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Influence of endplate avulsion and Modic changes on the inflammation profile of herniated discs: a proteomic and bioinformatic approach

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Abstract

Purpose The aim of this observational radiographic and proteomic study is to explore the influence of both Modic change (MC) and endplate avulsion (EPA) on the inflammation profile of herniated discs using a proteomic and bioinformatics approach.

Methods Fifteen nucleus pulposus (NP) harvested from surgery underwent LC-MS/MC analysis, the proteome was subsequently scanned for inflammatory pathways using a bioinformatics approach. All proteins that were identified in inflammatory pathways and Gene Ontology and present in > 7 samples were integrated in a multiple regression analysis with MC and EPA as predictors. Significant proteins were imputed in an interaction and pathway analysis.

Results Compared to annulus fibrosus tear (AFT), six proteins were significantly altered in EPA: catalase, Fibrinogen beta chain, protein disulfide-isomerase, pigment epithelium-derived factor, osteoprotegerin and lower expression of antithrombin-III, all of which corresponded to an upregulation of pathways involved in coagulation and detoxification of reactive oxygen species (ROS). Moreover, the presence of MC resulted in a significant alteration of nine proteins compared to patients without MC. Patients with MC showed a significantly higher expression of clusterin and lumican, and lower expression of catalase, complement factor B, Fibrinogen beta chain, protein disulfide-isomerase, periostin, Alpha-1-antitrypsin and pigment epithelium-derived factor. Together these altered protein expressions resulted in a downregulation of pathways involved in detoxification of ROS, complement system and immune system. Results were verified by Immunohistochemistry with CD68 cell counts.

Conclusion Both EPA and MC status significantly influence disc inflammation. The beneficial inflammatory signature of EPA illustrates that endplate pathology does not necessarily have to worsen the outcome, but the pathological inflammatory state is dependent on the presence of MC.

Keywords Modic changes · Endplate avulsion · Disc herniation · Proteomics · Bioinformatics

IRB approval: The study was performed after approval of the IRB committee.

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Introduction

Herniation of the lumbar intervertebral disc is a highly prevalent disease, during which the herniated disc compresses the adjacent nerve root. Patients with herniated discs experience debilitating back pain and often excruciating leg pain that radiates down the dermatome. The severity of these symptoms can vary widely between patients irrespective of the degree of compression. In search for an explanation for this wide variety in symptoms, research has focussed on inflammation of the intervertebral disc.

Disc inflammation seems to function as a two-edge sword [1]. In which it shows beneficial effects on one end: For example, through a resorption process of the herniated material initiated by macrophages, which is associated with a faster regression of the disc material [2]. On the other ends, inflammation may lead to exacerbation of pain symptoms, through sensitization of the nerve root by pro-inflammatory cytokines excreted in the disc [3]. Because of the clinical significance of disc inflammation, it is vital to understand the pathophysiology of these different effects of inflammation. However, little is known regarding what influences the type of disc inflammation that patients experience.

Recently, there has been an increase in attention for pathology of the endplate, how it may influence the course of the herniation and the rate of recovery. Most research on this topic has focussed on vertebral endplate signal changes on MRI, more commonly referred to as Modic changes (MC) [4, 5]. MC represent inflammatory or fibrotic changes in the endplate [6] and have been associated with a slower rate of recovery [7, 8]. This could be explained by intrusion of cartilage pieces of the disrupted endplate in the herniated nucleus pulposus, which subsequently prevent neovascularisation and macrophage infiltration in the disc [9, 10]. Moreover, others have associated MC with detrimental effect of infiltrating macrophages on clinical outcomes [1]. Taken together, the presence of MC seems to be an indication that the type of inflammation has gone from the beneficial type, toward a type of inflammation that may exacerbate pain symptoms and reduce the rate of recovery. However, the current evidence on this is still inconclusive.

A different, relatively underexposed, pathology of the endplate is endplate avulsion. A disc can herniate in two ways: Either through an annulus fibrosus tear (AFT) or through an endplate avulsion (EPA) [11]. During the latter, the annulus fibrosus is torn from the endplate due to a defect of the endplate. This pathology also resulted in pieces of cartilage in the herniated disc but only has a moderate association with MC [11]. At present, it remains unknown whether EPA has similar effects on inflammation of the disc or whether these concepts should be completely separated.

