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Extracellular vesicles derived from progenitor cells for the treatment of IVD degeneration: a translational study

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Alla mia famiglia, ai miei nonni,

a Rosario, a Simba,

e a tutti coloro che hanno creduto in me…

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Abstract

Low back pain (LBP) is a common musculoskeletal problem and represents the leading cause of disability and loss of working ability with a strong impact on patients'quality of life in addition to a critical socioeconomic burden on public health worldwide [1]. In healthy conditions intervertebral disc (IVD) acts as a shock absorber between the vertebrae allowing for flexion-extension, lateral flexion and rotation movements [2]. The most frequent symptoms associated to pathological alterations is LBP which affects more than 80% of the population. With aging, the progressive decline of the disc due to chronic processes such as dehydration, inflammation, ability to resist compressive loads, exhaustion of the endogenous cell population and degradation of the extracellular matrix (ECM) lead to the IVD degeneration (IDD) [3].

Although IDD in many cases is asymptomatic, it may be lead to serious problems also associated with chronic LBP, sciatica, segmental instability, spinal stenosis, hypertrophy or ossification of the facets, peripheral neuropathy and disc herniation or prolapsed [4-6].

However, current approaches to treat IDD are based on conservative or surgical procedures with the aim to relieve pain but are not able to change the natural history of the disease [7]. This thesis aims to illustrate the rationale behind a regenerative cell-free approach for IDD as well as the therapeutic potential of paracrine factors derived from mesenchymal stem cells (MSCs) such as extracellular vesicles (EVs).

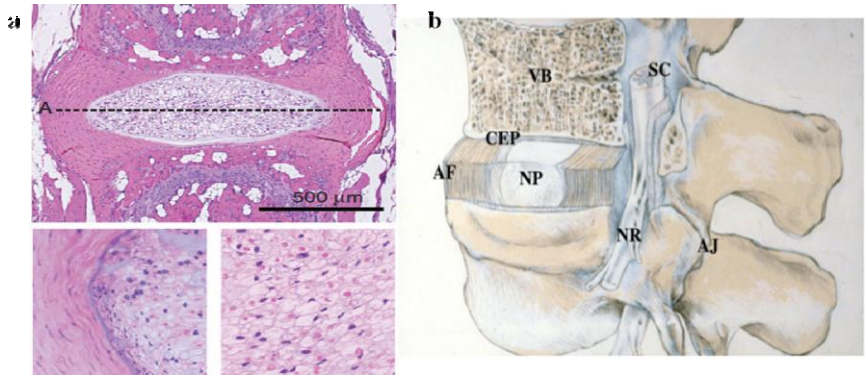


Figure 1. Morphology of spinal segment and organization of intervertebral disc regions. **a**) Histological analysis of a mouse-tail IVDs. **b)** The figure shows the nucleus pulposus (NP) surrounded by the lamellae of the annulus fibrosus (AF) and separated from the vertebral bodies (VB) by the cartilaginous end-plate (CEP); the spinal cord (SC), the nerve root (NR), and the apophyseal joints (AJ) [5-6].

1. INTRODUCTION

1.1 INTERVERTEBRAL DISC DEGENERATION

1.1.1 Epidemiology

The Global Burden of Disease study reported that LBP is the leading cause of disability worldwide compared to any other medical conditions [8,9]. The incidence of LBP is problematic to estimate since the first mild signs of the disease already begin to be present in 20% of adolescents and symptoms tend to recur over time [10,11]. Furthermore, the incidence increases with age, indeed up to 84% of adults suffer of LBP at least once in their life [12]. Patients who continue to have back pain beyond 4 weeks may have subacute (4 to 12 weeks duration) or chronic (> 12 weeks duration) back pain that causes disability by triggering both psychological and social awkwardness for the patients [13]. Another factor adversely related to LBP is the enormous economic burden due to lost profits and lack of productivity. It is estimated that in the United States, approximately $100-200 billion annually are spent for healthcare expenditures, hospitalization, diagnosis and treatment of LBP [14-16]. The degeneration of the IVD tissue begins before the degeneration of other muscles and skeletal tissue and is often asymptomatic [17]. The height of IVD gradually decreases and this leads to bio-mechanical alteration of the affected segment and other spinal structures, such as ligaments, joints and muscles. In the long term, serious consequences to this condition may be disc herniation or spinal stenosis, that is the narrowing of the spinal canal with compression of the nerves which is the main cause of pain [18,19].

The prevalence of IDD depends on other factors such as aging, excessive or impaired mechanical load, obesity, genetic predisposition, diet, smoking, trauma and inflammatory diseases [20-27]. Although IDD is the cause of approximately 50% of all cases of LBP in young adults; however not all cases of IDD result in LBP [28]. Due to the complexity of exact etiology and the multifactorial nature of IDD, the development of personalized therapies for patients with LBP is the biggest challenge for physicians.

1.1.2 The Structure of the Intervertebral Disc

The IVD is the largest avascular organ in the human body located between the vertebrae of spine and consists of three distinct anatomical regions (Fig. 2): 1) the nucleus pulposus (NP), 2) the annulus fibrosus (AF) and 3) the cartilage endplates (CEP) which anchor the discs to the adjacent vertebrae [29-30].

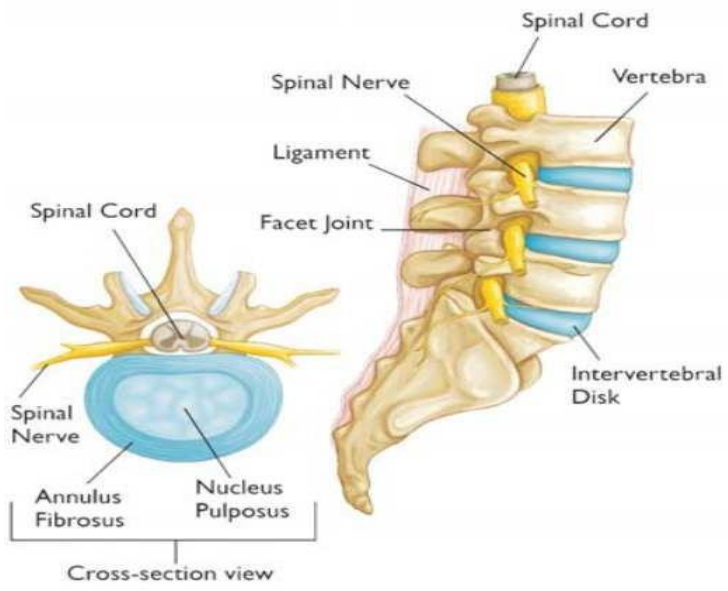


Figure 2. Intervertebral disc (IVD) anatomical structure.

1.1.2.1 Nucleus pulposus

The NP is a gel-like structure in the center of the IVD surrounded by thick layers of radially aligned collagen fibers constituting the AF and is a heterogeneous tissue derived from the notochord, an embryonic structure common to all vertebrates [31]. The vacuolated notochordal cells, also known as nucleopulpocytes, in the IVD of children and young adults degenerate with aging by becoming chondrocytes-like smaller cells identified as nucleus pulposus cells (NPCs) (Fig. 3) [32,33]. NPCs have phenotypic markers such as Forkhead Box F1 (FOXF1), paired box 1 (PAX1) and Keratin 19 (KRT19) [34,35]. Moreover, several studies have demonstrated the existence of a population of progenitor stem cells within the IVD, expressing the tyrosine protein kinase receptor Tie2 ($Tie2^+$) and the disialoganglioside GD2 ($GD2^+$) with a superior

capacity to proliferate, produce ECM, form spheroid colonies, self-renew, and multipotent differentiation capabilities, both *in vitro* and *in vivo* model [36]. The $Tie2^+$ $GD2^+$ populations decrease with age. However, the ligand of the Tie2 receptor, angiopoietin-1 (ANG-1), can stimulate the formation of NP progenitor cell colonies and decrease apoptosis rate [37].

NP is composed of 66% to 86% water, collagen type II, elastin and proteoglycans which are responsible for the considerable imbibition and swelling pressure. Proteoglycans have a central protein *core* with peripheral chains of glycosaminoglycans (GAGs) containing keratan sulfate and chondroitin sulfate. Among these, aggrecan and versican bind to hyaluronic acid and in combination with collagen type II and collagen type IX compose the ECM responsible for maintaining the integrity of the NP. Furthermore, collagen provides tensile strength and anchors the tissue to the bone while aggrecan is mainly involved in water retention within the tissue [38]. The internal hydrostatic pressure generated by the high content of proteoglycans and water confers to NP the function as a "shock-absorbing cushion" by distributing the forces throughout the spine to reduce the loads arising from body weight and muscle activity, the risk of fractures and degenerative changes [39-40]. Conversely, a hypothetical solid NP would transmit a force to the lower vertebral body, thus increasing the risk of trauma and losing its cushioning capacity [41].

1.2.2.2 Annulus Fibrosus

The AF is a fibrous connective tissue less rich in water, composed of an inner and an outer part that differ mainly in collagen composition. The outer AF contains mostly collagen type I, while the inner AF provides collagen type II and proteoglycans [42]. However, with increasing distance from the NP, the amount of collagen type II decreases to be replaced by collagen type I. Furthermore, another important difference between the two layers is the presence of morphologically distinct cells. The inner AF contains fibrocartilaginous cells described as round, while in the outer are fibroblast-like which form concentric sheets also known as "lamellae" with the function of a capsule containing the NP (Fig. 3) [43]. This structure highly organized is similar to car tires, made up of 15-25 "radial layers" oriented at about 60° to the vertical axis,

allowing an optimal resistance to twisting, flexion and extension movements of the spine compared to a longitudinal configuration [41]. This represents an excellent balance between strength and flexibility.

1.2.2.3 Cartilage endplate

More external, the CEP, a thin layer of hyaline cartilage sits between the vertebrae and disc. Unlike NPCs, the AF and CEP cells remain relatively stable throughout life [31]. Type II collagen-rich CEP acts as a filter that prevents the leakage of macromolecules from the subchondral bone of the vertebra and is important to monitor disc hydration under mechanical loads [44,45]. On the other hand, as both NP and AF are avascular tissues with an oxygen tension between 1 and 5% [46], their function is extremely related to diffusion of oxygen and nutrients through the capillaries present at the periphery of the disc [47,48]. This is a crucial concept since a reduced nutrient supply affect drastically the number of viable cells in the disc, leading to degeneration [49].

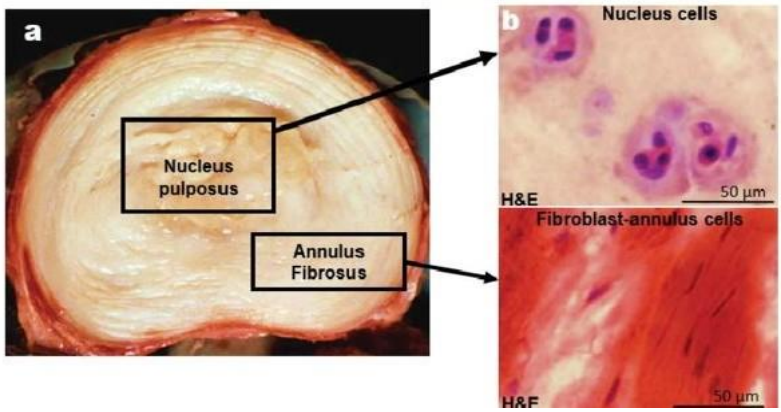


Figure 3. **a)** Healty adult lumbar intervertebral disc (IVD) showing the nucleus pulposus (NP) and lamellae of the annulus fibrosus (AF) **b)** Nucleus-rounded and annulus-fibroblastic cells morphology stained with hematoxylin and eosin (H&E) [33].

1.1.3 Pathophysiology of IDD

Degenerative processes include alterations in the architecture and biochemical configuration of the disc. With aging and advancing degeneration, the NP generally appear more fibrotic and less gelatinous, the lamellae are irregular and increasingly disoriented, the network composed of elastin and collagen fibers gradually deteriorates and emerge tiny fissures [50,51]. Another prominent change concerns cells morphology, cellularity and NP-AF boundary that becomes more difficult to distinguish making the disc structure disorganized and less flexible [52]. Specifically in the degenerated NP, the rate of cell proliferation is altered leading to cluster formation, as shown in figure 4 [6]. Although it was also reported that over 50% of cells in the adult discs are necrotic, cell apoptosis is also increased in the degenerated disc and dead cell debris are not easily removed since there is no vasculature and resident immune cells [53-55]. Furthermore, a senescent state could be established following specific triggers such as oxidative stress or DNA replication and damage. Senescent cells stop to replicate in response to mitogenic stimulation and produce aberrant levels of cytokines and enzymes that degrade the matrix [56]. During IDD, a significative change is the loss of proteoglycans which are degraded in smaller fragments. The main consequences are the loss of glycosaminoglycans (GAGs), degradation of aggrecan, dehydration and a prominent reduction in the mechanical properties and load-bearing capacity of the IVD [57,58].

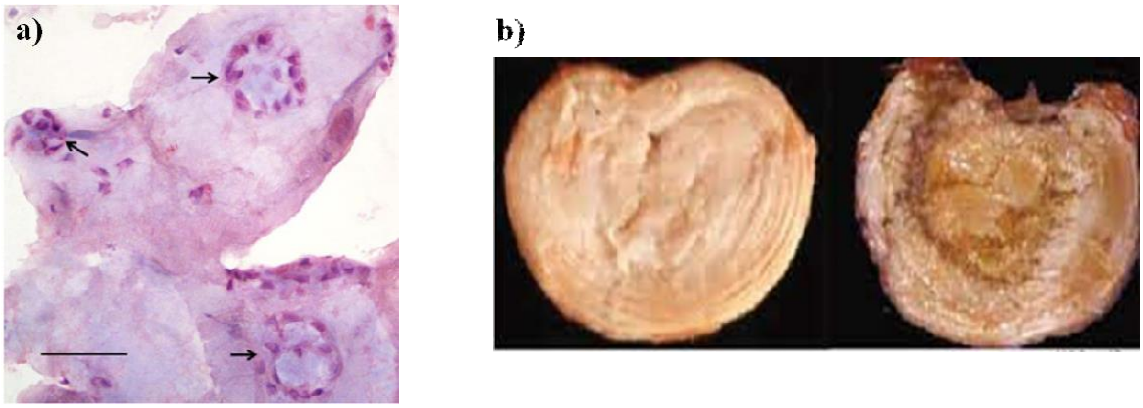


Figure 4. a) Cell clusters (arrows) are common in degenerated intervertebral discs (IVD) [6]. **b)** On the left a normal lumbar intervertebral disc and degenerated disc on the right, in which the nucleus is desiccated and the annulus is highly disorganized [57].

Collagen type II is denatured due to enzymatic activity and substitute by collagen type I which results in decreased elasticity and mechanical integrity of disc tissue. Other components, such as fibronectin, may increase their content with progression of degeneration by reflecting the cell's response to an altered microenvironment. The upregulation of enzymes such as cathepsins, matrix metalloproteinases (MMPs) and the disintegrins and metalloproteinases with thrombospondin motifs (ADAMTSs) lead to the degradation of the ECM [57,59-61].

Spinal structures can be strongly influenced by the shift from anabolism to catabolism in the ECM. This affects disc physiology and leads to functional changes such as increased susceptibility to injury (Fig. 5). For example, the CEP becomes thinner by allowing serum proteins and inflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor (TNF), to diffuse into the matrix fissured due to altered permeability affecting native cells by accelerating the process of degeneration [62,63].

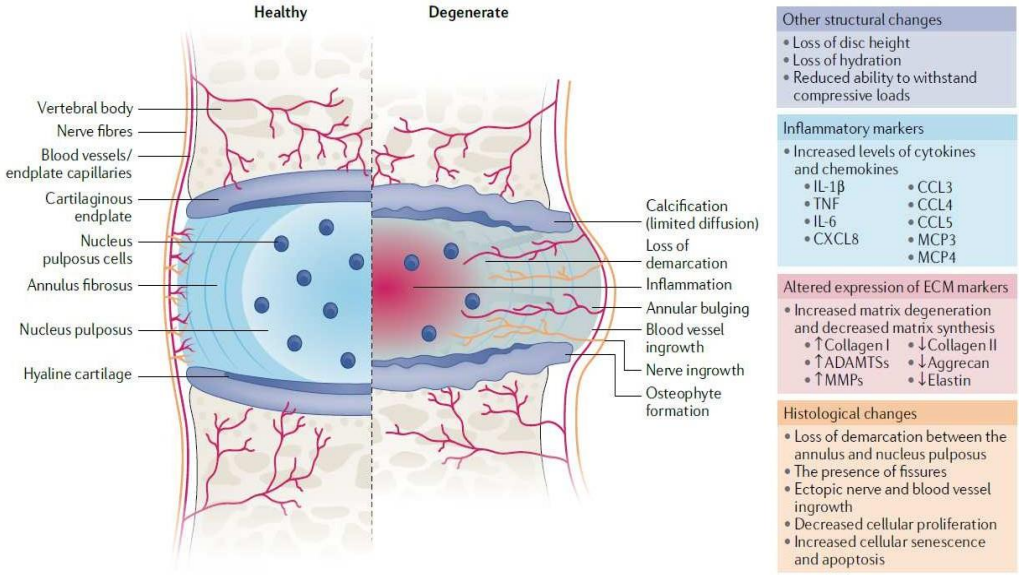


Figure 5. Schematic representation of healthy and degenerated intervertebral (IVD) [69].

The factors aforementioned in combination with inflammation can contribute to the development of pain. The causes of pain during the degenerative process are complex [64]. Frequently, chronic pain is mainly due to hypertrophy of the surrounding tissue structures which generate compression of the spinal nerve roots or also occurs due to the formation of osteophytes and the growth of tiny neural endings in the IVD [65].

Cleft in AF caused by degeneration or trauma leads to a bulging disc or herniation, in which internal disc material protrudes into the spinal canal and causing pain [66].
Another critical aspect of the painful *sequelae* in IDD is the ingrowth of the ectopic sensory nerve fibers and blood vessels in the innner AF and NP [67]. Convincing evidence including avascular environment and changes in tissue integrity can favour neo-innervation and neo-angiogenesis by meaning peripheral neuropathy that produces pain, weakness, and numbness due to nerve damage [68] However, the full complexity of the pain mechanisms underlying these conditions remains unclear.

