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TAILORED PVA/ECM SCAFFOLDS FOR
FOCAL ARTICULAR CARTILAGE DEFECTS

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ABSTRACT

Focal chondral defects impair considerably patients’ quality of life and may predispose for osteoarthritis. The strong association between age and increasing incidence of osteoarthritis marks it as an age related disease. However, osteoarthritis can be also consequence of other concomitant disorders; among these, the hereditary disease haemophilia stands out. The articular problems of patients with haemophilia begin still in infancy, when minor injuries result in recurrent haemarthroses that may predispose for haemophilic arthropathy. The lack of efficient modalities of treatment has prompted research into tissue engineering (TE), whose basic approach depends upon the interaction between cells, scaffolds and signalling factors to create in vitro a biological tissue construct to implant in vivo, mimicking the tissue of interest. Engineering cartilage is no exception to this approach. Such TE strategies are still adopted in orthopaedic surgical practice, providing for autologous chondrocytes implants with or without a supporting matrix in order to promote cartilage regeneration. Conversely, in patients with haemophilia, current available strategy can only slow the progression of joint damage, without recovery of tissue integrity.

The aim of this work was twofold. At first, a novel supporting structure to treat focal articular defects was manufactured and characterized. Then, the feasibility of using haemophilic chondrocytes for autologous cartilage TE was considered.

By a controlled chemical oxidation, 1% or 2% hydroxyls of the synthetic polymer polyvinyl alcohol (PVA) backbone were oxidized to carbonyls. Oxidation was verified by 2,4-dinitrophenylhydrazine assay and covalent binding with lysozyme. After physical cross-linking of polymeric solutions, 1% and 2% oxidized PVA scaffolds were evaluated and compared to neat PVA scaffolds. Scanning Electron Microscopy (SEM) micrographs showed oxidation to affect hydrogel surface continuity. Moreover, increasing carbonyls content, physical and biodegradation properties were modulated. In particular, mechanical properties, hydrodynamic radius of particles, thermal characteristics, and crystallinity degree of PVA hydrogels decreased with oxidation rate. Conversely, swelling behavior and protein release were enhanced, suggesting oxidized PVA potentiality as protein delivery system. Most important, biocompatibility and biodegradability of PVA scaffolds increased along with oxidation. After 12-week in vivo
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implantation, hydrogels did not elicit severe inflammatory reactions. Nevertheless, a little lymphomonocytic infiltration by CD3+ and F4/80+ cells suggested a role for inflammatory populations in implant reabsorption.

Afterwards, Non-Haemophilic and Haemophilic chondrocytes were isolated and cultured. After a morphological evaluation through optical microscopy, cells were compared for the expression of specific mRNAs (COL2A1, COL9A3, COMP; ACAN; SOX9) by RT-PCR and specific surface markers (CD44; CD49c; CD49e; CD49f; CD151; CD26; CD73) by flow cytometry. RT-PCR results confirmed the expression of target genes and any immunophenotypic difference was observed despite haemophilic chondrocytes were exposed to blood in vivo which is one of the major responsible of cytotoxicity. Flow-cytometry showed that both subcultures consisted of CD44+/CD49c+/CD49e+/CD151+/CD73+/CD49f-/CD26− cells. High expression of adhesion molecules (e.g. CD44, CD49c, CD49e) involved in cell-cell or cell-matrix interactions, revealed high chondrogenic capacity. As it is well known PVA inability in sustaining cell adhesion, a bio-hybrid composite scaffold was than obtained combining the biomechanical properties of 1% oxidized PVA with an alternative matrix source that is decellularized Wharton’s jelly (W’s J). The hydrogel itself and the more specific decellularized articular cartilage (AC) matrix, combined with 1% oxidized PVA, were used as controls. Both cell populations behavior was evaluated after seeding cells on scaffolds. According to SEM micrographs and Thiazoyl Blue Tetrazolium Blue (MTT) proliferation assay, W’s J matrix showed a singular attitude in sustaining adhesion and proliferation of both cell populations.

Our results highlighted oxidized PVA as a smart biomaterial useful for manufacturing scaffolds with customizable mechanical behaviour, protein-loading ability and biodegradability. Moreover, this study contributes to the definition of haemophilic chondrocytes phenotype, providing new potential markers to characterize them. Our preliminary evidences support the chance of using haemophilic chondrocytes for autologous implant in haemophilic patients. One percent-oxidized PVA/W’s J may be considered as an innovative and easily available scaffold for cartilage restoration both in Haemophilic and Non-Haemophilic patients.
RIASSUNTO

I difetti condrali focali compromettono significativamente la qualità della vita dei pazienti predisponendo all'osteoartrite. Eziologicamente sussiste una forte associazione tra l'età del paziente e l'incidenza di osteoartrite, consentendo di identificarla come una malattia legata all'invecchiamento. L'osteoartrite tuttavia può essere anche conseguenza di patologie concomitanti; tra queste l'emofilia, coagulopatia ereditaria. I problemi articolari nei pazienti emofilici esordiscono già nell’infanzia, quando danni minori possono esitare in emartri ricorrenti predisponendo all’artropatia emofilica.

L’assenza di trattamenti soddisfacenti per efficacia ha spinto la ricerca nell’ambito dell’ingegneria tissutale, il cui approccio di base si fonda sull’interazione tra cellule, scaffolds e fattori di crescita. L’obiettivo è di creare in vitro costrutti biologici funzionali, capaci di mimare il tessuto d’interesse dopo impianto.

Alcune strategie di ingegneria tissutale sono già adottate in chirurgia ortopedica. Esse prevedono l’impianto di condrociti autologhi come tali o supportati da matrici al fine di promuovere la rigenerazione e quindi l’integrità del tessuto compromesso. Di esse tuttavia i pazienti emofilici non possono beneficiare, disponendo ad oggi di approcci volti a rallentare solamente la progressione del danno senza favorirne il recupero.

Lo scopo di questo lavoro di Tesi è stato duplice. Dapprima è stato realizzato e caratterizzato un nuovo scaffold funzionale al recupero del danno cartilagineo focale. Successivamente, è stata valutata la possibilità di utilizzare i condrociti del paziente emofilico nella prospettiva di un impianto autologo.

Mediante una reazione chimica di ossidazione, l’1% o il 2% dei gruppi ossidrilici presenti sul backbone del polimero sintetico polyvinyl alcohol (PVA) sono stati ossidati a gruppi carbonilici. L’avvenuta ossidazione è stata verificata mediante saggio con 2,4-dinitrofenilidrazina e binding covalente di lisozima. A seguito di cross-linking fisico delle soluzioni polimeriche, scaffolds in PVA ossidato all’1% ed al 2% sono stati quindi valutati e confrontati con scaffolds in PVA non ossidato.

La microscopia elettronica a scansione ha rivelato come l’impiego di soluzioni polimeriche ossidate influenzi la continuità superficiale degli idrogeli risultanti. Inoltre, aumentando il contenuto in carbonilici, anche le proprietà fisiche e di biodegradazione risultano modulate. In particolare, la meccanicità degli scaffolds, il raggio idrodinamico
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delle particelle, le proprietà termiche ed il grado di cristallinità degli idrogeli di PVA diminuiscono all’aumentare del grado di ossidazione. Diversamente, il rigonfiamento ed il rilascio proteico aumentano, suggerendo potenzialità di \textit{protein-delivery system}. Anche le caratteristiche di biocompatibilità e biodegradazione sono state considerate. Dopo 12 settimane di impianto sottocutaneo \textit{in vivo}, gli idrogeli non hanno provocato gravi reazioni infiammatorie. Tuttavia, una limitata infiltrazione linfomonocitaria da parte di cellule CD3$^+$ e F4/80$^+$ ha suggerito un ruolo delle popolazioni infiammatorie nel riassorbimento dell’impianto: all’aumento del grado di ossidazione è stato riscontrato un incremento del tasso di degradazione degli \textit{scaffolds}.

I condrociti da paziente emofilico e non emofilico sono stati quindi isolati e messi in coltura. Dopo valutazione morfologica mediante microscopia ottica, le cellule sono state comparate per l’espressione di specifici mRNA (COL2A1; COL9A3; COMP; ACAN; SOX9) attraverso RT-PCR; e per l’espressione di \textit{marker} di superficie caratteristici (CD44; CD49c; CD49e; CD49f; CD151; CD26; CD73) attraverso analisi di citofluorimetria. I risultati di RT-PCR hanno confermato l’espressione dei geni \textit{target}; inoltre differenze immunofenotipiche non sono state osservate tra i tipi cellulari sebbene i condrociti da paziente emofilico fossero stati esposti \textit{in vivo} al sangue, tra i maggiori responsabili di citotossicità. La citofluorimetria ha mostrato dunque che entrambe le popolazioni presentavano cellule con immunofenotipo CD44$^+$/CD49c$^+$/CD49e$^+$/CD151$^+$/CD73$^+$/CD49f$^+$/CD26$. L’elevata espressione di molecole di adesione (e.g. CD44, CD49c, CD49e) coinvolte in interazioni cellula-cellula o cellula-matrice, ha suggerito un alto potenziale condrogenico.

Essendo nota l’inadeguatezza del PVA nel promuovere l’adesione cellulare, è stato realizzato uno scaffold bio-ibrido composito combinando le proprietà meccaniche del PVA ossidato all’1% con una matrice extracellulare decellularizzata non tessuto specifica: la gelatina di Wharton (W’s J). L’idrogel tal quale e la più specifica matrice da cartilagine articolare decellularizzata, combinata con il PVA ossidato all’1%, sono stati usati come controllo.

Il comportamento di entrambe le popolazioni cellulari è stata valutata dopo semina sugli \textit{scaffolds}. Immagini di microscopia elettronica a scansione ed il saggio di proliferazione con Thiazolyl Blue Tetrazolium Blue (MTT) hanno mostrato come la matrice da W’s J sostenga in modo singolare l’adesione e la proliferazione di entrambe le popolazioni cellulari.
I risultati di questo lavoro di Tesi hanno consentito di identificare nel PVA ossidato un biomateriale intelligente per la realizzazione di scaffolds con proprietà meccaniche, di protein-loading, e di biodegradazione modulabili. Inoltre, questo studio ha contribuito a definire il fenotipo dei condrociti da paziente emofilico, provvedendo a fornire nuovi potenziali marker per caratterizzarli e suggerendo la possibilità di impianto autologo. Lo scaffold composito PVA ossidato 1%W’J potrebbe infine essere considerato come una struttura innovativa per il recupero del danno cartilagineo sia in pazienti affetti da osteoartrite idiopatica che secondaria.
INTRODUCTION

1. Impact of musculoskeletal disorders on the individual

Over the past century, global priorities in health have been largely focussed on communicable diseases. With the World's population growth, increased average age and decreased death rates, people are now living longer and becoming increasingly susceptible to non-communicable diseases, including musculoskeletal (MSK) disorders (March et al., 2014).

According to Global Burden of Disease (GBD) Study (Lim et al., 2012), regarding 187 countries and 21 regions of the world for the years 1990 and 2010, MSK disorders prove to be the second most common cause of disability world-wide, measured by years lived with disability (YLDs) (Lim et al., 2012; Storheim et Zwart, 2014). Affecting one in four adults across Europe, they influence all aspects of life through pain and by limiting activities of daily living with also an enormous economic impact on society through both direct health expenditure related to treating the sequelae of the conditions and indirectly through loss of productivity (Woolf et al., 2012).

Even though MSK conditions are a diverse group of disorders with regard to pathophysiology, they are linked anatomically and by their association with pain and impaired physical function (Woolf et al., 2010). They may involve a number of different anatomical structures such as bone, joints and the periarticular structures which includes muscles, tendons, ligaments or bursae (Mody et Brooks, 2012).

In the burden estimates, there were five major defined conditions: i. osteoarthritis (OA); ii. rheumatoid arthritis; iii. gout; iv. low back pain; v. neck pain (March et al., 2014).

Articular cartilage (AC) plays a vital role in the function of the MSK system: frictionless motion between the articular surfaces of diarthrodial joints as well as loading distribution depends on its unique properties. Damage or degeneration of this remarkable tissue decreases mobility and frequently causes pain with movement and, in the most severe instances, deformity, severe chronic pain and increasing disability (Buckwalter 1998; Cohen et al., 1998; Al Maini et al., 2014). Due to the poor intrinsic ability of this tissue for repair, injuries to AC are one of the most challenging issues of MSK medicine (Vinatier et al., 2009a; Vinatier et al., 2009b).
2. Basic science of articular cartilage

In cartilage, under normal physiological conditions, degradation and synthesis of extracellular matrix (ECM) molecules are maintained in a state of balance; any disruption results in cartilage degeneration (Lane Smith et al., 2000).

In order to understand better AC diseases and developing treatments, its normal morphology and functioning must be well-understood (Cohen et al., 1998).

2.1 Anatomy

Hyaline AC is aneural, avascular and alymphatic connective tissue that covers the articulating ends of diarthrodial joints. It is composed of a single type of cell, chondrocytes, responsible for the production, organization and maintenance of an abundant and highly complex ECM.

Articular cartilage ECM consists primarily of high concentration of proteoglycans, entangled in a dense network of collagen fibres, and a large amount of water (Poole 1997; Alexopoulos et al., 2005; Rogers et al., 2006; Martel-Pelletier et al., 2008).

Chondrocytes that form only 1–5% of cartilage volume, receive their nutrition by diffusion through matrix, which represents only about 20% of the tissue wet weight (Bhosale et Richardson 2008). Water, mainly of extracellular origin, and inorganic salts dissolved in it (i.e., sodium, calcium, and potassium chloride) constitute most of the remaining tissue and are unevenly distributed depending on deep. Water highest concentration (80%) is found near the cartilage surface, decreasing gradually to reach about 65% in the deep zone. Beyond assuring nutrients diffusion from the synovial fluid, it is essential for tissue lubrication and resiliency. The maintenance and flow of water in the tissue relies on its interaction with ECM macromolecules (Martel-Pelletier et al., 2008).

2.2 Macromolecules of cartilage extracellular matrix

The ECM of AC is a unique environment (Gao et al., 2014). Chondrocytes form the macromolecular framework of cartilage tissue matrix from three classes of molecules: a) collagens; b) proteoglycans; c) non-collagenous proteins (Buckwalter et Mankin 1998) (Fig. 1).
Figure 1. Three classes of proteins exist in articular cartilage: collagens (mostly type II collagen); proteoglycans (primarily aggrecan); and other noncollagenous proteins (including link protein, fibronectin, cartilage oligomeric matrix protein) and the smaller proteoglycans (biglycan, decorin and fibromodulin). The interaction between highly negatively charged cartilage proteoglycans and type II collagen fibrils is responsible for the compressive and tensile strength of the tissue, which resists load in vivo. Abbreviation: COMP, cartilage oligomeric matrix protein.

a) Collagens
Collagens form the endoskeleton of AC. In particular, collagen types found in it are: type II, VI, IX, X, and XI. Despite type II collagen accounts for 90-95%, all contribute to the mature matrix arrangement. Type II, IX, and XI collagens form a fibrillary meshwork that lend tensile stiffness and strength to tissue. In particular, high amount of carbohydrate groups in collagen type II allows more interaction with water than other types. Type VI collagen forms part of the matrix immediately surrounding the chondrocytes and may help them to attach to the macromolecular framework of the matrix. Type X is closely related to the hypertrophied cells in calcified cartilage layer providing structural support and aiding in cartilage mineralization (Cohen et al., 1998; Salter, 1998; Temenoff et Mikos, 2000; Bhosale et Richardson, 2008).

b) Proteoglycans
Proteoglycans are protein polysaccharide molecules produced inside the chondrocytes and secreted in the matrix, providing a compressive strength to the AC. There are two major classes of proteoglycans found in AC, large aggregating proteoglycan monomers (or aggrecans) and small proteoglycans including decorin, biglycan and fibromodulin.
The subunits of proteoglycans are called as glycosaminoglycans (GAGs). These are disaccharide molecules, with main two types, chondroitin sulphate and keratin sulphate. GAGs are bound to the protein core by means of sugar bonds, to form aggrecan molecule. Link protein stabilizes this chain with a central hyaluronic acid chain to form an intricate structure of the GAG molecule.

Proteoglycans maintain the fluid and electrolyte balance in the AC. These macromolecules have negatively charged sulphate and carboxylate groups, which in turn attract only positively charged molecules and repel the negative molecules. This increases the total concentration of inorganic ions (i.e., sodium) inside the matrix, thereby increasing osmolarity of the AC, thus creating a Donnan effect (Temenoff et Mikos, 2000; Bhosale et Richardson, 2008).

c) Non-collagenous proteins
In contrast to proteoglycans, glycoproteins have only a small amount of oligosaccharide associated with the protein core. These polypeptides help to stabilize the ECM matrix and aid in chondrocyte-matrix interactions. Both anchorin CII and cartilage oligomeric matrix protein (COMP) anchor chondrocytes to the surrounding matrix; in particular, COMP may have also value as a marker of turnover and degeneration of cartilage. Other non-collagenous proteins commonly found in most tissues, such as fibronectin and tenascin, are observed even in AC, and are believed to perform similar functions as the glycoproteins (Buckwalter et Mankin, 1998; Temenoff et Mikos, 2000).

2.3 Ultrastructure of articular cartilage: zones and regions
Chondrocytes organize the collagen, proteoglycans and non-collagenous proteins into a unique and highly specialized tissue arranged in different zones and regions.

2.3.1 Zones
The composition, structure and functions of chondrocytes vary depending on the depth from the surface of the cartilage. Morphologically there are four named zones, from top to bottom (Youn et al., 2006; Bhosale et Richardson 2008): a) superficial zone; b) transitional zone; c) deep zone; d) calcified cartilage zone. These distinct tissue zones exhibit different biomechanical properties and rates of cell metabolic activities, suggesting differential adaptations to distinct biomechanical roles (Quinn et al., 2005). Each zone possesses attributes necessary to make AC as a completely strong, durable,
and more able to withstand shear and axial forces through a joint. From the superficial to the deep zone, cell density progressively decreases, whereas cell volume and the proportion of proteoglycan relative to collagen increases (Fig. 2) (Goldring et Marcu 2009).

**Figure 2.** Schematic image demonstrating chondrocyte organization in the three main zones of uncalcified cartilage: superficial zone; transitional zone; deep zone. Note changes in collagen fibre orientation from the superficial to the deep zone. Chondrocyte orientation also changes from the superficial zone (densely packed, flat) to the deep zone (columnar orientation perpendicular to the surface). The tidemark represents a relative change from the deep zone to the zone of calcified cartilage.

a) **Superficial zone**

The superficial zone protects deeper cartilage layers from shear stresses and makes up approximately 10% to 20% of AC thickness. It is made of two distinct layers. An acellular sheet of mainly collagen fibers (the *lamina splendens*) that covers the joint and deeply a second layer composed of flattened chondrocytes, parallel to the articular surface. The ECM in this area has less proteoglycan than the other zones and more densely packed collagen fibres, fibronectin and water. It is in contact with synovial fluid and is responsible for most of the tensile properties of cartilage, which enable it to resist the sheer, tensile, and compressive forces imposed by articulation (Poole, 1997; Martel-Pelletier et al., 2008).
b) **Transitional zone**
Immediately deep to the superficial zone is the transitional zone, which provides an anatomic and functional bridge between the superficial and deep zones. The transitional zone includes chondrocytes that are spherical; the ECM in this area has larger collagen fibrils, more proteoglycan and less collagen and water than in the previous zone.

c) **Deep zone**
The deep zone is responsible for providing the greatest resistance to compressive forces, given that collagen fibrils are arranged perpendicular to the articular surface. It contains the largest diameter collagen fibrils, the highest proteoglycan content, and the lowest water concentration. The cells are rounded, like in the transitional zone, but are stacked in columns perpendicular to the articulating surface and parallel to the collagen fibres. The deep zone represents approximately 40-60% of AC volume (Poole, 1997; Sophia Fox et al., 2009; Goldring et Marku 2009).

d) **Calcified zone**
The calcified zone plays an integral role in securing the cartilage to bone, by anchoring the collagen fibrils of the deep zone to subchondral bone. In this zone, the cell population is scarce and chondrocytes are hypertrophic. In some places, they seem to be completely surrounded by calcified ECM, indicating their little metabolic activity (Sophia Fox et al., 2009).

2.3.2 **Regions**
In addition to zonal variations in structure and composition, the AC matrix consists of several distinct regions based on proximity to the chondrocytes. Hence, the ECM can be divided into: a) pericellular region; b) territorial region; c) inter-territorial region (Youn et al., 2006) (Fig. 3).

a) **Pericellular region**
The pericellular region is a thin layer adjacent to the cell membrane that completely surrounds the chondrocyte. It contains mainly proteoglycans, as well as glycoproteins and other non-collagenous proteins. This matrix region may play a functional role to initiate signal transduction within cartilage with load bearing.
b) **Territorial region**
The territorial region surrounds the pericellular matrix; it is composed mostly of fine collagen fibrils, forming a basketlike network around the cells. This region is thicker than the pericellular matrix and it may protect chondrocytes against mechanical stresses, contributing to the resiliency of the AC structure and withstanding substantial loads.

c) **Inter-territorial region**
The inter-territorial region is the largest of the three regions. With its abundant proteoglycans and randomly oriented bundles of large collagen fibrils, it contributes most to the biomechanical properties of AC (Sophia Fox et al., 2009; Temenoff et Mikos 2000).

