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2021 Outstanding Paper Award Winner

Modic changes are associated with activation of intense inflammatory and host defense response pathways – molecular insights from proteomic analysis of human intervertebral discs

S Rajasekaran, PhD[a,*], Dilip Chand Raja Soundararajan, S FNB (Spine)[a], Sharon Miracle Nayagam, MSc[b], Chitraa Tangavel, PhD[b], M Raveendran, PhD[c], Pushpa Bhari Thippeswamy, FRCR[d], Niek Djuric[e], Sri Vijay Anand, K S MS[a], Ajoy Prasad Shetty, MS[a], Rishi Mugesh Kanna, FNB (Spine)[a]

[a] *Department of Spine Surgery, Ganga Hospital, 313, Mettupalayam road, Coimbatore, India*
[b] *Ganga Research Centre, No 91, Mettupalayam road, Coimbatore 641030, India*
[c] *Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore 641003 India*
[d] *Department of Radiology, Ganga Hospital, 313, Mettupalayam road, Coimbatore, India*
[e] *Department of Neurosurgery, Leiden University Medical Center, Albinusdreef 2, 2300 RC Leiden, the Netherlands*

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ABSTRACT

BACKGROUND CONTEXT: Patients with modic changes (MC) form a distinct clinical subset with reports of higher intensity of pain, poor clinical and surgical outcomes and higher incidence of recurrence. MC also is an independent risk factor for increased post-operative surgical site infection.

PURPOSE: This study aimed *to investigate the biological changes at molecular level, in discs with MCs*. We also aim to identify biological biomarkers and potential targets for molecular therapy.

STUDY DESIGN: Experimental analysis

MATERIALS AND METHODS: Nucleus pulposus (NP) from 24 patients undergoing microdiscectomy for disc herniation [14 discs with MC and 10 without modic changes (NMC)] were procured. The overall expression of proteins, biological processes, protein-protein and metabolite interactions were analysed and compared. *Host defense response proteins (HDRPs) and immunological pathways activated in patients with MC were documented and analysed.*

RESULTS: Label-free proteomic approach with stringent filters revealed a total of 208 proteins in MC and 193 in NMC groups. 45 proteins were specific to MC; 30 to NMC and 163 common to both. Downregulated proteins in MC belonged to components of extracellular matrix such as collagens (COL- 6A1, 6A2, 6A3, 11A1, 12A1, and 20A1), and proteoglycans (versican (VCAN), and biglycan (BGN)). Inflammatory molecules [plasminogen (PLG), angiogenin (ANG), fibroblast growth factor-binding protein 2 (FGFBP2), tetranectin (CLEC3B), cartilage acidic protein 1 (CRTAC1), kininogen (KNG-1), chitinase-3-like protein 2 (CHI3L2), and ferritin (FTL) were expressed only in the MC group. The significantly altered pathways in MC included Fc Fragment of IgG Receptor IIIa (FCGR3A)-mediated phagocytosis, regulation of Toll-like receptors (TLR) by endogenous ligand, neutrophil and platelet degranulation.

50 HDRPs were identified in the study, 14 of which were specific to MC and included acute phase reactants, antimicrobial peptides, complement cascade proteins, inflammatory molecule and

FDA. Device/drug status: Not applicable

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*Corresponding author. Department of Orthopaedics and Spine Surgery, Ganga Hospital, 313, Mettupalayam road, Coimbatore, India. Tel.: +(91 98-43022325; fax: +(91) 42-24383863.

E-mail address: rajasekaran.orth@gmail.com (S. Rajasekaran).

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stress response proteins. Metabolite-protein interaction analysis revealed a significant interaction between 19 proteins, specifically involving ubiquitin mediating proteasome degradative pathway and an association with the metabolite-glutamic acid in the MC group. Accumulation of glutamic acid in MC discs was confirmed by quantitative amino acid analysis using High-performance liquid chromatography.

CONCLUSION: *Our study confirms that MC represents an intense inflammatory status and activation of host defense response and immunological pathways*. Downstream effects leading to ubiquitin mediated proteasomal degradation of ECM proteins and the resulting metabolites such as glutamic acid *could cause excessive pain and needs further investigation.*

CLINICAL SIGNIFICANCE: We have documented the expression of inflammatory molecules, immune mechanisms and host defense response proteins which throw molecular insights into the pathological mechanisms of MC. Further, ubiquitin mediated proteasomal degradation and accumulation of glutamate in discs with MC might serve as targets for molecular therapy. © 2021 Elsevier Inc. All rights reserved.

Keywords: Bacterial infection; Host defense response; Intervertebral disc degeneration; Low Back Pain; Modic changes; Proteomics

Introduction

There is mounting evidence that clinical and functional outcomes of patients with chronic low back pain (LBP) are inferior in those with MC compared to patients without MC in both the lumbar and cervical spine [4–6]. MCs have been variably correlated to aging, smoking, mechanical trauma, inflammation, degeneration, genetic single nucleotide polymorphisms (SNPs), and infection [1–3], but the exact pathophysiological mechanisms underlying these changes still remain elusive. To date, no study has investigated why such a difference occurs despite identical treatment modalities but this knowledge is critical to overcome the poor clinical results and surgical outcomes in lumbar disc disease.

We have performed a comparative proteomic analysis of intervertebral discs with and without MC in the current study. Further, we have investigated the underlying molecular mechanisms to identify candidate biomarkers and molecular targets which may improve outcomes in patients with MC.

Materials and methodology

This study was approved by Institutional Review Board and was conducted according to the guidelines and ethical norms laid by Indian Council of Medical Research (ICMR). After obtaining informed consent, nucleus pulposus (NP) from 24 patients undergoing microdiscectomy for disc herniation were procured. The demographic details and grades of degeneration are mentioned in (**Table 1**). Tissue samples were retrieved under aseptic conditions and immediately snap-frozen at -196˚C using liquid nitrogen and stored for further proteomic analysis. Frozen samples were subjected to in-gel based label-free mass spectrometric analysis, as reported earlier. In order to unravel molecular mechanisms in patients with modic changes, a comparative proteomic analysis was performed between 14 discs with MC and 10 without modic changes (NMC).

Cryopreserved tissues were thawed on ice, aliquoted and subjected to in-gel based tryptic digestion as described earlier in our reports [7–9]. Purified tryptic peptides were then subjected to label-free mass spectrometric analysis and the output (.raw/.msf) files were subjected to identification of total proteins using Proteome Discoverer vs 1.4 with in-built SequestHT and Mascot search algorithms. The spectral counts of proteins were relatively quantified by normalized spectral abundance factor (NSAF) method [10]. Stringent filter (≥5 PSM and 30% sample positivity) were applied for further analysis. To understand the biological process involved in pathogenesis, pathway enrichment analysis was performed using Reactome database v.3.7 followed by comparison using Funrich, functional enrichment annotation tool with customized database ‘Reactome’ and their statistical significance was determined by Bonferroni test.

To unveil metabolic regulations, metabolite-protein interaction (MPI) network analysis was made using STITCH (Search Tool for Interactions of Chemicals) vs 5.0 [11]. MPI analysis of specific proteins were done by integrating predictions from active sources ‘expression’ ‘databases’ with confident network edges. The interaction scores were imported into cytoscape vs 3.8.3 with installed ANIMO (Analysis of Networks with Interactive Modeling) plugin for analysis of incoming/ outgoing signals. All the analysis were corrected using Bonferroni test for assessing their significance and further validated using quantitative amino acid analysis, for which around 200mg of intervertebral disc NP tissues were weighed, pulverized using liquid nitrogen and suspended in 1ml of sterile double deionized water. Subsequently, the mixture was subjected to incubation for 1 hour at room temperature with continuous mixing, followed by centrifugation at RT @10,000 x g, for 15 min to remove interfering aggregates. To deproteinize the samples, 2% acetonitrile (v/v) was added prior to quantitative analysis of extracted amino acids using Shimadzu UHPLC N-Series with RF-20A. Following a brief spin, 2μl

Table 1
Demographic and clinical phenotypes of study population considered for this study

Study group	Age	Sex	Levels	MODIC changes	Pfirmann grade	Mean age of the subjects ± SD
MC	21	M	L5S1	2	4	35 ± 11.04
MC	29	F	L5S1	2	3	
MC	31	M	L5S1	2	3	
MC	32	F	L4L5	2	4	
MC	32	M	L4L4	2	4	
MC	34	F	L4L5	1	4	
MC	37	M	L3L4	2	4	
MC	37	M	L4L5	2	4	
MC	38	F	L4L5	2	4	
MC	38	M	L4L5	2	4	
MC	40	F	L4L5	1	4	
MC	43	M	L5S1	2	4	
MC	43	F	L4L5	2	4	
MC	34	M	L4L5	2	4	
NMC	15	M	L4L5		2	36 ± 19.44
NMC	16	F	L5S1		4	
NMC	26	F	L4L5		4	
NMC	26	M	L5S1		4	
NMC	27	M	L4L5		3	
NMC	28	M	L5S1		4	
NMC	40	M	L4L5		3	
NMC	45	M	L4L5		3	
NMC	67	F	L5S1		3	
NMC	70	F	L4L5		5	

++MC- Modic Changes; NMC- Non-Modic Changes.

of deproteinized sample extracts were loaded onto HPLC column (Phenomenex Gemini 5 μm NX-C-18 110 Å (250 mm X 4.6 mm ID) (IICMS/LCC-266). Amino acids were eluted with an increasing gradient of 25mM dipotassium hydrogen phosphate anhydrous in sodium azide (A) and methanol: acetonitrile: water (40:45:15) (B) solvents. A constant flow of 1.0 mL/min was provided to separate amino acids through gradient elution.

To identify specific host defense mechanisms, a comprehensive list of 263 well established host defense response proteins (HDRP) (*Supplementary-Table-1*) was built and their expression were compared between MC and NMC groups. To visualize the concentration of HDRPs between conditions, supervised hierarchical clustering was made by using complete linkage method and distance metrics was calculated by Euclidean distances with the help of R-packages. GOnet (https://tools.dice-database.org/GOnet/) with its human ontology version 2019/07/01 was used to analyze enriched biological processes.

