nucléocapside présentes dans l'ECM ou à la surface cellulaire. Les GAG sont des chaînes sulfatées polysaccharidiques linéaires comprenant les familles Heparine / Heparan Sulfate (Hep / HS), Chondroïtin Sulfate (CS) et Keratan Sulfate (KS). L'acide hyaluronique (HA) est un GAG non sulfaté particulier non associé à la protéine de nucléocapside. Dans le cartilage, l'un des principaux composants de la MEC est l'aggrécane, une PG CS / KS qui forme un agrégat par interaction HA. Au cours du processus de vieillissement, les changements dans la qualité des PG ouvrent la voie à l'arthrose et les études se concentrent sur les aggrécanes et le catabolisme CS depuis 60 ans. Les niveaux d'expression CS, la taille de la chaîne, les schémas de sulfatation ont évolué au cours de l'arthrose, affectant les propriétés mécaniques de l'ECM. Cependant, les traitements basés sur la visco-supplémentation avec des injections locales de CS ont démontré leur limite puisque la réparation du cartilage n'est pas induite. Bien qu'ils soient rarement trouvés dans le cartilage adulte, les HSPG sont également exprimés par les chondrocytes et leur pertinence a été démontrée principalement au cours du développement osseux. Les chaînes HS sont des régulateurs homéostatiques très importants car elles peuvent lier et réguler l'activité de plusieurs protéines liant l'héparine (HBP) (facteurs de croissance, cytokines, chimiokines, morphogènes), les protégeant contre la protéolyse et potentialisant leur liaison à leurs récepteurs. Ces interactions fournissent un stock de facteurs régulateurs qui peuvent être libérés par une dégradation sélective des chaînes HS également. Tous ces effets régulateurs sont médiés par le schéma de sulfatation / acétylation complexe des chaînes HS, mais aucune donnée n'est disponible sur cet aspect pendant l'arthrose. Dans ce contexte, l'objectif de cette thèse était de caractériser l'évolution de la signature chimique et de la fonctionnalité du SH au cours de l'arthrose, dans le cartilage et les liquides synoviaux, deux compartiments importants impliqués dans l'évolution physiopathologique de cette maladie dégénérative associée au vieillissement. En collaboration avec les équipes rhumatologiques et orthopédiques de l'hôpital Henri Mondor, une évaluation quantitative de la quantité de HS et de CS dans le contrôle par rapport à l'arthrose du cartilage humain et des échantillons synoviaux a été corrélée à la gravité des dommages structurels. Selon les outils de la plate-forme glycomique de laboratoire du CRRET, des changements structurels sur les compositions de disaccharides sulfatés HS et CS ont été observés en utilisant la HPLC. Ces caractéristiques structurelles ont été corrélées à des changements fonctionnels sur les affinités HBP, tels que FGF-2, VEGF et PTN, par le biais d'un test de compétition basé sur ELISA. Enfin, les GAG de l'OA ont des capacités différentes pour moduler les propriétés (prolifération d'adhésion, phénotype ...) des cellules



souches mésenchymateuses, des chondrocytes, des fibroblastes et des cellules endothéliales. Ces résultats apportent clairement la preuve que des modifications des structures et des fonctions HS pourraient être impliquées dans l'évolution de l'homéostasie du cartilage et ouvrir la voie à des processus pathologiques altérés pendant l'arthrose. Dans la section de discussion de cette thèse, nous essayons de proposer quelques hypothèses sur le type de propriétés que HS modulent et la famille de HSPG qui pourrait apporter de telles chaînes HS dans le cartilage et la synoviale. Ceci est important car il devrait permettre d'identifier quels types de voies de signalisation et de cascade moléculaire de régulation pourraient être impliqués. De plus, nous essayons de mettre en évidence un rôle potentiellement pertinent de ces signatures HS spécifiques dans le processus inflammatoire chronique qui se déroule pendant l'arthrose, entre le cartilage et la synovie. Ce projet est clairement positionné comme une recherche fondamentale qui permettra d'acquérir des connaissances sur les mécanismes régulant les interactions cartilagineuses / matricielles au cours de l'arthrose. Cependant, toutes les données glycomiques que nous allons décrire devraient également permettre de développer plus de projets translationnels dans le futur: Nous proposons de discuter que les stratégies thérapeutiques cellulaires actuellement utilisées pour traiter l'arthrose, basées sur des cellules souches mésenchymateuses ou des chondrocytes, pourraient être optimisées avec des produits glycaniques ( mimétiques ou antagonistes) sur la base d'une meilleure compréhension du rôle biologique de l'HS dans le cartilage.

Mots clés: Arthrose, Cartilage, Chondrocyte, Glycosaminoglycanes, Matrice extracellulaire, Héparine / Héparane Sulfate, Sulfatation.

# INTRODUCTION

# Chapter 1: PROTEOGLYCANS AND GLYCOSAMINOGLYCANS OF THE ECM

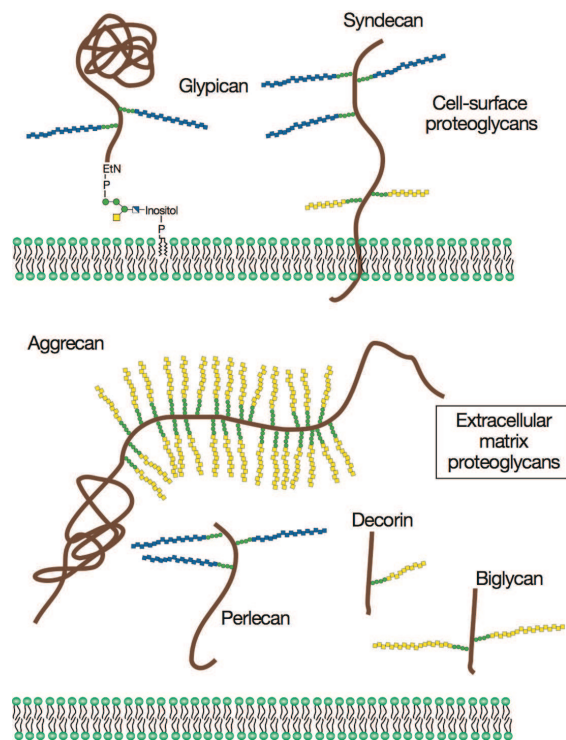
The Extracellular Matrix (ECM) is the non-cellular component present within all tissues and organs, and provides not only essential physical scaffolding for the cellular constituents but also initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis. The ECM is composed of two main classes of macromolecules: fibrous proteins and Proteoglycans (PGs) (Järveläinen et al., 2009). The main fibrous ECM proteins are collagens, elastins, fibronectins and laminins (Alberts et al., 2007)\*.

PGs fill the majority of the extracellular interstitial space within the tissue in the form of a hydrated gel. PGs have a wide variety of functions that reflect their unique buffering, hydration, binding and force-resistance properties. PGs could be presented intracellularly, at the cell surface or in the ECM. Their biological roles are highly diversified, as yet rather poorly understood. They can have effects on various cellular processes such as cell adhesion, motility, and proliferation and tissue morphogenesis. Most of these effects depend on binding of PGs to the Glycosaminoglycan (GAG) chains, which are able to play essential roles in the architecture of the ECM and in the regulation of cellular functions and tissue homeostasis by interacting with Heparin Binding Growth Factors (HBGFs) and to potentiate their half-life, activities and interactions with high affinity receptors. That is why over the past few decades more and more attention was attracted to glycol-structures of cell surface or ECM. Any changes in both the organization of the ECM or in its GAG components will further affect the cell and tissue integrities, making the tissue potentially vulnerable.

GAGs can be altered by post-translational modifications of their residues resulting in enormous structural diversity. This structural diversity is the basis for the wide range of biological activities of GAGs. Thus, the structural analysis of GAGs is key to understanding their biological functions (Tsang et al., 2010).

## 1. Proteoglycans, structures and functions

Proteoglycans (PGs) are one of the largest components of the ECM; consist of a core protein to which one or more GAG chains are covalently attached (**Figure 1**). The number of GAG chain substituents (generally on serine residues) on a protein core may vary from one (like decorin) to > 100 (like aggrecan) (Parish, 2006). The chains are negatively charged because of the presence of sulfates and uronic acids (Ly et al., 2010).



**Figure 1: Structure of PGs.**

PGs consist of a protein core which is shown in brown and one or more covalently attached GAG chains instance for aggrecan HS in blue and CS/DS in yellow. Adapted from the Book of Essentials of Glycobiology by Jeffrey D Esko et al., 2009.

The protein component of PGs is synthesized by ribosomes and translocated into the lumen of the rough endoplasmic reticulum. Glycosylation of the PG occurs in the Golgi apparatus in multiple enzymatic steps. First, a special link tetrasaccharide is attached to a Ser side chain on the core protein to serve as a primer for polysaccharide growth. Then sugars are added one at a time by glycosyl transferase. The completed PG is then exported in secretory vesicles to the ECM of the tissue (Vynios, 2014). Due to the complexity of the macromolecules, their biosynthesis can be examined separately, that is, biosynthesis of core proteins and biosynthesis of carbohydrate chains. The studies have shown that core protein biosynthesis follows the general rules of protein biosynthesis, with the modifications required for protein secretion. The addition of N-linked oligosaccharides close to the N-terminal end becomes very early, which are synthesized onto dolichol phosphate, as it has been supported by experiments using tunicamycin as inhibitor of N-glycosylation (Vynios, 2014). Therefore, all the enzymatic machinery needed for N-linked oligosacchride precursor biosynthesis and processing after its incorporation onto the core protein participates during the translation step. It has also been shown that once core protein is synthesized, it spends enough time to move to the Golgi apparatus for GAG biosynthesis.

There are four major classes of proteoglycans with distinct forms and functions: the intracellular, cell-surface, pericellular and extracellular proteoglycans (Iozzo and Schaefer, 2015a).

These PGs are not only structural component, also through their interactions with proteins, PGs mediate many biological processes including cell–cell and cell–matrix interactions, growth factor sequestration, chemokine and cytokine activation, microbial recognition, tissue morphogenesis during embryonic development, cell migration and proliferation. These interactions are due to the core protein but also to the GAG chains. Also PGs can be named ‘full-time’ like five classes of HSPGs that always carry HS chains. For example, syndecan-1 has five attachment sites for GAG but not all of the sites are used equally. Other proteoglycans can be ‘part-time’ that may exist with or without a GAG chain or with only a truncated oligosaccharide (Parish, 2006).

### **Proteoglycan function**

PG can function as co-receptors according to their ability to bind and present ligands to their respective signaling receptors. They are able to regulate signaling in a cell autonomous and non-cell autonomous manner through regulated ectodomain shedding and localizing signaling. Through a combination of these functions, PG signaling co-receptors mediate essential roles in regulating physiological and pathophysiological processes in a tissue and context dependent manner, including the effects on adhesion, migration and invasion (Couchman, 2010).

PG can be either transmembrane receptors or Glycosylphosphatidylinositol (GPI) anchored, have a large Extracellular Domain (ECD) with GAG attachment sites, a transmembrane domain and either no cytoplasmic domain or a short cytoplasmic domain (200 amino acids or less) that lacks intrinsic kinase activity. The most commonly found modifications on the PG are heparan sulfate and chondroitin sulfate. The heparin sulfate modifications of PG signaling co-receptors can mediate interactions with Fibroblast Growth Factor (FGF) or ECM proteins (Andres et al., 1992; Burgess and Maciag, 1989). PG can also bind ligands and extracellular matrix proteins through motifs in their core proteins. By modulating ligand binding, PG signaling co-receptors can regulate ligand function.

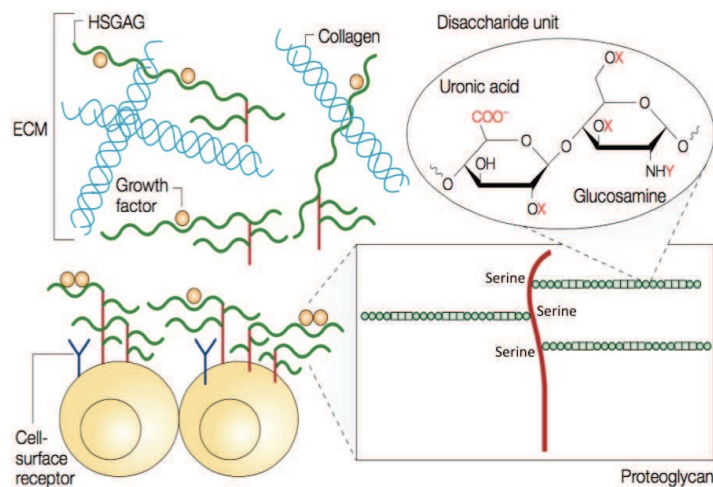
Aside from the GPI-anchored members of the glypican family, PG have short cytoplasmic domains that do not contain intrinsic enzymatic activity. However, these cytoplasmic domains are often serine/threonine rich, are frequently phosphorylated and often have PSD-95/Dlg/ZO-1 (PDZ) binding motifs at their extreme carboxy terminus. Through these motifs, the cytoplasmic domains of PG signaling co-receptors interact with scaffolding molecules to regulate the internalization, trafficking and signaling of these co-receptors and their associated receptors.

PG can directly or indirectly regulate attachment of cells to the ECM via integrins and through regulation of the formation of focal adhesions. The most well-characterized PG signaling co-receptors in terms of regulating cell-matrix adhesion are the syndecans, which are necessary for the formation of stable focal adhesions on fibronectin coated substrates through their interactions with integrins and fibronectin (Tumova et al., 2000). In addition to regulating migration via alterations in adhesion, PG can provide specific directional cues based on their environment ultimately affecting physiological processes including cell migration and cancer cell

dissemination and metastasis. For example, syndecans have a specific role in regulating the directional migration of neural crest cells during development *in vivo*, with the Planar Cell Polarity (PCP) pathway, a non-canonical Wnt signaling pathway, interacting with Syndecan-4 to control the direction in which cell protrusions are generated (Carmona-Fontaine et al., 2008).

PGs can also be classified according to size and GAG composition, which are consisting of Heparan Sulfate Proteoglycans (HSPGs), Chondroitin Sulfate Proteoglycans (CSPGs), Keratin Sulfate Proteoglycans (KSPGs) and Small Leucine-Rich Proteoglycans (SLRPs) (**Table 1**).

HSPGs act as co-receptors for heparin-binding growth factors and cytokines (van Horssen et al., 2002). Two major classes of HSPG are syndecans, which have a transmembrane domain in their core proteins, and its cytoplasmic domain contains sequences that bind to cytoskeletal proteins and serve as substrates for cellular kinases, acting in this way as signaling molecules able to bind various soluble and insoluble effectors that promote cell adhesion to ECM and to adjacent cells, and glypicans, which are attached to the cell membrane by Glycosyl phosphatidylinositol (GPI)-anchors (Papy-Garcia and Albanese, 2017) (**Figure2**).



**Figure 2: Structure and biology of heparan-sulphate proteoglycans.**

HSPGs exist at the cell surface and also in the ECM, where they are bound to protein core (red lines) to form proteoglycans. Owing to the highly hydrophilic nature of the HSPG chains. The biosynthesis of HSPG leads to regions of aggregate chemical character within the polysaccharide chain. The green squares indicate highly sulphated regions of the polysaccharide chain, whereas the green circles indicate regions of undersulphation. Zooming in further, all HSPG consist of a disaccharide unit of uronic acid (either iduronic acid or glucuronic acid) that is attached to a glucosamine. All potential sites of modification are shown in red. The diversity of chemical sequences that is contained within the polysaccharide chain enables HSPG to bind to and modulate the activity of various growth factors (orange circles), chemokines and enzymes at the cell surface and in the ECM (Sasisekharan et al., 2002).

CSPGs like aggrecan and small PG such as decorin, biglycan, epiphygan and fibromodulin (Iozzo, 1998). Aggrecan is the common type of PGs in the cartilage tissue (Wu et al., 2010). Decorin is involved in the regulation of important biological functions like matrix organization, cell adhesion, migration and proliferation (Gallagher, 1989; Iozzo, 1999). Biglycan is found in many connective tissues, such as skin, bones and blood vessels (Bianco et al., 1990; Fisher et al., 1989).

Lumican, keratocan and decorin are major KSPG which could be found in several tissues (Blochberger et al., 1992; Funderburgh, 2000).

The extracellular matrix also contains the largest class of proteoglycans, the so-called SLRPs that are the most abundant products in terms of gene number. These SLRPs can function both as structural constituent and as signaling molecules, especially when tissues are remodeled during cancer, diabetes, inflammation and atherosclerosis. SLRPs interact with several Receptor Tyrosine Kinases (RTKs) and Toll-like receptors, thereby regulating fundamental processes including migration, proliferation, innate immunity, apoptosis, autophagy and angiogenesis (Iozzo and Schaefer, 2015a). It is suggested that SLRPs would be directly involved in regulating organ size and shape during embryonic development and homeostasis (Iozzo and Karamanos, 2010).

Proteoglycans	Core protein(KD)	Number of GAG chain	Tissue distribution	
CS	Aggrecan family			
	Aggrecan	208-220	~100	Secreted; cartilage
	Versican/PG-M	265	12-15	Secreted; connective tissue cells; aorta; brain
	Neurocan	145	1-2	Secreted; brain
	Brevican	96	0-4	Secreted; brain
	SLRPs			
	Decorin	36	1	Secreted; connective tissue cells
	Biglycan	38	1-2	Secreted; connective tissue cells
	Other examples			
	Leprecan	82	1-2	Secreted; basement membranes
	Type IX collagen, $\alpha$ 2 chain	68	1	Secreted; cartilage; vitreous humor
	Phosphacan	175	2-5	Membrane bound; brain
	Thrombomodulin	58	1	Membrane bound; endothelial cells
	CD44	37	1-4	Membrane bound; lymphocytes
	NG2	251	2-3	Membrane bound; neural cells
	Invariant chain	31	1	Membrane bound; antigen-processing cells
Serglycin	10-19	10-15	Intracellular granules; myeloid cells	
HS	Perlecan	400	1-3 HS	Secreted; basement membranes; cartilage
	Agrin	200	1-3 HS	Secreted; neuromuscular junctions
	Collagen type XVIII	147	2-3 HS	Secreted; basement membranes
	Syndecans 1-4	31-45	1-2 CS	Membrane bound; epithelial cells and fibroblasts



			1-3 HS	
	Betaglycan	110	1 HS	Membrane bound; fibroblasts
			1 CS	
	Glypicans 1-6	~60	1-3 HS	Membrane bound; epithelial cells and fibroblasts
	Serglycin	10-19	10-15 heparin/CS	Intracellular granules; mast cells
KS I	SLRPs			
	Lumican	37		Secreted; broad
	Keratocan	37		Secreted; broad, but sulfated only in cornea
	Fibromodulin	59		Secreted; broad
	Mimecan	25		Secreted; broad, but sulfated only in cornea
	Other examples			
	SV2	80		Membrane bound; synaptic vesicles
KSII	Claustrin	105		Membrane bound; CNS
	Aggrecan (human)	200		Secreted; cartilage

**Table 1: Example of HSPGs, CSPGs and KSPGs.**

After Book of Essentials of Glycobiology by Jeffrey D Esko et al., 2009

The interaction between GAGs and surrounding proteins can have profound physiological effects on processes such as hemostasis, lipid transport and absorption, cell growth and migration, and development. Binding to GAGs can result in immobilization of proteins at their sites of production and in the matrix for future mobilization, regulation of enzyme activity, binding of ligands to their receptors, and protection of proteins against degradation. GAGs and the PGs they form are large negatively charged molecules that attract cations and water molecules, leading to the formation of a hydrated gel. These molecules comprise the ‘ground substance’ in connective tissues that provides support while permitting cell migration and diffusion of nutrients and soluble signaling molecules within the extracellular matrix.

An important additional function provided by many PGs is binding of growth factors, cytokines, chemokines and morphogens. Attachment of these soluble mediators to PGs can influence their activity in several ways. For proteins in the FGF family, association with proteoglycans containing heparin or heparan sulfate greatly enhances their biological activity. Many of the chemokines also display significant affinity for PGs containing heparin and heparan sulfate. Immobilization of chemokines on to PGs of the extracellular matrix allows establishment of solid-phase gradients that can direct the migration of leukocytes traveling through tissue. These interactions provide a stock of regulatory factors that can be released by selective degradation of the heparin chains also. All these regulatory effects are mediated through the complex sulfation/acetylation pattern of HS chains.

## 2. Glycosaminoglycans structures and biosynthesis

Glycosaminoglycan (GAG) are linear, sulphated, negatively charged polysaccharides with molecular weights of roughly 10–100 kDa (Mende et al., 2016). Two main types of GAGs can be distinguished: Non-sulphated GAGs include Hyaluronic acid (HA), which is not associated to core protein and sulphated GAGs include Chondroitin Sulfate (CS), Dermatan Sulfate (DS), Keratan Sulfate (KS), Heparin and Heparan Sulfate (HS) which are associated to a core protein. GAG chains are composed of disaccharide repeating units (**Figure 3**). The repeating units are composed of uronic acid (D-glucuronic acid or L-iduronic acid) and amino sugar (D-galactosamine or D-glucosamine) (Mende et al., 2016). Hence, GAGs species differ according to the type of hexosamine, hexose or hexuronic acid unit that they contain, as well as the geometry of the glycosidic linkage between these units (Taylor and Gallo, 2006). CS and DS, which contain galactosamine, are called galactosaminoglycans, whereas heparin and HS, which contain glucosamine, are called glucosaminoglycans. At physiological pH, all carboxylic acid and sulphate groups are deprotonated, giving GAGs very high negative charge densities (heparin has the highest negative charge density of any known biomolecule) (Gandhi and Mancera, 2008).

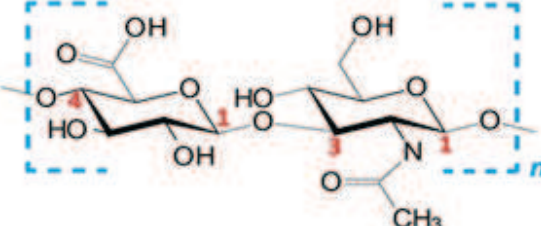
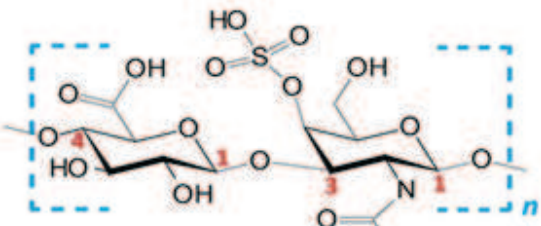
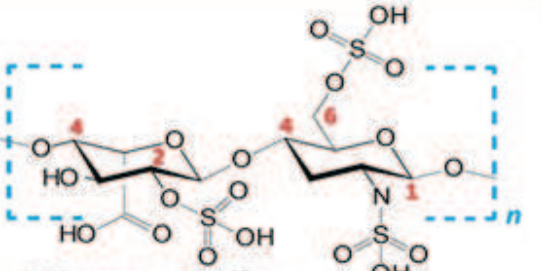
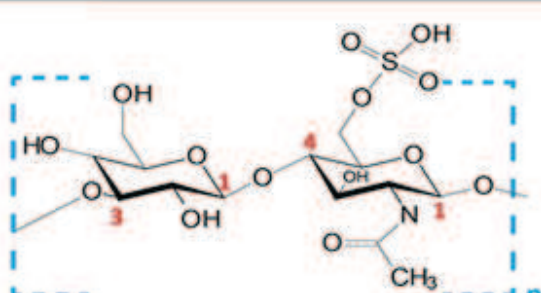
 <p><b>D-glucuronate (GlcA)</b>      <b>GlcNAc</b></p>	<p><b>Hyaluronates:</b>  composed of D-glucuronate (GlcA) plus GlcNAc, linkage is <math>\beta(1,3)</math></p>
 <p><b>D-glucuronate (GlcA)</b>      <b>GalNAc-4-Sulfate</b></p>	<p><b>Chondroitin 4- and 6-sulfates:</b>  composed of D-glucuronate (GlcA) and GalNAc-4- or 6-sulfate, linkage is <math>\beta(1,3)</math>  (the figure contains GalNAc 4-sulfate)</p>
 <p><b>L-iduronate-2-sulfate</b>  <b>N-sulfo-GlcNAc-6-sulfate</b></p>	<p><b>Heparin and Heparan sulfates:</b>  composed of L-iduronate(DoA: many with 2-sulfate) or D-glucuronate (GlcA: many with 2-sulfate) and N-sulfo-D-glucosamine-6-sulfate, linkage is <math>\alpha(1,4)</math> if DoA, <math>\beta(1,4)</math> if GlcA. heparans have less overall sulfate than heparins</p>
 <p><b>D-galactose</b>      <b>GlcNAc-6-Sulfate</b></p>	<p><b>Keratan sulfates:</b>  composed of galactose plus GlcNAc-6-sulfate, linkage is <math>\beta(1,4)</math></p>

Figure 3: Structure of glycosaminoglycans.

After <https://themedicalbiochemistrypage.org/glycans.php>

With the exception of hyaluronic acid, GAGs are invariably attached to core proteins, collectively known as proteoglycans (Prydz and Dalen, 2000). Interestingly, the core proteins may also serve functions independently of the glycan chains (Saied-Santiago and Bülow, 2017).

## 2.1. Heparin and Heparan sulfate

Heparin and heparin sulfate share a common biosynthetic route, in which nonuniform modifications are made to the chains resulting in sequence diversity (Esko and Lindahl, 2001). The uronic acid can be either  $\beta$  1-4 Glucuronic Acid (GlcA) or  $\alpha$  1-4 Iduronic acid (IdoA), and these can be sulfated at position 2. The amino sugar is a D-glucosamine, as either N-acetylglucosamine (GlcNAc) or N-sulfated  $\beta$ -glucosamine namido (GlcNS) in position 2 or as some evidence suggests it may remain unsubstituted. Furthermore, glucosamine derivatives can be O-sulfated at position 6 and, more rarely at position 3 (**Figure 3**).

The difference between HS and heparin is quantitative and not qualitative. Often, HS chains also contain domains of extended sequences having low sulphation and higher level of acetylated glucosamine compared with heparin, as illustrated in (**Table 2**). The non-sulphated regions that have a GlcA- GlcNAc (acetylated glucosamine) sequence are the most common in the HS chain, with IdoA-containing sulphated regions (called S-domains) usually of about 5–10 disaccharides. There are also relatively minor proportions of mixed sequences, which contain both GlcNSO<sub>3</sub> and GlcNAc (called NA-domain). A substantial proportion of the HS chain may consist of alternating GlcA–GlcNAc residues with no sulphate substitution (Gandhi and Mancera, 2008). Heparin is known to be highly evolutionarily conserved with similar structures found in a broad range of vertebrate and invertebrate organisms such as turkey (Warda et al., 2003a), whale (Ototani et al., 1981), camel (Warda et al., 2003b), mouse (Bland et al., 1982), human (Linhardt et al., 1992), lobster (Hovingh and Linker, 1982), shrimp (Dietrich et al., 1999), mussel (Dietrich et al., 1999), marine clam species (Pejler et al., 1987) and crab (Medeiros et al., 2000). Heparin is synthesized by and stored exclusively in mast cells, whereas HS is expressed on different cell surfaces and in the ECM as part of a PG (Gandhi and Mancera, 2008). Both of them have been shown to regulate activities of locally synthesized proteolytic enzymes, morphogens, chemokines and growth factors, ECM proteins and the cell surface proteins of pathogens (Wang et al., 2017).

	Heparan sulfate	Heparin
Size	10–70 kD	7–20 kD
Sulfate/hexosamine ratio	0.8–1.8	1.8–2.6
GlcNSO <sub>3</sub>	40–60%	≥80%
Iduronic Acid	30–50%	≥70%
Protein cores	Perlecan, agrin, syndecans, glypicans, ...	Serglycine
Site of synthesis	Virtually all cells	Mast cells

**Table 2: Different structure between Heparan sulfate and Heparin.**

After Book of Essentials of Glycobiology by Jeffrey D Esko et al., 2009.\*

HS consists of repeating units of GlcNAc and GlcA that are assembled to the non-reducing end of the GAG by HS copolymerases, exostosin 1 (EXT1) and exostosin 2 (EXT2) (Lin et al., 2000; Sugahara and Kitagawa, 2002). Heparin is distinguished from HS by being more heavily modified, although HS can contain heparin-like regions. Heparin is much less abundant in vivo than HS and exists primarily in mast cells (Rose and Page, 2004).

Modification reactions on the HS chain occur while the chain is being polymerized and begins with the N-deacetylase/N-sulfotransferase (NDST) enzymes that remove the N-acetyl groups and replace them with sulfate groups. This modification is a prerequisite for all subsequent modifications (Sugahara and Kitagawa, 2002). The activity of the HS 2-O-sulfotransferase (HS2ST) is specific for HS/heparin. HS 6-O-sulfotransferases add 6-O- sulfates to N-sulfated glucosamines and three isoforms of this enzyme have been described (HS6ST1, 2, and 3). Each enzyme has slightly different substrate specificities and expression patterns. Finally, the most unique sulfation modification of HS takes place with addition of a 3-O-sulfate to the glucosamine residue by HS 3-O-sulfotransferases. Six different isoforms of this enzyme have been described (HS3ST1, 2, 3A, 3B, 4, and 5) (Kusche-Gullberg and Kjellén, 2003a; Sugahara and Kitagawa, 2002). As with the HS6STs, each HS3ST prefers specific substrate sequences and have been suggested to be involved in producing HS sequences important in specific biological processes (**Figure 4**).

HS chains degradation occurs both at the intracellular and extracellular levels. Intracellular catabolism takes place after membrane associated HSPG endocytosis and transfer to the lysosome wherein they undergo degradations. During this catabolic process, lysosomal heparanases cleave the HS chains from the PGs core and degrade them completely to small HS

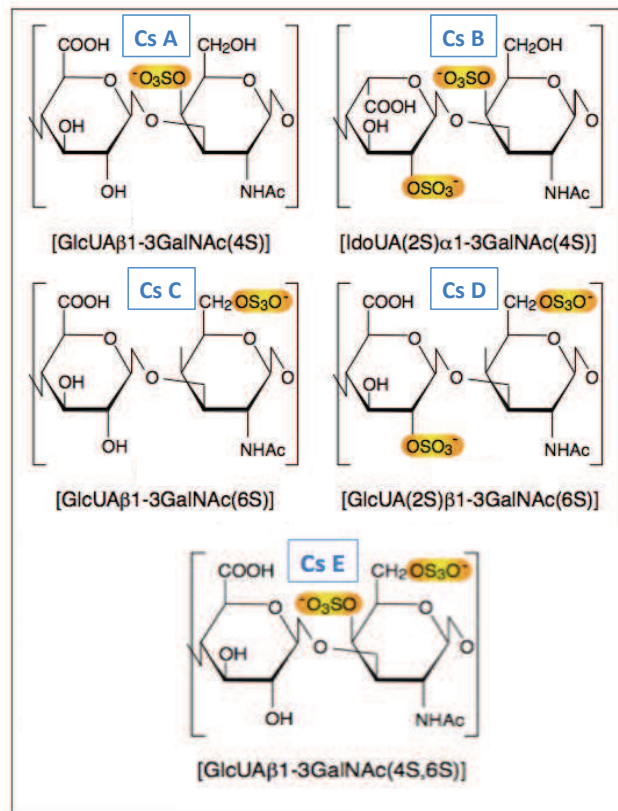
oligosaccharide fragments (Fuller et al., 2006). Whether these HS oligosaccharides can play intracellular functions remains to be explored. Extracellular degradation of HSPGs is carried out by extracellular heparanase, an endoglycosidase expressed in limited extent in most tissues but overexpressed during inflammation or during other pathological stages. Extracellular heparanase degrades basement membrane HSPGs at sites of injury or inflammation, allowing extravasation of immune cells into nonvascular spaces resulting in the release of factors that regulate cell proliferation and angiogenesis. According to the biological roles of HSPGs at these locations, i.e., integration of matrix structures, modulation of growth factor activities, etc., the elucidation of their metabolic turnover by the regulation of HS biosynthetic and catabolic enzymes is a vast field of study in the understanding of tissue homeostasis (Papy-Garcia and Albanese, 2017).

## 2.2. Chondroitin sulfate

The chains of chondroitin sulfate / dermatan sulfate consist of a succession of forty to more than one hundred disaccharide units composed of Glucuronic Acid (GlcA) or Iduronic Acid (IdoA) associated with a binding of the type  $\beta$ 1-3 (if GlcA) or  $\alpha$ 1-3 (if IdoA) to an N-acetylgalactosamine (GalNAc). The amino sugar in Cs may be sulphated on carbons 4 or 6 or on the non-acetylated nitrogen (Taylor and Gallo, 2006) (**Figure 3**).

They are linked to carrier proteins through a tetrasaccharidic moiety covalently bound to an l-serine residue to form PGs.

CSPGs participates in important biological processes such as regulation of cells behaviors (Malmström et al., 2012; Yamada and Sugahara, 2008) (growth, proliferation, and migration), development and repair of the Central Nervous System (CNS), (Ambrosius et al., 2009; Malmström, 1984) or cellular recognition (Mizumoto and Sugahara, 2012; Thelin et al., 2012). These critical biological activities seem to be governed by the finely tuned repartition of the negative charges borne by the sulfate and carboxylate groups of their CS chains (Ly et al., 2011). The sulfation patterns expressed all along the CS chains would encode information decipherable by positively charged domains of protein receptors. Also CS is capable of electrostatic interaction with positively charged Platelet-Derived Growth Factors (PDGFs) and in particular with Fibroblast Growth Factor (FGF), Insulin-like Growth Factor (IGF), Vascular Endothelial Growth Factor (VEGF) and Transforming Growth Factor beta (TGF $\beta$ ), resulting in stabilization and reduced degradation of growth factors in solution (Sandri et al., 2016). For example, during wound healing, CS/DS is reported to be the dominating GAG in wound fluid (Penc et al., 1998). FGF2 and FGF7 are two important growth factors during wound repair and they have been shown to preferentially bind to indoleamine 2,3-dioxygenase A (IdoA)-containing motifs in CS/DS, promoting proliferative processes (Thelin et al., 2013). Depending on their modifications and the specific sulfation patterns of the disaccharide units, CSs are classified into 5 different types (Mende et al., 2016) (**Figure 4**).



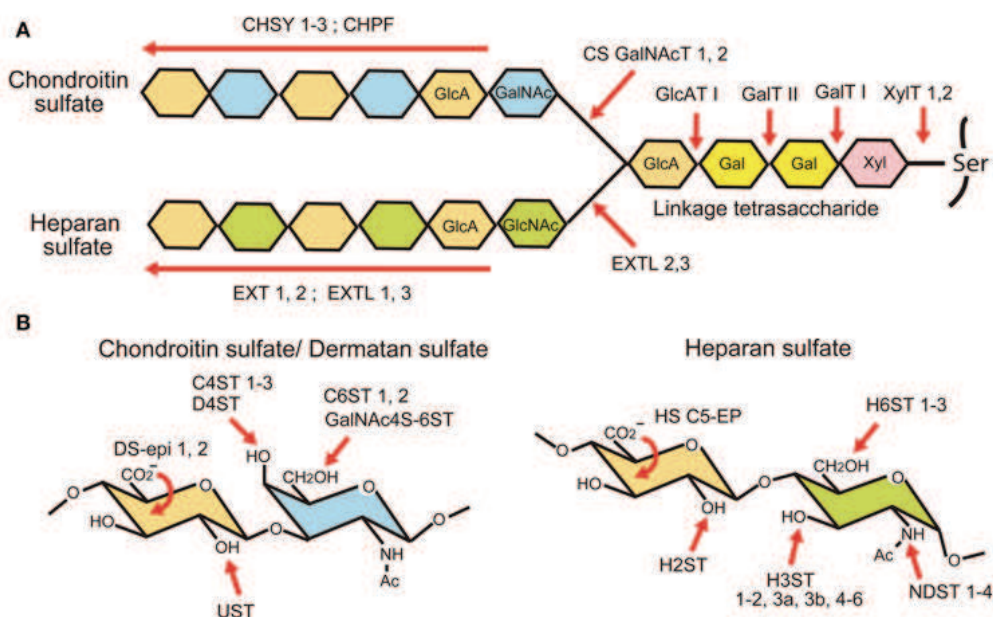
**Figure 4: Different type of chondroitin sulfate.** After (Sugahara et al., 2003).

CS are composed of repeating disaccharide units of GalNAc and GlcA, added on to the nonreducing end of the growing CS chain (Silbert and Sugumaran, 2002a), by two distinct proteins GlcA transferase and GalNAc transferase whereas, chondroitin synthase, appears to possess both activities (Kusche-Gullberg and Kjellén, 2003a). Modifications of the CS chain include epimerization of the C5 carbon of GlcA to form IdoA; this delineates the CS chain as DS, providing DS with a sugar modification (epimerization) similar to HS. In addition to epimerization, sulfation modifications may occur at the 2-position of the IdoA (and, to a lesser extent, GlcA) and at the 4- position or the 6-position of the GalNAc (Silbert and Sugumaran, 2002a). Unlike HS, CS/DS do not undergo N-deacetylation on the GalNAc.

Modifications of the CS/DS chains occur while the chain is undergoing polymerization although the details of CS/DS biosynthesis have not been worked out as well as they have for HS. Early in chain modification, 6-O-sulfation occurs by the action of chondroitin 6-sulfotransferase (C6ST) onto nonsulfated GalNAc residues in the medial/trans Golgi region (Habuchi, 2000a; Kusche-Gullberg and Kjellén, 2003a; Silbert and Sugumaran, 2002b). Next, 4-O-sulfation of the GalNAc takes place in the later trans Golgi. However, this apparently can occur concurrently with epimerization to produce DS. There exists 4-sulfotransferases (STs) that differentiate between CS and DS (C4ST and D4ST), but the epimerase prefers 4-O-sulfated substrates, suggesting that the enzymes are working at the same time. Three C4STs have been described (C4ST1, 2 and 3) that

prefer GlcA-rich regions (Habuchi, 2000a). Alternatively, one D4ST has been identified that is specific for the IdoA regions present in DS (Silbert and Sugumaran, 2002a). It has been suggested that D4ST works immediately after epimerization to prevent back-epimerization (Kusche-Gullberg and Kjellén, 2003a). Another enzyme, GalNAc4-6ST adds sulfates onto GalNAc already modified by C4ST (Habuchi, 2000b; Kusche-Gullberg and Kjellén, 2003b; Silbert and Sugumaran, 2002a). However, 6-O-sulfation on to 4-sulfated-GalNAc was unaffected due to the presence of the GalNAc4-6ST enzyme. The final step in CS/DS synthesis is the addition of 2-sulfates on IdoA by the 2-O-sulfotransferase (CS/ DS2ST). Only one CS/DS2ST enzyme has been identified and it prefers to add 2-sulfates to IdoA next to 4-sulfated GalNAc (Taylor and Gallo, 2006) (**Figure5**).

There are several steps in the degradation process of CS chains. It starts with the cleavage of the polymer into smaller fragments, initially tetra- and hexasaccharides, but ultimately disaccharides. The process continues with the removal of the sulfate groups, and ends with the final degradation of the disaccharides into individual sugars.



**Figure 5: Biosynthesis of Chondroitin sulfate (CS) and heparan sulfate (HS).**

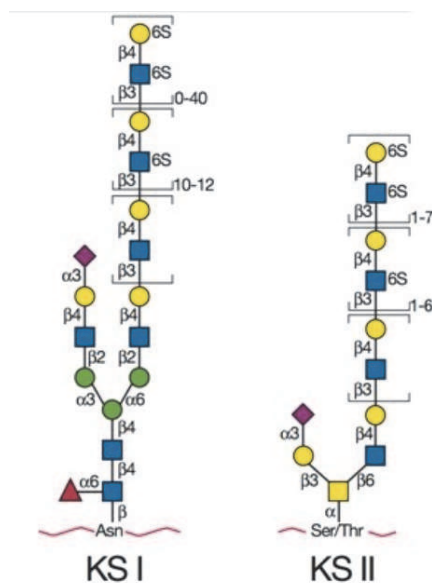
(A) CS and HS chains are covalently attached to the PG core proteins through a common linkage tetrasaccharide. It is considered that the biosynthesis of a chondroitin chain starts with the addition of an N-acetylgalactosamine (GalNAc) residue to the linkage tetrasaccharide by CS N-acetylgalactosaminyltransferases (CSGalNAcT1, 2). After that, glucuronic acid (GlcA) and GalNAc residues are co-polymerized by Cs synthases (CHSY-1, -2, -3) and chondroitin polymerization factor (CHPF). When an N-acetylglucosamine (GlcNAc) residue is added to the linkage tetrasaccharide instead of GalNAc by EXTL 2 or 3, a heparan chain is polymerized by EXT family members. (B) After polymerization, they are modified by sulfation and epimerization reactions by many GAG modifying enzymes. Adapted from (Maeda, 2015).



### 2.3. Keratan sulfate

KS is formed by alternating 3-linked -galactose (Gal) and 4-linked N-acetyl-glucosamine (GlcNAc) units (**Figure 3**). Although both units can be 6-O-sulfated, this modification occurs more often at the GlcNAc units. KS is the only GAG type, which does not bear an acidic residue such as glucuronic acid commonly seen in CS, DS, HA, and heparin/HS; or iduronic acid commonly seen in DS and heparin/HS. Instead of these acidic units, KS has a neutral sugar Gal, and this characteristic gives to KS a less acidic potential in solution, once sulfation is the only acidic component of its structure (Pomin, 2015).

KS chains can be either N-linked to asparagine residues (named as KS I) or O-linked to serine or threonine residues (named as KS II) of the core proteins to form KSPG (**Figure 6**). While KS I occurs more often at the corneal tissue, KS II happens more frequently at the cartilages and bones (Funderburgh, 2002). Both KS I and II possess a mixture of non-sulfated (Gal-GlcNAc), mono-sulfated (Gal-GlcNAc6S), and di-sulfated (Gal6S-GlcNAc6S) disaccharide units within their chains (Esko et al., 2009).



**Figure 6: Keratan sulfates structure.**

KS contains a sulfated poly-N-acetylglucosamine chain linked to either Asn or Ser/Thr residues. The actual order of the various sulfated and nonsulfated disaccharides occurs somewhat randomly along the chain. Not shown are sialic acids that may be present at the termini of the chains and fucose residues attached to N-acetylglucosamine units. KS I is found on an N-glycan core structure, whereas KS II is found on an O-glycan core-2 structure. Adopted from (Esko et al., 2009).\*

The poly-N-acetylglucosamine of KS I, can be quite long (~50 disaccharides, 20–25 kD). At least two classes of sulfotransferases, one or more GlcNAc 6-O-sulfotransferases, and one Gal 6-O-sulfotransferase catalyze the sulfation reactions. These enzymes, like other sulfotransferases, use activated sulfate (PAPS [3'-phosphoadenyl-5'-phosphosulfate]) as a high-energy donor. GlcNAc 6-O sulfation occurs only on the nonreducing terminal N-acetylglucosamine residue, whereas sulfation of galactose residues takes place on nonreducing terminal and internal galactose residues, with a preference for galactose units adjacent to a sulfated N-acetylglucosamine. Sulfation of a nonreducing terminal galactose residue blocks further elongation of the chain, providing a potential mechanism for controlling chain length. Only one

galactose sulfotransferase has been identified, whereas multiple sulfotransferases catalyze the sulfation of N-acetylglucosamine residues. Mutations in the corneal GlcNAc 6-O-sulfotransferase result in macular corneal dystrophy. The relationship of enzymes involved in KS I and KS II sulfation is unclear. Bacterial keratanases degrade KS at characteristic positions. In animals, KS is degraded in lysosomes by the sequential action of exoglycosidases ( $\beta$ -galactosidase and  $\beta$ -hexosaminidase) after removal of the sulfate groups on the terminal residue by sulfatases (Esko et al., 2009)

The KSPGs can be either primarily composed of KS chains like the family of small leucine-rich ECM PGs such as keratocan, mimecan, lumican, fibromodulin, osteomodulin, and osteoadherin, or just containing few KS chains, as the least abundant GAG type, like aggrecan, which is largely composed of chondroitin sulfate (Funderburgh, 2002; Greiling, 1994)

## 2.4. Hyaluronic acid

HA consists of repeating disaccharides composed of N-acetylglucosamine and glucuronic acid (**Figure 3**). It is the largest polysaccharide found in vertebrates that is neither sulfated nor covalently linked to protein so it does not occur as a PG. It has essential roles in development, tissue organization, cell proliferation, signaling reactions across the plasma membrane, and microbial virulence (Esko et al., 2009). Native HA has a very high molar mass, usually in the order of millions of Daltons (10<sup>5</sup> to 10<sup>7</sup> Da) with an extended length of about 300nm before being progressively degraded into smaller fragments during its catabolism and eventual lysosomal degradation (Misra et al., 2015). Due to its negative charge at neutral pH, hyaluronan attracts water; it can contain up to 10,000 times its weight of water, which contributes to the formation of loose and elastic matrices facilitating cellular migration.

HA is in connection with the major PG in the ECM of cartilage named aggrecan which is linked to core protein. Each aggregate consists of central long HA chain with up to 100 aggrecan molecules. Aggregates through their interaction with HA, stabilize their position within the matrix (in parallel with their entrapment by the collagen framework) and give to cartilage its mechanical properties (Pearle et al., 2005; Rizkalla et al., 1992).

There are three known hyaluronan synthases (HASes) HAS1, HAS2 and HAS3 that produce HA and that are conserved evolutionally in mammalian species (Heldin et al., 2013; Itano and Kimata, 2002; Jiang et al., 2007) (**Figure 7**). HAS1 is expressed during gastrulation and early neurulation, HAS2 exhibits a defined regulatory function and is specifically expressed within developing heart and skeleton as well as in emergent neural crest cells, whereas HAS3 is expressed in a restrictive manner within developing teeth and hair follicles (Spicer and McDonald, 1998). HAS's have been found to produce variations in HA chain length; HAS1 and HAS3 produce 2  $\times$  10<sup>5</sup> to 2  $\times$  10<sup>6</sup> Da whereas HAS2 synthesizes larger HA in excess of 2  $\times$  10<sup>6</sup> Da.

Hyaluronan synthases are unique in that they possess two enzymatic components, one to add on the GlcA and another to add on the GlcNAc. Unlike the other GAGs, addition of the

monosaccharide occurs at the reducing end of the growing HA chain on the inside of the membrane, and the growing polysaccharide is extruded out through the HAS complex into the extracellular space. This unique synthesis mechanism allows for unrestrained polysaccharide length, accounting for how HA exists in vivo in excess of 106 Da (Weigel et al., 1997). Although HA is not covalently attached to a PG intracellularly, HA possesses a variety of protein binding partners extracellularly, and is most likely not found in vivo as a free GAG (Zhuo et al., 2001).

### **3. Functional interaction of GAGs with HBGFs**

GAGs were recently identified as key regulators of cell behavior, particularly by their abilities to bind and potentiate the activity of Heparin Binding Proteins (HBP) such as Growth Factor (GF) or chemokines. They constitute niches of GF storage in specific ECM compartments or “niches” and they are involved in chemotaxis gradient of chemokines. GAGs can therefore compartmentalize HBP, protecting them from proteolysis. So they can be a sort of selective reserve of available factors, by allowing for example a better cell survival, especially in pathological conditions of inflammation and tissue degradation. This is clearly established that the whole family of HS can bind all GFs having an affinity for heparin, such as FGF, VEGF, Pleiotrophin (PTN), but also numerous cytokines involved in inflammatory processes such as Interleukin-1 (IL-1), Interleukin-6 (IL-6), TGF $\beta$ , Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ ). Moreover, depending on their fine structure, GAGs and especially HS can facilitate and optimize the dimerization of growth factors and their presentation to their high affinity cell receptors and thereby potentiate their activities (Coppi et al., 2007). HSPG or CSPG alone can constitute also low affinity co-receptor for HBP, thus transducing different signaling pathways. The sulfation patterns of GAGs disaccharides are then critical in determining the binding capacity and specificity for HBP (Bayliss et al., 1999). The functions of these GAGs can eminently vary also depending on their acetylated group content, and on the length of the GAG chains linked to PGs. We will describe below structural features of GAG that are necessary for their binding affinity to HBP family.

#### **3.1. FGF**

Fibroblast Growth Factors (FGF) are well-established secreted signaling proteins that play critical roles in development, angiogenesis, wound healing and various endocrine-signaling pathways. The FGF family contains 22 structurally and evolutionarily related proteins including FGF1–FGF23 in humans and mice. Ranging in size from 17 to 34 kDa and sharing 13–71% amino acid identity (Plichta and Radek, 2012). (Ornitz and Itoh, 2015a).

FGFs are HBP because it was demonstrated that their interactions with cell-surface-associated HSPG are essential for FGF signal transduction. They are key players in the processes of proliferation and differentiation of wide variety of cells and tissues. The mammalian FGF receptor family has 4 members, FGFR1, FGFR2, FGFR3, and FGFR4. The FGFRs consist of three extracellular immunoglobulin-type domains (D1-D3), a single-span trans-membrane

domain and an intracellular split tyrosine kinase domain. FGFs interact with the D2 and D3 domains, with the D3 interactions primarily responsible for ligand-binding specificity (**Figure 7**). HS binding is mediated through the D3 domain. A short stretch of acidic amino acids located between the D1 and D2 domains has auto-inhibitory functions. This 'acid box' motif interacts with the heparan sulfate-binding site to prevent receptor activation in the absence of FGFs (Plichta and Radek, 2012).

One of the most familiar FGFs is Basic Fibroblast Growth Factor (FGF2). It is involved in the transmission of signals between the epithelium and connective tissue, induces angiogenesis (Dailey et al., 2005) and influences growth and differentiation of a wide variety of tissue including epithelia (Bikfalvi et al., 1997).

Another subgroup of FGF family is FGF18 which has a number of functions in the developing and adult organism including a key role in skeletal development (Brown et al., 2014) and has undergone clinical trials for the treatment of osteoarthritis (Fortier et al., 2011).

GAGs, particularly HS, play a critical role in FGF signaling by facilitating the formation of the FGF-FGFR complex and enhancing FGF oligomerization (Raman et al., 2005) and mediating the cellular response to FGF (Nayak et al., 2015). FGFs have a high affinity for HS, and as such, their interaction stabilizes FGFs against thermal denaturation and proteolysis (Powers et al., 2000). In addition, HS limit the ability of FGFs to diffuse and be released into interstitial spaces, thus generating a reservoir of growth factors for the surrounding environment (Ornitz and Itoh, 2001)(Beenken and Mohammadi, 2009).

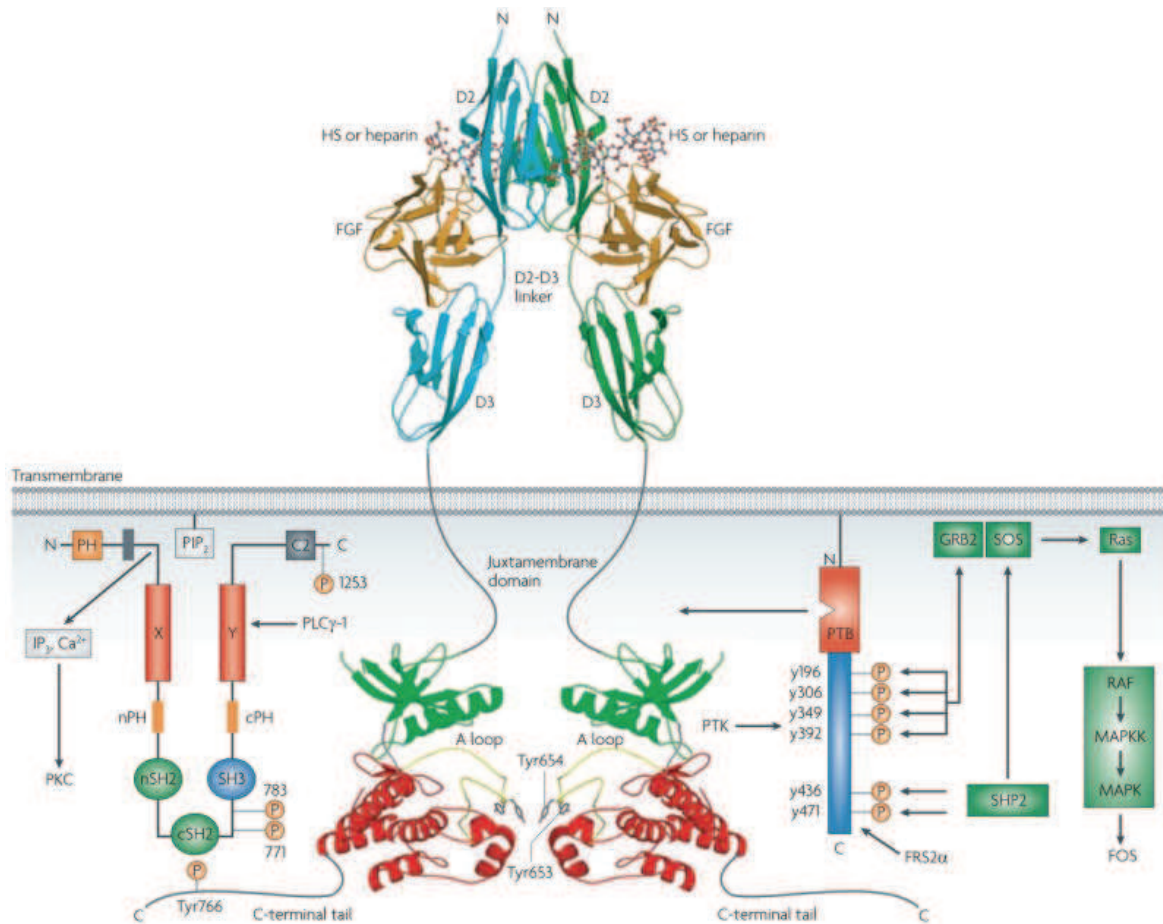
The role of HS in GF signaling and specifically the definition of the HS chemical signature, has been most thoroughly studied in relation to the FGF family. In fact, the sulfation pattern and length of HS chains are very important in the regulation of FGF signaling. In general, high levels of sulfation of HS chains facilitate FGF signaling and the formation of ternary complexes with FGFs and FGFRs (Patel et al., 2008). Oligosaccharides with eight or more sugar residues are active, but shorter HS chains can also facilitate the formation of ternary complexes with FGFs and FGFRs (Hadari et al., 1998; Ornitz et al., 1995; Patel et al., 2008).

Cleavage of the HSPG core protein also modulates FGF signaling. The cleavage by serine proteinases possibly facilitate FGF signaling by releasing FGFs that were sequestered at the cell-surface (Shimokawa et al., 2011). In addition, the cleavage by endoglycosidases such as heparanase possibly modulates FGF signaling (Patel et al., 2007). For example, FGF10 in the basement membrane, that is released by heparanase, promotes FGF signaling in branching morphogenesis (Ornitz and Itoh, 2015b).

Out of the 22 FGF members identified, most paracrine FGFs show significant affinity for HS, generally implicated with signaling through cognate high-affinity tyrosine-kinase receptors (FGFRs). However, the endocrine FGFs (FGF15/19, FGF21, and FGF23) interact only weakly with HS (Itoh et al., 2015).

Saccharide interaction with both the growth factor and its receptor in signaling complexes was inferred from the finding that receptor activation leading to mitogenesis required 6-O- sulfate in

addition to N- and 2-O-sulfate groups, whereas N- and 2-O- sulfate groups sufficed for binding of FGF2 alone (Li and Kusche-Gullberg, 2016).



**Figure 7: Fibroblast growth factor receptor signaling.**

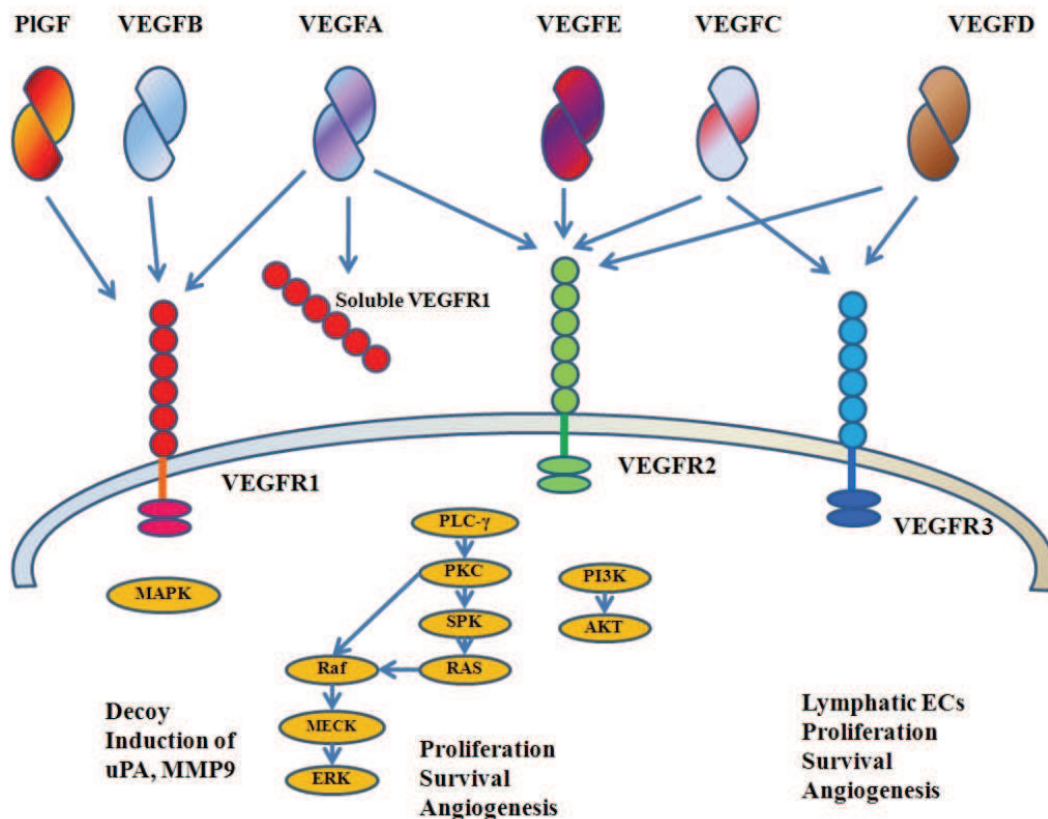
Structurally unresolved regions are shown as grey lines. Amino-terminal and carboxy-terminal lobes of the kinase domain are coloured green and red, respectively. The two major intracellular targets, phospholipase (PLC)  $\gamma$ 1 and FGFR substrate 2 $\alpha$  (FRS2 $\alpha$ ), are shown. Adopted from (Beenken and Mohammadi, 2009)

### 3.2. VEGF

Vascular endothelial growth factor (VEGF) is now accepted as a dominant mediator of angiogenesis (Ferrara and Adamis, 2016). VEGF is a 46–48 KD glycoprotein composed of different isoforms (VEGF121, VEGF165, VEGF189, and VEGF206) through alternate mRNA splicing, and which differ in their binding to the extracellular matrix and to the receptors (Dvorak, 2005; Neufeld et al., 1999a). The best-characterized VEGF isoform is the 165-amino

acid form (VEGF<sub>165</sub>), that is an HBP. The binding of VEGF<sub>165</sub> to its cell surface receptors on vascular endothelial cells is strongly modulated by cell surface-associated HSPG (**Figure 8**).

VEGF binds to the tyrosin kinase receptors VEGF receptor 1 (VEGFR-1) and 2 (VEGFR-2) which are strongly expressed by endothelial cells (Neufeld et al., 1999b; Robinson and Stringer, 2001). These receptors are characterized by seven extracellular immunoglobulin-like domains including a ligand-binding region, a membrane spanning domain and an intracellular tyrosine kinase domain (**Figure 9**).



**Figure 8: VEGF family members and receptors.**

VEGFA binds both VEGFR-1 and VEGFR-2. PIGF and VEGFB bind only VEGFR-1. VEGFC and VEGFD bind VEGFR-2 and VEGFR-3. After (Sa-Nguanraksa and O-Charoenrat, 2012).

VEGF plays a role in tumor growth and in pathological angiogenesis, but also in the control of blood vessel development and in vasculogenesis in embryos. VEGF acts mainly on endothelial cells by stimulating their proliferation, their migration, and the induction of various genes involved in tissue remodelling. However, a few other cell types also express VEGF Receptors (VEGFR) including monocytes and specialized macrophages (Fenwick et al., 1999), osteoblasts (Smith et al., 2003), and Osteoarthritic (OA), but not resting, chondrocytes (Le Jan et al., 2012).

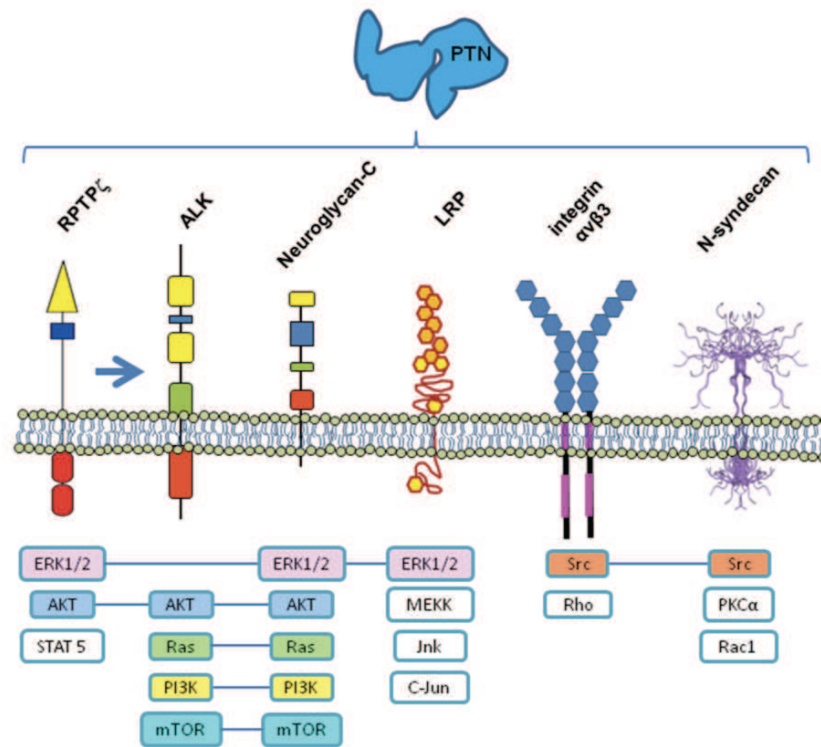
Elimination of HS *N*-sulfation on endothelial cells and pericytes was shown to result in reduced production of both HS and CS resulting in severely delayed angiogenesis, and resulted in increased CS biosynthesis. These results demonstrate a functional overlap between CS and HS in the support of VEGF-induced sprouting angiogenesis (Le Jan et al., 2012).

Many studies on inhibition of tumour angiogenesis via targeting of relevant angiogenic growth factors have shown promising primary results both *in vitro* and *in vivo* for therapies using heparin and HS-based oligosaccharides (Ferro et al., 2007; Mousa et al., 2006). These compounds competitively bind to HBGF (like VEGF?), preventing their interaction with cell surface HSPGs and cytokine receptors and hence inhibit angiogenesis (Ferro et al., 2007). Low molecular weight heparin, for example, has been demonstrated to be potent inhibitors VEGF-mediated human microvascular endothelial cell proliferation (Norrby, 2000). Recent evidence has implicated the significance of HS oligosaccharides on suppression of endothelial cell migration, tube formation, and signaling induced by VEGF165 and FGF2 (Cole et al., 2010).

### 3.3. PTN

Pleiotrophin (PTN) is a secreted cell signaling cytokine that acts as growth factor associated with the extracellular matrix. PTN is an 18-KD protein that has 168 amino acids (González-Castillo et al., 2014). It was discovered by several laboratories nearly 25 years ago; thus, it initially was named differently such as: Heparin-binding growth factor 8 (HBGF-8) (Milner et al., 1989), Heparin-binding growth-associated molecule (HB-GAM) (Merenmies and Rauvala, 1990; Milner et al., 1989), Heparin-binding neutrophil factor (HBNF) (Kovesdi et al., 1990), Osteoblast-specific factor 1 (OSF-1) (Takeshita et al., 1993) and Heparin affinity regulatory peptide (HARP) (Courty et al., 1991).

PTN is a potent mitogenic cytokine that acts through several proteoglycan receptors. First isolated in neural tissues, it not only plays an active role in the development of the central nervous system, but also is produced during tissue repair and regeneration. In addition, the expression of PTN is important in hematopoietic stem cell maintenance (Yeh et al., 1998). PTN is elevated in a number of cancer cell lines, and the expression level often correlates with the metastatic abilities of these cancer cells (Hatziapostolou et al., 2005a; Kong et al., 2012)



**Figure 9: Receptors and signaling pathways possibly involved in PTN signaling.**

All of these membrane receptors could function as a multi-molecular complex coordinated to transduce the PTN signal into the cell by different signaling pathways. Adapted from (González-Castillo et al., 2014).

PTN recognizes several receptors (**Figure 9**). The HSPG N-syndecan, the CSPG, Protein Tyrosine Phosphatase Zeta (PTPRZ), the Anaplastic Lymphoma Kinase (ALK) and the LDL Receptor-Related Protein (LRP) (Meng et al., 2000; Raulo et al., 1994). N-syndecan is considered to be crucial for the activity of PTN during neural development, whereas PTPRZ has been associated with the ability of PTN to facilitate cell proliferation and growth under both normal and pathological situations (Hatzia Apostolou et al., 2005b; Himburg et al., 2012; Meng et al., 2000; Mikelis et al., 2009).

PTN together with Midkine (MK) form a family of HBP that are normally expressed during embryogenesis (Kadomatsu and Muramatsu, 2004). The heparin-binding activity of MK and PTN postulated that HSPGs could be members of the receptor complex of the both. Indeed, the syndecan family members exhibit binding ability as to MK and PTN (Lim et al., 2013; Perez-Pinera et al., 2008).

The heparin-binding sites are also active in recognition of CS chains, especially E-type CS (Kreuger et al., 2006; Li et al., 2010; Meng et al., 2000), the extracellular domain of PTPRz carries CS chains, and its intracellular domain exhibits protein tyrosine phosphatase activity. The K<sub>d</sub> (the equilibrium dissociation constant between the antibody and its antigen) of PTPz as to MK is 0.56 nM, while chondroitinase digestion increases it to 8.8 nM, indicating that the chondroitin sulfate chain is necessary for high affinity (Meng et al., 2000).



MK and PTPz used Phosphoinositide 3-kinase (PI3-kinase) and Extracellular Regulated Kinases (Erk) transduction pathway for osteoblast cell migration and neuronal survival (Bernard-Pierrot et al., 2001; Li et al., 2010; Mikelis et al., 2011).

ALK, is another receptor for MK and PTN (Deshauer et al., 2015; Dunlevy et al., 1998), active in anchorage-independent cell growth (Deshauer et al., 2015) The LRP receptor, is a member of the LDL receptor family that accomplishes endocytosis of a diverse array of ligands (Maeda et al., 2003).

The receptors described above, namely N-syndecan, PTPz, ALK, and LRP, might be differentially utilized for specific biological activities of MK and PTN, or might cooperate with each other to enhance intracellular signals. The functional interaction and complex formation among these receptors is an important and complex issue that remains to be clarified.

CRRET laboratory have already showed that PTN binds to GAGs with a Kd of 13nM for heparin and 51nM for DS. Co-treatment of bovine epithelial cells with PTN and HS, CS-A or DS resulted in potentiation of PTN-induced proliferation (Vacherot et al., 1999). The binding of PTN to heparin induces a conformational change of the protein in the  $\beta$  sheets of the protein (Kilpelainen et al., 2000).

Some study showed that PTN has a strong affinity for heparin, promote neurite outgrowth, and has mitogenic (Muramatsu, 1993). cell transformation and neo-vascularization activities (Kovesdi et al., 1990). the interactions of PTN with heparin, is an analog of HS from N-syndecan (Raulo et al., 2005). Both TSR domains are needed to maintain high heparin affinity, but the C-terminal TSR domain (CTD) has a higher affinity for intact heparin than the N-terminal TSR domain (NTD). However, the C-terminal tail of PTN, despite having a large number of basic amino acids, does not contribute significantly to heparin binding (Raulo et al., 2005).

Identification of the specific saccharide binding sequences of PTN was possible by co-purification experiments of PTN and oligosaccharides. It was determined that PTN preferentially binds to chondroitinase B-resistant CSs.. (Bao et al., 2005).

Some studies showed that PTN prefers over sulfated CS structures enriched in 4,6-O-disulfated GalNAc (CSE) or 2-O-sulfate-GlcUA-6-O-sulfate-GalNAc rather than CS containing only 4-O-sulfated GalNAc (CSA), and the affinity of the interaction is also dependent on the size of CS ligand. However, no experimental information on how PTN domains contribute to binding CS is available (Ryan et al., 2016).

## 4. Role of GAG in different pathologies and tissues

According to their ability to interact with HBGF and to potentiate their half-life, activities and interactions with high affinity receptors, GAG play essential roles in the architecture of the ECM and in the regulation of cellular functions and tissue homeostasis. Any changes in both the organization of the ECM or in its GAG components will therefore affect the cell and tissue integrities, making this tissue potentially vulnerable.

### 4.1. Muscles Pathology

Our lab has investigated GAG content in Duchenne Muscular Dystrophy (DMD). Immunostaining targeted to specific GAG species showed greater deposition of CS/DS in DMD patient biopsies versus control. A selective accumulation of CS/DS in DMD of muscles was confirmed by biochemical quantification assay. Whereas no changes in the amount of HS were observed between control and DMD patients. In addition to the accumulation of CS/DS in DMD, high-performance liquid chromatography analysis demonstrated a modification of the sulfation pattern of CS/DS disaccharide units in DMD muscles. An enriched proportion of MonoS units and probably a specific increase in 4-O-sulfation of GalNAc residues were observed, consistent with the upregulation of the CHST11/C4ST-1 enzyme. This study suggest that GAGs could represent a new and original therapeutic target for improving the success of gene or cell therapy for the treatment of muscular dystrophies (Negroni et al., 2014).

In addition we demonstrated that muscular ischemia is associated with modification of expression of enzymes involved in GAG biosynthesis, which correlated with significant changes in HS and CS structural features such as size and sulphation pattern. We demonstrate that the sulphation level of GAGs and specifically of HS species strongly increases immediately after ischaemia induced in rat skeletal muscle. These major structural changes are associated to modifications of GAG abilities to bind growth factors FGF2 and IL8 over the time, both HBPs released from the damaged myofibres, and to modulate cell activity in vitro. Moreover, a CS hallmark of injury is maintained as well after the regeneration process. Finally, the relevance of the role of this glycanic matrix remodeling was showed, since a GAG mimetic treatment accelerated muscle repair after ischaemia. We also demonstrated modification of expression of enzymes involved in GAG biosynthesis induced during ischaemia, which are correlated with significant changes in HS and CS structural features such as size and sulphation pattern (Chevalier et al., 2015). This suggests that GAG structural modification could be associated to the modulation of growth factor activities driving muscle repair after ischaemia.

### 4.2. Brain Pathology

In Alzheimer's disease (AD), sulphated HS accumulate at the intracellular level in disease neurons where they co-localize with the neurofibrillary pathology, while they persist at the

neuronal cell membrane in normal brain. However, it is unknown whether HS are involved in the mechanisms leading to the abnormal phosphorylation of tau in AD and related tauopathies. Recently the CRRET lab; had investigate the implication of a specific sulfation pattern of HS in the molecular and cellular mechanisms leading to the abnormal phosphorylation of both wild-type tau and of tau carrying the mutation P301L (hTauP301L) responsible of AD (Alonso et al., 2004; Williams et al., 2006). It was demonstrated that abnormal phosphorylation of Tau protein was associated to intracellular accumulation of a specific 3-O-sulfated HS. Moreover this is associated to an increase of the expression of the HS3ST2 enzyme involved in this specific sulfation pattern. The CRRET lab showed that the chemical inhibition of HS sulphation, or the down- regulation of HS3ST2 expression in cells, strongly avoids the hyperphosphorylation of tau induced by oxidative stress or by the hTauP301L mutation. Finally, inhibiting the expression of *hs3st2* in a FTDP-17 zebrafish model of tauopathy avoids the abnormal phosphorylation of tau at several AD-related epitopes resulting in tauopathy arrest and animal functional recovery. In conclusion, these results position intracellular 3-O-sulphated heparan sulphates, and the enzymes responsible of their high sulphation in neurons, as central modulators of tau abnormal phosphorylation before it occurs. This opens a wide area of research in the field of glyco-neurobiology, positioning HS3ST2 and its products as potential key players in the development and evolution of the tau pathology, with the therapeutic consequences that this implies (Sepulveda-Diaz et al., 2015).

