

# Can Scoliotic Discs Be Controls for Molecular Studies in Intervertebral Disc Research? Insights From Proteomics

Global Spine Journal  
2022, Vol. 12(4) 598–609  
© The Author(s) 2020  
Article reuse guidelines:  
sagepub.com/journals-permissions  
DOI: 10.1177/2192568220959038  
journals.sagepub.com/home/gsj



S. Rajasekaran, MS, DNB, FRCS, MCh, PhD<sup>1</sup> ,  
Chitraa Tangavel, MSc, MPhil, PhD<sup>2</sup>, K. S. Sri Vijay Anand, MS, FISS<sup>1</sup>,  
Dilip Chand Raja Soundararajan, MS, DNB, FNB<sup>1</sup>,  
Sharon Miracle Nayagam, MSc<sup>2</sup>, R. Sunmathi, MTech<sup>2</sup>,  
M. Raveendran, MSc, PhD<sup>3</sup>, Ajoy Prasad Shetty, MS, DNB<sup>1</sup>,  
Rishi Mugesh Kanna, MS, MRCS, FNB<sup>1</sup> ,  
and B. T. Pushpa, DNB, FRCR<sup>1</sup> 

## Abstract

**Study Design:** Proteomic analysis of human intervertebral discs.

**Objectives:** To compare the characters of scoliotic discs and discs from magnetic resonance imaging (MRI)–normal voluntary organ donors controls used in disc research employing proteomics and establish “true controls” that can be utilized for future intervertebral disc (IVD) research.

**Methods:** Eight MRI-normal discs from 8 brain-dead voluntary organ donors (ND) and 8 scoliotic discs (SD) from 3 patients who underwent anterior surgery for adolescent idiopathic scoliosis were subjected to tandem mass spectrometry, and further analysis was performed.

**Results:** Mass spectrometry identified a total of 235 proteins in ND and 438 proteins in the SD group. Proteins involved in extracellular matrix integrity (Versican, keratins KRT6A, KRT14, KRT5, and KRT 13A1, A-kinase anchor protein 13, coagulation factor XIII A chain, proteoglycan 4) and proteins involved in transcription and DNA repair (Von Willebrand factor A domain-containing 3B, eukaryotic initiation factor 2B, histone H4, leukocyte cell–derived chemotaxin 2) were found to be downregulated in SD. Inflammatory proteins (C3, C1S), and oxidative stress response proteins (peroxiredoxin-2,6, catalase, myeloperoxidase, apolipoprotein E) were found to be upregulated in SD. These changes were reflected at the pathway level also.

**Conclusion:** Findings of our study confirm that scoliotic discs have an abundance of inflammatory, oxidative stress response proteins, which are either absent or downregulated in the ND group indicating that scoliotic discs are not pathologically inert. Furthermore, this study has established MRI-normal discs from voluntary organ donors as the “true” control for molecular studies in IVD research.

## Keywords

degenerative disc disease, proteomics, controls, disc, scoliosis, adolescent idiopathic scoliosis, tandem mass spectrometry, basic science

## Introduction

Disc degeneration (DD) is attributed to 40% of low back pain (LBP),<sup>1</sup> which is the single most common cause of disability worldwide.<sup>2</sup> Also, DD is the most researched subject in spinal disorders. Despite being extensively researched, the intricate mechanisms involved in homeostasis, the etiology, and pathomechanisms involved in disc degeneration are still unclear. The

<sup>1</sup> Ganga Hospital, Coimbatore, Tamil Nadu, India

<sup>2</sup> Ganga Research Centre, Coimbatore, Tamil Nadu, India

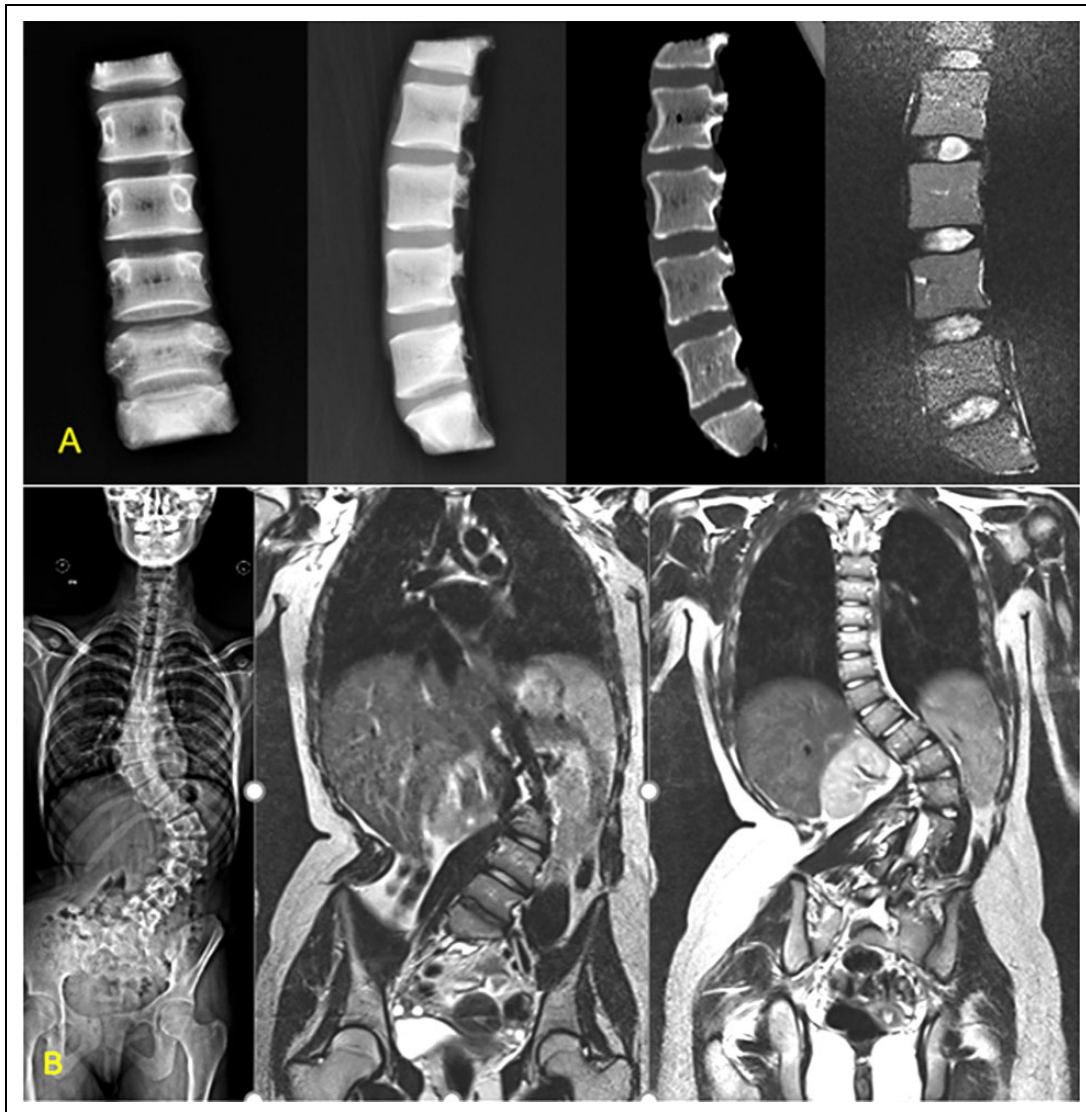
<sup>3</sup> Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

### Corresponding Author:

S. Rajasekaran, Department of Spine Surgery, Ganga Hospital, 313, Mettupalayam Road, Coimbatore, 641043, Tamil Nadu India.  
Email: rajasekaran.orth@gmail.com



Creative Commons Non Commercial No Derivs CC BY-NC-ND: This article is distributed under the terms of the Creative Commons Attribution-Non Commercial-NoDerivs 4.0 License (<https://creativecommons.org/licenses/by-nc-nd/4.0/>) which permits non-commercial use, reproduction and distribution of the work as published without adaptation or alteration, without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (<https://us.sagepub.com/en-us/nam/open-access-at-sage>).



**Figure 1.** Imaging of the study groups. (A) Normal group: Brain-dead voluntary organ donors. Imaging shows no degenerative changes. (B) Scoliosis group: Adolescent idiopathic scoliosis—magnetic resonance imaging showing normal discs.

most important reason for this elusiveness has been, lack of appropriate controls for making a precise comparison against degenerated discs. The inclusion of appropriate controls representing the original living status of intervertebral disc (IVD) will shed more light on the molecular mechanisms underlying the development of DD. Current research on disc degeneration uses heterogeneous controls for comparison. The commonly employed controls include scoliotic discs, traumatic discs,<sup>3</sup> discs from cadavers,<sup>4</sup> or discs from voluntary organ donors.<sup>5</sup> Also, results from animal studies have been extrapolated to humans due to a lack of appropriate human controls. However, the results from these studies have led to poor reproducibility and contributed to confusion rather than clarity. Hence, there is a need for defining appropriate controls for inclusion in any anatomical and molecular studies aiming at understanding the etiology of DD. In this study, we sought to compare the protein profiles of magnetic resonance imaging (MRI)–normal IVD

harvested from brain-dead voluntary organ donors against the most commonly used control discs obtained from scoliosis patients by employing proteomics strategy and establish “true controls” that can be utilized for future IVD research.

