Cell Therapy
in Intervertebral Disc Degeneration

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« ἕν οἶδα, ὅτι οὐδέν οἶδα »

Σωκράτης (?)

¨I know that I know nothing¨

(attributed to) Socrates

Στην οικογένειά μου,
το συνεχές όπου οι στιγμές μου ορίζονται

To my family,
the continuum in which my moments are defined

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ABSTRACT

Background: Chronic low back pain (LBP) is the leading cause of disability worldwide. Intervertebral disc degeneration (IDD) is central in the pathogenesis. The injection of bone marrow-derived mesenchymal stromal cells (BM-MSCs) into degenerate intervertebral discs (IVDs) has been proposed as an alternative therapy. The aims of these studies were to investigate the iron labeling of human BM-MSCs *in vitro* and in an animal model, to assess the feasibility and efficacy of the intradiscal injection of autologous, iron-labeled BM-MSCs in patients with LBP and IDD, and to examine the survival of these cells post-injection.

The studies: In **studies I and II** BM-MSCs from human donors were labeled with iron sucrose (Venofer®). In study I, histology showed labeling of 98.1% of the cells. Flow cytometry showed good viability and somewhat lower expression of MSCs´ surface markers (CD105) for the labeled cells. Cells cultured in the pellet mass system revealed: (i) traceability of labeled cells 28 days post-labeling and (ii) production of extracellular matrix (ECM). Immunohistochemistry (IHC) detected ECM components (coll2A1 and C6S). qRT-PCR (pellets) showed no differences between labeled and non-labeled cells for genes of chondrogenesis, ECM production and surface proteins.

In **study II**, the *in vitro* trilineage differentiation capability of the labeled cells was confirmed by detection of (i) GAGs (chondrogenesis) in pellets and (ii) calcium deposits (osteogenesis) and (iii) lipid droplets (adipogenesis) in cell cultures. Furthermore, a lapine animal model was used. Human BM-MSCs were injected in IVDs of 12 healthy animals ($25x10^4$cells/IVD). One IVD received labeled and one non-labeled cells and the animals were sacrificed 1- and 3-months post injection. The presence of human cells and the traceability of iron-labeled cells were confirmed by means of IHC and histology respectively. Cell viability per IVD (all cells) decreased by 25% at 3 months for the injected IVDs (regardless of the labeling).

In **study III**, ten patients from the waiting list for lumbar surgery were recruited to receive an intradiscal injection of autologous, expanded, iron-labeled BM-MSCs in degenerate IVDs ($1x10^6$ cells/IVD). The up to 2-year follow-up comprising of

patient-reported outcome measures (PROMs) and magnetic resonance imaging (MRI) controls at regular intervals, revealed no adverse events and no evident amelioration of the PROMs on a group level over time. Five of the patients chose to proceed with the originally planned surgical procedure within 2 years from the injection.

Study IV was a longitudinal evaluation of the MRI investigations of the patients enrolled in study III. Injected and adjacent lumbar levels were assessed for multiple qualitative (Pfirrmann grade, IVD and endplate homogeneity, Modic changes) and quantitative (IVD height and angle, IVD signal intensity) parameters. A detailed baseline characterization was performed. No significant changes over time were seen.

In **study V**, explanted tissues from injected IVDs were harvested from 4 patients from study III that proceeded to lumbar surgery, 8- (3 patients) or 28-months (1 patient) post-injection. Histological assessment showed the presence of iron-labeled cells in tissues explanted 8 months post-injection, with signs of metabolic activity in their vicinity. Expression of genes related to chondrogenesis (SOX9), ECM synthesis (COL2A1) and proliferation (PCNA) was confirmed by IHC investigations.

Conclusions: Iron sucrose labeling of BM-MSCs does not markedly affect cell viability and functionality. Intradiscal injection of autologous, expanded, iron-labeled BM-MSCs was a safe procedure. PROMs did not improve significantly in the present cohort; 5/10 patients could forgo surgery for a minimum of 2 years. Longitudinal MRI investigations revealed no adverse effects on the treated or the adjacent levels and no amelioration. Labeled BM-MSCs could be detected in IVD tissues explanted 8 months post-injection, indicating survival and engraftment of the injected cells in the IVDs.

Keywords: Low back pain, mesenchymal stromal cell, intervertebral disc, intervertebral disc degeneration, degenerative disc disease, cell therapy

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SAMMANFATTNING PÅ SVENSKA

Kronisk ländryggsmärta är en folksjukdom som drabbar människor världen runt och i nästan alla åldrar. Utöver det personliga lidandet, innebär kronisk ländryggssmärta stora samhällskostnader. Den exakta orsaken till smärtan är inte känd och genesen tycks vara multifaktoriell. Diskdegenerationen i ländryggens intervertebrala diskar anses dock vara en stor bidragande faktor. De befintliga behandlingsalternativen erbjuder inte lindring till alla patienter och innefattar ibland relativt omfattande kirurgisk åtgärd. Nya, biologiska, minimal-invasiva metoder har föreslagits, bland dessa är injektion av benmärgsderiverade mesenkymala stromala celler (BM-MSCs) till disk. BM-MSCs är multipotenta celler och skulle kunna bidra till lindring av smärtan genom olika verkningsmekanismer, bland annat genom att producera för disken nödvändiga substanser, genom att stödja andra celler eller genom att modifiera immunsystemets respons. Några få studier har genomförts med preliminärt positiva resultat men väldigt lite är känd kring olika aspekter runt denna typ av behandling t.ex. cellernas öde efter injektionen.

Syftet med studierna var att: 1) utveckla en metod för att kunna märka BM-MSCs med järn för att om möjligt detektera dem i histologiska vävnadsundersökningar, 2) kunna injicera autologa (kroppens egna), odlade och järnmärkta BM-MSCs i diskar hos patienter med långvarig ländryggssmärta (som stod på kö för diskprotes eller steloperation) för att i första hand utvärdera säkerheten med denna typ av behandling, 3) att för de som valde att genomgå den ursprungligt planerade operationen efter att ha erhållit injektion av celler i disken, undersöka förekomsten av de injicerade cellerna.

I studier I och II odlades BM-MSCs från donatorer och cellerna märktes med järn. Järnmärkningen påverkade cellerna minimalt och märkta celler kunde detekteras i pelletodlingar (3D odlingar) upp till 28 dagar efter märkningen. Därefter utvärderades metoden i en kaninmodell. Humana BM-MSCs injicerades i två ryggdiskar, den ena injicerades med märkta och den andra med omärkta celler. Djuren avlivades 1 eller 3 månader och de märkta cellerna kunde detekteras i vävnadsprover vid båda tillfällena.

I studie III injicerades autologa, odlade, järnmärkta BM-MSCs till 1 eller 2 diskar på 10 patienter med kronisk ryggsmärta och med konstaterad diskdegeneration som satts upp på väntelista för öppen ryggkirurgi. Patienterna inkluderades i studien efter informerat samtycke. Uppföljningen upp till 2 år

visade inga bieffekter av behandlingen. Ingen förbättring avseende smärta, funktion eller livskvalitet kunde ses på gruppnivå med hjälp av patient rapporterade utfallsmått (PROMs). Under de 2 första åren efter injektion valde 5 av 10 patienter att gå vidare till öppen operation.

Patienterna följdes upp även med magnetkameraundersökningar (MR) upp till 2 år efter injektionen. Studie IV var en detaljerad, longitudinell utvärdering av dessa med fokus på de injicerade och till dem angränsande diskar. En erfaren radiolog bedömde samtliga MR undersökningar avseende ett flertal kvalitativa och kvantitativa parametrar. Ingen säker förändring av vare sig injicerade eller angränsade diskar kunde påvisas.

I studie V undersöktes diskvävnad från 4 patienter som opererades med öppen ryggkirurgi efter cellinjektion. Tre patienter opererades 8 månader och en 28 månader efter diskinjektion. Märkta celler påvisades i vävnadsprover hos de 3 första och olika markörer indikerade att cellerna hade anpassade sig och var metaboliskt aktiva i diskvävnaden.

Sammanfattningsvis kunde vi etablera en metod för att kunna märka BM-MSCs och detektera dem i vävnadsprover. Vi utförde en intradiscal injektion av BM-MSCs hos patienter med kronisk ländryggssmärta utan att proceduren visade några bieffekter. MR kontroller visade ingen förändring, vare sig försämring eller förbättring, och detsamma gällde även PROMs. De injicerade cellerna kunde detekteras i vävnadsprover hos 3 patienter 8 månader efter injektionen med tecken på att cellerna anpassade sig i diskmiljön.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Papadimitriou N, Thorfve A, Brantsing C, Junevik K, Baranto A and Barreto Henriksson H (2014). "Cell viability and chondrogenic differentiation capability of human mesenchymal stem cells after iron labeling with iron sucrose." Stem Cells Dev 23(21): 2568-2580.

II. Papadimitriou N, Li S and Barreto Henriksson H (2015). "Iron sucrose-labeled human mesenchymal stem cells: in vitro multilineage capability and in vivo traceability in a lapine xenotransplantation model." Stem Cells Dev 24(20): 2403-2412.

III. Papadimitriou N, Hebelka H, Hingert D, Baranto A, Barreto Henriksson H, Lindahl A, Brisby H. “Intra discal injection of iron-labeled autologous mesenchymal stromal cells in patients with chronic low back pain. A feasibility study with 2 years follow up.” *Manuscript, submitted*

IV. Papadimitriou N, Hebelka H, Waldenberg C, Lagerstrand K, Brisby H. “Longitudinal MRI evaluation of disc and adjacent tissues up to 2 years after intervertebral disc injection of autologous mesenchymal stromal cells.” *Manuscript*

V. Henriksson H B,Papadimitriou N, Hingert D, Baranto A, Lindahl A and Brisby H (2019). "The traceability of mesenchymal stromal cells after injection into degenerated discs in patients with low back pain." Stem Cells Dev 28(17): 1203-12

CONTENTS

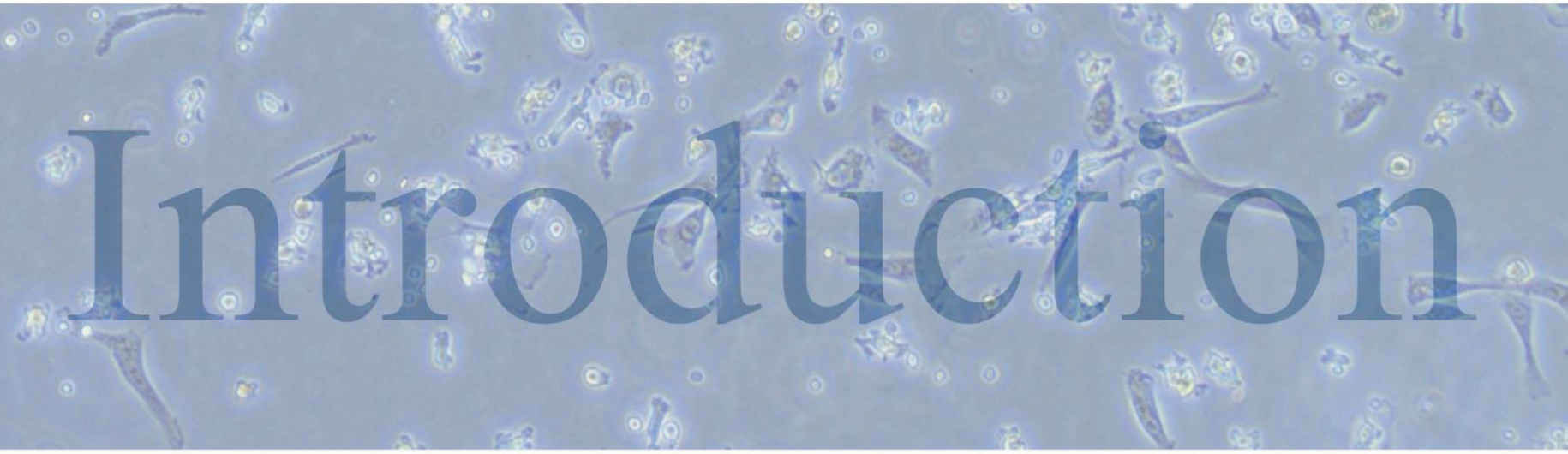
ABBREVIATIONS

ADAMTS	A Disintegrin And Metalloproteinases with ThromboSpondin Motifs
AF	Annulus Fibrosus
ATMP	Advanced Therapy Medicinal Product
AT-MSC	Adipose Tissue derived Mesenchymal Stromal Cell
BM-MSC	Bone Marrow-Derived Mesenchymal Stromal Cell
CEP	Cartilaginous Endplate
CFU-F	Colony Forming Unit-Fibroblastic
CSF	Cerebrospinal Fluid
CT	Computer Tomography
DDD	Degenerative Disc Disease
DMEM-LG	Dulbecco's Modified Eagle's Medium with Low Glucose
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EQ-5D-3L	European Quality of Life-5 dimensions-3 levels
EQ-VAS	European Quality of Life-Visual Analogue Scale
FACS	Fluorescence Activated Cell Sorting
FGF	Fibroblast Growth Factor

GAG	Glycosaminoglycan
GMP	Good Manufacturing Practice
HA	Hyaluronic Acid
HIF-1a	Hypoxia-Induced Factor 1 Alpha
HIZ	High Intensity Zone
HLA-DR	Human Leukocyte Antigen – DR Isotype
HRP	Horseradish Peroxide
IASP	International Association for the Study of Pain
ICHOM	International Consortium for Health Outcomes Measures
IDD	Intervertebral Disc Degeneration
IHC	Immunohistochemistry
IL-1	Interleukin-1
ISCT	International Society for Cellular Therapy
IVD	Intervertebral Disc
LBP	Low Back Pain, chronic
MMP	Matrix Metalloproteinase
MPC	Mesenchymal Precursor Cell
MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MSC	Mesenchymal Stromal Cell
NICE	National Institute for Health and Care Excellence

NIH	National Institutes of Health
NP	Nucleus Pulposus
NRS	Numerical Rating Scale
ODI	Oswestry Disability Index
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PROM	Patient-Reported Outcome Measure
PROMIS	Patient-Reported Outcome Measurement Information System
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROI	Region of Interest
SF-36	Short Form-36
SOX9	Sex determining Region Y-box 9
SPIO	Superparamagnetic Iron Oxide
Swespine	Swedish Spine Registry
T1W	T1 Weighted
T2W	T2 Weighted
TDR	Total Disc Replacement
TLIF	Transforaminal Lumbar Interbody Fusion
TNF-a	Tumor Necrosis Factor - Alpha
TSE	Turbo Spin Echo

TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
VAS	Visual Analog Scale
YLD	Years Lived with Disability
7-AAD	7-Aminoactinomycin-D



Introduction

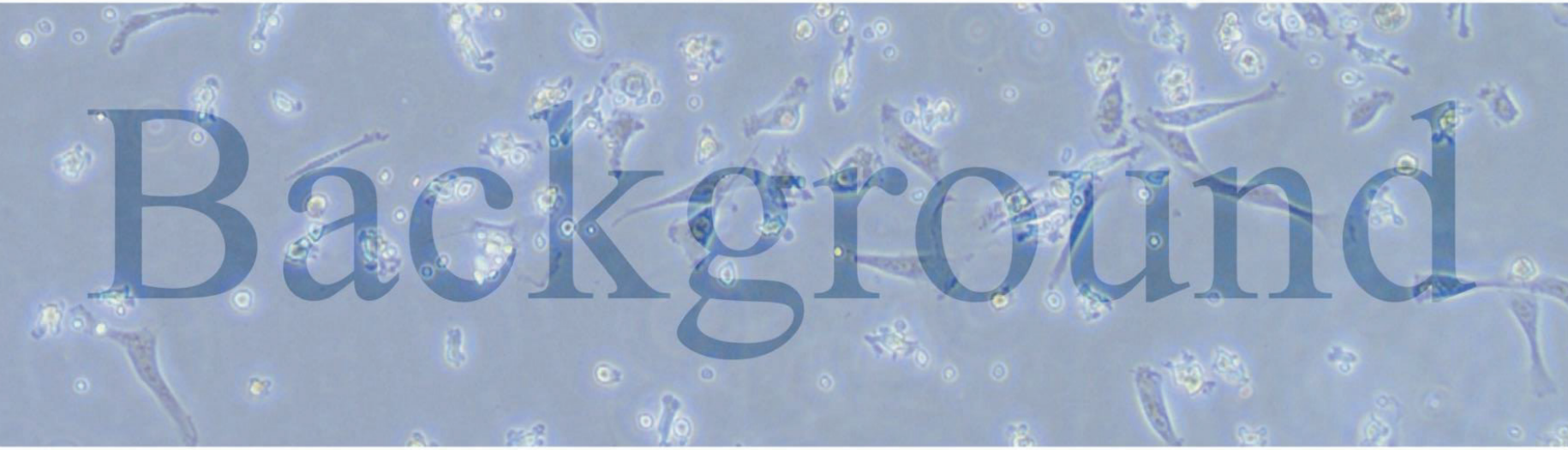
1. INTRODUCTION

Chances are, dear reader, that you, a member of your family or a dear friend have already experienced an episode of low back pain. It is namely so, that the lifetime prevalence of low back pain alone has been reported to be as high as 80% [1, 2].

It doesn't have to be dramatic, for in the majority of cases the annoyance is transient. For some patients, though, low back pain can lead to a crippling, chronic condition, seriously affecting the quality of their everyday life. In fact, chronic pain patients (not exclusively low back pain) score worse on quality of life questionnaires than patients with malignancies [3].