### 4.3. Lung Pathology

Altered ECM turnover is a hallmark of several pulmonary diseases, including Idiopathic Pulmonary Arterial Hypertension (IPAH), Pulmonary Fibrosis (PF), Asthma or Chronic Obstructive Pulmonary Disease (COPD), which underlines the importance of ECM homeostasis for proper lung function (Laurent et al., 2007; Noble and Jiang, 2006; Rabinovitch, 2001). Mice deficient in one of the enzymes involved in HS biosynthesis have abnormal phenotypes. Despite the fact that the defects observed are characteristic of the missing/mutated enzyme, significant overlapping lung phenotypes have been observed for some HS mutants (Smits et al., 2010). HS binds and modulates various classes of proteins, which are involved in the pathogenesis of emphysema. These include proteases and protease inhibitors (Gadek and Pacht, 1990; Snider, 1992), enzymes involved in neutralizing reactive oxygen species like superoxide dismutase (Lookene et al., 2000) and xanthine oxidase (Adachi et al., 1993), chemokines (Finkelstein et al., 1995) and growth factors/cytokines (Timens, W et al., 1997). In case of IPAH, it has been shown that there is a significantly increased content of HA. However, the content of the sulphated GAG, HS, DS or CS was decreased, indicating an increased ratio of non-sulphated to sulphated GAG (Papakonstantinou et al., 2008). In case of PF, two studies identified increased amounts of HA, HS, DS and CS lung tissue specimens, however, the increase of HA was significantly more than others (Papakonstantinou et al., 2008; Westergren-Thorsson et al., 2017).

#### 4.4. Aging

Biochemical and immunohistological studies from the CRRET lab recently demonstrated that, HS species was increased during aging process in the left ventricle of rat's myocardium. ELISA-based competition assays showed altered capacities of the aged myocardial GAGs to bind FGF-1, FGF-2, and VEGF but not HB EGF. Mitogenic assays in cultured cells showed an age-dependent decrease of the elderly GAG capacities to potentiate FGF-2 whereas the potentiating effect on VEGF165 was increased, as confirmed by augmented angiogenic cell proliferation in Matrigel plugs. Moreover, HS disaccharide analysis showed considerably altered 6-O-sulfation with modest changes in N- and 2-O- sulfations. Together, these findings suggest a physiological significance of HS structural and functional alterations during aging. This can be associated with an age-dependent decline of the extracellular matrix capacity to efficiently modulate not only the activity of resident or therapeutic growth factors but also the homing of resident or therapeutic cells. Furthermore, these results were observed also in adult and aged human hippocampus since levels and composition of GAGs, especially HS, are increased. Moreover, GAGs from aged hippocampus showed altered capacities to regulate trophic factor such as FGF-2 and Brain-derived Neurotrophic Factor (BDNF), activities without changing their capacities to protect cells from A $\beta$ 42 toxicity, compared to adult hippocampus GAGs. Finest analysis of disaccharidic sequences indicated also that specific sulfation pattern are modified and would be involved in these functional effect, according to modified ability to interact with HBF (Huynh et al., 2012a).

According to these results in skeletal and cardiac, muscle and in brain, during pathologies or aging, my host lab propose the hypothesis that GAG modifications during injury or pathology and repair process associated, is not only a marker of matrix remodeling, but could also be involved in cells functionality, necessary for homeostasis, therapy or repair, or inversely could be involved in cell dysfunctionality. In this context, we were interested to the role of GAG on another pathology, osteoarthritis, that affect a tissue very rich in GAG, namely the cartilage, and that could be linked to aging and/or inflammatory process.

## Chapter 2: OSTEOARTHRITIS (OA)

### 1. Definition of OA

#### **Socio-economical impact**

Osteoarthritis (OA) is the first cause of chronic functional disabilities in developed countries with about 250 million people in the world suffering from this disease (Murray et al., 2012) and more than 40 million people across Europe are affected by this disease (WHO Scientific Group on the Burden of Musculoskeletal Conditions at the Start of the New Millennium, 2003). In France, a recent population-based survey in people from 40 to 75 years reported that the prevalence of knee OA ranged from 2.1% (40–49 years) to 10.1% (70–75 years) for men and from 1.6 to 14.9% for women, respectively (Guillemin et al., 2011). Today, OA involves around 9 to 10 million French people (Le Pen C 2005)\*. In France and other developed countries, it is the first cause of medical consultation after cardio-vascular disorders (Le Pen C 2005)\*. It usually appears after the age 40 and becomes generalized around the age of 65. With 9 millions of consultations per year, 14 millions of medical prescriptions and 300 000 radiographic examinations, OA costs very expensive for French population and consequently for French social security system (Rhumatologie, Serge Perrot, ed Med-Line 2002)\*. In a study performed on 2010, it has been shown that annual cost of OA for each French patient per a general practitioner medicine is about 755 euros (Grange L. et al., 2012)\*. It has been reported that the estimated overall costs per patient for OA treatments reach 19,000 €/year in the world (Puig-Junoy and Ruiz Zamora, 2015).

Beyond the huge health care costs for treatment of OA, there is no treatment that can repair the cartilage and stop the progress of OA. Existing therapies, based on HA and CS injections, are symptomatic and pursue only pain alleviation with no effect on slowing disease progression and on restoring cartilage and chondrocytes functions. In parallel, new therapeutic strategies are currently based on stem cells, but these fragile cells are injected in an inflammatory microenvironment detrimental to their survival and clinical efficacy. Therefore, a more suitable alternative is highly mandatory.

#### **Symptoms**

The main symptom of OA is joint pain that is worse with activity and relieved by rest. In severe cases, the pain may also occur at rest or at night. The pain usually occurs near the affected joint; however, in some cases, the pain may be referred to other areas. For example, the pain of OA of the hip may actually be felt in the lower thigh or at the knee.

Morning stiffness is a common symptom of OA. This stiffness usually resolves within 30 minutes of rising, but it may recur throughout the day during periods of inactivity. Some people note a change in symptoms related to the weather.

OA may cause a type of joint swelling called an effusion, which results from the accumulation of excess fluid in the joint.

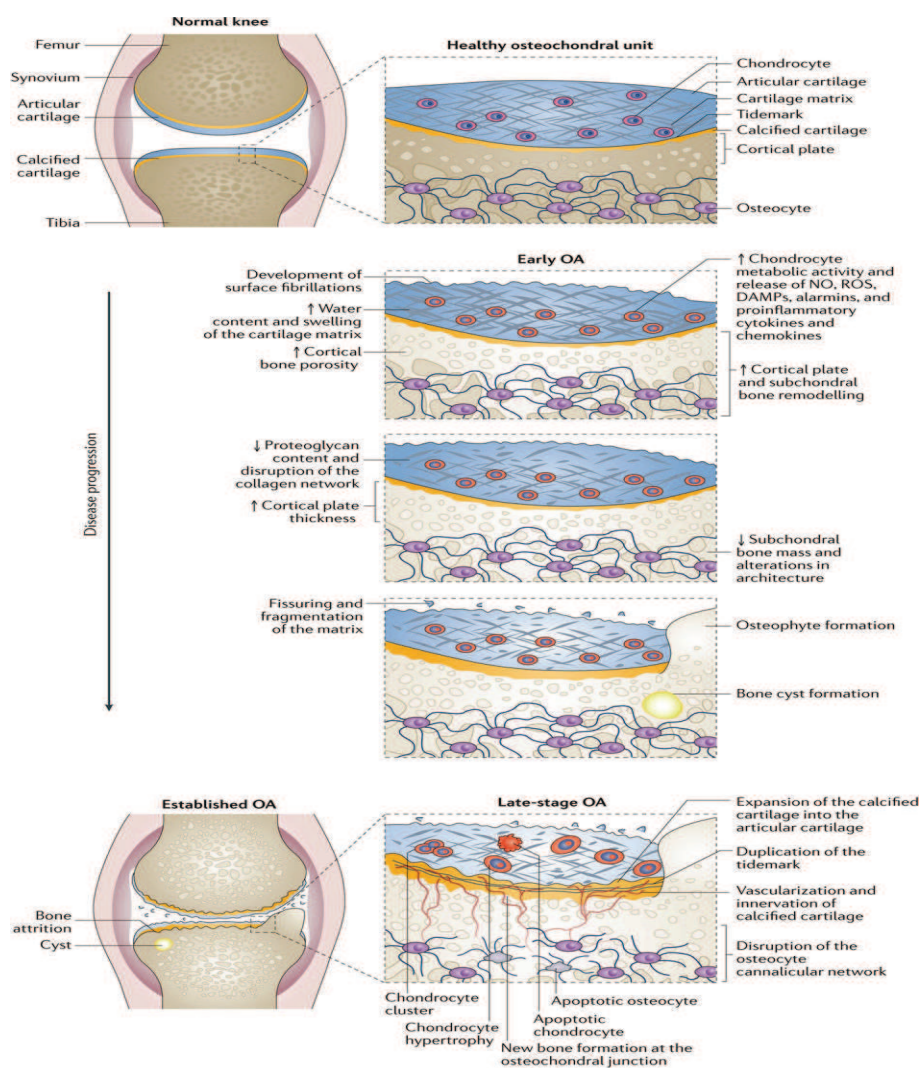
Movement of a joint affected by OA may cause a crackling or grating sensation called crepitus. This sensation likely occurs because of roughening of the normally smooth surfaces inside the

OA often causes outgrowths of bone called osteophytes or bone spurs. These bony protuberances can be felt under the skin near joints and typically enlarge over time.

OA does not affect all joints equally. The condition most commonly affects the fingers, knees, hips, and spine; it rarely affects the elbow, wrist, and ankle.

### **Resume of patho-physiological steps**

During the evolution of the OA process the compositions, functional properties, and structures of all of the tissues undergo marked alterations: the synovial joint, articular cartilage, subchondral and metaphysical bone, synovium, ligaments, joint capsules, and the muscles that act across the joint (Goldring and Goldring, 2016). The loss of cartilage integrity caused by the loss of PGs, which normally maintains cartilage matrix compressive stiffness, will increase the overload of the joint, feeding back to an elevation of bone formation as the joint attempts to adapt to the greater loads. Ultimately, this positive feedback loop will promote the continued loss of cartilage integrity, allowing deterioration to progress to clinically evident OA. In early-stage of OA, subchondral plate becomes thinner. Cartilage degeneration is started by increase of chondrocyte metabolic activity and release some proinflammatory and catabolic cytokines. In the same time increase of water content happens as well as decrease of PG content and disruption of the collagen network. Subchondral trabecular bone also deteriorates, with increased separation and thinner trabeculae. At the same time, microdamage begins to appear in both calcified cartilage and subchondral bone, which will persist throughout the whole pathological process. In late-stage of OA, calcified cartilage and subchondral plate become thicker, with reduplicated tidemarks and progressive non-calcified cartilage damage. Chondrocyte cluster, hypertrophy and apoptotic happen. Subchondral trabecular bone becomes sclerotic. The sclerosis of periarticular mineralized tissues may be a biomechanical compensational adaptation to the widespread cysts and microdamage in subchondral bone, which render subchondral bone structure more weaker (**Figure 10**) (Roach, 2008).

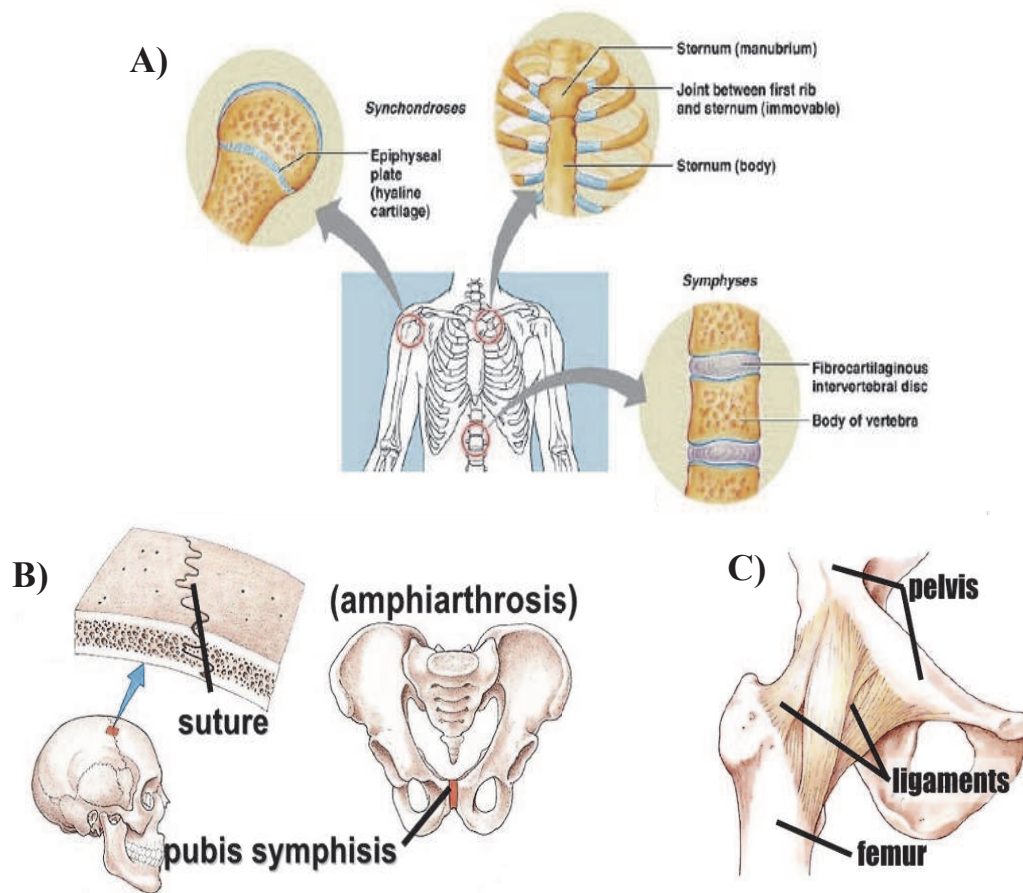


**Figure 10: Progression of OA.**

Adapted from (Goldring and Goldring, 2016).

## 2. Human joints and cartilage composition

A joint or articulation (or articular surface) is the connection made between bones in the body, which link the skeletal system into a functional whole. Three classes of joint design are identified (**Figure 11**): 1) Synovial or Diarthrodial joints which articulate with free movement, have a synovial membrane lining the joint cavity, and filled by synovial fluid; 2) Amphiarthrosis joint is a type of continuous, slightly movable joint like symphysis, intervertebral disks of vertebral bodies, distal tibiofibular articulation, sacroiliac joint articulation with pelvic bones); and 3) Synarthrosis joint which are found only in the skull (suture lines), is a type of joint which permits very little or no movement under normal conditions, yet permit growth in childhood and adolescence (Tom, 2017).



**Figure 11: Three classes of joint design.**

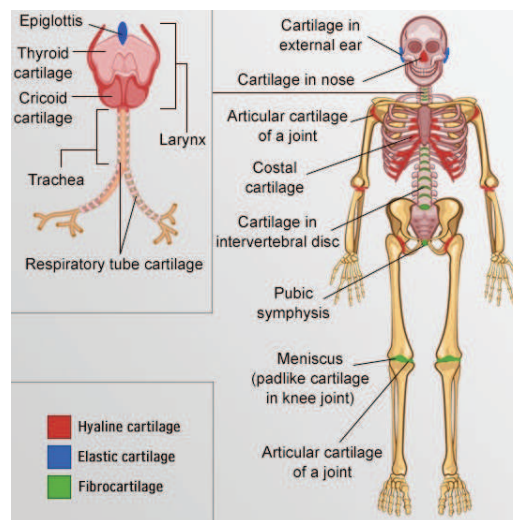
A): Amphiarthrosis (Slightly movable joint), B): Synarthrosis (Immovable joint), C): Synovial or Diarthrodial (Freely movable joint). Adapted from: <https://www.slideshare.net/slideshow/skeletal-system2>

Cartilage is hyalin cartilage and an avascular tissue that consists of an extensive ECM (about 95%) that is produced and maintained by chondrocytes (about 5%) (Roughley 2002)\*. All cartilage matrix are composed predominantly of aggrecan and Type II collagen. Type II collagen is primarily responsible for tensile strength, while aggrecan molecules, when entrapped within the Type II collagen lattice, provide compressive strength. The highly sulfated GAG side chains of CS and KS from aggrecan enable the matrix to structure large amounts of water and thereby create a large osmotic pressure (Ehrlich et al., 1998). Long chains of HA are present throughout the matrix and serve to specifically bind aggrecan and link protein to retain the aggrecan molecules within the cartilage ECM (Caplan, 1984). Together, these components help to retain water within the ECM, which is critical to maintain its unique mechanical properties (Sophia Fox et al., 2009).



## 2.1. Different type of cartilage

Different types of cartilage tissue are present throughout the body at various sites (**Figure 12**). They are classified histologically into hyaline, elastic, and fibrocartilaginous. (Naumann et al., 2002) (**Figure 13**). Type II collagen is the principal molecular component in healthy articular cartilage, but collagens III, VI, IX, X, XI, XII, and XIV all contribute in smaller amounts to the mature matrix. The main PG present in cartilage is aggrecan, which is a large chondroitin sulfate CS and KS proteoglycan. Other PGs found in cartilage include the syndecans and glypican, the decorin, biglycan, fibromodulin, lumican, epiphygan, and perlecan (Umlauf et al., 2010) which will be explained in chapter 3.



**Figure 12: Different places in the body that three types of cartilage are found.**

<https://www.buzzle.com/articles/understanding-the-types-of-cartilage-in-the-human-body.html>

### Hyaline cartilage

Hyaline cartilage is the most common type of cartilage, which is commonly associated with the skeletal system. It can be found at many places in the human body including nose, between the joints such as knees, hips, and shoulder as articular cartilage and at places where the ribs attach to the sternum bone. It is characterized by its type II collagen and high concentration of aggrecan (Ross & Pawlina, 2015)\*.

### Elastic cartilage

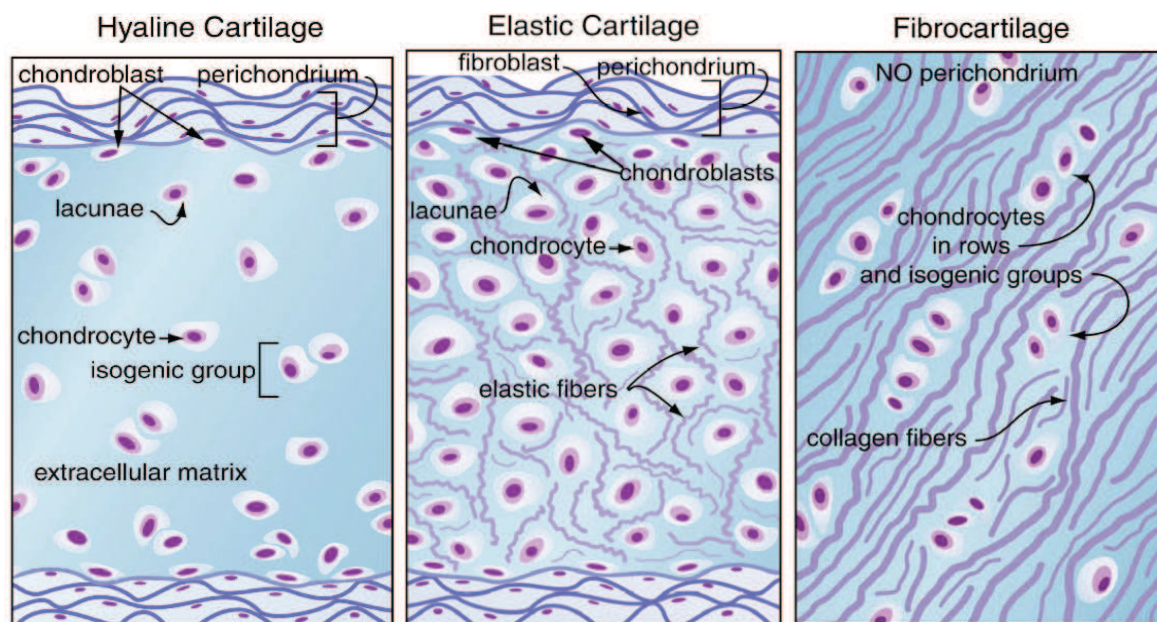
Elastic cartilage has yellow color, which is quite springy due to the presence of many irregular networks of elastin protein fibers in the matrix. Elastin fibers are responsible for its ability to spring back into shape immediately after being deformed. It resembles the hyaline cartilage to some extent. It has chondrocytes surrounded with a thin collagenous network of cartilage-ECM,

and the dense networks of elastic fibers are found between these regions. The differentiating factor between the two types of cartilage is the presence of elastin fibers that are embedded in the ground substance. Perichondrium is also found around this type of cartilage. It helps in forming and maintaining the shape of certain structures of the body. It is a supportive, elastic tissue that provides firmness and flexibility. It can be found at the cartilaginous structure in the external ears, pharyngotympanic (Eustachian) tubes, parts of the nose, larynx and epiglottis (Ross & Pawlina, 2015)\*.

### **Fibrocartilage**

Fibrocartilage is the toughest type of cartilage, which has white color. It has the ability to tolerate heavy weights. The defining feature when it comes to the histology is that it contains fine collagen fibers spread in a rows or layers, but with gaps between the lacunae and the bundles of collagen fibers. The number of chondrocytes is quite less, and the cells are embedded in the matrix material between the fibers, and not on the fibers. Unlike the other cartilage types, it is composed principally of type I collagen and has a lower proteoglycan content. This cartilage is very effective as a shock absorber due to its ability to resist compression forces. It provides support to the surrounding structures that are attached to it. It is found at different parts of human body such as in the intervertebral discs in the spine, menisci in the knees and pubic symphysis, which is place where the hip bones join at the front of the body (Ross & Pawlina, 2015)\*.

All the three types of cartilage present in the human body play an important role, especially the articular hyaline cartilage that ensures friction-free movement of the joints. The other two types are also important, as they act as a cushion for certain bones, provide support to the surrounding structures, and resist the compression forces. These can get damaged due to injuries or get worn down with age. Cauliflower ear, slipped disc and OA are examples of damage to the elastic cartilage, fibrocartilage, and hyaline cartilage, respectively.



**Figure 13: Different types of cartilage tissue classified histologically into hyaline, elastic, and fibrocartilaginous.**

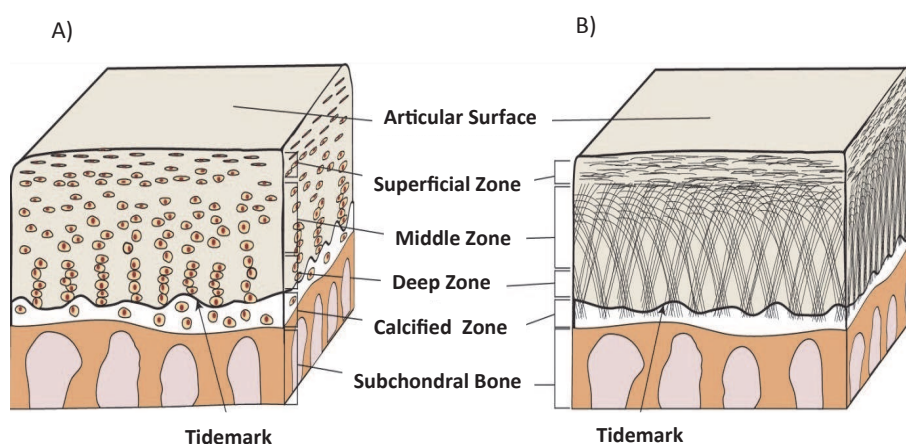
After: [http://www.auburn.edu/academic/classes/zy/hist0509/html/Lec05Bnotes-cart\\_bone\\_bloo.html](http://www.auburn.edu/academic/classes/zy/hist0509/html/Lec05Bnotes-cart_bone_bloo.html)- Copyright R. Nims & S.C. Kempf 12/2000

## 2.2. Normal articular cartilage homeostasis

Articular cartilage is hyaline cartilage with about 2 to 4 mm thick which is an avascular tissue nourished by diffusion from the vasculature of the subchondral bone and from the synovial fluid. It is about 80% water, and it is hypocellular compared with other tissues; chondrocytes constitute only 2% of its total volume (Rizkalla et al., 1992; Stockwell et al., 1983). Most of the dry weight of cartilage consists of type II collagen and the large aggregating proteoglycan, aggrecan. However several noncollagenous proteins and glycoproteins also seem to play a role in cartilage-matrix organization (Dudhia, 2005; Eyre et al., 2006). Together, these components help to retain water within the ECM, which is critical to maintain its unique mechanical properties (Sophia Fox et al., 2009). Mature articular cartilage is a heterogeneous tissue with four distinct regions: (1) the superficial tangential zone, (2) the middle zone, (3) the deep zone, and (4) the calcified zone, which is located immediately below the tidemark and above the subchondral bone (Misra et al., 2015; Poole et al., 1982a) (**Figure 14**).

The thin superficial (tangential) zone protects deeper layers from shear stresses and makes up approximately 10% to 20% of articular cartilage thickness, associated with type II and IX collagen (Sophia Fox et al., 2009), a high concentration of the small proteoglycan decorin and a low concentration of aggrecan. This zone is in contact with synovial fluid and is responsible for most of the tensile properties of cartilage, which enable it to compressive forces imposed by articulation. The middle zone represents 40% to 60% of the total cartilage volume, and it contains

PGs and thicker collagen fibrils which are arranged in radial fashion (Sophia Fox et al., 2009). In this zone chondrocytes are spherical with low density. Functionally, the middle zone is the first line of resistance to compressive forces. The deep zone is responsible for providing the greatest resistance to compressive forces. In this zone, chondrocytes frequently are grouped in columns or clusters and cell density progressively decreases from the surface to the deep zone, where it is one-half to one-third the density in the superficial zone (Stockwell, 1972). Chondrocytes in the deep and middle zones have a cell volume that is twice that of superficial chondrocytes (Wong et al., 1997). Water is progressively decreases from 75% to 80% in the superficial zone to 65% to 70% with increasing depth. Amount of collagen is decreased in the middle and deep zones, and type I collagen may be synthesized, in addition to type II collagen (Muir et al., 1970; Stanescu et al., 1976). With increasing depth, the proportion of PG increases to 50% of the dry weight (Maroudas et al., 1969; Ratcliffe et al., 1984; Wong et al., 1997)14,17-19. The calcified zone is formed as a result of endochondral ossification and persists after growth plate closure as the histologically defined tidemark (Lane et al., 1977). The calcified zone plays an important role as a mechanical buffer between uncalcified articular cartilage and subchondral bone. In this zone, the cell population is rare and chondrocytes are hypertrophic (Sophia Fox et al., 2009).



**Figure 14: Illustrations showing the zonal features of human articular cartilage and subchondral bone.**

A) (Chondrocyte organization): The superficial zone chondrocytes are small and flattened, which are orientated parallel to the joint surface. The middle zone chondrocytes are rounded and oriented randomly. The deep zone chondrocytes are grouped in columns or clusters. B): Collagen orientation in articular cartilage. In the superficial (tangential) zone, small and medium-sized collagen fibrils aggregate into bundles that are aligned parallel to the joint surface. In the middle and deep zones, collagen fibrils aggregate into larger bundles and are aligned more perpendicular to the joint surface. <https://www.intechopen.com/books/osteoarthritis-progress-in-basic-research-and-treatment>

In addition to zonal variations in structure and composition, the matrix consists of several distinct regions based on proximity to the chondrocytes, composition, and collagen fibril diameter and organization. The ECM can be divided into pericellular, territorial, and interterritorial regions. The pericellular matrix is a thin layer adjacent to the cell membrane, and it completely surrounds the chondrocyte. It contains mainly PGs, as well as glycoproteins and other noncollagenous proteins. This matrix region may play a functional role to initiate signal transduction within cartilage with load bearing (Eggle et al., 1985). The territorial matrix surrounds the pericellular matrix; it is composed mostly of fine collagen fibrils, forming a basketlike network around the cells (Guilak and Mow, 2000). This region is thicker than the pericellular matrix, and it has been proposed that the territorial matrix may protect the cartilage cells against mechanical stresses and may contribute to the resiliency of the articular cartilage structure and its ability to withstand substantial loads. The interterritorial region is the largest of the 3 matrix regions; it contributes most to the biomechanical properties of articular cartilage. This region is characterized by the randomly oriented bundles of large collagen fibrils, arranged parallel to the surface of the superficial zone, obliquely in the middle zone, and perpendicular to the joint surface in the deep zone. Proteoglycans are abundant in the interterritorial zone (Sophia Fox et al., 2009).

### **2.3. Cartilage components**

#### **Water**

Water is the major component of articular cartilage, about 80% of wet weight of the cartilage is formed by water, with 80% being in the superficial zone and 65% in the deep zones (Bhosale and Richardson, 2008). Approximately 30% of this water is associated with the intrafibrillar space within the collagen, although a small percentage is contained in the intracellular space. The rest is contained in the pore space of the matrix (Guccione et al., 1994). Inorganic ions such as sodium, calcium, chloride, and potassium are dissolved in the tissue water (Lai et al., 1991; Linn and Sokoloff, 1965). The relative water concentration decreases from about 80% at the superficial zone to 65% in the deep zone (Poole et al., 1982b). The circulation of water through the cartilage and across the articular surface helps to transport and distribute nutrients to chondrocytes and providing lubrication.

#### **Collagens**

Collagen is the most abundant structural macromolecule in ECM, and it makes up about 60% of the dry weight of cartilage (Kelley et al. 2013)\*. It consists of 3 polypeptide chains that form a triple helix along at least part of their length. They can be divided into fibrillar collagens (types I, II, III, V and XI), which form the framework of the tissue, and non-fibrillar collagens. Type II collagen represents 90% to 95% of the collagen in ECM and forms fibrils and fibers intertwined with proteoglycan aggregates, whereas types I, IV, V, VI, IX, and XI present in a minor ratio and they help to form and stabilize the type II collagen fibril network (Sophia Fox et al., 2009). For

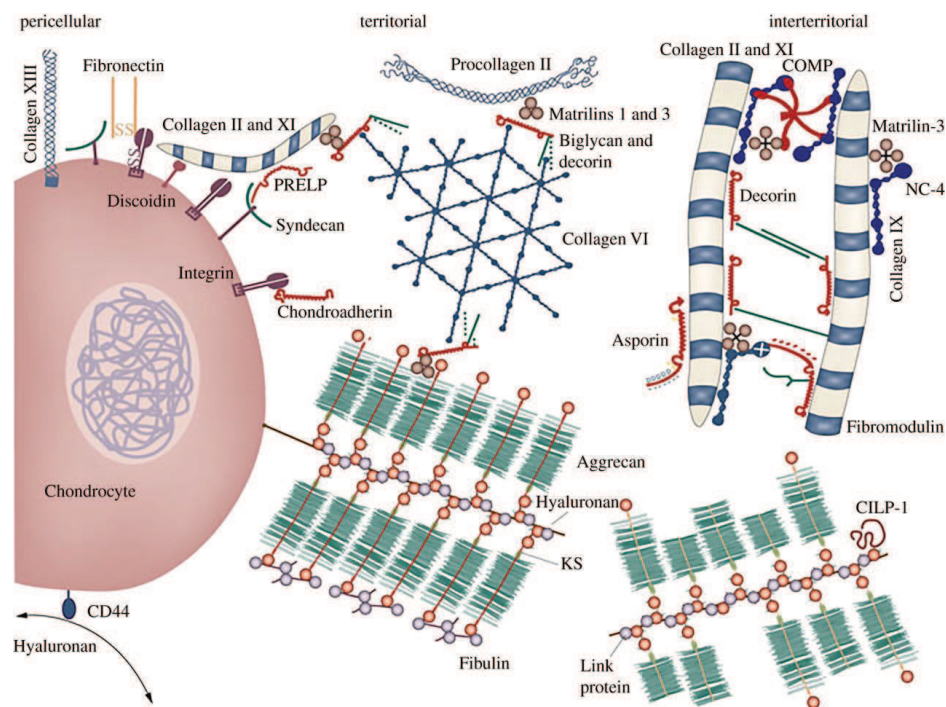
example collagen VI plays a role in cell attachment and interacts with other matrix proteins, such as hyaluronan, decorin, and biglycan (Aigner et al., 2007). Type IX collagen is a PG and a collagen because it contains a CS chain attachment site in one of the noncollagen domains and destruction of type IX collagen accelerates cartilage degradation and loss of function (Maroudas et al., 1991).

### **Proteoglycan**

In articular cartilage, PGs represent the second-largest group of macromolecules in the ECM and account for 10% to 15% of the wet weight (Sophia Fox et al., 2009) such as aggrecan, decorin, biglycan, bromodulin and etc. The largest in size and the most abundant by weight is aggrecan, which is characterized by its ability to interact with HA (Hunziker et al., 1997) (Figure 15). Aggrecan provides cartilage with its osmotic properties, which are critical to its ability to resist compressive loads. Cartilages also contain non-aggregating proteoglycans, which are characterized by their ability to interact with collagen rather than hyaluronic acid. They are much smaller in size and possess only a few DS (decorin and biglycan) or KS (fibromodulin and lumican) chains (Roughley 2002)\*. They mediate the interactions between adjacent collagen fibrils, or with other matrix components. Cartilage also contains perlecan, a heparan sulfate proteoglycan normally associated with basement membranes. The role of PGs in OA will be completely explained in chapter 3.

### **Noncollagenous Proteins and Glycoproteins**

Although a number of noncollagenous proteins and glycoproteins are found within articular cartilage, their specific function has not been fully characterized. Some of these molecules (such as bronectin and CII, a chondrocyte surface protein) likely play a role in the organization and maintenance of the macromolecular structure of the ECM (Sophia Fox et al., 2009).



**Figure 15: The molecular arrangement of extracellular matrix in cartilage.**

Two major load-bearing macromolecules are present in articular cartilage: collagens (mainly, type II) and proteoglycans (notably, aggrecan). Smaller classes of molecules, such as noncollagenous proteins and smaller proteoglycans, are present in smaller amounts. The interaction between the highly negatively charged cartilage proteoglycans and type II collagen provides the compressive and tensile strength of the tissue. Adapted from (Lindahl, 2015).

## Chondrocyte

The chondrocyte is the only cell type in articular cartilage. Immature articular cartilage also has stem cells but adult articular does not. Chondrocytes are highly specialized, metabolically active cells that play a unique role in the development, maintenance, and repair of the ECM. Chondrocyte originates from mesenchymal stem cells and constitute about 2% of the total volume of articular cartilage (Alford and Cole, 2005). They are different in shape, number, and size, depending on the anatomical regions of the articular cartilage. Their terminal differentiation determines the character of the cartilage (hyaline, fibrous, or elastic). They are flatter and smaller in superficial zone and generally have a greater density than that of the cells deeper in the matrix. Each chondrocyte establishes a specialized microenvironment and is responsible for the turnover of the ECM in its immediate vicinity. This microenvironment essentially fixes the chondrocyte within its own matrix and so prevents any migration to adjacent areas of cartilage. They are responsible for the synthesis of the two major constituents of the matrix, collagen, PG and enzymes that degrade matrix components (collagenase, neutral proteinases, and cathepsins) (Gold et al., 2003). This double function places the chondrocyte in the role of regulating cartilage synthesis and degradation (Ross E. Petty 2016)\*. Unfortunately, chondrocytes have limited

potential for replication, a factor that contributes to the limited intrinsic healing capacity of cartilage in response to injury.

## 2.4. Chondrogenesis and ossification

There are 2 main types of bone defined by their embryonic origin and their type of ossification:

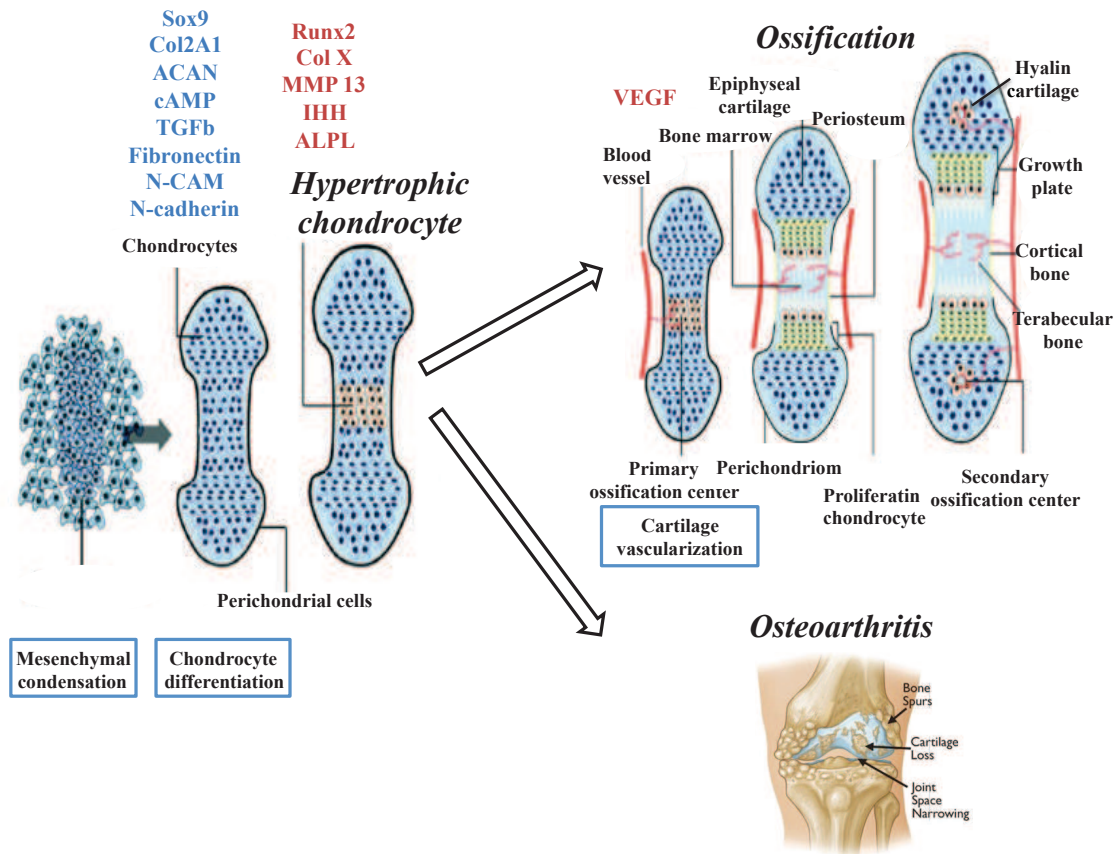
Flat bone from ectodermic origine, are formed by membranar or endoconjonctive ossification. This implique the osteoblastic diferenciacion of mesenchymal stem cells, that permit a direct ossification of the matrix devoided of a cartilaginous step. Flat bones are those of the calvaria and the face.

Short and Long bones are from mesodermic origin, and are formed and growing according to and Endochondral (or cartilaginous) ossification; This consist of multiple steps including the condensation of mesenchymal cells which express collagens I, III and V (Goldring, 2012), differentiation of those cells into chondrocytes, proliferation, hypertrophy, and apoptosis of the chondrocytes, followed by vascular invasion, and calcification, these complex programs are sequentially and exquisitely regulated during development (Nishimura et al., 2008). During limb development, the resting chondrocytes form the cartilage at the ends of the opposing bones with the intervening interzones formed during cavitation or they undergo proliferation, then proceed to terminal differentiation to hypertrophy, and apoptosis to permit endochondral ossification, in which calcified hypertrophic cartilage is resorbed and replaced by bone (Xie et al., 2014) (**Figure 16**).

The proliferating chondrocytes express collagen VI and matrilin 1 and are under control of the Parathyroid Hormone-related Peptide (PTHrP)/ Indian Hedgehog (IHH) axis. The hypertrophic zone is characterized by expression of collagen X and calcification of the matrix. Matrix remodeling involving Matrix Metalloproteinase (MMP) 9, 13, and 14 and vascularization mediated by VEGF and VEGF receptors are required to convert the nonvascularized and hypoxic tissue to bone through the actions of osteoclasts and osteoblasts. A similar sequence of events occurs in the postnatal growth plate, leading to rapid growth of the skeleton (Onyekwelu et al., 2009). These processes are subject to complex regulation by intermediate role of the FGF, TGF $\beta$ , Bone morphogenetic proteins (BMP) and Wnt signaling pathways (Goldring, 2012). SOX9 and Runt-related Transcription Factor 2 (RUNX2) are two crucial transcriptional regulators which are essential for articular cartilage formation and hypertrophic maturation, respectively (Lefebvre and Smits, 2005; Wuelling and Vortkamp, 2011). Moreover, RUNX2 is subject to direct inhibition by SOX9, and TGF $\beta$  and BMP signals differentially regulate Wnt/ $\beta$ -catenin signaling through activation of RUNX2 (Yoon et al., 2006). Recent studies indicate that epidermal growth factor receptor signaling is involved in regulating endochondral ossification in the developing growth



plate and its ligand TGF $\alpha$  suppresses articular chondrocyte phenotype through activating Rho/ROCK and MEK/ ERK signaling (Appleton et al., 2010) (**Figure 16**).



**Figure 16: The process of chondrogenesis of MSCs and endochondral ossification.**

Chondrogenesis is initiated by the condensation of MSCs, and cell-cell contact. The expression of cAMP, TGF $\beta$ , Fibronectin, N-CAM and N-cadherin is involved in this process and these factors are necessary for chondrogenic induction, marked by the expression of chondrogenic genes: SOX9, ACAN, COL2A1. Mature chondrocytes begin secreting cartilage matrix primarily consisting of collagen II and GAGs, which are the main components of cartilage. Chondrocytes from in vitro chondrogenesis of MSCs or in vivo cartilage could undergo hypertrophic differentiation, which is characterized by an increase in cell volume and the expression of hypertrophic markers (RUNX2, Collagen X, MMP13, IHH and ALPL). In vivo, physiological endochondral ossification and pathological osteoarthritis could be initiated after remodeling, mineralization of the extracellular matrix, and apoptotic death of chondrocytes. After (Karsenty and Wagner, 2002; Long and Ornitz, 2013; Xie et al., 2014).

## 3. What is OA?

### 3.1. Aging cartilage

Often there is difficulty to discriminate between the effects of aging itself and diseases such as OA that become more common with increasing age (Carrington, 2005; Loeser, 2006a). In both cases, biochemical changes in composition of matrix result in changes in cartilage structure.

With aging, zonal changes happen in the distribution of chondrocytes; however, the total number of chondrocytes remains unchanged. In the superficial zone, chondrocytes begin to disintegrate, whereas the deeper layers have an increased number of cells. Some of the cells, in particular in the deeper layers of the cartilage, start expressing markers of chondrocyte hypertrophy including expression of collagen type X.

The concurrent change in matrix composition and increased calcification is likely changing the biomechanical properties of the tissue and further contributing to its progressive destruction. Decrease in the hydration of the matrix happens by increasing age, with a corresponding increase in compressive stiffness. This may have implications for the underlying subchondral bone, which may feel increased forces as the cartilage loses its ability to undergo reversible deformation. The thickness of articular cartilage, could be noted on Magnetic Resonance Imaging (MRI) (Burstein and Gray, 2006).

TGF $\beta$  has an important role in cartilage homeostasis and normally signals to the Activin Like Kinase 5 (ALK5) receptor leading to intracellular activation of smad2 and 3 molecules. With aging, the ratio between the ALK5 and alternative ALK1 receptor appears to change, leading to preferential activation of smad1 and 5 molecules within the cell by TGF $\beta$ -ALK1 binding at the cell surface (van der Kraan et al., 2010). This specific cascade is associated with increased MMP13 production and with terminal differentiation of the chondrocytes, two features that may contribute to osteoarthritis.

### 3.2. Cellular aspect (Hypertrophy in Articular Chondrocytes)

It has been demonstrated that the development of OA is due to activation of hypertrophic differentiation of articular chondrocytes. Healthy articular cartilage is a stable tissue that has the potential to resist hypertrophic differentiation and maintain the normal phenotype through an unknown mechanism (Mackie et al., 2008), but during hypertrophic differentiation of chondrocytes in OA, chondrocytes lose the stable phenotype and gain the expression of hypertrophic phenotypic markers such as RUNX2, Collagen type X, MMP13, IHH and Alkaline phosphatase (ALPL) (Zhong et al., 2015).

Chondrogenic hypertrophy is known by a more than 10-fold increase in cell volume and ECM structural remodeling (Bush et al., 2008). Increase in cell volume affects cell's normal function (Chao et al., 2006). This enormous volume increase involves changes in both intracellular and extracellular osmolarity, ECM degradation around the cell, and an increase in the amount of organelles per cell (Mackie et al., 2011). Osmotic swelling has been shown stereologically to be responsible for most of the cell volume increase. Swelling can be the result of either an increase in cytoplasmic concentration or a decrease in extracellular osmolarity followed by aquaporin-mediated movement of water to re-establish iso-osmotic conditions (Wang et al., 2017).

Collagen type X, which is considered as the standard marker for chondrocyte hypertrophy, and MMP13 or collagenase-3 are two most important markers during chondrocyte hypertrophy.

The function of collagen type X is still unknown but its presence at the sites of chondrocyte hypertrophy and calcification suggest its role during early stage of endochondral bone formation. Collagen type X is normally not expressed in human healthy articular cartilage but is expressed at mRNA and protein level in human OA cartilage (Eyre, 2002). Particularly, in the proximity of lesions, collagen type 10alpha1 (COL10A1) has been shown to be upregulated. In a study by Fukui et al., focusing on the regional and zonal differences in gene expression in OA cartilage, it was demonstrated that overall expression of COL10A1 was elevated but that expression showed significant local variation. Notably, the expression of COL10A1 was higher in the less degenerated than in the more degenerated areas (Fukui et al., 2008). However, a number of expression array studies, both in human and experimental models of OA, have failed to reveal elevated COL10A1 expression in OA cartilage. Sampling differences, zonal differences and stage dependent alterations might explain these differences. An alternative explanation could be chondrocyte hypertrophy-like changes are not a general phenomenon in human OA but only observed in a subgroup of patients (Brew et al., 2010).

Collagenase-3 or MMP13, is also frequently shown to be elevated in OA cartilage (Shlopov et al., 2000). Up regulation of MMP13 by more than 40-fold in OA cartilage compared to normal cartilage was shown by microarray and RT-PCR. Moreover, in some studies that do not have

reported a clear increase of COL10A1 expression an increased expression of MMP13 in OA cartilage was demonstrated (Wang et al., 2004) This means that MMP13 and type X collagen expression are uncoupled and MMP13 expression can be augmented by different pathways rather than chondrocyte terminal differentiation (van der Kraan and van den Berg, 2012).

### **3.2.1. Signaling Pathways in Hypertrophy**

Several signaling pathways have been involved in regulation of hypertrophy-like changes in chondrogenesis of MSCs and chondrocytes. Based on recent literature, the most important related pathways are WNT, Bone morphogenetic protein (BMP)/TGF $\beta$ , PTHrP, IHH, FGF, IGF and Hypoxia-inducible factor (HIF) signaling pathways (Kronenberg, 2003). In each single pathway, several distinct subtypes are involved in the regulation of chondrocyte differentiation and hypertrophy. **Table 3** describes different signaling pathways and their main functions involved in chondrocyte hypertrophy.

Signal	Subtypes	Main Functions	References
WNT	WNT3a, WNT5a, WNT5b	Promotes chondrogenic differentiation; delays chondrocyte hypertrophy	(Blom et al., 2009; Dong et al., 2006; Hill et al., 2005; Miller, 2002; Yano et al., 2005)
	WNT4, WNT8, WNT9a	Blocks chondrogenic differentiation; promotes chondrocyte hypertrophy	
	WNT11	Promotes chondrogenic differentiation; stimulates RUNX2 and IHH expression	
	WNT16	Upregulation is accompanied by the downregulation of FRZB	
BMP/TGF- $\beta$	BMP2 & BMP4	Induces chondrocyte hypertrophy	(Caron et al., 2013; Miyazawa et al., 2002; Miyazono et al., 2004; Shu et al., 2011; Steinert et al., 2009; Weiss et al., 2010; Zhang et al., 2009)
	BMP7	Maintain chondrogenic potential and prevents chondrocyte hypertrophy	
	TGF- $\beta$	Promotes chondrogenic differentiation; inhibits chondrocyte hypertrophy	
	PTHrP	Blocks hypertrophy by stimulating Nkx3.2 and prevent RUNX2 expression	
	IHH	Promotes chondrocyte hypertrophy; Stimulates proliferating chondrocytes to produce PTHrP	
FGF	FGF2	Promotes expression of RUNX2	(Chia et al., 2009; Davidson et al., 2005; Ellman et al., 2013; Hung et al., 2007; Lewandoski et al., 2000)
	FGF8	Catabolic mediator with a pathological role in rat and rabbit articular cartilage	
	FGF9	Promotes chondrocyte hypertrophy	
	FGF18	Promotes chondrocyte proliferation and differentiation in the early stages of cartilage development	
IGF	IGF-1	Promotes chondrocyte proliferation and maturation; augments chondrocyte hypertrophy	Hunziker et al., 1994; Longobardi et al., 2009)
HIF	HIF-1 $\alpha$	Potentiates BMP2-induced SOX9 expression and cartilage formation, while inhibiting RUNX2 expression and endochondral ossification	(Saito et al., 2010; Yang et al., 2010; Zhang et al., 2015)
	HIF-2 $\alpha$	Increases expression of collagen X, MMP13 and VEGF	

**Table 3: Signal pathways of chondrocyte hypertrophy.**

In normal chondrocytes, signal pathways are regulated by their antagonists (DKK1 and FRZB for WNT, GREM1 for BMP) or other signal factors to get a fine balance to maintain the chondrocyte normal phenotype. The most important transcription factor regulating chondrocytes is SOX9, which is responsible for the expression of main chondrocyte makers including collagen type II and aggrecan. Subsequently, the effects of cascade pathways result in activating the transcription

factor RUNX2, which regulates the transcription of hypertrophic markers like collagen X, MMP-13, VEGF and IHH.

### **3.2.2. Cellular senescence and the senescence-associated secretory phenotype**

Cellular senescence is one of the most important points of ageing, and chondrocytes have many features that are characteristic of senescent cells during ageing and during OA.

The notion that OA catalysis the development of senescence proves that cellular stresses might have a critical role in establishing this phenotypic state. However, up to now the difficulty is how to define chondrocyte senescence and determine the underlying mechanisms that could be potentially used for therapeutical aspects. In vitro, we can characterize cellular senescence by studying the morphological changes and a significant decrease in cell proliferation after monolayer expansion, but defining cellular senescence in vivo has proved to be more complex.

Two main criteria of senescent cells are stress-induced permanent proliferative arrest and resistance to both mitogenic and oncogenic stimuli; unfortunately, both are very difficult to assess in chondrocytes. Chondrocytes have low proliferation rate, which disable us to measure and interpret their real impact on further reduction in proliferation. Lineage tracing experiments in mice models demonstrated that proliferation of particular chondrocyte subpopulations could maintain cartilage tissue (Kozhemyakina et al., 2015). Therefore, the loss of proliferation in these cells via senescence could contribute to OA progression. The other criterion that could help us study senescence is its ability to prevent the division of transformed cells (Muñoz-Espín and Serrano, 2014). Chondrosarcomas typically derive from the growth plate and perichondrium but not from articular cartilage, suggesting that articular cartilage might be inherently unlikely to undergo uncontrolled growth. This characteristic presents a challenge for the identification of senescent chondrocytes on the basis of an increased resistance to oncogenic transformation (Bové et al., 2010).

Another important feature of cellular senescence is the production of Senescence-Associated Secretory Phenotype (SASP). SASP is defined by the production of high levels of proinflammatory cytokines and matrix-degrading enzymes (Freund et al., 2010). Indeed, the SASP seems to be regulated independently from cell cycle arrest (Coppé et al., 2011). Some of the most highly upregulated SASP-related factors, such as Interleukin 1  $\alpha$  (IL-1 $\alpha$ ), IL-6 and Monocyte Chemoattractant Protein 1 (MCP-1, also known as CC chemokine ligand 2 (CCL2)) are found in OA cartilage (Tsuchida et al., 2014), hindering efforts to determine whether the level of a particular mediator is due to senescence or a result of OA. Moreover, the cell type that is responsible for producing these cytokines is also difficult to determine. Since, these factors are also found in the synovial fluid, they could be produced by cells other than chondrocytes, for instance, cells in the meniscus, synovium or bone (Loeser et al., 2012).

More detailed understanding of how chondrocytes enter the senescent state might enable researchers to develop treatments suitable for preventing such a phenotypic switch during OA. One model proposes that senescence occurs during conditions characterized by simultaneous signals for cell cycle arrest and cell growth, and aged cartilage tissue has the potential to provide both these cues (Demidenko and Blagosklonny, 2008).

Among the factors that could alter gene expression patterns in chondrocytes are DNA damage and oxidative stress. These factors could occur with ageing and in the OA tissue microenvironment (Loeser et al., 2012; Rose et al., 2012) and cause telomere attrition due to targeted DNA damage (Tsuchida et al., 2014). Of importance, stem cells and postmitotic cells such as neurons and chondrocytes can be particularly susceptible to the accumulation of cellular damage during the long interval between replicative events (Jurk et al., 2012; Liu and Rando, 2011). One common consequence of accumulating DNA damage is the upregulation of cell cycle inhibitors, like Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A or known as p16<sup>INK4A</sup>) and cyclin-dependent kinase inhibitor 1 (known as p21), which mediate the stable arrest that is associated with senescence (Sharpless and Sherr, 2015). Evidence correlating ageing and OA with increased p16<sup>INK4A</sup> gene expression in chondrocytes has been suggested by mechanistic study (Philipot et al., 2014). This is in accordance with a murine model in which by using muscle stem cells, increased p16<sup>INK4A</sup> expression with age, made cells more likely to undergo senescence during subsequent proliferation after injury (Sousa-Victor et al., 2014).

The second extremely important signal in senescence is the presence of growth factors that are either directly secreted from damaged cartilage or synthesized at high levels by chondrocytes during OA. For instance, bFGF is released from damaged cartilage tissue (Vincent et al., 2002), and can be found in focal clusters of chondrocyte proliferation that develop near areas of cartilage damage (Hoshiyama et al., 2015). As a consequence of signalling by inflammatory cytokines associated with OA, expression of bone BMP-2 is increased (Fukui et al., 2003), thereby stimulating enhanced turnover of extracellular matrix (Blaney Davidson et al., 2007a). Although their capacity for proliferation indicates that these chondrocytes are not senescent, cells that enter the cell cycle in the context of potentially damaging stimuli have higher probability to enter senescence as opposed to returning to quiescence (Johmura et al., 2014; Krenning et al., 2014). Regarding the results showing senescence occurs after monolayer expansion of chondrocytes, provide further evidence that status of increased proliferation correlates with emergence of the senescent phenotype.

### **3.2.3. Oxidative stress and the mitochondria**

Mitochondrial dysfunction is a hallmark of ageing that has attracted particular attention in the context of OA. The free radical concept suggests that cell damage happening as a result of excessive levels of Reactive Oxygen Species (ROS) substantially contributes to the development of the ageing phenotype and to the progression of age-related diseases (Jones, 2015). However, in addition to cell damage, increased levels of ROS produced as a result of age-associated oxidative stress also promote disease by disturbing homeostatic physiological cell signaling (Hui et al., 2016).

Using murine model, it has been demonstrated that mice with post-traumatic OA had increased intracellular and mitochondrial superoxide generation. This was associated with downregulation of mitochondrial superoxide dismutase 2 (SOD2) expression. Furthermore, in this same study,

SOD2 loss resulted in a remarkable increase of age-related cartilage degeneration (Koike et al., 2015).

One of the mechanism by which ROS regulates cell signalling is via oxidative post-translational modifications of particular thiol groups in proteins, which possess reactive cysteines (Klomsiri et al., 2011). Initially, cysteine oxidation results in the formation of a cysteine sulfenic acid (Cys-SOH) in a process known as S-sulfenylation. It has recently been shown; chondrocytes from patients with OA had an increased basal level of S-sulfenylation in comparison to those of healthy controls. ROS can induce sulfenylation of multiple chondrocyte proteins such as tyrosine kinase SRC; consequently, it increases the production of MMP-13. Collectively, these studies highlight ROS as crucial secondary signalling molecules in chondrocytes that requires further study in the context of ageing and OA (Wood et al., 2016).

#### **3.2.4. Dysfunctional energy metabolism**

Another important hallmark of ageing is dysregulated nutrient sensing. 5'-AMP Activated Protein Kinase (AMPK) is a key regulator of cell metabolism and energy balance that is activated by stressors that enhance the cellular AMP/ATP ratio (Hardie and Ashford, 2014). The activity of AMPK and its regulatory upstream kinase, Serine/Threonine-Protein Kinase STK11 (also known as Liver Kinase B1 (LKB1)), is reduced in cartilage from aged mice and mice with OA, as well as in bovine chondrocytes after dynamic compression-induced biomechanical injury (Petursson et al., 2013). Similarly, OA-associated reductions in AMPK activity have been observed in human chondrocytes and cartilage (Terkeltaub et al., 2011), and this reduced activity has been associated with substantially reduced mitochondrial biogenesis (Wang et al., 2017). Interestingly, AMPK could also modulate key homeostatic signalling pathways via regulation of autophagy, a cellular process that removes damaged and dysfunctional organelles and proteins (Hardie and Ashford, 2014; Lotz and Caramés, 2011).

Carames B et al, have recently shown that the expression of key autophagy proteins was remarkably decreased in cartilage from aged mice compared with that of young mice, and these changes were associated with enhanced levels of apoptosis and cartilage degeneration (Caramés et al., 2015). Similar results were also found in a mouse model of post-traumatic OA, claiming, dysfunctional autophagy as a key mechanism in both ageing and OA (Caramés et al., 2010).

### **3.3 Anabolic homeostasis**

Growth and differentiation factors generally are considered as positive regulators of homeostasis of mature articular cartilage because of their capacity to stimulate chondrocyte anabolic activity and, in some cases, inhibit catabolic activity. Numerous anabolic growth factors stimulate synthesis of proteoglycans by chondrocytes, aggrecan and type II collagen, induce synoviocyte and MSC proliferation, drive chondrogenic differentiation of MSCs, and decrease the catabolic effects of cytokines such as interleukin-1 and the MMP (Loeser, 2006b). The best characterized



anabolic factors in the context of their production and action in articular cartilage include IGF-I and members of the FGF and TGF $\beta$ / BMP families (Lories and Luyten, 2005). Many of these factors also regulate chondrogenesis and endochondral ossification during skeletal development. In adult cartilage, their expression declines with age, a risk factor for OA, and their activities are downregulated (Loeser, 2006b). **Table 4** shows summary of the effect of anabolic growth factors on chondrocytes/cartilage and synovium.

Growth factor	Chondrocytes/cartilage	Synovium	References
TGF $\beta$ 1	Stimulates synthesis of ECM Decreases catabolic activity of IL-1 and MMPs	Causes synovial proliferation and fibrosis Induces chemotaxis of inflammatory leukocytes to synovium  Induction of osteophyte formation	(Bakker et al., 2001; Baltzer et al., 2009; Elshaier et al., 2009; Irrgang et al., 2001; Wakitani et al., 1995)(Sun et al., 2010)
BMP-2	Stimulates synthesis of ECM  Partial reversal of dedifferentiated phenotype in OA  Increased ECM turnover (increased aggrecan degradation)	Presumed role in maturation of osteophytes  Multiple injections lead to synovial fibrosis  Stimulates synovial thickening in experimental OA	(Blaney Davidson et al., 2007b; Elshaier et al., 2009; Gobbi and Bathan, 2009; Irrgang et al., 2001; Wakitani et al., 1995)
BMP-7	Stimulates ECM synthesis  Decreases cartilage degradation through decreasing activity/ expression of numerous ILs and MMPs	Decreases expression of MMPs and aggrecanase  Does not appear to cause osteophyte formation or synovial fibrosis	(Badlani et al., 2009; Goodrich et al., 2007; Schmidt et al., 2006; Schnabel et al., 2007)
IGF-I	Stimulates ECM synthesis  Decreases matrix catabolism except in aged and OA cartilage	Protective effect on synovium resulting in decreased thickening and decreased evidence of chronic inflammation	(Fan et al., 2010; Lee and Shin, 2007; Miyakoshi et al., 2005; Wu and Hill, 2009)
FGF2	Decreases aggrecanase activity Antagonizes PG synthesis  Upregulates MMPs	Induces synovial proliferation  Inflammatory and induces osteophyte formation when used alone	(Ekenstedt et al., 2006; Gouttenoire et al., 2004; Middleton et al., 1996; Smyth et al., 2009)
FGF18	Increases chondrocyte proliferation and stimulates ECM in vitro and  in injured joints but not in normal joints	Induces synovial thickening  Enlargement of chondrocytes in experimental OA	(Ekenstedt et al., 2006; Ellman et al., 2008; Mishra et al., 2009)
PDGF	No adverse effect in normal joints	No adverse effect in normal joints	(Hayashi et al., 2008; Maehara et al., 2010)

**Table 4: Summary of the effect of anabolic growth factors on chondrocytes/cartilage and synovium.**

### 3.3.1. TGF $\beta$ /BMP Superfamily

Activities of the TGF $\beta$ /BMP superfamily in the skeleton were first discovered by Marshall Urist as constituents of demineralized bone that induced new bone formation when implanted into extraskelatal sites in rodents (Reddi, 2003). These bioactive morphogens subsequently were extracted, purified, and cloned and were found to regulate the early commitment of mesenchymal cells to chondrogenic and osteogenic lineages during cartilage development and endochondral bone formation. The TGF $\beta$ /BMP superfamily includes activins, inhibins, müllerian duct inhibitory substance, and nodal, glial-derived neurotrophic factor, OP-1 (or BMP-7), and Growth Differentiation Factors (GDFs), also called Cartilage-Derived Morphogenetic Proteins (CDMPs) (Pogue and Lyons, 2006a). In addition to regulating cartilage condensation and chondrocyte differentiation, members of this superfamily play key roles in site specification and cavitation of synovial joints and in the development of other organ systems. Many of these factors, including BMP-2, BMP-6, BMP-7, BMP-9, TGF- $\beta$ , and CDMP-1, are able to induce chondrogenic differentiation of mesenchymal progenitor cells *in vitro*. They also may have direct effects on mature articular chondrocytes *in vivo* and *in vitro*.

#### **Transforming Growth Factor- $\beta$**

TGF $\beta$  was named on the basis of its discovery as a factor that could transform cells to grow in soft agar. TGF $\beta$  is not a potent inducer of chondrocyte proliferation; however, it controls early mesenchymal cell condensation, as well as chondrocyte differentiation at early and late stages of chondrogenesis and endochondral ossification. TGF $\beta$  can control both inhibition and stimulation of the synthesis of aggrecan and type II collagen *in vitro*.

Different subtypes of TGF $\beta$  like; TGF $\beta$ 1, TGF $\beta$ 2, and TGF $\beta$ 3 generally are considered as potent stimulators of proteoglycan and type II collagen synthesis in primary chondrocytes and cartilage explants *in vitro* (Grimaud et al., 2002). Microarray analysis of chondrocytes *in vitro* demonstrates that TGF $\beta$  is able to counteract the expression of numerous IL-1–induced genes involved in cartilage injury (Takahashi et al., 2005).

Although intra-articular injection of TGF $\beta$  stimulates proteoglycan synthesis and limits cartilage damage in inflammatory arthritis models, injection or adenovirus-mediated delivery of TGF- $\beta$ 1 may result in side effects in joint tissues, such as osteophyte formation, swelling, and synovial hyperplasia (Bakker et al., 2001). Administration of agents that block TGF $\beta$  activity, such as the soluble form of TGF $\beta$ RII, inhibitory SMADs, or the physiologic antagonist, latency-associated peptide-1, increases proteoglycan loss and cartilage damage in an experimental model of OA.(Scharstuhl et al., 2002) A more recent finding that TGF $\beta$  induces expression of A Disintegrin And Metalloproteinase with Thrombospondin motifs (ADAMTS) (a disintegrin and metalloproteinase [ADAM family] with thrombospondin-1 domains)-4 in primary human chondrocytes and promotes the degradation of aggrecan suggests that it may be involved in normal turn-over of proteoglycans in mature cartilage (Moulharat et al., 2004).

Both TGF $\beta$  isoforms and TGF $\beta$  receptors are broadly expressed in cartilage, bone and synovial tissues. However, TGF $\beta$  signaling plays quite different role in these tissues. In synovial fibroblasts, TGF $\beta$  signaling is an inducer of synovial tissue fibrosis characterized as fibroblast proliferation and types I and III collagen accumulation (Fang et al., 2016). Moreover, attraction of inflammatory leukocytes to the synovial lining, and induction of osteophyte formation was also reported by this growth factor (Bakker et al., 2001; Blaney Davidson et al., 2007b). Levels of TGF $\beta$  measured in synovial fluids of OA and RA patients may reflect anabolic processes in cartilage and other joint tissues. TGF $\beta$  may promote anabolism by inducing the expression of Tissue Inhibitors of MMP (TIMP).

Given these serious safety concerns that are not components of other growth factor-based strategies, TGF $\beta$ 1 therapy is not presently a viable option for use in the articular environment.

### **Bone Morphogenetic Proteins**

Another large subclass of TGF $\beta$  superfamily are BMPs which are crucial for normal appendicular skeletal and joint development (Pogue and Lyons, 2006b). The isolation and cloning of the first BMP family members from bone opened the door for further investigation on cartilage-derived BMPs, or CDMPs such as CDMP-1, CDMP-2, and CDMP-3 which are classified as GDF-5, GDF-6, and GDF-7. The BMPs could be divided into four distinct sub-categories based on the similarity of primary amino acid sequences:

- (1) BMP-2 and BMP-2B (BMP-4), which are 92% identical in the 7-cysteine region;
- (2) BMP-3 (osteogenin) and BMP-3B (GDF-10);
- (3) BMP-5, BMP-6, BMP-7 (OP-1), BMP-8 (OP-2), BMP-9 (GDF-2), BMP-10, and BMP-11 (GDF-11); and
- (4) BMP-12 (GDF-7 or CDMP-3), BMP-13 (GDF-6 or CDMP-2), BMP-14 (GDF-5 or CDMP-1), and BMP-15.

BMP-1 is not a member of this family but is an astacin-related MMP that cleaves the BMP inhibitor chordin and acts as a procollagen C-proteinase (Li et al., 1996).

Among those mentioned BMPs, BMP-2, -4 and -7 are very important for us since they have been shown to stimulate differentiation of mesenchymal stem cell into chondrocytes and promote the differentiation of hypertrophic chondrocytes (Zhou, 2011). Moreover, BMP-2 and BMP-7 are able to oppose many of the detrimental effects of IL-1 on chondrocyte metabolism in vitro and in vivo (Vinall et al., 2002).

### **Bone Morphogenetic Protein-2**

BMP-2 is expressed in normal and OA articular cartilage, and it is a molecular marker, along with collagen type II and FGF receptor 3 (FGFR3), for the capacity of adult articular chondrocyte cultures to form stable cartilage in vivo (Dell'Accio et al., 2001; Gouttenoire et al., 2010).

Data from in vitro studies indicate that BMP-2 stimulates matrix synthesis and interestingly is capable of reversing chondrocyte dedifferentiation as indicated by an increase in synthesis of cartilage-specific collagen type IIB in dedifferentiated/OA chondrocytes (Gouttenoire et al., 2004). The effect of BMP-2 on MSCs is similar to that of TGF $\beta$ 1 with increased ECM production and decreased expression of collagen type 1 (Fan et al., 2010). When injected into murine knees, chondrocytes induced by BMP-2 were found in the location where the growth plates meet the joint space (van Beuningen et al., 1998). In the rabbit trochlear groove, BMP-2-impregnated collagen sponges implanted into full-thickness cartilage defects enhance cartilage repair compared with empty defects or defects filled with collagen sponge alone. The increased repair remained evident 1 year after implantation (Arai et al., 1997). In a mouse model of IL-1-induced cartilage degeneration, BMP-2 enhanced cartilage matrix turnover as evidenced by increased aggrecan degradation and increased collagen type II and aggrecan expression (Blaney Davidson et al., 2007a). An increase in matrix turnover might indicate a reparative response after cartilage injury or OA, but it is unclear how an increase in a catabolic mechanism such as aggrecan degradation would be of benefit in a cartilage repair procedure.

On the other hand, intra-articular injection of BMP-2 induced fibrosis of the synovium and synovial thickening, suggesting distinct effects of BMPs in synovial inflammation and joint pathology (Bramlage et al., 2006).

### **Bone Morphogenetic Protein-7**

BMP-7 has been investigated for its capacity to regenerate articular cartilage and currently appears to be one of the best growth factors for cartilage repair treatments (Chubinskaya et al., 2007). Like other anabolic growth factors, BMP-7 stimulates cartilage matrix synthesis and decreases catabolic activity of numerous catabolic cytokines, including IL-1, IL-6, IL-8, MMP-1, and MMP-13 (Elshaier et al., 2009). Moreover, BMP-7 can prevent retinoic acid-induced dedifferentiation of articular chondrocytes (Nishihara et al., 2003).

However, unlike other chondrogenic growth factors, these effects of BMP-7 are OA or age related (Chubinskaya et al., 2007). BMP-7 is synthesized by chondrocytes and its gene and protein expression decrease with aging and cartilage degeneration, but degenerate cartilage, via an unknown mechanism, is still able to respond to the anabolic cues of BMP-7 (Chubinskaya et al., 2002). BMP-7 inhibits MSC proliferation but stimulates ECM synthesis in both synovial and bone marrow-derived MSCs (Shen et al., 2010). Furthermore, chondrogenic differentiation of MSCs is increased when BMP-7 is in combination with TGF $\beta$ 3.

BMP-7 acts hand in hand with other anabolic growth factors such as IGF-I (Loeser et al., 2003). In animal studies, BMP-7 appears effective in regeneration of osteochondral or focal chondral defects (Chubinskaya et al., 2007). Administration of BMP-7 is also promising for the treatment of OA (Hayashi et al., 2008). In a rabbit anterior cruciate ligament (ACL) injury model, BMP-7 reduced cartilage degeneration (Badlani et al., 2008) and decreased expression of MMPs in the cartilage and synovial membrane compared with untreated limbs (Badlani et al., 2009). Although BMP-7 is highly effective in stimulating bone repair, it does not appear to lead to osteophyte formation when administered into a joint nor does it stimulate uncontrolled fibroblast proliferation leading to joint fibrosis. To sum up, the data suggest that BMP-7 therapy would lead to remarkable clinical improvement in cartilage repair; however, the addition of IGF-I might result in even greater healing potential.

### **3.3.2. Fibroblast Growth Factor family**

Members of the FGF family, including FGF-2, FGF-4, FGF-8, FGF-9, FGF-10, and FGF-18, together with the FGF receptors, FGFR1, FGFR2, and FGFR3, coordinate patterning and cell proliferation during chondrogenesis and endochondral ossification in embryonic and postnatal growth plates. Two members of the FGF family have been investigated for their role in cartilage homeostasis, FGF-2 and FGF-18 (Maddaluno et al., 2017). Among them the most extensively studied is FGF-2, also known as basic FGF, which is a potent mitogen for adult articular chondrocytes (Trippel, 2004), but findings on its effects on the synthesis of cartilage matrix are opposite, showing stimulation, inhibition, or no effect on proteoglycan synthesis (Loeser et al., 2005; Veilleux and Spector, 2005).

#### **Fibroblast Growth Factor-2**

Early studies suggested that low concentrations of FGF-2 could stimulate chondrocyte mitogenesis and proteoglycan synthesis, whereas high concentrations might have opposite effects. One study on rabbit model demonstrated improved healing of osteochondral lesions using a highly porous scaffold soaked in low-dose FGF-2 (10lg/mL) compared with higher doses of FGF-2 (100 lg/mL) or scaffold without FGF-2, suggesting an inverse dose response (Maehara et al., 2010) More recent studies showing that FGF-2 stored in the adult cartilage matrix is released with mechanical injury or with loading suggest a mechanism for modulating chondrocyte proliferation and anabolic activity (Vincent et al., 2004).

Although FGF-2 and FGF-9 stimulate the expression of Sox9 and increase the activity of the SOX9-dependent, chondrocyte-specific enhancer in the type II collagen gene (Schaefer et al., 2003), FGF-2 could interfere with anabolic activities of IGF-I and BMP-7 in vitro (Loeser et al., 2005). FGF-2 stimulates, whereas IGF-I inhibits, expression of the matrix Gla protein, which is a marker of chondrocyte survival during endochondral ossification (Stheneur et al., 2003).

Therefore FGFs and FGF receptors are important regulators of cartilage homeostasis during prenatal and postnatal life. In cartilage, FGF-2 is found in high doses in the pericellular matrix of

cartilage (Chia et al., 2009). On loading, FGF-2 binds to cell surface receptors and activates anabolic pathways leading to decreased aggrecanase activity but no apparent change in proteoglycan content. In FGF-2 KO mice, accelerated OA was observed in both control mice and in those undergoing transection of the anterior horn of the medial meniscus. In the surgical OA group, subcutaneous administration of FGF-2 ameliorated the OA to a level not different from control mice. However, recent evidence suggests that FGF-2 selectively activates FGF receptor 1 (FGFR1) to exert catabolic effects in human articular chondrocytes via upregulation of matrix-degrading enzyme production, inhibition of ECM accumulation and proteoglycan synthesis (Ellman et al., 2013). FGF-2 treatment of bone marrow-derived MSC monolayer cultures results in enhanced proteoglycan synthesis and cell proliferation (Stewart et al., 2007) However, given the numerous potentially deleterious effects on the articular environment, using FGF-2 in cartilage repair is a matter of question.

Some studies suggest that FGF-2 administration results in synovial proliferation through protection from apoptosis (Ellman et al., 2008). Furthermore, animal models suggest intra-articular administration of FGF-2 results in inflammation and osteophyte formation (Miyakoshi et al., 2005) and does not aid in healing of cartilage defects (Siebert et al., 2003).