1.1.4 Aetiology of IDD

The underlying causes of IDD are complex and multifactorial. Several evidences have demonstrated that genetic predisposition, aging, mechanics, exposure to trauma and an hostile avascular milieu with limited nutritional exchange in the NP are involved in the progression of the disease [70,71].
Furthermore, there is a lack of optimal animal models as there are significant anatomical differences between the human IVD and animals usually used as laboratory models. Indeed, the NP in rodents is populated by notochord cells throughout adulthood, while these cells, as mentioned, disappear in humans after infancy. Otherwise, sheep and canine species rapidly lose notochord cells early in life, similar to what is observed in humans, making these models more suitable for preclinical validation [72].

1.1.4.1 Genetics factors

A genetic influence exists among the different causes of IDD. The molecular mechanism triggering the pathology and the role played by epigenetics are not yet known, therefore the identification of specific genetic modifications is necessary [73]. Indeed, there are evidences both of heritability and some polymorphisms of genes coding for the components of the ECM which can influence the course of the degenerative process by altering the morphology of the matrix and consequently the mechanical function of the discs [74,75]. Among the structural components, mutations

associated with collagen and inflammatory cytokine have been extensively evaluated in IDD. Some examples are the polymorphisms of the COL1A1 gene strongly associated with a change in the intensity of the disc signal on magnetic resonance imaging (MRI) [76]. Similarly, Trp2 allelic variants of collagen type IX found in chains α2 and α3 affect MRI signal intensity in NP with a 4-fold increase in annular tears in patients older than 30 years [77,78]. Furthermore, Solovieva *et al.* reported the effect of a gene-gene interaction between Trp3 allele in COL9A3 with IL-1β polymorphism reflecting the role of immunomodulators in IDD [79]. Collagen type XI polymorphisms have been associated with a higher risk of herniation [74] Specifically, a sequence variation in COL11A2 intron 9 was related to an increased risk of disc bulges compared to people without this polymorphism.

Kawaguchi *et al.* indicated that a variable number of tandem repeats in the CS1 domain of aggrecan gene (ACAN) leads to a reduction of chondroitin sulfate, dehydration and reduction of disc height [80].

As previously mentioned, pro-inflammatory cytokines are key factors that increase expression of MMPs and ADAMTSs. It has been demonstrated that a combination of single nucleotide polymorphisms (SNPs) of MMP-3, MMP-2, MMP-9 and IL-1β results in a higher risk of IDD development [81,82]. Others gene alterations involved in IDD concern factors involved in cell differentiation and viability such as growth differentiation factor 5 (GDF5), vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) [83,84]. However, the exact mechanisms of how these variants influence cell fate and IDD are still unclear and require further investigation. One promising aspect is the Genome-wide Association Study (GWAS), a method that testing for variants that span the entire genome [85].

1.1.4.2 Mechanical stress

During daily life the IVD is exposed to various forces and acts as a shock absorber between the vertebral segments. However, many epidemiological studies support the concept that risk factors such as strenuous physical work, smoking and the induced endplate vessels' atherosclerosis, obesity, inappropriate posture and lack of physical activity can cause micro-lesions within the disc that wear out the disc tissue over time

and lead to chronic pain [28,86]. The abnormal mechanical loads and microscopic structural damage contribute to blood vessel formation and increased chemo-attraction of immune cells by pro-inflammatory cytokines released from native cells [61,87]. A pro-inflammatory environment and a moderate mechanical loading act synergistically by changing the size and architecture of the IVD leading to herniated disc or endplate defects [88].

IVD cells activity and ECM composition is profoundly affected by mechanical load intensity, duration and frequency [89]. When intradiscal pressure decreases due to dehydration of the NP, catabolic events are predominant: the ratio of MMPs/tissue inhibitors of metalloproteinases (TIMPs) is reversed, prolonged loading leads to mitochondrial damage and excessive ROS production and NPCs' apoptosis [90]. The effect of different loading on IVD cells has been the subject of several studies. Interestingly, the static compressive load increases cell death and matrix degradation. Conversely, dynamic compressive load elicits anabolic responses and enhances the diffusion of nutrients and growth factors from vertebral body to inside the NP [91].

Furthermore, many studies demonstrated that mechano-transduction signalling pathways are altered in AF and NP cells of degenerated IVD compared to healthy disc, suggesting that mechano-biological alterations play a significant role in the development of IDD [92].

1.2.4.3 The role of microenvironment in IDD

A strong limit in the field of disc regeneration is represented by the harsh microenvironment that is established during the degenerative process with conditions of hypoxia, nutritional deprivation, acidity, hyperosmolarity, increased rigidity of the ECM, and inflammation [93].

Nutrient supply is a key factor to ensure the normal function and structure of the disc. As already discussed, the disc is an avascular structure and the cell nutrient supply strictly depends on the capillaries arising in the vertebral bodies, penetrating the subchondral region and ending in the CEP, via diffusion through the ECM [94]. However, glucose and oxygen transport into the IVD, as well as the removal of cellular metabolic waste products, might be impaired by CEP calcification and subsequent

capillary obliteration, thereby accelerating the progress of IVD degeneration [65,95] as for example, it is often seen in scoliotic discs [96]. Glucose deprivation impairs NPCs proliferation and survival as well as proteoglycan and collagen synthesis [97,86]. Furthermore, the limited blood circulation establishes an hypoxic microenvironment with oxygen concentrations falling from 19.5% in AF to below 1% in degenerated NP [98]. Conversely to the limited availability of glucose, when oxygen tension falls below 5% the proliferation or survival of IVD cells is not affected due to their constitutive expression of hypoxia-inducible factor (HIF)-1α [99]. Novais *et al.* recently demonstrated that hypoxia and HIF-1α positively regulate cell proliferation, energy metabolism, response to oxidative stress, inhibit osteogenic potential, autophagy, cell apoptosis and down regulate collagen I synthesis [100,101]. Hypoxia also promotes the differentiation capacity of disc progenitor cells as shown in an *in vivo* study in which the chondrogenic differentiation of rat NPCs was stimulated [102].

A decrease in the amount of glucose and oxygen leads to a consequent increase in the concentration of lactic acid. Cell activity *in vitro* has been reported to be very sensitive to oxygen and pH [103]. Acid-sensing ion channels (ASICs) pick up pH fluctuations and regulate transmembrane exchange of Ca^{2+} ions according to changes in extracellular levels of H^{+} [104]. In the long term, the accumulation of lactic acid generated within the IVD drives to a lowering of the pH from 7.0-7.2 to 5.6 depending on the degree of severity'disease promoting cell death, decrease in aggrecan and SOX-9 content, up-regulation of MMPs, ADAMTSs and ASICs thus suggesting the importance of these channels in the regulation of IVD cells within the *milieu* acid [105].

A further factor making the disc microenvironment unfavorable is osmolarity. The osmolarity levels under physiological conditions are equal to approximately 400 mOsm but it is needed to emphasize that IVD cells are exposed to a wide range of mechanical loadings shifting rapidly tissue osmolarity. For istance, during daily activities it can decrease of 20-25% while under rest, the range is between 430 and 500 mOsm/L [49,106]. During IDD, loss of proteoglycans is associated with a hypo-osmotic shift (300 mOsm) which can promote inflammation [107]. Conversely, during high mechanical loading, osmolarity increases to approximately 500 mOsm leading to the activation of the osmo-sensitive transcription factor TonEBP (tonicity-responsive

enhancer binding protein) with the role to check intracellular osmotic stress under hypertonic stress [108,109].

IVD *milieu* of degenerated discs appear compromised by the presence of inflammatory cytokines such as TNF-α, IL-1β, IL-8 and IL-6, C-C motif chemokine ligand (CCL), nitric oxide synthase 2 (NOS-2), prostaglandin E2 (PGE2) and IFNγ that can remodel ECM metabolism, induce cell apoptosis, autophagy and senescence [110]. In particular, IL-1β and TNF-α resulted highly expressed in degenerative IVDs compared non-degenerative IVDs. It was widely demonstrated that IL-1β and TNF-α are involved in the progression and early stage of IDD by regulating several signal transduction pathways correlated to inflammatory response such as, IVD cell proliferation, senescence, apoptosis, autophagy, ECM degradation and oxidative stress, thus representing a target for the treatment of IDD [111]. Furthermore, it has been demonstrated that IVD progenitor cells adapt their metabolism to changes in the microenvironment by increasing ECM synthesis and the activity of anaerobic glycolysis by generating energy, reducing reactive oxygen species (ROS) and consume of oxygen [98]. Under inflammatory conditions, NP progenitor cells can undergo neurogenic differentiation and secrete both pro-inflammatory cytokines and pain-related neurotrophic factors, such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF), resulting in innervations and discogenic pain [112,113].

Despite the hostile microenvironment and avascularity, bacteria were detected within the disc tissue. It is still controversial the presence of a microbiome as IVD is considered an aseptic tissue, however, this suggestion was proposed as a potential regulator of inflammation and disc cell catabolism [114]. A systematic review by Granville Smith *et al.* reported in 27 of 34 research studies the presence of bacteria in human IVDs while in other studies it was attributed to the presence of *Cutibacterium acnes* contamination, a Gram-positive anaerobic bacterium that is part of the natural skin microbiome [115] It is recent discovered that human IVD cells stimulated with *C. acnes* express catabolic cytokines, suggesting that a bacterial infection could trigger IDD [116]. Further investigations are needed to understand whether a true microbiome exists within the disc, or bacteria are involved in the catabolic events of IDD, and if the gut microbiome could be involved [117].

Taken together, all these factors in addition to negative influence on the physiological activities of the resident IVD cells, can compromise the success of treatments such as cell therapy through MSCs injection (Fig. 6) [118-120].

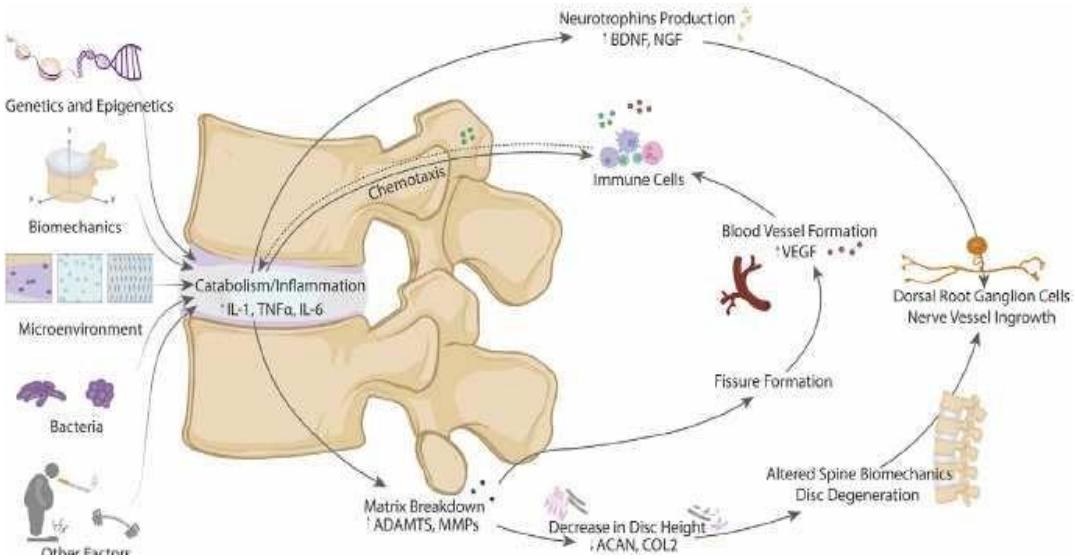


Figure 6. Main aetiological factors inducing to intervertebral disc degeneration (IDD) [113].

1.2 CLINICAL PRESENTATION

Patients with lumbar disorder often have familiarity with a myriad of symptoms including radicular pain and weakness. When examining patients with presumed lumbar IDD, it is important to exclude other potential pain-related etiologies and symptoms such as fever, fatigue, weight loss, which may be indicative of other disorders [121]. The pain may disappear in few weeks or persist for long time. One study followed 605 patients for 2 years with LBP with or without sciatica, results showed 54% of patients with chronic pain at 6 months follow-up [122]. Radicular pain is often associated with prolapse or herniated disc with a prevalence between 2% and 4%, especially in male subjects with an age between 30 and 50 years [123]. The main sign of herniation is a swelling of the disc due to partial or complete rupture of the external AF following a strong mechanical injury (Fig. 7). Beyond LBP the symptoms are leg pain due to compression of neural structures in the spinal canal [124]. Occasionally, spontaneous

disc resorption may occur leading to improvement or even to the ending of lumbar pain [125]. In patients over 60 years, the cause of LBP usually is spinal stenosis, mainly at the level of L4-L5 vertebrae as a result of hypertrophy of the facet joints and ligamentum flavum and/or spondylolisthesis characterized by the forward sliding of a portion or the entire vertebral segment. In addition, spinal stenosis can cause chronic mechanical compression resulting in axonal damage or nerve root ischemia [123]. Notable, both NP herniation and spinal stenosis are often incidentally identified by radiological diagnosis in asymptomatic patients.

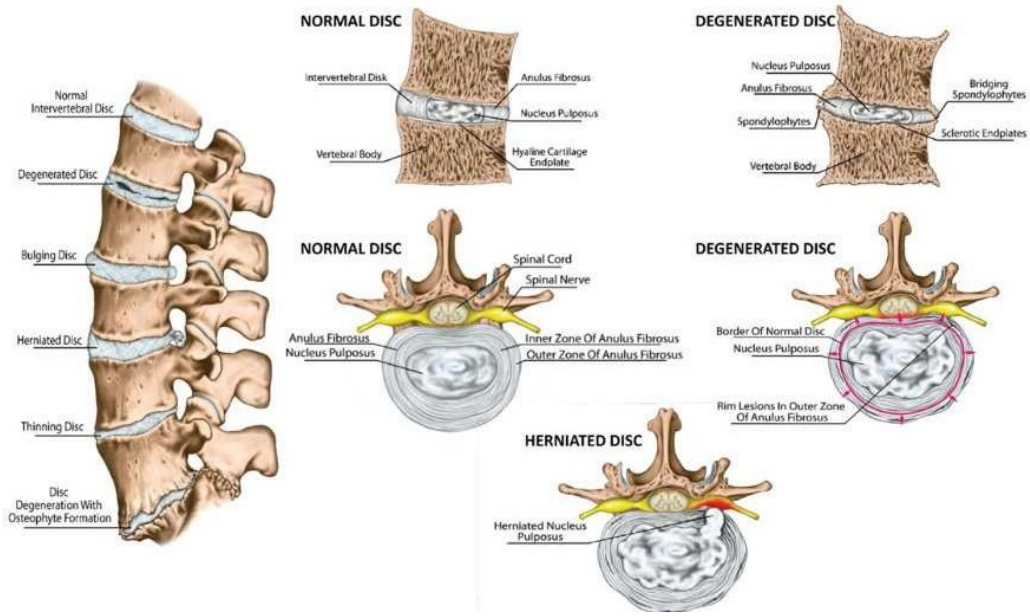


Figure 7. Sagittal and transversal views of the healthy, degenerated and herniated intervertebral disc (IVD) [27].

1.2.1 Diagnosis

Although, a large proportion of LBP cases are asymptomatic or resolve without a diagnosis, most guidelines recommend history and physical examination to identify specific clinical entities, due to the difficulties to distinguish between aging changes and degenerative changes [126]. Conversely to chronic LBP, the use of imaging techniques is strongly recommend for acute LBP when signs of severe or progressive neurological deficits persist [127]. Vertical radiograph is the first step in the imaging strategy to rule out deformity, fracture, or metastatic cancer as the triggering cause of back pain. Prior

to magnetic resonance imaging (MRI), the "gold standard" system modality to visualize the IVD was discography, which provides injection of a contrast medium in IVD, under fluoroscopy [128]. In patients who presents contraindications to MRI, computed tomography (CT) has a high capacity to detect osteophytes, intradiscal gas (empty disc), calcifications and annular lacerations [129]. Actually, among imaging modalities, MRI is the “gold standard” for the evaluation of degenerative disc disease. MRI scan findings include T1-weighted and T2-weighted sagittal images to detect potential foraminal or extraforaminal disc herniation, disc space narrowing, endplate sclerosis, "void" phenomenon within the disc and osteophytes. When IVD progressively becomes fibrotic due to degeneration and the distinction between AF and NP area is less evident, a uniform low signal on T2-WI is detected within the disc and it is often correlated with a drastic reduction of disc height [130-132]. Interestingly, there are several approaches in clinical practice to evaluate IDD. The Thompson Grading Scale is conducted by X-ray radiographic inspection of the disc to determine the extent of morphological degeneration (Table 1). It is a grading system ranging from grade 1 (non-degenerate) to grade 5 (severely degenerate) [133].

Table 1. Thompson Grading Scale [136].

Grade	Nucleus	Anulus	Endplate	Vertebral Body
I	Bulging gel	Discrete fibrous lamellas	Hyaline, uniformly thick	Margins rounded
II	White fibrous tissue peripherally	Mucinous material between lamellas	Thickness irregular	Margins pointed
III	Consolidated fibrous tissue	Extensive mucinous infiltration; loss of anular demarcation	Focal defects in cartilage	Early chondrophytes or osteophytes at margins
IV	Horizontal clefts parallel to endplate	Focal disruptions	Fibro-cartilage extending from subchondral bone, irregularity and focal sclerosis in subchondral bone	Osteophytes less than 2 mm
V	Clefts extend through nucleus and annulus	-	Diffuse sclerosis	Osteophytes greater than 2 mm

Modic classification is a reliable and reproducible method used to characterize radiologically disc changes and includes three types of changes (Fig. 8) [134-136]:

i. Type I, shows an increment of signal on the T2 and decreased signal intensity on the T1 indicative of medulla edema.

ii. Type II is characterized by fatty infiltration of the marrow as demonstrated by T1 and T2 hyperintense images.

iii. In type III occurs hypointense signals on the T1 and T2 which correspond to endplate sclerosis.

