## 1) Executive Summary.

Back pain is the major cause of years lived with disability world-wide and poses a huge economic and social burden on European countries, amounting to around 1.5% GDP. Its pathogenesis is poorly understood; diagnosis and treatment are subjective with rates of surgery varying 25 fold between different countries for the same symptoms.

Back pain is closely associated with intervertebral disc degeneration, hence the disc is the focus for research into this disorder even though many people with disc degeneration are symptom-free. The goal of the Genodisc project was to investigate the relationship between back pain and disc degeneration by studying patients with disc-degeneration related disorders rather than the general population. The aims were to improve phenotyping of the ailments associated with intense back pain for the purpose of improving quality of treatment, to further understanding of degeneration, to develop preventative measures and investigate possibilities of repair.

To improve diagnosis of these disorders, clinicians from 5 large specialist spine centres based in different EU countries defined the major clinical phenotypes, and characterised the clinical phenotypes of 2573 chronic back pain patients. The phenotypes were to MRI scores and answers to questionnaires on lifestyle, co-morbidities, disability and pain and outcome. This information is all collected in a central database and is available for association studies in collaboration with consortium members. Disc degeneration is mainly genetic in origin hence DNA was collected from all patients. New genes associated with disc herniation were identified through a pooling GWAS study and DNA will be stored for future study as technologies develop.

Scientists also investigated stress pathways involved in the progression of disc degeneration. Analysis of molecular mechanisms involved found that mechanical signaling played less of a role than stresses arising from aberrant nutrient transport into the disc, and from degradation of the macromolecules of the disc tissue itself.. Osmotic and oxidative stresses induced premature senescence which promotes an inflammatory cell phenotype and appears a one pathway to disc degeneration.

Bioengineers working on a large database of the mechanical and anatomical features of degenerate discs showed that moderate degeneration does not necessarily induce spinal instability but that all degradative changes affect disc biomechanical behaviour. They developed needle-based probes to measure cell viability in disc tissue and to measure fixed charge density and swelling pressure non-destructively, both of which can provide new diagnostic information on the state of the disc.

A programme aimed at early prevention of back pain through strengthening trunk muscles was tested on 1371 Hungarian school children has proved so successful it will be rolled out through the whole country. Genetic variants associated with muscle phenotypes were also successfully determined.

The possibility of repairing degenerate discs by injecting cells into the disc or by suppressing degradation by injection of growth factors is of much current interest. Scientists however found that osmotic and inflammatory conditions found in degenerate discs, suppressed cellular repair. Nutrient pathways were investigated in 222 discs using post-contrast MRI to determine the proportion of patients' discs which are nutritionally able to support new implanted cells. From information on the Genodisc database, ~90% of symptomatic degenerate discs examined are not suitable candidates for an injection-based approach to cellular-repair because of either aberrant nutrient transport or problems maintaining implanted cells because of fissures in the annulus.

Through these collective results, the Genodisc team have suggested new approaches for the diagnosis and prevention of back pain and its associated disorders that could benefit sufferers in the future. The information gathered has been published in peer-reviewed journals and the data collected is stored in a central database that is available for further association studies in collaboration with consortium members.

# 2) Summary description of the project context and the main objectives

## 2.1 Summary description of the project context

Back pain is an enormous clinical, social and economic problem; indeed a recent large international study on global burden of diseases, finds that this disorder is the leading cause of long-term disability world[1]. The costs of low back pain alone are as great as those of coronary artery disease or depression costing around 1.5% of the GNP in industrialized countries.

Within Europe, this disorder is one of the most common and costly of clinical problems and yet diagnosis of the primary problem is probably poorer than for any other condition. At present, there is no clear diagnosis in approximately 85% of major spinal cases and no clinical consensus on indications or methods of treatment. Surgery is the only current medical intervention on offer and is used even when no clear diagnosis can link the patient's symptoms to pathological changes. There are up to twenty fold differences in rates of surgical procedures between different centres. This represents the largest coefficient of variation seen with any surgical procedure[2] and implies that a significant number of people are either over- or under-treated. Diagnosis and stratification are thus one of the key issues for improving the clinical situation.

Another key issue is improving the understanding of the causal pathways of back pain; such understanding is necessary for the development of rationale strategies for prevention and treatment. Back pain is strongly linked to degeneration of the intervertebral disc and may arise from the disc itself. The discs occupy around one third of the length of the spine, carry load and act as the joints of the spine, degeneration of one or more discs places inappropriate loads on other structures such as apophyseal joints, ligaments, muscles and vertebral bodies which may also be a source of pain. The causes of intervertebral disc degeneration, its effects on spinal mechanics and means of prevention and regeneration of this tissue are thus the main focus of research on back pain.

The discs degenerate earlier than any other tissue in the body but the causes for this are poorly understood. Twin and family studies have shown that disc degeneration is highly genetically linked[3]. Several potential candidate genes have been identified[4] by association studies and GWAS but the most of the findings have not been replicated. Importantly, of the polymorphisms studied, each has only very modest effects. This suggests that disc degeneration is both complex and polygenic. It is likely that degeneration involves multiple, interacting genetic and environmental determinants. Disc degeneration is however often asymptomatic. A significant proportion of the population has degenerated and even prolapsed discs but remains free of pain and symptoms[5;6]. The features of disc degeneration which lead one person but not another to low back pain have not been identified. It is important to ascertain if certain disorders can be targeted by different therapies and also to determine which patients will benefit from biological therapy. Understanding how polymorphisms lead to degeneration will help to identify pathways for prevention and help to find out if the potential for successful repair is genetically determined.

The processes of disc degeneration are however partially understood even if the initiating causes are not. In most cases, degeneration appears to arise from inappropriate cellular activity. The disc is populated by a small number of resident cells with cell type varying among the different regions of the disc. In normal discs, cells produce matrix macromolecules to maintain disc structure but during the process of degeneration they tend to produce mainly agents involved in degradation of matrix macromolecules i.e. the matrix metalloproteinases (MMPs). The pattern of

MMP production is very variable and may be disorder specific; in degenerate discs, the matrix components are degraded and fragments leach out of the tissue. Cracks and fissures develop as the disc is no longer able to maintain tissue turgor under load and its altered biomechanical properties affects other structures of the spinal column such as the ligaments, muscles, vertebral bodies and facet joints, all of which may be a pain source

One of the factors which complicates study of back pain is that animal models are largely inappropriate because of factors such as differences in anatomy and cell populations; animals commonly used for studying disease mechanisms, such as mice, hence provide very limited useful information in this area[7]. Likewise, since it is pain rather than disc degeneration alone which is the problem to the patient, animal models are of very limited use.

***Our concept is thus to focus on disc degeneration in humans which is linked to pathology, pain and disability. We aim to improve the health of back pain sufferers by providing clearly defined phenotypes of different back pain disorder; these will help focus treatments more efficiently and help under cellular pathways to these different disorders. Defining phenotypes will improve diagnosis, target appropriate treatments and help prevent development of chronicity.***

## 2.2 The main objectives of the research

The overall aim of the project is to improve treatment of pathologies linked to degeneration of the intervertebral disc by developing clearer objective phenotyping as a pathway to improved diagnosis and stratification of different back pain disorders. Improving diagnosis will work towards better targeted treatments and faster treatment of acute conditions helping to prevent development of chronicity. It will also aid in development of preventative programmes and in understanding the biological and biomechanical bases of these disorders which will aid in developing rationale therapies.

*Diagnosis*

This project will address the need for improved diagnosis by:

> ***Selecting well-defined phenotypes.*** We will identify and develop protocols for selecting populations suitable for investigating disc degeneration and its associated disorders.
>
> ***Developing clearer diagnostic tools.*** We will use modelling analyses to aid development of clear diagnostic tools such as biomarkers and imaging parameters.
>
> ***Testing new diagnostic criteria.*** The diagnostic tools developed for assessing repair possibilities will be assessed in the final stages of the project

*Prevention*

This project will aim to identify pathways for prevention by:

> ***Identifying genes associated with disc-degeneration linked pathologies.*** We will carry out pooled genome screens of a carefully chosen patient population and use microarray profiling to better identify genes associated with specific pathologies.
>
> ***Uncovering pathological pathways.*** We will examine, experimentally, specific pathways that could alter functioning of the intervertebral disc and its cells.

***Designing preventative strategies:*** We will design exercise programmes to improve trunk muscle strength and test effects on back pain in young people

*Repair*

This project will enhance the current state of research in terms of 'repair' by:

***Learning through modelling.*** We will use computer and experimental modelling to show how the function of the tissue matrix and spinal units will alter with disease and identify when certain disorders could be targeted by different therapies.

***Developing strategies for repair.*** By genotyping and careful diagnosis and assessment of patients, it will be possible to develop strategies for determining which patients will benefit from biological therapy and successful repair of degenerate matrix within the disc. Involvement of diagnostic measures will determine which percentage of patients could be selected for treatment.

## 3) The main S & T results/foregrounds

The main S &T results and foregrounds have been reported as deliverables. Results from the S&T deliverables are summarised below under the main aims of the project.

**DIAGNOSIS**

### 3.1 Phenotype

The focus of Genodisc is on distinct, disc-related pathologies associated with pain and disability. These clinical phenotypes include lumbar disc herniation with radiculopathy, lumbar spinal stenosis with neurogenic claudication, and spondylolisthesis. Multi-level endplate defects, such as Schmorl's nodes, seen on MR imaging comprise another distinct phenotype of interest.

The aim here was to define clear clinical phenotypes; this is critical for success in identifying associated genetic polymorphisms, functional genetic pathways, biomechanical analysis and diagnostics.

The distinct clinical phenotypes identified by Genodisc are given below.

**Identification of Phenotypes**

The primary selection is "patients who seek secondary care for their back pain or spinal problem". The patients will be assigned to an appropriate clinical group at the end of the collection period when all the back-up data (such as MRI) has been received. The patients within a particular group will be as homogeneous as possible. The following are essential diagnostic criteria for the different groups:

| **Group** | **Phenotype** | **Diagnostic Criteria** | |
|---|---|---|---|
| | | ***Essential Criteria*** | ***Optional Criteria*** |
| 1 | Disc herniation ***AND*** radiculopathy (sciatica) | • Leg pain (dermatomal pattern, radicular pain)<br>• Disc prolapse (MRI)<br>• Limitation of straight leg racing | • Neurological deficit (e.g. motor loss, reflexes or sensation) |
| 2 | Juvenile disc herniation (no exceptional trauma cases – everyday leisure trauma cases only) | • Back pain<br>• MRI pathology<br>• Disc herniation<br>• <25 years old<br>• no spondylolysis | |

| | | | |
|---|---|---|---|
| 3 | Central lumbar spinal stenosis | • At least one level stenotic (MRI)<br>• No neurological deficit<br>• Pain relief with sitting<br>• Pain worse with standing/walking<br>• No scoliosis >15° | |
| 4 | Spondylolisthesis<br>(* degenerative MRI keep in group 2) | • Lytic/Isthmic (MRI) | |
| 5 | Osteochondrosis<br>(Scheurmann's disease) | • Endplate Irregularity in at least 3 levels | • Vertebral wedging<br>• Round back<br>• Pain<br>Schmorl's nodes |
| 6 | Non-specific low back pain | • Exclusion from all other groups<br>• No high disability<br>• Pain | Further stratification will be based on imaging and other clinical data |

**Imaging**
For every patient recruited to the study an 'in-house' clinical MRI, including T1 and T2 sequences, is obtained and analysed. Subjects' MRIs `re stored on CDs in DICOM format and evaluated by UH partner in Edmonton and Keele partner in Oswestry.

**Questionnaires and Medical History**
The following three finalised multi-centre forms were completed for each partner (see Deliverable 2.1)

- **Diagnosis Sheet** (including participant's symptoms and classification data)
- **Participant Survey** (including family history questions)

### 3.2 Modelling Database

*In vitro database*
A database of spinal *in vitro* experiments, which provides modelling data, was created using flexibility data from all spine specimens tested so far in Centre 4 (Ulm, Germany). 203 segments (from 111 donors) which had been tested for flexibility under pure moment loads of ±7.5Nm and for which radiographs were accessible were selected for inclusion.

The database was used to investigate the influence of intervertebral disc degeneration on lumbar spine rotational stability.

For example, radiographic degree of disc degeneration and range of motion and neutral zone was fit to a statistical model, which accounted for the influence of the spinal level. The mean estimates showed a continuous decrease of the range of motion from grade 0 to 3 in flexion/extension (by 3.1°, $p<0.05$) and lateral bending (by 3.4°, $p<0.05$). Only in axial rotation the range of motion tended to continuously increase. The neutral zone was affected in a similar way but to a smaller degree by the degree of degeneration. This suggests that early stages of intervertebral disc degeneration do not necessarily cause instability contrary to earlier assumptions.

These results [8] may be relevant for defining the indications for dynamic stabilisation procedures. They also serve for the validation of the FEM calculations.

### 3.3 Needle Osmometer

Aggrecan is one of the main macromolecular components of the intervertebral disc and loss of aggrecan is one of the first signs of intervertebral disc degeneration. Restoration of aggrecan, and hence disc height and turgor, is a major objective of biological therapies. Consequently, much effort

has gone into attempting to measure it but with limited success. By focusing on swelling pressure which arises from the osmotic properties of aggrecan, we have developed a needle osmometer that can be used to measure swelling pressure in tissue samples. This is a potential non-distructive diagnostic tool for determining how swelling pressure changes both in experiments on tissue explants and tissue-engineered constructs and in pathological tissues.

Although this needle osmometer was originally intended for diagnostic use, concurrently with the diagnostic procedure discography which aims to identify which discs are the source of pain by injecting a radio-opaque dye into the disc a study published during the first year of Genodisc found that discography increased the risk of subsequent herniation and surgery even in non-symptomatic discs[9]. Surgeons are now very unwilling to insert needles into discs until the pathway to degeneration arising from needle puncture has been identified, hence the osmometer cannot at present be used diagnostically as hoped. While it is still of use in experimental and animal studies, the potential demand is much lower and the cost of protecting its IP by taking out a patent could not be justified.

*Design and setup of needle osmometer.*

The osmometer design is based on the premise that differences in swelling pressure across a semi-permeable membrane are reflected in the flux of fluid into or out of the probe. To this aim, a microdialysis probe equipped with a 6 kDa semi-permeable poly(ethersulfone) membrane (Fig. 1a) was used as the needle osmometer. Measurements were carried out by filling the probe with known solution (saline or 5% PEG) and measuring the rate of movement of the fluid interface, representing the flux (Fig. 1b).