### **Fibroblast Growth Factor-18**

Although there is less literature regarding the role of FGF-18 and cartilage homeostasis, it appears more promising than FGF-2 and elicits several anabolic effects on chondrocytes (Ellsworth et al., 2002). It has been shown that FGF-18 and FGF-9 increase matrix synthesis by mature chondrocytes (Shimoaka et al., 2002). In a rat model of rapid and severe OA, intra-articular administration of FGF-18 resulted in reduced cartilage degeneration scores with increased cartilage thickness of the tibial plateau, but also increased synovial thickness and chondrophyte formation (Moore et al., 2005). In another study, Yoshifumi Mori et al, revealed that injection of FGF-18 causes decreased glycosaminoglycan release and depletion from the cartilage, and enhanced proliferation of articular chondrocytes. FGF-18 was strongly expressed in the articular cartilage chondrocytes of adult rats. In this work, a once-weekly injection of recombinant human FGF-18 (rhFGF-18) given 3 weeks post-surgery prevented cartilage degeneration (Mori et al., 2014)

### **3.3.3. The anabolic role of IGF-1**

IGF-1, also known as somatomedin C, was first discovered as a serum factor controlling sulfate incorporation by articular cartilage in vitro and was later found to have the specific capacity to stimulate or maintain chondrocyte phenotype and homeostasis through its capacity to stimulate proteoglycan synthesis, promote chondrocyte survival, and oppose the activities of catabolic cytokines in cooperation with other anabolic factors such as BMP-7.(De Ceuninck et al., 2004; Jenniskens et al., 2006; Loeser et al., 2003). It transmits signals via the type 1 IGF-1 receptor (IGF1R), which is expressed in the proliferating and prehypertrophic zone chondrocytes of growth plates (Parker et al., 2007). It has been shown that IGF-1 stimulates chondrocyte growth

at all stages of differentiation (Hunziker et al., 1994). High level of IGF-1 was detected in articular cartilage of OA patients (Middleton et al., 1996).

Specific IGF-Binding Proteins (IGFBPs) that do not recognize insulin also regulate IGF-I activity. Chondrocytes at different stages of differentiation express IGF-I and IGF receptors and different arrays of IGFBPs, providing a unique system by which IGF-I can exert different regulatory effects on these cells. IGFBP-2 seems to be a positive regulator in chondrocytes because its induction by TGF $\beta$  or estrogen is associated with increased proteoglycan synthesis (Morales and Hunziker, 2003). Binding of IGFBP-3 to IGF-I is thought to regulate negatively the anabolic functions of IGF-I, although IGFBP-3 may directly inhibit chondrocyte proliferation in an IGF-independent manner.

In OA cartilage, the normal anabolic function of IGF-I may be interrupted because chondrocytes from animals with experimental arthritis and from patients with OA are hypo-responsive to IGF-I, despite normal or increased IGF-I receptor levels. This hyporesponsiveness has been attributed to increased levels of IGFBPs that may interfere with IGF-I actions (Morales, 2002). Disturbances in the balance of IGF-I to IGFBPs that have been reported in OA and RA joints may contribute to defective chondrocyte responses to IGF-I (Neidel, 2001). Small-molecule inhibitors of IGF-I/IGFBP interactions that could restore IGF-I-dependent proteoglycan synthesis in cartilage have been proposed for treatment of OA. Although IGF-I can oppose the effects of inflammatory cytokines that promote cartilage degradation and inhibit proteoglycan synthesis, these cytokines increase the production of IGFBP-3 by chondrocytes (Olney et al., 1995)

In animal models, IGF-I has led to enhanced repair of extensive cartilage defects and protection of the synovial membrane from chronic inflammation (Fortier et al., 2002).

#### **3.3.4. Platelet-derived Growth Factor**

PDGF appears to be the first growth factor present in a wound and initiates connective tissue healing through the promotion of collagen and protein synthesis. The primary effect of PDGF seems to be its mitogenic activity to mesoderm-derived cells such as fibroblasts, vascular muscle cells, glial cells and chondrocytes. The most important specific activities of PDGF include angiogenesis and chemotaxis for fibroblasts and collagen synthesis.

Evidence to support the use of PDGF in cartilage repair is extrapolated from the role of PDGF in wound healing or stimulation of matrix synthesis in growth plate chondrocytes (Fortier et al., 2011). In vivo, when injected into the knee of skeletally immature rats, no adverse effects were noted in the cartilage or synovial membrane (Iannitti et al., 2011).

#### **3.4. Catabolic factors in OA**

The catabolic environment of OA is characterized by increases in degradative enzymes and proinflammatory cytokines.

Matrix MetalloProteinase (MMPs) play a major role in the degradation of the osteoarthritic joint. It has been shown that MMP-1 and MMP-3, an aggrecanase, are present in high levels in the synovial fluid of OA. Moreover, although detected at very low levels in the OA synovial fluid (287-fold lower than MMP-1 over 14,000-fold lower than MMP-3), the collagenase MMP-13 has been shown to be implicated due to the high gene expression in OA cartilage (Yoshihara et al., 2000).

The OA synovial fluid is full of proinflammatory cytokines and chemokines also, including IL-8 (Kaneko et al., 2000; Koch et al., 1991), MCP-1 (Koch et al., 1992; Stankovic et al., 2009), Macrophage Inflammatory Protein 1a (MIP-1a) (Koch et al., 1994; Suzuki et al., 1999) , Regulated and Normal T cell Expressed and Secreted (RANTES) and VEGF (Lee et al., 2001) among others. These proteins have been found at detectable levels, but the concentration of IL-1 $\beta$  and TNF $\alpha$  in the synovial fluid of OA is not stable. Patients have been reported with extremely low or considerably high concentrations of IL-1 $\beta$  or TNF $\alpha$  in their synovial fluid (Okamoto et al., 1997).

The difficulty in OA research is due to the fact that OA is not a single disease, but rather a complex combination of metabolic processes affecting the joint and surrounding tissues (Kahle et al., 1992). **Table 5** shows summary of the effect of catabolic growth factors on chondrocytes/cartilage and synovium.

Growth factor	Chondrocytes/cartilage	Synovium	References
MMPs	Degrade native collagens and proteoglycans in cartilage matrix	Induce proinflammatory cytokines like TNF $\alpha$ , Facilitating infiltration of proinflammatory cells	(Hamada et al., 2016; Okada et al., 1992; Yang et al., 2017; Zeng et al., 2015)
IL-1 $\beta$	Inhibit anabolic factors Stimulate chondrocytes to release several MMPs and aggrecanases Down regulate the synthesis of GAGs and collagen II (COL2)	Induce proinflammatory cytokines like IL-6, IL-8, MCP-1 and RANTES	(Alaaeddine et al., 2001; Goldring and Goldring, 2007; Guerne et al., 1990; Lotz et al., 1992; Malfait et al., 2002; Villiger et al., 1992)
IL-6	IL-6 or/and sIL-6R, inhibit type II collagen production in rabbit articular chondrocyte Induce activation of (JAK1), JAK2 and 1 (STAT1)/STAT3 In bovine articular chondrocytes. Down regulate the expression of type II collagen, aggrecan core, and link proteins Increase expression of MMP1, MMP3, and MMP13 as well as ADAMTS4 and ADAMTS5/11	Pro or anti-inflammatory effect	(Legendre et al., 2003; Orita et al., 2011; Stannus et al., 2013)
TNF $\alpha$	Activate the production and release of MMPs,	Pro or anti-inflammatory effect	(Kobayashi et al., 2005)
IL-17 IL-18	Induce catabolic responses by stimulate the production of IL-6, iNOS, COX-2, and MMPs.	Promotes IL-6, TNF $\alpha$ , IL-1 $\beta$ production, and proinflammatory cell survival	(Benedetti and Miossec, 2014; Joosten et al., 2003; Shahrara et al., 2010)
VEGF	Plays an important role in osteophyte formation and chondrocyte degradation	Increase in synovial fluid	(Murata et al., 2008; Nagao et al., 2017; Yuan et al., 2014)(Saetan et al., 2014)

**Table 5: Summary of the effect of catabolic growth factors on chondrocytes/cartilage and synovium.**



### 3.4.1. Enzymes

MMPs are a family of functionally and structurally related zinc endopeptidases that cleave proteins of the extracellular matrix, including collagens, elastin, matrix glycoproteins and proteoglycans (Bourboulia and Stetler-Stevenson, 2010) and are considered to be responsible for much of the degeneration of articular cartilage. Most MMPs are composed of three distinct domains (Zitka et al., 2010):

- 1) An amino-terminal propeptide involved in the maintenance of enzyme latency;
- 2) A catalytic domain that binds zinc and calcium ions and
- 3) A hemopexin-like domain that is located at the carboxy terminal zone of the protease and that plays a role in substrate binding.

All of the members of the MMP family have vast substrate specificities, but their main substrates include gelatinases, collagenases, matrilysins, stromelysins, membrane-type MMPs, and metalloelastase (Hemmann et al., 2007). Generally, MMPs can degrade any extracellular matrix component, including proteoglycans, vitronectin, fibronectin, laminin, and collagens. MMP-1, MMP-2, and MMP-9 are key members of the MMP family.

MMP-1, MMP- 2, and MMP-9 protein expression levels have been reported to be either correlated with OA or not related at all to OA indicating their important role in pathophysiology of this disorder (Heard et al., 2012; Xue et al., 2014). Accordingly, a study on 1408 OA patients revealed the elevated levels of those three MMPs (Zeng et al., 2015).

Chondrocytes synthesize and secrete MMPs, which are activated outside the cells via activation cascades. An important cascade in cartilage is initiated by plasmin, the product of plasminogen activator activity, which may be produced by the chondrocyte; plasmin activates latent stromelysin (MMP-3), an activator of latent collagenases. In early studies, chondrocytes were among the first identified sources of tissue inhibitors of metalloproteinases-1 (TIMP-1), and they are now known to synthesize additional TIMPs. Chondrocytes are assumed to be a major source of the TIMPs and MMPs detected in synovial fluids, where they reflect an adaptive response to the local imbalance caused by increased production of active MMPs by chondrocytes and other joint tissues. Collagenases 1, 2, and 3 (MMP-1, MMP-8, and MMP-13); gelatinases (MMP-2 and MMP-9); stromelysin-1 (MMP-3); membrane type 1 MMP (MT1)-MMP (MMP-14); and the aggrecanases, ADAMTS-4 and ADAMTS-5, specifically degrade native collagens and proteoglycans in cartilage matrix (Zeng et al., 2015) MMPs, aggrecanases, and the cleavage fragments generated by them are localized in regions of cartilage degradation (Little and Fosang, 2010)and are detected in synovial fluids and cartilage from OA and RA patients (Bonjour et al., 2014). Expression of MMP-13 in OA and RA cartilage and its ability to degrade type II collagen more effectively suggest a major role for this enzyme in cartilage degradation. Postnatal overexpression of constitutively active MMP-13 in cartilage in mice produces OA-like changes in knee joints (Neuhold et al., 2001), and knockout of the gene encoding MMP-13 protects cartilage against surgically induced OA (Little et al., 2009). Deficiency of Discoidin Domain Receptor 2 (DDR2), a collagen receptor whose activation is associated with upregulation of MMP- 13, attenuates development of OA induced by destabilization of the medial meniscus

surgery (Polur et al., 2010; Xu et al., 2010). Deficiencies of the RUNX2 and Hypoxia-Inducible Factor-2 alpha (HIF2 $\alpha$ ) transcription factors that are key regulators of MMP-13 also protect against OA development or progression (Yang et al., 2010). MMP-10, similar to MMP-3, activates pro-collagenases and is produced by the synovium and chondrocytes in response to inflammatory cytokines (Troeborg and Nagase, 2012). MMP-14, produced principally by the synovial tissue, is important for synovial invasiveness, and antisense mRNA inhibition of this membrane proteinase has been shown to reduce cartilage destruction (Rutkauskaite et al., 2005). Several MMPs, including MMP-3, MMP-8, MMP-14, MMP-19, and MMP-20, are capable of degrading proteoglycans. Members of the reprotolysin-related proteinases of the ADAM (a disintegrin and metalloproteinase) family, particularly ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs -4) and ADAMTS-5, are now regarded as the principal mediators of aggrecan degradation (Yang et al., 2017). The activities of MMPs and aggrecanases are complementary; however, ADAMTS-5 is associated with increased susceptibility to OA, as shown in Adamts5-deficient mice (Glasson et al., 2005a). TIMP-3 is a potent inhibitor of ADAMTS-4 and ADAMTS-5 in vitro (Wayne et al., 2007) and TIMP-3 deficiency results in mild cartilage degradation similar to that seen in patients with OA (Sahebjam et al., 2007). A recent study indicates that syndecan-4 by controlling the synthesis of MMP-3 is a positive effector of ADAMTS-5 activation (Echtermeyer et al., 2009a).

Cathepsin B also may have a role in extracellular degradation of collagen telopeptides, collagens IX and XI, and aggrecan. Cathepsin K is expressed in synovial fibroblasts on the cartilage surface at the pannus-cartilage junction and is upregulated by inflammatory cytokines (Hou et al., 2002). MicroRNAs are novel endogenous regulators in cartilage (Sondag and Haqqi, 2016). MicroRNA-140 downregulates ADAMTS-5 expression in normal cartilage and its expression is reduced in OA cartilage and is suppressed by exposing chondrocytes to IL-1 $\beta$ . MicroRNA-140 knockout mice are predisposed to age-related OA development, and overexpression of microRNA-140 in chondrocytes is protective against surgically induced OA (Miyaki et al., 2009).

### 3.4.2. IL-1 $\beta$

Many studies have been focusing on the role of IL-1 $\beta$  both in vitro and in vivo. It has already been demonstrated that the addition of IL-1 $\beta$  or TNF $\alpha$  to chondrocytes can downregulate the synthesis of major ECM components and inhibit the anabolic factors (Goldring et al., 1994; Saklatvala, 1986).

It was shown that when human OA chondrocytes were treated with IL-1 $\beta$ , aggrecan and COL2A1 gene expression was remarkably downregulated nearly two- to three folds compared to control group (Shakibaei et al., 2005). This is in accordance with other works showing the same results in porcine cartilage explants (Nietfeld et al., 1990), and rabbit articular cartilage (Chadjichristos et al., 2003). In addition to decreasing the anabolic activity, IL-1 $\beta$  has been shown to increase catabolism. IL-1 $\beta$  can stimulate chondrocytes to release several MMPs, including MMP-1, MMP-3, and MMP-13, and aggrecanases, including aggrecanase 1 and 2 (ADAMTS-4 and ADAMTS-5). These degradative enzymes with aggrecanase and collagenase activity have been

shown crucial in OA development (Malfait et al., 2002). Stimulation of chondrocytes with IL-1 $\beta$  has been shown to induce the production of many of proinflammatory cytokines commonly found in the OA synovial fluid, including IL-6 (Guerne et al., 1990) IL-8 (Lotz et al., 1992) MCP-1 (Villiger et al., 1992) and RANTES (Alaaeddine et al., 2001). It could be assumed that stimulation of chondrocytes with IL-1 $\beta$  produces an environment similar to the OA synovial fluid. In addition, due to IL-1 $\beta$ , this increase in cartilage breakdown factors in combination with the reduction in repair capabilities mimics many aspects of clinical OA. This dual role makes IL-1 $\beta$  appear to be a logical target for OA therapies.

### 3.4.3. IL-6

IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation, and oncogenesis. IL-6 is produced by many different cell types, including T cells, B cells, monocytes, fibroblasts, keratinocytes, and endothelial cells (Naka et al., 2002). As immunological point of view the role of IL-6 is very controversial. In OA patients, the level of IL-6 in the synovial fluid is quite high. As the result of this high level, its regulatory action might be crucial to the progression of OA. The exact role of IL-6 in OA is also controversial since this cytokine could be pro or anti-inflammatory depending on the context. Stannus et al. reported that an increase in IL-6 concentrations in the serum led to a significant increase in joint pain when standing (Stannus et al., 2013). Conversely, Pelletier et al. did not found similar correlation between IL-6 and Pain (Pelletier et al., 2010).

IL-6 levels were investigated in the synovial fluid from patients with increasing severity of joint destruction as measured by the Kellgren-Lawrence (KL) grading scale. In this study, the authors found a significant inverse relationship between the KL grade and concentration of IL-6 (Orita et al., 2011). This could indicate that IL-6 represents a pro-anabolic factor able to modulate cartilage damage early in the disease, but is decreased as OA progresses.

IL-6 and soluble IL-6 receptor (sIL-6R) have been demonstrated to modulate the balance of anabolism and catabolism in vitro. In an in vivo study using rabbit articular chondrocytes, IL-6 or/and sIL-6R, inhibited type II collagen production (Porée et al., 2008). In bovine articular chondrocytes, IL-6 and sIL-6R induced activation of Janus Kinase 1 (JAK1), JAK2, and Signal Transducer and Activator of Transcription 1 (STAT1)/STAT3. These conditions remarkably downregulated the expression of type II collagen, aggrecan core, and link proteins (Legendre et al., 2003). This diminished anabolic activity is followed by an increase in MMP and a disintegrin and metalloproteinase with thrombospondin motifs ADAMTS expression. Primary bovine chondrocytes were stimulated with IL-6 and sIL-6R for 24 h. Expression of MMP1, MMP3, and MMP13 as well as ADAMTS4 and ADAMTS5/11 were markedly increased (Legendre et al., 2005). These decreases in anabolic activity combined with the increased degradative activity are hallmarks of OA, suggesting that other cytokines could provide the stimulus needed for the progression of OA.

#### 3.4.4. TNF

TNF $\alpha$  is also one of the cytokines with much controversiality. Although famously known as pro-inflammatory cytokine, recently its anti-inflammatory property has been reported (Leclerc et al., 2016). TNF $\alpha$  has long been hypothesized to be important in the progression of OA, although the role of TNF $\alpha$  in cartilage degradation is generally regarded as less clear.

TNF is known as a multifunctional proinflammatory cytokine that is involved not only in various physiological processes but also in pathological processes, including inflammation, immunoregulation, proliferation, and apoptosis. TNF $\alpha$  and TNF $\beta$  (lymphotoxin) are members of the TNF superfamily. Some of the biological properties of TNF $\alpha$  and TNF $\beta$  suggest that these cytokines may be involved in the destruction of cartilages (Fernandes et al., 2002). TNF $\alpha$  levels are elevated in OA patients' synovial fluid, synovial membrane, subchondral bone, and cartilage (Abraham and Kroeger, 1999). However, TNF $\beta$  levels are detected differently in inflammatory and autoimmune diseases. TNF $\alpha$  activates the production and release of MMPs, which leads to matrix breakdown and could explain cartilage degeneration (Kobayashi et al., 2005). In a study in 2012, it has been shown that there is a close relationship between *TNFA* -G308A and *TNFB* +G252A polymorphisms and individual susceptibility to OA (Han et al., 2012). The pro-angiogenic role of TNF $\alpha$  and its interaction with vascular endothelial growth factor will be explained in detail in further part.

#### 3.4.5. Other cytokines

Since several other proinflammatory cytokines are also known to be very important in development of OA, it is necessary to explain them briefly. Among other factors, IL-17 and IL-18 are two additional cytokines that are potentially inducers of catabolic responses in chondrocytes. IL-17 and IL-18 stimulate the production of IL-6, Nitric Oxide Synthases (iNOS), cyclooxygenase-2 (COX-2), and MMPs. It has been depicted that adenoviral overexpression of IL-17 induces loss of cartilage proteoglycan in the joints. Other studies by recruiting animal models demonstrated that IL-18 deficiency or blockade reduced cartilage destruction and inflammation (Goldring and Goldring, 2004).

### 3.5. Inflammation in OA

Innate immunity refers to primitive mechanisms of defense, which are nonspecific and come into play immediately or shortly after an antigen's appearance in the body. These mechanisms include physical barriers such as skin, chemicals in the blood, and immune system cells that attack foreign cells in the body.

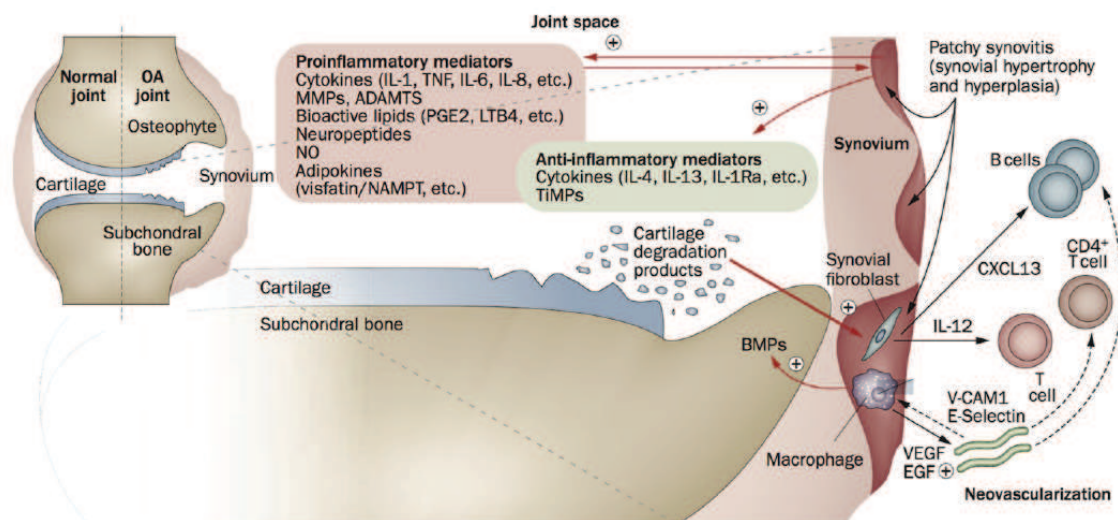
However, the important question is: how does innate immunity, which serves as our first line of defense, lead to inflammation and joint disorders? The answer lies in how the innate immune system reacts to changes that take place in the joint over time. Unlike the adaptive immune system, innate immunity relies on recognition of conserved motifs produced by pathogens or damage within the body (Chen and Nuñez, 2010).

Damage to products of cellular and cartilage ECM from trauma, microtrauma (from repetitive overuse) or normal aging makes Damage-Associated Molecular Patterns (DAMPs) that activate the innate immune system (Liu and Rando, 2011; Scanzello et al., 2008). DAMPs could be in form of fragments generated from proteins, proteoglycans or remnants of cellular breakdown, such as uric acid (Chen and Nuñez, 2010; Scanzello and Goldring, 2012a). DAMPs provoke an inflammatory response through interaction with Particle Recognition Receptors (PRR), such as Toll-Like Receptors (TLR), on the surface of immune cells, or with PRRs in the cell cytoplasm, such as Nod-Like Receptors (NLRs) (Sokolove and Lepus, 2013).

### **3.5.1. Synovial inflammation**

Synovial inflammation is likely to contribute to disease progression in OA, as proved by the correlation between biological markers of inflammation and the progression of structural changes in OA (Clark et al., 1999; Sowers et al., 2002). Histologically, the OA synovium shows hyperplasia with an increased number of lining cells and a mixed inflammatory infiltrate mainly consisting of macrophages (Benito et al., 2005).

The synovium is a specialized connective tissue that lines diarthrodial joints, surrounds tendons and forms the lining of bursae and fat pads. In synovial joints, the synovium seals the synovial cavity and fluid from surrounding tissues. The synovium is responsible for the maintenance of synovial fluid (SF) volume and composition, mainly by producing lubricin and HA. Through the synovial fluid, the synovium also aids in chondrocyte nutrition (together with subchondral bone), as articular cartilage has no intrinsic vascular or lymphatic supply (Scanzello and Goldring, 2012b). The normal synovium has two layers. The outer layer, or subintima, is up to 5 mm thick and consists of multiple types of connective tissues: fibrous (dense collagenous type), adipose (found mainly in fat pads) or areolar (loose collagenous type). This layer is rich in type I collagen and microvascular blood supply, accompanied by lymphatic vessels and nerve fibres, but is relatively acellular. The inner layer, or intima, lies next to the joint cavity and consists of a layer of 1–4 cells, only 20–40 µm thick. These synoviocytes have been identified by immunohistochemical and cytochemical methods as macrophages and fibroblasts; the latter is the dominant cell population in healthy synovium (Smith, 2011).



**Figure 17: Involvement of the synovium in OA pathophysiology.**

Products of cartilage breakdown released into the synovial fluid are phagocytosed by synovial cells, amplifying synovial inflammation. In turn, activated synovial cells in the inflamed synovium produce catabolic and proinflammatory mediators that lead to excess production of the proteolytic enzymes responsible for cartilage breakdown, creating a positive feedback loop. The inflammatory response is amplified by activated synovial T cells, B cells and infiltrating macrophages. To counteract this inflammatory response, the synovium and cartilage may produce anti-inflammatory cytokines. In addition to these effects on cartilage inflammation and breakdown, the inflamed synovium contributes to the formation of osteophytes via BMPs. Adopted from (Sellam and Berenbaum, 2010).

### 3.5.2. Synovial Membrane

The synovial membrane (SM) is a semi-permeable membrane, which is responsible of controlling molecular traffic into and out of the joint space, maintaining the composition of SF, which is critical in preservation of normal physiologic state of articular cartilage.

The cellular elements of the SM are a major source of SF components; these components contribute to the unique functional properties of articular surfaces and modulate chondrocyte activity. Two important molecules produced by synovial lining cells are lubricin and HA.

Lubricin is critical to normal joint function; it decreases friction by providing boundary lubrication of congruent articular surfaces under high contact pressure and near-zero sliding speed. Furthermore, it has an important role in preventing chondrocyte apoptosis and in synovial cell adhesion and proliferation; therefore, controlling the integrity of articular cartilage surfaces in diarthrodial joints (Szychlinska et al.). Lubricin reduces pathologic deposition of proteins at the articular surface (Rhee et al., 2005).

Lubricin-knockout mice show clinical and radiologic signs of joint disease and histologic abnormalities in their articulating joints with increasing age (Rhee et al., 2005). In case of OA, the concentration and average molecular weight of HA, and the concentration of lubricin in SF are altered (Scanzello and Goldring, 2012b), which deversely affects integrity of cartilage. Waller KA et al, showed that joint friction and cellular apoptosis was greater in lubricin-knockout than

wild-type mice. The addition of lubricin in the *in vitro* bovine explant cartilage-on-cartilage bearing system significantly lowered the coefficient of friction and chondrocyte apoptosis in superficial layers of cartilage, thereby confirming its crucial role in preventing cartilage degeneration (Waller et al., 2013).

During OA progression, the synovial membrane is also a source of pro-inflammatory and catabolic products, including MMPs and aggrecanases, which play a role in articular matrix degradation. Therefore, alterations in the SM can result in decreased concentrations of cartilage-protecting factors, and increased production of factors that contribute to the degradation of the articular matrix.

Under normal conditions, high molecular weight molecules like lubricin and HA are not readily permeable, while small molecules such as growth factors and cytokines can circulate through the SM. This permits the retention of high molecular weight (MW) lubricating molecules within the joint, while preventing high MW plasma proteins from entering and becoming deposited on the articular surface or altering the viscosity and composition of the SF. The permeability of the membrane can change in situation of synovial alteration like inflammation and hyperplasia. This change in permeability probably contributes to the decreased concentrations of HA and lubricin observed in SF in articular disease. In the setting of arthritis, elevation of HA is observed peripherally in the serum (Goldberg et al., 1991), and serum HA concentrations have been used as a marker of synovitis (Mazières et al., 2006).

At histological level, synovium in OA patients is characterized by sublining fibrosis, synovial lining, hyperplasia and stromal vascularization (Blom et al., 2007). Due to presence of variety of cytokines and cell adhesion molecules, there is a huge influx of leukocytes from the vascular compartment (Belcher et al., 1997). Many studies have demonstrated that T cells and macrophages are the most predominant immune cells in OA synovium, whereas mast cells, B cells and plasma cells are also found in fewer extents (Bock et al., 2001; Borzi et al., 2000). The presence of macrophages in synovium has been already confirmed. These cells could cluster and form Multinucleated Giant Cells (MGCs) for improved phagocytosis, and are increased in similar numbers in inflamed OA and RA synovia compared with non-inflamed OA and post-mortem controls (Blom et al., 2007).

Molecules from degraded hyaline cartilage release into the synovial cavity and start synovial inflammation in OA (**Figure 17**). Early in knee OA, damage to the meniscus may also release tissue debris, although molecules released from subchondral bone may also play a role. Synoviocytes begin to react by producing pro-inflammatory mediators. In result, they attract immune cells, increase angiogenesis and induce a phenotypic change in chondrocytes (Ng et al., 2010).

Macrophages are known to be the most important responsible of innate immune activation and cytokine production in the OA joint, but other cells including synoviocytes and chondrocytes also play an important role (Bradley et al., 1996).

### 3.5.3. The role of macrophages

Macrophages, as their name implies, are major phagocytic cells of the body, but they also possess several other important roles, such as starting inflammation, resolving inflammation, and restoring and repairing tissue damage (Mosser and Edwards, 2008; Murray and Wynn, 2011).

Macrophages can be activated in different ways. One of the primary ways is through activation of PRRs, which in turn activate a number of intracellular pathways, such as NF- $\kappa$ B. It is known that both TNF $\alpha$  and IL-1 $\beta$  have functional NF $\kappa$ B elements on their promoters and that in various macrophage models, there are both NF $\kappa$ B dependent and NF $\kappa$ B independent ways of inducing TNF $\alpha$  and IL-1 $\beta$  (Bondeson et al., 1999; Hayes et al., 1999). Another activation ways of macrophages is through inflammasome-mediated pathways (Dinarello, 2010). Inflammasomes are large multimeric intracellular protein complexes that help process caspase-1, which is responsible for producing the mature forms of several pro-inflammatory cytokines such as IL-1 $\beta$  (Schroder and Tschopp, 2010). NACHT, LRR and PYD domains-containing protein 3 (NLRP3) is the most profoundly researched inflammasomes (Schroder and Tschopp, 2010).

In a study on OA patients without gout the involvement of uric acid activated NLRP3 inflammasomes in the pathogenesis of OA was suggested (Denoble et al., 2011). In this study, synovial fluid uric acid concentrations correlated with the concentrations of two cytokines, IL-18 and IL-1 $\beta$ , known to be produced by uric acid activated inflammasomes, and synovial fluid IL-18 was associated with OA progression. Therefore, there is a high degree of correlation between uric acid crystal deposition and cartilage lesions (Muehleman et al., 2008), and evidence for inflammasome activation in association with uric acid in OA. It has been hypothesized that the chronic low-grade inflammasome activation helps the progression of OA (Denoble et al., 2011).

Experimental studies on macrophages have revealed the involvement of these cells in both reduction of inflammation and progression of OA. Depletion of macrophages from a cell culture of human OA synovium significantly reduced the inflammatory response, including cytokine response and the activity of proteolytic enzymes, such as matrix metalloproteases and aggrecanases (Bondeson et al., 1999). In addition, in vivo depletion of synovial macrophages via intra-articular injection of clodronate has led to less MMP activity and less cartilage damage in mouse model of OA (Blom et al., 2007).

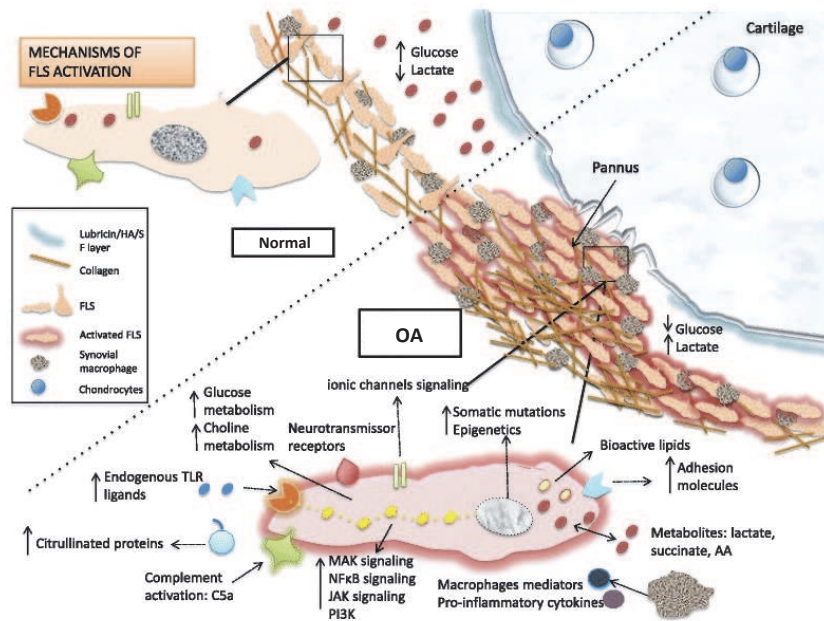
Macrophages themselves secrete growth factors, such as TGF $\beta$ , which can ameliorate cartilage repair (Blaney Davidson et al., 2007a). However, intra-articular injections of TGF $\beta$  into the knees of mice can lead to fibrosis and extensive osteophyte formation; this response was abolished via injection of clodronate beforehand which successfully depleted macrophages from the synovial lining (van Lent et al., 2004). Thus, the application of macrophages seems to be an attractive approach in OA therapy.



#### 3.5.4. Fibroblasts like synoviocytes

Fibroblast-like synoviocytes (FLS), also known as synovial fibroblasts or type B synoviocytes, are the predominant cell type comprising the structure of the synovial intima. They are organized in two to three layers of cells and constitute 75–80% of all synoviocytes in normal human synovium (Wilkinson et al., 1992). FLS interact with the ECM by expression of different molecules, including  $\alpha1\beta1$  integrin,  $\alpha2\beta1$  integrin (Agarwal and Brenner, 2006), and cadherin-11, a calcium-dependent adhesion molecule, collagens and lubricin (that was explained in SM parts), which are important in maintaining the internal joint hemostasis. In between, macrophage-like synoviocytes (MLS) or type A synoviocytes are located in this stromal cell network (Lee et al., 2007). FLS were recently proven to be an essential factor in the formation of a normally organized synovial lining. They have an intrinsic capacity to establish a three-dimensional complex synovial lining architecture characterized by the multicellular organization of the compacted synovial lining and the production of SF constituents (Lee et al., 2007).

During pathology of OA, due to the changes in proliferative and processes, significantly increases the number of FLS is happend. These cells, together with other immune cells such as macrophages, lymphocytes, neutrophils, mast cells, dendritic cells and platelets, create an inflammatory environment in the synovium, attract more immune cells to the damaged place and thus contribute to the joint destruction (Chang et al., 2010). In addition, these cells can secrete a number of pro-inflammatory signalling molecules, especially IL-6 and IL-8 and MMPs, which may directly affect other cells such as chondrocytes that produce additional cytokines and proteolytic enzymes, which eventually increase cartilage degradation (Baker et al., 2010) (**Figure 18**).



**Figure 18: Main mechanisms of FLS activation.**

The pro-inflammatory environment in OA, including high levels of cytokines, growth factors, and infiltrating inflammatory cells, strongly activates FLS. Other stimuli, such as danger-associated molecular patterns (DAMPs), microparticles, activation of calcium channels or stimulation through synovial nerves, complement, and antibodies are also important triggers of inflammatory signaling pathways, metabolic shifts, and epigenetic changes. Adapted from (Bustamante et al., 2017).

### 3.6. Angiogenesis in OA

Healthy adult joint cartilage does not contain blood vessels and nerves. In contrast, OA cartilage may be invaded by blood vessels from the subchondral bone. The mechanisms underlying cartilage angiogenesis in OA are unclear but may involve hypertrophic chondrocyte differentiation (Pesesse et al., 2011). Many research labs are focusing to identify the mechanism behind cartilage angiogenesis. Why normal cartilage does not have any blood vessel? The cliché answer is that we do not know the exact mechanism behind this, but we know the presence of high concentrations of anti-angiogenic molecules such as Chondromodulin-1 (ChM-1) and Thrombospondin-1 (TSP-1) in the cartilage (Hiraki and Shukunami, 2000; Pfander et al., 2000). In OA, cartilage angiogenesis could be one of the causes of osteophyte development, subchondral bone remodeling, and cartilage mineralization.

#### Angiogenesis at macroscopic level

In OA, the invasion of blood vessels from the subchondral bone is evident from early stages of the disease and subsequently leads to the loss of tidemark integrity (Walsh, 2004). This is followed by the formation of new bone at the osteochondral junction (Moskowitz and Goldberg, 1987). This process is considered to initiate as a tissue response due to the altered biomechanical

environment in the diseased joint. Moreover, sensory nerve formation is observed in OA, which explains pain development, due to the fact that angiogenesis and innervation are regulated by similar mechanisms (Autiero et al., 2005; Ferretti et al., 2003; Zukowska et al., 2003).

Histologically, chronic synovitis is observed in patients undergoing joint replacements. Macrophage infiltration, endothelial proliferation and increased angiogenesis in the synovium apparently contribute to the disease progression (Costa et al., 2004; Myers et al., 1990; Szekanecz and Koch, 2007). It is believed that inflammation and angiogenesis are at least at some points share the same mechanism (Mehrad et al., 2007; Silvestre et al., 2008). In case of OA, inflammation induced angiogenesis is considered as an important contributor to disease progression.

### **Vascular endothelial growth factor**

VEGF has three major effects on bone development: (a) induction of angiogenesis in intra-membranous or enchondral bone development, (b) chemotactic migration of osteoclastic cells to the hypertrophic cartilage and osteoblastic activation, and (c) direct effects on osteoprogenitor cells by promoting differentiation to osteoblast and increasing mineralization of the regenerated bone (Cebi et al., 2010). In embryonic development, VEGF-dependent signalling is critical for the regulation of growth plate morphogenesis and the coupling between cartilage and bone formation. Accordingly, mice lacking VEGF in chondrocytes develop impaired embryonic bone development, angiogenesis and impaired removal of hypertrophic chondrocytes (Zelzer et al., 2004).

However, VEGF is not expressed in healthy adult cartilage, therefore, the tissue is essentially avascular. The VEGF signalling mechanism in the adult is important for tissue repair processes such as fracture healing, however, in articular cartilage the expression of VEGF and subsequent VEGF dependent signalling in OA can be viewed as a mis-attempt of the body in repair processes within the joint.

The expression of VEGF and its receptors in OA has been already shown (Lingaraj et al., 2010a). There is evidence supporting that high VEGF levels in both plasma and synovial fluids are positively correlated with the radiographic severity of knee osteoarthritis, suggesting increased expression of VEGF occurs in osteoarthritis cartilage, and this may stimulate the growth of blood vessels from subchondral bone into articular cartilage, thereby contributing to OA progression (Saetan et al., 2014). Furthermore, Quan Yuan, et al. revealed that VEGF expression levels strongly correlate with the pathogenesis of osteoarthritis (Yuan et al., 2014). Moreover, it has been reported that VEGF plays an important role in osteophyte formation and development during OA through increasing angiogenesis (Murata et al., 2008). Thus, VEGF could be considered as a promising biomarker to assess disease severity in osteoarthritis

Pufe et al. reported the expression of VEGF and receptors in tissue derived from OA patients to be dependent on mechanical loading (Pufe et al., 2005). This means that the appearance of blood vessels is associated to an altered mechanical response of cartilage in OA compared to healthy

cartilage that permits a high degree of mechanical loading. VEGF expression has been associated with the induction of catabolic mediators that are involved in matrix degradation. The upregulation of matrix MMP1 and 3 particularly was observed in chondrocytes derived from OA patients (Nagao et al., 2017) and in immortalized chondrocytes treated with VEGF (Pufe et al., 2004).

Blood vessel and nerve growth are linked by common pathways that involve the release of proangiogenic factors, such as VEGF,  $\beta$ -nerve growth factor and neuropeptides. Proangiogenic factors might also stimulate nerve growth, and molecules produced by vascular cells could both stimulate and guide nerve growth. As sensory nerves grow along new blood vessels in osteoarthritic joints, they eventually penetrate noncalcified articular cartilage, osteophytes and the inner regions of menisci. Angiogenesis could, therefore, contribute to structural damage and pain in OA (Mapp and Walsh, 2012).

### **Hypoxia**

Lacking blood vessels, articular cartilage is characterized by a low oxygen tension. Since hypoxia is a positive regulator of angiogenesis, healthy cartilage is considered to have the ability to control the mechanism of hypoxia. Nevertheless, in OA oxygen tension appears to be even lower compared to healthy cartilage (Lund-Olesen, 1970; Murata et al., 2008), which likely contributes to the angiogenic processes. The pronounced hypoxic situation in OA articular cartilage results in enhanced expression of hypoxia-induced factor 1 $\alpha$  (HIF-1  $\alpha$ ), a positive stimulator of VEGF and Angiopoietins. HIF-1 $\alpha$  is a master regulator of hypoxia-regulated genes due to the presence of hypoxia response elements in the promoter of HIF-1 $\alpha$  dependent genes (Zhao et al., 2013).

### **3.7. Bone remodeling in OA**

Subchondral sclerosis is a hallmark and important sign of the progression of OA. One role of subchondral bone in the joint is to distribute forces, and to adapt in ways that maintain conformation of the joint and prevent stress concentrations. Articular cartilage is designed for loadbearing, and the high water content of cartilage allows it to deform under compressive loads without failure. However, it is mechanically less capable of withstanding tension or shear stresses that occur at the edges of the joint contact regions. High tensile and shear stresses at these margins predispose the cartilage to splitting or fibrillation. This predisposition could be exacerbated by inhomogeneities in the density and stiffness of the underlying subchondral bone, which would cause cartilage to deform more in regions overlying less dense parts of the subchondral plate than in regions over denser portions of the plate. The junction between the stiffer regions and the less stiff regions of the subchondral plate are sites of stress concentration at which the cartilage is more likely to fail in tension.

Early studies using a rabbit impulsive loading model suggested that increases in bone volume and catabolic changes in cartilage were concurrent (Radin et al., 1984). Non-human primates that develop OA spontaneously present with a thickened subchondral plate prior to the development

of apparent cartilage damage (Carlson et al., 1994, 1996) as does a well-established guinea pig model (Kuyinu et al., 2016). Other models in which OA is experimentally induced also demonstrate that bone changes occur earlier than cartilage deterioration (Pastoureau et al., 2010). This finding suggests a temporal relationship between the changes in bone and cartilage during OA progression.

### **Bone remodelling in early-stage disease**

In early OA, the mineral apposition rate, is increased (Amir et al., 1992). There is also an increase in the initiation of new remodelling sites within the subchondral bone. This reduces the thickness of the subchondral plate (Intema et al., 2010). Thinning of the subchondral plate early in OA has been shown in two different canine models of OA in which OA was induced by either creating grooves on the femoral condyles or transecting the Anterior Cruciate Ligament (ACL) (Sniekers et al., 2008) and in a rabbit model (Bellido et al., 2010).

Bettica P et al. in a cohort of women aged 45–64 years showed that resorption markers are considerably elevated in individuals with progressive OA, but not in those with non-progressive forms of OA (Bettica et al., 2002). Resorption markers are also elevated in young and middle-aged adults (aged 27–56 years) with early-stage OA, but without clinical symptoms (Bolbos et al., 2008). The increased rates of bone remodelling in early-stage OA may cause changes in joint shape and load transmission that predispose to progressive cartilage loss.

### **Bone remodeling at molecular level**

Increase in TGF $\beta$ , IGF, IL-1, IL-6 and prostaglandin E2 protein levels have been detected in degenerative cartilage (Mansell et al., 2007), but all these proteins are products and stimulators, of bone remodelling. In vitro experiments depicted that osteoblasts from joints of patients with OA produce more IL-6 and prostaglandin E2 than osteoblasts taken from individuals without OA (Massicotte et al., 2002). It has been suggested that normal repetitive loading of joints can create microcracks in the subchondral plate, even in non-diseased joints (Bani Hassan et al., 2016). Microcracks are the initiating reason for new remodelling events (Plotkin, 2014) and might stimulate osteocytes in the region of damage to produce Receptor Activator of NF $\kappa$ B Ligand (RANKL) and downregulate osteoprotegerin (OPG), an inactivating receptor for RANKL, to induce bone resorption (Kennedy et al., 2012; Nakashima et al., 2011). A decreased OPG/RANKL ratio has been observed in animal models of OA (Bellido et al., 2010), consistent with increased bone remodelling; RANKL and its isoforms have been shown to be differentially expressed in subchondral bone osteoblasts taken from patients with OA (Tat et al., 2008). However, dysregulation of the OPG/RANKL axis could also occur in other cell populations. For example, a reduced OPG/RANKL ratio has been observed in chondrocytes taken from patients with OA (Kwan Tat et al., 2009).

### **Vascular invasion in bone remodeling**

Increased bone remodelling is inevitably associated with vascular invasion, and in some cases, the vessels can invade the deep layers of articular cartilage. Increased vascularity can occur under stimulation of angiogenic factors such as VEGF, which are elevated in the synovial fluid of a major proportion of patients with OA, as already has been explained (Lingaraj et al., 2010b). These factors induce chondrocytes to synthesize and secrete different MMPs and other catabolic enzymes such as ADAMTS, and prevent the association of MMPs with their inhibitors (Luyten et al., 2006). The combined effect of vascular invasion of the articular cartilage, which provides a pathway for catabolic agents, and failure to inhibit matrix-degrading enzymes, creates an ideal condition for the degradation of the cartilage. Secondly to vascular invasion and failure to inhibit enzymes, cartilage fibrillation diminishes the mechanical integrity of the cartilage matrix, which can create a positive feedback loop as bone remodelling continues to occur to help the joint adapt to the altered loads.

### **Subchondral bone in late-stage disease**

Radiological and pathological studies of bone in OA clearly demonstrate increased apparent density and increased bone volume in late-stage disease. An increase of up to 15% in bone mineral density and up to 30% in bone volume in patients with OA compared with those without OA was reported (Chan et al., 2014).

Lower bone mineralization as a result of accelerated bone remodelling may be exacerbated by an altered osteoblastic phenotype, which impairs normal mineralization of bone once it has been deposited. Altered mineralization has been linked to endogenous production of TGF $\beta$  by OA osteoblasts, which is elevated 3 fold (Chan et al., 2011a). Studies also showed that osteoblasts from joints of patients with OA secrete a collagen type I homotrimer composed of  $\alpha$ 1 chains, which is phenotypically distinct from the normal heterotrimer formed by two  $\alpha$ 1 chains and one  $\alpha$ 2 chain (Chan et al., 2011b; Couchourel et al., 2009). This abnormal collagen formation might contribute to the impairment of proper tissue mineralization.

The relationships between bone remodelling, mineralization and mechanical consequences have been well demonstrated both biologically and mechanically. Mansell and Bailey demonstrated that OA was associated with increased collagen content signifying increased bone formation, but with a significantly reduced calcium to collagen ratio (Mansell and Bailey, 1998). Li and Aspden revealed the effects of greater bone volume and lower tissue mineralization, which were to increase apparent density of the subchondral bone, but to significantly decrease both the material density and the mechanical stiffness (Li and Aspden, 1997). Some researchers have suggested that the lower modulus of the subchondral bone can increase cartilage deformation and accelerate cartilage loss (Karsdal et al., 2008), an hypothesis which is directly in contrast with the idea that increased subchondral stiffness is the driving force for progressive cartilage degradation.

## 4. Current therapeutic treatment in OA

Today, there is no consensus regarding the ideal treatment for OA. However, several treatment methods have been used with the aims of pain relief and improvement of patients' functional abilities. These treatments could be divided in two parts: 1) non-pharmacological methods such as physiotherapy, occupational therapy, weight loss and exercise, and 2) pharmacological methods like oral, intra-articular injection, immunotherapy, gene therapy and cellular therapy. Surgery is only considered for severe cases when conservative therapy is ineffective because of the invasive trauma and higher risks.

Pharmaceutical therapy is the most commonly used OA treatment option aimed mainly at pain relief and anti-inflammation. The traditional drugs are limited to control OA symptoms, but none can reverse the damage in the OA joint. Moreover, the traditional drugs are always overwhelmed by its high incidence of negative effects.

Studies of new OA drugs (mainly biologic agents) with more effectiveness and fewer side effects are underway. In addition, regenerative therapy holds the possibility of repairing and regenerating damaged or lost tissues to restore the original structure and function (Mason and Dunnill, 2008). It has already been applied in the orthopedic clinic for several decades and promising outcomes have been achieved (Tang et al., 2012). In recent years, pilot clinical studies, show the usefulness of regenerative therapy in the treatment of OA, suggesting its potential to be translated from bench to bedside. Among pharmacological treatments, those based on cell therapy has raised much attention as an alternative approach towards tissue repair and regeneration.

Mesenchymal stem cells (MSCs) have been considered as an ideal regenerative cellular therapeutic for degenerative musculoskeletal conditions like OA (Sampson et al., 2015). These cells are found in a variety of tissues and have the ability to rapidly proliferate and differentiate to musculoskeletal lineages including bone and cartilage (Kolf et al., 2007). A significant body of research has also demonstrated that these cells orchestrate important immunologic functions through modulation of the local inflammatory response (Khosravi et al., 2017a, 2017b). Taken together, these factors support the ability of MSCs to deter degenerative joint disease and might be a potential replacement therapy for classical surgical interventions in future.

### 4.1. Non pharmacological treatment

Patient education should be the first step in OA therapy, then, weight control and physical therapy further improves the physical and mental condition of the patient. Obesity and, higher Body Mass Index (BMI) have been associated with an increased risk of progression of OA of the knee (Cooper et al., 2000). One study showed that a reduction in the percentage of body fat, rather than weight, may be significant in reducing pain from OA of the knee (Toda et al., 1998). Combination of weight loss and exercise can be superior to either intervention alone (Messier et al., 2004). Walking, swimming and home-based exercise also could be beneficial (Bentley and Dowd, 1984). There is some information in the literature that heat and cold applications should be superficially performed in patients with knee OA as a non-pharmacological method for

controlling pain. They are available to help treatment due to the presence of very few side effects, facility of implementation, and their non-invasiveness (Rie Kurose et al., 2015).

## **4.2. Pharmacological treatment**

### **4.2.1. Oral treatment**

#### **Glucosamine and CS**

Glucosamine and CS that are slow acting anti-arthritis drugs, which recommended by the European Association of Rheumatology (EULAR) and have been used for over 40 years. Glucosamine can be extracted from chitin, found primarily in the exoskeleton of crustaceans (crabs, prawns, and lobsters), as well as in the cell membranes of mushrooms and in the case of CS can be extracted from the cartilaginous tissue of cows, pigs, birds, and fish (sharks) (Jerosch, 2011).

Indeed, these drugs belong to the class of slow-acting anti-arthritis agents and have demonstrated an analgesic effect (often low) and a slowing down in the progression of the disease, although it is difficult to situate in terms of clinical relevance the impact of a 0.10 mm minimal slowdown of radiographic joint space narrowing over 2 to 3 years (Fransen et al., 2015). They could be sold as dietary supplements in North America and is a prescription drug under the regulation of the European Medicine Agency (EMA) in Europe (Henrotin et al., 2014a).

CS is mostly administered orally and also intramuscular injection has been reported. CS was shown to have various effects from anti-inflammatory and anti-catabolic to anti-apoptotic, and also anti-oxidant properties (Henrotin et al., 2010a). Absorption of CS could depend on the position and percentage of sulfate groups, which could be changed generally in relation to specific animal sources. For instance, desulfated chondroitin in mouse has a very rapid uptake, with a peak occurring within 15 min (Kusano et al., 2007), whereas shark CS in male healthy volunteers has a slower uptake, peak at 8.7h (Volpi, 2003). CS from bovine trachea presents a rapid increase in plasma of male healthy volunteers with a peak of 15h (Volpi, 2002). Also with combination with glucosamine, they can active on cellular and molecular aspects of the inflammatory reaction and finally treatment of synovitis could be happened (Henrotin et al., 2014b). The role of CS in vitro and in vivo will be explained completely in chapter 3.

#### **Non-steroidal anti-inflammatory drugs**

Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) are a type of anti-inflammatory drug that are very commonly taken to reduce pain, decrease fever and in higher doses, decrease inflammation. They are considered by many authors to be the first choice medications for pharmacological treatment of OA (da Costa et al., 2017). The most prominent members of this group of drugs are aspirin, ibuprofen, diclofenac and naproxen. NSAIDs work through nonspecific inhibition of



cyclooxygenase isoforms 1 and 2 (COX-1 and COX-2). COX-1 is constitutively expressed in renal and gastrointestinal (GI) tissues. COX-2 is inducible in inflammatory responses. The major side effects of NSAIDs are gastro-intestinal bleeding, renal dysfunction, and blood pressure elevation. Because GI tissues have a higher expression of COX-1, a selective COX-2 inhibitor might spare patients the GI side effects. NSAIDs may have deleterious effects on disease progression by increasing subchondral bone destruction and preventing cartilage matrix repair. Because COX-2 inhibitors offer the possibility to block cytokine-inducible prostaglandins without affecting physiologic levels due to COX-1 activity, they may slow cartilage and bone destruction (Rao and Knaus, 2008).

#### **4.2.2. Intra-articular injection**

##### **Corticosteroids**

Intra-articular (IA) corticosteroid injections are frequently used to reduce swelling and thereby reduce knee stiffness and pain. In common practice, they are diluted in local anesthetic to provide immediate relief, ensure accurate drug delivery and allow even dispersal of the drug within the joint due to the larger volume (Schumacher, 2003). Those corticosteroids commonly used in IA injections include Hydrocortisone Acetate (HCA), Methylprednisolone Acetate (MPA) and Triamcinolone Acetonide (TCA). Some studies suggest that IA steroid injections should be used as a short term bridging treatment to resolve acute painful pending further intervention such as surgery or physiotherapy.

One study have shown (Bannuru et al., 2009) in the treatment of knee osteoarthritis that intra-articular corticosteroids appear to be relatively more effective for pain than hyaluronic acid in the first four weeks, but in the long term hyaluronic acid has greater efficacy. Nevertheless, corticosteroids doses are cheaper than hyaluronic acid preparation, but the efficacy of these drugs seems to last less longer than hyaluronic acid preparations, with more relevant side effects, which can offset the initial saving (Merolla et al., 2011; Qvistgaard et al., 2006). All together it seems that HA based therapy is the better choice by looking to all advantages and disadvantages and therefore, could be involved in future of OA treatment.

##### **Hyaluronic acid/hyaluronan**

High molecular weight HA has been shown to improve joint lubrication and it is recognized as a common clinical treatment for OA to improve synovial lubrication by reducing the progression of wear and inflammation. One of the most important receptors of HA is CD44, which has been shown to have a positive influence on a large number of cellular pathways including stimulating differentiation of chondrocytes (Ehlers et al., 2001), chondrocyte proliferation (Patti et al., 2001) and regulation of cell migration (Cai et al., 2017). The positive effect of HA could be discussed in several aspects as follow:

1) HA is favorable to the deposition of ECM which is essential in cartilage tissue engineering (Amann et al., 2017). It has been shown that HA is quite a suitable scaffold for the growth of chondrocyte. HA-based scaffolds can induce or promote stem cell differentiation (Prè et al., 2016). This is in accordance with other studies showing that ECM secretion, by the chondrocytes, results in an increase of collagen II and aggrecan deposition (Lebourg et al., 2013; Yoo et al., 2005). Furthermore, one in vitro experiments demonstrated that HA-based scaffold can induce stem cells into cartilage in basal medium without the addition of growth factors (Meng et al., 2014).

2) HA can induce stem cells and cartilage progenitor cells to differentiate into chondrocytes. Chung and Burdick demonstrated in both in vivo and in vitro experiments that methacrylated HA is capable of providing a microenvironment that facilitates differentiation of mesenchymal stem cells into cartilage, and the effect can be enhanced in the presence of certain cytokines such as TGF $\beta$ 3 (Chung and Burdick, 2009a).

Co-culturing human MSCs with human articular chondrocytes in HA hydrogels could enhance the functional properties of tissue-engineered cartilage and inhibit the hypertrophic propensity of MSCs by decreasing the expression of collagen type X during chondrogenesis compared to MSC cultures alone (Chung and Burdick, 2009b).

3) HA can also act as a cell carrier, wrapping stem cells and chondrocytes in the injected area so that the cells can proliferate and differentiate in situ (Skaalure et al., 2014). reported that HA deposition would occur in the areas of cartilage injury and it would attract exogenous mesenchymal stem cells to the injured area (Lammi et al., 2001).

4) Some papers indicate that HA involves in the maintenance of chondrocyte phenotype, which is interesting since chondrocyte can lose its phenotype during in vitro culture (Kim et al., 2015). Chen et al. demonstrated that HA and platelet-rich plasma can restore the down-regulation of cartilage gene expression induced by IL-1 $\beta$  and TNF $\alpha$ , including SOX-9, collagen type II and aggrecan (Chen et al., 2014).

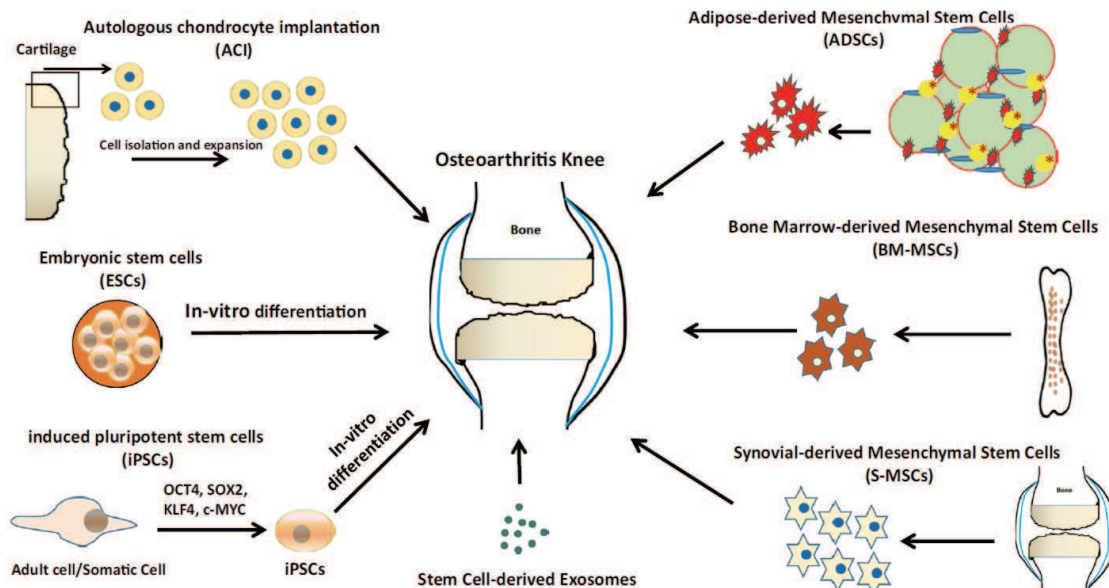
Currently, HA injections account for 25% of treatment costs associated with non-operative care for knee OA higher than any other treatment (Weick et al., 2016). Two studies proposed that HA treatments have few safety-related events and a better side-effect profile than NSAIDs orally (Bannuru et al., 2015). HA injection can reduce the necessary dose of NSAIDs during at least, 6 months period following the injection (Patti et al., 2001). The reduction in NSAIDs use is another important clinical achievement with significant health economic consideration (Sturkenboom et al., 2002). Not only direct costs (NSAIDs purchasing), but also the indirect costs associated with management of NSAIDs side effects, are saved.

Although HA injection is safe, but is restricted by the relatively high cost of the treatment (National Collaborating Centre for Chronic Conditions (UK), 2008). Another part of the problem

is that only one session treatment is typically proven to be efficient for just one year (Thomas et al., 2017) and patients should be injected again to reduce pain. Also it is not useful method for those patients with advanced OA (according to K-L system), they are less likely to respond (Bowman et al., 2018). In addition all patients undergoing IA injection should be consented for the risk of infection, although this is a rare event (incidence reported between 1 in 3,000 to 1 in 50,000) (Creamer, 1999) and lipoatrophy at the site of injection (estimated 0.6% of patients) (Kumar and Newman, 1999). Therefore, Although HA treatment stands as a good therapeutic progression, its weakness towards already advanced OA makes us to consider it in a back to back treatment, for example, joined or followed by cell therapy to fully benefits from its catabolic effects.

### 4.3. Cellular treatment

Cell therapy or cellular treatment is a technology that relies on replacing diseased or dysfunctional cells with healthy, functioning ones. Whole blood transfusions, packed red cell transfusions, platelet transfusions, bone marrow transplants, chondrocyte transplants and organ transplants are all forms of cell therapy. Cell therapy may be applicable to some types of cancer, neurological diseases, spinal cord injuries, inflammatory disorders, autoimmune diseases and diabetes. A great range of cells can serve in cell therapy including blood and bone marrow cells, mature and immature solid tissue cells, adult and embryonic stem cells. Different cell- based therapy approaches has been using over the last decade. **Figure 19** depicts a brief of different cellular treatments proposed to control OA.



**Figure 19:** Schematic diagram illustrating the current clinical approaches to cell-based therapy for cartilage tissue engineering. Adopted from (Burke et al., 2016).

### 4.3.1. Chondrocyte

#### **Autologous chondrocyte implantation**

Autologous chondrocyte implantation (ACI) was first reported in 1994 for treatment of focal cartilage defects in the tibiofemoral and patellofemoral compartments (Brittberg et al., 1994). First, sampling of between 200 and 300 milligrams cartilage is taken from a less weight bearing area of the medial or lateral femoral condyle of the patient. Then the matrix is removed enzymatically and the chondrocytes isolated. These cells are grown in vitro about four to six weeks, until there are enough in number to reimplant on the damaged area of the articular cartilage. In the next step chondrocytes are applied on the damaged area during an open-knee surgery (also called arthrotomy). These autologous cells should adapt themselves to their new environment by forming new cartilage.

Even if it is the first articular cartilage repair method using tissue engineering, the problem of sufficient number of cells cannot be secured (Harris et al., 2010). In addition, another difficulty is their derivation requires additional surgical procedures leading to possible complications at the donor site (Lee et al., 2000).

#### **Matrix-induced autologous chondrocyte implantation**

Matrix-induced autologous chondrocyte implantation (MACI) is a novel technique of cartilage recovery. It is a one-step procedure that combines microfracture with the fixation of a biological scaffold, such as a porcine collagen matrix. This matrix covers the blood clot, permitting the ingrowing of MSCs to differentiate into the chondrogenic lineage. The matrix acts as a temporary structure to allow the cells to be seeded and establish a 3-dimensional structure (Lee et al., 2014). Moreover, due to the concerns associated with donor site morbidity and the de-differentiation and limited lifespan of chondrocytes, novel cartilage repair strategies are in need necessary.

### 4.3.2. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stromal cells that first identified in 1966 by Alexander Fridenstein (Friedenstein et al., 1966). They are non-hematopoietic self-renewal cells that have the ability of multipotent differentiation. They could differentiate into mesodermal and non-mesodermal cells like chondrocytes, osteocytes, cardiomyocytes, adipocyte and neural cells (Mazor et al., 2014). These cells that are known for their adherence capacity to plastic, neither express the hematopoietic markers such as CD34, CD45 and CD14, nor endothelial markers like CD31 and CD144. Additionally, they do not express MHC II molecules like HLA-DR, and co-stimulation molecules like CD80 and CD86. However, they do express markers such as CD90, CD73, CD105, CD146 and CD29, plus a poor expression of MHC I molecules (Wang et al., 2015).

MSCs are now known to reside in connective tissues, notably adipose tissue (AT) (Zuk et al.,

2002), the placenta (Fukuchi et al., 2004), the umbilical cord (Nguyen et al., 2011), dental pulp (Gronthos et al., 2000), tendons (Bi et al., 2007), trabecular bone (Nöth et al., 2002), the perichondrium (Arai et al., 2002), the periosteum (Nakahara et al., 1991), the synovium and synovial fluid (De Bari et al., 2006), and the infrapatellar fat pads (Jones et al., 2002). MSCs may occur in virtually all postnatal organs and tissues. Bone marrow (BM) and adipose tissues (AT) are two main sources of MSCs used in cell therapy because of the high expansive potentials of such cells and the availability of reproducible isolation procedures (Crisan et al., 2008). Also synovial joints consist of number of different tissues and MSCs have been isolated from most of them.

The pathology of OA (**Figure 20**), shown in the left semicircle) includes inflammation, recruitment of inflammatory cells, and production of the proinflammatory cytokines like IL-1, IL-6, and TNF $\alpha$ . Articular chondrocytes within diseased cartilage synthesize and secrete proteolytic enzymes, MMPs and aggrecanases that degrade the cartilaginous ECM and ultimately trigger apoptotic cell death of differentiated chondrocytes. Repair of OA by MSCs (**Figure 20**), the right semicircle) is based on the fact that MSCs secrete a broad spectrum of bioactive molecules. These include the secretion of anti-inflammatory cytokines such as TGF $\beta$ , Indoleamine 2, 3-Dioxygenase (IDO), and TNF-Stimulated Gene 6 (TSG6). Moreover, M1 sub-population of macrophages is transformed into M2 anti-inflammatory macrophages. MSCs also secrete anti-proteinase cytokines such as TIMP-1 and TIMP-2 and anti-apoptotic growth factors like VEGF and IGF1. MSCs enhance neocartilage formation by secreting BMP, FGF, IGF, sex determining region Y box 9 (SOX9), Signal Transducer and Activator of Transcription 1 (STAT-1), and TGF $\beta$  (Wang et al., 2015) (**Table 6**), summarizes the recent application of MSCs harvested from different sources in treatment of OA.

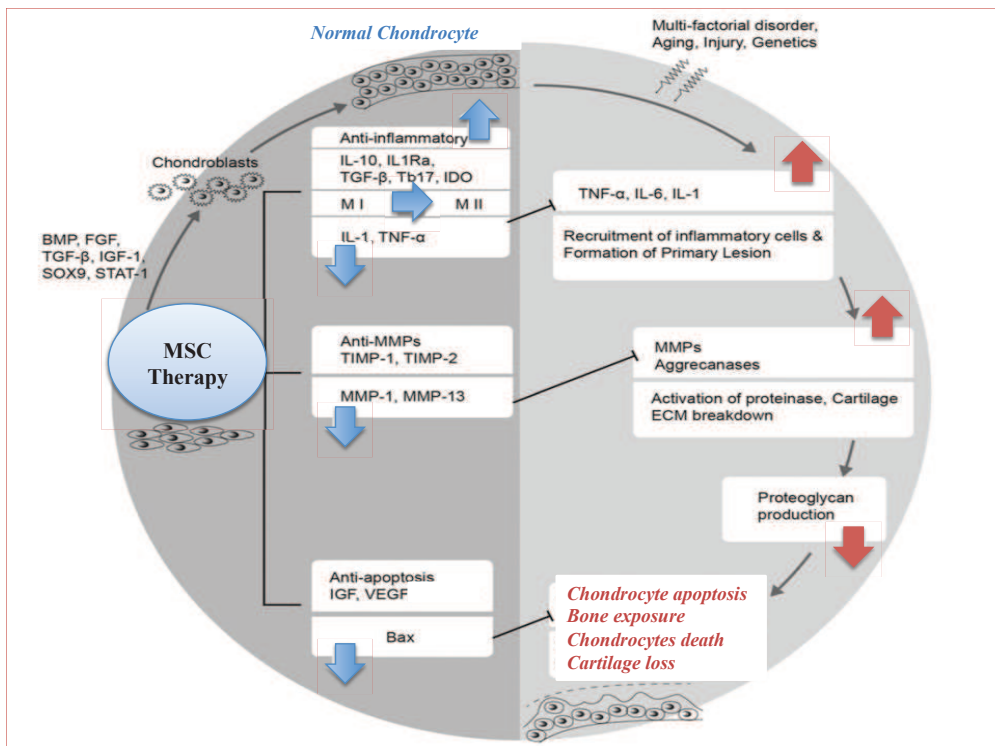


Figure 20: The potential therapeutic mechanisms of MSCs for osteoarthritis (OA).

### Bone Marrow-Derived-MSCs

Bone Marrow-Derived-Mesenchymal Stem Cells (BM-MSCs) are multipotent stem cells with a high proliferation capacity and the ability to differentiate into cells of the connective tissue lineage, such as adipocytes, osteoblasts and chondrocytes, both in vitro and in vivo. Human BM-MSCs are found in stem cell niches in bone marrow. It has several markers containing positive and negative markers (Dominici et al., 2006). The positive markers include CD166, CD90, CD105 and Stro-1 and should be negative for hematopoietic markers, such as CD14, CD34, CD45 and HLA-DR (Nishimura et al., 2008). BM-MSCs may be the most appropriate source for treatment of OA because of the availability of large quantities of cells and the chondrogenic differentiation property of such cells. Moreover, OA is associated with progressive and often severe inflammation. BM-MSCs not only contribute structurally to tissue repair but also possess potent immunomodulatory and anti-inflammatory effects (Khosravi et al., 2017a, 2017b). BM-MSCs secrete a broad spectrum of bioactive molecules with immunoregulatory (Uccelli et al., 2007) and/or regenerative activities (Caplan and Dennis, 2006). Via direct cell–cell interaction or by secretion of various factors, BM-MSCs exert powerful effects on local tissue repair by modulating the local environment and activating endogenous progenitor cells.

These properties mean that BM-MSCs are promising candidates for cell therapy of OA (Wang et al., 2015). Due to their vast advantages, they have being used more than other sources of MSCs

in OA therapy. **Table 6** depicts their application in OA treatment starting from beginning of 21<sup>st</sup> century until 2017. In contrast, there are some negative points to use BM-MSCs. For instance, the yield of adult stem cells from patients most likely to require such cartilage therapies may be insufficient, as BM-MSCs from older patients show lower proliferation rates in culture (Dexheimer et al., 2011) and BM-MSCs from patients with OA demonstrate reduced chondrogenic differentiation (Murphy et al., 2002).

### **Adipose-Derived MSCs**

Another source of MSCs is adipose-derived MSCs (AD-MSCs) which are defined by their functional abilities of differentiation into variety of lineages such as chondrocytes, osteoblasts or adipocytes. They differ from hematopoietic stem cells by the expression of mesenchymal markers (CD90, CD105, CD73), while lacking expression of CD34, CD45, CD14 or the human Major Histocompatibility Complex class II (MHC II). AD-MSCs have a phenotypic heterogeneity through the releasing of trophic factors such as Hepatocyte Growth Factor (HGF), IL-6, Macrophage Colony-Stimulating Factor (M-CSF), TGF $\beta$ 1, TNF $\alpha$ , and VEGF (Pers et al., 2016).

Intra-articular injection of AD-MSCs prevented OA onset in a collagenase-induced murine knee OA model and reduced synovitis, osteophyte formation, and cartilage degeneration (ter Huurne et al., 2012). AD-MSCs in co-culture with OA chondrocytes significantly reduced the expression of TIMP-3 and MMP-13 and in co-culture with synoviocytes significantly increased TIMP-1 expression, decreased ADAMTS5 and did not affect ADAMTS4, TIMP-3 or MMP-13. These data provided another evidence of AD-MSCs efficiency to reduce catabolic factors secretion by OA synoviocytes and OA chondrocytes and suggested possible beneficial effects of AD-MSCs in prevention of cartilage from degradation during OA (Manferdini et al., 2015).

### **Synovial Membrane-Derived MSCs**

Synovial Membrane-Derived MSCs (SM-MSCs) from patients with OA were much more than those from healthy one and increased according to postinjury period or severity (Sekiya et al., 2012). These cells that are considered to be derived from synovial tissue are positive for CD44, CD73, and CD90. Some studies showed that intra-articular injection of the SM-MSCs promoted meniscus regeneration and protected articular cartilage by arthroscopic and histological observations in pig (Nakamura et al., 2013), rat (Katagiri et al., 2013), or porcine (Hatsushika et al., 2014) massive meniscal defect models. In another recent study to investigate the effects of single or repetitive intra-articular injections of SM-MSCs on a rat OA model, Ozeki N et al. showed that, not single but periodic injections of SM-MSCs maintained viable cells without losing their MSC properties in knees and inhibited OA progression by secretion of trophic factor such as PRG-4, BMP-2, and BMP-6 genes encoding chondroprotective proteins, and TSG-6 encoding an anti-inflammatory one (Ozeki et al., 2016).