## Materials and Methods

### Sample Collection

The study was conducted after getting proper ethical clearance and approval from the institutional review board. Eight L4-L5 discs from 8 brain-dead voluntary organ donors screened for devoid of low back pain formed the normal discs group (ND), whereas scoliotic disc group (SD) consists of 8 discs from 3 adolescent idiopathic scoliosis (AIS) patients who underwent anterior deformity correction procedure (Figure 1). All the samples were collected in aseptic operating room conditions,

washed with 1X phosphate buffered saline (PBS) for the removal of bloodstains and snap-frozen in liquid nitrogen before storing at  $-80^{\circ}\text{C}$ .

### Protein Extraction and Prefractionation

Total protein was extracted from 200 mg of nucleus pulposus (NP) component of human intervertebral discs by radio immunoprecipitation assay buffer (RIPA) followed by 2% sodium dodecyl sulfate (SDS) buffer.<sup>6,7</sup> Both membrane and cellular protein fractions were extracted and subjected to precipitation using protein sample-methanol-chloroform-water in the ratio (1:4:1:3). Purified proteins were quantified using bicinchoninic acid (BCA) assay before prefractionation. About 100  $\mu\text{g}$  of purified total proteins were loaded onto 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for the separation based on molecular weight.

### In-Gel Tryptic Digestion

Prefractionated protein samples resolved in the form of small bands were cut and chopped finely into 1.5-mm gel pieces. Then, they were subjected to reduction using 25 mM dithiothreitol (DTT) to convert all cysteine disulfide bond into cysteine-free sulfhydryl groups, followed by alkylation using 55 mM iodoacetamide (IAA).<sup>5</sup> Alkylated pieces were washed with 100 mM ammonium bicarbonate before digesting the proteins with 600 ng of trypsin per protein band. The digestion was set at  $37^{\circ}\text{C}$  for 16 hours. Digested tryptic peptides were extracted from gel bands using 0.1% trifluoro acetic acid (TFA) and acetonitrile (ACN) on sonication (15-second pulsation on and off for 5 cycles). Extracted peptides were purified using Agilent c18 stage columns as per manufacturer's instruction and subjected to ESI-LC-MS/MS (electrospray ionization-liquid chromatography-tandem mass spectrometry) analysis.

### Tandem Mass Spectrometry

Purified trypsin-digested peptides were reconstituted with 0.1% trifluoroacetic acid/formic acid (TFA/FA) in 10% ACN before injecting into Orbitrap Velos Pro Mass Spectrometer at a concentration of 1000 femtomoles (fmol) per duplicate run as described earlier.<sup>8</sup> The raw files (.raw/.msf) obtained from the machine were searched against annotated UNIPROT consortium for identification of total proteins.

### Data Processing

The MS/MS raw data acquired from Orbitrap Velos Pro Mass Spectrometer were searched against universal human proteome databases for the global identification of proteins with the help of Proteome Discoverer v 1.4 available with Mascot and SEQUEST search engines. The peptide spectrum matches (PSMs) from SequestHT and Mascot were postprocessed using the Percolator algorithm. The peptides with rank one and having a  $q$ -value  $<0.01$  were considered for protein identification.

**Table 1.** Demographic Details of the Samples Considered for This Study.

Group	Age, years	Sex	Disc level	Pfarrmann grade	Modic
ND	13	Male	L4-L5	1	0
ND	22	Male	L4-L5	2	0
ND	28	Male	L4-L5	1	0
ND	32	Female	L4-L5	2	0
ND	43	Male	L4-L5	2	0
ND	45	Male	L4-L5	2	0
ND	55	Female	L4-L5	2	0
ND	63	Male	L4-L5	2	0
SD	13	Female	T12-L1	2	0
SD	13	Female	L1-L2	2	0
SD	13	Female	L2-L3	2	0
SD	13	Female	L3-L4	2	0
SD	15	Female	T12-L1	2	0
SD	29	Male	T12-L1	2	0
SD	29	Male	L2-L3	2	0
SD	29	Male	L3-L4	2	0

Abbreviations: ND, normal disc; SD, scoliotic disc.

### Bioinformatics Analysis

Total proteins were analyzed to remove the duplicate, unreviewed entries for further analysis. Spectral counts obtained by LC/MS-MS were further normalized by the normalized spectral abundance factor (NSAF) method.<sup>9</sup> Comparative proteome analysis was performed by FunRich, version: FunRich 3.0, a stand-alone software used for functional analysis, to understand the shared homology of proteins between groups.<sup>10</sup> Gene ontology was performed using Panther GO-slim classification tool version 14.0<sup>11</sup> and David Database version 6.7<sup>12</sup> to catalog proteins based on their molecular, biological, and cellular functions. Pathway enrichment analysis was performed using ShinyGO version 0.61<sup>13</sup> database with interface access to KEGG pathways along with Reactome pathway browser<sup>14</sup> version 3.6 to understand the pathomechanisms.

### Statistical Analysis

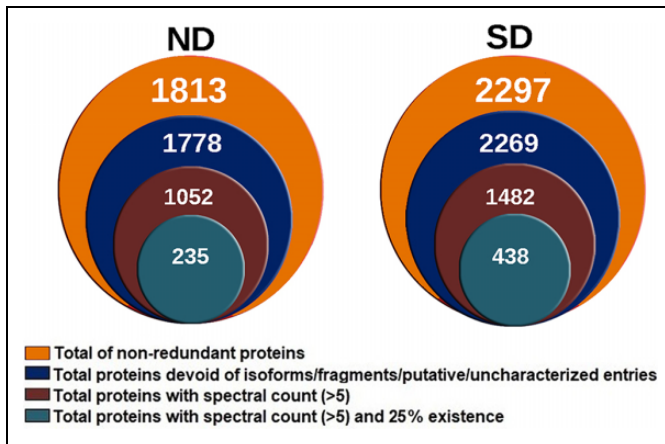
Differences in protein expression between ND and SD groups were analyzed using SPSS software version 25. Based on the normality assessment, independent  $t$  tests for 2 variables and Mann-Whitney  $U$  tests were used in case of normality assumption violation. Two-tailed alpha was set at 0.05.

### Data Availability

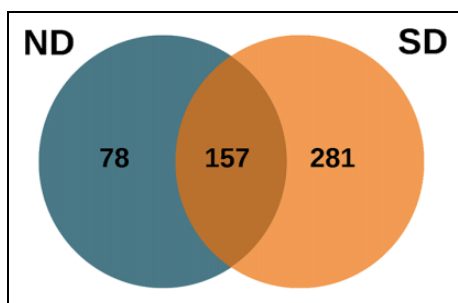
Raw data sets of this experiment have been deposited to the Proteome Xchange Consortium via the PRIDE<sup>15</sup> partner repository with identifier PXD018968 and project DOI 10.6019/PXD018968.

### Results

Human IVDs from the groups (ND and SD) examined in this study comprised mixed gender populations, as listed in Table 1.



**Figure 2.** Stacked Venn diagrams showing the total number of proteins at various levels of stringency. The innermost circle shows total proteins selected at a high stringency cutoff considered for the entire study. At high stringency, the normal disc (ND) group has 235 proteins, and the scoliotic group (SD) group has 438 proteins in total.



**Figure 3.** Venn diagram showing the number of unique and common proteins identified in normal disc (ND) and scoliotic disc (SD) groups. There were 157 proteins found common to both the groups with 78 unique in ND and 281 unique in SD groups. The diagram was generated with FunRich, version 3.0. FunRich 3.0 is a stand-alone software used for functional analysis.

The age (mean  $\pm$  SD) of the subjects was  $37.6 \pm 16.9$  years in the ND group and  $19.25 \pm 8.10$  in the SD group.

### Mass Spectrometry Identified More Number of Proteins in Scoliotic Discs

Tandem mass spectrometric analysis of ND and SD identified a total of 1813 proteins in ND and 2297 proteins in SD (Figure 2). Total proteins were verified with their peptide sequences using the UNIPROT database, and uncharacterized/putative proteins were removed (Supplementary File 1). Applying stringent statistical criteria (cutoff PSM  $\geq 5$  and the presence of a protein in at least 2 [25%] samples) identified a total of 235 proteins in ND and 438 in the SD group.

Comparative analysis of proteins revealed that 157 (30.23%) proteins were found to be common between ND and SD groups (Figure 3). Out of 157 proteins, 35 (22.29%) proteins were core matrisomal proteins, 19 (12.10%) were

matrisomal associated proteins and remaining 103 (65.06%) were comprised of keratins, immune regulators, proteases and proteins coding for basal metabolisms such as such keratinization, cornification, homeostasis, protein processing, and maturation. About 78 (15.31%) proteins were found only in the ND group, and 281 (54.45%) were found only in the SD group.