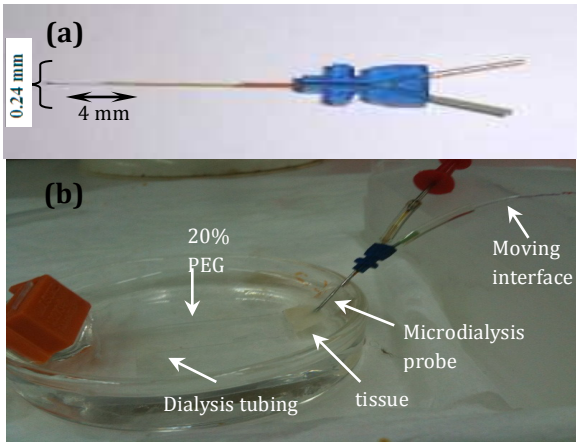



**Fig. 1.** A needle osmometer based on a microdialysis probe. The probe (MAB-4, Microbiotech/se), equipped with a 6kDa cut-off semi-permeable polyethersulfone membrane (4mm in length and 0.24mm in diameter) **(a)** was filled either with 5% PEG or saline and equilibrated vs concentrated PEG solutions or tissue slices as shown here **(b)**. Tissue was placed in a dialysis tubing to prevent dehydration, and the probe was introduced into tissue through a metal sleeve. Movement of the fluid interface was visualized by an internal air bubble.

*Determination of swelling pressure via flux measurements*

Details of the development and validation of measurements are given in the relevant publication[10].

As expected, in all cases studied, the *flux* increased proportionally with increase in swelling pressures and FCD values. Flux was correlated with the respective swelling pressure and data were fitted in a polynomial curve ($r^2$>0.93) as shown in Fig 2 below. Hence measurements of flux provide a non-destructive measurement of swelling pressure and hence of GAG content – the only alternative means of measurement is by digestion of the tissue followed by biochemical analysis.

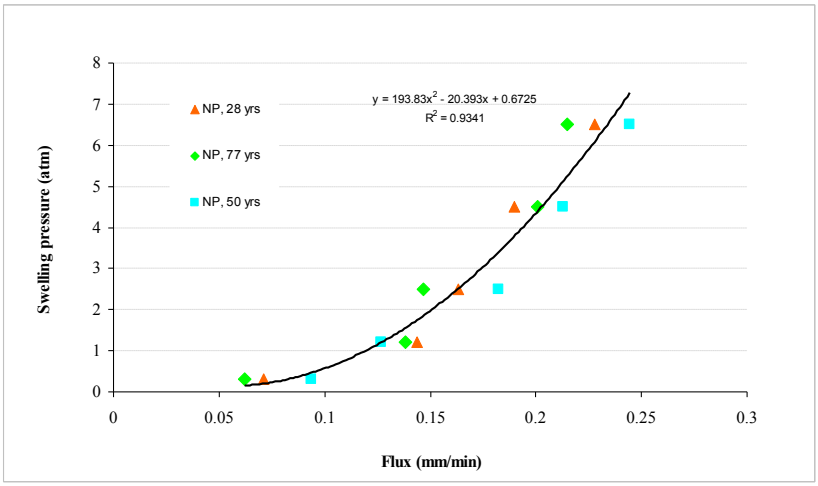



**Fig. 2.** Correlation of the flux with the swelling pressure of disc specimens aged 28, 50 and 77 yrs. An average polynomial curve was fitted to the data.

## 3.4 Database

The Genodisc central database includes diagnostic and self-report data for the 2514 study participants recruited (see Table 1), exceeding the initial recruitment target of 2250. The targeted sample size for the clinical cohorts of patients diagnosed with lumbar disc degeneration, lumbar spinal stenosis and spondylolisthesis exceeded initial recruitment targets, however many subjects had mixed spinal pathologies that may necessitate further subgrouping.

The database contains information on each patient recruited in relation to age, height, weight, gender, health history and co-morbidities and answers to questionnaires on pain, disability, mood and on relevant environmental factors such as type of employment and leisure activities and on smoking. It also contains information from the diagnostic questionnaire and on features of each patient's MRIs. Information was anonymised to researchers by the treating clinical team, with each patient identified only by an individual genodisc and ID number. All the information recorded from each patient is stored in the central database which is maintained by a data-base manager in Budapest (partner 8).

Screen shots of the contents of the data base are shown in Deliverable D6.1.

The database is available to all Genodisc partners together with the anonymised MRIs which are stored on disc.

A summary of the patients recruited and whose information has been entered on to the database is shown in Table 1.

| | Kettering | Oxford | Oswestry | Budapest | Milan | Ljubljana | TOTAL |
|---|---|---|---|---|---|---|---|
| ***Total*** | ***256*** | ***390*** | ***240*** | ***1246*** | ***364*** | ***18*** | ***2514*** |
| **Clear, distinct pathologies'** | | | | | | | |
| **Lumbar Disc Herniation (LDH)** | **64** | **102** | **53** | **383** | **197** | **12** | **811** |
| LDH extrusion or sequestration | 62 | 90 | 40 | 288 | 132 | 10 | 622 |
| LDH & radicular pain | 60 | 93 | 43 | 366 | 166 | 12 | 740 |
| LDH & neurological deficit | 33 | 48 | 9 | 208 | 52 | 8 | 358 |
| **Lumbar Spinal Stenosis (LSS)** | **28** | **62** | **93** | **113** | **24** | **2** | **322** |
| LSS & neurogenic claudication | 22 | 30 | 51 | 61 | 7 | 2 | 173 |
| **Lumbar Spondylolisthesis (LS)** | **22** | **3** | **16** | **55** | **37** | | **133** |
| LS/lytic | 13 | 9 | | 41 | 28 | | 91 |
| LS/degenerative | 9 | 9 | 3 | 14 | 8 | | 43 |
| **Mechanical or non-specific LBP** | 96 | 10 | 16 | 22 | | | 144 |
| **Other** | **3** | **25** | | **247** | **58** | | **333** |
| Scoliosis | 3 | 21 | 4 | | 1 | | **29** |
| Other/FBS | | | | 212 | | | 212 |
| Other/osteochondrosis | | | | | 25 | | 25 |
| **Exclusion diagnoses** (e.g. tumor) | **2** | | **2** | **60** | **2** | | **66** |
| **"Mixed pathologies"** | | | | | | | |
| LDH+LSS+LS | | | | | | | 52 |
| LDH+LSS | | | | | | | 229 |
| LDH+LS | | | | | | | 23 |
| LS+LSS | | | | | | | 111 |
| LDH+Mechanical | | | | | | | 60 |
| LSS+Mechanical | | | | | | | 36 |
| LDH+LSS+Mechanical | | | | | | | 30 |
| LSS+Scoliosis | | | | | | | 13 |
| LSS+LS+Scoliosis | | | | | | | 7 |
| "yet to be classified" | 4 | 15 | 3 | 14 | 14 | 1 | 51 |

**Table 1. Summary showing clinical phenotype of patients recruited.**

**PREVENTION**

## 3.5 Senescent Phenotype

Normal cells have a finite replicative capacity, i.e. they can undergo only a limited number of cell divisions, after which they remain metabolically active but are unable to proliferate; a phenomenon called in vitro ageing or replicative senescence. Beyond serial subculturing, cells exposed to a number of insults, such as oxidative stress, radiation, inflammatory cytokines, various chemicals or even overexpression of certain oncogenes, can enter a state of permanent growth arrest termed "stress-induced premature senescence" (SIPS). Senescent cells exhibit distinct morphological alterations in culture, such as an inflated and irregular shape, accompanied by enlarged and lobulated nuclei. Their main functional characteristic is their inability to proliferate, due to the activation of the p53/p21$^{WAF1}$/pRb axis. In addition, senescent cells show an altered gene expression profile, leading to a "pro-inflammatory" phenotype, marked by the overexpression of matrix metalloproteases, growth factors and cytokines and other inflammatory molecules. Due to this "pro-inflammatory"

phenotype senescent cells can affect tissue renewal and proper function and thus they can contribute to the ageing process and the development of age-related pathologies. In support of the latter, it has been shown that the percentage of senescent cells increases with age, at sites of chronic wounds or age-related diseases, e.g. osteoarthritis and atherosclerosis.

There is an increase in the proportion of senescent cells in herniated discs compared to non-herniated discs in cell clusters, with also higher percentage of senescent cells in degenerated vs. non-degenerated discs. The aim of the study was the molecular characterization of in vitro senescent intervertebral disc cells, the role of physical stresses in this process and, furthermore the investigation of the functional features of senescent cells that may contribute to disc degeneration.

The study was performed on **human** and **bovine** intervertebral disc cells from **annulus fibrosus** and **nucleus pulposus**. The cells were subjected to serial subculturing in order to achieve replicative senescence. In addition, the role of exogenous **stresses** in the senescent process has been investigated. In human cells the effect of **oxidative** stress has been studied. Moreover, nucleus pulposus cells were subjected to serial subculturing under continuous **hyperosmotic** conditions in order to investigate the possibility of SIPS (stress-induced premature senescence) induction by these conditions.

**Results show that human** IVD cells have a very low proliferative rate and lifespan comparable to that of other human adult cell strains, i.e. approx. 30 cell population doublings. Accordingly, serial subculturing in order to achieve replicative senescence in IVD cells is a lengthy process requiring several months.

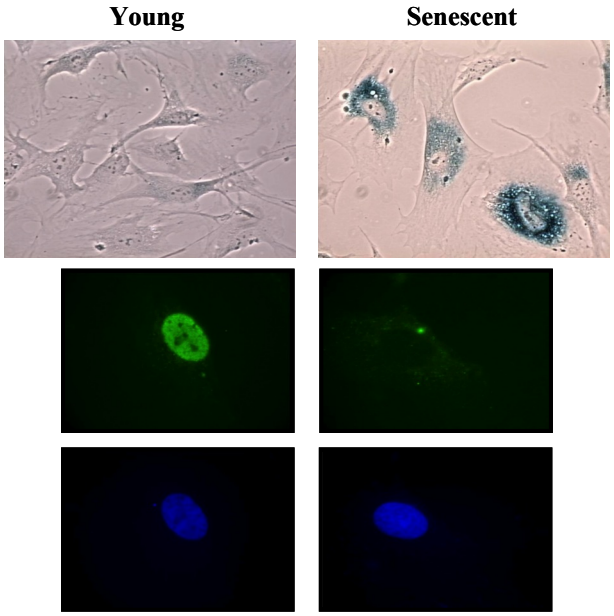



**Figure 1.** Senescence IVD cells characterized by senescence-associated β-galactosidase staining (above) and their inability to proliferate, as shown by BrdU incorporation (below).

Serial subculturing of human IVD cells resulted in senescent cells, as judged by using several established criteria, such as cellular and nuclear morphological alterations, inability to proliferate and expression of specific markers, e.g. cell cycle inhibitors.

As the rate of cell proliferation in IVD is extremely low, it is rather unexpected that the high percentage of senescent cells is due to serial cell doublings. Instead, it seems that exogenous stresses, IVD cells are continuously exposed to, may provoke premature senescence. In this vein, we have studied the effect of oxidative stress, as recent publications indicate the accumulation of carboxymethyl-lysine in aged IVDs, a marker of oxidative stress. Accordingly, we have exposed early passage IVD cells to repeated subcytotoxic doses of $H_2O_2$ and found that this activates a DNA damage response and leads to premature senescence, as shown by the inability of cells to proliferate, SA-βgal staining, and the expression of specific protein markers. Similar results were obtained with bovine

IVDs. We have also studied the effect osmotic stress, on the proliferation and senescence of intervertebral disc cells. To this end, bovine IVD cells were exposed to hyperosmotic conditions. We found that hyperosmolality inhibits cell proliferation by blocking the cells in the G1 and G2 phases of the cell cycle and activates the p38MAPK pathway (thus blocking the cells in the G2 phase of the cell cycle) and the ATM/p53/p21$^{WAF1}$/pRb axis, leading to G1 arrest.

In order to characterize the specific molecular signature of senescent cells, we have collected RNA from young, replicative senescent and stress-induced senescent human IVD cells and investigated the expression of specific genes known to be overexpressed in senescent cells from other tissues or in aged or degenerated IVDs. Our data indicate that senescent IVD cells express a pro-inflammatory phenotype, as shown by the overexpression of IGFBP3 and the down regulation of aggrecan, TIMP0-1 and TIMP-2 genes.

Furthermore, we found that hyperosmolality provokes chromatin alterations and a DNA damage response in IVD cells, as well as an increased DNA repair in these cells. As DNA damage response is a central motif in replicative- and stress-induced-senescence, we investigated the effect of hyperosmotic conditions on the lifespan of IVD cells. Indeed, we have found that hyperosmolality decreased considerably the lifespan of all IVD cell strains used. We characterized these cells in terms of their ability to proliferate. To this end, we measured BrdU incorporation into young and pre-senescent annulus fibrosus and nucleus pulposus intervertebral disc cells by immunofluorescence. BrdU is an analogue of thymidine that gets incorporated in the nuclei of cells during DNA synthesis. In senescent cells, which are unable to proliferate, BrdU gives a characteristic perinuclear staining and is not integrated into the nuclei. Senescent cells are characterized by morphological alterations such as cell enlargement and nuclear lobulation. Pre-senescent annulus fibrosus and nucleus pulposus cells labeled with DAPI had bigger and more acanonical in shape nuclei (Figure 2).

Finally, total protein extracts from young and pre-senescent nucleus pulposus intervertebral disc cells were subjected to western blot analysis with antibodies against cell cycle regulators that are known to be up-regulated during senescence (Fig. 1). The cyclin-dependent kinase inhibitors p21$^{WAF1}$ and p16$^{INK4}$ were used as molecular markers of senescence, while the phosphorylation status of the retinoblastoma protein Rb was used as a marker of the G0/G1 arrest, which is observed in senescent cells. The phosphorylation of the known stress MAP kinase p38 was also examined.