Cell Origin	Method of Cell Delivery	Outcome	Follow-Up & Duration	Reference
Human BM-MSCs/type 1 collagen from porcine tendon	MSCs embedded in collagen gel transplanted into the articular cartilage defect	Improve arthroscopic and histological grades	42 weeks	(Wakitani et al., 2002)PM
Human BM-MSCs/ceramic ankle prosthesis	Transplanted into ankle osteoarthritis patients	Improve clinical scores, no inflammatory reaction	2 months–2 years	(Ohgushi et al., 2005)PM
Human BM-MSCs/collagen gel	MSCs embedded within a collagen gel, transferred to the mice articular cartilage defect	Defect became filled with hyaline-like cartilage tissue	1 year	(Kuroda et al., 2007)PM
BM-MSCs from Iliac crest	Intra-articular injection	Promote cartilage regeneration and reduction pain	24 weeks	(Centeno et al., 2008)PM
Human BM-MSCs	BMSCs were implanted beneath a patch	Improve physical function	2 years	(Nejadnik et al., 2010)PM
BM-MSCs from Iliac crest	Intra-articular injection	Promote cartilage regeneration and reduction pain	6- 12 months	(Buda et al., 2010)PM
Human BM-MSCs/platelet-rich fibrin glue	MSCs placed on platelet-rich fibrin glue and transplanted into full-thickness cartilage defects	Improve physical function and symptoms, improve in RHSSK score, MRI showed filling of cartilage defects and complete surface congruity with native cartilage	1 year	(Haleem et al., 2010)PM
Human AD-MSCs/HA, platelet-rich plasma, calcium chloride, dexamethasone	Intra-articular injection	Increase meniscus cartilage volume on MRI, less pain	12 weeks	(Pak, 2011)PM
Human BM-MSCs	Intra-articular injection	Walking time before pain onset improved in three patients but unchanged in one, VAS pain scores improved in all patients	6 months	(Davatchi et al., 2011)PM
Human BM-MSCs/collagen scaffold	BMSCs were implanted into defects in combination with a three-dimensional collagen	Improve KOOS and IKDC scores, arthroscopic assessment showed good filling of defects, tissue stiffness was good, tissue was	30-31 months	(Kasemkijwattana et al., 2011)PM



	scaffold	incorporated into adjacent cartilage		
Infrapatellar fat pad-derived MSCs/ platelet-rich plasma	Intra-articular injection	Improve the mean Lysholm score, the Tegner activity scale, and the VAS score	12-18 months	(Koh and Choi, 2012)PM
Human BM-MSCs	Intra-articular injection	Less pain and improve walking distance up to 6 months postinjection, continued slight improvement to 12 months postinjection, MRI showed an increase in cartilage thickness in three patients	1 year	(Emadedin et al., 2012)PM
Murine AD-MSCs	Intra-articular injection in collagenase-induced murine knee OA model	Reduce synovitis, osteophyte formation, and cartilage degeneration	14-43 days	(ter Huurne et al., 2012)PM
Murine MSCs of C57BL/6 or MRL/MpJ superhealer mice	Intra-articular injection	Prevent post-traumatic arthritis	8 weeks	(Diekman et al., 2013)PM
Autologous activated peripheral blood stem cells with growth factor addition/ preservation, along with HA, in conjunction with arthroscopic microdrilling and mesenchymal cell stimulation	Intra-articular injection	Improve WOMAC and KOOS scores, electron microscopy revealed cell attachment and proliferation, histological analysis demonstrated the presence of hyaline cartilage	6 months	(Turajane et al., 2013)PM
Human infrapatellar fat pad-derived MSC/ platelet-rich plasma	Intra-articular injection	Decrease Western Ontario and McMaster Universities Osteoarthritis Index scores, improved Lysholm scores, decreased VAS scores	24-26 months	(Koh et al., 2013)PM
Human BM-MSCs	Intra-articular injection	Rapid and progressive improvement of algofunctional, quantitative MRI T2 mapping showed that diseased cartilage area decreased, improved cartilage quality in 11/12 patients	1 year	(Orozco et al., 2013)PM
Human BM-MSCs	Intra-articular injection	Improve pain levels and function	2 years	(Teo et al., 2013)PM

<b>Pig SM-MSCs</b>	<b>Intra-articular injection</b>	<b>Better hyaline cartilaginous-tissue regeneration</b>	<b>12 weeks</b>	<b>(Nakamura et al., 2013)PM</b>
<b>Rat SM-MSCs</b>	<b>Aggregates transplantation of SM-MSCs on the site of meniscal defects.</b>	<b>Meniscus regeneration</b>	<b>12 weeks</b>	<b>(Katagiri et al., 2013)PM</b>
<b>Human BM-MSCs/HA</b>	<b>Intra-articular injection</b>	<b>VAS scoring showed that patients with osteoarthritic changes who received MSCs experienced significant reductions in pain</b>	<b>2 years</b>	<b>(Vangsness et al., 2014)PM</b>
<b>Human AD-MSCs</b>	<b>Intra-articular injection</b>	<b>Improve cartilage regeneration and pain reduction</b>	<b>6 months</b>	<b>(Jo et al., 2014)PM</b>
<b>Porcine SM-MSCs</b>	<b>Intra-articular injection</b>	<b>Promot meniscus regeneration</b>	<b>16 weeks</b>	<b>(Hatsushika et al., 2014)PM</b>
<b>Human SM-MSCs</b>	<b>Intra-articular injection</b>	<b>Improve pain scores</b>	<b>52 months</b>	<b>(Sekiya et al., 2015)PM</b>
<b>Murine SM-MSCs</b>	<b>Intra-articular injection of Sca-1 + GFP + synovial cells from C57BL6 or MRL/MpJ into C57BL6 mice</b>	<b>Mouse synovial MSCs purified with Sca-1 have the capacity for chondrogenic differentiation in vitro and in vivo so increased cartilage repair was observed</b>	<b>4 weeks</b>	<b>(Mak et al., 2016)PM</b>
<b>Human AD-MSCs</b>	<b>Intra-articular injection</b>	<b>Improve pain levels and function</b>	<b>6 months</b>	<b>(Pers et al., 2016)PM</b>
<b>Rat SM-MSCs</b>	<b>Repetitive intra-articular injections</b>	<b>Inhibit OA in rat knee</b>	<b>12 weeks</b>	<b>(Ozeki et al., 2016)PM</b>
<b>Murine BM-MSC-derived MPs and Exosome</b>	<b>Intra-articular injection</b>	<b>Re-establishing chondrocyte homeostatic state, protecting chondrocytes from apoptosis and stimulating macrophage polarization towards anti-inflammatory phenotype.</b>	<b>6 weeks</b>	<b>(Cosenza et al., 2017)PM</b>
<b>Stromal vascular fraction</b>	<b>Intra-articular injection</b>	<b>Reduction in pain levels by</b>	<b>3and 6</b>	<b>(Bansal et al., 2017)PM</b>

(SVF) from BM-MSC		decreasing total WOMAC score	months 1, 1,5 and 2 years.	
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**Table 6: Summary of Therapeutic Applications of Stem Cells in Osteoarthritis.**

### **Induced pluripotent stem cells**

Induced Pluripotent Stem Cells (iPS) are a type of pluripotent stem cell that can be generated directly from adult cells. The iPS technology was pioneered by Shinya Yamanaka’s lab in Kyoto, Japan, who showed in 2006 that the introduction of four specific genes encoding transcription factors could convert adult cells into pluripotent stem cells (Takahashi and Yamanaka, 2006). He was awarded the 2012 Nobel Prize along with Sir John Gurdon "for the discovery that mature cells can be reprogrammed to become pluripotent. Pluripotent stem cells hold promise in the field of regenerative medicine (Mahla, 2016). IPSs have pluripotency and the potential for self-renewal.

They have the potential to provide an abundant cell source for tissue engineering, as well as generating patient-matched in vitro models to study genetic and environmental factors in cartilage repair and osteoarthritis (Diekman et al., 2012). Recent study has made it possible to generate integration-free iPS cells and to differentiate iPS cells toward chondrocytes (Tsumaki et al., 2015). As an alternative approach, chondrocytic cells can be induced directly from dermal fibroblasts without going through the iPS cell stage. In 2011, Hiramatsu et al (Hiramatsu et al., 2011) generated in vitro polygonal chondrogenic cells from adult dermal fibroblast cultures by ectopic expression of reprogramming factors (c-Myc and Klf4) and one chondrogenic factor (SOX9). Namely, this approach could lead to the preparation of hyaline cartilage directly from skin without generating iPS cells. In one study hyaline cartilage was generated from human iPS cells in immunodeficiency rats and immunosuppressed mini- pigs (Fransen et al., 2015).

#### **4.3.3. Human Embryonic Stem Cells**

Because of their unlimited capacity for self-renewal while maintaining potential for chondrogenic differentiation, Human Embryonic Stem Cells (hESC) could provide essentially limitless numbers of cells with potential for cartilage repair, and may offer an ideal alternative cell source for cartilage repair compared to MSCs, or to the adult chondrocytes used in ACI. In order to achieve this potential, procedures for efficient directed differentiation of pluripotent cells into the chondrogenic lineage are needed. Protocols have been reported for chondrogenic differentiation of hESC under various conditions, typically involving supplementation with growth factors such

as TGF $\beta$  and BMPs. Most approaches utilize an intermediate step involving Embryoid Body (EB) formation (Oldershaw et al., 2010). Embryoid bodies contain cells from all three embryonic germ layers including ectoderm and endoderm, which may introduce non-chondrogenic cell types, potentially requiring cell sorting or FACS to purify the mesenchymal progenitors (Jones et al., 2002). Procedures have also been reported in which either EB steps, co-culture with other cell types, cell sorting or prolonged culture periods in monolayer are used to derive an MSC-like intermediate from the hESC, which may then be directed into the chondrogenic lineage (Kopher et al., 2010; Mahmood et al., 2010).

Promising results have been obtained in a recent studies evaluating in vivo articular cartilage repair by chondrocytes derived from hESC (Perera et al., 2012). In one study, the hESC-derived chondrocytes were embedded in a hyaluronan hydrogel and surgically implanted into articular cartilage defects generated in rats. After 12 weeks, the defect was filled by hyaline cartilage-like repair tissue with good integration with the adjacent cartilage (Toh et al., 2010). Some potential problems noted in the study included lowlevel induction of type X collagen expression by the repair tissue, and surprising gradual loss of the human cells, despite chemical immune suppression to avoid graft rejection. Nonetheless, this encouraging study demonstrates the potential in vivo efficacy of a hESC-based cartilage repair strategy. Improvements will likely be realized through use of chondrogenic hESC generated in protocols which minimize potential heterogeneity (Gong et al., 2010), as well as through appreciation of the signals which control chondrocyte differentiation and hypertrophy, and a better understanding of hESC in graft-host responses.

#### **4.4. Surgical treatment**

Surgical treatment should be considered when OA symptoms are severe, other treatments do not succeed and quality of life is notably compromised. There are several different types of surgery which include: total knee arthroplasty to replace the knee joint with an artificial one, knee osteotomy to align bones and reduce friction and partial knee arthroplasty to remove loose pieces of cartilage.

Total Knee Replacement Surgery (Total Knee Arthroplasty): involves cutting the arthritically damaged ends of the tibia and femur and capping both with prostheses.

Partial Knee Replacement Surgery often called unicompartmental knee replacement involves replacing only one of the knee's three "compartments":

- 1) The medial compartment refers to the inner knee,
- 2) The lateral compartment refers to the outer knee,
- 3) The patellofemoral compartment is at the front of the knee, where the patella meets the femur.

Compared to total knee replacement, partial knee replacement is less invasive, so it is usually less

painful and requires less recovery time (Rönn et al., 2011).

Knee Osteotomy (Tibial Osteotomy or Femoral Osteotomy) which is appropriate for a limited number of younger (under age 60 or so), physically active people whose poor knee alignment has caused a great deal of wear-and-tear on just one side of the knee joint, a condition called unilateral knee arthritis or asymmetrical knee arthritis. During knee osteotomy the surgeon cuts into the femur or tibia and either: removes a small wedge of bone, or adds a small wedge of natural bone graft or artificial bone material (Sterett et al., 2010).

# Chapter 3: ROLE OF PGs AND GAGs IN NORMAL CARTILAGE AND OA

## 1. Proteoglycans

Although there are an abundant amount of PGs in the limb, only a small number of them are present in the cartilage. Inherent changes in the metabolism of chondrocytes occur in OA, but one consistent pathogenic feature of this arthropathy is the loss of matrix macromolecules from the cartilage (Knudson and Knudson, 2004). This loss may be due to increased degradation via elevated MMP activity as discussed in chapter 2, inhibition of biosynthesis as will be discussed further in this chapter and/or an inhibition of PG retention due to deficits in HA. In this chapter, we described the role of the major PG involved in adult cartilage homeostasis with specific focus on HSPG. Our objectif is to try to make a link between functional effect decipted for these HSPG in cartilage and OA, with potent GAG structural and functional information collected during this thesis project.

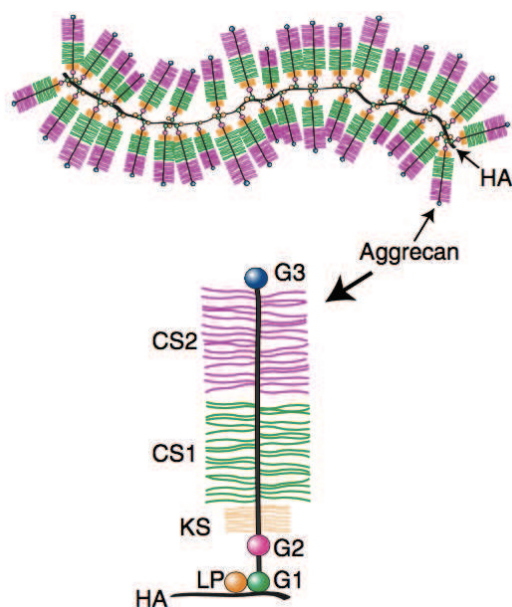
### 1.1. Aggrecans

Aggrecan is characterized by its ability to interact with HA to form large PG aggregates via link proteins (Hunziker et al., 1997). These large aggregates generate a densely-packed, hydrated gel enmeshed in a network of reinforcing collagen fibrils and other PGs and glycoproteins (Roughley and Mort, 2014a). The N-terminal domain contains four link protein-like modules or PG tandem repeats in addition to the Ig-like repeat. The entire link module is ~100 amino acids in length and has a characteristic consensus sequence with four disulfide-bonded Cys residues. These modules form two globular domains known as G1 and G2 (Kiani et al., 2002). The G1 domain is related to link protein and to the other G1 domains of the hyalectans, both in terms of structural domains and subdomains (Kiani et al., 2002). The G1/hyaluronan/ link protein ternary complex is very stable thereby immobilizing the aggrecan into enormous complexes that maintain a stable network and provide mechanical properties to cartilage. An interglobular region, between G1 and G2, has a rod-like structure and harbors several protease-sensitive sites involved in the partial degradation of aggrecan in arthritis and other inflammatory diseases.

Following the G2 domain is a relatively small region containing numerous KS chains. This domain is not well conserved and its size significantly varies among species. Next, is the largest domain of aggrecan, which contains the GAG-binding region. This protein domain is encoded by a single, very large (~4 kb) exon with ~120 Ser-Gly dipeptide repeats, which can generate >100 covalently linked CS chains. The concentration of negatively charged forces within aggrecan accounts for its ability to hold large amount of water, not only in cartilage, but also in the intervertebral disc and brain. Moreover, electrostatic repulsion forces generated by the numerous

negatively- charged CS and KS chains of aggrecan provide the equilibrium compressive modulus (a measure of stiffness) of cartilage.

The G3 module of aggrecan contains 2 EGF-like repeats, a C-type lectin domain and a complement regulatory protein (CRP) domain. Notably, the EGF repeats can be alternatively spliced in part because in rodents exon 13 is a pseudoexon. Moreover, in rodent brain, the most common aggrecan species lacks both EGF repeats (Morawski et al., 2012). As in the case of other hyalactans, the C-type lectin domain of aggrecan binds simple sugars, such as fucose and galactose, in a  $\text{Ca}^{2+}$ -dependent manner. Thus, aggrecan G3 may serve as a binding domain for the galactose present on collagen type II or other extracellular matrix or cell surface constituents. Moreover, the G3 domain of aggrecan interacts with tenascins, fibulins and sulfated glycolipids (Aspberg et al., 1997). Thus, aggrecan could bridge and interconnect various constituents of the cell surface and extracellular matrix via its C-terminal G3 domain (**Figure 21**).



**Figure 21: Structure of proteoglycan aggregates.**

The proteoglycan aggregate is depicted as a central HA filament with aggrecan and link proteins (LP) attached to it. The domains of the aggrecan core protein are indicated. G1, G2 and G3, globular regions; IGD, interglobular domain; KS CS1 and CS2. Adapted from (Roughley and Mort, 2014b).

### **Aggrecan and OA**

Aggrecan loss associated with OA has been attributed to the action of aggrecanases ADAMTS-4 and ADAMTS-5 (as described in chapter 2 already), which have the highest specific activity for aggrecan cleavage in vitro, both are expressed in human OA cartilage, and the expression of both is localized in areas of aggrecan depletion (Tortorella et al., 2002).

Recent published studies have shown that aggrecan in cultured explants from mice lacking the

ADAMTS-5 catalytic domain is resistant to breakdown induced by catabolic cytokines (Glasson et al., 2005b; Stanton et al., 2005). Furthermore, the aggrecan and cartilage were preserved in situ in mice from both OA and inflammatory arthritis models. In contrast, mice lacking the ADAMTS-4 catalytic domain showed normal susceptibility to cytokine-induced aggrecan cleavage in vitro; similarly, mice in the OA model showed normal aggrecan and cartilage loss (Glasson et al., 2004). Ablation of the gene encoding ADAMTS-1, another member of the ADAMTS family that is expressed in cartilage but has significantly weaker aggrecanase activity compared with that of ADAMTS-4 and -5, had no effect on the preservation of aggrecan in vitro or in vivo (Little et al., 2005). This information highlights at least two facts. First, the probable role of other enzymes involved in aggrecan breakdown and second, the probability of the role of other PGs involved in pathophysiology of OA such as HSPGs. These PGs will be explained in detail in further paragraphs.

## 1.2. Agrin

Agrin is an HSPG that was initially identified as a crucial regulator of differentiation on the Neuromuscular Junction (NMJ). Neuronal agrin (splice variant: y4, z8) expressed by presynaptic motor neurons bind to low-density Lipoprotein Receptor-related Protein 4 (LRP4) expressed by skeletal muscle in a complex with the Muscle-Specific Kinase (MuSK) and Amyloid Precursor Protein family members APP and APLP2 resulting in MuSK activation (Eldridge et al., 2016a). This triggers the formation of post-synaptic structures via the induction of the aggregation of Acetylcholine Receptors (AChRs), ensuring neuromuscular synapse formation and maintenance (Choi et al., 2013; Gesemann et al., 1995; Zhang et al., 2008).

Agrin protein is not confined to the NMJ but is expressed in several tissues including muscle (Lieth et al., 1992), kidney (Groven et al. 1998), brain (O'Connor et al., 1994)), immune system (Khan et al., 2001) and cartilage (Eldridge et al., 2016b). In other tissues, such as lung, agrin (splice variant: y0, z0) plays a role in mechanotransduction by linking the cell cytoskeleton to other basement membrane components including  $\alpha$ -Dystroglycan (DAG1) and Laminin- $\gamma$ 1 (LAMC1) through either direct binding or indirectly through integrins.

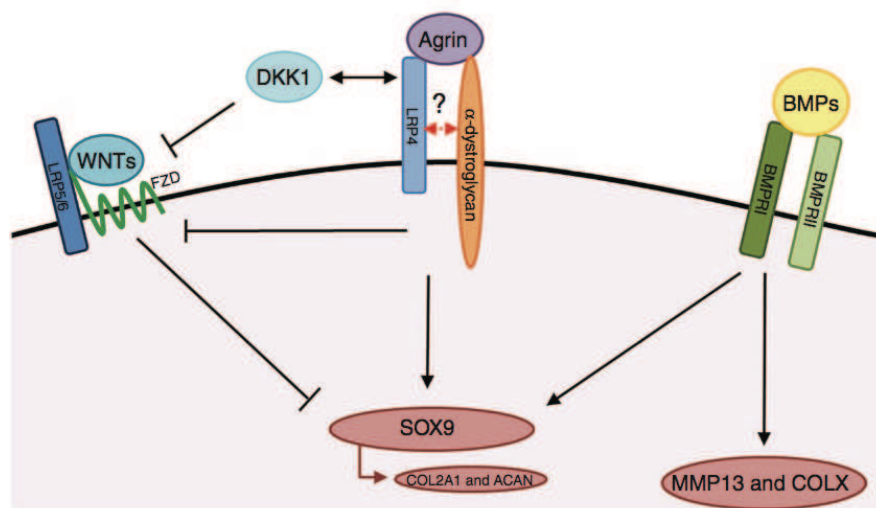
### **Agrin and OA**

Agrin plays a critical role in skeletal growth and endochondral bone formation (Eldridge et al., 2016c). As agrin deficient mice died at birth due to respiratory failure, their early postnatal development was analyzed in animals in which perinatal death was prevented by specific transgenic expression of neural agrin in motor neurons. These transgenic mice exhibit severe postnatal growth retardation due to compositional and structural alterations of cartilage formation (Eldridge et al., 2016c). A compressed growth plate region is observed, with changes most prominent in the hypertrophic zone, not caused by reduced viability of hypertrophic chondrocytes but by a decrease of proliferation and an impaired hypertrophic differentiation of chondrocytes. These changes in the hypertrophic zone are more pronounced with age, suggesting that late but



not early steps on bone morphogenesis are agrin dependent. Moreover, agrin was demonstrated to be highly expressed by chondrocytes and deposited in the growth plate of cartilage, in the zone of proliferating and prehypertrophic chondrocytes

Agrin strongly promotes chondrocyte differentiation and cartilage formation *in vivo*, as a novel potent anabolic growth factor (Eldridge et al., 2016a). Agrin expression was detected in normal cartilage but was progressively lost in OA, as demonstrated in human osteoarthritic cartilage and following destabilisation of the medial meniscus in different mice models (Dell'accio et al., 2008; Glasson et al., 2007). *In vitro*, agrin knockdown affect chondrocyte differentiation through downregulation of the cartilage transcription factor SOX9. Conversely, exogenous agrin expression supported cartilage differentiation in three-dimensional cultures *in vitro* and in ectopic cartilage formation assays in nude mice *in vivo*. In the context of cartilage differentiation, agrin used an unusual receptor repertoire requiring both LRP4 and  $\alpha$ -dystroglycan. The muscle-specific kinase MuSK, necessary to mediate the agrin's postsynapse inducing activity at the NMJ, was expressed also by chondrocytes but if agrin could exerts its effect via MuSK activation is not demonstrated (Figure 22).



**Figure 22: Agrin and LRP4 are central to a chondrogenic signalling network.**

Agrin is a paracrine signal that supports chondrocyte differentiation through a pathway requiring both LRP4 and  $\alpha$ -dystroglycan which upregulates SOX9 and its transcriptional targets COL2A1 and ACAN. Agrin-induced chondrocyte differentiation is not associated with chondrocyte hypertrophy. LRP4 interacts with other pathways regulating chondrocyte differentiation including WNTs and BMPs. Adapted from (Eldridge et al., 2016a).

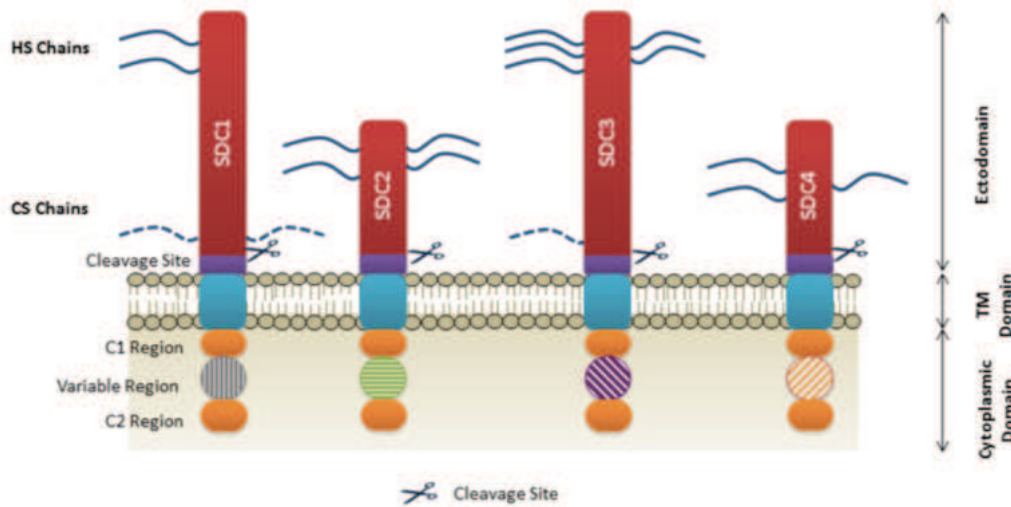
### 1.3. Syndecans

Chondrocytes also express cell surface proteoglycans, like syndecans (SDCs). The syndecans may carry two or more HS chains, alone or in combination with CS, allowing interactions with a variety of ligands including FGF, VEGF, PDGF, BMP-2 and Ihh (Tkachenko et al., 2005). These interactions contribute to the presentation of these factors to their corresponding receptor facilitating the signal transduction (Pap and Bertrand, 2013). For example, the presence of HS is

vital for the ability of FGF-2 to bind FGFR-1, allowing the stability of the ligand–receptor complex. Syndecans can also promote the formation of morphogen gradients throughout the matrix and restrict their diffusion along the surface of receiving cells, thereby preventing the loss of these factors to the extracellular space (Pap and Bertrand, 2013). Inversely, these interactions may also lead to sequestration of the soluble factor, inhibiting its activity.

The mechanisms that initiate signalling upon binding of ligands to the GAG chains of syndecans are not fully understood. However, it has been suggested that the dimerization of syndecans is fundamental to signal transduction (Pap and Bertrand, 2013).

Each syndecan family member has specific spatial and temporal patterns of expression. In vivo studies revealed that each member is expressed at different times during development and on specific cell types (Kim et al., 1994). SDC1 is predominantly expressed early on during development and is present on epithelial and mesenchymal tissues; relevant to cartilage, SDC1 expression is modulated in OA (Salminen-Mankonen et al., 2005) and is shown to promote activation of ADAMTS-4 (Gao et al., 2004). SDC2 is expressed by cells of mesenchymal origin as well as neuronal and epithelial cells. Whereas, SDC3 is most commonly linked with neuronal and musculoskeletal tissues and is shown to be involved in the process of endochondral differentiation (Shimazu et al., 1996), whilst SDC4 is found virtually within every cell type (Kim et al., 1994). Structurally, all members are composed of a short cytoplasmic domain containing two highly conserved regions (C1 and C2) that flank the variable region, followed by a Transmembrane (TM) domain and an ectodomain (**Figure 23**) (Iozzo and Schaefer, 2015b). Syndecans exhibit a modest core protein of approximately 20–45 kDa, with a short cytoplasmic domain lacking intrinsic kinase activity. This being said, syndecans are involved in cytoplasmic signalling. In regards to protein sequence homology, SDC1 and SDC3 form one subfamily, and SDC2 and SDC4 form the other (Oh and Couchman, 2004). SDC1 and SDC3 have the ability to bind HS and CS side chains, whereas SDC2 and SDC4 are thought to exclusively bind HS chains (**Figure 23**).



**Figure 23: The core-domain structure of syndecans.**

The extracellular domain is depicted in red and both heparan and chondroitin sulphate chains are indicated by the blue lines. Cleavage sites are indicated by purple blocks, followed by a short transmembrane domain in blue. Each syndecan has a short cytoplasmic domain in which a variable region is flanked by two highly conserved regions termed C1 and C2. Cleavage of the ectodomain can occur via numerous metalloproteinases such as MMP2, 3, 7 and 9, as well as proteases thrombin and plasmin. Adopted from (Binch et al., 2016a).

## Syndecans and OA

The expression of different syndecans during skeletal development and remodelling, together with the normal to mildly affected skeletal phenotypes of single syndecan gene knockout mice, suggest that, under normal conditions, syndecan family members constitute modulators of cellular functions that can be compensated for by other syndecans or by other, as yet poorly characterized, mechanisms (Pap and Bertrand, 2013). However, several studies provided some evidence of a specific role of each syndecan in developmental and pathological cartilage modifications.

SDC1 binds to various extracellular-matrix proteins, including types I, III and V collagens, fibronectin, thrombospondin and tenascin. It also binds to bFGF. Barre et al., provided evidence that both apparently intact and heavily damaged areas of OA cartilage, as well as their respective isolated chondrocytes expressed syndecan-1. The mRNA level of SDC1 was found to be lower in cartilage extracts from altered areas than in intact parts of the tissue. Inversely, SDC1 appeared up regulated in chondrocytes derived from heavily damaged samples compared to cells from apparently intact areas. To explain these conflicting results, Barre et al. suggested that the differences observed in this in-vitro system between chondrocytes from each area might only reflect the fact that they react differently to the adhesion on plastic dishes (Barre et al., 2000).

In mice developing early-onset OA related to a deletion mutation in the type II collagen gene, a transient upregulation of SDC1 mRNA synthesis was observed at the age of 6 months coinciding with early stages of cartilage degeneration and a period of attempted repair. Immunostaining for SDC1 was most intense in chondrocytes of superficial and intermediate zones of articular cartilage adjacent to defect areas (Salminen-Mankonen et al., 2005).

Some studies in human chondrocytes have suggested that SDC1 was closely associated with activation of ADAMTS-4, which is a major contributor to the pathogenesis of OA. Recently, Kong et al. confirmed this hypothesis showing that the synthetic cannabinoid WIN-55, 212-2 mesylate (WIN-55), known as a chondroprotective molecule, inhibits ADAMTS-4 activity in OA chondrocytes by decreasing the mRNA stability/expression of SDC1 (Kong et al., 2016).

No specific data are available concerning the role of SDC2 in normal and pathological cartilage. In tumour metastases, suppression of MMP-2 activity was shown to depend directly on the HS chains of SYND2. Indeed, HS removal (by heparitinase-I) from cells with low metastatic potential promoted MMP-2 activation, whereas MMP-2 activation was suppressed by transfection into highly metastatic cells of wild-type SYND2, but not of mutant SYND2 that lacked GAG-attachment sites. How SYND2-dependent MMP-2 activation affects cartilage degradation, however, has not yet been investigated, but similar mechanisms might also have a role in this context (Pap and Bertrand, 2013).

SDC3 is one of the major HSPGs expressed during the progression of limb cartilage differentiation. Shimazu et al. found that proliferating immature cartilage from chick embryo tibia and sternum contained significant amounts of syndecan-3 mRNA, whereas mature hypertrophic cartilage contained markedly lower transcript levels. Treatment with FGF-2 for 24 h stimulated both syndecan-3 gene expression and chondrocyte proliferation, which was counteracted by cotreatment with heparinase I or III. These results indicate that SDC3 represents a regulator of the proliferative phase of chondrocytes during endochondral ossification (Shimazu et al., 1996). Seghatoleslami et al. demonstrated that SDC3 antibodies inhibited chondrogenic differentiation and cartilage-specific gene expression in high-density micromass cultures of limb mesenchymal cells, confirming that SDC3 plays an important role in regulating the onset of limb chondrogenesis (Seghatoleslami and Kosher, 1996). The effects of SDC3 in chondrocyte proliferation seem to be linked to Ihh (Shimo et al., 2004). Inversely, Fisher et al. showed that overexpression of SDC3 dramatically impairs the ability of BMP2 to promote cartilage differentiation, possibly by restriction of its interaction with its receptors (Fisher et al., 2007).

On the other hand, SDC3 has been detected in approximately 80% of chondrocytes in the upper zone of severely affected osteoarthritic cartilage, as compared with only 20% of normal chondrocytes (Fisher et al., 2007). In line with the proposed role of SDC3 in early limb formation and chondrocyte differentiation, it has been suggested that this increased expression of syndecan 3 reflects the proliferative response of the chondrocytes (Pap and Bertrand, 2013).

Of the members of the syndecan family, SDC4 is the best characterised in terms of structure and biological activity. During embryonic endochondral ossification, SDC4 promoter activity is specifically induced in chondrocytes at all phases of differentiation. However, its deletion is associated with only minor changes in bone development, including reduced proliferation and decreased turnover in the uncalcified cartilage of the epiphysis, but this did not lead to a growth phenotype at birth (Bertrand et al., 2013). SDC4 encompasses a protein kinase C (PKC)- $\alpha$ -binding motif within its variable region. This feature is of importance because PKC- $\alpha$ -dependent signaling is particularly active during chondrogenesis, and the selective inhibition or down-regulation of PKC- $\alpha$  blocks differentiation of mesenchymal cells to chondrocytes. It was also demonstrated that the HS side chains of SDC4 were involved in controlling the expression of another critical factor, SOX9 that is involved in the regulation of aggrecan and collagen II expression (Binch et al., 2016b). This indicates that structural changes in HS chains during OA could probably alter its interaction with SOX9 and therefore, the regulation of aggrecan and collagen II expression.

Echtermeyer et al. investigated the expression of SDC4 and its regulation of ADAMTS-5 in OA cartilage. They note a strong upregulation of SDC4 in human and rodent OA cartilage. Analysis of osteoarthritic changes in mice revealed a correlation between collagen type X and SDC4 staining in dedifferentiated hypertrophic chondrocytes. SDC4 null mice were protected from development of post-traumatic OA and maintain a healthy level of cartilage ECM and tissue integrity. Similarly, injection of SDC4 –specific antibodies into the knee joint of wild-type mice with surgically induced osteoarthritis protected from typical osteoarthritic changes. Importantly these authors report a concomitant decrease in ADAMTS-5 activity in SDC4 knockout mice, leading to the exploration of molecular mechanisms that could control this phenomenon. These studies identified that SDC4 impacts ADAMTS-5 activation by direct binding and through MMP-3 via the regulation of MAPKinase ERK1/2 phosphorylation (Echtermeyer et al., 2009b).

Oh et al. published conflicting results related to the expression of SDC4 depending on the OA status. Indeed, they showed no significant differences of the expression of SDC4 between medial femoral cartilage with severe degenerative changes and lateral femoral cartilage that appeared grossly normal. On the other hand, they observed a significant positive correlation between expression levels of SDC4 and VEGF in cartilage explant suggesting a close relationship between them (Oh and Couchman, 2004).

Altogether, the data above suggest that syndecans are important modulators of cellular function that become particularly relevant under stress conditions. Inflammation is certainly one such condition and, therefore, it is not surprising that interest has grown in the question of how syndecans are regulated under inflammatory conditions, and how syndecans, in turn, regulate the inflammatory response. In fact, some evidence exists that different inflammatory stimuli modulate the cell-surface expression of syndecans. In particular, SDC4 has been shown to be induced by inflammatory stimuli such as TNF and IL-1 in various cellular systems (Pap and Bertrand, 2013). Similarly, inflammatory cytokines have been shown to induce the expression of

SDC2 on different cells. Data for inflammatory regulation of other syndecans are more complex; for instance, inflammatory cytokines such as TNF have been shown to reduce levels of syndecan 1 both by decreasing mRNA levels and by promoting its shedding from the cell surface (Pap and Bertrand, 2013).

Data from studies in the human TNF transgenic (hTNFtg) mouse model of Rheumatoid Arthritis published in 2012 suggest that SDC4 contributes to the severity of destructive inflammatory arthritis: hTNFtg mice deficient in SDC4 exhibited significantly reduced cartilage destruction as compared with hTNFtg mice carrying SDC4. Investigations of synovial fibroblasts from these animals revealed that loss of SDC4 impairs the ability of these cells to attach to and to invade cartilage. These observations certainly require further studies but might suggest that inhibition of SYND4 could indeed be a worthwhile principle to treat certain inflammatory disorders, particularly inflammatory joint destruction.

#### 1.4. Glypicans

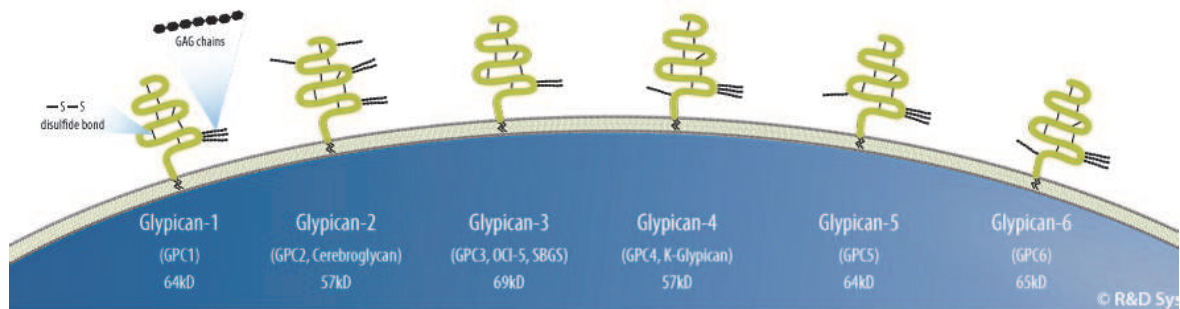
Glypicans is a cell surface HSPGs, and is linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) linkage. The six members of the glypican (GPC1-6) (**Figure 24**) are characterized by a cysteine-rich globular ectodomain carrying 2–3 HS chains, and a GPI moiety anchored to the plasma membrane (Filmus, 2001). No release of GPI has been documented, but its catabolism has been described to be mediated by endocytosis driven lysosomal degradation (Yanagishita and Hascall, 1992). The biological importance of GPC in BM niche has been suggested by several data.

For instance GPC-3 deficient mice are characterized by altered hematopoiesis, decreased osteoclast differentiation and a delay in endochondral ossification. This delay is associated to a marked reduction in calcified trabecular bone and an abnormal persistence of hypertrophic chondrocytes (Viviano et al., 2005). Interestingly, GPC-3 expression is associated to RUNX2 expression and differentiation (Haupt et al., 2009). Thereafter, RUNX2 enhances expression of genes related to proteoglycan-mediated signaling, including FGFRs and HSPGs (SDC-1, SDC-2, SDC-3, GPC-1) (Teplyuk et al., 2009).

Sisson et al demonstrate that GPC-4 activity is required cell autonomously in the chondrocytes and suggests a novel mechanism for Wnt5b and GPC-4 regulation of chondrocyte behavior that differs from their collaborative action of controlling cell movements during gastrulation (Sisson et al., 2015).

#### Glypicans and OA

GPC may also interact with other ligands involved in cartilage differentiation, particularly Ihh (Capurro et al., 2017). Ihh is a master regulator of bone development, coordinating chondrocyte proliferation, chondrocyte differentiation and osteoblast differentiation (Kronenberg and Chung, 2001).

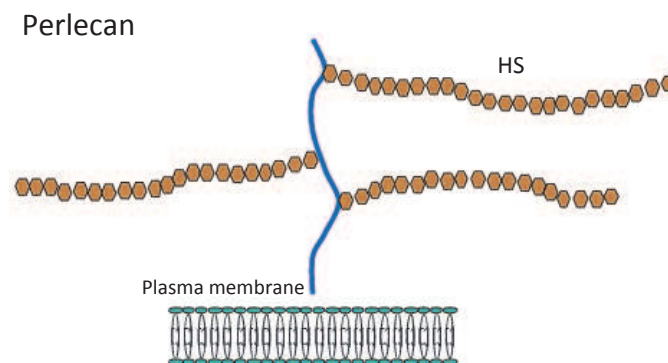


**Figure 24: The six members of the vertebrate glypican family share a characteristic structure.**

Glypicans are anchored to the cell surface via a GPI linkage, have a conserved pattern of 14 cysteine residues, which contribute to intramolecular disulfide linkages, and display GAG attachment sites predominantly near the membrane. Alternative names and molecular weight of the core proteins are also indicated. After (Rosenberg et al., 1997).

## 1.5. Perlecan

Perlecan (**Figure 25**) like glypican and syndecan is one of the tree cell surface HSPGs with a large core protein of 467 kDa, is the more abundant HSPG during initial step of skeletal formation (Arikawa-Hirasawa et al., 1999). It has five major protein modules. The N-terminal domain contains the GAG attachment sites. Domain II is homologous to the LDL receptor, domain III exhibits homology with laminin, and domain IV contains 21 immunoglobulin repeats resembling N-CAM. The C-terminal domain V contains EGF-like motifs and two LRE tripeptides that could mediate laminin binding.



**Figure 25: Structure of perlecan.**

As perlecan is the major PG in basement membranes it was surprising when it was demonstrated in cartilage (Handler et al., 1997; SundarRaj et al., 1995), a tissue organized without a basement membrane. Perlecan can undergo self-aggregation, and can interact with laminin, nidogen and fibronectin. Integrins have been proposed to function as cell surface receptors for perlecan. Perlecan in adult articular cartilage is enriched in the pericellular matrix and is a CS/HS hybrid PG (SundarRaj et al., 1995). Perlecan is found in cartilage after the expression of type II collagen

and aggrecan. Perlecan coated onto dishes promoted chondrogenesis and maintains chondrogenic differentiation, perhaps working in concert with SOX9 (Handler et al., 1997).

### **Perlecan and OA**

As perlecan is the major PG in basement membranes it was surprising when it was demonstrated in cartilage (Handler et al., 1997; SundarRaj et al., 1995), a tissue organized without a basement membrane. Perlecan can undergo self-aggregation, and can interact with laminin, nidogen and fibronectin. Integrins have been proposed to function as cell surface receptors for perlecan. Perlecan in adult articular cartilage is enriched in the pericellular matrix and is a CS/HS hybrid PG (SundarRaj et al., 1995). Perlecan is found in cartilage after the expression of type II collagen and aggrecan. Perlecan coated onto dishes promoted chondrogenesis and maintains chondrogenic differentiation, perhaps working in concert with SOX9 (Handler et al., 1997).

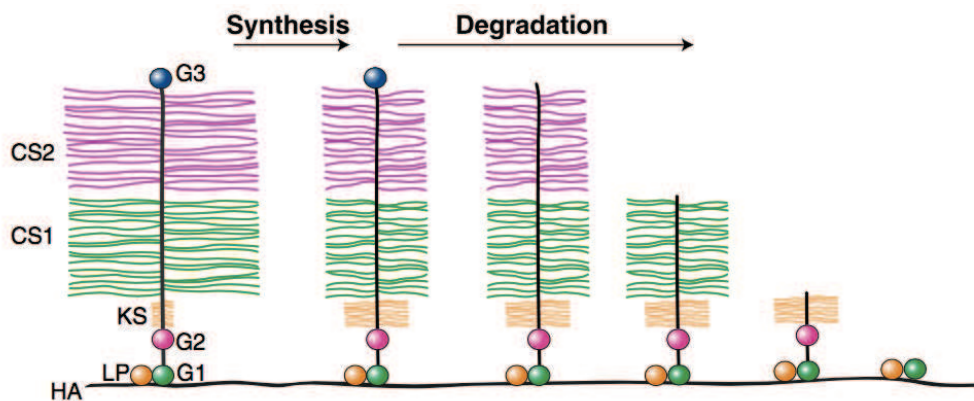
The perlecan knock-out mouse (Arikawa-Hirasawa et al., 1999; Costell et al., 1999) exhibits skeletal abnormalities, defective endochondral ossification, but no early defects in the formation of cartilage anlagen, so in vivo the early events of condensation and chondrocyte differentiation are apparently unaffected by the perlecan deficiency. However, in the knockout animals, there is disorganization of the growth plate, including a disruption of the chondrocyte columnar organization and a reduction of the fibrillar collagen network suggesting the role of perlecan in matrix structure. It has also been suggested that perlecan may influence chondrocyte metabolism via signaling receptors or by modulating the expression of FGFs (Handler et al., 1997).

Moreover soluble perlecan enhances VEGF-165 activity and receptor phosphorylation in human BM endothelial cells in vitro (Muthusamy et al., 2010). According to these properties, perlecan take part of bioengineering strategies aiming to respond to large manufacturing and clinical needs to expand MSC whilst retaining their multipotency (Decarlo et al., 2012; Muthusamy et al., 2010; Nakamura et al., 2014; Nigro et al., 2010).

## **2. Characterization of CS chains during aging and in OA cartilage**

The structure of aggrecan does not remain stable during life, but undergoes extensive changes due to variations in synthesis both within the chondrocytes and the ECM. The cellular changes due to synthesis take place during fetal life and postnatal growth. They affect mainly the length and sulfation pattern of the CS and KS chains on the aggrecan core protein (Brown et al., 1998; Plaas et al., 1997; Roughley and White, 1980a; Roughley et al., 1987). With increasing age, the CS chains become shorter in length. The major change in sulfation pattern affects CS. In the human, the degree of sulfation increases throughout fetal life, and by birth 4 and 6- sulfation of the N-acetyl galactosamine residues occurs in equal abundance. Following birth, the sulfation pattern slowly changes to predominantly 6-sulfation (**Figure 26**).





**Figure 26: Age-related changes in aggrecan structure.**

Changes in aggrecan structure due to synthesis and degradation are depicted. Changes due to synthesis result in an increase in chondroitin sulfate length and a decrease in keratan sulfate length. Changes due to degradation result in truncation of the aggrecan core protein by removal of the G3 region, cleavage within the GAG-attachment region, and cleavage between the G1 and G2 regions. Adapted from (Roughley and Mort, 2014c).

## 2.1. OA Treatment by CS

The ability of CS to slow down the development of OA has been demonstrated in several clinical trials with significant positive results (Michel et al., 2005; Volpi, 2007). On biochemical basis, the effect of CS in patients with OA is due to reactions involved in its anti-inflammatory activity, stimulation of the synthesis of PGs and HA, and the decrease in catabolic activity of chondrocytes inhibiting the synthesis of proteolytic enzymes, nitric oxide, and other substances that contribute to damage cartilage matrix and cause death of articular chondrocytes (Monfort et al., 2008). All these effects were demonstrated on in vitro studies and in animals in vivo models described in the 2 following chapters.

## 2.2. In vitro effect of CS on OA

In various models of cartilage culture or of isolated chondrocytes, CS has demonstrated the capacity to stimulate the synthesis of PG, aggrecanases, and HA at a high molecular weight. In cultures of human OA chondrocytes, CS inhibits collagenolytic activity and the synthesis of MMP-3, and it counteracts the negative effects of IL-1 $\beta$  on PG, collagen type 2, and PGE2 synthesis. CS is also able to prevent the apoptosis of chondrocytes induced in vitro by NO. Additionally, CS interacts with elastase of human leukocytes and that it determines a partial inhibition of activity (elastase is a potent mediator of cartilage degradation). The effects of CS on various mediators of inflammation and the degradation of cartilage can probably be explained on the basis of its capacity to reduce the nuclear translocation of transcription factor NF- $\kappa$ B induced by IL-1 $\beta$  (Chan et al., 2005a).

On the other hand, protection of the joint structure may be explained by the fact that in chondrocytes, CS diminishes IL-1 $\beta$ -mediated increase in MMP-2, MMP-3, MMP-9, MMP-13 and MMP-14 (Orth et al., 2002). Moreover, it has been documented that hyaluronan and mixtures of low concentrations of CS and glucosamine are able to prevent the release of MMP-3 and MMP-13 triggered by fibronectin fragments (Homandberg et al., 2004, 2006).

In chondrocytes, CS diminishes ERK1/2 phosphorylation and abrogates the phosphorylation of p38MAPK induced by IL-1 $\beta$ . Also chondroitin disaccharides sulfated at positions 4 and/or 6, (Ddi-4S, Ddi-6S and Ddi-4, 6S) reduce IL-1 $\beta$ -induced NF- $\kappa$ B nuclear translocation to a similar extent as CS (Jomphe et al., 2008a). On the other hand, CS decreases nitroprusside-induced apoptosis of the chondrocytes probably by preventing p38MAPK activation (Jomphe et al., 2008a). Use of glucosamine is based on studies performed on animal models and in vitro studies that showed that the joint metabolism became normalized during the healing of chondral lesions, along with slight anti-inflammatory action (Bassler et al., 1998a; McCarty, 1994). CS and glucosamine sulfate exert beneficial effects on the metabolism of in vitro models of cells derived from synovial joints (Henrotin et al., 2014c).

Bassler et al, demonstrated the treatment of human articular cartilage **chondrocytes** with glucosamine sulfate (GS) in vitro induces a significant increase of PG amounts measured in clusters and in culture media. In that work, GS did not affect cell proliferation nor coll II production by human chondrocytes cultivated in clusters. Their results showed that GS did not change the level of production of type II procollagen in another type of culture (Bassler et al., 1998a).

Recently, Wang et al developed a 3D hydrogel platform with varying biochemical and mechanical properties using methacrylated sGAG molecules, HS and CS, as biochemical cues. This enables a direct comparison of the effects of HS and CS doses on 3D MSC chondrogenesis, as well as elucidating how matrix stiffness further influences stem cell responses to ECM cues. Their results suggest that CS is promising in enhancing MSC chondrogenesis, especially in soft hydrogels (~7.5 kPa). Moreover, they demonstrated that CS-containing hydrogels at low mechanical stiffness can provide a promising scaffold for enhancing MSC-based cartilage tissue regeneration (Wang and Yang, 2017). **Table 7** summarizes some examples of in vitro experiments, which used CS to control OA.

In vitro experiments provide additional information regarding the potencies of CS to affect **synovial inflammation** in OA. Jin et al, demonstrated that CS inhibits the TLR-mediated IL-6 secretion by macrophage-like cells (Jin et al., 2011). This effect was shown to be dependent on CS structure, i.e., sulfation sites and size. Finally, CS was able to affect the metabolism of synovial fibroblasts by the regulation of hyaluronate synthase (David-Raoudi et al., 2009), the suppression of ADAMTS-4 and -5, and the recovery of TIMP-1 expression (Imada et al., 2010).

In synovial membrane CS inhibits some inflammatory markers of synovitis, including cell

infiltration, fibrosis and proliferation of the synovial lining cells (Monfort et al., 2005). As CS is able to modulate the function of synovial fibroblasts as well as that of chondrocytes, therefore, it is promising a multifunctional chondroprotective material for OA.

In the subchondral bone, resorption and bone formation is tightly regulated by a molecular triad composed of osteoprotegerin (OPG)/receptor activator of NF- $\kappa$ B (RANK)/RANK ligand (RANKL). The RANKL (localized on osteoblasts) and its receptor RANK (localized on osteoclasts), enhances osteoclastogenesis, whereas OPG (produced by osteoblasts) inhibits this osteoclastogenesis by binding to RANKL. The ratio of OPG to RANKL pay a key role in regulating bone metabolism: a high ratio promotes bone formation, while a low ratio favors bone resorption. CS or combination of CS and glucosamine controls over it and decreases the resorption activity (Tat et al., 2007).

Chondroitin Sulfate	System and duration	Result	Reference
10-400 mg/ml	Monolayer synovial cells	Stimulation by 11% of hyaluronate synthesis during the growth phase Increase by 88% during the stationary phase	(Verbruggen and Veys, 1977)
Bovine 100-1000 mg/ml	Human articular chondrocytes in clusters stimulated with IL-1 $\beta$ Up to 32 days	Significant reduction of IL-1 $\beta$ -stimulated production of PGE <sub>2</sub> Increase of PG and type II collagen production	(Bassleer et al., 1998b)
C poly S 10 mg/ml	Human articular chondrocytes in agarose gel stimulated with IL-1 $\beta$ 1 week	Stimulation of aggrecan, hyaluronan and type II collagen due to MMPs downregulation	(Wang et al., 2002)
CS-4 1.0 and 2.0 mg/ml	Human skin fibroblast	Anti-oxidant action of CS-4 by increased cell viability and inhibition of DNA fragmentation	(Campo et al., 2004)
20 mg/ml	Bovine articular cartilage explants stimulated with IL-1 $\beta$ 24 to 48h	Reduction of IL-1 $\beta$ -induced MMP-13	(Chan et al., 2005b)
20 mg/ml	Bovine articular cartilage explants stimulated with IL-1 $\beta$ 24 to 48h	Suppression of IL-1 $\beta$ -induced NO synthase, COX-2 and mPGES-1 gene expression	(Chan et al., 2005c)
10 1000 mg/ml	Human OA chondrocytes stimulated with IL-1 $\beta$ 48h	Inhibition of MMP-3	(Monfort et al., 2005)

25 mg/ml	Human articular chondrocytes stimulated with LPS Up to 72h	Inhibition of MMP-13 by the reduction of p38 and Erk1/2 activation	(Holzmann et al., 2006)
200 mg/ml	Human OA subchondral bone osteoblasts Up to 48h	Reduction of proresorptive agents Reversion of vitamin D3 effect on OPG:RANKL ratio	(Tat et al., 2007)
Chondrosulf or porcine 10 or 100 mg/ml	Rabbit articular chondrocytes stimulated with IL-1b 20 h	Increase PG synthesis Inhibition of ADAMTS-5 expression	(Tahiri et al., 2008)
Purified bovine CS-4 and CS-6 200 mg/ml	Rabbit chondrocytes stimulated with IL-1b 48h	Reduction of NF-kB nuclear translocation  Prevention of Erk1/2 and p38 activation Reduction of nitroprusside-induced apoptosis	(Jomphe et al., 2008b)
Avian CS 1 100 mg/ml	Bovine articular chondrocytes in hypoxic alginate beads 24 48 h	Decrease of IL-1b-induced MMP-1, -3, -13 and ADAMTS-4 and -5 Reduction of IL-1b-induced COX-2 and iNOS Reduction of PGE <sub>2</sub> production but not NO Slight increase of GAG synthesis Counteract IL-1b suppression of TGFb receptors	(Legendre et al., 2008)
Mix of CS A, CS B and CS C 10 50 mg/ml	Bovine cartilage explants 24h	Effective at joint lubricant Diffusion into cartilage	(Katta et al., 2009)
Avian CS 100 mg/ml	Human OA fibroblast-like syn- viocytes 48 h with pretreatment	Regulation of hyaluronan synthase	(David-Raoudi et al., 2009)
Bovine CS-4 and shark CS-6 0.5 and 1.0 mg/ml	Mouse articular chondrocytes stimulated with LPS 24h	Anti-apoptotic effect of both CS Inhibition of inflammatory mediators, iNOS and MMPs more evident with CS-4 than with CS-6	(Campo et al., 2009a)
Bovine CS-4 and shark CS-6 25 and 50 mg/ml	Mouse articular chondrocytes stimulated with LPS 24h	Inhibition of inflammatory cytokines (MyD88 and TRAF-6), inhibition of activation of NF-kB and iNOS by both CS Effect through TLR4 (confirmed with specific antibody experiment)	(Campo et al., 2009b)

CS-6 and CS-4 from shark origin 10-100 mg/ml	Bovine cartilage explants 4 weeks	Reduction of GAG content	(Bian et al., 2009)
Shark (mainly CS-6) and porcine (mainly CS-4) 1 100 mg/ml	Human articular chondrocytes in alginate beads and synovial fibroblast stimulated with IL-1b 6 or 4 days	Different effect of both CS  Suppression of IL-1b-induced ADAMTS-4 and -5 in both cell types Suppression of IL-1b-induced MMP- 13 and increase expression of aggrecan in chondrocytes Recovery of TIMP-3 in chondrocytes and TIMP-1 in synoviocytes by por- cine CS	(Imada et al., 2010)
CsA and CsC, Between 0 to 120 µg/ml	Macrophage-like cells	CS inhibited the TLR-mediated IL-6 secretion by macrophage-like cells	(Jin et al., 2011b)
Cs (0–10% w/v) % of weight of solution in the total volume	MSCs stimulated with Cs	CS is good in enhancing MSC chondrogenesis, especially in soft hydrogels	(Wang and Yang, 2017)

**Table 7: In vitro effects of chondroitin sulfate.**

After (Henrotin et al., 2010b).

### 2.3. In vivo effect of CS

Some studies have shown that CS reduces pain and improves articular function, prevents joint space narrowing of the knee, (Uebelhart et al., 1998, 2004) reduces joint swelling and effusion (Clegg et al., 2006). Pain decreasing and swelling may be explained by an anti-inflammatory effect of CS, probably through diverse mechanisms such as diminishing the expression of Phospholipase A2 (PLA2) (Ronca et al., 1998) of Cyclooxygenase-2 (COX-2), and the concentrations of prostaglandin E2 (PGE2) (Chan et al., 2005c). Moreover, in joints CS reduces the concentrations of pro-inflammatory cytokines, such as TNF $\alpha$  (Campo et al., 2003a) and IL-1 $\beta$  (Chou et al., 2005), and systemic and joint concentrations of NO (Orth et al., 2002) and Reactive Oxygen Species (ROS) (Campo et al., 2003b).

Promising results observed after in vitro administration of Cs, provoked more and more researchers to apply this GAG in different OA in vivo models. **Table 8** summarizes some examples of the in vivo experiments conducted in animal models of OA using Cs as a treatment.

Chondroitin Sulfate	Model	Result	Reference
80 mg/day oral or i.m.	Rabbit model of cartilage injury induced by chymopapain	Significant increase of PG production	(Verbruggen and Veys, 1977)
1000 mg/kg	Collagen-induced arthritis in mouse	Reduction of synovitis parameters: cell infiltration, fibrosis and proliferation of lining cells	(Omata et al., 2000)
1 ml/kg (i.p.) 25 mg/kg (orally)	Collagen-induced arthritis in rat	Treatment began after arthritis onset (day 10) Protection against oxidative stress Reduction of plasma levels of TNF $\alpha$ and myeloperoxidase	(Campo et al., 2003b)
1200 mg/kg	Collagen-induced arthritis in rat	Reduction of IL-6 levels	(Cho et al., 2004)
0.3 mg/day	Spontaneous OA in mouse 12 days	Significant reduction of apoptotic chondrocytes after 30 days	(Caraglia et al., 2005)
Dietary supplement (18 mg/g)	Freund's adjuvant-induced arthritis in rat	Prevention of MMP-9 increase Reduction of IL-1 $\beta$ in joint tissues but not in serum	(Chou et al., 2005)
CS-4 (bovine trachea) 30 120 mg/kg (i.p.)	Collagen-induced arthritis in mouse For 25 days	Inhibition of NF-kB activation Inhibition of MMP-13 Inhibition of caspases-3 and -7 activation	(Campo et al., 2008)
CS (300–900 mg kg <sup>-1</sup> )	Rat adjuvant artheritis	Reduced C-reactive protein, phagocytic activity and the intracellular oxidative burst of neutrophils	(Bauerova et al., 2011)
Cs (200 mg kg <sup>-1</sup> )	Guinea pigs spontaneously developing OA	Protected cartilage by inhibition of MMP-3 mRNA	(Taniguchi et al., 2012)

**Table 8: In vivo effects of chondroitin sulfate.**

After (Henrotin et al., 2010b).

## 2.4. CS as a biomarker of OA

As early as the year 1963, a decreased chondroitin sulfate was found in OA cartilage (Bollet et al., 1963; Roughley and White, 1980b).

On the other hand, the aggrecan fragments that diffuse into the synovial fluid have been used as biomarkers in OA patients, premise that a higher concentration of fragments in the synovial fluid reflects enhanced degradation within the cartilage (Roughley and Mort, 2014d). Another study showed that the ratio of CS isomers in synovial fluid in hip OA varies with the severity of disease (Yamada et al., 1999).

Furthermore, it has been shown that there is a significant negative correlation between levels of C6S and age. A significant inverse correlation was also observed between the ratio of C6S to C4S and age. Results of analysis of covariance in which age was covariate showed that the ratio of C6S to C4S in advanced and terminal stage OA was lower than that in early stage OA.

### 3. HS in OA

Unlike Cs, which has been highly studied and administered in OA treatment, much less is shown about HS. A simple bibliographic study on the role or/and application of Cs in OA brings up several hundreds of papers explaining the advantages and possibilities of this GAG in OA treatment. However, there is only very limited numbers of publications dedicated to the role of HS in OA, studying it as a biomarker or targeting it as a potential OA treatment.

Among few studies performed on the role of HS, they have highlighted the important role of HS in cartilage development during embryogenesis, mostly indirectly via serving as a binding reservoir for soluble factors (reviewed in Kirn-Safran et al., 2004; Pike et al., 2006; Rider, 2006).

Among those soluble factors, the interaction of TGF $\beta$  cytokine superfamily and HS has important roles in morphogenesis, organogenesis, tissue maintenance and wound healing. So far, around one-quarter of these cytokines have been shown to bind to heparin and HS. Well-established examples are the TGF $\beta$  isoforms 1 and 2, and the BMPs -2 and -4. Furthermore, researchers have been investigating the heparin/heparan sulphate-binding properties of BMP-7, which is a representative of a distinct BMP subgroup from that of BMPs -2 and -4. Interestingly, several of the various specific BMP antagonist proteins also bind to heparin and heparan sulphate. Much remains to be learnt about the nature and role of GAGs interactions in the TGF $\beta$  superfamily, but previous works performed on different tissues suggest that these cytokines do not share a single highly conserved heparin/heparan sulphate-binding site (Rider, 2006).

Different studies suggest that HS plays an important role in hemostasis of normal cells specially cartilage. In a recent study, Chen et al, used an in vitro human MSC chondrogenic differentiation model to study the role of exogenous HS on TGF $\beta$ 3-induced chondrogenesis and TGF $\beta$ /Smad signaling. Their results suggest that exogenous HS clearly potentiates TGF $\beta$ 3-induced chondrogenic differentiation of hMSCs by modulating the expression mode of TGF $\beta$  receptors and by activating the downstream Smad signaling pathway (Chen et al., 2016).

Moreover, HS has been reported to have inhibitory effects on aggrecan degradation in bovine synovium explants cultures (Vankemmelbeke et al., 2001), and may thus have some potential toward cartilage treatments.

It has been previously reported that HS 6-O endosulfatases (Sulfs) were expressed in articular cartilage, and that the Sulf-1 knockout mouse developed severe knee OA. In a one of kind, recent study performed in 2017, Sulf-1 injection into the mouse OA knee significantly suppressed GAG loss and MMP-13 expression. Erk1/2 signalling pathway activation was significantly reduced by

Sulf-1 and FGF2. These findings indicate that Sulf-1 prevents cartilage degeneration by suppressing MMP-13 via an effect on FGF2/Erk1/2 signalling (Otsuki et al., 2017).

## **4. Enzyme of GAG biosynthesis involved in OA**

Recent biochemical and molecular biology advances have led to the understanding of a series of enzymes responsible for the synthesis and modification of GAG chains, and the elucidation of their properties in OA. Furthermore, functional analyses of GAGs using model organisms have revealed unexpected roles of these molecules (Yamada et al., 2011).

### **4.1. Enzyme deficiencies in OA**

In contrast to catabolism of OA cartilage, the synthesis side of OA has not received much attention. This is important because the repair potential of cartilage is gaining more attention as target for OA treatment. The initial event in response to injury by chondrocytes is an attempt to repair the damaged matrix by upregulation of matrix synthesis including PGs.

As explained in previous section several enzymes are involved in biosynthesis of GAGs therefore, any change (at gene or protein level) in configuration of these enzymes could lead to severe disorders including.

In a study performed in 2012, in order to investigate whether the appearance in the circulation of the soluble form of Xylt1 can be used as a biomarker to predict onset of posttraumatic OA, a significant increase in serum Xylt1 levels was reported shortly after meniscectomy. This was positively correlated with severe cartilage damage evaluated by histological assessment at later time points in mice (McCoy et al., 2012). In another study, to examine the role of Xylt1 in OA disease, it has been found that Xylt1 regulates GAG synthesis in human cartilage during early onset and late stage of OA disease and that expression of Xylt1 gene is regulated by the proinflammatory cytokine IL-1 $\beta$  and by the cartilage anabolic factor TGF- $\beta$ 1. Interestingly, forced expression of this enzyme by gene transfer in late stage OA cartilage enhanced GAG synthesis and stimulate cartilage repair (Venkatesan et al., 2012).

In one study, using *Csgalnact1*<sup>-/-</sup> mice, knock-out mouse model of CS glycosyltransferases, analysis of their cartilage showed that it contain a lower amount of CS and a reduced volume of the ECM, where aggrecan is mainly processed. These observations suggest that CSGalNAcT-1 is necessary for normal levels of endochondral ossification, and the decrease in CS amount in the growth plate by its absence causes a rapid catabolism of aggrecan (Sato et al., 2011).

A loss-of-function mutation in C6ST-1 (CHST3) gene (Carbohydrate Sulfotransferase 3) causes human Omani-type spondyloepiphyseal dysplasia, a severe chondrodysplasia with major involvement of the spine (Unger et al., 2010a). The patients with Omani-type spondyloepiphyseal dysplasia caused by a missense mutation (R304Q) have a short stature; severe kyphoscoliosis; OA in elbow, wrist, and knee joints; secondary dislocation of the large joints; rhizomelia; fusion of carpal bones; and mild brachydactyly (Thiele et al., 2004). 6-O-Sulfation on GalNAc residues in CS chains was barely detected in fibroblasts and urine obtained from the patients (van Roij et al., 2008). Furthermore, Superti-Furga and colleagues (Unger et al., 2010b) have demonstrated



that additional CHST3 mutations cause autosomal recessive Larsen syndrome, chondrodysplasia with multiple dislocations, humerospinal dysostosis, and Desbuquois syndrome. These observations suggest that the degree of 6-O-sulfation deficiency in CS varies depending on the substituted amino acids in C6ST-1. The clinical spectra are similar to those seen in other skeletal dysplasias caused by defective sulfation of GAGs.

It was demonstrated that the expression of the 6-O-sulfotransferase 2 (6-OST2), an enzyme involved in the C6 sulfation of N-sulfo-D-glucosamine from HS, is decreased in OA as compared to normal cartilage and that 6-OST2 gene inactivation inhibits FGF-2 expression and anabolic activity on chondrocytes. These data suggest a link between level of HS sulfation and the decrease of chondrocytic activities in OA.

Mutations caused by disturbed HS synthesis often have overlapping phenotypic features, characterized by growth disorders and skeletal deformities. For example, ECM defects that cause bone and musculoskeletal defects (and additional defects) include Desbuquois dysplasia type 2, the progeroid variant of Ehlers–Danlos syndrome, Larsen-like syndrome, and multiple osteochondromas (MO). These diseases are associated with mutations in the genes encoding XylT1 and XylT2, GalT1, GalT2, GlcAT1, EXT1, and EXT2, respectively (Mizumoto et al., 2013).

Changes in sulfation of cartilage GAGs as mediated by sulfatases can regulate GF signaling. In a study aiming to analyze expression patterns of extracellular sulfatases Sulf-1 and Sulf-2 in articular cartilage and chondrocytes of OA, a significantly higher Sulf-1 and Sulf-2 mRNA expression was reported as compared with normal human articular cartilage (Otsuki et al., 2008). Indeed, this increased Sulf expression may change the sulfation patterns of HS and GF activities and thus contribute to abnormal chondrocyte activation and cartilage degradation in OA.

## AIMS OF STUDY

During the aging process, changes in PGs quality pave the way for OA development and studies are focus on aggrecans and CS catabolism since 60 years. CS expression levels, chain size, sulfation patterns evolve during OA, affecting the mechanical properties of ECM. However, treatments based on visco-supplementation with CS local injections have demonstrated their limit since cartilage repair is not induced. Although they are rarely found in adult cartilage, HSPGs are expressed by chondrocytes also and their relevance was demonstrated mainly during bone development. HS chains are very important homeostatic regulators because they are able to bind and regulate the activity of several heparin-binding proteins (HBP) (growth factors, cytokines, chemokines, morphogens), protecting them against proteolysis and potentiating their binding to their receptors. These interactions provide a stock of regulatory factors that can be release by selective degradation of the HS chains too. All these regulatory effects are mediated through the complex sulfation/acetylation pattern of HS chains. Whereas HS role are explored during cartilage development in which chondrocytic differentiation pathway are important and regulated by numerous HSPG, few data are available on the role of HS during pathological evolution of cartilage in OA.

The main goal of my scientific project during my PhD was to characterize the evolution of HS chemical signature and functionality in cartilage during OA. In collaboration with Rheumatology and Orthopedic clinical teams from Henri Mondor Hospital, coordonated by Professor Xavier-Jean Chevalier, we sought to perform a quantitative and qualitive evaluation of HS in control versus OA human cartilage samples and try to correlate it to the structural damage severity. According to the tools of the CRRET's lab glycomic platform, extraction of GAGs from control and OA cartilage human samples were performed to identify structural changes on HS and CS sulfated disaccharides compositions. Finally differents functional tests were performed to measure GAG binding affinities to HBP and GAG abilities to potentiate chondrocytes properties. According to these experiments, we were able to demonstrate for the first time that GAG extracted from OA cartilage, and specifically HS, induce hypertrophic phenotype of chondrocytes. This work was performed in collaboration with Pr. Martine Cohen Solal, the chef of rheumatology department, of Lariboisiere Hospital, Inserm UMR-1132 and Dr. Eric Hay. Some part of these data was presented as poster during the international joint meeting of the German and French societies for matrix biology, in which it won the "Best poster Awards". This work is the subject of article 1, currently submitted for publication.

During this project I also analyzed functional properties of commercial CS from 3 different biosources, that were kindly provided by a pharmaceutical partner, that we will called X company. These CS were called XA, XB, XC, and were compared to HS and CS purified from normal and OA cartilages. These results are of interest because they suggest distinct functionality of these 3 CS that could be linked to their disaccharides structural signature of sulfation pattern.

They confirm the data obtained on CS purified from cartilage, indicating that CS have important functionality in cartilage. These results are presented in complementary data of article 1.

In the second part of my PhD project, we were interested in another very important compartment of OA which is the synovial fluid.

In fact, Synovial Membrane (SM) inflammation is now recognized as an integral part of the pathophysiological process of OA. Two types of synoviocytes with different functions exist: The macrophage-like synoviocytes (MLS) and the fibroblast-like synoviocytes (FLS) which produce SF and the ECM of the synovial.

By the absence of any data from the literature and in view of the growing interest on FLS involvement in OA, and since we demonstrated different HS and CS signatures in cartilage from OA, we decided to explore if the same or some different HS signature in sulfation pattern could be observed in SF and SM.

The aim of this study was divided in three aspects: first, developing a protocol for the primary culture of FLS from human healthy and OA samples from SF and SM, and confirm the characterization of these cells as synoviocytes. Second, extraction, quantification of HS and analyze their sulfation pattern. The difficulty of this kind of analysis, is that it is very difficult to obtain “normal synovial fluid” because it is very few amount and not ethical to harvest this fluid from healthy patient. In this context we decided to obtain information from synoviocytes cultured from OA synovial fluid or healthy synovial membrane. We sought also to compare our results with GAG extractions from OA and CT serum. These structural characterizations permit to identify specific disaccharidic profiles of HS in OA SF and OA synoviocytes. These results on GAG (HS, CS and KS) structural characterization from SF and SM samples are currently the object of a second article, presented here.

Third, according to the specific sulfation pattern of HS that we observed in OA SF and synoviocytes, we decided to begin an exhaustive study on the expression profile of HS biosynthesis enzymes during OA. Moreover, some preliminary data from a master student of the lab suggest that this sulfation pattern could be due to the TGF $\beta$  signaling pathway and so linked to the pro-inflammatory role of these cells. These final data are not presented in the current version of the manuscript (article 2) because they will be patented.

During my PhD, my skills on biochemical purification and characterization of GAG, permitted me to participate in two other collaborative projects of Pr. Patricia Albanese. I co-signed two articles that are presented in Annexes.

a) The first of this project was based on a collaborative work with Dr. Sylvia Cohen-Kaminsky, DR2 CNRS, co-director of Inserm UMR S999, based on Marie Lannelongue Hospital. This Inserm unit is the French Reference Center for patients suffering from Pulmonary Arterial Hypertension (PAH). This rare pathology is associated to the progressive obstruction of

the distal precapillary pulmonary vasculature that contributes to the increase in pulmonary vascular resistance and arterial pressure. This vascular remodeling involves endothelial cell dysfunction, smooth muscle cell proliferation and altered extracellular matrix (ECM). Among ECM, sulfated GAG such as HS and CS are essential components in lung homeostasis (1)(2) and in PH lung, altered ECM participates to lung arterial remodeling. We hypothesized that changes in GAG could alter their bioactivity as selective binding to growth factors and cell proliferation, thus leading to vascular remodeling. Then we performed a fine structural and functional characterization of these GAG, from lung samples of human patients and controls and from lung samples of rat. In vivo experimental PH in rat induced with a single injection of Monocrotaline (MCT). We revealed specific HS accumulation associated to selective increases of some disaccharides sulfation patterns for PAH human and rat lung of PAH as compare to CT. These structural modifications were correlated to significant functional modifications of GAG binding to growth factors (VEGF, FGF-2, PDGF) and to increased cell proliferation in the presence of the altered GAGs. We propose that these specific changes of HS sulfation patterns in the lung are early alteration in disease progression that could maintain a chronic inflammatory and vascular remodeling process leading to deregulated vascular cell proliferation through abnormal binding to growth factors. These changes may rely on deregulation of glycanic targets in the pathological lung, of interest to study for future therapeutic intervention. This paper is under publication and was presented as a poster to the 6th world congress in PAH, in Nice 2018.

b) Finally the 2d project presented in annex is based on a collaborative work with Valerie Vanneaux and Jerome Larghero, from Cell Therapy Unit of AP-HP, Hopital Saint Louis. Paris, France. This project was based on a strategy of Tissue engineering for the esophagus replacement using a decellularised matrix and Mesenchymal Stem Cells. In this project our role was to characterize the GAG structure and composition of the de-cellularized matrix as a quality parameter to maintain for optimizing the capacity of the scaffold to be reseeded with MSC. This article is in preparation and we propose in annex the results sections concerning the GAG characterization.

# RESULTS

# Chapter 1

## HS in OA cartilage

- **Article 1**

**Heparan sulfates from human osteoarthritic cartilage display increased sulfation pattern, altered protein binding capabilities and pro catabolic effects on chondrocytes.**

**Shamdani S, Chantepie S, Flageollet C, Henni-Chebra N, Jouan Y, Hay E, Cohen-Solal M, Papy-Garcia D, Chevalier X, Albanese P.**

- **Result**
- **Supplementary data for article 1**
- **Complementary Results on commercial CS**
- **Discussion**

# Structural and functional characterization of matrix heparan sulfates and chondroitin sulfates of the human cartilage during osteoarthritis.