### Scoliotic Disc Proteins Exhibit Significant Alterations in Their Biological and Molecular Functions

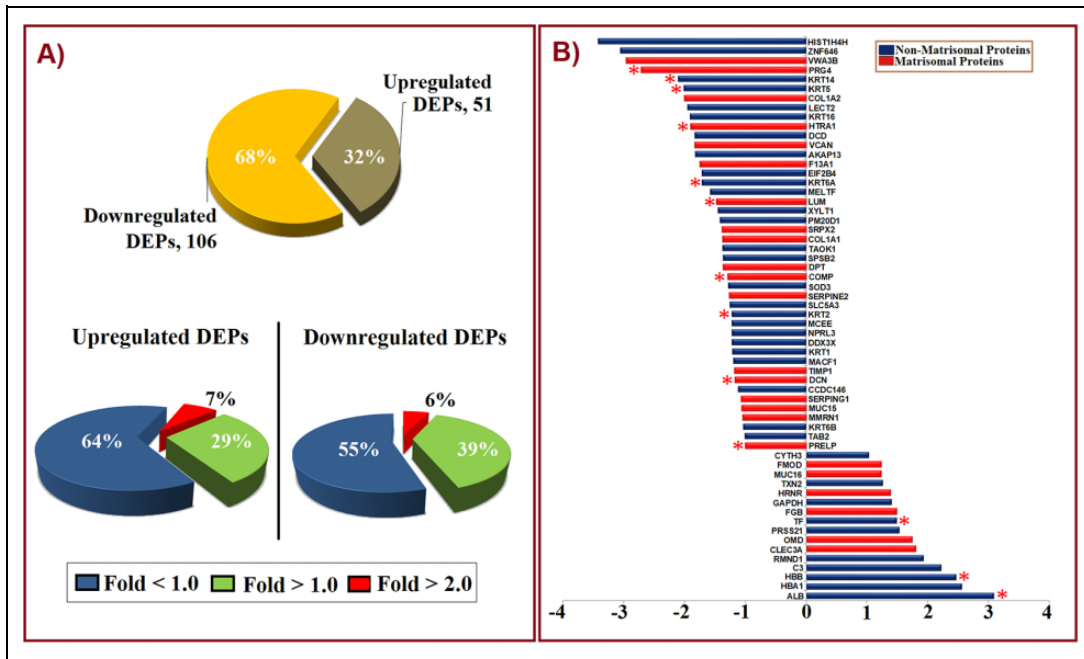
To elucidate the possible mechanisms in ND and SD groups, differential expression analysis was conducted, revealing 60 proteins to be differentially regulated. Out of 157 proteins, 51 (32%) proteins were found to be upregulated, and (106) 68% proteins to be downregulated in SD (Figure 4A). About 60 differentially expressed proteins (DEPs) with  $\log_2 FC \geq \pm 1.0$  (FC is fold change) were considered for the analysis (Figure 4B). Altogether, 13 proteins were found with significant  $p$ -value  $< 0.05$ , as shown in Table 2. The proteins that were upregulated in SD above 2.0-fold include blood proteins such as ALB, HBB, and HB1, and complement protein (C3), confirming the regulation of inflammatory cascades. Proteins such as proteoglycan IV (PRG4), Von Willebrand factor A domain containing 3B (VWA3B), histone H4 (HIST1H4H), zinc finger protein (ZNF646), keratin 15, keratin 4 were found to be downregulated for 2.0 fold and above in SD.

On their functional characterization of the biological process using David database, the upregulated DEPs in SD group were involved in hydrogen peroxide catabolic process, cellular oxidant detoxification, response to hypoxia, positive regulation of reactive oxygen species and complement activation along with other basal metabolic processes (Figure 5). Whereas, the downregulated DEPs were involved in positive regulation of NF- $\kappa$ B transcription activity, negative regulation of endopeptidase activity, and negative regulation of beta-amyloid activity enunciating normal metabolism involving cell survival, proliferation, and differentiation.

Pathway enrichment analysis using the Reactome pathway browser version 3.6 detected more than 800 pathways with more than 100 pathways having significant  $P$  value ( $P < .05$ ) (Supplementary File 2). Notable examples, namely, extracellular matrix (ECM) proteoglycans, ECM organization, integrin cell surface interactions, along with complement cascades and coagulation cascades involving proteins such as clusterin (CLU), complement C1S (C1S), complement C3 (C3), fibrinogen,  $\alpha$ 1-antitrypsin (A1AT), protein C inhibitor (PCI), and other SERPIN family-related proteins (Figure 6).

### Specific Proteins Expressed in Normal Disc and Scoliotic Disc Differ Strikingly

Characterization of specific proteins based on gene ontology to understand their functionality by Panther GO-slim classification tool version 14.0 revealed interesting events in ND and SD groups, as shown in Figure 7.



**Figure 4.** Characterization of proteins based on their regulation (up and down). (A) Pie chart describing the contribution of up and down-regulated proteins in terms of fold change (log<sub>2</sub> FC). More proteins were downregulated (68%) in the scoliotic disc (SD) group when compared against the normal disc (ND) group, and upregulated proteins contributed to 32% of total 157 common proteins. Pie charts also depict the contribution of differentially expressed proteins (DEPs) between fold changes (>1.0 and <3.5) in both up- and downregulation. (B) Bar chart showing DEPs with log<sub>2</sub> FC ≥ ±1.0 (up- and downregulation) in SD when compared with ND group considered under this study. \*Indicates statistical significance (P < .05), using t test.

**Table 2.** Differentially Expressed Proteins (DEPs) That Are Statistically Significant With log<sub>2</sub> FC > ±1.0.

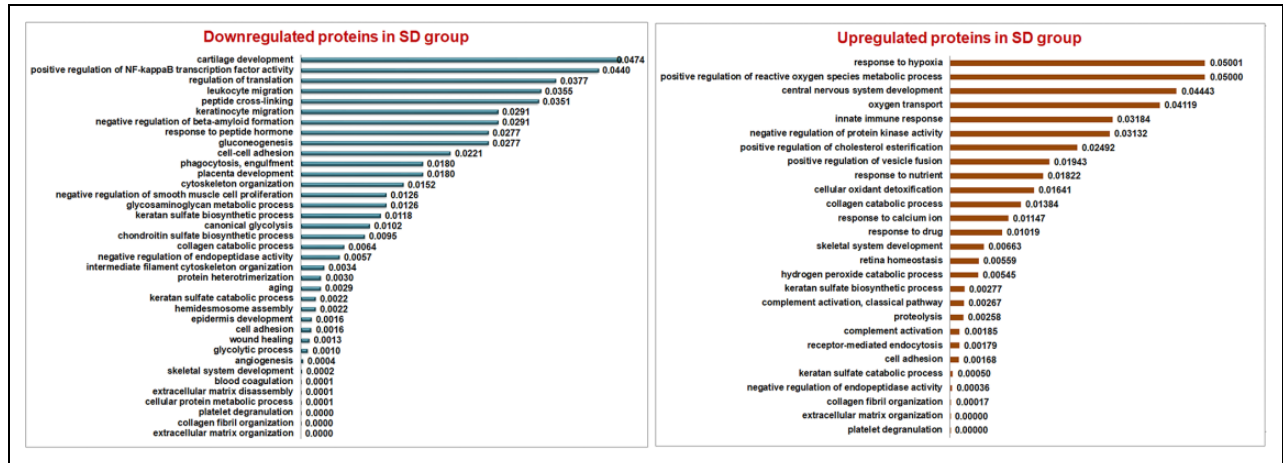
Protein name	Gene symbol	Expression in our data, P value	Implication in disease
Hemoglobin beta	HBB	Higher in SD group, .002	Mutations of HBB are associated with beta-thalassemia sickle cell anemia
Albumin	ALB	Higher in SD group, .045	Blood protein
Serotransferrin	TF	Higher in SD group, .021	Iron binding transport protein
Proteoglycan IV	PRG4	Higher in ND group, .003	Proteoglycan; regulates joint lubrication and synovial homeostasis
Lumican	LUM	Higher in ND group, .008	Helps in collagen fibril organization
Decorin	DCN	Higher in ND group, .013	Regulates autophagy and inhibits angiogenesis
Prolargin	PRELP	Higher in ND group, .040	Anchors basement membranes to underlying connective tissue
Cartilage oligomeric matrix protein	COMP	Higher in ND group, .010	Binds matrix proteins and polymerization collagen type II fibers
Serine protease HTRA1	HTRA1	Higher in ND group, .011	Tumor suppressor
Keratin 2	KRT 2	Higher in ND group, .010	Cytoskeletal protein
Keratin 5	KRT5	Higher in ND group, .020	Cytoskeletal protein
Keratin 6A	KRT6A	Higher in ND group, .026	Cytoskeletal protein
Keratin 14	KRT14	Higher in ND group, .045	Cytoskeletal protein

Abbreviations: FC, fold change; ND, normal disc; SD, scoliotic disc.