Even if you haven't met someone suffering from low back pain it might be interesting to hear that it is ranked worldwide as the leading cause of disability [4]. And assuming that you pay your taxes and are interested in where countries allocate resources, you might be surprised to find out that a study in the Netherlands showed that the annual societal cost for a single patient can mount up to over 18 000 Euros [5].

Current treatment options, obviously, do not always meet patients' needs. This thesis is an attempt to expand our knowledge on novel therapeutic modalities.


Background

2. BACKGROUND

The Problem(s)

Low back pain is the leading cause of years lived with disability (YLDs) worldwide and for all age groups [4], affecting even adolescents [6]. Usually defined as pain experienced between the lower rib cage and the gluteal crease [7, 8], it has an enormous socioeconomic cost that is well described predominantly in Western societies [5, 9-11]. Although the natural course of low back pain is not clear [12], it has been estimated that approximately 10% of patients can develop chronic low back pain [13]. The burden of low back pain is predicted to grow with the ageing population prompting *The Lancet* to publish a "call for action" viewpoint paper in 2018 [14].

Low back pain is considered to be a complex condition where a multitude of factors contribute to its pathogenesis, including genetic predisposition, psychological and social factors, co-morbidities, and pain-processing mechanisms [15-18]. A single nociceptive source of pain is usually not readily identifiable [12, 15], but the degenerate intervertebral disc is widely considered to play a central pathogenetic role [16].

There are several terms in the literature concerning low back pain which are in addition changing over time [8]. A usual distinction is between "specific" low back pain that is, pain that can be explained (by trauma, infection, malignancy) and "non-specific" low back pain, where the pain generator cannot be identified. The latter is thought to account for up to 90% of the cases [12, 19].

The International Association for the Study of Pain (IASP) is moving away from the concept of non-specific pain and is adopting the definition of chronic primary pain (and chronic primary low back pain therein) as pain that persists or recurs for longer than three months, causes emotional stress and/or functional disability and cannot be explained by another diagnosis [20]. The National Institutes of Health (NIH) in the USA proposed two questions to define chronic low back pain, namely pain duration over three months and experiencing pain at least half the days in the past six months [21].

In the context of this thesis the abbreviation LBP will be used for chronic low back pain.

The Intervertebral Disc (IVD)

The IVD is a complex structure connecting adjacent vertebral bodies, absorbing and distributing loads and allowing motion between the vertebrae. It consists of the nucleus pulposus (NP), the annulus fibrosus (AF) and the cartilaginous endplates (CEP). The nucleus is the core of the IVD, surrounded by the annulus and anchored to the vertebrae cranially and caudally by the endplates [22, 23].

The NP is a gelatinous structure optimized to resist compressive forces. The extracellular matrix (ECM) consists mainly of type II collagen fibers, randomly organized and is rich in highly aggregated proteoglycans (glycosylated proteins), predominantly aggrecan, that provide the necessary osmotic properties that render the NP resistant to compression [24, 25]. The proteoglycans are covalently attached to anionic glycosaminoglycans (GAGs) such as hyaluronan, chondroitin and keratan sulfate. It is the GAGs that trap water, providing hydration and swelling pressure to the tissue [26]. NP cells are relatively sparse, approximately 4×10^6 cells/cm^3 and often described as chondrocyte-like cells in the adult human NP [27, 28].

The AF surrounds the NP and is biomechanically optimized to offer resistance to tensile forces. It is a predominantly fibrous tissue consisting of up to 25 concentric lamellae of alternating oblique collagen fibers (mainly type I) interspersed with proteoglycans [22, 23].

The CEPs consist of hyaline cartilage and serve as anchors of the IVD to the subchondral bone of the adjacent vertebrae [23]. Nutrition of the NP is dependent upon the CEPs [29].

The IVD resists and distributes mechanical loads and at the same time permits mobility between adjacent vertebrae in all planes. The healthy NP is practically incompressible and remains pressurized within the compartment defined by the AF and the CEPs. Compressive forces along the spine increase the pressure within the NP which is transmitted towards the AF, causing the latter to “stretch”. The healthy AF is optimized to withstand large tensile loads. The loads that the IVD is expected to resist are considerable and vary with different positions of the body and alternating activities and are maximized at flexion [30]. The pressure within the NP can mount up to 2.4 MPa [31].

The healthy, adult IVD contains no blood vessels or nerve endings, except possibly in the outer lamellae of the AF [13]. The IVD and the NP in particular are dependent upon diffusion through the endplates for nutrition, oxygenation and removal of metabolic waste products. NP cells thereby have a harsh environment of acidic and hypoxic conditions [13, 29]. ***Figure 1*** offers a graphical illustration of the healthy and the degenerate IVD.

The interaction of cells, ECM and biomechanical stress is instrumental in the homeostasis of the IVD [22, 32].

The cells of the IVD

The NP is of notochordal origin and gets enclosed during organ development in somatic mesenchyme that forms the AF and the CEP as well as the vertebrae [33].

The origin of the cells of the mature NP is debated. In adult humans the large, round, vacuole-containing NP cells of the IVD are replaced by smaller cells that are described as chondrocyte-like cells [34] although they have distinct characteristics from chondrocytes, including those of the CEP. Such an example is the capacity of NP cells to synthetize ECM with a GAG to hydroxyproline (collagen component) ratio >20:1 compared to a ratio of 2:1 of the cells of the CEP [35, 36]. It has been proposed that these cells are of mesenchymal origin, migrating either from the CEP or from niches of transient amplifying cells located at the periphery of the disc. Mounting evidence, however, suggests though that the chondrocyte-like cells of the adult, healthy NP, or at least a subset therein, are of notochordal lineage [34, 37, 38]. There is evidence to support that the adult human NP is home to two different cell populations, one of notochordal origin and one of mesenchymal, comprised of cells derived from the AF or the CEP [33].

The fibroblast-like cells of the AF and the cartilage cells of the CEP are of mesenchymal origin [13, 39].

In both the AF and NP precursor cells have been detected in investigations, even in tissues of human origin [39-42].

The environment of the NP

The environment of the IVD and the NP in particular is among the harshest in the human body. The NP is the largest avascular structure in the human body. NP cells are dependent on diffusion through the CEPs for oxygenation, nutrition and removal of metabolic waste products and survive therefore in an acidic, hypoxic, hyperosmotic environment [13, 29, 43, 44]. It has been proposed that the nutrient supply regulates the cell density [45]. Several cellular adaptations have been described, for example the constitutive expression in NP cells of hypoxia-induced factor 1 alpha (HIF-1a), a transcriptional factor that shifts the cell metabolism to a glycolytic pathway [46-48]. Changes in the precarious homeostatic balance can affect the cells [49].

Even the complex mechanical loading of the spine affects the NP cells. Studies on explants have shown that loading conditions can have deleterious [50] or beneficial effect [51] on the IVD, influencing production of matric components and cell survival [52]. This infers even a cellular mechanism of the NP cells for sensing the alternating loads through interaction with the ECM [50, 53].

IVD degeneration

There are no universally accepted definitions of intervertebral disc degeneration (IDD) and the degenerative disc disease (DDD) something that is highlighted even in the literature [54]. Adams and Roughley proposed the following:

“The process of disc degeneration is an aberrant, cell-mediated response to progressive structural failure. A degenerate disc is one with structural failure combined with accelerated or advanced signs of aging. Early degenerative changes should refer to accelerated age-related changes in a structurally intact disc. Degenerative disc disease should be applied to a degenerated disc, which is also painful.”[24].

In the definition above, it is the structural damage that ultimately induces degeneration. Vergroesen *et al.* propose a degenerative circle where degeneration can be induced by cues from the cells, the extracellular matrix or biomechanical factors in a positive feed-back loop [22].

IVDs begin to degenerate earlier than most other tissues, as early as in the second decade of life [55], and this degeneration is considered to be part of the normal aging [22] with no clear boundaries between the aging process and the DDD that results in pain [22]. Genetic predisposition has been recognized as a major contributing factor [56, 57] as well as environmental factors such as smoking [58, 59], co-morbidities as diabetes [60] and obesity [61, 62] as well as injuries and aberrant loading [63]. Even a developmental origin has been suggested [64].

The degenerative process affects the cells, the ECM and the biomechanics of the IVD, all of which are interdependent. Alterations of the endplates such as sclerosis hamper diffusion of nutrients and metabolic products [29, 65, 66]. Senescent and apoptotic cells cannot support homeostasis and may contribute to a catabolic shift [32, 67, 68]. Cell clusters can be seen in degenerative IVDs [69]. Excreted inflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-a) [70, 71] and lytic enzymes such as matrix metalloproteinases (MMPs) and ADAMTS (a disintegrin and metallo-proteinases with thrombospondin motifs) induce inflammation and matrix breakdown and remodeling [32, 72]. In a mildly degenerated disc, the pH can be as low as 6.7 [44, 73]. Aggrecan cleavage and reduced synthesis leads to reduced hydration and decreased intradiscal pressure [13]. Collagen type II is gradually denaturated and replaced by collagen type I. As the NP loses its height and becomes more fibrous and less hydrated, it gets depressurized [74] and the compressive, hydrostatic stress increasingly shifts towards a shear one, affecting the cells in a negative manner [75]. Clefts and fissures appear in the AF and neovascularization occurs, followed by ingrowth of nerve fibers, predominantly in the outer layers of the AF [13, 22, 76]. As the degenerative process evolves the AF begins to bulge, and the spinal segment is destabilized. At later stages hypertrophy of the longitudinal ligaments and osteoarthritis of the facet joints can be seen [26]. Alterations of the ECM are present in all stages and subtypes of IVD degeneration [77].

The ingrowth of nerve fibers into fissures of the AF and the presence of products of the inflammatory process have been proposed as putative nociceptive pain signals in the development of LBP [70, 76, 78] although it is conceded that the process is more complex, involving peripheral and central nervous system sensitization [79, 80]. Even pathologies of the subchondral bone and the CEPs have been proposed as contributing etiological factors [81, 82]. ***Figure 1*** graphically depicts the healthy IVD and some common features of IDD.

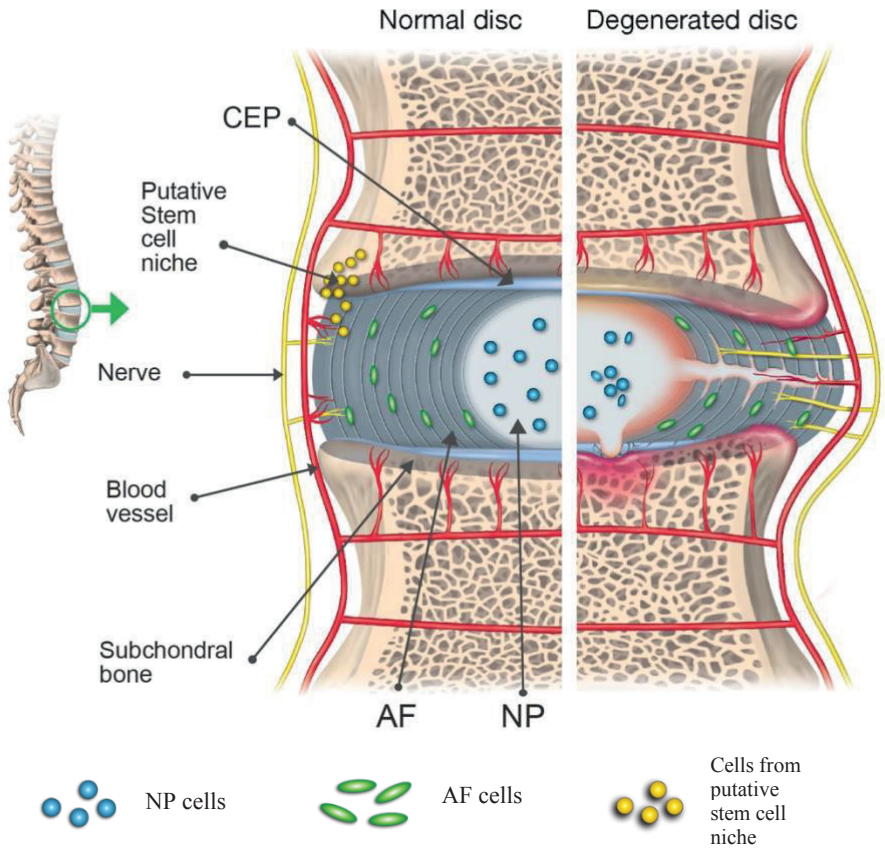


Figure 1. *Graphical illustration of a normal IVD (left) and an IVD presenting some of the most common features of degeneration (right).*
Left: *The different anatomical structures and regions are depicted, including the putative stem cell niche of the perichondrium. AF: annulus fibrosus. NP: nucleus pulposus. CEP: cartilaginous endplate.*
Right: *Common features of IDD. The NP cells form clusters and become senescent. The boundary between the NP and the AF is less distinct. Fissures and clefts appear in the AP where ingrowth of blood vessels and nerves takes place. The CEP becomes more calcified, gets thinner, may break and lead to herniation of the NP into the subchondral bone. The subchondral bone may show signs of inflammation, sclerosis or fat infiltration. The loss of hydration of the extracellular matrix, the depressurization of the NP and the structural disruption of the IVD may lead to loss of disc height and bulging of the IVD.*

Imaging of the IVD degeneration

Magnetic resonance imaging (MRI) is the radiological modality most commonly used for imaging of the degenerative lumbar spine today [83]. It is based on the application of a static magnetic field that aligns the spinning nuclei of the hydrogen atoms. Thereafter a second magnetic field is applied and excites the nuclei out of their equilibrium position. As they fall back to their "relaxed" condition a signal is produced, recorded and transformed to a grayscale image. Depending on the excitation impulses applied and the time between the excitation and the recording of the signal, different tissues can have different signal intensities on different sequences. The two fundamental relaxation times are T1 and T2. Different signal acquisition parameters produce images relying on either one and are referred to as T1-weighted (T1W) or T2-weighted (T2W) respectively [84].

Multiple parameters of the IVD and the surrounding tissues can be examined with MRI. Decrease of disc height is a parameter considered a hallmark of disc degeneration on MRI but even in plain radiography and computed tomography (CT) [85]. IVD bulging or prolapse can also be estimated on MRI. MRI can further provide an estimate of the hydration of the IVD [86]. Annular fissures can be depicted as well as changes in the CEPs and the subchondral bone of the vertebrae [83]. Most of the information derived from conventional MRI controls is qualitative, although there are methodologies to extract quantitative measurements [87].

Unfortunately, MRI findings as described in conventional radiological reports do not correlate well to the clinical course of the degenerative disease [88, 89], as positive MRI findings present even in controls of asymptomatic individuals [90-92]. On a population basis though, large studies suggest a clear link between IDD and LBP [93, 94].

The method most commonly used to assess the degree of degeneration on MRI images is the Pfirrmann classification [95, 96]. It is a 5-grade ordinal scale based on evaluation of T2W MRI images with regard to signal intensity, distinction between NP and AF, and disc height. An 8-level modified version of it has also been introduced [97].

Current treatment options

A multitude of different treatment modalities are available for LBP [98], including pharmacological regimes [99], non-pharmacological interventions, such as physiotherapy, cognitive behavioral therapy and multimodal pain teams [100], and surgery, usually spinal fusion or disc prosthesis (total disc replacement, TDR) [101-103]. In recent years the role of surgery has been challenged in the treatment of LBP [104, 105]. Most treatment guidelines focus on non-pharmacological and pharmacological therapy and surgery, which, if at all recommended, is reserved only for patients with LBP refractory to non-surgical treatment [98]. The guidelines published by the National Institute for Health and Care Excellence (NICE) in the UK, advise against surgery, stating that lumbar fusion surgery may be considered in the setting of a clinical trial while TDR is not recommended [106, 107]. The 2018 report of the Swedish Spine Registry (Swespine) shows that 7% (631 patients) of all procedures under 2017 were performed on a DDD indication [108] while the 2020 report shows that this percentage has remained stable, at 7.6% (749 patients) when assessing the procedures performed during 2019 [109]. It is of importance to remember that even non-surgical therapy does not always help patients [12, 110].

The existence of this multitude of proposed therapies and the ongoing debate about the optimal treatment suggest the need for novel, preferably less invasive approaches.

The rationale for biological strategies

The link between IVD degeneration and LBP and our growing understanding of the biological processes involved have led to the concept of trying to decelerate or reverse the degenerative process in an effort to alleviate the symptoms, namely LBP [111]. In theory possible approaches could include local or systemic administration of agents enhancing the homeostatic functions of resident cell populations, local implantation of a cell population that could boost the existing IVD cells or undertake the maintenance or/and regeneration of the IVD and finally implantation of some kind of biomaterial (eventually laden with exogenous cells) in order to ameliorate the mechanical properties of the IVD [32, 111].

In recent years several of the above-mentioned strategies have been investigated [112, 113]. One of the approaches that has gained much attention and has translated into clinical investigations is the use of mesenchymal stromal cells (MSCs) to repopulate degenerate IVDs.

MSC, a brief history of an abbreviation

Recapitulating a long line of work originating in the 1960s and 1970s [114-117], Owen and Friedenstein proposed in 1988 the term "stromal stem cell" for a cell type residing in the bone marrow [118]. This cell had been discovered through serial *in vivo* transplantations with the use of diffusion chambers and *in vitro* monolayer cultures in clonal density. Initially described as a colony forming unit – fibroblastic (CFU-F) this non-hematopoietic cell displayed self-renewal capacity and could give rise to different tissues. The realization that *in vitro* manipulation led to differentiation of this precursor cell type into different mature cell types, even of different embryological lineages, led Caplan to using the term "mesenchymal stem cell" and the acronym MSC [119]. In the laboratory such cells could be isolated from different tissue sources by means of adherence to the plastic material of tissue culture flasks and by detection of surface markers, none of which were though unique for any given cell type.