Hence, the aim of this study was to explore the effects of both MC and EPA on the inflammatory signature of the herniated disc using a proteomic and bioinformatics analysis of nucleus pulposus samples. Through exploration of these concepts, we may improve our understanding of different types of inflammation in the disc. This could result in a better prognosis and altered treatment strategies that will not just focus on nerve root compression but will also take the patients inflammation status into account.

Materials & methods

Patient population

IRB approval and informed consent were obtained. Fifteen consecutive patients between age group of 18–65 years suffering from single-level disc herniation who were planned for microdiscectomy in a single institution following a fair trial of conservative management were included in the study. However, patients undergoing epidural steroid injections were excluded as it might influence the findings of the study. Candidates were counselled to withhold NSAIDs for 48 h before sample procurement and those who had consumed oral steroids within a 2-week period were excluded. Patients diagnosed with pre-existing inflammatory and auto-immune disorders such as inflammatory bowel diseases, psoriasis, rheumatoid arthritis, ankylosing spondylitis and thyroiditis were also excluded. Transplant recipients, cancer patients and other who were on immunomodulators or immunosuppressive agents were also excluded.

Sample collection

Samples were harvested during surgery, after the removal of the herniated disc material, nucleus pulposus material was separated from annulus fibrosus material and directly transferred to sterile cryopreservation vials, and snap frozen in liquid nitrogen before transport to the research laboratory. MRI scans were performed pre-surgery by a 1.5 Tesla scanner, and both sagittal T1- and T2-weighted images of the lumbar spine were obtained. Image evaluation of endplate status was dichotomized into an intact endplate (AFT group), and an avulsed endplate (EPA group). Evaluation of MC status was according to the criteria of Modic et al. [4, 5].

Sample processing

Around 200 mg of nucleus pulposus tissues was pulverized with the help of liquid nitrogen, resuspended in 1 ml of RIPA (Radio Immunoprecipitation Assay) buffer to extract soluble/hydrophilic proteins by centrifugation at 10,000 g for 20 min at 4 °C. The supernatant has the soluble proteins,

and the pellet contains hydrophobic protein. The pellet was processed further by adding 2% SDS buffer, vortexed, heated at 70 °C for 10 min followed by centrifugation at 10,000 xg for 10 min at RT. Further, the proteins were subjected to clean up to remove the interfering agents such as glycans, salts, detergents using ice-cold organic solvents—methanol-chloroform in the ratio of (1 volume of sample: 4 volumes of methanol: 1 volume of chloroform: 3 volumes of high purity water). The pellet obtained after clean-up was resuspended in 100 µL of 25 mM Tris-HCl buffer pH 7.4 with 0.1% SDS. The protein yield was estimated using BCA assay. On average, our protein yield is 0.5–1 mg/100 mg of tissue after extraction and clean-up. Around 100 µg of estimated protein was pre-fractionated on 10% SDS-PAGE, prior to in-gel digestion in a keratin-free platform. Colloidal Coomassie-stained protein bands were chopped in 2*3 mm size (approximately 10 bands obtained from one single lane), destained using 25 mM NH₄HCO₃ in 50% ACN, and then, they were subjected to digestion using trypsin at a concentration of 600 ng/band for 16 h at 37 °C. The tryptic digested peptides were extracted with 0.1% trifluoroacetic acid (TFA) and pooled into three fractions which were desalted using C18 tips and finally 1000 fm of total peptides per each pool were loaded in duplicates for ESI-LC-MS/MS analysis. [12].

Bioinformatics analysis

MS/MS raw data acquired from Orbitrap Velos Pro Mass Spectrometer were analyzed by Proteome Discoverer v1.4 using Mascot (Matrix Science, London, UK; version 2.4.1.0) and inbuilt SequestHT search algorithm. The peptide spectrum matches (PSMs) from SequestHT, and Mascot was post-processed using the Percolator algorithm. The peptides with rank one and having a *q*-value < 0.01 were considered for protein identification. A more detailed description of bioinformatic analysis was published earlier [13].

Quantitative analysis

Out of the proteomic database, all proteins with > 2 unique peptide or 1 unique peptide with a PSM ≥ 10 were included in the analysis [14]. These selected proteins were subsequently integrated in a Gene Ontology and Pathway enrichment analysis using both STRING and DAVID databases with a false discovery rate of 0.05, which allowed us to identify all pathways and protein functions that are involved in inflammatory processes. Moreover, since mass spectrometry will regularly fail to detect proteins that are expressed in low quantities, our results will contain a large amount of missing data for the less abundant proteins. Therefore, only proteins that were expressed in at least eight samples were integrated in the statistical analysis.