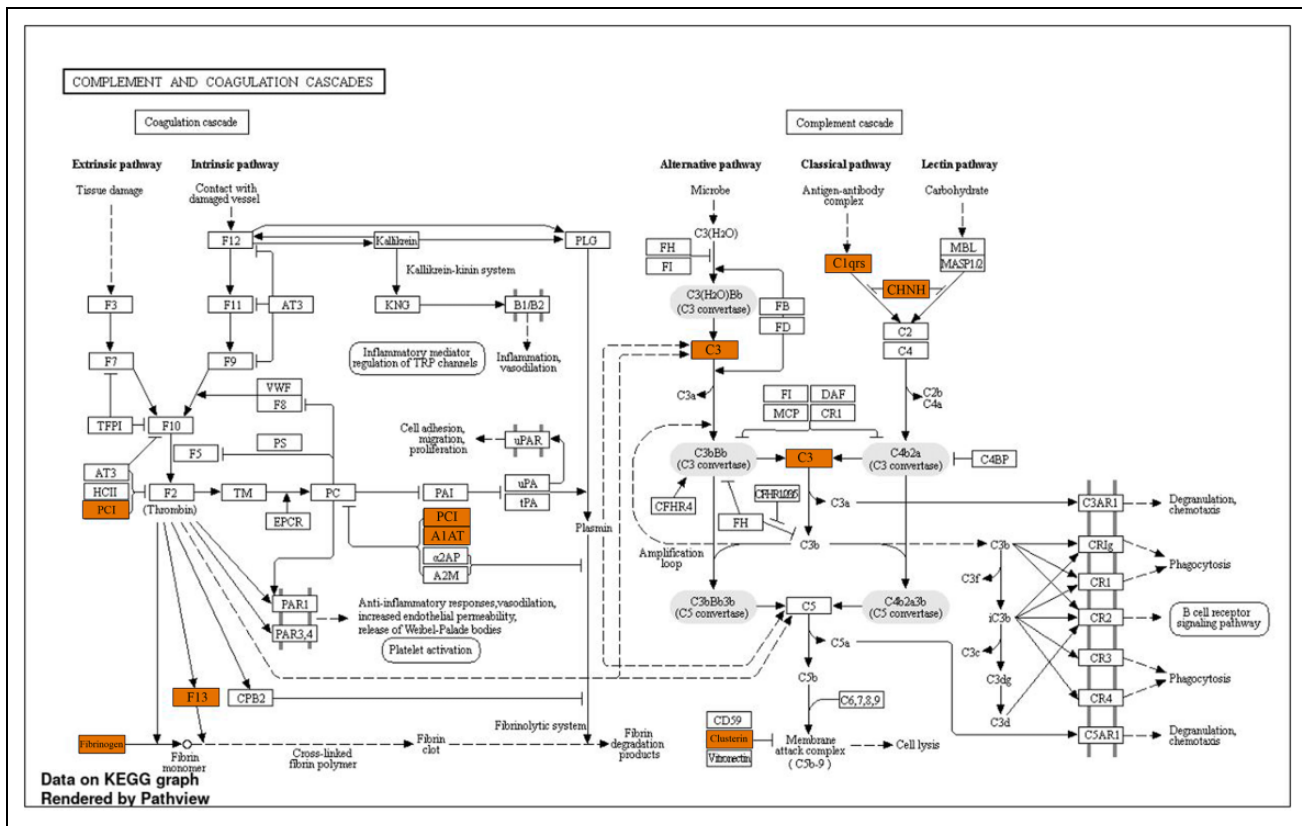
Proteins involved in molecular functions, namely, catalytic activity (41%), transporter activity (12%), and structural molecule activity (6%) were found to be predominant in ND (Figure 7A). Whereas the proteins involved in the above functions were significantly reduced and proteins involved in regulation activities like binding (44%) molecular function regulator activity (6%), and transcription regulation activity (2%) were found to be activated in SD. Under protein class, cytoskeletal proteins were found to be comparatively decreased

in SD (6%), which was 23% in the ND group; whereas defense-immunity proteins were strikingly abundant in SD group (8%) in comparison with the ND group (2%) (Figure 7B).

Classification based on biological process derived using ShinyGo version 6.0, reveals unlike events between ND and SD groups. In NDs, most cytoskeletal proteins are associated with keratinization, cornification, and vesicle-mediated transport. Other proteins codes for basal metabolism like cell communication, adhesion, and cell differentiation. In SD group, proteins



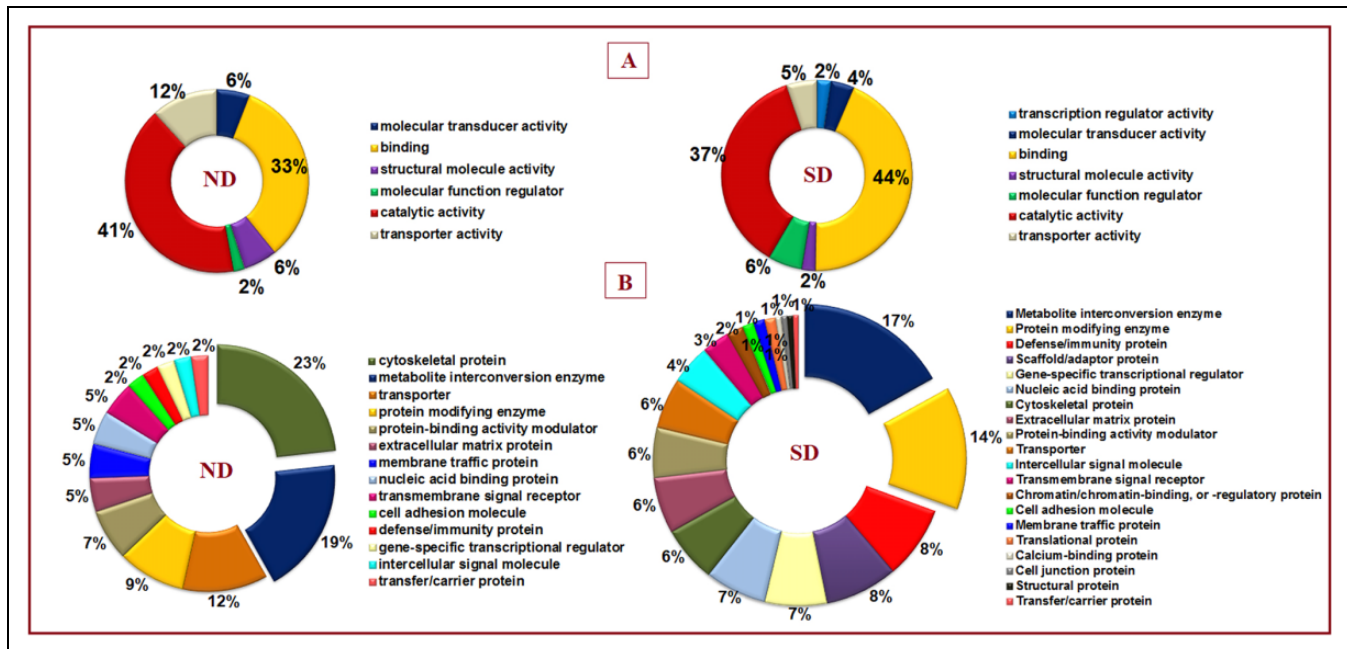
**Figure 5.** Biological process involved in both upregulated (A) and downregulated (B) differentially expressed proteins (DEPs) in the scoliotic disc (SD) group, using David database version 6.8 that were significantly enriched ( $P < .05$ ). Downregulated proteins in the SD group were found to be actively involved in the basal metabolic process such as cartilage development, regulation of translation, cell-cell adhesion, cytoskeletal organization, collagen catabolic process and extracellular matrix organization. Upregulated proteins in SD were found involved in stress-induced inflammatory processes such as response to hypoxia, cellular oxidant detoxification, hydrogen peroxide catabolic process and complement activation.