As understanding grew it became more and more evident that, although a genuine stem cell exists, the majority of the cells isolated by bulk bone marrow cultures did not live up to the stringent criteria of the definition [120]. It became evident that the cells isolated from the bone marrow resided in the stromal fraction, as part of the hematopoietic niche. The term "stromal" gained ground and the acronym could be retained. The International Society for Cellular Therapy (ISCT) issued criteria for characterizing these cells *in vitro*, based on adherence to plastic, a set of specific surface antigens (markers) and *in vitro* differentiation into the osteogenic, chondrogenic and adipogenic lineages [121].

As the pleiotropic mechanisms of action of these cells unraveled and our knowledge expanded, it was realized that the *in vitro* behavior of these cells was not necessarily analogous to their *in vivo* role. Caplan introduced the term "medicinal signaling cells" retaining the same acronym [122]. The term of skeletal stem cell was introduced to describe the *bona fide* stem cell residing in the stroma of the bone marrow [123].

This is a valid, ongoing and conceptually important debate that extends beyond mere onomatology. During the course of these studies, we shifted from "mesenchymal stem cell" in studies I and II to "mesenchymal stromal cell" in studies III, IV and V, acknowledging that the isolation procedure used does not secure the isolation of a stem cell but rather of a population of progenitor cells residing in the stroma of the bone marrow. In the literature, including studies referenced throughout the present thesis, the terms are sometimes used arbitrarily and some authors prefer using combinations such as "stem/stromal/precursor" to avoid the controversy. In the remainder of this work the MSC acronym shall refer to "mesenchymal stromal cell" with regard to the studies comprising this thesis, unless otherwise stated.

A few more words on MSCs

MSCs, initially described as colony forming units in monolayer cultures of bone marrow [114], are multipotent cells, capable of differentiation into different lineages. The 2006 position paper of the ISCT [121] for identification of MSCs relied on tri-lineage *in vitro* differentiation capability, adherence to plastic in standard culture conditions and a phenotype as described by a set of surface markers: MSCs must express (>95% +) markers CD73, CD90 and CD105 and lack expression (<2% +) of markers of cells of the hematopoietic lineage, namely CD45, CD34, either CD14 or CD11b, CD79a or CD19, and HLA-DR (human leukocyte antigen-DR isotype). The paper has been revised in 2019 to include functional definitions of the investigated cells, including "annotation of origin and a robust matrix approach to demonstrate relevant functionality" [124].

Although the MSCs are capable of differentiating into different cell lineages and thus providing a source of cells for tissue homeostasis and repair, they seem to exert their actions also through other mechanisms [125]. They have a paracrine role [126], supporting resident cells through excreted agents, including exosomes [127, 128] and are shown to have immune-modulating effects [128, 129].

Precursor cells, often ascribed the "MSC" acronym have been isolated from a variety of different tissues, including bone marrow, adipose tissue, synovium, umbilical cord, lung, amniotic fluid and dental pulp [126]. Of particular interest is the fact that there seems to be a population of progenitor cells even in the IVD [130], with their number decreasing with age and degeneration [131].

MSCs have the ability to respond to the microenvironment around them, for example to the elasticity of the substrate used for cell culture [132] or the pH [133].

MSCs have been tested in various clinical trials in orthopedic applications with no serious adverse effects reported [134]. Even when evaluating the safety of MSC therapies as a whole, no safety concerns have been raised [135, 136].

MSCs and IVD degeneration, the evidence for cell therapy

Experimental studies have yielded positive results using co-cultures of MSCs and IVD cells [137-140]. Animal studies have confirmed that implantation of MSCs in IVDs in disc injury or degeneration models is feasible, safe and yields positive results in terms of survival of the implanted cells and matrix production [141-143]. Results from different studies have shown that the MSCs could exert their action in different ways, by differentiating towards chondrocyte-like cells of the NP [144], by affecting the resident cell population [129], or a combination of both [137].

In order to study the fate of the injected MSCs in animal models, a variety of labeling agents can be used. The investigation is often performed on histology sections after the animals have been sacrificed. Superparamagnetic iron oxides (SPIOs) produce a traceable signal on MRI and can be therefore used for the *in vivo* tracking of cells in animal models [145], offering even the possibility of histological confirmation of the iron content [146]. Such agents have been available for clinical use for the detection by MRI of liver pathologies or metastases in lymph nodes [147], but their commercial failure led to discontinuance of production.

The clinical translation of MSC cell therapy

The laboratory and pre-clinical experimental findings have encouraged a few reported cohort clinical human trials employing MSCs injected into degenerative IVDs in order to ameliorate LBP [148-154]. A phase III trial is expected to be completed under 2021 [155], having published results on a safety and efficacy cohort of 100 patients [156]. Even a phase II/III study is ongoing [157].

Other studies have reported similar approaches, using for example bone marrow concentrate, platelet-rich plasma alone or in combination with stromal vascular fraction (product of liposuction) for intradiscal injections in degenerate IVDs [158-161]. Cell therapy applications have been investigated in the context of disc herniation or adjacent level disease after lumbar fusion surgery [162, 163]. Although undoubtedly interesting, these studies cannot be directly compared to studies investigating the use MSCs in patients with IDD.

The efficacy of each treatment can be assessed regarding the possible effects on the tissue by methods such as MRI. The need to try and better assess the outcome from the patients' perspective is one of the reasons that has led to the growing use of patient-reported outcome measures (PROMs) in the past decades [164, 165]. Overall quality of life, function or specific symptoms such as pain can be assessed. Among the most frequently used are the visual analog scale (VAS) and the numerical rating scale (NRS) for assessment of pain, the Short-Form-36 (SF-36) and the European Quality of Life-5 dimensions (EQ-5D) questionnaires for quality-of-life assessment, the Oswestry Disability Index (ODI) for assessment of the functional status of the lumbar spine [166].

A brief presentation of the studies that have investigated MSCs as therapeutical agents against LBP is necessary in order to set the scene for this thesis and facilitate discussion and comparison.

Yoshikawa et *al.* [152] reported a case series of 2 patients, treated with autologous, expanded, bone-marrow derived MSCs (BM-MSCs), embedded in a collagen sponge (a solution of 1 x 10^5 cells/ml was used). At 2 years favorable results were reported for pain and disability as well as increased signal intensity on T2W MRI.

Orozco *et al.* [151] presented a pilot cohort of 10 patients treated with percutaneous, intradiscal injection of autologous, expanded BM-MSCs (10±5 x 10^6 cells/disc). At 12 months VAS and ODI had improved, with a reported treatment efficacy of 71% (compared to an ideal response to treatment). MRI showed no difference of disc height but a statistically significant increase in IVD signal intensity on T2W MRI (normalized to the signal intensity of the healthy IVDs).

Pang *et al.* [154] reported a case report of 2 patients who were treated with intradiscal injection of allogeneic, umbilical cord derived MSCs (1 x 10^7

cells/disc). Pain alleviation and function amelioration were reported, with a 2-year follow-up.

Elabd *et al*. [149] reported a case series of 5 patients treated with a percutaneous, intradiscal injection of autologous BM-MSCs, expanded under hypoxic conditions. The cells (15.1–51.6 x 10^6 MSCs/disc) were suspended in autologous platelet lysate prior to injection. At follow-up (4-6 years) the overall improvement in quality of life was reported between 10%-90% and seemed to correlate with the number of cells used. MRI showed no adverse effects, maintenance or slight decrease of disc height and improvement of posterior bulging in 4/5 patients.

Noriega *et al.* [153] published the results of the 12-month follow up of their study of percutaneous intradiscal injection of allogeneic BM-MSCs from healthy donors. The 24 patients were randomized in a treatment group, receiving 25 x 10^6 MSCs per disc, and in a control group (sham infiltration of paravertebral muscles with local anesthetic). The total efficacy of the treatment reached 28%. A subgroup of patients in the treatment group that responded very well to the treatment could be identified (responders). IVD signal intensity improved in the treatment group (not reaching statistical significance) and the Pfirrmann grading showed amelioration. The same group published recently a report with a follow-up of 3.5 years [167], with an even higher treatment efficacy for the treated group (60% for pain and 71% for ODI) and a maintained amelioration of the Pfirrmann grade. A larger, phase II/III multicenter trial with a similar set up is ongoing [157].

Kumar *et al.* [150] employed autologous, expanded, adipose-tissue derived MSCs (AT-MSCs) that were delivered with a hyaluronic acid derivative as a carrier. A total of 10 patients were recruited and divided in 2 groups, one receiving 2 x 10^7 cells/IVD and one 4 x 10^7 cells/IVD. During the 12-month follow-up 6 patients (3 from each group) reached >50% amelioration in reported pain and ODI. Quantitative MRI showed improvement of IVD hydration in 3 of these patients.

Centeno *et al.* [148] reported on 33 patients treated with autologous BM-MSCs that were expanded in hypoxic conditions (5% oxygen) and injected in platelet lysate at concentrations varying from 1.73 x 10^6 to 4.5 x 10^7 cells per IVD. A platelet lysate epidural injection was performed 2 weeks prior to as well as 2 weeks after the MSC injection. Pain reduction and functional amelioration were reported.

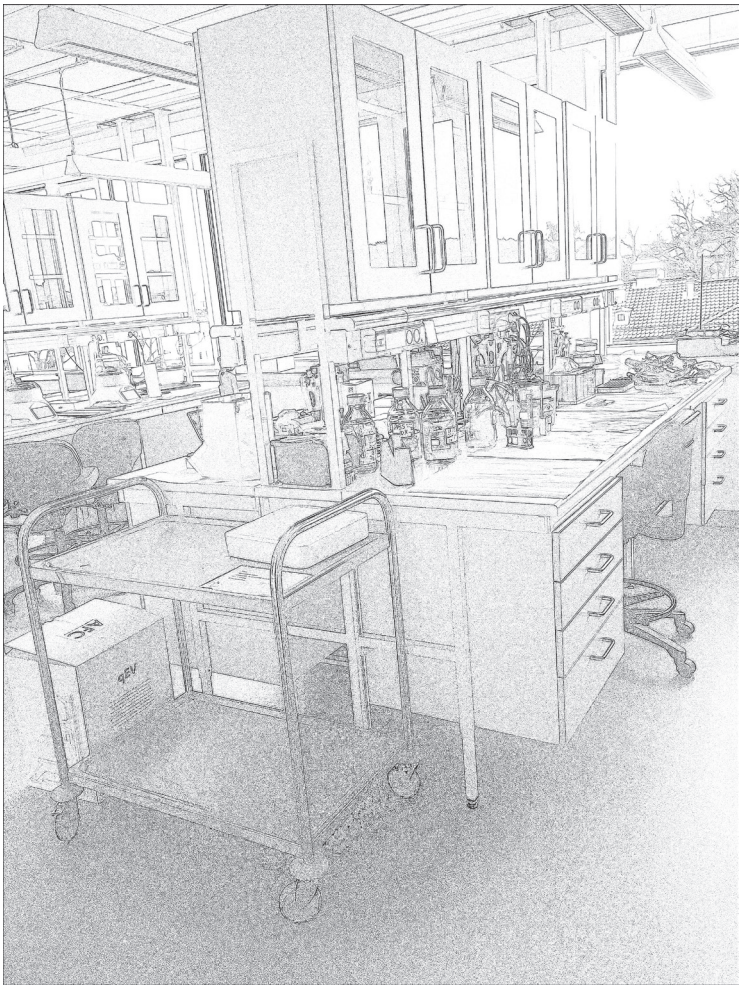
Amirdelfan *et al.* [156] reported on a phase II safety and efficacy study on the use of allogeneic mesenchymal precursor cells (MPCs) combined with hyaluronic acid (HA). The cells were derived from a single healthy donor using proprietary methods. A total of 100 patients were randomized into receiving a saline, placebo injection, injection of HA alone, or a combination of cells with HA at two different concentrations, either 6 x 10^6 or 18 x 10^6 cells per IVD. The treatment was deemed to be safe, including control of possible immunologic host reaction and the groups treated with MPCs showed improvement in pain and function compared to the control groups. No evident changes on MRI were detected. A phase III study comparing the low MPC dose (6 x 10^6) with and without HA to placebo is ongoing [155]. Both studies are funded by a private company, Mesoblast Ltd, and will occasionally be referred to by the company´s name.

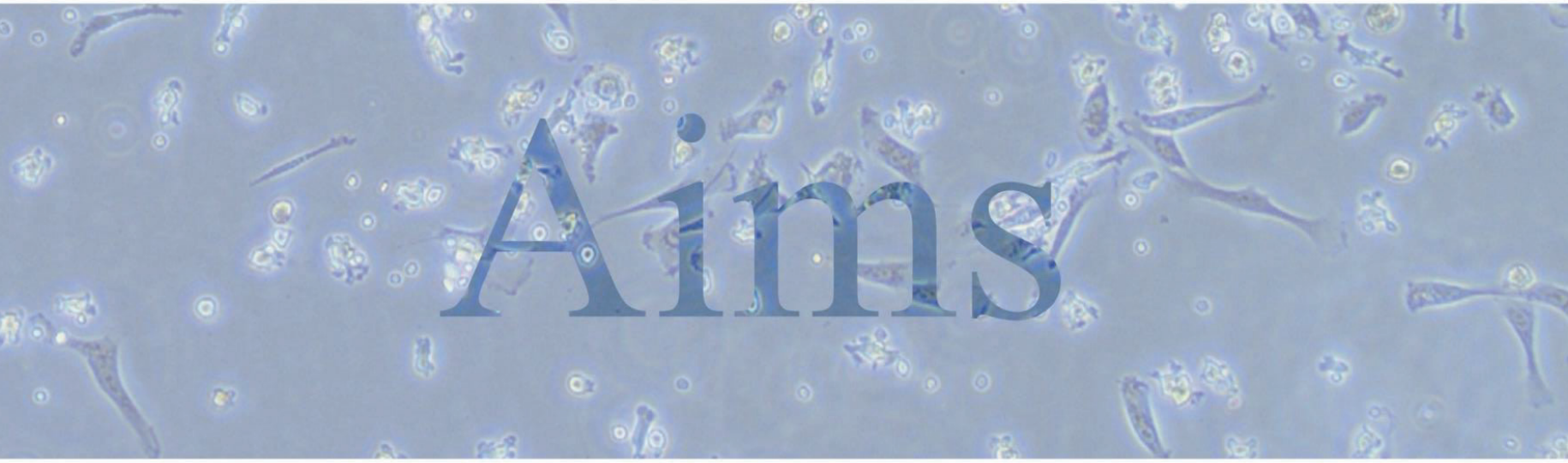
The above-mentioned studies display a striking heterogeneity in terms of type and number of MSCs used, the use or not of a carrier, the duration of the symptoms prior to the intervention and the outcome measures used to evaluate the results clinically and radiologically, thus impeding a direct comparison.

The efficacy of cell therapy for LBP, the optimal type and number of cells, the appropriate patient selection and the timing of the intervention, all remain unsolved research questions.

A major question that has not been addressed in clinical trials is the fate of the intradiscally-injected MSCs. In aforementioned pre-clinical *in vivo* animal studies, the procurement and examination of tissue samples has offered us valuable insight into the possible mechanisms in play. The availability of human tissue samples from IVDs is extremely limited and at the beginning of the studies in this thesis no cell-labeling agent was available for clinical use.

A method of labeling BM-MSCs and a clinical study design where retrieval of an IVD injected with the labeled cells would be ethically defendable could promote our understanding of the biological processes in place.




Aims

3. AIMS

The overall aim of the studies was to investigate the iron labeling of human BM-MSCs both *in vitro* and in an animal study, to assess the feasibility and safety of the intradiscal injection of autologous, iron-labeled BM-MSCs in patients with LBP and IDD and to examine the fate of these cells after the injection.

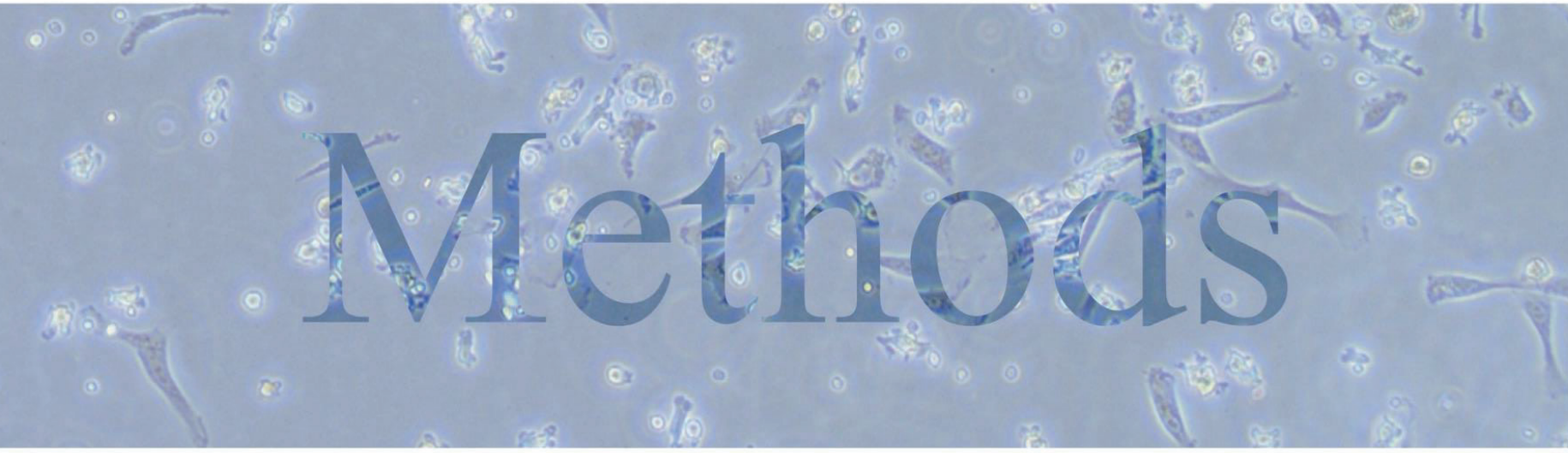
The specific aims of the studies comprising this thesis were the following:

- To investigate whether iron sucrose could be used for labeling and tracking of human BM-MSCs and to assess the labeling process with regard to uptake, tracing and possible effects of the labeling on cell viability and phenotype *in vitro* and *in vivo* (studies I and II).