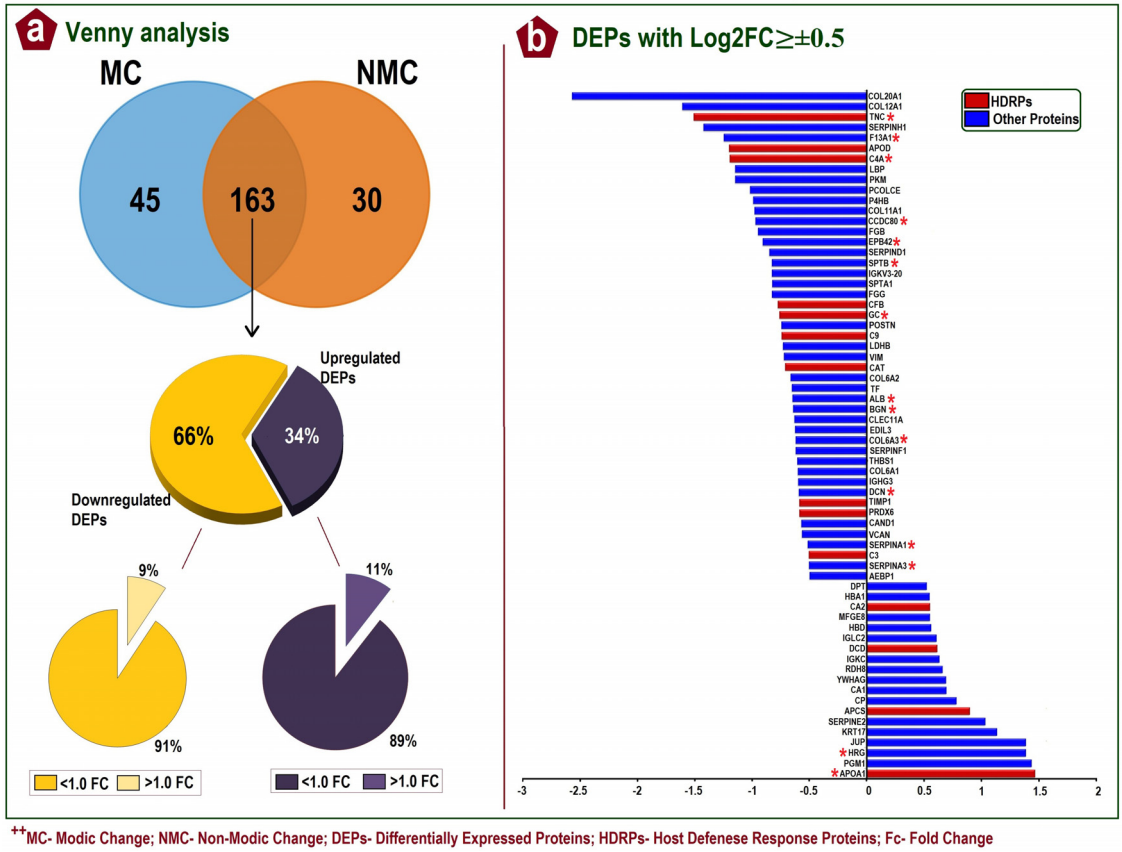
Statistical difference between conditions was analyzed using SPSS software vs.25.0 with the help of t-tests/ Mann-Whitney U tests (in the case of normality violation) and two-tailed alpha was set at 0.05 for all the tests.

Results

Label-free proteomic approach revealed a total of: 585 proteins in MC and 536 proteins in NMC group respectively. By applying a stringent filter on peptide spectral matches (PSMs) (≥5) and sample positivity (≥30%); this list narrowed to 208 proteins in MC and 193 in NMC groups respectively. Draw Venn tool, was used to depict a comparative proteomic analysis (http://bioinformatics.psb.ugent.be/webtools/Venn/) which showed 45 proteins specific to MC; 30 specific to NMC and 163 common to both as shown in Fig. 1A. Among 163 differentially expressed proteins (*Supplementary-Table-2*), 66 proteins were found to have a log2FC±0.5 variation with 14 proteins showing statistically significant differences ($p < .05$) as shown in Fig. 1B.

Differentially expressed proteins

Among the 66 differentially expressed proteins expressed with log2FC ≥±0.5, 19 were upregulated and 47 were downregulated in MC group (Fig. 1B). Acute phase reactants produced in response to trauma, or infection such as apolipoprotein A1 (APOA1), serum amyloid P-component (APCS), and ceruloplasmin (CP) were upregulated in MC. Immune system responses to antigenic exposure viz dermcidin (DCD) representing innate immunity and immunoglobulins [IGKC - immunoglobulin kappa constant (IGKC) and immunoglobulin Lambda Constant 2 (IGLC2)] representing adaptive immunity were upregulated in MC. Cytoprotective proteins in response to inflammation such as carbonic anhydrase (CA2), milk fat globule-epidermal growth factor 8 (MFGE8), serpin peptidase inhibitor clade E member 2 (SERPINE2) were again upregulated in MC.



++MC- Modic Change; NMC- Non-Modic Change; DEPs- Differentially Expressed Proteins; HDRPs- Host Defenese Response Proteins; Fc- Fold Change

Fig. 1. **Comparative Proteomics**- **(A)** Venn diagram representing total number of unique and common proteins between MC and NMC discs. About 163 were present in both the conditions with varying abundances. When compared with NMC, out of 163 proteins, 56 proteins (34%) were upregulated in MC and 107 proteins (66%) were found to be downregulated in MC. Pie chart depicts the contribution of differentially expressed proteins (DEPs) between fold changes (>1.0 and <1.0) in both up and downregulation. **(B)** Bar chart showing 66 differentially expressed proteins (DEPs) with log2FC ≥±0.5 (Up and Downregulation) in MC when compared to NMC considered in this study. *indicates statistical significance ($p<0.05$), using t-test/ MW-U test (in case of normality violation) using SPSS vs 25.0.

Most of the downregulated proteins in MC belonged to components of extracellular matrix such as collagens (COL- 6A1, 6A2, 6A3, 11A1, 12A1, and 20A1), proteoglycans (versican (VCAN), and biglycan (BGN)). Proteins essential to control infection [tenascin C (TNC), lipopolysaccharide binding protein (LBP)] and inflammation [serine protease inhibitors (SERPIN − A1, A3, D1, H1 and F1), vimentin (VIM), and catalase (CAT)] were downregulated in MC. Central component of the complement system (C3) and a protein of the terminal membrane attack complex (C9) were also downregulated.

Proteins specifically expressed in MC

Around 75 proteins were expressed specifically in either of MC/NMC group. Out of these 75 proteins 45 were specific to MC group and 30 proteins to NMC group respectively. Interestingly out of 45 MC specific proteins 14 mapped under host defense response mechanisms (Table 2) in contrast to only two proteins mapped in NMC under this category. The remaining 31 MC specific proteins include mainly immunoglobulins [IGHV3-7, IGLC1, IGKV3-15, IGKV3D-11, IGKV4-1, IGKV2-40, IGHV3OR16-13), metabolic enzymes [adenylate kinase isoenzyme 1 (AK1), glyceraldehyde-3-phosphate dehydrogenase, testis-specific (GAPDHS), ribonuclease 4 (RNASE4), flavin reductase (NADPH) (BLVRB)], nucleosome components [Histone H2A type 1-H (HIST1H2AH), histone H4 (HIST1H4A and purine nucleoside phosphorylase (PNP)], and inflammatory proteins [plasminogen (PLG), angiogenin (ANG), fibroblast growth factor-binding protein 2 (FGFBP2), tetranectin (CLEC3B), cartilage acidic protein 1(CRTAC1), kininogen (KNG-1), chitinase-3-like protein 2 (CHI3L2), ferritin (FTL).

Pathway enrichment analysis to depict its biological role

To understand the significant biological basis underlying MC and NMC, total proteins of MC- 208; NMC- 194 were included as input for the pathway enrichment analysis using Reactome database web browser vs 3.7 and the significantly altered pathways were ranked according to their p-values as shown in (Supplementary-Table-3 and Fig. 2**).** The significantly altered pathways in MC condition include FCGR3A-

Table 2
Clinical Implications of specific proteins of MC and NMC conditions

S. No	Gene Symbol	UNIPROT Protein name	Specific to	HDRP	Clinical implication
1	SOD3	Extracellular superoxide dismutase [Cu-Zn]	MC	Yes	Downregulate MAPK signalling pathway and NF-κB transcription factors thereby controlling inflammatory responses.
2	LGALS8	Galectin	MC	Yes	Potent immune suppressor reported in CSF; one of its isoform GAL-8M is produced in response to bacterial LPS stimulus and returns to normal once LPS is removed.
3	TNFAIP6	Tumor necrosis factor-inducible gene 6 protein	MC	Yes	Known to produce inflammatory effect in animal models of arthritis, cerebral and myocardial infarction.
4	C1S	Complement C1s subcomponent	MC	Yes	Pathogen clearance by classical pathway triggered via binding of pattern recognition molecule C1 complex (consisting of C1Q that has C1R and C1S proteases) to immunoglobin patches on the target pathogen.
5	UBB	Polyubiquitin-B	MC	Yes	Part of UPS complex and involved in degradation and clearance of misfolded proteins
6	CLEC3A	C-type lectin domain family 3 member A	MC	Yes	In vitro studies provide evidence of antimicrobial activity especially with peptides derived from CLEC3A towards septic arthritis.
7	S100A1	Protein S100-A1	MC	Yes	Released when there is inflammation or cellular stress. Regulation of P13/AKT signalling pathway reported in neuronal cells; reported as a pro-inflammatory molecule in Alzheimer disease.
8	CHI3L1	Chitinase-3-like protein 1	MC	Yes	Induced expression observed in inflammatory diseases and certain type of cancers.
9	TIMP3	Metalloproteinase inhibitor 3	MC	Yes	Physiological regulator of inflammation and controls metalloproteases involved in ECM turnover.
10	C1QB	Complement C1q subcomponent subunit B (Fragment)	MC	Yes	Part of C1Q molecule, involved in clearing the apoptotic debris. Upon binding to the apoptotic cells it supresses the dendritic and macrophages that mediate cellular proliferation.
11	APOA2	Apolipoprotein A-II	MC	Yes	Reported to maintain host responses to LPS by suppressing the inhibitory activity of LPS binding protein.
12	C8B	Complement component 8, beta polypeptide, isoform CRA_b	MC	Yes	Part of membrane attack complex (MAC) expressed as a result of pro-inflammatory trigger.
13	PLA2G2A	Synovial phospholipase-A2	MC	Yes	Increased secretion during inflammation and promotes wnt signalling. Present in abundance in biological fluids with inflammatory diseases (arthritis, sepsis and myocardial infarctions).
14	APOH	Beta-2-glycoprotein 1	MC	Yes	Multifunctional glycoprotein involved in transport of lipids into the circulatory system; binds to lipid moiety of bacteria as a host defence protein against bacterial infections; Inflammatory protein in systemic lupus erythematosus.
15	AK1	Adenylate kinase isoenzyme 1	MC	No	Key enzyme functions as immune modulator; assess the risk of pathogenesis due to oxidative stress such as neurodegenerative and metabolic disorders.
16	GAPDHS	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	MC	No	Malonylation of GAPDH which in turn promotes TNFα transcription leading to inflammation.