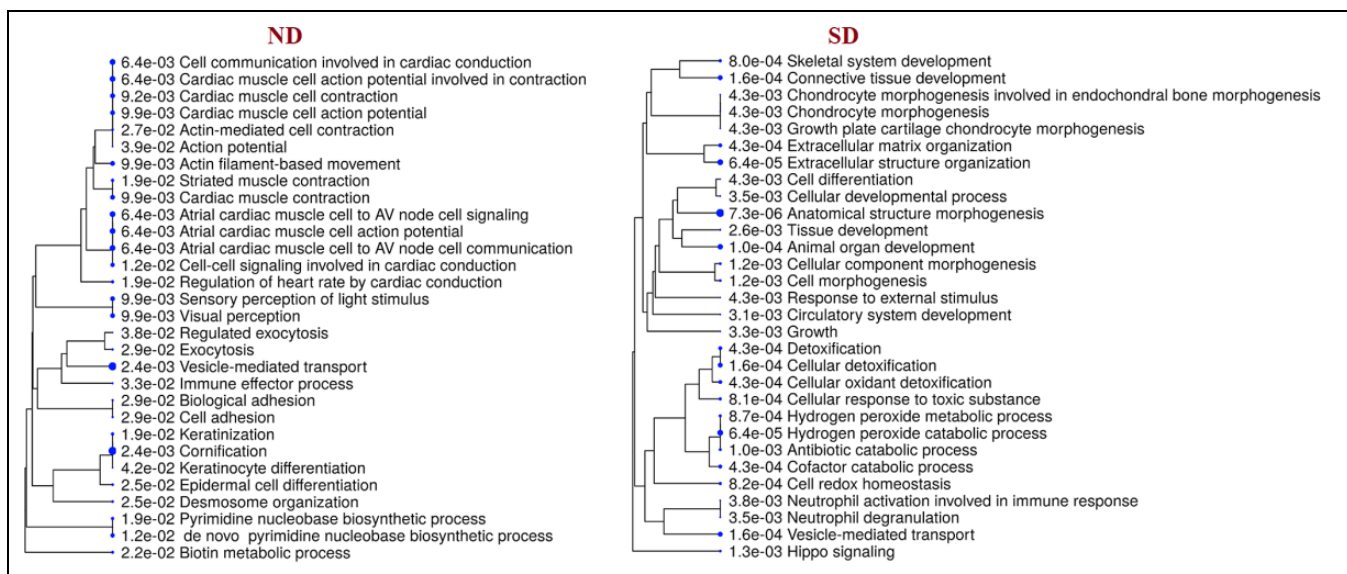
**Figure 6.** Pathway view created for complement and coagulation cascades using the KEGG database in ShinyGo version 6.0 with colored boxes indicating proteins present in our study.

such as peroxiredoxin-2 (PRDX2), peroxiredoxin-6 (PRDX6), catalase (CAT), apolipoprotein-E (APOE), myeloperoxidase (MPO), glutathione S-transferase P1 (GSTP1), hemoglobin A2 (HBA2), hemoglobin subunit delta (HBD), haptoglobin

(HP) that influence redox homeostasis and reactive oxygen species were found to be shared among significant process responsible for detoxification, and other catabolic processes such as neutrophil degranulation and neutrophil activation (Figure 8).



**Figure 7.** Gene ontological classification of normal disc (ND) and scoliotic disc (SD) groups derived using Panther GoSlim classification tool version 15.0. (A) Molecular function and (B) protein class to understand the class and function of unique proteins. Under molecular function, the majority of proteins were found to be involved in binding and catalytic activity with varied abundances in each group. Under protein class of ND group, proteins belonging to cytoskeletal, metabolite interconversion enzyme, and transporter classes are seen more. But in the SD group, the majority of proteins are seen grouped under metabolite interconversion, protein modifying and defense/immunity protein classes.



**Figure 8.** A hierarchical clustering tree demonstrates the correlation among significant biological process identified for unique proteins of normal disc (ND) and scoliotic disc (SD). The biological process with many shared genes is clustered together. Bigger dots indicate more significant  $P$  value. In the ND group, cornification, vesicle-mediated transport, action potential, actin filament-based movement are all enriched. In the SD group, anatomical structure organization, detoxification, cellular detoxification, hydrogen peroxide catabolic process, and neutrophil degranulation are enriched.

## Discussion

Disc degeneration is a highly researched subject in spinal pathologies. However, huge knowledge gaps exist with regard

to its etiopathogenesis. Disc degeneration is often diagnosed late when patients present with symptoms. At this time, the irreversible pathological changes have already occurred, and surgical treatment remains the mainstay of treatment.

Molecular studies on disc degeneration have utilized these disc samples with advanced degeneration acquired during the surgery. However, the availability of a disc sample with “no” degeneration or “early” degeneration has been sparse. This lack of availability of a true biologically inert control for comparative studies has plagued research on disc degeneration. Few authors have utilized discs from anterior scoliosis surgeries, fractures of spine, or cadavers as control discs in their studies. However, the validity of these studies remains questionable.

Scoliotic discs are the most commonly available and frequently used controls. Though both the scoliotic discs and discs from voluntary organ donors appear normal on MRI, the results from our study indicate substantial differences between scoliotic discs and control discs from voluntary organ donors. The differences were noted in terms of differential expression of common proteins, biological processes, molecular functions, pathways involved, and the presence or absence of unique proteins. Three findings were notable: (1) Proteins involved in basal metabolic processes, cellular functions, and structural proteins were downregulated in the SD group. (2) SD group had upregulation of inflammation cascade proteins and downregulation of its regulators. (3) SD group had upregulation of reactive oxygen species (ROS) and oxidative stress response proteins.

### ***Downregulation of Structural and Repair Proteins in the SD Group***

Many proteins that are involved in the maintenance of ECM and cellular process were downregulated in the SD group. Versican is a major proteoglycan and component of the ECM along with aggrecan. It is involved in cell adhesion, proliferation, migration, and plays a central role in ECM maintenance. It also binds hyaluronic acid and plays a role in cell adhesion.<sup>16</sup> Other proteoglycans, namely, lumican (LUM), decorin (DCN), and prolargin (PRELP), along with collagen 1A2, 1A1 were also downregulated in SD group indicating ECM disorganization.

Keratins KRT6A, KRT14, KRT5, and KRT 13A1 were downregulated in the SD group. Keratins play a role in enhancing mechanical properties by organizing filaments and also are involved in the wound healing process.<sup>17,18</sup> A-kinase anchor protein 13(AKAP 13A) or Rho guanine nucleotide exchange factor 13 is the guanine nucleotide exchange factor (GEF) for the RhoA small GTPase protein.<sup>19,20</sup> It activates Rho, a major regulator of the cell actin cytoskeleton.<sup>21</sup> Coagulation factor XIII A chain (F13A1) plays a role in stabilizing the fibrin clot. It also cross-links  $\alpha$ -2-plasmin inhibitor, or fibronectin, to the alpha chains of fibrin. The defects of this gene have been implicated in bleeding diathesis and impaired wound healing.<sup>22,23</sup> Proteoglycan 4 (PRG4) or lubricin have a unique role in cell-cell and cell-ECM interactions.<sup>24</sup> Decreased levels of lubricin have been noted in the synovial fluid of patients with rheumatoid arthritis and osteoarthritis.<sup>25</sup> Downregulation of keratins, AKAP 13A, F13A, PRG4, and cytoskeletal proteins indicates reduced structural integrity in scoliotic discs.

Another finding was the downregulation of proteins that are involved in transcription, translation, and DNA repair in the SD group. Von Willebrand factor A domain containing 3B (VWA3B) are intracellular proteins that are thought to function in transcription, DNA repair, ribosomal, and membrane transport. Von Willebrand factor also acts as a receptor for proteasome-mediated degradation for ubiquitin-like proteins.<sup>26-28</sup> Eukaryotic initiation factor 2B (EIF2B) is a GTP exchange factor necessary for protein synthesis. Stress responses lead to increase protein synthesis during which stress response proteins are produced, these proteins activate the kinases, which leads to phosphorylation of the eIF, and this reduces the activity of EIF2B. Mutations in EIF2B leads to phosphorylation of eIF, which is reported in neurological diseases such as leukodystrophy with vanishing white matter in the brain (VWM) and ovarioleukodystrophy.<sup>29,30</sup> Histone H4 (HIST1H4H) is a core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machinery, which requires DNA as a template. They play a vital role in transcription regulation, DNA repair, DNA replication, and chromosomal stability.<sup>31</sup> Leukocyte cell-derived chemotaxin 2 (LECT2) seems to be related to the cell cycle or repair process following damage to a variety of cells and stimulates the growth of chondrocytes and osteoblasts.<sup>32</sup> Zinc finger protein 646 (ZNF646) also has a possible role in transcriptional regulation. The downregulation of these proteins indicates a defective transcription and DNA repair, which could lead to premature cell death.<sup>33</sup>