![Diagram of cartilage matrix](image)

**Figure 3.** The cartilage matrix surrounding chondrocytes in healthy articular cartilage is arranged into zones defined by their distance from the cell: the pericellular matrix; the territorial matrix; the interterritorial matrix. Abbreviations: CILP-1, cartilage intermediate layer protein 1; COMP, cartilage oligomeric matrix protein; CS, chondroitin sulfate; KS, keratan sulfate; PRELP, proline-arginine-rich end leucine-rich repeat protein (Heinegård et Saxne, 2011).

### 2.4 Articular cartilage metabolism
Chondrocytes are responsible for the regulation of ECM turnover by synthesizing and secreting cartilage-specific matrix proteins. In adulthood, these large round cells no longer divide, and are called post-mitotic. Therefore, the cartilage has a low turnover rate and a very limited ability for self-repair.
Articular cartilage is avascularised, and chondrocytes function with a hypoxic (physioxic) metabolism: nutrients and oxygen are supplied to the cells primarily by diffusion from the synovial fluid and subchondral bone. From the most superficial to the deepest layers, it is estimated that the oxygen gradient ranges from 10 to 1%, respectively. In these conditions of hypoxia, chondrocytes metabolize glucose into energy substrates via the anaerobic glycolysis pathway. Glucose is a sugar essential for the proper functioning of cellular machinery and in particular, it is essential for cartilage, being required for GAGs synthesis. Like oxygen, it diffuses from the synovial fluid and into the chondrocyte through glucose transporters called GLUT (GLUT1, 3, 5, 9, 10 and 11) (Fig. 4).

**Figure 4.** Model of glucose diffusion and transport in AC. Glucose is delivered to the chondrocyte via the synovial microcirculation and take up by GLUT proteins. Intracellular glucose is accumulated in two distinct pools; the metabolic pool used for glycolysis and the structural pool used for synthesis of ECM macromolecules.

The chondrocytes maintain the surrounding matrix by modulating the balance between the synthesis and degradation of various ECM components in physiopathological situations. This balance is controlled by the relative amounts of cytokines and growth factors in the cartilage or synovial fluid. Chondrocytes synthesize ECM molecules, but also factors involved in ECM degradation such as metalloproteinases (MMPs)
collagenase, gelatinase, stromelysin and the cathepsins (cathepsin B and D). Collagenase degrades native helical collagen fibrils at a single site. Gelatinase degrades denatured type II and type IV collagen; it also has significant activity against fibronectin, elastin, and collagen types V, VII, X, and XI. The role of stromelysin is to degrade the protein core of aggrecan. All metalloproteinases are secreted as latent proenzymes that require activation extracellularly. Cathepsins are active in the degradation of aggrecan. Cartilage cells do not directly interact with each other but are linked to the ECM by surface receptors such as integrins. Hence, the chondrocyte has a close relationship with its microenvironment and is sensitive to changes in it.

The development of disease such as osteoarthritis is associated with dramatic changes in cartilage metabolism. This occurs when there is a physiological imbalance of degradation and synthesis by chondrocytes (Sophia Fox et al., 2009; Demoor et al., 2014) (Fig. 5).

**Figure 5.** Factors affecting cartilage anabolism and catabolism.

### 2.5 Biomechanical function

Articular cartilage is a thin layer of specialized connective tissue with unique viscoelastic properties. Its principal functions are to provide a smooth, lubricated surface for low friction articulation and to facilitate the transmission of loads to the underlying subchondral bone. Articular cartilage is unique in its ability to withstand high cyclic loads, demonstrating little or no evidence of damage or degenerative change.

The biomechanical behaviour of AC is best understood when the tissue is viewed as a biphasic medium. Its consists of two phases: a fluid phase and a solid phase. Water is the
Introduction

principal component of the fluid phase, contributing up to 80% of the wet weight of the tissue. The solid phase is characterized by the ECM, which is porous and permeable. The relationship between proteoglycan aggregates and interstitial fluid provides compressive resilience to cartilage through negative electrostatic repulsion forces. The initial and rapid application of articular contact forces during joint loading causes an immediate increase in interstitial fluid pressure. This local increase in pressure causes the fluid to flow out of the ECM, generating a large frictional drag on the matrix. When the compressive load is removed, interstitial fluid flows back into the tissue. The low permeability of AC prevents fluid from being quickly squeezed out of the matrix. The two opposing bones and surrounding cartilage confine the cartilage under the contact surface. These boundaries are designed to restrict mechanical deformation.

Articular cartilage is viscoelastic and exhibits time-dependent behaviour when subjected to a constant load or deformation. Two types of mechanisms are responsible for viscoelasticity in AC: flow dependent and flow independent. The flow-dependent mechanism depends on interstitial fluid and the frictional drag associated with this flow. The drag resulting from the interstitial fluid is known as biphasic viscoelastic behaviour. The flow-independent component of viscoelasticity is caused by macromolecular motion—specifically, the intrinsic viscoelastic behaviour of the collagen-proteoglycan matrix. As a result, the fluid pressure provides a significant component of total load support, thereby reducing the stress acting upon the solid matrix.

Articular cartilage also exhibits a creep and stress-relaxation response. When a constant compressive stress is applied to the tissue, its deformation increases with time, and it will deform or creep until an equilibrium value is reached. Similarly, when cartilage is deformed and held at a constant strain, the stress will rise to a peak, which will be followed by a slow stress-relaxation process until an equilibrium value is reached. Because AC tends to stiffen with increased strain, it cannot be described by a single Young’s modulus. Rather, the modulus of the tissue depends on the time at which the force measurement was taken during a stress-relaxation test, which was common practice in the preliminary studies of mechanical testing on AC. The current method is to apply a known strain, which is immediately followed by a peak in measured force and a slow stress-relaxation process; the force/stress value is recorded when it has reached equilibrium. This process is repeated across a range of strain values, and the equilibrium modulus is calculated as the slope of the stress-strain curve.
Mechanical force has long been appreciated as a regulator of musculoskeletal tissues, and may be the most important single environmental factor responsible for joint homeostasis. The complex composition and organization of cartilage through the middle zones of cartilage contributes significantly to its shear-resistant properties. Stretching of the randomly distributed collagen fibrils provides cartilage with its shear stress response. The tensile force-resisting properties derive from the precise molecular arrangement of collagen fibrils. The stabilization and ultimate tensile strength of the collagen fiber are thought to result from the intra- and intermolecular cross-links (Sophia Sophia Fox et al., 2009)

3. Osteoarthritis features

Amongst MSK disorders, OA is the most common joint disease (Buckwalter et al., 2006) and is recognized as a major cause of pain and disability (Buckwalter et al., 2004). It develops most commonly in the absence of a known cause of joint degeneration, a condition referred to as primary or idiopathic OA. Less frequently, it develops as a result of joint degeneration caused by injuries or a variety of hereditary, inflammatory, or developmental, metabolic disorders, a group of conditions referred to as secondary OA (Buckwalter et al., 2000; Beris et al., 2005) (Tab. 1).

| Table 1. Proposal for differentiation of clinical phenotypes of osteoarthritis. |

<table>
<thead>
<tr>
<th>AGE</th>
<th>POST-TRAUMATIC (ACUTE OR REPETITIVE)</th>
<th>PAIN</th>
<th>METABOLIC</th>
<th>GENETIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE</td>
<td>Old (&gt;65 years)</td>
<td>Young (&lt;45 years)</td>
<td>Variable</td>
<td>Middle-aged (45-65 years)</td>
</tr>
<tr>
<td>MAIN CAUSATIVE FEATURE</td>
<td>AGE, chondrocyte senescence</td>
<td>Mechanical stress</td>
<td>Inflammation, bony changes, aberrant pain perception</td>
<td>Mechanical stress, adipokines, hyperglycaemia, oestrogen/progesterone imbalance</td>
</tr>
<tr>
<td>MAIN SITE</td>
<td>Hip, knee, hand</td>
<td>Knee, thumb, ankle, shoulder</td>
<td>Hip, knee, hand</td>
<td>Knee, hand, generalised</td>
</tr>
<tr>
<td>INTERVENTION</td>
<td>No specific intervention</td>
<td>Joint protection, joint stabilisation, prevention of falls, surgical interventions</td>
<td>Pain medication, anti-inflammatory drugs</td>
<td>Weight loss, glycaemia control, lipid control, hormone replacement therapy</td>
</tr>
</tbody>
</table>

3.1 Pathogenesis of idiopathic osteoarthritis

In addition to the involvement of several joint tissues, OA has long been mainly characterised by a failure of the repair process of damaged cartilage due to biomechanical
and biochemical changes in the joint. Cartilage is non-vascularised, so this restricts the supply of nutrients and oxygen to the chondrocytes that are responsible for the maintenance of a very large amount of ECM. At an early stage, in an attempt to effect a repair, clusters of chondrocytes form in the damaged areas and the concentration of growth factors in the matrix rises. This attempt subsequently fails and leads to an imbalance in favour of degradation. Increased synthesis of tissue-destructive proteinases (matrix MMP and agrecanases), increased apoptotic death of chondrocytes, and inadequate synthesis of components of the ECM, lead to the formation of a matrix that is unable to withstand normal mechanical stresses. Consequently, the tissue enters a vicious cycle in which breakdown dominates synthesis of ECM. Since AC is aneural, these changes do not produce clinical signs unless innervated tissues become involved. This is one reason for the late diagnosis of osteoarthritis.

Although the pathophysiology of osteoarthritis has long been thought to be cartilage driven, recent evidence shows an additional and integrated role of bone and synovial tissue, and patchy chronic synovitis is evident in the disease. Synovial inflammation corresponds to clinical symptoms such as joint swelling and inflammatory pain, and it is thought to be secondary to cartilage debris and catabolic mediators entering the synovial cavity. Synovial macrophages produce catabolic and pro-inflammatory mediators and inflammation starts negatively affecting the balance of cartilage matrix degradation and repair. This process in turn amplifies synovial inflammation, creating a vicious cycle. Synovial inflammation happens in early as well as late phases of OA and is seldom as severe as in rheumatoid arthritis, but it might add to the vicious cycle of progressive joint degeneration. The main characteristics of OA are changes in the subchondral bone. Osteophyte formation, bone remodelling, subchondral sclerosis, and attrition are crucial for radiological diagnosis. Several of these bone changes take place not only during the final stage of the disease, but also at the onset of the disease (Bijlsma et al., 2011) (Fig. 6).
Figure 6. Schematic drawing of an osteoarthritic joint. The different tissues involved in clinical and structural changes of the disease are shown on the left. Note that cartilage is the only tissue not innervated. On the right the bidirectional interplay between cartilage, bone, and synovial tissue involved in osteoarthritis is shown, and the two-way interaction between this interplay and the ligaments and muscles. In the interplay between cartilage, bone, and synovial tissue one of the tissues might dominate the disease, and as such should be targeted for treatment (Bijlsma et al., 2011).

3.2 Pathogenesis of haemophilic arthropathy

Haemophilia is an X-linked heritable coagulopathy with an overall prevalence of approximately 1 in 10,000 individuals. The most common form is factor (F) VIII deficiency, or haemophilia A, which comprises approximately 80% of cases. Factor IX deficiency, or haemophilia B, comprises approximately 20% of cases (Knobe et Berntorp 2011). It generally affects males on the maternal side, however, FVIII and FIX genes are prone to new mutations, and as many as 1/ of all cases are the result of spontaneous mutations where there is no prior family history.

The severity of haemophilia is classified according to the amount of circulating functional clotting factor: patients with <1% have severe disease, those with 1–5% are moderate, and those with >5% are classified as mild. The characteristic phenotype of haemophilia is the bleeding tendency. Patients with severe haemophilia experience frequent spontaneous bleeding episodes, in contrast to those with moderate and mild haemophilia in whom trauma or surgery is usually required to provoke haemorrhage. Although bleeding can occur at almost any site, haemarthrosis (intra-articular bleeding) is the most common clinical manifestation, and the ankles, knees and elbows are most frequently affected (Tab. 2) (Raffini et Manno, 2007).
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<table>
<thead>
<tr>
<th>SITE OF BLEEDING</th>
<th>APPROXIMATE FREQUENCY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemarthrosis</td>
<td>70% - 80%</td>
</tr>
<tr>
<td>▪ More common into hinged joints: ankles, knees and elbows</td>
<td></td>
</tr>
<tr>
<td>▪ Less common into multi-axial joints: shoulders, wrists, hips</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>10% - 20%</td>
</tr>
<tr>
<td>Other major bleeds</td>
<td>5% - 10%</td>
</tr>
<tr>
<td>Central nervous system</td>
<td>&lt;5%</td>
</tr>
</tbody>
</table>

Table 2. Approximate frequency of bleeding at different sites

The consequence of repeated extravasation of blood into joint cavities hesitates in haemophilic arthropathy which is characterized by two main features (i.e., chronic synovitis and cartilage destruction).

Haemophilic synovitis is a proliferative disorder of the synovial tissue. Within hours of the extravasation of blood into a joint, distension of the joint capsule occurs, followed by an acute reaction of the synovial tissue with infiltration of polymorphonuclear cells and later monocytes and lymphocytes. This acute episode of haemarthrosis is resolved in about one week, the blood being progressively removed from the joint space by the synovial lining cells and invading macrophages. However, after repeated episodes of intra-articular bleeding, the blood removal capacity is exceeded and blood stays longer in the joint space. This leads to the deposition of iron contained in red blood cells in the synovial membrane. With each successive haemorrhage, there is a progressive accumulation of iron, as haemosiderin in the synovial membrane. This is postulated to be a major trigger of chronic synovitis, iron being involved in both synovial cell proliferation and vascular cell proliferation in the subsynovial layer. Although a normal synovial membrane is thin and mostly avascular, the proliferation of the synovium and neovascularization of the subsynovial layer result in an inflamed, villous, friable and highly vascular synovial tissue, more susceptible to further haemorrhage with minimal stress, which sets up a vicious circle. The overgrowth of the synovial membrane also causes pain and mechanical dysfunction of the joint. The cartilage destruction results from the production of enzymes and cytokines by inflammatory cells which have infiltrated the synovial membrane. Furthermore, it is favoured by the mechanical distension of the joint capsule and the increase in pressure in the joint space caused by the presence of blood which induces the apoptosis of chondrocytes and an inhibition of proteoglycan synthesis. The cartilage is thereby unable to restore the synthesis of the cartilage matrix, leading to a long-lasting joint damage. With time, a crippling arthritis
develops and the final result is a fibrotic and destroyed joint. In summary, haemophilic arthropathy shows characteristics of both inflammatory and degenerative joint disease (Fig. 7) (Lafeber et al., 2008).

**Figure 7.** General scheme of the pathogenesis of haemophilic arthropathy (Lafeber et al., 2008).

### 4. Classification of cartilage injuries

Both in primary or secondary OA, the early stages of disease are difficult to diagnose. Joint structure and function are typically altered substantially before symptoms cause patients to seek medical care; that is, the osteoarthritic process begins long before OA presents as a clinical disease. The insidious onset and “silent” progression of OA not only obscure an early diagnosis, but also delay treatment that may help prevent further cartilage destruction and joint failure (Matyas et al., 2004).

To guide management decisions and understand the prognosis of AC lesions it is important and necessary to document and grade them.

Cartilage lesions are determined by the depth of the lesion (chondral versus subchondral), the size, and the location of the defect (Baghaban Eslaminejad et Malakooty Poor, 2014). The clinical classification systems are based on the morphological gross appearance of the defect. Outerbridge described four grades of cartilage damage in chondromalacia, with grade I for softening of the surface, grade II for fissuring without reaching the subchondral bone, and grades III and IV for defects going to or beyond the subchondral plate and exposing bare bone (Nehrer et Minas, 2000; Beris et al., 2005).
With increasing interest in cartilage injuries and repair techniques, the International Cartilage Repair Society (ICRS) developed its own classification system (Visual Assessment Scale) to more accurately describe chondral defects (Mainil-Varlet et al., 2010; Ozmeriç et al., 2014). ICRS grade I lesions include those that demonstrate softening or those with superficial fissures present. Grade II injuries describe defects that have a depth less than 50% of the tissue thickness. Defects that have a depth greater than 50% of the tissue thickness are designated as grade III, while ICRS grade IV lesions are those that are full thickness, extending to or through the subchondral bone plate (Strauss et al., 2011).

Concerning classification of haemophilic arthropathy, several systems have been developed to quantify and monitor the degree of haemophilic arthropathy based on clinical and radiological findings. The two most widely used systems based on conventional radiological findings are: a) the Petterson score, in which the joint disease is classified based on its stage of development; b) the Arnold–Hilgartner scale, in which the joint is scored based on a summation of radiological changes. Recently, magnetic resonance imaging (MRI) has been used as a more sensitive imaging technique that can detect changes that are not visualised by conventional radiographs, and several scoring methods using MRI have been proposed (Raffini et Manno, 2007).

4.1 Partial-thickness defects
The partial-thickness (or chondral) defects of AC resemble the clefts and fissures observed during the initial stages of OA (Fig. 8 A,B). Defects of this nature in mature tissue do not heal spontaneously (Zhang et al., 2009). This failure is thought to be due to the fact that they only damage AC but do not penetrate into the underlying subchondral bone, rendering the defect site inaccessible to blood cells, and progenitor cells of the bone marrow space. Thus, the defect site lacks fibrin clots and other self-healing responses. In mature tissue, a limited repair process take place in response to the trauma within the tissue immediately adjacent to the site of the defect. The nature of this repair response has been investigated and it has been observed that the cells adjacent to the wound margins undergo cell death. After twenty-four hours, however, there is an increase in cell proliferation or chondrocyte cluster formation. Concurrent with this proliferation is also an increase in matrix synthesis and catabolism. However, this response is short lived and there is failure to repair the defect. It has also been observed that cells can be induced to migrate from the synovia membrane and subsynovial space across the articular surface to
the lesion and, under the influence of growth factors, can fill the defect with a repair tissue (Hunziker, 2001). However, in the absence of a fibrin matrix and mitogenic factors, these ‘synovial cells’ fail to fill the defect void due in part to the anti-adhesive properties of proteoglycans. Hence, is not only the absence of access to the bone marrow cells that prevents the repair of partial thickness defects, there are clearly other mechanisms involved that remain to be fully elucidated (Redman et al., 2005).

### 4.2 Full-thickness defects

Full-thickness (or osteochondral) defects penetrate the entire thickness of AC, beyond the calcified zone, and into the subchondral bone (Fig. 8 A,C). Unlike partial-thickness defects, these are accessible to mesenchymal progenitor cells, macrophages, and blood cells, all of which are involved in a spontaneous immune response and a healing process after injuries. Briefly, immediately following injury, the defect void is filled with a fibrin clot and trapped platelets in turn, release various bioactive factors (i.e., PDGF; TGF-β), that stimulate vascular invasion functional to promote following migration of undifferentiated mesenchymal progenitor cells into the fibrin clot (Steinert et al., 2007). Also an inflammatory response is activated. Next, mesenchymal stem cells (MSCs) from bone marrow migrate into the defect, gradually replacing the fibrin clot and completely filling the defect after one week. Many of these MSCs can differentiate into chondrocytes later, which secrete a proteoglycan-rich ECM and repair the damaged cartilage tissue. However, it has been observed that fibrous, not hyaline, tissues with weaker mechanical properties and higher permeability are formed in defect sites. Consequently, the spontaneous repair process in full-thickness defects is only transient and imperfect, and tissue degeneration eventually occurs. Hence, the cartilage tissue often becomes hypertrophic and is finally replaced by the progressive deposition of subchondral bone (Temenoff et Mikos, 2000; Steinert et al., 2007; Zhang et al., 2009). Repair of full-thickness cartilage defects depends mainly on the patient age, defect size and location. Only small full-thickness defects are repaired by formation of hyaline cartilage (Baghaban Eslaminejad et Malakooty Poor, 2014).
Figure 8. (A) Schematic drawing demonstrating partial and full-thickness defects of articular cartilage. Arthroscopic image of a partial-thickness (B) and full thickness (C) joint defect.

5. Current management of articular cartilage defects

Given the debilitating nature of severe joint pain, scientists and surgeons have tried for decades to repair or regenerate lost cartilage, but there has been little success due to the complex properties of the tissue and its essential function in the body (Temenoff et Mikos 2000). The management of AC defects continues to be one of the most challenging clinical problems for orthopaedic surgeons (Bedi et al., 2010; Baghaban Eslaminejad et Malakooty Poor, 2014).

Actually, cartilage repair strategies include: a) conservative/palliative treatments; b) surgical treatments (Neherer et Minas 2000) (Fig. 9).
5.1 **Conservative treatments**

Palliative options focus on the relief of mechanical symptoms in the cartilage injured patient (Williams et Brophy 2008). In the early phase of a chronic, symptomatic cartilage defect, conservative measures can be helpful. Nonsteroidal anti-inflammatory drugs can calm the inflammatory response of activated cartilage damage as well as intra-articularly administered corticosteroids. In addition, physiotherapeutic modalities (i.e., ultrasound, iontophoresis, thermal therapies) can decrease the symptoms of cartilage damage. The intra-articular application of hyaluronate provides lubrication of the joint surface and can improve joint function (Neherer et Minas 2000).

Despite palliative options are used clinically to reduce pain and maintain joint movement, in many cases, surgical approaches are necessary.