Statistical analysis

Data analysis was performed using SPSS software version 25. Effects of EPA and MC status on protein expression were analysed using a two-way ANOVA with interaction term, for this analysis, protein expression (normalized PSM) was Log₁₀ transformed, assumptions of no-outliers and homogeneity of variance had to be met. For all significant ANOVA results, post hoc tests were performed to assess differences for individual groups using Mann–Whitney U tests. Two-tailed alpha level was set at 0.05. Samples with missing values were excluded from the analysis.

Interaction analysis

All significant proteins were integrated in an interaction analysis using string database. Subsequently, up or down-regulations of relevant pathways corresponding to the identified interactions are evaluated.

Verification by immunohistochemistry

Twenty additional disc samples were harvested during discectomy surgery for a lumbar HNP's. Samples were embedded in formalin and subsequently fixed in paraffin. In order to quantify the degree of inflammation, macrophages were identified with CD68. 5-µm paraffin slices were rinsed in ethanol and methanol solutions after which they were stained with CD68 (DAKO, Denmark), using a three-step indirect method. CD68 was cooked in citrate pH 6.0 buffer, after which an avidin–biotin complex technique was performed with the Vectastain ABC-Elite Kit (Vector Lab USA) and the appropriate biotinylated antibodies. Visualization was done with DAB solution (Sigma). All samples were counterstained with Harris haematoxylin and accompanied by a positive control (atherosclerosis tissue). For evaluation, samples were photographed using a Philips ultra-fast scanner. Cells were evaluated based on morphological features, and only positively stained macrophages were photographed and included in the cell counting process, chondrocytes were excluded. Cells were counted using Image-J.

Results

Patient characteristics

Out of the 15 included patients, six patients were characterized as AFT on MRI (Mean age 45.2 ± 19.2 SD, 33% male) and nine patients as EPA (Mean age 32 ± 5.8 SD,

67% male). Mann–Whitney U test showed that neither the difference in age ($p=0.224$) nor sex ($p=0.205$) was significant.

Moreover, eight patients did not show any MC on MRI (Mean age 41.5 ± 17.9 SD, 50% male), and seven patients did show MC, (Mean age 32.4 ± 5.3 SD, 43% male). Again, the differences in age and sex were not significant. (Age: $p=0.908$, Sex: $p=0.447$). In addition, the distribution of EPA in patients with MC was similar as in patients without MC (Fisher exact: $p=0.608$). Lastly, neither EPA ($p=0.747$), nor MC ($p=0.800$) was associated with the extent of disc degeneration according to classification by Pfirrmann et al. [15]. All disc herniations were characterized as the extruded type according to Fardon et al. [16]. An overview of all patient characteristics can be found in Table 1.

Pathway analysis

The Gene Ontology and Pathway analysis identified 31 pathways that were involved in inflammation-related processes (Supplementary table S1). In these 31 pathways combined, 147 inflammation-related proteins were identified. Out of which 41 were eligible for statistical analysis (Supplementary table S2).

Comparing protein expression

Out of the 41 proteins, six proteins were significantly affected by EPA status. The presence of EPA resulted in significantly higher levels of catalase (CAT) ($p=0.005$) and FGB ($p=0.007$), Protein disulfide-isomerase (P4HB)

($p=0.031$), pigment epithelium-derived factor (SERPINF1) ($p=0.023$) and osteoprotegerin (TNFRSF11B) ($p=0.014$), and significantly lower expression of antithrombin-III (SERPINC1) ($p=0.002$) (Fig. 1A, Table 2).

Furthermore, the presence of MC patients resulted in significantly higher expression of clusterin (CLU) ($p=0.019$) and lumican (LUM) ($p=0.029$) and significantly lower expression of complement factor B (CFB) ($p=0.022$), (P4HB) ($p=0.029$), periostin (POSTN) ($p=0.012$) and Alpha-1-antitrypsin (SERPINA1) ($p=0.047$),

Moreover, in contrast with the presence of EPA, the presence of MC resulted in lower expression levels of SERPINF1 ($p=0.029$), CAT ($p=0.035$) and FGB ($p<0.001$) (Fig. 1B, Table 2). At last, KRT1 showed a statistical interaction effect in which KRT1 was only downregulated in MC patients with AFT but not in those with EPA ($p=0.047$). An overview of all subgroup changes is shown in Fig. 1C (AFT/MC-, EPA/MC-, AFT/MC+, EPA/MC+).