Table 2 (***Continued***)

S. No	Gene Symbol	UNIPROT Protein name	Specific to	HDRP	Clinical implication
17	HIST1H2AH	Histone H2A type 1-H	MC	No	Accumulation of histone proteins signals promotion of senescent cells leading to chronic inflammation.
18	PLG	Plasminogen	MC	No	Key molecules involved in regulation of macrophage polarization and phagocytosis of apoptotic cells to resolve inflammation along with its receptor.
19	MYCLP1	Putative myc-like protein MYCL1P1	MC	No	Overexpressed in many type of cancer cells, widely known as activators of tumorigenesis
20	IGHV3OR16-13	Protein IGHV3	MC	No	Involved in positive regulation of B-cell activation
21	ANG	Angiogenin	MC	No	Stress activated protein upregulated in human ocular diseases such as DR, AMD, RP and uveitis.
22	FGFBP2	Fibroblast growth factor-binding protein 2	MC	No	Secreted by cytotoxic lymphocytes and reported as a potential biomarker for Acute Myocardial Infarction.
23	IGHV3-7	Immunoglobulin heavy variable 3-7	MC	No	Involved in positive regulation of B-cell activation
24	IGLC1	Immunoglobulin lambda constant 1	MC	No	Involved in RET signalling helps in axon guidance
25	STOM	Erythrocyte band 7 integral membrane protein	MC	No	Pivotal in stabilisation of mature RBC and as well clear the damaged protein by vesiculation.
26	HIST1H4A	Histone H4	MC	No	Its presence induces neutrophil activation and inflammatory responses; hydrogen peroxide production; cell adhesion; IL-8 generation and degranulation.
27	KRT33B	Keratin, type I cuticular Ha3-II	MC	No	Deamidated protein, being explored for its functionality in hair diseases.
28	PCOLCE2	Procollagen C-endopeptidase enhancer 2	MC	No	Glycoprotein present in ECM; In mice, it's an important component of HDL system involved in reverse cholesterol transport where the cholesterol is returned to liver for excretion and is classified as atheroprotective.
29	IGKV3-15	Immunoglobulin kappa variable 3-15	MC	No	Participates in antigen recognition of humoral immunity
30	POTEE	POTE ankyrin domain family member E	MC	No	Anti-inflammatory molecule negatively regulates the stress response by attenuating NF-KB signals; also suppresses vascular injury in-vivo. In contrary upregulation in CRC cells has been used as novel biomarker for diagnosis.
31	IGKV3D-11	Immunoglobulin kappa variable 3D-11	MC	No	Participates in antigen recognition of humoral immunity
32	IGKV4-1	Ig kappa chain V-IV region Len	MC	No	Participates in adaptive immunity
33	ANK1	Isoform Er16 of Ankyrin-1	MC	No	Highly abundant in the immune microglial cells which are the key regulators in Alzheimer's disease.
34	IGKV2-40	Immunoglobulin kappa variable 2-40	MC	No	Participates in adaptive immunity
35	QSOX1	Sulfhydryl oxidase 1	MC	No	Reported as tissue derived biomarker that promotes lung cancer.
36	CLEC3B	C-type lectin domain family protein 3/ Tetranectin	MC	No	Reportedly, increased expression is positively correlated with the fibrosis in ischemic heart disease which in contrary with the serum concentration levels.
37	CRTAC1	Cartilage acidic protein 1	MC	No	ECM protein capable of forming amyloid-like structures associated in disease milieu.

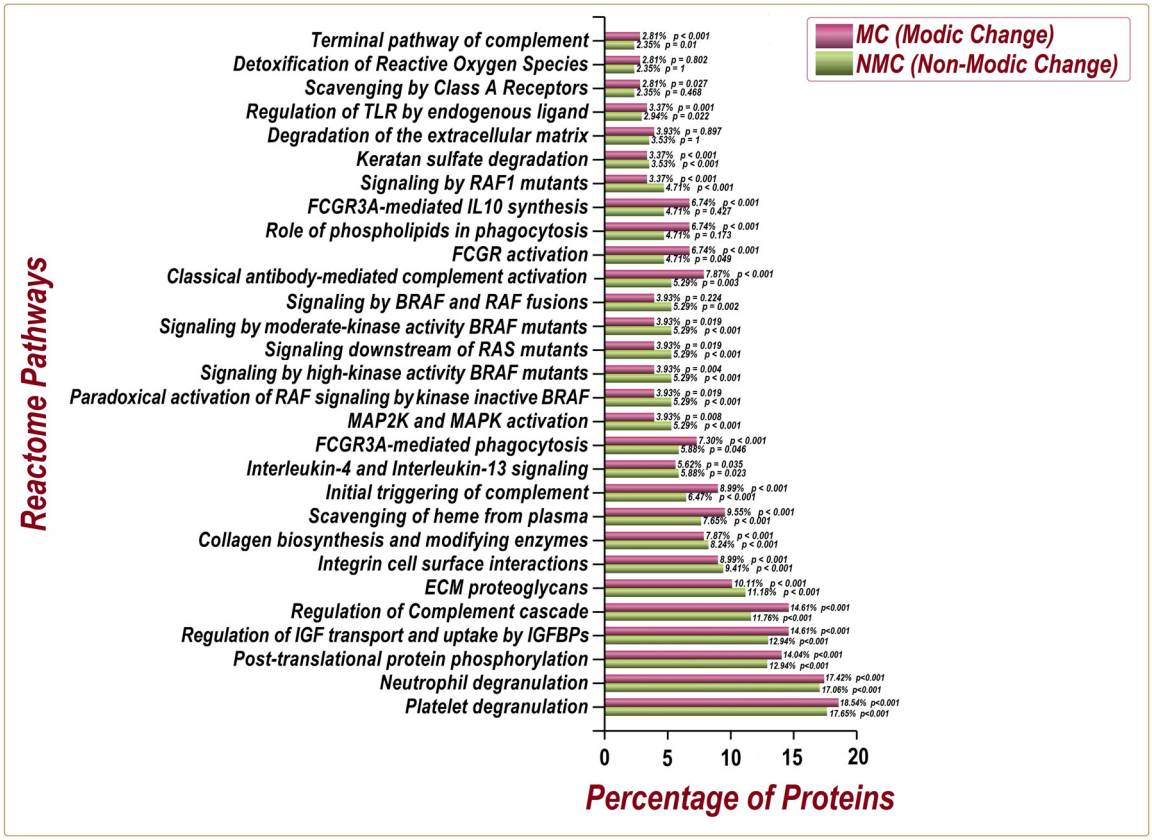
Table 2 (***Continued***)

S. No	Gene Symbol	UNIPROT Protein name	Specific to	HDRP	Clinical implication
38	COL1A1	Collagen alpha-1(I) chain	MC	No	Main structural protein of the ECM in musculoskeletal tissues. Known as protease resistant; associated fibrotic and connective tissue pathology; age-related diseases.
39	RNASE4	Ribonuclease 4	MC	No	Known as immune modulators. Possess antimicrobial activity that is secreted upon injury targeting damaged cells to be cleared from the inflammatory site.
40	BLVRB	Flavin reductase (NADPH)	MC	No	Part of redox cycle, bilirubin converted into biliverdin through ROS which could be a promising therapy for oxidative-stress mediated diseases
41	KNG1	Kininogen-1	MC	No	During *S.pyogenes* infection, KNG1 mediates inflammatory response mechanism
42	PNP	Purine nucleoside phosphorylase	MC	No	Deficient levels causes lymphopenia in humans
43	HBA2	Hemoglobin A2	MC	No	Higher levels lessens the severity of multiple sclerosis
44	CHI3L2	Chitinase-3-like protein 2	MC	No	Stress response protein in IVDD
45	FTL	Ferritin	MC	No	Vital inflammatory marker, as it arises from damaged cells
46	PON1	Serum paraoxonase/ arylesterase 1	NMC	No	Low levels are observed during oxidative stress which are associated with severity of the IVD disease
47	IQGAP1	IQGAP1 protein	NMC	No	Crucial for MAPK-driven microbial invasion
48	COL5A1	Collagen type V, alpha 1	NMC	No	Higher expression levels were observed in tumour cells
49	HSPA5	Endoplasmic reticulum chaperone BiP	NMC	Yes	Part of neuroinflammation and tumour cells produce high levels of HSPA5
50	ACTN1	Alpha-actinin-1	NMC	No	Significantly increased levels were observed in synovial tissues of RA
51	ANXA6	Annexin A6	NMC	No	Acts either as tumour suppressor or promoter based on the malignancy of cancer
52	C1R	Complement C1r subcomponent	NMC	Yes	Absence of C1R resulting in resolving inflammation caused due to clearance of apoptotic cells
53	MYH9	Myosin-9	NMC	No	Mediates TLR in platelets under the influence of Cγ-calpain-myosin 9-Rab7b axis
54	ATP5B	ATP synthase subunit beta, mitochondrial	NMC	No	Involved in electron transport process of respiratory chain
55	FBLN1	Fibulin-1	NMC	No	Simultaneous expression of ADAMTS-1 and FBLN1 induces antitumoral effect in breast cancers
56	MDH2	Malate dehydrogenase	NMC	No	Key protein in central oxidative pathway
57	GPI	Glucose-6-phosphate isomerase	NMC	No	Increased levels were observed in hypoxia-induced angiogenesis in RA
58	COL14A1	Collagen alpha-1(XIV) chain	NMC	No	Found expressed in young intervertebral discs known as regulator of fibrillogenesis.
59	XIRP2	Xin actin-binding repeat-containing protein 2	NMC	No	Xin upregulation is significantly and positively correlated with severity of muscle damage
60	MSN	Moesin	NMC	No	Transduces all LPS-induced signals by blocking monocytes response to LPS
61	ATP5F1B	ATP synthase subunit beta, mitochondrial	NMC	No	Involved in electron transport process of respiratory chain
62	CALR	Calreticulin	NMC	No	Inhibits LPS-induced inflammatory osteoclastogenesis in murine cells