5.2 **Surgical treatments**

A variety of surgical techniques has been developed to reduce joint pain, improve joint function and delay the onset of OA (Bark et al., 2014). In particular, these treatments can be classified into: repair, reconstruction and regeneration techniques (Vaquero et Forriol, 2012).
5.2.1 Repair techniques

The repair methods help to form fibrocartilaginous tissue, thereby facilitating the access of blood vessels and of osteoprogenitor cells that are capable of achieving chondrogenesis. These techniques include: a) arthroscopic lavage and debridment; b) stimulation of the bone marrow.

a) Arthroscopic lavage and debridment

Arthroscopic lavage involves the visually guided introduction and removal of saline solution into the knee joint to “washout” any excess fluid and loose bodies. In comparison, debridement may include the introduction of saline into the joint, in addition to the smoothening of bone surface without any further intervention, or in combination with other procedures such as abrasion, partial or full meniscectomy, synovectomy, or osteotomy (Health Quality Ontario, 2005) (Fig. 10). The cleaning process may relieve symptoms, but effects are temporary (Vaquero et Forriol, 2012).

![Figure 10. Cartilage repair technique through arthroscopic debridment.](image)

b) Stimulation of the bone marrow

These systems are intended to stimulate cell migration and cytokine expression to repair the cartilage. They include Pridie perforations, abrasion using a burr as far as the bleeding subchondral bone, and microfractures. Bone marrow stimulation is the most frequently used technique for treating small symptomatic lesions of the AC in the knee. This technique involves perforation of the subchondral plate in order to recruit MSCs from the bone marrow space into the lesion. MSCs are able to differentiate into fibro-chondrocytes, which contribute to fibrocartilage repair of the lesion (Fig. 11). However, the overall concentration of the MSCs is quite low and declines with age. The formation of a stable
blood clot that maximally fills the chondral defect is important and it has been correlated with the success of bone marrow stimulation procedures (Bedi et al., 2010).

![Steps of the microfracture technique.](image)

**Figure 11.** Steps of the microfracture technique. (A) Damaged cartilage is removed; (B) Awl is used to make holes in the subchondral bone; (C) Healing response brings new, healthy cartilage cells; (D) Microfracture in human patients.

### 5.2.2 Reconstruction methods

Reconstruction methods are intended to fill the injury with autologous AC transplantation or allografts by arthroscopy or a mini-arthrotomy approach. These techniques include: a) mosaicplasty; b) osteochondral allografts; c) synthetic plugs.

a) **Autologous osteochondral grafts (mosaicplasty)**

Autologous osteochondral mosaic transplantation technique is one of the recently evolved methods to create hyaline or hyaline-like repair tissue in the pathologic area (Bartha et al., 2006). It consists in transplantation of multiple, small-sized, cylindrical osteochondral grafts harvested from the relatively less weight bearing periphery of the patellofemoral joint (Fig. 12). The transplanted hyaline cartilage should, in theory, survive the procedure and result in a more durable surface than that provided by fibrous repair tissue. Donor site repair by the natural healing processes should result in filling of the tunnels with cancellous bone and coverage of the surface with reparative fibrocartilage (Szerb et al., 2005).
b) Osteochondral allografts

The advantage of allografts is that they are adaptable, as grafts can be designed for lesions of any shape or size, and they can be obtained from weight-bearing areas so that they are identical in form and curvature to the injured area. Moreover, they can be harvested without endangering the donor site. The disadvantage is that they have to be used in a short period of time, since they must be kept fresh in serum, and this is only possible for a few weeks after extraction, because cryopreserved cartilage is a matrix with few viable cells, and this affects the recovery of the cartilage morphology. There is also a risk of immune reactions and the transmission of disease (Vaquero et Forriol, 2012) (Fig. 13).
c) Synthetic plugs

Biphasic cylindrical scaffolds made of synthetic co-polymers greatly facilitate the techniques for filling osteochondral defects. The plug is designed to provide the benefits of marrow stimulation together with structural support to allow regeneration of AC to the same height as that of the surrounding articular surface. The advantage is that the right thickness and length can be chosen to fit the dimensions of the gap. In addition, such plugs can be combined with stem cells or growth factors (Vaquero et Forriol, 2012).

5.2.3 Regenerative techniques

Regenerative methods make use of bioengineering techniques to develop hyaline cartilage tissue. These techniques include: a) autologous chondrocytes implantation (Fig. 14 A,B,C); b) matrix assisted chondrocytes implantation (Fig. 14 A,B,D).

![Figure 14](image)

**Figure 14.** (A) A full-thickness focal chondral lesion. (B) The lesion is debrided to ensure healthy, stable margins for integration of the host tissue with the neotissue. (C) ACI. The debrided lesion is filled with 12–48 million autologous chondrocytes and covered with a periosteal flap or mixed collagen type I and type III membrane. (D) MACI. The autologous chondrocyte population is expanded in vitro and then seeded onto an absorbable 3D (collagen types I and III or hyaluronic acid) matrix prior to implantation. The cell-seeded scaffold is then secured into the lesion with fibrin glue.

a) Autologous Chondrocytes Implantation (ACI)

Biopsies of AC are taken from the low weight-bearing area of the patellofemoral joint and the autologous chondrocytes are harvested and expanded ex vivo for the re-implantation into debrided areas of the damaged weight-bearing surface. Early surgical procedures using this technique involved the suturing of a periosteal flap over the transplanted cells to retain them at the site of implant (Fig. 15A); later, collagen
membranes replaced the periosteal flap to reduce operating time and complications involving graft hypertrophy (Oldershaw 2012; Chiang et Jiang, 2009) (Fig. 15 B). ACI has demonstrated significant and durable benefits for patients in terms of diminished pain and improved function. However, despite the promising clinical results, the use of ACI carries a number of limitations, essentially related to the complexity of the surgical procedure (Marlovits et al., 2006).

**Figure 15.** ACI technique. Cells are retained in the site of implant with a periostal flap removed from the medial tibia (A) or with a collagen membrane (B).

b) *Matrix Assisted Chondrocytes Implantation (MACI)*

The first generation ACIs were associated with complications and problems due to cartilage hypertrophy and ossifications resulting from the use of periosteam. Subsequently, the second generation known as MACI introduced seeded membranes and biomaterials such as type I collagen, a matrix based on hyaluronic acid and type I/III collagen. Since this type of membranes were used, the hypertrophy was reduced to 5% of all cases, and the problems disappeared 3–6 months after the operation when the membrane was reabsorbed (Vaquero et Forriol, 2012) (Fig. 16).
5.3 Management of haemophilic arthropathy

In haemophilia, the aim of primary or secondary prophylaxis (depending on their onset, before or after joint damage) is to prevent recurrent bleeding into joints and the development of chronic arthropathy in later life. When started early, and at most after two joint bleeds, the result is predictably excellent if there is compliance with the primary prophylaxis regimen. Conversely, once joint damage has occurred, because of recurrent bleeding, secondary prophylaxis can only retard, but not prevent, ongoing joint damage (Van Den Berg et al., 2006).

Treatment recommendations for patients who develop chronic synovitis and arthropathy should be made after careful consideration of all potential options. Conservative options aimed at minimising bleeding and/or controlling pain should be evaluated prior to surgical intervention (Raffini et Manno, 2007).

The management of haemophilic arthropathy, which develops after repeated episodes of joint haemorrhage and accounts for the major morbidity in haemophilia provides for: a) physiotherapy; b) analgesia; c) synovectomy; d) steroid injections; e) hyaluronic acid injections; f) joint arthroplasty; g) arthrodesis; h) osteotomy.

a) Physiotherapy

Physiotherapy is an integral component of comprehensive haemophilia care and plays a role both in the prevention and treatment of joint disease (Astermark et al., 2014). Physiotherapy can be used acutely, after a haemarthrosis or surgery, as well as for patients with chronic synovitis or arthropathy. After a careful musculoskeletal assessment, treatment is individualised and often administered in conjunction with factor replacement. Goals of therapy include restoration or maintenance of range of motion, muscle strengthening, prevention or treatment of articular contracture, pain management,
increased exercise tolerance and improved balance and coordination (Raffini et Manno, 2007).

b) *Analgesia*
Non-steroidal anti-inflammatory drugs (NSAIDs) are often used to treat arthritic pain in Non-Haemophilic patients. They act by inhibiting cyclooxgenase (COX) enzymes resulting in both an analgesic and anti-inflammatory effect. This large class of drugs includes aspirin, traditional NSAIDS such as ibuprofen, and the newer selective COX-2 inhibitors.

Traditional NSAIDS are used sparingly in patients with bleeding disorders because of their anti-platelet effect and the concern for increased bleeding. Conversely, the use of COX-2 inhibitors seems to be effective as they do not interfere with platelet function (Rattray et al., 2006). Although narcotics may be effective, long-term use may lead to dependence (Raffini et Manno, 2007).

c) *Synovectomy*
Once a target joint has developed, it may be difficult to stop the cycle of repeated haemarthrosis with prophylactic clotting factor infusions (secondary prophylaxis). Patients who continue to bleed despite a trial of prophylaxis, and those in whom prophylaxis is not available or feasible, are candidates for interventions aimed at halting this cycle. Synovectomy, which entails excision or destruction of the friable synovium, is an approach that is frequently used to manage patients who experience recurrent haemarthrosis. This may be achieved by direct surgical excision during open or arthroscopic synovectomy, or by injection of a radioactive or chemical agent that causes fibrosis or sclerosis of the synovium, also called synoviorthesis. Patients with advanced arthritic changes, severely narrowed joint space, decreased range of motion, and pain are less likely to benefit from synovectomy, and joint arthroplasty may be considered.

There are many types of synovectomy: a) open synovectomy; b) arthroscopic synovectomy; c) Radionucleotide synoviectomy; d) chemical synovectomy (Raffini et Manno, 2007).

d) *Steroid injections*
Intra-articular injections of steroids have been used to transiently decrease pain and inflammation in patients with chronic synovitis, and may be useful as a palliative measure (Raffini et Manno, 2007).
e) **Hyaluronic acid injections**

Hyaluronic acid has become an increasingly common agent for intra-articular injection for patients with osteoarthritis. Hyaluronic acid is a natural occurring viscous substance that is a fundamental component of the cartilage matrix. Intra-articular injection of this substance can improve pain in patients with arthritis, although its mechanism of action in not well understood. Improvements in pain or function after 3–5 injections in patients with haemophilic arthropathy have been reported in approximately 75% of patients (Raffini et Manno, 2007).

f) **Joint arthroplasty**

Patients with haemophilia who develop severe arthropathy may experience relentless pain, loss of motion and functional disability. If conservative management fails (analgesics, orthotics and physical therapy) these patients may benefit from total joint replacement. However, despite these improvements in pain and functional mobility, arthroplasty in haemophilia has been hampered by a high-complication rate. Complications include postoperative haemarthrosis, wound infection, joint sepsis and prothestic loosening. The frequency of these complications varies, but infection has been the single biggest problem. The long-term survival of joint replacement in haemophilia has been reported to be 90% at 5 years and 83% at 10 years. These outcomes are inferior to those observed in patients with osteoarthritis, in whom the 10–15 year prosthetic survival ranges from 90–95% (Raffini et Manno, 2007).

g) **Arthrodesis**

Arthrodesis is a procedure in which the synovium is removed and the joint is fused to prevent further motion. This approach, by its nature, reduces mobility but involves less extensive surgery and rehabilitation than joint replacement; it may be preferred for relief of pain in other joints for which joint replacement surgery is not routinely available (Raffini et Manno, 2007).

h) **Osteotomy**

Corrective osteotomy may be performed in patients with haemophilic joint disease and axial deformities, particularly around the hip, knee and ankle. While this may not restore range of motion, long-term results after osteotomy for haemophilic arthropathy of the knee showed improved pain in the majority of patients. This may be an option for patients...
in whom joint replacement is not available or currently desirable (Raffini et Manno, 2007).

6. Tissue engineering: a novel approach

To prevent progressive joint degeneration, surgical approaches are often the only option. However, in spite of total joint replacement success, other treatments for repair of cartilage damage are often inadequate, and rarely restore full function or return the tissue to its native normal state (Tuli et al., 2003; Mazor et al., 2014; Hubka et al., 2014). The lack of efficient modalities of treatment has prompted research on TE solutions (Mollon et al., 2013). Through this approach, regeneration of cartilage is pursued combining chondrogenic cells, scaffold materials and environmental factors to guide tissue formation (Getgood et al., 2009; Kim et al., 2011; Vinatier et al., 2009b) (Fig. 17). Autologous chondrocyte implantation yet discussed, represents one of the first TE applications for the regeneration of the AC surface.

![Figure 17. Requirements for cartilage tissue engineering. Cells, such as chondrocytes or mesenchymal stem cells, are expanded ex vivo and subsequently mixed with morphogens (growth and differentiation factors in hypoxic environment) on a 3D scaffold to initiate differentiation. The engineered scaffold will lead to cartilage formation after cells have differentiated, either after a period of ex vivo culture or after implantation in vivo (Vinatier et al., 2009b).]

6.1 Cell sources for cartilage engineering models

The ideal cell source for cartilage TE is one that can easily be isolated and expanded, and which synthesizes abundant cartilage-specific ECM components, (i.e., aggrecan and type II collagen) (Johnstone et al., 2013). To date several cell sources have been investigated
as potential candidates (Nesic et al., 2006). However, the most interesting are chondrocytes and MSCs derived from a variety of tissues (Kock et al., 2012; Seo et al., 2014). Chondrocytes are the native, differentiated cell type, and MSCs are the precursor or progenitor cells that possess the ability to differentiate into functional chondrocytes (Hubka et al., 2014).

6.1.1 Differentiated chondrocytes
Adult chondrocytes have been isolated from various sources like articular cartilage, ear cartilage, nasal septum, ribs. Among these, chondrocytes from hyaline cartilage have been considered the most obvious cell source. They are characterized by a rounded morphology and the production of ECM molecules such as type II collagen and sulfated glycosaminoglycans (GAGs) (Vinatier et al., 2009b; Kock et al., 2012; Hubka et al., 2014). Moreover, differentiated chondrocytes maintain and remodel cartilage matrix tissue by a careful balance of catabolic and anabolic processes involving MMPs and tissue inhibitors of metalloproteinases (TIMPs).

6.1.2 Mesenchimal stem cells
Mesenchymal stem cells are multipotent progenitor cells. They exhibit vast mesodermal differentiation potentials able to give rise to osteocytes, adipocytes, chondrocytes, myoblasts, and tenocytes. In addition, they are able to differentiate into nerve cells and hepatocytes and can be considered as partly pluripotent (Kristjánsson et Honsawek, 2014). MSCs are adult stem cells and, unlike embryonic stem cells, they do not show unlimited self-renewal capacity and cannot be maintained and expanded indefinitely in vitro. (Pelttari et al., 2008; Vinatier et al., 2009b). Under normal culture conditions, MSCs display a fibroblast-like morphology, are adherent to plastic, and can form colonies from single cells referred to as colony-forming fibroblast units. They display the surface antigens CD73, CD90, and CD105, while lacking the expression of the haematopoietic antigens CD11b, CD14, CD34, CD45, CD79, CD19, and HLA-DR (Kristjánsson et Honsawek, 2014).

Recently, MSCs have been considered as an attractive source of cells for cartilage engineering (Pelttari et al., 2008; Vinatier et al., 2009b). Although they reside predominantly within the bone marrow, MSCs can be found in numerous tissues. In particular, MSCs isolated from adipose tissue, muscle (Adachi et al., 2002), periosteum
(Fukumoto et al., 2003) and synovium (Yokoyama et al., 2005) have been still investigated for cartilage TE approaches.

6.2 Biomaterials in cartilage tissue engineering

The use of matrix scaffolds in TE has paved the way for the use of functional tissue substitutes in the treatment of cartilage defects (Redman et al., 2005). Beyond biocompatibility, scaffolds intended for cartilage regeneration should fulfill many requirements, including adequate nutrient transport, adhesion to the defect site, minimally invasive implantation or injection, and degradability. Furthermore, one of the most important requirements is the ability to provide the proper mechanical function (i.e., compressive, shear, and tensile properties), either a priori or through directed tissue formation. Both synthetic and natural materials have been explored as potential scaffolds in a variety of forms, including hydrogels, sponges, and fibrous meshes, for cartilage regeneration. Of these various material structures, the most commonly explored are hydrogels, water-swollen networks crosslinked by either covalent or physical methods (Stoop, 2008; Kim et al., 2011).

6.2.1 Synthetic materials

Synthetic polymers have been widely used for TE since they are more controllable and predictable in mechanics and degradation rate than natural ones. Synthetic polymers currently explored for cartilage repair are: a) poly(α-hydroxy esters); b) Carbon fibres; c) Dacron and Teflon (Vinatier et al, 2009a).

a) Poly(α-hydroxy esters)

Polylactic acid (PLA) and polyglycolic acid (PGA) are derived from alpha hydroxypolyesters (Li et al., 2006); with their copolymers poly(lactic acid-co-glycolic acid) (PLGA) they are the most widely investigated for cartilage TE because of their biocompatibility and Food and Drug Administration approval for clinical application since 1990 (Lee et Shin 2007). PLA and PGA are degraded either by hydrolysis, or specific cleavage of oligopeptides. Their degradation products are however partially cytotoxic and these polymers induce important immunological reactions. Originally, they were developed to form resorbable suture wire (vicryl™) and medical devices (screw, plates). Since twenty years, they are tested alone or mixed with other matrices for cartilage TE. Various forms of these polymers, from the fine fibrillar layer to the sponge,
have been developed (Vinatier et al, 2009a). PGA polymers provide the best in vitro results, with a cellular density near of that found in vivo and a continuous production of type II collagen (Freed et al., 1993).

b) *Carbon fibres*
Carbon fibres are inert and therefore did not induce specific biological answer. They were used, without success, to fill rabbit cartilage defects in order to improve the spontaneous repair. The neo-tissue was fibrous and exhibited only weak mechanical properties. Despite these unsatisfactory results, carbon fibers have been applied in human with very variable results (Hunziker, 2002).

c) *Dacron and Teflon*
Dacron (polyethylene terephthalate) and Teflon (polytetrafluoroethylene) have been used to improve spontaneous repair of AC in rabbit. Results have reported the formation of a repair tissue, which was either a vascularized fibrous tissue or a fibrocartilage (Messner, 1993). Due to an increased rigidity of joint after resurfacing with Teflon (Defrere et Franckart, 1992) and immunological reaction observed when these matrices was used as suture wires, these matrices seem not adequate.

Unless specifically incorporated, synthetic polymers do not benefit from direct cell-scaffold interactions, which can play a role in adhesion, cell signaling, directed degradation, and matrix remodelling. In addition, degradation by products may be toxic or elicit an inflammatory response.

Among the synthetic biomaterials stands out Bio-Seed®-C (BioTissue Technologies, Freiburg, Germany). It is a porous 3D scaffold made of polyglycolic acid (PGA), polylactic acid (PLA) and polydioxanone that has been seeded with autologous chondrocytes embedded within fibrin gel. Bio-Seed®-C has been reported to induce the formation of hyaline cartilage, which is associated with a significant clinical improvement of joint function (Vinatier et al., 2009b).

6.2.2 *Natural materials*
Natural materials used in cartilage TE can be classified as: a) protein-based scaffolds; b) carbohydrate-based scaffolds.
Protein-based scaffolds include collagen membranes or gels, fibrin glue (FG), and platelet-rich plasma (PRP), whereas, carbohydrate-based scaffolds include hyaluronic acid, alginate, agarose, and chitosan (Haleem et Chu, 2010).

a) Protein based scaffolds

i. Collagen

Collagen-based membranes are among the mostly used matrices for cartilage engineering. Collagen is naturally degraded by collagenases and serines proteases and its degradation is controlled locally by the cells of the tissue. These collagen matrices implanted alone improve the spontaneous repair process of osteochondral defects in the rabbit. They are however generally associated with chondrocytes or MSCs (Vinatier et al., 2009a).

ii. Fibrin

Fibrin is a major component of blood clots. It can be used to adhere other engineered cartilage onto the recipient site, as a stand alone scaffold, or as a growth factor. Its utility is much limited by its inferior mechanical properties, the possibility of evoking immune and inflammatory responses, and its inability to allow host cells migration (Chiang et Jiang, 2009; Stoop et al., 2008).

iii. Platelet Rich Plasma

Recently, among blood derivatives, there has been a remarkable increase in the use of PRP to facilitate healing in a variety of pathological MSK conditions. The theoretical advantage of this autologous blood product rests in the concentrated platelets and associated quantity of platelet-derived growth factor and other mitogenic factors that may promote the healing of chondral injuries. PRP increases MSCs proliferation and chondrogenic differentiation but also proteoglycan and collagen production (Bedi et al., 2010).

b) Carbohydrate based-scaffold

i. Hyaluronic acid

Hyaluronic acid is a non-sulphated GAG that makes up a large proportion of cartilage ECM. In its unmodified form, it has a high biocompatibility and plays an important role in determining the biophysical microenvironment for chondrocyte growth and proliferation. Since unmodified hyaluronic acid does not possess sufficient mechanical strength, cross-linking by esterification has been used to optimise its biomechanical
properties. Matrices composed of hyaluronan have been frequently used as a carrier for chondrocytes or bone-marrow-derived MSCs (Stoop et al., 2008).

ii. Chitosan, alginate and agarose

Other naturally occurring polymers such as chitosan, alginate and agarose are extensively used in \textit{in vitro} applications. However, their role in \textit{in vivo} cartilage reconstruction is still very limited.

