GENODISC Report Summary

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Final Report Summary - GENODISC (Disc-degeneration linked pathologies: novel biomarkers and diagnostics for targeting treatment and repair)

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 five 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.

Project Context and 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

Research and Innovation

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.

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.

Project Results:

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.

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 less than 0.05) and lateral bending (by 3.4°, p less than 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.

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 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.

3.4 Database

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.

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/p21WAF1/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.

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, TGFß and TNFa. 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.

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: d(D/L)/dt = ki-kT(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 (kT) as a function of age, we made use of the racemization rate (ki) values, which were available for collagen and aggrecan. Using the relationship for half-life (?1/2): ?1/2=ln(2)/kT, we were able to determine half lives of both aggrecan and collagen of normal and degenerate disc as a function of age. Since no ki 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. For collagen and aggrecan, data were pooled for NP and AF (for normal or degenerate) as no significant difference (p less than 0.05) was observed in their rate of D-Asp accumulation. Since no significant difference (p less than 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 10-4 per year for normal collagen and 5.18 10-4 per year for degenerate collagen. Data pooled by the decade show a significant difference (p less than 0.05) between average values of D/L-Asp of normal and degenerate tissues only between ages 50 and 60 years 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 (greater than20 years) until a plateau was reached above 60 years of age. 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.

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±3.1×10-4 per year (R=0.95) and 11.7 ±3.8×10-4 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 less than 0.05 level. The D-Asp linear accumulation rate of the combined data sets is 12.3 ±2.6×10-4 per year (R=0.79). Above 50 yrs of age, the differences between the normal and degenerate tissue was statistically significant (p less than 0.05).

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

Research
and Innovation

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.

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 (greater than +/- 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), 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.

3.11 Muscle and School Exercise Programme

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.

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. Muscle status was measured using the 12 exercise test. The mean muscle score in the total cohort was 9.1+1.7. Less than 20% of the children were in muscle balance at the time of the first measurement. Muscle score was significantly (p less than 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+1.2) and not changed in the control group. 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.

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. 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. '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. Back muscle score was significantly associated with one individual SNP (rs3782905) and with no haploblock.

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 (greater than60%) 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.

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.

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. 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. and 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-ß (TGF ß) 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 but is expensive. However, without healthy and functional cells, a therapeutic effect of injecting growth factor(s) will not be achieved.

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 greater than80 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. 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.

The percentage of discs from each cohort with breaches of the annulus fibrosus increases wth degeneration grade; these discs with annulsu breachesd not be suitable for cell therapy for NP repair, as any tear may lead to leakage of the cell product introduced into the disc. 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;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. 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 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).

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 funneled into a better understanding of pain mechanisms in back pain patients so that we know better where and how to target treatment strategies.

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 GBP 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.

List of Websites:

http://www.physiol.ox.ac.uk/genodisc/index.html

Related information

Result In Brief	• Disc degeneration under the microscope
Documents and Publications	• Final Report - GENODISC (Disc-degeneration linked pathologies: novel biomarkers and diagnostics for targeting treatment and repair)

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