Table 2 (***Continued***)

S. No	Gene Symbol	UNIPROT Protein name	Specific to	HDRP	Clinical implication
63	ACTC1	Actin, alpha cardiac muscle 1	NMC	No	Polymorphisms lead to chronic inflammatory cardiomyopathy
64	VCP	VCP protein	NMC	No	Helps in formation of tER
65	LCP1	Plastin-2	NMC	No	Actin-binding protein. Plastin-deficient PMN lacks killing bacterial pathogens.
66	FLNA	Filamin-A	NMC	No	Increased expression of FLNA in advanced atherosclerotic plaques of human carotid arteries
67	HIST2H2AC	Histone H2A type 2-C	NMC	No	H2A, an important component of NETs possessing antimicrobial activity
68	DYNC1LI1	Cytoplasmic dynein 1 light intermediate chain 1	NMC	No	Adaptor protein that regulate dynein function
69	BMI1	Polycomb complex protein BMI-1	NMC	No	Cell differentiation and proliferation
70	NOA1	Nitric oxide-associated protein 1	NMC	No	Known for disc abnormalities. Causes oxidative stress in age-related diseases
71	MIF4GD	MIF4G domain-containing protein	NMC	No	Potential regulator of p27-dependent cell proliferation in HCC
72	TNRC6B	Trinucleotide repeat-containing gene 6B protein	NMC	No	Involved in RNA-mediated gene silencing
73	YWHAB	14-3-3 protein beta/alpha	NMC	No	Adapter protein involved in many signalling cascades
74	ASPN	Asporin	NMC	No	Binds with TGF-beta and BMP-2 and negatively regulates their activity; potential drug for DDD
75	ATP5F1A	ATP synthase subunit alpha, mitochondrial	NMC	No	Natural drug target for antimicrobial/ antitumor peptides

++MAPK- Map Kinase; NF-κB- Nuclear factor kappa B; CSF- Cerebro Spinal Fluid; LPS- Lipopolysaccharide; UPS- Ubiquitin/ Proteasome system; P13/AKT- Intracellular signalling pathway; ECM- Extracellular Matrix; TNF- Tumour Necrosis Factor; DR- Diabetic Retinopathy; AMD- Age-Related Macular Degeneration; RP- Retinitis Pigmentosa; RET-; RBC-; IL-8- Interleukin 8; HDL- High Density Lipoprotein; CRC- Colorectal Cancer; IVDD- Intervertebral Disc Disease; RA- Rheumatoid Arthritis; tER- transitional endoplasmic reticulum; BMP- Bone Morphogenetic Protein; DDD- Degenerative Disc Disease.



++TLR- Toll-like Receptors; RAF- Rapidly Accelerated Fibrosarcoma; FCGR3A- Fc gamma Receptor III-A; IL-10- Interleukin 10; FCGR- Fc gamma Receptor; BRAF- B-RAF; RAS- Rat Sarcoma; MAP2K- Mitogen-activated Protein Kinase Kinase; MAPK- Mitogen-activated Protein Kinase; ECM- Extracellular Matrix; IGF- Insulin-like Growth Factor 1; IGFBPs- Insulin-like Growth Factor-binding Protein; p- probability value.

Fig. 2. Comparative pathway enrichment analysis of total proteins using Reactome database vs 3.7 of the total proteins across both the conditions- MC and NMC**.** Significantly enriched pathways with p-value <0.05 are compared between the conditions and the illustrative image was created using FUNRICH, functional enrichment tool vs. 3.1.3 (http://www.funrich.org/). The p-value is calculated by the intersection of input genes against the background sets/ genes found in the database or in the pathways based on predicted molecular evidences using a Fisher's exact test and multiple test correction is applied to all annotated genes used as default. Interestingly, infection-mediated/immune-influenced pathways such as FCGR3A- mediated phagocytosis (role in host-defence mechanisms the uptake and destruction of infectious pathogens); scavenging of heme from plasma (clearing of free heme released by erythrocytes during infection associated with intravascular hemolysis); regulation of TLR by endogenous ligand (active upon tissue damage during infectious and inflammatory mechanisms) and immune responsive pathways- initial triggering of complement; regulation of complement cascade; neutrophil and platelet degranulation were enriched in MC. Whereas, discs with NMC showed pathways that mediate chronic inflammation: Signalling by BRAF and RAF fusions; Signalling by moderate kinase activity BRAF mutants; Signalling downstream of RAS mutants; Signalling by high-kinase activity BRAF mutants; paradoxical activation of RAF-signalling by kinase-inactive BRAF, MAP2K and MAPK.

mediated phagocytosis (role in host-defence mechanisms the uptake and destruction of infectious pathogens) (**Fig. 3**); FCGR3A-mediated IL10 synthesis; scavenging of heme from plasma; regulation of TLR by endogenous ligand; role of phospholipids in phagocytosis and immune responsive pathways - initial triggering of complement; regulation of complement cascade; neutrophil and platelet degranulation. Specific pathways of MC are enlisted under **Table 3**. In contrast discs with NMC showed enrichment of other pathways: transduce extracellular signals mediating inflammation such as signaling by BRAF and RAF fusions; signaling by moderate kinase activity BRAF mutants; signaling downstream of RAS mutants; signaling by high-kinase activity BRAF mutants, paradoxical activation of RAF-signaling by kinase-inactive BRAF, MAP2K and MAPK activation.

Interactive network analysis of specific proteins of MC and NMC

To understand the mechanism of functional modulators, protein-metabolite interactions were studied using Search Tool for Interactions of Chemicals (STITCH) database vs 5.0 for specific proteins in discs with MC and NMC and incoming / outgoing signals were predicted using Cytoscape vs. 3.8.3 with installed ANIMO plugin using UPPAAL running in the background.

In MC, out of 45 specific proteins subjected to interactions, 19 were found to have associations with a clustering coefficient of 0.848 (Fig. 4A). Strong associations were found between POTE ankyrin domain family member E (POTEE), hemoglobin subunit beta (HBB), spectrin beta, erythrocytic (SPTB), histone (HIST1H2AH),

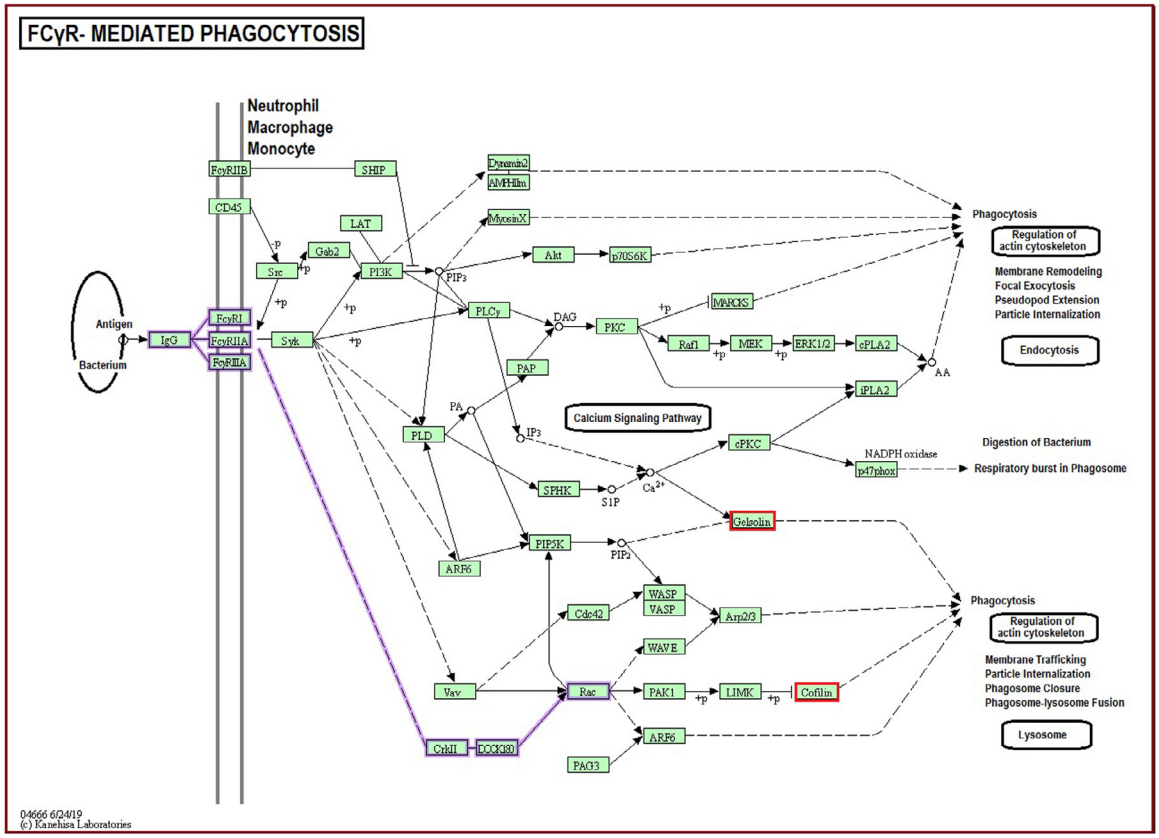


Fig. 3. KEGG pathview representing the significantly enriched pathway FCGR3A-mediated phagocytosis (p-value, 0.001) in Modic discs (MC). Phagocytosis helps in clearance of invading foreign particles where Fc-gamma receptors recognize IgG-coated targets- opsonized pathogens or other circulating invaders with their varying IgG affinity and intracellular trafficking. Recognized particles are processed and later diffused by reactive oxygen species. Highlighted proteins are present in our study (red colour). Violet colour represents cytoskeletal regulation mediated by viruses and bacteria in the host.

galectin (LGALS8), ubiquitin (UBB and UBC), stomatin (STOM), quiescin Q6 sulfhydryl oxidase 1 (QSOX1), and C1 complex. The proteins UBC, UBB, immunoglobulin lambda-like polypeptide 5 precursor (IGLL5), 26S proteasome non-ATPase regulatory subunit (PSMD4), proteasome 26S subunit, non-ATPase (PSMD5) and kininogen-1 (KNG1) were found to be associated with glutamic acid. However, higher number of proteins was found interacting with ubiquitin suggestive of its role in proteasome degradative pathway leading to the degradation of major ECM protein in the disc such as aggrecan and an association with glutamic acid.