Type	T1	T2	Histopathology
1	Hypointense	Hyperintense	Bone marrow edema
2	Hyperintense	Hyperintense	Fatty replacement
3	Hypointense	Hypointense	Sclerosis

Figure 8. Classification of Modic Changes. Modic 1 (A,B): L3–L4 representing bone edema (white arrows). Modic 2 (C,D): L3–L4 representing fat degeneration and red bone marrow conversion to yellow bone marrow (white arrows). Modic 3 (E,F): L5–S1 representing subchondral bone sclerosis (white arrows) [136].

An even more useful approach for imaging studies is the Pfirrmann classification which is based on a five-level rating scale useful to classify the degree of IDD according to mid-sagittal T2-WI (Table 2). This system takes into account the signal intensity of the NP, the distinction between the inner and outer fibers of the AF, and the height of the disc [137].

Table 2. Pfirrmann classification grading scale used to assess the severity of degenerative changes within the NP of the disc [136].

Grade	Structure	Distinction of Nucleus and Anulus	Signal Intensity	Height of Intervertebral Disc
I	Homogeneous, bright white	Clear	T2-w Hyperintense, isointense to cerebrospinal fluid	Normal
II	Inhomogeneous with or without horizontal bands	Clear	T2-w Hyperintense, isointense to cerebrospinal fluid	Normal
III	Inhomogeneous, gray	Unclear	Intermediate	Normal to slightly decreased
IV	Inhomogeneous, gray to black	Lost	Intermediate to hypointense	Normal to moderately decreased
V	Inhomogeneous, black	Lost	hypointense	Collapsed disc space

Unfortunately, there are many contradictions between the signs and/or symptoms of IDD and the imaging outcomes applied as diagnostic tools. Indeed, a disc is considered radiologically "normal" when it does not present structural modifications, trauma or aging; on the other hand, clinically "normal" means asymptomatic patient, even if imaging results can show congenital or aging alterations [132].

In addition to mapping using T1-weighted and T2-weighted sequences, a non-invasive and innovative MRI approach is T1ρ and T2 mapping based on quantitative maps of relaxation time constants, able to discriminate local biochemical changes within the discs in the early stages of IDD. As shown in Figure 9, T1ρ revealed an increased sensitivity to interactions between macromolecules of the ECM and water allowing to identify early disc degeneration using a color-coded map [138].

One revolutionary aspect both in the diagnostic and therapeutic field of IDD may be the use of artificial intelligence (AI). The multidisciplinary approach consisting of many imaging data together with emerging technologies could assist and improve the diagnosis and treatment of LBP [139].

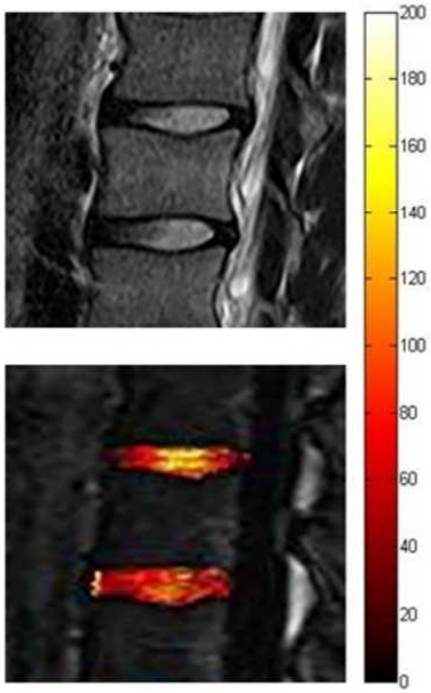


Figure 9. Color red in lower panel (T1ρ images) indicated early disc degeneration at the levels L3–L4 relative to L2–L3, despite in the upper panel (T2-weighted image) the signal intensity of the two discs were comparable.

1.2.2 Treatment of IDD

The current treatment options for IDD are predominantly limited to pain relief by physical therapy, use of drugs, opioids, epidural steroid injections or surgical approaches. These treatment strategies are able to relieve symptoms and improve quality of life rather than to exacerbate the disease, although many patients respond have little or no long-term benefits in the short term generally [140].

1.2.2.1 Conservative treatment

Non-drug treatment mainly includes rest, physiotherapy, acupuncture, Yoga, manual therapy and psychotherapy. Other passive physical modalities, such as the use of ultrasound, transcutaneous electrical nerve stimulation, traction, shortwave, diathermy, are generally used to reduce muscle spasm and improve mobility in combination with pharmacological treatments [141]. US guidelines, recommend non-pharmacological

care as the first therapeutic option and adopt pharmacological support only in patients which not react to non-pharmacological treatments. These guidelines highlighted the importance of physical exercise in recovering paraspinal muscle strength and encouraged to avoid bed rest by continuing with normal activities including work. Physiotherapy is suggested when recovery is slow or for patients with risk factors due to persistent disabling pain [142,143]. Currently, there are no guidelines recommending targeted exercise programs, however, the training should be prepared on the individual needs of patients with persistent LBP. In this regard, Papalia *et al*. in a systematic review reported a significant increase in the prevalence and intensity of LBP during the COVID-19 pandemia related to long quarantine periods, the reduced rate of physical activity and prolonged sitting time without adequate ergonomic supports during smart-working [144].

The guidelines also recommend to consider psychological aspects of therapies for patients who have a poor response to first-line therapy and who are functionally impaired by pain [145].

In recent decades, there has been less emphasis on pharmacological and surgical treatments in clinical practice, by favouring alternative approaches focused on both the functional and psychosocial needs of patients.

1.2.2.2. Drug therapy

Currently, guidelines recommend pharmacological treatment only following an inadequate response to first-line non-pharmacological approaches.

The commonly used drugs for the treatment of LBP are non-steroidal anti-inflammatory drugs (NSAIDs), opioids, painkillers, muscle relaxants, benzodiazepines, antidepressants, corticosteroids, antiepileptic drugs ect. NSAIDs are widely used to treat patients with LBP. The Guidelines of the American College of Physicians recommend a careful use of non-selective and selective COX-2 NSAIDs for acute or subacute LBP due to gastrointestinal and cardiovascular problems [143,146]. Opioids are used primarily in patients with acute, severe pain attacks whereas it has shown only modest pain relief in patients with chronic LBP. Although, opioids may be more effective than other analgesics for both neuropathic and non-neuropathic pain, the dosage and duration

of therapy are controversial due to their potential dependence [147]. As adjunctive therapy, muscle relaxants and benzodiazepines may relieve muscle spasm but with an increased risk of central nervous system adverse effects such as lethargy and vertigo. Antiepileptic drugs such as gabapentinoids and tricyclic antidepressants are also recommended for the treatment of neuropathic pain [148]. Epidural steroid injections are another pharmacological option to attenuate LBP by inhibiting the production of inflammatory molecules, which can be a source of pain. However, their long-term beneficial effects are still unclear [149].

In clinical practice the severity of pain, the duration of symptoms, and cost of treatment should be taken into account because it may represent the only therapeutic strategy in patients with multiple areas of pain, or who cannot undergo surgery due to high risk of complications.

1.2.2.3 Gene Therapy

Gene therapy originated as modality to treat rare genetic diseases with the goal of replacing defective genes with functional copies. A benefit of this strategy in IDD is that the alteration of the recipient cell's DNA affects not only cell metabolism but induces a constitutive expression of the inserted target gene. Exogenous genetic material, such as ECM components or immune-modulators, is transferred into target cells, to modulate the molecular mechanisms triggering the disease and the transgene is transcribed into mRNA and translated into the wanted protein product [150,151]. The three main approaches employed in IDD are through viral vectors, non-viral vectors and gene editing. The advantage of a viral vector is that it can replicate and proliferate efficiently in cells, but its safety needs to be confirmed [152]. An example of a viral vector is the retrovirus (single-stranded RNA) which can efficiently transfect IVD cells. For the first time Wehling and colleagues performed a retrovirus-mediated transfer of the bacterial enzyme marker gene β-galactosidase (LacZ) and human IL-1 receptor antagonist cDNA in bovine endplate chondrocytes. The results showed that despite the low rate of transduction, the expression of the IL-1 receptor antagonist transgene of interest was significantly increased compared to controls after 48 hours [153]. Adenovirus vectors showed good stability *in vitro* and are easy to purify. Nishida *et al.*

performed the first adenovirus-mediated gene transfer of LacZ marker transgene in New Zealand white rabbits. This study showed an efficiency rate of nearly 100% with no immune response up to 3 months post-transduction [154]. Furthermore, lentiviruses are rather chosen to carry a relatively large genetic amount, adeno-associated viruses have low immunogenicity while little is still known about the role of baculoviruses in IDD [152].

Non-viral vectors including inorganic, silicon and natural polymeric nanoparticles have a number of advantages related to their little volume, high gene transduction, low or lack of cytotoxicity and immunogenicity [155]. In last decade, EVs caught the attention of many groups of research as potential carriers. EVs are lipid bilayer membrane structure released by many types of cells through paracrine mechanism with a low immunogenicity and good cytocompatibility. These vesicular structures have an active biological role in intercellular communication by delivering cell membrane and cytoplasmic proteins, lipids and RNAs. Several studies demonstrated the therapeutic potential of EVs in the treatment of IDD by promoting cell proliferation, inhibiting NPCs apoptosis and ECM synthesis and delaying IDD [156]. However, this topic will be well explored in the next chapter.

Finally, a more recent discovery is the technology of gene editing by DNA nuclease which modifies the genome in a site-specific manner. CRISPR/Cas9 system is based on the use of a sort of molecular scissor, the Cas9 protein, which makes specific modifications to the target DNA. Following cutting, it is possible to eliminate or replace damaged sequences from the target DNA by correcting disease-causing mutations and carefully controlling the phenotype of the treated cells [157]. For instance, in IDD CRISPR/Cas9 system can directly inhibit the expression of cytokine receptors or markers of senescence to attenuate the disease.

Thus, gene therapy shows excellent potential in the treatment of IDD but there are still several challenges including long-term safety and stability of *in vivo* expressed genes, low efficiency of target gene transfer, changes in biological characteristics of target cells and ethical issues.

1.2.2.4 Surgical treatment

When non-surgical treatments are ineffective and clinical and imaging findings indicate associated symptoms with herniated discs or spinal stenosis, alternative approaches such as decompression surgery, spinal fusion, IVD replacement surgery and endoscopic resection of degenerated disc tissue are used [158]. For herniated disc, surgery results in more rapid relief of radiculopathy than conservative treatment although no substantial differences were observed after 1-2 years [159]. Similarly a recent randomized clinical trial showed encouraging results in patients with secondary sciatica compared to conservative treatment [160].

Spinal decompression is indicated for people with radicular pain not improved by conservative treatments and with correlation between radiological evidences and symptoms. One systematic review reported that in patients with lumbar spine stenosis pain was improved at 2-4 years follow-up by decompression surgery [161].

Patients with LBP or refractory spondylosis might undergo to lumbar fusion. In patients treated with lumbar laminectomy with or without fusion, the incidence to have recurrent LBP is from 10% to up 40%. The causes could be different, both related to the complications of the surgery and the patient [162].

Disc replacement is generally limited to people with predominantly discogenic pain in one or two segments and may have, short-term but not clinically, meaningful benefits [163]. With the development of biomaterials and the advent of artificial IVD, the disc replacement has became successful. At present, a large number of studies have tested the safety and efficacy of this treatment options with a clinical efficacy comparable to spinal fusion by providing increased mobility of the lumbar segments that IVD fusion surgery cannot provide [164]. Other options include intradiscal electrothermal injection therapy radiofrequency myeloplasty, percutaneous discectomy, with the common goal of reducing nerve root compression and pain. Chemical nucleolysis injection using ozone, for example, can reduce NP herniation and pain-triggering inflammatory reaction [140].

In addition, in the last years, robot-assisted minimal invasive surgery has attracted many physicians for the low risks of damage to neurovascular structures, high precision and stability [165].

1.2.2.5 Cell-based therapy

In cell therapy the drug injected in IVD is the cell itself. The use of cell-based therapies for IDD involves injecting cells into the region of the NP to repair or at least modulate the degenerated environment. In the current scenario, MSCs are considered safe, easy to collect from healthy and young donors as well as from autologous sources and show a strong therapeutic relevance in the field of regenerative medicine [166,167]. MSCs are non-hematopoietic pluripotent stem cells obtained from various tissues such as: bone marrow, adipose tissue, umbilical cord, Wharton jelly, placenta, amniotic fluid, dental pulp and other sources. When cultured *in vitro*, they have the ability to plastic-adhere and to differentiate into adipocytes, osteoblasts and chondroblasts. They are also considered immune-privileged cells since they do not express major histocompatibility complex-II (MHC-II) and express surface markers such as CD90, CD105, CD73 [168]. To date, MSCs are widely exploited in regenerative medicine due to their potential to repair tissue damage directly and to their immunomodulatory properties by producing a "cocktail" of paracrine factors, defined as MSCs secretome including EVs, cytokines, growth factor ect. The composition of the MSCs secretome and EVs content was studied to identify the key molecules responsible of the pro-regenerative effects of MSCs consisting in modulation of the immune system, inhibition of cell death and fibrosis, promotion of tissue remodeling and recruitment of other cells [169]. Interestingly, a large amount of data has suggested how *in vitro* preconditioning of MSCs can influence and potentiate the therapeutic potential of the MSCs secretome. The preconditioning has evaluated a serious of factors such as: 3D culture, drugs, inflammatory cytokines, growth factors such as insulin-like growth factor 1 (IGF-1), growth differentiation 6 (GDF-6), fibroblast growth factor (bFGF or FGF2), transforming growth factor β (TGF-β), bone morphogenetic proteins (BMPs), and hypoxia [170].

In a recent review Williams *et al.* reported the cellular sources currently applied in IDD treatment research which include NPCs, bone marrow mesenchymal stem cells (BMSCs), fat-derived mesenchymal stem cells (ADMSCs), NP mesenchymal stem cells (NPMSCs), Wharton jelly umbilical cord mesenchymal stem cells (WJ-MSCs) and pluripotent stem cells (IPSCs) [171]. *In vitro* studies have extensively demonstrated the efficacy of MSCs in the recovery of NPCs isolated from degenerated IVDs by

enhancing ECM expression and synthesis, promoting the up-regulation of phenotypic markers of the NPCs, inhibiting cell death BMSCs, reducing cellular aging and TGF-β/NF-κB signal transduction [172-174]. On the other hand, numerous preclinical and clinical studies have tested the safety and efficacy of IDD treatment in a variety of animal models using different types of MSCs. Sakai *et al.* collected the basis for the follow-up study with BMSCs in IDD after observing these cells can delay IDD in the rabbit [175]. The results encourage the application of cell therapy as MSCs were able to restore homeostasis in the degenerate environment of IVD by: 1) differentiating into NP-like cells 2) supporting resident cell to recruit local progenitor cells to induce endogenous repair 3) exerting paracrine signaling capable of promoting anabolic switch in native cells [35,176].

Orozco *et al.* are the authors of the first clinical pilot study using the injection of autologous BMSCs to evaluate the recovery of pain and disability after 3 months of treatment in ten patients unresponsive to conservative treatment for 6 months with lumbar IDD and chronic LBP. BMSCs of enrolled patients were harvested and isolated from bone marrow of the iliac crest. 3-4 weeks after the first procedure, the patients received a second intradiscal BMSCs injection. Follow-up occurred at 1 week, 3, 6 and 12 months after injection. LBP-associated disability was assessed using the visual analogic scale (VAS) and Oswestry Disability Index (ODI). Although MRI showed increases in water content 12 months after the injection, disc height did not improve [177]. Elabd and colleagues, similarly, recruited five patients with IDD who received intradiscal transplantation of autologous BM-MSCs isolated and cultured in hypoxic conditions. The data showed clinical improvement at 4-6 years of follow-up. The limitation of these studies was the small number of patients and the absence of intermediate time points even if the treatment with BM-MSCs has been shown to be safe in the long term [178]. The first randomized controlled trial (RCT) conducted by Noriega *et a.l* evaluated the feasibility and safety of allogeneic BM-MSCs use in a cohort of 24 patients with IDD in a phase I/II study where the control group was injected with a sham infiltration. In their 1-year follow-up, the authors recorded a significant improvement in VAS and ODI values and Pfirrmann classification compared to the baseline. Additionally, the researchers identified a group of non-responders (58% of the treatment group) in whom treatment was ineffective [179]. Another phase I

clinical trial in 2017 enrolled 10 patients unresponsive to conventional treatment for 3 months, with a Pfirrmann score 3-4 to 1 or 2 levels. Three weeks before the injection, participants underwent abdominal liposuction. ADSCs were isolated, expanded and subsequently injected into IVD tissue of patients in combination with hyaluronic acid. Two doses of ADSCs were tested, but no significant difference was found between them. After 1 year, the VAS pain and ODI scores of six patients improved significantly, and three of these patients had intervertebral disc rehydration. As in previous studies, no adverse events were reported. Moreover, the authors emphasized that potential causes of treatment failure are related to careful patient selection [180]. In a prospective phase I study, Comella *et al.* evaluated the effect of an intradiscal injection of autologous stromal vascular fraction (SVF) mixed with platelet-rich plasma (PRP). The safety and efficacy of ADSCs administration, involved 15 patients with IDD. Patients showed improvements in several parameters including lumbar flexion and significant reduction in VAS at 2 and 6 months. In a study published in 2015, Pettine *et a.l* evaluated the effects of BMSCs injection in 26 patients with discogenic LBP. 12 months post injection, 21 of 26 patients showed clinically meaningful improvements in ODI and VAS scores, and eight patients improved the Pfirrmann grade with better treatment efficacy related to the concentration of cells injected at higher doses [181].