- To assess the feasibility of injecting autologous, iron-labeled, expanded BM-MSCs in degenerate IVDs in patients with LBP waiting for lumbar surgery (fusion or TDR) and evaluate the intervention with regard to preparation of the cell product, adverse events (both clinically and radiologically) and clinical outcome with the use of PROMs (study III).

- To perform a longitudinal evaluation of multiple parameters on the acquired radiological investigations (MRI) and describe possible changes during follow-up (study IV).

- To examine the presence of labeled MSCs in histological preparations of IVDs injected with MSCs and thereafter explanted during lumbar surgery. Further, to attempt to indirectly assess the function of the injected cells (study V).


Methods

4. METHODS

4.1 THE OVERALL CONCEPTUAL FRAME OF THE PRESENTED STUDIES

As suggested by the aims, iron sucrose (Venofer®, Vifor Pharma Nordiska, Solna, Sweden) was evaluated as a possible cell tracer for human BM-MSCs. In **study I** cells from human donors were labeled. Both labeled and non-labeled cells were characterized by surface markers and investigated for uptake and viability. The cells were subsequently cultured in a three-dimensional chondrogenic system for up to 28 days in order to test the capacity to detect the signal in an *in vitro* biological system and assess the functionality of the cells. Tracing of the labeled cells was attempted as well as a comparison of cell functionality between labeled and non-labeled cells.

In the first part of **study II** labeled and non-labeled MSCs from human donors were compared for their differentiation capacity into the chondrogenic, adipogenic and osteogenic lineages. In the second part, iron-labeled and non-labeled cells from the same human donor were injected in IVDs in a lapine animal model. The animals were sacrificed 1 and 3 months after the injection, the lumbar spines harvested and controlled for the presence of the human cells, the capacity of the method to detect the iron label and the cell viability in an *in vivo* model.

Having established a labeling method for MSCs **study III** was planned and performed. Patients from the waiting list for surgery due to LBP attributed to IDD in one or two levels of the lumbar spine were recruited to a feasibility cohort. MSCs were isolated from bone marrow aspirates and expanded *ex vivo*. An intradiscal injection of autologous, iron-labeled MSCs into degenerate IVDs was performed and the patients were followed up longitudinally by means of PROMs and MRI controls at regular intervals up to 2 years after the injection.

MRI investigations were routinely reviewed by the hospital´s on duty radiologist in study III, with focus on adverse events and major radiological changes. A longitudinal, thorough evaluation of multiple radiological

parameters was performed in **study IV** in order to describe possible changes that could be attributed to the intervention.

During the course of the follow-up after the intradiscal cell injection, patients could opt to proceed with the surgical intervention that was originally planned, either transforaminal lumbar interbody fusion (TLIF) or TDR. As part of the surgical procedure the affected IVDs were removed and would otherwise be discarded. These IVD tissues were harvested and investigated for the presence of injected MSCs. The results of this investigation were presented in **study V**.

During the course of studies I, II, III and V different tissue samples (bone marrow, lapine lumbar spines, human IVD fragments) were harvested, processed and examined. Different types of cells (human BM-MSCs and cells from lapine IVDs) were isolated, expanded, characterized and investigated. An array of different laboratory techniques was employed, with some of them being used in more than one study. The parameters assessed included cell viability, morphology, spatial distribution in tissue samples, functionality, gene expression and detection of products of the cellular metabolic activity (for example cell surface proteins or components of the ECM). Furthermore, in studies III and IV a longitudinal clinical and radiological assessment of patients was performed.

To facilitate presentation and understanding of the role of each technique, first a brief outline of the pre-clinical and clinical *in vivo* studies in relationship to the laboratory methods will be presented. **Table 1** summarizes the techniques used within each study. Thereafter, a more detailed description of the different techniques follows, alongside **table 2** which provides an overview of the molecules used in the different investigations. Finally, the methodology used for the clinical and radiological follow up will be outlined.

4.2 OUTLINE OF THE IN VIVO STUDIES

4.2.1 The pre-clinical study – Lapine model in study II

Twelve New Zealand female white rabbits of 3 months of age (skeletally mature) were used. BM-MSCs from one human donor, both iron-labeled and non-labeled, were prepared. In each animal one IVD level was injected with iron-labeled and one with non-labeled cells (250 000 cells, 10 µl suspension

per level). The injections were performed with the animals anesthetized, with the help of an image intensifier and a 22-gauge needle. The animals were sacrificed 1 month (6 animals) and 3 months (6 animals) post-injection. The lumbar spines of 8 animals (4 from each time point) were harvested *en bloc* with surrounding tissues, prepared, sectioned (coronal plane, 5-7 μm sections) and examined by histology for morphology, detection of GAGs and iron-labeled cells and by immunohistochemistry (IHC) for the determination of presence of human cells (with a mouse anti-human nuclei primary antibody). The harvested lumbar spine tissue from 4 animals (2 from each time point) were used for isolation of IVD cells (of both lapine and human origin) and control of viability.

4.2.2 The clinical studies III and V

BM-MSCs were harvested from 10 patients and expanded using autologous serum. The cells were characterized by surface marker profiling after isolation and prior to injection of the final product (MSC markers: CD90, CD105, CD166, hematopoietic markers: CD34 and CD45 were used). Labeling with iron sucrose was performed. The cell cultures were controlled for possible contamination by bacterial cultures (BacT/ALERT system) of the culture medium from the last medium change and of samples from the final product. Pellet mass cultures of labeled and non-labeled cells were also performed.

The MSCs to be injected were suspended in culture medium (F12, Thermo Fischer Scientific, MA, USA) without antibiotics and 20% autologous serum at a concentration of 1 x 10^6 cells/ml.

The intradiscal injection was performed percutaneously, under sterile conditions, with the help of fluoroscopy and the use of a 22G discography needle. Approximately 1 x 10^6 cells per IVD were injected. A single dose of antibiotics was administered intravenously. ***Figure 2*** presents a fluoroscopy image and a photograph from this procedure.

Some of the patients were at later time points operated upon as originally planned and the injected IVDs were perioperatively removed as a step of the surgical procedure. Samples from 4 patients, in form of large tissue fragments, were harvested and investigated in study V. The samples were sectioned and the sections serially numbered. Every 20th section was stained with Alcian

blue van Gieson for the assessment of tissue and cell morphology and the accumulation of ECM (GAGs and collagen). The next consecutive section was stained with Prussian blue and controlled for the presence of iron-labelled cells. Areas with clusters of iron-positive cells were defined as regions of

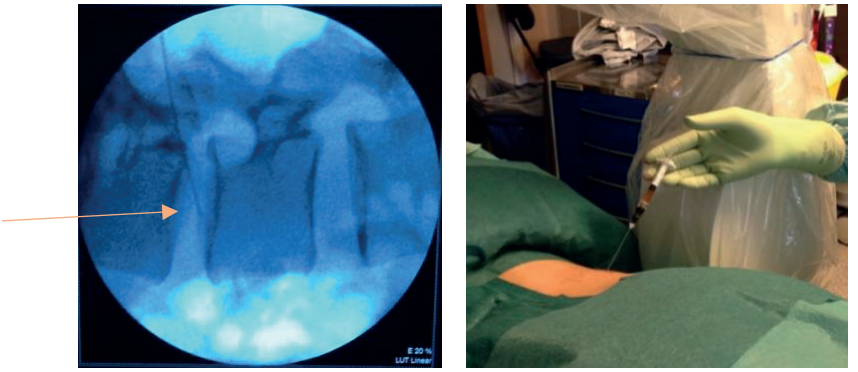


Figure 2. *The percutaneous injection of the BM-MSCs was performed under fluoroscopy control. Positioning of the needle in the center of the IVD was controlled in two planes (lateral and coronal). Left: Lateral fluoroscopy projection. The arrow is pointing at the tip of the needle, in the IVD during the procedure. Right: The syringe with the cell product just prior to injection.*

interest (ROIs). Adjacent, consecutive sections were then controlled with IHC for the expression of SOX9 (marker of chondrogenesis), COL2A1 (collagen synthesis), proliferation marker PCNA, CD68, a marker of macrophages [168] and presence of apoptotic cells (TUNEL). ROIs with cluster of cells were identified in 2 patients, both operated 8 months after the injection. Sections from 4 different tissue depths were investigated for each from the 4 patients. ***Figure 3*** is a graphical illustration of the procedure. In ***Figure 4*** the timeline of studies III, IV and V is depicted.

The cell product for the performed injections was prepared by the Cell and Tissue Laboratory, at the Department of Clinical Chemistry, Sahlgrenska University Hospital under GMP (Good Manufacturing Practice) conditions.

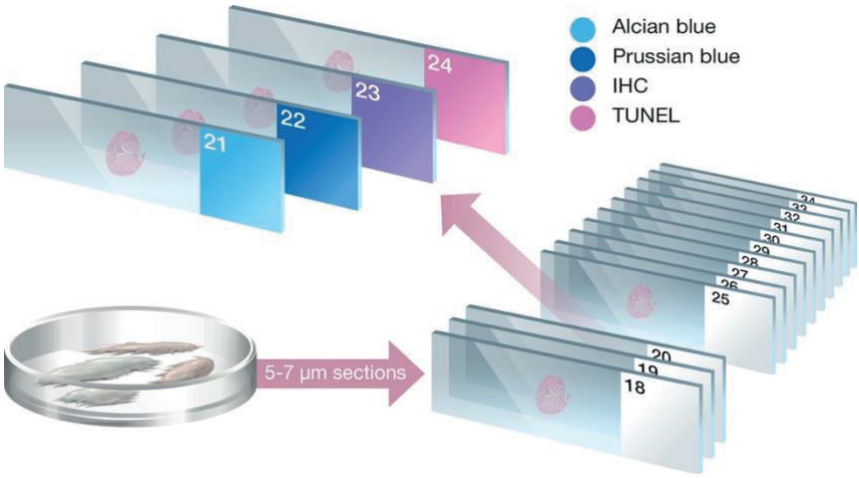


Figure 3. *Graphical illustration of the experimental set-up in study V. The explanted tissue fragments from IVDs injected with iron-labeled BM-MCSs were prepared and sectioned consecutively. The sections were numbered. Every 20th section was stained with Alcian blue van Gieson and the next section was stained with Prussian blue. In the cases where clusters of iron-positive cells were detected regions of interest were defined and investigated by IHC and the TUNEL assay in adjacent sections. Sections from 4 different tissue depths were investigated*

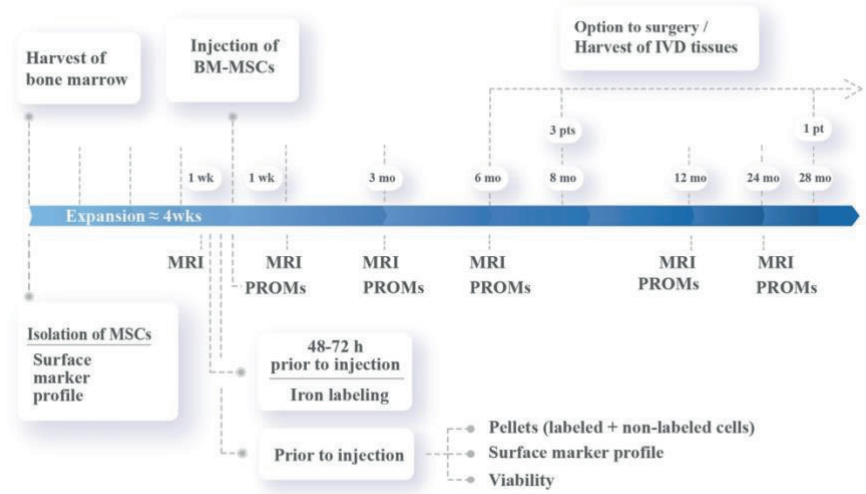


Figure 4. *A graphical illustration of the timeline in studies III, IV and V showing the key events. The time from harvest of bone marrow and isolation of BM-MSCs to the injection was approximately 4 weeks. Iron labeling was performed 2-3 days prior to implantation and the cell product was thereafter controlled. Follow-up consisted of regular MRI controls and PROMs. After the 6-month time-point, patients could opt to proceed with the surgical procedure that was originally planned. During surgery, the injected IVD, that would otherwise be discarded, was harvested and examined. (wk: week, wks: weeks, mo: months, pt: patient, pts: patients)*

Table 1. Parameters assessed and techniques employed in studies I, II, III and V

Study	Tissue harvest	Surface marker profile (FACS)	Uptake of Fe^{2+}	Cell viability	Pellet mass culture	qRT-PCR	IHC Fluorescence	Histology
I	BM-MSCs 7 donors	CD105 CD166 CD34 CD45	Cytospin Prussian blue	FACS after labeling	4 donors IHC Histology (Detection Of labeled MSCs)	(Pellets) ACAN CD105 CD166 SOX9 COL2A1 COL2B	(Pellets) SOX9 Coll2A1 C6S	Pellets
II	BM-MSCs 3 donors Lapine lumbar segments 12 animals	CD105 CD166 CD34 CD45		FACS of cells from explanted lapine IVDs	3 donors Chondrogenesis		(Pellets) Coll2A1 (Lapine IVDs) Human nuclei	Pellets Explanted lapine IVDs Osteogenesis, Adipogenesis.
III	BM-MSCs 10 patients	CD90 CD105 CD166 CD34 CD45		FACS Trypan blue (prior to implantation)	10 patients Ability of MSCs to form pellets			Pellets
V	IVD tissues from surgery 4 patients						SOX9 PCNA Coll2A1 CD68 TUNEL	Yes

4.3 HARVESTING AND PREPARATION OF TISSUES

4.3.1 Harvesting of MSCs from bone marrow (studies I, II and III)

Bone marrow aspiration was performed from the posterior iliac spine, in the prone position, with the use of a 14.5G bone biopsy needle. The needle was repositioned several times during aspiration to ensure maximal yield [169].

For donors in studies I and II aspiration was performed during lumbar spine surgery under general anesthesia and through the surgical incision. For patients in study III aspiration was performed percutaneously under local anesthesia.

4.3.2 Isolation of MSCs from bone marrow and monolayer cultures (studies I, II and III)

Isolation of MSCs from bone marrow aspirate in studies I, II and III was performed by centrifugation in a Ficoll density gradient [170] and seeding in plastic cell culture flasks. Expansion medium consisted of Dulbecco's modified Eagle's medium with low glucose (DMEM-LG) with the addition of antibiotics, L-glutamine, b-FGF (fibroblast growth factor) and 10% human serum (autologous in study III). Cells were incubated at 37°C, 7% CO_2 and 93% air. At approximately 90% confluence cells were detached using trypsin and reseeded at a density of approximately 15 x $10^3/cm^2$. In study III no antibiotics were used after the last passage before the implantation in the IVDs.

4.3.3 Iron labeling of MSCs (studies I, II and III)

Iron labeling of MSCs in studies I, II and III was performed by incubating the cells for 16 hours in serum-free DMEM-LG with the addition of iron sucrose (Venofer®) at a concentration of 1 mg/ml [138].

4.3.4 Harvesting and preparation of lapine lumbar spine tissues (study II)

Lumbar spine tissues were harvested *en bloc* from the sacrificed animals as described. The samples were immersed in 4% formaldehyde, embedded in paraffin and decalcified with the use of a 12.5% EDTA (ethylenediaminetetraacetic acid) solution. The IVD tissues could be then consecutively sectioned on the coronal plane and serially numbered. Prior to analysis the sections were deparaffinized by immersion in xylene solution (2 x 10 min) and rehydrated in ethanol solutions (99% - 95% - 70%, 5 min in each solution and then rinsed with PBS – phosphate buffer saline, pH 7.4).

4.3.5 Harvesting and preparation of IVD tissues from patients (study V)

During surgery (either TLIF or TDR) in patients who chose to be operated according to the original planning, IVDs injected with MSCs were harvested as part of the surgical procedure in large tissue fragments. Tissue samples were immersed in 4% formaldehyde, imbedded in paraffin and decalcified (12.5% EDTA solution). Sections with a 5-7 μm thickness could then be prepared. These paraffin sections were deparaffinized with immersion in xylene (2 x 10 min) and rehydrated with ethanol solutions (99% - 95% - 70%, 5 min in each solution, then rinsed with PBS) prior to analysis by histology or immunohistochemistry.

4.4 LABORATORY TECHNIQUES

4.4.1 Histology

Standard histology sections were stained with Alcian blue van Gieson for morphological studies and detection of GAGs by light microscopy. GAGs appear blue and collagen (all types) pink with this staining [171].

Prussian blue staining was used in order to detect iron deposits which appear blue when assessed with light microscopy [172].

Specific dyes were used in study II to assess the osteogenic (von Kossa [173]) and adipogenic (oil red [174]) differentiation potential of the MSCs and are presented later.