In NMC, only 15 proteins had interactions with clustering coefficient 0.801(Fig. 4B). Stronger associations were found between heat shock proteins- (HSP90B1-heat shock protein 90 B1, HSPA5- heat shock protein A5); CALR- calreticulin, actin molecules and ATP transporters- (ATP5A1, ATP5AB, ATP5C1, ATP5D) which all in-turn activating/triggering RAF1 (RAF proto-oncogene serine/threonine-protein kinase, an important member of inflammatory signals).

These results were validated by quantitative amino acid analysis of 18 different proteinogenic and non-proteinogenic amino acids using Shimadzu UHPLC N-Series with RF-20A. Except isoleucine (below detection limit), all other amino acids showed a good separation profiles in representative sample of MC condition (Fig. 5A**).** The results demonstrated that valine was the predominant amino acid in MC condition with leucine in NMC. Majority of amino acids were found elevated in MC condition with the exception of glycine, alanine, phenylalanine, and lysine being higher in NMC condition (Fig. 5B). Glutamic acid, a main excitatory neurotransmitter associated with the sensation of pain was found in higher concentration (4.471 ppm) in MC group, when compared to 2.446 ppm in NMC group.

Profiling of host defence response proteins

Categorization of proteins based on their functions identified 50 host defense response proteins (HDRPs) across discs with MC and NMC. The relative abundance transformed into Z-score for each protein is represented in a heat map as shown in Fig. 6A. Based on supervised hierarchal clustering analysis, the proteins were clustered using complete linkage method and the distances were measured by Euclidean distances. The heat map illustrating an overall enriched presence of HDRPs in MC is indicative of

Table 3.
Biological significance of Reactome pathways specific to MC condition

S. No	Reactome pathway	No. of genes (found/ total) with its ratio	Entities pValue	Participating proteins	Biological significance
1	CD22 mediated BCR regulation	9/72 (0.005)	5.13E-04	IGHM; IGKC; IGKV3-15; IGKV4 1; IGLC1; IGLC2; IGHV3-7; IGKV1D-33; IGKV3-20	Regulator of adaptive and innate TLR-mediated B cell responses
2	Innate Immune System	75/1331 (0.091)	6.80E-04	SERPINA3; SERPINA1; FRMPD3; TNFAIP6; GDI2; HP; HBB; C8B; CLU; HAPLN1; ACTB; IGHG3; C4B; IGHG4; C4A; IGHG1; IGHG2; PNP; IGLC1; QSOX1; LBP; IGLC2; IGHV37; PGM1; FGB; CAP1; FGA; DCD; ANXA2; S100A1; FGG; KRT1; IGKV1D-33; PKM; CAT; IGKV41; SERPING1; CHI3L1; ALDOA; PPIA; VCL; CFB; FTL; C1QB; APCS; C1S; CFH; A1BG; C3; VTN; C5; CAND1; C6; TTR; UBB; IGKC; C9; IGKV3-15; STOM; APOB; HSPA8; GSN; JUP; PLA2G2A; LYZ; PRDX6; TF; IGKV3-20; HSPA1A	Non-specific defense mechanism that evades invading foreign pathogenic cells
3	Role of phospholipids in phagocytosis	12/129 (0.009)	0.002	IGHG3; IGHG4; IGHG1; IGHG2; IGKC; IGKV3-15; IGKV4-1; IGLC1; IGLC2; IGHV3-7; IGKV1D-33; IGKV3-20	Generate essential second messengers and the phospholipases- PLA, PLC, PLD are known to inititate antibody (IgG) mediated phagocytosis
4	FCGR3A-mediated IL10 synthesis	12/141 (0.010)	0.005	IGHG3; IGHG4; IGHG1; IGHG2; IGKC; IGKV3-15; IGKV4-1; IGLC1; IGLC2; IGHV3-7; IGKV1D-33; IGKV3-20	IL10 immunoregulatory cytokine performs dual function either as protective or pathological mediator. During pathology, igG induce IL10 through FcγRs and kills phagocytic cells.
5	FCGR3A-mediated phagocytosis	13/157 (0.011)	0.005	IGKV1D-33; ACTB; IGHG3; IGHG4; IGHG1; IGHG2; IGKC; IGKV4-1; IGKV3-15; IGLC1; IGLC2; IGHV3-7; IGKV3-20	Phagocytosis via fcγRs subsequently activates Rac GTPases and Cdc42 which induces the phagocyte's NADPH oxidase leading to killing mechanism
6	Leishmania phagocytosis	13/157 (0.011)	0.005	IGKV1D-33; ACTB; IGHG3; IGHG4; IGHG1; IGHG2; IGKC; IGKV4-1; IGKV3-15; IGLC1; IGLC2; IGHV3-7; IGKV3-20	Leishmania infects millions of population but resides in macrophages
7	FLT3 signaling by CBL mutants	2/7 (4.76E-04)	0.006	UBB	c-Cbl, a proto-oncogene involved in RTK signaling, acting through its ubiquitin ligase activity and as a

Table 3. (***Continued***)

S. No	Reactome pathway	No. of genes (found/ total) with its ratio	Entities pValue	Participating proteins	Biological significance
					platform for several signaling adaptor molecules
8	Crosslinking of collagen fibrils	3/24 (0.002)	0.008	COL1A1; COL1A2; PCOLCE	In pathological conditions, dietary inhibition of lysl oxidase results in reduced strength of tendons
9	Myoclonic epilepsy of Lafora	2/11 (7.47E-04)	0.015	UBB	Reported in brain disorder and decline in intellectual function
10	Glycogen synthesis	3/26 (0.002)	0.017	UBB; PGM1	Normal cellular functioning pathway
11	Anchoring fibril formation	2/15 (0.001)	0.027	COL1A1; COL1A2	Procollagen dimerization
12	FCERI mediated NF-kB activation	9/175 (0.012)	0.029	UBB; IGKC; IGKV3-15; IGKV4-1; IGLC1; IGLC2; IGHV3-7; IGKV1D-33; IGKV3-20	Highly critical for proinflammatory cytokine production during mast cell activation that lead to allergic inflammatory diseases
13	Regulation of actin dynamics for phagocytic cup formation	13/158 (0.011)	0.031	IGKV1D-33; ACTB; IGHG3; IGHG4; IGHG1; IGHG2; IGKC; IGKV4-1; IGKV3-15; IGLC1; IGLC2; IGHV3-7; IGKV3-20	Involved in actin cytoskeletal organization
14	Role of LAT2 /NTAL/ LAB on calcium mobilization	8/107 (0.007)	0.031	IGKC; IGKV3-15; IGKV4-1; IGLC1; IGLC2; IGHV3-7; IGKV1D-33; IGKV3-20	Regulation of mast cell calcium responses
15	Maturation of protein E	2/8 (5.43E-04)	0.034	UBB	Translation of structural proteins
16	Retinoid metabolism and transport	6/79 (0.005)	0.036	TTR; APOA2; APOA1; APOA4; APOB; HSPG2	Normal cellular functioning pathway
17	FCERI mediated Ca+2 mobilization	8/129 (0.009)	0.039	IGKC; IGKV3-15; IGKV4-1; IGLC1; IGLC2; IGHV3-7; IGKV1D-33; IGKV3-20	Increase in intracellular calcium in mast cells leads to mast cell degranulation
18	Late endosomal microautophagy	5/35 (0.002)	0.043	HSPA8; UBB; HBB; VIM	Non-selective autophagic pathway
19	G2/M Checkpoints	7/154 (0.010)	0.048	YWHAE; HIST1H4A; UBB; YWHAQ; YWHAZ; YWHAG	Normal cellular functioning pathway
20	Chylomicron remodeling	4/17 (0.001)	0.050	APOA2; APOA1; APOA4; APOB	Plasma lipoprotein remodeling

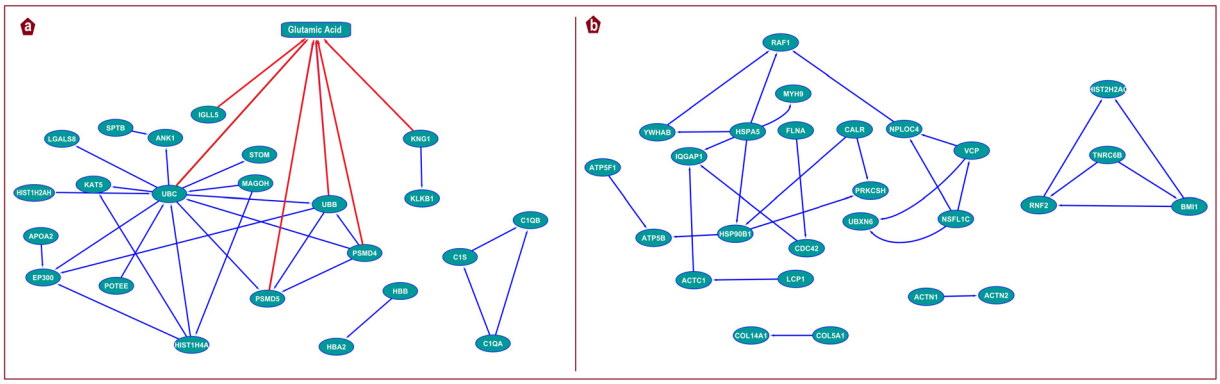


Fig. 4. Metabolite-protein interaction (MPI) network analysis of proteins specific to MC and NMC using STITCH database vs. 5.0. **(A)** With 45 specific proteins in MC given as input, only 19 had interactions with other predicted chemicals/small molecules/ metabolites with a clustering coefficient 0.848. Stronger associations were found between glutamate (metabolite, a neurotransmitter), and other proteins such as ubiquitin, proteasome subunits. **(B)** Non-Modic Change (NMC) had 30 specific proteins, in which only 15 had interactions with other molecules with a clustering coefficient 0.801. Stronger associations are found between RAF1 (oncogene, mediator of inflammatory signals), heat shock proteins and ATP subunits. The predicted incoming/ triggering events were predicted using cytoscape vs. 3.8.3 with installed ANIMO plugin having UPPAAL running in the background. Network nodes are better illustrated as either cylinders (chemicals) or ellipses (proteins, i.e. predicted functional partners). Edges with protein-protein interactions are shown in blue, metabolite/chemical-protein interactions are shown in red. NMC had exhibited no interactions with any other chemicals/ metabolites.

infection-mediated immune response where these HDRPs demonstrate a wide range of utility in bridging between innate and adaptive immunity. Venny analysis was done to know the shared homology of HDRPs between conditions-MC and NMC discs, which showed 34 proteins common to each other with 14 proteins specific to discs with MC and two proteins-[HSPA5 (heat shock protein 5) and C1R (complement C1r)] specific to NMC (Fig. 6B).

