UNDERSTANDING THE GENETIC AND MOLECULAR BASIS OF DISC DEGENERATIVE DISEASE IN HUMANS

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COIMBATORE – 641 003

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Thesis submitted in partial fulfillment of the requirements for the award of the degree of

DOCTOR of PHILOSOPHY IN BIOTECHNOLOGY

to the Tamil Nadu Agricultural University, Coimbatore - 641003.

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CERTIFICATE

This is to certify that the thesis entitled **"UNDERSTANDING THE GENETIC AND MOLECULAR BASIS OF DISC DEGENERATIVE DISEASE IN HUMANS"** submitted in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Biotechnology to the Tamil Nadu Agricultural University, Coimbatore is a bonafide record of research work carried out by **Ms. R. VEERA RANJANI** under my supervision and guidance and no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes.

Date: 27.01.2015

Place: Coimbatore. **(Dr. R. GNANAM)**

Chairperson

Approved by

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: **(Dr. V. PARANIDHARAN)**

Dedicated to Appa

Acknowledgement

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(R. VEERA RANJANI)

Abstract

ABSTRACT

UNDERSTANDING THE GENETIC AND MOLECULAR BASIS OF DISC DEGENERATIVE DISEASE IN HUMANS

By

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2014

Disc Degenerative Disease (DDD) is a major causative reason for Low Back Pain (LBP) caused by degeneration of lumbar intervertebral disc. LBP is one of the most important socioeconomic diseases and one of the most important health care issues today. It places an enormous economic burden on society by several means *viz.*, direct medical cost and indirect costs such as insurance, loss in production due to disability etc. There is a growing opinion that disc degeneration has more genetic basis than environmental factors. Hence, the present study was undertaken with an aim of analyzing the genetic and molecular basis of DDD through detailed clinical studies in Indian population.

In this work, attempts were made to identify candidate genes associated with DDD and characterize proteomic and metabolomics events associated with DDD. Firstly, to understand the genetic basis of DDD, 71 SNPs spread among 40 selected candidate genes were genotyped in 809 individuals from India and their association between the prevalence of SNPs and various DDD related phenotyping parameters viz., disc bulge, annular tears, Modic change, Schneiderman's score, Schmorl's Node, total end plate score and Pfirman score was studied. Association analysis based on individual disc levels revealed 33 different genes having significant association with DDD. Out of 33

different genes, 16 genes viz., *LEPR, NGFB, COX2, FN1, COL9A1,TAC1, IL6, MMP1, MMP7, MMP10, CHST3, VDR, CALM1*, *CILP*, *MMP2* and *ADAMTS5* were found to have significant association with DDD at 1 % significance level. Association study based on whole disc scoring revealed a significant association of 17 different genes (*CILP, VDR, MMP7, TAC1, IL6, ADAMTS5, IGF1R, MMP1, COL9A1, IL1RN, MMP20, MMP10, CALM1, FN1, SKT, COX2* and *NGFB*) with DDD. Association study using young adults as the study population based on whole disc scoring revealed 15 different genes having significant association with DDD. Out of these 15 genes, five genes *viz.*, *MMP7, COL9A1, CALM1, MMP1* and *ADAMTS5* have a significant association with DDD at 1 % significance level. In short, this study has validated the association between DDD and putative candidate genes involved in DDD.

Proteomic analysis of IVD tissue using 2D-PAGE revealed the expression of about 143 proteins and 30 abundant proteins were identified through MALDI-TOF. An improved method for extracting proteins from IVD tissues in humans suitable for 2D PAGE analysis was standardized by using 100 kDa MWCO filters. Major proteins were, apolipoprotein A-I, alpha-tubulin N-acetyltransferase isoform 4, huntingtin-interacting protein 1-related proteins etc., The improved protein extraction method established in this study have provided a promising avenue for the analysis of constituent proteins of IVD, and hence the identification of candidate genes playing roles in DDD and possibly a cure for DDD in humans. Metabolic profiling in the control and affected IVD through GC-MS resulted in the detection of 75 (hydrocarbons, organic acids, amines, carbonyl compounds) different chemical compounds. Significantly, the presence of lactic acid was found to be correlated to the progression of DDD.

Abbreviations

LIST OF ABBREVIATIONS

Aa	: Amino acid
ADAMTS5	: A disintegrin and metalloproteinase with thromombospondin motifs 5
ADH	: Alcohol dehydrogenase
AF	: Annulus Fibrosus
AGC	: Aggrecan
Bp	: Base pair
BMP	: Bone morphogenetic protein
cDNA	: Complementary DNA
CALM	: Calmadulin
CHST3	: Carbohydrate (Chondroitin 6) Sulfotransferase 3
CILP	: Cartilage intermediate layer protein
COL	: Collagen
COMP	:Cartilage Oligomeric Matrix Protein
COX2	: Cyclo Oxygenase 2
CS	: Chondroitin Sulphate
DDD	: Disc degenerative disease
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleotide triphosphate
ECM	: Extracellular matrix
EDTA	: Ethylene Diamine Tetra Acetic acid
EP	: End plate
g	: Gram(s)
GLI1	: Glioma - associated oncogene homolog 1 (zinc finger protein)

Hr	: Hour(s)
IGF1R	: Insulin like growth factor 1 receptor
IL	: Interleukins
IVD	: Intervertebral disc
kbp	: Kilo base pair
kDa	: Kilo Dalton
LBP	: Low back pain
LDD	: Lumbar disc degeneration
LEPR	: Leptin receptor
Trp	: tryptophan
M	: Molar
mg	: Milligram
$MgCl_2$	: Magnesium Chloride
Min	: Minute(s)
mL	: Millilitre
mm	: Millimetre
mM	: Millimolar
MALDI-TOF	: Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass spectrometry
MMPs	: Matrix metalloproteinase's
MRI	: Magnetic resonance imaging
MS	: Mass Spectrocopy
N	: Normality
ng	: Nanogram
NGFB	: Nerve growth factor beta polypeptide
NP	: Nucleus Pulposus
PCR	: Polymerase chain reaction

PG	: Proteoglycan
pmol	: picomolar
rpm	: Revolutions per minute
s	: Seconds
SD	: Standard Deviation
SKT	: Sickle tail
SNPs	: Single nucleotide polymorphisms
TAC1	: Tachykinin 1
TAE	: Tris acetate EDTA
TE	: Tris-EDTA
TEPS	: Total end plate score
Temp	: Temperature
TEPS	: Total end plate score
V	: Volts
VDR	: Vitamin D Receptor
VNTR	: Variable Number Tandem Repeats
v/v	: Volume/Volume
w/v	: Weight/Volume
2D PAGE	: Two Dimensional Polyacrylamide Gel Electrophoresis
µg	: Microgram
µL	: Microlitre
µm	: Micrometre
µM	: Micromolar
χ^2	: Chi-Square

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Introduction

CHAPTER I

INTRODUCTION

Low back pain (LBP) is a highly prevalent musculoskeletal disorder in mankind, caused by degeneration of intervertebral discs (IVD) of the lumbar spine (Solovieva *et al.,* 2004). The prevalence of low back pain in the society is reported to be around 60 per cent and 80 per cent of the population suffers at least one episode of low back pain in their life (Seki *et al.,* 2005). Frequency of occurrence of Degenerative Disc Disease (DDD) is becoming common and at least 30 per cent of people aged 30 to 50 years will have some degree of disc degeneration (Boden *et al*., 1990). The main anatomical structure implicated in LBP is the intervertebral disc (IVD) and the pathogenic process is its degeneration (Luoma *et al*., 2000). Progression of DDD may lead to further complications viz., disc prolapse and damage to neural structures of the spine (Boos *et al*., 2002). DDD seems to be a result of combined insult of mechanical, environmental and genetic factors (Jill *et al*., 2003). The environmental and mechanical factors that have been associated with DDD are aging, smoking, obesity, repetitive mechanical stress and atherosclerosis (Kauppila *et al*., 1997; Jones *et al*., 1998).

Several studies have indicated the role of strong genetic influence in the causation of DDD in addition to the environmental influences. A similar degree of disc degeneration irrespective of environmental influence was first shown in the case of "twins" and family studies (Battie *et al*., 1995a, Sambrook *et al*., 1999). After this study, a "candidate gene" approach was used in subsequent studies to understand the role of genetic factors. These candidate genes had been selected based on our knowledge on biology and biochemistry of intervertebral discs, *viz*., constituent proteins of bone matrix, cellular receptors and enzymes involved in bone and tissue remodeling. Based on this, several candidate genes have been shown to be associated with disc degeneration to varying extent. The first report on the influence of genetic polymorphism over etiology of DDD was reported in Vitamin D receptor gene in 1998 (Videman *et al*., 1998). Following this, several other attempts were made to relate DDD with genetic polymorphisms in Collagen IXA, Interleukins*, CILP*, *MMP2*, *MMP3* etc., All these studies focusing on genetic association

with DDD have been made only during the last decade and the results revealed a weak but significant association of genetic polymorphisms of these genes with DDD.

Existing studies on genetic influence of DDD have the following disadvantages:

- Each report has followed different and non-specific phenotyping protocols
- Focused on a single SNP of candidate genes
- No age criteria is considered while recruiting the study population
- Candidate genes were not selected based on systematic whole genome analysis
- No attempts have been made in Indian population, which constitute one sixth of world's population

Even at an international level, only a few research groups have attempted to study the genetic association of DDD. In India, till date there are no reports that have documented the role of genetic polymorphism in the putative candidate genes in the etiopathogenesis of DDD. Back pain and DDD is quite common in India and despite being one sixth of the world's population, the genetic association of DDD has not been studied in the Indians. Therefore, high throughput genomic, proteomic and metabolomics studies are needed in order to identify candidate genes that are associated with DDD, and shed light on the proteomic and metabolomics events that characterize the DDD.

Taking advantage of improved diagnostic modalities like, MRI which can accurately document the severity of DDD and define the phenotype specifically and the availability of genome sequence information, the present study was undertaken with the following objectives:

1. Understanding the molecular genetic basis of DDD through association analysis in 809 individuals from India,
2. Understanding the relation between age and DDD through association analysis in 543 young individuals selected from Indian Population,
3. Profiling proteins abundantly expressed in the intervertebral discs through 2D-PAGE and
4. Metabolic profiling of intervertebral disc tissue by Gas Chromatography - Mass Spectrometry (GC-MS**).**

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

Low back pain (LBP) has a lifetime prevalence of up to 84 % (Walker *et al*., 2000) and is the main cause of disability worldwide (Vos *et al*., 2013 and Kovacs *et al*., 2014). Low back pain from degenerative disc disease (DDD) is one of the most common disorders seen in general and orthopaedic practices (Chan *et al*., 2006). The main anatomical structure involved in DDD is intervertebral disc. Intervertebral discs are the anchors of the spine, ensuring flexibility and movement between vertebral bodies. The intervertebral disc is situated in between adjacent vertebrae. The disc is basically a composite structure made up of three different tissues; the central core is called the nucleus pulposus which is surrounded by the multilayered fibers of annulus fibrosus and the cartilaginous end plates. The DDD has been attributed to the cumulative effects of environmental factors such as occupation, sporting activities and smoking (Caplan *et al*., 1966). There are reports revealing the association between genetic influences and disc degeneration (Sambrook *et al*., 1999).

Human spine

The human spine is a mechanical structure that performs three fundamental biomechanical functions simultaneously (White and Panjabi, 1990). First, it transfers the weight (and resultant bending moments) of the head, trunk and any weights being lifted to the pelvis. Second, it allows sufficient physiological motion between the head, trunk and pelvis. Third and most important, it protects the delicate spinal cord from the potential damaging forces (and moments) resulting from the physiological motions and trauma (White and Panjabi, 1990). Figure 1 shows the schematic of the human spine, which is divided into three main regions. The upper region, with seven vertebrae, is called the 'Cervical Spine'; the middle region, with twelve vertebrae, is called the 'Thoracic Spine' and the lowermost, with five vertebrae, and is called the 'Lumbar Spine'. At the distal end of the spine, there is a basin shaped structure called the 'pelvis' that supports the spinal column and is made of the 'sacrum' and the 'coccyx' with fused

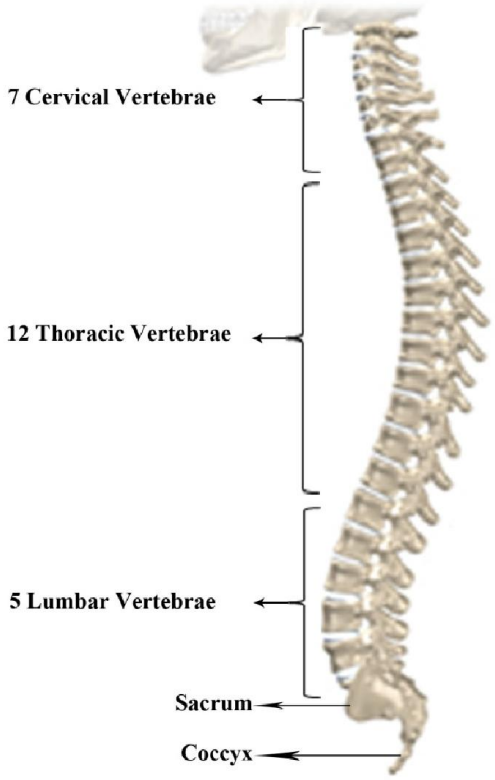


Figure 1. Schematic representation of human spine

vertebrae. Human spine is not an exactly straight structure, but has specific curvature, as seen from Figure 1. The spine in the cervical and in the lumbar region is slightly convex anteriorly while in the thoracic and sacral region, it is slightly convex posteriorly. The specific shape allows the increased flexibility while maintaining the overall spinal stability. It also facilitates increased shock absorbing capacity along with adequate stiffness (White and Panjabi, 1990). The vertebra is the primary weight bearing area. It also provides the resting place to the intervertebral disc, which separates the 5 adjacent vertebrae and acts as a cushion between them. There is a large hole in the center part (spinal canal) which is covered by 'Lamina'. The spinal cord runs through this spinal canal. There is a protruded process in the central posterior region, called 'Spinous Process', which can be felt by running our hand down the back. There are pairs of 'Transverse Processes' which are orthogonal to the spinous process and provide attachment for the back muscles. There are also four facet joint associated with each vertebra. Four facet joints in two pairs (superior and inferior) interlock with adjacent vertebrae and provide the stability to the spine. An intervertebral disc is situated in between adjacent vertebrae. The discs are referred with respect to the vertebrae levels, between which they are located. Thus, the T12/L1 disc is located between the 12th thoracic and 1st lumbar vertebrae while the L3/L4 disc is located between the 3rd and 4th lumbar vertebrae. Lower back pain is associated with the degenerative lumbar intervertebral disc disease and the discussion henceforth always refers to the lumbar spine (Figure 2)

2.1. Structure of intervertebral Disc

The intervertebral disc is the largest avascular tissue in the human body (White and Panjabi, 1990). It constitutes about one third height of the entire spinal column. The disc is primarily made of three different tissues; the central jelly-like portion is 'Nucleus Pulposus' (NP), which is surrounded by the outer laminated structure of the 'Annulus Fibrosus' (AF). The thin cartilaginous endplates with multiple perforations are in between the disc and a vertebra. Figure 3 shows the schematic of the lumbar intervertebral disc (IVD).

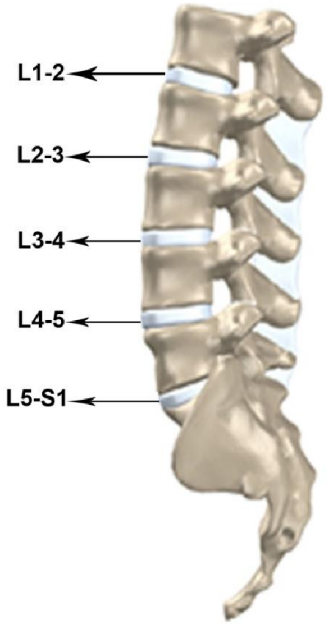


Figure 2. Schematic structure of lumbar spine

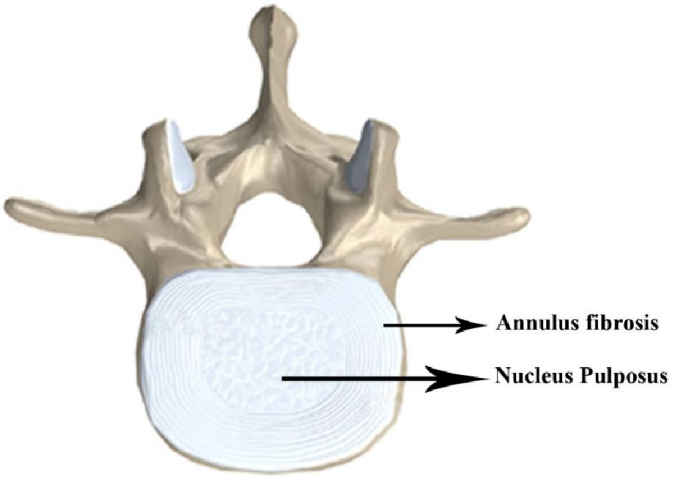


Figure 3. Structure of intervertebral disc

2.1.1. Nucleus Pulposus (NP)

The center of the IVDs consists of a gelatinous core with chondrocyte-like cells derived from notochord. In the normal IVDs, proteoglycan and water make up the highly hydrated core of NP and it is confined by concentric lamellae of AF. The water content of the NP that provides the IVD with hydraulic pressure to withstand the compressive force dissipated to the spine so that it is not deformed by the loading. The nucleus pulposus comprises about half of the healthy intervertebral disc and is essentially water in a matrix of proteoglycan, collagen and other matrix proteins (Iatridis *et al*., 1996). The water content of the nucleus is very high at birth (90 % or so) and then decreases at older age to 70 per cent or even less (Buckwalter, 1995). The high water content of the nucleus is mainly due to the presence of hydrophilic proteins called Proteoglycans (PGs) (Bushell *et al*., 1977). PGs are the most abundant macromolecules present in the nucleus, accounting as much as 65 per cent of the dry weight at young age, which may decrease to as low as 30 per cent (Buckwalter, 1995 and Iatridis *et al*., 1997). PGs consist of sulfated glycosaminoglycans side chains covalently bonded to core proteins. These molecules have the ability to attract and retain water due to ionic carbonyl and sulphate groups on the glycosaminoglycans chains (Urban and McMullin, 1988; Bogduk and Twomey, 1991 and Best *et al*., 1994). These large molecules with their negatively charged sulfate groups are not free to diffuse out of the nucleus. They are highly hygroscopic as some of the PGs are linked to hyaluronic acid, a longer chain of very hydrophylic nonsulfated glycosaminoglycan (Ghosh, 1988; Mooney, 1989 and Ray, 1992). Collagen comprises about 20 per cent of the dry weight, while a variety of noncollagenous proteins and elastin account for the rest of its dry weight. The external load acting on the disc determines the equilibrium water content of the disc. As the load increases, the pressure inside the nucleus also increases and the water is squeezed out into the vertebrae through perforated endplates. When the load on the disc decreases, the pressure within the nucleus also decreases and the water returns. Such a mechanism essentially creates an effective pump, providing a circulation path for the inflow of water, nutrients and outflow of metabolic waste (Bao *et al*., 1996).

2.1.2. Annulus Fibrosus (AF)

The annulus fibrosus gradually differentiates from the periphery of the nucleus to form outer boundary. The annulus is tough, outer fibro-cartilaginous layer of the disc. Water accounts for 60 to 70 per cent of the total annulus mass (Bogduk and Twomey, 1991). The annulus is often compared to the laminated automobile tire. The collagen fibers of the annulus are laid down in 15 to 20 multiple plies. The annulus fibers insert into the superior and inferior vertebral bodies (Ray, 1992). The fibers in the alternate layers are oriented in the opposite direction, with an angle of ± 30º with respect to the radial direction. Depending on location within the disc, the fibers are connected to vertebral endplates or directly to the vertebra. Because of this specific structure, the annulus essentially binds the adjacent vertebrae together and play major role in resisting of torsion (Ghosh, 1988 and Keller *et al*., 1987). It is the compressibility of the annulus, which accommodates the bending and twisting of the intervertebral disc. The outer annulus is primarily made of type I collagen while type II collagen is predominant near the nucleus. Other types of collagen, such as type V, VI and IX are also present in the annulus along with minor amount of type III collagen (Bogduk and Twomey, 1991).

2.1.3. Vertebral endplates

The cartilaginous endplates essentially separate the disc from the vertebral bodies. The endplates are recognizable as discrete entities at an early stage in the development of the axial skeleton and remain as cartilaginous endplates during the subsequent ossification of the vertebrae (Taylor and Twomey, 1988). The cartilaginous component of the endplates consists of a gel of hydrated PG molecules that is reinforced by a network of collagen fibrils (Maroudas *et al*., 1975). The cartilage in these endplates resembles the chemical structure of the adjacent portion of the disc (Bogduk and Twomey, 1991). Hyaline cartilage is the major component of the end plate, which is approximately 1 mm thick. The collagen content is highest, but the PGs and water content are lower as compared to the adjacent nuclear and annulus regions. In addition to serving as a semipermeable membrane that facilitates the diffusion of the solutes from vertebra to the disc, the end plate also prevents the nucleus pulposus from bulging into the adjacent vertebral body

(Moore, 2000) while absorbing the hydrostatic pressure resulting from mechanical loading of the spine (Broberg, 1983). By connecting with AF, EP provided good anchorage for the disc to adjacent vertebral bodies. With blood supply from the capillary beds running through the cartilaginous EP at young age or after the EP became avascular by the first decade of life, nutrient delivery can be made possible through the limited diffusion via EP into other parts of IVD. This process of nutrient supply to disc cells is believed to influence metabolism of disc cells and hence their viability which will eventually affect the integrity of IVD.

2.2. Function of the Intervertebral Disc

The disc serves primarily as a joint that holds two vertebrae together and provides flexibility. It also serves as "shock absorber" while simultaneously providing support to the spine. Both the EP and outer part of the AF anchor firmly to adjacent vertebrae such that the disc is not displaced easily during movement. Moreover, the AF and EP help to confine the NP within their own boundaries. Any leakage of the NP would be detrimental to the disc. Both the AF and EP are also the only sites of nutrient provision to the disc (Urban *et al*., 2004). The alternating layers and fibrous nature of the AF allows stretching and movement to occur multi-dimensionally, including flexion, extension and rotation to a limited degree. On the other hand, the soft and highly hydrated characteristics of the NP allows for a response to compression more readily.

When the disc is loaded, the NP deforms to expand radially and pushes against the AF. Here the compressive force is converted to tensile stress that is built up by the fibrous wall of the AF. Lamellae slide to expand at a small degree for dissipating stress while further expansion is then restricted. At the same time, some water is forced out from the NP during compression and thus the proteoglycan concentration increases. This in turn increases the swelling pressure in the NP that can resist further compression. When the load is removed, the AF and NP restore their original shapes as water is drawn back from the surroundings (Humzah and Soames, 1988; Walker and Anderson, 2004; Adams and Roughley, 2006).

2.3. Nutrient Supply

The IVD is the largest avascular tissue in the body. Blood vessels are present only in the disc margins which include the EP and periphery of the AF, with the former serving as the major route of nutrient transport (Urban *et al*., 2004). The distance between the center of NP and the nearest blood vessel can be as far as 7 to 8 mm (Urban *et al*., 2004). In addition, the IVD consists of an extensive matrix of collagens and highly negatively charged proteoglycans, which implies a difficult nutrient transport environment as it has been shown in other tissue that removal of proteoglycans increased solute diffusivity (Boubriak *et al*., 2000). Thus, achieving effective nutrient supply as well as waste removal is difficult but important to maintain each individual cell, and keep the whole disc healthy. Like many other cells, glucose and oxygen are consumed by disc cells for energy production while lactic acid is generated as a by-product. Solutes such as amino acids and sulphates are also required for matrix production. Transport of these small molecules into and out of the disc is mainly by diffusion (Holm *et al*., 1981) between the surrounding blood vessels and the disc center. The charge of a solute also affects its ability in entering the disc. Cations such as sodium can penetrate more readily into the disc than anions due to the negatively charged proteoglycans (Urban and Maroudas, 1979). For larger solutes such as growth factors and proteases, it is thought that the effect of the concentration gradient becomes less prominent and fluid movement plays a dominant role in the transport (Urban *et al*., 2004). Apart from cell viability, nutrition is also important for matrix integrity. It has been reported that a lowered pH, which is likely to result from accumulation of lactic acid, reduces the rate of matrix synthesis but not active matrix protease production, giving a net effect of enhanced matrix degradation (Razaq *et al*., 2003). Thus, the risk of having disc degeneration is increased.

2.4. Anatomy of the intervertebral disc

The intervertebral discs lie between the vertebral bodies, linking them together. They are the main joints of the spinal column and occupy one-third of its height (Jill*et al*.,, 2003). The intervertebral disc is composed of at least three elements. The central portion of the disc contains the nucleus pulposus, which is composed of cells from the primitive notochord. The outer portion of the disc is the annulus fibrosis, and it is composed of concentric layers of intertwined annular bands. These annular bands are arranged in a

specific pattern to resist forces placed on the lumbar spine. The annular bands are subdivided into inner fibres, which are connected to the cartilaginous endplate, and outer sharpy fibres, which are attached to the vertebral body. Intervertebral discs provide stability in the spine by anchoring the vertebral bodies to each other. In addition to this support, they also allow movement between the vertebrate, giving the spine its flexibility, and they absorb loads applied to the spine (Eyre and Muir, 1977). The discs have a specialized structure that enables them to perform all these functions. Intervertebral discs are largely a cellular and contain an abundant extracellular matrix of proteoglycans and collagens (Buckwalter, 1995).

The tissue is best described as a specialized form of fibrocartillage. The outer layer of disc, the annulus fibrosus, has a characteristic ring-like structure consisting mainly of densely packaged collagen I fibres (Guiot andFessler, 2000), while collagen I accounts for 70 % of dry weight of the annulus, it is virtually absent from the interior structure of the disc, the nucleus pulposus, this, in turn is rich in collagen II, Which accounts for about 20 % of its dry weight, and it also contains minor amounts of two other cartilage collagens, types IX and XI is also found in small amounts in annulus (Eyre and Muir, 1977). Likewise both the annulus and nucleus contain small amounts of collagen VI and minor amounts of collagen III and V are present throughout the disc (Nerlich *et al*., 1998). While proteoglycans account for only a few percent of the dry weight of the annulus fibrosus, they make up as 50 % the dry weight of the nucleus, the major structural component in which is Aggrecan, a large aggregating proteoglycan. Small amounts of a number of other matrix proteins such as elastin, biglycan, decorin, fibromodulin and versican have been found in disc. While the collagens provide tensile support for the disc, the extensively hydrated proteoglycans give the tissue its resistance to compressive forces.

2.5. DDD as a complex trait

Given the number of genes identified to be associated to disc degeneration, the high population prevalence of disc degeneration and the wide spectrum of disease severity, it is clear that disc degeneration is a complex trait disorder. DDD has a wide spectrum of degenerative features as phenotypes have quite high and variable prevalence in different

populations (Battie and Videman, 2006). This implicates the underlying complexity of etiology of IVD degeneration. The inheritance of DDD is complex in nature. The evidences from the familial studies and the results of the experimental results done with twins have produced many responsible factors for the disease. Moreover, many other environmental factors are also found to be associated with the disease incidence.

2.5.1. Definition of Disc Degeneration

Till now, there is no standard definition of disc degeneration and different studies may use different degenerative features of the disc to define disc degeneration. Common degenerative features of the disc are signal intensity loss in MRI scan, loss in disc height, annular tear, bulge, herniation and osteophyte formation (Battie and Videman, 2006).

2.5.2. Degenerative Disc Disease

As the human life progresses, significant changes occur in the lumbar disc components. Intervertebral disc degeneration can be defined as the loss of normal disc architecture accompanied by progressive fibrosis. At birth, the water content of the annulus fibrosus is about 80 per cent and that of the nucleus pulposus is about 90 per cent. This water content decreases eventually up to as low as 70 per cent or less, in case of nucleus (Vernon-Roberts, 1992). With age, nuclei transform from gelatinous substance (90 per cent water) into more solid-like structure. A further decrease in the number of healthy nuclear cells also takes place. In the annulus fibrosus, macroscopic changes are not readily discernible unless nuclear changes are advanced. However, microscopic changes such as, fragmentation of fibers, mucinous degeneration of fibers leading to cyst formation and focal aggregation of the collagen to form round aggregates of amorphous material, are observed in early stage of degeneration (Vernon-Roberts,1992). Reduction in the disc height, to a limited extent, occurs during adult life as the water content of the nucleus reduces. This disc narrowing is also associated with the bulging of the annulus towards the circumference of the disc (Kirkaldy-Willis *et al*., 1978). Loss of disc height is clinically important because it eventually leads to nerve root opening (Bao *et al*., 1996;). The salient features of intervertebral disc degeneration are the loss of gelatinous nucleus pulposus, gradual disappearance of the originally well-defined border between the nucleus and annulus, coarsening of the annulus lamellae,

progressive fibrosis and later fissuring of the annulus fibrosus with the deposition of the aging pigment (Coventry *et al*., 1945; Friberg and Hirsch, 1949; Harris and Macnab, 1954 and Van de Hooff, 1964).

With age and degeneration, total proteoglycan content decreases while the keratin sulfate/chondroitin ratio increases. It is suggested that degradation occurs in the hyaluronic acid binding region and that proteoglycan synthesis is slower in IVDD (Lipson, 1996). It was also proposed that the decrease in PGs content results from cell death due to lower pH. Because of this, the nucleus is unable to retain enough water for generation of intradiscal pressure as in the case of the normal discs. The load transfer mechanism is clearly altered in the case of a dry nucleus. Because of this, the end plates are subjected to reduced pressure at the center and the more pressure around the periphery. The stress distribution in the annulus is also altered significantly. Outer annulus layers of the degenerated disc experience circumferential stresses which are near zero or tensile. In the inner layers, the fiber stress is compressive. The circumferential stress is very small, annular stress is tensile and peripheral stress nearly vanishes (Shirazi-adl *et al*., 1984). Essentially, the nucleus does not perform its function of load transfer and the load transfer occurs through an end plate – annulus – end plate route. The annulus is subjected to abnormal stresses (mostly compressive), although it is naturally structured to support tensile stresses. Because of this altered load mechanism, the annulus is more prone to injuries and cracks/fissures first develop into the annulus.

With continued degeneration, the central nucleus may migrate through the crack developed in the annulus towards the periphery. The migration of the nucleus material is referred to as ‘disc herniation’. The migrated material may impinge on the nerve root. The contact of the migrated nucleus with the nerve root irradiates debilitating back pain. Also, the herniated material elicits an inflammatory response because of the avascular nature of the nucleus (Bao *et al*., 1996). The reduction in the disc volume leads to instability, resulting in the growth of bone, endplates and ligaments to compensate for this volume loss.

It is difficult to distinguish between the effects of aging from that of degeneration on the biomechanical behavior of the lumbar disc. The biomechanical behavior of the disc is dependent upon its state of degeneration which in turn depends upon the age. It was found that disc degeneration first appears in males in the second decade and in females a decade later. It was also observed that by age 50, almost all lumbar intervertebral discs (97 per cent) are degenerated, though not all are symptotatic (Miller *et al*., 1988).

2.6. Aging and Degeneration of IVD

It is generally accepted that aging of IVD or age-related changes of IVD is difficult to distinguish or even indistinguishable from degeneration of IVD or pathological changes of IVD (Eyring, 1969; Eyre, 1979; Urban *et al*., 2000; Cassinelli and Hall, 2001; Fardon and Milette, 2001; Raj, 2008). Despite differentiating them is difficult, some suggested that disc degeneration is inevitable process of aging and such process may occur in accelerated rate due to possibly genetic factors and environmental factors (Cassinelli and Hall, 2001). In the extreme case of early-onset disc degeneration, it is more likely that the manifestation of degeneration is the result of predisposing factors on susceptibility genes than normal aging process. In such accelerated case of disc degeneration, it is postulated that aging process and environmental influence are unlikely to be accumulated to a considerable level that could leads to disease outcome. It is thus accounted to be rather pathogenic with potentially major genetic involvement.

2.6.1. Age related changes of IVD

After 40 years of age, most human IVD demonstrate degenerative lesions, especially for cervical and lumbar levels (Eyre, 1979). Aging causes inevitable and gradual changes in disc ECM composition. Subtle changes are metabolic and biochemical changes which influence the anabolic and catabolic processes. These will affect the synthesis and breakdown of ECM components. The effect of subtle changes will accumulate and once they reach a certain level, observable histological as well as various morphological changes appear. These apparent changes in the structure of IVD may be a part of normal aging but some individuals demonstrate them at relatively earlier age. In aging, environmental factors are believed to be predominated. Nonetheless, variation in age of

IVD degeneration manifestation, particularly early-onset with accelerated degeneration compared with others of similar age, cannot be solely explained by aging. This suggests other factors, possibly genetic ones could be identified to answer why there are cases of accelerated signs of aging or possibly pathological IVD degeneration.

2.6.1.1. Changes in nucleus pulposus

NP of infant or immune IVD is white and opaque with gel-like appearance. However, as one ages, the NP gets less hydrated and more fibrous. The color changes from white to yellowish brown as the non-enzymatic glycosylation produces accumulate. When an individual is young, there is a great distinction in structure between AF and NP. However, as one becomes older, the boundaries between the two components get less conspicuous. Subsequently, cleft and fissures appear and the IVD loses its height and distorts. At the final stage of aging, the ECM disappeared and replaced by disorganized scar tissue (Urban *et al*., 2000). Water content changes with age as well and it is about 85 to 90 per cent in young IVD but decreases to 70 per cent with aging (Modic *et al*., 1984).

It can be found that drying out of gelatinous NP, solidification of NP as well as cleft formation are common symptoms of aging disc. These symptoms are caused by subtle changes such as loss of sulfated glycosaminoglycans and increase in non-collagenous proteins. As the total collagen content is not appreciably altered during aging, the whole process is not as simple as fibrosis of tissue caused by increased deposition of collagens. For the loss of sulfated glycosaminoglycans, it has been suggested the condition is particularly severe for proteoglycans rich in chondroitin sulfate.

2.6.1.2. Changes in annulus fibrosus

In young individuals, AF is an organized structure with discrete lamella-like layers of fibrous tissue. There is good distinct between AF and NP. As age progresses, the distinction becomes increasingly blur, especially at the boundary. The annulus rings thicken and turn disorganized. Later clefts may form in various ways. Concentric tears are fissures that in a plane roughly parallel to the curve of the periphery of the IVD, creating space between adjacent lamella. Transverse tears are fissures running horizontally and usually limited to rupture of the outer annulus. On the other hand, radial tears are those extending from the NP outward to the periphery of the AF

(Yu *et al*., 1989; Fardon and Milette, 2001 and Adams and Roughley, 2006). Water content decreases from 78 to 70 per cent as one ages (Modic *et al*., 1984).

2.6.1.3. Changes in end plates

As the IVD ages, vascularity decreases due to reduced number of the blood vessels in the EP. By the end of third decade of life, they seem to be disappeared (Cassinelli and Hall, 2001). Nevertheless, as early of two years of age, mild degenerative changes are observed such as matrix degeneration in EP (Roberts *et al*., 2006). It is regarded as the spine's weak link in compression (Adams and Roughley, 2006). Damage in EP can decompress the adjacent NP and transfers the load onto AF, causing it to bulge into the nucleus cavity. Schmorl's node is the lesion that NP herniated through damaged EP with subsequent calcification (Adams and Roughley, 2006).

2.6.2. Cellular level or histological changes

Change at cellular level is a typical symptom of IVD degeneration. Through histology, increased disc cell proliferation, cell cluster formation and increased cell death are commonly seen in degenerated IVD (Roberts *et al*., 2006). Histological changes across the whole life have been studied by Boos *et al*. (2002). From the study, the changes firstly appear at EP and subsequently to NP and then AF. With diminished blood supply at EP as the starting point (begins soon after birth and reaches maximum before the first decade of life), it is followed by breakdown of tissue at NP by the second decade of life.

2.6.3. Biochemical changes

Proteoglycans are the major ECM component of IVD. As early as childhood, proteoglycan fragmentation takes place. The extent is especially prominent at NP and the water content is affected accordingly. Proteoglycan fragments are trapped inside the IVD since NP is encapsulated by surrounding AF and EP. As a result, it is suggested that although the fragmented proteoglycans are structurally different from the original aggregates, they may remain functionally similar to those intact proteoglycan aggregates. Reduced matrix turnover in order IVD enables collagen fibrils to become crosslinked with one another and this further inhibits matrix turnover and repair. The resulting

retention of damaged macromolecules leads to compromised tissue strength (Adams and Roughley, 2006).

2.6.4. Metabolic changes

Matrix synthesis decreases constantly with age despite some fluctuations at certain stage. Reduced matrix synthesis may be explained by reduced cell density. Collagen and MMP synthesis also demonstrate age-related changes (Adams and Roughley, 2006).

2.6.5. Structural or morphological changes

Cellular level, biochemical and metabolic changes are subtle changes. On the other hand, there are more profound changes in the structure of IVD during aging or degeneration.