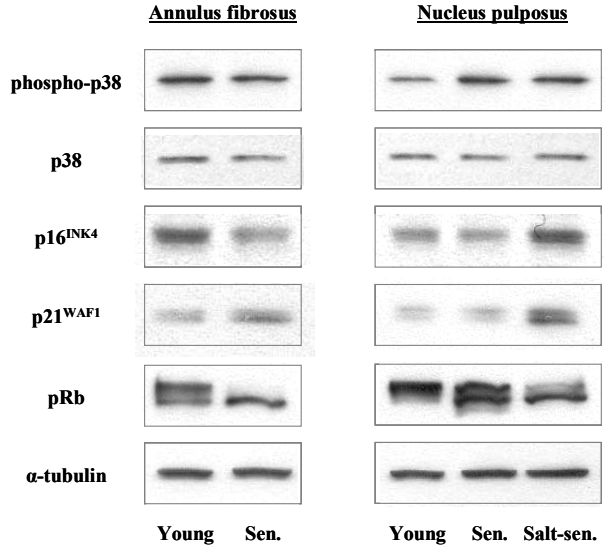



**Figure 2.** Western blot analysis of total lysates from young and pre-senescent annulus fibrosus and nucleus pulposus cells. In salt-senescent nucleus pulposus cells both p21$^{WAF1}$ and p16$^{INK4}$ were over-expressed, the retinoblastoma protein was hypophosphorylated and p38 MAPK was activated by phosphorylation. Pre-senescent annulus fibrosus and nucleus pulposus cells deriving after serial subculturing were not positive for all the above senescence-associated markers.

Serial subculturing of annulus fibrosus, nucleus pulposus intervertebral disc cells and human fibroblasts under iso-osmotic and hyperosmotic conditions indicated that hyperosmotic stress is able to interfere with cell proliferation and to provoke stress-induced premature senescence.

In conclusion, exogenous stresses accelerate the senescence process in IVD cells. These cells express a pro-inflammatory phenotype that can contribute to the degeneration of this tissue

**3.6 Cytokines**

Cytokines are small secreted proteins or glycoproteins that act as cell-signaling and regulatory molecules. They include molecules with diverse functions such as members of the interleukin family, TGFβ and TNFα. Each cytokine, following binding to its cell-surface receptor, results in a cascade of intracellular signalling which can alter the cellular behaviour, for example, upregulating and/or downregulating any of several genes and their transcription factors. This might result in the further production of other cytokines, an increase in the number of surface receptors for other molecules, production of proteinases or the suppression of their own effect by feedback inhibition. Intervertebral disc cells have long been known to be capable of synthesising several cytokines.

In other cell types, cells exhibiting signs of senescence, whether replicative or premature stress-induced (see Deliverable 4.1), have frequently been shown to have a pro-inflammatory-phenotype. As such, they remain viable but synthesise molecules they would not normally do, such as proteases or cytokines. We have examined intervertebral disc cells (both bovine and human) cultured in vitro in 'normal' culture conditions and also exposed to various stress factors such as serum starvation, glucose deprived, via the addition of staurosporine, ultraviolet treated or with exposure to free radical oxygen molecules (via $H_2O_2$). Cells have been monitored for their response in terms of the rate and type of cell death they undergo and the possible pathways involved. In addition, we have assessed the production of cytokines by human disc cells exposed to physiological-type environmental stresses, with low glucose levels or in serum starvation conditions. Measurement of cytokines by immunoblotting techniques for IL-6, IL-8 and TGFβ demonstrated that when cells from herniated or degenerate discs were cultured in vitro in conditions with greater levels of serum and/or glucose, that there were increased levels of cytokine production.

Assessments of in vivo production of cytokines by human intervertebral disc cells was undertaken by investigating surgical disc samples from patients with different clinical presentations of herniations (protrusion, extrusion or sequestration) in comparison to discs obtained at autopsy. Immunohistochemistry demonstrated a varying incidence of cells staining positively for a range of cytokines and pro-inflammatory markers. Semi-quantitative assessment of this indicated that all discs had some staining for mediators IL1-β, IL-6, iNOS, and MCP-1, whilst 94%, 91%, 89% and 70% had some for TSG-6, TNFα, thromboxane synthase and IL1-α, respectively. Quantitative assessment of the levels of TNFα, IL1-β and IL-6 using ELISA showed that the cells within different types of disc herniation appeared to have a different cytokine profile, with those in sequestrated disc producing particularly high levels of IL-6.

3.7 **Degeneration Biomechanical Model**

**Aim.** A wide range of clinical scenarios of disc degeneration, in which the most common macroscopic degenerative changes of the intervertebral disc (water loss, disc height loss, endplate calcification, osteophytses) are present both individually and in various combinations, was investigated by means of finite element models.

All the considered macroscopic changes were found to be mechanically relevant; they should be taken into account by grading systems for disc degeneration whenever possible. This work has led to the four publications listed which give details of models[8;11-13]. The reported findings might provide a basis for discussion about the choice of appropriate treatments for degenerative disc disease, both conservative and surgical, for specific clinical cases.

**3.8 Molecular Ages**

The molecular half-lives of structural macromolecules (collagens, elastin and aggrecan) in the disc in relation to degeneration grade were determined. This data provides information on the stability of the matrix in relation to effects of degradation. It is also a critical factor in the development of strategies for tissue repair. To measure half-lives, we have used the racemization of aspartic acid as a 'molecular age' marker.

Structural molecules were extracted; Aggrecan (A1) was extracted by means of associative/dissociative CsCl gradient centrifugation. Collagen was purified using sequential enzymatic treatments and analyzed for purity using hydroxyproline analysis. Elastin was extracted by a stepwise strategy for removal of tissue components using trypsin digestion, CNBr, and collagenase. Elastin was assessed for purity using partial amino acids analyses and proteomic methods. The isomers, L- and D- of aspartic acid were measured using high performance liquid chromatography (HPLC), after suitable derivatization of the target macromolecules.

Accumulation of D-Asp was used to assess protein turnover. The time rate of change of the amount of D-Asp in a protein is given by the equation: $\mathbf{d(D/L)/dt = k_i - k_T(D/L).}$ Where: values of d(D/L)/dt were derived from the derivative of a polynomial fit of the values of D/L obtained as a function of time. In order to estimate the protein turnover ($k_T$) as a function of age, we made use of the racemization rate ($k_i$) values, which were available for collagen and aggrecan. Using the relationship for half-life ($\tau_{1/2}$): $\tau_{1/2} = \ln(2)/k_T$, we were able to determine half lives of both aggrecan and collagen of normal and degenerate disc as a function of age. Since no $k_i$ value was available for elastin, its turnover was not calculated but its longevity was demonstrated.

Results show that for all proteins tested, in vivo accumulation of D-Asp (expressed as the ratio D/L-Asp) increased with age (Fig. 1). For collagen and aggrecan, data were pooled for NP and AF (for normal or degenerate) as no significant difference ($p<0.05$) was observed in their rate of D-Asp accumulation. Since no significant difference ($p<0.05$) was noted in D/L Asp accumulation between degenerate tissues of different grades of degeneration or different pathologies, D/L data for degenerate specimens were pooled and compared to healthy tissue.

For **collagen**, a linear fitting of the increase of D-Asp as a function of age resulted in an accumulation rate of $6.74\times10^{-4}$ per year for normal collagen and $5.18\times10^{-4}$ per year for degenerate collagen. Data pooled by the decade show a significant difference ($p<0.05$) between average values of D/L-Asp of normal and degenerate tissues only between ages 50 and 60 years (Fig. 2a). Except for collagen from dentin, collagen obtained from IVD experiences the most rapid accumulation of D-Asp compared to cartilage and skin.

For **aggrecan**, an increase of D/L-Asp with age was non-linear after maturity (>20 years) until a plateau was reached above 60 years of age (Fig. 1b). The relatively large scatter is probably due to the heterogeneity of the aggrecan preparation. The measured values of D/L-Asp are consistently and significantly lower in the degenerate aggrecan as compared to normal over all ages .Data pooled by the decade show that differences in D/L-Asp accumulation are more marked between normal and degenerate tissue and than as a function of age (Fig. 2b).

For **elastin**, accumulation of D-Asp increased with age in normal IVD samples. Due to the tendency of the data from degenerate discs to plateau between ages 50-80, linear regression in this case was only applied until the mid-50s. Performing linear regression on the complete data set resulted in a marginally poorer R-factor (R=0.8). Fitting the linear increase of D-Asp as a function of age resulted in accumulation rates of $16.2\pm3.1\times10^{-4}$ per year (R=0.95) and $11.7\pm3.8\times10^{-4}$ per year (R=0.84) respectively for elastin obtained from normal (grades 1-2, at all ages) and degenerate discs (grades 3-5, until the mid-50s). We found no statistically significant difference between these two rates at the $p<0.05$ level. The D-Asp linear accumulation rate of the combined data sets is $12.3\pm2.6\times10^{-4}$ per year (R=0.79). Above 50 yrs of age, the differences between the normal and degenerate tissue was statistically significant ($p<0.05$).

The half life values found for collagen and aggrecan decrease with age. For **collagen**, we found that the average half life values for collagen obtained from normal discs are 81±24.7 year between ages 22 to 30, 138.7±29.8 between ages 30-50 years, and 231.6±63 years between ages 50 to 62 (Fig. 3b). Turnover of collagen from degenerate discs may be more rapid than that found for normal discs; however statistical analysis leaves this point uncertain.

For **aggrecan**, mean half-life values were determined for normal and degenerate specimens.(Fig 3b)

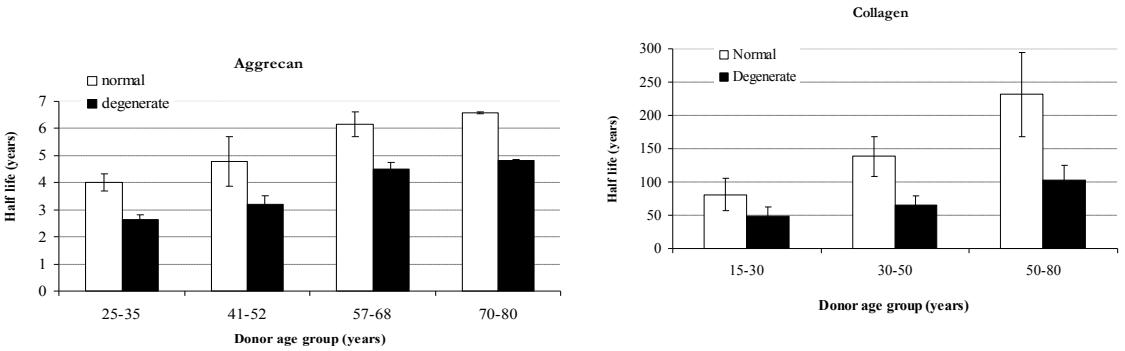



**Fig. 1. Half life values for (a) aggrecan and b (collagen) from normal and degenerate human intervertebral discs as a function of donor age.**

Here we have determined molecular half-lives of structural macromolecules, i.e. collagens, elastin and aggrecan in the disc in relationship to degeneration. No significant difference ($p<0.05$) was noted in D/L Asp accumulation between grades of degenerate tissues, D/L data for degenerate specimens were pooled and compared to healthy tissue. Accumulation of D-Asp increases with age for aggrecan, collagen and elastin all derived from IVD, suggesting these are all long-lived, metabolically stable proteins. Our data suggest that – in spite of the pronounced differences - half life values found for collagen and aggrecan are not constant, but increase with age, suggesting a dynamic turnover of these molecules.

## 3.9 Gene Expression

We used microarrays and proteomics to delineate differences between normal and pathological discs in cellular responses to stresses. Because of lack of baseline human disc tissue we examined this problem by simulating pathological conditions in a model system, we compared gene expression under normal and pathological conditions. As loss of proteoglycan with consequent effect on extracellular osmolarity is the first and major biochemical change in disc pathology, we used fall in osmolarity as our major degenerative signal. We then investigated pathological discs from patients for expression markers identified in model systems.

Briefly, nucleus pulposus cells were extracted from adult bovine caudal discs by collagenase digestion. They were cultured in alginate beads to maintain phenotype for 6 days with serum DMEM, for 1 day without serum and then a further 2 days in serum free DMEM over a range of osmolarities. Osmolarity was altered by NaCl addition from that seen in severely degenerate discs (268mOsm) to that seen in mildly degenerate discs (350mOsm) and that seen in normal discs (461 mOsm) Total RNA was extracted from the cells and gene expression analysed using the AffymetrixGeneChip Bovine Genome Array. Robust multiarray analysis (RMA) normalizes used the PM probe sets. The arrays were subsequently normalized in GeneSpring GX 11.0.2, using RMA and a paired t test used to identify differentially expressed genes which had a ≥1.5 fold change between two conditions selected. Genes were annotated with gene names, gene symbols and Gene Ontology using NetAffx, the Affymetrix annotation tool (www.netaffx.com).

Proteomic and genetic analysis of human discs from patients was studied using a proteome macroarray study, where protein expression level of 36 different cytokines was determined. Twelve patients with MRI-diagnosed lumbar disc herniation were included into the study. Half of them (6) had had radicular pain for less than 6 weeks, while the others had had symptoms for more than 3 months (chronic pain). Each patient underwent lumbar microdiscectomy when the sequestered tissue fragment was removed. The tissue sample was immediately washed in saline three times and snap-frozen in liquid nitrogen. We lyophilized the disc tissue samples and measured their cytokine profile using a Proteome Profiler Array/Human cytokine Array Panel (R&D Systems) according to the developer's instructions.We performed a gene expression analyses on 11 further disc herniation samples (5 acute and 6 chronic sequesters). The fresh-frozen samples (500mg) were pulverized under liquid nitrogen using a freezer-mill 6750 (SPEX Certiprep). Total RNA and cytokine expressed by qPCR.

Results from the model system found a total of 1375 probe sets reported as expressed at a significantly different levelin normal versus degenerate discs. The 20 genes with the highest fold-change, are given below (Table 1). Among those genes were MMP1 and DKK1 which are up-regulated in the normal relative to degenerate discs and GPNMB, NID1 and B3GALT2 which down-regulated in normal relative to degenerate discs.. The function of other genes differentially regulated are not well understood and require further investigation.