Functional analysis of HDRPs common to both conditions

Among the 34 common HDRPs, 4 were found to show significant differential expression between conditions using t-tests/ MW-U tests such as TNC (tenascin-regulates inflammatory axis during TLR signaling); C4A (complement C4A- anaphylatoxin help in degranulation of mast cells); GC (vitamin D binding- modulate host defense); and ApoA1 (apolipoprotein A1- initiate innate host defense) as shown in Fig. 7A. Characterization based on biological process analyzed using GOnet (https://tools.dice-database.org/GOnet/) (Fig. 7B, Supplementary-Table-4) revealed significant enrichment of complement activation-alternate pathway, complement activation-classical pathway, hydrogen peroxide catabolic process, defense response to other organisms, response to stress, immune effector process remains proof for infection and inflammation mediated mechanisms with a p-value threshold of $\leq$ 3.26e-7. Other significant proteins in lesser order involved in cytolysis: Apolipoprotein A1 (APOA1); Complement proteins C5, C6, C9; lysozyme (LYZ); Inflammatory response- haptoglobin (HP); Complement proteins (C3) and (C5); metalloproteinase inhibitor 1 (TIMP1); serum amyloid P component (APCS); C4A; C4B; peroxiredoxin 2 (PRDX2); LYZ; and perlecan (HSPG2).

HDRPS specific to MC group

The 14 HDRPs found specific to MC included acute phase reactants [Protein S100-A1,and apolipoproteins (APO A2 & H)], antimicrobial peptides [Galectin (LGALS8), C-type lectin domain family 3 member A (CLEC3A), chitinase-3-like protein 1(CHI3L1) and synovial phospholipase-A2 (PLA2G2A)], complement cascade (C1S, C1QB, and C8B), inflammatory molecule [tumour necrosis factor-inducible gene 6 protein (TNFAIP6) and polyubiquitin-B (UBB)] and stress response [extracellular superoxide dismutase (SOD3)].

Discussion

Low back pain associated with Modic changes form a discrete subgroup, as evidenced by the distinct clinical profile and surgical outcome in these patients [4–6,12–15]. Despite many studies documenting an intense inflammatory milieu in discs with MC, the exact pathophysiology leading to recurrent episodes of severe and disabling LBP and poor surgical outcomes have not been clearly documented so far [16]. Recent studies have suggested evidence for bacterial infection in MC using advanced technologies such as fluorescence in situ hybridization (FISH) and confocal laser scanning microscopy [17,18] but this is not universally accepted. In a previous prospective cohort study we followed up patients undergoing microdiscectomy for lumbar disc herniations and demonstrated poorer clinical and functional outcomes in patients with MC at one year following surgery [19]. Unravelling the molecular mechanisms in the etiopathogenesis of MC and their biological basis is important. Our study is the first to investigate the differences at molecular level amongst discs with and

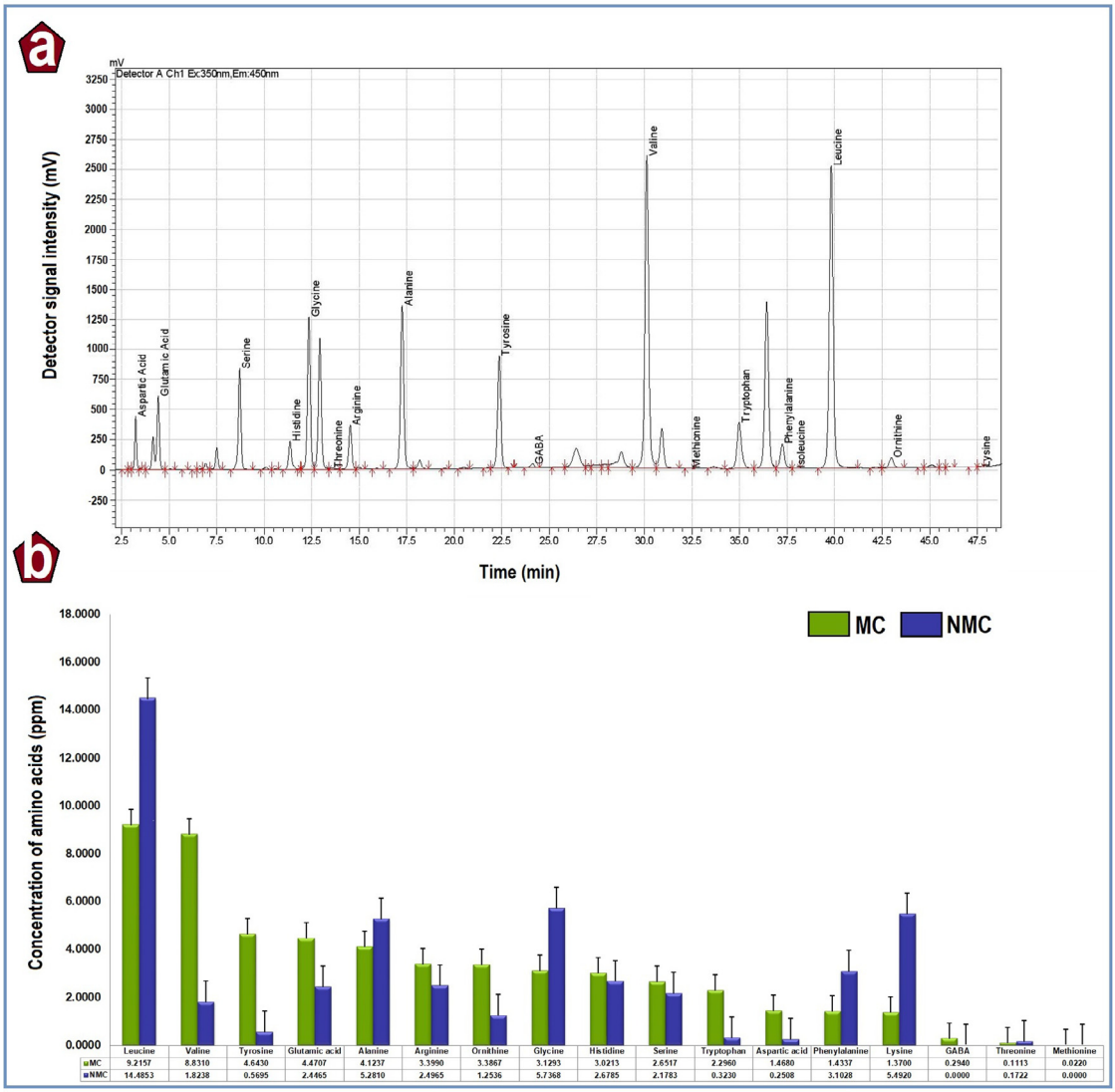


Fig. 5. Profiling of extracted amino acids. (A) Chromatogram of extracted amino acid in a sample representing MC condition. (B) Bar chart representation of mean concentration of amino acids observed across conditions; error bars represent standard errors. Valine and Leucine were found to be the dominant amino acids in MC and NMC conditions respectively. Glutamic acid, a neurotransmitter responsible for sensation of pain, was found elevated in MC.

without modic changes using high throughput proteomic sequencing.

In the current study, we have performed proteomic analysis on the disc samples with and without modic changes and performed a systematic analysis. We first investigated the overall proteomic constitution and documented the differences in discs with MCs. Biological pathway analysis was then performed to identify the metabolic profile. Having identified MC as an independent risk factor for developing SSI in a previous study, we specifically analyzed the role of bacterial etiology by comparing the expression of host-defense response proteins/pathways (HDRPs)[15]. To identify candidate biomarkers and molecular targets for possible therapeutic interventions, we then performed a protein-protein-metabolite interaction analysis which revealed activation of Ubiquitin mediated proteasome degradation pathway and an association with glutamic acid, which was later confirmed by quantitative amino acid analysis using high performance liquid chromatography.

Proteomic phenotype of MC is distinct

We found 45 proteins specific to MC; 30 specific to NMC and 163 common to both. In this study we included herniated discs (which represents a loss in structural integrity of ECM) in both MC and NMC groups. Despite having the same amount of degeneration by Pfirmann grading in MRI, we found the extent of ECM matrix breakdown in nucleus pulposus to be higher in MC as evidenced by the significant downregulation of collagens (COL- 6A1, 6A2, 6A3, 11A1, 12A1, and 20A1), and proteoglycans (versican (VCAN), and

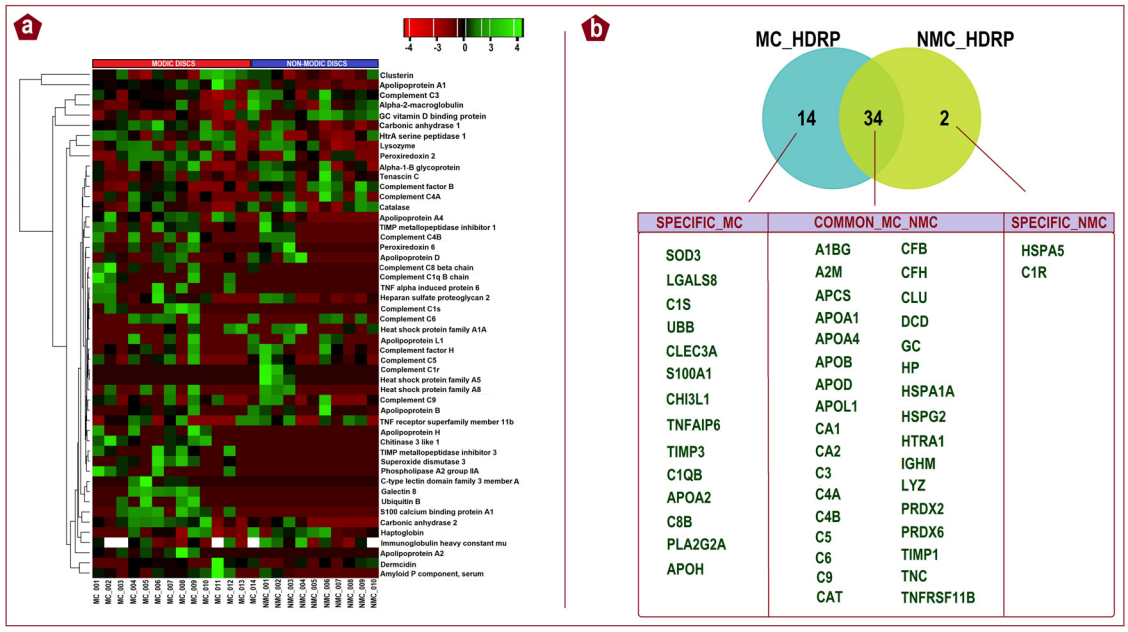


Fig. 6. Profile of HDRP in MC and NMC. (A) Heat map representation of relative abundance of 50 host defense response proteins (HDRPs) across 24 individual samples (MC- 14 samples; NMC- 10 samples) after supervised hierarchical clustering using complete linkage method. Their distance metrics were calculated by Euclidean distance. Left part of heat map shows MC and NMC (right) with the relative abundance based on the spectral count transformed into Z-score. In comparison with NMC, expression of HDRPs was more abundant in MCs which suggests clearance of cellular debris and infection-mediated immune response. (B) Venn diagram showing common and specific HDRPs between conditions- MC and NMC. About 34 (68%) were common, 2 (4%) specific to NMC and 14 (28%) specific to MC.