2.6.5.1. Annular tears

Annular tears represent fissures at AF and there are various types of annular tears that distinguishable by their regions of occurrence and ways of fissure (Fardon and Milette, 2001). The first type is concentric tear that creates fluid-filled spaces between adjacent lamella. The second type is transverse tears that are located horizontally between the AF and ring apophysis. The third type is radial tear that the fissure is extended from NP to the surface of disc. This type of tear creates the necessary but not sufficient condition for disc herniation (Yu *et al*., 1989). The three types of annular tears are believed to be independent of age and one another (Vernon-Roberts *et al*., 1997). As illustrated by a study in sheep (Osti *et al*., 1990) tears of the outer annulus might have a role in further developing into clefts and progression in disc degeneration. It was suggested that at early stage of disc degeneration, water content of NP may retain relatively normal water content and hence NP dehydration may be the consequence of biochemical changes in proteoglycan content in response to other early degenerative features such as annular tears.

2.6.5.2. Bulging and Herniation

Disc with contour of the outer AF extends, in the horizontal plane, beyond the edges of the disc space with over 50 per cent of the circumference of the disc (>180^{o}), can be described as bulging disc. It can be symmetrical or asymmetrical. This kind of structural change is not considered to be a form of herniation (Fardon and Milette, 2001).

For herniation, it is defined as localized displacement of disc material beyond the normal margins of the IVD space. There are two types of herniation – protrusion and extrusion. Protruded disc is a disc in which the greatest plane, in any direction, between the edges of the disc material beyond the disc space is less than the distance between the edges of the base, when measured in the same plane. Extruded disc is, otherwise, a herniated disc material in which, in at least one plane, any one distance between the edges of the disc material beyond the disc space and that within the disc space. Extrusion may further developed into sequestration if the displaced disc material has lost completely any continuity with the parent disc (Fardon and Milette, 2001).

2.6.5.3. Schmorl's nodes

It is also regulated as intervertebral herniation. A portion of the IVD, commonly the NP displaces through the EP into the centrum of the vertebral body (Fardon and Milette, 2001). Accumulation of trabecular microdamage was suggested to the reason why NP bulges into the vertebral bodies (Adams and Roughley, 2006).

2.6.5.4. Disc height reduction and osteophyte formation

With increasing age, the proteoglycan and water content of NP gets reduced and it increases the stiffness of NP as it becomes more fibrous. Reduction in nucleus pressure and vertical loading causes the AF to bulge radially outward. This will ultimately result in the narrowing of disc height.

The disc height determinate the separation of adjacent neural arches. This may explain why reduced disc height is associated with osteophytes around the margins of the vertebral bodies (Adams and Roughley, 2006). Anterior and lateral marginal vertebral osteophytes were found in all studied individuals over the age of forty and it is considered to be consequence of normal aging. On the other hand, posterior osteophytes were only found in minority of individuals over the age of eighty and hence it is not regarded as inevitable outcome of aging (Fardon and Milette, 2001).

2.6.5.5. Internal disc disruption

In old IVDs, there is a common feature that inner AF collapses into the NP with anterior being more severely affected than posterior. Besides, it is also commonly observed in old IVDs that the cartilaginous EP detaches from the underlying bone and it was suggested to be the outcome of the loss of internal pressure that presses it against the bone when it was young (Adams and Roughley, 2006).

2.6.7. Degenerative Marrow Changes

The relationship among the vertebral body, endplate, annulus, and disk has been studied (Masaryk *et al*., 1986 and Modic *et al*., 1988) by using both degenerated and chymopapain-treated disks as models. Signal intensity changes in vertebral body marrow adjacent to the endplates of degenerated discs are a common observation on MR images and appear to take three main forms.

Type I changes demonstrate decreased signal intensity on T1-weighted images and increased signal intensity on T2-weighted images and have been identified in approximately 4 per cent of patients scanned for lumbar disease, approximately 8 per cent of patients after discectomy (Ross *et al*., 1996 and Ross *et al*., 1998) and in 40 per cent to 50 per cent of chymopapain treated disks, which may be viewed as a model of acute disc degeneration (Masaryk *et al*., 1986). Histopathologic sections of discs with type I changes show disruption and fissuring of the endplate and vascularized fibrous tissues within the adjacent marrow, prolonging T1 and T2. Enhancement of type I vertebral body marrow changes is seen with administration of gadopentetate dimeglumine that at times extends to involve the disc itself and is presumably related to the vascularized fibrous tissue within the adjacent marrow.

Type II changes are represented by increased signal intensity on T1-weighted images and isointense or slightly hyper intense signal on T2-weighted images and have been identified in approximately 16 per cent of patients at MR imaging. Discs with type II changes also show evidence of endplate disruption, with yellow (lipid) marrow replacement in the adjacent vertebral body resulting in a shorter T1.

Type III changes are represented by decreased signal intensity on both T1 and T2-weighted images and correlate with extensive bony sclerosis on plain radiographs. The lack of signal in the type III change no doubt reflects the relative absence of marrow in areas of advanced sclerosis. Unlike type III, types I and II changes show no definite correlation with sclerosis at radiography (Modic *et al*., 1988). This is not surprising when one considers the histology; the sclerosis seen on plain radiographs is a reflection of dense woven bone within the vertebral body, whereas the MR changes are more a reflection of the intervening marrow elements.

Similar marrow changes have also been noted in the pedicles. While originally described as being associated with spondylolysis, they have also been noted in patients with degenerative facet disease and pedicle fractures. Again, the changes are probably a reflection of abnormal stresses, be they loading or motion (Morrison *et al*., 2000 and Ulmer *et al*., 1995).

DDD is still a poorly understood phenomenon because lack of availability of precise definition of healthy, ageing and degenerated discs (Rajasekaran *et al*., 2008). Decreased nutrition paves way for the incidence of DDD and the status of the endplate (EP) plays a crucial role in controlling the extent of diffusion. The vascular channels in the subchondral plate have muscarine receptors but the possibility of enhancing diffusion pharmacologically by dilation of vessels has not been probed. Although it is well accepted that EP damage will affect diffusion and thereby nutrition, there is no described method to quantify the extent to quantify the extent of EP damage. Precise definitions with an objective method of differentiating healthy, ageing and degenerated discs on the basis of anatomical integrity of disc physiological basis of altered nutrition will be useful. This information is urgent necessity for better understanding of DDD and also strategizing prevention and treatment.

The experiment was conducted by Rajasekaran *et al*. (2008), in seven hundred and thirty plates of 365 lumbar discs from 73 individuals (26 healthy volunteers and patients and 47 patients) with age ranging from 10 to 64 years were evaluated by pre contrast and 10 to 64 years were evaluated by pre contrast and 10 min, 2, 4, 6 and 12 hrs post contrast MRI after IV injection of 0.3 mmol/kg of Gadodiamide. End plates were classified according

to the extent of damage into six grades and an incremental score was given each category. Disc degeneration was assessed by Pfirmann's grading and discs were considered to be healthy if they were grade I, II or III and degenerated if they were grade IV or V.

DDD was diagnosed on the basis of signal intensity changes within the nucleus pulposus (NP) of the IVDs of the lumbar spine and graded using the Schneiderman's classification scheme (Schneiderman *et al*., 1987). Grade 0 was used to indicate normal disc with hyperintense signal within the NP, grade 1 for a slight decrease in signal intensity in the NP, grade 2 for a generalized hypointense NP and grade 3 for a hypointense NP with disc space narrowing.

2.7. Genetics of Disc Degenerative Disease

LDD was once widely accepted to be contributed mainly from age, gender, occupation, cigarette smoking and exposure to vehicular vibration while its association with factors such as height, weight and genetics was less certain (Frymoyer 1991). Such view has changed since the presentation of monozygotic twin studies, where genetics has been reported to explain most of the variability in LDD, whilst other factors only have a modest effect on it (Battie *et al*., 1995a & b). Since then, more evidence has been reported showing the strong relationship between genetics and LDD, thus opening up a new area of research.

Currently, genetic risk factors of LDD are identified mainly through the candidate gene approach, where genes are selected based on the proved or inferred function of the encoded protein in the disc and their polymorphisms are tested for the correlation with disc degeneration. As LDD is a complex disease, each risk factor may contribute mildly to the disease and cannot be used to explain all cases of LDD. A possible method to circumvent this situation is to study any interactions among the risk factors.

2.8. Family and twin studies

Twin studies have shown nearly 80 per cent heritability in disc degeneration in lower lumbar levels suggesting that disc degeneration may be primarily explained by genetic influences (Battie *et* al., 1995a & b and Sambrook *et* al., 1999). Additional support has been provided by epidemiological studies indicating more than four times increased risk

for degenerative disc disease in patients with relatives that have undergone spinal surgery (Simmons *et al*., 1996, Matsui *et* al., 1998). Despite the fact that the etiology of LDD is not fully characterized, studies have suggested the possibility of familial predisposition to LDD and the genetic component of LDD. In the study carried out by Postacchini *et al*. (1988), which primarily investigated on the possible role of genetic susceptibility in the etiology of discogenic LBP, results suggested relatives of discogenic LBP patients were having a significantly higher chance of having discogenic LBP than those of asymptomatic subjects. Considerable familial predisposition to early onset sciatica and disc herniation has been proven as well. It was found that the relative risk for lumbar disc herniation before the age of twenty-one years was five times greater in patients with a positive family history (Matsui *et al*., 1992; Scapinelli, 1993). In addition, a synchronous lumbar disc herniation in adult female monozygotic twins was reported, further highlighting the role of genetic factors (Bhardwaj and Midha, 2004).

2.9. Association Studies

There are a number genetic risk factors identified in various populations that are associated with LDD (Table 1). These include the *Taq*I and *Fok*I polymorphisms of vitamin D receptor (*VDR*) (Videman *et al*., 1998), Trp2 and Trp3 alleles of alpha 2 and alpha 3 chains of type IX collagen respectively (Annunen *et al*., 1999 and Paassilta *et al*., 2001), CS1 polymorphism of aggrecan (Kawaguchi *et al*., 1999), Sp1 polymorphism of type I collagen (Pluijm, *et al*., 2004), 5A/6A allele of matrix metalloproteinase 3 (*MMP3*) (Takahashi *et al*., 2001), a functional single nucleotide polymorphism of cartilage intermediate layered protein (*CILP*) (Seki *et al*., 2005) and D14 allele of asporin (*ASPN*) (Song *et al*., 2008). Among all these risk factors, *Taq*I polymorphisms of *VDR*, Trp2 allele of type IX collagen and D14 allele of *ASPN* can be replicated in several populations (Kawaguchi *et al*., 2002; Jim *et al*., 2005 and Cheung *et al*., 2006). An immediate observation from these association studies is that all the associated genes, except *VDR*, encode ECM structural molecules including collagen types I and IX, aggrecan, asporin and CILP, or proteins involved in the metabolism of ECM including MMP. This reflects the importance of the ECM during degeneration of the IVD. Rajasekaran *et al*., (2013) reported that 58 SNPs in 35 potential candidate genes were

Table 1. List of previous genetic association studies on disc degeneration showing the lack of uniformity of phenotype criteria

S. No	Candidate gene	Study population	Phenotype	Author
1	*COL9A2*	Germany, 288 patients	Biopsy of disc material from patients who underwent discectomy	Knoeringer *et al*. (2008)
2	*Aggrecan,* VNTR polymorphism	Finland, 132 men	MRI features such as signal intensity of nucleus pulposus, disc bulge, disc height	Solovieva *et al*. (2007)
3	Taq I vitamin D receptor	China, 804 volunteers	MRI features such as Schneiderman's score, Schmorl's nodes, HIZ	Cheung *et al*. (2006)
4	*COL9A3*	Finland, 135 subjects	MRI features of disc bulge and decrease in disc height	Solovieva *et al*. (2002)
5	*GCH1*	England, 69 patients undergoing surgical treatment of lumbar DDD	MRI features including desiccation, broad-based bulging, and collapse, with or without gross fissuring and Modic-type end-plate changes	Kim *et al*.(2010)
6	*HAPLN1*	Japan, 622 postmenopausal women	Radiographic features of spinal degeneration such as degree of end-plate sclerosis, osteophyte formation, and disc space narrowing	Urano *et al*. (2011)
7	*TSG6*, *TNF*α, *IL1*α and *IL1*β	58 discs from 54 patients, who underwent discectomy	Discs graded macroscopically for their degree of degeneration	Roberts *et al*. (2005)
8	*COL9A1*, *COL9A2*, and *COL9A3*	Finland, 804 patients	MRI features of Schneiderman's classification, disc herniations, annular tears, and end-plate herniations (Schmorl's nodes)	Jim *et al.* (2005)
9	Vitamin D receptor	205 young adults	MRI: Schneiderman's score	Kawaguchi *et al*. (2002)

S. No	Candidate gene	Study population	Phenotype	Author
10	Aggrecan, VNTR region	North China 197 patients, compared patients with or without LDH	All symptomatic LDH patients confirmed with MRI/CT	Cong *et al*. (2010)
11	Vitamin D receptor	Finland, 85 pairs of twins	MRI: disc bulging and disc height narrowing	Videman *et al*. (1998)
12	*COL2A1*	Mice	Spine tissues were studied using radiographic analyses; conventional, quantitative, and polarized light microscopy and immunohistochemistry	Sahlman *et al*. (2001)
13	*SOX9* transcription factor	Discs from 12 normal donors and 25 surgical patients	Discs graded macroscopically for their degree of degeneration using Thomson grade	Gruber *et al*. (2005)
14	*MMP3*, *TIMP1*, and *COX2*	720 women	Radiographic features such as osteophytes, disc space narrowing	Valdes *et al.* (2005)
15	Aggrecan	64 young women	MRI: Schneiderman score	Kawaguchi *et al*. (1999)
16	*IGF1R*	Japan, 434 postmenopausal women	Radiographic features such as end-plate sclerosis, osteophytes, and narrowing of disc spaces	Urano *et al*. (2008)
17	*COL9A3* and *COL9A2*	Finland, 1832 men	MRI signal intensity in nucleus pulposus, disc bulge, disc height reduction	Solovieva *et al*. (2006)
18	*COL9A2*	159 patients with sciatica	Sciatica compared with MRI features such as normal disc, bulge, contained herniation, noncontained herniation, and sequestration	Karppinen *et al*. (2002)

evaluated in 332 subjects from Indian Population. Eleven of the 58 SNPs provided evidence of association. *AGC1*, *ADAMTS5*, *NGFB*, *IL1B*, *IL18RAP*, *MMP10*, *GLI1* and *MMP 13* were associated. Since candidate genes are selected based on the hypothesis that normality of the disc is affected, a good understanding of the selected genes in the process of disc degeneration is required.

2.9.1. Candidate gene association studies

Epidemiological evidence from twins had certainly provided strong ground to believe in genetic inheritance as a significant contributor to disc degeneration. On top of this, research groups from several countries had also successfully identified a number of mutations or polymorphisms as genetic risk factors in several candidate genes (Table 2).

2.9.1.1. Matrix metalloproteinase's

Matrix metalloproteinase's (MMPs) are a group of enzymes that take part in degrading the major structural components of IVD and are known to be involved in normal turnover and pathological degradation of the extracellular matrix in connective tissue (Borden and Heller, 1997; Roberts *et al*., 2000). Song *et al*. (2008) demonstrated that individuals with the presence of D allele for the -1607 promoter polymorphism of *MMP1* are about 1.5 times more susceptible to develop DDD compared with those having G allele only. Also association was identified individuals over 40 years of age.

Dong *et al.* (2007) has been documented the increased expression and activity of *MMP-2* in degenerative discs of Chinese population. The polymorphism -1306C/T was identified in the promoter region of *MMP-2* gene was reported to influence gene transcription and expression. The C/T transition at -1306 disrupts Sp1-binding site and results in decreased transcriptional activity, whereas the presence of the Sp1 promoter site in the -1306C allele may enhance transcription. Therefore, MMP-2 protein expression would be higher in individuals who carry the CC genotype than those who carry TT or CT genotype.

MMP-3 has a potential role in proteoglycans degradation and has been indicated to take part in IVD degeneration (Goupille *et al*., 1998). A common 5A/6A polymorphism in the promoter region of human *MMP-3* has been identified, with the 5A allele having twice as

Table 2. Genetic association studies on low back disorders

Gene	SNP	Region	Consequence	Population	References
CHST3	A/C allele at rs4148941and C/T allele at rs4148949	3' UTR	Reduced expression of CHST3 mRNA (miR-513a-5p)	South Chinese, Finnish and Japanese	Song *et al*. (2013)
CILP	C>T	-	Not known	Finnish	Kelempisioti *et al*. (2011)
IL6	-	Promoter	Not known	Finnish	Kelempisioti *et al*. (2011)
SKT	-	-	Not known	Finnish	Kelempisioti *et al*. (2011)
MMP-9	-1562T >C	promoter	Not known	Northern Chinese	Sun *et al*. (2009)
MMP-1	1607G/D	Promoter	Not known	Southern Chinese	Song *et al*. (2008)
MMP-2	1306C >T	Promoter	Not known	Chinese	Dong *et al*. (2007)
COL9A2	+22C >T	Exon 19	Gln326Trp	Finnish, Chinese	Annunen *et al*. (1999); Jim *et al.* (2005)
CILP	+1184T >C	Exon 8	Ile395Thr	Japanese	Seki *et al. (*2005)
IL-1A	-889C >T	promoter	Not known	Finnish	Solovieva *et al*. (2004)
IL-1B	3954C >T	Exon 5	Not known	Finnish	Solovieva *et al*. (2004)
MMP-3	5a/6a polymorphism	Promoter	Not known	Japanese	Takahashi *et al*. (2001)
COL9A3	+52C >T	Exon 5	Arg103Trp	Finnish	Paassilta *et al. (*2001)

Gene	SNP	Region	Consequence	Population	References
Aggrecan	VNTR	Exon12	Not known	Japanese	Kawaguchi *et al*. (1999)
Vitamin D receptor	+2T >C	Exon 2	Met2Thr	Finnish	Videman *et al. (*1998)
	T352C	Exon 9	Ile352Ile	Finnish	Videman *et al. (*1998; 2001)
COL11A1	TT/GT/GG	Intron 1	Not known	Dutch	Uitterlinden *et al.* (1998); Grant *et al*. (1996)
IL-6	-597G >A	promoter	Not known	Finnish	Terry *et al*. (2000)
	-174G >C	promoter	Not known	Finnish	Fishman *et al*. (1998)
	+15T >A	Exon 5	Asp162Glu	Finnish	Noponen-Hietala *et al*. (2005)

much promoter activity as the 6A allele (Ye *et al*., 1996 and Takahashi *et al*., 2001). The 5A5A and 5A6A genotypes were found to associate with degenerative changes of lumbar IVDs in an elderly group of individuals aged more than 64 years, suggesting that the 5A allele may be a risk factor for the acceleration of degenerative changes in lumbar IVDs in the elderly (Takahashi *et al*., 2001).

Many studies have demonstrated that matrix metalloproteinase-9 (*MMP-9*) is involved in the development of lumbar disc disease (LDD). (Sun *et al.*, 2009) has been identified the expression and activity of *MMP-9* are significantly enhanced in degenerative discs. The study was conducted in the young adult population in north China. The result showed that polymorphism -1562C/T in the promoter region of MMP-9 gene alters the transcriptional activity of this gene. The identified genotypes were correlated with the presence of lumbar disc degeneration on MRI. The frequency of the *MMP-9* -1562T genotype in patients with LDD was significantly higher than in healthy controls. Moreover, an association was found between this genotype and more severe grades of disc degeneration observed on MRI scan. These results indicated that the -1562C/T polymorphism of the *MMP-9* gene is associated with a high risk of degenerative disc disease in the young adult population in North China.

2.9.1.2. Aggrecan

Aggrecan is the major proteoglycan component in cartilage and the nucleus pulposus of the intervertebral disc. Its key function is to maintain hydration of the disc structure, attracting water molecules through the highly negatively charged GAG moieties, which are mainly chondroitin sulphate (CS) chains. Thus, from the structural integrity point of view and the associated loss of water content in a degenerating disc, this extra cellular matrix molecule is considered as a good candidate for genetic association studies (Roughley *et al*., 2006). Within the aggrecan core protein, CS chains are present in two adjacent regions, the CS1 and CS2 domain. In the human AGC1 gene, the region coding for the CS1 domain exhibits size polymorphism, commonly known as variable number of tandem repeats (VNTR) in exon 12, ranging from 13 to 33 repeats (Kawaguchi *et al.,* 1999). Additional studies of the VNTR polymorphism or other

single nucleotide polymorphisms (SNPs) identified through the Hap map project are needed to validate AGC1 as a disposing gene for DDD in much larger scale studies (The International HapMap Consortium, 2005).

2.9.1.3. Vitamin D receptor (VDR)

The vitamin D receptor (VDR) has an important role in the biological function of vitamin D as it binds particularly the active form of vitamin D (1,25(OH) 2D3) and produces a number of biological effects. VDR has also been suggested to have an important role in certain bone disorders, such as osteoporotic fractures, alterations in bone mineral density and femoral neck stress fractures (Smith *et al*., 1973, Morrison *et al*., 1994). There are two intragenic *VDR* gene polymorphisms, named *Taq*I and *Fok*I, have been shown in several separate studies to be associated with disc degeneration.

A *Taq*I polymorphism in exon 9 of *VDR*, more precisely the tt (corresponding CC) genotype, has been reported to associate with low bone density, degenerative changes in knee joints, disc degeneration, disc height narrowing, disc bulging, annular tears, and disc herniation (Spector *et al*., 1995; Uitterlinden *et al*., 1997; Jones *et al*., 1998; Videman *et al*., 1998; Videman *et al*., 2001, Kawaguchi *et al*., 2002 and Cheung *et al*., 2006). The *Fok*I polymorphism in exon 2 (T2C) of the *VDR* gene results in the synthesis of two VDR mRNAs (Arai *et al*., 1997; Miyamoto *et al*., 1997) and there is some evidence that the shorter receptor protein (C allele) may play a more active role in VDR-responsive gene expression than does the full-length receptor protein (T allele) (Jurutka *et al*., 2000). The TT genotype, designated as ff, has been found to increase the risk for disc degeneration, bulging and disc height compared to the CC (FF) genotype (Videman *et al*., 1998).

2.9.1.4. Collagen IX

Annunen *et al*. (1999) reported association between DDD and a gene called *COL9A* encoding the collagen IX, an extra cellular matrix molecule present in the cartilage and the nucleus pulposus of IVD. Collagen IX is proposed to act by bridging molecules important for the maintenance of tissue integrity of the matrix in the presence of tryptophan amino acid. They were able to detect two mutations in the alleles of this gene namely *COL9A2* (Jim *et al*., 2005) and *COL9A3* (Paassilta *et al*., 2001).

COL9A2 - α-helix (326) - xxxx-**trp**-xxxx - no risk

xxxx-**glu**-xxxx – DDD susceptible

COL9A3 - α-helix (103) - xxxx-**trp**-xxxx - no risk

xxxx-**arg**-xxxx – DDD susceptible

2.9.1.5. Collagen I A1

COLIA1 codes for the α 1(I) chain of the collagen I molecule, the major collagen in bone matrix. Collagen I also present in the annulus fibrosus of the intervertebral disc providing tensile strength (Feng *et al*., 2006). The study was conducted in Dutch population (Grant *et al*., 1996 and Uitterlinden *et al*., 1998), showed that the Sp1 polymorphism (TT/GT/GG) in intron 1 of the COLIA1 for the binding site for the transcription factor Sp1 is associated with DDD. The individuals who carry TT genotype had a higher risk for disc degeneration than individuals with GT and GG genotype.

2.9.1.6. Cartilage intermediate layer protein

Cartilage intermediate layer protein (CILP) was identified as a matrix constituent of human articular cartilage that appeared to be unregulated in OA. The protein was found to be restricted in its distribution within specific zones in the intermediate layer of cartilage and thus the name CILP (Lorenzo *et al*., 1998). In a recent case control association study of twenty candidate genes using sequence variations selected from the Japanese SNP Database and the Applied Biosystem SNP resource, a function SNP in *CLIP*, +1184T → C, in Exon 8 was shown to be associated with DDD in a Japanese population (Seki *et al*., 2005). The allelic change resulted in amino acid substitution Ile395Thr.

Amino acid substitution

CILP (395 a. a) xxxx-**Thr**-xxxx - no risk

CILP (395 a. a) xxxx-**Ile**-xxxx – DDD susceptible

2.9.1.7. Interleukins

There is numerous evidence that inflammatory factors may be involved in the genesis of LBP. Herniated discs spontaneously produce a variety of pain inducing substances such as nitric oxide, pro inflammatory cytokines, as well as cyclooxygenenase-2, phospholipase A_2 (Kang *et al*.,1997; Ahn *et al*., 2002). Even though sequence variations in the genes coding for several cytokines have been mainly implicated in autoimmune or infectious, recent studies suggest an association of LBP and disc degeneration with the polymorphisms in the *IL1* locus (Solovieva *et al*., 2004). Importantly, several sequence variations in cytokine genes have been shown to have a functional significance with potential influences on the inflammatory responses.

Solovieva *et al*. (2004) reported the single nucleotide polymorphism (-889C>T) of *IL-1A* gene in the promoter region and *IL-1B* gene polymorphism (3954C >T) in Exon 5 regions of Finnish population. This study suggests the interleukin may be risk factor for the lumbar disc disease. In the same Finnish population, three SNPs were identified in the IL-6 gene where as two in the promoter and one was in Exon 5 region. Terry *et al*. (2000) reported the -597G>A polymorphism and -174G>C polymorphism was identified in the promoter region by Fishman *et al*. (1998). In a recent association study, a function SNP in IL-6, +15T>A, in Exon 5 was shown to be associated with DDD in a Finnish population (Noponen-Hietala *et al*., 2005). The allelic change resulted in amino acid substitution Asp162Glu.

2.9.1.8. Nerve growth Factor

NGF was the first neurotropic cytokine to be described. It is both a growth and a survival enhancement factor for nerves, particularly non-myelinated fibres. NGF consists of two chains, α and β, of which *NGFβ* is responsible for its neurotropic properties. NGF exerts its effects through high-affinity (trk-A) and low-affinity (p75) receptors. Expression of the neurotropic factor *NGFβ* only in pain level IVD and then only in those pain level IVD that have nonmyelinated nerves actively growing into the deep layers of the annulus fibrosus and the nucleus pulposus. Evidence implicating trk-A in the regulation of neuronal pain networks which might explain the biological advantage of selective expression of the trk-A receptor in situations, such as disc degeneration, where pain might be

part of a protection reaction against further tissue damage (Freemont *et al*., 2002). Aoki *et al*. (2005) has been reported that inflammatory mediators, nerve growth factor (NGF), and a decrease of proteoglycan content have the potential to promote nerve ingrowth into the disc. Furthermore, recent reports suggested that the levels of inflammatory mediators and NGF are higher in painful discs than in asymptomatic discs. Nerve ingrowth into painful discs may therefore be regulated by multiple factors, including factors as yet unidentified.

2.9.1.9. A disintegrin and metalloproteinase with thrombospondin motifs (*ADAMTS5*)

Several members of the *ADAMTS* (a disintegrin and metalloprotease with thrombospondin-like repeat) family of enzymes (*ADAMTS*1, 4, 5, 8, 9 and 15) are known to be capable of cleaving aggrecan at the Glu 373/Ala 374 site, but *ADAMTS4* and *ADAMTS5* (aggrecanase-1 and aggrecanase-2, respectively) seem to be the most active aggrecanases. Which aggrecanase is responsible for aggrecan degradation during human articular cartilage destruction, however, remains unclear. Aggrecan is the major proteoglycan in cartilage, endowing this tissue with the unique capacity to bear load and resist compression. In arthritic cartilage, aggrecan is degraded by one or more 'aggrecanases' from the *ADAMTS* (a disintegrin and metalloproteinase with thrombospondin motifs) family of proteinases. *ADAMTS*1, 8 and 9 have weak aggrecan-degrading activity (Stanton *et al*., 2005).

Wu and his coworkers reported that the genetic polymorphisms of *ADAMTS-5* may be associated with susceptibility to LDD in Chinese Northern Han population (Wu *et al*., 2014). The involvement of *ADAMTS-5* gene in LDD has been proved in vivo and in vitro. Patel *et al*. (2007) reported that abundant levels of *ADAMTS-5* in human cadaveric IVDs. Le Maitre *et al*., (2007) have reported the relationship between expression of *ADAMTS-5* and altered phenotype of IVD cells during degeneration, which was also proved by Zhao (Zhao *et al*., 2011). Use of *ADAMTS-5* siRNA was effective in suppressing the degeneration of the nucleus pulposus in rabbit model, which further proved the participation of *ADAMTS-5* in the process of DDD (Wu *et al*., 2014).

2.10. Implications of genetics and molecular Studies of DDD

These association studies support the fact that genetics plays a critical role in DDD. However, how each genetic risk factor contributes to the underlying molecular mechanism of the degenerative process still requires investigation. Genetics can give vital clues and provide knowledge to possible biological processes of DDD, while understanding disc metabolism can help to uncover potential risk factors for further genetics research. This leads to the need for a comprehensive understanding on the molecular components of the disc and their corresponding changes from normal to aging and disc degeneration, together with any mechanisms involved including cellular activities, ECM structure and maintenance.

2.11. Implications of proteomics and metabolomics studies of DDD

Proteomics, defined as the large-scale analysis of proteins, is emerging as a powerful field with large promise for un-locking many of the pathophysiologic mechanisms of disease. As a whole, proteomics encompasses many technical disciplines including light and electron microscopy, array and chip experiments, genetic read-out experiments, and mass spectroscopy (MS). However, of these various disciplines, MS-based proteomics is proving to be the technique of choice for high throughput analysis of complex protein samples.

The explosive development in MS-based proteomics has been made possible by several recent advances in the biomedical sciences. In the 1990s, biological mass spectroscopy evolved as a tool for rapid and powerful large-scale protein analysis and enabled scientists to overcome the limitations of protein analysis imposed by two-dimensional gel electrophoresis (Goldring, 2000). This rapidly evolving technology combined with the completion of the Human Genome Project in July 2000 and public access to the entire human genome have defined the beginning of this new era in biomedical research.

Still, proteomics, MS-based proteomics included, has many significant technical challenges to overcome. Mass spectroscopy of individual proteins has enabled us to

develop the ability to identify almost any protein, analyze the protein for the presence of post-translational modifications (PTM's), characterize its protein-protein interactions and provide structural information about the specific protein in gas-phase experiments. The potential of proteomics promises a high-throughput simultaneous analysis of many proteins in a specific physiologic state. As of yet, the advances in proteomics have translated into very few clinically useful applications.

Nevertheless, each technological breakthrough that permits a new type of measurement or improves the quality of data analysis expands the range of potential applications for this very promising field. Gobezie and his coworkers reported that using of MS-based proteomics and a novel experimental design to explore the potential of this technology for analysis of the complex mixture of proteins. And to identify specific biomarkers and potentially new etiologic factors in these diseases (Gobezie *et al*., 2012).

New technology and techniques for combining mass spectroscopy, or tandem mass spectroscopy, allow us to achieve unprecedented sensitivity and specificity for identifying individual proteins within complex protein mixtures. Hence, the goal of determining the proteome (a profile of all proteins expressed in the extracellular and/or intracellular environment) of body tissue in specific disease states is becoming a reality.

The implementation of proteomics technology will permit us to identify protein profiles and potential new etiologic proteins involved in the pathogenesis rheumatoid and osteoarthritis (Gobezie *et al*., 2012), similarly for low back pain too. The novel candidate proteins identified using these proteomics techniques will yield valuable therapeutic targets for new drug development.

In recent years, a novel analytical technology, metabolomics is widely used in the modern research of medical science. Metabolomics adopts a "top-down" strategy to reflect the function of organisms from terminal symptoms of metabolic network and understand metabolic changes of a complete system caused by interventions. As a holistic approach, metabolomics technology, including nuclear magnetic resonance, gas

chromatography-mass spectrometry, and liquid chromatography-mass spectrometry, favorable to express the meaning of basic theories of most of the diseases in medical science. Therefore, it is to be believed that metabolomics technology will greatly benefit to development for the research of disc degenerative disease in the light of modern sciences.

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

The present investigation was undertaken on "Understanding the genetic and molecular basis of disc degenerative disease in humans" was conducted at Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University. The collection of blood samples, disc tissues and MRI scanning of all the individuals were done at Ganga hospital, Coimbatore during 2011-2014. Sequenom analyses were performed at The University of Hong Kong, Hong Kong. The materials used and methodologies adopted are detailed as below:

I. Genetic studies of disc degenerative disease

3.1. Recruitment of study subjects

The Spine Unit, Ganga hospital identified 809 study population individuals of both sexes (455 male and 354 female) for control and affected category with early disc degeneration and suitable controls. All the members of the study population were evaluated by MRI (Magnetic Resonance Imaging) scan to identifying the various changes to the different components of the disc namely the nucleus pulposus, annulus fibrosus, end plate, disc bulge and presence of nodes.

Degenerative changes of the nucleus pulposus in plain MRI was recorded according to Pfirman grading (Rajasekaran *et al.,* 2008). Dong *et al*. (2007) reported the changes in the end plate in T1 and T2 sequences in plain MRI. Diffusion changes in the various parts of the disc were found by serial post-contrast MRI studies. A non-ionic contrast Gadiodamide (dye) was given and MRI images were obtained in all planes to document the diffusion of gadiodamide in various regions of the disc. Five serial scans were done over a period of 12 h and documented the quantity and temporal sequence of diffusion. These MRI results were graded according to Ganga Hospital Score (Total End Plate Score - TEPS) and other grading was correlated with the clinical symptoms.

All the 809 individuals were phenotyped by different phenotypic classification such as, disc bulge, HIZ/annular tears, Modic change, Schneiderman's score, Schmorl's Node, Total end plate score and Pfirmann score.

3.2. Phenotyping the individuals by MRI

This study involves seven highly selective phenotypes that depict degenerative changes in three different components of the disc (Table 3).

Table 3. Selection of phenotypes based on the disc component affected and the parameters used for quantifying disc degeneration

S. No	Phenotype	Disc component affected	Parameter
1.	Disc Bulge	Annulus fibrosus	Bulging of disc
2.	Annular tears		Hyperintense zones
3.	Pfirmann score	Nucleus pulposus	Pfirmann grading
4.	Schneiderman's score		Schneiderman's grading
5.	Schmorl's node	Vertebral end plate	Based on presence of nodes
6.	Modic change		Modic classification
7.	End plate damage		Total end plate score

3.2.1. Disc Bulge

Phenotypes were done according to the shape of the disc. For normal disc, score was given as 1 and 2 for bulged disc (Figure 4).

3.2.2. Annular tears / HIZ

Phenotypes were done according to appearance of annular tears. For normal annulus, score was given as 1 and 2 for collapsed annulus (Figure 5).

3.2.3. Modic change

Phenotypes were done according to Modic classification and grade pattern was 1, 2 and 3. (Table 4 and Figure 6). Disc with no change was given as score 0.