Among natural materials, such products are still used in clinical practice. Membranes formed of type I and III collagens are clinically available for autologous chondrocyte implantation; such membranes include MACI\textsuperscript{®} (Verigen, Lever-kusen, Germany), Maix\textsuperscript{®} (Matricel, Hezoenrath, Germany) and Chondro-gide\textsuperscript{®} (Geistlich Biomaterials, Wolhusen, Switzerland). This collagen gel enables the 3D culture and \textit{in vivo} implantation of human autologous chondrocytes and of bone marrow MSCs. Of the polysaccharide-based biomaterials, Hyalograft\textsuperscript{®} C, a tissue-engineered graft, consists of autologous chondrocytes that are associated with a hyaluronic-acid-based matrix termed HYAFF-11\textsuperscript{®} (Fidia Advanced Biopolymers, Abano Terme, Italy). This concept has shown a clinical improvement of cartilage function in humans (Vinatier et al., 2009b).

6.2.3 ECM-based scaffolds

One approach that has become very popular and appealing for tissue repair is the use of decellularized tissues. The principle of this technology involves decellularization of an allogenic or xenogenic homologous organ (to remove tissue antigenicity) followed by recellularization of the resultant three-dimensional scaffold with autologous stem and progenitor cells, which would hopefully assemble and organize into a structural and functional unit. The three-dimensional decellularized tissue consists exclusively of the component molecules of ECM, which provide cues that affect cell migration and proliferation. The use of ECM as a bioactive scaffold to promote functional tissue reconstruction has been used yet in many clinical applications including MSK repair (Faulk et al., 2014; van Osch, 2014). Because the native ECM guides organ development, repair and physiologic regeneration, it provides a promising alternative to synthetic scaffolds and a foundation for regenerative efforts (Song et Ott, 2011).

Advantages of these materials are that many of them are natural bodily constituents that provide a natural adhesive surface for cells and carry the required information for their
activity. Moreover, their degradation products are physiological and therefore non-toxic (Vinatier et al., 2009b).

### 6.2.4 Hydrogels

Various polymers, including natural, synthetic and natural/synthetic hybrid polymers, have been used to make hydrogel scaffolds via chemical or physical crosslinking (Scotti et al., 2010). Hydrogels have been used as an important class of tissue-engineering scaffolds because they can provide a soft tissue-like environment for cell growth and allow diffusion of nutrients/cellular waste through the elastic hydrogel network (Zhu et Marchant, 2011). As they exhibit a high water content, close to that found in 3D-like ECMs, hydrogels are particularly useful in TE approaches especially for cartilage TE (Vinatier et al., 2009b; Seo et al., 2014). Moreover, they have advantages over other types of polymeric scaffolds, such as easy control of structural parameters, high water content, promising biocompatibility and adjustable scaffold architecture (Zhu et Marchant, 2011). In the field of tissue engineering, hydrogels have many different functions. They are applied as space filling agents, as delivery vehicles for bioactive molecules, and as three-dimensional structures that organize cells and present stimuli to direct the formation of a desired tissue (Drury et Mooney, 2003).

### 6.2.5 Polyvinyl alcohol

Polyvinyl alcohol (PVA) is a semi-crystalline synthetic polymer produced by the partial or full hydrolysis of polyvinyl acetate (PVAc). The amount of hydroxylation determines the physical characteristics and chemical/mechanical properties of PVA (Baker et al., 2012). Because of its excellent biocompatibility, low price, and easy processability, PVA has gained much attention over the past few decades for its biomedical and pharmaceutical applications as a matrix for TE and as a vehicle for controlled drug delivery. Aqueous PVA solutions can be chemically crosslinked through the formation of acetal linkages using difunctional crosslinking agents (i.e., formaldehyde and glutaraldehyde) or by electron beam or gamma irradiation. However, it is well known that these solutions can be transformed into hydrogel even via crystallite formation from repeated freezing thawing cycles, without any chemical crosslinkers that may lead to toxicity. Briefly, during exposure to cold temperatures water freezes, expelling PVA and forming regions of high PVA concentration. As the PVA chains come into close contact with each other,
crystallite formation and hydrogen bonding occur. These interactions remain intact following thawing and create a non-degradable three-dimensional hydrogel network (Fig. 18). By increasing the number of freeze–thaw cycles and modulating temperature ranges, the degree of polymer phase separation, crystallite formation, and hydrogen bonding can be increased (Holloway et al., 2011; Spiller et al., 2011). Hence, hydrogel mechanical properties can thus be tailored (Kim et al., 2015).

Figure 18. Schematic diagrams showing the mechanisms for (A) hydrogel formation of PVA solution by a freezing–thawing method (Kim et al., 2015).

Polyvinyl alcohol hydrogels have been extensively investigated as artificial AC (Grant et al., 2006). Values of compressive modulus, shear modulus, tensile modulus, and permeability were similar (Spiller et al., 2011), making these hydrogels attractive biomaterials for cartilage TE applications (Holloway et al., 2011). SaluCartilage™ (Salumedica, Smyrna, GA); Cartiva™ (Fig. 19), Cartiva SCI™ (Carticept Medical, Inc) are PVA devices that has been designed to mimic natural cartilage and still used in clinical to repair focal cartilage defects and osteoarthritic joints, while minimizing the resection of healthy tissue.

Figure 19. Cartiva™ implant before (A) and after (B,C) implantation.
6.3 Chondrogenic factors in cartilage engineering

A number of growth and differentiation factors that regulate cartilage development and homeostasis of mature AC have been identified. The most characterized factors which stimulate the anabolic activity in cartilage include members of Transforming Growth Factor (TGF)-β superfamily, Bone Morphogenetic Protein (BMP), Fibroblast Growth Factors (FGF), Insulin Growth factor (IGF)-1, Hedgehog (hh) and Wingless (Wnt) proteins (Vinatier et al., 2009a; Demoor et al., 2014).

7. Engineering of articular cartilage: three approaches

There are three possible approaches to the engineering of articular cartilage. According to the first, the repair tissue is engineered completely in vitro, the fully-differentiated construct being then implanted within the defect void. The advantage of this approach is that cell metabolism and differentiation are subject to better control in vitro than in vivo, if the appropriate bioreactor systems, growth factors and delivery systems are available. Its drawbacks are that the problems associated with the integration and the mechanical fixation of the repair tissue cannot be readily anticipated and solved. Moreover, the simulated mechanical-loading conditions of the ECM may not be ideally suited to the specific needs of the prospective repair site. In addition, biocompatibility and immunological problems are frequently associated even with this in vitro approach and in the absence of a scaffold. Furthermore, the natural curvature of the joint surface poses a challenge to the process of press-fitment whereby the engineered construct is introduced into the defect void.

The second approach, which is more frequently adopted, aims to engineer only the basic building block: a matrix scaffold containing a homogeneous population of cells, and signalling molecules that are entrapped within an appropriate delivery system. The vehicle-bound signalling molecules ensure that the desired differentiation process takes place in vivo and that chondrocytic activity within the repair tissue is sustained. According to this approach, the differentiation and the remodelling of the repair tissue occur in vivo under physiological conditions of mechanical loading. Repair tissue that is formed in situ is more likely to adhere to and integrate with native AC than is that produced in vitro, and it will also adapt naturally to the contour of the synovial joint, provided that appropriate measures are taken to promote the bonding of the implant that has been generated in culture. One of the disadvantages of this second approach is that cell activity is more difficult to control on a long-term basis. Moreover, appropriate measures must be taken
to avoid “contamination” from cells and signalling substances that are involved in the spontaneous healing response, since the tissue thereby formed would compromise the quality and the mechanical competence of the final repair-composite.

The third approach involves the direct application of exogenous growth factors (entrapped within a matrix) to the defect site; these then stimulate the intrinsic formation of cartilaginous tissue *in situ*. An appropriate matrix is first introduced into the defect to define the space that is to be repaired. The laying of this physical “track” is necessary to guide the movements of the intrinsic precursor cells, which have a limited spatial awareness. The growth factors are usually introduced into the matrix in two different states: as a freely-soluble agent, to stimulate the immediate recruitment, migration and proliferation of the precursor cells from their site of origin into the defect area; and in a vehicle bound form, for gradual delivery at a steady rate that is sustained for several weeks to stimulate the chondrogenic differentiation of the defect filling population of cells. Hence, an intelligent, internally-programmed matrix is applied to the defect. The freely-soluble signalling agent usually applied are IGF-1, bFGF or a TGF-b (at low concentration) (Hunziker et al., 2014).
Aim of the study

Cartilage degeneration is the hallmark of osteoarthritis. Amongst musculoskeletal disorders, osteoarthritis is the most common joint disease and is recognized as a major cause of pain and disability. It develops most commonly in the absence of a known cause of joint degeneration, a condition referred to as primary or idiopathic osteoarthritis. Beyond non-surgical conservative options, which can only provide symptomatic relief, osteoarthritic patients can take advantage from a large variety of surgical treatments that have been developed to reduce joint pain, improve joint function and delay the onset of osteoarthritis, sustaining cartilage recovery. Among these, TE approaches stand out (i.e. ACI, MACI). However, despite the promising clinical results, they still carries a number of limitations.

Less frequently, osteoarthritis develops as a result of joint degeneration caused by injuries or a variety of hereditary, inflammatory, or developmental, metabolic, and neurologic disorders, a group of conditions referred to as secondary osteoarthritis. A hereditary rare bleeding disorder, that is haemophilia, is characterized by recurrent haemarthroses that determine progressive cartilage damage leading to haemophilic arthropathy. In patients with haemophilia, current available orthopaedic strategies can only slow the progression of joint damage resulting in lesions that tend to be more extensive than focal. Arthroplasty becomes the only resolute option; however, it can results in a hospital stay painful and in several complications ranging from anemia, inhibitor development, anaphylactic reactions, haematomas and haemarthroses, up to infections. Interestingly to our purposes, any approach aims to promote cartilage recovery in haemophilics, at least in the early stages of cartilage damage.

Due to cartilage low self-ability repair, AC restoration represents a challenge of musculoskeletal TE. In fact, the employ of matrix scaffolds has paved the way for the use of functional tissue substitutes in the treatment of cartilage defects. Beyond biocompatibility, scaffolds intended for cartilage regeneration should fulfill many requirements, including adequate nutrient transport, minimally invasive implantation or injection, and degradability (Stoop, 2008).

Hydrogels have been used extensively in the field of TE, because they can provide a soft tissue-like environment. Among these, a synthetic polymer still used in orthopaedic surgery, is polyvinyl alcohol. In virtue of its ability in mimicking cartilage tissue, it has
Aim of the study

...gained a wide attention. However, it is employed merely as non-biodegradable prosthesis.

Established that, this work focused on polyvinyl alcohol, providing to:

i. chemically modify and characterize a novel synthetic polymer polyvinyl alcohol-based;

ii. manufacture and characterize for physico-mechanical properties, biocompatibility, biodegradation rate, loading ability, physically cross-linked scaffolds realized using chemically modified polymer solutions;

iii. promote scaffold ability in sustaining cell adhesion and proliferation through the development of a composite scaffold. This will be obtained combining chemically modified polyvinyl alcohol and a biological decellularized tissue from umbilical cord in comparison with AC extracellular matrix;

iv. isolate articular chondrocytes from cartilage of haemophilic patients and characterize them for their morphology, expression of specific m-RNA, immunophenotype in comparison with chondrocytes isolated from AC of Non-Haemophilic patients. Will be verified the chance of using them in tissue engineering approaches in the perspective of autologous implant;

v. verify composite scaffold ability in sustaining cell adhesion and proliferation assessing eventual differences among Haemophilic and Non-Haemophilic chondrocytes.
MATERIALS AND METHODS

1. Preparation of polymer solutions

1.1 PVA solution

PVA solution was obtained suspending in MilliQ water (MilliQ Academic system, Millipore, Bedford, MA, USA) a pre-weighted quantity of PVA powder (Molecular weight (Mw.) 146,000-186,000 Da, 99+% hydrolyzed) (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) (16% w/w solution). Powder suspension was then heated for 48 hours (h) at 100 °C, under stirring, until polymer was completely dissolved.

1.2 Oxidized PVA solutions

PVA oxidation was performed in 4 stages described below.

a) PVA dissolution.

A pre-weighted quantity of PVA powder (Mw. 146,000-186,000 Da, 99+% hydrolyzed) was suspended in MilliQ water (16% w/w solution). Powder suspension was then heated for 48 hours (h) at 100 °C, under stirring, until polymer was completely dissolved.

b) Preparation of oxidant solution.

Partial oxidation of PVA was obtained using potassium permanganate (KMnO₄) (Fluka, Basel, Switzerland) in dilute perchloric acid (HClO₄) (Fluka).

The required amount of KMnO₄, in the ratio of 10 mg salt per ml of water, was weighed accurately and dissolved in deionized water under stirring. The environment was acidified by addition of HClO₄ at 70%.

c) Oxidation of PVA.

The oxidant mixture was poured rapidly in PVA solution, stirred vigorously, and allowed to react in a thermostatic bath at 30 °C until complete discoloration of the polymer solution. Discoloration occurred in about 60 min.

d) Dialysis against deionized water.

Oxidized solution was poured in a membrane with 8,000 Da cut-off (SpectraPor, Philadelphia, PA, USA) and dialyzed extensively against deionized water under stirring for 48h. Water was replaced every 6 h.
For our purposes, were prepared PVA solutions with an oxidation degree of 1% and 2% respectively.

Typically, to prepare 1% oxidized (1% Ox) PVA, one gram of PVA was solubilized in 20 ml of MilliQ water and then 5 ml of KMnO₄ water solution [2.9 mg/ml] and 0.3 ml of 70% HClO₄ were added. 

To prepare 2% oxidized (2% Ox) PVA, the amounts of KMnO₄ and HClO₄ were doubled. The stoichiometry of the reaction is described by the following equation, referred to a hypothetical vinyl alcohol monomer (- CH₂ CHOH -):

\[ 2\text{KMnO}_4 + 5[\text{CH}_2 \text{CHOH}] + 6\text{HClO}_4 \rightarrow 5[\text{CH}_2 \text{CO}] + 2\text{Mn(ClO}_4)_2 + 8\text{H}_2\text{O} + 2\text{KClO}_4 \]

2. Oxidized polymer solutions characterization

2.1 2,4-Dinitrophenylhydrazine assay.

Highly sensitive assay for detection of carbonyls (aldehydes and ketones) involve derivatisation of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH) leading to the formation of a stable 2,4-dinitrophenyl (DNP) hydrazone product (Fig. 20). Derivatisation with DNPH is typically developed in solutions of 2 M hydrochloric acid (HCl) (Carlo Erba, Milan, Italy).

![Figure 20](image)

**Figure 20.** Mechanism for the reaction between 2,4-dinitrophenylhydrazine and carbonyl group of an aldehyde or ketone.

The classical approach involves carbonyl groups reaction with DNPH, followed by the spectrophotometric quantification of the acid hydrazones at 375 nm. It absorbs ultraviolet light so that the total carbonyl content can be quantified by a spectrophotometric assay; however, to give greater sensitivity and specificity, it can be coupled to fractionation by high-performance liquid chromatography (HPLC). Gel-filtration chromatography (GPC) by HPLC has proved to be a convenient and efficient technique in which DNP-carbonyl derivatives are separated by molecular weight, allowing a more specific
Materials and Methods

2.1 Quantification of oxidized PVAs carbonyls

2,4-Dinitrophenylhydrazine assay has been performed to quantify the oxidative modification of PVA backbone. Briefly, 10 mg of lyophilized 1% and 2% oxidized PVA were dissolved at 100°C in 1 ml of MilliQ water respectively. One-hundred microliters of these solutions were added to 900 µl of 10 mM DNPH (Fluka) solution in HCl 2.5 M. The PVA-phenylhydrazone derivative reached maximum yield after 48 h at 25°C and it was recovered by GPC on a PD-10 column (GE Healthcare, Cleveland, OH, USA). Mobile phase consisted of 70/30 (v/v) Phosphate Buffered Saline (PBS) (Gibco, Invitrogen Corporation, Paisley, UK) and methanol (Fluka) at 1 ml/min flow rate. The separation profile was monitored at 375 nm. The first eluted peak corresponded to hydrazones bound to PVA; it was collected and the absorption spectrum was registered using a spectrophotometer Jasco mod V630 (Jasco Corporation, Great Dunmow, Essex, GB). The amount of phenylhydrazone derivatives was evaluated from the absorbance (A) at 375 nm taking into account a molar absorbtivity (ε) of 22,000 (M x cm\(^{-1}\)).

2.2 Reductive amination.

Reductive amination is a selective and versatile technique to alkylate amines. It is accomplished by treating the protein with a simple aliphatic aldehyde or ketone and a reducing agent, usually sodium borohydride or sodium cyanoborohydride (which is more selective). It consists of two subsequent reactions:

a. condensation of an amine with an aldehyde/ketone to an imine, with abstraction of water
b. reduction of the imine to the corresponding amine

![Reduction of imine to amine](image)

2.2.1 Covalent binding of lysozyme

The covalent interaction between oxidized PVA and lysozyme (Sigma-Aldrich) was evaluated using the reducing agent sodium cyanoborohydride (NaBH₃(CN)) (Sigma-Aldrich). Twenty mg/ml of 1% Ox and 2% Ox PVA solutions were incubated with a lysozyme solution in PBS [13 mg/ml], at RT, in presence of NaBH₃(CN) [2.5 mg/ml]. At different end-points (4, 24, 50, 120 h) the solutions were analyzed by GPC using a high performance column Superose 6 10/300 GL and an HPLC AKTA (GE HealthCare, Little Chalfont, Buckinghamshire, UK). The absorption profiles of bound and free lysozyme were monitored at 280 nm with PBS as eluent and a flow rate of 0.5 ml/min.

3. Scaffold manufacture.

PVA, 1% and 2% oxidized PVA were used to prepare scaffolds by pouring solution of each between two plate glasses detached by spacers (2 mm). Physical cross-linking of polymer solutions occurred through a partially modified FT process according to Lozinsky (Lozinsky et al., 1995). Briefly, polymeric aqueous solutions cast into molds were exposed to FT: one cycle consisted in a freezing period (at -20°C) followed by a thawing one (at -2.5°C). Each period lasted 24 h; three cycles occurred.

4. Scaffold characterization

4.1 Morphological analysis by Scanning Electron Microscopy analysis.

Morphological analysis of PVA, 1% and 2% oxidized PVA scaffolds was performed by SEM. Samples were dehydrated by immersion in increasing concentrated alcohols (30% - 100%) (1h /alcohol) (Sigma-Aldrich). Hydrogels were then exposed to Critical Point Drying and gold sputtered and finally observed. Images were taken with a Stereoscan-205 S scanning electron microscope (Cambridge instruments, Pine Brook, NJ, USA) of CUGAS, Interdepartmental Service Centre at University of Padua.
4.2 Mechanical behavior and physical properties.

4.1.1 Stress-strain tests
Resilience is a measure a material’s ability to deform reversibly without energy loss. To evaluate the chance of using oxidized PVAs as mechanical support for cartilage regeneration, the resilience of chemically modified hydrogels was assessed in comparison to the neat polymer and to human AC tissues.

Five samples of neat PVA, 1% and 2% Ox PVA hydrogels were cut from cross-linked polymer sheets into strips (5 x 25 x 0.3 mm3, n = 5). In parallel, isometric samples were obtained from human AC specimens (5 x 25 x 0.3 mm3, n = 3). Hydrogels were evaluated both dried and swelled, after 24 h in PBS at 37 °C to simulate the exposure to body fluids.

The strips were clamped with tensile grips between opposing motors for stress-strain tests using a universal testing machine Bose® ElectroForce® TestBench system (Bose Corporation, Eden Prairie, Minnesota, USA) with an extended stroke actuator. The system was equipped with a 22 N load cell and tests were carried out at room temperature by subjecting each sample to a maximum elongation of 100% of the initial length (from 5 mm to 10 mm) with a strain rate of 0.5 mm/s. This parameter is of major importance: it can affect the measure because of the viscoelasticity of the samples. To minimize any viscoelastic effect, high strain rate is usually applied.

The load cell measured the force applied during consecutive loading/unloading cycles.

The instrument used is located at Department of Industrial Engineering, University of Padua. Data were collected with the WinTest® Controls software (Bose Corporation) and analyzed with Origin Pro 9.1 (Northampton, Massachusetts, USA).

4.1.2 Dynamic Light Scattering
Dynamic Light Scattering (DLS), also called Photon Correlation Spectroscopy, is a spectroscopic technique that indirectly determines particles size and size distribution of polymers, proteins and colloids by measuring their speed as they move in suspension.

Small particles in suspension undergo random thermal motion known as Brownian motion. This random motion is modeled by the Stokes-Einstein equation:

\[ Dh = \frac{kBT}{3\pi Dt} \]
where $D_h$ is the hydrodynamic diameter; $D_t$ is the translational diffusion coefficient; $k_B$ is the Boltzmann’s constant; $T$ is the thermodynamic temperature; $\pi$ is the dynamic viscosity.

Stokes-Einstein equation connects diffusion coefficient measured by dynamic light scattering to particle size.

Shining a monochromatic light beam, such as a laser, onto a solution with spherical particles in Brownian motion causes a Doppler Shift when the light hits the moving particle, changing the wavelength of the incoming light. This change is related to the size of the particle. It is possible to compute the sphere size distribution and give a description of the particle’s motion in the medium, measuring the diffusion coefficient of the particle and using the autocorrelation function.

Although the fundamental size distribution generated by DLS is an intensity distribution, this can be converted, using Mie theory, to a volume distribution or a distribution describing the relative proportion of multiple components in the sample based on their mass or volume rather than based on their scattering (Intensity).