4.4.2 FACS (Fluorescence Activated Cell Sorting)

FACS analysis was used in studies I, II and III in order to characterize the cells with respect to surface markers (antigens). In FACS analysis a light beam is directed at one cell at a time and scattering or fluorescence of the light can be detected [175]. Forward scatter (in the direction of the light beam) gives information about the size of the cell or particle examined. Side scatter reflects the granularity of the examined cell. Specific antibodies can be conjugated to fluorescent agents (usually fluorescein, phycoerythrin or peridin) and detection of fluorescence at different wavelengths is translated into information about the surface antigens. In studies I and II cells were tested for CD105 and CD166 (MSC markers) as well as CD34 and CD45 (markers of hematopoietic cells, HC). In study III an additional MSC marker, CD90, was investigated.

In studies I, II and III FACS was also used to measure cell viability by the 7-aminoactinomycin D (7-AAD) method [176]. 7-AAD binds strongly to DNA but cannot penetrate the cell membrane of live, intact cells, thus labeling only non-viable ones.

4.4.3 Cytospin

Cytospin centrifuge system results in a thin layer preparation from liquid medium, especially from hypocellular fluids, depositing cells evenly on glass

slides in a small, defined area, and thus facilitating examination of the cells [177]. In study I cytospin sample preparations of iron labeled MSCs were stained with Prussian blue staining for detection of iron deposits with light microscopy (blue color).

4.4.4 Culture systems for *in vitro* multilineage differentiation capacity of MSCs

1) Pellet mass culture system (Chondrogenesis)

The pellet mass culture system used in studies I, II and III is a chondrogenesis-inducing system for MSCs [178]. Chondrogenesis in this system is driven by cell-to-cell interactions in the three-dimensional environment, mimicking the *in vivo* conditions, and the addition of dexamethasone and transforming growth factor-β1.

Histology sections stained with Alcian blue van Gieson were used for describing morphology and detection of GAGs (blue color) to assess chondrogenesis by light microscopy.

Pellets from iron-labeled cells were additionally stained with Prussian blue in order to detect iron deposits (blue color) with light microscopy.

IHC was additionally performed on pellet sections in studies I and II, as presented separately.

2) Adipogenesis

A commercially available kit was used, STEMPRO® Adipogenesis Differentiation Kit. Standard histology sections were stained with oil red for the detection of lipid-containing vesicles (appearing red) in adipocytes with light microscopy.

3) Osteogenesis

Osteogenesis was induced with the use of β-glycerophosphate in two-dimensional cultures [179, 180]. Standard histology sections were stained with von Kossa staining for detection of calcium deposits (brown/black color).

4.4.5 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is a method that exploits the specificity of antibody-antigen reaction to identify an epitope (antigen) if present in the investigated tissue and relies on fluorescence for detection. IHC involves often 2 antibodies. The primary antibody binds to the epitope while the secondary one binds to the primary. Secondary antibodies are often labeled with fluorescent molecules that can be detected by fluorescent microscopy. Alternatively, secondary antibodies can be labeled with horseradish peroxide (HRP) or biotin. These molecules need the addition of a substrate (reporter molecule) that emits fluorescent light upon contact with the label of the secondary antibody [181, 182].

IHC was used in studies I, II and V. The investigated molecules can be seen in table 2.

4.4.6 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR is a method that can be used to detect and quantify specific mRNA sequences. After RNA isolation gene-specific primers are used that enable the synthesis of coding DNA (cDNA) sequences with the help of reverse transcriptase. Then the cDNA is amplified by PCR cycles. Automated detection and quantification of the end product is done in commercially available systems and by taking into account the number of PCR cycles the amount of the original mRNA can be calculated [183].

qRT-PCR was performed in study I for the detection of activation of multiple genes. RNA isolation was performed using a commercially available kit, following the manufacturer's protocol (Qiagen). Commercially available gene expression assays were used and 40 cycles of PCR were performed. Ct (cycle) values over 37 were considered not detected. Due to interaction or interference of the iron compounds with the polymerase enzymatic activity only detection of the target mRNAs was possible and not quantification [184]. The investigated genes can be seen in **tables 1** and **2**.

4.4.7 TUNEL Assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay detects DNA fragmentation occurring during apoptosis [185]. The terminal deoxynucleotidyl transferase is an enzyme that can attach deoxynucleotides to the 3-OH´ end of DNA breaks. The nucleotides used can be tagged with fluorescent agents and thus be detected. A commercially available kit was used according to the manufacturer's instructions (FragEL DNA Fragmentation Detection Kit). Fluorescence microscopy was used to evaluate results. The assay was used in study V in order to examine the presence of apoptotic cells in the explanted IVD tissues.

Table 2. Investigated molecules in the studies

Marker		Role	Technique / Study
CD90	Thy-1, cell membrane protein	MSC marker	FACS / III
CD105	Endoglin	MSC marker	FACS / I, II, III qRT-PCR / I
CD166 (protein) ALCAM (gene)	Transmembrane glycoprotein	MSC marker	FACS / I, II, III qRT-PCR / I
CD34	Transmembrane sialomucin	Marker of hematopoietic cells	FACS / I, II, III
CD45	Membrane protein	Marker of leukocytes	FACS / I, II, III
SOX9	Sex determining region Y-box 9	Transcription factor of chondrogenesis	IHC / I qRT-PCR / I
ACAN	Gene encoding aggrecan	Proteoglycan of the ECM	qRT-PCR / I
C6S	Chondroitin-6-sulfate	Glycosaminoglycan in newly synthesized ECM	IHC / I
Coll2A1 (protein) COL2A1 (gene)	Collagen 2A1	Chondrogenic marker, early ECM synthesis	IHC / I, II, V qRT-PCR / I
COL2B	Collagen 2B	Chondrogenic marker, mature ECM	qRT-PCR / I
PCNA	proliferating cell nuclear antigen	Marker of proliferation	IHC / V
CD68	Transmembrane glycoprotein	Marker of macrophages	IHC / V

4.5 THE PATIENTS, BASELINE DATA AND FOLLOW-UP - PROMS

4.5.1 Patients

Patients in studies III, IV and V were recruited from the waiting list of our clinical department for lumbar surgery (TLIF or TDR) due to LBP on the basis of IDD. Candidate patients had an age between 18-65 years, suffered from severe LBP proven refractory to non-surgical treatment, experienced no radiation of pain below knee level and had been diagnosed with IDD at one or two levels of the lumbar spine. Rheumatoid arthritis and other serious co-morbidities rendered candidate patients ineligible for inclusion in the study.

4.5.2 Baseline Data, Follow-up and PROMs

The collection of baseline data and the prospective clinical follow up was performed with the use of the questionnaires used by Swespine which has implemented the 2015 recommendations of the consensus proposal by the study group of the International Consortium for Health Outcomes Measurement (ICHOM) [186].

The questionnaires were completed prior to the injection of the MSCs and at 3-, 6-, 12- and 24-months post-injection during consultation at the outpatient clinic. On a few occasions patients chose to send in the questionnaires by post. MRI controls were performed at approximately the same time points, with an extra investigation taking place within one week after the injection.

Baseline data collected included weight and height, working status, smoking habits, duration of pain in the back and the leg, pharmacological and non-pharmacological treatment, co-morbidities and educational level.

At the beginning of the study intensity of LBP and leg pain (during the week prior to the visit) was recorded using a visual analogue scale (VAS). Following the aforementioned ICHOM recommendations, Swespine adopted an 11-point numerical rating scale (NRS) where 0 stands for “pain free” and 10 for “the worse thinkable pain”. Both scores have proven to be responsive in a LBP patient population [187] and have shown to be highly correlated in patients with chronic pain (not interchangeable though) [188]. This change left us with

reports in both PROMs. Observations in VAS were converted by the investigators to NRS as this was the PROM to be used in the future. The VAS value was measured in millimeters, converted to centimeters and rounded to a whole digit.

The EQ-5D and accompanying EQ-VAS (European Quality of life-Visual Analogue Scale) is a generic preference-based outcome measure [189, 190]. The version used in Swespine is the EQ-5D-3L, using 5 dimensions (mobility, self-care, usual activities, pain/discomfort and anxiety/depression) with 3 levels each (1=no, 2=some or 3=extreme problems). The resulting 5-digit code represents a health state. These health states can be converted to a single summary index using appropriate value sets. In study III, as in Swespine, the United Kingdom value set was used. EQ-5D has not been validated for use in LBP patients [186] but a recent review concluded that it performs well in this patient population [191]. One of the biggest benefits with the use of EQ-5D is its widespread use for health economic evaluations as it permits cost-effectiveness and value assessment [187].

The ODI questionnaire was first published in 1980 [192] and has since then been revised [193]. ODI is the most widely used disease-specific PROM for evaluating function of the spine [187]. It has been validated and found to be reliable and responsive in LBP populations [187]. It consists of 10 sections with 6 statements each. The maximum score for each section is 5. The first statement has a score of 0, the second a score of 1 and so forth. The section scores are added and presented as a percentage (or points out of 100 possible). Higher scores represent better functional outcomes.

4.6 RADIOLOGICAL EVALUATION

MRI controls were scheduled prior to the injection of the MSCs, within one week as well as 3-, 6-, 12- and 24-months post-injection. Patients who opted for lumbar surgery during the course of the study did not undergo later follow-up examinations.

The MRI control of the lumbar spine consisted of T2W turbo spin echo (TSE) axial and sagittal sequences and T1W TSE sagittal ones. A 1.5 scanner was used (Philips Healthcare, Best, The Netherlands). The acquisition parameters are presented in table 3.

Table 3. MRI acquisition parameters for T1W and T2W examinations

	T1W TSE	T2W TSE	T2W TSE
Imaging plane	Sagittal	Sagittal	Axial
Slice thickness (mm)	3.5	3.5	3.5
FOV (mm^2)	350x350	350x350	200x200
TR (ms)	405	3558	3625
TE (ms)	8	120	120
T1W=T1 Weighed; T2W=T2 Weighed; TSE=Turbo Spin Echo; FOV=Field of View; TR=Repetition Time; TE=Echo Time			

The MRI controls were reviewed routinely by the hospital's on-duty radiologist in order to detect gross radiological changes or adverse effects of the intervention. These reports are considered in study III.

An experienced radiologist reviewed the MRI examinations separately, evaluating multiple parameters in the injected as well as the adjacent lumbar segments. A thorough baseline characterization was performed as well as a detailed longitudinal follow-up. The results are reported in study IV. A brief presentation of the reviewed parameters follows.

The Pfirrmann classification, in the original 5-grade description, is an ordinal scale used to describe the degree of IVD degeneration [95, 96]. It is based on the assessment of the morphology and the signal intensity of the IVD on T2W MRI images, estimating the hydration of the tissue which is dependent on the proteoglycan content of the IVD.

High intensity zones (HIZ) represent annular tears associated with inflammatory processes in degenerate IVDs [194, 195].

Disc height, or rather loss of it, has been considered a hallmark of IVD degeneration on different radiological modalities. Considerable structural changes of the IVD are required though in order to result in changes in disc height [196]. Mean disc height at the midsagittal plane was calculated [197].

Disc angle was also used to describe and longitudinally follow-up lumbar segments. Disc angle was measured at the midsagittal plane [198, 199].

Modic changes [200] represent changes of the subchondral bone of adjacent vertebrae, both cranially and caudally and are detected by examining both T1W and T2W images [201]. Three different types have been described, thought to represent different stages of tissue pathology, namely inflammation (type 1), fat infiltration (type 2) and sclerosis (type 3).

A detailed qualitative analysis of the IVD, including the endplates, on T1W and T2W sequences was performed. Findings such as local signal inhomogeneity, endplate irregularities, Schmorl's nodes and IVD protrusion were described at baseline. At follow-up investigations the occurrence of new findings and/or changes from baseline were noted.

In addition, a quantitative assessment of changes in IVD signal intensity was attempted. For this reason, a custom-made analysis routine in MATLAB (R2016a, Mathworks®, Natick, MA, USA) was used. ROIs were manually delineated on T1W images. The ROIs included the injected as well as the IVD of the proximal adjacent level and a homogeneous region in the cerebrospinal fluid (CSF) which was used as a reference. The T1W ROIs were overlaid on T2W images and each ROI could then be segmented into 5 equal parts from ventral to dorsal. The mean signal intensity and the standard deviation could be calculated for the entire IVD as well as for the central segments (2-4) [202, 203]. All values were normalized to the value of the CSF ROI [204].

4.7 STATISTICAL ANALYSES

The description of variables was by mean and standard deviation or standard error of the mean. For comparisons of normally distributed data the Student's t-test and the effect size by Cohen's d were used (the latter in study IV). In study IV the Shapiro-Wilk test of normality was used to examine whether data was normally distributed. Effect sizes (by Cohen's d) were classified according to the original description as small small (d=0.2), medium (d=0.5) or large (d=0.8) [205]. For data not following a normal distribution non-parametric tests were employed. Friedman's 2-way analysis of variance by ranks for related samples was used in study III and the Wilcoxon Signed-ranked test in study IV. All tests were two-sided and the alpha was set at 0.05.

Given the small sample size in studies III and IV the statistical analyses should be interpreted with caution.

4.8 ETHICAL CONSIDERATIONS

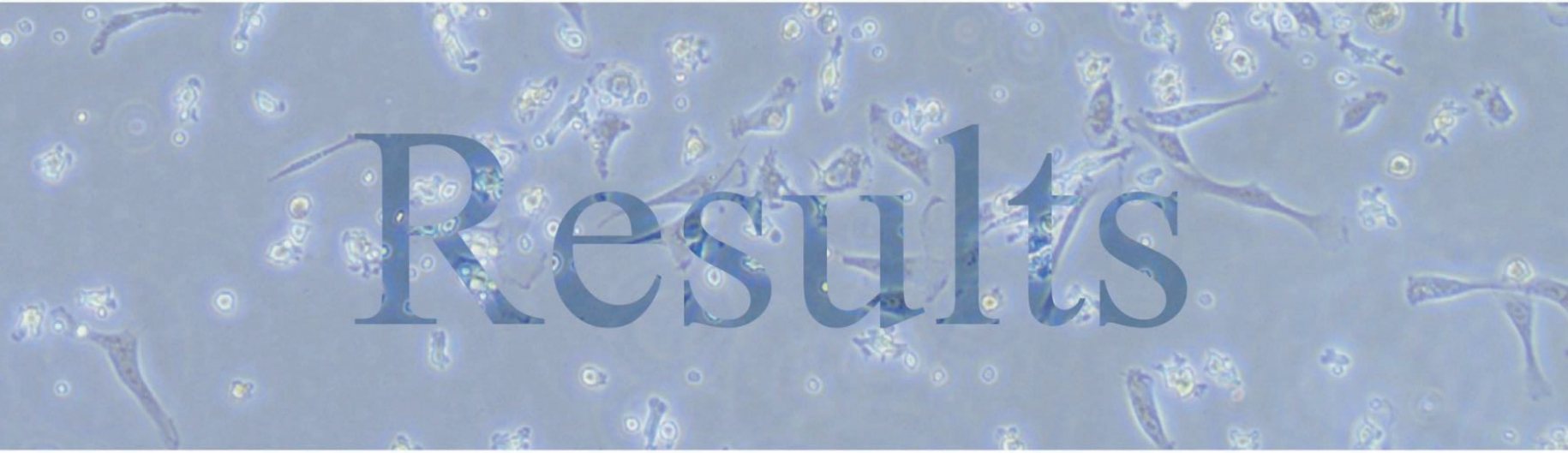
Studies I and II were approved by the regional ethics committee (reference no: 532-04).

The animal experiments in study II were approved by the regional animal ethics committee (reference no: 4-2014).

Study III was approved by the regional human ethics committee (reference no: 505-12).

The MSCs were produced under a hospital exemption approved by the Swedish Medical Products Agency (approval no: 5.9.2.-2016-049647) in compliance with the Advanced Therapy Medicinal Product (ATMP) regulation (EC) 1394/2007.

All donors in studies I and II, as well as patients in studies III, IV and V, provided their written informed consent.


Results

5. SUMMARY OF THE STUDIES / RESULTS

5.1 STUDY I

The capability of iron sucrose (Venofer®) to label human BM-MSCs as well as possible effects of the labeling on cell viability and function were examined in this study. Additionally, the ability to detect the cells after labeling over time in an *in vitro* biological system was assessed.

MSCs from 7 donors were harvested, isolated and expanded in monolayer cultures to passage 4. Iron labeling was performed and the MSCs were controlled for viability and surface marker profile by FACS. The percentage of cells labeled by the iron sucrose was determined by histological examination of cytospin preparations. MSCs from four of the donors were additionally cultured in a chondrogenic pellet mass culture system. The pellets were harvested at three different time points (7, 14 and 28 days) and examined by histology for detection of iron, presence of GAGs and cell morphology. IHC was employed to evaluate presence of SOX9 and ECM components (Coll2A1 and C6S). A qRT-PCR analysis was performed to assess the presence and activity of an array of genes. Cultures and pellets from non-labeled MSCs were used as controls.

Results

After the labeling procedure 98.1% of the BM-MSCs showed uptake of iron. The mean viability of the labeled cells was 92.7% compared with 94.6% for the non-labeled ones. The surface marker profile analysis directly after labeling showed that the MSC surface markers were detected at lower levels on the labeled cells, with the difference reaching statistical significance for CD105. CD34 and CD45 remained expressed at low levels in both labeled and non-labeled cells.

Iron-labeled cells were detectable in the pellet system up to 28 days post-labeling and showed no morphological differences when compared to non-labeled ones. GAG accumulation as observed in histology sections did not differ between pellets from labeled and non-labeled cells. Expression of SOX9 was somewhat lower in the pellets of the labeled MSCs without reaching a level of statistical significance and followed the same temporal pattern as the

non-labeled ones. No major differences were observed regarding Coll2A1 and C6S on IHC. In ***Figure 5*** an example of the result of IHC analysis for coll2A1from pellets can be seen.