Table 4. Modic classification of vertebral endplate changes

Grade	T1 MRI Signal	T2 MRI Signal
1	Hypo intense	Hyper intense
2	Hyper intense	Isointense or Hyper intense
3	Hypo intense	Hypo intense

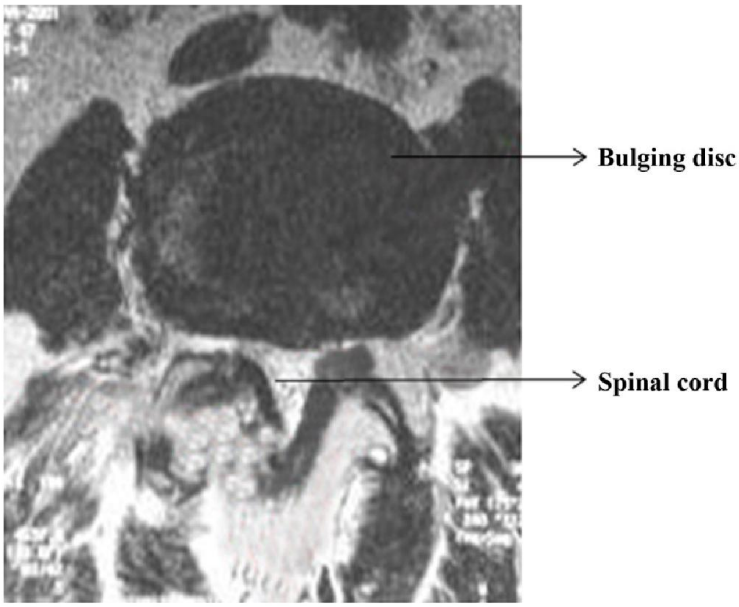


Figure 4. Axial T2 magnetic resonance image through the middle of a lumbar disc showing disc Bulge

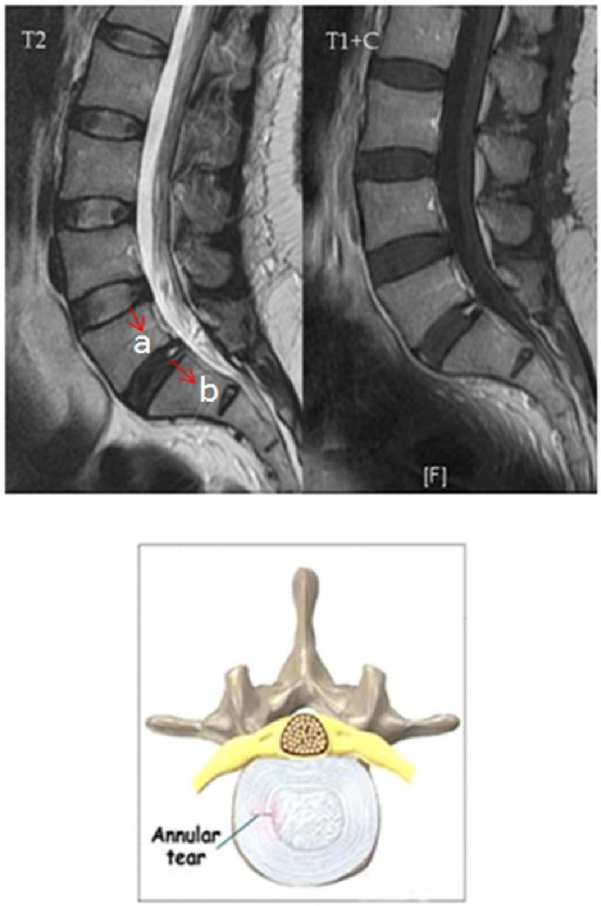
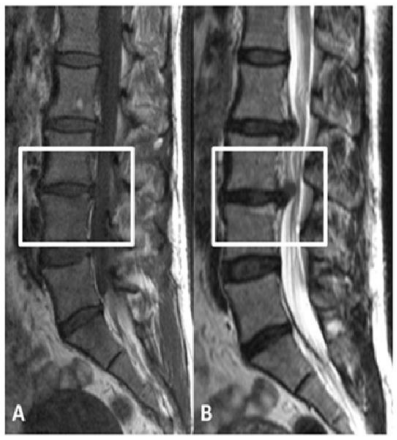
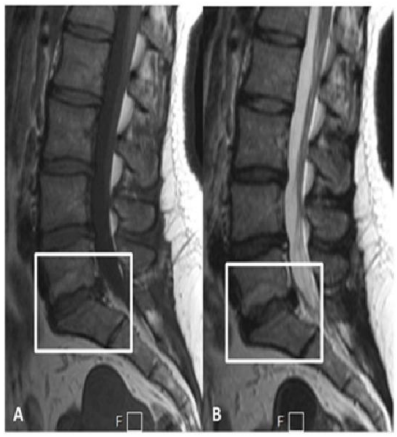


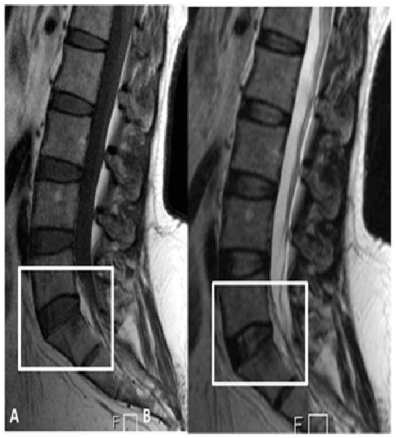
Figure 5. Sagittal MRI image of the lumbar spine indicating normal and collapsed view of annular Tears
a - normal b - collapsed



Type I Modic changes at L3-4 end plate
Hypointense T_1 signal, hyperintense T_2 signal



Type II Modic changes at L5-S1end plate
Hyperintense T_1 signal,hyperintense T_2 signal



Type III Modic changes at L5-S1 end plate
Hypointense T_1 signal, hypointense T_2 signal

Figure 6 . Modic Classification of lumbar spine

3.2.4. Schneiderman's score

Phenotypes were done according to Schneiderman's classification and grading was given as grade 0, 1, 2 and 3 (Table 5).

Table 5. Schneideman's classification of lumbar spine

Grade	Structure	Description
0	Normal	No narrowing of intervertebral disc, no osteophytes.
1	Minimal osteochondrosis or spondylosis	Minimal narrowing of intervertebral disc or minor osteophytes (<2 mm ventral or lateral)
2	Moderate osteochondrosis or spondylosis	Moderate narrowing (maximum half of adjacent unaffected discs) or moderate osteophytes (3 – 5 mm ventral or lateral, 1 – 2 mm dorsal)
3	Severe osteochondrosis or spondylosis	Severe narrowing (more than half of adjacent unaffected discs) or severe osteophytes (>5 mm ventral or lateral, >2 mm dorsal)

3.2.5. Schmorl's Node

Phenotype score was given according to presence of number of nodes present in the disc (Figure 7).

3.2.6. Total end plate score (TEPS)

Phenotypes were done according to the extent of damage of end plates into six grades (Table 6 and Figure 8).

3.2.7. Pfirmann score

Phenotypes were done according to Pfirman classification into 5 grades (Table 7 and Figure 9).

3.3. Survey of genetic polymorphism in the study population

3.3.1 Collection of blood samples

The blood samples were collected after MRI scanning examination from Ganga Hospital, Spine unit, Coimbatore. Blood samples were collected from 809 individuals in EDTA vacutainer tubes and stored at -80ºC for laboratory analysis. List of samples are given in annexure 1.

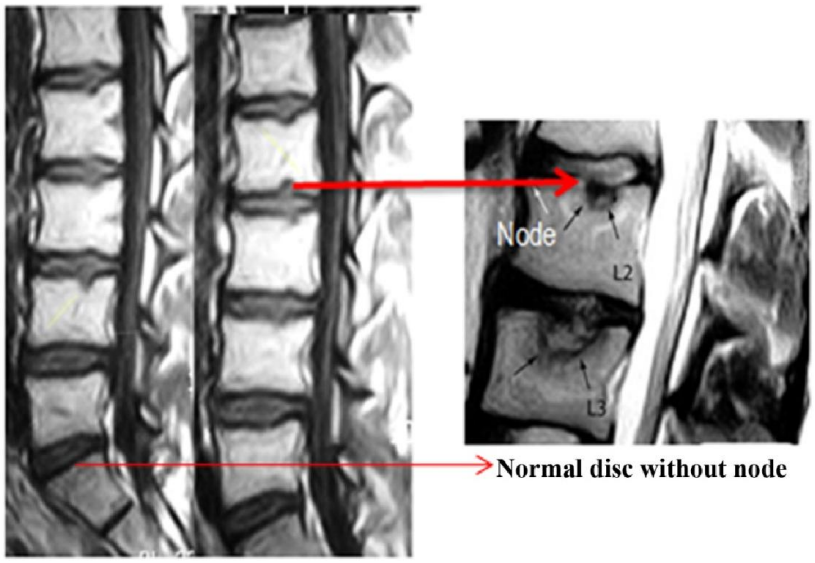


Figure 7. Sagittal MRI image of the lumbar spine showing the presence of Schmorl's Node

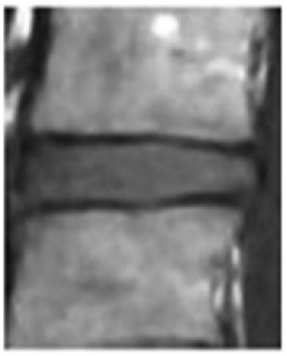
Table 6. Total end plate score classification of lumbar spine

Type	Characteristics	End Plate Score
I	No EP breaks/defects Uniform Hypointense band Symmetrically concave Not associated with Modic change	1
II	Focal thinning of the EP No EP breaks No Modic change	2
III	Focal disc marrow contacts Normal contour of EP maintained No Modic change	3
IV	Defect upto 25 % of width of EP Typical depression present Modic changes usually present	4
V	Defect upto 50 % of width of EP Typical depression present Modic changes usually present	5
VI	Complete EP damage Irregularity and sclerosis of EP Modic changes usually present	6

Table 7. Pfirmann classification of lumbar spine

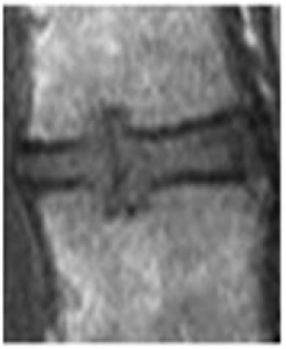
Grade	Structure	Distinction of Nucleus and Annulus	Signal Intensity	Height of Intervertebral Disc
1	Homogeneous, bright white	Clear	Hyperintense, isointense to cerebrospinal fluid	Normal
2	Inhomogeneous with or without horizontal bands	Clear	Hyperintense, isointense to cerebrospinal fluid	Normal
3	Inhomogeneous grey	Unclear	Intermediate	Normal to slightly decreased
4	Inhomogeneous grey to black	Lost	Intermediate to hypointense	Normal to moderately decreased
5	Inhomogeneous black	Lost	Hypointense	Collapsed disc space

Score 1

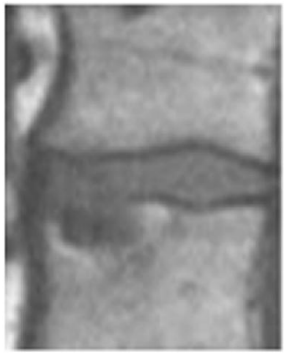


Score 2

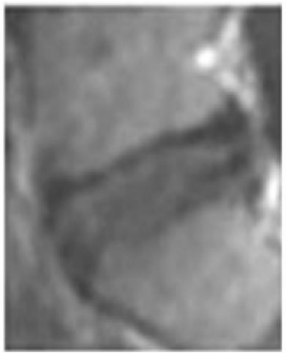
Score 3



Score 4



Score 5

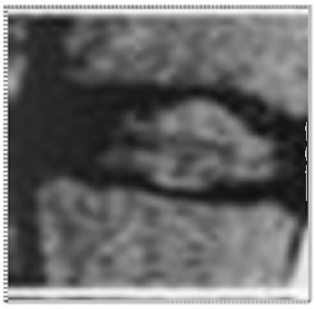


Score 6

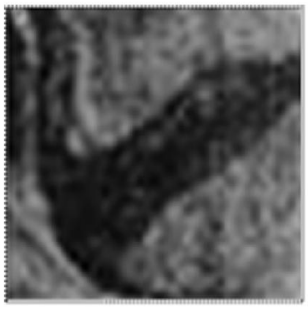
Figure 8. Characteristics of six different end plates based on severity of damag (TEPS Classification)

Score 1

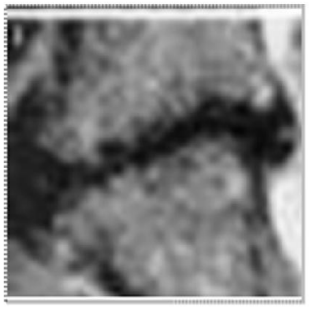
Score 2



Score 3



Score 4



Score 5

Figure 9. Pfirman grading of lumbar spine

3.3.2. Selection of putative candidate SNPs

Degenerative disc disease is a complex genetic disorder and mapping of disease associated genes by linkage analysis have not been successful in this. Therefore attention has focused on the rapid elucidation of a new class of genetic markers termed single-nucleotide polymorphisms (SNPs). Seventy-one putative potential candidate SNPs were selected for analysis based on previous genetic studies on DDD, candidate genes that encode for vital disc components, potential genes that have been implicated in other disease with multifactorial etiology, and genes involved in important intracellular signaling mechanisms (Table 8 and 9).

Table 8. Selection and analysis of 40 candidate genes and its functional zones

Functional Zones	**Candidate Genes**
Structural Genes - matrix	*COL9A2, COL11A1, COL22A1, COL9A1, AGC1, SKT, CHST3, GLI1, CILP, CALM1, IGF1R, COMP, HAPLN1*
Structural Genes - bone	*BMP5, BMP2, HHIP, VDR*
Apoptosis related/ neuronal genes	*NGFB, TAC1*
Degradative Genes	*MMP2, MMP7, MMP20, MMP9, MMP10, MMP1, MMP3, MMP12, MMP13, ADAMTS5, ADH2*
Inflammatory Genes	*IL18RAP, IL1A, IL1B, IL1F5, IL6, IL1F10, IL1RN, COX2,LEPR*

3.3.3. Isolation of genomic DNA from human blood

DNA was extracted from frozen human blood of both control and disease affected individuals using the protocol method along with modification suggested by Grimberg *et al*. (1989), as described below. The quality and quantity of DNA was checked by agarose gel electrophoresis and spectrophotometry. The final DNA concentration of all the samples was adjusted to 50 ng/µL.

Requirements

a**.** Blood samples in EDTA vacutainer tubes stored at -80ºC

b. Cell Lysis Buffer (CLB):

- 0.32 M sucrose
- 10mM Tris HCl (pH – 7.6)
- 5mM $MgCl_2$
- 1 % Triton X-100

Table 9. List of candidate genes analyzed in this study

S. No	Chromosome	SNP	Position	Genes
1.	1	rs1137100	65809029	*LEPR*
2.	1	rs8179183	65848540	*LEPR*
3.	1	rs1463035	103222784	*COL11A1*
4.	1	rs1337185	103317353	*COL11A1*
5.	1	rs2856813	115639442	*NGFB*
6.	1	rs4076018	115644333	*NGFB*
7.	1	rs5275	184909681	*COX2*
8.	1	rs2066826	184912550	*COX2*
9.	1	rs5277	184914820	*COX2*
10.	2	rs1420100	102403434	*IL18RAP*
11.	2	rs1800587	113259431	*IL1A*
12.	2	rs1143634	113306861	*IL1B*
13.	2	rs1143633	113306938	*IL1B*
14.	2	rs7575934	113533805	*IL1F5*
15.	2	rs2071375	113535438	*IL1A*
16.	2	rs579543	113606102	*IL1RN*
17.	2	rs11690459	113833538	*IL1F10*
18.	2	rs2234679	113875584	*IL1RN*
19.	2	rs7608342	215954325	*FN1*
20.	2	rs6709607	215964860	*FN1*
21.	2	rs1250240	216003259	*FN1*
22.	2	rs1250247	216007874	*FN1*
23.	2	rs1250258	216008430	*FN1*
24.	4	rs1229984	100458342	*ADH2*
25.	4	rs1812175	145794294	*HHIP*
26.	5	rs179851	82996626	*HAPLN1*
27.	6	rs966329	55798298	*BMP5*
28.	6	rs696990	71078947	*COL9A1*
29.	7	rs1800797	22732746	*IL6*
30.	7	rs1800796	22732771	*IL6*
31.	7	rs1800795	22733170	*IL6*
32.	7	rs2069837	22734552	*IL6*
33.	7	rs2066992	22734774	*IL6*
34.	7	rs1229434	97203778	*TAC1*
35.	7	rs3779470	97203867	*TAC1*
36.	8	rs2292927	139908094	*COL22A1*
37.	10	rs16924573	24644899	*SKT*
38.	10	rs4148941	73438998	*CHST3*
39.	10	rs4148949	73440657	*CHST3*
40.	11	rs1996352	101901457	*MMP7*
41.	11	rs1784438	101978396	*MMP20*
42.	11	rs1784430	101980023	*MMP20*

S. No	Chromosome	SNP	Position	Genes
43.	11	rs17099008	101987714	*MMP20*
44.	11	rs2701964	101999091	*MMP20*
45.	11	rs470154	102152520	*MMP10*
46.	11	rs11225422	102161528	*MMP10*
47.	11	rs2239008	102166290	*MMP1*
48.	11	rs491152	102171253	*MMP1*
49.	11	rs591058	102216548	*MMP3*
50.	11	rs672535	102261577	*MMP12*
51.	11	rs2252070	102331749	*MMP13*
52.	11	rs1940044	102421892	*MMP7*
53.	12	rs2228570	46559162	*VDR*
54.	12	rs2292657	56146199	*GLI1*
55.	12	rs2228224	56151588	*GLI1*
56.	14	rs2300496	89934601	*CALM1*
57.	14	rs3213718	89939666	*CALM1*
58.	14	rs1058903	89943551	*CALM1*
59.	15	rs2073711	63281265	*CILP*
60.	15	rs11856834	63283340	*CILP*
61.	15	rs2019185	63287488	*CILP*
62.	15	rs1551343	66872811	*ANP32A*
63.	15	rs1042631	87203243	*AGC1*
64.	15	rs11247361	97024915	*IGF1R*
65.	16	rs243865	54069307	*MMP2*
66.	19	rs3787049	18761512	*COMP*
67.	20	rs235770	6709765	*BMP2*
68.	20	rs2273073	6750882	*BMP2*
69.	20	rs17576	44073632	*MMP9*
70.	21	rs2249350	27244377	*ADAMTS5*
71.	21	rs162509	27247646	*ADAMTS5*

c. Nucleus Lysis Buffer (PLB):

- 10 mM Tris HCl (pH – 7.6)
- 10 mM NaCl
- 10 mM EDTA (pH – 8.0)
- Proteinase K@ 1mg/mL of PLB

*Preparation of buffers and concentrations are given in annexure 2

d. Chloroform: Isoamylalcohol 24:1 (v/v)

e. Ice-cold absolute ethanol (100 %)

f. Tris EDTA (TE) Buffer

Tris HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

(This was dissolved and made up to 100 mL, autoclaved and stored at 4^{o}C)

3.3.3.1. Extraction of genomic DNA

- About 250µL blood was taken in a 2 mL eppendorf tube and mixed with 4-fold excess of CLB buffer (1000µL). Centrifuged at 10000 rpm for 5 minutes then supernatant was discarded.
- Pellets were washed with 500 µL of CLB. Centrifuged at 10000 rpm for 5 minutes and supernatant was discarded.
- Pellet was washed with small aliquot of PLB buffer and centrifuged at 10000 rpm for 5 minutes and supernatant was discarded.
- About 60µL of PLB was added along with Proteinase K @1mg/mL. Incubated in water bath for 2 hours at 60ºC.
- After incubation, 100 µL of Chloroform: Isoamylalcohol (24:1) was added and centrifuged at 12000 rpm for 5 minutes.

- The upper layer was collected into the new eppendorf tube and double the volume (120 µL) of absolute ethanol was added and incubated for half an hour at -30ºC to precipitate the DNA.
- Centrifuged at 12000 rpm for 5 minutes and pellets were washed with 70 % ethanol and air dried
- Finally pellets were dissolved in 50 µl of 1X TE buffer and stored at -80ºC for further use.
- DNA quality was checked by 0.8 % agarose gel electrophoresis.

3.3.3.2. Agarose gel electrophoresis

Requirments

- Loading Dye

  Glycerol 50 % (v/v)
  Bromophenol blue 0.5 % (w/v)

- 10X TAE (Tris acetate EDTA buffer)

Tris Base	48.4 g
Glacial Acetic Acid	11.45 mL
0.5M EDTA ($Na_2.2H_2O$)	20 mL

(Dissolved in 800 mL of sterile water and made up to 1000 mL)

Protocol

- The Pyrex gel casting plate with open ends were sealed with cello tape and placed on a horizontal perfectly leveled platform.
- 0.8 % (0.8 g/100 mL) agarose was added to 1X TAE, boiled until the agarose dissolved completely and then allowed to cool. Ethidium bromide (DNA intercalating agent) was added when temperature reached 55-60^{o}C as a staining agent.

- Agarose gel was poured in to the gel casting tray. The comb was placed properly and allowed to solidify.
- The comb and the cello tape were removed carefully after solidification of the agarose.
- The casted gel was placed in the electrophoresis unit with wells towards the cathode and submerged with 1X TAE to a depth of about 1 cm.

Loading the DNA samples

- 2 µL of DNA sample dissolved in TE was pipetted onto a parafilm and mixed well with 2 µL of 10X loading dye by pipetting up and down several times.
- The gel was run at 80V for 1-1.5 hours and bands were visualized and documented using a gel documentation system (Model Alpha Imager 1200, Alpha Innotech Corp., USA).

3.3.3.3 Quantification of DNA

DNA was quantified by using Nanodrop (Nanodrop® Spectrophotometer ND-1000). The absorbance for all samples was measured at 260 nm as double stranded DNA has maximal absorbance at 260 nm.

Requirements

- NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, USA.)
- Sterile Milli-Q water
- TE buffer
- Total DNA Sample

Protocol

- The instrument was initialized with 2.0 µL of sterile milli-Q water.
- 2.0 µL of sterile milli-Q water was used as blank.
- 2.0 µL of unknown DNA was added to the instrument and the quantity measured.
- To ensure accuracy, the quantification of each sample was repeated twice.

3.3.4. SNP marker genotyping by Sequenom assay

Sequenom analysis was done at The University of Hongkong, Hongkong. The materials and methods used were as follows.

Genotyping was performed using the Sequenome® platform. The Mass ARRAY Assay Design software (Sequenom) was used to design amplification and allele-specific extension primers. The extension primer was designed to hybridize to the amplicon near the SNP site for the extension of a single base or a few bases depending on the genotype of the allele. PCR reactions were set up in 384 well plates at 6 μl total volume per reaction and the reaction mixture contains: 5 ng genomic DNA, 0.3 pmol each of specific forward and reverse primers, 200 μM of each dNTP, 3.25 mM $MgCl_2$ and 0.2 unit of Hot-Star Taq polymerase (5U/μL, Qiagen, Valencia, CA). The PCR condition was: 95^{o}C for 15 min, 45 cycles of 95^{o}C for 20 sec, 56^{o}C for 30 sec and 72^{o}C for 1 min, followed by 72^{o}C for 3 min. The treatment of PCR products with alkaline phosphatase and mass extend reactions were all performed according to manufacturer's (Sequenom) protocol. The final base-extension products were desalted using SpectroClean resin (Sequenom) mixed with 3-hydroxypicolinic acid and analyzed using a modified Brucker Autoflex MALDI-TOF mass spectrometer.

Sequenom analysis was done for all the 809 samples along with duplicate check and distributed into 96 well plates. All the samples were processed with 71 primers.

3.4. Association studies

The SNPs prevalent in all the gene of study population was predicted by using PLINK software. Association test was performed by using PLINK software based on the nature of SNPs. Probability value and odds ratio was estimated for all the seventy one genes, also tested their significance towards DDD.

II. Proteomic Studies

3.5. Sample detail

The IVD was obtained from a fifty two year old female who underwent spine surgery for lumbar disc prolapse at Spine unit, Ganga hospital, Coimbatore, India. It was approved by ethical clearance committee for utilizing the tissue sample for research purpose.

3.6. Protein extraction from IVD

Requirements

- Liquid nitrogen
- 4 M Guanidine HCl
- 50 mM Sodium acetate
- 65 mM DTT
- 10mM EDTA
- Mini-Protease inhibitor cocktail
- Ice-cold ethanol
- IEF rehydration buffer

Protocol

- IVD tissue samples were pulverized with liquid nitrogen and solubilized in 1 mL of 4 M guanidine HCl, 50 mM sodium acetate, pH 5.8, containing 65 mM DTT, 10 mM EDTA, and mini-protease inhibitor cocktail with shaking at 4^{o}C for 18 hours. (Belluoccio *et al*., 2006).
- Insoluble material was removed by centrifugation at 13000 x g for 10 min at 4^{o}C. This procedure effectively removes the cross-linked collagen II fibrils, (Heinegard *et al*., 1982), facilitating the analysis of less abundant proteins.
- The supernatant was collected and precipitated for 2 hours at – 20^{o}C using nine volumes of ice-cold ethanol, followed by centrifugation at 10000 x g for 20 min and two washes in 70 % ethanol to remove residual guanidine HCl and other salts.
- The precipitate were lyophilized in a freeze dryer for overnight and it is resuspended in IEF rehydration buffer composed of 5 M urea, 2 M thiourea, 2 % CHAPS, 2 % Ampholytes 4 - 7 (BioRad), 65 mM DTT, and 40 mM Tris, pH 8.0 and lysed at 37^{o}C for 1 hour with intermittent vortexing.
- The complex was centrifuged at 12000 rpm for 20 min at room temperature and supernatant was collected. Protein concentrations were measured by spectrophotometry using Bradford assay (Bradford, 1976).

3.7 Protein Profiling through 2D-PAGE

A novel sample preparation approach was followed to increase the amount of resolvable proteins through 2D-PAGE. Molecular weight cut off (MWCO) centrifugal filters were used to purify protein extract from contaminating lipids and polysaccharides. The protein extract was passed through 100 kDa MWCO centrifugal filter (Amicon, USA) and the filtrate was collected and used for 2D PAGE analysis.

3.7.1. First dimensional separation by isoelectric focusing

Requirements

- IPG strips
- Rehydration buffer (8 M urea, 2 % CHAPS, DTT)
- Reswelling tray
- IEF unit

Protocol

- IPG strips (BioRad Laboratories, USA) of 17-cm length and pH 4–7 were used and it is rehydrated overnight with 350 µl of rehydration buffer (8M Urea, 2 % CHAPS, DTT (7 mg per 2.5 mL of rehydration buffer) and 0.5 % (v/v) IPG buffer pH 4-7) containing the required quantity of proteins (150 µg) in a reswelling tray (GE Healthcare, USA) at room temperature.
- The strips were allowed 12-14 hours for rehydration and subjected to first dimension separation.
- Isoelectric focusing was performed at 20ºC with a GE Healthcare Lifesciences Multiphor II kit with following subsequent steps: 500 V for 30 minutes, 1000 V for 30 minutes and 3000 V for 10 hours.
- Prior to the second dimension, the IPG strips were equilibrated twice for 15min each in 20 mL/strip of equilibration solution containing 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS, 50mM Tris-HCl, 1 % DTT was added to the first equilibration solution.
- The second equilibration was performed in a solution modified by the replacement of DTT by 2 % (w/v) iodoacetamide.

3.7.2. Second dimensional separation by SDS-PAGE

Requirements

- Acrylamide
- Bis-acrylamide
- 1.5M Tris buffer
- SDS (10 %)
- TEMED
- Ammonium persulfate
- Isopropanol
- Agarose sealing solution

3.7.2.1. Preparation of the gel solution

The gel glass plates, spacer and comb were cleaned thoroughly with water and then with alcohol. The spacers were kept at the sides between the two glass plates. The gel mixture (12 %) was prepared as follows.

Acrylamide: Bis-acrylamide mixture	:	47.50 mL
1.5M Tris buffer (pH 8.8)	:	30.00 mL
SDS (10 %)	:	1.20 mL
Water	:	40.50 mL
TEMED	:	00.60 mL
Ammonium persulfate (10 %)	:	00.60 mL
Total	:	120.40 mL

Ammonium per-sulphate and TEMED were added finally, mixed well and the gel mixture was poured into the plate assembly. Isopropanol was layered over the surface to minimize exposure of the polymerizing solution to oxygen and thus to create a flat gel surface. The gel was allowed to polymerize for two hours. After polymerization of the gel, isopropanol was poured off and the gel surface was rinsed with running buffer.

Equilibrated IPG strips were rinsed with electrode buffer and placed on the top of SDS gel and overlaid with 2 mL of agarose solution.

3.7.2.2. Electrophoresis

- Slab gel apparatus was connected to a circulating water bath and temperature of circulating water was adjusted to 4°C.
- Gels were electrophoresed at constant current (15 mA) till the dye front reaches the bottom of the gel.
- Gels were removed from their gel cassettes and fixed in the fixative (4:1:5 of methanol:acetic acid:water).

3.7.2.3. Silver staining

- The gels were stained by silver staining method as described by Blum *et al.,* (1987).
- After fixing for one hour, gels were washed (30 % Ethanol) twice for 20 minutes each. Third washing was done with deionized water. Gels were sensitized for 1 min in 0.02 % thiosulfate reagent.
- Gels were washed thrice in deionized water (30 seconds each) and impregnated in 0.2 % Silver nitrate solution for 30-60 min.
- The gels were developed after thorough washing using deionized water (thrice) using a developer (30 g sodium carbonate and 5 mg of sodium thiosulfate in 1000 mL water) for 8 to 10 minutes.
- The reaction was stopped using a stopper reagent and rinsed again with deionized water. Silver stained gels were scanned using the Image master 2D platinum version 7, Scanner III (GE Healthcare, USA).

3.7.3. In-gel trypsin digestion of proteins

Abundant protein spots of different intensities and regions of the 2-D gel were selected for Peptide Mass Fingerprinting analysis. Samples were excised manually from a silver-stained 2-D gel and extracted by an addition of 10µl of the extraction buffer, followed by an addition of 10–15 µl of acetonitrile. Pooled extracts were dried in a freeze dryer and

the extracts were re-dissolved in 1µl of extraction buffer and 1µl of matrix solution (α-acyano- 4-hydroxycinnamic acid, HCCA) (Shevchenko *et al*., 1996).

3.7.3.1. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI - TOF)

Dried samples were targeted onto a MALDI-TOF plate. After drying the samples completely onto the targeting plate, MALDI-TOF was conducted using a Voyager- DE STR mass spectrometer (Applied Biosystems, Franklin Lakes, NJ, USA) equipped with delay ion extraction. Mass spectra were obtained over a mass range of 800–3,000 Da.

3.7.3.2. Protein identification by PMF

For identification of proteins, the peptide mass fingerprinting data were used to search against the NCBI database using the Mascot program (http://www.matrixscience.com). The following parameters were used for database searches: taxonomy, *Homo sapiens* (human); cleavage specificity, trypsin with one missed cleavage allowed; peptide tolerance of 100 ppm for the fragment ions; and allowed modifications, Cys Carbamidomethyl (fixed), and oxidation of Met (variable). The protein score was −10*log (P), where P is the probability that the observed match is a random event.

III. Metabolomic studies

3.8. Sample collection

Human intervertebral disc tissue was obtained from Spine unit, Ganga hospital, Coimbatore, India. For method development and validation, a representative healthy and affected disc tissue was snap-frozen immediately following surgery and then stored at -80^{o}C.

3.8.1. Metabolite extraction from disc tissue

Requirements

- Chloroform
- Methanol
- Toluene
- Sodium sulphate (anhydrous)
- MSTFA
- TMCS

Protocol

- About 20mg of the stored tissue weighed accurately and ground with liquid nitrogen. 1mL of a mono-phasic mixture of chloroform/methanol/water in ratio of 20:50:20 (v/v/v) was added to each sample. (Mal *et al*., 2009).
- The samples were ultrasonicated in an ultra-sonicator bath at ambient temperature (24–28^{o}C) for 100 min and then vortex-mixed for 2 min.
- The samples were subsequently centrifuged at 12000 rpm for 10 min and the supernatant was collected separately from each sample in different tubes.
- The collected supernatant was concentrated to complete dryness at 50^{o}C for 30 min.
- A 100 µL of toluene (kept anhydrous with sodium sulfate) was added to each of the sample extracts, vortex-mixed for 5 min and again evaporated to complete dryness using vacuum evaporator in order to eliminate any trace amount of water which might interfere with the GC/MS analysis.
- The dried samples were then derivatized by adding 100 µL of MSTFA with 1 % TMCS to each sample.
- The samples were then vortex-mixed for 2 min and incubated at 70^{o}C for 30 min. After incubation, samples were again vortex-mixed for 2 min and then transferred to glass vials for GC/MS analysis.

Experimental Results

CHAPTER IV

EXPERIMENTAL RESULTS

The present study was undertaken with an aim of understanding the genetic and molecular basis of Disc Degenerative Disease in humans. Eight hundred and nine individuals were genotyped for 71 putative candidate SNPs to study the association between genetic polymorphism in the candidate gene and DDD. Further, abundant proteins expressed in the IVD were profiled through 2D PAGE and metabolite profiling was carried out in the normal and degenerated discs with a view to identify molecular level changes during degeneration. The results obtained are detailed as below:

4.1. Understanding genetic basis of DDD

4.1.1. Study Population

The present study was conducted in 809 individuals. There was no gender bias in the selection of the study population (455 men and 354 women). The age of the patients ranged between 10 to 80 years with a mean age of 36.76±10.80. The study population was phenotyped for seven different parameters *viz*., disc bulge, annular tears, Modic change, Schneiderman's score, Schmorl's Node, total end plate score and Pfirmann score and the population was divided into cases and controls for each phenotypic trait and used for association analysis.

4.1.2. Clinical evaluation

Detailed clinical studies were conducted in all the individuals by the Spine Unit, Ganga hospital, Coimbatore through MRI, Scan. Severity of the DDD was scored based on the changes in the different components of the disc namely the nucleus pulposus, annulus fibrosus and end plate. Frequency of control and cases differed for each phenotypic trait (Figure 10 and Table 10). Out of 809 individuals, based on disc bulge nature, 474 were classified as DDD patients and 335 as control. 281 were classified as DDD patients and 528 as control based on the appearance of annular tears. 107 were classified as DDD patients and 702 as control by Modic change. According to Pfirman score, 494 were classified as DDD patients and 315 as control. According to presence of Schmorl's Node, 147 were classified as DDD patients and 662 as control. According to TEPS, 121 were classified as DDD patients and 688

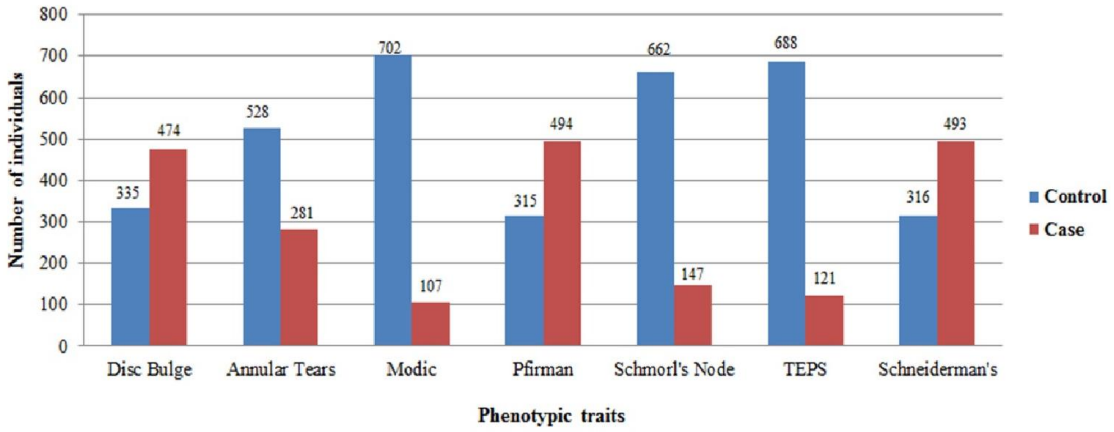


Figure 10. Prevalence of DDD in the Indian study population based on seven different phenotypic traits

Table 10. Demographics of cases and controls divided based on their phenotypes

Phenotypic traits		Number of subjects	Male	Female	Age (years)	
					Mean ±SD	Range
Total		809	455	354	36.76±10.80	10 - 80
Disc bulge	Controls	335	170	165	34.32±10.25	12 - 75
	Cases	474	290	184	38.48±10.85	10 - 80
Annular tears	Controls	528	288	240	36.04±11.43	10 - 80
	Cases	281	167	114	38.12±9.35	14 - 67
Modic change	Controls	702	398	304	35.93±10.54	10 - 71
	Cases	107	57	50	42.17±10.94	25 - 80
Pfirman score	Controls	315	159	156	33.46±10.08	10 - 71
	Cases	494	296	198	38.86±10.72	12 - 80
Schmorl's node	Controls	662	363	299	36.52± 10.90	10 - 80
	Cases	147	92	55	37.82±10.30	14 - 63
Total end plate score	Controls	688	376	312	35.64±10.06	10 - 75
	Cases	121	79	42	43.12±12.54	14 - 80
Schneiderman's score	Controls	316	160	156	33.48±10.08	10 - 71
	Cases	493	295	198	38.86±10.73	12 - 80

as controls. According to Schneiderman's score, 493 were classified as DDD patients and 316 as controls.

4.1.3. Association analysis between SNPs of candidate genes and various degenerative features of IVD

It has been demonstrated that with the help of the total end plate score it is possible to define the extent of disc damage (Rajasekaran *et al*., 2008). Similarly, it is possible to associate disc abnormalities with other degenerative features of IVD. In this study, changes in seven phenotypic features, *viz.,* Pfirman score, Modic change, Schneiderman's score, disc bulge, annular tears, Schmorl's Node and total end plate score were investigated. Frequencies of alleles, p value of χ^2 tests in various genetic models as well as the odds ratio based on allele frequency on various phenotypes, obtained using the software PLINK, were summarized in tables 13, 15 and 17.

There was wide variation in the results of the association analysis depending on the individual phenotype studied. In this study, association analysis carried out for the individual disc level, based on whole disc scoring for the total population and for the patients less than 40 years of age.