Average size and dispersion of neat PVA, 1% Ox and 2% Ox PVA micelles in water was evaluated by DLS using a Zetasizer Nano-ZS DLS instrument (Malven Instruments Ltd., Worcestershire, UK), under the control of a Peltier unit at 25 °C. Solutions containing 3 mg/ml of each sample were centrifuged at 22°C at 15,000 rpm for 1 h in a GS-15R centrifuge (Beckman, Brea, CA). A volume of 150 µl of each supernatant was analyzed; volume plots were used. The refraction indices considered were that of polystyrene and water for PVA and the dispersing medium, respectively.

### 4.1.3 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is a thermal analysis technique used to measure enthalpy changes due to changes in the physical and chemical properties of a material as a function of temperature. In a DSC the difference in heat flow to the sample and a reference at the same temperature, is recorded as a function of temperature. The reference is an inert material such as alumina, or just an empty aluminium pan. The temperature of both the sample and reference are increased at a constant rate. Since the DSC is at constant pressure, heat flow is equivalent to enthalpy changes. The heat flow difference between the sample and the reference can be either positive or negative. In an endothermic process, such as most phase transitions, heat is absorbed and, therefore, heat flow to the sample is higher than that to the reference. Hence enthalpy is positive. In an exothermic process,
such as crystallization, some cross-linking processes, oxidation reactions, and some decomposition reactions, the opposite is true and enthalpy is negative.

Thermal characteristics of PVA scaffolds were assessed by means of Differential Scanning Calorimetry (DSC 200 PC, Netzsch-Gerätebau GmbH, Selb, Germany). Samples were firstly air-dried for 24 h, about 10 mg were then placed in an aluminum pan and heated at a scanning rate of 10 °C/min up to 250 °C. Measurements were carried out in nitrogen atmosphere. Melting temperature ($T_m$) and enthalpy ($\Delta H_m$) were evaluated from the first heating scan; the crystallinity degree was calculated from the melting enthalpy as follows:

$$X_c = \frac{\Delta H_m}{\Delta H_0} \times 100$$

where $\Delta H_0$ is the melting enthalpy of 100% crystalline PVA (138.6 J/g) (Hassan et Peppas, 2000).

4.1.4 Swelling behavior

Immersed in an aqueous solution, a network of covalently cross-linked polymers imbibes the solution and swells, resulting in a hydrogel. The amount of swelling is affected by: mechanical forces, pH, salt, temperature, light, and electric field.

PVA and oxidized PVA hydrogels (5 x 2 x 30 mm) were separately immersed in 5 ml of PBS 1X solution 37 °C and 95% relative humidity for a total amount of 600 h. Every 24 h, PBS excess was removed by wiping hydrogels with filter paper and samples were weighed. The swelling ratio was calculated by the following equation:

$$\text{Swelling (\%)} = \frac{(W_s-W_d)}{W_d} \times 100$$

where $W_s$ is the weight of the swollen hydrogel and $W_d$ is the weight of the dry hydrogel (Del Gaudio et al., 2013).

5. Oxidized polyvinyl alcohol scaffolds as drug carrier

5.1 Bovine serum albumin adsorption and release

Protein release ability of PVA, 1% Ox and 2% Ox PVA scaffolds (7 mm diameter x 2 mm thickness) was investigated using Bovine Serum Albumin (BSA) (Mw. 66.5 kDa) (Sigma-Aldrich) as protein model. Scaffolds were soaked in 1 ml of BSA solution in PBS.
[40 mg/ml] for 24 h at 37 °C (pH 7.4). After the incubation period, each scaffold was transferred in 1 ml of fresh PBS which was collected and changed at predetermined end-points: 1, 24, 144 h. The absorption at 280 nm of washing solutions was recorded and the content of BSA was evaluated (ε = 0.66). The resulting data were normalized considering scaffolds weight after lyophilization.

5.2 TGF-β1 absorption and release
PVA supports were incubated in 1ml of TGF-β1 (Mw. 25 kDa) (Sigma-Aldrich) [50 ng/ml] in PBS for 48 h at 37 °C. Growth factor release at 24, 48 and 72 h was detected using a fluorimeter Jasco FP-6500 (Jasco Corporation). The spectra were recorded using a Suprasyl quartz cuvette with excitation and emission optical paths of 10 mm and 2 mm, respectively. Excitation wavelength was set at 280 nm, while emission spectra were acquired in the range between 290 and 450 nm.

5.2.1 Evaluation of TGF-β1 release on human primary chondrocytes.
The effect of TGF-β1 release from PVA and oxidized scaffolds was evaluated on a human articular chondrocyte (AC) population. Cells were isolated, cultured, and characterized as we described previously (Stocco et al., 2014). A 600 μl suspension of ACs at passage 2 was seeded in one well of a 24-multiwell plate (10,000 cells/cm²) and a culture plate insert (cut-off, 1µm) (BD-Falcon, USA) was placed in. PVA and oxidized PVA scaffolds were then introduced into inserts and soaked in 400 µl of complete medium. After 72 hours and 7 days from seeding, cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT) assay.

MTT is a yellowish solution which is converted to water-insoluble MTT-formazan of dark blue color by mitochondrial dehydrogenases of living cells. The blue crystals are soluble with acidified isopropanol and the intensity can be measured colorimetrically at a wavelength of 570 nm.

At the end points considered, once inserts with scaffolds were removed, wells were washed gently with PBS. After taking out the buffer, cells were treated with 500 µl of MTT (0.5 mg/ml) in basal medium for 4 h at 37 °C in humidified atmosphere containing 5% CO₂. Afterwards, MTT solution was removed and formazan precipitates were dissolved in 300 µl of 2-propanol acid (0.04 M HCl in 2-propanol) (Sigma-Aldrich). Dissolution occurred placing the 24 well plates on a tilting shaker (Asal, Milano, Italy) for 20 min at RT. Dissolved formazan precipitates were collected carefully and moved to
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6. In vivo study on scaffold biodegradability.
All animal procedures were approved by the ethical committee of Padua University, following the guidelines established by the National Institutes of Health. Animals were housed in a temperature-controlled facility, and were given laboratory rodent diet and water ad libitum. Twelve BALB/c mice were gas anesthetized by isoflurane and oxygen administration. The dorsa of the animals were shaved and sterile prepped with Betadine® (Bayer, Leverkusen, DE). A lombotomic incision of about 20 mm was performed on the right side of the dorsum using a N°. 10 surgical blade (Becton-Dickinson, Franklin Lakes, NJ, USA). A subcutaneous pouch was created using blunt dissection technique and polymers disks (7 mm diameter x 2 mm thickness) of PVA, 1% Ox and 2% Ox PVA (n=4 each group) were inserted in, and anchored to the latissimus dorsi muscle using TYCRON 4/0 sutures. Following implantation, the skin was stitched up using absorbable NOVOSYN 4/0 sutures. After surgery, animals were administered anti-inflammatory (Rimadil, 5 mg/kg) and antibiotic (Bytril, 5 mg/kg) therapy for 5 days and were allowed to recover in the cage. Twelve weeks from implantation, mice were euthanized by carbon dioxide asphyxiation. Implants and surrounding tissues were excised and scaffolds were preliminary analysed for their size and integrity, compared to not implanted controls. Moreover, samples were fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) (Sigma-Aldrich) for SEM investigation. Samples for histological and immunohistochemical analysis were frozen and included in OCT or fixed in 10% formalin solution in neutral PBS (Sigma-Aldrich) and embedded in paraffin.

6.1 Histological analysis of explanted samples
Samples included in OCT were cut into 10 μm-thick serial sections and stained with Haematoxylin-Eosin (Bio-Optica, Milano, Italy). They were mainly used for evaluation of PVA characteristics and state of degradation. Haematoxylin has a deep blue-purple colour and stains nucleic acids. Eosin is pink and stains proteins non-specifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining.
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Briefly, sections were stained with haematoxylin solution for 1 min at RT, and then the dye in excess was removed before washing slides in deionized water (2 rapid washes) and in tip water (1 wash/6 min). After drying the excess water, sections were stained with eosin solution for 5 sec at RT. The dye in excess was removed before washing slides in deionized water (1 min). Finally, air dried sections were mounted with resinous mounting medium before optical microscope analysis.

Paraffin embedded samples were previously fixed in 10% buffered formalin for 24 h; thereafter, samples were rinsed in tip water to remove excess formalin and dehydrated by a series of alcohol baths of increasing strengths. In particular, dehydration occurred using an automatic processor Leica TP1020 (Leica, Wetzlar, Germany) performing a dehydration program of 10 h (total). Samples were soaked in alcohol baths (2 cycles in alcohol 70%; 2 cycles in alcohol 95%, 3 cycles in alcohol 100%), then in xylene (3 cycles) and paraffin (2 cycles). Finally, specimens were paraffin embedded using Leica EG 1150 embedder and cut into 5 µm-thick serial sections. Sections were dewaxed by 2 cycles in xylene of 10 min each and then rehydrated by a series of alcohol baths of decreasing strengths (2 washes in 100% alcohol; 1 wash in 95% and 70% alcohol respectively) and 1 wash in deionized water.

6.2 Immunohistochemical analysis of explanted samples

Immunological characterisation of the cells identified in contiguity with PVA was carried out with the following antibodies diluted in PBS: anti-CD3 (Polyclonal Rabbit Anti-Human CD3, A0452, DAKO, Milan, Italy) diluted 1:500; anti-F4/80 (sc-26643-R, Santa Cruz Biotechnology, CA, USA) diluted 1:800. Sections were incubated in 1% hydrogen peroxide in deionised H₂O, to remove endogenous peroxidase activity and enhance antibody penetration into the tissue, and then washed in 0.01 M PBS. For both protocols, antigen unmasking was performed with 10 mM sodium citrate buffer, pH 6.0, at 90 °C for 10 min. Sections were then incubated for 30 min in blocking serum (0.04% bovine serum albumin (A2153, Sigma-Aldrich, Milan, IT) and 0.5% normal goat serum (X0907, Dako Corporation, Carpinteria, CA, USA) to eliminate unspecific binding, and then incubated for 1 hour at room temperature with the above primary antibodies. Primary antibody binding was revealed by incubation with anti-rabbit/mouse serum diluted 1:100 in blocking serum for 30 min at room temperature (DAKO® EnVision + TM Peroxidase, Rabbit/Mouse, Dako Corporation, Glostrup, Denmark) and developed in 3,3’-
diaminobenzidine for 3 min at RT. Lastly, sections were counterstained with haematoxylin. As negative control, sections were incubated without primary antibodies.

7. Cell cultures

Human articular chondrocytes were isolated from AC samples collected from two different patients categories: osteoarthritic and haemophilic donors. All patients underwent to total knee arthroplasty.

7.1 Cartilage harvest and chondrocyte isolation

Human AC samples were collected from donors who underwent total knee arthroplasty; only tissue from joints without signs of degenerative changes was used. The cartilage specimens were kept in basal medium DMEM and Nutrient Mixture F12, ratio 2:1, until further processed (within 24 h of sample collection). For chondrocyte isolation, cartilage was washed in PBS containing 2% of penicillin/streptomycin, minced finely and digested with 0.1% collagenase B in basal medium at 37 °C for 22 hours. The resulting cell suspension was collected and centrifuged at 1500 rpm for 5 min. Isolated cells were then seeded on 25 cm²-flasks (BD Falcon) at high density with complete medium as described below.

7.2 Chondrocyte culture medium

Chondrocytes were cultured at 37 °C in humidified atmosphere containing 5% CO₂ with complete medium: DMEM/F12 (2:1) (Gibco) added with 10% fetal bovine serum (FBS) (Gibco), 0.4 μg/ml hydrocortisone, 8 ng/ml cholera toxin, 5 μg/ml insulin, 24 μg/ml adenine, 0.5 μg/ml transferrin, 136 pg/ml triiodothyronin, 1% Penicillin/Streptomycin solution (Sigma-Aldrich). The medium was changed at the sixth day and then every 3-4 days.

8. Chondrocyte characterization

Before testing Haemophilic chondrocytes for their potential use in autologous cartilage restoration, a detailed characterization of cell population was performed to assess their distinctive properties according to literature. Chondrocytes isolated from AC samples of Non-Haemophilic patients were used as control.
8.1 Optical microscopy analysis.

Cell cultures were daily observed by optical microscope DM/IL (Leica) and pictures were taken with a camera Nikon Digital Sight Ds-SMCc (Nikon Corporation).

8.2 Reverse Transcriptase - Polymerase Chain Reaction

Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) is a sensitive method for the detection of specific mRNA expression levels. Traditionally RT-PCR involves two steps: the RT reaction and PCR amplification. RNA is first reverse transcribed into cDNA using a reverse transcriptase and the resulting cDNA is used as templates for subsequent PCR amplification using primers specific for one or more genes.

Briefly, the method provide:

A) RT-Reaction
a. Isolation of the template RNA.

b. Priming for reverse transcription. To generate cDNA using the enzyme reverse transcriptase (RT), a primer is annealed to the template RNA. The primer can be gene specific primers, random primers or oligo-dT primers.

c. First strand synthesis. The first strand of cDNA is synthetized using RT beginning at the primer annealing site.

d. RT adds complementary nucleotide bases to the mRNA strand creating a strand of cDNA.

e. Removal of RNA. The template strand of RNA is removed by treatment with RNase H. The cDNA can now be used for amplification by PCR.

B) PCR reaction

f. The oligonucleotide primer is allowed to anneal to the template cDNA.

g. Taq polymerase adds complementary nucleotides beginning at primer annealing site.

h. The resultant product is a double stranded cDNA

i. The three step process of denaturation, primer annealing and extension are repeated to yield a detectable PCR product. The product can be visualized on an ethidium bromide stained agarose gel following electrophoresis.

RT-PCR can also be carried out as one-step RT-PCR in which all reaction components are mixed in one tube prior to starting the reactions.
8.2.1 Expression of specific mRNA

Extraction of total RNA

mRNA was extracted using Trizol® Reagent (Sigma-Aldrich), a monophasic solution of phenol and guanidine isothiocyanate. Monolayer cultures of articular chondrocytes from Haemophilic and Non-Haemophilic patients were detached by treatment with trypsin-EDTA (Sigma-Aldrich), washed with PBS and finally centrifuged at 1500 rpm for 5 min. Cell pellets, obtained after the removal of washing buffer, were then treated first with 1 ml of Trizol for 5 min. at RT and then with 200 μl of chloroform (Sigma-Aldrich). For a better separation of RNA, the samples were shaken gently for 15 sec. and afterwards incubated at RT for 3 min. Later, samples were centrifuged at 12000 rpm for 15 min. at 4°C to obtain phase separation. Samples were resolved as a lower phase containing proteins, white interphase containing DNA and an upper aqueous phase containing RNA. Aqueous phase was collected carefully and RNA was precipitated by adding 500 μl of isopropanol (Sigma-Aldrich) (0.5 ml per 1 ml of Trizol used) and shaking by hand the sample. After 10 min. of incubation, samples were centrifuged at 1200 rpm for 10 min. at 4°C., the supernatant was removed and the resulting pellet was washed with 1 ml of cold 75% ethanol and then centrifuged at 8600 rpm for 5 min. at 4°C. After the careful removal of the supernatant, the pellet was air dried for 10 min. and resuspended in 10 μl of RNase-free water (Sigma-Aldrich). RNA was quantitated by spectrophotometric analysis and then samples were stored at -80°C.

Spectrophotometric quantitation of extracted RNA

After extraction of total RNA, it was quantitated by spectrophotometric analysis. The absorbance of 1 μl of each sample was measured at the wavelength of 260 nm. using the spectrophotometer NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). At the same time, samples purity was also evaluated considering absorbance at 280 and 230 nm which is ascribable to absorbance wavelength of proteins and carbohydrates respectively. Gene expression studies were performed using RNA samples whose 260/280 ratio were in the range of 1.8 - 2.0.

One Step RT-PCR

Reverse transcription and specific amplification of RNA in cDNA were performed in a single tube using QIAGEN® OneStep RT-PCR Kit (Qiagen, Hilden, Germany). Its provides a blend enzymes: Sensiscript and Omniscript Reverse Transcriptases and
HotStarTaq DNA Polymerase, that allow both reverse transcription and PCR amplification to take place in the same reaction mix in a "one-step" reaction which is temperature-dependent. During reverse transcription, the HotStarTaq DNA Polymerase is inactive; while, during amplification, to the deactivation of the reverse transcriptase, corresponds DNA polymerase activation at a temperature of 95°C. The reaction mixture (25 μl) was prepared in ice using 1 μl of RNA at a concentration of 30 ng/μl, 3 μl forward primer (5 μM), 3 μl reverse primer (5μM), 1 μl dNTP mix (10mM), 1 μl Qiagen® One Step RT-PCR Enzyme Mix, 1 μl RNase inhibitor (125 U), 5μl 5X buffer and RNase-free water. One Step RT-PCR was performed with the thermal cycler iCycler iQ™ (Bio-Rad). Specific oligo primers (Life technologies, Carlsbad, CA, USA) designed on Gene Bank sequences (Tab. 3) were used and the expression of HPRT was considered as internal control.

Agarose gel Electrophoresis
The electrophoretic analysis of PCR reaction products was performed by running samples on 2% agarose gel prepared in 1X TBE buffer (tris, 0.04mM Borate, 0.001M EDTA, pH 8). For loading, 6 μl of amplified product were mixed with 2 μl of loading dye (Sigma-Aldrich). As a reference marker to the molecular weights between 100 and 1000 bp, the PCR 100 bp Low Ladder was used. The bands of amplified samples were visualized by staining with Gel Red (0.1μl/ml) and exposure to UV light. Images were acquired with Gel Doc 2000 (Bio-Rad).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’ → 3’</th>
<th>Reverse primer 3’ → 5’</th>
<th>GenBank accession</th>
<th>Base pair (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen, type II, alpha 1 (COL2A1)</td>
<td>F: CCSSGCCAGAGGCAATATAGCGGT</td>
<td>R: GAATGCAAGGGAGGCGTGAAG</td>
<td>NM_001844.4</td>
<td>127</td>
</tr>
<tr>
<td>Collagen, type IX, alpha 3 (COL9A3)</td>
<td>F: AATCAGGCTCTGAAAGAGCTCATAAAA</td>
<td>R: CCGGCCACACCCCGCTCTTTCAT</td>
<td>NM_001853.3</td>
<td>99</td>
</tr>
<tr>
<td>Cartilage oligomeric matrix protein (COMP)</td>
<td>F: CCGAGGAGGTGACGGGAGAATTGAG</td>
<td>R: TGGCCTGGAAGGCACGCCTTGA</td>
<td>NM_000095.2</td>
<td>132</td>
</tr>
<tr>
<td>Aggrecan (ACAN)</td>
<td>F: GGCTGGCTGCTCCCCTGAAGAAG</td>
<td>R: GGGAGGCCAAGTGAAGGAAGAT</td>
<td>NM_001135.3</td>
<td>162</td>
</tr>
<tr>
<td>Transcription Factor SOX9 (SOX9)</td>
<td>F: CGGGGCGAGAGCCCTGGAAGA</td>
<td>R: ATGTGGCGTCTGCTCCGTG</td>
<td>NM_000346.3</td>
<td>178</td>
</tr>
<tr>
<td>Hypoxantine phosphoribosyl transferase 1 (HPRT1)</td>
<td>F: ATGAGAGGGACTGACAATGGTTCTTCT</td>
<td>R: TTGAGGACACAAGGGGCTCAATG</td>
<td>NM_000194.2</td>
<td>79</td>
</tr>
</tbody>
</table>

Table 3. Primers for RT-PCR.
8.3 Flow cytometry evaluation

To identify specific immunophenotype of articular chondrocytes from haemophilic patients in comparison with chondrocytes from Non-Haemophilic patients, flow cytometry analysis were performed. Flow cytometry is a technology that simultaneously measures and then analyses multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle’s relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence. A flow cytometer is made up of three main systems: fluidics, optics, and electronics (Fig. 21).

- The fluidics system transports particles in a stream to the laser beam for interrogation.
- The optics system consists of lasers to illuminate the particles in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors.
- The electronics system converts the detected light signals into electronic signals that can be processed by the computer.

![Flow Cytometry Diagram](image)

**Figure 21.** The basic components of a flow cytometer.

In the flow cytometer, particles are carried to the laser intercept in a fluid stream. Any suspended particle or cell from 0.2–150 µm in size is suitable for analysis. Cells from
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Solid tissue must be disaggregated before analysis. The portion of the fluid stream where particles are located is called the sample core. When particles pass through the laser intercept, they scatter laser light. Any fluorescent molecules present on the particle fluoresce. The scattered and fluorescent light is collected by appropriately positioned lenses. A combination of beam splitters and filters steers the scattered and fluorescent light to the appropriate detectors. The detectors produce electronic signals proportional to the optical signals striking them. List mode data are collected on each particle or event. The characteristics or parameters of each event are based on its light scattering and fluorescent properties (Fig. 22). The data are collected and stored in the computer. This data can be analysed to provide information about subpopulations within the sample.

**Figure 22.** Cytofluorimetric analysis of cells. Characteristics of each event are based on its light scattering and fluorescent properties.

Light scattering occurs when a particle deflects incident laser light. The extent to which this occurs depends on the physical properties of a particle, namely its size and internal complexity. Factors that affect light scattering are the cell's membrane, nucleus, and any granular material inside the cell. Cell shape and surface topography also contribute to the total light scatter.
Forward-scattered light (FSC) is proportional to cell-surface area or size. It is a measurement of mostly diffracted light and is detected just off the axis of the incident laser beam in the forward direction by a photodiode. FSC provides a suitable method of detecting particles greater than a given size independent of their fluorescence.