### ***Upregulation of Inflammatory Cascade Proteins and Downregulation of Its Regulators in the SD Group***

Many proteins of complement cascades and innate immune systems such as complement C1s (C1s), complement C3 (C3), IGKC, IGHG1, and other SERPIN family-related proteins were upregulated in SD group. C3 is the chief component of the complement system and plays a vital role in the innate immune response. Complement-mediated inflammation is important not only in immunological defense reactions but also in the clearance of damaged/apoptotic cells.<sup>34</sup> This process is ensured by an alternate pathway, where tissue damage activates the complement cascade, and through a constant spontaneous process known as tick over, C3 complement protein is produced.<sup>35</sup> Excessive complements have been implicated in inflammaging<sup>5</sup> and many disorders like autoimmune disorders, Alzheimer's, disease, macular degeneration, and Crohn's disease.<sup>36</sup> Clusterin (CLU) is an important regulator of complements. CLU prevents uncontrolled membrane attack complex activity and thus plays a significant role in controlling terminal complement-mediated damage to host cells and chronic inflammation.<sup>37</sup> The downregulation of CLU was noted in the SD group. Similarly, SERPING1 or plasma protease inhibitor of C1, was downregulated in SD group. SERPING1 exhibits anti-inflammatory role by inhibition of complement system proteases (C1r, C1s, mannose-binding protein-associated



serine protease 2-MASP2) and the plasma kallikrein-kinin system proteases.<sup>38</sup> It also inhibits chymotrypsin.<sup>39</sup>

The SERPINE1, Plasminogen activator inhibitor-1 protein, was downregulated in SD group. It is an inhibitor of serine protease and also plays a vital role in signal transduction, cell adhesion, and migration.<sup>40</sup> It also inhibits plasmin-mediated breakdown of the ECM.<sup>41</sup> In contrast, C-type lectin domain family 3 member A (CLEC3A), which was upregulated in the SD group, enhances tissue plasminogen activator-mediated plasminogen activation.<sup>42</sup> Serine protease 21 (PRSS21) is a member of the trypsin family that cleaves peptide bonds subsequent to a positively charged amino acid (lysine or arginine).<sup>43</sup> The presence of proteins involved in the coagulation cascade in SD group indicates a response to tissue injury. Exposed collagen as a result of injury could activate coagulation cascades.<sup>44</sup>

The coexistence of complement and coagulation cascade mediates innate immunity with coagulation enzymes activating complement cascades and both functioning as a proteolytic cascade.<sup>45</sup> Serine proteinases, along with activators and inhibitors, are majorly responsible for their interlink between coagulation and complement systems, and the dysregulation in coagulation or complement activities would, in turn, activate inflammatory cascades.<sup>46</sup>

In biological processes, positive regulation of NF- $\kappa$ B transcription factor was noted in our study. NF- $\kappa$ B is a transcription factor involved in inflammatory and immune responses with additional roles in cell survival, proliferation, and differentiation. NF- $\kappa$ B can stimulate the expression of the immunoglobulin  $\kappa$  light chain in B cells and play a fundamental role in lymphocyte development and activation, and it is essential for innate and adaptive immune responses.<sup>47,48</sup> Neutrophil activation and degranulation were among the biological processes uniquely found in the SD group. Neutrophils are essential regulators of an inflammatory response that acts as the first line of defense mechanism against inflammatory tissue damage either through degranulation or respiratory bursts.<sup>49</sup> These above findings indicate a pro-inflammatory state in scoliotic discs.

### *Upregulation of Reactive Oxygen Species and Oxidative Stress Response Proteins in the SD Group*

Proteins involved in redox homeostasis like PRDX2, PRDX6, CAT, APOE, MPO, and GSTP1 were upregulated in SD group. Oxidative stress induces inflammation and vice versa. PRDX2 is a ubiquitous redox-active intracellular enzyme. Once released, the extracellular PRDX2 acts as a redox-dependent inflammatory mediator, triggering macrophages to produce and release tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ).<sup>50</sup> Ye et al,<sup>51</sup> in their proteomic analysis of normal and degenerated discs, documented the presence of PRDX2 in degenerated discs and hypothesized an oxidative injury to have a possible role in disc degeneration. PRDX6, is the only peroxiredoxin capable of reducing phospholipid hydroperoxides and also plays crucial roles in lung phospholipid metabolism, lipid peroxidation repair, and inflammatory signaling.<sup>52</sup> Myeloperoxidase is a

potent enzyme with antibacterial activity and is implicated in autoimmune disorders.<sup>53</sup> Catalase is a crucial enzyme and plays a role in reducing ROS and counteracting TNF- $\alpha$ -mediated apoptosis.<sup>54</sup> Decreased catalase has been associated with ligamentum flavum hypertrophy.<sup>55</sup> ROS-mediated oxidative injury to NP cells resulting in premature cell death have been well documented in the literature.<sup>56-58</sup> Rajasekaran et al<sup>5</sup> also documented the presence of peroxiredoxin, CAT, and MPO in degenerated discs but not in MRI-normal voluntary organ donor discs.

APOE is involved in cholesterol transport protein linked with atherosclerosis, neurodegenerative disorders like Alzheimer's disease.<sup>59</sup> Recently, knock out mice models of APOE have documented a premature intervertebral disc degeneration.<sup>60,61</sup> GSTP1 is an intracellular detoxification enzyme that catalyzes the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione.<sup>62</sup> GSTP1 also acts as a critical ligand-binding protein with a role in regulating kinase pathways.<sup>63</sup> Oxidative stress activates the mitogen-activated protein kinase (MAPK) signaling pathway and induces apoptosis by a pathway involving c-Jun N-terminal kinase (JNK) and pro-apoptotic proteins.<sup>64</sup> GSTP1 interacts directly with JNK and regulates cell death.<sup>65</sup> These findings reflected in the evaluation of biological processes where positive regulation of ROS, cellular oxidant detoxification processes were found to be upregulated in the SD group. The disparity between production and assimilation of ROS through detoxification causes increased oxidative stress response in the host leading to inflammation.<sup>66</sup> The unique presence of oxidative stress response proteins in the SD group serves as evidence for inflammatory response leading to tissue damage.

The above findings indicate that though scoliotic discs were looking "normal" in MRI, they had many biological processes indicating inflammatory and oxidative stresses. Also, the decreased proteoglycans, proteins involved in transcription and DNA repair indicate impaired structural integrity and cellular functions in scoliotic discs.

Stress profilometry studies on scoliotic discs have demonstrated abnormally high stresses in scoliotic discs on comparison with nonscoliotic control discs.<sup>67</sup> Moreover, these stresses were asymmetrical, stresses in the concave annulus were higher than in the convex annulus. These abnormal stresses could alter the biology of the discs and could have been responsible for our findings. Brown et al<sup>68</sup> documented the fragmentation of small leucine-rich proteoglycans (SLRP) in scoliotic discs and degenerated discs alike, indicating a breakdown of the ECM in scoliotic discs.<sup>68</sup> Rajasekaran et al,<sup>69</sup> in their study using postcontrast MRI, found decreased diffusion and cell viability in scoliotic discs in comparison to controls. They also noted calcification, neovascularization, and matrix degeneration in scoliotic discs providing evidence for tissue injury and early degeneration.<sup>69</sup>

Calcification process may lead to impairment of nutrient supply and metabolism of the disc and promote degeneration. A study by Hristova et al<sup>70</sup> demonstrated calcification in degenerate discs and scoliotic discs and postulated that

mineralization in scoliosis discs reflect a premature degenerative process. Osteomodulin, a protein found in abundance in the SD group, is implicated in the biomineralization processes and functions by binding of osteoblasts via the alpha(V)beta3-integrin. It has been claimed to be a biomarker to monitor early changes in subchondral bone metabolism in osteoarthritis.<sup>71</sup>

### Significance and Limitation of the Study

Ours is the first study to evaluate the validity of scoliotic discs as controls for IVD research. Here we establish MRI-normal voluntary organ donors as the precise controls for IVD research and recommend it for future studies. Our study also questions the validity of other studies that have employed inappropriate controls. Additionally, our study identified many downregulated proteins in scoliotic discs, which play a role in maintaining the structural integrity of the disc. These proteins can be further probed for their role in the etiopathogenesis of adolescent idiopathic scoliosis. The limitation of the study was that no separate analysis of concave versus convex discs was done.

### Conclusion

In a first of its kind study, we evaluated the controls used in IVD research for their preciseness. Our findings indicate that scoliotic discs have an abundance of inflammatory, immune response, oxidative stress, and antioxidant proteins and are not biologically inert. Discs from MRI-normal voluntary organ donors are in a state of homeostasis and are void of pathological processes. Hence, we establish it as the ideal control for IVD research and recommend its use in future studies. Also, our study questions the validity of earlier studies that have utilized other controls.

### Acknowledgments

The authors thank Ms Alishya Maria Jose for her help in data collection and maintenance and Mr Balakrishnan for his support in preparing figures.


### Declaration of Conflicting Interests


The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The project was funded by Ganga Orthopaedic Research & Education Foundation, Coimbatore, Tamil Nadu, India.

### ORCID iD

S. Rajasekaran, MS, DNB, FRCS, MCh, PhD  <https://orcid.org/0000-0001-6043-006X>

Rishi Mugesh Kanna, MS, MRCS, FNB  <https://orcid.org/0000-0001-5817-4909>

B. T. Pushpa, DNB, FRCR  <https://orcid.org/0000-0001-8451-1729>

### Supplemental Material

Supplemental material for this article is available online.