In another recent phase II RCT, 100 patients suffering from chronic LBP were recruited. Amirdelfan *et al.* compared 2 doses of allogeneic mesenchymals precursor cells while the control group was administered only the saline and hyaluronic acid vehicle. Treated patients showed significantly greater improvement in LBP and ODI than control groups [182]. An improvement in VAS and ODI was also reported in two patients treated with allogeneic UC-MSCs [183]. Finally, a randomized placebo-controlled phase IIb study to evaluate the efficacy of allogeneic BMSCs in the degenerated discs of 112 patients has been just concluded [184,185]. However, the hostile microenvironment of the IVD can drastically compromise MSCs survival. Thus, incorporating cells into biomaterial scaffolds, hydrogels, and vectors can be possible to bypass this issue supporting cells engrafment, proliferation and differentiation in the IVD [186]. Biomaterials such as natural collagen, chitosan and hyaluronic acid, as well as synthesized carbon fibers, hydrogels, polylactic acid, polyglycolic acid have low immunogenicity, non-toxicity, degradability and specific mechanical support properties [187]. Recently, Vadalà *et al.*

described a new surgical approach to repair NP damage via transpedicular approach without AF rupture in an IDD preclinical ovine model. This type of surgery could represent an useful and simple method to reach NP with the wanted biological therapy and may contribute significantly the translation of therapies into the clinical practice [188].

Although encouraging results have been achieved in the application of MSCs for the treatment of IDD, there are still some points to clarify. The main limitations are the suitable selection of donor cells, the lack of standardized protocol for culture procedures, the choice of patients to be treated in relation to the degree of the pathology, the unwanted differentiation into other cell lines and the formation of osteophyte, related to the potential migration of MSCs or their leakage from the IVD. In addition, the route, timing and adequate dose of treatment are still controversial. Furthermore, the results obtained in animal models cannot be directly translated to humans due to different biomechanical properties, different IVD structure, size, cellularity, shorter life span and non-physiologic onset of IDD in animals [189,190]. New therapeutic methods are emerging, including cell-free treatment strategies. MSCs can generate EVs that deliver anabolic messages (miRNA, long non-coding RNA) to recipient cells with equivalent therapeutic effects.

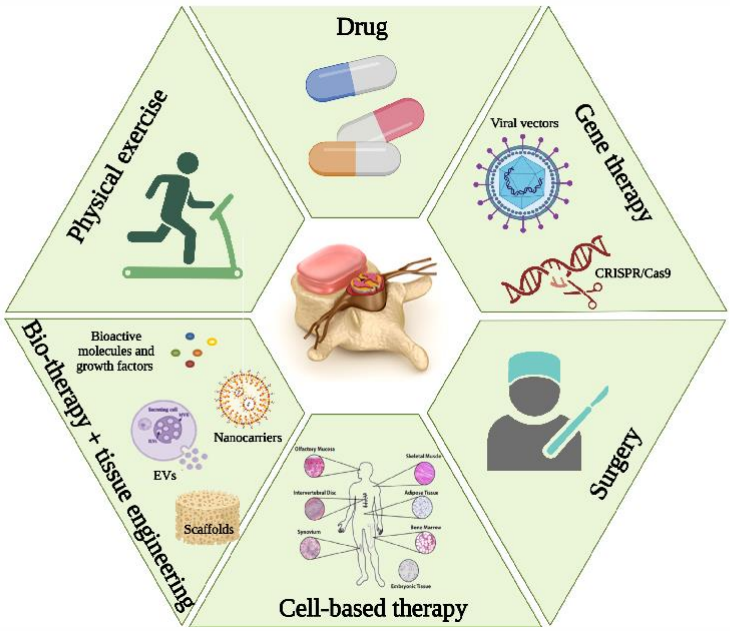


Figure 10. Summary of treatment approaches in intervertebral disc degeneration (IDD). Created with BioRender.com

2. POTENTIAL THERAPEUTIC ROLE OF EVs IN IDD

2.1 EVs NOMENCLATURE

The role of paracrine activity of MSCs in promoting IVD regeneration has been extensively studied. A crosstalk between MSCs and NPCs is mediated by the secretion of trophic factors including EVs. Such exosomes and microvesicles may “reprogram” target cells thanks to their ability to transport proteins, lipids and nucleic acids [191]. Hingert *et al.* investigated the content of peptides present in the conditioned medium of human MSCs and their effects on both degenerated disc cells and MSCs pellets. The results of the mass spectrometry analysis revealed 129 secreted peptides which included growth factor modulators, anti-apoptotic and anti-angiogenic factors, MMPs inhibitors [192]. In a co-culture system, increased mRNA expression of the genes SOX-9, COL1A1, COL2A1 and ACAN were observed by explaining the mechanism of BMSCs and NPCs interaction in a 3D environment, excluding cell-cell direct contact [193]. Although the classification of EVs is constantly evolving, based on their size, content and formation mechanisms, they are suddivided into three main categories: apoptotic bodies, microvesicles and exosomes (Fig. 11) [194].

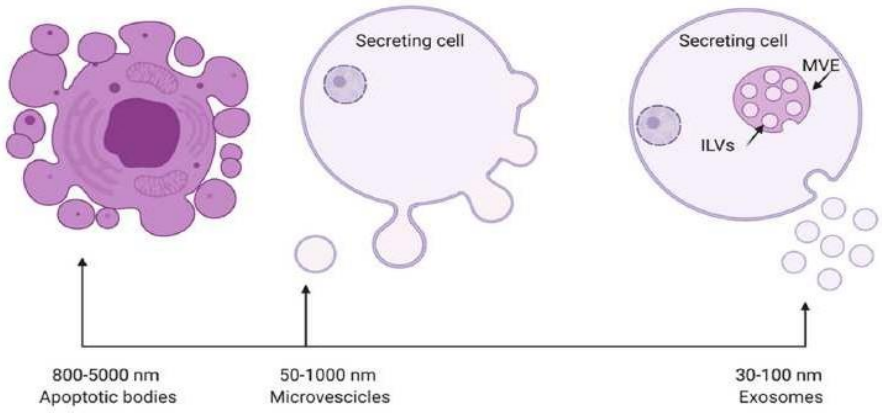


Figure 11. Extracellular vesicles distinguished by size: apoptotic bodies (800–5000 nm), microvesicles (50–1000 nm) and exosomes (30–100 nm) [195].

The largest EVs are the apoptotic bodies released during apoptosic process with a heterogeneous morphology. Microvesicles were first isolated from platelets and originally described as “platelet dust” with a diameter of 50–1000 nm, a spherical structure and expelled through budding of the plasma membrane. In contrast, exosomes have endosomal origin with a size range of 30–130 nm and a cup-shaped morphology (Table 3). However, the overlapping range of size may lead to a confused terminology, therefore further standardized protocols are expected to establish an appropriate nomenclature for EVs [195]. Exosomes were first discovered during maturation of sheep’s reticulocyte in which the release of transferrin receptors were correlated with a small vesicle [196]. This class of EVs arouses major interest in biology since the biogenesis mechanism makes a “unique” intracellular content and determins their function, once secreted into the extracellular space [197].

Table 3. General features of exosomes, microvesicles and apoptotic bodies [195]

	Exosomes	Microvesicles	Apoptotic Bodies
Size	30–100 nm	50–1000 nm	800–5000 nm
Morphology	Cup-shaped	Spherical structures	Heterogeneous
Content	Coding RNA, noncoding RNA, proteins, lipids, DNA	RNA, proteins, lipids, cytosol	Proteins, lipids, DNA, RNA, cytosol
Biogenesis	By exocytosis in which ILVs within the lumen of MVEs fuse with the plasma membrane to release ILVs	By outward budding and fission of the plasma membrane and the sub-sequent release of vesicles into the extracellular space	Outward blebbing of the cell membrane

ILVs = intraluminal vesicles; MVEs = multivesicular endosomes.

2.1.1 EVs biogenesis and cell-cell communication

Microvesicles or ectosomes are formed by budding of plasma membrane. This biogenesis requires the intervention of Ca^{2+} dependent enzymes such as flippases, floppases and scramblases which cause various molecular rearrangements within the plasma membrane, including exposure of phosphatidylserine from the inner leaflet to the cell surface driving membrane curvature and favouring budding and formation of

microvesicles. Lipid components such as cholesterol, cytoskeletal elements and small GTPases of the RHO-associated protein kinase (ROCK) induce biogenesis of microvesicles [191,194,198].

The first step of exosome biogenesis is the endocytic formation of an early endosome that incorporates cell surface and soluble proteins of the extracellular environment, which can subsequently mature into a late endosome and generate multivesicular bodies (MVBs). Loading and biogenesis of exosomes are regulated by endosomal sorting complex required for transport (ESCRT). ESCRT consists of 4 complexes: ESCRT-0 involved in the clustering of cargo in a ubiquitin dependent manner on membrane of the MVBs; ESCRT-I and –II induce ILVs budding; ESCRT-III drives ILVs cleavage by proteins such as TSG101 and ALIX while ATPase VPS4 enables complex dissociation and recycling ESCRT-III [199]. Exosomal biogenesis can also occur by ESCRT-independent mechanisms. Neutral sphingomyelinase inhibition has been shown to lead to impaired exosome secretion by limiting ceramide synthesis which is involved in invagination of the MVB's limiting membrane to form ILVs [200]. Similarly to ceramide, phosphatidic acid (PA) is required for exosome biogenesis to induce inward bending and thus the formation of ILVs. Therefore, phospholipase D2, responsible of the hydrolysis of phosphatidylcholine to PA, is enriched in exosomes [201]. On the other hand, four-transmembrane domain proteins of the tetraspanin family CD9, CD81, CD63 and heat shock protein 70 (Hsp70) are involved in ESCRT-independent biogenesis. Tetraspanins have been recognized as surface markers in exosomes [202]. Endosomal membrane budding is also promoted by the syndecan–syntenin–ALIX pathway. Indeed, syndecan heparan sulphate proteoglycans are connected to their cytoplasmic adapter syntenin, which interacts with ALIX (component of ESCRT III) [203]. The fate of MVBs is counterbalanced by their fusion alternatively with the plasma membrane and the release of exosomes in the extracellular *milieu* or with the autophagosome promoting their degradation. The process of degradation of damaged cellular components in the lysosomes provides the maintaining of cellular homeostasis [204]. The molecular mechanisms that regulate this balance and the secretion process are still unclear. The small GTPase proteins of the RAB family (RAB11, RAB27 and RAB35) control different steps of intracellular vesicular trafficking from vesicle budding to mobility towards plasma membrane through cytoskeleton interaction. In

addition, SNARE proteins are probably involved in fusion of these MVBs with the plasma membrane [205, 206]. It is probable that the EVs released from the parental cells interact with the target cells by: i) direct stimulation of ligands bound to the surface receptor**;** ii) direct membrane fusion with transfer of activated receptors to recipient cells and iii) internalization via endocytosis and epigenetic reprogramming of target cells via delivery of functional proteins, lipids and RNA (Fig. 12) [208-210]. Several mediators, for example, integrins on extracellular vesicles can interact with intercellular adhesion molecules (ICAMs) or with ECM proteins, fibronectin and laminin, present on the surface of recipient cells. In addition, the exosomal tetraspanins, heparan sulfate proteoglycans, lectins and lipids could regulate cell targeting [211-213]. The spontaneous formation of a protein "*corona"* has recently been detected around EVs composed of numerous molecules that are adsorbed to the surface of EVs during release from the originating cell by non-specific mechanisms. Among these proteins, specific ligands of surface receptors (i.e integrins) can facilitate or disfavour the uptake of nanoparticles [214].

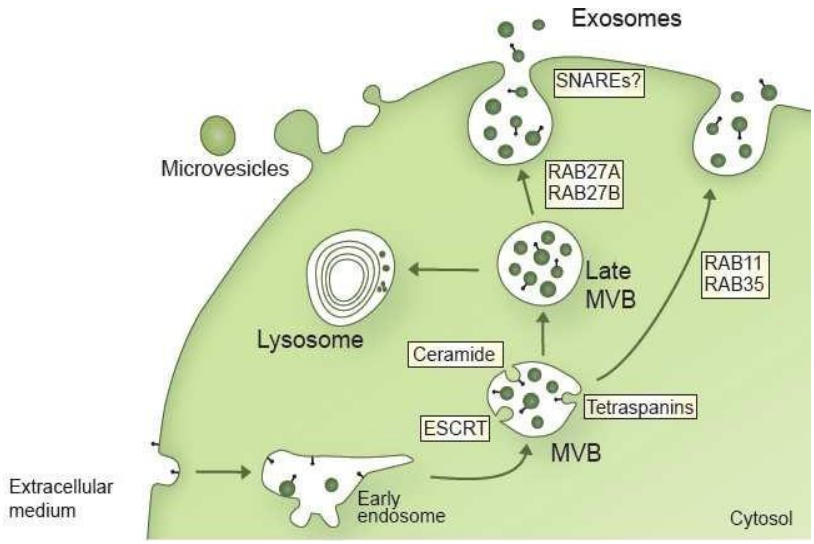


Figure 12. Schematic representation of the EVs biogenesis and release of exosomes. Microvesicles budding from the plasma membrane. Exosomes are formed as ILVs by budding into early endosomes and MVBs. through the ESCRT machinery or ESCRT- indipendent mechanism [198].

2.2 EVs CHARACTERIZATION AND ISOLATION

Several techniques have been suggested for the identification and isolation of EVs, in particular, exosomes. Transmission electron microscopy (TEM) is used to characterize the morphology of the EVs which usually appear as cup-shaped entities (Figure 13). The study of size and quantity is performed by nanoparticle tracking analysis (NTA) which detects individual vesicles under Brownian motion. Furthermore, their phenotype could be rapidly determined by Western blot or flow cytometry to identify surface markers such as tertaspanins CD9, CD63 and CD81 or Alix, TSG101, HSP70 [215].

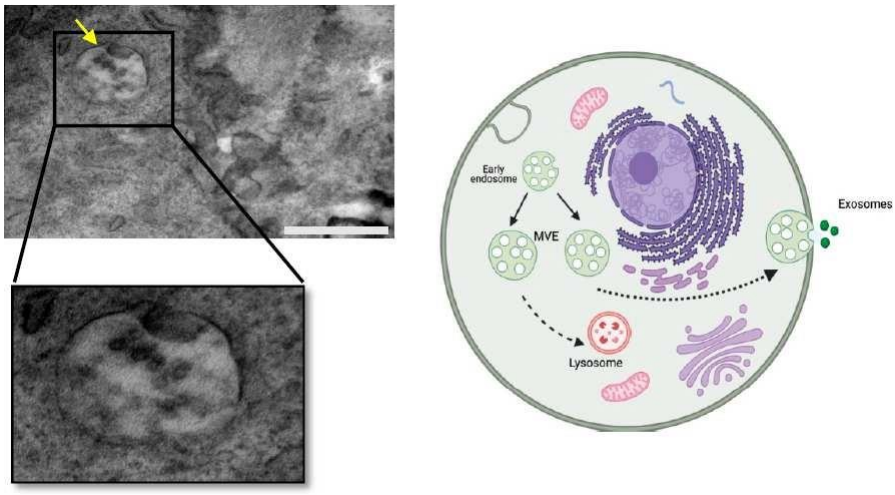


Figure 13. Visualization of MVEs by electron microscopy (scale bar = 1 μm). Biogenesis process of ILVs by budding into early endosomes. MVBs release exosomes by fusion with the plasma membrane or fuse with lysosomes. ILVs = intraluminal vesicles; MVBs = multivesicular bodies [195].

In general, EVs can be isolated from various body fluids (i.e. semen, plasma, urine, blood, saliva, breast milk, amniotic fluid, ascites fluid, cerebrospinal fluid, bile, and conditioned cell culture media) by differential centrifugation, size exclusion chromatography (SEC), immune affinity capture, tangential flow filtration and commercially available kits or microfluidic technologies [198]. A series of centrifugation steps removing cells and microvesicles, followed by high-speed ultracentrifugation and subsequent density gradient purification on a sucrose cushion is the best method to obtain a highly pure exosome fraction. However, this technique has limitations for exosomes derived from urine and serum since the final product contains

a mixture of multiple components, possibly co-sedimentation of protein aggregates [216]. It has been proposed that the combination of tangential flow filtration and SEC may become the standard for many laboratories in the near future. Ultrafiltration devices use dead-end filtration, in which the medium is applied perpendicular to the membrane to flush the entire volume while the SEC allows to efficiently eliminate the contaminating materials [217, 218]. A disadvantage of high-pressure filtration could be the fragmentation of larger micro particles into smaller vesicles. Another method is based on immunoaffinity pull-down with the use of antibody-loaded magnetic beads that select specifically vesicular surface markers but with low yields [219]. In recent years, several commercially available kits have emerged to enrich exosomes, reducing greatly operation time resulting to be efficient, reliable, and reproducible when compared to other methods [220].

However, further challenges include reproducibility and consistency of product batches, and inadequate quality control and standardization. Therefore, much more research is needed for robust validation of these methods to be successfully implemented in clinical diagnosis.

2.3 EVs AS THERAPEUTIC TOOL

EVs may represent a natural "bio-nanomedicine". With the rapid progress of nanotechnology, EVs have new applications: as biomarkers for the screening and diagnosis of various pathologies and as nanocarriers of drugs or biomolecules for therapeutic purposes [221]. By recurring to proteomics and biophysical techniques it is possible, for diagnostic purposes, to quantify and analyze the cargo carried by EVs isolated from biological fluids [222]. In this regard, the role of microRNAs and small interfering RNAs is relevant in cell targeting [223].

EVs can also serve as “packaging” for drug delivery. Genetic engineering allows to modify cells before EVs production thus by incorporating secreting cells-derived mRNA or protein [224]. The efficiency of encapsulation of EVs depend on the physicochemical properties, carrier characteristics and active components of the cargo [225]. Another modality involves the incorporation of exogenous load into EVs through co-incubation, sonication, heat shock and electroporation [226]. For example, chemical

modification of the EVs'membrane by increasing chemokine ligand expression on tumor cell-derived EVs promoted their internalization into tumor cells expressing the chemokine receptor [227]. Likewise for MSCs, EVs can also be combined with tissue engineering to achieve tissue repair and regeneration. Hyaluronic acid, hydrogel and bioink have been tested for the treatment of early osteoarthritis (OA) and for osteochondral defect [221, 228].