**Table 1 The 20 most differentially expressed genes -normal versus most pathological condition.**
Fold change up-regulated

| Gene | Fold | Function of encoded gene | Probe Set ID[a] |
|---|---|---|---|
| MMP1 | 16.0 | collagenase 1, matrix degradation, | Bt.72.1.S1_at |
| DKK1 | 12.0 | negative regulation of Wnt receptor | Bt.13880.1.S1_at |
| CLCA2 | 10.6 | chloride channel modulator | Bt.15970.1.A1_at |
| CD96 | 10.3 | adhesive interactions of activated T and NK cells | Bt.20940.1.S1_at |
| ID1 | 8.2 | negative regulation of transcription | Bt.1730.1.A1_at |
| LYZ | 7.5 | Lysozyme | Bt.209.3.S1_at |
| THBD | 6.9 | thrombomodulin | Bt.471.1.S1_at |
| CA2 | 5.8 | carbonic anhydrase | Bt.22854.1.S1_at |
| C5 | 5.8 | activation of MAPK activity | Bt.5622.1.S1_at |
| ARG2 | **5.6** | arginase type II | Bt.8624.1.S1_at |
| **Fold Change down-regulated** | | | |
| **Gene** | **Fold** | **Function of encoded gene** | **Probe Set ID** |
| GPNMB | 20.0 | Transmembrane glycoprotein, osteoactivin | Bt.9807.1.S1_at |
| LRRC17 | 7.8 | Negative regulator of osteoclast differentiation | Bt.4886.1.A1_at |
| NID1 | 6.0 | cell-matrix adhesion | Bt.8106.1.S1_at |
| B3GALT2 | 5.5 | galactosyltransferase | Bt.7447.1.A1_at |
| FGL2 | 4.8 | fibrinogen-like 2 | Bt.9202.1.S1_at |
| PTGDS | 4.7 | prostaglandin biosynthetic process | Bt.1645.1.S1_at |
| GCSH | 4.6 | glycine catabolic process | Bt.4195.1.S1_at |
| LGALS1 | 4.5 | Apoptosis | Bt.5472.1.S1_at |
| GCLC | 3.8 | cysteine metabolic process | Bt.19499.1.S1_at |
| CA9 | 3.8 | Carbonic andydrase | Bt.27842.1.S1_at |

The table below shows the differential expression of metalloproteinases and their inhibitors between normal and pathological discs; these proteins are actively involved in tissure degradation and thus are of pathological interest. When comparing gene expression in moving from normal to degenerate conditions, we showed that MMP-2, MMP-3 and MMP13 genes are upregulated in degenerate conditions.These are proteases involved in proteoglycan and glycoprotein degradation wherease MMP13 is thought to be the main collagenase involved in tissue degradation. By contrast, expression of MMP1, MMP9, MMP11, MMP16, ADAMTS1, ADAMTS5, TIMP1 and TIMP3 were all down regulated under in degenerate conditions. MMP-1 also known as interstitial collagenase or collagenase-1, may be involved in normal tissue turnover rather than degradation. ADAMTS1 and ADAMTS5 also involved in aggrecan turnover were differentially affected.

**Table 2. Metalloproteinase genes differentially expressed (fold change) under different pathological conditions.** This table lists the genes that were statistically altered in normal compared compared to pathological conditions;
↑: upregulation; ↓: downregulation; - :no difference

| | Very pathological versus normal | Mildly versus moderately pathological | Moderately pathological versus normal |
|---|---|---|---|
| ***MMP1*** | 16↑ | - | 11.6↑ |
| ***MMP2*** | 2.2↓ | 1.7↓ | - |
| ***MMP3*** | 1.9↓ | 1.7↓ | - |
| ***MMP9*** | 3.1↑ | - | 2.6↑ |
| ***MMP11*** | 2.3↑ | 1.9↑ | - |
| ***MMP13*** | 3.1↓ | 4.5↓ | - |
| ***MMP16*** | 1.6↑ | - | - |
| ***ADAMTS1*** | 2.8↑ | - | 3.4↑ |
| ***ADAMTS5*** | - | - | 2↑ |
| ***TIMP1*** | 1.9↑ | - | 1.7↑ |
| ***TIMP3*** | 1.9↑ | 1.9↑ | - |

On patient samples from the cytokine proteome array, we identified 6 cytokines (Rantes, IFN-g, G-CSF, IL-6, IL-8, GRO-a) with significantly different expression in the acute and chronic herniations (Figure 3). The gene expression analysis of these cytokines resulted in similar results. Five of the six target genes expressed differently in the acute and chronic sequesters (Figure1).

**Figure 1 Significantly different expression of 6 cytokines in acute and chronic disc herniation**

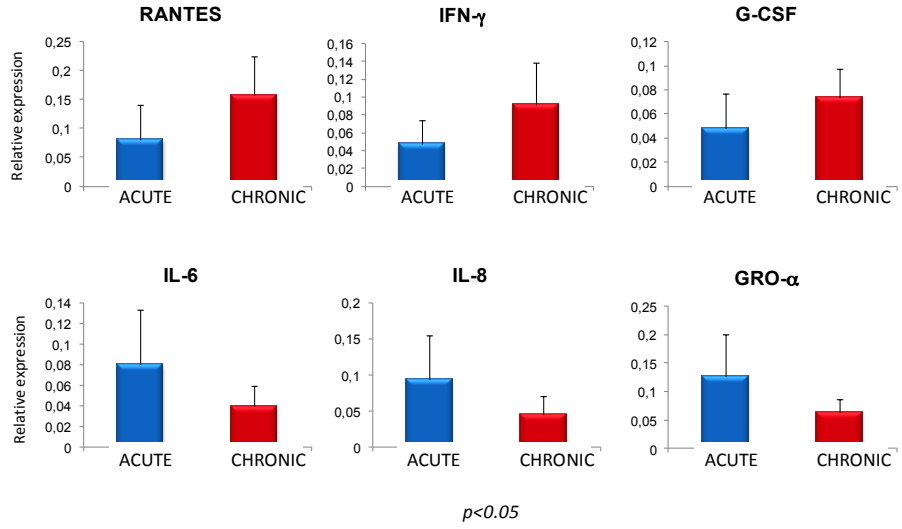
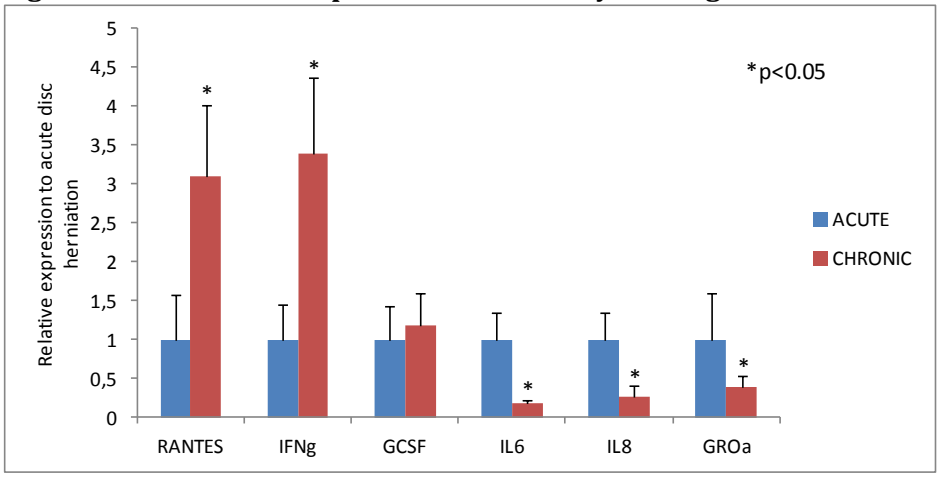



**Figure 2 Difference in expression of the six cytokine gene**



Using microarrays in model systems have found many differences in gene expression between normal and pathological discs. Of these, upregulation of matrix metalloproteinases is of most interest as these proteases are involved in degradative change. The results seen in this regard were validated by protein assays (Deliverable 3.2) and are in agreement with reports of protease upregulation in pathological tissues in the literature. As well as these known genes, transcriptomic analysis drew attention to other genes with strong differential expression between normal and pathological tissues, whose role in the degradation of the disc has not previously been explored and now should be urgently investigated.

The results of the protein array and gene expression on patients require further analysis, but our results suggest the response of the immune system to the herniated disc tissue change with time. The cytokines above have been previously published in relation to the intervertebral disc or herniated disc but their origin is not clear (i.e. the disc cells themselves or the immune cells infiltrating the sequestered tissue can produce these molecules). The change in the cytokine profile can be associated with the change of the inflammatory reaction generated around the herniated disc tissue and it can be related to the alteration of the symptoms that we clinically notice in disc herniation patients. The inflammatory reaction can be also associated with the ”spontaneous” absorption of the sequestered fragment, but it requires further investigation. We assume that the determined cytokines (or a subset of them) can be used as biomarkers in the future to distinguish biologically acute from chronic disc herniation which can modify the treatment in the future or can help to select patient for non-invasive biological therapy (i.e. immune system modulation).

**3. 10 Diagnostic Genes**

In order to find target genes for diagnosis or as drug targets, we have used the approaches delineated in WP3 and WP5. Genome wide association analysis has been successfully applied to many different chronic conditions of complex etiology, but not for disc degeneration or related spinal conditions; the first published gwas study was this autumn (2012) and found one novel association , to the PARK2 gene, which had not been suspected to play a role in disc degeneration. To reduce costs, we used pooling ofl DNA from cases and controls and compare allele frequencies in the pools rather than individually genotyping every person in the sample. Pooling has now become a technologically viable alternative to individual genotyping to identify variants associated with disc degeneration, which offers also substantial cost savings. We proposed an efficient, cost-effective multi-stage design GWA pooling study to identify a number of SNPs involved in disc degeneration.

DNA from Hungarian lumbar disc herniation samples and controls was prepared and analysed at the Technology Center, Finnish Institute for Molecular Medicine (successor to the Finnish Genome Centre). In the final pools (with 12 replicates) there were 203 cases and 203 controls with equal amount of DNA from each subject. These were run on a Illumina OmniExpress chip, genotyping a total of 709,358 single nucleotide polymorphisms on chromosomes 1 to 22. The data were cleaned by requiring that the cases and controls have successful genotypes on all 12 replicates. The individual success rate for genotyping per pool was 99.34% or more. Next, we excluded SNPs in which the variability of allele frequencies was too great (> +/- 0.05 from the mean).

Two sets of analyses were conducted. Candidate gene analysis was based on published work and a systematic review (Eskola P et al, Plos One, in press and table 2 below), we aimed to replicate earlier candidate gene associations. Eskola et al identified six genes (ASPN, COL11A1, GDF5, SKT, THBS2 and MMP9) as having moderate level of evidence in lunbar disc degeneration (with a multitude of phenotypes having been assessed in different studies). We were able to assess three of the same snps in our dataset, and none showed differences between cases and controls (largest allele frequency difference was 0.029). We also examined two other candidates, Aggrecan and Vitamin D receptor. Aggrecan rs 1042631 was associated with disc degeneration in TwinSpine. Here the minor allele frequency (MAF) in cases was .1794 and in controls 0.1257, p=0.09, so some suggestion, but for Vit D receptor rs731236 the case MAF 40.7 and control 40.3, p=0.98.

A recent paper (Olsen et al, J Neurosci. 2012 Jul 18;32(29):9831-4) implicated the mu opioid receptor 1 (OPRM1) functional variants A188G in the degree of pain intensity associated with lumbar disc hernation. We found that the OPRM1 G allele has a higher frequency in the Hungarian cases than

the controls (.189 vs .135). The control frequency is the same as reported for European populations, while the higher case frequency in our pooled DNA analysis may be due to those with more and persistent pain being selected into Genodisc.

Gene discovery was based on examining the largest effects in our data set. We computed an effect size measure proportional to the variance (difference between case and control allele frequencies squared), but penalized very large or very small allele frequencies (which are more subject to error and have less power) by multiplying by p(1-p), where p is the allele frequency. The largest effect sizes; case-control allele differences greater than 0.1 are shown in Table of SNPs.

Table of best SNPs (rs number, chromosomal location and position, allele frequency difference and frequencies in cases and controls, and whether it is found in intragenically (gene name is given) or intergenically. *SNPs in regulatory regions (as identified in the ENCODE project) are highlighted in yellow.*

| rs number | Chromosome | position | Alllele frequency difference | Case mean allele frequency | Control mean allele frequency | Gene |
|---|---|---|---|---|---|---|
| rs7604513 | 2p12 | 76741847 | 0,1509 | 0,516 | 0,667 | intergenic |
| rs991725 | | 76785084 | 0,1658 | 0,380 | 0,545 | intergenic |
| rs313305 | 2q14.3 | 126082102 | 0,1591 | 0,554 | 0,713 | intergenic |
| rs10048673 | 2q34 | 213083282 | 0,1509 | 0,417 | 0,568 | ERBB4 |
| rs10201602 | 2q36.3 | 228245434 | 0,1651 | 0,310 | 0,475 | intergenic |
| rs1675382 | 3p14.3 | 57945243 | 0,1421 | 0,386 | 0,528 | intergenic |
| rs524164 | 3q22.3 | 138405597 | 0,1502 | 0,514 | 0,664 | PIK3CB |
| rs7690099 | 4q28.3 | 132080596 | 0,1449 | 0,449 | 0,594 | intergenic |
| rs10512920 | 5p15.31 | 7402561 | 0,1639 | 0,545 | 0,708 | ADCY2 |
| rs17710639 | 5p15.1 | 17808002 | 0,1514 | 0,520 | 0,672 | intergenic |
| rs33699 | 5q14.3 | 87491545 | 0,1652 | 0,575 | 0,740 | TMEM161B |
| rs4571506 | 5q14.3 | 87756918 | 0,1618 | 0,459 | 0,621 | intergenic |
| rs10069193 | 5q21.1 | 101716620 | 0,1438 | 0,280 | 0,424 | SLCO6A1 |
| rs435021 | 5q22.1 | 110650965 | 0,1721 | 0,367 | 0,539 | CAMK4 |
| rs42322 | 7p15.1 | 28690496 | 0,1499 | 0,343 | 0,493 | CREB5 |
| rs9641147 | 7q21.3 | 94368896 | 0,1499 | 0,347 | 0,497 | intergenic |
| rs2061847 | 8p23.3 | 2158537 | 0,1476 | 0,528 | 0,381 | intergenic |
| rs10106620 | 8p23.2 | 4662898 | 0,1824 | 0,500 | 0,683 | CSMD1 |
| rs2187983 | 8q21.3 | 91526934 | 0,1847 | 0,533 | 0,718 | intergenic |
| rs4330650 | 8q21.3 | 91684009 | 0,1701 | 0,461 | 0,631 | TMEM64 |
| rs6471246 | 8q21.3 | 91692022 | 0,1499 | 0,500 | 0,650 | TMEM64 |
| rs13439805 | 8q23.3 | 117594864 | 0,1556 | 0,451 | 0,607 | intergenic |
| rs1830877 | 8q24.23 | 139109143 | 0,1531 | 0,489 | 0,335 | intergenic |
| rs2889293 | 9p24.2 | 2435209 | 0,1500 | 0,493 | 0,643 | FLJ35024 |
| rs4127335 | 9p24.1 | 8220067 | 0,1857 | 0,465 | 0,651 | intergenic |
| rs2784611 | 9p23 | 10551203 | 0,1442 | 0,365 | 0,510 | PTPRD |
| rs639949 | 9p21 | 32490675 | 0,1505 | 0,360 | 0,510 | DDX58 |
| rs657454 | 9p21.1 | 32534851 | 0,1496 | 0,284 | 0,434 | intergenic |
| rs1330351 | 9q33.1 | 117840922 | 0,1589 | 0,456 | 0,615 | TNC |
| rs522540 | 10q24.2 | 99647665 | 0,1545 | 0,534 | 0,380 | CRTAC1 |
| rs4539296 | 11q22.3 | 105373672 | 0,1565 | 0,365 | 0,522 | intergenic |
| rs7302861 | 12q13.13 | 52974463 | 0,1515 | 0,724 | 0,573 | intergenic |