4.1.3.1) Association between candidate SNPs and DDD at individual lumbar disc level for 809 individuals

Study population were classified as case and control based on Pfirman, Modic, Schneiderman's, disc bulge, annular tears, Schmorl's Node and TEPS at individual lumbar disc level (Table 11a and 11b). Association analysis was done for all the seven different phenotypes at individual lumbar disc level (Table 12). The results of the analysis as follows

i) SNPs associated with disc bulge

The analyses of association of various SNP markers were tested by individual disc level with disc bulge for this study (Table 13a). At lumbar level 1, rs2019185, rs11856834 and rs2073711 of *CILP* was significantly associated with a p value of 0.01008, 0.03618 and 0.03954 respectively. At level 2, *CILP (*rs2073711, s11856834, rs2019185); *MMP20* (rs1784438) and *IL1F5* (rs7575934) were significantly associated. At level 3, *TAC1*

Table 11a. Prevalence of DDD by different phenotypic traits in the population at individual disc level

Case / Control	Lumbar disc level	Disc bulge	Annular tears	Pfirman score	Schmorl's Node	TEPS	Schneiderman's score
Control	L1-2	777	799	656	737	622	635
Case		32	10	153	72	187	174
Control	L2-3	753	784	630	727	616	606
Case		56	25	179	82	193	203
Control	L3-4	679	755	541	739	605	529
Case		130	54	268	70	204	280
Control	L4-5	427	618	387	737	503	372
Case		382	191	422	72	306	437
Control	L5-S1	445	645	417	757	464	399
Case		363	164	392	52	345	410

Table 11b. Prevalence of DDD by Modic change in the population at individual disc level

Case / Control	Lumbar level	Modic Change	Case / Control	Lumbar level	Modic Change
Control	UL1	800	Control	LL1	801
Case		9	Case		8
Control	UL2	797	Control	LL2	790
Case		12	Case		19
Control	UL3	790	Control	LL3	768
Case		19	Case		41
Control	UL4	777	Control	LL4	764
Case		32	Case		45
Control	UL5	779	Control	LL5	773
Case		30	Case		36

Table 12. Number of SNP markers associated with DDD for seven different phenotypes at individual lumbar disc level

S.No	Disc level	Disc bulge	Annular tears	Modic changes		Schneiderman's score	Schmorl's Node	Total end plate score	Pfirman score
				Upper level	Lower level				
1	L1-2	3	2	2	-	3	2	1	2
2	L2-3	5	7	3	4	5	1	1	5
3	L3-4	6	6	-	2	2	6	1	2
4	L4-5	6	5	7	2	3	4	-	3
5	L5-S1	6	4	3	2	1	4	3	1

Table 13a. Summary of association analysis of putative candidate genes with disc bulge at individual lumbar disc level

Lumbar disc level	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
1	15	*CILP*	rs2019185	T	C	0.5172	0.3520	0.010080**	1.972
	15	*CILP*	rs11856834	T	C	0.5517	0.4133	0.036180*	1.747
	15	*CILP*	rs2073711	C	T	0.5517	0.4156	0.039540*	1.730
2	15	*CILP*	rs2019185	T	C	0.4904	0.3486	0.003617**	1.798
	15	*CILP*	rs11856834	T	C	0.5278	0.4103	0.017050*	1.607
	15	*CILP*	rs2073711	C	T	0.5283	0.4127	0.020150*	1.594
	2	*IL1F5*	rs7575934	T	C	0.0556	0.1282	0.038930*	0.400
	11	*MMP20*	rs1784438	A	G	0.2685	0.3675	0.026760*	0.632
3	7	*TAC1*	rs3779470	T	C	0.2389	0.1691	0.012180*	1.542
	2	*FN1*	rs1250247	C	G	0.4103	0.4952	0.016930*	0.709
	2	*FN1*	rs1250240	A	G	0.4068	0.4889	0.020330*	0.717
	20	*MMP9*	rs17576	A	G	0.3941	0.4731	0.025260*	0.724
	1	*LEPR*	rs8179183	C	G	0.1496	0.1006	0.026920*	1.572
	2	*FN1*	rs1250258	C	T	0.4068	0.4810	0.035940*	0.740
4	15	*CILP*	rs11856834	T	C	0.4542	0.3882	0.009560**	1.311
	15	*CILP*	rs2019185	T	C	0.3908	0.3304	0.014930*	1.300
	15	*CILP*	rs2073711	C	T	0.4542	0.3921	0.014990*	1.290
	2	*FN1*	rs1250258	C	T	0.4380	0.4963	0.024230*	0.791
	2	*FN1*	rs1250247	C	G	0.4506	0.5087	0.025130*	0.792
	2	*FN1*	rs1250240	A	G	0.4456	0.5025	0.027570*	0.796
5	1	*NGFB*	rs2856813	A	G	0.5092	0.4362	0.004976**	1.341
	7	*TAC1*	rs1229434	A	G	0.4894	0.4182	0.005977**	1.333
	21	*ADAMTS5*	rs162509	G	C	0.3632	0.2972	0.006682**	1.349
	15	*CILP*	rs2019185	T	C	0.3933	0.3314	0.013010*	1.308
	15	*CILP*	rs11856834	T	C	0.4502	0.3941	0.028440*	1.259
	15	*CILP*	rs2073711	C	T	0.4500	0.3981	0.043080*	1.237

** Significant association with p value <0.01 * Significant association with p value <0.05

*(*rs3779470*)*; *FN1* (rs1250247, rs1250240, rs1250258); *MMP9* (rs17576); *LEPR* (rs8179183) and at level 4, *CILP (*rs2073711, s11856834, rs2019185); *FN1* (rs1250247, rs1250240, rs1250258) were significantly associated. At level 5, *NGFB* (rs2856813); *ADAMTS5* (rs162509) *TAC1 (*rs1229434); *CILP (*rs2073711, s11856834, rs2019185) and *MMP7 (*rs1996352) were significantly associated. Among them *CILP (*rs2019185) from level 1, level 2 and level 4, *NGFB* (rs2856813), *ADAMTS5* (rs162509) and *TAC1* (rs1229434) from level 5 were strongly associated with the p value of 0.01008, 0.003617, 0.00956, 0.004976, 0.006682 and 0.005977 respectively.

ii) SNPs associated with HIZ/Annular tears

The analyses of association of various SNP markers were tested by individual disc level with annular tears for this study (Table 13b). Markers from *MMP10* (rs470154) and *MMP1* (rs491152) at level 1; *VDR* (rs2228570), *MMP7* (rs1996352 and rs1940044), *CHST3* (rs4148941 and rs4148949), *CILP* (rs2019185) and *CALM1* (rs3213718) at level 2; *COX2* (rs5275), *TAC1* (rs3779470), *IGF1R* (rs11247361), *COL11A1* (rs1463035 and rs1337185) and*MMP7* (rs1996352) at level 3; *IL1RN* (rs2234679), *IL1B* (rs1143633); *CILP*(rs2019185, rs2073711 and rs11856834) at level 4; *ADAMTS5* (rs2249350 and rs162509), *MMP1* (rs2239008) and *COL9A1* (rs696990). Among them rs470154 of *MMP10,* rs491152 of *MMP1* at level 1; rs2228570 of *VDR* at level 2; rs5275 of *COX2* and rs3779470 of *TAC1* at level 3; rs2249350 of *ADAMTS5,* rs2239008 of *MMP1* and rs696990 of *COL9A1* at level 5 were significantly associated with p value of 0.006329, 0.007757, 0.002482, 0.001767, 0.007447, 0.000771, 0.003046 and 0.003680 respectively.

iii) SNPs associated with Modic change

The analyses of association of various SNP markers were tested by individual disc level with annular tears for this study (Table 13c). A marker of *IL6, ANP32A, LEPR, MMP20, IGF1R, COX2, VDR, FN1,* ADAMTS5, *IL1B, CHST3, CALM1* and *MMP1* were found to be significant. Among them rs2069837 of *IL6* from upper level 1, rs2066826 of *COX2* from upper level 4; rs4148941 and rs4148949 of *CHST3* from upper level 5; rs3213718 and rs2300496 of *CALM1,* rs8179183 of *LEPR* from lower level 2and rs491152 of *MMP1* from lower level 3 were the most significant markers associated with Modic change with

Table 13b. Summary of association analysis of putative candidate genes with annular tears at individual lumbar disc level

Lumbar disc level	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
1	11	*MMP10*	rs470154	A	C	0.3571	0.1184	0.006329**	4.138
	11	*MMP1*	rs491152	T	C	0.3571	0.1217	0.007757**	4.011
2	12	*VDR*	rs2228570	T	C	0.4167	0.2077	0.002482**	2.725
	11	*MMP7*	rs1940044	T	C	0.3500	0.1901	0.011650*	2.295
	10	*CHST3*	rs4148949	T	C	0.3000	0.4979	0.013510*	0.432
	10	*CHST3*	rs4148941	C	A	0.3000	0.4925	0.016290*	0.442
	11	*MMP7*	rs1996352	C	T	0.3750	0.2170	0.017530*	2.165
	15	*CILP*	rs2019185	T	C	0.5250	0.3538	0.025920*	2.018
	14	*CALM1*	rs3213718	T	C	0.5250	0.3638	0.036950*	1.933
3	1	*COX2*	rs5275	C	T	0.2000	0.3855	0.001767**	0.399
	7	*TAC1*	rs3779470	T	C	0.3000	0.1740	0.007447**	2.035
	15	*IGF1R*	rs11247361	G	C	0.4028	0.2687	0.012950*	1.835
	1	*COL11A1*	rs1337185	C	G	0.0139	0.0967	0.018120*	0.132
	11	*MMP7*	rs1996352	C	T	0.3333	0.2156	0.018810*	1.819
	1	*COL11A1*	rs1463035	G	A	0.0417	0.1340	0.022790*	0.281
4	2	*IL1RN*	rs2234679	C	G	0.3171	0.2487	0.012900*	1.402
	2	*IL1B*	rs1143633	A	G	0.2994	0.2405	0.031220*	1.350
	15	*CILP*	rs2019185	T	C	0.4074	0.3449	0.037730*	1.306
	15	*CILP*	rs2073711	C	T	0.4695	0.4073	0.043630*	1.288
	15	*CILP*	rs11856834	T	C	0.4665	0.4054	0.047310*	1.282
5	21	*ADAMTS5*	rs2249350	A	C	0.1918	0.1175	0.000771**	1.781
	11	*MMP1*	rs2239008	A	G	0.1448	0.2235	0.003046**	0.588
	6	*COL9A1*	rs696990	G	A	0.4349	0.3440	0.003680**	1.468
	21	*ADAMTS5*	rs162509	G	C	0.3793	0.3130	0.030450*	1.341

** Significant association with p value <0.01 * Significant association with p value <0.05

Table 13c. Summary of association analysis of putative candidate genes with Modic changes at individual lumbar disc level

Lumbar disc level	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
Upper level 1	7	*IL6*	rs2069837	G	A	0.3889	0.1517	0.005661**	3.557
	15	*ANP32A*	rs1551343	G	A	0.4444	0.2306	0.032950*	2.669
Upper level 2	1	*LEPR*	rs8179183	C	G	0.2500	0.1060	0.024310*	2.812
	11	*MMP20*	rs17099008	C	A	0.0833	0.0188	0.024700*	4.734
	15	*IGF1R*	rs11247361	G	C	0.0833	0.2782	0.033930*	0.236
Upper level 4	1	*COX2*	rs2066826	A	G	0.2742	0.1445	0.005084**	2.237
	12	*VDR*	rs2228570	T	C	0.0807	0.2187	0.009368**	0.313
	2	*FN1*	rs7608342	A	G	0.5645	0.4146	0.019210*	1.830
	15	*IGF1R*	rs11247361	G	C	0.1452	0.2807	0.019280*	0.435
	21	ADAMTS5	rs162509	G	C	0.4516	0.3206	0.031220*	1.745
	2	*IL1B*	rs1143634	T	C	0.2419	0.1439	0.033310*	1.898
	2	*FN1*	rs6709607	C	T	0.5484	0.4178	0.041630*	1.692
Upper level 5	10	*CHST3*	rs4148941	C	A	0.6786	0.4799	0.003518**	2.288
	10	*CHST3*	rs4148949	T	C	0.6786	0.4853	0.004544**	2.239
	21	*ADAMTS5*	rs162509	G	C	0.4655	0.3204	0.020820*	1.847
Lower level 2	14	*CALM1*	rs3213718	T	C	0.5789	0.3626	0.006324**	2.417
	14	*CALM1*	rs2300496	A	C	0.5526	0.3445	0.007915**	2.350
	1	*LEPR*	rs8179183	C	G	0.2368	0.1049	0.009789**	2.647
	2	*FN1*	rs1250258	C	T	0.6316	0.4651	0.042360*	1.971
Lower level 3	11	*MMP1*	rs491152	T	C	0.2368	0.1179	0.002149**	2.323
	11	*MMP1*	rs2239008	A	G	0.1184	0.2135	0.046910*	0.495
Lower level 4	12	*VDR*	rs2228570	T	C	0.1163	0.2188	0.024160*	0.470
	1	*COX2*	rs2066826	A	G	0.2273	0.1450	0.035970*	1.734
Lower level 5	12	*VDR*	rs2228570	T	C	0.1520	0.2227	0.022070*	0.626
	11	*MMP20*	rs17099008	C	A	0.0388	0.0169	0.035790*	2.354

** Significant association with p value <0.01 * Significant association with p value <0.05

a p value of 0.005661, 0.005084, 0.009368, 0.003518, 0.004544, 0.006324, 0.007915, 0.009789 and 0.002149 respectively.

iv) SNPs associated with Schneiderman's score

Markers from *CALM1, COX2, FN1, SKT, GLI1, IL1B* and*IL18RAP* were found significantly associated on Schneiderman's score (Table 13d). Among them rs2300496 of *CALM1* from level 1, 2 and *3,* rs1250247 and rs1250240 of *FN1* from level 2*,* rs3213718 of *CALM1* from level 3 was the most significant markers associated with p value of 0.004475, 0.007811, 0.002139, 0.002714, 0.006698 and 0.006076 respectively.

v) SNPs associated with Schmorl's node

Markers from *MMP7, COX2, MMP20, BMP2, MMP12, COL11A1, HAPLN1, IL1F10, TAC1, MMP2* and *IL1B* were found to be significant with Schmorl's node (Table 13e). Among them, rs1996352 of *MMP7* at level 1, 2 and 5; rs243865 of *MMP2* at level 4 was the most significant markers associated with p value of 0.000425, 0.000425, 0.002383 and 0.006609 respectively.

vi) SNPs associated with total end plate score (TEPS)

The analyses of association of various SNP markers were tested by individual disc level with total end plate score for this study (Table 13f). A marker of *MMP7* at level 1; *ADAMTS5* at level 2 and 3; *NGFB, COX2* and *MMP1* at level 5 were found to be significant with a p value of 0.03574, 0.03154, 0.02204, 0.03534, 0.03626 and 0.03852 respectively.

vii) SNPs associated with Pfirman score

Marker from *CALM1, FN1, IL1B, GLI1, SKT* and *IL18RAP* and were found significantly associated with Pfirman score (Table 13g). Among them rs2300496 of *CALM1* at level 1 and 3; rs1250247 of *FN1* at level 2; rs3213718 of *CALM1* at level 3 was the most significant marker associated with p value of 0.008117, 0.002569, 0.003531 and 0.005356 respectively.

Table 13d. Summary of association analysis of putative candidate genes with Schneiderman's score at individual lumbar disc level

Lumbar disc level	Chromosome	Gene	Marker	P Value
1	14	*CALM1*	rs2300496	0.004475**
	14	*CALM1*	rs3213718	0.014850*
	1	*COX2*	rs5275	0.041320*
2	2	*FN1*	rs1250247	0.002714**
	2	*FN1*	rs1250240	0.006698**
	14	*CALM1*	rs2300496	0.007811**
	14	*CALM1*	rs3213718	0.010190*
	2	*FN1*	rs1250258	0.014660*
3	14	*CALM1*	rs2300496	0.002139**
	14	*CALM1*	rs3213718	0.006076**
4	10	*SKT*	rs16924573	0.020570*
	12	*GLI1*	rs2228224	0.038250*
	2	*IL1B*	rs1143634	0.046130*
5	2	*IL18RAP*	rs1420100	0.023080*

** Significant association with p value <0.01

* Significant association with p value <0.05

Table 13e. Summary of association analysis of putative candidate genes with Schmorl's node at individual lumbar disc level

Lumbar disc level	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
1	11	*MMP7*	rs1996352	C	T	0.3380	0.2091	0.000425**	1.932
	1	*COX2*	rs2066826	A	G	0.2170	0.1430	0.019640*	1.665
2	11	*MMP7*	rs1996352	C	T	0.3010	0.2113	0.000425**	1.609
3	11	*MMP20*	rs1784438	A	G	0.2740	0.3697	0.022040*	0.643
	11	*MMP7*	rs1996352	C	T	0.2950	0.2133	0.024700*	1.539
	20	*BMP2*	rs235770	T	C	0.5070	0.4092	0.025380*	1.486
	11	*MMP12*	rs672535	T	C	0.3220	0.4136	0.032020*	0.673
	11	*MMP20*	rs1784430	G	A	0.2780	0.3669	0.033910*	0.664
	1	*COL11A1*	rs1463035	G	A	0.1850	0.1237	0.036350*	1.607
4	16	*MMP2*	rs243865	T	C	0.2140	0.1310	0.006609**	1.809
	11	*MMP7*	rs1996352	C	T	0.3000	0.2131	0.018340*	1.582
	5	*HAPLN1*	rs179851	G	A	0.3570	0.4560	0.025040*	0.663
	2	*IL1F10*	rs11690459	A	C	0.0880	0.1543	0.038880*	0.530
5	11	*MMP7*	rs1996352	C	T	0.3400	0.2126	0.002383**	1.905
	1	*COX2*	rs5277	C	G	0.0940	0.0421	0.013040*	2.371
	11	*MMP20*	rs1784438	A	G	0.2550	0.3689	0.018210*	0.585
	2	*IL1B*	rs1143634	T	C	0.0750	0.1529	0.030130*	0.452

** Significant association with p value <0.01 * Significant association with p value <0.05

Table 13f. Summary of association analysis of putative candidate genes with total end plate score at individual lumbar disc level

Lumbar disc level	Chromosome	Gene	Marker	P Value
1	11	*MMP7*	rs1996352	0.03574*
2	21	*ADAMTS5*	rs2249350	0.03154*
3	21	*ADAMTS5*	rs2249350	0.02204*
5	1	*NGFB*	rs2856813	0.03534*
	1	*COX2*	rs2066826	0.03626*
	11	*MMP1*	rs2239008	0.03852*

* Significant association with p value <0.05

Table 13g. Summary of association analysis of putative candidate genes with Pfirmann score at individual lumbar disc level

Lumbar disc level	Chromosome	Gene	Marker	P Value
1	14	*CALM1*	rs2300496	0.008117**
	14	*CALM1*	rs3213718	0.021260*
2	2	*FN1*	rs1250247	0.003531**
	2	*FN1*	rs1250240	0.010250*
	14	*CALM1*	rs3213718	0.011650*
	14	*CALM1*	rs2300496	0.011770*
	2	*FN1*	rs1250258	0.024920*
3	14	*CALM1*	rs2300496	0.002569**
	14	*CALM1*	rs3213718	0.005356**
4	2	*IL1B*	rs1143634	0.020700*
	12	*GLI1*	rs2228224	0.028350*
	10	*SKT*	rs16924573	0.038630*
5	2	*IL18RAP*	rs1420100	0.015960*

** Significant association with p value <0.01
* Significant association with p value <0.05

4.1.3.2) Association between candidate SNPs and DDD (based on whole disc scoring) in 809 individuals.

Association analysis has been done for all the seven different phenotypes based on whole disc scoring (Table 14). The results of the analysis as follows

i) SNPs associated with disc bulge

The analysis of association of various SNP markers with disc bulge (Table 15a) revealed that rs11856834, rs2019185 and rs2073711 (markers of *CILP*); rs2228570 (markers of *VDR*); rs1940044 (marker for *MMP7*); rs1229434 (marker for *TAC1*) and rs2069837 (marker for *IL6)* and were significantly associated with a p value of 0.01083, 0.01236, 0.01896, 0.04986, 0.03277, 0.02199 and 0.0397respectively.

ii) SNPs associated with HIZ/Annular tears

HIZ is an indication of annular tear. The analysis of association of various SNP markers with annular tears (Table 15b) revealed that rs2249350 (marker for *ADAMTS5*), rs11247361 (marker for *IGF1R*), rs2239008 (marker for *MMP1*), rs696990 (marker for *COL9A1*) and rs2234679 (marker for *IL1RN*) were found to be significant. Among them rs2249350 of *ADAMTS5* were significantly associated with p value of 0.01325.

iii) SNPs associated with Modic change

The analysis of association of various SNP markers with Modic change revealed that markers from *VDR* and *MMP20* were found to be significant (Table 15c). Among them rs2228570 of *VDR* was the most significant marker associated with p value of 0.02207.

iv) SNPs associated with Schneiderman's score

The analysis of association of various SNP markers with Schneiderman's score (Table 15d) revealed that rs2300496 and rs3213718 (markers for *CALM1*); rs16924573 (marker for *SKT*); rs1250247 and rs1250240 (markers for *FN1*); rs5275 (marker for *COX2*) and rs4076018 (marker for *NGFB*) were significantly associated with p value of 0.02658, 0.04353, 0.04865, 0.033878, 0.04869, 0.03955 and 0.04867 respectively**.**

Table 14. Number of SNP markers associated with DDD for seven different phenotypes based on whole disc scoring

S. No	Phenotype	No. of associated SNP markers
1	Disc bulge	7
2	Annular tears	5
3	Modic change	2
4	Schneiderman's score	7
5	Schmorl's Node	3
6	Total end plate score	-
7	Pfirman score	3

Table 15a. Summary of association analysis of putative candidate genes with disc bulge based on whole disc scoring

S.No	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
1	15	*CILP*	rs11856834	T	C	0.4458	0.3802	0.01083*	1.312
2	15	*CILP*	rs2019185	T	C	0.3844	0.3215	0.01236*	1.317
3	15	*CILP*	rs2073711	C	T	0.4458	0.3851	0.01896*	1.284
4	12	*VDR*	rs2228570	T	C	0.2303	0.1877	0.04986*	1.295
5	11	*MMP7*	rs1940044	T	C	0.1761	0.2203	0.03277*	0.757
6	7	*TAC1*	rs1229434	A	G	0.4740	0.4142	0.02199*	1.274
7	7	*IL6*	rs2069837	G	A	0.1385	0.1773	0.03970*	0.746

* Significant association with p value <0.05

Table 15b. Summary of association analysis of putative candidate genes with annular tears based on whole disc scoring

S.No	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
1	21	*ADAMTS5*	rs2249350	A	C	0.1615	0.1161	0.01325*	1.467
2	15	*IGF1R*	rs11247361	G	C	0.3154	0.2540	0.01116*	1.353
3	11	*MMP1*	rs2239008	A	G	0.1788	0.2245	0.03842*	0.752
4	6	*COL9A1*	rs696990	G	A	0.3966	0.3424	0.03722*	1.262
5	2	*IL1RN*	rs2234679	C	G	0.2950	0.2470	0.04372*	1.276

* Significant association with p value <0.05

Table 15c. Summary of association analysis of putative candidate genes with Modic change based on whole disc scoring

S.No	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
1	12	VDR	rs2228570	T	C	0.1520	0.22270	0.02207*	0.626
2	11	MMP20	rs17099008	C	A	0.0388	0.01687	0.03579*	2.354

* Significant association with p value <0.05

Table 15d. Summary of association analysis of putative candidate genes with Schneiderman's score based on whole disc scoring

S.No	Chromosome	Gene	Marker	P Value
1	14	*CALM1*	rs2300496	0.026580*
2	14	*CALM1*	rs3213718	0.043530*
3	10	*SKT*	rs16924573	0.048650*
4	2	*FN1*	rs1250247	0.033878*
5	2	*FN1*	rs1250240	0.048690*
6	1	*COX2*	rs5275	0.039550*
7	1	*NGFB*	rs4076018	0.048670*

* Significant association with p value <0.05

v) SNPs associated with Schmorl's node

The analysis of association of various SNP markers with Schmorl's node (Table 15e) revealed that rs1784438, rs1784430 (markers for *MMP20*) and rs11225422 (marker for *MMP10*) was found to be significantly associated with a p value of 0.03143, 0.03871 and 0.04271 respectively.

vi) SNPs associated with Pfirman score

The analysis of association of various SNP markers with total end plate score (Table 15f) revealed that rs2300496, rs3213718 (markers for *CALM1*) and rs1250247 (marker for *FN1*) was found to be significantly associated with a p value of 0.03293, 0.04167 and 0.03402 respectively.

4.1.3.3) Association between candidate SNPs and DDD for the young adults (less than 40 years of age) based on whole disc scoring in 543 individuals

The young adult populations (543 individuals) were classified as case and control based on Pfirman, Modic, Schneiderman's, disc bulge, annular tears, Schmorl's Node and TEPS at individual lumbar disc level (Table 16). Association analysis has been done for all the seven phenotypes based on whole disc scoring for the young adults (Table 17). The results of the analysis as follows

i) SNPs associated with disc bulge

The analysis of association of various SNP markers with disc bulge (Table 18a) revealed that rs1940044 (marker of *MMP7*); rs7575934 (marker of *IL1F5*); rs3787049 (markers of *COMP*) and rs8179183 (marker of *LEPR*) were significantly associated with a p value of 0.000388, 0.0159, 0.3776 and 0.03697 respectively.

ii) SNPs associated with HIZ/Annular tears

The analysis of association of various SNP markers with annular tears (Table 18b) revealed that rs696990 (marker for *COL9A1*), rs2249350 (marker for *ADAMTS5*), rs162509 (marker for *ADAMTS5*) and rs11247361 (marker for *IGF1R*) were significantly associated with a p value of0.006066, 0.006465, 0.02507 and 0.04342 respectively.

Table 15e. Summary of association analysis of putative candidate genes with Schmorl's node based on whole disc scoring

S.No	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
1	11	*MMP20*	rs1784438	A	G	0.3050	0.3732	0.03143*	0.737
2	11	*MMP20*	rs1784430	G	A	0.1511	0.1072	0.03871*	1.482
3	11	*MMP10*	rs11225422	G	A	0.3058	0.3703	0.04271*	0.749

* Significant association with p value <0.05

Table 15f. Summary of association analysis of putative candidate genes with Pfirman score based on whole disc scoring

S.No	Chromosome	Gene	Marker	P Value
1	14	*CALM1*	rs2300496	0.03293*
2	14	*CALM1*	rs3213718	0.04167*
3	2	*FN1*	rs1250247	0.03402*

* Significant association with p value <0.05

Table 16. Prevalence of DDD by different phenotypic traits for the young adults

S. No	Phenotypes	Control	Case
1.	Disc Bulge	254	289
2.	Annular Tears	367	176
3.	Modic	492	51
4.	Pfirman	243	300
5.	Schmorl's Node	453	90
6.	TEPS	491	52
7.	Schneiderman's	243	300

Table 17. Number of SNP markers associated with DDD for seven different phenotypes for the young adults (less than 40 years of age) based on whole disc scoring

S. No	Phenotype	No. of associated SNP markers
1	Disc bulge	4
2	Annular tears	4
3	Modic change	5
4	Schneiderman’s score	8
5	Schmorl’s Node	1
6	Total end plate score	-
7	Pfirman score	6

Table 18a. Summary of association analysis of putative candidate genes with disc bulge for the young adults (less than 40 years of age) based on whole disc scoring

S.No	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
1	11	*MMP7*	rs1940044	T	C	0.1489	0.2363	0.000388**	0.566
2	2	*IL1F5*	rs7575934	T	C	0.0959	0.1450	0.015900*	0.626
3	19	*COMP*	rs3787049	T	C	0.0130	0.0336	0.027230*	0.378
4	1	*LEPR*	rs8179183	C	G	0.1278	0.0869	0.036970*	1.540

** Significant association with p value <0.01 * Significant association with p value <0.05

Table 18b. Summary of association analysis of putative candidate genes with annular tears for the young adults (less than 40 years of age) based on whole disc scoring

S.No	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
1	6	*COL9A1*	rs696990	G	A	0.4303	0.3416	0.006066**	1.456
2	21	*ADAMTS5*	rs2249350	A	C	0.1727	0.1111	0.006465**	1.670
3	21	*ADAMTS5*	rs162509	G	C	0.3567	0.2872	0.025070*	1.376
4	15	*IGF1R*	rs11247361	G	C	0.3171	0.2565	0.043420*	1.346

** Significant association with p value <0.01 * Significant association with p value <0.05

iii) SNPs associated with Modic change

The analysis of association of various SNP markers with Modic change revealed that markers from *MMP1, MMP20, VDR, SKT* and *AGC1*were found to be significant (Table 18c). Among them rs491152 of *MMP1* was the most significant marker associated with p value of 0.005842.

iv) SNPs associated with Schneiderman's score

Markers from *CALM1, MMP7, FN1, COMP*and *COX2* were found significantly associated with Schneiderman's score (Table 18d). Among them rs2300496 *of CALM1* and rs1940044 of *MMP7* were the most significant marker associated with p value of 0.004651 and 0.007937 respectively.

v) SNPs associated with Schmorl's node

Using Schmorl's node as phenotype, rs1996352 of *MMP7* was associated significantly with p value of 0.02452 respectively (Table 18e).

vi) SNPs associated with Pfirman score

Markers from *CALM1, FN1 MMP7*and*COMP* were found significantly associated with Pfirman score (Table 18f). Among them rs2300496 of *CALM1* and rs1940044 of *MMP7* was the most significant marker associated with p value of 0.004651 and 0.007937 respectively.

In summary, 17 genes *viz., CILP, VDR, MMP7, TAC1, IL6, ADAMTS5, IGF1R, MMP1, COL9A1, IL1RN, MMP20, MMP10, CALM1,FN1, SKT, COX2* and *NGFB* were associated at 5 % significant level with DDD at all the five lumbar disc levels. 33 genes were associated with DDD at individual disc level, out of 33 markers, 16 markers *viz*., *LEPR, NGFB, COX2, FN1, COL9A1,TAC1, IL6, MMP1, MMP7, MMP10, CHST3, VDR, CALM1, CILP, MMP2 and ADAMTS5* was associated at 1 % significant level. Fifteen genes were associated with DDD for the young adult population. Out of 15 genes, five genes *viz*., MMP7, COL9A1, CALM1, MMP1 and ADAMTS5 was associated at 1 % significant level.

Table 18c. Summary of association analysis of putative candidate genes with Modic change for the young adults (less than 40 years of age) based on whole disc scoring

S.No	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
1	11	*MMP1*	rs491152	T	C	0.2000	0.1070	0.005842**	2.087
2	11	*MMP20*	rs1784438	A	G	0.2600	0.3736	0.024780*	0.589
3	12	*VDR*	rs2228570	T	C	0.1224	0.2186	0.026280*	0.499
4	10	*SKT*	rs16924573	A	G	0.1400	0.0797	0.040720*	1.880
5	15	*AGC1*	rs1042631	T	C	0.2100	0.3050	0.048030*	0.606

** Significant association with p value <0.01 * Significant association with p value <0.05

Table 18d. Summary of association analysis of putative candidate genes with Schneiderman's score for the young adults (less than 40 years of age) based on whole disc scoring

S.No	Chromosome	Gene	Marker	P Value
1	14	*CALM1*	rs2300496	0.004651**
2	11	*MMP7*	rs1940044	0.007937**
3	2	*FN1*	rs1250247	0.010840*
4	14	*CALM1*	rs3213718	0.015980*
5	19	*COMP*	rs3787049	0.026800*
6	2	*FN1*	rs1250240	0.035610*
7	2	*FN1*	rs1250258	0.047550*
8	1	*COX2*	rs5277	0.047900*

** Significant association with p value <0.01 * Significant association with p value <0.05

Table 18e. Summary of association analysis of putative candidate genes with Schmorl's node for the young adults (less than 40 years of age) based on whole disc scoring

S.No	Chromosome	Gene	Marker	Allele 1	Allele 2	Frequency of allele 1		P Value	OR
						Case	Control		
1	11	*MMP7*	rs1996352	C	T	0.2907	0.2121	0.02452*	1.523

** Significant association with p value <0.01 * Significant association with p value <0.05

Table 18f. Summary of association analysis of putative candidate genes with Pfirman score for the young adults (less than 40 years of age) based on whole disc scoring

S.No	Chromosome	Gene	Marker	P Value
1	14	*CALM1*	rs2300496	0.004651**
2	11	*MMP7*	rs1940044	0.007937**
3	2	*FN1*	rs1250247	0.010840*
4	14	*CALM1*	rs3213718	0.015980*
5	19	*COMP*	rs3787049	0.026800*
6	2	*FN1*	rs1250240	0.035610*

** Significant association with p value <0.01 * Significant association with p value <0.05

4.2. Profiling Proteins Expressed in Human Intervertebral Discs

Intervertebral disc contains abundant of proteoglycans in the extracellular matrix which needs high salt content for separation and purification. A new method for extracting the proteins with guanidium hydrochloride, precipitating with nine volumes of ice cold ethanol and followed by two ethanol washes to remove the excess guanidium hydrochloride salt. In 2D-PAGE analysis, it resulted in poor resolution and horizontal streaking due to poor solubility of the proteins (Figure 11a). The protein isolation was modified through trial and error method to overcome these problems. To improve the solubility and resolution of the proteins for 2D-PAGE analysis, contaminating proteoglycans were removed by using MWCO filters. First, to remove the large aggregating proteoglycans, samples were passed through 50 kDa MWCO (Molecular weight cut off centrifugal filter). Figure 11b shows that this 50 kDa MWCO filter was proven to remove large aggregates effectively.

Only 72 spots (Figure 11b) were identified while using 50 kDa MWCO filter. Further improvement has to be done to increase the number of abundant spots. Therefore, 100 kDa MWCO filters were used in order to increase the abundant spots with better resolution (Figure 11c). Upon silver staining of the 2D gels, about 143 proteins spots were detected consistently. This shows the dramatic increase in the number of abundant spots in the filtered extract of 100 kDa filter. This may be due to the removal of the high molecular weight proteoglycan aggrecan and hyaluronan from the protein sample, which interferes with IEF.

Abundant protein spots (30 spots) were cut out from the gel and analyzed through MALDI-TOF for peptide mass fingerprinting (Figure 12). The identity of the protein spots were revealed by MASCOT analysis with *Homo sapiens* as taxonomy filter. Out of 30 protein spots identified, 25 spots were with significant matches. The peptide mass fingerprinting of those remaining 5 spots did not generate significant matches. This may be due to less amount of protein obtained from the particular spot. Identified protein name, its localization, theoretical and experimental molecular weight, iso-electric point, sequence coverage and score were given in table 19.