Sara Shamdani, Sandrine Chantepie, Nadia Henni Chebra, Dulce Papy-Garcia, Xavier Chevalier\*, and Patricia Albanese

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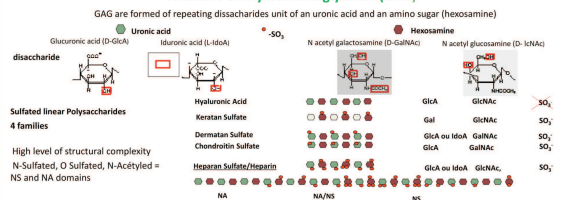
## Introduction

Osteoarthritis (OA) is the most prevalent joint disease characterized by degradation of articular cartilage, subchondral bone remodeling, and joint inflammation. Cartilage ECM consists of collagens, glycoproteins, and proteoglycans (PGs), that are composed of sulfated polysaccharides named Glycosaminoglycan (GAG) which are linked to core proteins. In cartilage, studies are focused on the biomechanical properties of GAG chains from the major PG aggregan, that are consist of chondroitin sulfate (CS), keratan sulfate (KS) and hyaluronic acid (HA). CS expression levels and sulfation patterns evolved during OA, whereas few data are available on heparan sulfate (HS) structural features and functionality. According to their chemical complexity, HS are known to bind and regulate the activity of several heparin binding growth factors, cytokines, chemokines, morphogens, protecting them against proteolysis and potentiating their binding to their receptors. These interactions provide a depot of regulatory factors that can be liberated by selective degradation of the HS chains also. Then, it was recently demonstrated that HS chains can participate to the regulation of homeostasis during physiopathological events of degenerative process in tissues.

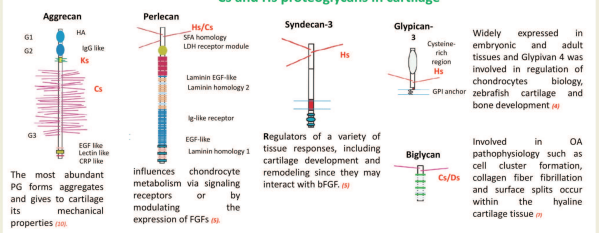
## Hypothesis

A detailed understanding of the evolution of the chemical structure and functionality of GAG, HS as compared to CS, during the development of OA in human cartilage, is a key element which will permit to understand some new physiopathological processes of this degenerative tissue and further propose the development of new therapeutic targets and strategies of this pathology. We proposed such original studies of relationship between structural features of GAG and their functional impacts on HBGF and cells, according to the tools of a glycomic technological platform, some of which are presented here.

### Structure of Glycosaminoglycans (GAG)



### Cs and Hs proteoglycans in cartilage



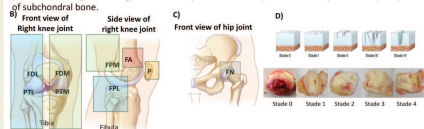
### Human Samples

A) Patient's descriptions, macroscopic classification of OA severity according to Beignaud-Locker (B.L.).

Sample	Sex	Age (year)	Severity of OA (B.L. classification)	Sample	Sex	Age (year)	Sample	Sex	Age (year)
1	Female	65	3	1	Female	52	1	Female	78
2	Female	73	4	2	Female	53	2	Female	79
3	Female	74	4	3	Female	55	3	Female	82
4	Female	83	3	4	Female	63	4	Female	83
5	Female	89	4	5	Female	74	5	Female	87
6	Male	65	4	6	Female	80	6	Female	91
7	Male	69	3	7	Female	81	7	Male	89
8	Male	73	4	8	Male	58	8	Male	60
9	Male	79	3	9	Male	60	9	Male	79
10	Male	80	4	10	Male	79	10	Male	79
11	Male	85	4	11	Female	n=7	11	Female	n=6
n=11	n=5	n=6	n=4	n=10	n=3	n=3	n=7	n=6	n=3

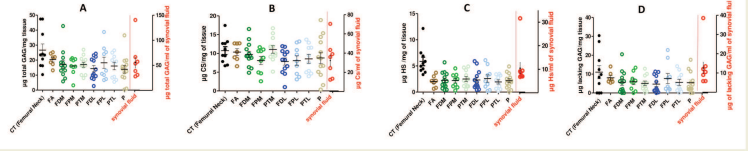
B) And C) Schematic representation of knee and hip joint's anatomy (respectively). FA: Femur anterior, FDM: Femur distal medial, FDL: Femur distal lateral, FPM: Femur posterior medial, PPL: Femur posterior lateral, PTM: Plateau tibial medial, PTL: Plateau tibial lateral, P: Patella and FN: Femoral neck.

D) Appearance, consistency and picture of normal cartilage (stage 0) and 4 stages of OA severity from patient: 1. Cartilage softening, 2. Cracks or superficial erosions, 3. Cracks or deep ulceration, 4. Exposure of subchondral bone.



### Quantification of total GAG, Cs and Hs

GAGs were extracted and quantified from 8 parts of knee and synovial fluid (SF) from patients with OA and one part from controls (CT) with efficient methods based in the selective complexation of sulfated GAGs with dimethylmethylene blue (DMB).



Analysis of GAG amount ( $\mu\text{g}/\text{mg}$  of tissue) indicate that whereas total GAG amount (A) is slightly decrease in all OA cartilage part as compared to CT, this is significantly due to HS decrease (C), whereas CS amount modification is less significant (B). Remaining GAG, that should correspond to HS (D, characterization in progress), are also significantly decrease.

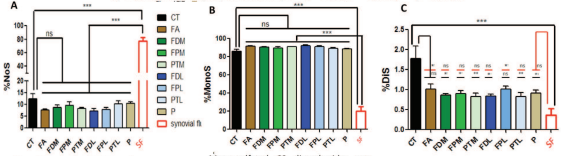
Analysis of HS, CS and KS repartition (%) among total GAG (100% panel E) confirmed that HS fraction was decreased in 7 part of OA cartilage (between 30 to 14%) compared to CT (25-3%) and Patella (22%). Whereas KS fraction is not modified significantly between CT and OA samples, CS fraction is increased (between 50 to 60%) in OA part compared to CT (43%) and Patella (47%). Analysis of HS/CS ratio indicate that whereas in CT cartilage the HS/CS ratio is around 1/2 (and for patella also), this ratio is between 1/2 and 1/4 in OA cartilage, confirming the clear decrease of HS.

Analysis of GAG repartition in SF suggest that CS fraction in SF (58%) is the same than in OA cartilage (between 50 and 60%). However analysis of HS/CS ratio suggest a 1/1 ratio as observed in CT cartilage. HPLC analysis of CS and HS disaccharide units are in progress to characterize further chemical signature of these synovial GAG.

All together these data clearly demonstrated a relevant decrease of HS amount in OA cartilage compared to control.

### HPLC analysis of Cs disaccharides sulfation pattern in GAG chains

The statistical significance of differences was determined (i) by one way ANOVA test between the group of 8 OA parts and CT or SF; (ii) by t-test between the group of CT and SF. p value  $< 0.05$ ,  $** < 0.005$ ,  $*** < 0.0001$ .

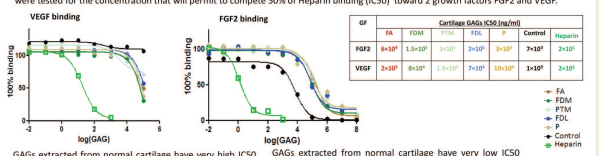


No-sulfated unit of OA cartilage were slightly decreased (between 7 to 10%) compared to CT cartilage (12%).

Surprisingly SF pattern is very different than those of cartilage tissue since is significantly enriched in No-sulfated CS unit (80%) as compared to CT and OA patient, whereas Mono- and Di-sulfated units are very low.

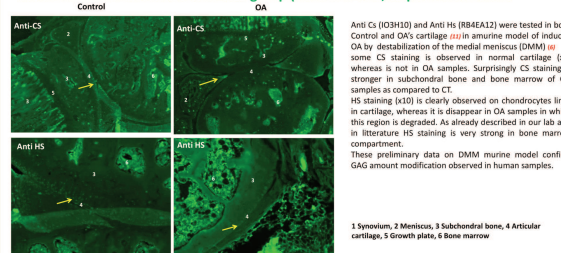
### ELISA-based binding assay of GAG toward HBGF

GAG and specifically HS are regulators of cell behavior, according to their abilities to bind and potentiate the activity of HBGF. This interaction can be measured by a modified ELISA based competition binding assay. GAG extracted from all parts of patients and controls were tested for the concentration that will permit to compete 50% of Heparin binding (IC50) toward 2 growth factors FGF2 and VEGF.



GAGs extracted from normal cartilage have very high IC50 value ( $3 \times 10^4$  ng/mL) and so low binding affinity to VEGF (as compared to heparin  $2 \times 10^3$  ng/mL). Very interestingly, these IC50 decrease until  $10 \times 10^4$  ng/mL for GAGs extracted from OA patients suggesting that GAG affinity toward VEGF is increased in OA.

### Immunohistochemistry collaboration with Pr. M. Cohen Solal group (Inserm U1132, Hôpital Lariboisière)



### Conclusion & Perspective

- GAG amount and specifically HS are decreased during OA.
- The CS and HS disaccharidic unit sulfation pattern evolve, with a loss of di-sulfation motif
- GAG affinity for VEGF is increased during OA, whereas affinity for FGF2 is decreased

Further HPLC studies will be focused on characterization of structural features of HS, as well as RO-PCR analysis of expression pattern of specific genes encoding for the enzymes involved in HS and CS biosynthesis. This would permit to identify new targets to a better understanding of OA. Moreover, result on GAG binding affinities that evolved in OA suggest that GAG from OA could have relevant effects on distinct cells populations, such as chondrocytes or mesenchymal stem cells. This hypothesis will be tested on in vitro culture cells experiments.

Such differences on GAG structures and functionality could be not only a cause of a degenerating cartilage but a consequence also of adaptive or repair process with functional impact in the evolution of OA that remain to be identified.

Today, there is no treatment capable of stopping anatomically the progress of OA. Most treatments focus on reducing the pain, by means of analgesics and/or nonsteroidal anti-inflammatory drugs, or CS and glucosamine sulfate (GS) injections. Clinical trials on beneficial effects of CS and GS protocols are always under debate because of the difficulty to demonstrate their significant functional effects. So the last opportunity for patient remains the implementation of a total articular prosthesis. New strategies based on injection of chondro-protective cells such as chondrocytes and MSC are under studies, but will be limited by the fragility of these cellular products, not only during its production in vitro but also after injection in non-vascularized cartilage in vivo. In that way we suggest that it will be of interest to associate such cell based therapy to matricial one, as soon as the role of GAG, and specifically HS, will be clearly elucidated in the physio-pathological process of OA.

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## Article 1

### **Heparan sulfates from human osteoarthritic cartilage display increased sulfation pattern, altered protein binding capabilities and pro catabolic effects on chondrocytes.**

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Key Words; Heparan sulfate, sulfation, cartilage, chondrocytes, osteoarthritis

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## Abstract

Osteoarthritis (OA) is the most prevalent joint disease characterized by degradation of extracellular matrix (ECM) of articular cartilage, subchondral bone remodeling and synovitis. Although the Glycosaminoglycans (GAGs) component of the cartilage ECM is mainly composed of Chondroitin Sulfates (CS) and Keratan sulfates (KS), other GAGs as Heparan Sulfates (HS) are also present in minority. In general, CS are mostly known to assure ECM structural stability, while HS are recognized as central functional regulators of cell behavior, principally because of their capacities to interact with several growth factors, cytokines and other tissue repair effectors. Regardless of the importance of HS in the regulation of cell behavior, investigations aiming to study their functional changes in OA have never been considered. The aim of this work was to investigate HS and CS structural and functional changes in cartilage. We focused on their effects on relevant cell properties, according to their abilities to interact with heparin-binding proteins (HBP). We purified and compared HS, CS and KS from human macroscopically normal (CT) versus OA cartilage samples. We studied changes in amounts, structures and functional properties of HS, regarding to their capacities to interact with HBP, such as FGF-2 and VEGF, involved in OA physio-pathological pathways or in articular chondrocytes cell behavior. We demonstrated that during OA HS sulfated disaccharides composition is altered, and this is associated to functional changes on HS HBP binding capacities. Finally, we show that HS from OA cartilage, and not from normal counterpart, are able to induce hypertrophic and pro catabolic phenotype on murine chondrocytes. These results show important structural and functional changes of HS in OA cartilage, paving the way for a future research directed to a better understanding of the role of GAGs in the pathological processes leading to OA.

## Introduction

Osteoarthritis (OA) is the most frequent form of articular disease, characterized by degradation of the joint cartilage associated with subchondral bone remodeling and an associated synovitis [1]. Biological, genetic, biochemical, nutritional and mechanical factors contribute towards the etiology of OA [2][3]. The impact of this disease has gradually gained importance with the increase of aging and obesity of the population [4]. Then OA is one of the main causes of limitation and reduction of quality of life among the population over the age of 50 years. This is mainly because none efficient treatments are currently available to induce repair of the cartilage and because cartilage self-repair capacity is poor. Thus, in order to identify new therapeutically targets, it has become necessary to better understand the mechanisms involved or leading to the OA disease process.

Articular cartilage is an avascular tissue composed of an abundant extracellular matrix (ECM), produced by a unique cell type, chondrocytes, that are obtained from the condensation and differentiation of mesenchymal cells. Cartilage performs biomechanical functions within the joint, such as absorption of mechanical stress and reduction of friction of the articular surface, enabling painless motion. These biomechanical competences are sustained by an ECM very rich in fibrillary proteins such as collagens and in proteoglycans, as Chondroitin Sulfate Proteoglycans (CSPG) aggrecans. As a result of mechanical injuries or of catabolic processes during aging, chondrocytes become hyper-activated, triggering their own hypertrophy and the mineralization process, characteristic steps of OA disease (ref). As the pathological degradative processes advance, ECM remodeling take place with metalloproteinases activation and growth factors degradation, all contributing to increase the abnormal differentiation phenotype and altered metabolism of chondrocytes [3, 5, 6]. ECM degradation is also associated to further subchondral bone remodeling and osteophytes formation [7]. Finally, increase of angiogenesis signaling molecules have shown to contribute to the local and chronic inflammatory process, possibly by inducing local differentiation of chondrocytes towards hypertrophic state (ref). In this complex context, detailed knowledge of the ECM impact on the chondrocyte properties and its relationship with tissue repair effectors is an important requirement for the better understanding of the physiopathologic mechanisms leading to OA and the concomitant identification of new targets for the generation of new therapeutic strategies to treat this disease.

Among ECM components, glycosaminoglycans (GAG) are long anionic polysaccharides composed of disaccharides building blocks that form highly complex structure. The structural complexity on GAG chains is due to the heterogeneity in the different GAGs disaccharide compositions, their glycosidic linkages and notable, on the extremely diverse sulfation patterns characteristic of some GAGs subfamilies like HS. All together, these factors regulate the GAGs interactions with surrounding molecules in the ECM. Four GAG subfamilies are distinguished according to their repeating disaccharide units: heparan sulfate (HS), chondroitin sulfate (CS), keratan sulfate (KS) and hyaluronic acid (HA). Sulfated GAG (HS, CS and KS) are covalently

linked to core protein to form proteoglycans (PG) whereas the non-sulfated HA is free in the ECM. PG, as aggrecans, and HA interact with proteins to strengthen the architecture and equilibrium of the ECM. Aggrecans are composed of a core protein to which are attached about a hundred of CS and KS chains and its N-terminal domain contains a specific globular domain that allows its binding to HA. The main function of this CS/KS PG is to assure the water retention in the cartilage via the sulfate groups, thereby maintaining under pressure the more rigid network of collagen fibers. Although the CS/KSPG Aggrecan is the major PG in the cartilage matrix, other PGs are also present and implicated in the functional regulation of cell properties through the abilities of their GAG moieties to specifically interact with heparin-binding proteins (HBP), including growth and/or differentiation factors, cytokines, chemokines, morphogens, and enzymes [8]. It's particularly the case of HS, which, according to their complex structural characteristics, are able to interact with a great number of HBP and to regulate their cell behaviour modulatory function. For example specific interaction of HS with HBP like Fibroblastic Growth Factor (FGF) -2, eminently vary depending on their sulfated and acetylated group contents, and on the length of the HS sulfated domain (ref) interacting with the growth factor. HS not only protect HBP from their proteolytic degradation and trigger their activities [9, 10], they also provide matrix storage sites for HBP prior to their binding to cell surfaces. This facilitates both the formation of local HBP gradients and the synchronous coordination of motility and cell adhesion [11-13]. Fine changes in GAG structures affect their capacities to regulate trophic factors activities and their functionalities, particularly by regulating cell properties, making a tissue potentially vulnerable during physiological processes, as previously shown for aging [14, 15] or in pathologies including Alzheimer disease [16], myelofibrosis [17], ischemia [18] or muscular dystrophie [19]. Studies on ECM remodeling during OA have mainly focused on the investigation of imbalance between synthesis and degradation of aggrecan, the major CS/KS proteoglycan in cartilage [3]. The reduction of size and the loss of sulfated CS and KS chains from aggrecans compromise both the functional and structural integrity of the cartilage matrix and ultimately render the tissue unable to resist to the compressive loads applied during joint articulation [20]. Over the time, this process leads to irreversible cartilage erosion. Changes in the CS chains quality during the aging process has been considered as central for OA in cartilage. The importance of structural and moisturizing functions of CS in healthy and OA cartilage is well known. However, though HS are regarded as low represented members of the GAG family in cartilage, their structural evolution and biological functionality remains to be explored during the time course of the OA. In this work, we hypothesize that quantitative and qualitative evolutions of the chemical structures and biological functions of HS, are key functional events during the development of the patho-physiological process of OA. After extraction of GAGs from control and OA cartilage human samples, we demonstrated that changes in HS sulfation pattern impact their abilities to bind to FGF-2 and VEGF, two HBP relevant in cartilage homeostasis. The extracted HS showed to modify these HBP functionalities on human mesenchymal stem cell properties induce hypertrophic phenotype of murine chondrocytes together with an increase in their catabolic activities.

## MATERIALS AND METHODS

### Patient's characterization

Human cartilage explants were harvested from 11 OA patients undergoing total knee replacement surgeries and from 7 Femoral Head after neck fracture considered as control donor, with informed patient consent and according to Legal authorization of the Ethics Committee of the Rheumatology Department of Henri Mondor Hospital (N° 07-34). Personal and clinical data were collected. All parts, Femur (F), Plateau Tibial (PT) and Patella (P), were recovered within 1-4h after ablation from the joint and were evaluated according to the extent of wear, using a five point grading system (0-4) (Hitchcock et al. 2006). Harvested parts were immediately frozen at -80 until used.

### GAGs extraction from articular cartilages

Samples from each cartilage part were cut with a scalpel and separated according to the different areas of the resting cartilage: femoral part, tibial plateau part and and patella part. Samples were then freeze-dried during the night. GAGs were extracted according to an adaptation of our previously published protocol (ref Huynh et al. 2012). Freeze-dried samples were weighted and homogenized. The dried tissue was suspended in 1 mL of a digestion buffer (Tris-HCl 50 mM, NaCl 10 mM, MgCl<sub>2</sub> 3 mM, Triton X-100 1%, pH 7.9) to a final concentration of 50 mg ( $\pm$  0.05) of tissue per mL of buffer. Samples were then treated with proteinase K (Sigma, final concentration 100  $\mu$ g/mL) at 56°C for 24 hours, followed by the enzyme inactivation at 90°C for 30 min. DNA was then digested by DNase I (QIAGEN, 10 U/mL of sample) overnight at 37°C. Samples were filtered (PALL life science ODM02C34) and centrifuged (12 000 rpm, 10 min, at 6°C). The filtrates were diluted 1:1 with 4M NaCl and vortexed for 30 min at room temperature (rt). Samples were treated with TCA (SIGMA-ALDRICH) to final 10% and cooled at 4°C for 15 min, centrifuged (12 000 rpm, 10 min, 4°C) and the supernatants recovered. Lipids were eliminated by chloroform extraction (1:1) and centrifugation (12 000 rpm, 10 min, 8°C). The aqueous fractions were dialyzed (Thermo scientific 3.500 MWCO) against a buffer (1X: Tris 50 mM, CH<sub>3</sub>COO-Na 50 mM, CaCl<sub>2</sub> 2 mM, pH 7) and then against pure H<sub>2</sub>O. Samples were frozen at -80°C and freeze-dried. Finally, samples were re-suspended in pure H<sub>2</sub>O to a desired tissue weight/H<sub>2</sub>O volume concentration and stored at -20°C until use. Extraction/quantification quality controls (QC) were included in each experiment. These QC were prepared from freeze-dried cartilage samples in where a known quantity of GAGs, similar to that expected in most samples, was spiked. This was made in order to calculate the extraction yield and at the end of the processes.

### Dimethylmethylene blue (DMMB) assay of total sulfated GAGs

Extracted total sulfated GAGs were quantified according to 1-9 dimethyl-methylene blue (DMMB, Sigma Aldrich) assay as previously described (Barbosa et al. 2003). Briefly, total GAG extracts were diluted to 100  $\mu$ L with pure H<sub>2</sub>O and 1 mL of DMMB solution was added. Samples were stirred for 30 min and centrifuged (12 000 rpm, 15 min). Samples supernatants were discarded and pellets were dissolved in 500  $\mu$ L of a decomplexing solution. GAGs in the decomplexing solution were quantified by measuring the absorbance (650 nm) in a fixed volume from the mixture using a micro plate reader (TECAN). Total GAG quantities in the samples were determined according to the linear portion of a standard / calibration curve generated in the same condition with known concentrations (2.5 – 30  $\mu$ g/mL) of standard Cs-A (Sigma C9819). For each sample, CS, KS, and HS amount were determined after chondroitinase ABC, heparitinases I/II/III, and keratanase treatments, respectively.

#### Quantification of CS

CS was enzymatically digested by chondroitinase ABC (Chse ABC) treatment as previously described (Barbosa et al, 2003). Briefly, total GAG extracts were diluted in a glycanase digestion buffer (CH<sub>3</sub>COO-Na 50 mM, CaCl<sub>2</sub> 2mM). Chondroitinase ABC (30 mU, SIGMA C3667) was added and samples were incubated at 37°C for 1 hour. The residual HS and KS were then determined by following the DMMB protocol as described above. A standard calibration curve with known CS (SIGMA H7640) concentrations (2.5 – 30  $\mu$ g/mL) was included in each experiment. The amount of CS in samples was calculated as the difference with total GAG quantified in the same sample.

#### Quantification of HS

HS were chemically digested by nitrous acid treatment as previously described [21]. Briefly, total GAG samples were completed up to 100  $\mu$ L with H<sub>2</sub>O. NaNO<sub>2</sub> (SIGMA S-2252) was added (100  $\mu$ L, 0.25 M,) followed by addition of acetic acid (VWR 20103.364) (100  $\mu$ L, 33%). Samples were incubated at RT, for 1 hour and the reaction was stopped by adding 100  $\mu$ L of ammonium sulfamate (14%) (SIGMA 228745). After the chemical HS digestion, remaining GAGs (CS and KS) in the samples were determined by following the DMMB protocol as described above. A standard calibration curve with known HS (SIGMA H7640) concentrations (2.5 – 30  $\mu$ g/mL) was included in each experiment. The amount of HS in samples was calculated as the difference with total GAG quantified in the same sample.

## Quantification of KS

KS were quantified following 2 strategies. First, HS and CS were digested as described above and the remaining GAGs (KS) were quantified. Briefly, total GAG samples were completed up to 100  $\mu$ L with the glycanase digestion buffer and were then treated by Chse ABC (30 mU) and a heparitinases (Iduron) cocktail (Hep I, Hep II and Hep III), at 37°C for 1 h. Remaining GAGs (KS) in the samples were determined according to DMMB protocol as described above. The KS quantification after CS and HS digestions was confirmed in total GAGs samples treated by Keratanase. Briefly, total GAG samples were completed up to 100  $\mu$ L with the glycanase digestion buffer and Keratanase (Amsbio PS170615) was added. Samples were incubated in a 37°C for 1 h and remaining GAGs amount were determined according to the DMMB protocol as described above. A calibration curve, made of known KS concentrations (2.5 – 30  $\mu$ g/mL) was included and processed in each experiment. The amount of KS in samples was calculated as the difference with total GAG quantified in the same sample.

## Structure of HS and CS by HPLC analysis

Extracted GAGs were first used to determine the global HS and CS disaccharides composition by HPLC. Briefly, total GAGs samples were treated with 20 mU of Chse ABC (SIGMA C3667) for 1h 30 at 37°C followed by heparitinases I, II and III (Iduron) cocktail treatment (0.25mU of each, overnight at 37°C. After filtration, samples were injected to HPLC system set as previously described [14]. Then, CS composition was specifically analyzed by digesting the total GAG samples with Chase ABC and without treatment with the heparitinases cocktail. This allowed determination of the HS disaccharide composition by subtraction of the CS peaks from HS+CS signals. This was performed to avoid chromatography column clotting resulting from CS digestion that interfered with HS analysis along. The HPLC analysis was performed as previously described [14]. Briefly, after total extracted GAGs filtration, 50  $\mu$ l of sample was automatically loaded through a Proteomix SAX-NP5 column eluted by a solvent gradient consisting of eluent A (NaCl 20 mM) and eluent B (NaCl 1 M) at 1 ml/min flow rate. The gradient was 0–5 min (100% A), 5-25 min (from 100% to 0% A by steps), 25–29 min (0% A), 30–37 min (100% A). The column effluent was mixed (1:1) in line with 2% of 2-cyanoacetamide solution and a 25 mM of NaOH, supplied at 0.5 mL/min each. The mixture passed through a reaction coil (10-m length, 0.5-mm internal diameter) set in an oven at 120 °C, followed by a cooling coil (3-m length, 0.25-mm internal diameter). The effluent was monitored at 346 nm of excitation and 410 nm of emission with a fluorescence detector (JASCO; FP-1520). Identification of peak was performed using commercial HS and CS disaccharides standards (Iduron). Moreover, 3 controls of digestion using commercial GAG were included in each experiment to validate complete enzymatic digestion. Results were expressed as % of area of each peak to the sum area of all peaks. external standard.

## Heparin/Glycosaminoglycan ELISA competition assay towards HBP

The capacities of the cartilage extracted GAGs to interact with HBP were evaluated by an ELISA-based competition assay as described by Najjam et al [22]. The tested HBP were Recombinant Human Basic FGF-2 (PROMOKINE, C-60240) and Recombinant Human VEGF-165 (PROMOKINE C-64420). Briefly, ELISA 96-well plates (Costar) were coated overnight at 4°C with heparin-BSA complex in Tris-HCL 50 mM, EDTA 12.7 mM, PH 7.4, and washed 3 times with PBS, 0.05%, Tween 20 (washing step). Wells were then saturated with BSA 3% in PBS (Sigma A7030) for 1 h at rt. Then, HBP diluted in BSA 1%/PBS were added to wells (FGF-2 at 2.5 ng/well; VEGF-165 at 5 ng/well) together with increasing concentration of extracted GAG (serial dilution from 0, 0.01 to 100 000 ng/mL in PBS) in duplicate. Competition of the HBP binding to immobilized-heparin or to the soluble GAGs was performed for 1 h in the plate. Heparin (Sigma) at concentrations ranging from 0.01 to 1000 ng/mL was used as a control of the GAGs competitive binding. After washing, the HBP remaining bond to heparin was targeted by an specific antibody (anti-FGF2 Mouse Monoclonal IgG diluted 1/2000, R&D systems MAB233; anti-VEGF Rabbit Polyclonal IgG, diluted 1/500 PROMOKINE PK- AB815-64420) for 1h at rt, followed by a HRP-conjugated secondary antibody (HRP-AffinPure Donkey Anti-Mouse IgG 1/5000, Jackson ImmunoResearch 715-035-151; HRP-AffiniPure Goat Anti-Rabbit IgG 1/2000, Jackson ImmunoResearch 111-035-144). Peroxidase activity was measured by the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB, SI254783, Thermo Scientific, France) substrate as described by manufacturer's protocol. The maximum binding (100%) was determined in presence of the HBP and in absence of extracted GAG and IC50 was defined as the concentration of GAG able to displace 50% of the HBGF binding to immobilized heparin. Finally, GAGs IC50 values were determined for each HBGF.

#### Human Mesenchymal Stem Cells culture, adhesion and proliferation.

Human Mesenchymal Stem Cells (hMSCs) were purchased from ABCell-Bio (France). Flow cytometry analyses were performed to confirm hMSC phenotype as previously described [23]. Phenotype was positive for CD90, CD105, CD73, and negative for CD34 and CD45 (data not shown). Then hMSCs were expanded in  $\alpha$ -modified Eagle's medium ( $\alpha$ MEM) (Life technologies, France) containing 10% fetal bovine serum (FBS) (StemCell Technologies, France) supplemented with 0,5% Cifloxacin (Bayer Pharma, France). Culture medium was changed twice per week and cultures were maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. When cells reached 80-90% confluence, they were detached using trypsin/EDTA (PAA Laboratories, Austria) and then replated at 1000 cells/cm<sup>2</sup>. Cells expanded between passages 2 to 5, were used as responsive cells. HMSC were seeded at density of 40000 cells/well in 24 well plates, in absence or presence of increasing doses of extracted GAG. Cells were numerated by MTT (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) colorimetric assay 3 hours after seeding, as the non-optimal time of adhesion, to evaluate the ability of GAGs to inhibit or potentiate hMSC adhesion. Briefly 400  $\mu$ L of MTT (0.5 mg/mL) was added to each well and plates were

incubated at 37°C in 5% CO<sub>2</sub> for 3h. The formazan crystals were dissolved in 100 µL of DMSO, and the absorbance was determined spectrophotometrically at 570 nm. For proliferation assays, hMSC were seeded at density of 20000 cells/well in 24 well plates, in absence or presence of increasing doses of extracted GAG. Cells were numerated by MTT colorimetric assay 7 days after.

#### Murine primary chondrocyte culture

Murine articular chondrocytes were harvested from 6-day-old mice as described [24], and seeded in 6 wells plates for amplification. At day 6, primary chondrocytes are treated with recombinant mouse interleukin 1β (IL-1β, R&D systems ref 401-ML-00) at 1 ng/ml, without and with GAG extracted from human CT versus OA cartilage samples at 2,5 µg/ml, for 24 hours. Heparin was used as control. Then cells are harvested for RQ-PCR analysis: RNA from chondrocytes were recovered using Quiazol (QIAGEN 79306). RT was performed using high capacity cDNA reverse transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) in 4 independent experiments, to analyzed pattern of expression of anabolic (Coll II Agg sox9 ) catabolic (Mmp-3 and 13 Adamts 4 and 5) and hypertrophic (VEGF Coll X) markers, according to primers sequence already validated [25]. Averaged threshold cycle (Ct) values were normalized to the averaged Ct value of RPL13A house keeping gene. Adjusted average Ct values were used to calculate relative expression versus the control. Cell cultures supernatant were used for western blot analysis. Antibodies were obtained for MMP-3 (1:500, ab52915 Abcam Cambridge, UK), MMP-13 (1:3000, abcam Cambridge, UK), for the revelation a secondary anti body against rabbit IgG horseradish peroxydase was used (Jackson ImmunoResearch West Grove, PA, USA).

#### Statistical analysis

Values are expressed as mean ± SEM. The statistical significance of differences between the various groups was determined by t-test or one-way analysis of variance (ANOVA) and group-to-group comparisons were made by two tails unpaired student's t-test. All graphs and values were analyzed using GraphPad Prism 5 software. Each sample was tested 3 times in each experiment and each experiment was repeated at least 3 times. A p value < 0.05 was considered to be statistically significant. Note that \*\* < 0.005, \*\*\* p < 0.0001.



## RESULTS

### Clinical data

Clinical data from OA patients and control were collected. Cartilage was collected from femoral head of healthy donors and of different parts of knee of OA patients characterized according to KL grading system. (Table 1)

The control (CT) cartilage, obtained from a femoral neck fracture, presented a normal macroscopic appearance.

### Sulfated GAG levels in cartilage are decreased in OA.

Levels of total sulfated GAGs were analyzed in the cartilage extracts from CT and OA patients. As shown in fig. 1A, the basal amount of total sulfated GAG in CT individuals was 25  $\mu\text{g}$  per mg of dried cartilage. This total sulfated GAG content was decreased by more than 1.5 folds in cartilage samples obtained from OA femoral condyle (16.3  $\mu\text{g}/\text{mg}$ ), OA tibial plateau (16  $\mu\text{g}/\text{mg}$ ), and OA patella (14.4  $\mu\text{g}/\text{mg}$ ) recuperated from Knee OA during total joint prosthesis (Fig 1A). Differential quantification of CS, KS and HS species in the freeze-dried tissue showed basal levels of CS around 9.5 (SEM= +/- 0.9)  $\mu\text{g}/\text{mg}$  and 10.5  $\mu\text{g}/\text{mg}$  (SEM= +/- 1.4) of KS. No significant differences were observed in CS amount from OA femur, tibial plateau and patella as compared to CT (Fig 1B). However, significant decrease of KS was observed in cartilages samples from OA femoral condyle (5.6  $\mu\text{g}/\text{mg}$ ), OA tibial plateau (4.3  $\mu\text{g}/\text{mg}$ ), and OA patella (3  $\mu\text{g}/\text{mg}$ ) (Fig 1C) compared to CT. Quantification of HS species showed that the most affected GAGs were HS; the basal level of HS in normal macroscopic cartilage was 4.8  $\mu\text{g}/\text{ml}$ , and it was significantly decreased (2 folds) in cartilage samples from OA femoral condyle OA tibial plateau and OA patella (Fig 1D). Total GAGs extracted from cartilage from different parts of knee of OA patients (25  $\mu\text{g}/\text{mg}$ ) were significantly reduced as compared to total GAGs collected from cartilage of healthy donors (15.6  $\mu\text{g}/\text{mg}$ ) (Fig 1E). Furthermore, the analysis of the percentage of each GAGs species on total amount (Fig 1F) indicates that in OA patients the percentage of CS is increased (56%) as compared to control (38%), whereas the percentage of HS (14%) and KS (29%) were decreased as compared to control HS (20%) and KS (42%).

### Sulfation pattern of CS and HS disaccharides units evolved during OA

Total GAGs, as well as HS levels, were shown to be altered in OA tissue. To investigate whether HS presented altered sulfation patterns in the pathology, we analysed relative composition of HS

disaccharides by HPLC analysis. We decided to compare it to disaccharides CS composition, as it was already described. KS disaccharides analysis has not been performed since structural variation is minimal and structural-functional relevance is unknown. CS analysis indicates that in control cartilage, CS disaccharides are mainly monosulfated (85%), whereas only 13% of CS disaccharides are Non-Sulfated and 1.2% are disulfated. In OA samples, the fraction of Mono-sulfated disaccharide of CS increase significantly to 90% (Fig 3B). This is associated to a decrease of no-Sulfated disaccharides (Fig 3A, from 13 to 8%) and di-sulfated disaccharides (Fig 3C, from 1.2% to 0.8%) forms of CS. HPLC analysis of HS disaccharides indicates that in CT cartilage, HS disaccharides are mainly monosulfated (Fig 3E, 71%), whereas 26% are Non-Sulfated (Fig 3D) and 1.8% are di-sulfated (Fig 3F). In OA samples, the fraction of Mono-sulfated disaccharides is not modified significantly (Fig 3E), but a 2 folds decrease of Non-Sulfated HS disaccharides from 26% to 14% is observed (Fig 3D), as well as a significant increase of di-sulfated disaccharides from 1.8% to 4% (Fig 3E), this is particularly relevant in femoral and tibial plateau part as compared to patella.

### **GAGs from cartilage display differential binding affinities toward growth factors during OA**

The capacity of GAGs to regulate cells functions is largely related to their abilities to bind to HBPs. An ELISA-based competitive binding assay was used to investigate if structural modifications of GAGs in OA cartilage are associated to distinct abilities to bind HBPs relevant in cartilage homeostasis such as FGF-2 and VEGF. In this test IC<sub>50</sub> of GAG were defined as the concentration of GAG that permit to inhibit 50% of the HPB binding to immobilized heparin. The determined IC<sub>50</sub> values (in µg/ml) for each studied HBP are showed in table 2. A high IC<sub>50</sub> value corresponds to a low affinity, since big concentration of GAG is necessary to compete for HBP binding to heparin, and inversely.

Total GAGs extracted from CT cartilage were able to bind to FGF2 and to inhibit its binding to heparin with EC<sub>50</sub> values of 11µg/mL (Table 2). This value was taken as relative values (CT = 100%) to compare binding capacities of extracted GAGs form OA samples (femoral condyle, tibial plateau, pattela and mean from all OA samples) in figure 3. IC<sub>50</sub> values obtained from total GAG extracted from OA samples compared to normal CT were shown to increase around 8 folds toward FGF2 (82 µg/mL) (Table 2), suggesting corresponding decrease of binding affinities of this growth factor as illustrated in Figure 3, panel A and C respectively. Whereas total GAGs extracted from CT cartilage were not able to bind to VEGF and to compete even at 1000 µg/ml, GAG extracted from OA samples were able to bind to VEGF with IC<sub>50</sub> mean at 136 µg/ml (Table 2). To illustrate this gain of binding affinity IC<sub>50</sub> value of CT GAG across VEGF was considered up to 1000 µg/ml and take as 100%.

Same binding assays were performed with purified HS and mix of CS and KS (devoid of HS).

This fine analysis reveals that binding of total GAG to FGF2 is related to HS binding affinity for this growth factor, whereas CS/KS mix extracted from cartilage samples are not able to bind to FGF2 and to compete. As for total GAG, HS affinity for FGF-2 was decreased, as the IC50 value was increased between CT (34 µg/ml) and OA (80 µg/ml). Concerning VEGF, binding of total GAG to this growth factor is also linked to of its affinity for HS. Accordingly, CS/KS mix extracted from cartilage samples are neither able to bind to VEGF nor to compete with it. IC50 value of HS toward VEGF increase 3 times in OA as compared to CT, suggesting that HS structural modification are associated to a loss of binding affinity toward VEGF, as observed for FGF-2. However, HS extracted from normal cartilage have a higher binding affinity toward VEGF (IC50 value: 13 µg/ml) that was not observed in total GAG extract.

In summary, OA total GAG showed to loss their affinities toward FGF2 and to increase affinity toward VEGF. The affinities toward FGF-2 and VEGF are found to be mainly due to HS. However, in total GAG from CT cartilage, the presence of CS/KS is able to inhibit HS binding to VEGF, and this inhibitory effect of CS/KS is loss during OA.

### **GAG from OA cartilage lose their abilities to induce adhesion and proliferation of MSCs**

In order to investigate the functional activities of GAGs on different cells, total GAGs extracted from CT and OA cartilage were used in a dose effect manner to compare their abilities to modulate Mesenchymal Stem Cells (MSCs) adhesion and proliferation in vitro. We chose those MSCs according to their potential ability to repair matrix. In the adhesion test, the total number of MSC initially seeded per well was considered as 100%. Basal condition was defined in the absence of GAGs, as the number of cells that adhered 3 h after seeding. Under this condition, only 55% of MSCs initially seeded were adherents. Treatment with GAGs from CT cartilage increased adhesion in a dose dependent manner, reaching 92% of MSC adhesion (Fig 4A). Importantly, GAG from OA used at the same concentrations did not induce any significant effect on MSC adhesion, as compared to cells without GAG, indicating that the GAGs adhesion function is altered in GAGs from OA cartilage.

Dose-effect experiments were then conducted to investigate the effect of GAG from OA on MSCs proliferation. MSC numbering was performed 7 day after seeding. Cells cultured in the absence of GAGs were considered as the control of proliferation (100%). Increasing doses of GAG from CT cartilage in culture medium induced a significant increase of 30% of cell numbers, whereas no significant difference was observed with GAG from OA cartilage (Fig 4B). These results suggest that GAGs from OA patients have lost their capacity to induce cellular functionalities related to adhesion and proliferation, at least for MSCs.

### **GAGs from OA cartilage induce hypertrophic phenotype of murine articular chondrocytes.**

Hypertrophic phenotypes of chondrocytes are a characteristic of OA cartilage, that could be induced *in vitro* by addition of IL-1 $\beta$  in culture media. Thus, we investigate whether GAGs extracted from OA and CT cartilage have capacities to modulate such hypertrophic phenotype in primary cultured of murine articular chondrocytes. Cells were harvested from femoral heads, condyle and tibial plateau of normal mice. These primary cultures were performed alone or associated to 1 ng/ml of IL-1 $\beta$ . Chondrocytes were characterized by the relative expression of markers measured by real-time PCR at passage 0. Anabolic chondrocyte markers were coll2 and aggrecan; catabolic markers were MMP3 and MMP13, and hypertrophic markers were VEGF and CollX. Expression pattern of chondrocytes cultured 24 h in presence of the extracted GAG were compared to basal levels of expression in chondrocytes alone, referred as 1 DDCT. Treatment of cells with the CT GAG added in the basal culture media (without IL-1 $\beta$ ) did not induce modulation of MMP3, MMP13, coll2, Aggrecan, collX, and VEGF expressions, indicating that these CT GAGs do not alter cartilage chondrocyte phenotype (Fig 5). However, addition of the same dose of GAG extracted from OA cartilage significantly increased the expression of MMP3 (1000 DDCT); MMP13 (25 DDCT) and VEGF (2 DDCT) and significantly decreased expression of coll2 (0.7 DDCT) and Aggrecan (0.7 DDCT) (Fig 5). This suggests a rupture of the equilibrium between anabolic and catabolic markers toward the catabolic pathway. These results are sustained by the observation that addition of GAG from OA patient induced a significant 2 folds increase of VEGF expression (2 DDCT), again suggesting the induction of a hypertrophic phenotype by the OA GAGs.

### **HS GAG from OA cartilage induces hypertrophic phenotype of murine articular chondrocyte**

Next we sought to investigate the effect of each sulfated GAG separately. In order to observe the modulatory effect of these GAGs on hypertrophic phenotype of cell, primary culture of murine articular chondrocytes was performed. Then HS, CS and KS were purified by enzymatic digestion and tested *in vitro*. Interestingly, we noticed that HS is the most active GAG to induce the expression of MMP3 and MMP13 compare to KS with remarkably lesser extent and finally CS with the lowest effect on our interest factors (Fig 6). Of importance, while there is no effect of total GAG on induction of MMP13, we observed an upregulating effect of KS alone (Fig 6). We hypothesize then, CS and HS might have an inhibitory effect on KS.

## DISCUSSION

OA is the most prevalent joint disease in the population, for which there is not curative treatment. OA is characterized by an enhanced catabolic metabolism of the articular cartilage, with degradation of ECM. Most studies in OA cartilage ECM have been concentrated on one of its major glycanic component that is the CS family of GAG. However, HS, the most important glycanic regulators of cell behavior, are also present in the cartilage, in where they might excerpt important regulatory roles. In order to investigate the implication of HS in the OA articular cartilage, we studied and compared structural and functional changes of total GAGs, CS, HS and KS in the cartilage from OA. In this study we demonstrated that HS are present in human cartilage and that their concentration is decreasing and their functionalities are strongly altered, during OA. The origins of macroscopic normal CT cartilage was from Hip which differs from the OA samples due to difficulty to harvest normal samples from healthy knee joints. We show that the sulfation pattern of the HS disaccharide is increased in OA and this is associated to changes in the HS binding affinities toward HBP involved in cartilage homeostasis, such as FGF2 and VEGF. This was associated with altered GAGs functionalities on cells: GAG, and particularly HS, from OA cartilage are unable to induce adhesion and proliferation of hMSC, but significantly induce a hypertrophic phenotype in murine articular chondrocytes. It is well know that during OA chondrocytes are surrounded by an abnormal ECM, which induces phenotypic and metabolic alterations. Our original data demonstrate for the first time that HS are strongly involved on these ECM phenotypic alterations that might possible results in remodeling and phenotypic alterations in the human disease.

Since many years quantitative and structural modifications of GAG and PG have been described during the articular aging process in humans, but are merely focus on CS/KS PG such as an Aggrecans which are mostly associated to structuration and water retention on the diseased tissue. [26-29]. Change in aggecan composition influence chondrocyte behavior through specific binding receptors, which can ultimately affect its metabolism, phenotype and properties. When the CS/KSPG are degraded, the collagen network becomes accessible to the enzymatic activity. Furthermore GAG synthesis is defective, characterized by a shorter aggrecan size, a lower number and shorter size of CS and KS chains and a modification in the ratio of the chondroitin 6-sulfate to chondroitin 4-sulfate ratio [30] This increased "turnover" is related to a high enzymatic activity, linked to the presence of many pro-inflammatory factors such as cytokines IL-1b, TNF $\alpha$  and IL-6 [31]. Thus, during the aging process, it's well known that changes in the quality of CSPG pave the way for OA and our results confirm this global decrease of CS sulfation.

Because HS have strong modulatory activity on the cell behavior of in most tissues, we hypothesized here that HS might also be altered in OA cartilage, and that they can centrally modify the properties of cartilage cells and ECM homeostasis. If effective, HS chain structural and functional modifications can alter HBP activities, and consequently alter the transduction of cellular pathway and properties. Even if it's clear that the HS and CS sulfation patterns are

implicated in regulating signaling pathway (for review, ref 2014), studies demonstrating the involvement of HS in the imbalance of anabolic versus catabolic pathway during OA processes are scarce (ref). This lack of studies could be explained since HS content in adult cartilage is low as compared to CS. Another limitation might be the difficulty to extract and purify HS from the CS rich cartilage, so mainly studies are focused on the core protein rather than HS chain description. According to an adapted protocol that permitted to us to purify sufficient amount of HS from human samples, we were able to demonstrate for the first time a significant decrease of HS amount in OA cartilage as compared to CT. This quantitative modification was associated to qualitative alteration of the glycanic molecules, which showed a significant increase of mono- and di-sulfated HS amount. This indicates that HS levels are reduced in OA cartilage, but the existing glycans bear increased levels of sulfation. Our data confirmed previous works reporting an increase of mono-sulfated CS in OA, characterized by an increased GalNAc 6-O-sulfation [32]. However, although alteration on the sulfation patterns of CS were here observed, they did not modify CS functionality, as did the altered HS.

The functional strength of HS is associated to their chemical structure, which is particularly complex and depends of several families of enzymes including the anabolic glycosyl transferases (EXTs and EXTLs), epimerase, and sulfotransferases (NDSTs and HSTs), and the catabolic heparanase and HS 6-O-sulfatase Sulfs. Xx. To sustain our hypothesis of a link between HS sulfation and modifying activity of GAG in OA, studies from Lotz et al also demonstrate that changes in sulfation of GAG can regulate growth factor signaling in cartilage: Sulf1 and Sulf2 are the two HS 6-O-endosulfatases that edit the sulfation of multiple HSPG, regulating the activity of multiple growth factors. Thus, Sulf1 and Sulf2 are essential for mammalian development and cell survival [33]. Increased expression of Sulf1 and Sulf2 has been detected in human OA tissues and in aging mouse as compared with normal joints [34]. But spontaneous cartilage degeneration and surgically induced OA were significantly more severe in Sulf1(-/-) and Sulf2(-/-) mice compared with wild-type mice. Indeed, Sulfs were shown to enhance bone morphogenetic protein (BMP) /Smad 1/5 but also to inhibit FGF/Erk1/2 signaling in articular chondrocytes, participating to the maintenance of cartilage homeostasis [35]. More recently, it was demonstrated that intra-articular injection of Sulf1 in mouse OA model following surgical destabilisation of the medial meniscus have the potential to prevent cartilage degeneration by suppressing MMP-13 via an effect on FGF2/Erk1/2 signaling [36]. Although binding of either growth factor or tissue repair effect to HS has not been investigated in these mutants, the results are in agreement with a role of HS sulfation pattern in regulating the activity of cell signaling pathways during OA. These data suggest that de-sulfation of HS induced by Sulf could have a protective effect during OA, which is in accordance with our observation of the presence of over-sulfated HS during OA.

Interestingly, we show that the cartilage GAGs capacities to bind to FGF2 are strongly associated with HS and that these binding capacities are lost in OA. The sulfation level on HS is increase during OA, so therefore we hypothesis that sulfation modifications of HS during OA are associated to modification of binding affinities towards FGF2. This is in accordance with other

studies demonstrating that FGF-2 binding affinity to heparin is mainly regulated by 2-O and 2-O sulfation [37] FGF-2 is produced endogenously and sequestered in the articular cartilage ECM [38]. Upon cartilage injury, FGF-2 is released from its bound matrix and subsequently activates the ERK signaling pathway [39]. The outcome of this event is controversial: it has been associated to catabolic effects in human articular chondrocytes via upregulation of matrix degrading enzyme, such as MMP13 or aggrecanase, inhibition of ECM accumulation and PGs synthesis, and clustering of cells characteristic of arthritic states, whereas chondro-protective role were shown in murine cartilage and OA models [40]. However, we can hypothesize that during OA, the decrease of available HS and the modification of HS sulfation pattern that is associated to a decrease of FGF-2 binding affinity, would be involved in the decrease of FGF-2 sequestration in the ECM, therefore associates to an increase of FGF2 bioavailability for its catabolic activity on chondrocytes (Fig 6 )

Accordingly, we show that whereas total GAGs extracted from CT cartilage were not able to bind to VEGF, they gain an ability to bind to VEGF during OA. Interestingly, VEGF is an essential chondrocyte survival factor during bone formation, skeletal growth and postnatal homeostasis. It has apparently a destructive role in OA. First, increased VEGF expression correlates with increased OA severity, and VEGF could be induced by an array of catabolic triggers in chondrocytes. Second, VEGF can affect the chondrocytes, via angiogenesis leading to osteophyte formation, through induction of matrix proteases and to differentiation toward a hypertrophic state. Third, in surgically induced knee OA in mice, a model of post-traumatic OA in humans, increased expression of VEGF is associated with catabolic processes in chondrocytes and synovial cells. Finally, conditional knock-down of VEGF attenuates induced OA. Furthermore intra-articular injection of anti-VEGF antibodies suppresses OA progression, and oral administration of the VEGFR2 kinase inhibitor Vandetanib attenuates OA progression. It's already demonstrated that VEGF binding affinity to its receptor and further activation pathways are regulated by 6-O sulfated HS. Then, we suggest that during OA, increased sulfated pattern of HS, associated to increase binding affinity to VEGF and its activity could increase hypertrophic state of chondrocytes.

To go further in this hypothesis we decided to test the effect of cartilage GAG on murine articular chondrocytes and demonstrated for the first time that GAG from OA cartilage merely, are able to modify the catabolic/anabolic balance of normal chondrocyte. Moreover, increased expression of VEGF was also observed suggesting the induction of a hypertrophic signaling. More strikingly the same experiment performed with purified extract of HS, CS or KS, clearly demonstrates clearly that this effect is mainly due to HS and not Cs or KS. These results demonstrated for the first time that pure HS could have functional effect on catabolic phenotype of murine chondrocytes. It remains to clarify whether one particular PG core protein is carrying the deleterious HS chains or if these chains are present in all HSPG expressed in the OA articular cartilage.

Numerous HSPG are presents in the cartilage, some are secreted in the matrix as perlecan, or linked to the cell surface, as syndecans and glypicans. HSPG have important biological activities, which has been mainly demonstrated mainly during bone and cartilage development [41]. The importance of the HS chains in cartilage hemostasis, particularly in OA, can be associated to observation made when focusing to the PG core proteins: i) Syndecan are transmembrane HSPGs involved in migration, proliferation and differentiation of chondrocytes, according to their ability to interact and modulate FGF, BMP2 and TGF B-1 [42] ii) SDC-3, the major HSPGs expressed during chondrogenesis, can impair the ability of BMP2 to promote cartilage differentiation, iii) loss or blockage of SDC4, which is expressed in OA cartilage, protect against rapid cartilage degeneration in mouse OA models [43]. Iv) Glypican-3 is widely expressed in embryonic and adult tissues morphogenesis and Glypican 4 is involved in regulation of chondrocytes biology, zebrafish cartilage and bone development [44]. Finally, v) perlecan is a HS/CSPG identified as a regulator in cartilage development and maintenance. It influences chondrocyte metabolism via signaling receptors or by modulating the FGFs expression and sequestration in the pericellular ECM [Vincent et al., 2007]. Perlecan is expressed in normal cartilage [45] and upregulated in cells from the area adjacent to the main OA defect [46]. Perlecan is an extracellular matrix HSPG suggested to stabilize ECM during OA, and synovial perlecan plays an important role in osteophyte development in OA [47]. All these HSPGs can affect anabolism/catabolism balance in cartilage even if being present in different compartments during OA. They could be good candidates to explore transduction pathways associated to modification of HS chains.

Finally, the known functional roles of HS, associated to their capacities to potentiate growth factors activities, cytokines or chemokines and to protect them from proteolysis, places HS as a sort of reserve of available factors in ECM niches, by allowing for example a better cell survival, especially in pathological conditions of inflammation and tissue degradation. We previously demonstrated that fine changes in GAG species (HS and CS) composition and structure could affect the cells and tissue integrities (muscle, brain, bone marrow and bone...), [14] and potentially participating to pathological processes [18, 19]. Clearly, our results demonstrate for the first time that total GAG extracted from OA cartilage, and specifically their HS content can importantly modify affinities to at least 2 HBPs involved in the catabolic process of OA such as FGF2 and VEGF. In this context, we propose a model of action of the altered HS chains depending on their fine structure and we hypothesis that these HS chains could be associated to perlecan.

## **Conclusion**

Abnormal ECM is already known to affect differentiation phenotype and metabolism of chondrocytes, and the role of HS are probably underestimated, since, we demonstrate that changes in HS amount, structure, binding quality and cellular functionality, could pave the way for OA during aging process. In this context, HS antagonists could constitute a new therapeutic strategy to favor cartilage repair.



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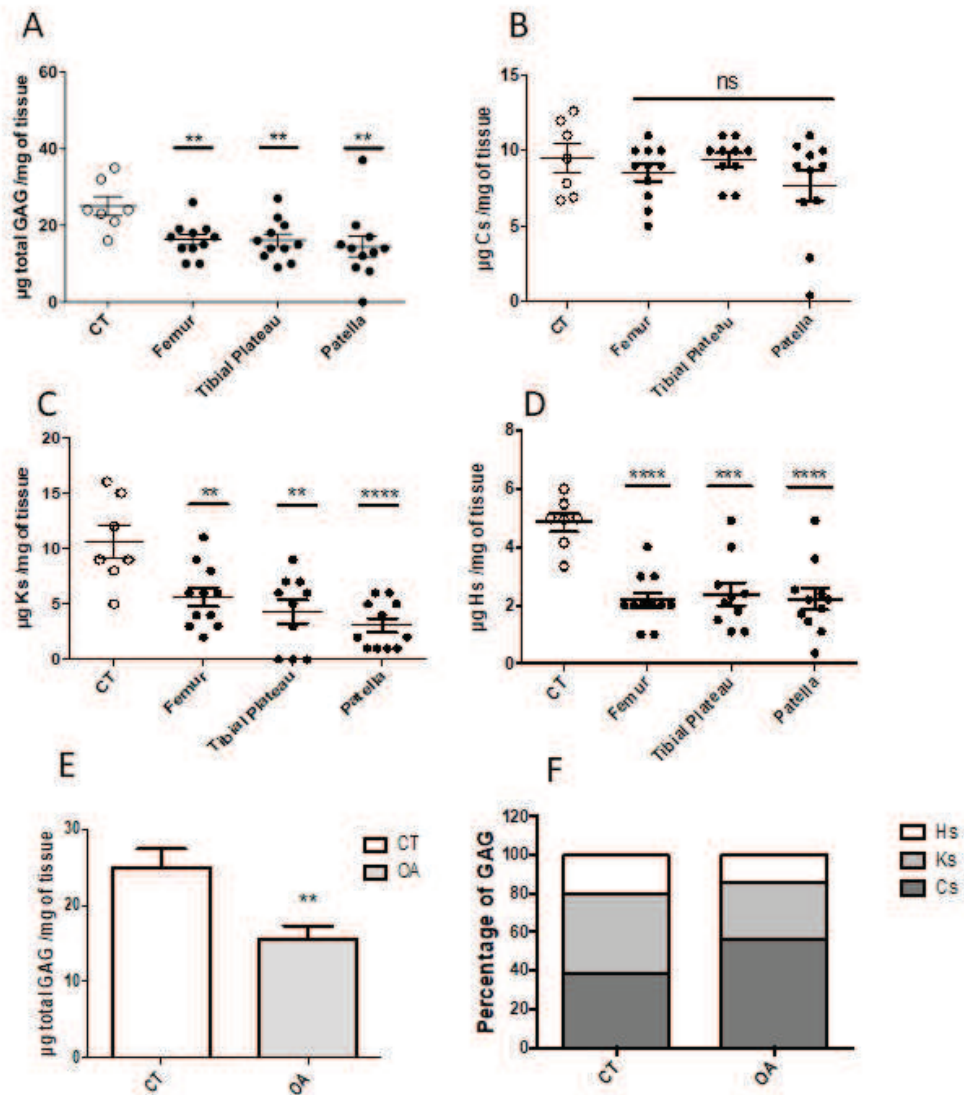
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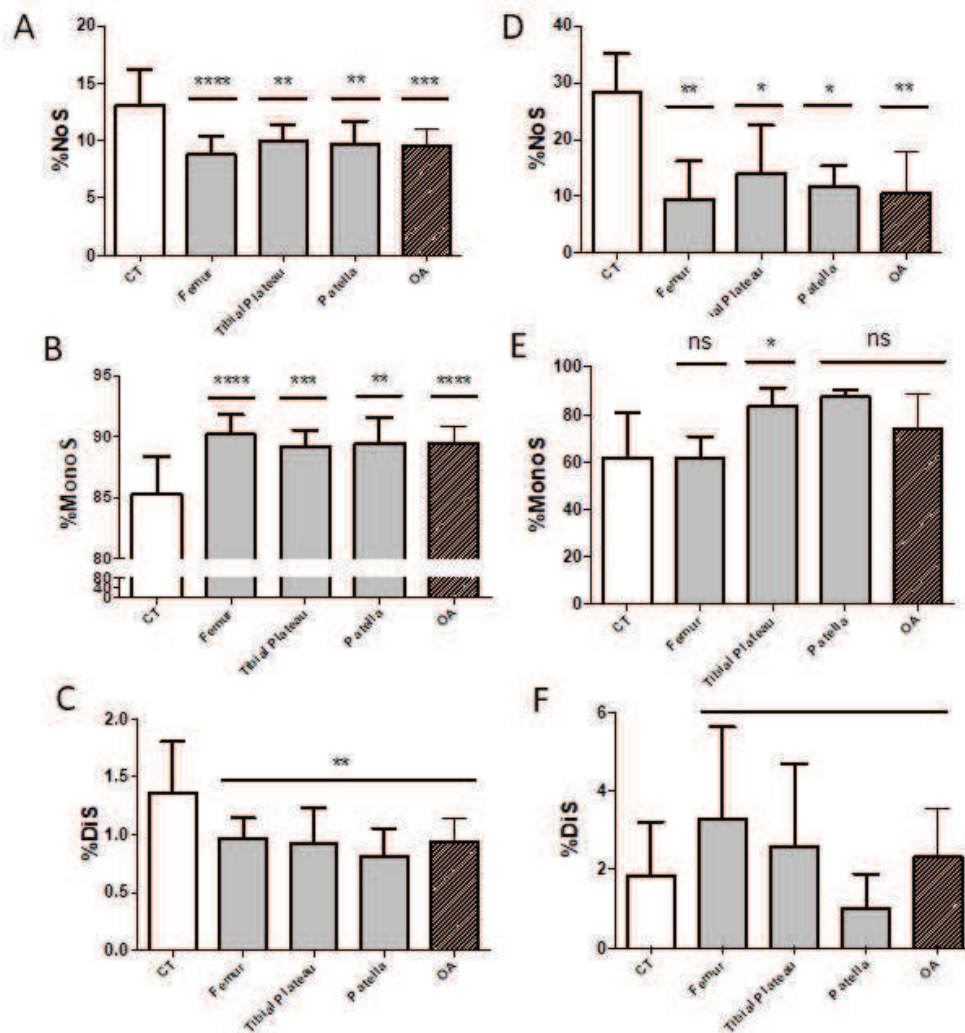
## Figures of article 1

Clinical Characteristics	CT		OA	
	Female	Male	Female	Male
Sex				
Age (year)	82 ± 5	88	78 ± 13	74 ± 7
Length (cm)	160 ± 1	NA	156 ± 2	178 ± 3
Weight (kg)	50 ± 6	NA	66 ± 4	96 ± 24
OA severity (K-L global)	2 ± 0	1	4 ± 0	4 ± 0
Smoking condition	NA	NA	No	No
Professional athlete	NA	NA	No	No
Menopause	Yes	NA	Yes	NA
Treatment				
Analgesics	NA	NA	No	Yes
AINS *	NA	NA	No	No
Corticoid	NA	NA	No	No
Hyaluronic Acid	NA	NA	Yes	No
Period of symptoms(Year)	NA	NA	12 ± 4	9 ± 3
Mechanical pain	NA	NA	Yes	Yes
Effusion	NA	NA	No	NA
Evaluation of pain/100	NA	NA	75 ± 7	65 ± 7

**Table 1: Patient's descriptions and treatments.** All informations are expressed as means +/- SEM, comes from average of all patients and healthy donors. Female CT n= 6, Male CT n= 1, Female OA n= 5 and Male OA n= 6. Kellgren–Lawrence (KL) Radiographic classification of knee osteoarthritis 0-4: 0 normal, 1 doubtful, 2 minimal, 3 moderate and 4 severe (Pettersson et al., 1997). Evaluation of pain 0-100: 0-20 very low or low, 20-40 mild, 40- 60 moderate, 60-80 high and 80-100 very high pain (Bortoluzzi et al., 2013). \*AINS= Anti Inflammatory Treatment



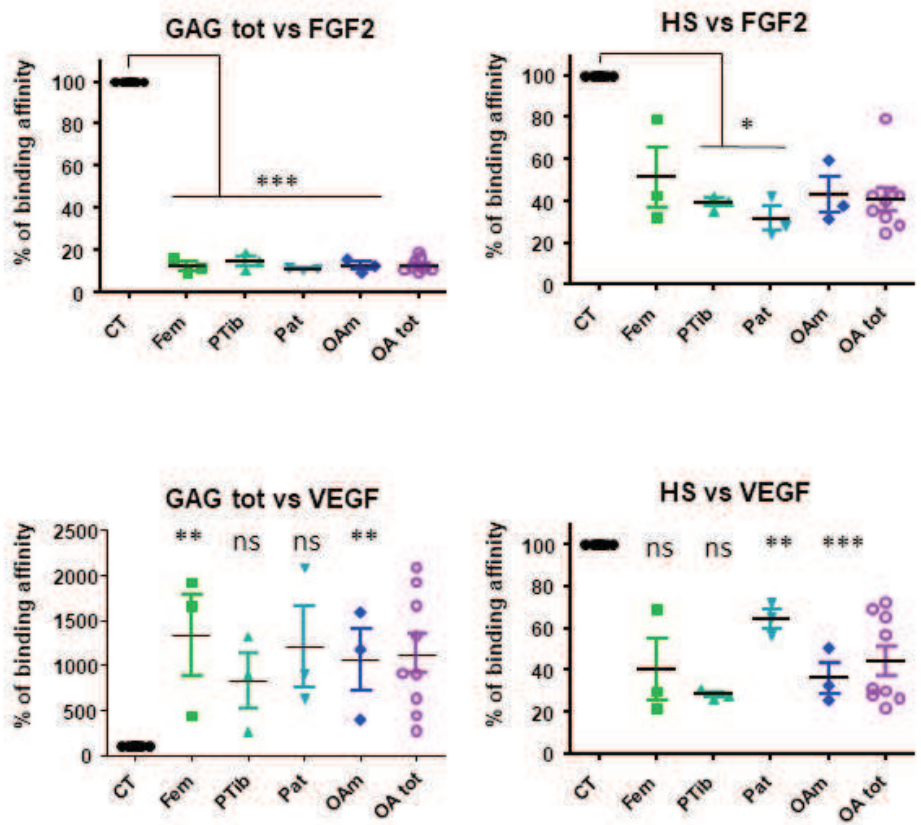
**FIGURE 1.** Total sulfated GAGs were extracted and quantified according to the enzymatic digestion protocol. GAG levels were normalized in  $\mu\text{g}$  of total GAGs by mg of dried tissue. A) Total sulfated GAGs level of cartilage from healthy donor and OA patients. B)  $\mu\text{g}$  of Cs per mg of tissue. C)  $\mu\text{g}$  of Ks per mg of tissue. D)  $\mu\text{g}$  of Hs per mg of tissue. Data are shown for CT ( $n = 7$ ) and OA ( $n = 11$ ). E) Total sulfated GAGs levels in OA and normal articular cartilage (CT). F) Percentage of different fraction of GAG from OA and normal articular cartilage (CT), chondroitin sulfate (Cs), Keratan sulfate (Ks) and Heparan sulfate (Hs). Data are shown for CT ( $n = 7$ ) and OA ( $n = 11$ ).



**FIGURE 2.** HPLC analysis of sulfation pattern of CS (panel A, B, C) and HS (panel D, E F) disaccharides units in GAG chains. CS purified from control compared to CS purified from OA patients. HPLC analysis allowed to determine the relative composition in each disaccharide units GAG species, namely the non sulfated (NoS), the monosulfated (MonoS), the disulfated (DiS) and the trisulfated (TriS) after enzymatic digestion. The statistical significance of differences between the groups was determined by one way anova test. All values were analyzed and all graphs were made using Graph Pad Prism 5 software. Each sample was analyzed 2 times and each analysis was repeated 2 times. A p value < 0.05 was considered to be statistically significant. Note that \*\* < 0.005, \*\*\* p < 0.0001. Data are shown for CT (n = 6) and OA (n = 7).

IC50 (µg/ml)	CT	Femur	P. Tibial	Patella	OA
<b>Total GAG</b>					
FGF-2	11 +/- 4	88 +/- 23	75 +/- 23	91 +/- 1	84 +/- 20
VEGF	NoC	113 +/- 76	187 +/- 125	106 +/- 36	136 +/- 78
<b>HS</b>					
FGF-2	34 +/- 14	74 +/- 31	88 +/- 8	110 +/- 30	82 +/- 25
VEGF	13 +/- 2	39 +/- 19	45 +/- 3	21 +/- 2	38 +/- 12
<b>CS + KS</b>					
FGF-2	NoC	NoC	NoC	NoC	NoC
VEGF	NoC	NoC	NoC	NoC	NoC

**Table 2 : IC50 value of purified total GAG, purified HS, and mix of CS and KS according to their ability to compet for heparin binding to FGF-2 and VEGF.** GAG were extracted for CT cartilage, and OA cartilage. Dinstinct GAG from Femur, P.Tibial and Patella were tested an Elisa competitive binding assay. IC50 doses were determined in µg/ml as the concentration of tested cartilage GAG able to compet for 50% of the binding of HBF to Heparin, and used to calculate % of binding affinity represented in Figure 3. NoC : No competition.



**Figure 3 : % of binding affinity of total GAG and purified HS toward FGF-2 and VEGF.** IC50 value determined in Elisa binding assay and resume in table 2, were used to calculate GAG binding affinity. IC50 value of GAG from CT cartilage were considered as 100% of binding. Then % of binding affinity of distinct GAG from OA Femur, P.Tibial and Patella were calculated according to these 100% values.



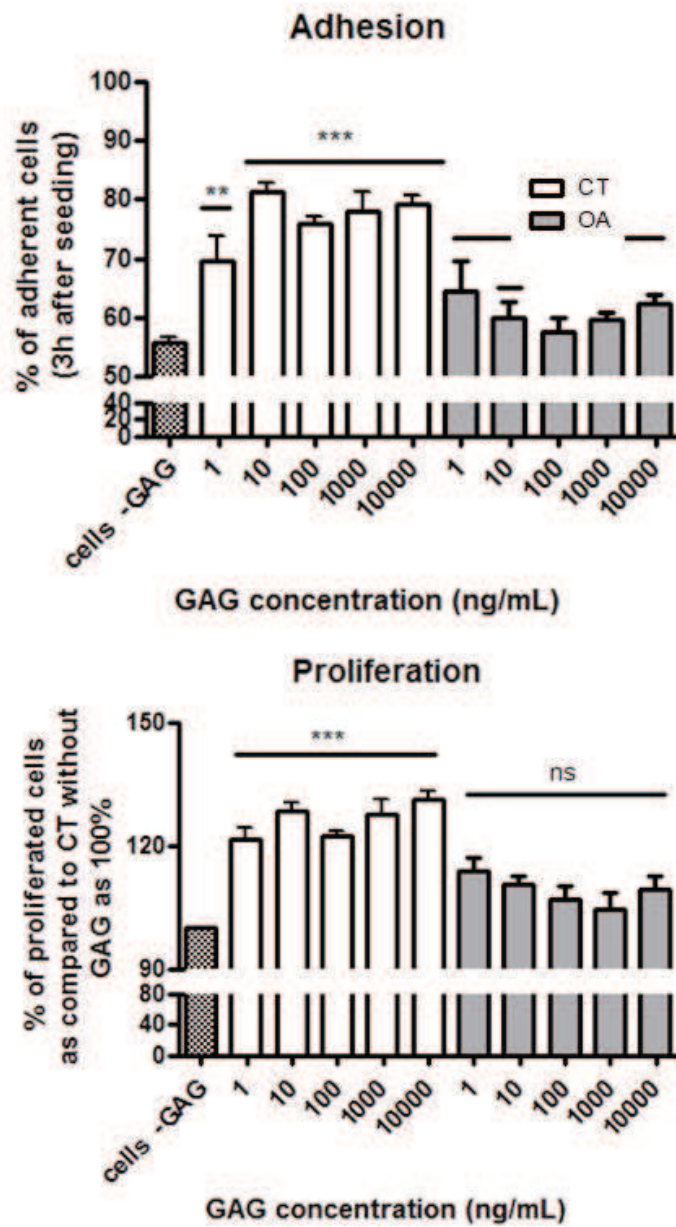
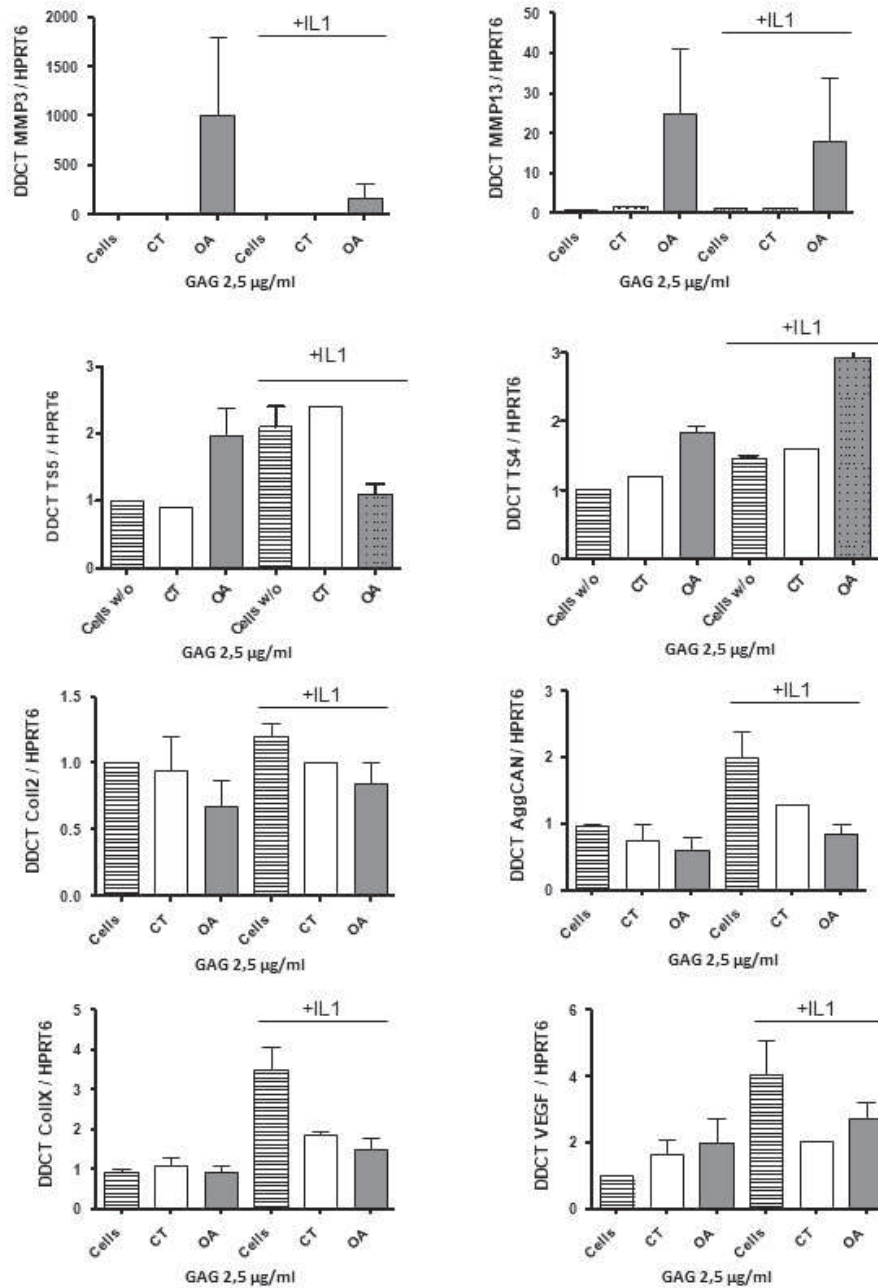
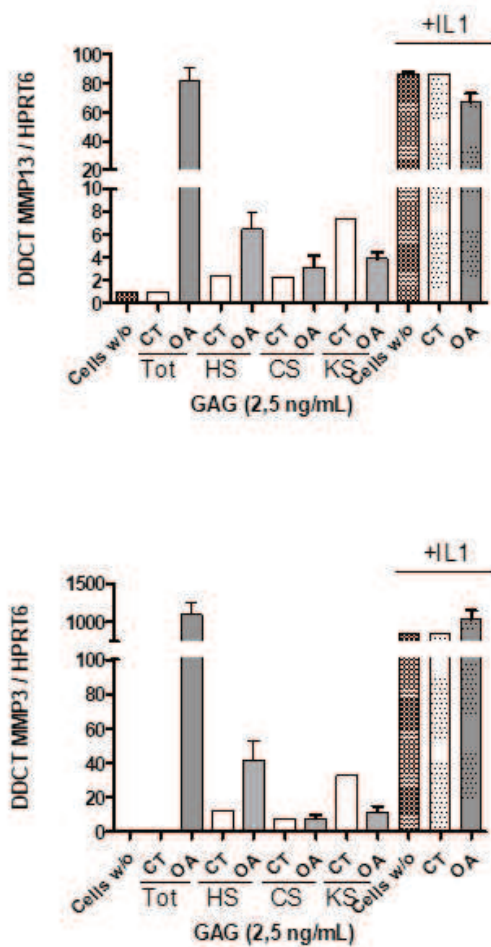


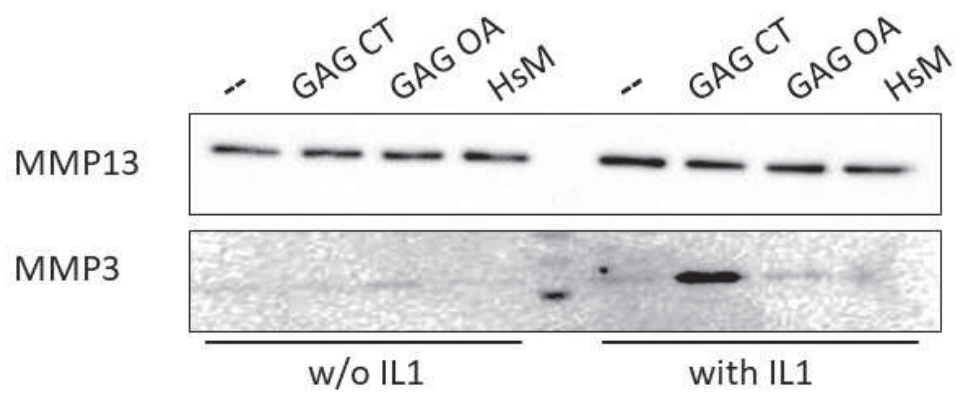
FIGURE 4. Effect of increasing concentration of GAGs from healthy donors (CT, n = 4) and OA patients (n =4). on MSCs adhesion (A) and proliferation (B).