| | | | | | | |
|---|---|---|---|---|---|---|
| rs4550315 | 12q23.3 | 106121578 | 0,1537 | 0,345 | 0,498 | intergenic |
| rs7336290 | 13q12.13 | 27612154 | 0,1492 | 0,476 | 0,625 | intergenic |
| rs803815 | 13q21.33 | 71536609 | 0,1500 | 0,513 | 0,663 | intergenic |
| rs7330047 | 13q22.2 | 76925888 | 0,1468 | 0,401 | 0,548 | intergenic |
| rs4924385 | 15q14 | 40037021 | 0,1490 | 0,438 | 0,587 | FSIP1 |
| rs516937 | 17p13.3 | 706009 | 0,1711 | 0,490 | 0,319 | NXN |
| rs7237066 | 18q11.2 | 23504200 | 0,1424 | 0,347 | 0,490 | intergenic |
| rs8095374 | 18q21.1 | 43793488 | 0,1619 | 0,668 | 0,506 | C18orf25 |
| rs10460040 | 18q21.1 | 45735556 | 0,1578 | 0,792 | 0,634 | ZBTB7C |
| rs12955021 | 18q21.2 | 48686563 | 0,1474 | 0,400 | 0,547 | intergenic |
| rs10502916 | 18q21.2 | 48745119 | 0,1491 | 0,648 | 0,499 | intergenic |
| rs394843 | 22q11.21 | 18467483 | 0,1416 | 0,353 | 0,495 | MICAL3 |
| rs738791 | 22q11.23 | 24117525 | 0,1669 | 0,465 | 0,632 | MMP11 |
| rs5757312 | 22q13.1 | 39255591 | 0,1469 | 0,447 | 0,594 | intergenic |

In summary we have identified 20 Genes putatively associated with lumbar disc herniation, as well as 25 other SNPs, several of which are in regulatory regions and hence active.

## 3.11 Muscle and School Exercise Programme

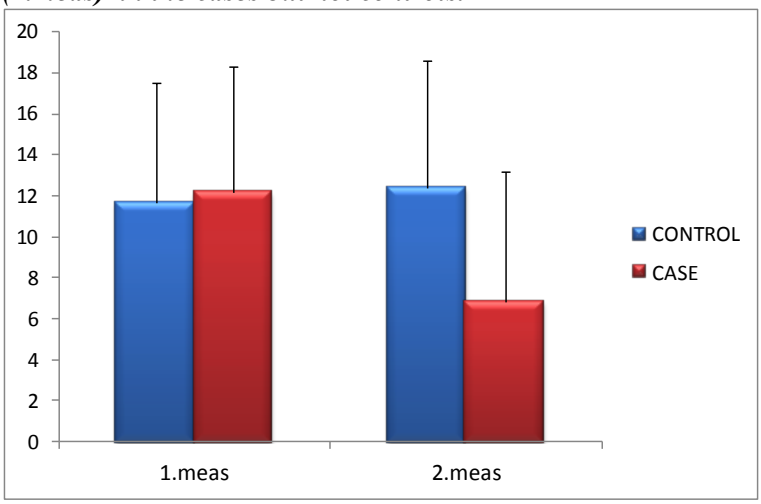
| Variable | | Mean, % |
|---|---|---|
| Sex (boy/girl) | | 49%/51% |
| Age (year) | | 9.79+1.2 |
| Weight (kg) | | 36.2+9.7 |
| Height (cm) | | 141.4+9.4 |
| BMI (kg/m$^2$) | | 17.9+3.3 |
| Dominant hand (right/left) | | 89%/11% |
| Gripping force (dom) (kg) | | 14.8+4.2 |
| Gripping force (nondom) (kg) | | 13.8+3.9 |
| No. of gyms/w | 2 | 32% |
| | 3 | 37% |
| | 4 | 17% |
| | 5 | 14% |

The efficacy of a special school exercise program focusing on the early prevention of degenerative spinal disorders and the exploration of genetic markers associated to the efficacy of the early prevention was determined. Eight schools in three Hungarian cities were involved into the study during this period. Altogether 1371 pupils were included into the study, 1180 subjects were measured twice with 6 months apart and parents of 982 students gave the permission for the saliva collection and DNA analyses..

**Table1 Descriptive statistics of the study cohort**

Students underwent physical examination, non-invasive spinal functional capacity evaluation and muscle exercises. They also completed lifestyle questionnaires. After the first test all pupils have participated in a special activity program focused on the training of trunk and lower extremities muscles built in the gymnastics for 6 months. In the second test period effect of spine exercises was determined. Third of the pupils formed the control group in the first study year, when they did not do the exercise program in the 6-month long study period. These children have got the prevention program during the next school year. The posture problems were determined following the protocol of a standard physical examination (Table 4). Muscle status was measured using the 12 exercise test. The mean muscle score in the total cohort was 9.1$\pm$1.7. Less than 20% of the children were in muscle balance at the time of the first measurement. Muscle score was significantly ($p<0.05$) associated with weight, number of gyms, quality of gyms and presence of neck hyperlordosis, back hyperkyphosis and scoliosis. After 6 months of schoolbased prevention program, the muscle score was significantly higher in the intervention group (10.7$\pm$1.2) and not changed in the control group (Figure 1). The incidence of posture problems decreased in the intervention group while deterioration in the spinal curvatures was observed in the control group during the school year.

**Figure 1 Results of the Matthias test** ***(decrease in trunk inclination – y axis – means improvement in spinal functional capacity) showing the significant increase in muscle strength after 6 months exercise (2.meas) in the cases but not controls.***




The results of the Matthias test – quantitatively measured with the Spinal Mouse device – showed the significant improvement of the spinal global functional capacity due to the prevention program (Figure 1). As a consequence, this programme will be unrolled across all primary schools in Hungary.

3.12 **VDR Polymorphisms**

The aim was to investigate VDR polymorphisms in relation to muscle strength.

We performed an individual genotyping study using a Sequenom MassArray system at the University of Helsinki (Partner 5). After the detailed literature review and SNP selection work, 55 SNPs in 14 candidate genes. Among these SNPs 7 were inside the Vitamin D receptor gene (VDR). We analyzed the association of these VDR SNPs (and the haplotypes constructed by these SNPs) and two muscle related phenotypes; the hand grip strength and the back muscle score in schoolchildren. ANOVA was used for the individual SNP analyses while log-likelihood ratio tests were applied to investigate the effect of the VDR haplotypes.

We used the data of the first measurements in the prevention project. Table 1 shows the description of the study cohort (only children with all genotyped VDR SNPs were included into the study). Figure 1 shows the linkage disequilibrium among the VDR variants. We identified two haploblocks inside the gene, and the genetic effect of these haplotypes was also studied. We found three individual SNPs, significantly associated with the grip force of the dominant hand and one SNP associated with the grip force of the non-dominant hand (Table 2). 'ACT' haplotype constructed by the three SNPs located in the 3' part of the gene (rs1544410, rs731236, rs10783215) were associated with the highest hand grip strength in both hands (Table 5, Figure 2). Back muscle score was significantly associated with one individual SNP (rs3782905) and with no haploblock..

**Table 1 Study cohort (N=788)**

| Variable | Mean (SD) |
|---|---|
| Age (y) | 9.8 (1.2) |
| Sex (m/f %) | 51% / 49% |
| Weight (kg) | 36.0 (9.1) |
| Height (cm) | 141.2 (9.1) |
| BMI (kg/m2) | 17.8 (3.0) |
| Dominant hand (righ/left %) | 91% / 9% |
| Grip strength dominant (N) | 147.5 (42.1) |
| Grip strength non-dominant (N) | 137.3 (38.3) |
| Back muscle score | 9.4 (1.8) |

**Figure 1 Linkage disequilibrium map about the genotyped SNPs**

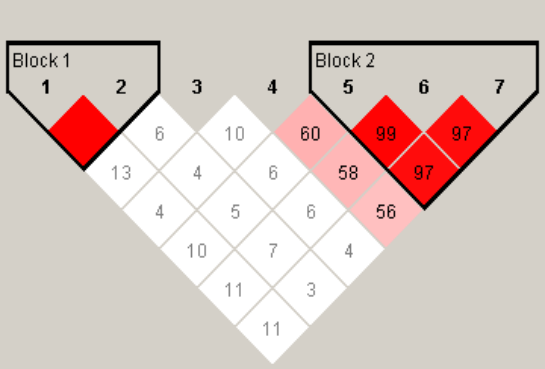


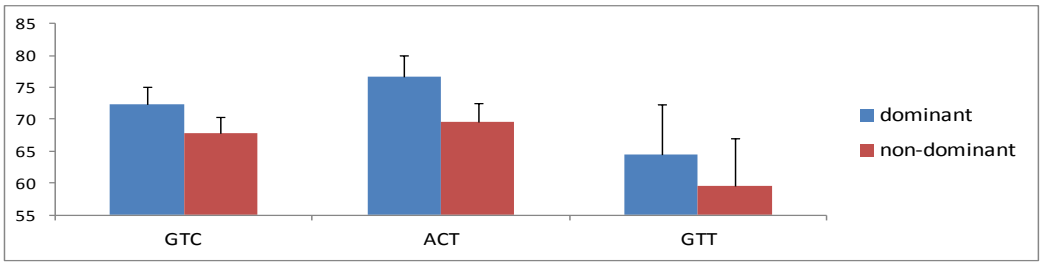

**Table 2 Genotyped VDR variants and their association with muscle phenotypes (p-values of the ANOVA models are represented)**

| rs number | Conventional name | Alleles | Region | Success rate (%) | Grip force dominant | Grip force non-dominant | Back muscle performance |
|---|---|---|---|---|---|---|---|
| rs11568820 | Cdx | G/A | promoter | 95.8 | 0.854 | 0.207 | 0.589 |
| rs4516035 | A1012G | T/C | promoter | 99.0 | ***0.009*** | ***0.034*** | 0.425 |
| rs2228570 | FokI | C/T | Exon 2 | 98.9 | 0.534 | 0.606 | 0.397 |
| rs3782905 | DdeI | C/G | Intron 2 | 98.2 | 0.278 | 0.248 | ***0.002*** |
| rs1544410 | BsmI | G/A | Intron 8 | 98.9 | ***0.010*** | 0.169 | 0.397 |
| rs731236 | TaqI | T/C | Exon 9 | 98.9 | ***0.039*** | 0.116 | 0.252 |
| rs10783215 | - | C/T | 3' UTR | 99.4 | 0.372 | 0.655 | 0.482 |
| | | | | | | | |

**Table 3 Effect of VDR haplotypes on muscle phenotypes (*p<0.01, **p<0.001)**

| Haplotype | Frequency | Grip force dominant** | Grip force non-dominant* | Back muscle performance (ns) |
|---|---|---|---|---|
| GTC | 52.7 | 72.3 (69.5-75.1) | 67.9 (65.5-70.4) | 4.7 (4.6-4.8) |
| ACT | 36.5 | 76.6 (73.1-80.1) | 69.5 (66.4-72.6) | 4.7 (4.6-4.9) |
| GTT | 9.5 | 64.5 (56.6-72.3) | 59.5 (51.8-67.1) | 4.6 (4.3-4.8) |

**Figure 2 The effect of VDR haplotypes on hand grip strength**




Our results suggest that VDR and its genetic variants can play a significant role in muscle function and different muscle phenotypes can have genetic background. The biological importance of the VDR variants in degenerative spinal disorders should be further investigated.

# REPAIR

## 3.13 Drug Delivery

*This work was carried out in collaboration with Professor Shirazi-Adl, École Polytechnique, Montréal and his students.*

Infections of the intervertebral disc have devastating consequences. To prevent such infections occurring, antibiotics are routinely administered prophylactically by intravenous injection before disc surgery. They are also administered intravenously to treat infections if they arise. In order for successful prophylaxis and treatment, drug levels must remain above critical levels throughout the disc for a defined time. However at present there is little information on how antibiotics penetrate into the disc; there is no basis for deriving satisfactory drug dosing regimes.

We have thus developed a computational model which can predict how drug levels throughout the disc vary with time in relation to disc properties, properties of the drug and dose administered. We tested this model against data from two published studies, both of which measured concentrations of a solute in the blood and in the disc after an intravenous injection. The first study examined movement of radioactive tracer sulphate into animal discs for six hours after injection. The second study examined concentrations of the antibiotic cephazolin in discs removed at surgery up to 150 minutes after pre-operative administration of the drug. In both cases, the computational model simulated the experimental results very well. We thus took the models further and examined the effect of endplate calcification on transport of the drugs into the disc. It has now been established than in many cases, the endplate of degenerate and aged discs calcifies thus impeding transport into the disc from the blood supply arising in the vertebral bodies. The simulation shows that even partial blockage of the endplate dramatically reduces transport of drugs into the disc[14].

The simulations show clearly that, as expected from diffusion theory and in agreement with published experimental data:-

(i) transport into the centre of the disc is slow, taking many hours to reach maximum concentrations in the centre of human discs. Animal studies, especially on animals such as rabbits whose discs which are much smaller than those of humans overestimate the speed and degree of drug penetration to a very great extent

(ii) after intravenous injection of a solute, apart from areas close to the disc's margins, concentrations throughout even healthy human discs are very low compared to blood values and in the case of antibiotic administration, a very large percentage of the disc (>60%) does not reach concentrations required to inhibit infection

(iii) administration of drugs into degenerate discs should take account of fall in permeability of the endplate route into the disc; rate of penetration and final concentrations will consequently be much lower than in normal discs.

In summary the simulations show that adequate dosing of drugs, growth factors or tracers into the large human lumbar discs cannot be achieved via an intravenous route without very high doses or many hours of perfusion.