### References

1. Luoma K, Riihimäki H, Luukkonen R, Raininko R, Viikari-Juntura E, Lamminen A. Low back pain in relation to lumbar disc degeneration. *Spine (Phila Pa 1976)*. 2000;25:487-492. doi:10.1097/00007632-200002150-00016
2. GBD 2017 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet*. 2018;392:1789-1858. doi:10.1016/S0140-6736(18)32279-7
3. Sarath Babu N, Krishnan S, Brahmendra Swamy CV, Venkata Subbaiah GP, Gurava Reddy AV, Idris MM. Quantitative proteomic analysis of normal and degenerated human intervertebral disc. *Spine J*. 2016;16:989-1000. doi:10.1016/j.spinee.2016.03.051
4. Sharp CA, Roberts S, Evans H, Brown SJ. Disc cell clusters in pathological human intervertebral discs are associated with increased stress protein immunostaining. *Eur Spine J*. 2009;18:1587-1594. doi:10.1007/s00586-009-1053-2
5. Rajasekaran S, Tangavel C, Anand KSSV, et al. Inflammation determines health and disease in lumbar discs-evidence from differing proteomic signatures of healthy, aging, and degenerating discs. *Spine J*. 2020;20:48-59. doi:10.1016/j.spinee.2019.04.023
6. Valkova N, Yunis R, Mak SK, Kang K, Kültz D. Nek8 mutation causes overexpression of galectin-1, sorcin, and vimentin and accumulation of the major urinary protein in renal cysts of jck mice. *Mol Cell Proteomics*. 2005;4:1009-1018. doi:10.1074/mcp.M500091-MCP200
7. Ericsson C, Nistér M. Protein extraction from solid tissue. *Methods Mol Biol*. 2011;675:307-312. doi:10.1007/978-1-59745-423-0\_17
8. Rajasekaran S, Tangavel C, Aiyer SN, et al. ISSLS prize in Clinical Science 2017: is infection the possible initiator of disc disease? An insight from proteomic analysis. *Eur Spine J*. 2017;26:1384-1400. doi:10.1007/s00586-017-4972-3
9. Zybaylov B, Mosley AL, Sardi ME, Coleman MK, Florens L, Washburn MP. Statistical analysis of membrane proteome expression changes in *Saccharomyces cerevisiae*. *J Proteome Res*. 2006;5:2339-2347. doi:10.1021/pr060161n
10. Pathan M, Keerthikumar S, Ang C-S, et al. FunRich: An open access standalone functional enrichment and interaction network analysis tool. *Proteomics*. 2015;15:2597-2601. doi:10.1002/pmic.201400515
11. Mi H, Muruganujan A, Ebert D, Huang X, Thomas PD. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res*. 2019;47(D1):D419-D426. doi:10.1093/nar/gky1038
12. Dennis G Jr, Sherman BT, Hosack DA, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol*. 2003;4:R60. doi:10.1186/gb-2003-4-9-r60
13. Ge SX, Jung D, Yao R. ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics*. 2020;36:2628-2629. doi:10.1093/bioinformatics/btz931

14. Fabregat A, Sidiropoulos K, Viteri G, et al. Reactome pathway analysis: a high-performance in-memory approach. *BMC Bioinformatics*. 2017;18(1):142. doi:10.1186/s12859-017-1559-2
15. Perez-Riverol Y, Csordas A, Bai J, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res*. 2019;47(D1):D442-D450. doi:10.1093/nar/gky1106
16. Andersson-Sjöland A, Hallgren O, Rolandsson S, et al. Versican in inflammation and tissue remodeling: the impact on lung disorders. *Glycobiology*. 2015;25:243-251. doi:10.1093/glycob/cwu120
17. Atkinson SD, McGilligan VE, Liao H, et al. Development of allele-specific therapeutic siRNA for keratin 5 mutations in epidermolysis bullosa simplex. *J Invest Dermatol*. 2011;131:2079-2086. doi:10.1038/jid.2011.169
18. Chan YM, Yu QC, LeBlanc-Straceski J, et al. Mutations in the non-helical linker segment L1-2 of keratin 5 in patients with Weber-Cockayne epidermolysis bullosa simplex. *J Cell Sci*. 1994;107(pt 4):765-774.
19. Dutt P, Nguyen N, Toksoz D. Role of Lbc RhoGEF in  $\alpha$ 12/13-induced signals to Rho GTPase. *Cell Signal*. 2004;16:201-209. doi:10.1016/S0898-6568(03)00132-3
20. Diviani D, Soderling J, Scott JD. AKAP-Lbc anchors protein kinase a and nucleates  $\alpha$ 12-selective Rho-mediated stress fiber formation. *J Biol Chem*. 2001;276:44247-44257. doi:10.1074/jbc.M106629200
21. Thumkeo D, Watanabe S, Narumiya S. Physiological roles of Rho and Rho effectors in mammals. *Eur J Cell Biol*. 2013;92:303-315. doi:10.1016/j.ejcb.2013.09.002
22. Hayashi T, Kadohira Y, Morishita E, Asakura H, Soury M, Ichinose A. A case of acquired FXIII deficiency with severe bleeding symptoms. *Haemophilia*. 2012;18:618-620. doi:10.1111/j.1365-2516.2012.02763.x
23. Inbal A, Oldenburg J, Carcao M, Rosholm A, Tehranchi R, Nugent D. Recombinant factor XIII: a safe and novel treatment for congenital factor XIII deficiency. *Blood*. 2012;119:5111-5117. doi:10.1182/blood-2011-10-386045
24. Jay G. Lubricin and surfacing of articular joints. *Curr Opin Orthop*. 2004;15:355-359. doi:10.1097/01.bco.0000136127.00043.a8
25. Kosinska MK, Ludwig TE, Liebisch G, et al. Articular joint lubricants during osteoarthritis and rheumatoid arthritis display altered levels and molecular species. *PLoS One*. 2015;10:e0125192. doi:10.1371/journal.pone.0125192
26. Berry KN, Brett TJ. Structural and biophysical analysis of the CLCA1 VWA domain suggests mode of TMEM16A engagement. *Cell Rep*. 2020;30:1141-1151.e3. doi:10.1016/j.celrep.2019.12.059
27. Rani N, Aichem A, Schmidtke G, Kreft SG, Groettrup M. FAT10 and NUB1L bind to the VWA domain of Rpn10 and Rpn1 to enable proteasome-mediated proteolysis. *Nat Commun*. 2012;3:749. doi:10.1038/ncomms1752
28. Springer TA. Complement and the multifaceted functions of VWA and integrin I domains. *Structure*. 2006;14:1611-1616. doi:10.1016/j.str.2006.10.001
29. Ibitoye RT, Renowden SA, Faulkner HJ, Scolding NJ, Rice CM. Ovarioleukodystrophy due to EIF2B5 mutations. *Pract Neurol*. 2016;16:496-499. doi:10.1136/practneurol-2016-001382
30. Wong YL, LeBon L, Basso AM, et al. eIF2B activator prevents neurological defects caused by a chronic integrated stress response. *Elife*. 2019;8:e42940. doi:10.7554/eLife.42940
31. El Kennani S, Adrait A, Shaytan AK, et al. MS\_HistoneDB, a manually curated resource for proteomic analysis of human and mouse histones. *Epigenetics Chromatin*. 2017;10:2. doi:10.1186/s13072-016-0109-x
32. Nagai H, Hamada T, Uchida T, Yamagoe S, Suzuki K. Systemic expression of a newly recognized protein, LECT2, in the human body. *Pathol Int*. 1998;48:882-886. doi:10.1111/j.1440-1827.1998.tb03855.x
33. Cassandri M, Smirnov A, Novelli F, et al. Zinc-finger proteins in health and disease. *Cell Death Discov*. 2017;3:17071. doi:10.1038/cddiscovery.2017.71
34. Mollnes TE, Fosse E. The complement system in trauma-related and ischemic tissue damage: a brief review. *Shock*. 1994;2:301-310. doi:10.1097/00024382-199410000-00012
35. Ricklin D, Reis ES, Mastellos DC, Gros P, Lambris JD. Complement component C3—the “Swiss Army Knife” of innate immunity and host defense. *Immunol Rev*. 2016;274:33-58. doi:10.1111/imr.12500
36. Tichaczek-Goska D. Deficiencies and excessive human complement system activation in disorders of multifarious etiology. *Adv Clin Exp Med*. 2012;21:105-114.
37. Naponelli V, Bettuzzi S, Clusterin. In: Barnum S, Schein T, eds. *The Complement Facts Book*. 2nd ed. Academic Press; 2018:341-349. doi:10.1016/B978-0-12-810420-0.00032-8
38. Davis AE 3rd, Mejia P, Lu F. Biological activities of C1 inhibitor. *Mol Immunol*. 2008;45:4057-4063. doi:10.1016/j.molimm.2008.06.028
39. Aulak KS, Davis AE, Donaldson VH, Harrison RA. Chymotrypsin inhibitory activity of normal C1-inhibitor and a P1 Arg to His mutant: evidence for the presence of overlapping reactive centers. *Protein Sci*. 1993;2:727-732. doi:10.1002/pro.5560020504
40. Itoh T, Hayashi Y, Kanamaru T, et al. Clinical significance of urokinase-type plasminogen activator activity in hepatocellular carcinoma. *J Gastroenterol Hepatol*. 2000;15:422-430. doi:10.1046/j.1440-1746.2000.02150.x
41. Carter JC, Church FC. Obesity and breast cancer: the roles of peroxisome proliferator-activated receptor- $\gamma$  and plasminogen activator inhibitor-1. *PPAR Res*. 2009;2009:345320. doi:10.1155/2009/345320
42. Lau D, Elezagic D, Hermes G, et al. The cartilage-specific lectin C-type lectin domain family 3 member A (CLEC3A) enhances tissue plasminogen activator-mediated plasminogen activation. *J Biol Chem*. 2018;293:203-214. doi:10.1074/jbc.M117.818930
43. Evnin LB, Vásquez JR, Craik CS. Substrate specificity of trypsin investigated by using a genetic selection. *Proc Natl Acad Sci USA*. 1990;87:6659-6663.
44. Zillmann A, Luther T, Müller I, et al. Platelet-associated tissue factor contributes to the collagen-triggered activation of blood coagulation. *Biochem Biophys Res Commun*. 2001;281:603-609. doi:10.1006/bbrc.2001.4399
45. Oikonomopoulou K, Ricklin D, Ward PA, Lambris JD. Interactions between coagulation and complement—their role in