The qRT-PCR examination was hampered because of the presence of iron and quantification was not possible. No clear pattern of impact of the labeling process could be seen, with variability of results between donors and time points. Of interest is the observation that the genes encoding CD105 and CD166 seemed to be activated in the pellet mass system at all time points.

These results support the use of iron sucrose as a tracer for BM-MSCs, as it demonstrates good uptake by the cells, does not affect cell viability and can be detected up to 28 days after labeling in an *in vitro* system. BM-MSCs retained their functionality as mirrored in the capability of forming pellets and producing ECM, although their surface marker profile was somewhat affected.

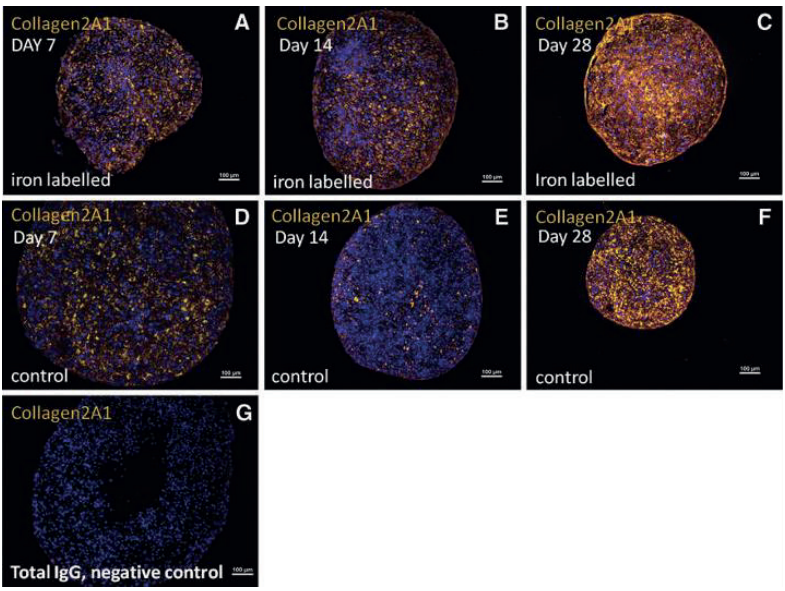


Figure 5. *Images from immunohistochemical staining of pellets for collagen2A1 (yellow color) from one of the donors. (A-C) Pellets from iron-labeled cells, days 7-28. (D-F) Pellets from non-labeled cells (controls), days 7-28. (G) Total immunoglobulin G, negative control. There was a more pronounced positive collagen2A1 staining at day 28 for both groups compared with the earlier time points. Nuclei stained with DAPI (blue). Images reproduced from Papadimitriou et al. 2014, Stem Cells and Development with permission from Mary Ann Liebert, Inc.*

5.2 STUDY II

In continuation of study I, the ability of iron labeled human BM-MSCs to differentiate into the chondrogenic, osteogenic and adipogenic lineages was tested. In addition, the traceability of the labeled cells in a pre-clinical *in vivo* system was assessed.

BM-MSCs from 3 donors were harvested, isolated and expanded in monolayer cultures to passage 4-5. Their surface marker profile was controlled as well as their viability. Following labeling with iron sucrose the trilineage *in vitro* differentiation capability of the cells was examined. For chondrogenesis the pellet mass system was used and assessed with histology (GAGs and detection of iron) and IHC (Coll2A1). Adipogenesis was evaluated with a commercially available kit and osteogenesis by standard protocols with the use of β-glycerophosphate.

For the *in vivo* investigation 12 New Zealand white rabbits were used. In each animal 1 IVD was injected with human, iron-labeled BM-MSCs and 1 with non-labeled cells. The animals were sacrificed 1 month (6 animals) and 3 months (6 animals) post injection. Harvested tissue from the lumbar spines of 8 animals (4 from each time point) were examined for morphology, production av ECM and detection of iron-labeled cells as well as for the presence of human cells. The harvested tissues from 4 animals (2 from each time point) were used for control of viability of the cells that were isolated from the IVDs.

Results

Iron-labeled BM-MSCs showed similar differentiation capacity into the 3 lineages compared to non-labeled cells. Pellets from iron-labeled cells showed somewhat weaker staining for GAGs with Alcian blue van Gieson than pellets from non-labeled cells, otherwise no differences were observed.

In the *in vivo* model, human BM-MSCs were detected by IHC at both time points in 7/8 animals. When comparing naïve IVDs to the ones injected with labeled or non-labeled cells no clear differences were observed with regard to ECM accumulation. Histology showed cells positive for iron deposits both in the AF and the NP regions in 4/4 animals sacrificed 1 month after the injection. In animals sacrificed 3 months post-injection, iron-positive cells were detected in the AF region in 4/4 animals and in the NP in 2/4. The cells had a migratory, elongated phenotype and were observed as solitary cells and/ or in small

clusters of 2 to 5 cells. Mean cell viability (for all isolated cells per IVD, of both human and lapine origin) at the one-month time point was similar in both groups (99% for IVDs injected with iron-labeled cells and 95% for IVDs injected with non-labeled cells) and comparable to the naïve IVD (99%). At the three-month time point the mean viability of cells from IVDs injected with iron-labeled cells was 73% compared with 77% for cells from IVDs injected with non-labeled MSCs and with 98% for cells from naïve IVDs. ***Figure 6*** is an example of histological analysis for the detection of iron-labeled cells.

Results from this study corroborate the use of iron sucrose as a BM-MSC tracer. The iron-labeled cells maintained their *in vitro* differentiation capacity into the chondrogenic, osteogenic and adipogenic lineages. Moreover, the label could be traced in an *in vivo* system up to three months post labeling (endpoint in this study).

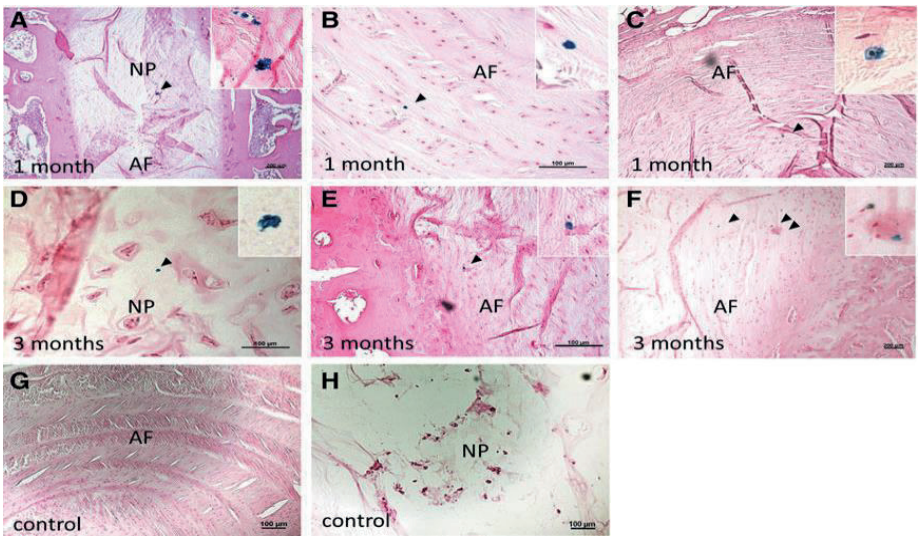


Figure 6. *Images from histological investigation for the presence of human iron labeled MSCs (iron deposits stained blue) in lapine IVDs. Top row (A-C) 1 month post-injection. Middle row (D-F) 3 months post-injection. Arrowheads indicate human cells positive for iron deposits. Enlarged images in upper right corners. Bottom row (G-H) negative controls, naïve IVDs. NP: nucleus pulposus. AF: annulus fibrosus. Staining: Prussian blue. Images reproduced from Papadimitriou et al. 2015, Stem Cells and Development with permission from Mary Ann Liebert, Inc.*

5.3 STUDY III

This study represents the translation of the pre-clinical results into a feasibility clinical trial. Patients with LBP refractory to non-surgical treatment were recruited from the waiting list for either TLIF or TDR. An injection of autologous, expanded, iron-labeled BM-MSCs into one or two IVDs was performed and the patients were followed-up for up to 2 years. During the course of the study and after the 6-months follow-up, patients could opt for the surgical procedure that was initially suggested and in that case their follow-up was discontinued.

The patient cohort consisted of 10 patients, 7 men and 3 women. Their mean age was 40 years, ranging from 26 to 53 years old. They all reported having had pain for over two years, which is the highest possible value that can be reported with the Swespine questionnaires. In 8 patients the injection was performed at the L4-L5 level, in 1 patient at the L5-S1 and in 1 patient both levels were addressed. Approximately 1 x 10^6 cells/IVD were administered.

Results

The yield of the bone marrow aspiration displayed considerable variation between patients with cell counts of mononuclear cells ranging between 0.65 – 3.8 x 10^6/ml (mean value 2.38 x 10^6 cells/ml). In order to reach the number of cells required for the intervention BM-MSCs underwent 1 to 3 passages and the mean time needed for expansion was 25.9 days, ranging from 21 to 44 days. The profile of the surface markers showed a clear increase of the MSC markers during expansion. Iron-labeled cells could form pellets that were somewhat more brittle than the ones from non-labeled cells and displayed less GAG accumulation as visually estimated by histology.

No complications or adverse events were reported. In one case, bacterial contamination of the cell culture was suspected and the process of the bone marrow aspiration and MSCs' expansion had to be repeated. Routine review of the MRI investigations showed no adverse effect of the injection at either the injected or adjacent levels.

Three patients opted for lumbar surgery at the 6-months timepoint and were operated within 8 months from the injection. Two more patients chose to be operated, with the procedures taking place 2.5 years and 3 years after the MSC injection. Five out of ten patients could forgo lumbar surgery for a minimum

of two years. PROMs showed no statistically significant changes over time on a group level, although numerically some improvement could be seen. In ***Figure 7*** the values for the ODI and NRS per patient over time are depicted.

These results support the safety of similar procedures and provide insight into the methodological challenges and the logistical needs to be met in larger scale trials.

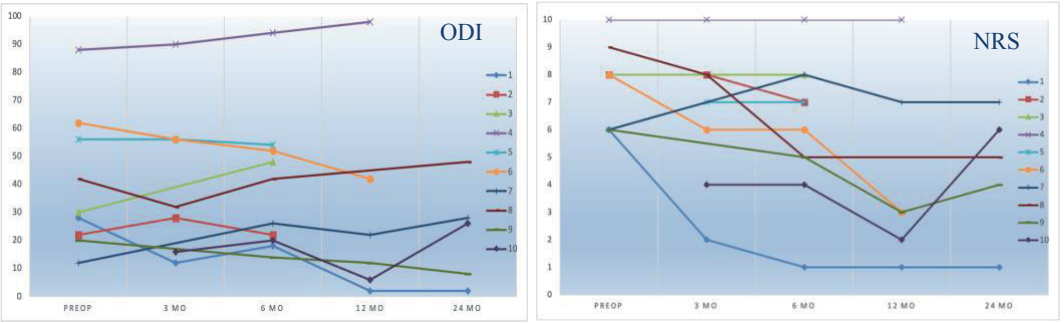


Figure 7. *Diagrams of the reported ODI (left) and NRS (right) scores per patient over time during the 2-year follow-up. Each line represents one patient.*

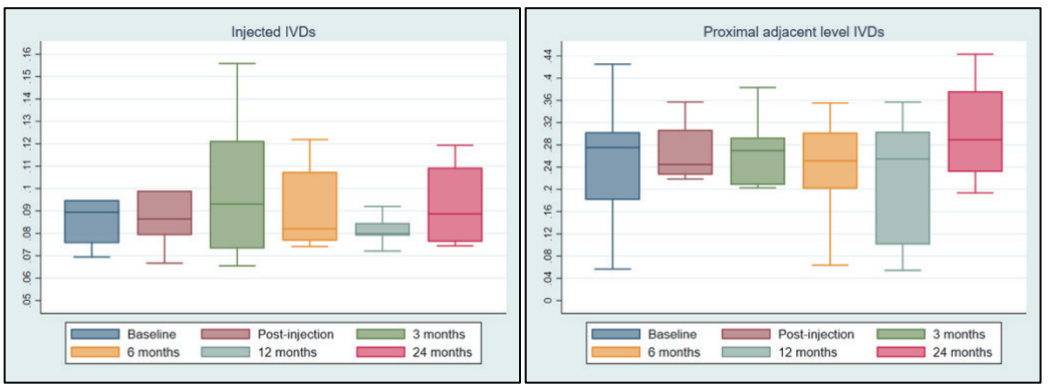


Figure 8. *Box-plot diagrams of the mean signal intensity in T2W images of the central part (segments 2-3-4) of the injected IVDs (left) and the IVDs of the proximal adjacent level (right) over time. No major changes over time were observed.*

5.4 STUDY IV

This study is a detailed, longitudinal evaluation of the MRI controls of the patient cohort from study III.

The MRI investigations were reviewed for multiple parameters regarding the injected and the adjacent lumbar levels. The examined parameters included Pfirrmann grading, Modic changes, HIZs, evaluation of the homogeneity of the nucleus and the endplates, disc height and angle. A quantitative evaluation of the IVD signal intensity on T2W sequences was performed using custom software.

Results

There were no signs of negative effects such as infections or tumor development. A thorough baseline evaluation was established regarding the parameters that were assessed. Only minor fluctuations could be observed during follow-up, with no distinct pattern of amelioration or deterioration that could be attributed to the intervention. When comparing the normalized signal intensity in the nucleus area of the IVD between baseline and at 6 months post-injection for the injected and the proximal adjusted levels no difference of statistical significance was seen, as illustrated even in ***Figure 8*** (page 54). A marginal effect size according to Cohen's d was noted for the injected and the proximal adjacent levels.

Results from this study confirm the safety of this therapeutic modality. Reviewed radiological parameters could be considered in future studies as part of the follow-up protocol.

5.5 STUDY V

This study describes the examination of explanted tissue fragments from IVDs injected with BM-MSCs in patients from the study III cohort. As aforementioned, patients recruited were already on the waiting list for lumbar surgery (TLIF or TDR) and could opt to proceed with the initially planned intervention. In that case the degenerate IVD, injected with BM-MSCs could be harvested and examined for the presence of iron-labeled cells. Three patients that were operated approximately 8 months and one that was operated 28 months after the injection were included.

The tissue fragments were examined in consecutive sections by means of histology (for ECM accumulation and detection of iron particles). In tissue samples from 2 patients (operated 8 months post injection) ROIs with iron-labeled in clusters could be identified. In these ROIs, and on adjacent sections, IHC was performed, as well as the TUNEL assay for apoptotic cells.

Results

Iron-labeled cells could be detected in tissues from the 3 patients operated within 8 months from the injection of MSCs but not in tissue samples from the patient that was operated 28 months post-injection. The cells were detected either in clusters or as solitary cells. ECM accumulation was observed in the vicinity of the detected cells as well as presence of Coll2A1. SOX9- and PCNA-positive cells could be identified in low numbers in samples from both investigated patients. A small number of apoptotic cells could be identified in samples from 1 patient and a few CD68-positive cells in samples from the other. In ***Figure 9*** images of the histological assessment for the presence of iron-labeled cells in the harvested IVD tissues can be seen.

These results show that the injected BM-MSCs retained the iron label for at least eight months. The detection of SOX9, Coll2A1 and PCNA in the vicinity of the cells offers an indication that the injected cells (or their progeny) remain metabolically active, adapting to their environment.