## 3.14 **Selection of low back pain patients who might benefit from cell therapies.**

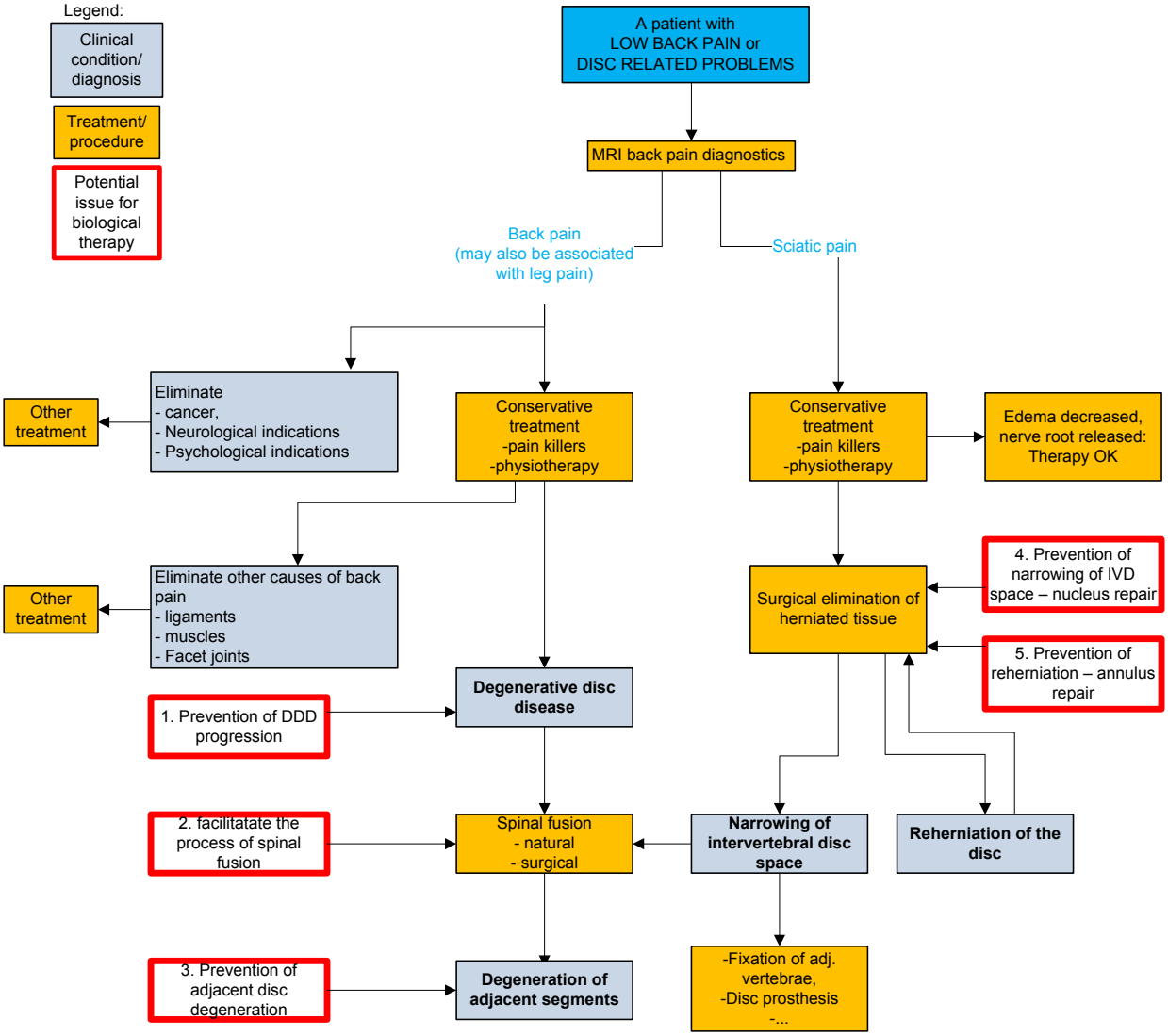
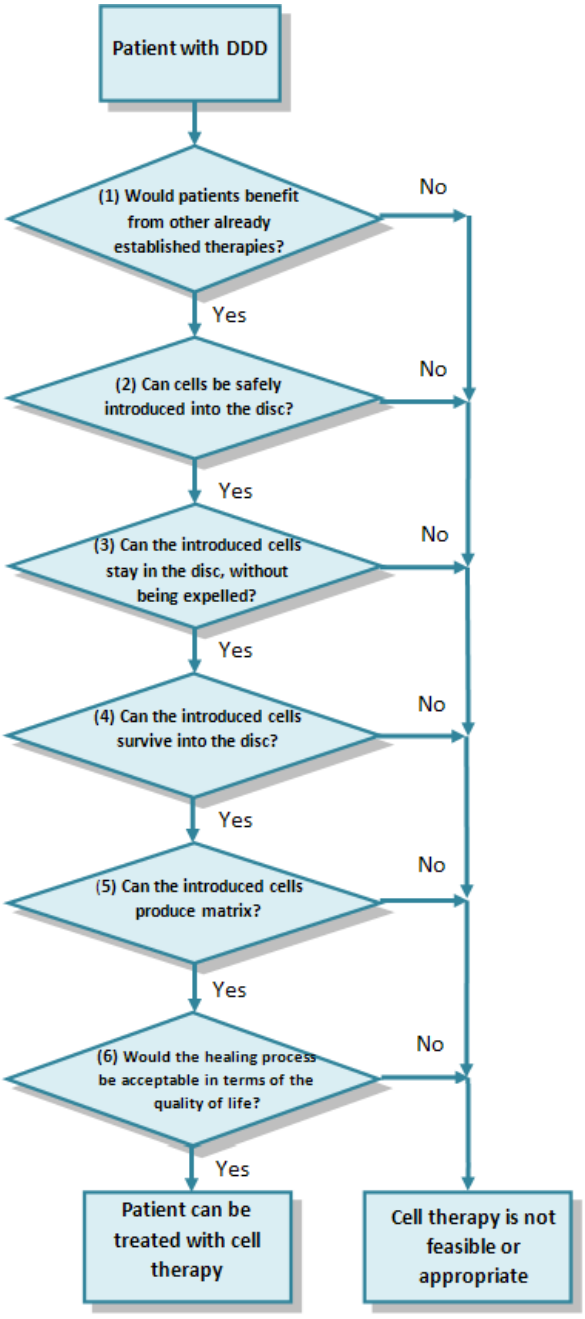



**Figure.1**: Diagram, showing diagnostic and treatment options during progression of IVD pathology. Targets for potential cell therapy approach are described in red fields

Cell therapies have been used for cartilage repair since 1994; development of similar therapies for treating low back pain are now a topic of much research interest. Most research in this area has concentrated on the choice of cell sources and how they can be manipulated for the purpose of restoring the nucleus in patients with low back pain resulting from disc degeneration. However there are clinical questions about patient selection which are not much discussed as well other technical issues which also require further investigation. Figure 1 above shows diagnostic and treatment options for back pain patients showing various decision points. It also indicates which patients could be considered for cell therapies.

However the state of the disc must meet criteria outlined in Figure 2 if cell therapies are to succeed; there are at least 2 known exclusion criteria. Moreover, even if the discs can potentially support implanted or stimulated cells, there are at present no diagnostic means to predict whether successful of a cell therapy will alleviate the patient's symptoms.

Figure 2 A diagnostic algorithm for selecting low back pain patients whose discs are in a state to benefit from a cell therapy treatment. Annotations showing some of the problems requiring solution before cell therapies are regarded as a routine treatment for treating degenerative disc disorders are given below and in Deliverable 7.3




1. Patient selection. At present, we cannot predict who will develop painful disc degeneration so uncertain benefits of preventative treatment is unwarranted and would require screening of a symptomless population;

2&3. Cells require injection into the disc without damaging the disc further and must be retained in the disc until anchored. Radial tears or fissures, identifiable on MRI are exclusion criteria

4.For implanted cells to survive, the nutrient supply must be adequate. Assessment at present is only possible by delayed contrast MRI as shown initially by Bydder[15] and Rajasekaran [16;17]. Inappropriate nutrient supply is also an exclusion criteria.

5. Matrix production is inhibited if the extracellular environment is inappropriate; acid conditions and low oxygen found in many degenerate discs are contra-indications but at present cannot be evaluated non-invasively.

6. As shown in Deliverable 6.2, the half-life of major macromolecules aggrecan, collagen and elastin are 10-100 years respectively so replacement of matrix in a human disc will be very slow and effects on quality of life of this slow healing have not yet been assessed.

If the discs meet inclusion criteria, the choice of an appropriate cell source is one of the prerequisites for the successful outcome of disc cell therapies. Implanted cells should be able to survive and, moreover, they should produce an appropriate, proteoglycan-rich matrix – an essential feature of nucleus pulposus tissue. However, whether these cells truly differentiate into disc cells is unclear as there are at present no disc-specific cell markers.

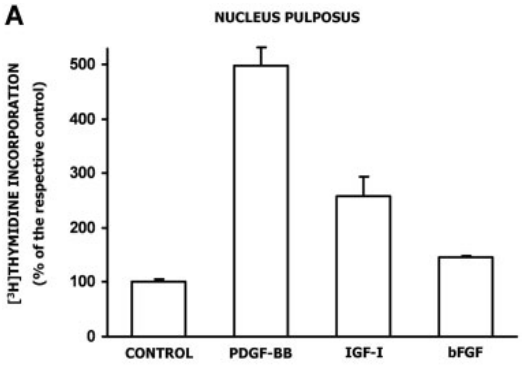
Cell sources for disc cell therapy in clinical use to date include autologous disc cells and Mesenchymal stem cells. Although some cell products are offered to patients in the US and Germany they have not been fully characterised or used in randomised controlled trials. Indeed there are several recent publications expressing concern about a boom in unproven procedures involving cell therapy (particularly with 'stem' cells) in many areas of medicine around the globe[14,15]. Apart from issues relating to cell survival and efficacy, delivery of these cells for cell therapy provides a challenge on its own.

Cell therapies, besides cellular implantation can also involve stimulation of resident cells. There is interest in the use of growth factors in treating degenerate intervertebral discs because they have been shown to have potentially beneficial effects on disc cells, both in *in vitro* and some *in vivo* model systems. We have shown that human disc cells from both the annulus fibrosus and nucleus pulposus cells respond to growth factors, such as platelet derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF) and transforming growth factor-β (TGF β) by activating intracellular signalling pathways (i.e. the MEK/ERK and the PI3K/Akt pathways) and subsequently cell proliferation. Interestingly, these pathways have also been found to be activated in disc specimens *in vivo*, most probably as a response to exogenous and autocrine growth factors

PRP is prepared from blood fraction, rich with platelets, that release large quantities of different growth factors. PRP preparations are variable, but yet they are accepted as treatment option and widely used for different clinical applications to facilitate regeneration of different structural tissues. We observed positive effect of PRP on chondrogenic differentiation of NP cells, however our comparison of PRP-supplemented medium with differentiation medium supplemented by TGFß1 has shown that PRP-medium was less powerful with regard to chondrogenic differentiation of both MSCs and NP cells (shown on both gene expression and protein level). PRP had a stronger influence on proliferation then on differentiation. The study was published in the Journal of Tissue Engineering and Regenerative Medicine[18].

It thus seems that if cell proliferation is the desired result, autologous PRP is the cheapest and safest option but may be ineffective in some patients; PDGF is the most potent (Fig 3) but is expensive. However, without healthy and functional cells, a therapeutic effect of injecting growth factor(s) will not be achieved. Hence a careful choice of patients appropriate for growth factor application may be dependent on factors shown in Figure 2

*Figure 3: DNA synthesis, reflecting cell proliferation, in human disc cells exposed to growth factors* [19]




.

**Using the criteria described above, we have assessed what proportion of some patient cohorts recruited to the Genodisc project would be appropriate for cell therapy, as an indication for patient cohorts in general as described below.**

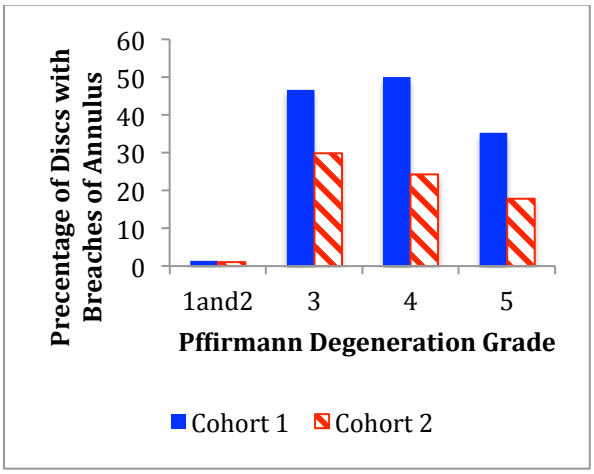
*We have found that just over 10% of lumbar discs in back pain patients referred to tertiary spinal centres remain after exclusion criteria are applied and hence could be considered for biological therapies to treat degenerative disc disease via repair or regeneration of the nucleus pulposus (NP).*

Over 2000 patients were recruited from 6 collection sites (Oxford, Kettering and Oswestry from the UK, Milan from Italy, Ljubljana in Slovenia and Budapest from Hungary). Clinical MR images of the 6 lumbar spinal levels (T12-L1) of each subject, were scored by a single very experienced radiologist (partner 3). Twenty eight features were scored using a numerical scale, including markers for breaches of annulus integrity by fissures or herniations. Reliability of the scores was assessed by replicating scoring of >80 spines. The number of discs of each grade in the MRIs was counted from a representative subgroup of spines. The proportion of discs with herniations of any type (i.e. protrusion, extrusion, sequestration) or with annular tears were assessed by degeneration grade using the Pfirmann grading scheme (Figure 2). There are differences in referral patterns in tertiary spinal centres from different EU countries. Since the proportion of patients who are eligible for treatment by cell therapy will vary from centre to centre, we have separated the Genodisc patients into two cohorts. Cohort 1 comprised patients recruited from Italy only, of which 54% had lumbar disc herniation. Cohort 2 comprised patients recruited from UK and Hungary, of which 24% had lumbar disc herniation.

Cohort 2 has a markedly higher proportion of moderately (Grade 4) and severely (Grade 5) degenerate discs in its patient population than Cohort 1 though, from either cohort, most discs examined were classified as either Grade 1 or Grade 2. Discs with Pfirrmann Grades 1 or 2 have no pathological changes and, in general, are not considered to be the source of pain. Most of the discs assessed, therefore, would not be suitable targets for treatment with cell-therapies.

Figure 2 shows the percentage of discs from each cohort with breaches of the annulus fibrosus in relation to degeneration grade; these would not be suitable for cell therapy for NP repair, as any tear may lead to leakage of the cell product introduced into the disc.