Interaction analysis

Out of the six proteins that were significantly up/down-regulated in EPA, four proteins revealed an interaction: CAT, P4HB, FGB and SERPINC1. The Reactome pathway analysis revealed that this corresponded to an upregulation in: fibrin clot formation and detoxification of reactive oxygen species (ROS). SERPINF1 and TNFR11B did not show direct interactions with any of the other five proteins (Fig. 2A).

Out of the seven proteins significantly up- or down-regulated in patients with MC, 2 interaction cascades were found. One cascade including CAT, P4HB, SERPINA1, FGB, CFB, and CLU, which were involved in several pathways defined by Reactome.org. Based on whether these proteins were up or downregulated in patients with MC, it could be concluded that the pathways involving complement/coagulation cascade, detoxification of ROS, and the immune system functions were all downregulated in MC patients. The other interaction cascade, which included POSTN, LUM and SERPINF1, interacted due to often reported co-expression but were not involved in the same pathway. Moreover, KRT1 was not involved in any of the interaction cascades (Fig. 2B). An overview of the relevant pathways and their involved proteins can be found in Table 3.

Verification by immunohistochemistry

Immunohistochemistry was in line with the proteomic results, EPA groups showed higher levels of CD68+ cells compared to the AFT: AFT/MC- had a median of 47 positive cells, AFT/MC+ of 198 positive cells, whereas EPA/MC- showed a median of 701 and EPA/MC+ a median of 225. Nevertheless, ANOVA showed that this difference

Table 1 Patients characteristics

Case ID	Age	Sex	Pfirrmann	MC	AFT/EPA
1	29	F	3	No	AFT
3	70	F	5	No	AFT
5	45	M	3	No	AFT
6	67	F	3	No	AFT
7	27	M	3	No	EPA
8	26	M	4	No	EPA
9	40	M	3	No	EPA
10	28	M	4	No	EPA
11	29	F	4	Type 1	EPA
12	34	M	4	Type 2	AFT
13	26	F	4	Type 2	AFT
14	32	M	4	Type 2	EPA
15	43	F	4	Type 2	EPA
16	31	M	3	Type 2	EPA
17	32	F	2	Type 2	EPA

Patient characteristics at baseline, age, sex, disc degeneration (Pfirrmann grade), AFT/EPA- and MC status are displayed for all patients