biglycan (BGN)). Biglycan has been previously documented to have regenerative potential in animal and cell-culture models and needs to be investigated for its ability to reverse or halt degeneration in human intervertebral discs [20]. The excessive ECM breakdown could be secondary to the products of immune and inflammatory pathways which were observed to be upregulated in MC group as discussed below.

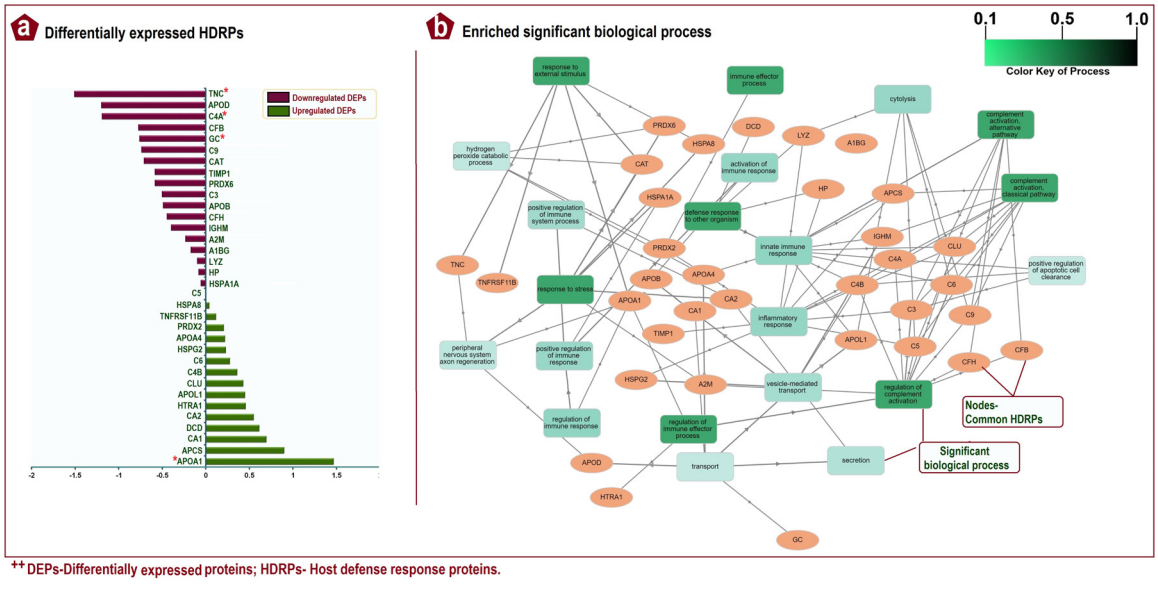


Fig. 7. Functional analysis of differentially expressed HDRPs across MC and NMC conditions – **(A)** Bar diagram represents 34 differentially expressed HDRPs in MC and NMC discs. *indicates statistical significance ($p<0.05$), using t-test/ MW-U test (in case of normality violation) using SPSS vs 25.0. B) Significant biological process of common HDRPs analysed using GOnet with a p-value threshold of ≤3.26e-7. Significant enrichment of complement activation-alternate pathway, complement activation-classical pathway, hydrogen peroxide catabolic process, defense response to other organisms, response to stress, immune effector process remains proof for infection and inflammation mediated mechanisms found indicating in-vitro pathogen associated infection/ inflammatory response.

Inflammatory profile in MC group

On analyzing the 45 proteins expressed only in MC group, 14 of them were host-defense response proteins which are known to initiate inflammatory responses to eliminate pathogens. The remaining 31 proteins mainly consisted of immunoglobulins and inflammatory mediators. The presence of excessive immunoglobulins indicate activation of adaptive immunity in response to chronic pathogenic exposure. On the other hand, we also identified Plasminogen, Angiogenin and Kininogen only in MC group, which have well established roles in inducing vasodilatation, chemotaxis and pain generation through bradykinin production [21,22]. Other strong inflammatory mediators such as Ferritin which has been implicated in systemic inflammations following infections were also present only in MC. The pro-inflammatory status results in accumulation of serine protease (HTRA1) which in turn causes proteolytic degradation of tissues leading to ECM breakdown and more symptoms in MC group.

We also observed the unique expression of TNFAIP6 in MC and upregulation of its receptor (TNFRSF11B) adding evidence to the presence of an intense inflammatory status in MC. Our findings are consistent with that of Ohtori et al. who noticed higher expression of tumor necrosis factor (TNF) and protein gene product 9.5 immunoreactive nerve fibres in MC using immunohistochemistry [23]. TNFA upregulates CRTAC1 expression in primary human articular chondrocytes and synovial fibroblasts causing inflammation and cartilage destruction. Interestingly CRTAC1 was also expressed only in MC group in our study. Deletion of CRTAC1 has provided an anti-inflammatory effect in mice models of inflammation and therefore both TNF and CRTAC1 are potential molecular targets to inhibit inflammatory response in MC group [24].

Stress response proteins

In this study, we also observed an increase in expression of stress response proteins in MC group, which is an inherent compensatory mechanism to tackle accumulated reactive oxygen species (ROS) occurring secondary to tissue oxidative stress following inflammation. Catalase (CAT) and superoxide dismutase (SOD3) expressed only in MC group and the upregulated clusterin (CLU) are crucial antioxidant enzymes that mitigate oxidative stress [25,26]. Peroxiredoxin 2 (PRDX2) is another efficient highly efficient redox protein that neutralizes hydrogen peroxide, rescuing cells from oxidative damage during inflammation. Their upregulation in a disc with MC signifies the amount of inflammatory and oxidative stress in these patients.

Biological process and pathways

To understand and capture the ongoing metabolic activity we analyzed the biological processes amongst both MC and NMC groups. Specific pathways activated amongst MC group mainly involved infection mediated inflammatory and immune responsive pathways. We observed FCERI mediated NF-kB activation which is a well-established antibacterial response resulting in phagocytosis and killing of pathogens in the accumulated macrophages [27]. Other significant immune response pathways observed in MC group included initial triggering of complement; regulation of complement cascade; neutrophil and platelet degranulation. FCGR3A-mediated IL10 synthesis and phagocytosis, Regulation of actin dynamics for phagocytic cup formation, and late endosomal microautophagy found in MC group adds up the evidence towards an active and ongoing antibacterial response.

Host defense response proteins in Modic changes

While the role of bacteria in MC is fiercely debated owing to the contamination theory, we have analyzed the expression of Host defense response proteins in addition to acute phase reactants and complement activation which form the three main pillars of antibacterial response. Out of the 50 HDRPs identified in this study, 29 (**Figs. 6B & 7A)** of them were either expressed only in MC or were upregulated. A heatmap (**Fig. 7A)** generated to compare the HDRPs expression between MC and the non-MC group clearly shows a significantly higher magnitude of expression of bacterial mediated stimulation of HDRPs in MC. While 14 of them were expressed only in MC group, only 2 were specific to NMC group, and 15 out of the 34 commonly expressed HDRPs were found to be upregulated.

Of notable importance was the upregulation of the antimicrobial peptide dermcidin (DCN). DCN is a first line host defense protein which is secreted form neutrophils or macrophages having intense proteolytic activity [7]. Other proteins induced following pathological stimuli such as acute phase reactants (APOA1, APOL1 and APOA4), and mediators of chronic inflammation like serum amyloid P-component (APCS) were also upregulated in MC group. Neutrophilic degranulation, also causes the release of antimicrobial peptides such as synovial-phospholipase-A2 (PLA2G2A)-an anti-bacterial protein to defend the host resulting in opsonisation, phagocytosis, and apoptosis especially in MC [28].Though complement cascade proteins were present in both groups, the presence of these proteins (C1S, C1QB and C8B) in MC alone and upregulation of (C6 and C4B) indicate complement activation.

S100A1, expressed only in MC discs, is a pro-inflammatory molecule that has an immense role in protecting the intra- and extracellular environments during infection. However, its uncontrolled activity has been found to result in many inflammatory and neurodegenerative diseases [29]. Similarly, LGALS8 and C-type lectin domain family three-member A (CLEC3A) are antimicrobial proteins having a well-established role in autophagy of both gram-negative and gram-positive bacteria, which were specifically expressed in the MC group [30].

While the above discussed proteins indicate activation of innate immune system, which represent an acute response, the presence of immunoglobulins (IGHV3-7, IGLC1, IGKV3-15, IGKV3D-11, IGKV4-1, IGKV2-40) and activation of FCGR3A mediated phagocytosis pathway in MC group represent the activation of adaptive immune system. This adds evidence for the existence of a chronic bacterial antigenic stimulus leading to induction and mediation of immune pathways which needs further investigation using multi-disciplinary research co-relating bacterial isolation from discs and proteomic analysis.