**Figure 4: Percentage of discs with loss of Annulus Integrity versus Degeneration Grade**




Very few Grade 1 or 2 discs show any breaches of the annulus but this is not the case for more degenerate discs. In general, the percentage of discs with loss of annulus integrity is similar for Grades 3 and 4 but less for Grade 5, probably because at this stage of severe degeneration, the disc space has almost collapsed and no features can be distinguished by MRI. We assessed the proportion of patients' discs with annular tears (as measured by MRI, which may underestimate them) in Cohorts 1 and 2 by Pfirrmann Grade (Figure 4);47% of the Grade 3 discs from Cohortand 30% of the Grade 3 discs from Cohort 2 would be unsuitable for cell therapies.

In addition both resident cells and implanted cells must have an adequate nutrient supply to be able to survive and function and repair/rejuvenate the disc [20]. In degenerate discs, pathways of nutrients to the disc are reduced with pathological changes interfering with nutrient transport into the disc. If resident cells failed to function appropriately, or died because of lack of nutrient supply, any implanted cells will suffer the same fate. It is imperative, therefore, that there be some means of assessing nutrient supply when determining if treatment by a cell therapy approach is feasible for any particular patient, as a fall in nutrient supply in degenerate discs is likely to be one of the main impediments to the success of any form of cell therapy.

Here, we have assessed nutrient pathways into 222 individual discs, via post-contrast MRIs using published methods [15;21]. Briefly, after intravenous injection of contrast medium (CM), diffusion of CM into the disc is followed by measuring the degree of enhancement with time relative to the MRI signal pre-contrast injection.

**Figure 5: Percent deviation of contrast medium enhancement in degenerate relative to normal discs**

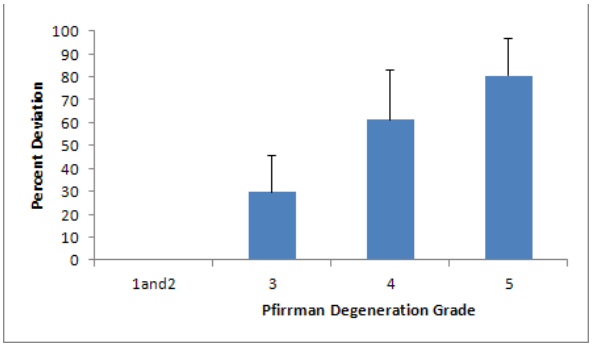



Figure 5 shows that of the discs likely to be a pain source, only Grade 3 discs are possible candidates for cell therapy on nutritional grounds. Not all Grade 3 discs, however, will have an adequate nutrient supply. Individual diagnostic tests to assess likely nutrient flow on each patient will be required before treatment by cell therapy can be advocated

We have put together the data described above to calculate the percentage of patients recruited to the Genodisc project that would be suitable for treatment by cell therapy, i.e. not excluded because of breaches of the annulus or insufficient disc nutrition. The result, shown in Table 1, is that just over 10% of Genodisc patients meet the inclusion criteria for cell therapy on the basis of MRI findings (although of course they may be inappropriate for other reasons as well).

**Table 1: Percentage of discs from two cohorts suitable for NP cellular repair**

| Cohort | % Grade 3 discs | % Grade 3 discs with intact annulus | % Discs which meet MRI inclusion criteria for cell therapy |
|---|---|---|---|
| **1** | 24 | 47 | **12.7** |
| **2** | 16 | 30 | **11.2** |

*Conclusions.* Few diagnostic or prognostic tests are available, at present, to determine if cell therapies for nucleus pulposus repair are likely to succeed. Even though there are strong associations between back pain and disc degeneration, many people, even with severely degenerate discs or with herniated discs, are pain-free [6;22]. Moreover, there is evidence that there is often central processing of pain - the symptom which drives patients to the clinic - or development of neuropathic pain [23;24]. Removal of the source of pathology, may not remove the pain and cell therapies must be targeted more precisely than at a Grade 3 degenerate disc if they are to be used effectively. Furthermore, matrix half-life in human discs is very slow, partly because of the low cell density even in healthy human discs; matrix regeneration by implanted cells would take years rather than months [25-27] and questions regarding quality of life and rehabilitation during this long process would need to be taken into consideration.

Cell therapy for the purpose of treating degenerate disc disease is a very attractive concept until the feasibility of its usefulness in a clinical setting is investigated. There are still many obstacles to be overcome. Some of these are way beyond our current capacity. At present, there is no acceptable diagnostic method of deciding whether an individual patient might benefit from cell therapy. This needs major advances in understanding back pain that have defied serious investigations over the last century. The potential that implanted cells will have to reverse the degeneration process and repair the intervertebral disc also remains to be determined. Moreover, it should be realised that disc regeneration and repair by cell therapies is likely to be very slow and thought should be given to appropriate rehabilitation protocols after implantation.

It is difficult to see how cell therapy can be introduced into widespread routine clinical practice for treatment of 'degenerative disc disease', taking all the considerations discussed here into account. Moreover, disc cell therapies are likely to be an expensive procedure because of the need for considerable expansion of cell sources [28]. These issues should be of relevance in guiding the distribution of rare resources for back pain research. Perhaps they should be funnelled into a better understanding of pain mechanisms in back pain patients so that we know better where and how to target treatment strategies.

# 4) Potential impact

## 4.1 Impact including socio-economic impact and the wider societal implications

Low back pain is a major economic and social burden on all societies including those of Europe and is the leading cause of long-term disability worldwide[1]. It is very poorly researched in relation to other major causes of disability such as depression, coronary heart disease and diabetes even though the economic costs are as high. A recent PubMed search (April, 2013) found 10 fold fewer publications on low back pain (43,973) than on diabetes (430,969) and 5 fold few than on depression (287,853) and coronary heart disease (244,761).

Treatment is arbitrary and varies from centre to centre as around 80% of back pain cases have no clear diagnosis[29]. Thus any study that aims to improve understanding of the causes and progression of disorders associated with back pain, and which clarifies diagnosis of the different pathways involved has the potential to have a significant impact on treatment and prevention of this major medical and social problem. Improving treatment of back pain would also have significant economic benefits as costs of back pain do not only included treatment but also costs of disability benefits and loss of productivity as chronic back pain strikes people of working age[30]. Low back pain, along with coronary heart disease and diabetes, is one of the most expensive of disorders; costs were around £12 billion in the UK in 2000 and are estimated to cost around 1.5% of GDP in EU countries[31]. The costs of health treatment of low back pain at a primary level in the UK are more than two fold those of matched controls[32] and do not take into account over-the-counter medications and interventions using chiropractors or other complementary medicine approaches.

As low back pain is a leading cause of disability, it destroys the quality of life of sufferers and impacts their families. Suffers from chronic low back pain are generally unemployed, often depressed. Low back pain impacts on all activities of daily life, employment, sport and family participation and overall emotional and cultural well-being. It thus markedly reduces the quality of life of sufferers and as affects a significant proportion of people in society and adds to its welfare burden.

*The research carried out in Genodisc* adds a significantly to current knowledge of the back pain disorders and will impact understanding of the progression and treatment of back pain in several ways. It thus has the potential to improve diagnosis, provide means of rational treatment and provide practical and economically viable means of prevention and thus impact on the economic and social costs of this major disorder.

**The main research impacts are described below**.

*(i) Clarification of back pain phenotypes.*
Back pain is at present often conflated with the term 'degenerative disc disease'. This non-specific term does not distinguish between disorders ranging from disc herniation to spinal stenosis and spondylosis all of which follow different pathways and require different approaches to treatment. The nosology is also confused as different terms for instance herniation and protrusion can be used for the same pathology. There are thus clear benefits to defining clearly the complex phenotypes of interest. Moreover, unless the phenotypes are clearly defined, the statistical basis of the epidemiology of the disorders and in particular their genetic bases can be difficult to uncover. Clinical phenotypes characterised in Genodisc are reviewed in a study in press in the European Spine Journal[33]. Genodisc, by clearly defining clinical phenotypes and their co-morbidities provides a basis for sound epidemiological and genetic studies as well as improving diagnosis and hence treatment.

(ii) *Advances in diagnosis.*
Present means of diagnosis concentrate on the intervertebral disc and its degenerative changes, usually codified by a degeneration grade which however misses many features which could contribute to pain and pathological changes. Apart from clinical examination, MRI of the spine is used as the major diagnostic tool but shows little ability to discriminate between symptomatic and non-symptomatic changes. Discography, the main tool used to diagnose discogenic pain, has not been able to predict clinical outcome. Current means of diagnosis are clearly inadequate.

A potential future alternative are miniature sensors which can be inserted into the disc and used in a non-destructive means to diagnose the state of the disc quantitatively. In Genodisc, a number of such sensors were developed. One measured viability of the disc cells. Knowing whether the cells has viable cells or not is essential for understanding the long-term prospects of disc health and also for decisions on whether biological disc repair approaches can utilised. Another sensor was developed for measurement of MMP13, a major enzyme involved in degradation of disc matrix. In principle the methods developed could be adapted to measure other degradative enzymes, inflammatory molecules, matrix degradation products, and molecules such as bradykinin involved in nociception. A sensor was also developed for measuring swelling pressure and osmolarity of the disc[10]. This parameter is essential for maintaining appropriate disc biomechanical behaviour and disc height; fall in swelling pressure is one of the first signs of disc degeneration. This device is potentially an important diagnostic tool.

Novel MRI techniques are also potentially of diagnostic use. Of these, the most potentially useful is post-contrast MRI. Here a contrast medium is injected intravenously and the change in contrast of the discs monitored over time. Movement of the contrast medium into the disc is a marker for transport of nutrients required to maintain cell viability and is a minimally invasive means of estimating whether discs have an adequate nutrient supply and hence whether they are able to maintain viable cells. In Genodisc, 222 discs were assessed using this method and it was found that nutrient transport was disturbed in severely degenerate but not in mildly degenerate discs. This method thus provides a potential diagnostic tool both for assessing possible pathways to disc degeneration and also for assessing possibilities of biological repair.

*New Biomechanics approaches.*
Current treatments for low back pain are based on the assumption that the mildly degraded disc is mechanically unstable and responsible for back pain and hence fusing or stabilising the mildly or moderately degenerate disc will relieve pain. Multiple devices to stabilise the spine have been

thus been developed and spinal fusion is thus regarded as the gold-standard treatment. However fusion does not necessarily relieve pain; indeed randomised controlled trials have shown that outcome for non-surgical treatments is the same as that for fusion[34;35;35]. Work carried out in Genodisc on an analysis of a large database of in vitro results on 203 motion segments harvested from 111 donors, showed that in most cases stability increased in flexion/extension and lateral bending as degree of degeneration increased[8]. Some few mildly degenerate motion segments however showed instability. These findings should have impact on the use of dynamic stabilization systems and fusion; their use should be should be restricted to cases in which the instability is actually proven.

*Degradation pathways*

Degeneration arises from degradation of the matrix macromolecules which constitute the tissue of the disc matrix and regulation of its biomechanical function. Understanding pathways of degeneration are thus essential for any rational intervention aimed at preventing further degeneration or reversing and repairing degenerate tissue. Degeneration is clearly a cellular process. How it is initiated is unclear although twin studies have shown that disc degeneration is strongly genetic[36;37]. The progress of disc degeneration involves upregulation of agents involved in matrix degradation[38], loss of swelling pressure[39] and matrix disruption. Studies in Genodisc had impact on the understanding of the biochemical and molecular processes of degeneration in the following ways.

Tissue turnover was measured using aspartic acid racemization and the molecules making up the matrix were found to be long-lived even when degraded. Molecular turnover rates of the major constituent matrix macromolecules were found to be particularly slow, especially in the case of collagen. Over a normal human life span, this slow turnover may compromise the structural integrity of the disc extracellular matrix essential for normal physiological functioning. Elastin[40], like collagen[26] was found to be metabolically stable and long-lived in both healthy and degenerate human discs with some signs of new synthesis in the latter, possibly as an attempt of repair of this structural molecule. Hence D-Asp content could be used as a novel marker for the over-all ageing process.

In Genodisc we investigated stresses which could induce an inflammatory and catabolic cell phenotype. Mechanical stress on its own in general tended to promote matrix production however when coupled with a more physiological environment, other factors such as extracellular pH, oxygen and particularly osmolarity tended to modify the cellular responses to mechanical stress. Indeed we found that the same mechanical stress could upregulate matrix production under physiological osmolarities but down-regulate it under osmolarities found in degenerate tissue[41]. This finding has important impacts on experimental design, showing that responses to external signals such as those from mechanical stresses or growth factors area governed by environmental conditions and misleading responses can be reported unless appropriate environmental conditions are taken into account.

The work in Genodisc showed also that osmolarity is a very important signal regulating cellular behaviour. Osmolarity is directly regulated by the concentration of aggrecan; osmolarity is low in degenerate discs because aggrecan is degraded and is lost in disc degeneration. Results show the importance of osmolarity and hence aggrecan in modulating cellular activity and maintaining tissue homeostasis; fall in aggrecan concentration leads to upregulation of matrix-degrading proteases and hence a feed-forward catabolic cascade arising. Thus initiation of aggrecan loss by any pathway will drive progression of disc degeneration. In addition, high osmolarity as found in healthy discs in vivo, has an has an anti-proliferative effect by delaying the cells at the G2/M and

G0/G1 phases of the cell cycle[42] and can be a factor in formation of cell clusters seen in disc degeneration.

Cell senescence has been identified in intervertebral disc cells. Senescent cells show a "pro-inflammatory" phenotype, marked by the overexpression of matrix metalloproteases, growth factors and cytokines and other inflammatory molecules. Due to this "pro-inflammatory" phenotype, senescent cells can adversely affect tissue renewal and proper function and thus they can contribute to the ageing process and the development of age-related pathologies. In Genodisc we have shown that exogenous stresses, particularly oxidative and osmotic stresses can contribute to premature senescence of disc cells, and accelerate the senescence process in IVD cells. As these cells express a pro-inflammatory phenotype, these stresses contribute to the degeneration of this tissue.

Thus the basic studies in Genodisc have improved understanding of cellular and molecular pathways leading to disc degeneration and potentially have impact in efforts to prevent and treat degeneration linked-disorders.

*Genetic basis*

Disc degeneration is known to be strongly genetic and there have been many candidate gene studies on populations[43]. However there have been few studies on patient cohorts and hence genes associated with symptomatic disc degeneration rather than asymptomatic disc degeneration have not been investigated in any depth. In Genodisc, DNA is available from all patients in the database. To date we have carried out a pooling GWAS on carefully genotyped patients and identified 49 novel genetic variants associated with painful disc herniation and DNA is available for further studies. Genodisc thus has the potential to impact on understanding the genetic bases of disc degeneration-linked disorders.