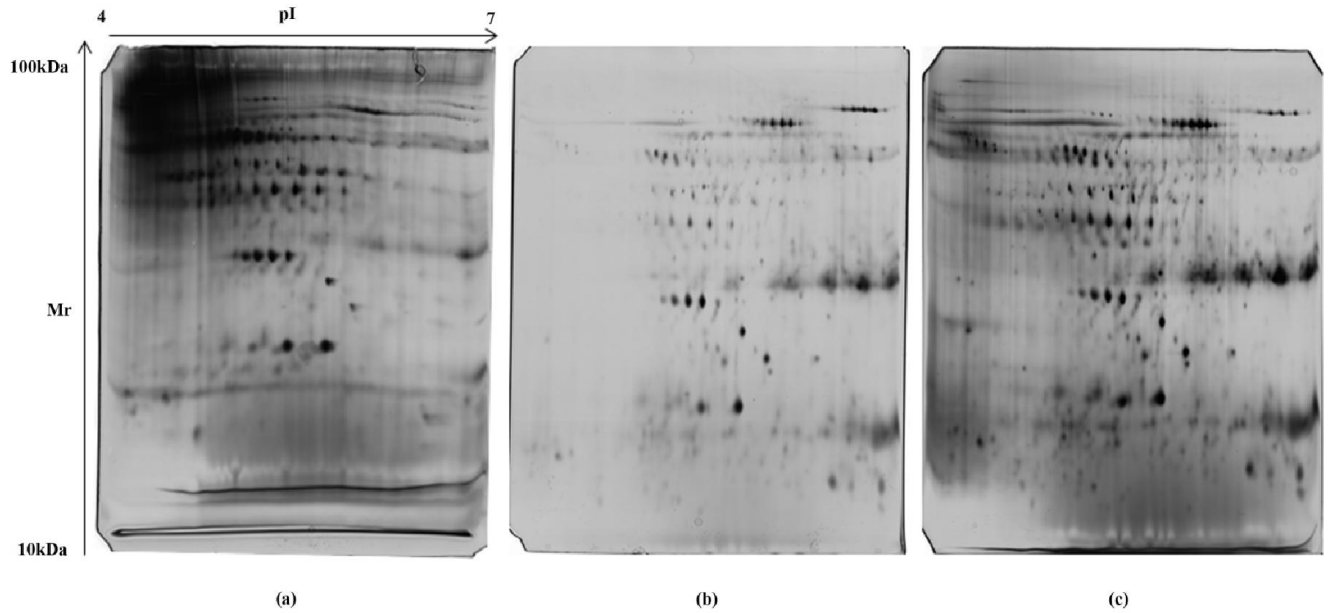


Figure 11. 2D PAGE protein profiles of intervertebral disc tissue
(a) 2D profile from unfiltered extract (b) using 50 kDa MWCO filter (c) using 100 kDa MWCO filter

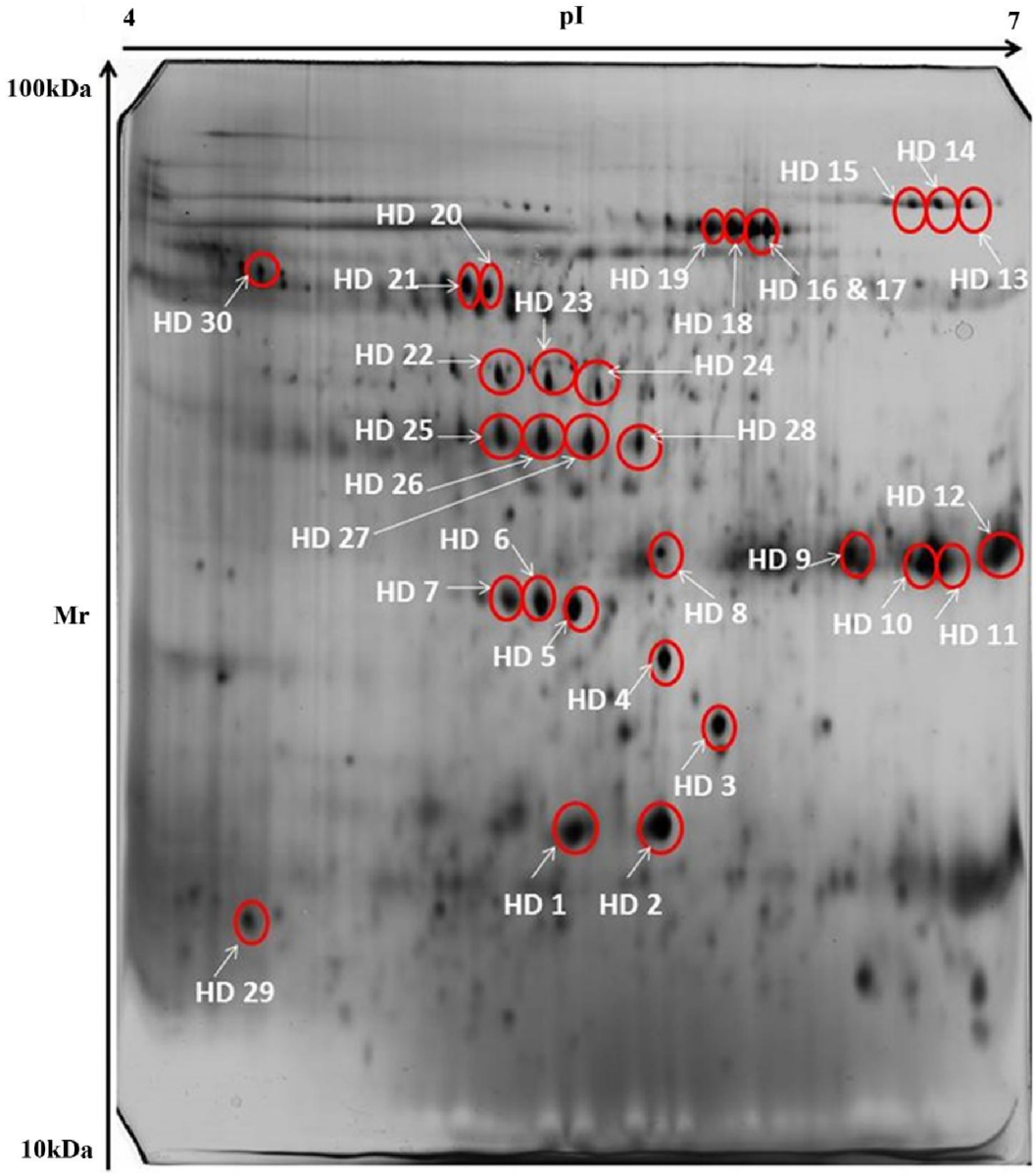


Figure 12. 2D PAGE protein profiles of intervertebral disc tissue extract filtered through 100kDa

Table 19. Identification of IVD proteins by MASCOT analysis

(A) Significant spots

Spot ID [1]	Experimental [2]		Coverage [3]	Number of peptides matched [4]	Theoretical [5]		Accession No	Protein Identity [6]
	pI	MW			pI	MW		
HD1	5.46	19	48	27	5.19	49.68	167887751	Vimentin variant 3
	5.46	19	25	16	5.21	53.54	19908424	Mutant desmin
HD2	5.77	19	31	15	5.04	41.32	205360981	Protein RIC-3 isoform a precursor [Homo sapiens]
HD3	5.98	24	11	38	5.62	414.17	119619469	Dystrophin (muscular dystrophy, Duchenne and Becker types), isoform CRA_c [*Homo sapiens*]
HD4	5.78	29	47	21	5.29	42.56	914833	Keratin type II
HD5	5.45	33	64	21	5.27	28.06	90108665	Chain A, Crystal Structure Of Lipid-Free Human Apolipoprotein A-I
HD6	5.33	33	49	15	5.27	28.06	90108664	Chain A, Crystal Structure Of Lipid-Free Human Apolipoprotein A-I
HD7	5.21	33	30	29	6.39	97.26	62088402	Promyelocytic leukemia protein isoform 1 variant [*Homo sapiens*]
HD9	6.46	38	27	14	9.07	61.10	119612556	PHD finger protein 20-like 1, isoform CRA_c [*Homo sapiens*]
	6.46	38	27	13	9.1	48.13	4929613	CGI-72 protein [*Homo sapiens*]
HD10	6.73	36	37	35	5.77	99.60	3721836	Huntingtin-interacting protein 1-related protein HIP1R [*Homo sapiens*]
HD12	6.90	38	30	11	9.53	43.85	3660517	Nebulette [*Homo sapiens*]
HD14	6.79	90	48	36	6.99	58.79	194387990	unnamed protein product [*Homo sapiens*]

Spot ID [1]	Experimental [2]		Coverage [3]	Number of peptides matched [4]	Theoretical [5]		Accession No	Protein Identity [6]
	pI	MW			pI	MW		
HD15	6.69	90	21	12	9.07	61.10	119612556	PHD finger protein 20-like 1, isoform CRA_c [*Homo sapiens*]
HD16	6.16	85	27	32	8.27	133.60	31455194	NUMA1 protein [*Homo sapiens*]
HD18	6.03	85	44	81	5.06	191.47	40788217	KIAA0336 [*Homo sapiens*]
HD19	5.95	85	28	15	9	56.65	119592961	Zinc finger protein 135 (clone pHZ-17), isoform CRA_d [*Homo sapiens*]
HD20	5.13	73	54	116	5.54	224.61	110624781	Myosin-13 [*Homo sapiens*]
HD21	5.05	74	64	6	9.37	13.71	159163872	Chain A, Solution Structure Of The Mitochondrial Ribosomal Protein L17 Isolog
HD22	5.17	59	42	11	9.84	35.22	332245896	Alpha-tubulin N-acetyltransferase isoform 4 [*Homo sapiens*]
	5.17	59	43	11	9.95	34.08	13543925	C6orf134 protein
HD23	5.35	58	63	20	8.96	32.98	39653325	PHD finger protein 20-like protein 1 isoform 3 [*Homo sapiens*]
HD24	5.54	57	25	19	5.82	99.44	121934188	KIF5C protein [*Homo sapiens*]
HD25	5.18	50	26	14	5.29	64.94	385198093	Shootin-1 isoform c [*Homo sapiens*]
HD26	5.33	50	64	28	9.53	43.85	3660517	Nebulette [*Homo sapiens*]
HD27	5.50	49	27	12	5.29	64.94	385198093	Shootin-1 isoform c [*Homo sapiens*]
HD28	5.69	50	39	15	8.61	33.14	4867999	Ku70-binding protein [*Homo sapiens*]
HD30	4.30	75	14	69	5.04	532.42	17426164	Macrophin 1 isoform 2 [*Homo sapiens*]

(B) Non-Significant spots

Spot ID [1]	Experimental [2]		Coverage [3]	Number of peptides matched [4]	Theoretical [5]		Accession No	Protein Identity [6]
	pI	MW			pI	MW		
HD8	5.77	38	61	6	9.37	13.71	159163872	Chain A, Solution Structure Of The Mitochondrial Ribosomal Protein L17 Isolog
HD11	6.80	37	34	5	4.67	11.37	134254718	Amyloid precursor-like protein 1 isoform 1 precursor [*Homo sapiens*]
HD13	6.90	90	33	4	6.07	13.37	296676	T-cell receptor V-beta 8.3 [*Homo sapiens*]
HD17	6.11	85	61	5	7.85	7.48	145938571	Immunoglobulin heavy chain variable region [*Homo sapiens*]
HD29	4.25	15	27	11	5.29	64.94	385198093	Shootin-1 isoform c [*Homo sapiens*]

[1] ID refers to the corresponding spot number in figure 2
[2]Experimental MW and p*I* calculated by Image Master 2D Platinum software image analysis.
[3]Amino acid sequence coverage and [4] number of peptides for the proteins identified by MASCOT.
[5] Theoretical MW and p*I* values were calculated using NCBI computed p*I*/MW.
[6]Proteins were identified by using the peptide masses from MALDI-TOF analysis, followed by data base search. Corresponding accession numbers for the identified proteins were obtained from NCBI (www.ncbi.nlm.nih.gov).

4.3. Metabolic profiling from intervertebral disc tissue

GC/MS analysis from this study led to the identification of 75 different metabolites (Figure 13 and 14) belonging to diverse chemical classes such as amino acids, organic acids, fatty acids, phenolics, silanes, nitriles, ethers, amines, azides, hydrocarbons, alcohols, carbonyl compounds, heterocyclic compounds, spiro compounds and metal complex (Table 20 and 21). The identities of selected metabolites were recognized by the NIST mass spectral library. In summary, this study has identified the seventy-five different chemical compounds in IVD.

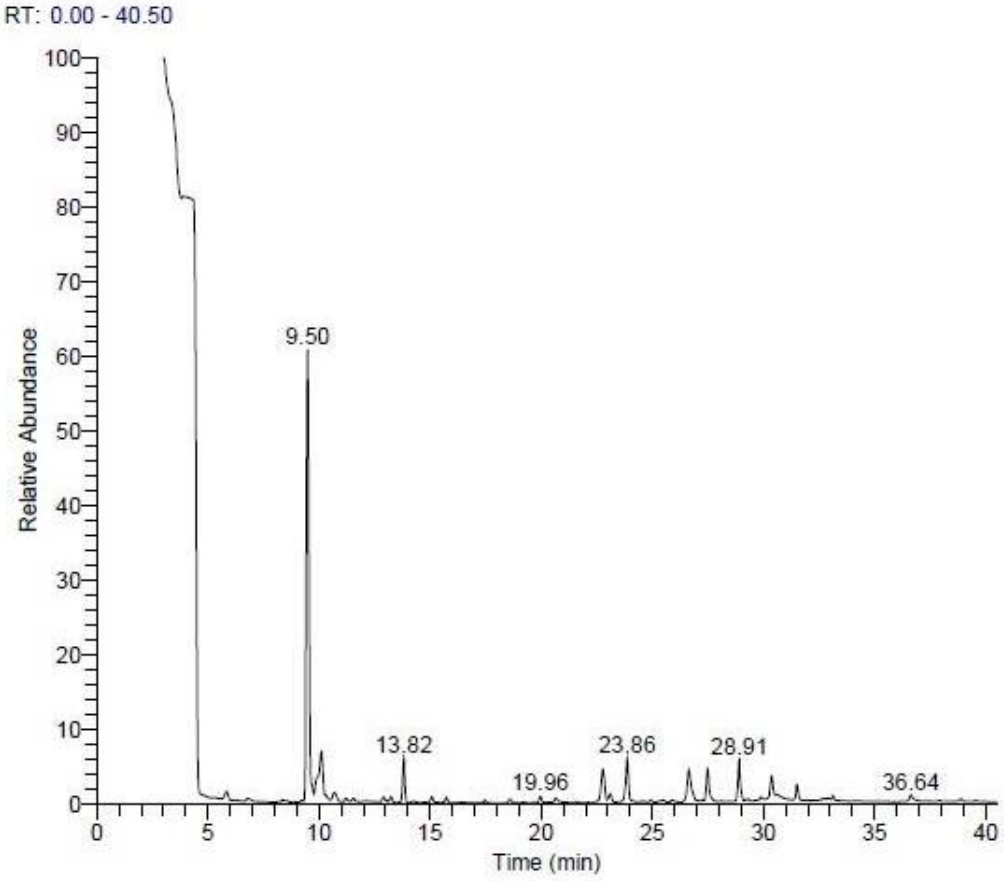


Figure 16. GC/MS chromatograms of control intervertebral disc

Table 20. Identified metabolites from control intervertebral disc tissue through GC/MS against NIST library search

S. No	Metabolites	Retention time (min)	Chemical groups identified
1	Cyclopropanecarboxylic acid, pent-2-en-4-ynyl ester	3.06	Organic acid and its derivatides
2	N1-(Formyl)-N2-(1-oxobut-2-en-1-yl)hydrazide	3.45	Azides
3	exo-5-hexyl-exo-4-oxa-tricyclo[5.2.1.0**2,6]dec-8-en-3-one	4.43	Carbonyl compounds
4	Oxime-, methoxy-phenyl-_	4.92	Carbonyl compounds
5	D-Lactic acid-DITMS	5.28	organic acid and its derivatives
6	Methyl 3-((aminocarbonyl)amino)-2-cyano-3-phenylpropenoate	5.87	Organic acid and its derivatides
7	2-[5-(2-hydroxyethyl)-2-thienyl]-4,4-dimethyloxazoline	6.28	Heterocyclic compounds
8	Decane, 2-methyl-	6.77	Hydrocarbons
9	1,2-Dioxetane, 3,4,4-trimethyl-3-[[(trimethylsilyl)oxy]methyl]-	7.38	Silanes
10	5,8-Diethoxy-7-methoxyquinoline	8.05	Heterocyclic compounds
11	Octanoic acid, trimethylsilyl ester	8.4	fatty acid (S)
12	1-Tert-buthoxy-6-trimethylsilyloxyhexane	8.79	Ethers
13	3-Ketovaleric acid, bis(trimethylsilyl)-	9.22	Amino acids
14	Tris(hydroxymethyl)aminomethane,O,O',O''-tris(trimethylsilyl) ether	9.5	Ethers
15	Benzocycloheptene, 3-hydroxy-	10.12	Hydrocarbons
16	4-Methyl-6-cyanothieno[2,3-b]pyridine	10.31	Heterocyclic compounds
17	[(2-deuterio)-s-isobutyl]-2-propenyl-sulfoxide	10.73	Hydrocarbon
18	N-Acryloylmorpholine	11.21	Heterocyclic compounds
19	4,5-Dihydro-4,5-trans-di-n-propyl-2-ethoxyimidazole	11.54	Heterocyclic compounds
20	Docosane	12.16	Hydrocarbon
21	N-Nitrosomethylethylamine (à-D2)	12.57	Amines
22	[6-(4-tert-Butylphenyl)-1,3,5-hexatriynyl]trimethylsilane	12.9	Silanes
23	Hexadecane (CAS)	13.24	Hydrocarbon
24	3-phenyl-1,2-naphthoquinone	13.82	Hydrocarbon
25	Dodecanoic acid (CAS)	14.26	fatty acid (S)
26	Eicosane, 10-methyl- (CAS)	14.72	Hydrocarbon
27	Isopropyl Dodecanoate	15.09	Organic acid and its derivatives
28	2-Methoxy-8-Chloro-Dibenzofuran	15.27	Heterocyclic compounds
29	Eicosane, 2-methyl- (CAS)	15.74	Hydrocarbon

S. No	Metabolites	Retention time (min)	Chemical groups identified
30	Oxirane, hexyl- (CAS)	15.98	Cyclic ether (Ethylene oxide)
31	Malonic acid, dodecyl 2-ethylbutyl ester	16.19	Organic acid and its derivatives
32	1-methoxymethyl-4-methylnaphthalene	16.41	Hydrocarbon
33	Nonadecanoic acid, 18-oxo-, methyl ester (CAS)	17.25	fatty acid (S)
34	17-Methyl-9-oxo-10-nor-14à-4,5-nitrilomorphinan	17.46	Heterocyclic compounds
35	Tetradecanoic acid (CAS)	18.59	fatty acid (S)(Myristic acid
36	2-(o-Hydroxymethylbenzyl)naphtho[2,3-b]thiophene	19.19	Heterocyclic compounds
37	Heneicosane	19.96	Hydrocarbon
38	(15à)-phylloclad-16-ene-15-carbaldehyde	20.65	Carbonyl compounds
39	Eicosane	21.55	Hydrocarbon
40	1-Hydroxy-17-(1-oxoethyl)-2-oxa-androst-4-en-3-one	22.14	Carbonyl compounds
41	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (CAS)	22.79	Organic acid and its derivatives
42	2-Methoxycarbonyl-3-phenylsulfonylhydroquinone	23.1	Hydrocarbon
43	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	23.86	Carbonyl compounds
44	Bis[1,2-di(2-thienyl)-1,2-ethenedithiolene]nickel	24.57	Metal complex
45	Dibutyl phthalate	24.93	organic acid and its derivatives
46	4-(4-Fluorophenyl)-2-methyl-6-methylthiobenzonitrile	25.51	Hydrocarbon
47	Diethyl [2- (4'-methylphenyl) ethyl] phosphonate	25.89	Organic acid and its derivatives
48	2,2-Dichlorocyclobuta[a]cyclopent[3,4-a]azulenone	26.64	Carbonyl compounds
49	1,3-Dimethoxy-5,7-dihydrodibenz[c,e]oxepine	27.48	Heterocyclic compounds
50	Hentriacontane	28.12	Hydrocarbon
51	Octadecanoic acid, propyl ester (CAS)	28.31	Fatty acid (S) (Stearic acid
52	10,10-dimethylanthrone hydrazone	28.53	Carbonyl compounds
53	1,3-Dimethoxy-5,7-dihydrodibenz[c,e]oxepine	28.91	Heterocyclic compounds
54	9,10-Dihydrocyclobuta[a]triphenylene-11,12-dione	29	Carbonyl compounds
55	Pulchelstyrene D	29.91	Hydrocarbon
56	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	30.36	Carbonyl compounds

S. No	Metabolites	Retention time (min)	Chemical groups identified
57	3,18-Epoxyandrosta-5,7-dien-17-ol, 4,4-dimethyl-3-methoxy- (13á)	31.25	Epoxide (Hydrocarbons)
58	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	31.5	Carbonyl compounds
59	2áBenzyl-8-oxo-4,6-dimethyl-3,5,7-trioxatetracyclo[7.2.1. 0(4,11).0 (6,10)]dodecane	31.94	Hydrocarbon
60	Pentacosane	32.87	Hydrocarbon
61	Di-(2-ethylhexyl)phthalate	33.14	Organic acid and its derivatives
62	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	33.32	Hydrocarbon
63	Nonacosane (CAS)	33.59	Hydrocarbon
64	03027205002 Flavone	34.27	Carbonyl compounds
65	10-Benzyloxy-1,8-dihydroxy-9(10H)-anthracenone	35.21	Carbonyl compounds
66	2-Pentoxy-tetrahydropyran	35.59	Heterocyclic compounds
67	N-(5à-Cholestan-3á-yl)-acetamide	36.17	Organic acids and its derivatives
68	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (CAS)	36.64	Fatty acid
69	Tetratetracontane (CAS)	37.31	Hydrocarbon
70	1,4-Cyclohexadiene-1,2-dicarboxylic acid, 4,5-dimethyl-, dimethyl ester	37.92	Organic acid and its derivatives
71	Acetic acid, 4,5-dihydroxy-10,13-dimethyl-3-oxohexadecahydrocyclop enta[a]phenanthren-17-yl ester	38.21	Organic acid and its derivatives
72	Methyl 4-(4-methoxybenzoyl)-4-methyl-pent-2-enoate	38.5	Organic acid and its derivatives
73	13-Docosenamide, (Z)-	38.87	Organic acid and its derivatives
74	2-[3-(Aminomethyl)-5,7-Dimethyl-1-Adamantyl]Ethanamine	39.56	Amines
75	Dihydromonticamine	39.93	Amines

Table 21. Identified metabolites from degenerated intervertebral disc tissue through GC/MS against NIST library search

S. No	Metabolites	Retention time (min)	Chemical groups identified
1	1-Benzyloxymethyl-1-Hydroxymethyl-2,5-Cyclohexadiene	3.1	Hydrocarbon
2	4-Chloro-4-(phenylsulfinyl)-3-heptanol isomer	3.51	Alcohols
3	(6á)-8a-(3',3'-Dimethylbut-1'-ynyl)-3,4,4a,5,6,8a-hexahydro-6-methoxy-3,3,6-trimethylnaphthalen-1(2H)-one	4.65	Carbonyl compound
4	4-[3-(Trimethylsilyl)-2-propinyl]oxy-2-butynoic acid	5.09	Organic acid and its derivatives
5	D-Lactic acid-DITMS	6	Organic acid and its derivatives
6	2-[5-(2-hydroxyethyl)-2-thienyl]-4,4-dimethyloxazoline	6.41	Heterocyclic compounds
7	Ethyl 3-(Trimethylsilyl)Propanoate #	6.84	Organic acid and its derivatives
8	3,7-Dioxa-2,8-disilanonane, 2,2,8,8-tetramethyl-5-[(trimethylsilyl)oxy]- (CAS)	7.22	Silanes
9	2-[4(or 5)-(2-Phenylimidazolyl)]propionitrile	7.59	Nitriles
10	3-Phenylnon-4-en-3-ol	8.06	Alcohols
11	Octanoic acid, trimethylsilyl ester	8.43	Organic acid and its derivatives
12	9,12,15-Octadecatrienoic acid,2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)- (CAS)	8.81	Organic acid and its derivatives
13	2-Aminoethanol, N-acetyl-, trimethylsilyl ether	9.04	Ethers
14	3-Oxovaleric acid TMS ether TMS ester	9.22	Amino acids
15	5-Benzoyl-1,2,3,4-tetrahydronaphthalene	9.48	Hydrocarbon
16	Dibenzo[c,e]thiin-2-thione	10.05	Carbonyl compound
17	4-Methyl-6-cyanothieno[2,3-b]pyridine	10.31	Heterocyclic compound
18	Eicosane (CAS)	10.65	Hydrocarbon
19	N-Acryloylmorpholine	11.21	Heterocyclic compound
20	Hexadecane, 2,6,11,15-tetramethyl- (CAS)	11.52	Hydrocarbon
21	1,2-Difluoro-3,4,5-trimethylbenzene	12.12	Hydrocarbon
22	N,N'-Dityrityl-1,5-diaminopentane	12.51	Hydrocarbon
23	Hexadecane (CAS)	13.22	Hydrocarbon
24	2-tert-Butyl-4-isopropyl-5-methylphenol	13.8	Phenolic
25	Dodecanoic acid (CAS)	14.3	Fatty acid (S)
26	2-(2-chinoxalinyl)-(all-às)cyclotetrathiophen	14.7	Heterocyclic compound
27	Dodecanoic acid, 1-methylethyl ester	15.07	Fatty acids

S. No	Metabolites	Retention time (min)	Chemical groups identified
28	Spiro(1,3-dioxolane)-2,3'-[5'-androsten-16'-trimethylsilyloxy)-	15.26	Spiro compounds
29	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	15.72	Hydrocarbon
30	11-Heneicosanone (CAS)	16.17	Carbonyl compound
31	1-methoxymethyl-4-methylnaphthalene	16.39	Hydrocarbon
32	Docosane (CAS)	16.57	Hydrocarbon
33	Octadecane (CAS)	17.46	Hydrocarbon
34	Tetradecanoic acid (CAS)	18.62	Fatty acid (S) (Myristic acid)
35	Tetradecanoic acid, trimethylsilyl ester (CAS)	19.17	Fatty acid (S) (Myristic acid)
36	1,1-Bis(p-tolyl)ethane	19.64	Hydrocarbon
37	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	19.95	Hydrocarbon
38	Methyltetrahydrofurolacellicolic anhydride	20.26	Organic acid and its derivatives
39	1-Amino-2-cyano-3,4-dihydro-4-ethoxycarbonyl-3-phenylpyrido[1,2-a]benzimidazole	20.64	Heterocyclic compounds
40	Hexadecanoic acid, methyl ester (CAS)	21.64	Fatty acid (S) (Palmitic acid)
41	4-phenyl-6-(p-methylbenzoyl)bicyclo[3.3.0]octa-3,7-dien-2-one	22	Carbonyl compound
42	10-Cyano-3-amino-12-(4'-methoxyphenyl)-2-oxopyrano[4,3-d]pyrido[1,2-a]benzimidazole	22.88	Heterocyclic compounds
43	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	23.88	Carbonyl compound (Spiro)
44	Heptadecanoic acid (CAS)	24.6	Fatty acid (S) (Margaric acid)
45	Dibutyl phthalate	24.93	Organic acid and its derivatives
46	Octadecanoic acid, methyl ester (CAS)	25.47	Fatty acid (S) Stearic acid
47	1-[1-[[(tert-butyldimethylsilyl)oxy]methyl]-1-methyl-2-oxoethyl]perhydroazine	25.9	Carbonyl compound derivatives
48	6,6-d2-.delta.2-5à-androsten-1-one	26.73	Carbonyl compound
49	10-Methylene-8-methoxy-3,4,4a,10-tetrahydro-2H,9H-anthracene-1,9-dione	27.13	Carbonyl compound
50	1-[1-[[(tert-butyldimethylsilyl)oxy]methyl]-1-methyl-2-oxoethyl]perhydroazine	27.52	carbonyl compound derivatives
51	Octadecane, 3-ethyl-5-(2-ethylbutyl)- (CAS)	28.12	Hydrocarbon
52	Ethanamine, 2,2'-oxybis[N,N-dimethyl- (CAS)	28.53	Amine

S. No	Metabolites	Retention time (min)	Chemical groups identified
53	1,3-Dimethoxy-5,7-dihydrodibenz[c,e]oxepine	28.92	Heterocyclic compounds
54	Acetyl-Ginsenol	28.98	Alcohols
55	Butyl 9-octadecenoate or 9-18:1	29.49	Organic acid and its derivatives
56	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	29.91	Carbonyl compound
57	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	30.37	Carbonyl compound
58	Hept-enyl-2-acetate	31.05	Organic acid and its derivatives
59	(S)-(2l,6l,3"x)-(1'E)-2-[3'-(3"-Oxocyclohexyl)-1'-propenyl]-6-methyl-3-(1-methylethyl)-1,3,2-oxazaphosphorinane 2-Oxide	31.52	Heterocyclic compounds
60	2áBenzyl-8-oxo-4,6-dimethyl-3,5,7-trioxatetracyclo[7.2.1.0(4,11).0(6,10)]dodecane	31.93	Hydrocarbon
61	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethylester	32.7	Organic acid and its derivatives
62	Di-(2-ethylhexyl)phthalate	33.13	Organic acid and its derivatives
63	Docosane, 11-decyl- (CAS)	33.34	Hydrocarbon
64	erythro-1,2-Epoxy-2-methyl-3-heptanol	33.68	Alcohols
65	7-(4'-Nitrophenyl)-5-imino-2-methyl-5H-thiazolo[3,2-a]pyridine-6-carbonitrile	34.05	Heterocyclic compounds
66	3-Formyl-1-oxyl-4-(pyren-1'-yl)-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole	34.52	Heterocyclic compounds
67	1'-Benzyl-3-methoxynaphtho[16,17-b]estra-1,3,5(6)-triene	34.99	Hydrocarbon
68	Cholan-24-oic acid, 3,12-dihydroxy-, (3à,5á,12à)- (CAS)	35.66	Organic acids
69	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethylester	36.67	Organic acid and its derivatives
70	3,5-dicyano-2,6-di(m-tolyl)-1-methylpyridine-4(1H)-thione	37.5	Heterocyclic compounds
71	2,6-Diisopropylanisole	37.91	Phenolic
72	13-Docosenamide, (Z)-	38.87	Organic acid and its derivatives
73	2,2,5,5-Tetramethyl-4-(p-methoxyphenyl)-3-oxazoline	38.97	Heterocyclic compounds
74	Benzoic acid, 2,4-dimethyl-, (3,5-dimethylphenyl)methylester	39.56	Organic acid and its derivatives
75	Hexadecanoic acid, hexadecyl ester (CAS)	39.93	Organic acid and its derivatives

Discussion

CHAPTER V

DISCUSSION

Low back pain is one of the most important socio-economic health care issues today. It places an enormous economic burden on society by several means *viz.,* direct medical cost and indirect costs such as insurance, loss in production due to disability etc. It has been estimated that 60 to 80 per cent of the people would, at some point in their life time, experience back pain (Andersson, 1999). Even though several environmental and constitutional risk factors have been implicated in this disease, their effects are relatively minor and recent studies on monozygotic twins have suggested that disc degeneration may be explained to a large extent by genetic factors (Ala-kokko, 2002). The main anatomical structure implicated in LBP is the intervertebral disc (IVD) and the pathogenic process is its degeneration, which predisposes disc herniation.

5.1. Genetics of disc degenerative disease

There is no standard definition of disc degeneration, therefore determinations of its presence and severity vary widely and are determined largely by the technology used to interrogate the disc. The challenge of developing more uniform case definitions of disc degeneration and accurate, reliable measures needs to be addressed to advance further studies in this area. It was believed that the LBP due to lumbar disc herniation was primarily due to aging, sex, nature of occupation, habits, and mechanical stress to IVD (Borenstein *et al*., 2001). Apart from environmental risk factors, such as lifting heavy loads, torsional stress, and motor vehicle driving, research findings also indicate that there is a significant genetic influence on both disc degeneration and back pain (Battie *et al*., 2007; Ala-kokko, 2002). Putative candidate genes *viz.,* collagen IX A2 *(COL9A2),* collagen IX A3 *(COL9A3),* Vitamin D receptor (*VDR),* Matrix metalloprotease-3 (*MMP3),* Matrix metalloprotease-7 (*MMP7) and* Interleukin-1 (*IL-1*) were found to be associated with DDD but with moderate to less level of significance (Chan *et al*., 2006; Zhang *et al*., 2008).

With this backdrop, attempts were made in this study to find the genes that are associated with DDD. In this regard, blood samples were collected from 809 individuals representing both cases and controls from the Spine unit, Ganga hospital, Coimbatore. All the members of the study population were evaluated by MRI (Magnetic Resonance Imaging) scan to identify the various changes in different components of the disc namely the nucleus pulposus, annulus fibrosus, end plate, disc bulge and presence of nodes. Individuals were classified as case and control based on Pfirman, Modic, Schneiderman's, disc bulge, annular tears, Schmorl's Node and TEPS. Frequency of case and control differed for each phenotypic trait. Out of 809 individuals, based on disc bulge nature, 474 were classified as DDD patients and 335 as control. 281 were classified as DDD patients and 528 as control based on the appearance of annular tears. 107 were classified as DDD patients and 702 as control by Modic change. According to Pfirman score, 494 were classified as DDD patients and 315 as control. According to presence of Schmorl's Node, 147 were classified as DDD patients and 662 as control. According to TEPS, 121 were classified as DDD patients and 688 as controls. According to Schneiderman's score, 493 were classified as DDD patients and 316 as controls.

In the present study, an association between the phenotypic changes observed in the lumbar disc and 71 SNPs (out of 87 possible SNPs involved in this pathway) in 40 candidate genes was established among Indian population.

5.1.1. Genes associated with DDD at individual disc level

The results of this association analysis based on individual disc levels revealed that 33different genes *viz*., *CALM1, ADAMTS5, MMP1, MMP2, MMP7, MMP9, MMP10, MMP20, COL9A1, COL11A1, NGFB, CILP, FN1, SKT, CHST3, VDR, COX2, TAC1, IL6, IGF1R, IL18RAP, IL1RN, BMP2, HAPLN1, GLI1, HHIP, IGFIR* and *LEPR* have significant association with DDD for all the phenotypes. Out of these 33 genes, 16genes (*LEPR, NGFB, COX2, FN1, COL9A1, TAC1, IL6, MMP1, MMP7, MMP10, CHST3, VDR, CALM1, CILP, MMP2 and ADAMTS5*) were strongly associated with DDD at 1 % significant level. *LEPR* was associated with Modic change (lower level 2); *NGFB* was associated with disc bulge (level 5). *COX2* was associated with annular tears (level 3) and

Modic change (upper level 4); *FN1* was associated with Pfirman grading (level 2) and Schneiderman's score (level 2). *COL9A1* was associated with annular tears (level 5); *TAC1* was associated with disc bulge (level 5) and annular tears (level 3). *IL6* was associated with Modic change (upper level 1); *MMP1* was associated with annular tear (level 1 and 5). *MMP7* was associated with Schmorl's node (level 1, 2 and 5); *MMP10* was associated with annular tears at level 1. *CHST3* was associated with Modic change at upper level 5; *VDR* was associated with annular tears at level 2 and Modic change at upper level 4. *CALM1* was associated with Pfirman grading at level 1 and 3, Schneiderman's score at level 1, 2 and 3, Modic change at lower level 2. *CILP* was associated with disc bulge at level 1, 2 and 4; *MMP2* was associated with Schmorl's node at level 4. *ADAMTS5* was associated with disc bulge at level 5 and annular tears at level 5.

5.1.2. Genes associated with DDD based on whole disc scoring

The results of association analysis based on whole disc scoring revealed the significant association of 17 different genes (*CILP, VDR, MMP7, TAC1, IL6, ADAMTS5, IGF1R, MMP1, COL9A1, IL1RN, MMP10, MMP10, CALM1, FN1, SKT, NGFB and COX2*) with DDD. Association at individual disc level were also analyzed in PLINK for all the seven phenotypic traits. Previous studies have also reported similar association for different genes (Table 1).

5.1.3. Genes associated with DDD for the young adults (less than 40 years) based on whole disc scoring

Association study for the young adults based on whole disc scoring revealed that 15 different genes. Out of these 15 genes, five genes *viz., MMP7*, *COL9A1*, *CALM1*, *MMP1* and *ADAMTS5* have a significant association with DDD at 1 % significance level. *MMP7* was associated with disc bulge, Schneiderman's score and Pfirman score with p value of 0.000388, 0.007937 and 0.007937 respectively. *COL9A1* was associated with annular tears with p value of 0.006066. *CALM1* was associated with Schneiderman's score and Pfirman score with p value of 0.004651 and 0.004651 respectively. *MMP1* was associated with Modic change with p value of 0.005842. *ADAMTS5* was associated with annular tears with p value of 0.006465. This analysis of young adults (less than 40 years) at all the disc level strongly shows that responsible for DDD is also by genetic cause.

5.1.4. Candidate genes associated with DDD

5.1.4.1. Matrix Metalloproteinases

Biochemically, disc degeneration is characterized by enhanced breakdown of the extra cellular matrix in intervertebral disc. The main components of disc ECM (extra cellular matrix), collagens and proteoglycans, are degraded by a specific class of proteolytic enzymes known as the MMPs. The MMPs are a family of proteolytic enzymes that participate in the degradation of all major matrix components, including glycoproteins, proteoglycans and collagen. Under normal physiological conditions, the efficiency of these enzymes is regulated by their own activation mechanism (positive stimulus) and by endogenous tissue inhibitors of metalloproteinases (negative stimulus). In various pathological processes, an imbalance between MMPs and TIMPs plays a pivotal role in tissue degradation and degeneration. According to their substrate specificity and gene structure, the MMPs can be classified into different groups: the collagenases, gelatinases, stromelysins and matrilysins. From the previous studies of evidence show that MMPs play a pivotal role in regulation of intervertebral disc homeostasis. Among the MMP family members, *MMP-2* is of particular importance due to its broad spectrum of proteolytic activity toward ECM components including gelatin, proteoglycans, fibronectin and elastin (Woessner and Nagase, 2000). The presence of *MMP-2* within normal and degenerative nucleus pulposus and annulus fibrosus cells was also demonstrated in several immunohistochemical studies (Roberts *et al*., 2000). Enhanced expression of *MMP2* has been found in aging and degenerative discs and *MMP2* production appears to increase when disc cells are exposed to abnormal physical stresses. In addition, Kozaci *et al*. (2006) reported that Pro-MMP-2 levels were higher at early stages of the degenerative disc disease, which were negatively correlated with the collagen content in herniated disc material.

Two SNPs rs491152 and rs2239008 of *MMP1*, rs243865 of *MMP2*, two SNPs rs1940044 and rs1996352of *MMP7*, rs17576 of *MMP9*, two SNPs rs470154 and rs11225422of *MMP10*, rs672535 of *MMP12*, three SNPs rs1784438, rs1784430 and rs17099008 of *MMP20* had a significant association with DDD in this study. *MMP7* belongs to the

group of Matrilysins, which mainly degrade the protein core of proteoglycans and small non-collagenous proteins. During intervertebral disc degeneration, normal matrix synthesis decreases and degradation of disc matrix increases. *MMP7* is known to cleave the major matrix molecules of the disc (proteoglycan aggrecan and collagen type II). Le Maitre and his coworkers investigated the localization of *MMP7* in different human intervertebral discs (non-degenerate, degenerated and prolapsed discs) to ascertain whether *MMP7* is up-regulated during disc degeneration. They found that the chondrocyte-like cells of nucleus and inner annulus were *MMP7* immuno-positive; little immunopositivity was observed in the outer annulus. Non-degenerate discs showed few immune-positive cells. A significant and progressive increase in the proportion of *MMP7* immuno-positive cells was seen in the discs with intermediate and severe degeneration. Prolapsed discs also showed more *MMP7* immunopositive cells (Le Maitre *et al*., 2006). So, abnormal expression of *MMP7* can alter the metabolism within the disc matrix, leading to early disc degeneration.

5.1.4.2. A Disintegrin and Metalloproteinase with ThromboSpondin motifs1 (*ADAMTS*)

The results from this study showed two SNPs rs2249350 and rs162509 of *ADAMTS5* had a significant association with DDD in this study.Aggrecan is the major proteoglycan in cartilage, endowing this tissue with the unique capacity to bear load and resist compression. In arthritic cartilage, aggrecan is degraded by one or more 'aggrecanases' from the ADAMTS (A Disintegrin and Metalloproteinase with ThromboSpondin motifs1) family of proteinases. *ADAMTS1*, *8* and *9* have weak aggrecan-degrading. Also, previous studies have revealed that several alleles (rs151058, rs229052, and rs162502) locating in the introns of *ADAMTS5* were significantly associated with DDD and hence these alleles were pathogenic (Wu *et al*., 2014). Similarly, Stanton and his coworkers reported that *ADAMTS5* is the major aggrecanase in mouse cartilage, both *in vitro* and in a mouse model of inflammatory arthritis and suggested that *ADAMTS5* may be a suitable target for the development of new drugs designed to inhibit cartilage destruction in arthritis, although further work will be required to determine whether *ADAMTS5* is also the major aggrecanase in human arthritis (Stanton *et al*., 2005).