2.3.1 Advantages over cell-therapy in IDD

Therapies with the application of MSCs-derived EVs avoid some problems of cell based-therapy. The abnormal differentiation of exogenous stem cells, limited survival in IVD microenvironment and the release of catabolic factors from dead cells with peripheral inflammation may generate harmful responses of endogenous cells [197]. Compared to MSCs, EVs have low immunogenicity by maintaining the regenerative capacity of stem cells as chemical messengers.

Compared to synthetic nanoparticles, EVs can provide a number of advantages since they have cell-based biological structures and functions. Natural biocompatibility, highest chemical stability, long-distance intercellular communication, fusion, targeted delivery, ability to penetrate tissue structures such as the blood-brain barrier with a low risk of systemic toxicity commonly found in other nanomaterials [229].

2.3.2 Disadvantages

EVs heterogeneity is the prickliest issue due to both the production/content of EVs closely related to the pathophysiological state of the secreting cell and the difficulty to purify a specific EVs subpopulation in line with current techniques [230]. Therefore, MSC-derived EVs should be studied and applied according to certain specifications. For this reason the International Society for Extracellular Vesicles (ISEV) recommends the use of the collective term "EV" unless the biogenesis pathway is demonstrated [231].

3. EXPERIMENTAL STUDY

3.1 BACKGROUND

The limitations of MSCs cell therapy are related to limited cell survival due to the hostile microenvironment of the disc [117-119]. Growing evidence suggests that MSCs exert their therapeutic effects through the secretion of paracrine signaling and bioactive molecules such as EVs [197, 232]. However, the mechanisms behind EVs therapy are not fully understood and needed further investigation. Indeed, the findings of several studies regarding the use of MSC-derived EVs, above all exosomes, in IVD regeneration and repair field demonstrated to revert ECM homeostasis, promote cell proliferation, reduce cell death, and attenuate inflammatory status [233].

3.1.1 Study hypothesis

EVs isolated from human WJ-MSCs may play an attractive role as an alternative cell-free therapy for IDD thanks to their regenerative potential through:

- attenuating inflammatory status
- stimulating matrix deposition
- restoring a healthy phenotype.

3.1.2 Study Objectives

Primary objective: The main objective of this study is to evaluate, the effects of human Wharton's Jelly MSCs (WJ-MSCs) derived EVs on degenerated hNPCs in an *in vitro* 3D model of disc degeneration.

Secondary objective: To explore the effects of WJ-MSCs derived EVs on hNPCs proliferation, viability, ECM production under inflammatory stimulation by mimicking the hostile intervertebral disc microenvironment.

3.2 MATERIALS AND METHODS

Isolation and culture of hNPCs

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Campus Bio-Medico University of Rome. Nucleus pulposus tissues were obtained from 10 patients undergoing discectomy for a lumbar disc herniation at Campus Bio-Medico University Hospital Foundation in Rome. All patients (n =10, mean age: 46 ± 16.26 years old, no comorbidities) provided their informed consent for the collection and use of surgical waste for research purposes. Nucleus pulposus specimens were washed three times with PBS, then cut into pieces and digested overnight at 37°C under gentle agitation in sterile Dulbecco's Modification of Eagle's Medium (DMEM; Corning, Corning, NY, USA) containing 1% penicillin/streptomycin, 1% glutamine (Sigma, St. Louis, MO, USA), 5% heat inactivated fetal bovine serum (FBS; Corning) and 0.01% collagenase type II (Worthington, Lakewood, NJ, USA). The digest was filtered through a 70- μm filter, the suspension was centrifuged at 300 × g for 5 min, and the isolated cells were cultured in DMEM with 10% FBS and 1% P/S and 1% glutamine incubated at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was replaced every 3 days, and cells were allowed to grow until reaching 80–90% confluence.

Isolation and characterization of Wharton's Jelly MSCs

Human WJ-MSCs were obtained from National Cancer Institute (Lithuania). Healthy donors (delivering mothers 18 – 44 years old) provided informed consent for the use of cord tissue in this experiment. MSC at 1–6th passages were seeded in DMEM (with low glucose, 10% FBS, 2mM L-Alanyl-L-Glutamine, 40 μg/ml Gentamicin) with a density of 4×10^3 cells/cm^2 on uncoated 5-layer cell culture Multi-Flasks and were cultured to 70–80% confluence. Then MSCs were washed three times using 50 mL of PBS without Ca^{2+} and Mg^{2+}, and replenished with DMEM with high glucose, without phenol red, 2mM L-Alanyl-L-Glutamine, 2% PRP lysate, 2 IU/ml Heparin, 40 μg/ml Gentamicin. Cells were cultured in standard conditions (7,5% CO2; 37 °C) for 1-2 days. For the detection of cell surface markers, MSCs were characterized by positive expression of

CD73, CD90 and CD105 and negative expression of CD45, CD34 and HLA-DR (Sony Biotechnology) using flow cytometry according to the manufacturer's instructions. Moreover, Alizarin red staining, Oil red O staining and Alcian blue staining were performed to confirm the multi-lineage differentiation potential of MSCs in osteogenic, chondrogenic, and adipogenic, respectively.

Isolation and characterization of WJ-MSCs derived EVs

When hMSCs reached 70–80% confluence they were washed with PBS and cultured in serum-free medium for an additional 24 hours at 37 ℃ in an atmosphere of 5% humidified CO_2. Conditioned medium was then collected, filtrated through 0,2 µm pore filter to remove cell debris and other extracellular vesicles higher than 220 nm. The filtered conditioned medium was subjected to tangential flow filtration using a VivaFlow 200 filter with a 100-kDa molecular weight cutoff (MWCO) and concentrated 10-fold (volume reduced 10-fold). Buffer is changed as normal PBS. The final product was sterile filtered using a 220-nm filter. The measurement of nanoparticle numbers and size distribution of EVs were analyzed by nanoparticle trafficking analysis (NTA) using the NANOSIGHT NS300 system (Malvern, UK) according to manufacturer's instructions. The morphology of the EVs was observed by transmission electron microscopy (TEM). Further, the EVs surface markers, such as CD9, CD81, and TSG101 (Invitrogen-Thermo Fisher) were detected by Western blotting assay (Figure 14).

EVs uptake by hNPCs

Purified WJ-MSC derived EVs were labeled with PKH26 (Sigma-Aldrich) for 5 min at room temperature. After washed in PBS and centrifuged at 110000 g for 90 min, the EVs were suspended in basal medium and coincubated with hNPCs for 4 h at 37 ℃. Then stained by Hoechst 33258 (Life Technologies - Thermo Fisher Scientific, US). Capture of images of EVs uptake was performed under confocal laser scanning microscope (Zeiss LSM700, Germany).

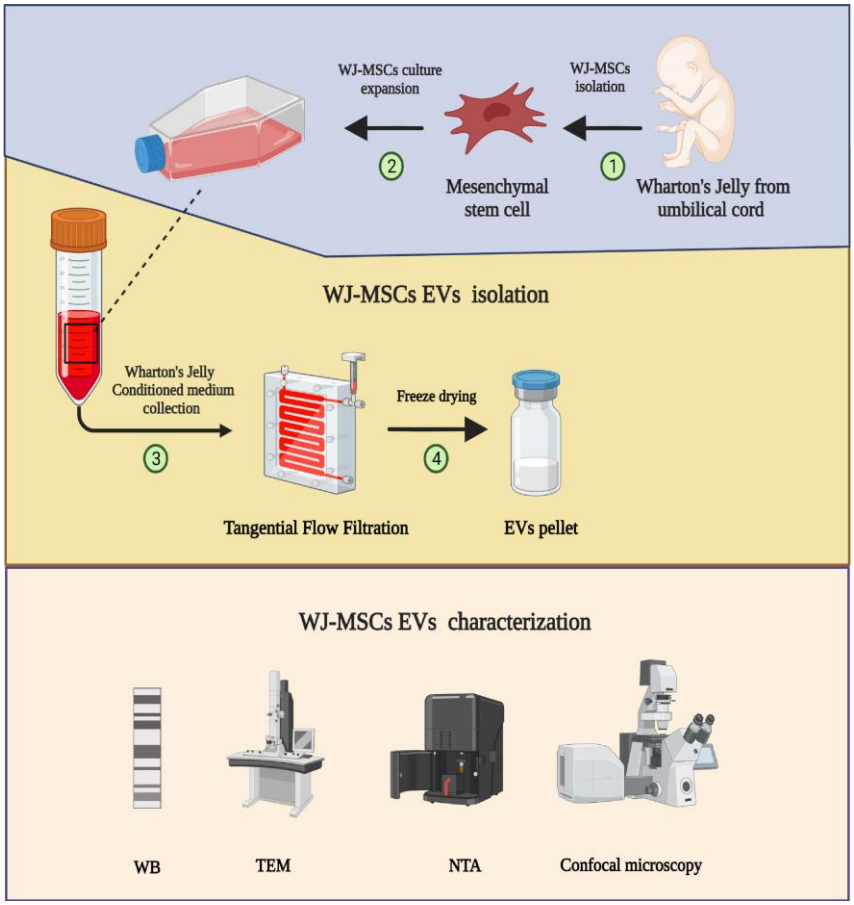


Figure 14. WJ-EVs were isolated by tangent filtration of Wharton's Jelly derived MSCs conditioned media. The EVs were quantified by BCA, vesicular morphology was characterized by TEM, WB analysis was performed for markers expression and NTA for vesicular size and quantification. Confocal microscopy was used to detect EVs PKH26-labeled uptake in hNPCs. Created with BioRender.com

hNPC metabolic activity

MTT assay (Sigma) was performed to evaluate hNPCs metabolic activity after EVs treatment at growing concentrations of 1, 10, 50, 100 and 200 µg/mL, according to the manufacturer's instructions. At the second passage, cells were transferred to 96-multi-well plates (5×10^3 cells/well). At 48 hours, after treatment as described above, DMEM was supplemented with 100 µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (c = 5 mg/ml) and cells were incubated for 4 h at 37°C. Thereafter MTT solution was removed. After addition of 100 µl of DMSO the

plates were incubated for 15 min at 37°C to dissolve the formazan crystals. Absorbance readings of DMSO extracts were performed at 570 nm with reference at 690 nm using a Tecan Infinite M200 PRO.

hNPCs encapsulation in alginate beads and treatment with EVs

hNPCs were then cultured in a three-dimensional culture system as previously described [234]. Briefly, adherent hNPCs at passage 2 were treated with trypsin-EDTA (Corning) washed and centrifuged at 1200 rpm for 5 minutes. Then, cell mixtures were resuspended in 1.2% low-viscosity sterile pharmaceutical grade alginate (Pronova Biopolymer, Drammen, Norway) solution at 4×10^6 cells/mL using a syringe with a 21-gauge needle into a 102 mmol/L calcium chloride solution to form semisolid beads. After 10 minutes of polymerization, the beads were washed. Cultures were then maintained in DMEM medium, 10% FBS in a 5% CO_2 and 95% air incubator. After two weeks, hNPCs in 3D were treated with either: i) DMEM with serum EVs-free as control; ii) chosen EVs concentrations at 10, 50 or 100 µg/mL; iii) interleukin 1β (10 ng/ml) to mimic inflammatory microenvironment of the disc; iv) pre-treatment with IL-1β for 24 hours and then co-incubation with EVs concentrations at 10, 50 or 100 µg/mL. The media were replenished every two days (Figure 15).

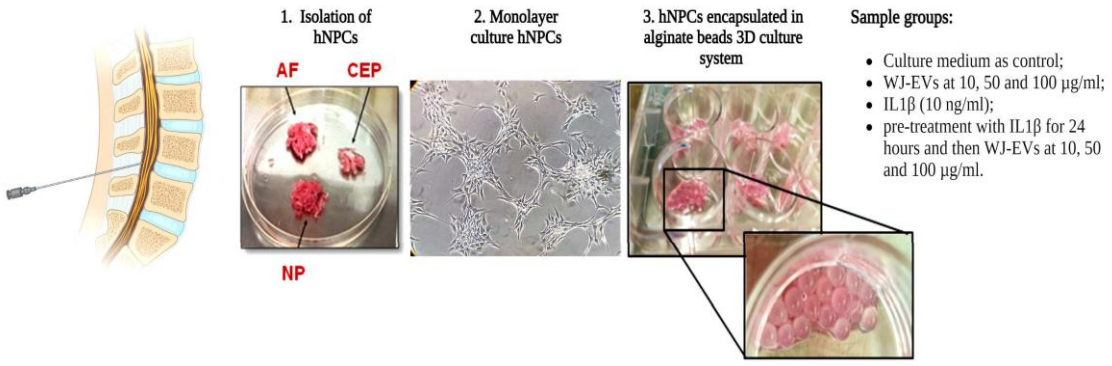


Figure 15. Human nucleus pulposus cells (hNPCs) isolated from surgical specimens, culture expanded *in vitro* and encapsulated in alginate beads. Created with BioRender.com

Cell count and viability

hNPCs in alginate beads were dissolved by incubation for 10 minutes at 4°C in 10 volumes of 55 mmol/L sodium citrate, 30 mmol/L EDTA, 0.15 M NaCl, pH 6.8 to analyze cell count and viability by CytoFlex instrument (Beckman Coulter) and using CytExpert Software, v.2.1. Cell count was assessed at 1, 4, 10 and 14 days expressed as event/µl. However, only after 24 hours to study the effect of EVs treatment on cell death, hNPCs were stained with Fixable Viability Dye conjugated with eFluor780 fluorochrome (Affimetrix eBioscience), using the following gating strategy (Figure 16). Similarly, cell viability was determined analyzing the membrane integrity through the the LIVE/DEAD Assay, following the manufacturer's instructions. hNPCs in 3D were incubated for 45 min with ethidium homodimer-2 and calcein acetoxymethylester (AM) at room temperature and washed 3 times with PBS and Hoechst 33258 (Life Technologies - Thermo Fisher Scientific, US) to stain the nuclei. After incubation, Green, red and blue fluorescence were detected using confocal laser scanning microscope (Zeiss LSM700, Germany) and quantified with ImageJ.

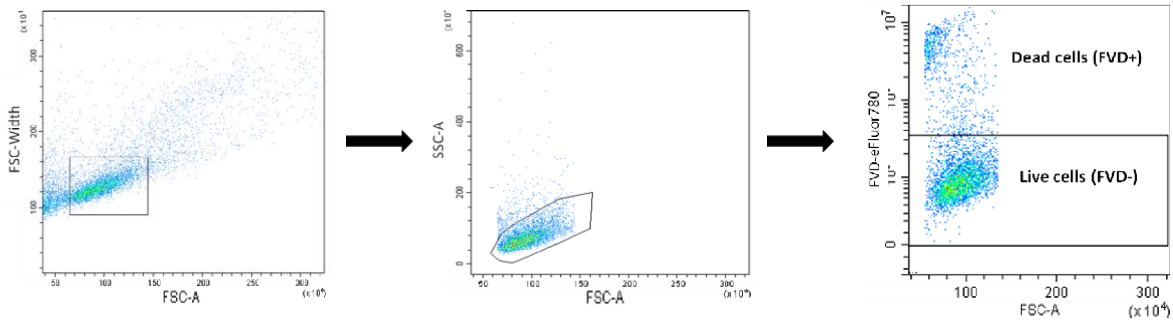


Figure 16. Gating strategy for NPCs viability. Cells are stained with Fixable Viability Dye conjugated with eFluor780 fluorochrome to exclude death cells (FVD+). Gating strategy based on Forward and Side scatter is shown.

1,9-dimethylmethylene Blue (DMMB)

After dissolving beads, hNPCs were digested with 100 µL of papain (Sigma) solution (0.25 mg/mL in 50mM phosphate buffer, pH 1.5 containing 5 mM cysteine–hydrochloride and 5 mM ethylenediaminetetraacetic acid) overnight with gentle shaking at 65 °C. GAGs were measured by reaction with DMMB (Polysciences, Warrington,

PA, USA) using chondroitin sulfate (Sigma) as a standard. Measurements of absorption were performed at a wavelength of 530 nm (Tecan Infinite M200 PRO). Data were expressed as GAG quantity normalized to DNA content using PicoGreen assay (Invitrogen, Carlsbad, CA, USA), comparing the percent variation between the control group and the experimental groups. DNA content was assessed using a standard curve based on known concentration of DNA to determine the DNA content. The sample fluorescence was measured using a microplate reader (Tecan Infinite M200 PRO) at 488 nm and 520 nm wavelengths, respectively.

Histological assessment

At seventh experimental day alginate beads previously fixed in 10% (v/v) phosphate-buffered formalin (Sigma, St. Louis, MO), paraffin embedded and cut into 5 μm sections according to standard procedures. Slides of consecutive sections were dewaxed, rehydrated, and stained with hematoxylin-eosin (H&E) and Alcian Blue (AB) to assess cell morphology and GAGs content, respectively.

Assessment of hNPC nitrite concentration

Griess reaction was performed to determine the nitrite concentration in the supernatant as an indicator of the production of nitric oxide (NO). Nitrites in the medium react with sulfanilic acid under acidic conditions forming a purple azo product which subsequently links to the naphthyl-ethylenediamine dihydrochloride suspended in water. After 1 week, each supernatant of treated hNPCs and control sample group was incubated with 20 μl Griess reagent (Invitrogen) for 30 min at dark. With a standard curve as the reference, nitrite concentration was calculated. The absorbance was then measured at 546 nm using a Tecan Infinite M200 PRO.

RNA Extraction and Gene Expression Analysis

Total RNA was extracted from pellets after alginate beads digestion using TRIzol reagent (Invitrogen). cDNA was produced using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) according to the

manufacturer's instructions. The mRNA levels of aggrecan (ACAN; Hs0153936), MMP-1 (Hs00899658), MMP-13 (Hs00233992), ADAMTS5 (Hs00199841) SOX-9 (Hs01001343), IL-6 (Hs00174131) NOS2 (Hs01075529) and GAPDH (Hs03929097) were measured through qRT-PCR using TaqMan Gene Expression Assays and TaqMan Universal Master Mix II with UNG-Real Time PCR System Instrument 7900HT FAST. qRT-PCR analysis for KRT19 was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). Primers for detecting differentiation-related genes are listed in Table 1. GAPDH was used to normalize the level of the interest gene. The expression level of each gene has been normalized to the expression of GAPDH and calculated as $2^{-\Delta\Delta Ct}$. Values in the experimental group were normalized to expression levels encountered in the control group, which was considered as a baseline.