**FIGURE 5.** RQ-PCR analysis of expression pattern of catabolic (MMP13, MMP3), anabolic (Coll2, Aggrecan) and hypertrophic markers on murine articular chondrocytes. Day 6 murine chondrocytes were cultivated for 24h in presence of GAG (2,5 µg/ml) extracted from CT and OA cartilage, in absence and in presence of IL1b. Level of expression of each markers were reported to HPRT6 housekeeping gene and compared to basal conditions as cell alone take as 1 value of DDCT.



**FIGURE 6 . RQ-PCR analysis of expression pattern of catabolic (MMP13, MMP3), anabolic (Coll2, Aggrecan) and hypertrophic markers on murine articular chondrocytes, in presence of purified fractions of HS CS and KS. Day 6 murine chondrocytes were cultivated for 24h in presence of total GAG (2,5 µg/ml) and corresponding HS, CS and KS fraction, extracted from CT and OA cartilage, in absence and in presence of IL1b. Level of expression of each markers were reported to HPRT6 housekeeping gene and compared to basal conditions as cell alone take as 1 value of DDCT.**



**FIGURE 6 . WB analysis of MMP13 and MMP3 expression in supernatant of murine articular chondrocytes, in presence of purified GAG.** Day 6 murine chondrocytes were cultivated for 24h in presence of total GAG (2,5  $\mu\text{g/ml}$ ), extracted from CT and OA cartilage, in absence and in presence of IL1b.

## Supplementary data of Article 1

### 1. Patients characterization

Human cartilage explants were harvested from 11 OA patients undergoing total knee replacement surgeries and from 7 Femoral Head after neck fracture considered as control donor. Table S1 and S2 present here Complete data details for each OA and CT patients respectively, as compared to table 1 from article 1 that is a resume of all the informations.

Clinical Characteristics	OA										
	A	B	C	D	E	F	G	H	I	J	K
Sex	Female	Female	Female	Female	Female	Male	Male	Male	Male	Male	Male
Age (year)	82	64	97	72	73	79	64	84	68	78	72
Lenght (cm)	155	158	NA	NA	NA	NA	180	NA	NA	175	178
Weight (kg)	64	69	NA	NA	NA	NA	98	NA	NA	72	120
Target knee	Left	Right	Right	Right	Left	Left	Right	Left	Left	Right	Left
Type of OA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
OA severity (K-L global)	4	4	4	4	4	4	4	4	4	4	4
Smoking condition	No	Yes	No	No	No	No	No	No	No	No	Yes
Professional athlete	No	No	No	No	No	No	Yes	No	No	No	No
Menopause	Yes	Yes	Yes	Yes	Yes	NA	NA	NA	NA	NA	NA
Treatment											
Analgesics	No	No	NA	NA	NA	NA	Yes	NA	NA	Yes	Yes
AINS*	No	No	NA	NA	NA	NA	No	NA	NA	Yes	No
Corticoid	No	Yes	NA	NA	NA	NA	No	NA	NA	No	No
Hyaluronic Acid	No	Yes	NA	NA	NA	NA	No	NA	NA	No	No
Period of symptoms(Year)	15	10	NA	NA	NA	NA	10	NA	NA	5	11
Mechanical pain	Yes	Yes	NA	NA	NA	NA	No	NA	NA	Yes	Yes
Effusion	No	No	NA	NA	NA	NA	Yes	NA	NA	NA	No
Evaluation of pain/100	70	80	NA	NA	NA	NA	60	NA	NA	NA	70

**Table S1.** Patient's (OA) descriptions and treatments. Kellgren–Lawrence (KL) Radiographic classification of knee osteoarthritis 0-4: 0 normal, 1 doubtful, 2 minimal, 3 moderate and 4 severe. Evaluation of pain 0-100: 0-20 very low or low, 20-40 mild, 40- 60 moderate, 60-80 high and 80-100 very high pain. \*AINS= Anti Inflammatory Treatment,

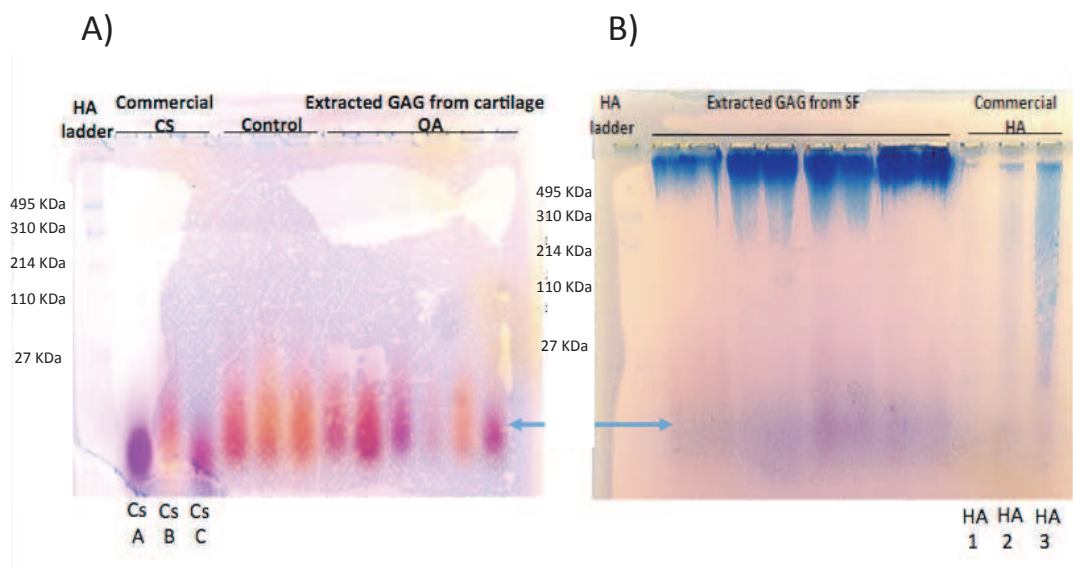
NA= Not Available.

Clinical Characteristics	CT						
	L	M	N	O	P	Q	R
Sex	Female	Female	Female	Female	Female	Female	Male
Age (year)	81	82	86	78	89	77	88
Length (cm)	NA	NA	NA	159	160	NA	NA
Weight (kg)	NA	NA	NA	46	54	NA	NA
Target knee	Right	Right	Right	Left	Left	Right	Left
Type of OA	NA	aging	aging	aging	aging	NA	aging
OA severity (K-L global)	2	1	2	2	2	2	1
Smoking condition	NA	NA	NA	NA	NA	NA	NA
Professional athlete	NA	NA	NA	NA	NA	NA	NA
Menopause	Yes	Yes	Yes	Yes	Yes	Yes	NA
<b>Treatment</b>							
Analgesics	NA	NA	NA	NA	NA	NA	NA
AINS*	NA	NA	NA	NA	NA	NA	NA
Corticoid	NA	NA	NA	NA	NA	NA	NA
Hyaluronic Acid	NA	NA	NA	NA	NA	NA	NA
Period of symptoms(Year)	NA	NA	NA	NA	NA	NA	NA
Mechanical pain	NA	NA	NA	NA	NA	NA	NA
Effusion	NA	NA	NA	NA	NA	NA	NA
Evaluation of pain/100	NA	NA	NA	NA	NA	NA	NA

**Table S2.** Healthy donor's descriptions and treatments. Kellgren–Lawrence (KL) Radiographic classification of knee osteoarthritis 0-4: 0 normal, 1 doubtful, 2 minimal, 3 moderate and 4 severe. Evaluation of pain 0-100: 0-20 very low or low, 20-40 mild, 40- 60 moderate, 60-80 high and 80-100 very high pain. \*AINS= Anti Inflammatory Treatment, NA= Not Available

## 2. Analysis of GAG's chain size and HA presence in cartilage during OA

Cartilage and synovial fluid are rich in HA, a non-sulfated GAG, that it is not detected by DMMB technic and so is not quantified in our extraction protocol. So it can be present and interfere with the functional activity of our purified GAG, HS, KS and CS. Moreover, a second important parameter for GAG properties is the GAG chain size. In order to verify the quality and chain size of the GAG extracted from cartilage as well as to verify the absence of HA contamination after extraction, we performed Agarose gel, to analyse and compare electrophoresis pattern, 10 µg of GAG were loaded in a 2% agarose gel and run in TAE 1X buffer for 150 min at 50 mV. After migration, the gel was stained with 0.005% Stains-All solution (Sigma-Aldrich) overnight in the dark and destained with water.



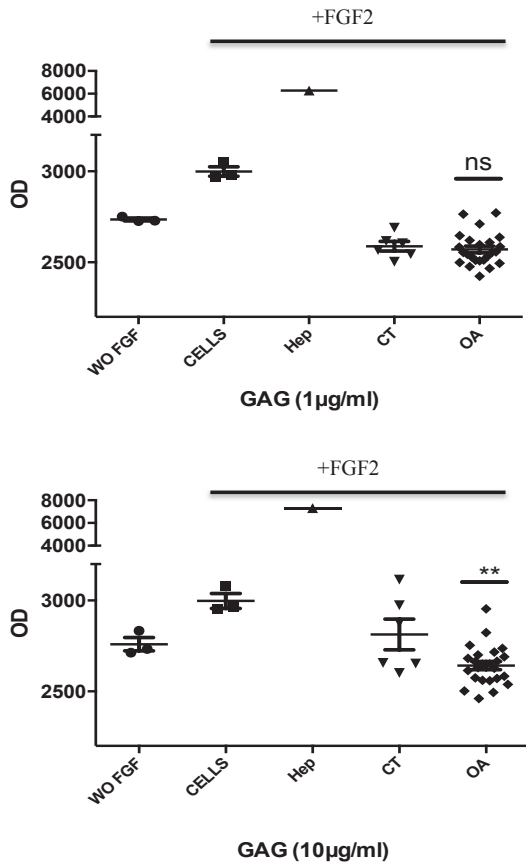
**Figure S1.** Electrophoresis pattern of GAG. 10  $\mu$ g of GAG were loaded on gel. (Panel A), HA ladder, CS from IBSA company, with different origin A, B and C, compared to GAG extracted from 3 CT cartilage samples, and 6 OA cartilage samples. (Panel B) 8 different GAG extracted from SF compared to increasing doses of commercial HA (1, 2 and 3; 5-10-20  $\mu$ g).

The analysis of the electrophoresis pattern is a qualitative data that complete the quantitative value that we obtained in paper 1. It confirmed that GAG extract from cartilage (panel A) are devoid of HA, that are not visible in upper side of the gel, as compared to GAG extracted from synovial Fluid and to commercial HA (panel B). Moreover it suggest that GAG from OA cartilage have slight higher size, and at least are not completely degraded, as it was described for CS: we do not observed a igrnificant decrease of HS size. A much more resolutive agarose gel should permit to confirm this information.. It's of interest to note also that XA, and XC, have lower size than XB and GAG from cartilage. Finally we observed clearly that GAG extracted from SF are very rich in HA. Even if this HA do not interfere with DMMB dosage, it is possible that it will interfere with HPLC analysis and maybe functional test of GAG on cells.

### **Effect of GAG extracted from CT and OA cartilage on Baf32 cell mitogenic assay**

According to the difference of GAG binding affinity to FGF2, observed between CT and OA condition (see article 1), we initially performed some experiments to test the effect of these GAG on cellular properties. For that we decided to test the synergic effect of GAG and FGF2 on the

proliferation of BAF32 cells that are fibroblastic cells dependent to heparine and FGF2 for their proliferation.



**Figure S3.** Human cartilage (Control CT versus OA), as compared to cells alone (without w/o FGF and without GAG), to Cells with FGF and without GAG (Cells), and to Cells with FGF and with Heparine (Hep) as a positive control of the experiment. Assay was performed using 96 well plates direct measurements of the cell viability with a fluorometric detection of the reduced products from PrestoBlue. *In brief, BAF32 were cultured in RPMI 1640 Glutamax medium supplemented with 10% SVF, Antibiotic-Antimycotic and 1 ng/mL final IL-3 (R&D systems 403-ML-010). Prior to mitogenic assay, cells are resuspended in starving medium based on RPMI 1640 Glutamax, 10% horse serum and seeded at 10.000 cells/well (100 µL) into 96 well plates overnight at 37°C, 5% CO<sub>2</sub>. Thereafter final concentration of 1 - 10 µg/mL of GAGs and 5 ng/mL FGF-2 are added. Controls are cells stimulated without any GAGs and with as compared to without FGF-2. Cells are incubated 72 hours at 37°C, 5% CO<sub>2</sub> (the doubling time of the cells are around 30 hours). Cell viability was measured after addition of 10 µL of PrestoBlue® in each well, incubation 4 hours at 37°C, 5% CO<sub>2</sub>, and direct Fluorometric detection at 544 ex /590 em.*



Proliferation of Cells is lightly increase in presence of FGF2 alone (CELLS) as compared to w/o FGF2 and clearly induce when Heparine is added to FGF2 as positive control (Hep) at each doses (1 and 10 µg/ml). We observe here that the addition of GAG from CT cartilage clearly inhibit proliferation to the basal level (w/o FGF and w/o GAG), and this inhibitory effect is significantly higher with GAG from OA cartilage at 10 µg/ml. This indicate that GAG from OA cartilage, has a significant inhibitory effect on cell proliferation induced by FGF2 compare to GAG from control cartilage, as well as compare to Heparine. **Interaction of** Proliferation of Cells is lightly increase in presence of FGF2 alone (CELLS) as compared to w/o FGF2 and clearly induce when Heparine is added to FGF2 as positive control (Hep) at each doses (1 and 10 µg/ml). We observe here that the addition of GAG from CT cartilage clearly inhibit proliferation to the basal level (w/o FGF and w/o GAG), and this inhibitory effect is significantly higher with GAG from OA cartilage at 10 µg/ml. This indicate that GAG from OA cartilage, has a significant inhibitory effect on cell proliferation induced by FGF2 compare to GAG from control cartilage, as well as compare to Heparine.

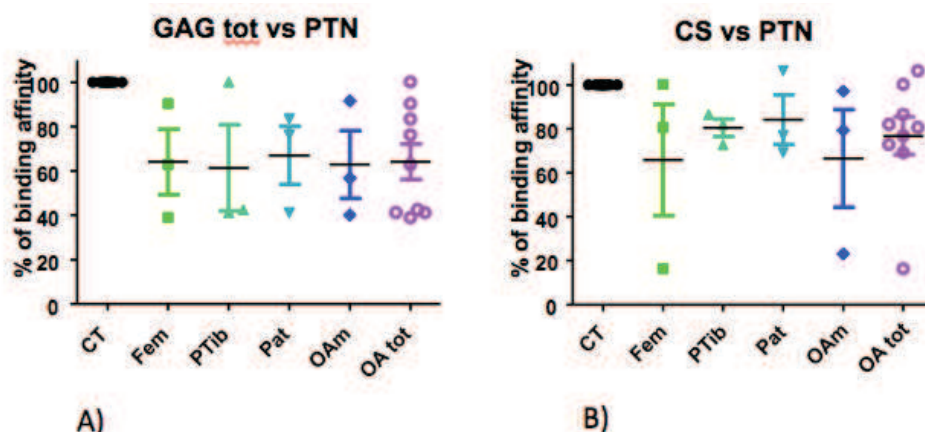
#### **Interaction of GAG from cartilage and PTN**

Previous data from CRRET lab have demonstrated that PTN is a heparin-binding factor that is able to induce hypertrophic phenotype of human mesenchymal stem cells induced to differentiate toward chondrocytic phenotype (Bouderlique et al., 2014). Moreover PTN is suggested to be associated to OA phenotype (see chapter 1). Then we decided to include this HBP as a regulatory factor of interest in our study.

As described in article I for VEGF and FGF2, total GAG extracted from CT and OA cartilage, as well as purified HS and CS/KS mix, were tested for their ability to interact with PTN, in elisa competitive binding assay. Results presented above are expressed as IC50 and % of binding as compared to CT samples as 100% basal level.

IC50 toward PTN (µg/ml)...	CT	Femur	P. Tibial	Patella	OA
<b>Of Total GAG</b>					
	266 +/- 29	465 +/- 143	511 +/- 164	437 +/- 139	473 +/- 126
<b>Of pure HS</b>					
	NoC	NoC	NoC	NoC	NoC
<b>Of pure CS + KS</b>					
	55 +/- 5	154 +/- 122	69 +/- 5	68 +/- 11	63 +/- 6

**Table S3:** IC50 value of total GAG, HS and CS/KS mix toward PTN, according to their ability to compete for heparin binding to PTN. NoC: No Competition. Capacities of cartilage extracted GAGs to bind PTN were evaluated by an ELISA based competition assay as described before in article and performed against 3ng/well of Recombinant Human Pleiotrophin/ PTN (R&D systems 252-PL-050). After washing step, the HBP remaining bond to heparin was targeted by a corresponding specific antibody (anti-PTN: Goat Polyclonal IgG diluted 1/500 R&D systems AF-252-PB), followed by a HRP-conjugated secondary antibody (HRP-AffiniPure Rabbit Anti-Goat IgG 1/5000, Jackson ImmunoResearch 305-035-045).



**Figure S2.** Percentage of total GAG and pure CS/KS mix binding affinity to PTN by ELISA binding assay. Total GAG (A), purified CS (B) Femoral head of control donors CT (n = 2) and three parts of knee of patients OA (n = 3), were tested toward PTN.

Total GAGs extracted from CT cartilage were able to bind to PTN and to inhibit its binding to heparin with EC<sub>50</sub> values of 266µg/mL (Table S3). IC<sub>50</sub> values obtained from total GAG extracted from OA cartilage is 473 µg/mL, corresponding to around 2 folds increase, suggesting a 2 fold decrease of binding affinity in OA, as illustrated in Fig S2.

Surprisingly we observed that purifiedHS extracted from control and OA cartilage samples are not able to compete to heparin binding to PTN, so they are not able to bind directly to PTN. However CS/KS mix from control cartilage are able to compete to heparin binding to PTN, with IC<sub>50</sub> value of 55 µg/ml, indicating that they're able to bind to PTN. Moreover the IC<sub>50</sub> is increased in some part of OA cartilage, suggesting a decrease of binding affinity of CS/KS mix toward PTN, as observed for total GAG.

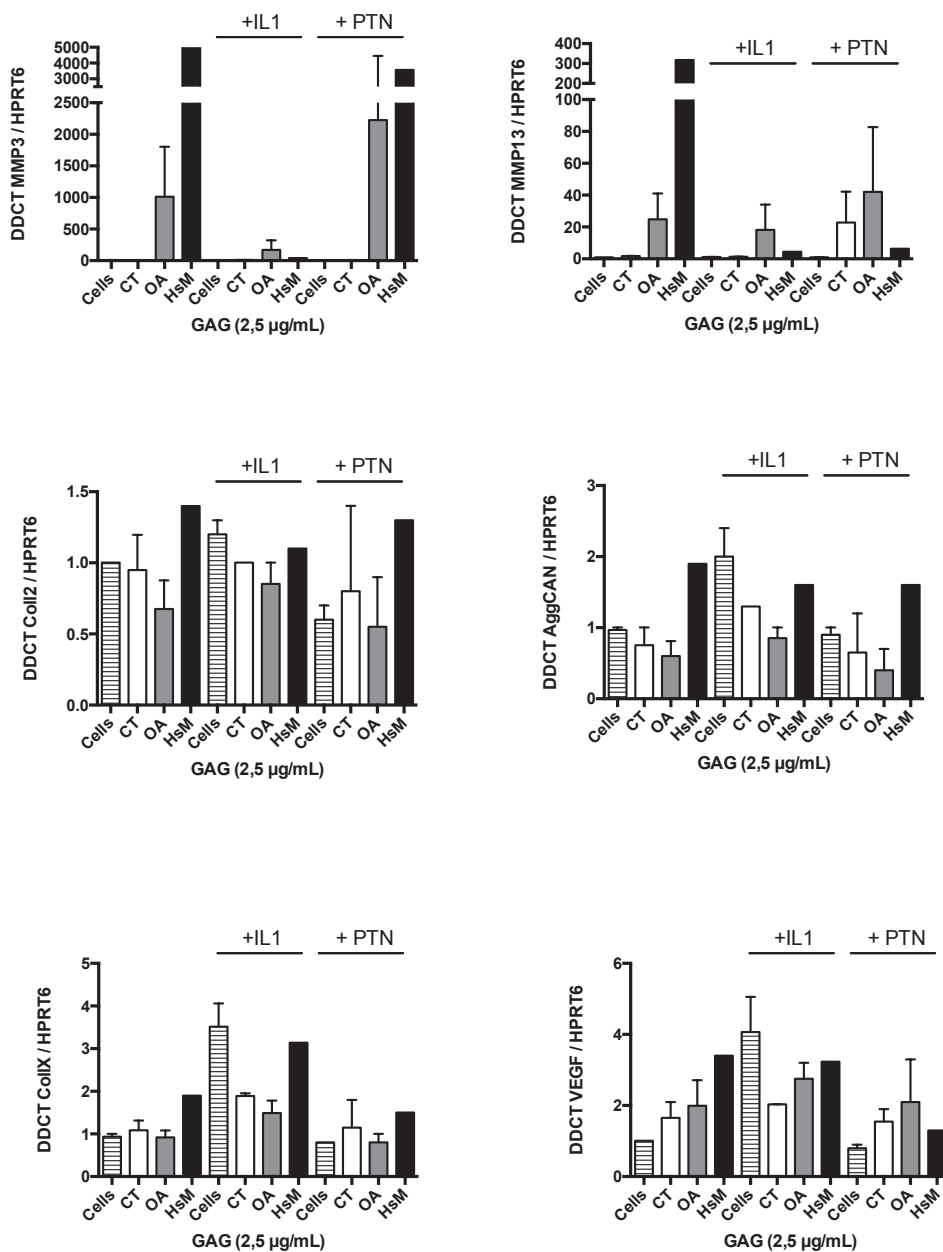
This reveals that total GAG binding to PTN it probably not due to HS binding affinity but to CS/KS mix. This is relevant according to data from the literatures that indicate the same information.

To go further in this functional aspect we decided to test the ability of cartilage GAG to potentiate PTN activity on cells. For that we decided first to test it on human Mesenchymal Stem Cells, as it was already published in the lab (Bouderlique et al) that PTN was able to induce hypertrophic phenotype during their chondrogenic differentiation. This aspect was performed by a Master 1 and a BTS student that I have mastered. But the difficulty of the 3D model of chondrocytic differencitaion of hMSC, do not permit to us to obtain results.

Finally we decided to test if PTN is able to induce catabolic and hypertrophic markers in murine chondrocytes cultures, as IL1b should be able to do. We presented here a first experiment that was performed with PTN added to the murine chondrocytes culture, alone or associated to control CT and OA cartilage, at 2,5 and 50 µg/ml doses. A positive control with addition of IL-1 alone or associated to control CT and OA cartilage, at 2,5 and 50 µg/ml doses was tested also. However we do not observed any differences with PTN and IL-1 alone on cells alone, suggesting that we have a problem with our cytokines during this first experiment. This does not permit to us to conclude on the synergic effect of CT versus OA

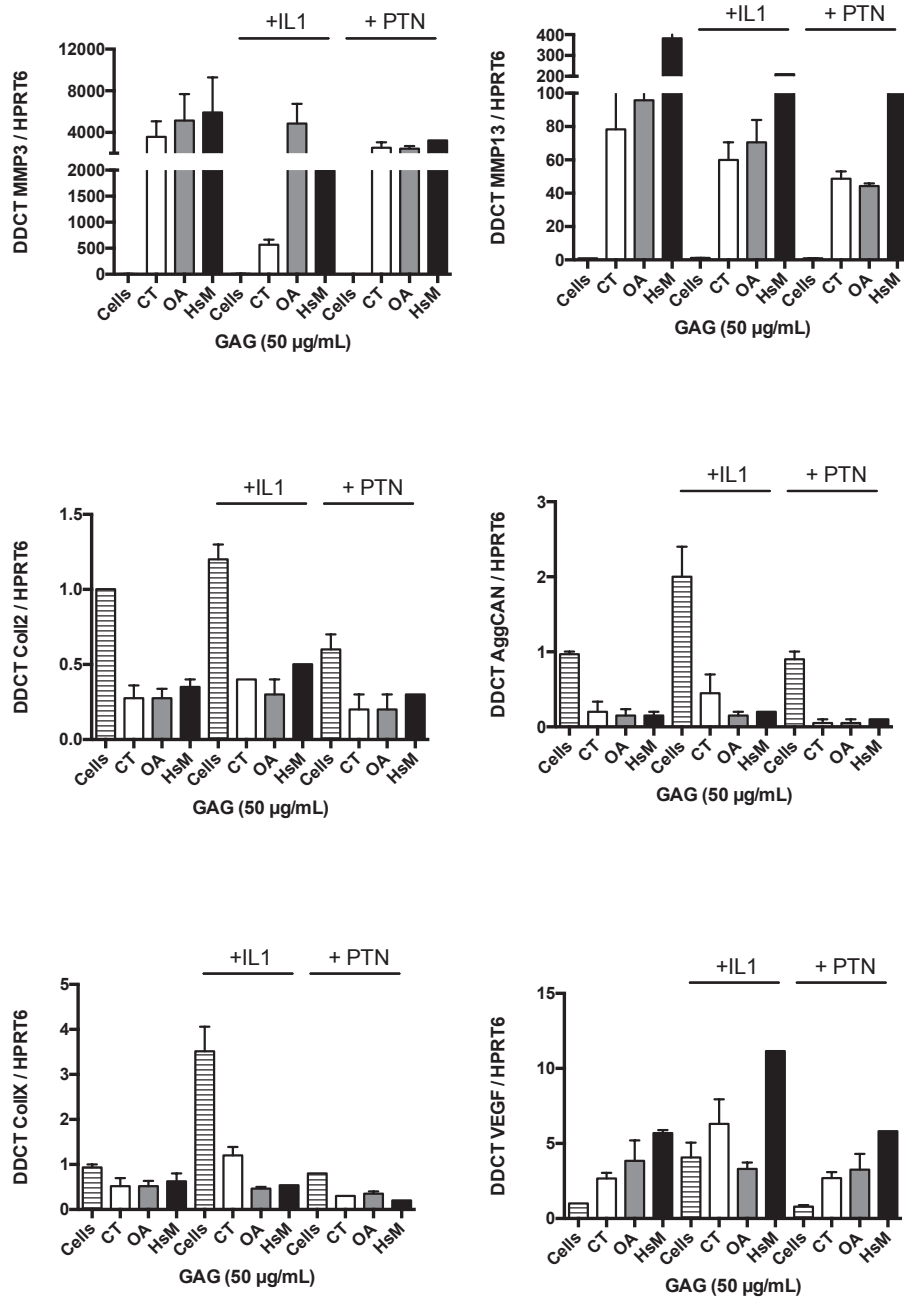
GAG on these HBP (IL-1 and PTN). This kind of experiment need to be performed a new time, maybe with murine PTN on murine cells instead of human one.

However, these data confirmed clearly the reslts described on article 1, on the facts that GAG from OA cartilage induce catabolic (MMP13 and MMP3 increased epression) and hypertrophic phenotypes (VEGF increased expression) of murine chondrocytes.



**Figure S4.** RQ-PCR analysis of expression pattern of catabolic (MMP3, MMP13), anabolic (Coll2, Aggrecan) and hypertrophic markers on murine articular chondrocytes. Day 6 murine chondrocytes were cultivated for 24h

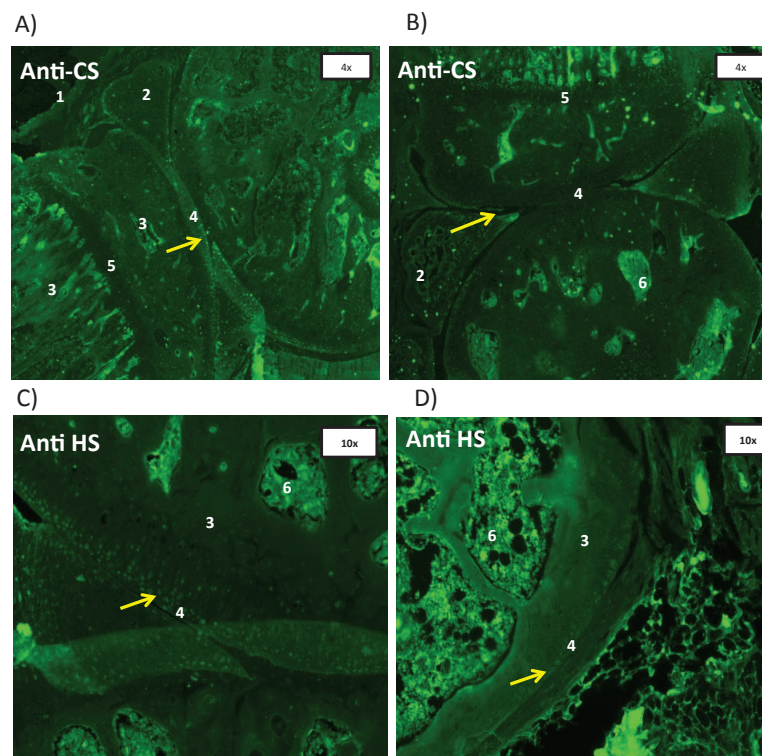
in presence of GAG (2,5  $\mu\text{g}/\text{ml}$ ) extracted from CT and OA cartilage and HsM, in absence and in presence of 1 ng/ml IL1b and PTN. Level of expression of each markers were reported to HPRT6 housekeeping gene and compared to basal conditions as cell alone take as 1 value of DDCT.



**Figure S5.** RQ-PCR analysis of expression pattern of catabolic (MMP13, MMP3), anabolic (Coll2, Aggrecan) and hypertrophic markers on murine articular chondrocytes. Day 6 murine chondrocytes were cultivated for 24h in presence of GAG (50  $\mu\text{g}/\text{mL}$ ) extracted from CT and OA cartilage and HsM, in absence and in presence of 1 ng/ml IL1b and PTN. Level of expression of each markers were reported to HPRT6 housekeeping gene and compared to basal conditions as cell alone take as 1 value of DDCT.

## Immunohistochemistry of Control and OA's cartilage in a murine model of induced OA by destabilization of the medial meniscus (DMM)

Final objective of the project is to propose some glycanic target as potential new therapeutic agent in OA, suitable to be tested in animal models. In that way, during my PhD we were interested to validate that the evolution of GAG pattern that we observed in human samples could be also observed on animal models of OA. Our collaboration with Martine Cohen-Solal team and Eric Hay, permit to have access to histological samples of a murine model of induced OA by destabilization of the medial meniscus (Latourte et al., 2017).



**Figure S8.** Anti CS (IO3H10) and Anti HS (RB4EA12) were tested in both Control and OA's cartilage in a murine model of induced OA by destabilization of the medial meniscus (DMM). Collaboration with Pr. M. Cohen Solal group (Inserm U1132, Hopital Lariboisiere).

Some CS staining is observed in normal cartilage (x4) whereas is not in OA samples. Surprisingly CS staining is stronger in subchondral bone and bone marrow of OA samples as compared to CT. HS staining (x10) is clearly observed on chondrocytes lines in cartilage, whereas it is disappear in OA samples in which this region is degraded. These preliminary data on DMM murine model confirm GAG amount modification in murin samples that could

## **Chapter 2**

### **HS in SF**

- **Article 2**

**Synoviocytes lining cells purified from Osteoarthritis patient present specific heparan sulfate sulfation patterns and functionality as well as GAG biosynthesis enzymes pattern of expression.**

**Shamdani S, Teboul-Core S, Ould-Braham T, Flageollet C, Henni-Chebra N, Chantepie S, Papy-Garcia D, Chevalier X, Albanese P.**

- **Result**

## Article 2

**Synoviocytes lining cells purified from Osteoarthritis patient present specific heparan sulfate sulfation patterns and functionality as well as GAG biosynthesis enzymes pattern of expression.**

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\$ equal contribution to this work

**Key Words; Heparan sulfate, disaccharides sulfation, Synoviocytes, osteoarthritis**

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## **Abstract**

Osteoarthritis (OA) is the most prevalent joint disease characterized by degradation of extracellular matrix (ECM) compounds of articular cartilage, subchondral bone remodeling and synovitis. ECM of the cartilage contains mainly large proteoglycans, namely aggrecans, with glycosaminoglycans (GAGs) chains that are Chondroitin Sulfates (CS) and Keratan Sulfate (KS) species. We recently demonstrated that rare Heparan Sulfates (HS) are also involved in physiopathological processes of OA, thanks to their abilities to interact with heparin-binding proteins (HBP) and to induce hypertrophic phenotypes of chondrocytes. Degradation fragments of GAG chains such as CS or KS are presents in synovial fluid (SF), but few data are available on HS chains amount, as well as their structures and functionality during synovitis in OA. The aim of this study was to characterize HS in SF and fibroblastic-like synoviocytes (FLS). We purified HS and CS from human SF and FLS from OA samples, and compared them to GAG species from normal and OA cartilage and serum. We identified extremely rare structural pattern on the HS sulfated disaccharides compositions of synovial fluid and synoviocytes from OA. Focus on the functional properties of these GAGs, on HBP involved in physio-pathological pathways during OA such as FGF-2 and VEGF; suggest specific binding affinities to these HBP. Moreover GAG from synoviocytes and OA serum are able to inhibit FGF-2 mitogenic activity on BAF32 cells according to a dose effect. Eventually, synoviocytes expressed specific pattern of GAG biosynthesis enzymes that could be involved in this particular sulfation pattern. These results confirm that increase of HS sulfation pattern in SF pave the way for altered pathological processes during OA.

## Introduction

The 2015 Global Burden of Study reports that osteoarthritis (OA) is the most notable non-communicable disease with total disability-adjusted life-years (DALYs) rising by 35% between 1990 and 2015 (1). However, despite its frequency, all currently treatments have only symptomatic effects and are unable to halt the progress of the disease. This ineffectiveness is most certainly explained by their oral route of administration. Intra-articular treatments including visco-supplementation and infiltration of corticosteroids have demonstrated their ability to reduce painful symptomatology. However, these treatments did not demonstrate any contribution to slowing down the anatomical evolution of joint damage. The surgical implant of an artificial joint is currently the only radical treatment for osteoarthritis of the lower limbs but exposes the patient to numerous complications and leaves 20% of cases with residual pain or discomfort (2). Mesenchymal stem cell injection strategies seem promising because they target cells with infinitely greater potential for tissue repair. However, the inflammatory microenvironment of the arthritis joint could be detrimental to these cellular grafts, survival and therefore to their effectiveness. Faced with all these limitations, the development of new therapeutic approaches seems essential.

Long considered as being a consequence of OA disease, inflammation of the synovial membrane (SM) is now recognized as an integral part of the pathophysiological process. Indeed, SM is found from the very first stages of the disease, laying the foundations for a highly rational approach to the development of therapeutic strategies targeting it. Delimiting the cavity of diarthrodial joints, MS is a loose connective tissue made up of synoviocytes arranged in one to four cell layers. There are two types of synoviocytes with different functions. The macrophage-like synoviocytes "clean" debris produced by worn-out cartilage and involve in immunological responses in pathological conditions. The fibroblast-like synoviocytes (FLS) are non-vascular and non-epithelial cells located within the deep layer of the intima that are responsible for the production of synovial fluid (SF) and the extracellular matrix of the synovial intima, including proteoglycans (PGs) (3).

Among the major components of the extracellular matrix (ECM), glycosaminoglycans (GAGs) present a very high structural complexity related to modifications of acetylation, N-sulfation, O-sulfation and epimerization of disaccharide units. These modifications can be homogeneous and repetitive as on the chondroitin sulfate (CS), dermatan sulfate (DS) and keratan sulfate (KS) chains or heterogeneous and thus define more or less sulfated or

acetylated specific regions, as with HS. Thus, HS have a unique molecular signature: non-substituted or low-substituted domains alternate with highly substituted regions, including very high levels of sulfation within the same chain. The presence of these highly sulfated domains determines most of their biological properties, as they contain all the necessary information in recognising and binding of their protein ligands. HS assure structural scaffold functions and relevant biological effects on cell growth, migration, and differentiation. These key regulators of cell behaviour are related to their ability to bind and potentiate the activity of heparin-binding growth factors (HBGF) such as Fibroblastic Growth Factor (FGF) or Vascular Endothelial Growth Factor (VEGF). HS can therefore store and compartmentalize particular cytokines or chemokines in ECM niches, protecting them from proteolysis.

The biosynthesis of HS requires coordinated and concerted action by numerous enzymes present in the membrane of the endoplasmic reticulum and in the compartments of the Golgi apparatus. As they mature, the HS chains undergo a series of major modifications conferring their molecular signature: C5 epimerization of glucuronic acid (GlcA) to iduronic acid (IdoA) by glucuronyl C5 epimerase, C2 sulfation of GlcA or IdoA (2-O-sulfation by 2-O sulfotransferase or HS2ST) or C6 sulfation of N-acetyl glucosamine (GlcNAc) or N-sulfo-D-glucosamine (GlcNS) (6-O-sulfation by 6-O sulfotransferase or HS6STs including 3 isoforms). Finally, the family of 3-O-sulfotransferases (HS3STs) - comprising 6 isoforms plus a variant resulting from alternative splicing (3OST1, 2, 3A, 3B, 4,5 and 6) - can lead to a rare C3 sulfation on less than 1% of GlcNS (5). Considering the importance of the complex structure of HS chains, a finely regulated control of gene transcription and translation of their biosynthetic enzymes – especially of this 3-O sulfation - might be a primary mechanism involved in the regulation of cells function.

Thanks to previous studies comparing the cartilage of healthy versus osteoarthritic knees cartilage, we already demonstrated a significant decrease in HS accompanied by an increase in their sulfation degree with an impact on their functional capacity to bind growth factors such as FGF2 and VEGF during OA. In the absence of any data from the literature and in view of the growing interest on FLS involvement in OA, we decided to conduct a study about HS sulfation pattern in synovial fluid and membrane. The objective of this study was threefold: first, to develop a protocol for the primary culture of FLS from human healthy and osteoarthritic SF and SM samples. Secondly, to extract, to quantify all HS and analyze their sulfation pattern. Third, to study the genomic expression profile of HS biosynthesis enzymes

## **Materials and Methods**

### **Collect of Synovial Fluid and Membranes from OA Patients and controls.**

Patients were recruited from orthopaedic and rheumatologic practices at Henri Mondor's Hospital (Créteil, Val de Marne). SF were obtained by needle aspiration from n = 18 patients with painful knee osteoarthritis. Synovial tissues were isolated from three patients undergoing total knee replacement surgery for symptomatic knee osteoarthritis. The only SM control sample was obtained from patients undergoing total hip replacement after femoral neck fractures. Demographic and radiological characteristics were collected at enrollment: age, sex, weight, height and body mass index (BMI), sporting activity (yes/no), history of joint injury on the symptomatic knee (yes/no) including anterior cruciate ligament (ACL) rupture or meniscectomy, current treatments or in the last three months (yes/non), including analgesics, non steroidal anti-inflammatory drugs (NSAIDs), slow-acting anti-rheumatic drugs (SAARDs), joint corticosteroid injection and hyaluronic acid injection, duration of painful symptoms and knee pain in the past month. Radiographs were scored according to the Kellgren and Lawrence (KL) classification by one investigator (STC). The study was carried out in accordance with L.1123-6 article of the French Health Code. The study protocol was approved by the local ethics committee. Informed consent was obtained from all subjects. As a matter of practicality, our patients were assigned a number based on their date of inclusion. As a matter of practicality, our patients were assigned a number based on their date of inclusion: cultures from MS were numbered from 2 to 5 - patient 5 being our only control, and those from LS were numbered 21,25,27,31,32,34,35 and 36 respectively.

### **Isolation of synoviocytes and cultures conditions**

FLS were isolated from synovial tissue and fluid according to adapted protocols from respectively the procedure published by Scanu and Rosengren and Al (6,7). From synovial membrane, briefly, cells were isolated by enzymatic digestion of synovial tissues obtained from patients undergoing total joint replacement surgery. Each tissue sample was minced into 1 mm<sup>2</sup> pieces and treated for 12 hours with 1 mg/ml of type VI collagenase (Sigma-Aldrich) in  $\alpha$ -MEM Eagle (Life technologies) medium containing 10% bovine serum albumin (BSA;

(100 g/ml) at 37°C in 5% CO<sub>2</sub> (later referred to complete  $\alpha$ -MEM Eagle medium). Dissociated cells were centrifuged at 250  $\times$  g, resuspended in  $\alpha$ -MEM Eagle medium, and plated at a high density (10<sup>6</sup> cells/cm<sup>2</sup>) in a suitable flask. From synovial fluid, briefly SF obtained from osteoarthritic knee was treated with hyaluronidase (10 UI/mL) (Sigma-Aldrich) for 45 min at 37°C. The solution was diluted 3 times in Phosphate Buffered Saline (PBS), before a 300-x g centrifugation. Pellet of cells were resuspended in complete  $\alpha$ -MEM Eagle medium and plated 10<sup>5</sup> cells/cm<sup>2</sup> in a suitable flask. For both samples, after overnight culture, non-adherent cells were removed, and adherent cells were cultured in complete  $\alpha$ -MEM Eagle medium supplemented with type fibroblast-growth factor 2 (FGF2) (10 ng /mL) in 5% CO<sub>2</sub>. Medium was changed every 2 or 3 days, until appearance of dense cellular clones at passage 0. Then cells were passaged and amplified by seeding at 2000 cells/cm<sup>2</sup> in appropriate flask. Synoviocytes from passages 0 to 6 were used in the experiments described here.

### **Phenotyping of FLS**

The purity of the cultured cell populations was analysed for all passages of all patients, prior to any experiment, by their particular morphology in optical microscopy, their ability to attach on the culture plastic but also thanks to their surface and intracellular protein expression determined by flow cytometry. To assess the relative composition of fibroblast like and macrophage like synoviocytes purity in culture, flow cytometry was performed. FLS were enzymatically dissociated, resuspended in FACS buffer (PBS 1X, 1% BSA) and immunostained with FLS surface protein (fluorescein isothiocyanate (FITC) conjugated CD55 or phycoerythrin (PE)-conjugated CD44 or CD90) or macrophage like synoviocytes FITC-conjugated CD14, CD45 and Alexa 647 CD68 (1/100e, Life Technologies). Cells were incubated with the primary antibody for 30 minutes at room temperature and washed three times. VSV-tagged antibody was used to analyse 2,6 and 3-O-sulfated HS structure (8). FLS were initially suspended in a solution containing HS4C3 antibody previously diluted in FACS buffer for 30 minutes (1: 50). After washing, cells were incubated for 30 minutes with a vesicular stomatitis virus (VSV)-tagged phage-display antibody against specific sulfated HS structure (1/200e), in FACS buffer. After washing, cells were incubated with rabbit anti-VSV glycoprotein antibody (Sigma-Aldrich) in FACS buffer, washed, and stained with a FITC-conjugated anti-rabbit antibody (1/200e, Sigma-Aldrich) in FACS buffer. Cells analysis was

performed using a FACS MACSQuant Analyser (Miltenyl Biotec). Results were expressed as a percentage of positive cells.

### **GAGs extraction from serum and synovial fluid.**

Previously centrifuged synovial fluid and non-centrifuged serum were suspended in a PK-DNase buffer 10X (Tris-HCl 500 mM, NaCl 100 mM, MgCl<sub>2</sub> 30 mM, pH 7.9) and 1% Triton X-100, to further perform GAG extraction according to adaptation of previously published protocol (ref Huynh et al. 2012). Samples were then treated with proteinase K (Sigma, final concentration 100 µg/mL) at 56°C for 24 hours, followed by the enzyme inactivation at 90°C for 30 min. DNA was then digested by DNase I (QIAGEN, 10 U/mL of sample) overnight at 37°C. Samples were filtered (PALL life science ODM02C34) and centrifuged (12 000 rpm, 10 min, at 6°C). The filtrates were diluted 1:1 with 4M NaCl and vortexed for 30 min at room temperature (RT). Samples were treated with TCA (SIGMA-ALDRICH) to final 10% and cooled at 4°C for 15 min, centrifuged (12 000 rpm, 10 min, 4°C) and the supernatants recovered. Lipids were eliminated by chloroform extraction (1:1) and centrifugation (12 000 rpm, 10 min, 8°C). The aqueous fractions were dialyzed (Thermo scientific 3.500 MWCO) against a buffer (1X: Tris 50 mM, CH<sub>3</sub>COO-Na 50 mM, CaCl<sub>2</sub> 2 mM, pH 7) and then against pure H<sub>2</sub>O. Samples were frozen at -80°C and freeze-dried. Finally sample were suspended in water according to the initial volume of SF or serum, and stored at -20°C until use. Extraction/quantification quality controls (QC) were included in each experiment. These QC were prepared from freeze dried cartilage samples in where a known quantity of GAGs, similar to that expected in most samples, was spiked. This was made in order to calculate the extraction yield and at the end of the processes.

### **Dimethylmethylen blue (DMMB) assay of total sulfated GAGs**

Extracted total sulfated GAGs were quantified according to 1-9 dimethyl-methylene blue (DMMB, Sigma Aldrich) assay as previously described (Barbosa et al. 2003). Briefly, total GAG extracts were diluted to 100 µL with pure H<sub>2</sub>O and 1 mL of DMMB solution was added. Samples were stirred for 30 min and centrifuged (12 000 rpm, 15 min). Samples supernatants were discarded and pellets were dissolved in 500 µL of a decomplexing solution. GAGs in the decomplexing solution were quantified by measuring the absorbance

(650 nm) in a fixed volume from the mixture using a micro plate reader (TECAN). Total GAG quantities in the samples were determined according to the linear portion of a standard / calibration curve generated in the same condition with known concentrations (from 2,5 to 30 µg/mL) of standard CS-A (Sigma C9819). For each sample, CS, KS, and HS amount were determined after chondroitinase ABC, heparitinases I/II/III, and keratanase treatments, respectively.

### **Quantification of CS**

CS were enzymatically digested by chondroitinase ABC (Chse ABC) treatment as previously described (Barbosa et al, 2003). Briefly, total GAG extracts were diluted in a glycanase digestion buffer (CH<sub>3</sub>COO-Na 50 mM, CaCl<sub>2</sub> 2mM). Chondroitinase ABC (30 mU, SIGMA C3667) was added and samples were incubated at 37°C for 1 hour. The residual HS and KS were then determined by following the DMMB protocol as described above. A standard calibration curve with known CS (SIGMA H7640) concentrations (2.5 – 30 µg/mL) was included in each experiment. The amount of CS in samples was calculated as the difference with total GAG quantified in the same sample.

### **Quantification of HS**

HS were chemically digested by nitrous acid treatment as previously described (Barbosa et al, 2003). Briefly, total GAG samples were completed up to 100 µL with H<sub>2</sub>O. NaNO<sub>2</sub> (SIGMA S-2252) was added (100 µL, 0.25 M,) followed by addition of acetic acid (VWR 20103.364) (100 µL, 33%). Samples were incubated at RT, for 1 hour and the reaction was stopped by adding 100 µL of ammonium sulfamate (14%) (SIGMA 228745). After the chemical HS digestion, remaining GAGs (CS and KS) in the samples were determined by following the DMMB protocol as described above. A standard calibration curve with known HS (SIGMA H7640) concentrations (2.5 – 30 µg/mL) was included in each experiment. The amount of HS in samples was calculated as the difference with total GAG quantified in the same sample.

## **Quantification of KS**

KS were quantified following 2 strategies. First, HS and CS were digested as described above and the remaining GAGs (KS) were quantified. Briefly, total GAG samples were completed up to 100  $\mu\text{L}$  with the glycanase digestion buffer and were then treated by Chse ABC (30 mU) and a heparitinases (Iduron) cocktail (Hep I, Hep II and Hep III), at 37°C for 1 h. Remaining GAGs (KS) in the samples were determined according to DMMB protocol as described above. The KS quantification after CS and HS digestions was confirmed in total GAGs samples treated by Keratanase. Briefly, total GAG samples were completed up to 100  $\mu\text{L}$  with the glycanase digestion buffer and Keratanase (Amsbio PS170615) was added. Samples were incubated in a 37°C for 1h and remaining GAGs amount were determined according to the DMMB protocol as described above. A calibration curve, made of known KS concentrations (2.5 – 30  $\mu\text{g}/\text{mL}$ ) was included and processed in each experiment. The amount of KS in samples was calculated as the difference with total GAG quantified in the same sample.

## **Structure of HS and CS by HPLC analysis**

Extracted GAGs were first used to determine the global HS and CS disaccharides composition by HPLC. Briefly, total GAGs samples were treated with 20 mU of Chse ABC (SIGMA C3667) for 1h 30 at 37°C followed by heparitinases I, II and III (Iduron) cocktail treatment (0.25mU of each, overnight at 37°C. After filtration, samples were injected to HPLC system set as previously described. Then, CS composition was specifically analyzed by digesting the total GAG samples with Chase ABC and without treatment with the heparitinases cocktail. This allowed determination of the HS disaccharide composition by subtraction of the CS peaks from HS+CS signals. This was performed to avoid chromatography column clotting resulting from CS digestion that interfered with HS analysis along. The HPLC analysis was performed as previously described (ref). Briefly, after total extracted GAGs filtration, 50  $\mu\text{l}$  of sample was automatically loaded through a Proteomix SAX-NP5 column eluted by a solvent gradient consisting of eluent A (NaCl 20 mM) and eluent B (NaCl 1 M) at 1 ml/min flow rate. The gradient was 0–5 min (100% A), 5-25 min (from 100% to 0% A by steps), 25–29 min (0% A), 30–37 min (100% A). The column effluent was mixed (1:1) in line with 2% of 2-cyanoacetamide solutions and a 25 mM of NaOH, supplied at 0.5 mL/min each. The mixture passed through a reaction coil (10-m length 0.5-mm internal diameter) set in an oven at 120 °C followed by a cooling coil (3-m



length, 0.25-mm internal diameter). The effluent was monitored at 346 nm of excitation and 410 nm of emission with a fluorescence detector (JASCO; FP-1520). Identification of peak was performed using commercial HS and CS disaccharides standards (Iduron). Moreover, 3 controls of digestion using commercial GAG were included in each experiment to validate complete enzymatic digestion. Results were expressed as % of area of each peak to the sum area of all peaks external standard.

### **Heparin/Glycosaminoglycan ELISA competition assay towards HBP**

The capacities of the SF extracted GAGs to interact with HBP were evaluated by an ELISA-based competition assay as described by Najjam et al. The tested HBP were Recombinant Human Basic FGF-2 (PROMOKINE, C-60240) and Recombinant Human VEGF-165 (PROMOKINE C-64420). Briefly, ELISA 96-well plates (Costar) were coated overnight at 4°C with heparin-BSA complex in Tris-HCL 50 mM, EDTA 12.7 mM, PH 7.4, and washed 3 times with PBS, 0.05%, Tween 20 (washing step). Wells were then saturated with BSA 3% in PBS (Sigma A7030) for 1 h at RT. Then, HBP diluted in BSA 1%/PBS were added to wells (FGF-2 at 2.5 ng/well; VEGF-165 at 5 ng/well) together with increasing concentration of extracted GAG (serial dilution from 0, 0.01 to 100 000 ng/mL in PBS) in duplicate. Competition of the HBP binding to immobilized-heparin or to the soluble GAGs was performed for 1h in the plate. Heparin (Sigma) at concentrations ranging from 0.01 to 1000 ng/mL was used as a control of the GAGs competitive binding. After washing, the HBP remaining bond to heparin was targeted by a specific antibody (anti-FGF2 Mouse Monoclonal IgG diluted 1/2000, R&D systems MAB233; anti-VEGF Rabbit Polyclonal IgG, diluted 1/500 PROMOKINE PK- AB815-64420) for 1h at rt, followed by a HRP-conjugated secondary antibody (HRP-AffinPure Donkey Anti-Mouse IgG 1/5000, Jackson ImmunoResearch 715-035-151; HRP-AffiniPure Goat Anti-Rabbit IgG 1/2000, Jackson ImmunoResearch 111-035-144). Peroxidase activity was measured by the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB, SI254783, Thermo Scientific, France) substrate as described by manufacturer's protocol. The maximum binding (100%) was determined in presence of the HBP and in absence of extracted GAG and IC<sub>50</sub> was defined as the concentration of GAG able to displace 50% of the HBGF binding to immobilized heparin. Finally, GAGs IC<sub>50</sub> values were determined for each HBGF.

### **Baf32 cell mitogenic assay.**

Baf32 cell mitogenic assay was performed using 96 well plates direct measurements of the cell viability with a fluorometric detection of the reduced products from PrestoBlue. In brief, BAF32 cells were cultured in RPMI 1640 Glutamax medium (Gibco 61870-010), supplemented with 10% SVF (Gibco 10270), Antibiotic-Antimycotic (Thermo 15240062) and 1 ng/mL final IL-3 (R&D systems 403-ML-010). Prior to mitogenic assay, cells are washed by centrifugation and resuspended in starving medium based on RPMI 1640 Glutamax, 10% horse serum (Gibco 26050-070) and antibiotic-antimycotic. After counting, cells are seeded at 10.000 cells/well (100  $\mu$ L) into 96 well plates in starving medium and incubated overnight at 37°C, 5% CO<sub>2</sub>. Thereafter 10  $\mu$ L of GAGs are added to final concentration between 0.1 - 10  $\mu$ g/mL and 10  $\mu$ L of FGF-2 (Invitrogen 68-8785-82) are added for a final concentration of 5 ng/mL. GAGs and FGF-2 are conditioned in starvation medium. Controls are cells stimulated without any GAGs and with as compared to without FGF-2. Cells are incubated 72 hours at 37°C, 5% CO<sub>2</sub> (the doubling time of the cells are around 30 hours). Cell viability was measured after addition of 10  $\mu$ L of PrestoBlue® in each well (Cell Viability Reagent, Invitrogen A13262), incubation 4 hours at 37°C, 5% CO<sub>2</sub>, and direct Fluorometric detection at 544 ex /590 em.

### **Quantitative analysis of gene expression pattern by reverse transcription-polymerase chain reaction.**

Total RNA was extracted using the RNeasy minikit (Qiagen) according to the manufacturer's instructions on a column genomic DNA digest using RNase free cDNase (Qiagen). Synoviocytes mRNA was evaluated for expression of 6-OST1, 6-OST3, 3-OST1, 3-OST2, 3-OST3A, 3-OST3B, Sulfatase 1 and 2. All expression levels were normalized to  $\alpha$ -tubulin, as screening of all samples determined no variability in  $\alpha$ -tubulin expression. Equal concentrations of RNA (1ng) from each sample were reverse transcribed into cDNA using affinity script multiple temperature (Agilent Technologies) for RT-PCR. Relative gene expression was determined by quantitative PCR using SYBR-Green based probes and primers purchased from Eurofins MWG (Table 1). All qPCR assays were run on the thermocycler AriaMx Real-Time PCR system (Agilent Technologies) using a protocol consisting of denaturation for 3 minutes at 95°C, 40 cycles for 5 s at 95°C, hybridization and

determined by comparing gene expression levels to those found at the first passage and by comparing gene expression between patients and control at the same passage.

### **Immunofluorescence analysis in vitro**

At each passage, 10 000 FLS were seeded into 8-well chamber slides (Ibidi). After fixation in PBS containing 4% paraformaldehyde, nonspecific binding was blocked with PBS and glycine for 15 minutes before permeabilizing the cells in PBS- 3% BSA (Sigma-Aldrich) - Tween 20 (0,02%) for one hour at room temperature. Cells were incubated overnight at 4°C with three anti-HS antibodies, selected for their recognition of differently sulphated epitopes - 10E4, EV3C3V and HS4C3 (respectively binding all HS and C5 epimerized and 2-0 sulfated units and 3-0 sulfated units) -, followed by an incubation for 1 hour with rabbit anti-VSV antibody. The cells were incubated with an Alexa-488-coupled anti-rabbit immunoglobulin antibody, washed three times in PBS-BSA-Tween and mounted with ProLong Gold (Life technologies). In addition, a negative control containing VSVr and tertiary AC alone was added. The slats were kept at 4°C away from light. In the immunofluorescence procedure referred to as “non permeabilized”, cells were not permeabilized or washed with Tween 20 (0,2%). Cells were visualized using a disk-scanning confocal microscope system, which includes an inverted microscope base (IX81; Olympus) and a disk-scanning unit (IX2-DSU; Olympus). In each experiment, pictures from different samples were taken consecutively using identical settings.

### **Statistical analysis**

Values are expressed as mean  $\pm$  SEM. The statistical significance of differences between the various groups was determined by t-test or one-way analysis of variance (ANOVA) and group-to-group comparisons were made by two tails unpaired student's t-test. All graphs and values were analyzed using GraphPad Prism 5 software. Each sample was tested 3 times in each experiment and each experiment was repeated at least 3 times. A p value  $< 0.05$  was considered to be statistically significant. Note that \*\*  $< 0.005$ , \*\*\* p  $< 0.0001$ . For descriptive analyses, qualitative variables are reported with absolute and relative frequencies, and quantitative variables with mean (SD) or median (quartile 1–3 [Q1–Q3]).

## RESULTS

### Patient characteristics

At the time of inclusion, the median age was 63.0 years (Q1-Q3: 56-70) (Table 1). Overall, 13/21 (62%) patients were women. The mean (SD) duration of painful symptoms was 30.4 (38.6) months, and mean (SD) knee pain intensity (VAS, 0-100 mm) in the past month was 63.1 (18) mm. The mean (SD) BMI was 27.4 (1.6). The median Kellgren and Lawrence score (Q1-Q3) was 3 (2-4). The SF and SM groups were comparable except for the longer duration of painful symptoms in the MS group (respectively 9.4 [8.4] months versus 70 [5] months). We were not able to collect the socio-demographic characteristics - apart from her age and X-rays - of our only control, patient 5 (MS of the femoral neck). The patient was 95 years of age and had moderate OA in the contralateral hip with a definite osteophyte.

### Characterization of FLS in vitro culture

In culture, FLS initially appeared thin and elongated by inverse microscopy. Overtime, they became thicker and formed increasingly dense cytoplasmic branches with neighbouring cells. Occasional cells had a stellar or pseudo-dendritic morphology. This cell differentiation occurred earlier in SF versus SM cells: from 3rd or 4th versus 5th or 6th passage (Figure 1A). In contrast, macrophage-like synoviocytes appeared rounder and smaller (data not shown).

When cells approached confluence at each passage, the dissociated cells were counted to determine the FLS doubling time (DT). DT was determined according to the formula  $DT = t \times \ln(2) / (\ln(N_f) - \ln(N_i))$ , (t: culture time (days) between each passage,  $N_f$ : final cell number,  $N_i$  initial cell number). Figure 2 suggest that the mean doubling time of FLS from SM was shorter than that of SF demonstrating an accelerated growth of these last ones (Figure 1B).

We carried out a total of 40 flow cytometry analyses in eleven patients at different passages, up to the 5th passage (patient 4, MS). Phenotypic characterization of cell cultures all along the passages clearly showed a positive expression of FLS markers such as CD55, CD44 and CD90 from the first days of culture in vitro, and a negative expression of the macrophagic

markers, CD14 and CD68. SF cells expressed FLS markers in greater numbers (respectively, a CD55 mean percentage of 77% versus 69%) (Figure 2).

### **Characterization of Sulfated GAG levels in synovial fluid and synoviocytes as compared to cartilage and serum from CT and OA.**

As it is not possible to obtain normal synovial fluid from healthy donors, we decided to perform a comparison of the GAG Structural characterization of SF from OA patient to GAG structural characterization from another physiological fluid of the same patient that is serum. Then we compared their structural composition of GAG to normal serum. It is clear that during pathology of OA, releasing of GAG into the SF happens but we did not know if in blood from OA patient also it happened or not. To gain this answer CT's serum was used to quantify GAG and to compare with OA SF and OA serum as well. The amount of total sulfated GAG from CT serum is (20  $\mu\text{g/ml}$ ), which is not big changing for OA serum (20  $\mu\text{g/ml}$ ). However we clearly observed an increase of GAG concentration in OA SF (47  $\mu\text{g/ml}$  of liquid) (Fig 3A). These results were put in parallel to those already described in cartilage: whereas in cartilage we observed a decrease in total GAG contents between CT and OA, it's not the case in serum between CT and OA. This suggests that serum maybe does not contain GAG based biomarkers of what is happening in cartilage. These results need to be complete by analyze of the literature, to be sure that nobody already described glycanic biomarkers of OA passing from cartilage to serum.

Differential quantification of CS, HS and KS bring distinct informations. In fact we observed that whereas total GAG concentration is 2 times higher in SF than in serum, this is not due to HS because HS species are around the same concentration in synovial fluid and in CT versus OA serum. However, we note a big difference in CS concentration since this was three time higher in OA SF than in OA and CT serum. Finally, another difference is observed in quantification of KS that is present in OA SF but no detected in CT and OA serum. Surprisingly, synoviocytes cells purified from synovial fluid do not expressed KS on their cell surface. In resume biggest difference in GAG composition between OA SF and OA Serum is due to highest concentration of CS and KS in SF as compared to HS, whereas HS concentration are the same. Moreover differences in Total GA, CS, HS, and KS concentration between CT and OA cartilage are not observed between CT and OA serum, suggesting that

This result confirms all result, which published previously about increase amount of KS in SF. Furthermore, the analysis of the percentage of each GAGs species on total amount (Fig 4) resumes these elements and clearly show that Cartilage and Synovial Fluid have roughly the same composition based on CS HS And KS species whereas Synoviocyte contains mainly HS and CS as Serum.

### **Sulfation pattern of CS and HS disaccharides units in OA SF is different to those of cartilage**

HPLC-fluorescence labeling methodology was used to analyze structural aspects on sulfated disaccharides compositions of GAG purified from Synovial Fluid. We decide to compare this profil to those of cartilage (OA and CT), and collect of data are in progress to compare t to those of serum (CT versus OA) and synoviocytes.

Analysis on purified CS disaccharides units are presented (in figure 5 upper panel) and demonstrate differences on sulfation pattern of CS from OA patients and SF as compared to controls (CT). Mono-sulfated CS disaccharides (Fig 5B) are the major unit in CS extracted from control cartilage (85%), and is significantly increase in OA (90%), whereas Dis-sulfated units (Fig 5C) are significantly decreased in OA part (1%) compare to CT (1,8%). Surprisingly SF pattern is very different since SF is significantly enriched in No-sulfated CS unit (Fig 5A) (80%) as compared to control (13%) and OA (9%) patient, whereas Mono- and Di-sulfated units are very low. Analysis on purified HS disaccharides units was performed also from OA patients and SF as compared to controls (CT). SF has significantly higher No-sulfated HS unit (Fig 5D) (65%) as compared to control (30%) and OA (15%) patient, whereas Mono- and Di-sulfated units are very low (30% and 2%) (Fig 5E and F) respectively.

### **GAGs from SF display differential binding affinities toward growth factors during OA**

The capacity of GAGs to regulate cells functions is largely related to their abilities to bind to HBPs. An ELISA-based competitive binding assay was used to investigate if structural modifications of GAGs in OA SF are associated to distinct abilities to bind HBPs relevant in cartilage homeostasis such as FGF-2 and VEGF. In this test IC<sub>50</sub> of GAG were defined as the concentration of GAG that permit to inhibit 50% of the HPB binding to immobilized heparin. The determined IC<sub>50</sub> values (in µg/ml) for each studied HBP are showed in table 2.

A high IC<sub>50</sub> value corresponds to a low affinity, since big concentration of GAG is necessary to compete for HBP binding to heparin, and inversely.

Total GAGs extracted from CT cartilage were able to bind to FGF2 and to inhibit its binding to heparin with EC<sub>50</sub> values of 11 µg/mL (Table 2). This value was taken as relative values (CT = 100%) to compare binding capacities of extracted GAGs from OA cartilage and SF in figure 6. IC<sub>50</sub> values obtained from total GAG extracted from OA SF compared to normal CT and were shown to decrease toward FGF2 (3 µg/mL) (Table 2), suggesting corresponding increase of binding affinities of this growth factor as illustrated in (Figure 6, panel A). Whereas total GAGs extracted from CT cartilage were not able to bind to VEGF and to compete even at 1000 µg/ml, GAG extracted from OA SF were able to bind to VEGF (Panel C), with IC<sub>50</sub> mean at 115 µg/ml (Table 2). To illustrate this gain of binding affinity IC<sub>50</sub> value of CT GAG across VEGF was considered up to 1000 µg/ml and take as 100%.

Same binding assays were performed with purified HS and mix of CS and KS (devoid of HS). This fine analysis reveals that binding of total GAG to FGF2 is related to HS binding affinity for this growth factor, whereas CS/KS mix extracted from SF are not able to bind to FGF2 and to compete. As for total GAG, HS affinity for FGF-2 was decreased, as the IC<sub>50</sub> value was increased between CT (34 µg/ml) and OA cartilage (80 µg/ml) which is not the case for OA SF by decreasing the IC<sub>50</sub> (6 µg/ml), it means increasing of binding from HS purified (Panel B). Concerning VEGF, binding of total GAG to this growth factor is also linked to of its affinity for HS. Accordingly, CS/KS mix extracted from cartilage and SF samples are neither able to bind to VEGF nor to compete with it. IC<sub>50</sub> value of HS toward VEGF increase 3 times in OA and 10 times in OA SF as compared to CT, suggesting that HS structural modification are associated to a loss of binding affinity toward VEGF (Panel D), as observed for FGF-2. However, HS extracted from normal cartilage have a higher binding affinity toward VEGF (IC<sub>50</sub> value: 13 µg/ml) that was not observed in total GAG extract.

In summary, OA SF total GAG showed to have affinities toward FGF2 and much more towards VEGF. The affinities toward FGF-2 and VEGF are found to be mainly due to HS. However, HS purified from OA SF have lost their ability to bind to VEGF it means the effect of CS/KS induced binding affinity of HS alone.

### **GAGs from OA Samples inhibit mitogenic activity of BAF32 cell lines**

In order to investigate the functional activities of GAGs from OA versus control samples in vitro, total GAGs extracted from were tested for their mitogenic activities on cellular tests dependants to HBP. Baf32 cells proliferation is dependant to FGF2 and Heparin: addition of one of these compounds independently in the medium did not induce cell proliferation, whereas addition of the two concomitantly induce 2,5 increase of cells in 24h of culture (Figure 8A from article HTAP). In this context, GAG extracted from OA versus CT cartilage, synovial fluid, synoviocytic cells, and serum were tested for their ability to synergise to FGF2 to induce proliferation of BAF32 cells. As illustrated in figure 6A, GAG from human CT and OA cartilage inhibit proliferation of BAF32 cells induced by FGF2, ( and as compared to the increasing effect of hep that stimulate it). We observed that GAG extracted from synoviocytes in culture has exactly the same effect than those of CT and OA cartilage. However, when we tested increasing amount of GAG purified from OA synovial fluid, OA serum and CT serum, we observed that with higher doses of 5µg/ml of these GAG have a clear inhibitory effect on the cells viability on this condition. .

These preliminary data on functional effect of GAG from synovial fluid remain to be confirmed but suggest that it will be of interest to characterize much more structural feature of HS and CS disaccharides that could be involved in these aspects.



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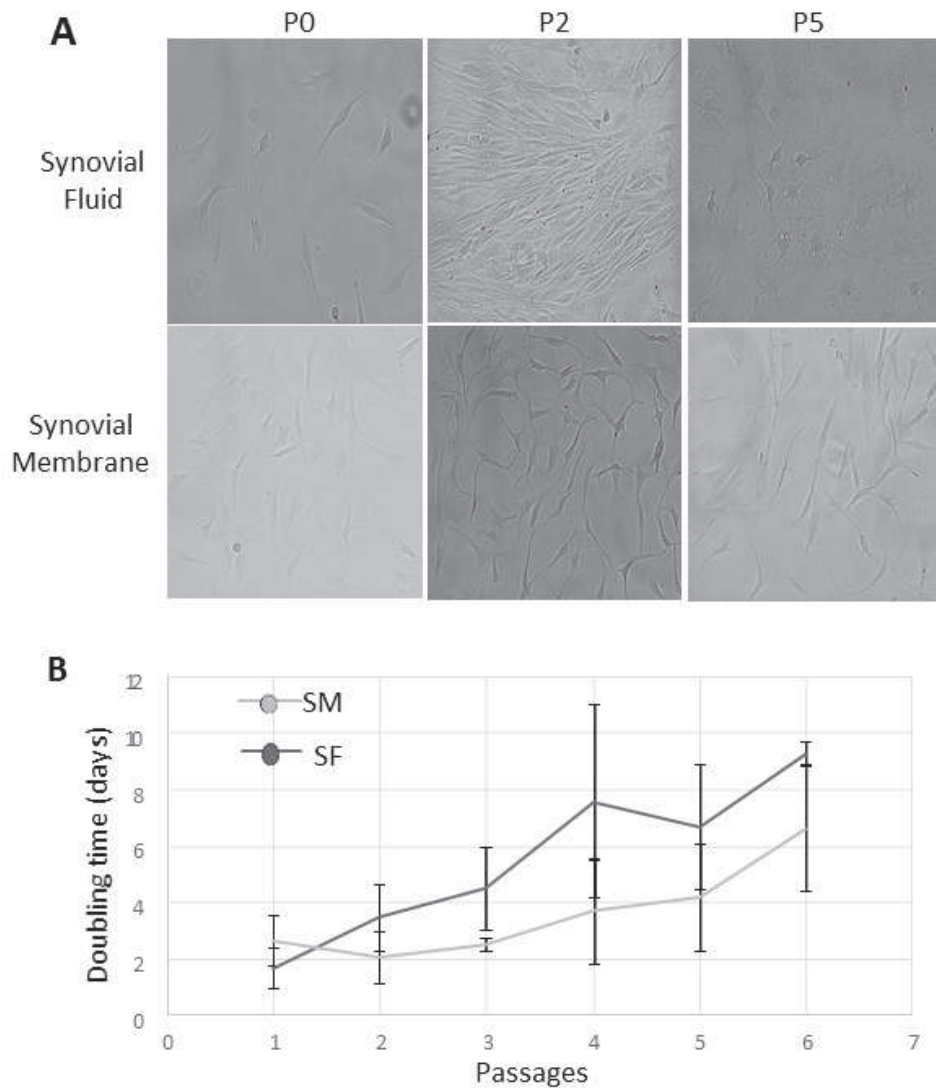
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## Figures

**Table 1. Characteristics of patients with knee osteoarthritis at inclusion**

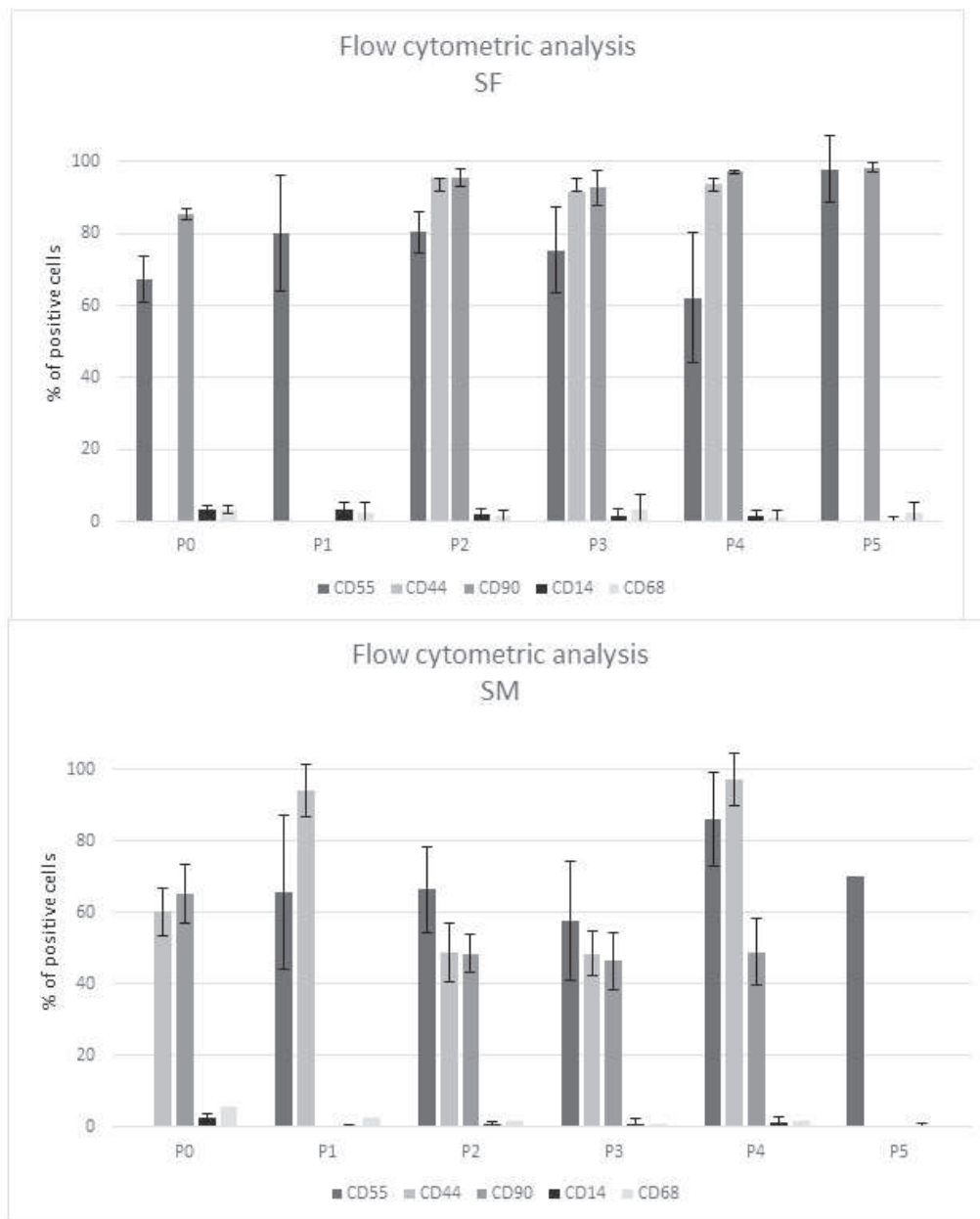
SF: synovial fluid, SM: synovial membrane; BMI: body Mass index; ACL: anterior cruciate ligament; NSAIDs: non steroidal anti-inflammatory drugs; SSARDs: slow-acting anti-rheumatic drugs ; VAS: visual analogue scale. \* In the case of SM, exclusion of femoral neck specimen. † Missing data from the control patient .