Side-scattered light (SSC) is proportional to cell granularity or internal complexity. SSC is a measurement of mostly refracted and reflected light that occurs at any interface within the cell where there is a change in refractive index. SSC is collected at approximately 90 degrees to the laser beam by a collection lens and then redirected by a beam splitter to the appropriate detector.

Correlated measurements of FSC and SSC can allow for differentiation of cell types in a heterogeneous cell population (Fig. 23).

![Figure 23](image)

**Figure 23.** Forward and side scatter data can be used to classify samples by size (FSC) and by internal complexity (SSC).

By using fluorochrome-conjugated antibodies, flow cytometry allows the identification of specific cell markers. Fluorochrome are fluorescent compound absorbs light energy over a range of wavelengths that is characteristic for that compound. This absorption of light causes an electron in the fluorescent compound to be raised to a higher energy level. The excited electron quickly decays to its ground state, emitting the excess energy as a photon of light. This transition of energy is called fluorescence. The range over which a fluorescent compound can be excited is termed its absorption spectrum. As more energy is consumed in absorption transitions than is emitted in fluorescent transitions, emitted wavelengths will be longer than those absorbed. The range of emitted wavelengths for a particular compound is termed its emission spectrum (Fig. 24).
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Figure 24. A typical fluorochrome absorption-emission spectral diagram is illustrated. Note the wavelength shift between excitation and emission.

The argon ion laser is commonly used in flow cytometry because the 488-nm light that it emits excites more than one fluorochrome. Various fluorochromes can be used, among these the most common are: fluorescein isothiocyanate (FITC); phycoerythrin (PE); peridinin chlorophyll protein (PerCP); allophycocyanin (APC) (Fig. 25).

Figure 25. Absorption (A) and emission (B) spectra of four common fluorochromes

When a fluorescent dye is conjugated to a monoclonal antibody, it can be used to identify a particular cell type based on the individual antigenic surface markers of the cell. In a mixed population of cells, different fluorochromes can be used to distinguish separate subpopulations. The staining pattern of each subpopulation, combined with FSC and SSC data, can be used to identify which cells are present in a sample and to count their relative percentages.
8.3.1 Immunophenotype characterization

Cells from Haemophilic and Non-Haemophilic patients (used as controls), at passage (P) 2 were first harvested by treatment with trypsin-EDTA, centrifuged at 1500 rpm for 5 min. and finally resuspended in PBS and 0.2% BSA. Hence, chondrocytes were stained with 2µl of phycoerythrin-conjugated antibodies, CD26, CD49c, CD44 and CD73; fluorescein isothiocyanate-conjugated antibodies, CD49e and CD151 and PerCP-Cyanine5-conjugated antibody, CD49f. Labeling occurred in 15 minutes at RT, in the dark. Isotypic antibodies served as controls. All the antibodies were purchased from BioLegend (San Diego, CA, USA), with the exception of CD151 and its isotype, purchased from Millipore (Billerica, MA, USA) (Tab. 4). At the end of the procedure, all samples were rinsed with PBS-BSA and centrifuged at 1500 rpm for 5 min. Analysis was performed on FACS Canto II (Becton Dickinson) resuspending samples in 200 µl of PBS-BSA. For each sample, at least 10,000 events were analysed. Results were expressed as percentage of positive cells compared to the isotype negative control.

<table>
<thead>
<tr>
<th>Antigen recognized</th>
<th>Isotype</th>
<th>Fluochrome</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD26 (peptidase IV)</td>
<td>IgG2a</td>
<td>PE</td>
<td>Ecto-enzyme</td>
</tr>
<tr>
<td>CD44</td>
<td>IgG1</td>
<td>PE</td>
<td>Adhesion molecule</td>
</tr>
<tr>
<td>CD49c (α3 integrin chain)</td>
<td>IgG1</td>
<td>PE</td>
<td>Adhesion molecule</td>
</tr>
<tr>
<td>CD49e (α5 integrin chain)</td>
<td>IgG2b</td>
<td>FITC</td>
<td>Adhesion molecule</td>
</tr>
<tr>
<td>CD49f (α6 integrin chain)</td>
<td>IgG2a</td>
<td>PerCP/Cy5.5</td>
<td>Adhesion molecule</td>
</tr>
<tr>
<td>CD73 (5’-nucleotidase)</td>
<td>IgG1</td>
<td>PE</td>
<td>Ecto-enzyme</td>
</tr>
<tr>
<td>CD151</td>
<td>IgG1</td>
<td>FITC</td>
<td>Tetraspanin</td>
</tr>
</tbody>
</table>

Table 4. Antibodies used for flow cytometry.
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9. Composite scaffold manufacture

Three different scaffold groups were investigated to analyse their ability in sustaining chondrocytes adhesion and proliferation: the PVA hydrogel alone, the PVA hydrogel combined with W’s J derived matrix; the PVA hydrogel combined with AC derived matrix.

Manufacture of scaffolds consists in a two-phase process: a) production of tissue-matrices; b) construction of the PVA-matrix composite scaffold.

9.1 Matrices manufacture

9.1.1 Matrices from umbilical cord

Ten fragments of umbilical cord of about 10 cm in length were obtained, with the mother’s consent, from full term caesarean deliveries and stored at -80 °C until use.

All samples were thawed at room temperature (RT) and rinsed four times in PBS containing 2% penicillin/streptomycin solution to remove contaminating red blood cells. After washing, the umbilical vessels were manually removed and the jelly was minced into small fragments that were all gathered in a 50 ml tube (BD Falcon). Fragments were then decellularized according to the detergent-enzymatic method by Meezan. (Meezan et al., 1975). Briefly, samples were soaked in: distilled water for 72 h at 4 °C, changing the aqueous solution every 2 h; 4% sodium deoxycholate for 4 h at RT; 2000 KU (Kunitz Units) DNase-I in 1 M NaCl for 2 h at RT.

The presence of cellular elements was verified through immunohistochemical staining: after each detergent-enzymatic cycle, 10 slides from each sample were mounted with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Burlingame, CA, USA) and analysed with a fluorescence microscope (Leica). As control, native jelly samples were stained.

Only after having ascertained the complete tissue decellularization, 1 g of tissue was put in a 50 ml tube (BD Falcon), soaked with 15 ml of 10% acetic acid solution (2.5 M) (Sigma-Aldrich) in deionized water (dH2O) and homogenized at 0 °C in a Ultra-Turrax homogenizer (Janke & Kunkel GmbH, Staufen, Germany, EU) 8 times/20 sec with intervals of 5 min. This stage was lead in ice bath. Thereafter, 400 ul of matrix solution were cast into each well of a 24-well cell culture plate (mould) and frozen at -20 °C before being lyophilized overnight using an under-vacuum evaporator (Speed Vac Concentrator Savant, Instruments Inc., Farmingdale, NJ, USA).
9.1.2 Matrices from articular cartilage tissue

Human articular cartilage tissue was obtained according to local Ethics Committee guidelines from 5 male donors who underwent total joint replacement surgery. Samples were stored at –80 °C until use. All samples were thawed at RT and rinsed four times in PBS containing 2% penicillin/streptomycin solution. After washing, tissue samples were minced into small fragments and treated as described previously for umbilical cord-derived ones. At the end of each cycle, the presence of cellular elements was verified through immunohistochemical staining with Vectashield mounting medium for fluorescence with DAPI; as control, native articular cartilage samples were used.

10. Quality assessment of ECM after decellularization treatment

10.1 Histologic evaluation

Once the absence of cellular elements was ascertained, specimens of each tissue underwent histological analysis. Wharton’s jelly and articular cartilage fragments, after being soaked in isopenthanol, were frozen in liquid nitrogen fumes and then were kept at –80 °C for 24 h before being ice-included and sliced in 7 µm serial slices using a cryomicrotome (Leica CM 1850 UV). These sections were fixed with acetone and stained with Masson trichromatic and Movat pentachromatic staining. Masson Trichrome Goldner with Light Green Kit (Bio-Optica) and Movat Pentachromic Staining Kit (Diapath, Bergamo, Italy, EU) were used respectively. As control, native articular cartilage and Wharton’s jelly samples were adopted.

Masson Trichrome Goldner is a recommended method for connective tissue. Four different stains are used: Weigert’s iron hematoxylin for nuclei, picric acid for erythrocytes, a mixture of acid dyes (acid fuchsin-”ponceau de xylidine”) for cytoplasm and light green for collagen. Briefly, sections were bring to distilled water and upon each one were put 6 drops of Weigert’s iron hematoxylin A solution (reagent A) and 6 drops of Weigert’s iron hematoxylin B solution (reagent B) which were left to act for 10 min. Without washing, the slides were drained and on the sections were put 10 drops of Picric acid alcoholic stable solution (reagent C) for 4 min. After a quick wash in distilled water, 10 drops of Ponceau acid fuchsin according to Masson (reagent D) were put for 4 min, followed by a
wash in distilled water. Then, 10 drops of phosphomolibdic acid solution (reagent E) were left for 10 min. Without washing, slides were drained and 10 drops of light green solution according to Goldner (solution F) were put on and left for 5 min. Finally, sections were washed in distilled water and mounted with resinous mounting medium (Bio-Mount HM). This method was useful to demonstrate nuclei and gametes (black); cytoplasm (red); collagen, mucus (green).

Movat pentachromatic stain is used to highlight collagen, muscular tissue, reticular fibres, mucines and fibrin. Eight different stains are used: alcian blue solution (reagent A), alkaline alcoholic solution (reagent B), hematoxylin alcoholic (reagent C1), solution iodure-iodide (reagent C2), ferric chloride (reagent D), sodium thiosulphate (reagent E), Briebrich scarlet – fuchsine acid (reagent F), phosphotungstic acid (reagent G), alcoholic safran (reagent H).

Briefly, sections were bring to distilled water and upon each one was put reagent A for 20 min. After washing with running water (5 min), reagent B was left for 60 min and then washed for 10 min. Reagent C was obtained mixing C1 and C2 and sections were stained with it for 15 min before washing with distilled water. Then, reagent D was put till elastic fibres become black and soon after it was washed at first with distilled water and subsequently with a 0.5% solution of acetic acid in distilled water. Afterwards, sections were stained with reagent E for 1 min which was than washed in running water (10 min) and then distilled water. Briebrich scarlet – fuchsine acid (reagent F) was put for 3 min, and washed with a 0.5% solution of acetic acid in distilled water. Soon after reagent G was put upon sections for 10 min, washed in a 0.5% solution of acetic acid in distilled water and then sections were dipped in absolute ethyl alcohol before adding reagent H for 15 min. Finally sections were dehydrated in absolute ethyl alcohol for 2 min and mounted with resinous mounting medium.

According to this method, nucleus and elastic fibres are black stained, collagen and reticular fibres appear yellow, mucines are blue, fibrinoid, fibrins are intense red coloured.

Mounted slides were observed using fluorescence microscope.

10.2 Protein quantitation assay of decellularized ECMs

Total ECM proteins were quantitated by bicinchoninic acid (BCA) method using the Pierce™ BCA Protein assay kit (Thermoscientific, Rockford, IL, USA).
The principle of this method is that protein can reduce Cu^{+2} to Cu^{+1} in an alkaline solution (the biuret reaction) and result in a purple colour formation by bicinchoninic acid which is formed by the chelation of two molecules of BCA with one cuprous ion (Fig. 26). The macromolecular structure of protein, the number of peptide bonds and the presence of four particular amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for colour formation with BCA. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000μg/mL). The BCA method is not a true end-point method; that is, the final colour continues to develop.

![Figure 26. The reaction of BCA with cuprous ion. Two molecules of BCA bind to each molecule of copper that had been reduced by a peptide-mediated biuret reaction](image)

Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as BSA. A series of dilutions of known concentration are prepared from the protein and assayed alongside the unknown(s) before the concentration of each unknown is determined based on the standard curve.

The analysis was performed on five different donor samples of W’s J and AC matrix homogenates, obtained as described previously. Acetic acid homogenates (1 ml) were centrifuged at 12,000 rpm for 5 min at 4 °C and protein pellets were dissolved in 1 ml of 1% sodium dodecyl sulphate (SDS). For the assay quantification, 8 dilutions of the stock standard BSA (2 mg/ml) and a blank solution were prepared. Subsequently, 25 μl of each dilution solution and the samples to be quantified were placed in 96-well plates and 200 μl of Working Reagent was added to each well. The Working Reagent solution was previously prepared by mixing 50 parts of Reagent A (sodium carbonate, sodium Bicarbonate reagent for the detention of BSA and sodium tartrate in 0.2 N NaOH), with
a part of reagent B (solution of copper sulphate to 4%). The plate was then incubated for 30 min at 37°C. The colorimetric reactions were analyzed at 562 nm using a Microplate autoreader EL 13 (BIO-TEK instruments Inc., Winooski, Vermont USA). The total protein amount was determined using a standard curve for BSA.

11. Preparation of PVA hydrogel composite scaffolds
An aqueous solution of 16 wt % PVA (Mw 146,000-186,000 Da, 99+% hydrolysed) was prepared by dissolving a pre-weighted quantity of PVA in deionized water and heating it for 48 hours at 90 °C, under stirring, until complete dissolution. The PVA solution was then slowly cooled down to RT. A quantity of 0.3 g of the PVA solution was cast into each well of a 24-well cell culture plate (mould) (BD Falcon, USA) and a thin matrix-layer, prepared as described in Paragraph 9.1, was then set down carefully upon each PVA solution just poured. A freeze-thaw treatment was used to physically cross-link the hydrogel and to embed the lyophilized matrix upon the hydrogel. Briefly, the coated plate was frozen at –20 °C and slowly thawed at –2.5 °C for 5 times. At the end of the freeze-thawing treatment, composite scaffolds were kept at –20 °C until use. As control, freeze-thawed PVA hydrogels without any coating were used.

11.1 Composite scaffold morphological characterization
Ox 1% PVA and Ox 1% PVA composite scaffolds morphology was investigated by SEM. Samples were fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 24 h and then dehydrated. Dehydration was performed by soaking each sample in increasing concentrate alcohols (30-95%) (1h/alcohol). After Critical Point Drying and gold sputtering, samples were observed by a scanning electron microscope (Stereoscan-205 S; Cambridge instruments, Pine Brook, NJ, USA) of CUGAS, Interdepartmental Service Centre at University of Padua.

12. Chondrocytes culture on scaffolds
Primary human chondrocytes from P2, isolated and cultured as previously described (Paragraphs 7.1 and 7.2), were used for seeding on scaffolds. PVA/W’s J and PVA/AC scaffolds were washed 4 times of 2 h each, in PBS solution containing 2% penicillin/streptomycin. The purpose was to disinfect supports and to remove any residual of acetic acid solution used to prepare matrices. Then they were incubated at 37 °C in basal medium over-night.
At the time of cell seeding, monolayer cultures of AC from Haemophilic and Non-Haemophilic patients were detached by treatment with trypsin-EDTA. Chondrocytes suspensions, after deactivation of trypsin with basal medium DMEM/F12 (2:1) added with 10% FBS, were centrifuged at 1500 rpm for 5 min. Hence, cell pellets were resuspended in complete medium. The resulting cell suspensions were divided into 4 tubes, as the cell seeding was carried out in 4 times interspersed with a break of 15 min each during which supports were maintained at 37 °C in cell culture incubator. At each time, scaffolds (previously placed in each well of a 24-multiwell) were seeded with 100 µl of cell suspension. After seeding, each sample was added in complete medium, to a final volume of 1000 µl. Scaffolds were seeded with chondrocytes (20,000 cells/cm²) and incubated at 37°C in a 5% CO₂ humidified atmosphere.

13. Evaluation of cell adhesion and proliferation on composite scaffolds

13.1 Scanning Electron Microscopy
After 7 and 14 days of Haemophilic and Non-Haemophilic articular chondrocytes growth on Ox 1% PVA; W’s J/Ox 1% PVA; AC/Ox 1% PVA scaffolds, cell adhesion was evaluated by SEM. Culture medium was removed and scaffolds on wells were washed gently with PBS. After taking out the buffer, samples were fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 24 h and then dehydrated. Dehydration was performed by immersion in increasing concentrated alcohols (30-95%) (1h min/alcohol) (1h/alcohol). After Critical Point Drying and gold sputtering, samples were observed by a scanning electron microscope (Stereoscan-205 S; Cambridge instruments, Pine Brook, NJ, USA) of CUGAS, Interdepartmental Service Centre at University of Padua.

13.2 Thiazolyl Blue Tetrazolium Blue assay
Proliferative activity of cells seeded on composite scaffolds and, in parallel, matrices potential in sustaining chondrocytes proliferation, was measured by Thiazolyl Blue Tetrazolium Blue (MTT).
After 7 and 14 days from seeding, Haemophilic and Non-Haemophilic articular chondrocytes proliferative activity on Ox 1% PVA; W’s J/Ox 1% PVA; AC/Ox 1% PVA
scaffolds, was measured. Cells grown on wells of a 24-multiwell plate were used as control.

Once culture medium was removed, scaffolds and cells on wells were washed gently with PBS. After taking out the buffer, hydrogels were moved to clean 24 well plates using a tweeze and then samples were treated with 500 µl of MTT (0.5 mg/ml) in basal medium for 4 h at 37 °C in humidified atmosphere containing 5% CO₂. Afterwards, MTT solution was removed and formazan precipitates were dissolved in 300 µl of 2-propanol acid (0.04 M HCl in 2-propanol). Dissolution occurred placing the 24 well plates on a tilting shaker (Asal, Milano, Italy) for 20 min at RT. Dissolved formazan precipitates were collected carefully and moved to a 96 well plates. Optical density was measured at 570 nm, using a Microplate autoreader EL 13 (BIO-TEK instruments Inc., Winooski, Vermont USA). Results were expressed as number of cells grown on seeded surface.
RESULTS

1. Oxidized polymers characterization

1.1 Carbonyl content quantification

Oxidized PVA carbonyl content was determined by DNPH assay. 2,4-dinitrophenyl hydrazine is typically used to determine carbonyl groups of aldehydes and ketones; reacting with carbonyls, it leads to the formation of hydrazone derivatives. Fig. 27 represents GPC chromatograms of PVA carbonyls derivatisation with DNPH: as shown, with the increasing of oxidation degree, a higher hydrazone amount was detected. Average carbonyl content was equal to 5 µmol/mg and 11.4 µmol/mg in 1% Ox and 2% Ox PVA, respectively. PVA was considered as negative control.

**Figure 27.** GPC chromatograms of PVA, 1% Ox and 2% Ox PVA after DNPH treatment. Peaks represent size exclusion configurations of high molecular weight phenylhydrazone derivatives eluted from a PD-10 column and monitored at 375 nm. Free DNPH profile is not shown. Mobile phase: PBS/Methanol 70/30 (v/v); flow rate: 1 ml/min. Data are representative of three independent experiments.

1.2 Lysozyme covalent binding

Sodium cyanoborohydride is a reducing agent that was used to establish a stable covalent interaction between carbonyls groups of oxidized PVA and amino groups of lysozyme, through reduction of intermediate Schiff’s bases, otherwise not visible. By GPC, we obtained chromatograms of 1% Ox (Fig. 28 A) and 2% Ox (Fig. 28 B) PVA incubated with lysozyme at different end-points (4, 24, 50, 120 h). Since 4 h of incubation, the
Results

typical band absorbing at 280 nm is recognizable. After 24 h, the lysozyme bounded to polymers reached about 22% (1% Ox PVA) and 45% (2% Ox PVA) of the initial total amount. Bounded lysozyme increased progressively up to 120 h.

Figure 28. GPC evaluation of covalent interaction between lysozyme and 1% Ox (A) and 2% Ox (B) PVA. Chromatograms show size exclusion configurations of bound and free lysozyme eluted from Superose 6 10/300 GL and monitored at 280 nm. Mobile phase: PBS; flow rate: 0.5 ml/min. Data are representative of three independent experiments.

2. Scaffold characterization

2.1 Morphological analysis by Scanning Electron Microscopy

SEM micrographs were obtained to characterize the superficial morphology of the scaffolds, investigating contingent differences ascribable to oxidation. According to this analysis, PVA scaffolds showed a smooth and regular surface (Fig. 29 A). Conversely, 1% and 2% oxidized PVA supports had a different morphology, characterized by a rough surface (Fig. 29 B,C).

Figure 29. SEM investigation of PVA (A), 1% Ox PVA (B) and 2% Ox PVA (C) scaffold surface morphology. Micrographs show that structural remodeling occurs with increasing oxidation degree. Magnification x8000.
2.2 Mechanical characterization.

The instrument expressed data stress-strain curves in N versus mm. However, it is more useful to convert applied force in MPa, dividing the force applied by the area of the resistant section of each sample, and expressing the deformation in %. The result is a stress-strain graph in which the slope of the curve in the elastic range directly indicates the elastic modulus of the material (Young's modulus). The slope of the curve is calculated by linear interpolation.

The stress vs. strain curves of PVA (Fig. 30 A,D) Ox 1% PVA (Fig. 30 B,E) and Ox 2% PVA (Fig. 30 C,F) were superimposable and no hysteresis was observed, suggesting high resilience for all samples. Moreover, increasing the oxidation degree, polymer elastic modulus decreased, showing a reduction in stiffness (Tab. 5). No significant difference (p < 0.05) was detected in the values of the elastic modulus between dried and swelled samples.

Conversely, considering cartilage tissue, the stress vs. strain curves were no longer superimposable showing a clear hysteresis (e.g., energy los): some of the work done during the loading phase was dissipated and could be not recovered upon uploading (Fig. 31). As shown in Tab. 5 higher values of the Young’s modulus are ascribable to cartilage tissue, in comparison to hydrogels.