Table 4 Quantitative real-time PCR primers.

Gene	Primer	Sequence (5′–3′)
KRT19	Forward	AACGGCGAGCTAGAGGTGA
	Reverse	GGATGGTCGTGTAGTAGTGGC
GAPDH	Forward	GAAGGTGAAGGTCGGAGT
	Reverse	GAAGATGGTGATGGGATTTC

Western Blot analysis

Total proteins were isolated from cell lysates using radioimmunoprecipitation assay buffer (RIPAbuffer; Sigma) for 30 min on ice, cleared by centrifugation for 30 min at 12000 g at 4 °C for 30 min and quantified using detergent compatible (DC) protein assay kit (Bio-Rad, Hercules, CA, USA). Each sample were loaded on 4-12% SDS-PAGE gels, transferred onto nitrocellulose membranes through the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA) and incubated in a blocking buffer (TBST 1X with 5% non-fat dry milk) for one hour. Membranes were incubated with primary antibody overnight shaking at 4 °C in TBST 1X with 1% non-fat dry milk. Anti-CD63 (mouse, 1:1000, Invitrogen), anti-CD9 (mouse, 1:1000, Invitrogen), anti-TSG101 (mouse, 1:1000, Invitrogen) anti-GRP75 (Rabbit, 1:2000, Proteintech) and anti-Tubulin (Rabbit, 1:5000, Sigma-Aldrich) were used. Anti-rabbit/mouse HRP-

conjugated antibody (1:10000, Abcam, Cambridge, UK) was used and the chemiluminescence signal detected using ChemiDoc (Bio-Rad, Hercules, CA, USA) and Quantity One so ftware (Bio-Rad, Hercules, CA, USA) to quantify the signal intensity of different bands. The experiments were performed in triplicate.

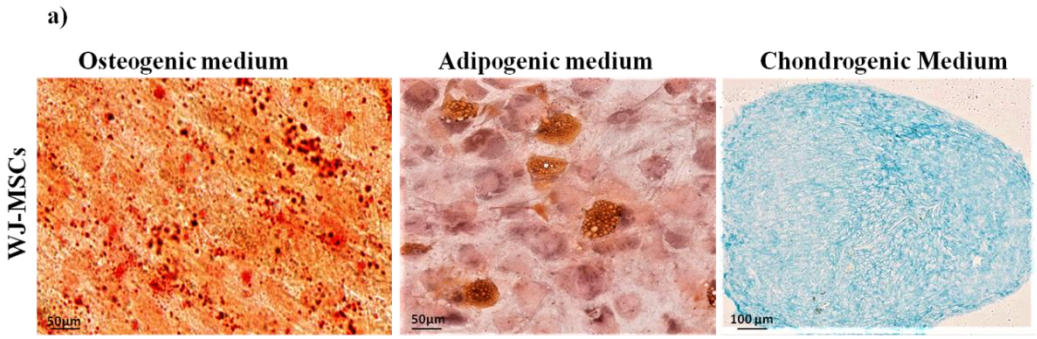
Statistical analysis

All quantitative data are expressed as means ± SD. The statistical analysis of the results was performed using one-way or two-way analysis of variance (ANOVA) with Dunnett's or Tukey's post-tests for multiple comparisons wherever applicable. Statistical significance was set as $p < 0.05$. Formal analysis was performed using Prism 8 (GraphPad, San Diego, CA, USA). Each experiment was performed in triplicate and representative experiments are shown.

3.3 RESULTS

Identification of human WJ-MSCs

Multilineage differentiation was confirmed by calcium deposits stained by Alizarin red staining after osteogenic differentiation and lipid droplets stained red by Oil red O staining after adipogenic differentiation (Figure 17a). Flow cytometric analysis showed that hWJ-MSCs were positive for CD90, CD73, and CD105 (> 90%) and negative for CD45 and HLA-DR (<5%), (Figure 17b) confirming the phenotypical characteristic of the isolated hMSCs.



b)

Marker	Results
CD73+	92,6%
CD105+	99,8%
CD90+	97,9%
CD45	0,04%
HLA-DR	0.99%

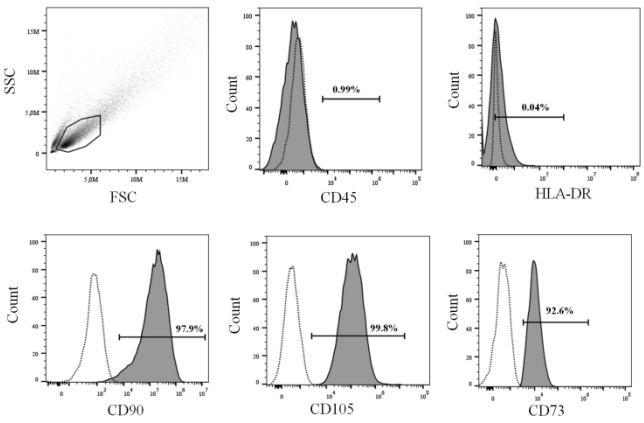


Figure 17. Identification of human Wharton Jelly MSC. **a)** Multilineage differentation of WJ-MSC into the osteogenic, chondrogenic, and adipogenic lineages was confirmed by Alizarin Red, Oil red O and Alcian Blue staining (scale bar: 50µm; scale bar: 100µm). **b)** Cell surface markers (CD90, CD105, CD73, CD45 and HLA-DR) detected by flow cytometric analysis.

Identification of MSC-EVs and uptake by hNPCs

EVs derived from WJ-MSCs were isolated with the methods above described. Results showed that the concentration of isolated EVs was 1.60 × 10^9 particles/mL, and the mean diameter of particles was 172.5 nm as measured by the NTA (Figure 18a). The morphology of these particles was confirmed by TEM analysis (Figure 18b). According to the WB results, the levels of protein expression of the EVs surface exosomal marker proteins such TSG101, CD9, and CD63 was much higher in EVs samples than cells (Figure 18c). Subsequently, under fluorescent microscopy PKH26-labeled EVs co-incubated with hNPCs were distributed throughout the perinuclear region of recipient cells as shown in Figure 18d, indicating that the nano-sized vesicles were internalized by hNPCs.

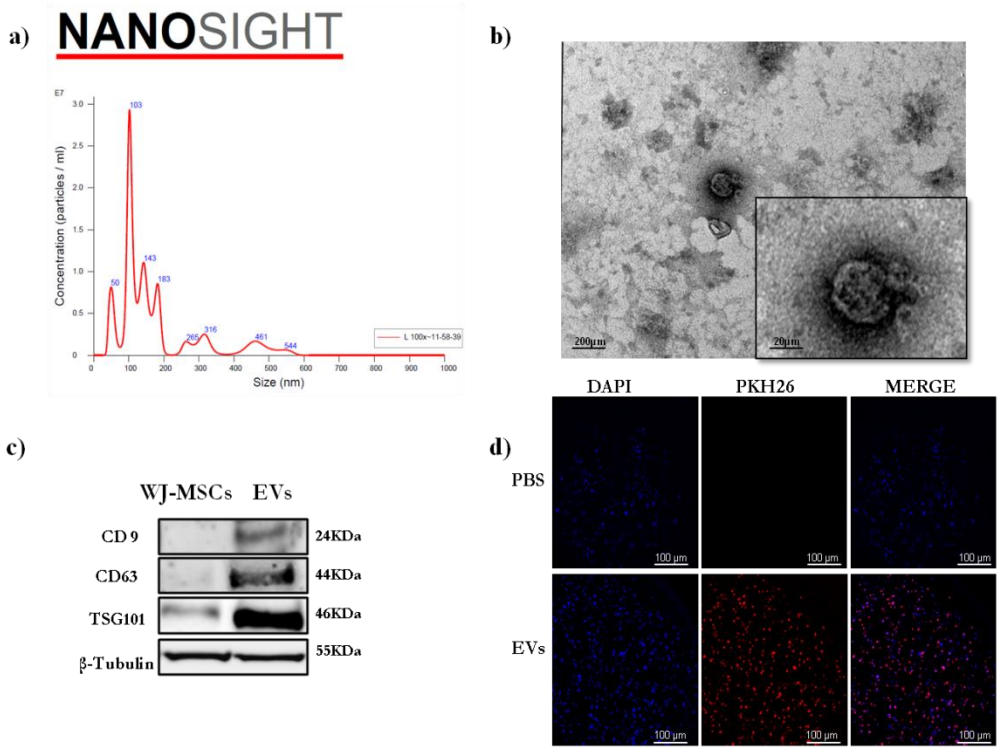


Figure 18. Identification of human Wharton Jelly MSC extracellular vesicles (WJ-MSCs) **a)** Particle size distribution of WJ-MSC EVs was measured by nanoparticle trafficking analysis (NTA). **b)** Image captured by transmission electron microscopy (TEM) of WJ-MSC-EVs morphology (Scale bar:200 nm; Scale bar: 20 nm). **c)** Western blot analysis of EVs protein markers CD63, CD9, TSG101, GRP75 and β-tubulin. **d)** Representative images of NP cells incubated with PBS or PKH26-labelled (red) WJ-MSC EVs. The nuclei of NP cells were stained by DAPI (blue). Magnification: 200 ×, scale bar: 100 µm.

Metabolic activity

Metabolic activity, measured with MTT assay, is an indirect measurement of cell status (Figure 19). hNPCs in the control group were assumed to have 100% mitochondrial activity. Significant differences in metabolic activity were encountered following treatments with EVs at 10 μg/mL (129.6 ± 10.05%; $p < 0.01$) and 100 μg/mL (140.2 ± 19.25%; $p < 0.05$) compared to the control group. Although a significant increase was also found in the group treated with 50 μg/mL (122.4 ± 17.88%) there was not a statistical difference compared to control. Therefore, we have chosen these concentrations for our further experimental investigations.

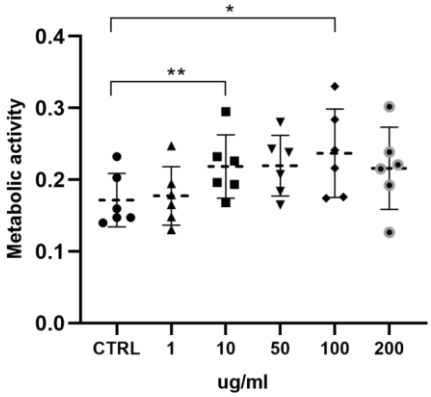


Figure 19. MTT assay for hNPCs metabolic activity assessment. WJ-MSCs derived EVs improved significantly metabolic activity in the experimental group treated with 10 μg/mL and 100 μg/mL. Data are expressed as percent variation between the control and experimental groups. n=5 *$p < 0.05$, **$p < 0.01$.

EVs promoted cell proliferation

Flow cytometry was used to determine cell viability and the presence of proliferating cells following WJ-MSCs derived EVs treatment at different doses. The results revealed an increase in cell content at 10 and 14 days after starting 3D cell culture (Figure 20). Specifically, at day 10^{th} , exposure to 10 and 50 μg/mL EVs led to a statistically significant increment in cell count (218.9 ± 16.58 events/μL and 224.7 ± 18.49

events/µ L, $p < 0.01$ and $p < 0.05$ respectively). At day 14th, the mean of hNPCs content after EVs treatments remained significantly higher at 50 µg/mL concentration (250.2 ± 22.69 events/µ L; $p < 0.001$) in comparison with the control group. Likewise, cell content in 50 µg/mL group was significantly higher compared to 100 µg/mL treatment ($p < 0.05$) both at 10 and 14 days. Although 10 and 100 µg/mL EVs treatments were not associated with significant increases in cell count at day 14th, long term improvements were observed compared to control group. Furthermore, in IL1-β stimulated hNPCs, EVs at 10 µg/ml counteract in a significant way the nocive effect of IL1β at almost all time points and the medium dose has a similar behaviour after 10 and 14 days from the start of treatment (Figure 20). Specifically, at 4th, 10th and 14th day the exposure to 10 µg/mL EVs increased significantly hNPCs number compared to IL1-β group (270.0 ± 21.14 events/µL; 246.0 ± 28.11 events/µL and 268 ± 21.11 events/µL $p < 0.05$, $p < 0.001$ and $p < 0.01$, respectively). Similarly, after pre-treatment with IL1-β for 24 hours EVs at 50 µg/ml both at 10th and 14th day, the mean of hNPCs content was significantly higher (238.0 ± 28.05 events/µL and 262.0 ± 32.35 events/µL; $p < 0.01$ and $p < 0.05$, respectively) in comparison with the stimulated group.

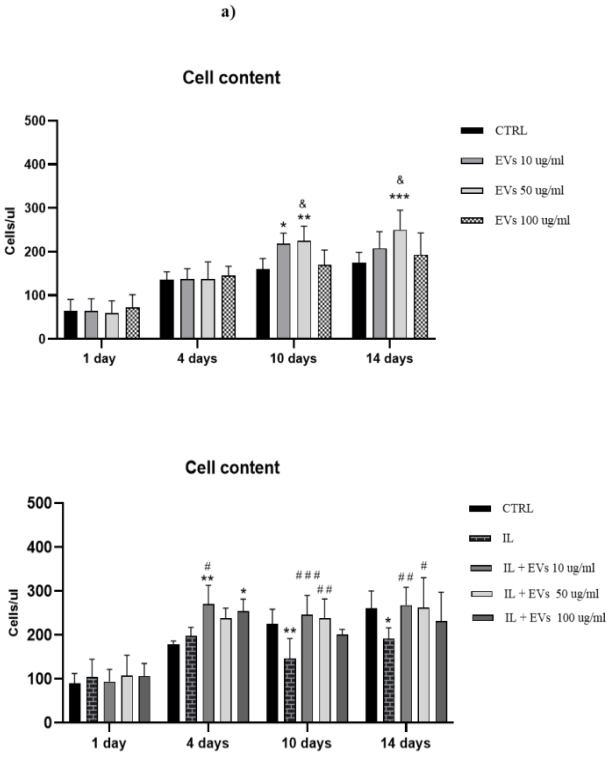
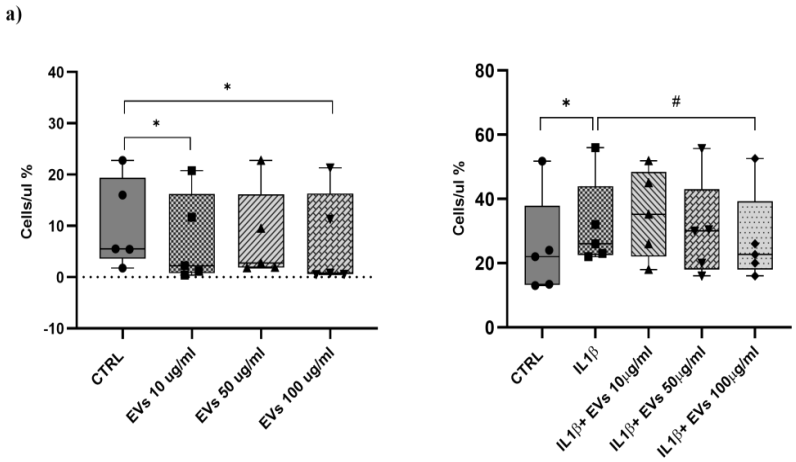


Figure 20. WJ-MSCs derived EVs promoted hNPCs content. Cell count increased after treatment with 10 and 50 µg/mL EVs at day 10, and 14, as compared with the control group.(n=5) *$p < 0.05$ and **$p <$

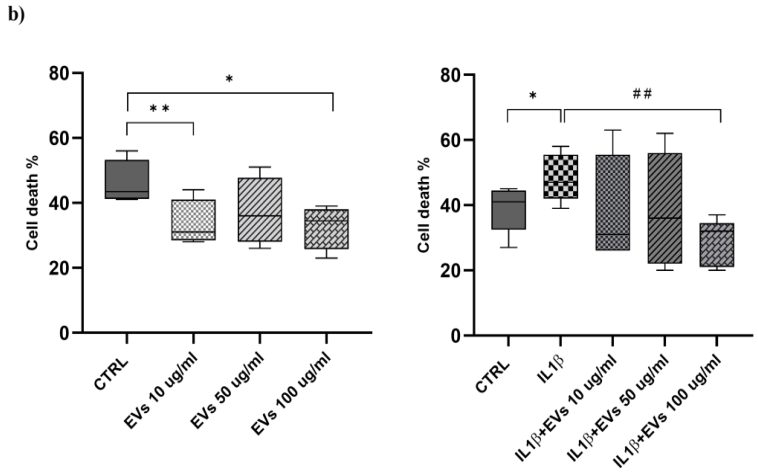
0.01; #p < 0.05 and ##p < 0.01 and ###p < 0.001. * compared to the control group and # compared to the IL1-β group at each timepoint.