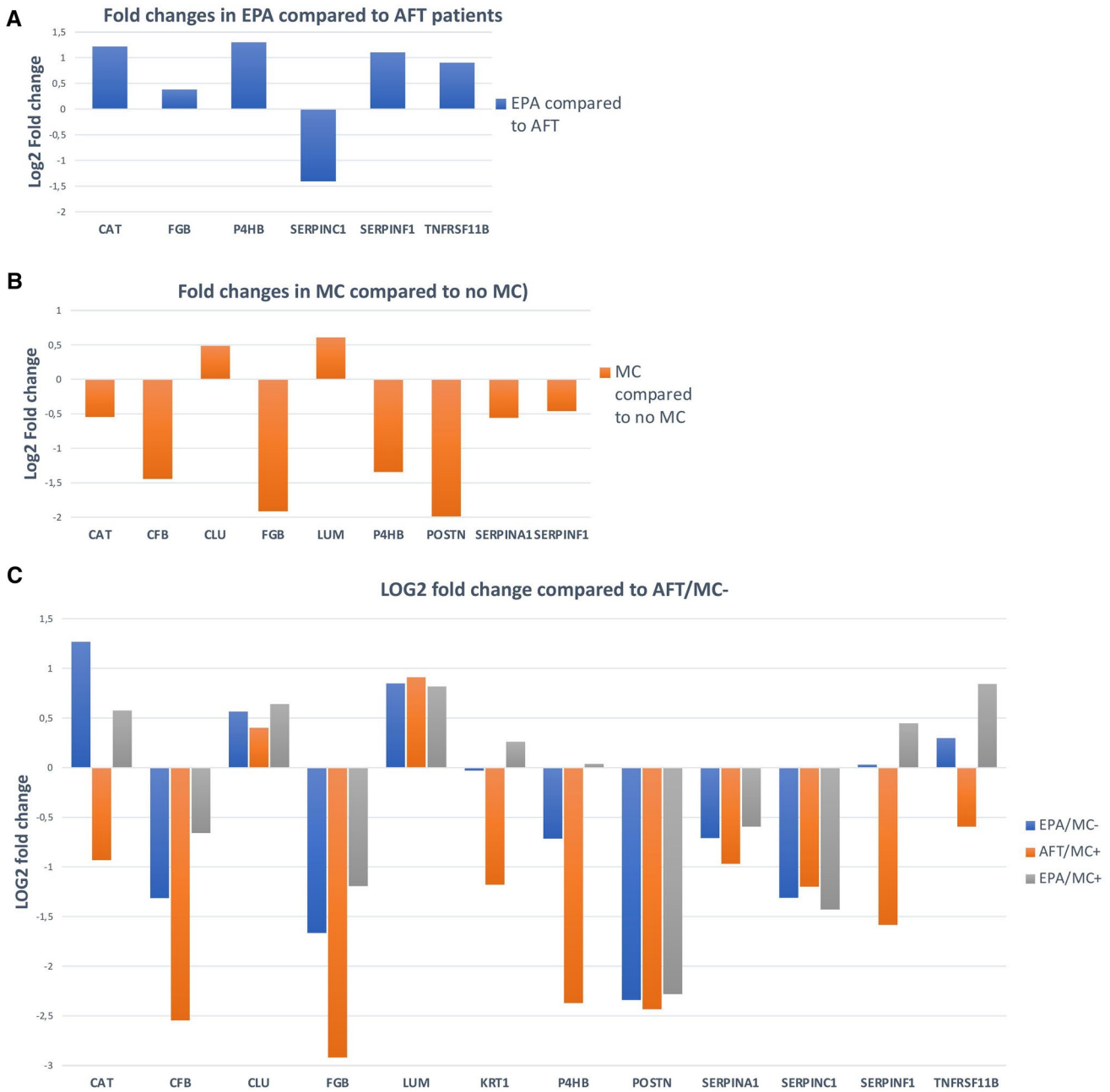


Fig. 1 Significantly altered protein expression. This figure illustrates the significantly different expressed proteins between AFT versus EPA and no MC versus MC, as assessed by a multiple regression with EPA and MC status as predictor. **A** shows the seven proteins that differed significantly between AFT and EPA. AFT is used as baseline to illustrate the Log2 fold changes in EPA as compared to AFT. **B** displays the nine proteins significantly altered in MC compared to no

MC. No MC is used as baseline to show the Log2 fold changes in MC compared to no MC. **C** shows the Log2 fold changes for all subgroups, AFT/MC- was used as a reference category to which AFT/MC-, EPA/MC- and EPA/MC+ were compared. All proteins symbols are shown on the x-axis, Log2 fold changes in protein expression (nPSM) are shown on the y-axis

was not significant ($p=0.20$). Neither was a significant difference seen in CD68 + cells for patients with and without MC ($p=0.52$). An example of the CD68 + cells shown in Fig. 3A, and the results in Fig. 3B and Table 4.