- inflammation. *Semin Immunopathol.* 2012;34:151-165. doi:10.1007/s00281-011-0280-x
46. Markiewski MM, Nilsson B, Ekdahl KN, Mollnes TE, Lambris JD. Complement and coagulation: strangers or partners in crime? *Trends Immunol.* 2007;28:184-192. doi:10.1016/j.it.2007.02.006
47. Liu T, Zhang L, Joo D, Sun SC. NF- $\kappa$ B signaling in inflammation. *Signal Transduct Target Ther.* 2017;2:17023. doi:10.1038/sigtrans.2017.23
48. Giuliani C, Bucci I, Napolitano G. The role of the transcription factor nuclear factor-kappa b in thyroid autoimmunity and cancer. *Front Endocrinol (Lausanne).* 2018;9:471. doi:10.3389/fendo.2018.00471
49. Lamy M, Mathy-Hartert M, Deby-Dupont G. Neutrophil-induced Oxidative Stress. In: Vincent JL, ed. *Yearbook of Intensive Care and Emergency Medicine.* Springer; 1996:83-95. doi:10.1007/978-3-642-80053-5\_8
50. Salzano S, Checconi P, Hanschmann EM, et al. Linkage of inflammation and oxidative stress via release of glutathionylated peroxiredoxin-2, which acts as a danger signal. *Proc Natl Acad Sci U S A.* 2014;111:12157-12162. doi:10.1073/pnas.1401712111
51. Ye D, Liang W, Dai L, et al. Comparative and quantitative proteomic analysis of normal and degenerated human annulus fibrosus cells. *Clin Exp Pharmacol Physiol.* 2015;42:530-536. doi:10.1111/1440-1681.12386
52. Arevalo JA, Vázquez-Medina JP. The role of peroxiredoxin 6 in cell signaling. *Antioxidants (Basel).* 2018;7:172. doi:10.3390/antiox7120172
53. Strzepa A, Pritchard KA, Dittel BN. Myeloperoxidase: a new player in autoimmunity. *Cell Immunol.* 2017;317:1-8. doi:10.1016/j.cellimm.2017.05.002
54. Li S, Yang X, Feng Z, Wang P, Zhu W, Cui S. Catalase enhances viability of human chondrocytes in culture by reducing reactive oxygen species and counteracting tumor necrosis factor- $\alpha$ -induced apoptosis. *Cell Physiol Biochem.* 2018;49:2427-2442. doi:10.1159/000493841
55. Yücetaş ŞC, Çakir T. Decreased catalase expression is associated with ligamentum flavum hypertrophy due to lumbar spinal canal stenosis. *Medicine (Baltimore).* 2019;98:e15192. doi:10.1097/MD.00000000000015192
56. Feng C, Yang M, Lan M, et al. ROS: Crucial intermediators in the pathogenesis of intervertebral disc degeneration. *Oxid Med Cell Longev.* 2017;2017:5601593. doi:10.1155/2017/5601593
57. Feng C, Zhang Y, Yang M, et al. Oxygen-sensing Nox4 generates genotoxic ROS to induce premature senescence of nucleus pulposus cells through MAPK and NF- $\kappa$ B pathways. *Oxid Med Cell Longev.* 2017;2017:7426458. doi:10.1155/2017/7426458
58. Feng C, Zhang Y, Yang M, et al. The matrikine N-acetylated proline-glycine-proline induces premature senescence of nucleus pulposus cells via CXCR1-dependent ROS accumulation and DNA damage and reinforces the destructive effect of these cells on homeostasis of intervertebral discs. *Biochim Biophys Acta Mol Basis Dis.* 2017;1863:220-230. doi:10.1016/j.bbdis.2016.10.011
59. Liu CC, Kanekiyo T, Xu H, Bu G. Apolipoprotein E and Alzheimer disease: risk, mechanisms, and therapy. *Nat Rev Neurol.* 2013;9:106-118. doi:10.1038/nrneurol.2012.263
60. Beierfuß A, Dietrich H, Kremser C, et al. Knockout of Apolipoprotein E in rabbit promotes premature intervertebral disc degeneration: a new in vivo model for therapeutic approaches of spinal disc disorders. *PLoS One.* 2017;12:e0187564. doi:10.1371/journal.pone.0187564
61. Zhang D, Jin L, Reames DL, Shen FH, Shimer AL, Li X. Intervertebral disc degeneration and ectopic bone formation in apolipoprotein E knockout mice. *J Orthop Res.* 2013;31:210-217. doi:10.1002/jor.22216
62. Donovan M, Saeed AM, Hyska M, Marin-Castano M, Lee WH. Oxidative stress modulates glutathione S-transferase Pi isoform (GSTP1) expression in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 2014;55:6319-6319.
63. Wang T, Arifoglu P, Ronai Z, Tew KD. Glutathione S-transferase P1-1 (GSTP1-1) inhibits c-Jun N-terminal kinase (JNK1) signaling through interaction with the C terminus. *J Biol Chem.* 2001;276:20999-21003. doi:10.1074/jbc.M101355200
64. Holley SL, Fryer AA, Haycock JW, Grubb SEW, Strange RC, Hoban PR. Differential effects of glutathione S-transferase p1 (GSTP1) haplotypes on cell proliferation and apoptosis. *Carcinogenesis.* 2007;28:2268-2273. doi:10.1093/carcin/bgm135
65. Mohammad J, Singh RR, Riggle C, Haugrud B, Abdalla MY, Reindl KM. JNK inhibition blocks piperlongumine-induced cell death and transcriptional activation of heme oxygenase-1 in pancreatic cancer cells. *Apoptosis.* 2019;24:730-744. doi:10.1007/s10495-019-01553-9
66. Pizzino G, Irrera N, Cucinotta M, et al. Oxidative stress: harms and benefits for human health. *Oxid Med Cell Longev.* 2017;2017:8416763. doi:10.1155/2017/8416763
67. Meir AR, Fairbank JC, Jones DA, McNally DS, Urban JP. High pressures and asymmetrical stresses in the scoliotic disc in the absence of muscle loading. *Scoliosis.* 2007;2:4. doi:10.1186/1748-7161-2-4
68. Brown S, Melrose J, Caterson B, Roughley P, Eisenstein SM, Roberts S. A comparative evaluation of the small leucine-rich proteoglycans of pathological human intervertebral discs. *Eur Spine J.* 2012;21(suppl 2):S154-S159. doi:10.1007/s00586-012-2179-1
69. Rajasekaran S, Vidyadhara S, Subbiah M, et al. ISSLS prize winner: a study of effects of in vivo mechanical forces on human lumbar discs with scoliotic disc as a biological model: results from serial postcontrast diffusion studies, histopathology and biochemical analysis of twenty-one human lumbar scoliotic discs. *Spine (Phila PA 1976).* 2010;35:1930-1943. doi:10.1097/BRS.0b013e3181e9a156
70. Hristova GI, Jarzem P, Ouellet JA, et al. Calcification in human intervertebral disc degeneration and scoliosis. *J Orthop Res.* 2011;29:1888-1895. doi:10.1002/jor.21456
71. Sanchez C, Mazzucchelli G, Lambert C, Comblain F, DePauw E, Henrotin Y. Comparison of secretome from osteoblasts derived from sclerotic versus non-sclerotic subchondral bone in OA: a pilot study. *PLoS One.* 2018;13:e0194591. doi:10.1371/journal.pone.0194591