EVs ameliorate hNPCs viability

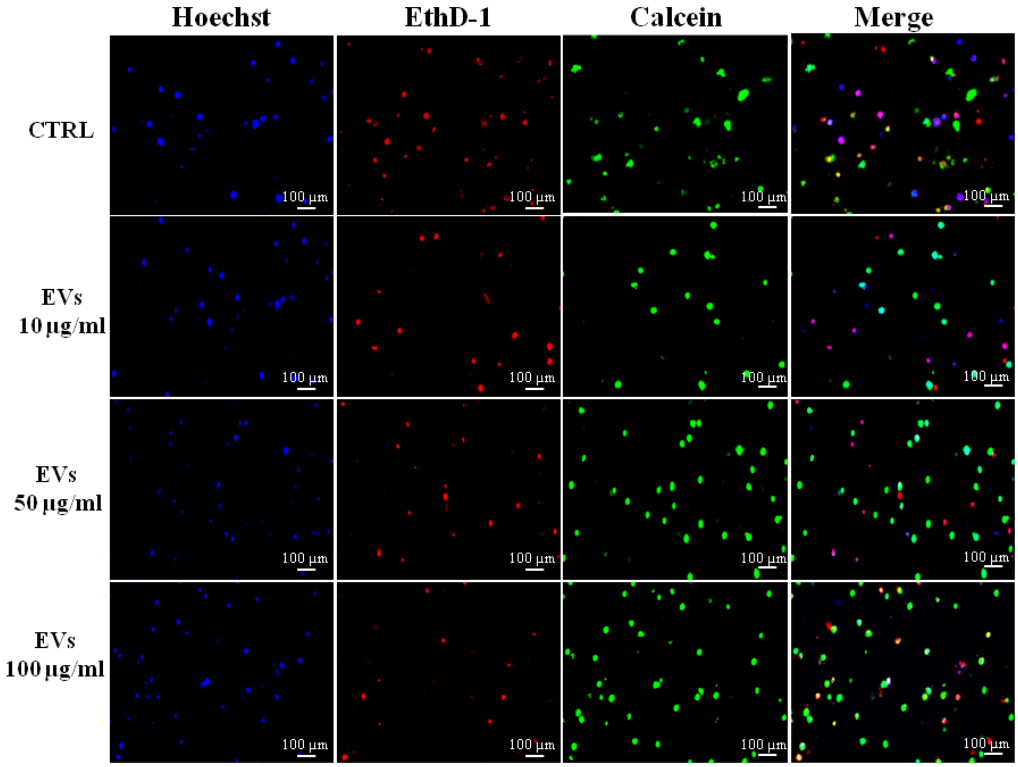
WJ-EVs stimulation was able to improve hNPCs viability. After 1 day, dead cell exclusion was performed with Fixable Viability Dye conjugated with eFluor780 fluorochrome through flow cytometry and significant lower numbers of dead cells were detected after treatment with EVs at 10 and 100 µg/mL compared to control (7.26 ± 8.83 events/µL and 7.73 ± 8.99 events/µL respectively; $p < 0.05$) (Figure 21a).



Moreover, in pre-treated hNPCs the percentage of dead cells was higher than control group (31.80 ± 14.07 events/µ L; $p < 0.05$), whereas cell death decreased significantly with WJ-EVs 100 µg/ml compared to IL1β sample group (27.45 ± 14.51 events/µ L; $p < 0.05$).



These results were confirmed by Live/Dead staining of hNPCs encapsulated in alginate beads as showed in figure 21b where green-fluorescent calcein-AM indicated intracellular esterase activity and red-fluorescent ethidium homodimer-1 indicated loss of plasma membrane integrity and blue-fluorescent Hoechst stained the nuclei. The number of dead cells normalized over the total number of the cells was reduced significantly by EVs at 10 and 100 µg/mL compared to the control group in basal conditions and by EVs at 100 µg/mL after inflammatory stimulation with IL1β.



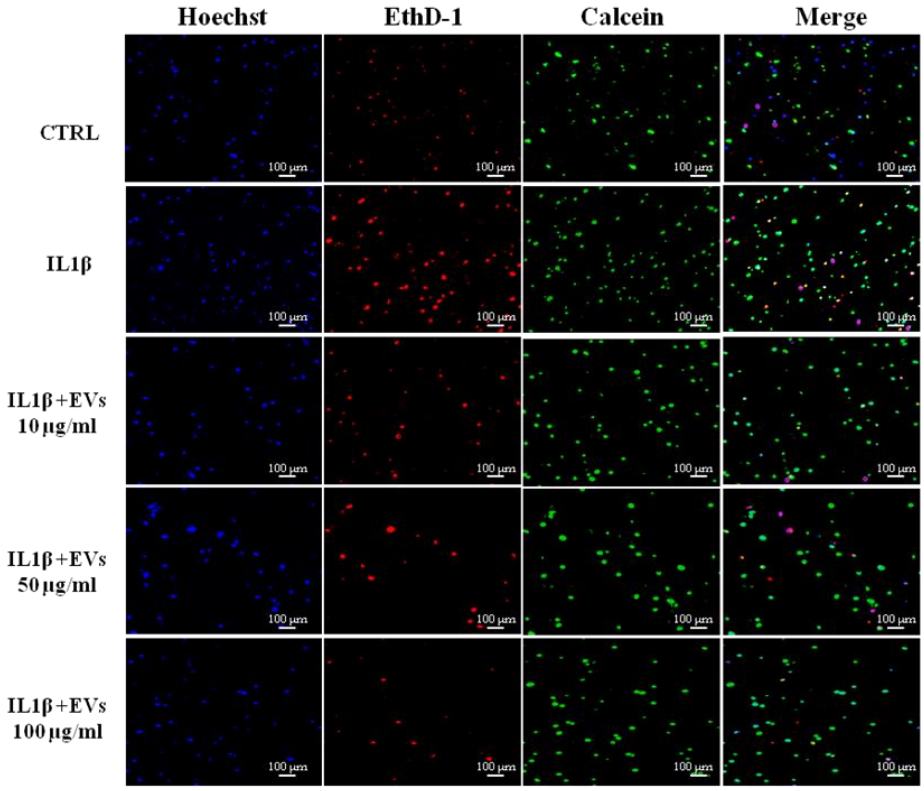
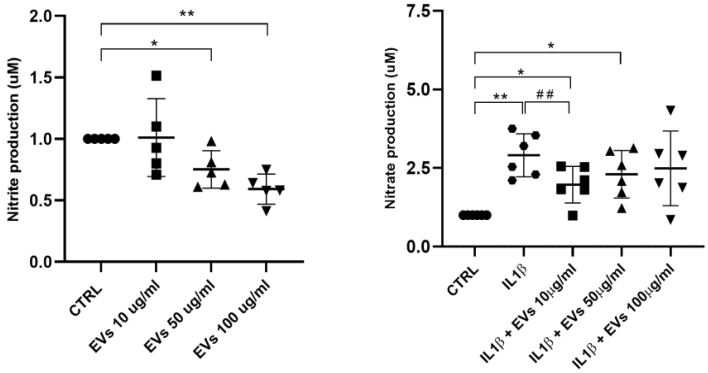


Figure 21. WJ-MSCs derived EVs attenuated cell mortality. **a)** After 1 day, EVs exposure reduced significantly hNPCs death at 10 and 100 µg/mL.(n=5) *p < 0.05 and **p < 0.01. **b)** Live/Dead assay showed a decrease of mortality after 10 and 100 µg/mL EVs treatment with and without IL1β stimulation.(n=5) *p < 0.05 and **p < 0.01; ##p < 0.01. Cell death results were expressed as mean percentage ± standard deviation for at least three independent experiments. Magnification: 200 ×, scale bar: 100 µm. * compared to the control group and # compared to the IL1-β group.

EVs attenuate nitrite induced oxidative stress

Endogenous nitrite release in supernatant by 3D-hNPCs was detected by Griess reaction (Figure 22). Considering the control group as 1, nitrite concentration was 1.01 ± 0.31 mM in the EVs 10µg/mL group, 0.75 ± 0.15 mM in the EVs 50 µg/mL group, 0.59 ± 0.12 mM in the EVs 100µg/mL group. Nitrite levels were significantly lower following exposure to 50 and 100 µg/mL EVs ($p < 0.05$ and $p < 0.01$, respectively). In the *in vitro* IDD model, inflamed hNPCs response to oxidative stress was mitigated by co-

incubation with all EVs concentration under exam. Compared to IL1-β group EVs 10µg/mL reduced significantly nitrite production (1.97 ± 0.58 mM, $p < 0.01$).



Interestingly, NOS2 mRNA levels were significantly lower in the 100 µg/mL group compared to the control group ($p < 0.001$).

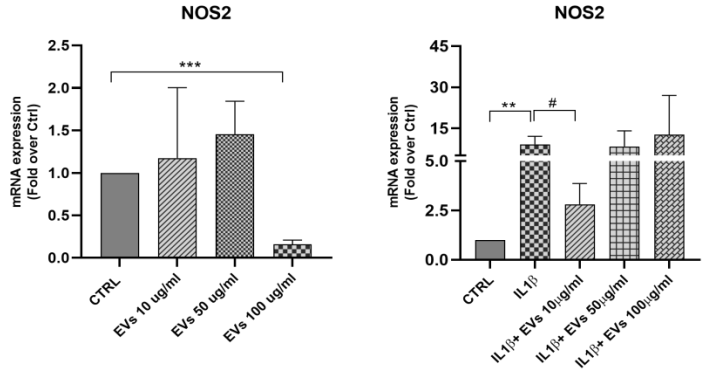
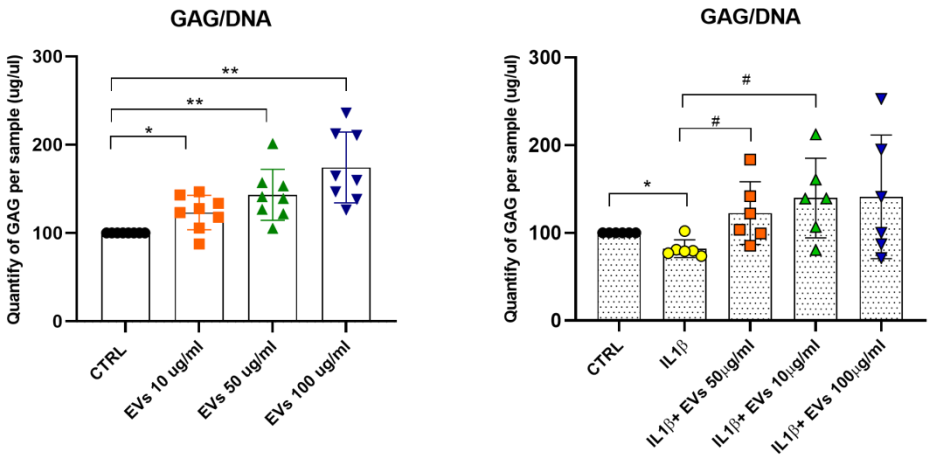


Figure 22. EVs led to a significant decrease of nitrite concentration released in cell supernatant after 7 days in groups treated with 50 and 100 µg/mL EVs compared to the control group both in presence and in absence IL1-β. NOS2 mRNA levels were significantly reduced by EVs 100 µg/mL group in basal conditions whereas by EVs 10 µg/mL after IL1-β treatment. (n=5) *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. #$p < 0.05$ and ##$p < 0.01$. * compared to the control group and # compared to the IL1-β group.

EVs enhanced ECM production

To investigate the role of EVs from WJ-MSCs in ECM production DMMB assay and AB histology staining were used at 7th day from the beginning of treatments. DMMB colorimetric assay resulted in an enhancement of GAG production normalized to DNA. Considering the GAG/DNA ratio in the control group as a baseline of 100%, three-dimensional hNPC cultures treated with all EVs concentrations (n=8) studied in a dose dependent manner showed significant higher levels of GAG compared to control (10µg/mL: 123.0 ± 19.43%; 50µg/mL: 143.2 ± 28.91%; 100µg/mL: 174.2 ± 40.12%; $p < 0.05$; $p < 0.01$) (Figure 23a). Similarly, in hNPCs pre-treated with IL1-β (n=6), GAG synthesis was enhanced in a dose dependent manner with an increment at EVs 10 and 50 µg/mL (139.8 ± 45.4% and 122.6 ± 35.7%; $p < 0.05$) compared to IL1-β sample group in which GAG/DNA ratio was reduced significantly.

a)



Histological assessment through AB staining confirmed these results, as showed in figure 23b, showing that EVs may promote ECM synthesis in degenerated hNPCs. Interestingly, sectioned beads of IL1-β stimulated hNPCs showed the presence of several cells cluster. As reported by Le Maitre *et al*., the number of NPCs cluster, the fissures and loss of distinction between NP and AF are degenerative features correlated to IDD [235].

b)

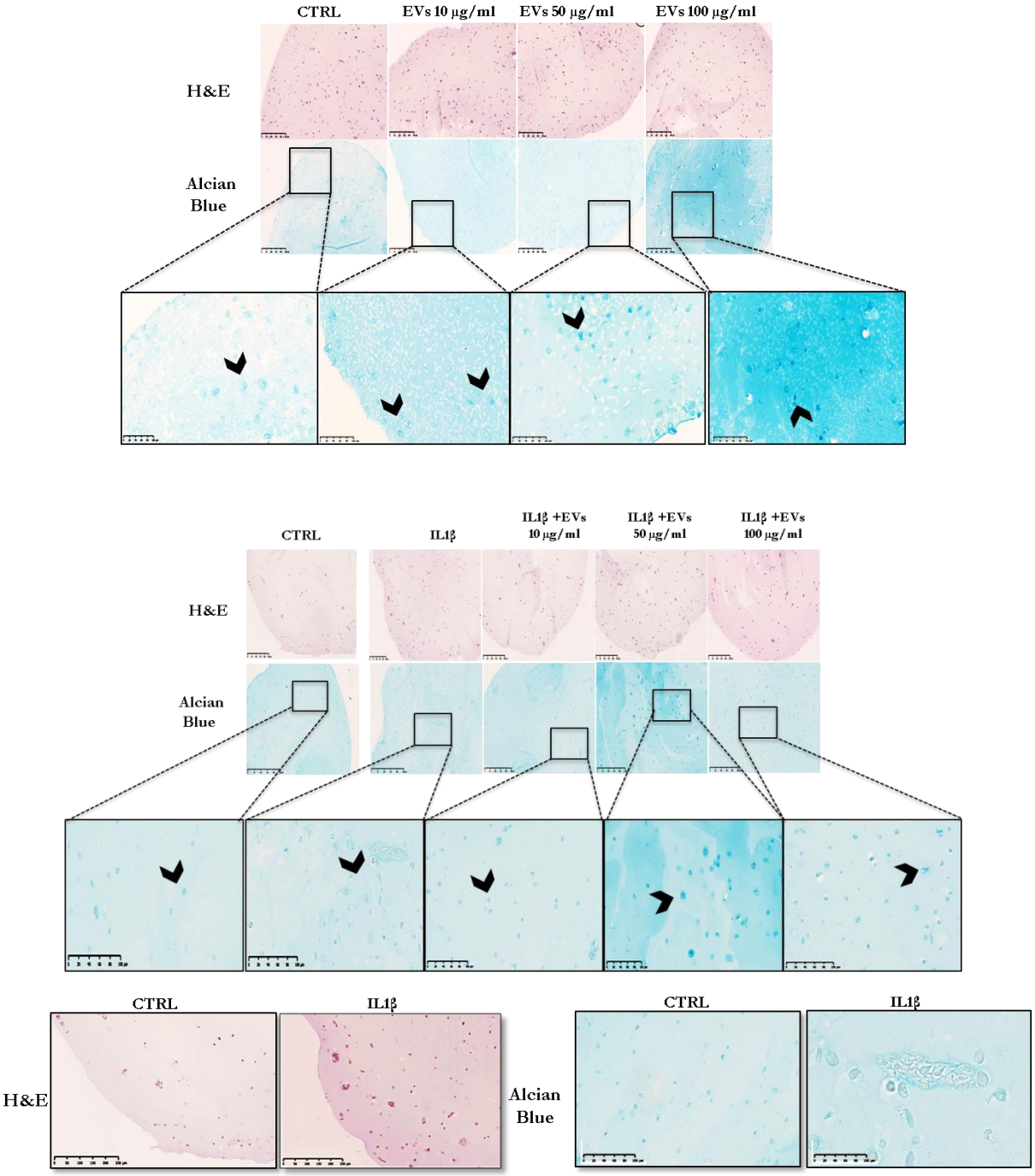
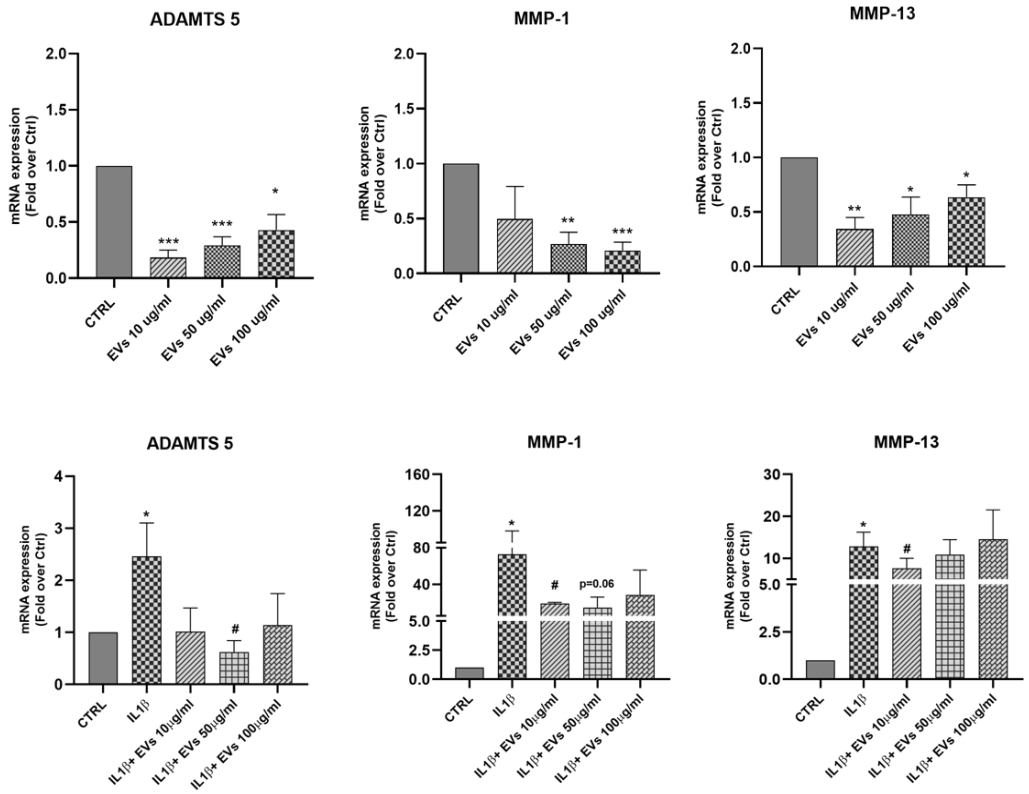


Figure 23. EVs increases GAG content in treated hNPCs. **a)** GAG/DNA ratio in hNPCs after EVs treatment demonstrated a significant dose dependent increase in all experimental groups. Data are expressed as GAG/DNA ratio percent variation between the control and experimental groups. *p < 0.05, **p < 0.01 and #p < 0.05. **b)** Alcian Blue staining of hNPCs alginate beads (n=3). Blue colour in histology sections identified proteoglycans synthesized. Scale bar: 500 µm; scale bar: 100 µm. * compared to the control group and # compared to the IL1-β group.