*Repair exclusion criteria*

Cell therapy for the purpose of treating degenerate disc disease is a focus of much research as insertion of cells or agents into the disc by a needle appears an attractive, minimally invasive method of treatment. Most work has concentrated on developing appropriate cell sources and on scaffold. There has been little work on examining the clinical issues such as which patients should be treated and would cells survive, produce matrix and repair degenerate discs of patients.

Work in Genodisc has shown that material injected into the disc cannot be retained in the tissue once it is loaded if the disc has fissures or annular tears. Thus any disc which has identifiable breaches of the annulus on MRI, would not be able to maintain injected material once the patient sat or stood up. Cell therapies would thus have to utilise methods of annulus sealing or other invasive procedures, thus losing most of their attraction. Discs with breaches of the annulus are thus excluded from treatment by minimally invasive cell therapies

In order for the disc cells to stay alive and function, they require an adequate nutrient supply. Nutrients are supplied to nucleus of the avascular disc by blood vessels from vertebral bodies which penetrate the subchondral plate and terminate in capillary loops at the cartilaginous endplate. In degenerate discs, the cartilaginous endplate tends to calcify and thus limits passage of nutrients to the disc cells and hence cell viability[20;44]. We examined nutrient supply of 222 discs in Genodisc and showed that while nutrient pathways were similar in normal and mildly degenerate discs, they were disturbed in degenerate discs as seen also by others[17]. Any discs

which had disturbed nutrients pathways should be excluded from treatments using cell therapies as the implanted cells would not survive.

Genodisc thus has the potential to impact the development of cell therapies for treating disc degeneration by providing diagnostic methods for determining which patients can benefit from cell therapy. It should be noted that of the discs examined in our database, around 90% of potentially symptomatic discs would not meet these inclusion criteria. It should also be noted that even if discs can support cell therapies, clinical benefit of this treatment should also be demonstrated.

*Prevention in young people.*
Back pain is now known to be a problem in young people. In Genodisc, an exercise programme was developed for the early prevention of degenerative spinal disorders. Spine disorders are correlated with weak trunk muscles so an exercise programme was developed to strengthen trunk muscles. Eight primary schools in three Hungarian cities were involved into the study during this period. Altogether 1371 pupils were included into the study. After 6 months of exercises designed to strengthen trunk muscles, muscle strength had increased significantly in an exercised cohort relative to a control non-exercised cohort. The incidence of posture problems decreased in the intervention group while deterioration in the spinal curvatures was observed in the control group during the school year. Overall there was significant improvement of the spinal global functional capacity due to the prevention program. This programme has the potential to have an impact on spine problems in young people; it demonstrates that a simple, easily administered exercise programme provides significant improvement in global spinal functional capacity within 6 months.

**4.2 Dissemination activities**
Dissemination of research by the Genodisc consortium has been to
(i) the scientific and clinical community through peer reviewed scientific papers and reviews, book chapters, editorials in spine journals, presentations at scientific meetings both oral and poster, published abstracts, invited plenary lectures, PhD theses,
(ii) communications to the public by workshops on back pain topics, newspaper articles, lectures to schools.
(iii) communications to patients, by posters in hospitals, workshops aimed at back pain patients, patient leaflets.

**4.3 Exploitation of results**
*(1) Disc database and DNA and serum samples*
*Database*. The anonymised database provides a unique resource on more than 2500 chronic back pain patients. The database contains more than 300 items of information on clinical phenotyping, quantitative and qualitative MRI assessment, patient characteristics such as age, gender, occupation, ethnicity, co-morbidities and answers to questionnaires.
The original anonymised MRIs are also available on disc for any further study. The database is available to all Genodisc partners for correlative studies.

*DNA* All patients provided samples of blood or saliva for extraction of DNA. This DNA has been used for pooling and validation studies. Each centre is responsible for storing DNA from its patients. No further studies are proposed at present. It is anticipated that as technologies improve, costs of genetic sequencing fall, and understanding of epigenetic changes increase that this DNA, of carefully phenotyped and imaged patients, will provide a valuable resource for uncovering genetic background to disc degeneration and back pain development.

Serum samples from a proportion of UK patients are available, stored appropriately, for evaluation of serum biomarkers. Again this is a valuable resource which will be utilised only when information and costs provide appropriate indications.

These resources area also open to outside researchers on the basis of a research proposal submitted through a post-Genodisc steering committee. This steering committee will maintain the database and oversee research and publications pertaining to the work carried out in Genodisc.

*2. Prevention*

The school exercise aimed at strengthening trunk muscles and hence improving global spinal function and diminishing the chances of back pain in adolescence, will be rolled out among all primary schools in Hungary. The long-term outcome will be followed up to see if the effects can be maintained and if back pain incidence diminishes among young people. Potential impact is high if this simple easily administered exercise programme for prevention of back pain is successful.

*3. Diagnosis.*

Needle-based diagnostic devices for measuring cell viability, levels of MMP13 and osmolarity were developed during Genodisc. However, studies on long-term effects of discography, published initially in 2009[45], have shown that the process of discography, which involves puncturing the disc using needles and injecting a contrast agent under pressure, may accelerate disc degeneration and herniation. Thus these devices cannot be used diagnostically in patients at present until it is determined whether needle puncture alone can promote degeneration as some animal studies indicate, or whether injection pressure and or presence of contrast medium causes the problem. The devices however have the potential to be of considerable benefit in *in vitro* and in animal studies as data can be obtained non-destructively, thus reducing numbers of experimental animals and speeding experimental in vitro studies.

Reference List


1. Vos T, Flaxman AD, Naghavi M et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012;380:2163-96.
2. Weinstein JN, Lurie JD, Olson PR et al. United States' trends and regional variations in lumbar spine surgery: 1992-2003. *Spine* 2006;31:2707-14.
3. Battie MC, Videman T. Lumbar disc degeneration: epidemiology and genetics. *J.Bone Joint Surg.Am.* 2006;88 Suppl 2:3-9.
4. Chan D, Song Y, Sham P et al. Genetics of disc degeneration. *Eur.Spine J* 2006.
5. Boden SD, Davis DO, Dina TS et al. Abnormal magnetic-resonance scans of the lumbar spine in asymptomatic subjects. A prospective investigation. *J Bone Joint Surg [Am]* 1990;72:403-8.
6. Elfering A, Semmer N, Birkhofer D et al. Risk factors for lumbar disc degeneration: a 5-year prospective MRI study in asymptomatic individuals. *Spine* 2002;27:125-34.
7. Butler WF. Comparative anatomy and development of the mammalian disc. In: Ghosh P, ed. *The Biology of the Intervertebral Disc*. Boca Raton: CRC Press, 1989:84-108.
8. Kettler A, Rohlmann F, RING C et al. Do early stages of lumbar intervertebral disc degeneration really cause instability? Evaluation of an in vitro database. *Eur.Spine J* 2011;20:578-84.
9. Carragee E, Cuellar JM, Hurwitz EL et al. Does Provocative Discography Cause Clinically Important Injury to the Lumbar Intervertebral Disc? A Ten-Year Matched Cohort Stud. The Spine Journal 11(10), S23-S24. 2011.t

10. Sivan SS, Merkher Y, Wachtel E et al. A needle micro-osmometer for determination of glycosaminoglycan concentration in excised nucleus pulposus tissue. *Eur.Spine J* 2013.
11. Galbusera F, Schmidt H, Neidlinger-Wilke C et al. The effect of degenerative morphological changes of the intervertebral disc on the lumbar spine biomechanics: a poroelastic finite element investigation. *Comput.Methods Biomech.Biomed.Engin.* 2011;14:729-39.
12. Galbusera F, Schmidt H, Neidlinger-Wilke C et al. The mechanical response of the lumbar spine to different combinations of disc degenerative changes investigated using randomized poroelastic finite element models. *Eur.Spine J* 2011;20:563-71.
13. Galbusera F, Mietsch A, Schmidt H et al. Effect of intervertebral disc degeneration on disc cell viability: a numerical investigation. *Comput.Methods Biomech.Biomed.Engin.* 2011;in press.
14. Motaghinasab S, Shirazi-adl A, Urban JP et al. Computational pharmacokinetics of solute penetration into human intervertebral discs - effects of endplate permeability, solute molecular weight and disc size. *J Biomech.* 2012;45:2195-202.
15. Bydder GM. New approaches to magnetic resonance imaging of intervertebral discs, tendons, ligaments, and menisci. *Spine* 2002;27:1264-8.
16. Rajasekaran S, Naresh-Babu J, Murugan S. Review of postcontrast MRI studies on diffusion of human lumbar discs. *J.Magn Reson.Imaging* 2007;25:410-8.
17. Rajasekaran S, Babu JN, Arun R et al. ISSLS Prize Winner: A Study of Diffusion in Human Lumbar Discs: A Serial Magnetic Resonance Imaging Study Documenting the Influence of the Endplate on Diffusion in Normal and Degenerate Discs. *Spine* 2004;29:2654-67.
18. Mietsch A, Neidlinger-Wilke C, Schrezenmeier H et al. Evaluation of platelet-rich plasma and hydrostatic pressure regarding cell differentiation in nucleus pulposus tissue engineering. *J Tissue Eng Regen.Med.* 2011.
19. Pratsinis H, Constantinou V, Pavlakis K et al. Exogenous and autocrine growth factors stimulate human intervertebral disc cell proliferation via the ERK and Akt pathways. *J Orthop Res* 2012;30:958-64.
20. Grunhagen T, Shirazi-adl A, Fairbank JC et al. Intervertebral disk nutrition: a review of factors influencing concentrations of nutrients and metabolites. *Orthop.Clin.North Am.* 2011;42:465-77, vii.
21. Nguyen-Minh C, Haughton VM, Papke RA et al. Measuring diffusion of solutes into intervertebral disks with MR imaging and paramagnetic contrast medium. *American Journal Of Neuroradiology* 1998;19:1781-4.
22. Kovacs FM, Abraira V, Gervas J et al. Overenthusiastic interpretations of a nonetheless promising study. *Transplantation* 2012;93:e6-e7.
23. Smart KM, Blake C, Staines A et al. Self-reported pain severity, quality of life, disability, anxiety and depression in patients classified with 'nociceptive', 'peripheral neuropathic' and 'central sensitisation' pain. The discriminant validity of mechanisms-based classifications of low back (+/-leg) pain. *Man.Ther.* 2012;17:119-25.
24. Giesecke T, Gracely RH, Grant MA et al. Evidence of augmented central pain processing in idiopathic chronic low back pain. *Arthritis Rheum.* 2004;50:613-23.
25. Liebscher T, Haefeli M, Wuertz K et al. Age-related variation in cell density of human lumbar intervertebral disc. *Spine (Phila Pa 1976.)* 2011;36:153-9.
26. Sivan SS, Wachtel E, Tsitron E et al. Collagen turnover in normal and degenerate human intervertebral discs as determined by the racemization of aspartic acid. *J.Biol.Chem.* 2008;283:8796-801.
27. Sivan SS, Tsitron E, Wachtel E et al. Aggrecan turnover in human intervertebral disc as determined by the racemization of aspartic acid. *J.Biol Chem.* 2006;281:13009-14.
28. Chang G, Kim HJ, Vunjak-Novakovic G et al. Enhancing annulus fibrosus tissue formation in porous silk scaffolds. *J.Biomed.Mater.Res A* 2009.
29. Waddell G. Low back pain: a twentieth century health care enigma. *Spine* 1996;21:2820-5.
30. Waddell G. Preventing incapacity in people with musculoskeletal disorders. *Br.Med Bull.* 2006;77-78:55-69.
31. Maniadakis N, Gray A. The economic burden of back pain in the UK. *Pain* 2000;84:95-103.
32. Hong J, Reed C, Novick D et al. Costs associated with treatment of chronic low back pain: an analysis of the UK General Practice Research Database. *Spine (Phila Pa 1976.)* 2013;38:75-82.
33. Battie MC, Fairbank JCT, Brayda-Bruno M et al. Disc degeneration related clinical phenotypes. Eur.Spine J. in press. 2013.
34. Fairbank J. Randomised controlled trial to compare surgical stabilisation of the lumbar spine with an intensive rehabilitation programme for patients with chronic low back pain: the MRC spine stabilisation trial (vol 330, pg 1233, 2005). *British Medical Journal* 2005;330:1485.
35. Fairbank J, Frost H, Wilson-MacDonald J et al. Randomised controlled trial to compare surgical stabilisation of the lumbar spine with an intensive rehabilitation programme for patients with chronic low back pain: the MRC spine stabilisation trial. *BMJ* 2005;330:1233.
36. Battie MC, Videman T. Lumbar disc degeneration: epidemiology and genetics. *J Bone Joint Surg.Am.* 2006;88 Suppl 2:3-9.
37. Sambrook PN, MacGregor AJ, Spector TD. Genetic influences on cervical and lumbar disc degeneration: a magnetic resonance imaging study in twins. *Arthritis Rheum.* 1999;42:366-72.

38. Crean JK, Roberts S, Jaffray DC et al. Matrix metalloproteinases in the human intervertebral disc: role in disc degeneration and scoliosis. *Spine* 1997;22:2877-84.
39. Urban JPG, McMullin JF. Swelling pressure of the lumbar intervertebral discs: influence of age, spinal level, composition and degeneration. *Spine* 1988;13:179-87.
40. Sivan SS, Van EB, Merkher Y et al. Longevity of elastin in human intervertebral disc as probed by the racemization of aspartic acid. *Biochim.Biophys.Acta* 2012;1820:1671-7.
41. Neidlinger-Wilke C, Mietsch A, Rinkler C et al. Interactions of environmental conditions and mechanical loads have influence on matrix turnover by nucleus pulposus cells. *J Orthop.Res.* 2012;30:112-21.
42. Mavrogonatou E, Kletsas D. Differential response of nucleus pulposus intervertebral disc cells to high salt, sorbitol, and urea. *J Cell Physiol* 2012;227:1179-87.
43. Eskola PJ, Lemmela S, Kjaer P et al. Genetic association studies in lumbar disc degeneration: a systematic review. *PLoS.One.* 2012;7:e49995.
44. Benneker LM, Heini PF, Alini M et al. 2004 Young Investigator Award Winner: vertebral endplate marrow contact channel occlusions and intervertebral disc degeneration. *Spine* 2005;30:167-73.
45. Carragee EJ, Don AS, Hurwitz EL et al. 2009 ISSLS Prize Winner: Does discography cause accelerated progression of degeneration changes in the lumbar disc: a ten-year matched cohort study. *Spine (Phila Pa 1976.)* 2009;34:2338-45.