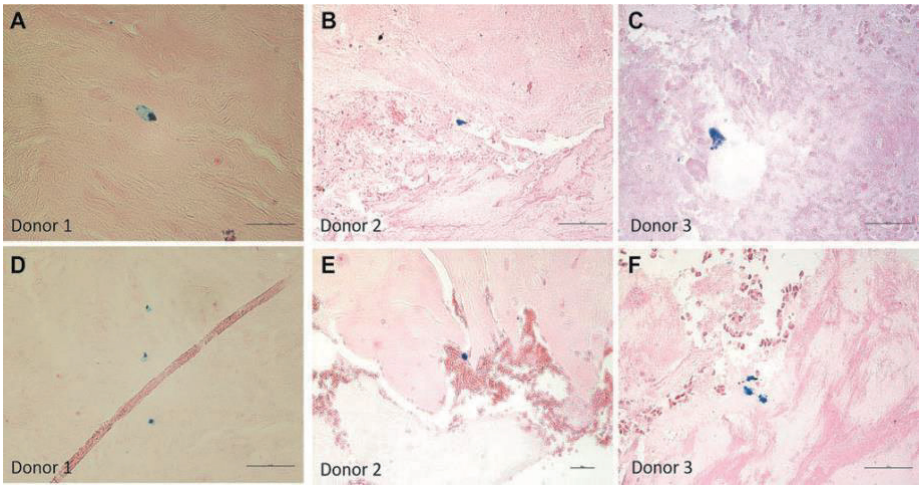
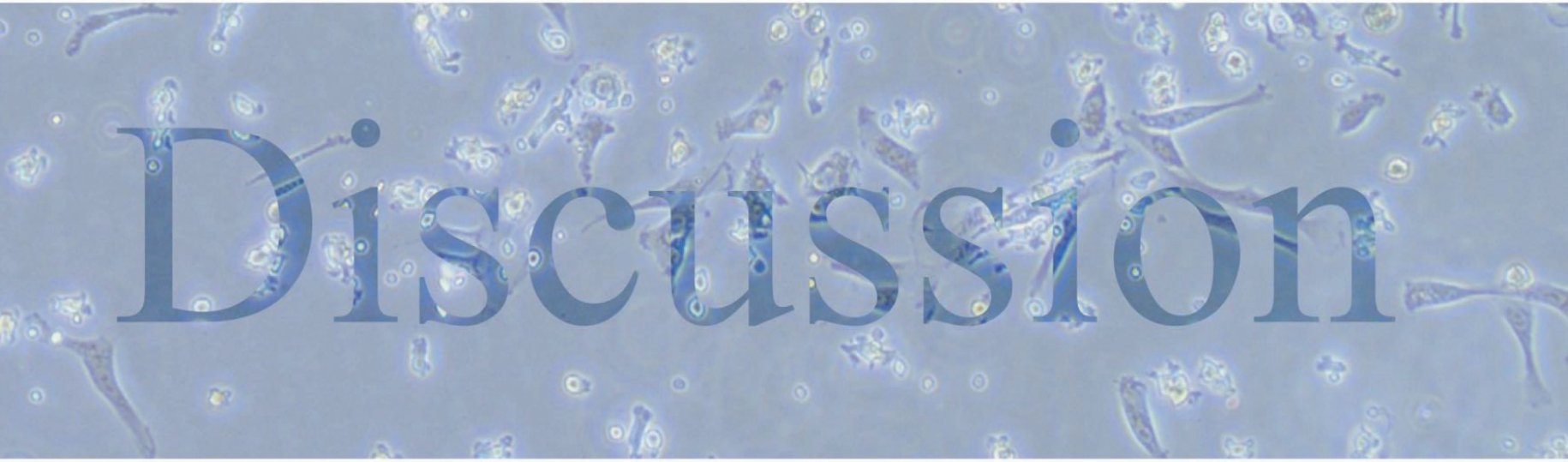


Figure 9. *Images from histological investigations for the presence of iron-labeled cells in explanted tissues from IVDs injected with iron-labeled BM-MSCs. Donors (patients) 1-3 were operated 8 months after the MSC injection. Iron deposits stained blue. Staining: Prussian blue. Images reproduced from Barreto et al. 2019, Stem Cells and Development with permission from Mary Ann Liebert, Inc.*


Discussion

6. DISCUSSION

Despite extensive research activity over the past decades in the field of MSCs as a whole, very few applications have translated into medical products. In Europe only one product has gained regulatory permission, for treatment of perianal fistulas in patients with Crohn's disease [206]. In that sense, the mere fact that there are 2 ongoing phase III and II/III studies for the treatment of LBP with cellular products can be seen as a positive sign. Less than ideal efficacy, costs, regulatory concerns, the procedural and logistic perplexity of some approaches [206, 207] alongside a less than perfect understanding of the biological processes involved [208] have been discussed as possible explanations to this translational delay.

Dowdell *et al*. [32] have described a conceptual escalation of possible biological interventions in the treatment of LBP with regard to the degree of IVD degeneration. In early stages, with somewhat healthy *in situ* cell populations, biomolecular interventions could be attempted in order to shift the metabolic equilibrium towards more anabolic pathways. In intermediate stages, repopulating the IVD may be indicated and in advanced stages tissue-engineered constructs may be needed. All of these possibilities are being explored. With our currently used diagnostic tools though, identifying patients with early degenerative changes and predicting which of them are going to progress to advanced DDD is a big challenge [89] and tissue engineering of IVD constructs has not yet advanced to the point of planning for clinical trials. This could in part explain why cell-based therapeutical approaches have gained momentum.

The available clinical studies in the literature that have employed MSCs in order to treat LBP involve a rather limited number of patients, in the majority of cases without a control group and lack the statistical power that would permit one to draw definitive conclusions. No serious adverse events have been reported. The overall results are promising when reduction of pain intensity and functional outcome are considered as well as findings from the radiological follow-up. But many questions are still open for discussion.

Which cells should be used?

When choosing the right cells availability, safety, ease of harvest (when applicable) and the potential of the cells to accomplish the task in question

should all be taken into consideration. Different cell types have been proposed. In a study comparing MSCs from different tissues, cells of synovial origin showed the greatest chondrogenic capacity, with BM-MSCs being second best [209]. Synovial MSCs though are not readily (neither easily) available in the numbers needed. Even in a comparison between BM-MSCs and AT-MSCs from the same donor the former showed better chondrogenic capacity [210]. BM-MSCs can be harvested from the iliac spine, a procedure with minimal morbidity [211]. In the study of Blanco *et al.* [212] the comparison of BM-MSCs and progenitor cells isolated from the IVD in humans showed that the two populations have similar characteristics, rendering thus BM-MSCs suitable for use in cell therapy applications. BM-MSCs are extensively investigated [213, 214] with regard to possible mechanisms of action when used in the setting of IDD [144, 215, 216]. Co-culture for example of MSCs with NP cells in a 3D-model showed positive effects in modulating catabolic induction when the cells were exposed to inflammatory and hypoxic conditions [140].

AT-MSCs are easier to harvest than their bone marrow-derived counterparts [217]. Theoretically they present somewhat lower chondrogenic capacity [210]. The putative pleiotropic action mechanism of MSCs though does render AT-MSCs legitimate candidates for cell therapy. In the clinical setting AT-MSCs have been used in the studies of Kumar *et al.* [150] with promising outcome. Our studies as well as those of Noriega *et al.* employ BM-MSCs. Mesoblast [156] uses what is defined as "allogeneic mesenchymal precursor cells" derived from bone marrow aspirate from one donor.

Autologous MSCs present no concerns of immunogenicity and have therefore been considered safer. Their harvest requires an extra procedure and they often have to be expanded *ex vivo* prior to application, adding to the logistic perplexity of the intervention. The initial yield of the harvest as well as the efficacy of the cells may vary between patients rendering the standardization of the procedure difficult. Such variation was evident even in study III where the time needed to expand the cells varied between 21 and 44 days.

As MSCs seem to be immune evasive, allogeneic MSCs have been proposed as an alternative source of cells. The use of allogeneic MSCs permits the "off the shelf" use of products that can be produced in large scale, without the need for an additional harvesting procedure and presents therefore an attractive business model [113]. Even meeting the requirements of regulatory agencies can be easier when it comes to quality controls of different batches of the

products [113, 218]. The candidate donors can be chosen on the basis of their age and the overall health status. The cells used must though undergo extensive expansion that might affect their efficacy. In addition, some concern arises from the fact that allogeneic MSCs can elicit immune responses in a few patients as shown in other than intradiscal local applications [219], although this does not seem to affect the overall safety profile [135, 136]. In the study by Amirdelfan *et al*. [156] no differences in immunological responses could be seen between the different groups over time.

Pang *et al.* [154] reported the use of umbilical cord-derived MSCs. There is ongoing research in this field, with a few reports even in orthopaedic applications [220], it is probably too early to draw any conclusions about the safety and efficacy of this cell-type in a clinical setting.

Study III was conceived as a feasibility cohort investigation. As BM-MSCs were more extensively studied, including in applications of labeling with iron-containing agents and evade the consideration of immunological reactions they were the cell population of choice.

A survey study in Europe on cellular and tissue-engineered therapies in all fields (excluding hematopoietic stem cell therapy for reconstitution of hematopoiesis) showed that BM-MSCs are preferred in 56% of the cases and that autologous cells are used by 61% of the institutions that took part in the inquiry [221].

How well do the cells do when expanded *ex vivo*?

It is widely accepted that the isolation, seeding and expansion of MSCs alters their phenotype [222-224]. At high passage numbers for example senescence is induced [225]. Alternative methods of isolation and/or expansion have been used in the laboratory, such as seeding of the bone marrow aspirate without prior density centrifugation [226] or expansion in lower cell density [227]. These methods have yet to be used in clinical trials in the field of IVD degeneration.

The BM-MSCs in study III underwent 1 to 3 passages, thus the risk for inducing cell senescence that could influence the clinical outcome was reasonably small. The ongoing Mesoblast study [155] employs a proprietary method of cell expansion, therefore no comparison can be attempted. The report of Amirdelfan *et al.* [156] discloses that the end product used is the result

of the expansion of the MSCs of a single donor implying large scale expansion. The ongoing clinical trial employing allogeneic BM-MSCs [157] states the use of cells from healthy donors without offering more details.

Does the label matter?

The iron sucrose labeling used in these studies affected somewhat the cells, primarily the detection of surface markers, predominantly CD105, directly after the labeling. Notably, the gene expression of the same markers did not seem to be disturbed. Functionally the cells were able to form pellets in the pellet mass culture system. Pellets formed showed less GAG production than pellets from non-labeled MSCs on histology. As this method has not been used in a clinical setting before, it is difficult to comment on the possible effect of the labeling on the overall clinical result. Studies performed on MSCs with other iron compounds, SPIOs, have not demonstrated serious affection of the MSCs [146, 228]. There is evidence from *in vitro* studies though that the chondrogenic capacity of MSCs can be compromised by SPIOs when the cells are exposed to high concentrations in a dose-dependent manner [229].

In study II a lapine animal model was used in order to examine the traceability of the labeled MSCs in an *in vivo* system. There is an ongoing debate regarding which animal model is best suited for the study of IDD with regard to the presence or not of notochordal cells in the skeletally-mature animals, naturally occurring IDD in some models as opposed to injury-induced degeneration in others, the different biomechanics between bipeds and quadrupeds or the size of the IVDs [230, 231]. The lapine model is among the ones most often used [232]. In the case of study II this established animal model was chosen as the animals are large enough to allow for a percutaneous intradiscal injection without the practical problems of housing bigger animals over a long period of time.

Do injected MSCs feel better in the company of a carrier?

The use of a carrier in cell injections is appealing. A carrier can help maintain the cells within the anatomical compartment where they are to reside [233]. The proper carrier could mimic the physical properties of the ECM providing proper biomechanical cues to the cells [234] and even assisting the *in situ* cell population to perform better. In addition, carriers can be laden with molecules known to enhance cell function. Experimental data suggest that the

combination of MSCs and a carrier has beneficial effect on degenerate IVDs [235].

Study III was an attempt to evaluate the feasibility and possible effect of cellular therapy, and providing a carrier could impede the evaluation of the effect of the cell activity per se. The iron labeling of the cells may have to some extent confounded the results; introducing one more factor could hamper interpretation of the outcome.

The use of a carrier has been tested in the study of Kumar *et al.* [150]. Even the ongoing Mesoblast study [155] applies the concept of cell therapy combined with a carrier, testing its efficiency against an injection of the carrier alone. In the published results [156] the combination of the carrier with cells seems to be performing better than the carrier alone.

Should the MSCs be prepared?

In the studies of Elabd *et al.* [149] and Centeno *et al.* [148] the MSCs were cultured under hypoxic conditions (5% O_2). Implanting the MSCs into the nutrient and oxygen deficient environment of the IVD can affect their viability and ability to produce ECM components [236]. Exposing the cells to hypoxic conditions prior to implantation (preconditioning) could enhance their chances of survival [237]. A similar approach, priming, employs growth factors in order to equip the MSCs to endure the harsh environment of the IVD [238].

The results of the two clinical studies mentioned are positive but somewhat confounded by the concomitant use of platelet lysate. Expansion under hypoxic conditions was considered for study III but was not applied in order to avoid confounding the result of the cell therapy per se.

How many cells is enough?

When injecting MSCs in the biologically, chemically and mechanically compromised milieu of the degenerate IVD, one has to consider the optimal number of cells to use. The answer is obviously dependent upon the original cell concentration of the healthy tissue but even on the extent to which the nutrient supply and the homeostatic balance are impaired. Pre-clinical models have shown the importance of the number of injected MSCs per IVD [239]. We know that the healthy, human NP contains approximately 4 x 10^6 cells/ml

[27, 28]. It is also logical to hypothesize that not all of the injected MSCs are going to survive, as implied even from results from the animal model in study II. In the compromised milieu of the degenerate IVD, with limited nutrient supply and reduced elimination of metabolic waste, injecting too many metabolically active cells could lead to increased cell death [240]. This was the rationale behind the choice of the dose of 1 x 10^6 cells/IVD in study III. In the published studies higher numbers of MSCs per disc have been used compared with study III with the exemption of the two cases presented by Yoshikawa *et al.* where the number of applied cells cannot be deduced. Elabd *et al.* report that an increasing number of injected cells correlates well with the clinical outcome, whereas Kumar *et al*. found no difference between the two groups receiving 2 x 10^7 and 4 x 10^7 cells per IVD. The study of Amirdelfan *et al.* compared two different doses, 6 x 10^6 cells/IVD and 18 x 10^6 cells/IVD, while the ongoing clinical Mesoblast trial uses the lower dose. Whether this choice was driven by a difference between the groups or manufacturing costs in the absence of a clinical difference is not clear.

The local environment (nutrient supply, ECM state of degradation, metabolic balance, inflammation) possibly controls the cell density that can be supported in the individual IVD [45, 240]. This implies that the therapeutic intervention could be tailored according to the conditions present or that a given intervention may have a temporal window of opportunity with regard to disease progression to ensure optimal result.

When is the right time?

The timing of an intervention against LBP may thus play paramount role for the clinical outcome. Animal studies utilizing disc puncture injury models have shown that timing of MSCs injection can affect the number of MSCs retained in the NP [241] or the overall efficacy of the treatment [242]. The degree of degeneration as mirrored in the pH of the extracellular environment has also been shown to affect BM-MSCs in an *in vitro* study where bovine BM-MSCs were cultured in environments of different acidity. A threshold of pH 6.8 was observed, under which the survival of the cells and their ability to synthesize components of the ECM were seriously compromised [73]. At the clinical level, recent work of Jess *et al.* showed that between patients enrolled in the same (non-surgical) treatment pathway, those with symptom duration over 12 months improved less than patients reporting symptom duration less than three months at enrollment [243].

All patients in our cohort reported pain duration of longer than two years at baseline, which is the highest possible value in the Swespine questionnaires. This could imply more advanced degeneration or greater pain sensitization. In the other clinical studies symptom duration of at least six months is reported. In all studies patients had "mild" degenerative changes in 1-2 lumbar IVDs on MRI, in most studies assessed by the original or the modified Pfirrmann grading system. Other radiological parameters that were considered vary between studies.

Who is the right patient?

Selecting the patients most suitable for the planned intervention is of paramount importance for a good outcome. Noriega et al [153] identified a subgroup of treated patients (5/12) that responded very well to the treatment whereas the remaining patients showed almost no amelioration. This remained consistent even throughout the long-term follow-up [167]. With such a small sample size one cannot generalize this observation, but it will be interesting to see the results of the ongoing phase II/III study. In study III only 1 patient reported an almost ideal response to therapy.

The pathogenesis of LBP is multifactorial and the intradiscal injection of MSCs addresses at best one of the putative nociceptive pain generators. At the moment we lack an adequate method of ensuring that the targeted IVD is the main source of nociceptive stimuli. The Pfirrmann grading system provides an ordinal scale to describe the continuum of IDD which might be better evaluated with quantitative MRI techniques [203, 244]. The overall radiological evaluation in clinical praxis today is probably inadequate to detect subtle but probably important changes in the IVD environment, structure and biomechanical properties and cannot help us stratify patients. As our understanding of the underlying pathology evolves evaluating combinations of MRI findings has been proposed as a predictor of worsening of LBP over time, thus identifying patients that could benefit from an early intervention [245, 246]. Moreover new, quantitative MRI methodologies are being assessed [247-249] and are starting to find their way into clinical research. Kumar *et al.* [150] used such a method to assess hydration of the IVD during the patients' follow-up. In study III the IVDs that were treated were classified as of Pfirrmann grade III or IV.

The psychosocial profile of the patients is seldom addressed in the clinical studies in this field. One the five dimensions of EQ-5D used in study III is

anxiety/depression but it is usually not assessed separately. With only three possible answers/levels in the case of the EQ-5D-3L and applied to a relatively small number of patients it is not the tool of choice for assessing the complexity of chronic pain. None of the studies available have used a more adapted tool for inclusion or stratifying.

The age of the donor/patient might affect the efficacy of the MSCs something that is important especially if autologous therapy is considered. Numbers of BM-MSCs obtained by bone marrow aspiration decline with age and the overall fitness of the cells is diminishing [250]. Additionally, a study of the transcriptome of human mesenchymal stem cells showed differences between MSCs from young (mean age 23 years) and old (mean age 73 years) donors, affecting even the MSCs' regenerative capacity which was lower for the cells from the older donors [251]. This has shown to be true even in a large animal (equine) model [252]. Donor variation even within the same age group has been observed, affecting the initial cell yield after harvest, but even the efficacy of the cells [210]. With the exemption of the case series of Yoshikawa et *al*., the available studies include cohorts of comparable mean age, thus this factor should not contribute to the disparity of the outcomes.

Evaluating the outcome

Evaluating the outcome of an intervention is not always straightforward. The disparities between the studies expand even in this field, both regarding the clinical and the radiological assessment.

All studies report on pain reduction, often using a VAS or an NRS and most assess function with the help of the ODI, and it seems like the overall results are positive. In the studies of Noriega *et al.* [153, 167] but even that of Amirdelfan *et al.* [156] there seems to be a subset of patients that responds very well to the treatment. The quality-of-life measurement that is most frequently employed is the SF-36. Reported results are mixed, with no deterioration though and interestingly enough, in the published Mesoblast study all four groups report improvement after treatment. In study III the EQ-5D was used, no evident change could be seen in the cohort.