EVs maintained hNPCs phenotype and dampen inflammatory and matrix catabolic marker expression

We next assessed genes related to anabolic, inflammatory mediators and ECM degradation by qPCR. WJ-MSC EVs significantly reduced the expression of ADAMTS5, MMP1 and MMP13 catabolic genes (Figure 24a). More specifically, ADAMTS5 mRNA levels significantly decreased following treatment with all investigated EVs doses (10 µg/mL: 0.18 ± 0.16, $p < 0.001$; 50 µg/mL 0.29 ± 0.19, $p < 0.001$; 100 µg/mL: mL 0.42 ± 0.34, $p < 0.05$). Similarly, gene expression of MMP-1 was downregulated by EVs at 50 and 100 µg/mL (0.26 ± 0.26, $p < 0.01$; 100 µg/mL: mL 0.208 ± 0.19, $p < 0.001$, respectively). Whereas a statistically significant decrement of MMP-13 expression was noted after exposure to all EVs concentrations (10 µg/mL: 0.34 ± 0.25, $p < 0.01$; 50 µg/mL: 0.47 ± 0.38, $p < 0.05$; 100 µg/mL: 0.64 ± 0.26, $p < 0.05$).

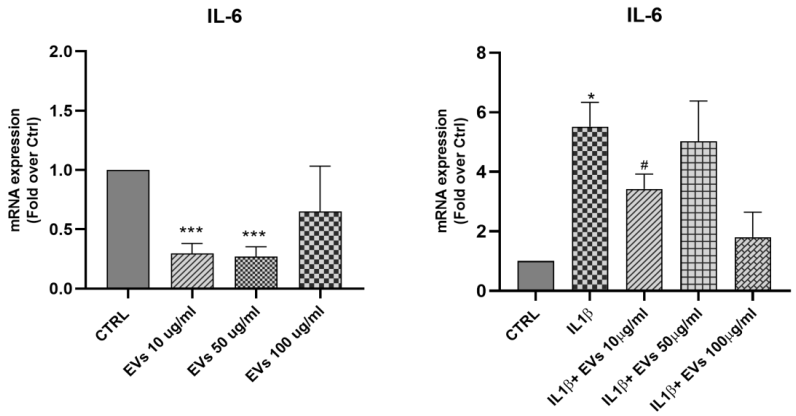
a)



When we simulated inflammatory microenvironment of degenerated IVD by adding IL1-β in our experimental conditions, mRNA levels of catabolic genes were significantly increased compared to un-treated hNPCs. However, ADAMTS5 gene expression decreased following treatment with EVs 50 µg/mL 0.62 ± 0.22, ($p < 0.05$). Similarly, MMP-1 and MMP-13 mRNA levels were downregulated after treatment with EVs at 10 µg/mL (18.92 ± 1.25 and 7.66 ± 5.8, $p < 0.05$, respectively).

Moreover, EVs were able to blunt the inflammation on hNPCs. IL-6 mRNA levels were significantly lower after exposure to 10 and 50 µg/mL (0.29 ± 0.205, $p < 0.001$; 0.26 ± 0.204; $p < 0.001$, respectively) in basal conditions while co-treatment with IL1-β plus EVs 10 µg/mL counteracted the detrimental effect of IL1-β (3.42 ± 0.99, $p < 0.05$) (Figure 24b).

b)



Interestingly, compared to levels of the untreated group, EVs-treated hNPCs expressed significantly more ACAN, SOX-9 and KRT19 mRNA (Figure 24c). Indeed, the experimental group displayed an increased mRNA expression of ACAN after exposure with 50 µg/mL (2.2 ± 0.30; $p < 0.05$) while for both SOX9 and KRT19 genes results were statistically significant only after treatment with 10 µg/mL (4.74 ± 0.36; $p < 0.01$ and 1.34 ± 0.06; $p < 0.05$, respectively), compared to the control. IL1-β treated hNPCs showed a drastic reduction of anabolic genes. However, all EVs concentration exerted a beneficial effect on ACAN and SOX9 phenotypic markers despite the lack of statistical significance (10 µg/mL: 0.32 ± 0.46, $p < 0.01$; 10 µg/mL 3.81 ± 1.7, $p < 0.05$, respectively). Moreover KRT19 expression increased in a dose dependent manner with a significant difference following the treatment with EVs 100 µg/mL (0.10 ± 0.03, $p < 0.01$).

c)

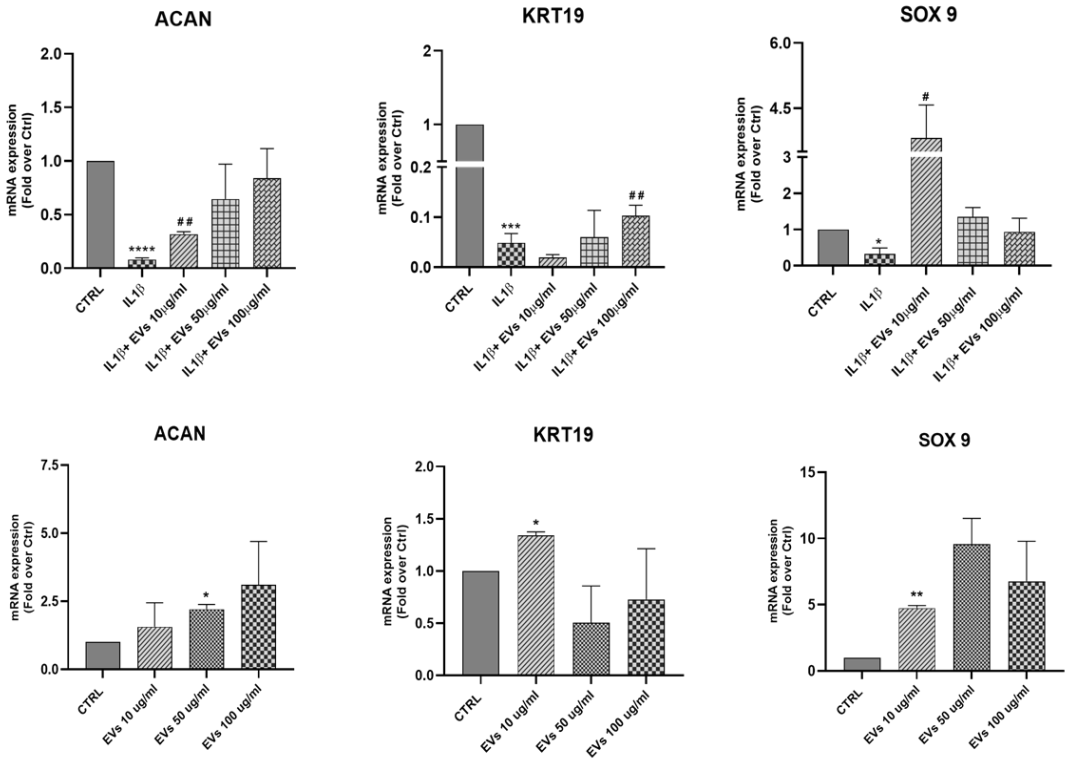


Figure 24. EVs isolated by WJ-MSCs maintain hNPCs phenotype and reduced catabolic gene expression levels. EVs treatment for 7 days resulted in a significant decrease of of ADAMTS-5, MMP-1, MMP-13 and IL-6 mRNA levels while increasing ACAN, SOX9 and KRT19 mRNA levels with and without IL1-β stimulation. Results were normalized based on GAPDH expression and calculated as fold change compared to the controls. (n=5) *p < 0.05, **p < 0.01, ***p < 0.001; ****p < 0.001. #p < 0.05, ##p < 0.01 . * compared to the control group and # compared to the IL1-β group.

3.4 DISCUSSION

The progression of IDD is generally characterized by a series of events such as dehydration with consequent deterioration of the ECM, inflammation, loss of vital cells and oxidative stress [6]. A safe and minimally invasive approach for IDD treatment involves the intradiscal injection of MSCs to exert a direct local effect despite the hostile microenvironment of IVD [5, 182]. However, recent evidences suggest that the therapeutic benefits of MSCs are mainly mediated by paracrine mechanisms [236, 237]. Interestingly, the MSCs can be preconditioned *in vitro* by different stimuli (i.e growth factors, biomaterials, drugs, 3D culture conditions, hypoxia) that improve the therapeutic potential [169, 238]. Among the secreted factors, the EVs have attracted attention as they act as messengers between the cells. The EVs carry cargo related also to the physiopathological state of the donor cell by modulating the behaviour of the target cells [195].

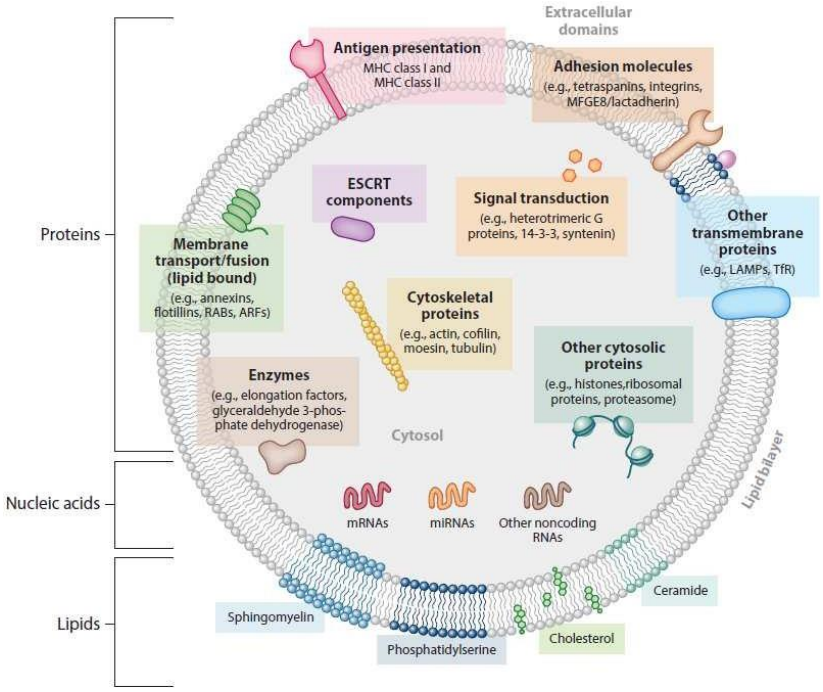


Figure 25. Schematic representation of the overall composition of EVs in terms of families of proteins, lipids and nucleic acids [201].

In this study, we reported how EVs isolated from WJ-MSCs in a 3D *in vitro* model promoted hNPCs cell metabolism, cell proliferation, GAG synthesis, and reduced the mRNA levels of catabolic and inflammatory markers. The microenvironment in the NP of degenerated discs is characterized by hypoxia, low glucose, hyperosmolarity, mechanical load, acidity and reduced supply of nutrients, also due to calcification of CEPs, leading to a decrease of cell viability and an increase of cell apoptosis [105]. In our study, treatment with EVs on hNPCs encapsulated in alginate beads promoted significantly cell proliferation from 4^{th} to 14^{th} experimental day with the lower (10µg/ml) and medium (50µg/ml) EVs doses both in basal and inflammatory conditions. Cell viability was assessed after 24 hours and all three different EVs concentrations attenuate hNPCs mortality. Conversely, after IL-1β stimulation the higher EVs dose (100 µg/ml) was able to reduce cell death compared to inflamed group. Previous studies have reported that EVs isolated from BM-MSCs increased the proliferation of 3D hNPC pellets from day 7 to day 28 [192], as well as exosomes from BM-MSCs at a concentration of 50 µg /ml significantly improved the proliferation rate of degenerated NPCs after 12 days, underlying that their effect was more effective compared to an indirect co-culture system between BM-MSCs and NPCs [239]. In several *in vitro* and *in vivo* IDD models induced by noxious stimuli including H_2O_2, LPS, IL-1β, TNF-α, acidic pH and high glucose MSCs-derived EVs have been shown to exert a pro-proliferation and anti-apoptosis effect. The mechanisms underlying these effects are often related to miRNAs transported by Evs to degenerated IVD cells. Cheng *et al*., in 2018 reported that MSC-derived exosomes containing miR-21 inhibited phosphatase and tensin homolog (PTEN); therefore, activated the phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathway in a rat model of IDD by preventing TNF-α-mediated apoptosis [240]. In addition to miR-21, others non-coding RNA such as miR-532–5p, miR-142-3p, miRNA-4450 and miR-199a enhance cell proliferation and attenuate apoptosis by targeting regulatory genes of downstream factors Bad, Bax and caspase-3 [241-244]. Moreover, a recent study revealed that BM-MSCs derived exosomes promote proliferation and counteract IL-1β-induced apoptotis in AF cells by triggering autophagy mediated by the PI3K/AKT/mTOR signaling pathway [245].

During degeneration, the homeostasis between oxygen free radical generation and antioxidant defense is disrupted. As a result, the formation of reactive oxygen species

(ROS) takes place. Our results demonstrated that EVs were able to decrease the release of nitrite, inducible nitric oxide synthase (iNOS) gene level and improved mitochondrial metabolism of hNPCs. However, the response in the IL-1β pre-treated groups was opposite compared to untreated hNPCs, probably for the presence of an altered environment influencing the beneficial effects of EVs. According to Xia *et al*. MSCs exosomes restored mitochondrial dysfunction caused by oxidative stress and improved cellular homeostasis by replacing damaged mitochondrial components and delivering proteins with antioxidant function [246]. Similarly, Liao *et al.* demonstrated that MSCs exosomes ameliorated advanced glycation end products (AGEs) -induced ER stress in disc cells both *in vitro* and in a rat tail model [247].

The synthesis of ECM components like GAGs represents an endogenous regenerative response by disc cells and our findings confirm that WJ-MSCs EVs increased GAGs production after 7 days in a dose dependent manner, measured by DMMB also supported by the Alcian Blue staining. GAGs and proteoglycans represent the supporting structure of the ECM which help to ensure disc integrity, nevertheless the unbalanced expression of MMPs or TIMPs may disrupt ECM [248]. Indeed, clinical studies showed that serum levels of MMP-1 were significantly higher in patients with severe IDD compared to patients with mild or moderate IDD [249]. It was widely demonstrated that after stimulation of NP cells with exosomes derived from BM-MSCs, they can restore the balance between catabolism and anabolism by up-modulating the expression of TIMP-1 and down-modulating MMP-1/MMP-3 [239]. Furthermore, EVs from iPSCs were found to downregulate ADAMTS-4 and MMP-3 by increasing the expression of type II collagen and aggrecan [250].In our experimental conditions, gene expression analysis suggested that WJ-MSCs EVs were able to down-regulate significantly the catabolic enzymes MMP-1, MMP-13 and ADAMTS-5 as well as IL-6 pro-inflammatory cytokine involved in the pathogenesis of IDD. Disc cells produce pro-inflammatory chemokines during the IDD process that can attract other leukocytes and produce pro-inflammatory cytokines including TNF-α, IL-1, IL-2, IL-6 and IL-8 which accelerate IDD progression [251]. In another study exosomal miR-410 targets the 3'UTR of NLRP3 inflammasome and reduces pyroptosis of LPS treated NPCs [252]. Previous studies had highlighted an anti-inflammatory role of exosomes derived BM-MSCs that attenuated H_2O_2-induced inflammation in NP cells. These results were

confirmed in an IDD rabbit model, reinforcing the concept of using EVs as a novel therapeutic strategy for IVD regeneration[246].

We also analyzed the mRNA levels of NP phenotypic markers ACAN, KRT19 and SOX9. The treated cells showed a higher expression level of these genes than the untreated group. Alginate bead was the 3D culture model used in this study and by mimicking the gelatinous structure of NP could promote the maintenance of the healthy NPCs phenotype [253].

This study has some limitations. First, results have been obtained through an *in vitro* experimental design. Although hNPCs showed to strongly respond to EVs, in our finding there was not investigated the EVs cargo involved in their beneficial effect. Second, hNPCs have been harvested from herniated disc tissues without considering the severity of degeneration degree, therefore the EVs effects should be different. Third, we used a 3D model that may re-differentiate hNPCs to a healthy phenotype so the effectiveness of EVs treatment may be influenced by our culture system.

3.4.1 Future challenges and conclusion

Although the promising therapeutic potential of EVs has been demonstrated, special caution should be exercised before using them in clinical trials [254]. To date, there are many challenges to overcome. First, the lack of uniformity and scalable purification and separation methods of EVs according to good manufacturing practices [197]; second, the selection of appropriate culture conditions to produce safer and more effective functionalized EVs. Third an omic approach to analyze EVs cargo should be contemplated as miRNAs are described the main mediators of EVs capability to regulate cells or tissues by inhibiting the post-transcriptional process of target genes. Even more challenging is the reproducibility in clinical practice. Furthermore, it is necessary to elucidate various critical issues such as pharmacological characteristics, biodistribution, pharmacokinetics of EVs, the optimal route of administration (local or systemic), dosage and timing [233]. Further studies are needed to clarify their mechanism of action.

In conclusion, our data demonstrate that EVs isolated from WJ-MSCs increased hNPCs proliferation, metabolic activity, ECM production and reduced cell death and

inflammation. These results suggested that EVs may represent a novel cell-free strategy for the repair and regeneration of IVD. Moreover, given a multiple advantages and functions, the EVs emerged as innovative tools for the development of a promising bio-nanomedicine.

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