5.1.4.3. Interleukin 6 (*IL6*)

The results from this study showed rs2069837 of *IL6* had a significant positive association with DDD in this study**.** IL6, inflammatory mediator and it is spontaneously produced by lumbar herniated disc and it is associated with neurological symptoms of DDD. *IL6* upregulates the *MMP*s and *ADAMTS5* gene expression and down regulate the collagen and aggrecan core protein expression. *IL 6* has direct effects on nucleus pulposus cells to reduce the proteoglycan synthesis and thus affects the disc integrity. Previous studies have reported that the inflammatory response is genetically determined (Solovieva *et al*., 2004), and it plays a crucial role in symptomatic DDD. Noponen-Hietala *et al.* (2005), have identified that a specific *IL6* haplotype predispose to sciatica, further supporting the important role of *IL6* in the mediation of sciatic pain. Since it is known that disc degeneration and herniation also occur among asymptomatic individuals, it may be that the occurrence of symptoms may depend on the individual's systemic inflammatory response to mechanical insults. In the future, the role and significance of IL6 variations should be studied in the different populations.

5.1.4.4. Calmodulin 1 (*CALM 1*)

Two SNPsrs2300496 and rs3213718 of *CALM1* had a significant association with DDD in this study. This is the first report on association of *CALM 1* with lumbar disc degeneration. The Calmodulin 1 gene encodes for a ubiquitous, eukaryotic Ca2+ binding protein and is the principal mediator of the calcium signals. It is an intracellular protein that interacts with several proteins involved in signal transduction. A different polymorphism (functional core promoter SNP -16C/T: rs12885713) was recently associated with hip osteoarthritis in the Japanese population (Mototani *et al*., 2005), but it did not show significant association in a British study (Loughlin *et al*., 2006). *CALM1* is expressed in cultured chondrocytes and articular cartilage, and its expression has been observed to be increased in osteoarthritis. Mechanical compression of articular chondrocytes is known to trigger changes in aggrecan expression, and such changes are dependent on calmodulin signaling. Disc degeneration is also characterized by disintegration and loss of extracellular matrix, end plate damage and associated with

varying degrees of osteophyte formation. Hence, Calmodulin could be an important mediator in the pathogenesis of disc degeneration too.

5.1.4.5. Cyclo-Oxygenase 2 (*COX 2*)

Three SNPs *viz*., rs5277 rs2066826 and rs5277 of the *COX2* gene had a strong positive association with DDD. *COX2* is an enzyme that is responsible for formation of important biological mediators such as prostaglandins, prostacyclin and thromboxane. These are mediators of inflammation and pharmacological inhibition of COX can reduce inflammation and pain. Variants of genes coding for inflammatory mediators such as interleukin 1, interleukin receptor 1, cyclooxygenase 2 and matrix metalloprotease have all been found to be associated with lumbar degenerative disc disease (Miyamoto *et al*., 2002). Pro-inflammatory cytokines are involved in extracellular matrix degradation and fibrosis of the disc, and also play a role in peripheral neuropathic pain. The *COX2* gene is considered to be involved in disc degeneration and herniation through the up regulation of PGE2. It is also reported to be involved in the peripheral modulation of pain. IL1 is possibly involved in disc degeneration by increasing the destruction of disc matrix through activating degradative enzymes and modulates pain by enhancing the activity of *COX2*.

5.1.4.6. Nerve Growth Factor Beta polypeptide (*NGFB*)

Two SNPs rs2856813 and rs4076018 of the *NGFB* gene was highly significant for the development of disc degeneration ($p<01$). Nerve growth factor (NGF) is critical for the survival and maintenance of sympathetic and sensory neurons. There is strong evidence demonstrating the role of NGF as an inflammatory mediator. Aoki *et al*. (2005) has been reported that inflammatory mediators, nerve growth factor (NGF), and a decrease of proteoglycan content have the potential to promote nerve ingrowth into the disc.

5.1.4. 7. Carbohydrate (Chondroitin 6) Sulfotransferase 3 (*CHST3*)

Two SNPs rs4148941 and rs4148949 of *CHST3* had a significant association ($p < 0.01$) with disc degeneration in this study. This is the first report on association of CHST3 with disc degeneration among Indian Population. CHST3 is an enzyme that catalyzes the

sulfation of proteoglycan and also contributes to the synthesis of extracellular matrix proteoglycans It is essential for the normal development of cartilage tissue. This enzyme modifies molecules called chondroitin sulphate proteoglycans, which are abundant in cartilage tissue. Sulfation of these molecules is a most critical step in cartilage formation. Song and his coworkers reported that this *CHST3* gene is a susceptible gene for causing lumbar disc degeneration (Song *et al*., 2013)

Disc degeneration is also characterized by gradual disintegration and loss of extracellular matrix. A variant of this gene can cause loss of proteoglycan and reduce the amount of water content in the nucleus pulposus of intervertebral disc that causes DDD. (Song *et al*., 2013). It indicates that a mutation of this particular gene leads to affect the hydration and loss of ECM in the disc tissue.

Song *et al*., 2013 identified that the susceptible allele of rs4148941 for lumbar disc degeneration in Southern Chinese family based data. 43 percent of DDD patients in Hong Kong carry this *CHST3* variant risk allele. Similarly in our Indian population also we could find this risk allele from *CHST3*. Together with this finding, it showed that strong evidence for the involvement of genetic factor (*CHST3*) in Disc Degenerative Disease.

5.2. Profiling proteins expressed in the IVD

Knowledge on the proteins expressed in the intervertebral disc (IVD) tissue in humans is limited and many components are yet to be identified. The presence of abundant proteoglycans and poorly soluble extracellular matrix (ECM) in IVD tissue poses a major technical challenge to profile proteins constituting IVD using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). In this study, we have optimized the extraction of IVD tissues for 2D-PAGE analysis and successfully resolved around 143 protein spots abundantly expressed in the IVD.

2D PAGE analysis, which separates proteins according to their molecular weight and isoelectric point, followed by detection with MALDI TOF, is a powerful technique for not only profile proteins, but also identify the differentially expressed proteins involved in diseases by comparing protein profiles of normal and diseased tissues.

During the 2D-PAGE analysis, the resolution of the proteins was very poor due to interfering substances, which affects isoelectric focusing (Figure 11a). To improve the protein separation and also to increase the number of detectable spots, we purified the proteins from the contaminants using MWCO centrifugal filters. This resulted in the detection of around 150 protein spots expressed in the IVD of human beings with better resolution and abundance (Figure 11c). Thirty different abundant proteins were excised out from the gel and subjected to MALDI-TOF analysis and the MALDI-TOF results were used for the search against database using MASCOT software, which resulted in the identification of 25 annotated proteins in human genome that share significant homology.

Most of the proteins identified in our experiments appear to be authentic proteins of IVD (Table 19). For example Vimentin (HD 1) is a type III intermediate filament (IF) protein abundantly expressed in mesenchymal cells capable of developing into connective tissues such as bone and cartilage. These intermediate filaments along with tubulin and actin based microfilaments comprise the cytoskeleton of a cell. This is expected to play a major role in maintaining the organization of extracellular matrix of IVD. Expression pattern of vimentin is divergent and its biological function during growth, differentiation, and morphogenesis must be broad and significant (Capetanaki *et al*., 1989).

Dystrophin (HD 3) is a cytoplasmic protein that connects the cytoskeleton of a muscle fiber to the surrounding extracellular matrix and it is responsible for neuromuscular disease such as Duchenne (DMD) and Becker (BMD) Muscular Dystrophies. Dystrophinplays a major role determining muscle fiber strength, muscle stiffness, sarcolemmal deformability and connections of costameres to nearby myofibrils.

Apolipopotein (HD 5 and HD 6), a component of high-density lipoproteins, supplies nutrition to the intervertebral disc. This cytoplasmic protein involves in endplate bone formation and also in the maintenance of extracellular matrix. Liu and his coworkers have reported that ApoA-IV (Apolipoprotein A IV) is up-regulated in the cerebro spinal fluid of Lumbar disc herniation patients (Liu *et al*., 2006). ApoE (Apolipoprotein E) deficiency may alter the integrity of the intervertebral disc causing impairment of the nutrition supply to the disc thereby leading to disc degeneration (Zhang *et al*., 2013).

Hence, expression of apolipoprotein may play an important role in the integrity of the intervertebral discs.

This study showed that Huntingtin-interacting protein 1-related protein (HIP1) (HD 10) and Alpha-tubulin N-acetyltransferase are present in IVD. HIP-1, localized in neuronal cells, is suggested to be a novel proapoptotic mediator and a molecular accomplice in the pathogenesis of Huntington's disease, a progressive neurodegenerative disorder (Hackam *et al*., 2000). Inappropriate apoptosis disrupts the functions of the intervertebral disc, which leads to the disc degeneration (Martin *et al*., 2002; Guo *et al*., 2011).Alpha-tubulin N-acetyltransferase (HD 22) is involved in the development of neuron and it is also a building block of neurofibrillary tangles found in a range of neurodegenerative disease including Alzheimer's (www.uniprot.org).

Further confirmatory studies are required to ascertain whether the proteins found in this study play real roles in disc degenerative disease. Hence, comparison of protein profiles from both control and affected tissue are required to obtain valuable insights about the intervertebral disc degeneration. Achieving superior resolution of proteins constituting IVD on a 2D-PAGE gel is a critical hurdle in the identification and analysis of IVD proteins and hence, dissection of the molecular mechanism of DDD. The improved protocol has been standardized for resolving IVD protein by 2D PAGE. About 143 abundantly expressed protein spots in the IVD have successfully resolved. This study has established the presence of 25 proteins with significant score in IVD tissues of DDD patients, indicating that these are authentic IVD proteins. Further studies are required to profile proteins from both normal (i.e., Control) and DDD patients in order to characterize the proteomic changes during IVD degeneration, and identification of candidate proteins playing a role in DDD, potential markers for DDD.

5.3. Metabolic profiling of IVD

GC/MS-based metabolomics is a powerful tool to elucidate the impact of low back pain thereby providing novel insights into the patho-mechanism underlying disc degenerative disease. This GC/MS based metabolomic approaches cover a large number of novel compounds. Unlike proteomics, metabolomics demands a large amount of manual

evaluation like the validation and peak correction of identified metabolites. Mass spectrometric analysis could identify the metabolites present in the disc tissue sample through NIST library search.

Interestingly, the presence of lactic acid in this study indicates that lactic acid might play a role in disc degeneration. Intervertebral disc is the largest avascular tissue in the body, its metabolism is mainly anaerobic, and thus lactate is produced at a significant rate. The higher lactate concentration reduces the pH in the disc (Diamant *et al*., 1968; Ohshima and Urban, 1992). Experimental data from other studies have showed that acidic pH adversely affect the supply of nutrients to IVD, cellular activity of IVD (Ishihara and Urban, 1999; Razaq *et al*., 2003) synthesis of proteoglycans (which play a major role in the load bearing capacity of the disc) (Ohshima and Urban, 1992),integrity of extracellular matrix of the IVD, even IVD viability (Bibby and Urban, 2004), and thus acidification of IVD due to lactic acid may play a role in disc degeneration (Ishihara and Urban, 1999; Razaq *et al*., 2003).

The presence of lactic acid in both control and degenerated disc indicates that quantification of lactic acid is necessary for correlating the quantity of lactic acid with the causation of IVD degeneration. Similarly further confirmatory studies are required to ascertain whether the compounds found in this study play real roles in disc degenerative disease. Quantification of the metabolites from both control and affected tissue can obtain valuable insights about the IVD degeneration.

GC/MS-based metabolic profiling study has profiled the metabolites present in both control and degenerated human IVD. This study revealed that some of these metabolites present in the human IVD might play an important role in disc degenerative diseases.

The present study is the first large set population based genomic study on the genetic basis of DDD in the Indian population. The Indian population forms one-sixth of the world's population and is ethnically different from other population groups such as Chinese and Caucasians on whom previous genetic studies have been done. For example, association studies of Trp2 and Trp3 alleles of collagen IX have shown that Trp allele

frequencies between Finns and Chinese were contrasting and gave the first indication that the genetic risk factors for DDD vary between ethnic groups.

Different research groups have used different phenotypes and have identified association with various genes. However, in this study, for the seven different phenotypes, significant association has been found with different SNPs. This indicates that different genes may affect the function and structure of the various parts of the disc complex. It is also important that a large patient population with common and highly specific phenotypes should be studied by several international groups to enhance the power and significance of these findings. In conclusion, disc degeneration seems to be multifactorial with a significant role for genetic polymorphisms.

As a whole, the findings of this thesis obtained from the three "omics" approaches, *viz.,* association analysis, 2D PAGE profiling of proteomics approach and GC-MS based metabolites profiling, have provided us novel insights, which contributes to characterization of the genetic factor and pathological mechanism of DDD in Indian population. Future research will aim at functional characterization of the DDD-associated candidate genes identified in this study for in-depth understanding of the genetics of disc degeneration.

Summary

SUMMARY

The present study was undertaken with aim of generating improved knowledge on the molecular genetic basis and biochemical basis of DDD through various genomic approaches viz., association analysis, protein and metabolite profiling in IVD. The summary of results obtained from the present study is as follows:

- The Indian study population comprising of 809 individuals was subjected to thorough clinical evaluation for various DDD related parameters viz., disc bulge, annular tears, Modic changes, Schneiderman's score, Schmorl's Node, total end plate score and Pfirman grading at Ganga Hospital, Coimbatore and classified into cases and controls.
- The percentage of male and female is 56.24 % and 43.76 % respectively. In the sample cohort, the age of the individuals ranged from 10 to 80 with the mean age of 36.
- Based on seven different phenotypic traits, 809 individuals were classified as cases and controls based on scoring at both whole disc and individual disc level.
- All the 809 individuals were genotyped for 71 different candidate SNPs using Sequenom assay. The analyses for validating the association between the SNPs and DDD was performed by PLINK software.
- Association study based on individual disc levels revealed the significant association of 33 different genes with DDD. Out of 33 different genes, 16 genes *viz*., *LEPR, NGFB, COX2, FN1, COL9A1,TAC1, IL6, MMP1, MMP7, MMP10, CHST3, VDR, CALM1, CILP, MMP2 and ADAMTS5* were found to have significant association with DDD at 1 % significance level.
- The results of association analysis based on scoring at whole disc level revealed the significant association of 17 different genes (*CILP, VDR, MMP7, TAC1, IL6, ADAMTS5, IGF1R, MMP1, COL9A1, IL1RN, MMP20, MMP10, CALM1, FN1, SKT, COX2* and *NGFB*) with DDD at 5 % significance level.
- Association analysis performed in a sub-set of young individuals (<40 years of age) based on whole disc scoring revealed the strong association of 15 different

genes with DDD. Out of 15 different genes, five genes *viz., MMP7*, *COL9A1*, *CALM1*, *MMP1* and *ADAMTS5* were found to have significant association with DDD at 1 % significance level.

- In summary, the results from these association studies (i.e., analyses of association between SNPs and DDD phenotype at individual disc levels, based on whole disc scoring and also in young adults) have identified 16 candidate genes *(LEPR, NGFB, COX2, FN1, COL9A1, TAC1, IL6, MMP1, MMP7, MMP10, CHST3, VDR, CALM1, CILP, MMP2)* that are strongly associated with DDD at 1 % significant level.
- An improved method for extracting proteins from IVD tissues in humans suitable for 2D PAGE analysis was standardized. 2D-PAGE analysis resulted in the detection of about 143 proteins expressed in human IVD.
- Thirty abundant proteins were identified by MALDI-TOF which includes, apolipoprotein A-I, alpha-tubulin N-acetyltransferase isoform 4, huntingtin-interacting protein 1-related proteins etc.,
- GC/MS-based metabolic profiling study has profiled the metabolites present in both control and degenerated human IVD. This study revealed that some of these metabolites present in the human IVD might play an important role in disc degenerative diseases.

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ORIGINAL ARTICLE

Genetic susceptibility of lumbar degenerative disc disease in young Indian adults

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Abstract
Purpose Although the exact mechanisms that lead to degenerative disc disease (DDD) are not well understood, a significant genetic influence has been found. Focusing on DDD that occurs in young adults can be valuable in determining the exact role of genetic predisposition to DDD.
Methods Patients (<40 years) with lumbar disc degeneration were evaluated with MRI imaging (1.5 Tesla) and genetic association analysis for 58 single nucleotide polymorphism (SNP) of 35 candidate genes was performed. Disc degeneration of individual discs of lumbar spine from L1 to S1 was graded by Pfirrmann's grading. The subjects were stratified into two groups based on Total Disc Degenerative Score (TDDS). Based on TDDS, the severity of DDD was classified as mild (Group A: TDDS <10) and severe (Group B: TDDS >10).
Results 695 Indian subjects including 308 with mild TDDS and 387 with severe TDDS were studied. The mean age of the patients was 29.6 ± 6.9 years in group A and 31.7 ± 6.1 in group B ($p < 0.05$). Five of the 35 candidate genes viz., rs1337185 of *COL11A* ($p = 0.02$), rs5275 ($p = 0.03$) and rs5277 ($p = 0.05$) of *COX2,* rs7575934 of *IL1F5* ($p = 0.04$), rs3213718 of *CALM1* ($p = 0.04$) and rs162509 of *ADAMTS5* ($p = 0.04$) were found to be significantly associated with severe TDDS.
Conclusion The study identifies specific SNP associations of five genes in young adults with severe lumbar disc degeneration. These five genes (*COL11A1*, *ADAMTS5*, *CALM*1, *IL1F5*and *COX2*) have different functions in the matrix metabolism, intracellular signalling and inflammatory cascade. This shows that disc degeneration is a complex disease with an intricate interplay of multiple genetic polymorphisms.

Keywords Disc degeneration · Genetics · Single nucleotide polymorphism · Young adults

Introduction

Degenerative disc disease (DDD) is characterised by nucleus dehydration and tears in the annulus leading to loss of disc height. It is a commonly observed radiologic phenomenon in the lumbar spine with increasing age [1]. But DDD can affect the young adults. Salminen et al. [2] reported that 35 % of people between the ages of 20 and 39 had evidence of degenerative disc disease. DDD in the young can lead to debilitating back pain and secondary neurological insults.

Despite extensive research, the aetiology and pathogenesis of DDD have not been clearly understood and studies in the last decade have implicated genetic predisposition in combination with environmental factors to play a central role in the etiopathogenesis of DDD [3, 4]. Genes encoding for the structural components of intervertebral discs, matrix turnover and organisation, inflammatory mediators like Interleukin genes or their receptors have all been associated with DDD [3–12]. An important element

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of genetic studies on DDD is the selection of study phenotype. The term DDD as a phenotype has been used synonymously with various terms like disc dehydration, signal intensity changes, reduction in disc height, annular tear, disc bulge, disc herniation, Modic changes, Schmorl's nodes, osteophyte formation, etc. [3–12]. This has resulted in multiple candidate genes being reported as having associations with DDD in different studies. For example, the Taq I polymorphism of vitamin D receptor gene has been associated with disc degeneration in three different studies. But different phenotypes have been studied in three studies (clinical and MRI assessment of lumbar disc degeneration like signal intensity, disc height, and disc bulging/herniations—Finnish twin study, young adults with/without back pain, and disc degeneration in MRI—Japanese study, disc degeneration based on Schneidermann's score, disc bulge/herniations, annular tears and Schmorl's nodes—South Chinese study) [6, 8, 12]. This indicates the importance of selecting a specific phenotype for disc degeneration.

In the present study, we selected a highly specific study phenotype, which is the presence of severe disc degeneration in the lumbar discs in young population of age less than 40 years. Since clinical symptoms such as back pain and sciatica may be initiated from one of the multiple pain generators of the spine, we selected the MRI feature of disc degeneration quantified by Pfirrmann's grading as the study phenotype. Pfirrmann's et al. [13] grading is a standard grading system to quantify the extent of disc degeneration. Severe DDD in the young people has clinical implications and socio-economic importance in terms of lost productivity. Since the incidence of disc degeneration and the influence of environmental factors on DDD increases with aging, it is possible that young patients with early severe disc degeneration would have a significant genetic predisposition. Focused efforts to understand the genetic basis of early severe disc degeneration in young adults would also help us to elucidate the potential genetic factors in the causation of DDD.

Materials and methods

Institutional review board approval was obtained before starting the study. The study population was recruited from patients of Indian origin presenting at the Spine Unit of a tertiary referral hospital. Specific inclusion and exclusion criteria were used to select the patients for the study. A patient was included in the study based on the following criteria: any sex, less than 40 years, no evidence of other spinal diseases (congenital, pathological, inflammatory and infective), no history of previous lumbar spine surgery and no history of significant spinal injury. MRI of the lumbar spine was performed in a 1.5T MRI scanner to examine the presence and extent of disc degeneration. All the patients were well informed about the study and consent was obtained from all the individuals included in the study. A total of 695 individuals of age less than 40 years representing both the sexes were studied.

Evaluation of the subjects [both cases (TDDS >10) and controls (TDDS <10)]

The study population was subjected to a detailed evaluation to document clinical symptoms, details about occupation, lifestyle and other environmental factors were collected through standard questionnaires. MRI evaluation included T1 and T2-weighted axial, and sagittal sections of the lumbar spine (T12—S1) (1.5 Tesla MRI, Siemens, Germany). Evaluation and phenotyping of the MRI images were performed by two independent observers and the inter-observer reliability was tested (kappa statistic of 0.84 ± 0.11). Any dispute on the score(s) given by the two observers was settled by consensus. Disc degeneration was diagnosed on the basis of signal changes in the intervertebral discs of the lumbar spine and graded using Pfirrmann's grading (Table 1). A Total Disc Degeneration Score (TDDS) was developed by adding the individual scores (Pfirrmann's grading) of all the five lumbar discs. Based on the TDDS, the severity of DDD among the subjects was classified as mild (Group A: TDDS <10) and severe (Group B: TDDS >10). An example of developing the TDDS is given in Fig. 1.

Isolation of genomic DNA

The blood samples were collected from the study population in EDTA containing tubes and stored at −80 °C for laboratory analysis. DNA was extracted from the frozen human blood and the quality and quantity of DNA was checked by agarose gel electrophoresis and spectrophotometry. Fifty-eight SNPs in 35 candidate genes were selected for analysis based on previous genetic studies on DDD (Tables 2, 3) [14]. Genotyping of SNPs of case and control samples was performed using the Sequenome® platform. The Mass ARRAY assay design software was used to design amplification and allele-specific extension primers. The extension primer was designed to hybridise to the amplicon near the SNP site for the extension of a single base or a few bases depending on the genotype of the allele. PCR reactions were set up in 384 well plates using 5 ng of genomic DNA as template. The final base-extension products were desalted using SpectroClean resin (Sequenom) mixed with 3-hydroxypicolinic acid and analysed using a modified BruckerAutoflex MALDI-TOF mass spectrometer. The SpectroAquire and MassARRAYTyper Software packages (Sequenom, San Diego, CA, USA) were

Table 1 Pfirrmann's grading of lumbar disc degeneration

Grade	Structure	Distinction of nucleus and annulus	Signal intensity	Height of intervertebral disc
I	Homogeneous, bright white	Clear	Hyperintense, isointense to cerebrospinal fluid	Normal
II	Inhomogeneous with or without horizontal bands	Clear	Hyperintense, isointense to cerebrospinal fluid	Normal
III	Inhomogeneous grey	Unclear	Intermediate	Normal to slightly decreased
IV	Inhomogeneous grey to black	Lost	Intermediate to hypointense	Normal to moderately decreased
V	Inhomogeneous black	Lost	Hypointense	Collapsed disc space

Pfirrmann et al. [13]

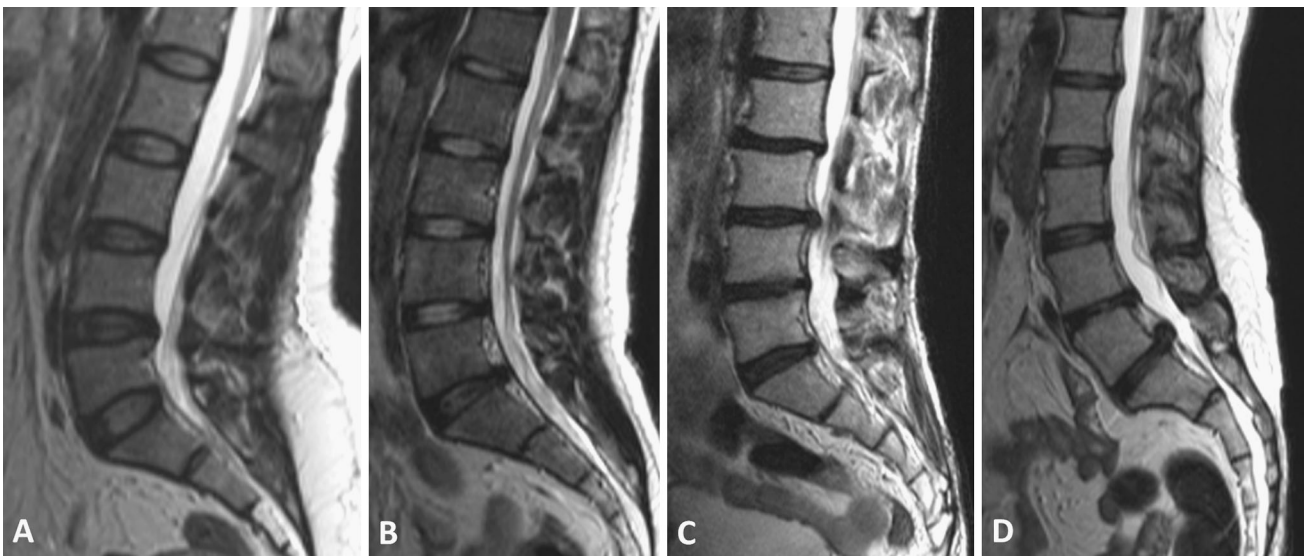

Fig. 1 Calculating Total Degenerative Disc Score (TDDS) in a sagittal MRI image of the lumbar spine. The Pfirmann's grades of each of the five lumbar discs are added together to develop the TDDS. Patient in **a** has a score of 10 (1, 1, 2, 4, 2), **b** has a score of 9 (1, 1, 2, 2, 3) and thus belong to mild TDDS. The patients in **c** and **d** have scores of 21 (3, 5, 4, 5, 4), and 17 (2, 3, 3, 5, 4), respectively, and hence belong to severe TDDS

used for interpretation and Typer analyser V3.4.0.18 was used to review and analyse all data.

PLINK, an association analysis software was used for all the downstream analysis and validation data. The SNPs prevalent in all the target genes of study population were predicted and association test was performed using PLINK software based on the nature of SNPs. Probability value and odds ratio were estimated for all the 58 SNPs, and also tested their significance towards DDD.

Results

The study population comprised of 695 subjects including 308 patients with mild TDDS (Group A) and 387 patients with severe TDDS (Group B). The mean age of the patients was 29.6 ± 6.9 years in group A and 31.7 ± 6.1 in group B ($p < 0.05$). The male:female ratio was as follows: 162:146 in group A and 228:159 in group B. The mean Pfirrmann's grade was 7.99 ± 2.1 (range 5–10) in group A and 13.7 ± 2.4 (range 11–25) in group B. Five of the 35 candidate genes showed significant association with severe TDDS. SNPs viz., rs1337185 of *COL11A* ($p = 0.02$), rs5275 ($p = 0.03$) and rs5277 ($p = 0.05$) of *COX2,* rs7575934 of *IL1F5* ($p = 0.04$), rs3213718 of *CALM1* ($p = 0.04$) and rs162509 of *ADAMTS5* ($p = 0.04$) were found to be significantly associated with severe TDDS (Table 4). Among the previously reported candidate genes, our analysis in the young Indian population had revealed that SNPs of *COL 9, SKT, CHST 3, CILP, IGFR, SOXp,*

Table 2 The study involved the selection and analysis of the following 35 candidate genes

Functional zones	Candidate genes
Structural genes—matrix	COL9A2, COL11A1, COL9A1, AGC1, SKT, CHST3, GLI1, CILP, CALM1, IGF1R, SOX9
Structural genes—bone	BMP5, BMP2, HHIP
Apoptosis related/neuronal genes	NGFB, TAC1
Degradative genes	MMP2, MMP7, MMP20, MMP10, MMP1, MMP3, MMP12, MMP13, ADAMTS5, ADH2
Inflammatory genes	IL18RAP, IL1A, IL1B, IL1F5, IL1F10, IL1RN, COX2

BMP, MMP 2-12, ADH2, IL1RN, AGC 1, NGFB, GLI, HHIP, TAC1 and IL1B were not significantly associated with severe TDDS.

Discussion

Though intervertebral discs degenerate much earlier than other musculoskeletal tissues, they are mostly asymptomatic. Disc degeneration in the lumbar discs has been reported to occur as early as in the second decade of life [15]. Boos et al. [15] demonstrated age-associated changes in morphology of discs, with discs from children as young as 2 years of age having some very mild cleft formation and granular changes to the nucleus. About 20 % of people in their teens have discs with mild signs of degeneration and this incidence increases with aging, so that around 10 % of discs in 50 years old and 60 % of discs in 70 years old people are severely degenerated [16]. Though disc degeneration starts in the young adulthood, severe disc degeneration characterised by the presence of TDDS >10 is uncommon in the young adults [2, 17]. Such advanced disc degeneration at a young age represents a different subset of patients with possibly different etiopathogenic mechanisms. Inclusion of these patients together with adult patients (who have disc degeneration due to aging) in genetic studies on disc degeneration could potentially confound the results. We studied young patients with severe disc degeneration of the lumbar spine and observed that SNPs of five genes have been significantly associated with severe disc degeneration. So far, genetic association studies on DDD have implicated several SNPs in various candidate genes. But these SNP associations are not always replicated uniformly in other studies. We believe that young adults with severe DDD should be considered as a separate study group in genetic association studies of DDD.

Table 3 The relevant SNPs in the various candidate genes and their positions in the respective chromosomes

Chromosome no	SNP	Position	Genes
1	rs12077871	40545737	*COL9A2*
1	rs1463035	103222784	*COL11A1*
1	rs1337185	103317353	*COL11A1*
1	rs2856813	115639442	*NFGB*
1	rs4076018	115644333	*NGFB*
1	rs5277	184914820	*COX2*
2	rs1420100	102403434	*IL18RAP*
2	rs1800587	113259431	*IL1A*
2	rs1143634	113306861	*IL1B*
2	rs1143633	113306938	*IL1B*
2	rs7575934	113533805	*IL1F5*
2	rs2071375	113535438	*IL1A*
2	rs579543	113606102	*IL1RN*
2	rs11690459	113833538	*IL1F10*
2	rs2234679	113875584	*IL1RN*
4	rs1229984	100458342	*ADH2*
4	rs1812175	145794294	*HHIP*
6	rs966329	55798298	*BMP5*
6	rs696990	71078947	*COL9A1*
7	rs1229434	97203778	*TAC1*
7	rs3779470	97203867	*TAC1*
10	rs16924573	24644899	*SKT*
10	rs4148941	73438998	*CHST3*
10	rs4148949	73440657	*CHST3*
11	rs1996352	101901457	*MMP7*
11	rs1784438	101978396	*MMP20*
11	rs1784430	101980023	*MMP20*
11	rs17099008	101987714	*MMP20*
11	rs2701964	101999091	*MMP20*
11	rs470154	102152520	*MMP10*
11	rs11225422	102161528	*MMP10*
11	rs2239008	102166290	*MMP1*
11	rs491152	102171253	*MMP1*
11	rs591058	102216548	*MMP3*
11	rs672535	102261577	*MMP12*
11	rs2252070	102331749	*MMP13*
11	rs1940044	102421892	*MMP7*
12	rs2292657	56146199	*GLI1*
12	rs2228224	56151588	*GLI1*
14	rs2300496	89934601	*CALM1*
14	rs3213718	89939666	*CALM1*
14	rs1058903	89943551	*CALM1*
15	rs2073711	63281265	*CILP*
15	rs1551343	66872811	*ANP32A*
15	rs1042631	87203243	*AGC1*
15	rs11247361	97024915	*IGF1R*
16	rs243865	54069307	*MMP2*
17	rs10852739	67421373	*SOX9 (p)*
20	rs235770	6709765	*BMP2*
20	rs2273073	6750882	*BMP2*
21	rs467691	27208461	*ADAMTS5*

Table 3 continued

Chromosome no	SNP	Position	Genes
21	rs229079	27219920	*ADAMTS5*
21	rs226794	27224226	*ADAMTS5*
21	rs2249333	27232045	*ADAMTS5*
21	rs2249350	27244377	*ADAMTS5*
21	rs162509	27247646	*ADAMTS5*
21	rs229077	28295339	*ADAMTS5*
X	rs4898	47329929	*TIMP1*

Table 4 Association analysis of SNPs between controls and cases

S. no	Chromosome	Gene	SNP	*p* value	Odds ratio
1	1	*COL11A1*	rs1337185	0.02433*	1.55
2	1	*COX2*	rs5275	0.03003*	1.29
3	1	*COX2*	rs5277	0.05002*	0.6182
4	2	*IL1F5*	rs7575934	0.04567*	0.719
5	14	*CALM1*	rs3213718	0.04347*	1.261
6	21	*ADAMTS5*	rs162509	0.04068*	1.281

* All *p* values were significant at <0.05

Total Disc Degenerative Score (TDDS)

It is critical that studies on disc degeneration should consider the radiologic features of all the five lumbar discs rather than concentrating on few discs. Systemic causes such as genetic predisposition to disc degeneration or metabolic causes should make all the lumbar discs equally susceptible to disc degeneration. Similarly changes that happen at one or two disc levels may not be comparable to generalised changes happening in all the lumbar discs. A patient with single level DDD of Pfirrmann's Grade 5 with other normal discs is possibly different from another patient with Grade 3 degeneration in all discs. Hence in the present study, we used the TDDS developed by adding the individual Pfirrmann's scores of all the five lumbar discs. The comparison was made between the mild (TDDS <10) and severe (TDDS >10) patients. We studied 58 SNPs in 35 potential candidate genes for their association with severe lumbar disc degeneration. In our study, we have found strong associations of two polymorphisms of COX2 and one each of CALM1, ADAMTS5, COL11A1 and IL1F5, with severe disc degeneration in young adults.

CALM1 (Calmodulin 1 gene)

The SNP rs3213718 of *CALM1* had a significant association ($p < 0.05$) with severe disc degeneration in our study. This is the first report on the association of CALM1 with lumbar disc degeneration. The Calmodulin 1 gene encodes for a calcium-binding protein which is a mediator of the calcium signals. It is an intracellular protein that interacts with several proteins involved in signal transduction. A different polymorphism (functional core promoter SNP-16C/T: rs12885713) was associated with hip osteoarthritis in the Japanese population [18], but it did not show significant association in a British study [19]. *CALM1* is expressed in cultured chondrocytes and articular cartilage, and its expression has been observed to be increased in osteoarthritis. Mechanical compression of articular chondrocytes is known to trigger changes in aggrecan expression, and such changes are dependent on calmodulin signalling. Disc degeneration is also characterised by disintegration and loss of extracellular matrix, end plate damage and associated with varying degrees of osteophyte formation. Hence, calmodulin could be an important mediator in the pathogenesis of disc degeneration.

Collagen XI (*COL11A1*)

Type XI collagen is a constituent of both annulus fibrosus and nucleus pulposus. In a study in Japanese population by Mio and his co-workers, a significant association between one of the type XI collagen genes, COL11A1, and lumbar disc herniation was identified. COL11A1, which encodes the α1 chain of type XI collagen, is normally highly expressed in discs, but the authors observed that the expression was decreased in patients with disc herniation [11]. The expression level was inversely correlated with the severity of disc degeneration. Allele rs1676486 of COL11A1 had the most significant association with lumbar disc herniation and the authors concluded that type XI collagen is critical for intervertebral disc metabolism. In our study, we observed that rs1337185 of COL11A1 gene was significantly associated with severe disc degeneration in young patients.

A disintegrin and metalloproteinase with thrombospondin motifs 5 (*ADAMTS5*)

In this study, SNP of ADAMTS5 was very significantly associated with severe disc degeneration. ADAMTS5 is a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) protein family. The enzyme functions as an aggrecanase to cleave aggrecan. It is considered to play an important role in matrix degradation and metabolism of extracellular disc matrix. A recent study, using a murine system suggested that ADAMTS5 was primarily responsible for the major turnover of aggrecan in mouse cartilage [20]. Accelerated destruction of the extracellular matrix of the intervertebral disc could lead to disc degeneration.

Interleukin 1 (IL1F5)

Interleukin 1 is possibly involved in disc degeneration by increasing the destruction of disc matrix through activating degradative enzymes. Lemaitre and his co-workers have shown that *IL-1* is not only capable of inducing the matrix changes characteristic of disc degeneration but that its expression is increased during degeneration, without a concomitant increase in its naturally occurring inhibitor *IL-1Ra* (IL-1 receptor antagonist) [21]. In our study, we observed that genetic polymorphism of *IL1F5* gene was significantly associated with severe DDD.

Cyclooxygenase 2 (*COX2*)

The rs5277 SNP of *COX2* had a strong positive association with severe disc degeneration. *COX2* is an enzyme that is responsible for formation of important biological mediators such as prostaglandins, prostacyclin and thromboxane. Variants of genes coding for inflammatory mediators such as interleukin one, interleukin receptor one, cyclooxygenase two and matrix metalloprotease have all been found to be associated with lumbar degenerative disc disease [9, 10, 22–24]. The *COX2* gene may be involved in disc herniation through the upregulation of prostaglandin E2 (PGE2) [22]. It is also reported to be involved in the peripheral modulation of pain [25]. IL1 possibly causes degeneration by activating degradative enzymes and inhibiting synthesis of proteoglycans. It also modulates pain by inducing PGE2 synthesis which in turn enhances the activity of phospholipase A2 and COX2. IL1 secreted by herniated discs, along with IL6 and TNF-α sensitise the nociceptors that innervate the disc [26, 27]. This could be involved in the pain generation in disc degeneration.