	SF n=18	SM† n=3	All n=21
<b>Age (years), median (Q1-Q3)</b>	60 (55-68)	69,5 (67-76)	63 (56-70)
<b>Sex (woman), n (%)</b>	11/18 (61)	2/3 (66)	13/21 (62)
<b>BMI (kg/m<sup>2</sup>), mean (SD)</b>	28 (1,5)	26 (1,1)	27,4 (1,6)
<b>Sport activities, n (%)</b>	5/18 (27)	0/3 (0)	5/21 (24)
<b>History of joint injury on the symptomatic knee (yes/no), n (%)</b>			
ACL rupture	0/18	0/3 (0)	0/21 (0)
Meniscectomy	1/18 (5)	0/3 (0)	1/21 (5)
<b>Current treatments or in the last three months (yes/no), n (%)</b>			
Analgesics	13/18 (72)	3/3 (100)	16/21 (76)
NSAIDs	6/18 (33)	0/3 (0)	6/21 (29)
SSARDs	0/18 (0)	0/3 (0)	0/21 (0)
Joint corticosteroid injection	16/18 (89)	1/3 (33,3)	17/21 (81)
Hyaluronic acid injection	14/18 (78)	1/3 (33,3)	15/18 (83)
<b>Duration of painful symptoms (months), mean (SD)</b>	9,4 (8,4)	79,3 (36,9)	30,4 (38,6)
<b>Knee pain in the past month (VAS 0-100 mm), mean (SD)</b>	60,1 (21)	70 (5)	63,1 (18)
<b>Kellgren-Lawrence classification, mean (SD)</b>	2 (1,5-3,5)	3 (3-3,5)	3 (2-4)



**Figure 1. Proliferative Characterisation of Fibroblastic-Like Synoviocytes (FLS) in vitro culture.**

**1A.** Appearance of FLS by phase light microscopy, isolated from Synovial fluid (SF, upper panels, example of patient 27) and from Synovial Membrane (MS, lower panel, example of patient 2). Cells at different passages : P0 right panel, P2 central panel, P5, right panel. Cells colony are observed at passage 0 (P0, right panel), P2 (central panel), P5 (right panel). **1B.** Doubling time of FLS isolated from SF and SM. Mean doubling time (measured in days) and standard error of the mean are reported.



**Figure 2. Phenotypic Characterisation of Fibroblastic-Like Synoviocytes (FLS) in vitro culture.** Percentages of CD antigen on FLS cells purified from Synovial Fluid (SF) and Synovial Membrane SM. CD55, CD44 and CD90 are specific of fibroblastic like synoviocytes and CD18 and CD68 are characteristic of macrophage like synoviocytes.

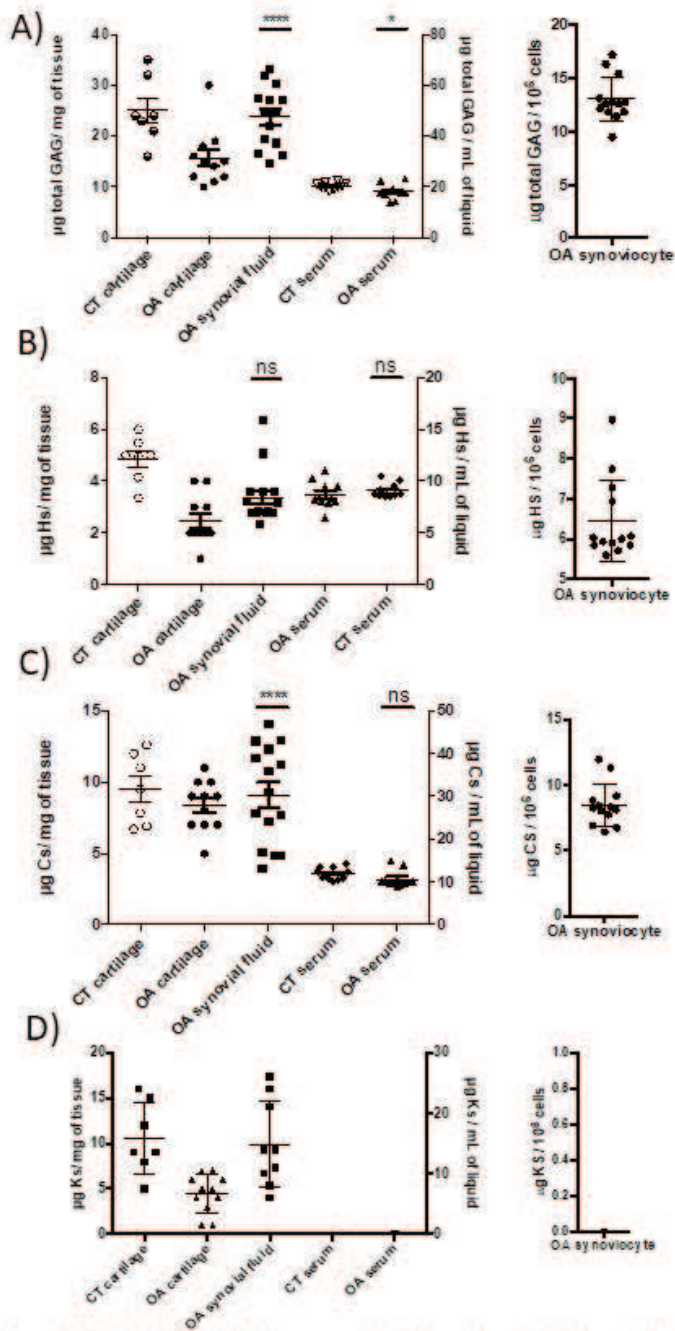


Figure 3. Total sulfated GAGs were extracted and quantified according to the enzymatic digestion protocol. GAG levels were normalized in  $\mu\text{g}$  of total GAGs by mg of tissue for CT and OA cartilage,  $\mu\text{g}$  of total GAGs by ml of liquid for CT and OA serum, synovial fluid (SF) and  $\mu\text{g}$  of total GAGs by  $10^6$  cell for synoviocyte (FLS). A) Total sulfated GAG B) HS C) CS D) KS. Data are shown for CT cartilage (n = 7) and OA cartilage (n = 11), CT serum (n = 10), OA serum (n = 11), synovial fluid (n=15) and synoviocyte (n=13).

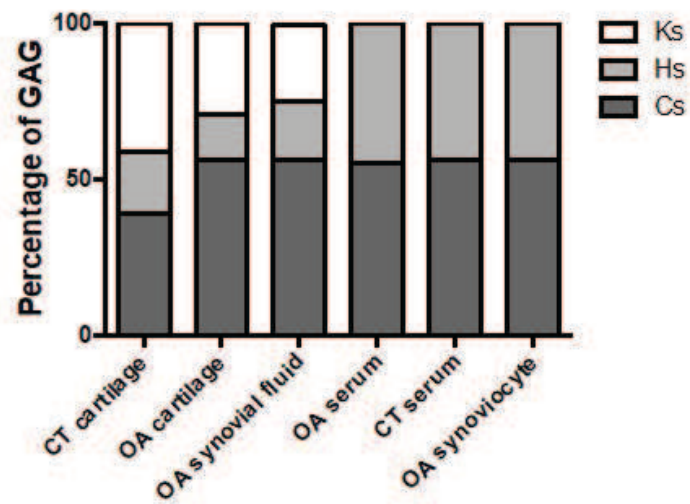
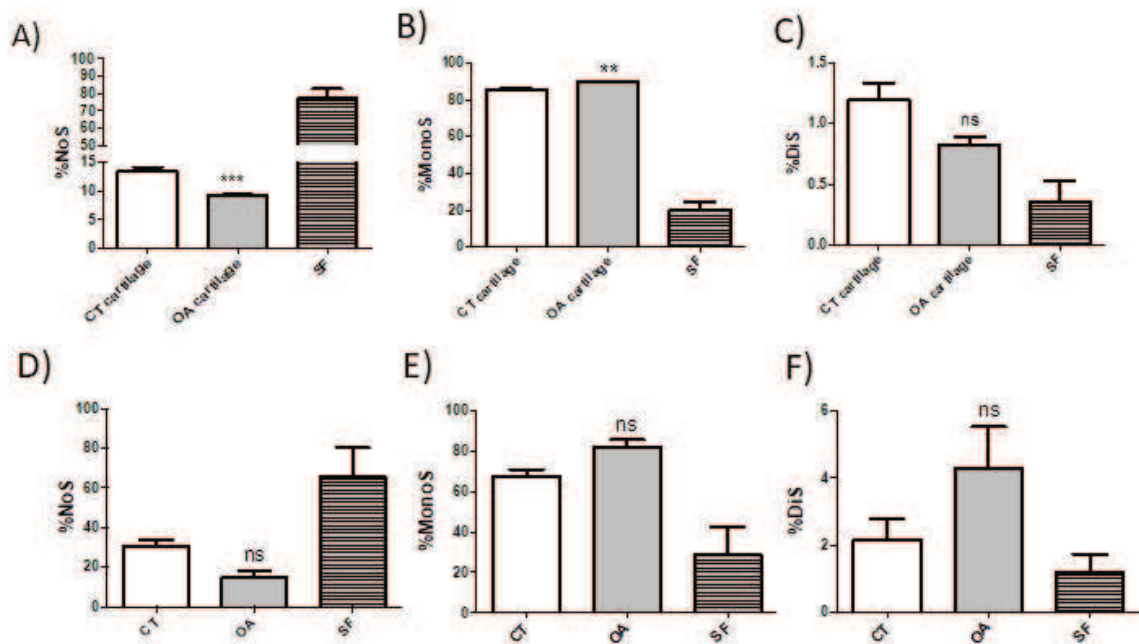


FIGURE 4. Percentage of different fraction of GAG from CT and OA cartilage, CT and OA serum, synovial fluid (SF) and synoviocyte (FLS).  
 KS: Keratan sulfate, HS: heparin sulfate, CS: chondroitin sulfate

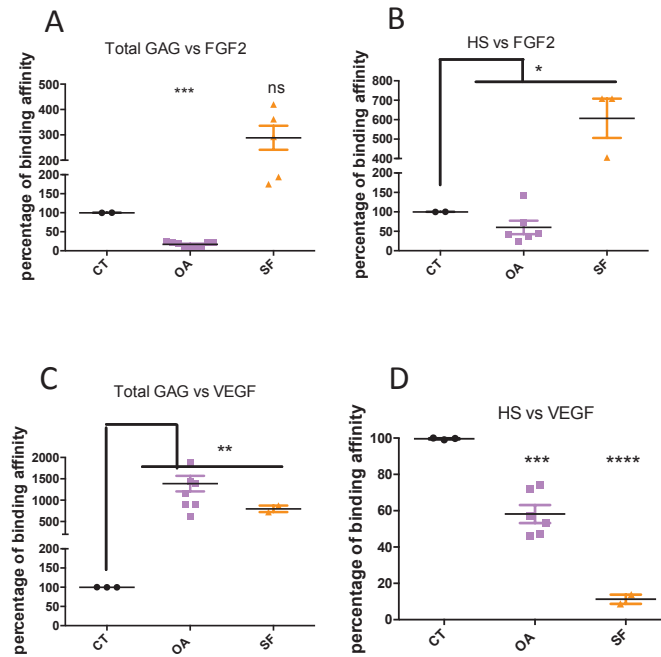


**FIGURE 5.** HPLC analysis of sulfation pattern of CS (upper panel A, B, C) and HS (lower panel D, E F) disaccharides units in GAG chains. HPLC analysis allowed to determine the relative composition in each disaccharide units GAG species, namely the non sulfated (NoS), the monosulfated (MonoS), the disulfated (DiS) and the trisulfated (TriS) after enzymatic digestion. The statistical significance of differences between the groups was determined by one way anova test. All values were analyzed and all graphs were made using Graph Pad Prism 5 software. Each sample was analyzed 2 times and each analysis was repeated 2 times. A p value < 0.05 was considered to be statistically significant. Note that \*\* < 0.005, \*\*\* p < 0.0001. Data are shown for CT (n = 6) and OA (n = 7), SF (n=10).

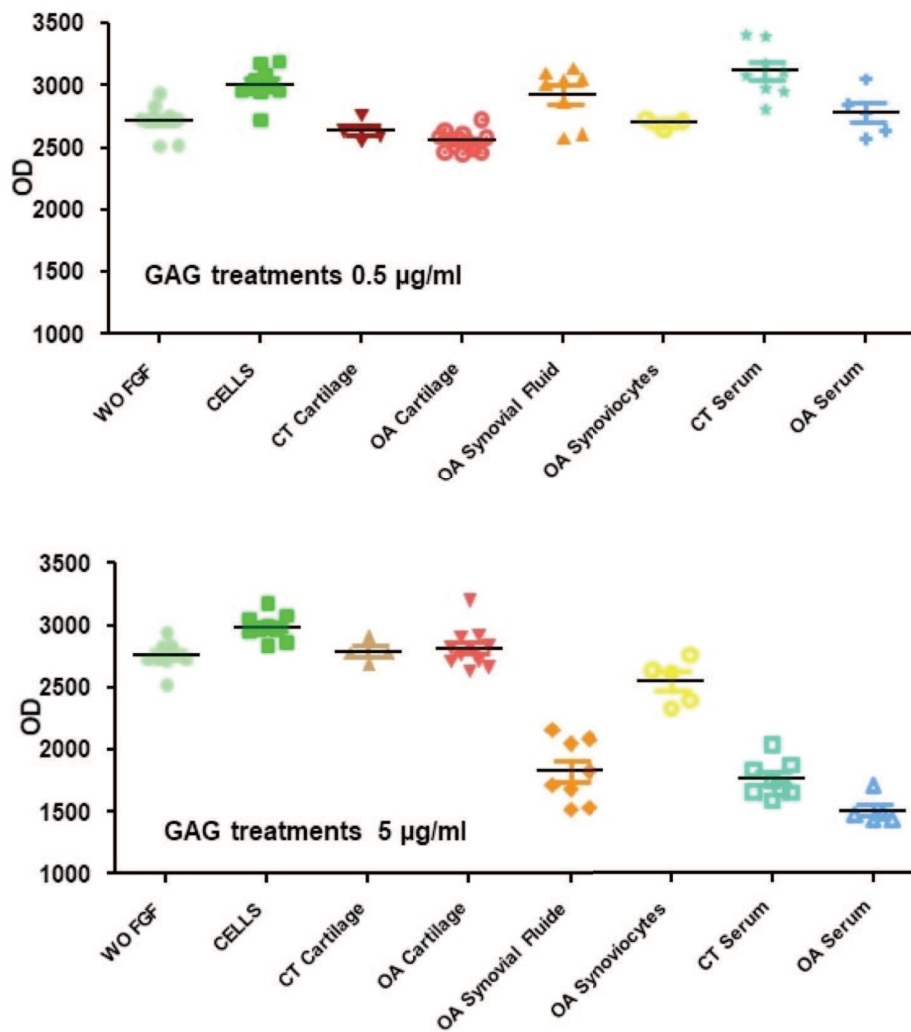
IC50 ( $\mu\text{g/ml}$ )	CT	OA	SF
<b>Total GAG</b>			
<b>FGF-2</b>	11 +/- 4	84 +/- 20	3 +/- 0
<b>VEGF</b>	NoC	136 +/- 78	115 +/- 121
<b>HS</b>			
<b>FGF-2</b>	34 +/- 14	82 +/- 25	6 +/- 2
<b>VEGF</b>	13 +/- 2	38 +/- 12	118 +/- 74
<b>CS+KS</b>			
<b>FGF-2</b>	NoC	NoC	NoC
<b>VEGF</b>	NoC	NoC	NoC

**Table 2 : IC50 value of purified total GAG, purified HS, and mix of CS and KS according to their ability to compete for heparin binding to FGF-2 and VEGF.** GAG were extracted for CT cartilage, OA cartilage and OA SF. IC50 doses were determined in  $\mu\text{g/ml}$  as the concentration of tested GAG able to compete for 50% of the binding of HBF to Heparin, and used to calculate % of binding affinity represented in Figure 6. NoC : No competition.





**Figure 6 : % of binding affinity of total GAG and purified HS toward FGF-2 and VEGF.** IC50 value determined in Elisa binding assay and resume in table 2, were used to calculate GAG binding affinity. IC50 value of GAG from CT cartilage were considered as 100% of binding. Then % of binding affinity of GAG from OA and GAG from SF were calculated according to these 100% values.



**Figure 6 : Cells proliferation assays on BAF32 fibroblastic cells dependant to FGF2 (5ng/ml) and GAG (0.5 and 5 µg/mL) extracted from differents samples CT and OA cartilage and serum , or Synovial Fluid ans Synoviocytes , as compared to cells alone (as 100%, cells without FGF-2 and without GAG). Cell proliferation was evaluated after 48 hours in culture, with PrestoBlue™ cell Viability Reagent.**

## **DISCUSSION and FUTURE DIRECTION**

OA is the most prevalent joint disease in the human population, for which no curative treatment has been developed. This disease is characterized by an imbalance between anabolic and catabolic factors in articular joint with degradation of ECM. Most of the studies on cartilage ECM, normal as compared to OA, have been concentrated on one of its major glycanic component that is the CS family of GAGs. However, HS is one of the most important glycanic regulators of cell behavior in most tissues, but that was not described as a relevant GAG chain in cartilage because rare. We hypothesis that if HS are also present in the cartilage, even in few amount, HS could be modified during OA, and that they can centrally modify the properties of cartilage cells and ECM homeostasis.

During my PhD thesis, we sought to investigate the implication of HS, structural and functional changes, during OA in two relevant compartment of this pathology: articular cartilage and synoviocytes from synovial fluid.

## **1. HS in articular Cartilage**

In the first part of my results, I have demonstrated for the first time that HS are not only present in human cartilage, but also very important in its pathological evolution during OA.

### **Some limits on HS studies in cartilage**

One of the difficulties that I have encountered during my thesis was to have access to some “good” control samples, standard to compare to OA Knee. Indeed, we do not have access to healthy human cartilage samples from healthy knee. Therefore, we decided to use macroscopic normal CT cartilage from Hip, obtained from Rheumatology Department of Henri Mondor Hospital (N° 07-34). This type of comparison is classically done in OA studies, and accepted in international publications on this topic. .

Even if it is clear that the HS and CS sulfation patterns are implicated in regulating signaling pathway, no study demonstrates the involvement of HS in the imbalance of anabolic versus catabolic pathway during OA processes. This lack of knowledge could be explained since HS content in adult cartilage is low as compared to CS. Very few published data were interested to this kind of GAG, tempting to decrease their potential role in cartilage.

Another limitation preventing further studies on HS is due to the difficulty to extract and purify HS chains from the CS-rich cartilage. Therefore, most of the studies on this kind of ECM component in cartilage, are much more concentrated on HSPG than on HS chains alone, because it's simplest to focus on the core protein rather than HS chain description, as proteomic tools are easily available than glycanic one. **Our solution for HS study is coming from a big work of** my host laboratory during previous years, to develop a more precise and yielding protocol to extract GAGs from different tissues, and specifically when I arrive in the lab, to purify HS from CS-rich tissue like cartilage. Thanks to this improved protocol, we were able to demonstrate for the first time a significant decrease of HS amount in OA cartilage as compared to controls.

### **HS are present in amount and quality in cartilage**

After extraction and quantification of GAGs from these samples, we have been able to demonstrate that even if HS amount is decreased in OA cartilage, this was associated to qualitative modification of the glycanic molecules, which showed a significant increase of mono- and di-sulfated HS amount. One question remaining in this HS chemical characterization is which kind of sulfation is involved; In fact, in HS purified from cartilage, enrichment of GAG extracts in CS and KS do not permit to us to clearly analyze HS sulfated disaccharides by HPLC, and do not permit to discriminate between N-sulfated, 2-O-sulfated and 6-O-sulfated HS, as we do it for lung extract (HTAP collaboration and article 3).

However this kind of information should be available according to the transcriptomic analysis of the pattern of expression of enzymes involved in these kinds of sulfation motifs.

In parallel, our data confirmed previous works reporting an increase of mono-sulfated CS in OA, that was previously characterized by an increased GalNAc 6-O-sulfation (Lauder et al., 2001).

All these modification of sulfation pattern were associated to changes in the HS and CS binding affinities toward HBP involved in cartilage homeostasis, such as FGF2 VEGF and PTN.

### **Functional interactions of HS with HBP**

Interestingly, we show that the cartilage GAGs capacities to bind to FGF2 are strongly associated with HS (whereas CS from cartilage are not involved) and that these binding capacities are lost in OA. The sulfation level on HS is increase during OA, therefore, we hypothesis that sulfation modifications of HS during OA are associated to modification of binding affinities towards FGF2. This is in accordance with other studies demonstrating that FGF-2 binding affinity to heparin is mainly regulated by 2-O sulfation (Balazs et al., 1967). FGF-2 is produced endogenously and sequestered in the articular cartilage ECM (Vincent et al., 2007). Upon cartilage injury, FGF-2 is released from its bound matrix and subsequently activates the ERK signaling pathway (Vincent et al., 2002). The outcome of this event is controversial: it has been associated to catabolic effects in human articular chondrocytes via upregulation of matrix degrading enzyme, such as MMP13 or aggrecanase, inhibition of ECM accumulation and PGs synthesis, and clustering of cells characteristic of arthritic states, whereas chondro-protective role were shown in murine cartilage and OA models (Li et al., 2012). However, we can hypothesis that during OA, in which we have established a decrease of available HS chains, the modification of their sulfation pattern that we observed by HPLC

Elisa. Finally, this lower affinity could induce a decrease of FGF-2 sequestration in the ECM matrix, and therefore be associated to an increase of FGF2 bioavailability for its catabolic activity on chondrocytes. Complementary elements on HSPG role in this hypothesis will be discussed in lower section.

Accordingly, we show that whereas total GAGs extracted from CT cartilage were not able to bind to VEGF, they gain an ability to bind to VEGF during OA. Interestingly, VEGF is an essential chondrocyte survival factor during bone formation, skeletal growth and postnatal homeostasis. But It has apparently also a destructive role in OA: (i), increased VEGF expression correlates with increased OA severity. VEGF could be induced by an array of catabolic triggers in chondrocytes (Murata et al., 2008). (ii), VEGF can affect the chondrocytes, via angiogenesis leading to osteophyte formation, through induction of matrix proteases and to differentiation toward a hypertrophic state. (iii), in surgically induced knee OA in mice, a model of post-traumatic OA in humans, increased expression of VEGF is associated with catabolic processes in chondrocytes and synovial cells. (iv) Finally, conditional knockdown of VEGF attenuates induced OA. Furthermore intra-articular injection of anti-VEGF antibodies suppresses OA progression, and oral administration of the VEGFR2 kinase inhibitor Vandetanib attenuates OA progression. It's already demonstrated that VEGF binding affinity to its receptor and further activation pathways are regulated by 6-Osulfated HS (Huynh et al., 2012b). Then, we suggest that during OA, increased sulfated pattern of HS, associated to increase binding affinity to VEGF and its activity could increase hypertrophic state of chondrocytes.

Pleiotropin (PTN) is a secreted heparin-binding peptide expressed in mesodermal and neuroectodermal cells during development, but rarely in adult tissues. Although PTN is abundant in fetal or juvenile cartilage, it is undetectable in mature cartilage. However, PTN is re-expressed in chondrocytes in early stages of osteoarthritis where it is detectable in situ and

cellular matrix synthesis, reduction of degrading matrix metalloproteases and induction of their inhibitors; PTN also slightly reduces pro-inflammatory factors, such as nitric oxide and vascular endothelial growth factor. Furthermore, PTN stimulates chondrocyte clustering and proliferation. (Mentlein, 2007). Some study aimed to investigate pleiotrophin levels and its receptor protein tyrosine phosphatase beta/zeta in the serum, cartilage and subchondral bone of osteoarthritis patients. Serum pleiotrophin levels and expression of pleiotrophin in chondrocytes and subchondral bone osteocytes significantly increased in osteoarthritis patients graded Ahlback II to III. Receptor protein tyrosine phosphatase beta/zeta was mainly detected in the subchondral bone osteocytes of patients with moderate osteoarthritis and as disease severity increased, in the osteocytes and bone lining cells of the distant trabeculae (Kaspiris et al., 2013). Few studies have reported a possible involvement of PTN in the pathophysiology of OA and very little is known about its role in rheumatoid arthritis (RA). A recent study had measured PTN level in the sera and synovial fluids from RA patients, knee OA patients and serum samples were withdrawn from healthy controls, to assess its relation to activity, functional class and radiological staging (Fadda et al., 2016). Demographic, clinical and serological data, as well as Functional and radiographic grades were prospectively assessed. Whereas there were no statistical significant differences on comparing the mean PTN level in sera of RA, OA patients and healthy controls, the mean synovial fluid level of PTN in both patient groups was significantly higher than mean serum level. Whereas some authors have suggested that PTN could mediate repair and protective processes in osteoarthritic cartilage, some recent data from our lab have suggested that PTN is able to induce hypertrophic differentiation of chondrocytes from human Mesenchymal Stem Cells. All these data suggest that the real role of PTN during OA is not so clear: a protective factor that will stimulate repair or at the inverse a pro-hypertrophic inducer? The data obtained during this thesis suggest that binding affinity to CS chains are modified during



should indicate to us which kind of role, catabolic or anabolic, PTN could have during OA according to this modified binding affinity.

### **New Cellular properties of HS during OA**

Modification in sulfation pattern and HBP binding affinities were associated to modified GAG functionalities on cells properties in vitro. We showed for the first time that GAG, and particularly HS, from Control cartilage lose their ability to induce adhesion and proliferation of hMSC during OA, but became able to significantly induce a hypertrophic phenotype in murine articular chondrocytes.

Chondrocytes in OA samples are surrounded by an abnormal ECM, which induces phenotypic and metabolic alterations. The results acquired during my thesis clearly demonstrate for the first time that HS are strongly involved on these ECM phenotypic alterations. This might possibly result in remodeling and phenotypic alterations in the human disease. To go further in this hypothesis we decided to test the effect of cartilage GAG on murine articular chondrocytes and demonstrated for the first time that GAG from OA cartilage merely, are able to modify the catabolic/anabolic balance of normal chondrocyte. Moreover, increased expression of VEGF was also observed suggesting the induction of a hypertrophic signaling. More strikingly, the same experiment performed with purified extract of HS, CS or KS, clearly demonstrates clearly that this effect is mainly due to HS and not Cs or KS. These results demonstrated for the first time that pure HS could have functional effect on catabolic phenotype of murine chondrocytes. It remains to clarify whether one particular PG core protein is carrying the deleterious HS chains or if these chains are present in all HSPG expressed in the OA articular cartilage.

## **HSPGs and their role in cartilage and OA**

Since many years quantitative and structural modifications of PG have been described during the articular aging process in humans. However, for most of the time researchers have been merely focused on CS/KS PG such as an Aggrecans which are mostly associated to structure and water retention on the diseased tissue. Change in Aggrecan composition influence chondrocyte behavior through specific binding receptors, which can ultimately affect its metabolism, phenotype and properties. When the CS/KS PG are degraded, the collagen network becomes accessible to the enzymatic activity. Furthermore, GAG synthesis is defective, characterized by a shorter Aggrecan size, a lower number and shorter size of CS and KS chains and a modification in the ratio of the chondroitin 6-sulfate to chondroitin 4-sulfate ratio. This increased "turnover" is related to a high enzymatic activity, linked to the presence of many pro-inflammatory factors such as cytokines IL-1 $\beta$ , TNF $\alpha$  and IL-6. Thus, during the aging process, it's well known that changes in the quality of CSPG pave the way for OA and our results confirm this global decrease of CS sulfation.

Numerous HSPG are presents in the cartilage, some are secreted in the matrix as perlecan, or linked to the cell surface, as syndecans and glypicans. HSPG have important biological activities, which have been mainly demonstrated during bone and cartilage development. The importance of the HS chains in cartilage homeostasis, particularly in OA, can be associated to observation made when focusing to the PG core proteins: i) Syndecan are transmembrane HSPGs involved in migration, proliferation and differentiation of chondrocytes, according to their ability to interact and modulate FGF, BMP2 and TGF B-1 (Knudson and Knudson, 2001). ii) loss or blockage of SDC4, which is expressed in OA cartilage, protect against rapid cartilage degeneration in mouse OA models (Echtermeyer et al., 2009a), suggesting that such expression of SDC4 is deleterious during OA. iii) Perlecan is a HS/CSPG identified as a regulator in cartilage development and maintenance. It influences chondrocyte metabolism

pericellular ECM (Vincent et al., 2007). Perlecan is expressed in normal cartilage (SundarRaj et al., 1995) and upregulated in cells from the area adjacent to the main OA defect (F. Tesche and Miosge, 2004). Perlecan is an extracellular matrix HSPG suggested to stabilize ECM during OA, and synovial perlecan plays an important role in osteophyte development in OA (Kaneko et al., 2013). All these HSPGs can affect anabolism/catabolism balance in cartilage even if being present in different compartments during OA. Then they could be good candidates, presents on the matrix for Perlecan, or presents on cell surface for syndecan, to explore the transduction pathways that would be associated to modification of HS chains.

## **2. HS in Synovial Fluid and Synoviocytes**

The renewal of ECM is important to retain cartilage properties and homeostasis. However, it is a slow process, the majority of constituents being stable over time. This renewal is ensured by the chondrocytes, which constantly maintain the balance between catabolism and anabolism. Thus, depending on the mechanical, physicochemical or inflammatory stress that applies to their direct environment, the chondrocytes will secrete either pro-anabolic stimuli [IGF-1; BMP, TGF- $\beta$ ] or pro-inflammatory cytokines, considered as pro-catabolic stimuli [IL-1 $\beta$  and TNF- $\alpha$ ] (Aigner et al., 2006).

In front of the cartilage, the synovium is a specialized connective tissue that seals the synovial cavity and fluid from surrounding tissues in diarthrodial synovial joints. Also called the synovial membrane (SM), it is a semi-permeable membrane, which is responsible of controlling molecular traffic into and out of the joint space, maintaining the synovial fluid (SF) volume and composition, mainly by producing lubricin and HA, which are critical in preservation of normal physiologic state of articular cartilage. Through the synovial fluid, the synovium also aids in chondrocyte nutrition (together with subchondral bone), as articular cartilage has no intrinsic vascular or lymphatic supply. The inner layer, or intima of

synovium lies next to the joint cavity and consists of a layer of 1–4 cells named synoviocytes that have been identified by immunohistochemical and cytochemical methods as macrophages and fibroblasts; the latter is the dominant cell population in healthy synovium (Smith, 2011). The cellular elements of the SM are a major source of SF components; these components contribute to the unique functional properties of articular surfaces and modulate chondrocyte activity.

Molecules from degraded hyaline cartilage during aging release into the synovial cavity and start synovial inflammation in OA. Early in knee OA, damage to the meniscus may also release tissue debris, although molecules released from subchondral bone may also play a role. Synoviocytes begin to react by producing pro-inflammatory mediators. In result, they attract immune cells, increase angiogenesis and induce a phenotypic change in chondrocytes (Ng et al., 2010). Chondrocytes produce additional cytokines and proteolytic enzymes, which eventually increase cartilage degradation and induce further synovial inflammation (Baker et al., 2010).

Then Cartilage and synovial fluid are two distinct compartments, but that are able to exchange important regulatory signals in the control of cartilaginous system, and these interactions are potentially deregulated during pathophysiological evolution of OA (Chevalier et al., 2013) (Figure 27). Spontaneously in our studies on cartilage we have also been interested in the evolutions of HS in the synovial fluid and in the fibroblastic like synoviocyte that constitute part of their cellular components. We clearly show that specific HS patterns are presents in synovial fluid and fibroblastic synoviocytes that are different to those observed in cartilage. Remaining questions are: (i) What is the link between these distincts glycanic signatures? (ii) Are they presented by the same family of HSPG? (iii) Are they taking part of the same signaling pathway in 2 different cells types, chondrocytes and synoviocytes? Or are they due to very distinct molecular regulatory pathway. (iv) On an other

fragment (digested by heparanase?) that are of interest during OA? All these questions remained to be addressed because our data are the first to clearly demonstrate HS pattern in synovium.

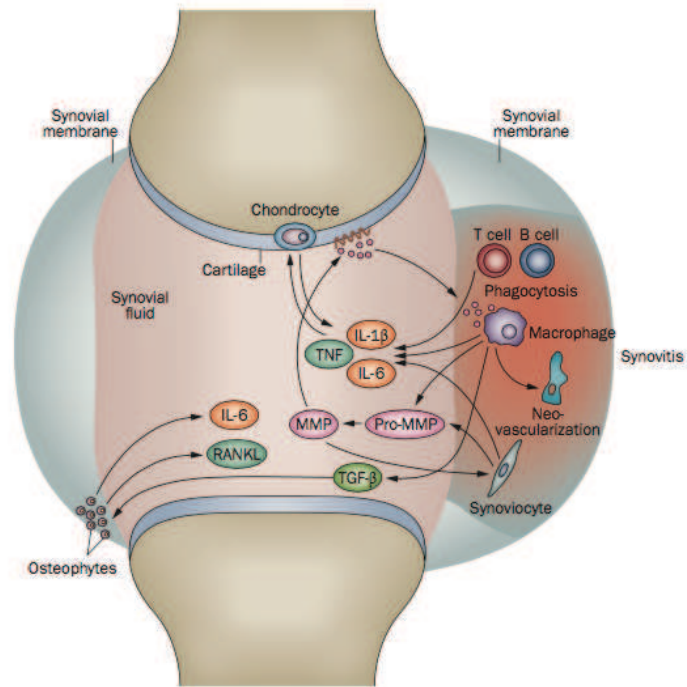
It is known that during synovial inflammation, the synovial fluid located at the interface between the cartilage and the synovial membrane can play a deleterious role, via the presence in it of a certain number of pro-inflammatory mediators, promoting chondrolysis. The induction of an inflammatory phenotype at the level of synoviocytes of type A and B (secretion of cytokines IL-1 $\beta$ , TNF- $\alpha$ , IL-6 or IL-8) but also of a pro-catabolic phenotype characterized by the secretion of metalloproteinases (MMP) such as MMP-1, MMP-3, MMP-13 or aggrecanase is also detrimental to the progression of osteoarthritis (Sellam and Berenbaum, 2010). In addition, arthroscopic studies show that in an osteoarthritic joint, cartilage and synovial involvement are located in the same area, suggesting paracrine communication between these tissues (Ayrat et al., 2005). One hypothesis highlights the role of cartilage debris released in synovial fluid (Silverstein et al., 2017). In addition to the role of clearing cartilaginous debris, the synovial fluid could also have an effect on the synovial membrane and cartilage (Koppikar et al., 2015). However, if pro-inflammatory factors are present, their synovial concentration remains very variable and their functional role on the surrounding cells, chondrocytes and synoviocytes, also depends on the accessibility to their cellular receptors.

No data in the literature currently describes the role that HS could play in these inflammatory factor modulation processes, most of which are HBP. However, our preliminary results suggest the presence of HS in the synovial fluid as well as in the membrane of synoviocytes in culture. In addition, we have demonstrated a very particular sulfation profile of these HS, currently described in very few tissues and only under inflammatory conditions. As with cartilage HS, this glycan signature is associated with changes in HS affinity for HBP. These

preliminary results make it possible to envisage an equally important role of synovial membrane HS in the pathophysiology of osteoarthritis.

The hypothesis that we propose is that the HS present in osteoarthritic synovial fluid could be derived from cartilage (products of degradation or neosynthesis) and/or in synoviocytes (neoproduced according to inflammatory stimuli). These HS could be involved in the activation of chondrocytes and synoviocytes via their binding to HBP modulators of inflammation, such as IL-1 and IL-6. It would therefore be interesting to look in particular if synovial fluid HS alone can induce an inflammatory (release of IL-6, IL-8, PGE<sub>2</sub>) and pro-catabolic (MMP-1, MMP-3, MMP -13) phenotype on synoviocytes and a hypertrophic phenotype (expression of RunX2, Col X, MMP-13) on chondrocytes in culture. These functional effects could be studied according to the different HSs collected from the synovial fluid. In other words, the type of HS's glycanic signature could influence their functionality.

As previously discussed, it's also important to identify which kind of HSPG bear these specific and functional HS chains. Because they are more rare in cartilage, the functional effects of HS chain PGs have been little studied so far in osteoarthritis. Recent data, however, suggest an important role for perlecan, an HSPG present in the peri-cellular matrix of cartilage and in synovial fluid during osteoarthritis: (i) its synthesis is increased in osteoarthritic cartilage (Tesche and Miosge, 2004), (ii) its selective inhibition in the synovial membrane decreases osteophyte formation in a murine model of destabilization of the medial meniscus (DMM) (Kaneko et al., 2013), (iii) its selective inhibition limits the capacity of differentiation of synoviocyte stem cells to a chondrocyte phenotype (Sadatsuki et al., 2017). Finally Shu et al. have shown that the binding domain of HS chains on perlecan is important for transmitting its effects (Shu et al., 2016). Then Perlecan need to be highlighted in our glycanic studies.



**Figure 27: Cytokine production in OA.**

OA is a whole-organ disease involving cytokine production by cartilage, synovial membrane, and bone. Cytokines such as TNF, IL-1 $\beta$  and IL-6 are produced by chondrocytes, macrophages, T cells and osteophytes in response to tissue damage. Pro-MMPs, released by synoviocytes and macrophages, are cleaved into MMPs and further contribute to tissue damage. IL-1 $\beta$ , TNF and IL-6 enter the bloodstream; OA is therefore a systemic disease. T cells and B cells are recruited by the cytokine milieu in the synovial fluid and contribute to local synovitis. Bone cells release several cytokines, most notably IL-6 and RANKL. Adapted from (Chevalier et al., 2013).

### **3. Other role of HS in Inflammatory process during OA**

Products of cartilage breakdown released into the synovial fluid are phagocytosed by synovial cells, amplifying synovial inflammation. In turn, activated synovial cells in the inflamed synovium produce catabolic and proinflammatory mediators that lead to excess production of the proteolytic enzymes including MMPs and aggrecanases, responsible for cartilage breakdown, articular matrix degradation, creating a positive feedback loop. The inflammatory response is amplified by activated synovial T cells, B cells and infiltrating macrophages. To counteract this inflammatory response, the synovium and cartilage may produce anti-inflammatory cytokines. In addition to these effects on cartilage inflammation and breakdown, the inflamed synovium contributes to the formation of osteophytes via BMPs. (Sellam and Berenbaum, 2010).

Damage to products of cellular and cartilage ECM from trauma, microtrauma or normal aging makes Damage-Associated Molecular Patterns (DAMPs) that activate the innate immune system (Liu and Rando, 2011; Scanzello et al., 2008). DAMPs could be in form of fragments generated from proteins, proteoglycans or remnants of cellular breakdown, such a uric acid (Chen and Nuñez, 2010; Scanzello and Goldring, 2012a). One of our question could be if GAG and HS sulfated disaccharides could be such as DAMPs.

Synovial inflammation is likely to contribute to disease progression in OA, as proved by the correlation between biological markers of inflammation and the progression of structural changes in OA (Clark et al., 1999; Sowers et al., 2002).

At histological level, synovium in OA patients is characterized by sublining fibrosis, synovial lining, hyperplasia with an increased number of lining cells and a mixed inflammatory infiltrate mainly consisting of macrophages (Benito et al., 2005) and stromal vascularization (Blom et al., 2007). Due to presence of variety of cytokines and cell adhesion molecules, there is a huge influx of leukocytes from the vascular compartment (Belcher et al., 1997).



Many studies have demonstrated that T cells and macrophages are the most predominant immune cells in OA synovium, whereas mast cells, B cells and plasma cells are also found in fewer extents (Bock et al., 2001; Borzi et al., 2000). The presence of macrophages in synovium has been already confirmed. These cells could cluster and form Multinucleated Giant Cells (MGCs) for improved phagocytosis, and are increased in similar numbers in inflamed OA and RA synovia compared with non-inflamed OA and post-mortem controls (Blom et al., 2007).

Macrophages are known to be the most important responsible of innate immune activation and cytokine production in the OA joint. Experimental studies on macrophages have revealed the involvement of these cells in both reduction of inflammation and progression of OA. Depletion of macrophages from OA synovium significantly reduced the inflammatory response, including cytokine response and the activity of proteolytic enzymes (Bondeson et al., 1999). In addition, *in vivo* depletion of synovial macrophages has led to less MMP activity and less cartilage damage in mouse model of OA (Blom et al., 2007). Macrophages themselves secrete growth factors, such as TGF $\beta$ , which can ameliorate cartilage repair (Blaney Davidson et al., 2007a). Thus, the application of macrophages seems to be an attractive approach in OA therapy. In our study we don't have yet analyse the HS signature of Macrophages purified from synovial fluid but this aspect could be of interest to determine the role of such glycanic signature in this cellular component of interest in cartilage homeostasis

HS are involved in many step of inflammation such as leukocytes recruitment to inflammatory sites through interaction with the inflamed endothelium that expressed numerous selectin (E-S- and P-), extravasation process through the blood vessel wall, and chemokine gradient formation (Parish, 2006). All these phenomena involved cellular HSPG such as syndecan and glypican family, as well as matricial HSPG such as Perlecan and agrin

migration through the interaction of their selectin with syndecan HSPG expressed on endothelial cells, they interact with chemokines (that are HBP also) on the underlying basement membranes, these chemokines being presented by HSPGs such as Perlecan. In addition, for leukocytes to traverse the subendothelial basement membrane, they need to solubilise the basement membrane components by deploying various proteases and the heparan sulfate degrading enzyme heparanase. During basement membrane solubilisation, heparanase then release growth factors associated to HSPG that will eventually contribute to a chronic inflammatory response by inducing angiogenesis and tissue remodeling. It's important to note that heparanase not only aids leukocytes invasion of the basement membrane but also regulate the inflammatory responses in several ways. It's seem highly probable therefore that vascular beds can modulate inflammatory responses by changing the chemical structure of the HS sulfated chains they express. Indeed there is good evidence that endothelial cells in both normal and inflamed synovium expres SDC3, but HS chains on SFC3 bind CXCL8 only under inflammatory conditions!! (Patterson et al., 2005).

Since, HS is associated with many aspects of inflammation, molecules that interfere with HS function have considerable potential as anti-inflammatory agents. It has been known for many years that heparin has anti-inflammatory activity (Ekre et al., 1992; Lider et al., 1990), most probably by interfering with HS-dependent stages of leukocyte extravasation (Johnson et al., 2005). A principal difficulty with heparin, however, is that it is structurally diverse and therefore has many effects in vivo, some of which are undesirable. As there is considerable evidence that different HS-binding proteins interact with unique saccharide sequences in HS (Esko and Selleck, 2002), it should be possible to design small HS mimetics that specifically disrupt various stages of leukocyte transmigration. In fact, several mimetics have already been developed that have potential anti-inflammatory activity by blocking heparanase activity (Khachigian and Parish, 2004), and by disrupting the interaction of chemokines (Freeman et

that specifically target certain HS–protein interactions.

An alternative to the use of HS mimetics is the synthesis of small peptides that resemble the HS-binding sites of cytokines, chemokines and adhesion molecules. This point is very interesting in case of known pro-inflammatory cytokines such as  $\text{INF}\gamma$  and  $\text{TNF}\alpha$  as their interaction with GAGs has already been revealed (Kenig et al., 2008). Since these two cytokines are widely involved in pathophysiology of many inflammatory disorders, it is remarkably important to find a possibility to regulate their action. One peptide of this type has already been produced that corresponds to the HS-binding region of  $\text{INF}\gamma$ . It interferes with the binding of  $\text{INF}\gamma$  and some chemokines to GAGs and is effective in delaying the rejection of skin allografts in rats (Fernandez-Botran et al., 2002). In the same line more studies are necessary to create GAG mimetics that could specifically interact with these mentioned cytokines and other pro-inflammatory cytokines in order to exploit them to prevent inflammatory disorders.

### **TNF $\alpha$ –GAGs interactions dilemma: good or bad cytokine in OA**

It has been recently shown that  $\text{TNF}\alpha$  induces heparan sulfate 6-O-endosulfatase 1 (Sulf-1) expression in fibroblasts (Sikora et al., 2016). In a dose- and time-dependent manner, via a mechanism involving NF- $\kappa$ B, ERK1/2 and p38 MAPK. Interestingly, it has been already discussed that intra-articular injection of Sulf-1 have the potential to prevent cartilage degeneration by suppressing MMP-13 via an effect on FGF2/Erk1/2 signaling (Otsuki et al., 2017). Therefore, one can imagine the possibility of a positive role for  $\text{TNF}\alpha$  during OA. This notion could be even more empowered after several recent studies showing the positive role of TNF/TNFR2 signaling pathway on regulatory T cell (Leclerc et al., 2016). Knowing

the important role of T cells and inflammatory macrophages in pathophysiology of OA, the boosting effect of TNF $\alpha$  on Treg functionality seems to be highly positive.

On the other hand, the presence of TNF $\alpha$  in OA could directly and indirectly, through VEGF cooperation, increase the angiogenesis and neovascularization of endothelial cells (Goukassian et al., 2007). This side of story does not seem to be of interest of OA treatment since this phenomenon increases the incidence of OA. Therefore, more studies are required to be performed to clearly understand the both side of the balance.

### **Which mechanisms are responsible for these modifications in sulfation patterns of HS?**

The functional strength of HS is clearly associated to their chemical structure, which is particularly complex and depends of several families of enzymes. These enzymes include the anabolic glycosyl transferases (EXTs and EXTLs), epimerase and sulfotransferases (NDSTs and HSTSs), and the catabolic heparanase and HS 6-O-sulfatase Sulfs. Some of these enzymes have numerous isoforms, which are differentially expressed in time and tissue. Very few data are available on the transcriptional and post-traductional mechanisms that are involved in the control of the specificity of the spatio temporal pattern of expression of these differents enzyme. However, according to the so stricking specificity of the function of their product, we could imagine that these patterns of expression are very tighly control. So one of the future research directions of my host lab, and specifically of P. Albanese on OA, is to study such kind of transcriptional control of the promoter of this enzyme, to try to make a link between a glycanic signature and a physiopathological process.

In that way, To sustain our hypothesis that a link exist between HS sulfation, GAG biosynthesis enzyme and modifying activity in OA, we referred to studies from Lotz el all demonstrating that changes in sulfation of GAG can regulate growth factor signaling in

cartilage. Lotz et al work on Sulf1 and Sulf2, the two HS 6-O-endosulfatases that edit the sulfation of multiple HSPG, regulating the activity of multiple growth factors. Thus, Sulf1 and Sulf2 are essential for mammalian development and cell survival (Lamanna et al., 2008). Increased expression of Sulf1 and Sulf2 has been detected in human OA tissues and in aging mouse as compared with normal joints (Otsuki et al., 2008). But spontaneous cartilage degeneration and surgically induced OA were significantly more severe in Sulf-1 (-/-) and Sulf-2 (-/-) mice compared with wild-type mice. Indeed, Sulfs were shown to enhance bone morphogenetic protein (BMP) /Smad 1/5 but also to inhibit FGF/Erk1/2 signaling in articular chondrocytes, participating to the maintenance of cartilage homeostasis (Otsuki et al., 2010). More recently, it has been demonstrated that intra-articular injection of Sulf1 in mouse OA model following surgical destabilisation of the medial meniscus have the potential to prevent cartilage degeneration by suppressing MMP-13 via an effect on FGF2/Erk1/2 signaling (Otsuki et al., 2017). Although binding of either growth factor or tissue repair effect to HS has not been investigated in these mutants, the results are in agreement with a role of HS sulfation pattern in regulating the activity of cell signaling pathways during OA. These data suggest that de-sulfatation of HS induced by Sulf could have a protective effect during OA, which is in accordance with our observation of the presence of over-sulfated HS during OA.

## **New strategies to overcome OA**

New strategies based on injection of chondro-protective cells such as chondrocytes and MSCs are under study, but will be limited due to the fragility of these cellular products, not only during its production in vitro but also after injection in non-vascularized cartilage in vivo. In that way we suggest that it will be of interest to associate such cell based therapy to matricial one.

According to the study of Vangsnæs et al, they demonstrated that by using of intra-articular injection of human mesenchymal stem cells with sodium hyaluronate (hyaluronic acid/hyaluronan), the effects of MSCs on osteoarthritic cartilage changes in the knee (Vangsnæs et al., 2014). This could be an explanation of the good effect of non-sulfated GAG that can promote therapeutic effect of MSCs on OA treatment. This study could be related to our result, which we showed that, increasing of sulfation pattern of HS during pathology of OA could be involved in induction of cartilage degradation. To be sure about this hypothesis first we have done some in vitro test to see what is the functional effect of GAG from OA (high sulfated GAG) compare to CT (low sulfated GAG). On MSCs. Interestingly, we observed that GAG from CT that are characterized as a low sulfated GAG (according to HPLC analysis) can induce adhesion and proliferation of MSCs which was not the case of OA GAG with higher level of sulfation. The next step should be use of purified HS from OA and low sulfated HS mimetic to be clear about functional effect of these two high sulfated and low sulfated HS alone on cells like MSCs and chondrocyte proliferation and adhesion.

This test should be confirmed in in vivo test by injection of HS (high and low sulfated) with cells (MSCs and chondrocyte) into the OA induced cartilage's mice.

My PhD thesis could help to clarify the role of GAG, and specifically HS, in the physiological process of OA, which is necessary for such treatments.





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## **Annex**

## **HS in HTAP**

- **Article 3**

**Heparan sulfate imbalance and fine structural modifications of heparan sulfate sulfation patterns in lung are associated with functional effects in Precapillary Pulmonary Hypertension.**

Albanese P, Dumas S J, **Shamdani S**, Ranchoux B, Chantepie S, Huinh M B, Henault E, Montani D, Girerd B, Dormuller P, Fadel E, Dartevelle Ph, Perros F, Humbert M, Papy Garcia D, Cohen Kaminsky S.

# Heparan sulfates imbalance and fine sulfation pattern modifications in lung are associated with functional effects in Precapillary Pulmonary Hypertension

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### Background

Pulmonary hypertension (PH) is associated to the progressive obstruction of the distal precapillary pulmonary vasculature that contributes to the increase in pulmonary vascular resistance and pulmonary arterial pressure. This vascular remodeling involves endothelial cell dysfunction, smooth muscle cell proliferation and altered extracellular matrix (ECM). Among ECM, sulfated glycosaminoglycans (GAG) such as heparan sulfates (HS) and chondroitin sulfates (CS) are essential components in lung homeostasis (1). They control cell migration and proliferation through selective binding of chemokines and growth factors, usually known as "Heparin Binding Protein" (HBP). The specificity of GAG interactions with HBP is strikingly controlled by post-translational modification of HS disaccharides such as sulfation (2).

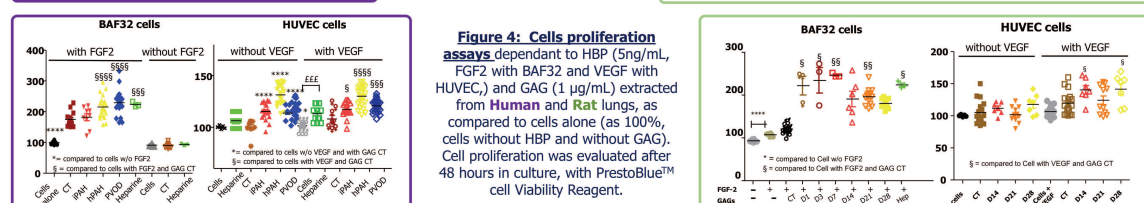
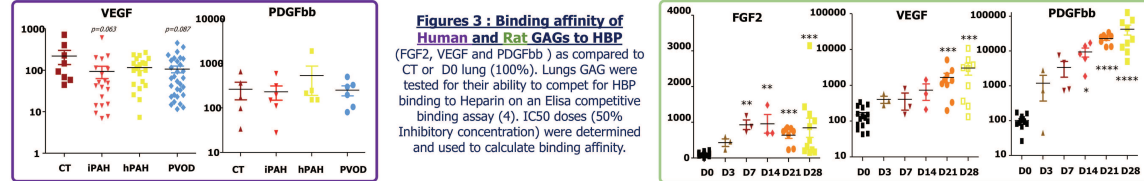
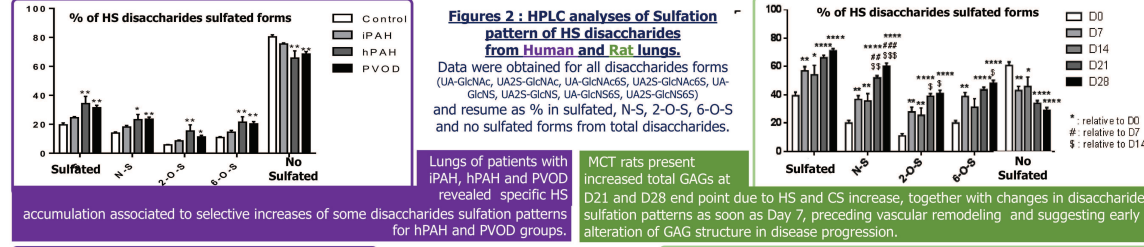
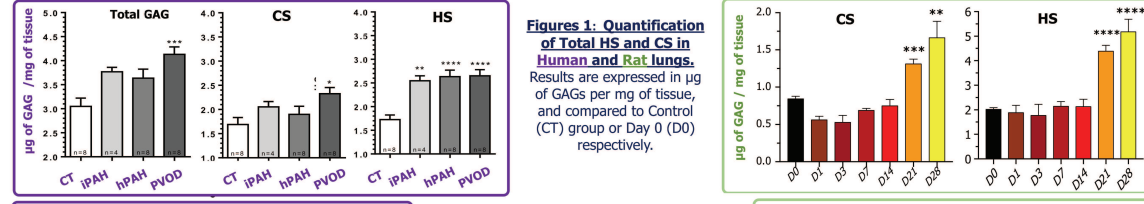
### Aims and objectives

In PH lung, altered ECM participates to lung arterial remodeling. We hypothesized that changes in GAG and specifically in HS patterns, composition and fine structure, could alter their bioactivity as selective binding to growth factors and cell proliferation, thus leading to vascular remodeling.

### Methods

Human and animals lung sampling were performed with approval of the appropriate ethical committees. **Patients with idiopathic (IPAH, n=5), heritable (hPAH, n=7) PAH, pulmonary veno-occlusive disease (PVOD, n=8) and controls (n=8)** were included in the study. Patients were diagnosed at the National referral Center for severe PH (Kremlin Bicêtre Hospital), and the lung samples collected at the time of transplantation were stored frozen in the PH tissue bank of Marie Lannelongue Hospital until use. **Experimental PH was induced with a single injection of Monocrotaline (MCT) in Rats, and lung samples were recovered according to a kinetic at D0, D1, D3, D7, D14, D21 and D28 (n=6 per days).** The glycomic platform skills of the CRRET lab (3,4) permit to performed a complete Quantitative (GAG total amount, HS/CS ratio), qualitative (disaccharidic sulfation pattern) and functional analysis (growth factor binding, cell proliferation) of GAGs extracted from human and animals lungs. ANOVA/Bonferroni statistical analysis were performed. \*\*\*\* P < 0,0001; \*\*\* P < 0,001. \*\* P < 0,01; \* P < 0,05

## Results



### Conclusions

Structural modification of HS sulfation patterns in Human and Rat experimental PH lungs are correlated to significant functional modifications of GAG binding to growth factors (VEGF, FGF-2, PDGF) and to increased cell proliferation in the presence of the altered GAGs. We propose that these specific changes of HS sulfation patterns in the lung could maintain a chronic inflammatory and vascular remodeling process leading to deregulated vascular cell proliferation through abnormal binding to growth factors. These changes may rely on deregulation of glycanic targets in the pathological lung, of interest to study for future therapeutic intervention.



## Article 3

**Heparan sulfate imbalance and fine structural modifications of heparan sulfate sulfation patterns in lung are associated with functional effects in Precapillary Pulmonary Hypertension.**

ALBANESE Patricia<sup>1</sup>, DUMAS Sébastien J.<sup>2,3</sup>, SHAMDANI Sara<sup>1</sup>, RANCHOUX Benoit<sup>2,3</sup>,  
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**Running title:** *Heparan sulfate sulfation changes in precapillary PH*

## **Abstract**

**Rational:** Sulfated glycosaminoglycans (GAGs) such as heparan sulfate (HS) and chondroitin sulfate (CS) are essential components of the extracellular matrix (ECM) in lung homeostasis. In pulmonary hypertension (PH), altered ECM participates to lung arterial remodeling.

**Objectives:** We hypothesized that changes in GAG and specifically in HS patterns, composition and fine structure in the lung could alter their bioactivity as selective binding to growth factors and effects on vascular cell proliferation, thus leading to vascular remodeling in PH lung. Here we deciphered the fine structure and bioactivity of GAGs both in human and experimental PH.

**Methods:** We performed quantitative (HS/CS ratio, total amount), qualitative (disaccharidic sulfation pattern) and functional analysis (growth factor binding, cell proliferation) of GAG extracted from human lungs patients with precapillary PH and controls, and of monocrotaline (MCT) PH rats. Patients with idiopathic (iPAH) and heritable (hPAH) pulmonary arterial hypertension as well as patients with pulmonary veno-occlusive disease (PVOD) were included.

**Measurements and main results:** Lungs of patients with iPAH, hPAH and PVOD, revealed specific HS accumulation associated to selective increase of disaccharide sulfation patterns for hPAH and PVOD groups. MCT rats presented increased total GAGs at end point, due to HS and CS increase, together with changes in disaccharide sulfation patterns sooner preceding vascular remodeling, and suggesting early alteration of GAG structure in disease progression. These structural modification of HS sulfation were correlated to significant functional modifications of GAG binding to growth factors (VEGF, FGF-2, PDGF) and to increased cell proliferation in the presence of the altered GAGs.

**Conclusion:** Specific changes of HS sulfation patterns could maintain a chronic inflammatory and vascular remodeling process leading to deregulated vascular cell proliferation through abnormal binding to growth factors. These changes may rely on deregulation of lung sulfation enzymes that could be targeted for future therapeutic intervention.

**Keywords:** glycosaminoglycan, lung, Pre-capillary PH, pulmonary Arterial hypertension, PVOD, heparan sulfate sulfation pattern, vascular remodeling,

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**Authors' contribution:**

**Running head:** Altered HS sulfation patterns in precapillary pulmonary hypertension

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## Introduction

Pulmonary arterial hypertension (PAH) is a rare, severe and devastating pulmonary vascular disease characterized by chronic increase in pulmonary artery pressure above 25 mmHg, due to a progressive obliteration of small pulmonary arteries, [1]. In PAH, remodeling of small pulmonary arteries is a very complex and multifactorial process ultimately leading to secondary right heart failure and death. PAH could be idiopathic, heritable, induced by drugs and toxins, or secondary to several conditions [2]. The majority of known genetic variations associated with PAH occur in bone morphogenetic protein receptor, type II (*BMPR2*) gene, encoding a type of transforming growth factor (TGF)- $\beta$  family of receptors. *BMPR2* mutations are responsible for the etiology of approximately 80% familial PAH and 30% idiopathic PAH [3-5]. Pulmonary veno-occlusive disease (PVOD) is a rare form of pulmonary hypertension (PH) characterised by preferential remodelling of the pulmonary venules, associated to a biallelic mutations in the *EIF2AK4* gene as the cause of heritable PVOD. PVOD and PAH share a similar clinical presentation, with features of severe precapillary PH. However PVOD carries a worse prognosis and life-threatening pulmonary oedema may occur following the initiation of PAH therapy. Thus it is important to differentiate these two conditions [6].

During the remodeling process of the pulmonary microcirculation in PAH, endothelial cell dysfunction (EC) and smooth muscle cell proliferation (SMC hyperplasia) are thought to mediate structural changes of the pulmonary vasculature. Moreover vascular remodeling of small pulmonary arteries leads to the reduction of the vessel lumen, which results from endothelial cell (intima), smooth muscle cell (media), and fibroblast (perivascular tissue) over proliferation in the pulmonary vessel wall [7]. It is increasingly evident that chronic inflammation plays a central role in PAH pathogenesis [8]. Triggers for the vascular remodeling include injury, inflammation, abnormal growth factor expression, and/or exposure to hypoxia [9]. The interplay among endothelial cells, vascular smooth muscle cells, pericytes and fibroblasts is controlled by heparin binding growth factor signaling pathways, that are critical for the development of a functional microvasculature and vascular remodeling and that could be involved in dysfunctions during the remodeling process of PAH. For example, FGF-2 is markedly overproduced by pulmonary endothelial cells in PAH and contributes to smooth muscle hyperplasia [10] and to altered endothelial phenotype by autocrine FGF-2 signaling [11]. Moreover in human lung tissues, numbers of pericytes (pericyte coverage) are increased in distal PAH pulmonary arteries and their proliferation

migratory and differentiation properties are regulated by FGF-2, IL-6 and TGF- $\beta$  growth factors [12]. During the last decades, therapeutic progresses have been made, addressing endothelial cell dysfunction. Drugs such as epoprostenol derivatives, endothelin receptor antagonists and phosphodiesterase type 5 inhibitors allow clinical, functional and hemodynamic improvement. However, prognosis remains poor with median survival <5 years and refractory cases are candidates for lung transplantation [13]. There is no approved anti-remodeling therapy approved so far in PAH. Therefore better understanding of PAH pathomechanisms is needed to identify new therapeutic avenues for PAH patient management.

As disturbances in extracellular matrix (ECM) production, deposition, and composition have been implicated in the pathophysiology of PAH [14-17]. Restoration of ECM balance has been suggested as a strategy to prevent or reverse vascular remodeling processes [18, 19]. The complex ECM network provides structural support for the cells in the vessel wall, enables intracellular communication, and promotes proliferation, migration, and apoptosis [20]. The ECM in human pulmonary vessels predominantly consists of structural fibrillary components such as collagen I and elastins, as well as glycoproteins including fibronectin, laminins, tenascin C (TnC) [21].

Next to these fiber-forming molecules (collagene and elastin) a large number of less abundant molecules are part of the ECM, including proteoglycans (PGs) and glycosaminoglycans (GAGs) know to play key roles on cell behaviour to maintain tissue homeostasis. GAGs are long anionic polysaccharides composed by specific disaccharides building blocks with high complexity of structures due to heterogeneity in glycosidic linkage, sulfation pattern and hydrophobic regions. Four GAG subfamilies are distinguished through their repeating disaccharide units: heparan sulfate (HS)/Heparin, chondroitin/dermatan sulfate (CS/DS), keratan sulfate (KS) and hyaluronic acid (HA). Heparin is structurally similar to HS, but has a higher degree of *N*- and *O*- sulfation. Sulfated GAGs are covalently linked to core protein to form proteoglycans (PGs) whereas HA, which is the only unsulfated GAG species, is free in the ECM. PGs and HA interact with proteins to strengthening the structure and equilibrium of the ECM. However, it is admitted that GAGs and especially HS, may have functional roles due to their particular structural features, regulating their interactions with surrounding heparin-binding proteins (HBPs). HS protect and trigger the activity of HBPs which include chemokines, cytokines and enzymes [22]. Sulfated GAGs thus provide matrix-bound or cell

their half-life and providing storage sites [23, 24]. Moreover, by immobilizing HBPs on the ECM and cell surfaces, GAGs restrain their diffusion and facilitate both the formation of local gradients and the synchronous coordination of motility and cell adhesion [25-27].

GAGs are of crucial importance for maintaining the architecture and functioning of the different lung compartments [28]. Recent data suggest that GAG structural changes, and specifically HS sulfation pattern, could be associated to pathological mechanism such as tissue injury or remodelling. Indeed, the dying cells release numerous enzymes degrading or modifying GAGs that induces a massive liberation of HBPs that are no longer protected by GAGs, and so rapidly degraded by proteases also. Moreover, as it was demonstrated that the fine structure of GAGs and their capacities to regulate trophic factors activities are tightly regulated during physiological processes such as aging [29, 30], it's assumed that such structure/function modulations are involved in pathological processes such as degenerative disease associated to strong tissue remodelling, during ischemia or dystrophy (chevalier, negroni), myelofibrosis (martinaud albanese) or Alzheimer disease (Sepulveda et al). To date hyaluronan, the only unsulfated form of GAG, is one of the GAG that has been investigated in PAH [31]. The finding that heparin fragments can modulate PH in a guinea pig experimental model, suggests to us a role for sulphated GAGs structurally related to heparin in disease pathogenesis.

The aim of this study was to characterize the modifications of the glycanic profiles of HS during PAH that could be associated to vascular remodeling in lung, and the consequences of growth factor binding to these GAGs and on vascular cell proliferation. This was performed combining in situ observations in explanted PAH patient lungs and dynamics of changes after induction of the pathology in an experimental model of pulmonary hypertension (PH) in rats. We hypothesized that delineating the changes in the expression and composition of sulfated GAGs in IPAH pulmonary arteries might lead to a better understanding of disease pathogenesis, and more specific therapy based on their processing enzyme targeting.

## Materials & Methods

### Collection of Human samples

Human lung specimens were obtained from PAH and PVOD patients during lung transplantation and from control subjects during lobectomy or pneumonectomy for localized lung cancers. In control lung specimens, the samples studied were located far away from the tumor areas. PAH and PVOD were diagnosed by cardiac catheterization at the National Reference Center for PAH, in a program approved by our institutional ethics committee, CPP IDF VII (Comité de Protection des Personnes Ile-de-France VII), and written informed consent for participation in the study was obtained (protocol N8CO-08- 003, ID RCB: 2008-A00485-50, approved on June 18, 2008). Lung histology was assessed by a pathologist, after transplantation, and confirmed the diagnosis of PH subgroups. Clinical characteristics of all study subjects, including baseline demographic information, pulmonary haemodynamics, New York Heart Association Functional Class (NYHA FC), 6-min walk distance (6MWD) and lung function testing, were retrieved from the Registry of the French PH Network. This Registry was established in accordance with French bioethics laws (Commission Nationale de l'Informatique and des Libertés) and all patients gave informed consent. The clinical and hemodynamic data of idiopathic PAH, heritable PAH and PVOD patients are summarized in Table 1.

Diagnosis of precapillary PH, defined as a mean pulmonary arterial pressure (mPAP)  $\geq 25$  mm Hg with a normal pulmonary capillary wedge pressure (PCWP) of  $\leq 15$  mm Hg, was confirmed in all patients by right heart catheterization. Right atrial pressure, mPAP, PCWP, and mixed venous oxygen saturation were recorded. Cardiac output (CO) was measured by using the standard thermodilution technique. Pulmonary vascular resistance was calculated as  $(mPAP-PCWP)/CO$ , expressed in Wood units. Acute vasodilator testing was performed during right heart catheterization by using inhaled nitric oxide, as previously described. Routine evaluation at baseline included medical history, physical examination, echocardiography, high-resolution CT of the chest, ventilation/perfusion lung scan, abdominal ultrasound, autoimmunity screening, and human immunodeficiency virus (HIV) serology. Age at diagnosis, modified New York Heart Association functional class, and 6-minute walk distance were also recorded. All patients tested for BMPR2 and EIF2AK4 mutations received genetic counselling and signed written informed consent.



## **Experimental model of pulmonary hypertension**

Six-week-old male Wistar rats (100g body weight) (Janvier Labs) received a single subcutaneous injection of saline or 60 mg.kg<sup>-1</sup> monocrotaline to induce experimental pulmonary hypertension. Organ sampling was performed at day 0 for control rats and 1 day, 3 days, 7 days, 14 days, 21 days and 28 days after monocrotaline injection for the others. For each time point, rats were submitted to right-heart catheterization under anesthesia (2% isoflurane (Baxter) mixed with air) to assess the absence or presence of pulmonary hypertension. Briefly, a 3.5 French umbilical vessel catheter connected to the pressure detector was introduced into the jugular vein and inserted into the right ventricle and then into the pulmonary artery. The signal obtained was amplified and analyzed automatically with the Cardiomax III physiological data acquisition system (Colombus Instrument). After exsanguination *via* the abdominal aorta, the heart was removed from the thoracic cage, the auricles were removed and the right ventricle was separated from the left ventricle associated with the septum. Each part of the heart was weighed, and the ratio of right ventricle-to-left ventricle plus septum weight (Fulton index) was calculated for each rat. The lung was removed and snap-frozen in liquid nitrogen. All organs were stored at -80°C.

All rats were maintained in a room with controlled temperature and humidity under a 12 h light/12 h dark cycle, with *ad libitum* access to standard chow and water. They were used in strict accordance with European Union regulations (Directive 2010/63/UE) for animal experiments and our institution's guidelines for animal care and handling. The procedures performed were approved by the local ethics committee, CEEA26 (Animal experimentation ethics committee no. 26) and the French Ministry of Higher Education and Research.

## **GAGs extraction and quantification**

Frozen human and rat lung samples were freeze-dried and suspended in GAGs extraction buffer as previously described [29], according to slight modifications. Briefly, samples were digested by proteinase K (Merck, France) and DNase (Qiagen, France) and then transferred to 4 mol/L NaCl final sample concentration and vigorously agitated for 30 min. Proteins were precipitated and supernatants were cleared by chloroform washing followed by dialysis (Spectrum, France) of the aqueous phase against extraction buffer and then pure water and stored at -20°C until use. Extraction/quantification quality controls (QC) were included in

each experiment. These QC were prepared from freeze dried cartilage samples in where a known quantity of GAGs, similar to that expected in most samples, was spiked. This was made in order to calculate the extraction yield and at the end of the processes. After freeze drying, species of the extracted GAGs were analyzed by specific digestion with chondroitinase ABC (Sigma-Aldrich, France) or by nitrous acid treatment and quantified according to the 1–9 dimethyl-methylene blue (DMMB) assay as previously described [32].