Figure 30. Stress-strain curves of neat PVA samples (A, D), ox 1% (B, E) and ox 2% (C, F) analyzed dried (A, B, C) or after immersion in solution saline (D, E, F).
Results

![Stress-strain curves of cartilage samples](image)

**Figure 31.** Stress-strain curves of cartilage samples

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<tr>
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Table 5. Values of the elastic modulus (MPa) of PVA, Ox 1% and Ox 2% PVA samples

### 2.3 Dynamic Light Scattering.

The dimension and the homogeneity of PVA, 1% Ox and 2% Ox PVA molecules were investigated in water solutions by DLS analysis. Size distribution of the polymer samples was reported as percent volume (Fig. 32). Mean hydrodynamic size values were of about 28 nm for PVA, 23 nm for 1% Ox PVA and 16 nm for 2% Ox PVA. The shift between the maximum of the three curves represents the decrease of particles mean size; the areas below the curves represent the extent of dimensional dispersion. Along with oxidation, the hydrodynamic radius of particles decreased. It is also noteworthy that 2% Ox PVA showed a broader size distribution peak in comparison with PVA and 1% Ox PVA, suggesting its higher degree of polydispersity.
Results

Figure 32. DLS analysis of PVA and oxidized PVA molecules: size distribution in percent volume. Along with oxidation degree, hydrodynamic radius of particles decreased. Note the broader distribution peak of 2% Ox PVA, ascribable to higher polydispersity. Data are representative of three independent experiments.

2.4 Differential Scanning Calorimetry.

The DSC analysis allowed to measure melting temperatures, enthalpies, and crystallinity degree of PVA gels. Thermograms are shown in Fig. 33, while results are summarized in Tab. 6. Samples were characterized by an endothermic broad band in the range between 80 and 160 °C, which can be ascribed to the evaporation of residual water. Following, a sharp peak can be observed, related to the melting of PVA, whose intensity decreases as oxidation rate increases. Oxidation seems to affect the PVA melting temperature and crystallinity degree, which show to lower as carbonyl content arises (Tab. 6).

Figure 33. DSC thermograms of PVA and oxidized PVA samples.
Table 6. Thermal properties of PVA gels. Hydrogel $T_m$ and $\Delta H_m$ slightly decrease along with oxidation degree, indicating a gradual loss of crystallinity, as shown by $X_c$, calculated values.

<table>
<thead>
<tr>
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<th>$T_m$ [°C]</th>
<th>$\Delta H_m$ [J/g]</th>
<th>$X_c$ [%]</th>
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<tr>
<td>PVA</td>
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</tr>
<tr>
<td>1% Ox PVA</td>
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<tr>
<td>2% Ox PVA</td>
<td>214.6</td>
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</tr>
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</table>

2.5 Swelling test.

Both 1% and 2% Ox PVA hydrogels showed rapid swelling ratio of 60% and 110% respectively in 24 h. Swelling then decelerated until an equilibrium was reached at approximately 70% (1% Ox PVA) and 40% (2% Ox PVA) mass increase after 288 h, remaining steady for the duration of the experiment. Conversely, PVA hydrogels displayed different swelling ratio, increasing in mass by about 20% within 24 h. Swelling continued, albeit at a slower rate until equilibrium was reached after approximately 288 hours (Fig. 34).

3. Protein release potential of PVA and oxidized PVA hydrogels.

BSA and TGF-β1 release, at different end-points, was investigated to assess the potential of scaffolds for controlled drug delivery. Results are shown in Fig. 35 (BSA) and Fig. 36.
Results

(TGF-β1): both oxidized hydrogels sustain protein delivery better than PVA; furthermore, a higher protein release was monitored for 2% Ox than 1% Ox PVA scaffolds. Considering BSA (Fig. 35), an initial exponential release was clear (within 24 h); afterwards, according to UV spectra, protein release was sustained up to 144 h.

Figure 35. BSA release profiles of PVA and oxidized PVA scaffolds. Protein release is higher for 2% Ox PVA and 1% Ox PVA compared to PVA itself. Data are representative of three independent experiments.

TGF-β1 release was evaluated measuring fluorescence intensity of loaded scaffolds washing solutions; the fractions after 72 hour-incubation were pooled and their fluorescence measured. In Fig. 36, emission spectra of the solutions after incubation with the three hydrogels are reported: the peak at nearly 300 nm is due to tyrosine, while the broad band on the right is ascribable to tryptophan. Fluorometric analysis highlighted that released TGF-β1 increased along with scaffold oxidation rate.
As TGF-β1 shows a promising potential in promoting chondrocyte proliferation (Leipzig et al., 2006), a MTT assay was performed on ACs co-cultured with loaded scaffolds. A significant increase of proliferative activity assessed effective loading and release. Seventy-two hours from seeding, cells co-cultured with 1% Ox and 2% Ox PVA showed a higher proliferation rate (p<0.01) compared to chondrocytes/PVA co-cultures. The same trend was recorded also at 7 d from seeding (Fig. 37). These results, beyond highlighting the effectiveness of oxidized PVA in loading and releasing proteins, allowed us to rule out any modified PVA cytotoxicity.

Figure 36. TGF-β1 release profiles of PVA and oxidized PVA scaffolds. Spectra are of washing solutions after 72 hours incubation.

Figure 37. Chondrocyte proliferation after 72 h and 7 d of co-culture with TGF-β1 loaded PVA, 1% Ox and 2% Ox PVA scaffolds (**: p<0.01 with respect to Ctrl; ▲▲: p<0.01 with respect to PVA).
4. **In vivo scaffold degradation.**

No implanted mice were euthanized or died prematurely due to scaffold related complications. Careful surveillance of the mice in the weeks after surgery did not show systemic signs of infection, neither local signs of rejection or inflammation. At the end of the 12-week period of *in vivo* implantation, preliminary evidences were gathered (Fig. 38). Size and integrity of explanted oxidized scaffolds were visually compromised (Fig. 38 F,I) compared to pre-implant supports (Fig. 38 D,G). In particular, degradation rate increased along with PVA oxidation degree. Conversely, PVA scaffolds did not show remarkable biodegradation: gross appearance after explant was similar to pre-implant one (Fig. 38 A,C).

![Figure 38](image)

**Figure 38.** Gross appearance of pre-implant (A, D, G) and explanted (C, F, I) PVA, 1% Ox and 2% Ox PVA scaffolds. In (B), (E), (H) implants are shown before removal (12-weeks after surgery).

SEM analysis of explants highlighted surface differences between PVA, 1% Ox and 2% Ox PVA scaffolds (Fig. 39 A,B). In particular, both PVA and 1% Ox PVA explants showed an identifiable polymeric structure, even though their surface appeared deteriorated in comparison to pre-implant controls. Conversely, 2% Ox PVA surface was no longer recognizable: SEM micrographs reveal the presence of disorganized collagen
fibers (Fig. 39 C) which should be attributed to scaffold reabsorption and substitution by new connective components.

![SEM investigation of PVA (a), 1% Ox PVA (b) and 2% Ox PVA (c) surface morphology at 12-weeks. Compared to pre-implantation controls (A,B,C). PVA (a) and 1% Ox PVA (b) explanted scaffolds show typical polymeric surface features. Otherwise, 2% Ox PVA explants (c) were substituted by collagen fibers. Magnification x8000.](image)

**Figure 39.** SEM investigation of PVA (a), 1% Ox PVA (b) and 2% Ox PVA (c) surface morphology at 12-weeks. Compared to pre-implantation controls (A,B,C). PVA (a) and 1% Ox PVA (b) explanted scaffolds show typical polymeric surface features. Otherwise, 2% Ox PVA explants (c) were substituted by collagen fibers. Magnification x8000.

### 4.1 Histological and immunohistochemical analysis.

OCT-embedded samples proved more useful for evaluation of PVA characteristics, as PVA underwent volume changes and modifications of its characteristics along the various processing phases of formalin-fixed samples. Also in frozen samples, PVA sections were very difficult to be obtained without ruptures, dislocations or folds. However, apart from these artefactual modifications, PVA texture was intact, without areas of degradation and reabsorption. Conversely, 1% Ox PVA showed partial degradation and 2% Ox PVA was nearly not visible, in spite of exhaustive serial sectioning of samples, due to almost complete degradation. On biomaterials, no cellular adhesion occurred. Furthermore, while PVA-hydrogel surfaces remained smooth and transparent, oxidized PVA sections were amorphous and opaque (Fig. 40).
Figure 40. Hematoxylin and Eosin staining of PVA scaffolds explanted after 12 weeks of in vivo subcutis implantation. Note the absence of degradation and cell infiltration of PVA scaffold, ruptures and folding being ascribable to post-sampling artefactual changes (A, D). Conversely, 1% (B, E) and 2% (C, F) Ox PVA appeared much more compromised after exposure to biological fluids, being the second one almost no more visible next to the implant site. Magnification: (A, B) x12.5; (C) x25; (D, E, F) x100.

Paraffin-embedded samples were mainly used for specific analysis of the tissues surrounding the implanted materials (Fig. 41). In all the samples, both from PVA and oxidized PVAs, severe inflammatory reactions were not present, but only slight infiltration of the connective tissue immediately superficial to the implanted material. Implant of 2% Ox PVA caused a slightly higher infiltration of the surrounding connective tissues. As it regards immunological characterization, most infiltrating cells were positive for CD3 or F4/80, consistently with lymphomonocytic nature of the infiltration. Sections incubated without primary antibodies showed no immunoreactivity, confirming the specificity of the immunostaining.
Figure 41. Histological and immunohistochemical analysis of PVA scaffolds explanted after three months of in vivo subcutis implantation. Note more numerous lymphomonocytic cells after implant of 2% oxidized PVA. Magnification: (A, D, G) x100; (B, C, E, F, H, I) x200.

5. Evaluation of acellular ECMs

5.1 Immunofluorescence and histological analysis

Umbilical cord W’s J and AC were completely decellularized with 3 and 7 detergent-enzymatic cycles respectively; DAPI staining was used to assure decellularization degree after each cycle. Cartilage tissue resulted more resistant to cell removal compared to W’s J; already one cycle induced an appreciable disappearance of cellular elements in the umbilical cord derived matrix. The histological sections of native and decellularized ECMs stained with DAPI are presented in Fig. 42 A,G and Fig. 42 B,H, respectively. ECMs morphology before and after the decellularization treatment was evaluated by means of Masson trichromic staining, which demonstrated a similar protein content of W’s J and AC samples. In particular, both matrices mainly consist of collagen fibers and mucus, as shown by the green staining of native (Fig. 42 C,I) and decellularized (Fig. 42 D,L) tissues.
Movat pentachromic staining allowed us to detect red fibrin and yellow collagen components in native W’s J (Fig. 42 E). Moreover, in native AC (Fig. 42 M) and acellular ECMs (Fig. 42 F,N), blue and yellow colors indicate the presence of mucins and collagen fibers respectively.

**Figure 42.** Histological evaluation of decellularized ECMs (B, D, F, H, L, N) versus native tissues (A, C, E, G, I, M). Magnification: x100.

### 5.2 Protein quantitation of decellularized ECMs

After ECM decellularization treatment, BCA assay was performed to control contingent sample-to-sample variations in total protein amount. Matrix homogenates of W’s J and AC, gained from different donors, were compared. Total protein content for W’s J and AC ECMs resulted of 29.2 and 24.8 mg per gram of tissue, respectively (mean values). No statistically significant difference was found between samples of each study group (Fig. 43).
6. Characterization of composite scaffolds

SEM micrographs were obtained to characterize the superficial morphology of scaffolds before chondrocyte seeding (Fig. 44). One percent oxidized PVA scaffolds showed a smooth surface (Fig. 44 A). Conversely, 1% Ox PVA/W’s J and 1% Ox PVA/AC scaffolds have a different surface morphology: the first is quite regular, smooth and with convolution-like structures (Fig. 44 B); the second has a more irregular spongy appearance (Fig. 44 C).

7. Characterization of chondrocyte monolayer cultures

7.1 Morphological analysis

Freshly isolated chondrocytes from AC of Non-Haemophilic (A) and Haemophilic (B) patients were small and round and they initially grown as a suspension culture. Six days
after AC enzymatic digestion, adherent cells were observed. At a subconfluence state, both chondrocytes populations showed the classic round or polygonal shape with small membrane extroflections (Fig. 45 a,b). Chondrocytes were expanded in culture up to passage 4, hereafter their proliferation rate started to decrease and their morphology changed to elongated fibroblast-like phenotype.

Figure 45. Gross appearance of human AC from Non Haemophilic (A) and Haemophilic (B) patients. Analysis by optical microscopy of human AC chondrocytes from Non-Haemophilic (a) and Haemophilic (b) patients. Images are referred to cells at a subconfluent state P2. Magnification: x100.

7.2 Expression of specific mRNA
Before seeding on 3D scaffolds, isolated human chondrocytes from Non-Haemophilic and Haemophilic patients were characterized for the expression of specific cartilage markers. Gene expression analysis by RT-PCR showed that both AC-derived cell populations are active in the transcription of typical chondrocyte mRNAs: collagen type II, and IX, cartilage oligomeric matrix protein, aggrecan, SOX9 (Fig. 46 A,B). The housekeeping gene HPRT1 was used as a control.
7.3 Immunophenotype

To define the immunophenotype of AC chondrocytes of Haemophilic and Non-Haemophilic patients, cell surface molecules expressed on cells were evaluated by flow cytometry. Chondrocytes of were cultured for 2 weeks in monolayer and P2 was investigated. The analysed cell surface molecules were classified into different categories according to their function: adhesion molecules (CD44; CD49c; CD49e; CD49f), receptors (CD151), and other surface molecules as ecto-enzyme molecules (CD26; CD73). Flow-cytometry showed that both subcultures consisted of CD44+/CD49c+/CD49e+/CD151+/CD73+/CD49f+/CD26- cells (Fig. 47).
Figure 47. Immunophenotype evaluation by flow cytometry of AC chondrocytes from Non-Haemophilic (A) and Haemophilic patients (B). Data are expressed as percentage of positive cells (blue and red profile respectively) compared to isotypic control (black profile).

8. Chondrocytes growth on 3D scaffolds
Haemophilic and Non-Haemophilic chondrocyte’s distribution and proliferative activity on scaffolds was evaluated by SEM and MTT assay.

8.1 Scanning Electron Microscopy analysis
According to SEM micrographs, at day 7 and 14 from seeding no cell adhesion and proliferation was visible on 1% Ox PVA scaffolds. On the contrary, Non-Haemophilic and Haemophilic chondrocytes were visible both on 1% Ox PVA/W’s J and on 1% Ox PVA/AC scaffolds at the same end-points.
In particular, at day 7 from seeding, both chondrocytes populations appeared well distributed on 1% Ox PVA/AC (Fig. 48 B,D) and 1% Ox PVA/W’s J (Fig. 48 A,C) scaffolds. At day 14, both chondrocytes populations extensively colonised scaffold surfaces, forming a homogeneous monolayer (Fig. 48 F,H,E,G).
8.2 Thiazoly Blue Tetrazolium Blue assay

According to MTT assay, 1% Ox PVA did not sustain cell adhesion and proliferation (not shown). Seven days from seeding, colonization of 1% Ox PVA/W’s J and 1% Ox
PVA/AC scaffolds occurred, even though such differences were observed. Considering matrices attitude in sustaining chondrocytes proliferation, exists a difference between W’s J and AC. In fact, Non-Haemophilic chondrocytes proliferate higher on AC matrix than on W’s J matrix (p≤0.05). Moreover, considering cell populations, Non-Haemophilic chondrocytes have a substantial growth in comparison with Haemophilic chondrocytes on AC matrix (p≤0.05). At day 14 from seeding any difference in proliferation rate was identified neither between matrices, nor between cells. Non-Haemophilic and Haemophilic chondrocytes growth on tissue culture-treated polystyrene plates of was considered as internal proliferation control (Ctrl) (Fig. 49).

Figure 49. Cell proliferation following seeding on 1% Ox PVA/W’s J and 1% Ox PVA/AC scaffolds (*p≤0.05: 1% Ox PVA/W’s J versus 1% Ox PVA/AC scaffold at 7 days from seeding; ▲p≤0.05: Non-Haemophilic chondrocytes versus Haemophilic chondrocytes at 7 days from seeding).
Articular hyaline cartilage is a soft tissue, which sustains the pressure between the hard ends of bones, and it is subjected to particularly complex loads affecting its development and maintenance in the body (Ahn et al., 2014; Jung et al., 2008). Because of its limited self-healing capacity, as it is an avascular and aneural tissue, even minor cartilage defects lead to mechanical joint instability and progressive damage (Jung et al., 2008; Demoor et al., 2014). In addition to idiopathic OA, some disorders may cause arthropathy; in such cases, OA is referred to as secondary.

Haemophilic arthropathy is a real burden for patients with haemophilia due to increased pain as well as decreased mobility and quality of life. Preventing bleeding through prophylaxis enables to avoid or reduce the clinical impact of MSK impairment from haemophilic arthropathy and the related consequences in psycho-social development and quality of life of these patients (Coppola et al., 2009). However, despite several advantages, there are some concerns about this strategy. These are mainly related to the high costs but also to the patient’s compliance to therapy (Tagariello et al., 2014) which frequently declines (Petrini et Seuser, 2009). Exposure to minimal amounts of blood or minimal number of bleedings are sufficient to result in irreversible cartilage damage (Roosendaal et al., 2008).

Cartilage damage is difficult to treat. Until now, considering Non-Haemophilic patients, many approaches have been investigated: arthroscopic repair procedures, marrow stimulation, osteochondral transfer, autologous chondrocytes transplantation (Farr et al., 2011), but average long-term results are unsatisfactory. A general drawback of these therapeutic strategies is that the newly formed tissue lacks the structural organization of cartilage; it has inferior mechanical properties compared to native tissue, and it is therefore prone to failure (Jung et al., 2008; Kock et al., 2012). Hence, the goal is to produce a repair tissue that has the same functional and mechanical properties of hyaline AC (Redman et al., 2005).

Considering haemophilic patients, as aforementioned, current strategies can only provide for pain relief, slowing the progression of damage. Joint replacement surgery is often necessary even though, sometimes, it raises several issues such as perioperative and
Discussion

postoperative bleeding, infection and heterotopic bone formation leading to disruption of physiotherapy, limp and limitation of walking distance (Asencio et al., 2014).

To favor the recovery of focal joint damage by promoting the formation of new cartilage tissue could be an interesting approach even in haemophilic patients. In particular, it could be a smart strategy in the perspective of slowing arthroplasty which in haemophilic patients is performed at younger age than osteoarthritics (Raffini et Manno 2007).

Cartilage restoration represents a challenge of MSK TE, despite that, the use of matrix scaffolds has paved the way for the use of functional tissue substitutes in the treatment of cartilage defects (Demoor et al., 2014)

Biomaterials play an important role in most TE strategies: their selection constitutes a key-point for the success of tissue defects treatment following diseases or trauma (Armentano et al., 2010). A wide range of natural and synthetic materials has been investigated as scaffolding. Amongst synthetic biomaterials, hydrogels are promising biomaterials for TE (Toh et Loh 2014) being widely studied for their potential use in soft tissue engineering (Gkioni et al., 2010) as space filling agents, vehicles for bioactive molecules, and three-dimensional structures for cell proliferation and specific induction (Drury et Mooney 2003). Among synthetic polymers, PVA hydrogels stand out: Food and Drug Administration (FDA) and Conformité Européenne (CE) already approved them for clinical use in humans, due to their excellent biocompatibility and safety (Jensen et al., 2011; Ino et al., 2013). They offer unique possibilities to build up engineered substitutes, which closely match human tissues for their elasticity and mechanical characteristics, water content, and accessibility to solutes (Alves et al., 2011). In particular, physically cross-linked PVA hydrogels became attractive to the field of TE for repairing and regenerating vessels (Conconi et al., 2014), heart valves (Jiang et al., 2004), cornea (Jiang et al., 2014) and cartilage (Gu et al., 1998; Jiang et al., 2011).

Despite PVA has an extensive history of biomedical applications, yet its current form suffers from significant shortcomings: physical cross-linking leads to stronger polymer-polymer chain interactions, loss of biodegradation rate and less controlled drug release (Fejerskov et al., 2013; Martinez et al., 2012). Moreover, when compared to other biocompatible polymers, PVA exhibits scant synthetic opportunities: beyond a limited choice of solvents, hydroxyl groups appear to be significantly disadvantaged in comparison with classic conjugation sites (Alves et al., 2011 Jensen et al., 2011). Hence, re-engineering of PVA to improve degradation and control drug release appears highly rewarding (Fejerskov et al., 2013). As reported in literature, PVA chemical modifications
can be attributed either to direct coupling or activation with 1,1-carbonyldiimidazole or 4-nitrophenylchloroformate of polymer hydroxyl groups. Both of them aim at introducing more convenient sites or functional groups for conjugation, chain extension, and/or substrate immobilization (Alves et al., 2011). Such groups include meth(acrylates) (Martens et al., 2002), thiol groups (Dicharry et al., 2006; Totani et al., 2008 Ossipov et al., 2008), carboxylic groups (Ruiz et al., 2001), halides (Baudrion et al., 1998), aminooxy (Ossipov et al., 2008), aldehyde (Ossipov et al., 2007), hydrazide (Ossipov et al., 2007). The present study reports the development and the characterization of new chemically oxidized PVA for biodegradable cross-linked scaffolds manufacture, able to vehicle proteins and promote tissue regeneration.