Kelempisioti et al. [17] investigated the associations of 19 SNPs in 16 candidate genes reported to be associated with disc degeneration among 538 young adults with a mean age of 19. Of the 538 individuals studied, 46 % ($n = 246$) had no degeneration, while 54 % ($n = 292$) had disc degeneration and 51 % ($n = 150$) of these had moderate degeneration. They compared the patients with moderate disc degeneration with those having no degeneration. The risk of DD was significantly higher in subjects with SNPs of Interleukin 6 (*IL6*), Sickle cell trait (*SKT*) and Cartilage Intermediate Layer Protein (*CILP*). They have concluded that *IL6*, *SKT* and *CILP* are possibly involved in the aetiology of disc degeneration among young adults. In the present study, we studied the above SNPs but could not identify any positive association. This could be possibly due to ethnic variations as the radiological methods and patient grouping are similar in both the studies. In genetic association studies, the strength of a study depends on its cohort size. The present study and the work by Kelempisioti et al. involved a moderate sample size. However, for the specific phenotype that we have selected, and considering the infrequent incidence of severe disc degeneration in young adults, the study population is a high number. It would be interesting and informative to have multi-centre studies on this subject which can provide a larger sample size.

Conclusion

The present study is the first of its kind towards understanding the genetic basis of degenerative disc disease in young Indian population. The Indian population forms one-sixth of the world's population and ethnically different from the other cohorts like Chinese, Caucasian in whom previous genetic studies have been done [6–8, 12, 28, 29]. This study identifies specific associations of six SNPs present in five different genes in young adults with severe lumbar disc degeneration. These five genes (*COL11A1*, *ADAMTS5*, *CALM1*, *IL1F5* and *COX2*) have different functions in the matrix strength and metabolism, intracellular signalling and inflammatory cascade, respectively. The results showed that disc degeneration is a complex disease with an intricate interplay of multiple genetic polymorphisms.

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Conflict of interest None.

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Intervertebral Disc Proteome Database in Human (TNAUPDB –Human Proteome)

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Abstract

Human intervertebral disc (IVD) proteome is an open accessible database of Tamil Nadu Agricultural University Proteome DataBase (TNAU PDB) that focuses on proteome of human intervertebral disc. Currently, the database contains reference maps of Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) of proteins obtained from degenerated intervertebral disc. The initial 2D-PAGE and MALDI TOF studies have revealed that abundantly expressed 30 protein spots. The database provides information about experimentally identified properties, such as molecular weight, pI value and protein identities obtained using MALDI-TOF and MASCOT analysis. This database runs on WAMP server with HTML as the front end and MySQL as the backend using PHP as interface and it is hosted in TNAU genomics domain. The basic intention of this database is to provide information about intervertebral disc proteome. Further information on differentially expressed proteins on comparison with healthy and degenerated intervertebral disc tissue will help us to gain insights into the underlying molecular mechanism in disc degenerative disease. This database will be a valuable biological resource for the treatment of disc degenerative disease.

Availability: The human intervertebral disc proteome database is freely available at http://www.tnaugenomics.com.

Keywords: Intervertebral disc, Disc degenerative disease, database, proteomics, proteome database, TNAUPDB, TNAU genomics.

Introduction

Low back pain (LBP) is a highly prevalent musculoskeletal disorder in mankind, caused by degeneration of intervertebral discs (IVD) of the lumbar spine (1). It is one of the leading causes of disability in the industrialized world including India. The prevalence of low back pain in the society is reported to be around 60 per cent and 80 per cent of the population suffers at least one episode of back pain in their life (2). The annual prevalence ranges from 15 to 45% with the point prevalence averaging 30% (3). The costs associated with this condition are enormous, including both direct medical costs and indirect costs, such as decreased productivity in the workplace. LBP is therefore not only a health problem but also a socio-economic problem. The second most frequent reason for visits to physicians, the fifth-ranking cause of admission to hospital and the third most common reason for surgical procedures (4).

Molecular mechanism(s) underlying LBP/DDD is still unclear mainly because of the difficulty in obtaining intact IVD tissue from patients for molecular studies. So far, only a very few attempts have been made to understand the molecular basis of DDD in humans. Recent reports are showing enough evidences to the involvement of genetic factors and a number of

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genes.However, functional studies of candidate genes will be an important step for testing whether a candidate gene is truly associated with DDD or not. Hence proteomic approach will be a better option to understand the molecular mechanism of disc degenerative disease.

Methodology

Database Content and Source *:* Proteins were extracted from the degenerated IVD was obtained from a fifty two year old female who underwent spine surgery for lumbar disc prolapse after getting permission from Ethical Clearance Committee. IVD tissue sample was pulverized using liquid nitrogen and proteins were extracted as explained by Belluoccio *et al.* (2006) with minor modifications. Extracted proteins from the single patient were subjected to 2D-PAGE analysis (Figure 1) and abundantly expressed proteins were analyzed using MALDI TOF analysis, and the putative functions of these proteins were annotated by MASCOT analysis. Hence, currently, the TNAUPDB human intervertebral disc proteome database consists of information on abundantly expressed proteins from degenerated intervertebral disc tissue.

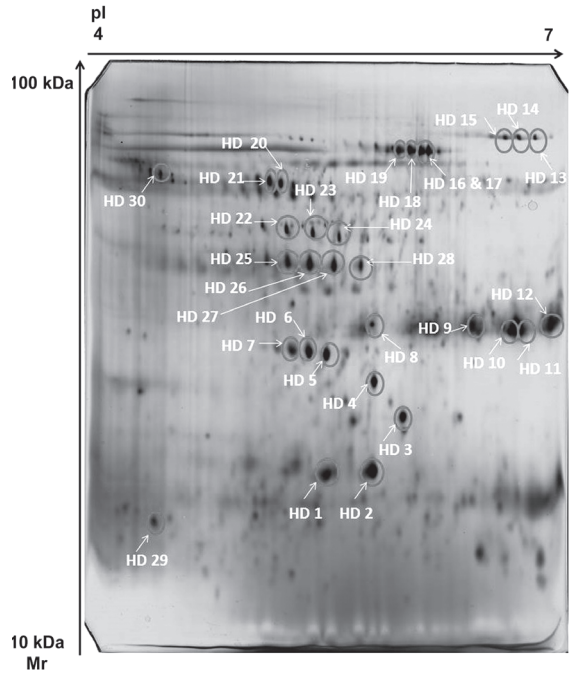

Fig. 1. 2D PAGE protein profiles of intervertebral disc tissue

Languages and softwares used *:* The front end of the web application is developed on HTML 5.0 (Hyper Text Markup Language) and the validations are done using javascript. The server side scripting was done on PHP 5.4 (Hypertext Pre Processor) and the application was connected to the database using MySQL 5.5.16. PhpMyAdmin provides a graphical user interface for the MySQL database. Web application was created by using wamp server 2.3 windows web development environment. Adobe Dreamweaver was used to link each spot in the gel image to the corresponding protein information.

Techniques behind the database *:* In order to provide effective information from biological data, advanced web query interface is created. Web query interface was created using HTML as the front end and MySQL as the back end with Hypertext preprocessor (PHP) as the interface. Database indexing is a method that is used to improve the speed of data retrieval operations on database table. Clustering method was used to retrieve group of similar object based on the maximum and minimum value. Data are stored as relational model database (5).

Database architecture *:* This human intervertebral disc database architecture is accumulated in three layers similar to black gram proteome database (6). Presentation layer, Application layer and Data storage layer as shown in the Figure 2. The database is built on a three layer architecture model consisting of presentation layer, application layer and the data storage layer. The presentation layer or the top most layer is created using HTML which serves as interface for the user to interact with the database. The middle layer or the application layer is created using PHP, which connects the user interface with that of the database. This application layer transfers the user's query to the database and retrieves the result from it. The

Fig. 2. Database architecture

bottom layer or the data storage layer (MySQL) contains the data tables from which the database results are retrieved.

Results and Discussion

Data flow *:* Intervertebral disc proteome database has been added as a sub link under TNAU genomics domain which has home page under the link http://www.tnaugenomics.com. From the homepage, it can be reached through the drop down link named TNAUPDB under the tab Databases. The flow of data and the procedure to access the database is shown in Figure 3.

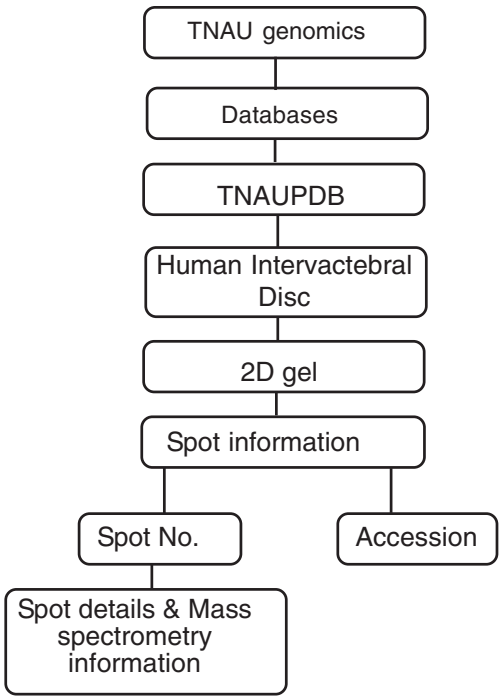

Fig. 3. Data flow

Database Schema *:* The database contains the protein information from intervertebral disc. Here, the 30 abundantly expressed IVD proteins identified using the 2D PAGE and MALDI-TOF (Table 1) are displayed using image analyzer software and the description of each spot are mapped to the corresponding spots using Adobe Dreamweaver. The reference 2D-PAGE gel shows the position of each identified protein. By selecting the spot number in the gel image, the entire information about that protein such as spot ID, molecular weight, isoelectric point, organism, tissue, homologous protein name, sequence coverage, theoretical molecular weight and isoelectric point, mascot score and mascot result can be obtained. The experimental protocols are listed and are freely available under the protocol section. Some of the major proteomics tools like Mascot, Compute pI/Mw tool in ExPASy and ExPASy Proteomics tools are displayed under proteome tools section. Similar advanced proteome databases are available for Arabidopsis and maize by Cornell university (7) and DynaProt 2D for dynamic online access to proteomes and two-dimensional electrophoresis gels (8).

Utility of the intervertebral disc proteome database to the biological community : Proteomics is one of the high throughput technologies in post genomics era. In the database, we have provided a snapshot of human IVD proteome. Further studies are required to dissect the functions of these differentially expressed proteins from both control

Table 1. Identification of IVD proteins by MASCOT analysis

Spot ID [1]	Coverage [2]	Accession No [3]
HD1	48	167887751
	25	19908424
HD2	31	205360981
HD3	11	119619469
HD4	47	914833
HD5	64	90108665
HD6	49	90108664
HD7	30	62088402
HD8	61	159163872
HD9	27	119612556
	27	4929613
HD10	37	3721836
HD11	34	134254718
HD12	30	3660517
HD13	33	296676
HD14	48	194387990
HD15	21	119612556
HD16	27	31455194
HD17	61	145938571
HD18	44	40788217
HD19	28	119592961
HD20	54	110624781
HD21	64	159163872
HD22	42	332245896
	43	13543925
HD23	63	39653325
HD24	25	121934188
HD25	26	385198093
HD26	64	3660517
HD27	27	385198093
HD28	39	4867999
HD29	27	385198093
HD30	14	17426164

[1] ID refers to the corresponding spot number in fig. 1

[2] Amino acid sequence coverage

[3]Corresponding accession numbers for the identified proteins were obtained from NCBI (www. ncbi. nlm.nih.gov).

and degenerated disc to understand their precise role in the disc biology and hence pave way for designing novel strategies to treat the disc degenerative disease. The primary users/ beneficiaries of this database will be spine surgeons, biochemist, students and other research scholars interested in treatment of disc degenerative disease.

Future developments : The intervertebral disc proteome database will be updated regularly. The scientists of TNAU Genomics and Proteomics lab will deposit and update their new data that become accessible on the web. In future, data on control IVD proteome and data on differentially expressed protein also will be made available.

Conclusion

TNAU PDB – Intervertebral disc Proteome database focuses on proteome of human IVD. Currently, data on degenerated IVD tissue proteome is made available, and the database will be updated routinely as and when new data are obtained. Hence, this database will be highly helpful to spine surgeons, biochemist, students and other researcher scholars interested in treatment of disc degenerative disease.

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Profiling of Metabolites from Human Intervertebral Disc through Gas Chromatography - Mass Spectrometry

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Abstract

This work aims to identify the metabolites present in the human Intervertebral Disc (IVD). Metabolomic analysis of human IVD tissue has not been extensively done to date. Knowledge on the metabolites present in the IVD tissue in humans is very limited and many compounds are yet to be identified. In this study, we have carried out the metabolic profiling for human IVD through Gas Chromatography/Mass Spectrometry (GC/MS). This is the first initial study that has compared the metabolites of control and diseased IVD. We have identified 75 different chemical compounds in IVD, and also metabolites that are unique to the diseased IVD, suggesting that some of these metabolites might play a role in disc degenerative disease.

Keywords: DDD, Human, Intervertebral Disc, Metabolite Profiling

1. Introduction

Low Back Pain (LBP) is a global health problem in which more than 40% is caused by lumbar intervertebra[1] disc degeneration1 and one of the most important health care issues today. About 60 % and 80 % of the global population experiences LBP at least once in their lifetime[2]. The annual prevalence ranges from 15% to 45% with the point prevalence averaging 30% [3]. Only a small proportion (approximately 20%) of LBP cases can be attributed with reasonable certainty to a pathologic or anatomical entity. Thus, diagnosing the cause of LBP represents the biggest challenge for doctors in this field. Recent reports are showing enough evidences to the involvement of genetic factors and a number of genes like Vitamin D Receptor (VDR)[4], Collagen – factors[5], Interleukins[6], MMP3 (Matrix metalloproteinase-3)[7], aggrecan[8] and cartilage intermediate layer protein[2] are reported to be associated with DDD. A few candidate genes, which have a weak association with DDD (viz., (Vitamin D receptor) VDR, (collagen IX A2) COL9A2, (collagen IX A3) COL9A3, (Matrix metalloprotease-3) MMP3 and Aggrecan), have been identified using genetic polymorphisms[9]. However, functional studies of candidate genes will be an important step for testing whether a candidate gene is truly associated with Disc Degenerative Disease (DDD) or not.Hence systematic analysis of genes expressed in IVD through high throughput genomic tools viz., microarrays, proteomics and metabolomics are needed which will shed light on the major events associated with DDD and lead

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to the identification of metabolic pathways involved in this degeneration.

2. Materials and Methods

The IVD of control and patient were obtained from spine surgery unit and used after getting permission from Ethical Clearance Committee with informed consent. For method development and validation, a representative control and affected disc tissue was snap-frozen immediately following surgery and then stored at – 80°C. About 20mg of the stored tissue weighed accurately and ground with liquid nitrogen. Then 1mL of a mono-phasic mixture of chloroform/methanol/water in ratio of 20:50:20 (v/v/v) was added to each sample[10]. The samples were ultrasonicated in an ultra-sonicator bath at ambient temperature (24–28°C) for 100 min and then vortex-mixed for 2 min. The samples were subsequently centrifuged at 12000 rpm for 10 min and the supernatant was collected separately from each sample in different tubes. The collected supernatant was concentrated to complete dryness at 50°C for 30 min. A 100 µL of toluene (kept anhydrous with sodium sulfate) was added to each of the sample extracts, vortex-mixed for 5 min and again evaporated to complete dryness using vacuum evaporator in order to eliminate any trace amount of water which might interfere with the GC/MS analysis.

2.1 Derivatization Procedure

The dried samples were then derivatized by adding 100 µL of MSTFA with 1% TMCS to each sample. The samples were then vortex-mixed for 2 min and incubated at 70°C for 30 min. After incubation, samples were again vortex-mixed for 2 min and then transferred to glass vials for GC/MS analysis.

2.2 GC/MS Analysis

The analysis was performed on a Thermo GC - Trace Ultra Ver: 5.0, Thermo Ms DSQ II. A DB 5 - MS capillary standard non - polar column (30 x 0.25mm; 0.25µm flim thickness, Thermo Scientific). Helium was used as the carrier gas at 1.0 mL per min and the injector split ratio was set to 1:5. An injection volume of 1µl was used and the solvent cut off time was 5 min.

The injector and source temperatures were kept at 250°C and 200°C, respectively. The oven temperature was kept at 60°C for 3 min, increased at 7°C min^{-1} to 140°C where it was held for 4 min and further increased at 5°C min^{-1} to 310°C where it remained for 6 min. The mass spectrometer was operated in Electron Impact (EI) ionization mode at 70 eV. Data acquisition was performed in the full scan mode from m/z 50 to 650 with a scan time of 0.5 s.

3. Results

3.1 Metabolite Identification and Data Processing

GC/MS analysis from this study led to the identification of 75 different metabolites (Figure 1 and 2) belonging

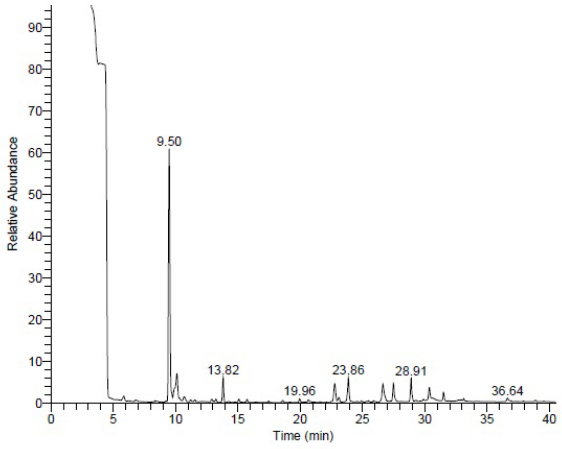

Figure 1. GC/MS chromatograms of control intervertebral disc.

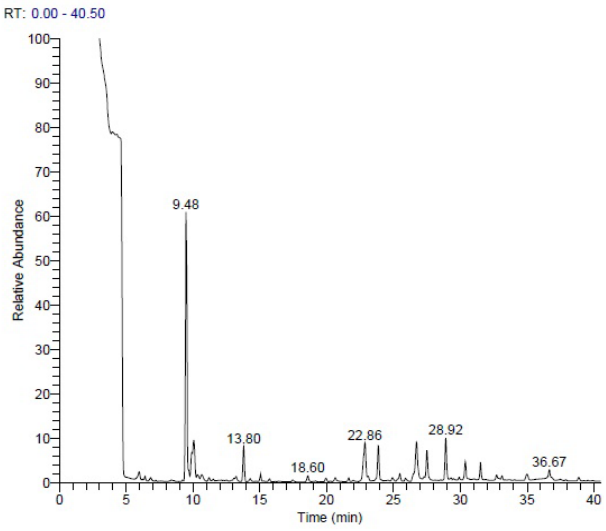

Figure 2. GC/MS chromatograms of degenerated intervertebral disc.

to diverse chemical classes such as amino acids, organic acids, fatty acids, phenolics, silanes, nitriles, ethers, amines, azides, hydrocarbons, alcohols, carbonyl compounds, heterocyclic compounds, spiro compounds and metal complex (Table 1 and 2). The identities of selected metabolites were recognized by the NIST mass spectral library.

4. Discussion

GC/MS-based metabolomics is a powerful tool to elucidate the impact of low back pain thereby providing novel insights into the pathomechanism underlying disc degenerative disease. This GC/MS based metabolomic approaches cover a large number of novel compounds.

Table 1. Identified metabolites from control intervertebral disc tissue through GC/MS against NIST library search

S. No	Metabolites	Retention time	Chemical groups identified
1	Cyclopropanecarboxylic acid, pent-2-en-4-ynyl ester	3.06	Organic acid and its derivatides
2	N1-(Formyl)-N2-(1-oxobut-2-en-1-yl)hydrazide	3.45	Azides
3	exo-5-hexyl-exo-4-oxa-tricyclo[5.2.1.0**2,6]dec-8-en-3-one	4.43	Carbonyl compounds
4	Methoxy-phenyl oxime	4.92	Carbonyl compounds
5	D-Lactic acid-DITMS	5.28	organic acid and its derivatives
6	Methyl 3-((aminocarbonyl)amino)-2-cyano-3-phenylpropenoate	5.87	Organic acid and its derivatides
7	2-[5-(2-hydroxyethyl)-2-thienyl]-4,4-dimethyloxazoline	6.28	Heterocyclic compounds
8	2-methyl Decane	6.77	Hydrocarbons
9	1,2-Dioxetane, 3,4,4-trimethyl-3-[[(trimethylsilyl)oxy] methyl]1,2-Dioxetane	7.38	Silanes
10	5,8-Diethoxy-7-methoxyquinoline	8.05	Heterocyclic compounds
11	Octanoic acid, trimethylsilyl ester	8.4	fatty acid (S)
12	1-Tert-buthoxy-6-trimethylsilyloxyhexane	8.79	Ethers
13	Bis(trimethylsilyl) 3-Ketovaleric acid	9.22	Amino acids
14	Tris(hydroxymethyl)aminomethane,O,O',O"-tris(trimethylsilyl) ether	9.5	Ethers
15	3-hydroxy Benzocycloheptene	10.12	Hydrocarbons
16	4-Methyl-6-cyanothieno[2,3-b]pyridine	10.31	Heterocyclic compounds
17	[(2-deuterio)-s-isobutyl]-2-propenyl-sulfoxide	10.73	Hydrocarbon
18	N-Acryloylmorpholine	11.21	Heterocyclic compounds
19	4,5-Dihydro-4,5-trans-di-n-propyl-2-ethoxyimidazole	11.54	Heterocyclic compounds
20	Docosane	12.16	Hydrocarbon
21	N-Nitrosomethylethylamine (à-D2)	12.57	Amines
22	[6-(4-tert-Butylphenyl)-1,3,5-hexatriynyl]trimethylsilane	12.9	Silanes
23	Hexadecane (CAS)	13.24	Hydrocarbon
24	3-phenyl-1,2-naphthoquinone	13.82	Hydrocarbon
25	Dodecanoic acid (CAS)	14.26	fatty acid (S)
26	Eicosane, 10-methyl- (CAS)	14.72	Hydrocarbon
27	Isopropyl Dodecanoate	15.09	Organic acid and its derivatives
28	2-Methoxy-8-Chloro-Dibenzofuran	15.27	Heterocyclic compounds
29	Eicosane, 2-methyl- (CAS)	15.74	Hydrocarbon

Continued ...

30	Oxirane, hexyl- (CAS)	15.98	Cyclic ether (Ethylene oxide)
31	Malonic acid, dodecyl 2-ethylbutyl ester	16.19	Organic acid and its derivatives
32	1-methoxymethyl-4-methylnaphthalene	16.41	Hydrocarbon
33	Nonadecanoic acid, 18-oxo-, methyl ester (CAS)	17.25	fatty acid (S)
34	17-Methyl-9-oxo-10-nor-14à-4,5-nitrilomorphinan	17.46	Heterocyclic compounds
35	Tetradecanoic acid (CAS)	18.59	fatty acid (S)(Myristic acid
36	2-(o-Hydroxymethylbenzyl)naphtho[2,3-b]thiophene	19.19	Heterocyclic compounds
37	Heneicosane	19.96	Hydrocarbon
38	(15à)-phylloclad-16-ene-15-carbaldehyde	20.65	Carbonyl compounds
39	Eicosane	21.55	Hydrocarbon
40	1-Hydroxy-17-(1-oxoethyl)-2-oxa-androst-4-en-3-one	22.14	Carbonyl compounds
41	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (CAS)	22.79	Organic acid and its derivatives
42	2-Methoxycarbonyl-3-phenylsulfonylhydroquinone	23.1	Hydrocarbon
43	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	23.86	Carbonyl compounds
44	Bis[1,2-di(2-thienyl)-1,2-ethenedithiolene]nickel	24.57	Metal complex
45	Dibutyl phthalate	24.93	organic acid and its derivatives
46	4-(4-Fluorophenyl)-2-methyl-6-methylthiobenzonitrile	25.51	Hydrocarbon
47	Diethyl [2- (4'-methylphenyl) ethyl] phosphonate	25.89	Organic acid and its derivatives
48	2,2-Dichlorocyclobuta[a]cyclopent[3,4-a]azulenone	26.64	Carbonyl compounds
49	1,3-Dimethoxy-5,7-dihydrodibenz[c,e]oxepine	27.48	Heterocyclic compounds
50	Hentriacontane	28.12	Hydrocarbon
51	Octadecanoic acid, propyl ester (CAS)	28.31	Fatty acid (S) (Stearic acid
52	10,10-dimethylanthrone hydrazone	28.53	Carbonyl compounds
53	1,3-Dimethoxy-5,7-dihydrodibenz[c,e]oxepine	28.91	Heterocyclic compounds
54	9,10-Dihydrocyclobuta[a]triphenylene-11,12-dione	29	Carbonyl compounds
55	Pulchelstyrene D	29.91	Hydrocarbon
56	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	30.36	Carbonyl compounds
57	3,18-Epoxyandrosta-5,7-dien-17-ol, 4,4-dimethyl-3-methoxy- (13á)	31.25	Epoxide (Hydrocarbons)
58	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	31.5	Carbonyl compounds
59	2áBenzyl-8-oxo-4,6-dimethyl-3,5,7-trioxatetracyclo[7.2.1. 0(4,11).0 (6,10)]dodecane	31.94	Hydrocarbon
60	Pentacosane	32.87	Hydrocarbon
61	Di-(2-ethylhexyl)phthalate	33.14	Organic acid and its derivatives
62	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	33.32	Hydrocarbon
63	Nonacosane (CAS)	33.59	Hydrocarbon
64	03027205002 Flavone	34.27	Carbonyl compounds
65	10-Benzyloxy-1,8-dihydroxy-9(10H)-anthracenone	35.21	Carbonyl compounds
66	2-Pentoxy-tetrahydropyran	35.59	Heterocyclic compounds
67	N-(5à-Cholestan-3á-yl)-acetamide	36.17	Organic acids and its derivatives

Continued ...

68	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (CAS)	36.64	Fatty acid
69	Tetratetracontane (CAS)	37.31	Hydrocarbon
70	1,4-Cyclohexadiene-1,2-dicarboxylic acid, 4,5-dimethyl-, dimethyl ester	37.92	Organic acids and its derivatives
71	Acetic acid, 4,5-dihydroxy-10,13-dimethyl-3-oxohexadecahydrocyclop enta[a]phenanthren-17-yl ester	38.21	Organic acids and its derivatives
72	Methyl 4-(4-methoxybenzoyl)-4-methyl-pent-2-enoate	38.5	Organic acids and its derivatives
73	13-Docosenamide, (Z)-	38.87	Organic acids and its derivatives
74	2-[3-(Aminomethyl)-5,7-Dimethyl-1-Adamantyl] Ethanamine	39.56	Amines
75	Dihydromonticamine	39.93	Amines

Table 2. Identified metabolites from degenerated intervertebral disc tissue through GC/MS against NIST library search

S. No	Metabolites	Retention time	Chemical groups identified
1	1-Benzyloxymethyl-1-Hydroxymethyl-2,5- Cyclohexadiene	3.1	Hydrocarbon
2	4-Chloro-4-(phenylsulfinyl)-3-heptanol isomer	3.51	Alcohols
3	(6á)-8a-(3',3'-Dimethylbut-1'-ynyl)-3,4,4a,5,6,8a-hexahydro-6-methoxy-3,3,6-trimethylnaphthalen-1(2H)-one	4.65	Carbonyl compound
4	4-[3-(Trimethylsilyl)-2-propinyl]oxy-2-butynoic acid	5.09	Organic acid and its derivatives
5	D-Lactic acid-DITMS	6	Organic acid and its derivatives
6	2-[5-(2-hydroxyethyl)-2-thienyl]-4,4-dimethyloxazoline	6.41	Heterocyclic compounds
7	Ethyl 3-(Trimethylsilyl)Propanoate #	6.84	Organic acid and its derivatives
8	3,7-Dioxa-2,8-disilanonane, 2,2,8,8-tetramethyl-5-[(trimethylsilyl)oxy]- (CAS)	7.22	Silanes
9	2-[4(or 5)-(2-Phenylimidazolyl)]propionitrile	7.59	Nitriles
10	3-Phenylnon-4-en-3-ol	8.06	Alcohols
11	Octanoic acid, trimethylsilyl ester	8.43	Organic acid and its derivatives
12	9,12,15-Octadecatrienoic acid,2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)- (CAS)	8.81	Organic acid and its derivatives
13	2-Aminoethanol, N-acetyl-, trimethylsilyl ether	9.04	Ethers
14	3-Oxovaleric acid TMS ether TMS ester	9.22	Amino acids
15	5-Benzoyl-1,2,3,4-tetrahydronaphthalene	9.48	Hydrocarbon
16	Dibenzo[c,e]thiin-2-thione	10.05	Carbonyl compound
17	4-Methyl-6-cyanothieno[2,3-b]pyridine	10.31	Heterocyclic compound
18	Eicosane (CAS)	10.65	Hydrocarbon
19	N-Acryloylmorpholine	11.21	Heterocyclic compound
20	Hexadecane, 2,6,11,15-tetramethyl- (CAS)	11.52	Hydrocarbon
21	1,2-Difluoro-3,4,5-trimethylbenzene	12.12	Hydrocarbon
22	N,N'-Dityrityl-1,5-diaminopentane	12.51	Hydrocarbon

Continued ...

23	Hexadecane (CAS)	13.22	Hydrocarbon
24	2-tert-Butyl-4-isopropyl-5-methylphenol	13.8	Phenolic
25	Dodecanoic acid (CAS)	14.3	Fatty acid (S)
26	2-(2-chinoxalinyl)-(all-às)cyclotetrathiophen	14.7	Heterocyclic compound
27	Dodecanoic acid, 1-methylethyl ester	15.07	Fatty acids
28	Spiro(1,3-dioxolane)-2,3'-[5'-androsten-16'-trimethylsilyloxy)-	15.26	Spiro compounds
29	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	15.72	Hydrocarbon
30	11-Heneicosanone (CAS)	16.17	Carbonyl compound
31	1-methoxymethyl-4-methylnaphthalene	16.39	Hydrocarbon
32	Docosane (CAS)	16.57	Hydrocarbon
33	Octadecane (CAS)	17.46	Hydrocarbon
34	Tetradecanoic acid (CAS)	18.62	Fatty acid (S) (Myristic acid)
35	Tetradecanoic acid, trimethylsilyl ester (CAS)	19.17	Fatty acid (S) (Myristic acid)
36	1,1-Bis(p-tolyl)ethane	19.64	Hydrocarbon
37	Hexadecane, 2,6,10,14-tetramethyl- (CAS)	19.95	Hydrocarbon
38	Methyltetrahydrofurolacellicolic anhydride	20.26	Organic acid and its derivatives
39	1-Amino-2-cyano-3,4-dihydro-4-ethoxycarbonyl-3-phenylpyrido[1,2-a]benzimidazole	20.64	Heterocyclic compounds
40	Hexadecanoic acid, methyl ester (CAS)	21.64	Fatty acid (S) (Palmitic acid)
41	4-phenyl-6-(p-methylbenzoyl)bicyclo[3.3.0]octa-3,7-dien-2-one	22	Carbonyl compound
42	10-Cyano-3-amino-12-(4'-methoxyphenyl)-2-oxopyrano[4,3-d] pyrido[1,2-a]benzimidazole	22.88	Heterocyclic compounds
43	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	23.88	Carbonyl compound (Spiro)
44	Heptadecanoic acid (CAS)	24.6	Fatty acid (S) (Margaric acid)
45	Dibutyl phthalate	24.93	Organic acid and its derivatives
46	Octadecanoic acid, methyl ester (CAS)	25.47	Fatty acid (S) Stearic acid
47	1-[1-[[(tert-butyldimethylsilyl)oxy]methyl]-1-methyl-2-oxoethyl]perhydroazine	25.9	carbonyl compound derivatives
48	6,6-d2-.delta.2-5à-androsten-1-one	26.73	Carbonyl compound
49	10-Methylene-8-methoxy-3,4,4a,10-tetrahydro-2H,9H-anthracene-1,9-dione	27.13	Carbonyl compound
50	1-[1-[[(tert-butyldimethylsilyl)oxy]methyl]-1-methyl-2-oxoethyl]perhydroazine	27.52	carbonyl compound derivatives
51	Octadecane, 3-ethyl-5-(2-ethylbutyl)- (CAS)	28.12	Hydrocarbon
52	Ethanamine, 2,2'-oxybis[N,N-dimethyl- (CAS)	28.53	Amine
53	1,3-Dimethoxy-5,7-dihydrodibenz[c,e]oxepine	28.92	Heterocyclic compounds
54	Acetyl-Ginsenol	28.98	Alcohols
55	Butyl 9-octadecenoate or 9-18:1	29.49	Organic acid and its derivatives
56	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	29.91	Carbonyl compound
57	3,7-dimethoxy-1,9-dimethyldibenzofuran-4-carbaldehyde	30.37	Carbonyl compound
58	Hept-enyl-2-acetate	31.05	Organic acid and its derivatives
59	(S)-(2l,6l,3")x)-(1'E)-2-[3'-(3"-Oxocyclohexyl)-1'-propenyl]-6-methyl-3-(1-methylethyl)-1,3,2-oxazaphosphorinane 2-Oxide	31.52	Heterocyclic compounds

Continued ...

60	2áBenzyl-8-oxo-4,6-dimethyl-3,5,7-trioxatetracyc lo[7.2.1.0(4,11).0(6,10)]dodecane	31.93	Hydrocarbon
61	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethylester	32.7	Organic acid and its derivatives
62	Di-(2-ethylhexyl)phthalate	33.13	Organic acid and its derivatives
63	Docosane, 11-decyl- (CAS)	33.34	Hydrocarbon
64	erythro-1,2-Epoxy-2-methyl-3-heptanol	33.68	Alcohols
65	7-(4'-Nitrophenyl)-5-imino-2-methyl-5H-thiazolo[3,2-a] pyridine-6-carbonitrile	34.05	Heterocyclic compounds
66	3-Formyl-1-oxyl-4-(pyren-1'-yl)-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole	34.52	Heterocyclic compounds
67	1'-Benzyl-3-methoxynaphtho[16,17-b]estra-1,3,5(6)-triene	34.99	Hydrocarbon
68	Cholan-24-oic acid, 3,12-dihydroxy-, (3à,5á,12à)- (CAS)	35.66	Organic acids
69	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethylester	36.67	Organic acid and its derivatives
70	3,5-dicyano-2,6-di(m-tolyl)-1-methylpyridine-4(1H)-thione	37.5	Heterocyclic compounds
71	2,6-Diisopropylanisole	37.91	Phenolic
72	13-Docosenamide, (Z)-	38.87	Organic acid and its derivatives
73	2,2,5,5-Tetramethyl-4-(p-methoxyphenyl)-3-oxazoline	38.97	Heterocyclic compounds
74	Benzoic acid, 2,4-dimethyl-, (3,5-dimethylphenyl)methylester	39.56	Organic acid and its derivatives
75	Hexadecanoic acid, hexadecyl ester (CAS)	39.93	Organic acid and its derivatives

Unlike to proteomics, metabolomics demands a large amount of manual evaluation like the validation and peak correction of identified metabolites. Mass spectrometric analysis could identify the metabolites present in the disc tissue sample through NIST library search.

Interestingly, the presence of lactic acid in our study indicates that lactic acid might play a role in disc degeneration. Intervertebral disc is the largest avascular tissue in the body, its metabolism is mainly anaerobic, and thus lactate is produced at a significant rate. The higher lactate concentration reduces the pH in the disc[11,12]. Experimental data from other studies have showed that acidic pH adversely affect the supply of nutrients to IVD, cellular activity of IVD[13,14], synthesis of proteoglycans (which play a major role in the load bearing capacity of the disc)[12], integrity of extracellular matrix of the IVD, even IVD viability[15], and thus acidification of IVD due to lactic acid may play a role in disc degeneration[13,14].

The presence of lactic acid in both control and degenerated disc indicates that quantification of lactic acid is necessary for correlating the quantity of lactic acid with the causation of IVD degeneration. Similarly further confirmatory studies are required to ascertain whether the compounds found in our study play real roles in disc degenerative disease. Our future studies will concentrate on the quantification of the metabolites from both control and affected tissue for obtaining valuable insights about the IVD degeneration.

5. Conclusion

Our GC/MS-based metabolic profiling study has profiled the metabolites present in both control and degenerated human IVD. Our study revealed that some of these metabolites present in the human IVD might play an important role in disc degenerative diseases.