When looking at the radiological evaluation then it is evident that MRI is the method of choice in all studies, but the exact technique applied, and the parameters assessed vary. The original Pfirrmann classification and the modified one, together with estimates of the hydration of the IVD on T2W

sequences have been used alongside ADC mapping and assessment of disc bulging. The results show overall no deterioration in the treated groups, and in some studies some amelioration is reported. When assessing MRI controls according to the Pfirrmann or the modified Pfirrmann it is important to consider the fact that although both scores have good inter- and intraobserver reliability, in the cases of disagreement between assessors, the difference is often of 1 grade [96, 97]. Orozco et *al.* [151] have used a technique to assess the signal intensity of the treated IVDs on T2W images similar to the one used in study IV. The healthy IVDs are used as a reference value (instead of CSF in study IV) and a statistically significant increase is reported at the 12 months follow-up. Noriega *et al.* [153, 167] report an amelioration on the Pfirrmann grading even at 3.5 years after the treatment. In study IV no changes could be seen, in accordance with the results of the Mesoblast report that employed though the modified Pfirrmann scale. The thorough radiological evaluation of study IV is not precedented in any of the other reports. Amirdelfan *et al.* [156] report on longitudinal evaluation of the adjacent lumbar levels and only Kumar *et al.* [150] comment on the presence of Modic changes at baseline.

Detecting the injected BM-MSCs in explanted IVD tissues

In study V iron labeled MSCs could be detected in IVD tissues explanted approximately 8 months after the injection. No other clinical studies of intradiscal MSC injection have examined explanted tissue samples and there has been no other indirect evidence of the injected cells surviving over time. This result is of importance as in other clinical MSC applications, where intravenous administration is used, the majority of the infused cells get trapped in the lungs and cleared by the immune system [218, 253]. It is thus thought that they exert their actions by modifying the immune response during this brief encounter. The detection of labeled cells in study V confirms results from animal studies showing that human BM-MSCs survive in IVDs for (at least) several months [141, 254] and has implications on deducing a possible mechanism of action.

The cells were observed as solitary cells or in clusters, which are observed in degenerate IVDs and are believed to represent an attempt to local “repair” [69]. Investigation by IHC indicated differentiation along the chondrogenic lineage (detection of SOX9) as well as synthetic activity (Coll2A1) in the vicinity of the iron-labeled cells. For technical reasons the same histological section could

not be both stained for the presence of iron and undergo IHC. The latter had to be performed on adjacent sections.

The mechanism of action in play cannot be determined on these few observations but our findings indicate that the cells have adapted to the environment and are metabolically active. The detected cells could be the injected BM-MSCs or their progeny, exerting their actions directly or by supporting the *in situ* cell population.

No iron-labeled cells were detected in the IVD tissues of the patient operated 28 months post-injection. One can only speculate when commenting on a single observation; possible explanations are that the cells did not survive, or that the iron concentration in the cells decreased with time below traceable levels.

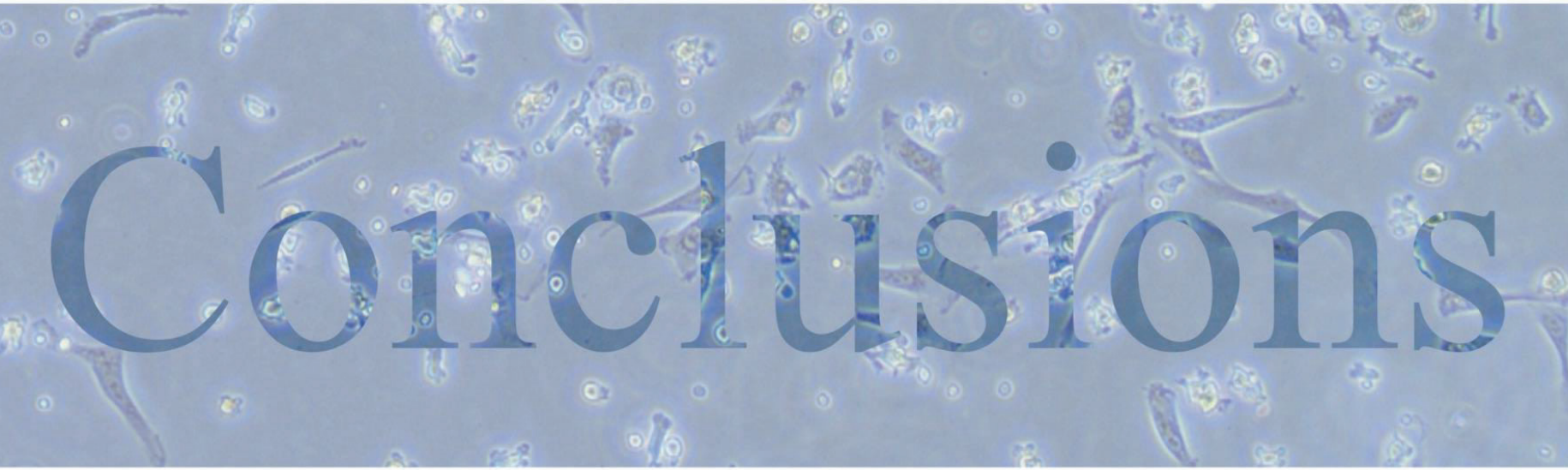
Limitations - Strengths

In studies I and II only one concentration of iron sucrose was used, based on previous data with the use of other iron compounds. In future investigations one could consider to test labeling the cells with different concentrations of the labeling agent in order to fine-tune the procedure and enhance the labeling without compromising cell functionality.

Study III was conceived, approved, and performed as a feasibility cohort study. The small number of included patients and the absence of a control group are clear limitations as the utilization of robust statistical methodology is precluded. Nevertheless, results from the study provide valuable insight in the procedural and logistical challenges of such an endeavor. The well-defined patient population and the characterization of the cells, including a functional matrix assay in the form of the pellet mass culture system, add to the value of the study. The clinical outcome may provide the basis for designing larger trials in the future.

The small number of included patients affects even study IV. Given the small number of patients and in an attempt to maximize the amount of information that could be extracted from the MRI investigations, one experienced radiologist evaluated all the controls. This of course could introduce bias in the assessment.

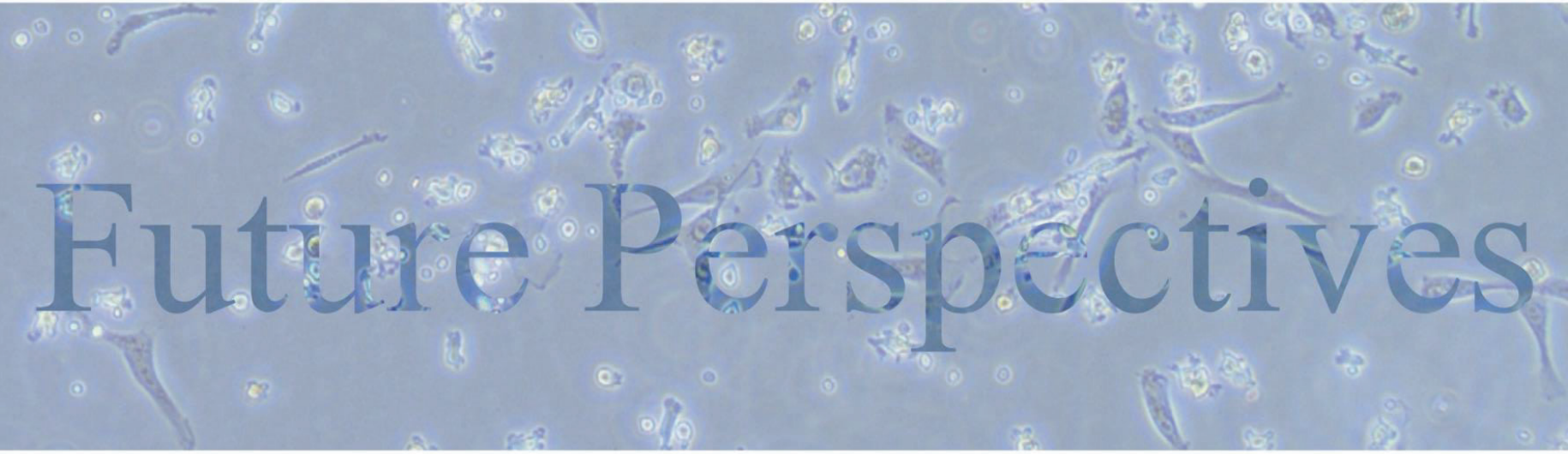
In study V tissue fragments from only four patients were available for examination. The paucity of tissue was a clear limitation as to the extent of investigations that could be performed, and the results are consequently not conclusive. Conversely, this is to the best of our knowledge the only study examining human explant tissues from IVDs that have been treated with MSCs, adding to our collective knowledge regarding similar procedures.


Conclusions

7. CONCLUSIONS

During the course of the presented studies a labeling method for BM-MSCs could be established. Autologous, expanded, labeled BM-MSCs were then injected intradiscally in patients with LBP and the patients were followed up for a minimum of two years. No adverse events occurred. Five of the patients proceeded at some point to surgery and tissue samples from the treated IVDs from four of them could be harvested and examined. The presence of labeled cells could be confirmed in tissues explanted within 8 months from the injection. PROMs showed no improvement of statistical significance and a thorough longitudinal radiological evaluation showed no negative effects of the treatment, and no evident amelioration either.

- Iron sucrose can be used as a labeling agent for BM-MSCs. The label is retained and can be traced several months post-labeling on histology sections.
- The intradiscal injection of autologous, expanded, iron-labeled BM-MSCs in degenerate IVDs is a feasible intervention with no adverse effects in a ten-patient cohort with a minimum of 2-year follow-up.
- PROMs did not change significantly for the cohort over time. Patients were recruited from the waiting list for lumbar surgery and 5/10 could forgo surgery for a minimum of 2 years.
- A longitudinal evaluation of the patients' MRI investigations showed no signs of adverse effects in the treated and the adjacent lumbar levels and no evident amelioration.
- Labeled cells could be identified in IVD tissues explanted 8 months after the injection, strongly indicating that the injected cells survive and suggesting that they are metabolically active.


Future Perspectives

8. FUTURE PERSPECTIVES

As low back pain is a complex, multifactorial entity it is logical to assume that the treatment must also take into account multiple aspects regarding not only local tissue compromise but even co-morbidities, psychosocial parameters, peripheral and central pain sensitization. Biological strategies, and MSC utilization therein, deserve to be considered as part of the therapeutical arsenal. Selecting the right patient, the right time to intervene, the right method to apply and the right tool to follow-up and evaluate the results is the challenge awaiting.

The phase III Mesoblast study is to report its results during 2021. This will hopefully be the first study to report on a patient sample large enough to permit drawing credible conclusions about the efficacy of the treatment. Robust research will give us better understanding of the mode and mechanisms of interaction of MSCs with native IVD cells and may help us refine our future therapeutic approaches [254]. The implementation of new methods such as proteomics may further help us understand the pathology involved and define targets for intervention [255].

Imaging technology is going to have a paramount role in the development of treatments. Novel quantitative MRI methodologies such as T2 mapping [202] and T1ρ [256], hold the potential of describing early degenerative changes and their progress in a continuous manner, permitting better understanding of the phenomenon of IDD, staging of the disease and stratifying of patients. New MRI techniques allow us to detect the results of mechanical loading on IVDs *in vivo* [199]. The translation into clinical praxis of proposed MRI sequences could offer us in the future a non-invasive measurement of biophysical, biochemical and biomechanical properties of the IVD such as the pH [257], GAG content [258] or stiffness [259]. Even the perfusion through the CEPs can be assessed by dedicated MRI sequences [260]. Functional MRI of the CNS could help us understand the mechanism behind chronic pain and help us evaluate different treatment modalities [261].

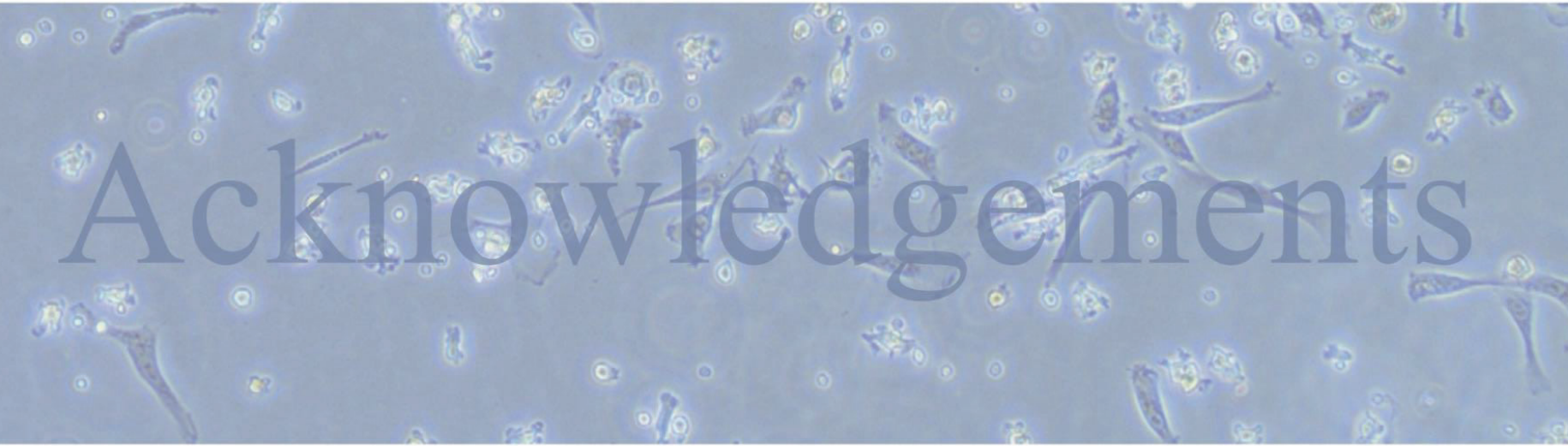
SPIOs are being investigated for clinical use [262]. Should a product become commercially available it would be of great interest to examine the possibility of tracking labeled cells in clinical trials with the help of MRI.

New methods of delivering MSCs are being explored for example via the CEP by transpedicular injection into the vertebrae [216, 263]. Cell-free biological treatment solutions that are based on our growing understanding of cell interactions, such as exosomes from MSCs [264, 265] are being explored. Even cell-free scaffolds are considered, or engineered constructs that are mimicking the tissue microenvironment [266-268]. Tissue engineering of IVD substitutes is an alternative approach that is being investigated [269], targeting probably patients with more advanced IVD degeneration than the ones deemed suitable for cell-based therapies. Our growing understanding of the IVD biology and the effect of loading for example, could help us improve even other interventions such as physiotherapy [270].

Cell therapy or other biological methods of treating LBP address at best the local, nociceptive stimuli. As the etiology of LBP seems to be multifactorial, other aspects should be taken into consideration when planning an intervention. Profiling of the candidate patients for an intervention should probably be included in a treatment algorithm in order to try stratifying patients to the optimal treatment. An example where this principle is applied is actually acute low back pain in the primary care setting in the United Kingdom where the STarT Back questionnaire is used to stratify patients into different treatment paths [106, 271].

The harmonization of the process of acquiring data, reporting and evaluating results, as proposed in the 2015 ICHOM statement, would be of great value in the future. The PROMIS® (patient-reported outcome measurement information system) initiative which was funded by the NIH is another such move in the right direction. It offers a collection of validated items [272] that can be combined in different questionnaires. It has already shown good performance in patients with back and neck pain [273] and in spine surgery populations [274]. The parallel PROsetta Stone® project is linking legacy measures to PROMIS®, and the linking to ODI is under way [275].

Nikolaj Bogduk noted in 2012: “Controversy outweighs conviction in the field of low back pain” [276]. Almost ten years later this holds still true but our growing understanding of the development of IDD and LBP combined with our expanding arsenal of treatment modalities, including cell therapy, will hopefully allow us to provide patients with better care in the not so distant future.



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INSANITY
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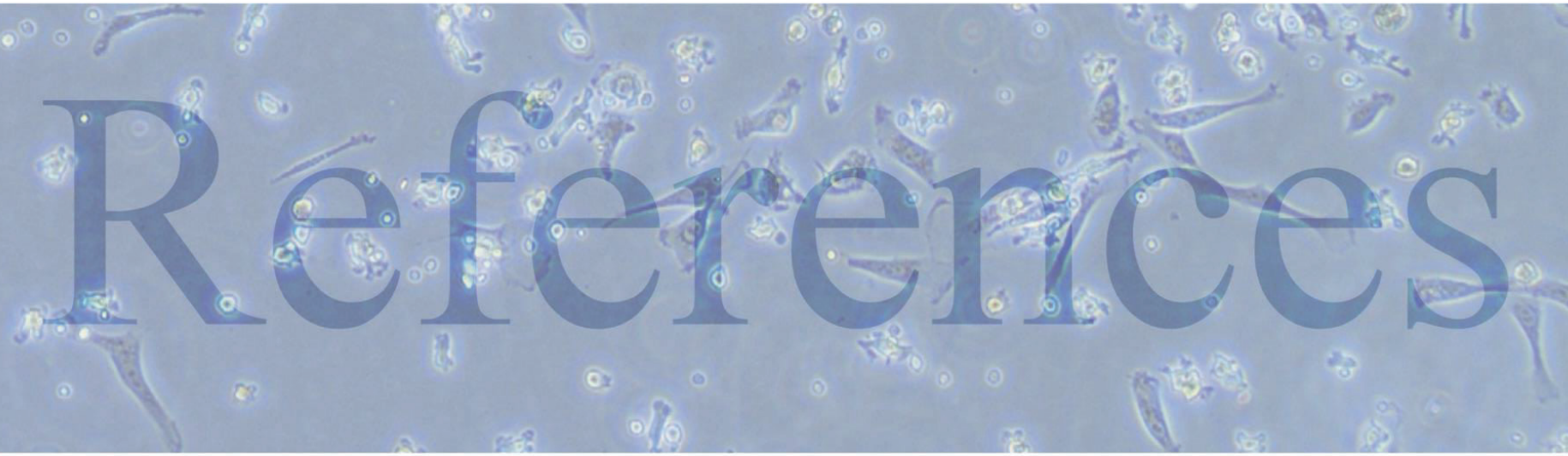

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