Table 2 Effect of AFT/EPA and MC status on protein expression

Protein gene symbol	N (AFT/EPA)	ANOVA AFT vs EPA	N (no MC/ MC)	ANOVA no MC vs MC	Interaction EPA*MC
A1BG	5/5	$F(1)=0.07, p=0.806$	6/4	$F(1)=2.39, p=0.172$	$F(1)=1.84, p=0.224$
A2M	6/9	$F(1)=0.13, p=0.724$	8/7	$F(1)=1.31, p=0.277$	$F(1)=0.01, p=0.927$
ACTB	2/7	$F(1)=0.10, p=0.796$	5/4	$F(1)=1.66, p=0.254$	$F(1)=0.422, p=0.545$
AGT	3/8	$F(1)=0.79, p=0.404$	6/5	$F(1)=3.68, p=0.097$	$F(1)=2.40, p=0.165$
ANXA2	6/8	$F(1)=0.38, p=0.553$	8/6	$F(1)=3.68, p=0.084$	$F(1)=0.169, p=0.690$
APOA1	5/7	$F(1)=0.66, p=0.440$	6/6	$F(1)=2.59, p=0.146$	$F(1)=0.37, p=0.561$
C3	6/9	$F(1)=0.21, p=0.659$	8/7	$F(1)=4.67, p=0.054$	$F(1)=0.50, p=0.496$
C5	4/5	$F(1)=4.92, p=0.077$	6/3	$F(1)=0.58, p=0.480$	$F(0)$
CA1	6/6	$F(1)=0.25, p=0.632$	6/6	$F(1)=2.77, p=0.135$	$F(1)=0.72, p=0.420$
CA2	6/2	$F(1)=0.65, p=0.456$	2/6	$F(1)=0.032, p=0.856$	$F(0)$
CAT	5/7	$F(1)=12.46, p=0.008^{**}$	6/6	$F(1)=5.35, p=0.050^{*}$	$F(1)<0.01, p=0.969$
CFB	6/7	$F(1)=4.25, p=0.069$	8/5	$F(1)=10.29, p=0.011^{*}$	$F(1)=3.23, p=0.106$
CLU	6/9	$F(1)=0.35, p=0.568$	8/7	$F(1)=5.63, p=0.037$	$F(1)=0.65, p=0.438$
COL2A1	6/8	$F(1)=0.16, p=0.695$	7/7	$F(1)=0.20, p=0.666$	$F(1)=2.00, p=0.666$
FGB	5/6	$F(1)=15.10, p=0.012^{*}$	5/6	$F(1)=52.01, p=0.001^{**}$	$F(1)=0.68, p=0.448$
FGG	6/8	$F(1)=1.55, p=0.241$	8/6	$F(1)=4.84, p=0.052$	$F(1)=3.86, p=0.078$
FN1	6/9	$F(1)=3.64, p=0.083$	8/7	$F(1)=1.86, p=0.20$	$F(1)=2.66, p=0.131$
GAPDH	6/9	$F(1)=0.22, p=0.381$	8/7	$F(1)=0.83, p=0.381$	$F(1)=0.45, p=0.514$
GSN	6/9	$F(1)=0.07, p=0.801$	8/7	$F(1)=1.79, p=0.208$	$F(1)=0.13, p=0.721$
HBB	6/9	$F(1)=0.13, p=0.724$	8/7	$F(1)=1.26, p=0.286$	$F(1)=2.07, p=0.178$
HP	6/9	$F(1)=0.01, p=0.912$	8/7	$F(1)=0.75, p=0.405$	$F(1)=2.13, p=0.172$
HPX	6/8	$F(1)<0.01, p=0.977$	8/6	$F(1)=1.22, p=0.295$	$F(1)=2.03, p=0.184$
HSPG2	5/3	$F(1)<0.01, p=0.994$	4/4	$F(1)=0.33, p=0.598$	$F(1)=1.12, p=0.350$
HTRA1	6/9	$F(1)=1.32, p=0.275$	8/7	$F(1)=1.20, p=0.296$	$F(1)<0.01, p=0.946$
KRT1	6/9	$F(1)=4.77, p=0.052$	8/7	$F(1)=1.95, p=0.190$	$F(1)=5.01, p=0.047^{*}$
KRT16	3/7	$F(1)=1.37, p=0.287$	4/6	$F(1)=0.43, p=0.54$	$F(1)=0.011, p=0.918$
KRT6A	2/6	$F(1)=0.92, p=0.381$	4/4	$F(1)=0.03, p=0.868$	$F(0)$
LUM	6/9	$F(1)=0.47, p=0.031$	8/7	$F(1)=6.14, p=0.031$	$F(1)=0.354, p=0.564$
LYZ	6/5	$F(1)=2.09, p=0.192$	5/6	$F(1)=0.14, p=0.716$	$F(1)=0.587, p=0.469$
P4HB	5/5	$F(1)=8.84, p=0.025^{*}$	6/4	$F(1)=7.02, p=0.038^{*}$	$F(1)=1.53, p=0.263$
PKM	4/4	$F(1)=0.06, p=0.823$	3/5	$F(1)=3.25, p=0.146$	$F(1)=0.07, p=0.801$
POSTN	6/6	$F(1)=1.60, p=0.241$	7/5	$F(1)=8.87, p=0.018^{*}$	$F(1)=0.11, p=0.752$
PRG4	4/9	$F(1)=1.01, p=0.341$	7/6	$F(1)=0.07, p=0.794$	$F(1)=0.42, p=0.534$
SERPINA1	6/9	$F(1)<0.01, p=0.953$	8/7	$F(1)=4.91, p=0.049$	$F(1)=0.32, p=0.586$
SERPINC1	5/6	$F(1)=16.43, p=0.007^{**}$	6/5	$F(1)=0.17, p=0.695$	$F(1)=0.93, p=0.373$
SERPINF1	6/8	$F(1)=7.74, p=0.019^{*}$	7/7	$F(1)=7.48, p=0.021^{*}$	$F(1)=1.44, p=0.258$
SERPING1	6/8	$F(1)=0.05, p=0.821$	8/6	$F(1)=2.69, p=0.132$	$F(1)=0.02, p=0.894$
THBS1	5/7	$F(1)=0.08, p=0.788$	7/5	$F(1)=0.30, p=0.598$	$F(1)=0.12, p=0.742$
TNFRSF11B	5/4	$F(1)=17.79, p=0.008^{**}$	5/4	$F(1)=0.20, p=0.673$	$F(1)=3.45, p=0.122$
VIM	5/5	$F(1)=0.05, p=0.824$	6/4	$F(1)=3.32, p=0.118$	$F(1)=1.28, p=0.301$
VTN	3/9	$F(1)=0.23, p=0.644$	6/6	$F(1)=1.48, p=0.258$	$F(1)=3.33, p=0.105$