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Annexures

Annexure 1

List of samples selected for analysis

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
1	Srinivasan	49	M	8121979	1
2	Vijayakumari	45	F	9010696	2
3	Harishkumar	20	M	9020233	5
4	Aswathi nambi	14	F	-	7
5	Nagarajan	58	M	9011261	8
6	Sindhu deepa	28	F	1140	17
7	Kanimozhi	23	F	9042240	19
8	Bhavani	27	F	8070598	22
9	Muthulakshmi	54	F	8112233	25
10	Manivel	39	M	8081224	27
11	Amudha	29	F	8111481	28
12	Sajith kumar	31	M	8112414	29
13	Dini deborah	25	F	8112855	30
14	Vinoth kumar	33	M	8100934	31
15	Manian	40	M	8112957	35
16	Pappathi	43	F	9061457	40
17	Umamaheswari	47	F	8112524	41
18	Sakthivel	38	M	8121714	44
19	Dhiraj kumar	21	M	8121236	45
20	Govindan	42	M	8112603	46
21	Revanna	38	M	9023001	48
22	Ramla	48	F	8080177	50
23	Sivaprasad	35	M	9020431	53
24	Anu unni	40	F	9022627	55
25	Muthulakshmi	40	F	9010725	57
26	Sumathi	32	F	8032335	58
27	Shankar ganesh	33	M	9021040	60
28	Rajesh george	42	M	-	61
29	Saraswathi	47	F	8112997	62
30	Prema	29	F	9022203	63
31	Majeed	29	M	8122000	64
32	Rama ganesan	54	F	8092075	65
33	Kathirvel	35	M	9030449	66
34	Harish varthudi	30	M	9022243	68
35	Deepak kumar	29	M	9010207	69
36	Jaga nathan	40	M	9012674	70

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
37	Balasubramaniam	37	M	8121073	72
38	Ramesh	39	M	9023180	74
39	Beeba john	58	F	7103865	75
40	Selvaraj	49	M	8102740	77
41	Maheswari	35	F	8112176	79
42	Vignesh	18	M	8120857	81
43	Sujatha ramesh	34	F	9023238	82
44	Sujith	30	M	8122108	84
45	Deepak krishna	38	M	9021906	89
46	Palanivel	48	M	8122974	91
47	Chitra	34	F	8123436	92
48	Balakrishna	44	M	9021478	93
49	Boopathy	42	M	8112254	94
50	Durai	41	M	8011209	95
51	Muthu kumar	33	M	9021701	98
52	Dinesh	22	M	9022786	100
53	Dinesh	43	M	9021091	101
54	Prakash kumar	30	M	9022052	104
55	Deepak hariraj	23	F	9022227	105
56	Rishta waheed	32	F	8041307	106
57	Sumathy	32	M	9020556	107
58	Ilayaraja	34	F	8121742	108
59	Sivagami	51	M	9020338	109
60	Mamta sharma	41	M	9012381	112
61	Arum kumar	40	M	9010020	115
62	Murali rama chandran	27	M	8120902	117
63	Abdul rahim	34	M	9013137	118
64	Jamosh	20	M	8120878	119
65	Harish kumar	38	F	9013152	120
66	Kalyan das	30	M	9020235	122
67	Rajeev antony	12	M	8120641	123
68	Helina jebaraj	28	F	9020336	125
69	Booma deki	58	M	8120526	126
70	Muralitharan	39	M	9031809	130
71	Parimala	57	F	8032514	132
72	Kumaran	36	M	7093230	133
73	Muralidharan	39	M	9011198	134
74	Allinosh pra vin	36	M	9030952	135
75	Haridass	50	M	9031637	136
76	Nandha kumar	42	M	9030757	137

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
77	Arun prasad	24	M	9030943	140
78	Navaneetham	50	F	7120012	142
79	Manoranjitham	43	F	8100188	143
80	Chandadevi	45	F	9022801	145
81	Nithyalakshmi	28	F	9021389	146
82	Vennila	42	F	8101056	147
83	Abdul raheed	33	M	9031672	148
84	Shanthamani	42	F	8110273	149
85	Valiveti srinivas	35	M	9031827	152
86	Ashik kumar	30	M	8123005	153
87	Siva reddy	42	M	8120768	154
88	Azim ibrahim	26	M	9013159	156
89	Kailasapathi	59	M	9031389	158
90	Savithri	46	F	8092808	159
91	Inadhullah	45	M	9031326	161
92	Srinivas	44	M	8121951	162
93	Ranganayaki	55	F	8090863	165
94	Meena krishnan	41	F	8123567	166
95	Akkammal	45	F	8102243	167
96	Marimuthu	31	M	9010737	168
97	Kissan kumar	21	M	7090488	170
98	Husha	23	F	8100770	171
99	Karthik badri	27	M	7094232	172
100	Sumitha prasanth	34	F	8101117	173
101	Ganga prasad	35	M	9020274	174
102	Chitra	34	F	8120003	175
103	Dakshni moorthy	32	M	8122538	177
104	Abdul lateef	40	M	9012843	178
105	Mohana nambiar	39	M	9020460	179
106	Manivel	32	M	9013038	180
107	Siva kumar	36	M	9020489	181
108	Raj ramzit	40	M	8121858	183
109	Pradeep	32	M	9031674	184
110	Anitha rajesh	32	F	902339	185
111	Ranganathan	40	M	8082554	186
112	Latha	26	F	9030927	187
113	Vijayalakshmi	53	F	9031988	188
114	Abdulla	56	M	8112526	189
115	Leela ramasamy	48	F	8120702	190
116	Perumal	18	M	9011961	192

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
117	Gopalsamy	44	M	9010385	193
118	Tamilarasi	35	F	7123714	194
119	Sundharam	50	M	8121382	195
120	Anandhraj	40	M	8113016	196
121	Sheefa	42	F	8120126	197
122	Jayaram	14	M	8120633	199
123	Amudha	46	F	8101282	201
124	Narasimman	23	M	8120166	205
125	Khaleel	49	M	8093498	209
126	Muneer	39	M	8112075	210
127	Sivasubramaniam	38	M	8121269	211
128	Syed akbar	31	M	8122830	213
129	Pooraj	42	M	9082456	215
130	Ayynar	26	M	9091639	216
131	Manjeet singh	32	M	9091807	218
132	Sarojini	71	F	9090911	219
133	Gururagavendiran	36	M	9083447	222
134	Sindana	21	F	9060269	223
135	Pappathy	52	F	1018	224
136	Jayakumar	25	M	11071425	225
137	Manoharan	37	M	9042264	226
138	Palani	57	M	9083288	228
139	Gokila	36	F	9060693	232
140	Dhanamani	48	F	9041179	234
141	Sudha	24	F	9053822	235
142	David durairaj	47	M	8020997	236
143	Paulraj	35	M	9012904	237
144	Parvathy	41	F	9052193	238
145	Padmalatha	49	F	9060974	240
146	Paneerselvam	41	M	9050804	241
147	Ravindranath	33	M	-	244
148	Flora mary anitha	37	F	9050506	245
149	Mr. Mani	40	M	9062053	246
150	Mohammed maiden	46	M	9082118	248
151	Sakkaravarthi	44	M	9061876	249
152	Naseema sulaiman	53	F	9062704	251
153	Arumugam	40	M	9061745	252
154	Madhusudhanan	18	M	9062128	253
155	Master. Arunprakash	17	M	8112103	255
156	Kavitha	29	F	9061204	257

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
157	Balamani	42	F	9061564	258
158	Lakkendra	29	M	8122059	259
159	Poovathal	35	F	9052756	260
160	Riyaz	28	M	9072645	261
161	Pushpa	28	F	9054012	262
162	Kathiravan	18	M	9042740	263
163	Balakrishnan	56	M	9053167	265
164	Manoharan	44	M	9062133	266
165	Anup nair	24	M	8020298	267
166	Nandhini	38	F	8023171	268
167	Jothilakshmi	39	F	8074087	271
168	Murugesan	47	M	9042202	275
169	Antony	41	M	9052835	276
170	Ravikumar	49	M	9061849	278
171	Elumalai	42	M	8032690	279
172	Krishnachand	61	M	8050904	280
173	Kesava mani	44	M	9041426	281
174	Venkatachalam	38	M	8062411	282
175	Vijayanirmala	37	F	9042399	283
176	Muralidharan	39	M	8092945	285
177	Arulvanan	35	M	9032004	286
178	Akshya hari	20	F	9052337	287
179	Kumar	33	M	9040236	288
180	Kalpana devi	30	F	9041190	289
181	Priyadharshini	26	F	9041953	290
182	Krishna kishore	43	M	9050851	291
183	Mohanraj	22	M	11081109	292
184	Kaliswari	37	F	9051037	293
185	Moosa muneer	50	M	9033600	294
186	Narayana naik	36	M	9033603	295
187	Mahalingam	23	M	9033689	296
188	Rasu	40	M	9040059	297
189	Abdul kalam azad	44	M	9051573	298
190	Hamsa	39	M	9032677	299
191	Loganathan	33	M	9101729	300
192	Muthammal	47	M	E-1064	301
193	Akila	27	F	9040629	302
194	Haris	24	M	9030529	303
195	Palanivel	52	M	9043594	305
196	Palanisamy	39	M	9052338	306

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
197	Karthikeyan	32	M	9033138	307
198	Nazreen banu	35	F	9031897	308
199	Murugan	38	M	9031240	309
200	Hariprakash	25	M	9090225	310
201	Lakshmanan	39	M	9083439	312
202	Sushila gahak	55	M	9031772	313
203	Rajendran	50	M	9040158	314
204	Raveedran	49	M	9032796	315
205	Madhaiyan	35	M	9012641	317
206	Mani	32	M	9032608	318
207	Selvaraju	41	M	9051325	319
208	Paramasivam	36	M	8100013	320
209	Sadidali kutty	42	M	9083297	323
210	Venkatachalam	45	M	9032031	324
211	Subramani	39	M	9030512	325
212	Priya harilal	24	F	8081646	326
213	Rahma bibi	56	F	9022787	327
214	Rekha	29	F	9023377	328
215	Manivel	39	M	9092424	329
216	Kasturi	48	F	10092083	330
217	Shylaja devi	43	F	8121959	331
218	Aswathy	18	F	9083224	333
219	Kavitha gupta	28	F	9082647	335
220	Raveendran	53	M	9093356	337
221	Sekar	44	M	9041094	338
222	Rajendran	46	M	11051684	341
223	Sennapanna	25	M	9092147	342
224	Duraisamy	31	M	9080432	343
225	Hariharan	31	M	9050924	344
226	Damodarasamy	65	M	9011799	345
227	Gowndarajan	36	M	9073891	346
228	Muthaiyan	40	M	9082134	347
229	Nagathulla	35	M	9082318	348
230	Meksonbalu	46	M	9042067	349
231	Solachana	40	F	11080966	350
232	Jaya prasath	48	M	9081122	351
233	Chelladurai	44	M	9081729	353
234	Ramalingam	34	M	9080991	354
235	Ramzi rizwan	34	M	9080412	356
236	Annamalai amma	56	F	9080176	358

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
237	Mrs. Sumathy	38	F	9080672	361
238	Gopal purdit	31	M	9042695	362
239	Ashraf	33	M	9072592	363
240	Narayanan	36	M	9073937	364
241	Poongothai	39	F	9080357	365
242	Mohammed kazzali	23	M	8112312	367
243	Chandrakrishnan	40	M	9032979	368
244	Ramyalakshmi	32	F	9050589	369
245	Saishankar	38	M	9081020	370
246	Saritha	29	F	9073612	371
247	Vijay	29	M	9081616	379
248	Faisal	30	M	9060990	381
249	Shiva kumar	43	M	9073191	382
250	Unni krishnan	37	M	9080423	383
251	Ahmed mohammed	45	M	9071082	388
252	Riyaz ahamed	28	M	9083645	389
253	Rajasekaran	46	M	9072556	390
254	Senthur pani	51	M	9062440	391
255	Navamani	27	M	9070139	392
256	Kumaran	47	M	9080960	393
257	Dinesh danniel	23	M	9071682	394
258	Murugadas	30	M	9071508	395
259	Thangavel	56	M	9061618	396
260	Kandha vadivelu	37	M	9020246	398
261	Vinitha premarajan	41	F	9063678	400
262	Supriya	23	F	9070154	401
263	Balkees	46	F	9062562	402
264	Ponnusamy	35	M	8110677	403
265	Varadharaj	36	M	9073145	404
266	Jansi rani	45	F	9072919	405
267	Ratheesh	24	M	9072622	407
268	Saraswathi	38	F	9072488	408
269	Prasanth	17	M	9063633	409
270	Ashok kumar	30	M	9072244	412
271	Rabiya	41	F	8040072	415
272	Sulaika razak	40	F	9072772	416
273	Farhan salim	15	M	9073198	417
274	Anoop kumar	23	M	7110007	418
275	Dr narasa raju	31	M	9071077	419
276	Merina elsa	10	F	7101565	422

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
277	Ramamoorthy	42	M	9063665	426
278	Mohammed ummar	30	M	9091054	427
279	Sanjay jain	37	M	9072605	429
280	Saravana kumar	31	M	9082572	430
281	Dr. Shalini priya	22	F	9072854	431
282	Sakthivel	37	M	9073601	432
283	Gunasekaran	48	M	9070417	433
284	Umanath bai	43	M	8011215	434
285	Vinoth kumar	28	M	9063896	436
286	Madhavi	39	F	9072744	437
287	Mohanan	51	M	9071081	438
288	Kadeeja kasim	43	F	10010896	439
289	Nafeesa hamsa	42	F	7111881	452
290	Murugesan	34	M	7112891	456
291	Neelaveni	38	F	9100073	460
292	Revathi	24	F	10010924	461
293	Mr. Kannan	32	M	532	463
294	Dr. Sibi	36	M	523	464
295	Karthick	31	M	9122099	465
296	Dhanalaxmi	35	F	9073738	466
297	Lakshmi	43	F	9080468	467
298	Kamarunisha	40	F	10012551	469
299	Dr. Geethan	32	M	527	472
300	Senthil kumar	25	M	10013535	473
301	Ramadas	40	M	526	474
302	Dr. Leo	33	M	497	475
303	Nafeez	32	M	540	476
304	Suresh	44	M	533	477
305	Karthick	29	M	524	478
306	Sumithra	23	F	514	479
307	Dr. Karunanidhi	34	M	499	480
308	Er. Prabhu	31	M	501	481
309	Kamalesh	42	M	9083313	482
310	Parameswaran	40	M	500	484
311	Puspha	36	F	508	485
312	Vijayalakshmi	43	F	516	491
313	Dr. Sathy reddy	28	M	531	492
314	Santha kumari	32	M	9011105	495
315	Akilandeshwari	24	F	520	496
316	Mr. Monika	25	F	518	498

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
317	Ravichandran	28	M	509	499
318	Sivagami	22	F	519	500
319	Uma	36	F	515	501
320	Dr. Ramalingam	34	M	529	502
321	Sabapathy	30	M	511	505
322	Appadurai	26	M	502	506
323	Dr.rishi	30	M	510	507
324	Sasirekha	38	F	9100885	509
325	Srinivasan	43	M	8110911	510
326	Jeyachandran	43	M	517	511
327	Senthil kumar	26	M	9093168	512
328	Thangavel	43	M	538	513
329	Krishnakumar	21	M	7090488	514
330	Ms. Deepa	26	F	503	515
331	Ms. Malarvizhi	29	F	504	516
332	Dr. Kamath	34	M	498	517
333	Maheswaran	31	M	491	518
334	Padmanaban	37	M	505	519
335	Palaniammal	42	F	9090572	529
336	Abdul rahman	36	M	9053286	530
337	Prabhakaran	21	M	9100240	531
338	Mrs. Lalitha	29	F	9100060	533
339	Janakala	27	F	9073882	535
340	Karuppusamy	32	M	9052880	537
341	Pradeep	32	M	9121583	538
342	Manju haridas	26	F	9101747	539
343	Sajina	26	F	9093668	545
344	A.y. Natarajan	38	M	8120214	547
345	Sathish	58	M	9101766	548
346	Ramani	32	M	9120481	549
347	Marakatham	35	F	9032815	550
348	Vasanthi	39	F	9020101	554
349	Gomathi	39	F	10023500	556
350	Ragukathuja	37	M	10030132	557
351	Mani	36	M	8092574	558
352	Safiasulaiman	39	M	10040895	560
353	Manimegalai	40	F	7102231	562
354	Sunandha hedge	32	M	9032802	564
355	Surendran	40	M	9041644	565
356	Sivakumar	40	M	9113883	566

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
357	Sreeman narayanan	60	M	10022369	568
358	Shanthi	49	F	10030051	569
359	Kandasamy	43	M	9042047	571
360	Chaitanya	17	M	10040681	572
361	Alfonsa	49	F	10031924	574
362	Seetha mahalakshmi	39	F	10030300	575
363	Mala shukla	43	M	9031736	576
364	Dhanalakshmi	35	F	8020191	580
365	Palaniammal	43	F	9091203	582
366	Afsal	28	M	9100864	583
367	Vinoth kumar	20	M	9070459	584
368	Chandra kala	33	F	9073306	587
369	Biju	32	M	9072695	588
370	Thenmozhi	29	F	9080677	589
371	Maarappan	32	M	9053719	590
372	Sangeetha belkavi	41	F	9072738	597
373	Abdul rahim	37	M	9113516	600
374	Vineesh	33	M	10032973	603
375	Baby	42	F	9113614	604
376	Aflah ebrahaim	39	M	9121784	609
377	Lakshmi	45	F	9091053	610
378	Kanimozhi	24	F	9092250	615
379	Balasubramanian	38	M	9092813	616
380	Ramasamy	42	M	7103440	617
381	Pradeep kumar thakur	42	M	9091298	619
382	Shylaja	28	F	9093669	620
383	Abdul hameed	42	M	9083208	627
384	Rajendran	46	M	9092003	628
385	Govindarajan	37	M	9061161	631
386	Kamalam	43	F	9082100	632
387	Sajath jabbar	26	M	9082250	633
388	Muthuraman	39	M	9093454	643
389	Muthuswamy	52	M	7121526	645
390	Vijayaraja	35	M	9090928	649
391	Prassanamuthy	29	M	9080998	654
392	Sulochana	40	F	9092643	656
393	Ahamed koya	66	M	10011443	658
394	K.kumar	31	M	9111262	659
395	Vasanthi	47	F	9020105	660
396	Gautam rajan	19	M	10033441	661

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
397	Nallathambi	36	M	8122581	665
398	Mythili	63	F	9102356	669
399	Suraj safarulla	35	M	9060347	672
400	Tamilarasi	32	F	C70	674
401	Stella mary	30	F	1267	675
402	Bhavani	26	F	C669	676
403	Parvathi	40	F	1072	678
404	Shila banu	27	F	942	679
405	Mumtaz	43	F	1076	680
406	Sabia banu	27	F	1457	681
407	Nagamani	34	F	2493	682
408	Manish agarwal	32	M	10070805	684
409	Vasantha	42	F	940	687
410	Jothimani	34	F	1982	688
411	Banu	28	F	749	689
412	Abdul majeed	49	M	9082123	692
413	Kaliamal	25	F	1044	696
414	Dhanalaxmi	24	F	973	697
415	Radha	28	F	1861	698
416	Noorjahan	46	F	1708	699
417	Dhanabagiyam	32	F	2907	700
418	Fathima	35	F	805	701
419	Saraswathi	23	F	893	702
420	Veeramuthu	46	M	920	703
421	Ayisha	38	F	1467	704
422	Chitra	39	F	754	706
423	Pankajam	22	F	1289	707
424	Rajeshwari	42	F	1835	708
425	Yasotha	28	F	1639	709
426	Ganesh	25	M	971	710
427	Eshwari	30	F	1840	711
428	Raigon	25	F	2484	713
429	Moideen bee	66	F	756	714
430	Sasikala	28	F	1287	715
431	Nagamani	22	F	3108	716
432	Sindhu	31	F	2115	733
433	Radhamani	28	F	1588	734
434	Thirumurthi	36	M	1776	752
435	Anitha	28	F	2482	762
436	Thangaraj	32	M	1006	764

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
437	Selvaraj	33	F	1928	767
438	Leelavathi	23	M	1397	768
439	Kennedy	35	M	737	769
440	Bagyavathi	28	F	1876	770
441	Jayamani	33	F	1424	773
442	Nagamma	31	F	2737	778
443	Vidhya	25	F	2065	780
444	Rathinam	37	F	2066	781
445	Leela	36	F	2329	782
446	Priya	24	F	2001	784
447	Rahima bee	40	F	1091	785
448	Harish kumar	20	F	9050811	789
449	Roshini ramesh	16	F	8023145	790
450	Anantha	32	F	L116	791
451	Vadivel	26	M	2028	793
452	Ramakrishnan	49	M	9111515	794
453	Rehika	26	F	9010713	796
454	Selvaraj	33	M	1494	797
455	Jain banu	27	F	10052586	798
456	Geetha	32	F	2114	801
457	Geethan	28	M	2432	802
458	Senthil kumar	26	M	871	803
459	Devi	43	F	1049	804
460	Chinna mani	50	F	1841	805
461	Kandhasamy	49	M	10044639	806
462	Jeenath	45	F	1196	807
463	Thirumurthi	25	M	3020	809
464	Jaya	51	F	2413	811
465	Jagannatham	34	M	794	813
466	Veeramani	33	M	L76	815
467	Daisy	37	F	B16	816
468	Karuppasamy	32	M	1132	817
469	Bagyalakshmi	42	F	1134	818
470	Selvi	40	F	1495	819
471	P. Selvaraj	29	M	1482	821
472	Arogyaraj	24	M	E3038	822
473	Muthukumar	28	M	868	823
474	Ravi	28	M	1855	824
475	Ranganathan	33	M	968	826
476	Rajkumar	38	M	2310	828

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
477	Ramesh	32	M	2294	829
478	Venkatesh	38	M	889	830
479	Seetha	27	F	1837	831
480	Ganesh	26	M	1680	832
481	Arumugam	28	M	2164	833
482	Rangasamy	47	M	10054300	834
483	Divya	14	F	10062677	835
484	Krithiga	15	F	10050296	838
485	Bathiran	34	M	2207	839
486	Mini	26	F	1670	841
487	Lakshmi. R	28	F	2892	842
488	Muthammal	47	F	1064	844
489	Lakshmi	32	F	1147	849
490	Fahmidha	14	F	9102026	850
491	Murugammal	44	F	1219	851
492	Bannammal	45	F	1871	853
493	Julie	37	F	1146	854
494	Balamani	39	F	3011	855
495	Andal	40	F	875	856
496	Rahameth	27	F	1131	857
497	Poonkodi	32	F	1650	858
498	Thilagavathi	30	F	1141	859
499	Kamalam	43	F	1460	860
500	Chinraj	40	M	1028	861
501	Murugamma	44	F	1423	862
502	Baby	42	F	2430	863
503	Swarnam	40	F	2063	864
504	Chinju	22	F	3104	867
505	Bincy	22	F	2982	869
506	Annamalai. M.r	48	M	2661	870
507	Nandhini	40	F	C724	871
508	Nagalakshmi	25	F	C2915	873
509	Maharani	24	F	1752	874
510	Shobi	32	F	3189	876
511	Sambath kumar	34	M	9072362	877
512	C mary	28	F	3054	879
513	Lallu mary	32	F	L111	880
514	Bijo mathew	22	M	3169	881
515	Priya	24	F	L181	882
516	Mrs. Jancy	45	F	2855	883

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
517	Jothimani	34	F	L198	884
518	Mrs. Jaya	40	F	L124	885
519	Jeeva	21	F	C3048	886
520	Selvanayagi	38	F	10020783	887
521	Meenambal	24	F	1573	888
522	Bagyalakshmi	42	F	8080418	889
523	Deepthi	22	F	2777	891
524	Janarthanan	45	M	10227/10	892
525	Sathya devi	24	F	1071	895
526	Valarmathi	35	F	1004	899
527	Jaya	40	F	1685	901
528	Shabeera banu	36	F	7103732	905
529	Srikumar	44	F	9040677	906
530	Sridevi	40	F	9041634	911
531	Madhavaraj	35	M	9042025	913
532	Kala	45	F	9013237	914
533	Mathivadhani	19	F	10053007	915
534	Venilla	60	F	9040128	916
535	Kalyani	18	F	10081904	917
536	Imthiaz ahmhad	20	M	9041569	920
537	Assainar	50	M	9042394	923
538	Palaniappan	48	M	9041439	924
539	Nagammal	56	F	10091466	928
540	Vasudevan	37	M	10081910	929
541	Radhakrishnan	54	M	10022410	933
542	Prema	36	M	10023125	934
543	Karthikeyan	35	M	10061186	935
544	Revathy	23	F	C3044	936
545	Geetha	45	F	9052364	937
546	Amutha ravi	40	F	10013724	938
547	Tamalarasi	47	F	9042357	939
548	Balamurugan	36	M	E969	949
549	Abdul majeed	53	M	10112998	952
550	Jayasudha	33	F	9022179	955
551	Shanthi	33	F	11052040	957
552	Chinnappan	57	M	10033020	958
553	Rita	44	F	E2425	963
554	Surendran	48	M	9093824	967
555	Thirumoorthy	21	M	9042052	969
556	Nagarajan	40	M	9071312	973

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
557	Abdul s	25	M	10124145	980
558	Abdulla	24	M	9063099	981
559	Abid hashim t	40	M	11041845	982
560	Anitha sarcar	45	F	9083262	983
561	Baijnath prasad	48	M	9071045	984
562	Chinnammal	42	F	11041617	985
563	Ibarahim kutty p	46	M	11023837	987
564	Jobaed ragib jaman	18	M	11040482	989
565	Malathi d	33	F	10100423	991
566	Mohammed rafeek	30	M	10122836	992
567	Raja s	27	M	11041564	993
568	Santhya dinesh kumar	25	F	11041611	995
569	Sesha narayanan	42	M	11042303	996
570	Shanthi	38	F	10090288	997
571	Chitra sathish	41	F	11043066	998
572	Kala (maya)	45	F	11043895	1001
573	Aysha banu	27	F	11034189	1002
574	Sivakumar ramachandran	37	M	11010027	1003
575	Sowmiyan	19	M	11041973	1004
576	Srinivasan k	35	M	10012353	1005
577	Sulaiman	23	M	11043044	1006
578	Vidhyarani k	60	F	8092645	1007
579	Vijitha k	14	F	7103753	1011
580	Zuhra	36	F	10120830	1012
581	Sunitha basanl	39	F	11041059	1013
582	Susheela	53	F	11041797	1014
583	Thangavel	46	M	10121408	1015
584	Thangavel m	45	M	10034846	1016
585	Valarmathi s	39	F	10060959	1018
586	Udhirasamy	50	M	11042796	1019
587	Sengodan	67	M	11043006	1020
588	Balakrishnan	31	M	11043113	1021
589	Arjun rai	57	M	11043058	1022
590	Eswari	49	F	11042825	1023
591	Aziz modi	42	M	11045037	1025
592	Murugesan	60	M	10044646	1026
593	Shanthi	45	F	10095069	1027
594	Apparao v	38	M	11030743	1029
595	Ashok kumar	25	M	11042767	1030
596	Bakiyalakshmi	45	F	11042779	1031

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
597	Balaji d	42	M	11041048	1032
598	Chander gsr	50	M	11040713	1033
599	Chinnaponnu s	36	F	10124626	1034
600	Firoz	40	M	11042894	1036
601	Gandhimathi	39	F	11041895	1037
602	Gandhimathi s	52	F	8052544	1038
603	Ganesan	36	M	11042917	1039
604	Geo	41	M	8121944	1040
605	Iyyappan h	36	M	10123021	1041
606	Jayabal	37	M	11042840	1042
607	Jayadoss t	66	M	10120681	1043
608	Jeevanandham m	44	M	11033843	1044
609	Jennathul firdouse	36	F	11041494	1045
610	Kadeeja ct	36	F	10075501	1046
611	Kandhayee m	70	F	10125098	1047
612	Karuppusamy	38	M	11044399	1049
613	Kavitha	36	F	10124549	1050
614	Malarvizhi k	37	F	11042935	1051
615	Mani d	54	M	10100967	1052
616	Manoharan r	38	M	11042641	1053
617	Manonmani	43	F	9072445	1054
618	Mekala r	30	F	11041637	1055
619	Muthaya	41	M	11042574	1056
620	Nafeez fathima	39	F	11032520	1058
621	Asharaf	35	M	11044607	1059
622	Niranchana	43	M	11021529	1060
623	Periyasamy k	55	M	11035087	1061
624	Perumal p	35	M	10101918	1062
625	Pushpa mega	33	F	11042518	1063
626	Ramlath vk	32	F	11021037	1065
627	Ranganayaki g	48	F	11010648	1066
628	Santhamani ts	50	F	8083263	1067
629	Saraswathi r	42	F	11041350	1068
630	Sasikala b	53	F	11012982	1069
631	Sekar n	44	M	11012478	1070
632	Selvi r	36	F	11040862	1071
633	Sivagami	38	F	11042855	1073
634	Sridevi n	30	F	10125080	1074
635	Srikanth	28	M	11031208	1075
636	Sudha c	35	F	11032258	1077

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
637	Sumathara c	27	F	11041355	1079
638	Arun k	26	M	11052414	1080
639	Balasubramani	37	M	11051092	1081
640	Eswari	48	F	11051685	1082
641	Jothi dashan	24	M	11051587	1085
642	Madhumathi	21	F	11012154	1087
643	Murugan	26	M	11052293	1088
644	Prathoesh	16	M	11051697	1089
645	Nirmala	31	F	11044089	1090
646	Ramachandra gupta	56	M	11052649	1091
647	Ravikumar	34	M	11052114	1092
648	Sajith joss	29	M	11051660	1093
649	Selvi	32	F	11044372	1094
650	Sundara rajan	45	M	11051251	1095
651	Vanjiappa gounder	75	M	11052309	1096
652	Vijayakumar	38	M	11030019	1098
653	Karupan	46	M	11052735	1099
654	Rajeswari	27	F	11052714	1100
655	Abdullah shivaz	26	M	11053060	1102
656	Ambika	46	M	11044142	1104
657	Alamelu mangai	34	F	11051592	1105
658	Balakrishnan	43	M	7110918	1106
659	Deepa pradeesh	30	F	11045015	1108
660	Gangadharan	41	M	11020014	1109
661	Hariharan	34	M	11051479	1111
662	Haris mohammed	38	M	11051435	1112
663	Hemalatha	27	F	10014174	1113
664	Hussaina	41	F	10092002	1114
665	Jaffer ali	50	M	11040884	1115
666	Jayanthi	33	F	10032812	1116
667	Janeeth	40	M	11044894	1118
668	Kandhasamy	29	M	11043149	1119
669	Kannammal	58	F	11050609	1120
670	Kannammal	71	F	11043034	1121
671	Kadeeja	55	F	11051902	1123
672	Kathiravan	45	M	11044715	1124
673	Kathiravan	36	M	11050567	1125
674	Lakshmanan	23	M	11043215	1127
675	Lakshmi	48	F	11011876	1128
676	Mohammed nasser	36	M	10092141	1130

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
677	Moideen kutty	46	M	11040509	1131
678	Murugesan	60	M	11043179	1132
679	Nandhakumar	49	M	10121603	1133
680	Narendran	39	M	9090531	1134
681	Naseera mustafa	32	F	11044244	1135
682	Noor najeema	40	F	11051466	1136
683	Padmavathy	34	F	11043364	1137
684	Palanivel	48	M	11044758	1139
685	Puthiya ali	42	M	11050779	1140
686	Rajakumari	55	F	11050419	1141
687	Rajamani	61	F	11043025	1142
688	Rajesh uk	39	M	11044591	1143
689	Yasotha	33	F	11050622	1165a
690	Priya	30	F	C3109	1167
691	Ramya	24	F	C3646	1168
692	Sreekumar	50	M	E 50	1169
693	Maruthupandi	36	M	C994	1170
694	Balamurugan	36	M	C969	1175
695	Mariyamma	38	F	C624	1178
696	Radhika	25	F	C3426	1179
697	Rama nandhini	34	F	C3571	1180
698	Ruban	29	M	C2142	1181
699	Kavitha	28	F	C1442	1182
700	Abitha	29	F	C3430	1183
701	Ajitha	23	F	C1793	1186
702	Dhamodaran	40	M	E214	1187
703	Muthumari	22	F	C2099	1188
704	Mubina	23	F	C502	1189
705	Anbuselvi	29	F	C1154	1191
706	S. Shanthi	34	F	E3440	1192
707	Tamilvannan	23	M	C2012	1193
708	Asha	20	F	C3572	1195
709	Kunjaram	60	F	D 2010	1196
710	Jayasudha	31	F	C1479	1197
711	Selvam	38	M	11083543	1198
712	Sangeetha	27	F	11072429	1199
713	Kumar	23	M	11073605	1200
714	Parvathi	51	F	11072194	1201
715	Palaniammal	45	F	11072748	1202
716	Vellingiri	48	M	11072515	1203

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
717	Saravanan	33	M	11081336	1204
718	Dhanapal	61	M	11071844	1205
719	Suseela jain	52	F	11080237	1206
720	Aarthy	22	F	11075248	1207
721	Kavitha	33	F	11080963	1208
722	Sankara narayanan	35	M	11074967	1209
723	Bindhu madavi	31	F	11064812	1210
724	Gandhari	21	M	11071144	1211
725	Muthukumar	42	M	11074671	1212
726	Shibu	23	M	11080275	1213
727	Mahalingam	59	M	11080618	1214
728	Selvaraj	57	M	11072859	1215
729	Tamilselvi	27	F	11081486	1216
730	Kranth kumar	31	M	11064364	1217
731	Rashidh	26	M	11075035	1218
732	Sowadadh	37	F	11050756	1219
733	Gautam	19	M	11053040	1220
734	Syed idrahim	48	M	11074493	1221
735	Mathiazhagam	50	M	11065254	1222
736	Kavin	20	M	11070403	1223
737	Jayammal	67	F	10080781	1224
738	Nagamanick	48	M	11075057	1225
739	Natesan	28	M	11073184	1227
740	Suaina gandhi	42	F	10091627	1229
741	Shakila	32	F	11080321	1230
742	Thanga thangaraj	59	M	11021588	1233
743	Sarveswaran	26	M	11064575	1234
744	Nachimuthu	80	M	11074651	1235
745	Rajathi	55	F	11071004	1236
746	Shameed ahmed	38	M	11074763	1237
747	Rakesh jain	54	M	11080239	1238
748	Narimuthu	39	M	11071454	1239
749	Dilip kumar	40	M	11075163	1240
750	Kanakasababathi	40	M	11074790	1242
751	R. Usha	49	F	11052660	1244
752	Arunachalam	40	M	11071333	1245
753	Karthick m	21	M	11073817	1247
754	Vinod	32	M	11052653	1248
755	Selvi. Rasika	14	F	11081113	1249
756	Kabeer	45	M	11080672	1250

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
757	Nivetha	18	F	10124729	1251
758	Narayanan jogwa	35	M	11081556	1252
759	Maheswari	39	F	11074842	1253
760	Arun kumar	23	M	9043777	1254
761	Maheswari	42	F	11081442	1255
762	Sivaraj mr	43	M	11064427	1256
763	Prakash	27	M	11073346	1257
764	Abdul hammid	52	M	11075519	1258
765	Selvi. Ruksana yousuf	13	F	11074815	1259
766	Vasantha kumari	63	F	11041934	1260
767	Ganesh	40	M	11070856	1261
768	Ramla	30	F	11070757	1262
769	Dileep kumar	32	M	11075163	1263
770	Vijaya kumari	43	F	11051445	1264
771	Prakasham	51	M	11075148	1265
772	Murugan	32	M	11065421	1266
773	Gandhimathi	43	F	11074031	1267
774	Shanmugavel	32	M	11074784	1268
775	Shima	35	F	11052911	1269
776	Archana bharathi	28	F	11080255	1270
777	Marimuthu	74	M	11072826	1271
778	Noyeel	37	M	11075197	1272
779	Rajalakshmi	28	F	10093431	1274
780	Sunil	29	M	11081896	1275
781	Deenan	53	M	11064467	1276
782	Meher bath	20	F	11063904	1277
783	Sivakumar	32	M	11073272	1279
784	Jayakodi	45	F	11073145	1280
785	Mustafa	51	M	11073751	1281
786	Radha	28	F	11065338	1282
787	K. Kasi	45	M	11081450	1283
788	Vasanthi	36	F	11074774	1284
789	Kulandhaisamy	62	M	11073602	1285
790	Mohan raj	37	M	10061144	1286
791	Rajan	41	M	11081228	1287
792	Anjali	32	F	11080993	1288
793	Angaleswari	29	F	11080989	1289
794	Subramaniam	52	M	7121910	1290
795	Rohit mittal	27	M	11073371	1293
796	Sowmiya	19	F	11072754	1294

S. No	Patient name	Age	Sex	Ganga ID[1]	TNAU ID[2]
797	Saathmani	65	F	11034040	1295
798	Sekar	44	M	11080240	1296
799	Venugopal rao	38	F	11041786	1297
800	Ramaraj	25	M	C2146	1298
801	Senthil	26	M	C1080	1300
802	Murugeshwari	22	F	C3363	1301
803	Chitradevi	29	M	D2013	1302
804	Selvaraj	36	M	11081688	1304
805	Sivaraj Dr	31	M	D14	1306
806	M.s. Nanjudan	54	M	E1790	1307
807	Baburajendran	34	M	268	1308
808	Rahmath	22	F	C1131	1309
809	Manikandan	24	M	E3398	1311

[1]**Ganga ID**: Ganga Hospital Identification number

[2]**TNAU ID**: Tamil Nadu Agricultural University Identification number

Annexure 2

CLB Buffer preparation

Components	Concentration	Stock solution	For 100ml	For 250ml
Sucrose	0.32M	---	10.953g	27.3825g
Tris HCl(7.6)	10mM	500mM	2ml	5ml
$MgCl_2$	5mM	100mM	5ml	12.5ml
Triton X -100	1%	---	1ml	2.5ml

PLB Buffer preparation

Components	Concentration	Stock solution	For 100ml	For 250ml
Tris HCl(PH-8.0)	10mM	1000mM	1ml	2.5ml
NaCl	10mM	5000mM	0.2ml	0.5ml
EDTA	10mM	500mM	2ml	5ml
Proteinase K	1mg/ml	---	100mg	250mg