Ubiquitin mediated proteasome degradation in Modic changes

In a retrospective case-control study of 1124 patients undergoing lumbar surgeries in a single-center, we found that the preoperative MC (odds ratio 2.725) was an independent risk factor for developing SSI [15]. Further, on comparing patients undergoing lumbar microdiscectomy in a prospectively followed up cohort of 209 patients we found that patients with preoperative MC had less favorable back pain, functional scores, and patient satisfaction [19]. Laustsen et al in a systematic review of literature of 14 articles involving 1652 surgical patients, confirmed a negative association between surgical outcomes and patients with MC [4]. However, to date there is no study which has analyzed the possible pathomechanisms barring association studies.

The overall expression of proteins in the current study revealed strong evidence towards infection mediated host defense response, inflammation and compensatory stress response. To identify potential targets for targeted molecular therapies, we subjected the proteins expressed in MC and non-MC group to a novel data analysis using STITCH database vs 5.0 which provides interactions between proteins, small molecules and chemicals, and found important leads. Many of the above described proteins such as apolipoprotein, kininogens, kallikrein, immunoglobulins and galectins were tightly interacting with ubiquitin (UBB and UBC) which activate formation of chaperones (PSMD4 & PSMD5) and finally proteasome formation. This finding implicates the activation of Ubiquitin mediated proteasome degradation which has immense potential in ECM breakdown if left unchecked.

The Ubiquitination machinery orchestrates a complex inflammasome activation following exposure to pathogenic bacteria [31]. Another interesting observation was the association of all the degradative pathway proteins with glutamic acid. Glutamic acid is an excitatory neurotransmitter, which on excess stimulation is known to cause seizures and many neuropsychic disorders [32]. Multiple studies have demonstrated their excrescence from herniated disc material and ability to have nociceptive effect on dorsal root ganglion of nerves [33]. Excessive glutamic acid production in CSF has been found in patients with bacterial infection. However, it remains unknown as to whether MC have any relation to their excess production, which requires metabolomic studies to compare and quantify glutamic acid production in patients with and without MC.

We performed quantitative estimation of 18 amino acids amongst discs with and without MC using high performance liquid chromatography which confirmed the excessive accumulation of glutamic acid (4.471 ppm) in MC group, when compared to 2.446 ppm in NMC group.

Role of glutamate

Histopathological analysis of damaged end plates show neovascularisation and ingrowth of nerve fibres [34]. While neovascularisation predisposes accumulation of inflammatory molecules, the ingrowth of nerve fibres subjects these patients to excessive nociceptive stimuli. However, it remained unknown as to which metabolite could lead to this phenomenon. The higher expression of glutamate, an excitatory neurotransmitter in MC found in the current study could sensitise these nerve fibres causing excessive pain. It would be interesting to investigate the efficacy of anti-glutaminergic therapy in improving clinical and functional outcomes in patients with MC. Improvement in chronic LBP following high dose of Fasinumab, a monoclonal antibody against nerve growth Factor, is yet another evidence to this phenomenon and needs further focussed research [35].

Molecular targets for improving outcomes

On analyzing the various biological pathways, we found strong evidence for uncontrolled inflammatory response. We found two main downstream targets which could have a positive impact on clinical outcomes. One being the ubiquitin mediated proteasome degradation, antibodies to which have already been developed some of which include Carfilzomib and Bortezomib, which also suppresses inflammatory signals including IL-6 and TNF-A secretion. Another target being anti-glutaminergic therapy due to excess accumulation of glutamic acid in patients with Modic changes documented in the study.

Limitation of the study

In this study, we have performed only molecular analysis of discs with Modic changes and compared it with those without Modic changes. A clinical outcome comparison has not been performed. All the discs harvested were from herniated samples in both the groups and whether non-herniated discs with MC differ have not been analysed. This study involved only 2 Type 1 MC and 12 Type 2 MC. Traditionally, Modic Type 1 changes were considered unstable and Type 2 were considered relatively unstable. However, recent studies have shown that Type 2 are not quiescent lesions and could involve pathological changes. Owing to lower prevalence of MC Type 1, we could not compare their molecular profile with MC Type 2. Further, though our study has clearly documented HDRPs, as one of the main biological changes

in MC, we have not investigated whether bacterial etiology has played a direct role in their activation. Future studies using advanced culture-independent approaches such as Next-generation sequencing (NGS) is necessary to investigate bacterial role in MC. The effect of antiglutaminergic therapy in MC needs further investigation.

Clinical implication

Modic changes have been strongly associated with degenerative disc disease and several aetiologies have been proposed including genetic, mechanical, inflammatory, and infections. Recently, there are increasing number of studies

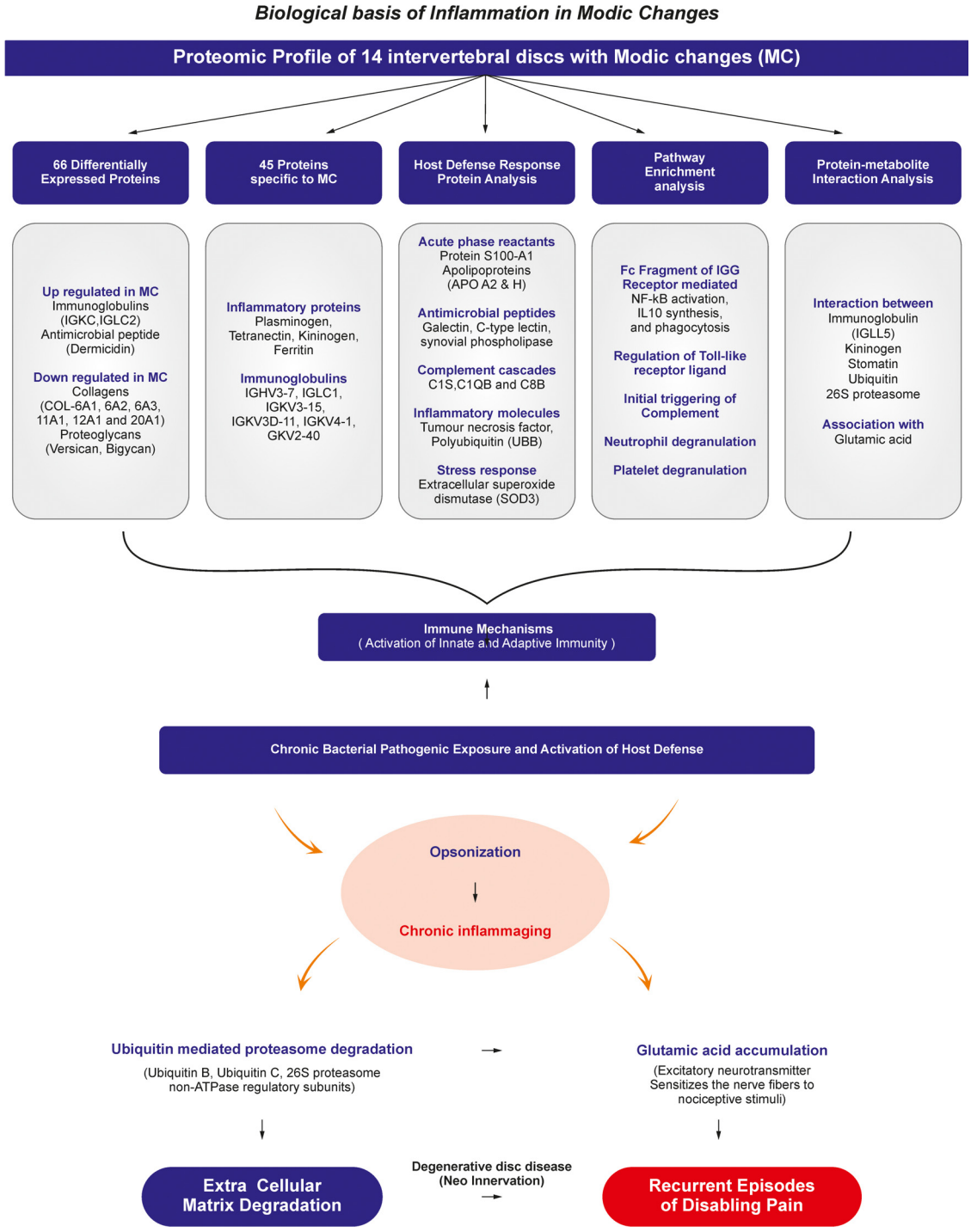


Fig. 8. The proteomic analysis of discs with Modic changes revealed significant bacterial mediated activation of both innate and adaptive immunity evidenced by the host defense response resulting in the accumulation of acute phase reactants, inflammatory proteins, complement proteins, immunoglobulins and related pathways. Ubiquitin mediated proteasome degradation leading to extra cellular matrix degeneration and resultant accumulation of glutamic acid are possible molecular targets for inhibition.

reporting poor clinical and functional outcomes in both conservative and surgical outcomes of patients with Modic changes. However, the exact biological basis causing these difference in outcomes had not been investigated on a molecular level. In this study, the overall proteomic constitution of MC group shows an intense inflammatory status and activation of host defense response proteins, acute phase reactants, complement proteins and Immunoglobins. More importantly, a significant association was found for Ubiquitin mediated proteasome degeneration, which leads to ECM breakdown and accumulation of glutamic acid, an excitatory neurotransmitter and potential pain generator. The findings have been confirmed by amino acid analysis, and the study provides potential targets for molecular therapy, at different levels ranging from TNF-A blockers to inhibit inflammation, monoclonal antibodies targeted against ubiquitin mediated proteasome degradation or downstream targets such as antiglutaminergic therapies for patients with MC.

Conclusion

Our study confirms that MC represents an intense inflammatory status with activation of host defense response and immune reactive pathways. Downstream effects leading to ubiquitin mediated proteasomal degradation of ECM proteins and the resulting metabolites such as glutamic acid were detected and confirmed in MC. Inhibition of Ubiquitin mediated proteasome degradation with specific antibodies or administration of antiglutaminergic therapy were identified as two possible therapeutic interventions in Modic changes Fig. 8.

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IRB approval

The study was performed after approval of the IRB committee.

Conflict of Interest

The authors declare that they have no conflict of interest.

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SR, DCR, CT and MR conceived and formulated the project. SMN, CT contributed to the design of the analysis; performed lab experiments and bulk of data analysis; DCR, KSVA, RMK and APS wrote and prepared the manuscript. All authors have read through and given the final approval of the submitted publication. We also acknowledge the efforts of Ms M Sujitha and Ms M Dhanalakshmi for assistance in LC−MS/MS experiments.

Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.spinee.2021.07.003.

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