### **Dimethylmethylene blue (DMMB) assay of total sulfated GAGs**

Extracted total sulfated GAGs were quantified according to 1-9 dimethyl-methylene blue (DMMB, Sigma Aldrich) assay as previously described (Barbosa et al. 2003). Briefly, total GAG extracts were diluted to 100  $\mu$ L with pure H<sub>2</sub>O and 1 mL of DMMB solution was added. Samples were stirred for 30 min and centrifuged (12 000 rpm, 15 min). Samples supernatants were discarded and pellets were dissolved in 500  $\mu$ L of a decomplexing solution. GAGs in the decomplexing solution were quantified by measuring the absorbance (650 nm) in a fixed volume from the mixture using a micro plate reader (TECAN). Total GAG quantities in the samples were determined according to the linear portion of a standard / calibration curve generated in the same condition with known concentrations (from 2,5 to 30  $\mu$ g/mL) of standard CS-A (Sigma C9819). For each sample, CS, KS, and HS amount were determined after chondroitinase ABC, heparitinases I/II/III, and keratanase treatments, respectively.

### **Quantification of CS and HS species**

Quantification of CS and HS were performed as previously described (Barbosa et al, 2003), and briefly resumed respectively. For CS quantification, total GAG extracts were diluted in a glycanase digestion buffer (CH<sub>3</sub>COO<sup>-</sup>Na 50 mM, CaCl<sub>2</sub> 2mM), and CS were enzymatically digested by chondroitinase ABC (Chse ABC) treatment (30 mU, SIGMA C3667) at 37°C for 1 hour. The remaining GAG amounts were determined by following the DMMB protocol as described above. The amount of CS in samples was calculated as the difference with total GAG quantified in the same sample. Efficiency of CS digestion was controlled with samples of know amount of CS untreated or treated with Chondroitinase. For HS quantification, total

chemically digestion of HS followed by addition of a same volume of acetic acid 33% (VWR 20103.364). Briefly, samples were incubated at RT, for 1 hour and the reaction was stopped by adding 1 vol of ammonium sulfamate (14%) (SIGMA 228745). The remaining GAGs were determined by following the DMMB protocol as described above. The amount of HS in samples was calculated as the difference with total GAG quantified in the same sample. Standard calibration curve with known HS (SIGMA H7640) and CS concentrations (2.5 – 30 µg/mL) was included in each experiment.

### **Structural characterization of HS and CS by HPLC analysis**

In order to characterize sulphated pattern of HS and CS disaccharides on GAG chains, HPLC- Anionic exchange Chromatographic technique allows relative distribution determinations (ref JBC 2012).. Disaccharides of purified HS or CS were obtained from total GAG extract from human lung sample and rat lung samples all along the MCT rat kinetic. HS and CS were digested in disaccharides by treatment with either heparinase I, II, and III cocktail (Iduron, U.K. 0.25 mU each, 16h, 37 °C) .or chondroitinase ABC (Sigma-Aldrich 20 mU, 90 min, 37°C). Samples were analyzed by HPLC as previously described [33] with some modifications. Briefly, 50 µL of sample was loaded onto a Propac PA-1 (Dionex) strong-anion exchange column eluted by a NaCl-solvent gradient. Post-column in-line modification was realized by mixing 2-cyanoacetamide solution (2% v/v) and 250 mmol/L NaOH supplied both at 0.25 mL/min. The mixture passed through a reaction coil set in an oven at 120°C, followed by a cooling coil and then fluorimetric monitoring was registered ( $\lambda=346$  nm excitation,  $\lambda=410$  nm emission fluorescence detector (JASCO; FP-1520). Peak corresponding to each Disaccharides sulfation pattern are identified by their retention time compared to a mix of external standards (Iduron, UK). Moreover, 3 controls of digestion using commercial GAG were included in each experiments to validate complete enzymatic digestion. Areas under curve were measured and the percentage of each disaccharide in sample was calculated as % of area of each peak relative to the sum of the total area for all disaccharide by sample.

### **GAG binding competition towards heparin-binding proteins**

Capacities of GAGs extracted from lung to interact with heparin binding protein FGF2,

Najjam et al. [34]. Briefly, ELISA plates (VWR, France) were coated with 0.5 µg/well of heparin-BSA complex. After washing (1X PBS – 0.05% Tween 20), wells were saturated with 3% BSA in 1X PBS. The tested HBP were Recombinant Human Basic FGF-2 (PROMOKINE, C-60240) and Recombinant Human VEGF-165 (PROMOKINE C-64420). Then, HBPs diluted in BSA 1%/PBS (FGF2: 0.5 ng/well; VEGF: 12 ng/well; PDGF-BB xx ng/ml) together with increasing concentration of extracted GAGs (serial dilution from 0, 0.01 to 100 000 ng/mL in 1X PBS) were simultaneously added into duplicate wells. Heparin (Sigma) at concentrations ranging from 0.01 to 1000 ng/mL was used as a control of the GAGs competitive binding. After 1h of binding competition and washing step, the HBP remaining bond to heparin was targeted by an specific antibody (anti-FGF2 Mouse Monoclonal IgG diluted 1/2000, R&D systems MAB233; anti-VEGF Rabbit Polyclonal IgG, diluted 1/500 PROMOKINE PK- AB815-64420) for 1h at rt, followed by a HRP-conjugated secondary antibody (HRP-AffinPure Donkey Anti-Mouse IgG 1/5000, Jackson ImmunoResearch 715-035-151; HRP-AffiniPure Goat Anti-Rabbit IgG 1/2000, Jackson ImmunoResearch 111-035-144). Peroxidase activity was measured by the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB, SI254783, Thermo Scientific, France) substrate as described by manufacturer's protocol. The maximum binding (100%) was determined in presence of the HBP and in absence of extracted GAG and and was used as a reference to determine the dose sufficient to displace 50% of binding of HBPs to immobilized heparin (IC50). Finally, IC50 values were normalized for each HBPs to the IC50 of GAGs extracted from control human and rat lung (IC50 reference=100%).

### **Baf32 cell mitogenic assay.**

Mitogenic assay was performed on Baf32 cells that are deficient in FGFR and need HS and FGF2 signaling to optimize their cell growth. Cell viability measurements are performed according to a fluorometric detection of the reduced products from PrestoBlue using 96 well plates. In brief, Baf32 cells were cultured in RPMI 1640 Glutamax medium (Gibco 61870-010), supplemented with 10% SVF (Gibco 10270), Antibiotic-Antimycotic (Thermo 15240062) and 1 ng/mL final IL-3 (R&D systems 403-ML-010). Prior to mitogenic assay, cells are washed by centrifugation and resuspended in starving medium based on RPMI 1640 Glutamax, 10% horse serum (Gibco 26050-070) and antibiotic-antimycotic. After counting, cells are seeded at 10 000 cells/well (100 µL) into 96 well plates in starving medium and

incubated overnight at 37°C, 5% CO<sub>2</sub>. Thereafter 10 µL of GAGs are added to final concentration between 0.1 - 10 µg/mL and 10 µL of FGF-2 (Invitrogen 68-8785-82) are added for a final concentration of 5 ng/mL. GAGs and FGF-2 are conditioned in starvation medium. Controls are cells stimulated without any GAGs and with as compared to without FGF-2. Cells are incubated 72 hours at 37°C, 5% CO<sub>2</sub> (the doubling time of the cells are around 30 hours). Cell viability was measured after addition of 10 µL of PrestoBlue® in each well (Cell Viability Reagent, Invitrogen A13262), incubation 4 hours at 37°C, 5% CO<sub>2</sub>, and direct Fluorometric detection at 544 ex /590 em.

### **VEGF-dependent Mitogenic Assay on HUVEC**

HUVECs were routinely maintained at 37 °C, 5% CO<sub>2</sub> in full EBM medium (Lonza), supplemented with: 2% SVF serum, Hydrocortisone, Ascorbic acid, GA1000, Heparin, EGF, IGF, VEGF and FGF, defined by the provider. For the mitogenic test, HUVECs were seeded into 96-well plates at a density of 5,000 cells/well in starving medium EMB-2 medium (Lonza) containing 0.5% FBS, Hydrocortisone, Ascorbic acid and GA1000, without heparin and growth factors, and incubated overnight at 37 °C in 5% CO<sub>2</sub>. GAGs were then added to wells at final concentrations of 0.5, 5, or 50 ng/ml, in the presence or not of VEGF165 (5 ng/ml, Thermofisher RF233613) and cells were incubated for an additional 72h. Then 25 µl of a 5 mg/ml MTT solution (The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma M2128-10G) was added to each well and the plate was further incubated at 37°C in 5% CO<sub>2</sub> for 3 hours. Thereafter the medium was carefully take out and 100 µL of the DMSO: Sorensen's buffer solution (9:1, v/v, pH: 10.5, Sorensen's buffer: 0.1 M glycine, 0.1 M NaCl) was added to each well. After vortex to dissolve, the absorbance was determined spectrophotometrically at 570 nm. (2015 van Tonder et al). Heparin (5 ng/ml) was used as a positive control, and its effect was considered as 100% effect.

### **Cell proliferation assays on hPASCs**

Cell proliferation assays were performed on control hPASCs (Lonza) between passages 4 and 7. These cells were obtained from healthy non-smokers with no alcohol dependence. Cells were cultured according to the supplier's instructions, at 37°C, under a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air. For proliferation assays, we added 5000 cells/well to SmGM-2 complete medium (10%) in a 96-well plate. After 24 h of adhesion, cells were starved of serum in minimal medium containing SmBM medium with 0.2% FBS and antibiotics. After 24 h of serum starvation, proliferation was stimulated with 5 ng.ml<sup>-1</sup> PDGF-BB (R&D Systems) with or without 100 ng.ml<sup>-1</sup> of GAGs extracted from patient or control lungs, in minimal SmBM supplemented with 0.2% FBS (Lonza) and 0.1 μM BrdU (Perkin Elmer) for 24 h. There were six replicates. Proliferation was quantified with the DELFIA® cell proliferation kit (Perkin Elmer), according to the instructions in the user manual. The fluorescence signal in the 96-well plate was read with an Envision 2103 plate reader (Perkin Elmer).

### **Statistical Analysis**

Two-tailed tests were used throughout statistical analysis. Unpaired t tests with or without Welch's correction were used for pairwise comparisons between groups, depending on variance heterogeneity. For multiple comparisons, we used one-way analysis of variance with Bonferroni correction after the evaluation of variance homogeneity in Brown-Forsythe tests, or Kruskal-Wallis tests followed by Dunn's tests, as appropriate. Differences were considered significant if  $P < 0.05$ . Statistical analysis was performed with Prism 6 (Graphpad).

## Results

### Clinical and Hemodynamic data of PAH and PVOD patients (Table 1)

### Clinical and Hemodynamic data of HAP in vivo animal model by MCT injection in rats

#### HS and CS sulfation levels are specifically increased in PAH and PVOD

The basal amount of total sulfated GAGs in human control lung was  $3.05 \pm 0.17$   $\mu\text{g}$  per mg of dried lungs and was significantly increased to  $3.85 \pm 0.11$   $\mu\text{g}/\text{mg}$  in PAH lungs (Fig 1 SUPP). Specific GAG depolymerisation by either CSase digestion or nitrous acid treatment permit to distinguish HS and CS species respectively. This indicates an equal repartition between HS ( $1.73 \pm 0.10$   $\mu\text{g}/\text{mg}$ ) and CS ( $1.69 \pm 0.15$   $\mu\text{g}/\text{mg}$ ) in control lungs. Moreover the increase of total sulfated GAG in PAH lung was characterized by a significant 0.5 time fold increase of CS species and a 2 times fold increase of HS species (Fig 1a SUPP). Sulfated GAG amount in control and PAH lungs were reduced to 0 after successive treatment of the samples with chondroitinase digestion and nitrous acid indicating that any other GAG species are present in the samples (data not shown). A second analysis of these data (lower panel of fig 1 SUPP) was performed to distinguish PVOD patients (n=8) from PAH group (n=12) and permit to observe a much more significant increase of total sulfated GAG in PVOD patients than in PAH ones (containing idiopathic and hereditary form of PAH). Whereas the increase in HS species is significant for PVOD patients as well as for PAH patients, it is of interest to note that the increase in CS was significant in the PVOD group only ( $P < 0.01$ : \*\*) as compared to PAH. These data suggest that pattern of HS and CS amount could be representative of specific PAH forms. For example the high increase in both CS and HS species in PVOD patients as compared to PAH groups may suggest that PVOD patients exhibit a higher remodeling of the extracellular matrix.

#### HS and CS sulfation levels are specifically increased during remodelling associated with pulmonary hypertension development in the monocrotaline rat model.

In order to confirm GAG modification highlighted in PAH and PVOD in human lungs, same analysis were performed on the animal model of PH, based on Monocrotaline injection in rats. The basal amount of total sulfated GAGs in control lungs was  $2.29 \pm 0.09$   $\mu\text{g}$  per mg of

dried healthy lungs at day 0 (Fig 2A). As compared to this basal level, a slight increase of Total GAG amount was detected as soon as day 1. This increase was confirmed at day 7 and day 14, with a 1,5 fold change ((3 µg/mg of weight tissue) when tissue remodelling and increased pressure were observed respectively. Finally, at day 21 and 28 when rat began to die, the GAG sulfation content was increased by 2 and 2,5 fold and reached 5.49±0.56 µg/mg. The specific GAG depolymerisation by either CSase digestion or nitrous acid treatment allowed the distinction among sulphated HS and CS species respectively (Fig 2b) and permit to observe that basal levels of HS and CS were 2.02±0.08 and 0.84±0.04 µg per mg of dried healthy lungs respectively at day 0. Thereafter the GAG increase corresponds to both CS and HS increase from day 7 to day 28.

### **Sulfation pattern of HS and CS disaccharides units are modified in PAH lung.**

HPLC analysis allowed determining the relative composition in each disaccharide units of HS or CS species, namely the non sulfated (NoS), the monosulfated (MonoS), the disulfated (DiS) and the trisulfated (TriS). It permits also to discriminate among each sulfation pattern of a disaccharide unit as the monosulfated HS (NS, 2S, 6S), disulfated HS (NS2S; NS6S, 2S6S) and finally more rare tri-sulfated HS in mammal tissue (NS2S6S) (fig3, panel B). HS chains from control lungs were composed by 80.4±1.4 % of NoS, 11.4±1.5 % of MonoS, 5.3±0.2 % of DiS and 2.9±0.2 % of TriS disaccharide units (Fig 4a). In PAH lung samples, NoS units were significantly decreased to 69.4±1.6 %, and it was inversely associated to a significant 1,7 fold increase of all sulfated HS, NS, 2-O-S and 6-O-S. Fine analysis of the monosulfated and disulfated pic indicate that all sulfated forms in C6 of glucosamine were increased significantly, it's the case of 6S, NS6S and 2S6S, whereas other species (NS, 2S, NS2S) were slightly but not significantly increased. Finally, distinct analysis of each PAH groups, iPAH, hPAH and PVOD, suggest that whereas C6 sulfation is characteristic of PVOD group, hPAH could be discriminate by increase level of 2-O sulfation of hexuronic acid (fig 4)

In control lung CS chains were composed by 27.0±4.2 % of NoS, 68.4±4.0 % of MonoS and 4,4±0.5 % of DiS units (Fig x, supplementary data). This suggest that in control lung CS are globally much more sulphated then HS. In PAH samples, a significant increase of non sulphated CS is observed concomitently to a decrease of monosulfated disaccharides. Disulfated and trisulfated CS are not modified. On the same way, distinct analysis of the patient groups permit to discriminate for PVOD.



### **Sulfation pattern of HS and CS disaccharides units evolved over the time in the monocrotaline-induced PH rat model**

Data from HPLC analysis of human samples were compared to the rat monocrotaline kinetics sampling. Basically, control lungs from rat present a 2 time higher level of sulphated HS (40%) as compared to human control lungs (20%), and inversely, lower level of nosulfated HS (60%) in rat as compared to human (80%). A decrease of non-sulphated fraction of HS was significantly observed as soon as day 7 and day 14 and clearly confirmed at day 21 and day 28. As observed in human samples, this decrease of non-sulfated HS was correlated to a clear increase of sulfated forms within the same kinetics, significantly also as soon as day 7 and all along the kinetic until day 28. As observed in human lungs, increased sulfation concern NS, 2-O-S and 6-O-S patterns. It's important to note that this modification of sulfation pattern precede the increase of HS amount.

### **GAGs from PAH lung and MCT lung display differential binding affinities toward growth factors**

The capacity of GAGs to regulate cells functions is largely related to their abilities to bind to HBPs. Binding affinity of lung GAGs toward heparin binding protein were evaluated according to Elisa binding competitive assays. FGF-2, PDGFbb and VEGF were tested as heparin binding growth factors involved in lung homeostasis and according to their ability to potentiate biological properties of respectively fibroblasts, smooth muscle and vascular endothelial cells involves in vascular remodelling during PH. Analysis of GAG extracted from human lungs do not demonstrated significant difference in binding affinity to PDGFbb, between CT and different PAH groups, iPAH, hPAH and PVOD as well (Fig6,A). Slight decrease of binding affinities toward VEGF were observed between CT lungs and iPAH hPAH and PVOD, but not significantly with  $p=0.063$ ,  $p=0.097$  and  $p=0.063$  statistical value respectively (Fig6B). Inversely, in the case of rat lungs from MCT model, binding affinity toward FGF2, PDGFbb and VEGF clearly increase as soon as day 3, and are significant at day 7 for FGF2 with a 9 fold increase (fig7B), at day 14 for PDGFbb with 90 fold increase (Fig7A) and at day 21 for VEGF with 10 times increase (fig 7C). It's important to note that these modifications of binding affinities take place timely in parallele to those of HS sulfation patterns.

### **GAGs from PAH Lung and MCT lung modulate mitogenic activity of different cell lines**

In order to investigate the functional activities of GAGs on different cells in vitro, total GAGs extracted from lungs were tested for their mitogenic activities on cellular tests dependant to HBP. First of all, Baf32 proliferation is dependant to FGF2 and Heparin: addition of one of these compounds independently in the medium did not induce cell proliferation, whereas addition of the two concomitantly induce 2,5 increase of cells in 24h of culture (Figure 8A). In this context, GAG extracted from lungs were tested for their ability to synergise to FGF2 to induce proliferation of BAF32 cells. As illustrated in figure 8A, GAG from human CT lungs are able to induce a 2 times fold increase of cells proliferation, suggesting that there are functional and able to interact with FGF2. Moreover, GAG extracted from iPAH are able to stimulate also BAF32 proliferation to the same extend than CT lungs, but proliferating effect of hPAH and PVOD is significantly increase 2 times as compared to CT lungs. Some of these results are confirmed with rat lungs (fig 8B): GAG extracted from lung at day 0 induce a slight increase of BAF32 proliferation (clearly lower than those induced by human CT lungs). However, as soon as day 1 after MCT injection, proliferating effect of GAG extracted from rat lung is clearly significant and maintain until day28, suggesting a synergic effect to FGF2 activity. This increasing activity on cells is clearly parallele to the increasing binding affinity to FGF2 demonstrated by elisa test (fig 7).

In the same way, similar mitogenic test was performed secondly on HUVEC cells, dependant to VEGF and heparin to proliferate (as illustrated in left panel of fig 9A). We observed that in presence of VEGF (right panel of fig 9A), GAG extracted from human CT lungs are not able to induce significant proliferation of HUVEC cells, whereas GAG extracted from PAH patient have a significant effect, even higher to those obtained with heparin. It's relevant to note also that proliferative effect is stronger for hPAH groups and PVOD, than for iPAH, suggesting a distinct functionality of the GAG from this patient. (Surprisingly these functionality differences on HUVEC cells are not correlated to binding difference to VEGF in elisa tests). A second result of interest is that GAG from PAH lungs are able also to stimulate HUVEC in absence of exogenous VEGF, and that the difference of efficiency between iPAH, hPAH and PVOD is maintained. Some of these results are confirmed with rat lungs (fig 9B): GAG extracted from lung at day 0 are not able to induce significant proliferation of HUVEC cells in presence of VEGF, whereas GAG from D14 after MCT injection, have significant proliferating effect maintain until day28. This proliferative activity is not observed in absence of VEGF and confirms a synergic effect of GAG from rat lung

with this HBP on HUVEC cells. This increasing activity on cells is clearly parallel to the increasing binding affinity to VEGF demonstrated by Elisa test, and as observed for FGF2.

## Discussion

Our data clearly demonstrate that Lungs of patients with iPAH, hPAH and PVOD presents specific HS accumulation with selective increase of disaccharide sulfation patterns for hPAH and PVOD groups. MCT rats presented increased total GAGs at end point, due to HS and CS increase, together with changes in disaccharide sulfation patterns also, and sooner preceding vascular remodeling. These biochemical characterizations of GAG structure suggest early and specific alteration of HS sulfation in each kind of human disease group progression. The originality of our data lies on the significant functional information that we brings on the ability of these modified sulfated HS to bind to growth factors (VEGF, FGF-2, PDGF) and to increased cell proliferation in the presence of the altered GAGs. We hypothesis that HS play a crucial role in lung ECM remodelling during PAH. Specific changes of HS sulfation patterns could maintain a chronic inflammatory and vascular remodeling process leading to deregulated vascular cell proliferation through abnormal binding to growth factors. These changes may rely on deregulation of lung sulfation enzymes that could be targeted for future therapeutic intervention.

Our results demonstrate that HS structure and functionality are compromised in PAH. According to the multiple functions described to these GAG, these may lead to a major disturbance in the coordination of effector molecules ranging from growth factors and morphogens to proteases and matrix molecules, thus compromising orderly tissue repair and regeneration, cumulating in the development of PAH. Our data are original because studies on HS in the lung pathology are rare, primarily due to a lack of appropriate tools to study them.

Twenty years ago, the American Thoracic Society stated that “future directions should explore the role of pulmonary parenchymal GAG in maintaining alveolar integrity ”(AST 1998). Proteoglycans containing glycosaminoglycans (GAGs) of the heparin and heparan sulfate families are produced by many cell types, including vascular ECs, and are integral components of the subendothelial matrix. The majority of PGs in lung tissue carry HS, DS or CS side chains. However the amount of each type of PG differs in the different compartments. For example, large cartilaginous CS/KS proteoglycans are the major species in the tracheobronchial tree whereas pleural lining of the lung contains mainly small DSPGs. Heparin, the GAG component of the PG serglycin, is abundant in mast cells whereas other

kuppelvelt 's review [28] resume the crucial importance of proteoglycans and GAGs for the architecture and functioning of the lung, focusing on HSPG according to their distribution, their strategic ultrastructural location [35] and their involvement during development stage [36]. These sulfated GAGs contribute to the regulation of a wide variety of biological functions, including cell proliferation, differentiation, and migration [37].

Three classes of HSPG can be discerned, (i) associated to basement membranes (perlecan, agrin and collagen XVIII); (ii) the GPI-anchored to the cell membranes Glypicans and (iii) the transmembrane syndecan, that are all expressed in distinct cell-, tissue- and specific patterns. In the lung, perlecan is associated with basement membrane-producing cells and is found in all vascularized and some connective tissue (e.g., cartilage) in which it ensures tissue integrity and interacts with FGF-2 via its HS chains (Smits 2004). Syndecan isoforms are expressed by vascular endothelial cells and smooth muscle cells, and can be found in all layers of the intima, the media and the adventitia. The different fine structures of HS associated to the core protein are generated in the Golgi apparatus through the coordinated actions of successive HS modification enzymes.

Alterations in proteoglycan/GAG structure and/or content have been observed in lung tissue derived from both patients with emphysema and animal models, although results tend to be contradictory [38-42]. In lung tissue from patients with severe emphysema a diminished staining for HSPG as well as for the interstitial proteoglycans decorin and biglycan has been reported [43]. Specific inhibition of HSPGs synthesis induces emphysematous lesions in rats [44]. In elastase induced emphysema in rats, HS is readily removed from the lungs by degradation of the protein core of HSPGs [45]. As a consequence, the protective protease (elastase) inhibiting capacity of HS may be lost [46].

Perlecan is the major HSPG in the vascular ECM that binds growth factors including FGF2 and PDGFbb and thus serve as modulator of signalling pathway: the HS chains may either sequester the HBP or present the ligand to facilitate receptor activation [47-49]. Perlecan HS can inhibit SMC proliferation in muscular arteries in the systemic circulation. In a recent work, Chang and colleagues [49] used a mouse strain that express perlecan deficient in HS (Hspg2d3d3). They demonstrated pulmonary vascular development is impaired in mice expressing HS deficient Perlecan (Hspg2) and that when challenged with hypoxia, the PH is less severe in these mice. All these data suggest that HS chains from Perlecan are necessary

to proliferation of SMC and normal vascular arborisation during development and could be involved also during pathological hyperproliferation of PASMC, through FGF2/FGFR1 interaction, during vascular remodelling in Lung PH.

These work clearly indicate that during normal development proliferative activity of HS perlecan chains are controlled, whereas during induced PH, HS chains are hyperproliferative on PASMC. Our data suggest that these changes from Good to Bad role of HS chains could be due to some modification in their sulfations patterns.

Much more precise information specifically about HS domains and structures in lung is very sparse but suggest large heterogeneity of structurally different HS chains [28] New tools are now entering the field, including antibodies defining specific HS domains [50-53] and sequencing techniques to establish the monosaccharide sequence of HS [54, 55].

It was shown that Heparin inhibit vascular remodeling associated with hypoxic pulmonary hypertension in vivo, This was due to an anti-proliferative effect of heparine on pulmonary artery smooth muscle cells that has been attributed to 3-O-sulfate on the internal glucosamine on synthetic pentasaccharide fragments. However, study from Garg et al [56] on delta-tetrasaccharides suggest that 3-O-sulfate of glucosamine residue is not critical in whole heparins for antiproliferative activity.

Proliferation of vascular pericytes (PCs), smooth muscle-like cells found in the distal microvasculature, contributes also to vascular remodeling in PH, but the factors controlling lung PC quiescence in normal states are poorly understood. It was demonstrated that exogenous heparin and HSPG inhibit rat lung PC proliferation in vitro as does pulmonary vascular subendothelial matrix, particularly its HS component [57]. Heparin inhibits the intracellular alkalinization that reduces PC proliferation. Heparin does not induce apoptosis in PCs, but maintains lung PCs in the G0/G1 growth phase. Heparin induces production of p21, a potent inhibitor of cyclin-dependent kinases, thereby potentially identifying a fundamental mechanism by which heparin inhibits proliferation in smooth muscle-like cells. These studies establish additional similarities between lung PCs and smooth muscle cells and provide further understanding of growth control in the lung microvasculature. They also further support the rationale that heparin-like molecules might be therapeutically beneficial in HTAP. PCs are found just beneath the endothelial layer, and they share the endothelial cell (EC) basement membrane (BM). This endothelium-synthesized unidentified extracellular

matrices components that inhibit vascular smooth muscle and pulmonary vascular PCs proliferation [58, 59].

Exogenous heparin markedly inhibits smooth muscle cell proliferation *in vivo* after arterial injury [60] and the pulmonary vascular remodeling caused by hypoxia [61]. Heparin is also known to inhibit the development of pulmonary hypertension *in vivo* [61]. Heparin and heparin-like molecules, including heparan sulfate proteoglycan (HSPG), bind to vascular smooth muscle cells and strongly inhibit their growth *in vitro* [59, 62-64].

Heparins from various commercial sources, structurally modified heparins. The effects of HSPGs, specific enzymes to degrade subendothelial BMs: both de-N-sulfation and carboxyl reduction of heparin reduced its growth inhibitory activity. Other sulfated GAGs, chondroitin sulfate and dermatan sulfate, also inhibited PC growth but they were less effective than heparin. Heparin has a higher degree of sulfation than the other sulfated GAGs studied, and this may explain its more potent antiproliferative effect. Furthermore, pentosan polysulfate, which is even more highly sulfated than heparin, has a more potent antiproliferative effect on smooth muscle cells.

The generation of knockout mice for various proteins involved in HS biosynthesis have highlighted HS role in lung development and pathology. Some of them induce severe lethal phenotype associated to pulmonary deficiency, such as respiratory failures in NDST-1<sup>-/-</sup> mice [65, 66], aberrant lung morphology in HS6ST-1 [67] breathing difficulties and enlarged airspaces in *sulf2*<sup>-/-</sup> mice [68].

HS6ST-1 is one of the isoform enzyme involved in 6-O-sulfation of HS. In Izvolsky et al 2008 study [69], adult HS6ST-1<sup>-/-</sup> mice show enlargement of the airspaces, associated with fragmented and irregularly deposited elastin in alveoli, a feature suggestive of pulmonary emphysema. The formation of lung alveoli is known to be regulated by Wnt5a, FGF-10, and BMP4, which bind to HS/heparin. HS6ST-1 is expressed strongly at the tips of branching tubules in the developing lung [70] and FGF-10 is preferentially bound to 6-O-sulfate residues rather than 2-O-sulfate residues in HS. A deficiency in HS6ST-1 is potentially relevant to impaired lung development, and suggest that 6-O-sulfation of HS is essential for homeostasis of alveoli. Decreased 6-O-sulfation may affect the signalling of growth factors and ECM proteins in the lung which impact alveolarization. This involvement of 6-O-sulfated HS during lung development clearly comfort our data on involvement of 6-O-sulfation of HS during vascular remodeling of PAH.

Very recently it was observed that idiopathic pulmonary fibrosis (IPF) is associated with increased amount of HS and CS in lung with modified structures according to increase of sulfation pattern, with a specific increment of 2-O, 6-O and N-sulfation of HS [71]. These highly sulphated GAGs were colocalized with perlecan in basement membrane of blood vessels and airways, as well as on the cell surface of spindle shaped cells in the alveolar interstitium. Our data on PAH and PVOD confirm that both amount and structure of GAG are altered in this lung pathology such as observed in IPF, but inform much more precisely on the kind of sulphated pattern of HS disaccharides involved in PAH. We were able to precise that specific disaccharides HS sulfation pattern could permit to discriminate between PAH groups, iPAH, hPAH and PVOD. Finally, independently on the fact that these glycanic studies were performed on distinct pulmonary pathologies, that involved different cellular and molecular targets, the input and novelty of our data lies on the functional informations that we demonstrate. Modified HS during human and rat PAH, have altered ability to bind to keys HBP as FGF2, VEGF and PDGbb, and associated to increase of the proliferation properties of endothelial and fibroblastic cells. This sustained our hypothesis that HS sulfation modification are associated with vascular remodeling during PAH.

The therapeutic potential of GAGs and HS in lung was already suggested. It was shown that heparin and sulfated polysaccharides could prevent elastase-induced emphysema in mice and hamsters [72-75]. Moreover, aerosolized hyaluronan had protective role against elastase-induced or smoke-induced lung damage[76]. Then, lung glycobiology exploration with its new tools had just begun and it should permit new way of research and futures discoveries of glycanic targets for the development of new therapeutics to treat lung pathologies.



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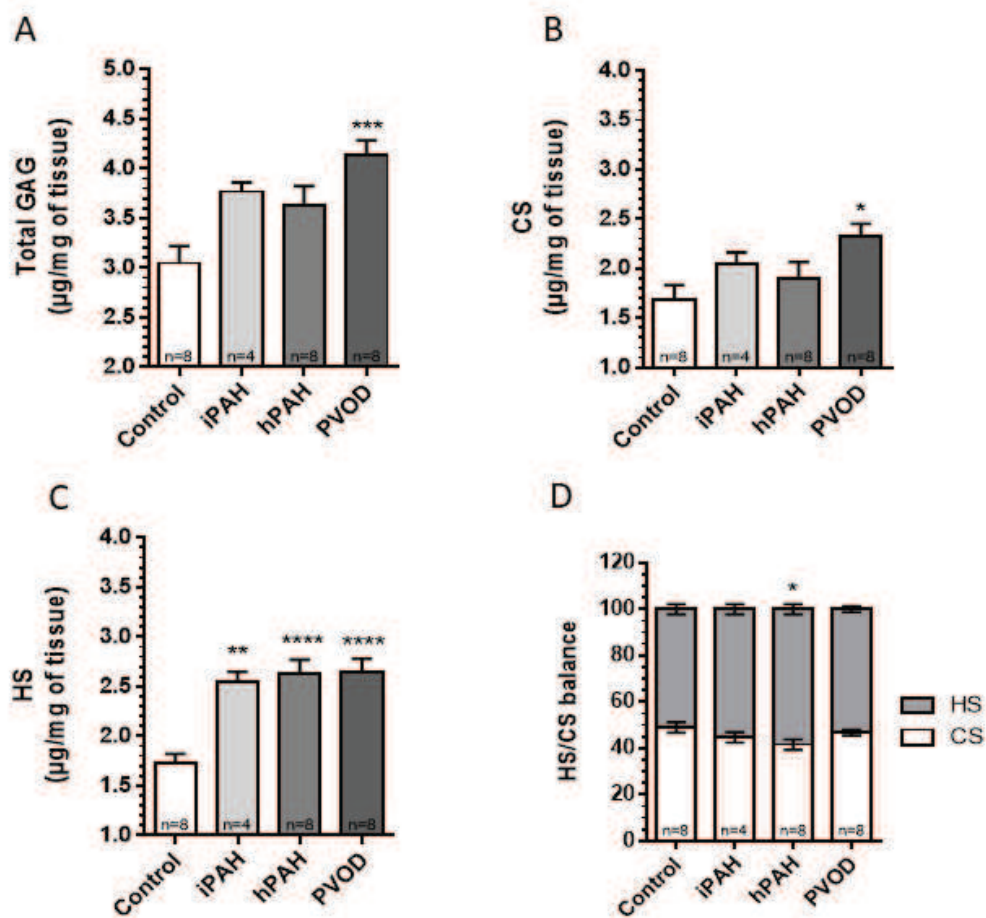
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**Table 1: Clinical and hemodynamic data of PAH patients.** Data expressed as mean  $\pm$  SEM unless otherwise indicated.

- NYHA: New York Heart Association
- Acute vasodilator response is defined by a fall in Ppa of at least 10 mm Hg, reaching an absolute value of Ppa under 40 mm Hg, associated with no change or an increase in CI.
- 6-MWD: 6-minute walk test
- mPAP: mean pulmonary arterial pressure
- CI: cardiac index
- PVRi: pulmonary vascular resistance index
- ERA: endothelin receptor antagonists
- PDE5-i: phosphodiesterase 5 inhibitors
- CCB : calcium channel blockers

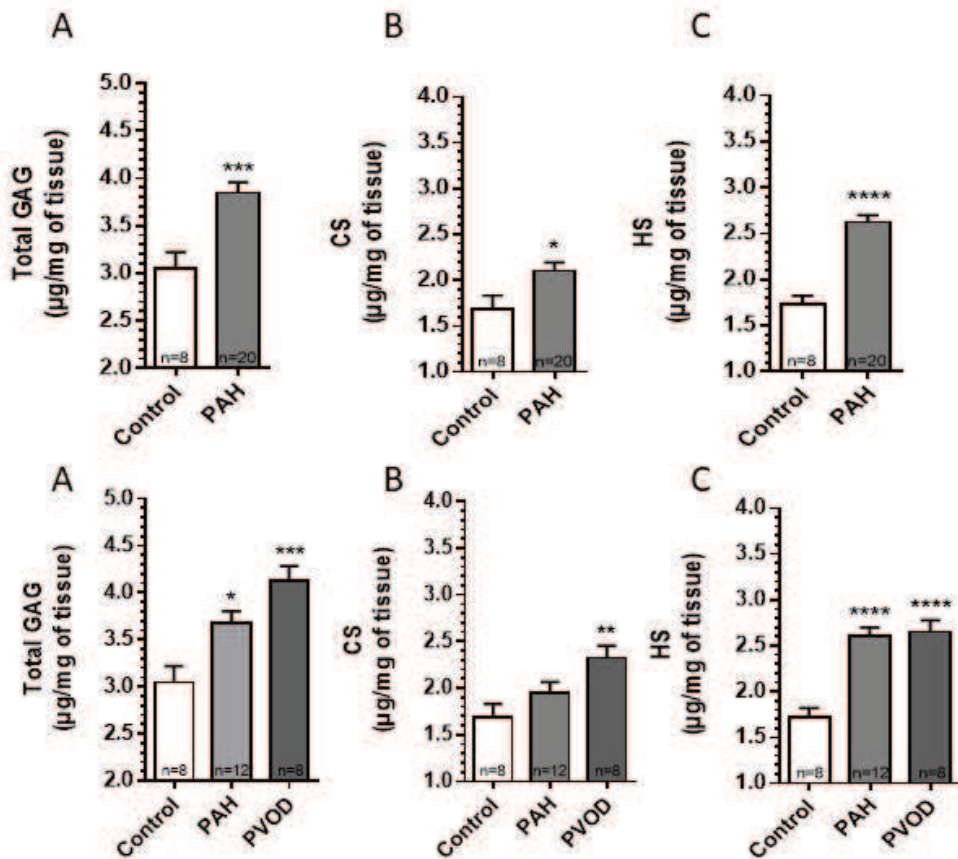
	PAH n=22	Controls n=23
Age, yrs	47.5 $\pm$ 3.3	38.2 $\pm$ 2.0
Sex, M/F	6 / 16	10 / 13
Heritabe/Idiopathic, n	7 / 15	
NYHA <sup>(a)</sup> functional class, n		
- I-II	15	
- III-IV	7	
Disease duration, yrs		
- 0.1-5	9	
- > 5	13	
Acute vasodilator response <sup>(b)</sup> , n	5	
6-MWD <sup>(c)</sup> , m	489 $\pm$ 19	
mPAP <sup>(d)</sup> , mmHg	47.23 $\pm$ 2.99	
CI <sup>(e)</sup> , l/min/m <sup>2</sup>	3.48 $\pm$ 0.18	
PVRi <sup>(f)</sup> , mmHg/l/min/m <sup>2</sup>	6.92 $\pm$ 0.67	
Specific PAH therapy, n		
- ERA <sup>(g)</sup>	3	
- PDE5-i <sup>(h)</sup>	1	
- ERA <sup>(g)</sup> + PDE5-i <sup>(h)</sup>	4	
- Prostanoids	1	
- PDE5-i <sup>(h)</sup> + Prostanoids	1	
- ERA <sup>(g)</sup> + PDE5-i <sup>(h)</sup> + Prostanoids	7	
- ERA <sup>(g)</sup> + CCB <sup>(i)</sup>	3	
- CCB <sup>(i)</sup>	2	

**Figure 1 : Total GAG, HS and CS amount in human iPAH, hPAH and PVOD Samples.** Sulfated GAGs were extracted and quantified according to chemical and enzymatic digestion protocol, normalized in  $\mu\text{g}$  of GAGs by  $\text{mg}$  of dried tissue. A) Total sulfated GAGs level of lung from healthy donor and iPAH, hPAH and PVOD patients. B)  $\mu\text{g}$  of Cs per  $\text{mg}$  of tissue. C)  $\mu\text{g}$  of HS per  $\text{mg}$  of tissue. D) HS/CS balance. Total GAG amount was considered as 100% and HS and CS ratio were determined and compared. Statistical analysis were performed according to ANOVA/Bonferonni test.

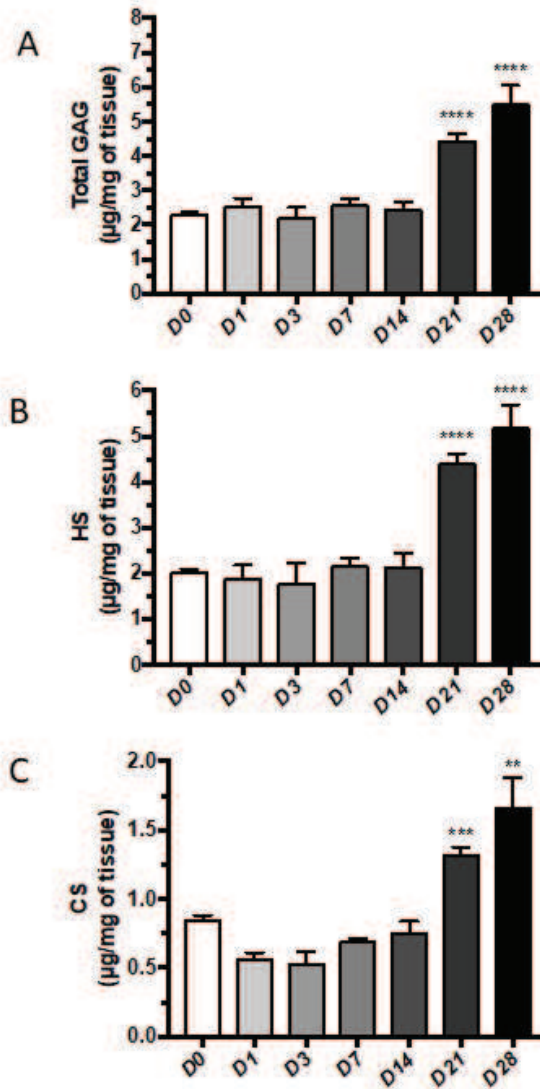




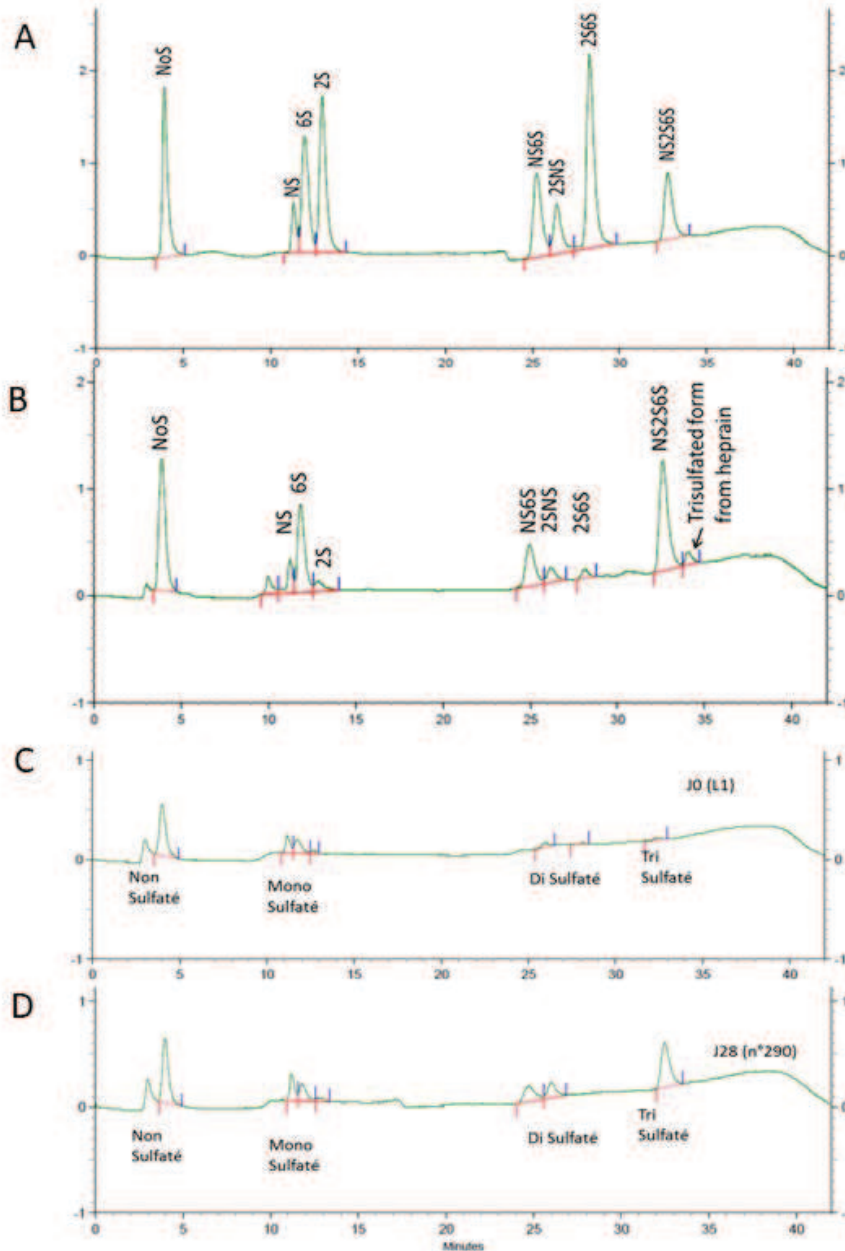
**Figure 1 SUPP : Total GAG, HS and CS amount in human PAH and PVOD Samples.** Sulfated GAGs were extracted and quantified according to chemical and enzymatic digestion protocol, normalized in  $\mu\text{g}$  of GAGs by mg of dried tissue. A) Total sulfated GAGs level of lung from healthy donor and PAH and PVOD patients. B)  $\mu\text{g}$  of Cs per mg of tissue. C)  $\mu\text{g}$  of HS per mg of tissue. Results are presented according 2 ways : upper panel, is a global analysis of all PH patient (PAH correspond to mixed iPAH, hPAH and PVOD patients); lower panel, PVOD are analysed separatlyn PAH corresponding to iPAH and hPAH. Statistical analysis are performed as compared to CT lung.



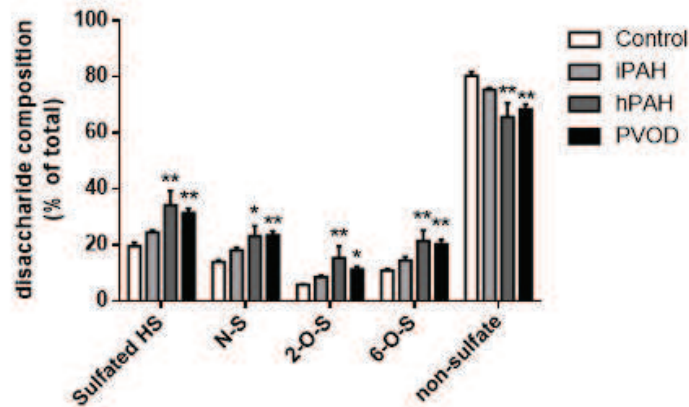
**Figure 2: Total GAG, HS and CS amount in rat lung samples.** Lung were sampled all along a cinetic from Day 0 to Day 28, after monocrotaline injection in rat. Sulfated GAGs were extracted, quantified according to chemical and enzymatic digestion protocol, normalized in  $\mu\text{g}$  of GAGs by mg of dried tissue. A) Total sulfated GAGs level B)  $\mu\text{g}$  of Cs per mg of tissue. C)  $\mu\text{g}$  of HS per mg of tissue. Statistical analysis were performed according to ANOVA/Bonferonni test.



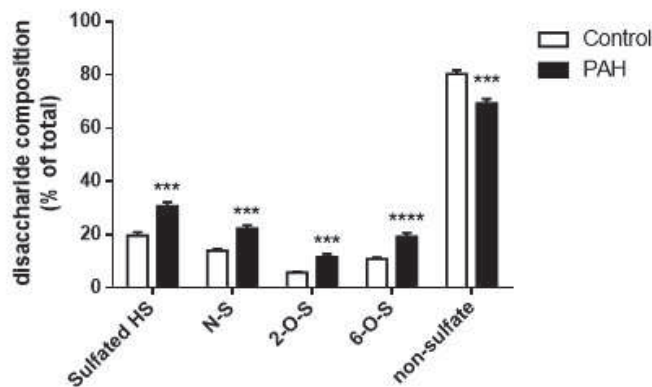
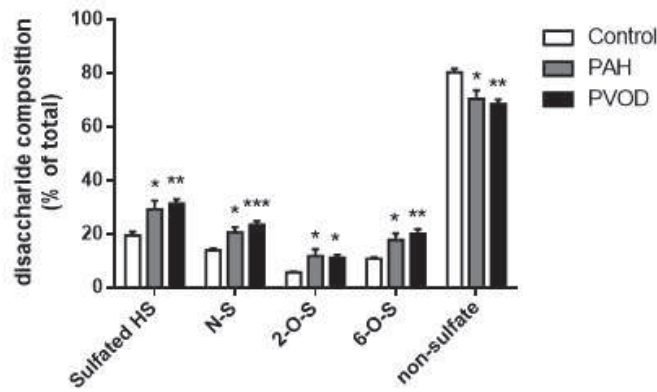
**Figure 3 : HPLC profiles of HS disaccharides** (A) Mixed commercial standards of sulfated and nosulfated (NoS) disaccharides; Monosulfated disaccharides (MonoS) as NS, 6S and 2S; Di-sulfated (DiS) disaccharides as NS6S; 2SNS and 2S6S; tri-sulfated (TriS) disaccharide NS2S6S. (B) Disaccharides profiles obtained after digestion of a mix of commercial heparine and HS used as Digestion control. Trisulfated form of heparin can be distinguish from trisulfated form of HS disaccharides; (C) Disaccharides profiles from HS extracted from lung of control rat at Day0; NoS, MonoS, DiS and TriS are represented (D) Disaccharides profiles from HS extracted from lung of MCTrat at Day0. NoS, MonoS, DiS and TriS are represented



**Figure 4 : HPLC analysis of HS disaccharides units sulfation pattern, from GAG chains purified from control (n= 6x) iPAH (n=3) hPAH (n=3) and PVOD (n=6x).** HPLC analysis allowed to determine the relative composition in each disaccharide units, namely : (NoSulfated) UA-GlcNAc; (Monosulfated) UA2S-GlcNAc, UA-GlcNAc6S, UA-GlcNS; (Disulfated) UA2S-GlcNAc6S, UA2S-GlcNS, UA-GlcNS6S, and (Trisulfated) UA2S-GlcNS6S. All forms are quantified as % as compared to total all forms. Analysis of HPLC profiles was presented according to the type of sulfated forms, global sulfated HS, versus N-S, 2-O-S, 6-O-S, and non-sulfated HS. Each sample was analyzed 2 times. The statistical significance of differences between the groups was determined by Anova/Bonferroni ou Kruskal Wallis/Dunn's for heterogeneous variances. p value < 0.05 was considered to be statistically significant. \*\*\*\* P<0,0001; \*\*\* P< 0,001. \*\* P<0,01; \* P<0,05.

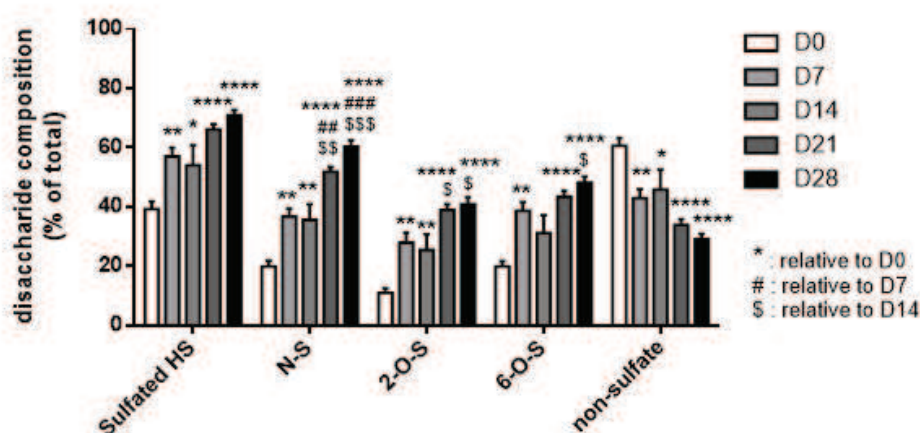


**Figure 4 SUPP1: HPLC analysis of HS disaccharides units sulfation pattern, from GAG chains purified from control (n= 6x) PAH (n=6) and PVOD (n=6x).** HPLC analysis allowed to determine the relative composition in each disaccharide units, namely : (NoSulfated) UA-GlcNAc; (Monosulfated) UA2S-GlcNAc, UA-GlcNAc6S, UA-GlcNS; (Disulfated) UA2S-GlcNAc6S, UA2S-GlcNS, UA-GlcNS6S, and (Trisulfated) UA2S-GlcNS6S. All forms are quantified as % as compared to total all forms. Analysis of HPLC profiles was presented according to the type of sulfated forms, global sulfated HS, versus N-S, 2-O-S, 6-O-S, and non-sulfated HS. Panel (A) compare control to PAH (mix iPAH and hPAH) and PVOD groups; whereas Panel (B) ) compare control to PAH (mix of all : iPAH hPAH and PVOD groups) Each sample was analyzed 2 times. The statistical significance of differences between the groups was determined by Anova/Bonferroni ou Kruskal Wallis/Dunn's for heterogeneous variances. p value < 0.05 was considered to be statistically significant. \*\*\*\* P< 0,0001; \*\*\* P< 0,001. \*\* P<0,01; \* P<0,05.

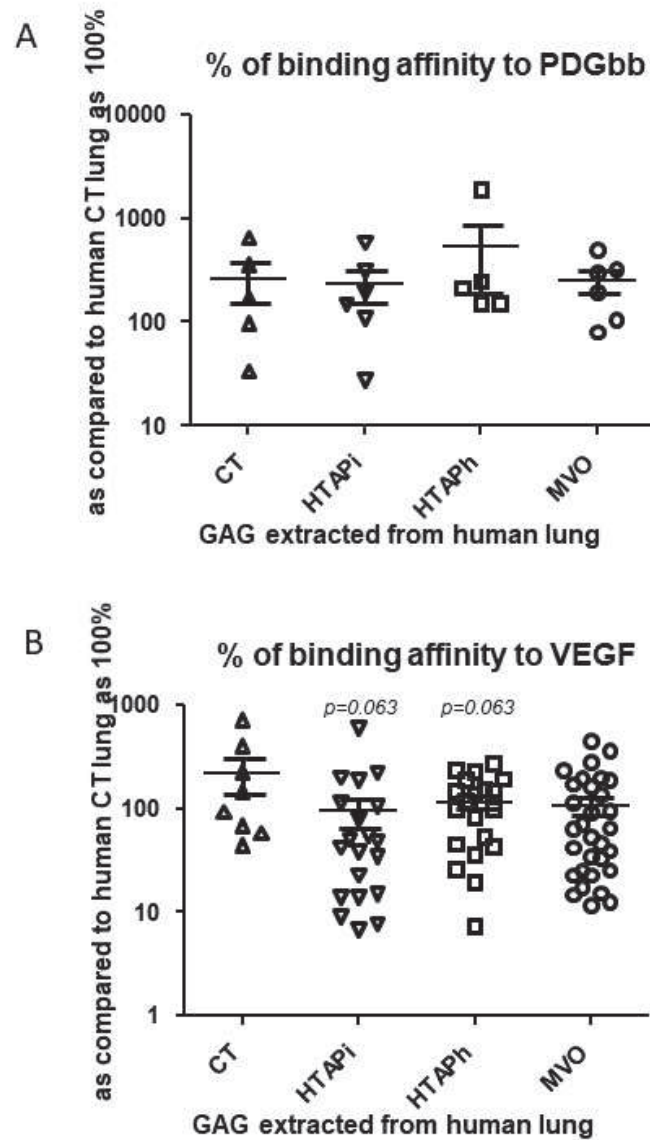


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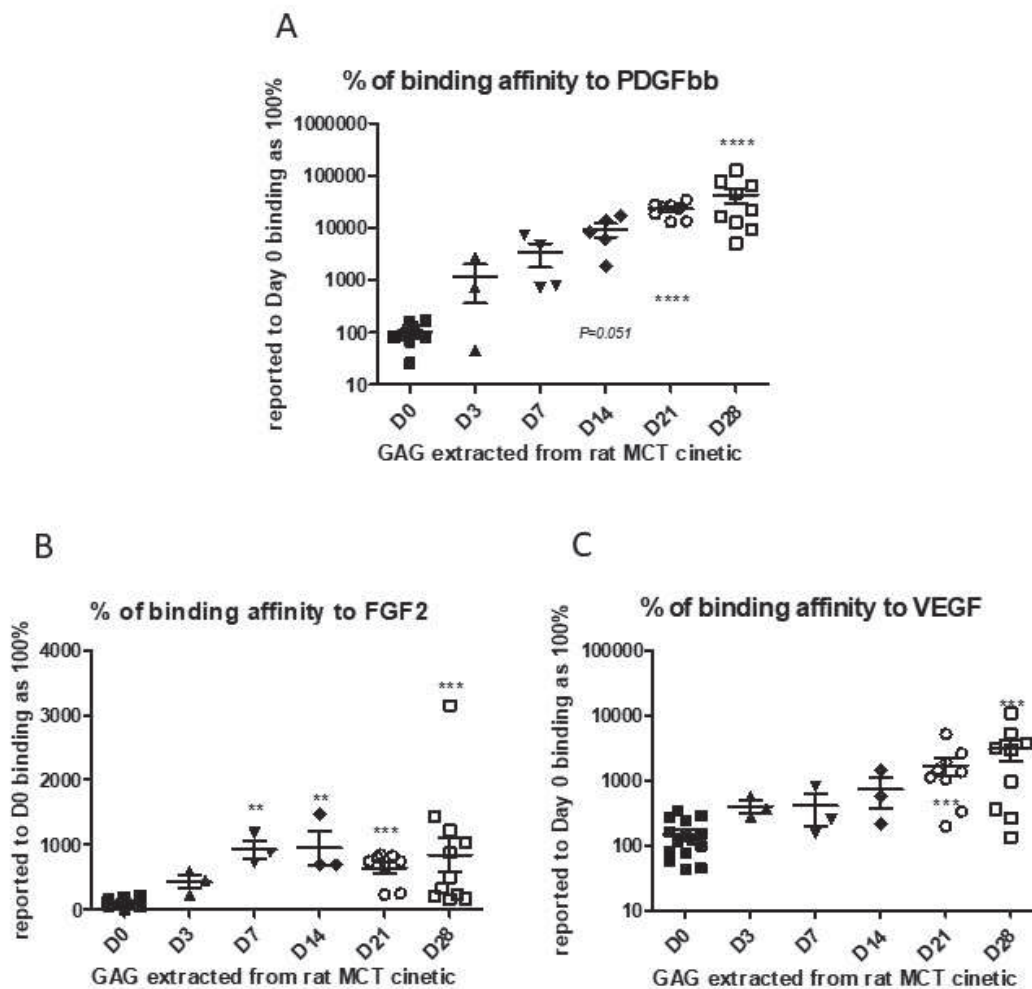
**Figure 5 : HPLC analysis of HS disaccharides units sulfation pattern, from GAG chains purified from rat lungs all along kinetic of Monocrotaline model.** HPLC analysis allowed to determine the relative composition in each disaccharide units, namely : (NoSulfated) UA-GlcNAc; (Monosulfated) UA2S-GlcNAc, UA-GlcNAc6S, UA-GlcNS; (Disulfated) UA2S-GlcNAc6S, UA2S-GlcNS, UA-GlcNS6S, and (Trisulfated) UA2S-GlcNS6S. All forms are quantified as % as compared to total all forms. Analysis of HPLC profiles was presented according to the type of sulfated forms, global sulfated HS, versus N-S, 2-O-S, 6-O-S, and non-sulfated HS. Each sample was analyzed 2 times. The statistical significance of differences between the groups was determined by ANOVA/Bonferonni statistical tests to compare data relative to \*D0; // D7; and \$D14. n=3 for D7, D14 et D28, n = x for D0 and D21. p value < 0.05 was considered to be statistically significant. \*\*\*\* P< 0,0001; \*\*\* P< 0,001. \*\* P<0,01; \* P<0,05.



**Figure 6 : Percentage of binding affinity of total GAG from human lungs toward PDGFbb (A) and VEGF (B).** IC50 value were determined according to Elisa competitive binding assay (and resume in table 2). They were expressed as  $\mu\text{g/ml}$ , as the concentration of tested GAG able to compete for 50% of the binding of HBP to Heparin) These IC50 values were used to calculate % of binding affinity represented in Figure 5 : IC50 value of GAG from CT Lung were considered as 100% binding. Then % of binding affinity from distinct GAG from PAH were calculated according to these 100% values. ANOVA/Bonferonni statistical tests were performed. \*\*\*\*  $P < 0,0001$ ; \*\*\*  $P < 0,001$ . \*\*  $P < 0,01$ ; \*  $P < 0,05$ .

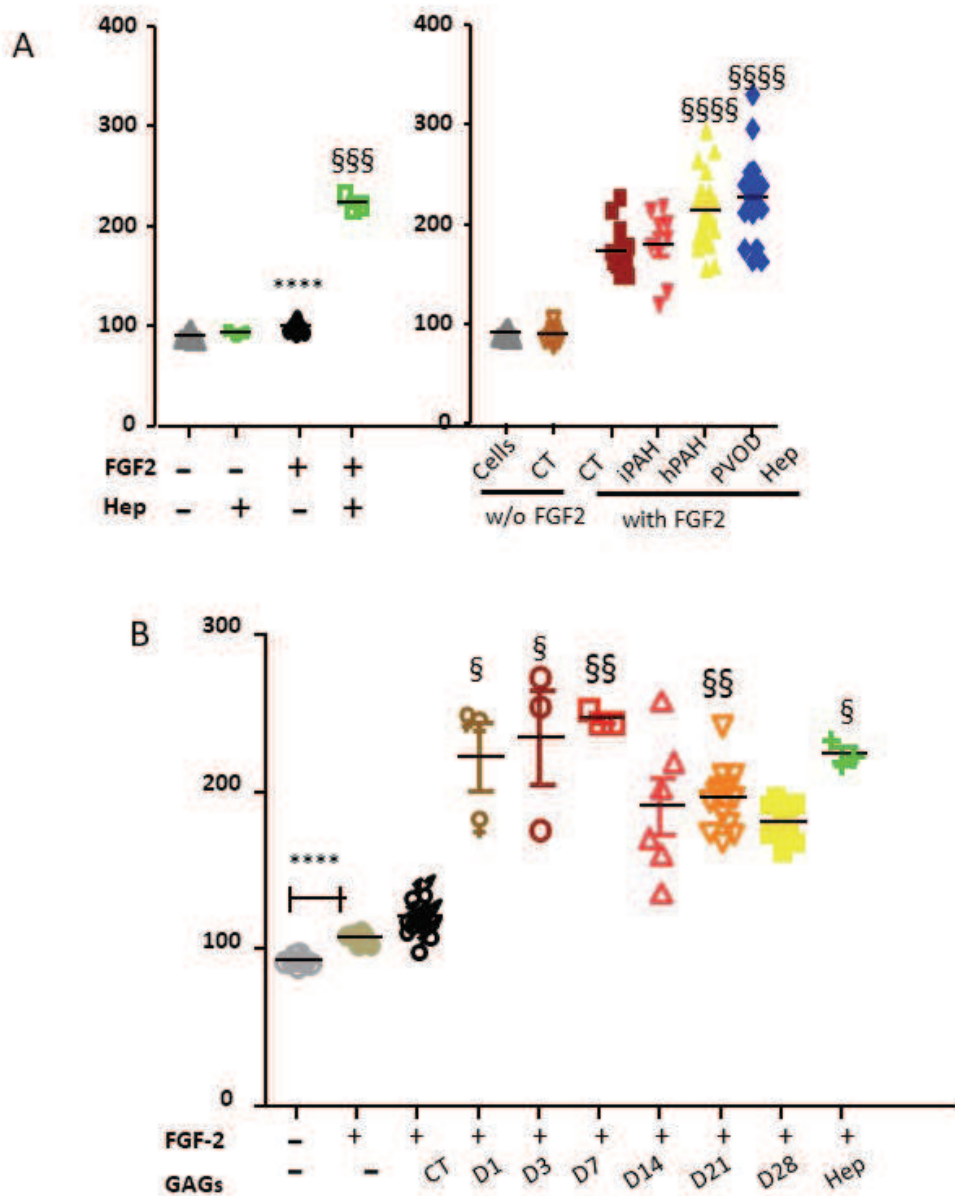


**Figure 7 : Percentage of binding affinity of total GAG from rat lungs toward (A) PDGFbb , (B) FGF2 and (C) VEGF, all along cinetic of Monocrotaline model . IC50 value were determined according to Elisa competitive binding assay (and resume in table 2). They were expressed as  $\mu\text{g/ml}$ , as the concentration of tested GAG able to compete for 50% of the binding of HBP to Heparin) These IC50 values were used to calculate % of binding affinity represented in Figure 5 : IC50 value of GAG from CT Lung were considered as 100% binding. Then % of binding affinity from distinct GAG from PAH were calculated according to these 100% values.**

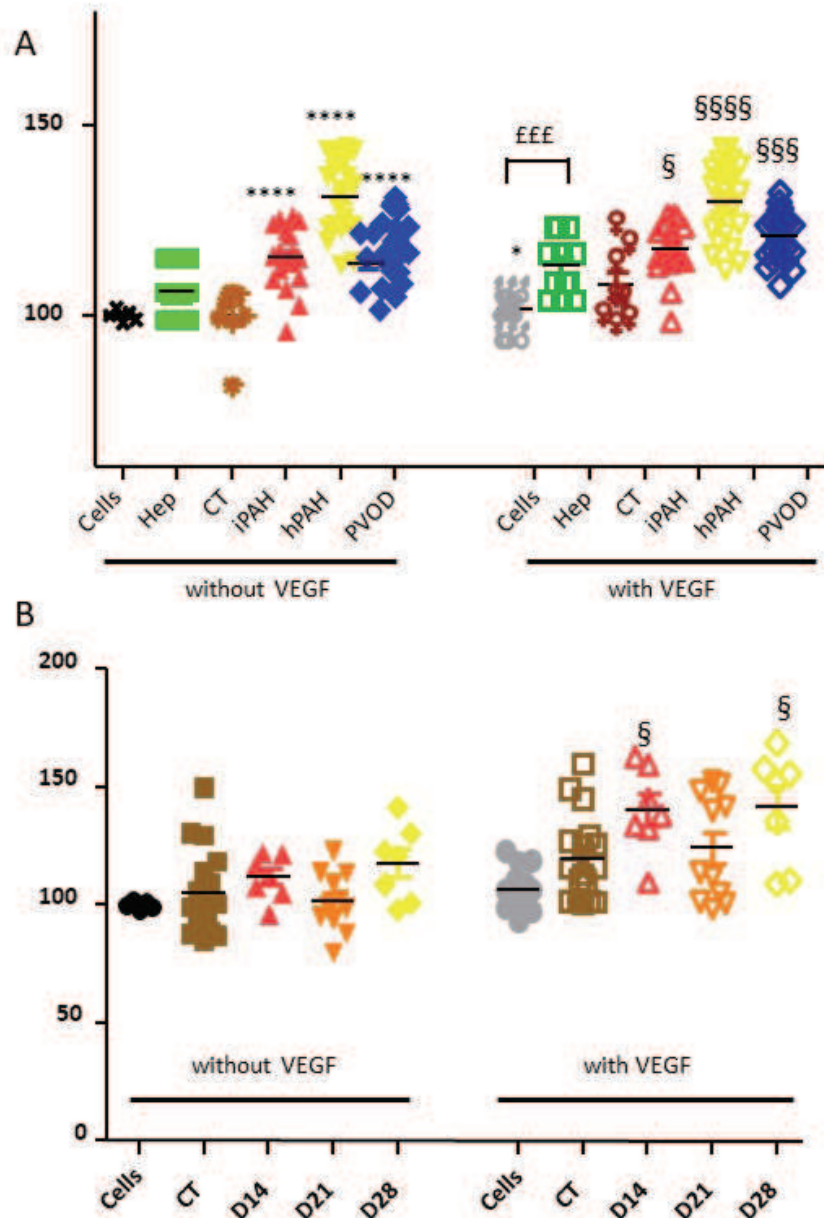




**Figure 8 : Baf32 mitogenic assays** dependant to FGF2 (5ng/mL) and GAG (1 µg/mL) extracted from **Human (panel A)** and **Rat (panel B)** lungs, as compared to cells alone (100%, cells without FGF2 and without GAG). Cell proliferation was evaluated after 48 hours in culture, with PrestoBlue™ cell Viability Reagent. ANOVA/Bonferonni statistical tests were performed. \*\*\*\* P< 0,0001; \*\*\* P< 0,001. \*\* P<0,01; \* P<0,05. \* : values were compared to cells alone without FGF2 and without GAG; § = values were compared to cells with FGF2 and with GAG from CT human lung or CT Day 0 rat lung. Heparine was used as positive control.



**Figure 9 : HUVEC mitogenic assays** dependant to VEGF (5ng/mL) and GAG (1 µg/mL) extracted from **Human (panel A)** and **Rat (panel B)** lungs, as compared to cells alone (100%, cells without VEGF and without GAG). Cell proliferation was evaluated after 48 hours in culture, with PrestoBlue™ cell Viability Reagent. ANOVA/Bonferonni statistical tests were performed. \*\*\*\* P< 0,0001; \*\*\* P< 0,001. \*\* P<0,01; \* P<0,05. \* ; values were compared to cells alone without VEGF and without GAG; § = values were compared to cells with VEGF and with GAG from CT human lung or CT Day 0 rat lung. Heparine was used as positive control.



## Article 4

### **Tissue engineering of the esophagus using a decellularised matrix and Mesenchymal Stem Cells**

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## **ABSTRACT**

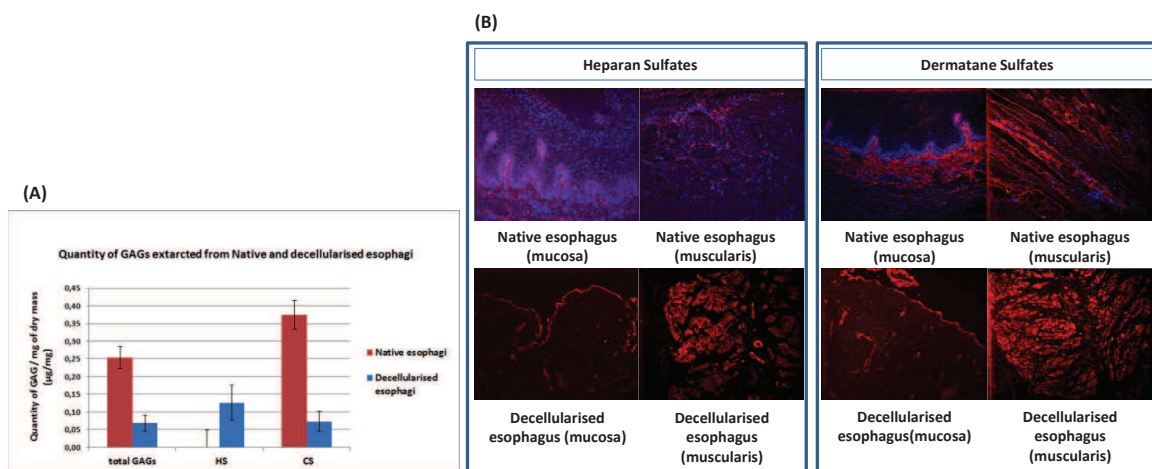
Tissue engineering appears as a promising alternative technique for esophageal replacement. A decellularised esophageal matrix combined with Mesenchymal stem cells (MSC) could be of interest for esophageal regeneration. The aim of this study was to develop a decellularised esophageal matrix seeded with bone marrow derived MSCs to promote tissue remodeling toward an esophageal phenotype. 10 cm porcine esophagi were decellularised in a bioreactor with SDS and DNase. The quality of the matrix was then studied in order to evaluate: the efficiency of the decontamination/decellularisation process; the mechanical properties and the capacity of the scaffold to be reseeded with MSC. Immunological and cytotoxicity assays were also performed, and the composition of the decellularized matrix was analysed. Antibiotics/antimycotic treatment efficiently decontaminated the esophagus. Immunohistochemical staining showed an efficient removal of the cells and their content from the esophagus while preserving the structure and the composition of the extracellular matrix. The scaffold did not induce any acute inflammatory reaction *in-vitro*, nor was cytotoxic for balb/3T3 cells. From a biomechanical point of view, the decellularised matrix had similar properties compared to the native esophagus. Finally, when reseeded with MSC, the cells adhered to the decellularised esophagus and began to infiltrate the scaffold after three days. Our results indicate that native esophagi were efficiently decellularised. Our process allowed preserving the general structure and composition of the extracellular matrix. It was also successfully seeded with MSC, making it a promising scaffold to be evaluated *in-vivo*.

**Keywords:** Decellularised scaffolds, Esophagus, Tissue Engineering, Mesenchymal Stem Cells.

## **GAG quantification and immunostaining**

Sulfated Glycosaminoglycans (GAGs) are important components of the extracellular matrix (ECM) that are involved in its scaffold according to their ability to interact with fibrillar proteins such as collagen. Moreover, GAGs are linked to core protein to form proteoglycans, expressed on the cell surfaces or secreted on the ECM. According to their structural complexity, the GAGs chains of PGs are involved in numerous regulatory processes since they are able to interact with heparin binding proteins (growth factor, chemokines, or enzymes and

delivering them to the cells. It is therefore important to evaluate the presence of these molecules in the decellularised matrix. We extracted the GAGs from native and decellularised esophagi and quantified the concentration of total GAGs per mg of dried tissue. The results showed that the native esophagus tissue has a low concentration of GAGs (as compared to their GAG contents evaluated in other tissue like brain, muscle, cartilage and the concentration of total GAGs decreases after decellularisation, probably due to the loss of GAG associated to cells. However the decellularisation process does not completely destroy total GAGs, some of them remaining in the ECM. The staining with antibodies showed that in the decellularised matrix, the main GAGs which are conserved in the decellularised matrix are the heparan sulfates and dermatan sulfates. However, there is a major loss of the chondroitin sulfates after decellularisation.



**Figure 4. Quantification and immunostaining of total GAGs, HS and CS in native and decellularised esophagi:** GAGs from native and decellularised esophageal samples were extracted and total GAGs, HS and CS were quantified using DMMB (A). Paraffin embedded slices of native and decellularised esophagi were immunostained with EV3CV3 to observe the remaining heparan sulfates (D) and with for dermatan sulfates (B). Abbreviations: DMMB: 1,9-Dimethyl-Methylene Blue zinc chloride double salt; GAG: Glycosaminoglycan; HS: Heparan Sulfate proteoglycans, CS: Chondroitin Sulfate