To our knowledge, this is the first study that considers oxidation of PVA through chemical agents in order to introduce more useful carbonyl groups in the polymer backbone. This should make PVA more attractive for TE approaches, implementing biomaterial potential. To confirm that oxidation reaction occurred, carbonyl groups were quantified by investigating their reaction with DNPH (Wehr et Levine, 2013). As expected, the amount of phenylhydrazone derivatives increased proportionally with carbonyl content, according to oxidation degree. Beyond chemical characterization, covalent interaction with lysozyme allowed us to predict the ability of oxidized PVA to form covalent binding even with RGD sequences or oligopeptides, useful to functionalize scaffold surface.

Following chemical characterization, scaffolds were manufactured. Supports were obtained after pouring into moulds polymer solutions that appeared less viscous as oxidation degree increased. Aqueous PVA, 1% and 2% PVA solutions were then transformed into hydrogels via crystallite formation by freezing-thawing cycles and without any chemical cross-linkers, that may lead to toxicity (Kim et al., 2015). Physical cross-linked PVA hydrogels show a rubbery elastic behaviour, non-toxicity, and biocompatibility. All this makes them more attractive than other synthetic polymers from a biomimetic perspective (Gupta et al., 2011). After cross-linking, oxidized PVA scaffolds appeared more and more transparent as carbonyl content increased. This can be appreciated observing pre-implant scaffolds in Fig. 38 (A,D,G). Moreover, as showed by SEM micrographs, oxidization process seemed to influence the resulting cross-linked hydrogel surface, appearing 1% and 2% Ox PVA less smooth and regular compared to neat PVA.

In TE, biomaterial selection and scaffold design are strictly dependent on the expected outcome. The choice of polymer is crucial in this regard, as it should be specifically
tailored for the target tissue to be regenerated or the therapeutic function to be achieved. Physical parameters (such as cross-linking density, mechanical strength and crystallinity) can surely affect polymer behavior in mimic biological tissues. Our study demonstrated that polyvinyl alcohol hydrogels prepared via a freeze-thaw method can be chemically modified in order to adjust their properties depending on specific TE applications.

As far as mechanical properties, PVA possesses better strength and stiffness than its chemically oxidized counterparts. This is why hydrogel stiffness results from the available hydroxyl groups involved in intra- and inter-molecular hydrogen binding (Gupta et al., 2011): so, altering the number of cross-linkable groups per chain, hydrogel mechanics changes. It is then reasonable to plan a tissue regenerative strategy based on the modulation of the mechanical response of PVA for the replacement of more or less resistant soft tissues, such as cartilage, tendons, gut, nerves and vessels.

Polymeric micelles are supramolecular assemblies of tens of nanometers in diameter, which can mimic naturally occurring biological transport systems such as lipoproteins and viruses. Recently, polymeric micelles as drug carriers have drawn increasing research interests, due to their various advantages in drug delivery applications (Shuai et al., 2004). In this regard, the study of the size and surface properties of polymeric micelles have crucial importance in achieving modulated drug delivery with remarkable efficacy. Because of its hydrophilic nature, PVA can form polymeric micelles in an aqueous milieu. The size and size distribution of particles obtained from 1% and 2% oxidized polymer solutions were estimated by DLS, in comparison with PVA itself. As showed by our data, 2% oxidation determines reduction in particles hydrodynamic radius and higher polydispersity. Considering that, DLS study allowed us to define the possibility to modulate PVA micelles characteristics with the aim to fit polymer drug-delivery capacity according to specific needs.

The hydrogen bonding between hydroxyl groups of PVA and water governs also the crystallinity of the bulk PVA material (Nagura et al., 1989): DSC analysis highlighted that oxidation treatment can influence even polymer thermal properties, such as melting temperature, enthalpy, and crystallinity. In particular, PVA itself was found to be more crystalline than its oxidized counterparts and, as such, it is expected to withstand degradation for a longer period since the crystalline regions are more resistant to hydrolytic attack than the amorphous regions (Santos et al., 1999). This means that thermal property study allowed us to predict a higher biodegradation rate for 1% and 2% Ox PVA in view of in vivo implant.
In addition to their promising biocompatibility characteristics, PVA hydrogels are particularly desirable in the biomedical field due to their sensitivity in the physiological or biological environment where they are used. In recent years, much research has focused on the development and analysis of environmentally responsive hydrogels that can exhibit swelling changes due to modification of intrinsic properties or external conditions (Peppas et al., 2000). When a biopolymer network is in contact with an aqueous solution or a biological fluid, the network starts to swell due to the thermodynamic compatibility of the polymer chains and water. The swelling force is counterbalanced by the retractive force induced by the cross-links of the network. Swelling equilibrium is reached when these two forces are equal. In this study, the maximum swelling index was rapidly reached by all investigated hydrogels by 24 h of incubation in PBS, with a major mass increase recorded by 2% Ox PVA; however, also 1% Ox PVA showed a significant PBS uptake. After 12 days, both 1% and 2% Ox PVA reached equilibrium, showing from then a stable swelling behaviour. By taking up large quantities of water solution, hydrophilic polymer as PVA can increase their degradation rates; considering that, the enhancement of PBS uptake capacity following oxidation treatment may ameliorate PVA biodegradation properties. On the other hand, the uptake of water is especially important in the area of drug delivery. For PVA hydrogels, gaining substantial swelling ability is the decisive parameter for controlling the release of drugs.

The overall analysis of oxidized PVA physico-chemical characterization clearly underlines a direct relationship between mechanical response and thermally-derived properties (i.e., crystallinity). In particular, we could speculate that modifying polymer crystallinity also affects swelling and degradation behavior, clearly showing that the proper modulation of chemical oxidation allows to tailor scaffold characteristics according to needs.

Synthetic polymers are the most widely used materials in TE as GF delivery carriers (Lee et Shin, 2007). Such examples concern polyethylene glycol (PEG) (Leslie-Barbick et al., 2009; Yang et al., 2013), polycaprolactone (PCL) (Cipitria et al., 2013; Kim et al., 2014), poly(DL-lactic acid) (PLA) (Yang et al., 2004; Kanczler et al., 2008), poly(d,l-lactide-co-glycolide) (PLGA) (Kaigler et al., 2006; Jeon et al., 2007; Jung et al., 2012; Rui et al., 2012) polyurethane (PUR) (Hafeman et al., 2008; Li et al., 2009).

The advent of protein therapeutic enhanced the need of controlled delivery systems with improved pharmacokinetic and pharmacodynamic properties (Censi et al., 2012). As demonstrated, the introduction of carbonyls on PVA backbone supported protein-loading...
ability of scaffolds. This should be ascribable to a possible Schiff-base interaction between the amino group of proteins and carbonyls of oxidized PVA and/or to the higher swelling ratio.

GFs are protein molecules specific for intercellular and cell-ECM signalling. Bolus administration of GFs would not be effective in tissue healing, since they are readily enzymatically digested or deactivated. Moreover, prolonged exposition of the bioactive molecules is necessary to support tissue regeneration which normally occurs in long time frames. Integrating controlled release strategies within hydrogels may lead to novel delivery platform able to control and guide tissue regeneration (Biondi et al., 2008; Stoop, 2008). Absorption and release of the model protein BSA by hydrogels was found to be dependent on polymer oxidation rate. After an initial peak, protein release by 1% Ox and 2% Ox PVA was sustained up to 144 h. Differently, unmodified PVA showed a significantly reduced BSA uptake and subsequent release. These evidences confirm that modified scaffolds have high potentiality for in situ protein/drug delivery applications.

To further underline this potentiality, we considered the effect of chemically modified PVA loaded with TGF-β1 on human chondrocytes. In vivo and in vitro data suggest that TGF-β1 is substantial (although probably not sufficient) for normal proliferation of chondrocytes (Beier et al., 2001). Evidences concerning opposite action of TGF-β1 (Serra et al., 1999) were later reconsidered by the same authors: the anti-proliferative effect of TGF-β1 on chondrocytes is likely indirect, mediated by the perichondrium, whereas the direct (paracrine or autocrine) effects of TGF-β1 on chondrocytes are mitogenic (Beier et al., 2001). With regard to our data, TGF-β1-loaded PVA showed enhanced GF release capacity when chemically oxidized. In parallel, biological test on AC chondrocytes highlighted significant increase (p<0.01) in cell proliferation after exposure to both oxidized PVA scaffolds loaded with TGF-β1, in comparison with unmodified PVA and untreated cells. Additional in vitro studies will be necessary for complete understanding of TGF-β1 effects on chondrocytes; however, it is clear that GF-loading capability of non-oxidized PVA is unsuitable to promote cell proliferation.

As we already stressed, we considered oxidation to promote biodegradation of crosslinked PVA: oxidation treatment is an important step in biodegradation (Hatanaka et al., 1996). Most biodegradable polymers suffer from a short half-life due to rapid degradation upon implantation, high stiffness, and limited ability to functionalize their surface with chemical moieties (Wang et al., 2010). Combining material chemistry and processing
technology, it is possible to tune scaffold degradation rate. The aim is to match tissue growth rate so that the regenerated tissue may progressively replace the scaffold.

In the present study, we evaluated *in vivo* behavior of the three different types of biomaterials after *subcutis* implantation. In particular, a 12-week time interval was considered in order to completely evaluate material biodegradability and possible inflammatory responses by the surrounding tissues. The gross analysis of explanted 1% Ox PVA showed a significant reduction in surface area, while 2% Ox PVA was barely recognizable. Conversely, unmodified PVA scaffolds retained their initial size and morphology (Fig. 38).

In spite of difficulties observed during explants sectioning, yet reported in literature (Allen et al., 2004), histological analysis of the samples confirmed the absence of severe inflammatory reactions, although mild lymphomonocytic infiltration was present in all analyzed samples. In particular, it is important to stress that lymphomonocytic cells appeared more numerous when 2% Ox PVA had been implanted, suggesting a possible role for inflammatory cells in implant reabsorption. In fact, 2% Ox PVA was nearly not visible as hydrogel disk, indicating considerable reabsorption and substitution by new connective components, as confirmed by SEM results.

Tailoring a biomaterial is a capital aspect to be considered for the design of medical devices. Oxidized PVA hydrogels present tunable degradation behaviour, resulting suitable polymers for *in vitro* engineering of various biological substitutes. By modifying PVA original physico-chemical properties, oxidation process showed to enhance degradation rate and polymer ability in binding (i.e., lysozyme covalent binding) or releasing proteins (i.e., BSA and TGF-β1), optimizing hydrogel potential to be used for tissue regeneration studies. It is well known that mechanical properties of PVA hydrogel are dependent on polymer concentration, freezing time and temperature, and the number of freezing-thawing cycles (Kim et al., 2015). Considering that, future studies will focus on fine tuning of such variables, in order to allow a whole management of oxidized PVA features for wider TE applications.

Unfortunately, cell adherence on PVA hydrogels is inhibited by its highly hydrophilic nature (Liu et al., 2010). Most synthetic material-based scaffolds suffer from poor anchorage of chondrocytes or stem cells, and incorporation of natural ECMs including collagen, fibrin and hyaluronic acid in synthetic scaffolds for cell attachment has been a popular approach studied extensively. Synthetic materials provide relatively high mechanical strength with a tunable degradation rate, whereas their
hydrophobicity/excessive hydrophilic nature and lack of cellular anchorage site are drawbacks for their application in TE (Park et Cho, 2010). Many authors demonstrated ECM-based scaffold efficacy in creating a more suitable microenvironment to sustain cellular adhesion and growth. Extracellular matrix is a reservoir of structural and functional proteins like collagens, glycoproteins, proteoglycans, mucins, elastic fibres as well as a known repository for a variety of GFs. As in vivo it is progressively degraded by proteinases, it can result in the exposure of new recognition sites with potential bioactivity (Brown et Badylack, 2014). Conversely, their weak mechanical properties make it difficult to use them in load-bearing regions such as cartilage. In most cases a combination of multiple components to address various features required for culturing chondrocytes is desired. Thus, these characteristics of naturally or synthetically originated materials drive us to attempt a combination of the two materials in order to afford higher mechanical strength, tunable degradation, and cellular attachment (Park et Cho, 2010).

After having studied and stated oxidized PVA characteristics, we decided to combine Ox 1% PVA mechanical properties with ECM features. Our aim was to provide a supportive biomimetic microenvironment for chondrocytes to produce AC, taking advantage from both PVA and ECM. In particular, we considered an alternative matrix source: we focused our attention to a new ECM represented by decellularized W’s J, in comparison with decellularized cartilage matrix.

The research of a new biological ECM useful in cartilage restoration arises from the need to identify an easily available resource suitable in sustaining chondrocytes adhesion and proliferation, even not specific. Each tissue and organ contains an ECM with unique composition that consists of the secreted products of resident cells (Brown et Badylack, 2014). The main components of W’s J were ECM proteins such as collagen and fibronectin. Previous studies demonstrated that W’s J contains growth factors such as insulin-like growth factor I (IGF-1), fibroblast growth factor (FGF), transforming growth factor β (TGFβ), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and ECM proteins (Hao et al., 2013). These peptides and growth factors induce W’s J cells to produce large amounts of collagen and glycosaminoglycans (Sobolewski et al., 2005) also typical components of cartilage matrix (Poole et al., 2001). The aim of decellularization treatment is to decrease the antigenicity of matrices, through an efficient removal of cellular and nuclear material, preserving its composition (Grandi et al., 2011).

Histological analysis of decellularized W’s J and AC ECM demonstrated the effectiveness of the treatment. A different number of detergent-enzymatic cycles, 3 and
7 cycles respectively, were performed. According to DAPI staining, native W’s J showed a higher cellular density in comparison with native AC; nevertheless, its complete decellularization was more easily to achieve. This may be related to a different tissue macroscopic aspect: while chondrocytes are deeply embedded in matrix, W’s J permits a better exposure of its cellular elements to sodium deoxycholate and DNase-I, as well as to the osmotic effect of deionized water. To control and quantify W’s J and AC batch-to-batch variations, decellularized ECMs were analysed as regard their total protein levels. Matrix homogenates, gained from different donors, showed a similar profile to BCA assay: no significant difference was detected between samples of the same group. According to this data, sample-to-sample variations are negligible. Extracellular matrix characterization before and after detergent-enzymatic treatment was also achieved by means of Masson trichrome and Movat pentachrome stainings. According to Masson trichrome, both W’s J and AC maintain their collagen and mucus content (deeply green appearance). Movat Pentachrome stain confirmed the concomitant presence of collagen and mucus, even after the treatment. The resulting green leading colour is due to the overlapping between yellow (referred to collagen and reticular fibres) and blue (referred to mucus). However, the detergent-enzymatic treatment seemed to remove or reduce fibrinoid elements expression. This ECMs characterization highlighted a similar histomorphology for W’s J and cartilage, supporting our theory.

The chief aim of many authors is to preserve tissue or organ histoarchitecture from a too aggressive decellularization treatment; on the contrary, we approached to ECMs in a different manner. We take advantage of matrices macromolecules instead of their superstructure. ECM homogenates are an interesting and innovative manner of working with matrices. Choosing an adequate mould and modulating the needed quantity, the liquid suspension obtained can be used to create tailored scaffolds. Furthermore, the lyophilization process they subsequently undergo, makes them easy to store. The two different lyophilized matrices realized were examined by SEM for their fine structure: cartilage derived one appeared more spongy than the W’s J analogue. Physical cross-linking of lyophilized matrices with PVA solutions led to three-dimensional composite scaffolds.

In collaboration with Transfusion Service, Regional Centre for Blood Diseases and Haemophilia Centre of Castelfranco Veneto General Hospital, Treviso, arose the interest for haemophilic arthropathy. In particular, the dialogue with the clinician has arisen the need to identify a strategy that differed from the approaches already used in clinical.
Prophylaxis replacement of clotting factor, which is the gold standard in haemophilia, prevents bleeding and joint destruction; however, it does not reverse established joint damage as well as all the other approaches, even surgical. Hence, the idea was to promote the regeneration of cartilage tissue translating in haemophilic patients TE strategies already used in non-haemophilic patients.

Beyond scaffold and adequate stimuli, also cells represent a key element in TE approaches. Cell quality could potentially influence the success of the strategy (Pestka et al., 2011). In the perspective of carrying out a TE approach for haemophilic arthropathy, investigating haemophilic chondrocytes phenotype is an unavoidable requirement. To our knowledge in literature, nothing is known about them, as any characterization has been performed yet. Stated that, AC chondrocytes from Non-Haemophilic patients were used as control.

Hence, before seeding chondrocytes on scaffolds, gene expression profile and phenotype of haemophilic and Non-Haemophilic chondrocytes was investigated through RT-PCR and flow citometry analysis.

The viscoelastic properties of AC arise from the composition of its ECM, which consists primarily of type II collagen, but also of collagen type IX and X and a proteoglycan termed aggrecan (ACAN) (Ono et al., 2013). Aggrecan is retained in cartilage by binding to long filaments of another glycosaminoglycan, hyaluronan (HA), which is synthesized at the plasma membrane level by an enzyme called hyaluronan synthase (HAS) (Takahashi et al., 2010). Moreover, one of the major non-collagenous proteins in the cartilage is COMP, which represents a useful marker of differentiation state of primary chondrocytes (Zaucke et al., 2001). The synthesis of this cartilage-specific ECM requires the expression of genes associated with the specific chondrocyte phenotype, controlled by the transcription factor SOX9 (Ono et al., 2013). According to RT-PCR analysis, both cell populations isolated for this study expressed specific cartilage markers at the mRNA level, showing a gene expression profile typical of articular chondrocytes.

Expanded chondrocytes were thus assessed by flow cytometry. We purchased antibodies against several CDs, typically used to characterize the phenotype of mesenchymal progenitor cells (Pittenger et al. 1999; Grogan et al. 2007) and recently introduced to determine the stage of differentiation of human AC (Grogan et al. 2007; Diaz-Romero et al., 2005). In this study we confirmed the expression of several articular chondrocyte surface markers: the hyaluronan receptor CD44, the ecto-enzyme CD73, the integrins α3 (CD49c), α5 (CD49e), and the tetraspanin CD151 (Grogan et al. 2007; Diaz-Romero et
al., 2005). According to Grogan and colleagues (Grogan et al., 2007), chondrocytes with marked chondrogenic capacity express high levels of the hyaluronan receptor CD44, the α3 integrin subunit CD49c, and the tetraspanin CD151. These are surface molecules involved in the early stages of cartilage development; all of them were present in the chondrocytes we investigated. Moreover, these proteins are responsible for establishing cell-cell and cell-matrix interactions. These processes are known to be important mediators of mesenchymal condensation, which is in turn necessary for initiation of chondrogenesis (DeLise et al., 2000). Hence, high expression levels of these membrane proteins might increase the propensity of the cells to differentiate and produce cartilage ECM. Markers characteristic of mesenchymal progenitor cells, i.e., CD44 and CD73 (Pittenger et al., 1999), have shown to be expressed in high-chondrogenic-capacity populations (Grogan et al. 2007). This suggests that within a chondrocyte culture, subpopulations with higher capacity to form cartilage might correspond to those with progenitor characteristics.

After characterization of scaffold histomorphology and chondrocyte gene expression profile and specific immunophenotype, we seeded a known cell amount of 20,000 cells/cm² on Ox 1% PVA, Ox 1% PVA/W’s J and Ox 1% PVA/AC supports. We evaluated chondrocyte adhesion and proliferation at two different end-points: 7 and 14 days. If Ox 1% PVA itself clearly demonstrated its absolute inability to sustain chondrocyte proliferation, cells on composite scaffolds revealed a progressive increasing growth trend. At 24 h from seeding, cells adhered on Ox 1% PVA/ECMs, which were able to sustain cell proliferation up to the last end-point considered (14 days). According to SEM micrographs, chondrocytes cultured on Ox 1% PVA/AC showed a more specific morphology and a more tidy orientation on the scaffold surface. In parallel, Ox 1% PVA/W’s J revealed a singular attitude to sustain cell proliferation despite its aspecific origin. Hence, as stressed also by MTT proliferation assay, our in vitro model confirmed the starting hypothesis regarding the possibility to use Wharton’s jelly in composite scaffolds that mimic articular cartilage.
CONCLUSIONS

This work led to the development and characterization of novel PVA-derived biomaterials obtained by polymer oxidation. Oxidized PVA polymers showed customizable mechanical behaviour, protein-loading ability, biocompatibility and biodegradability in vivo, making them suitable for TE applications. Moreover, Wharton’s jelly, usually believed a waste product and investigated in literature only as source of MSCs, promotes chondrocyte adhesion, representing an idoneous biomimetic microenviroment even though its aspecific nature. Combining hydrogel potentiality with Wharton’s jelly intrinsic properties we obtained a bio-hybrid scaffold, overcoming drawbacks related to the typical inadequacy of synthetic hydrogels in promoting cell adhesion and characteristic lack in mechanical properties of ECMs when used itself as scaffold.

This study contributed also to the definition of haemophilic chondrocytes phenotype. Our preliminary evidences highlighted the chance of using these cells for autologous implant. In vivo exposure to blood seems not to have altered their potential in synthetizing matrix-related proteins as well as surface markers typically involved in cell-cell and cell-matrix interactions. Finally, considering the impact of the primary osteoarthritis, our composite scaffold may be considered also as an innovative, easily available and inexpensive support for cartilage restoration even in Non-Haemophilic patients.

Further investigations are necessary to evaluate phenotype maintenance of chondrocytes, and hydrogels behaviour after implantation in the treatment of focal articular cartilage defects.
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