This Table displays the results of a two-way ANOVA in which EPA and MC status were used as predictor for the listed protein expression, interaction effects between the two predictors were also included. F values with degrees of freedom and p values are given. The first column lists the protein gene symbols, second and fourth column show the number of samples that expressed the protein in each subgroup. Column three and five showed the result of the ANOVA for the effect of EPA and MC, respectively. The last column shows whether there was a significant interaction effect between EPA and MC. * indicates $P<0.05$, ** indicates $P<0.01$, $F(0)$ indicates that no degrees of freedom were left for the interaction analysis

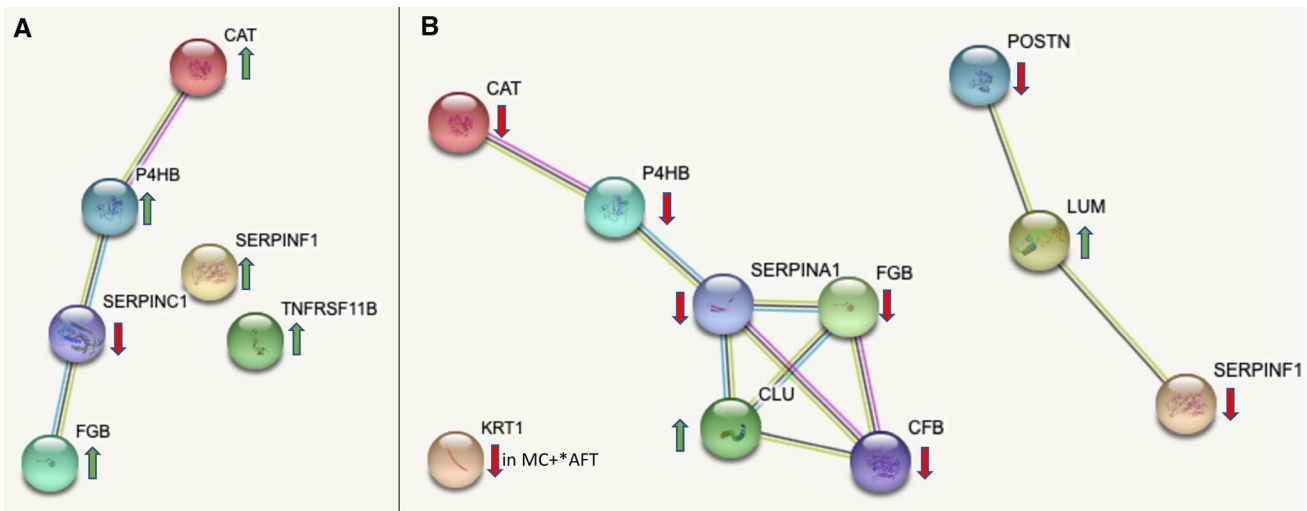


Fig. 2 Protein–protein interaction analysis by STRING database. This Figure illustrates the protein–protein interactions revealed by STRING database between AFT versus EPA and no MC versus MC, as assessed by a multiple regression with EPA and MC status as predictor **A** shows the interactions between the proteins that were significantly up or down regulated in EPA compared to AFT. Lines between the proteins illustrate a direct functional interaction between the two proteins, with either a stimulating or inhibiting effect, which connects four out of the six proteins in an interaction cascade. Arrows indicate up or downregulation in EPA compared to AFT. **B** shows the interac-

tions between the proteins that were significantly up or down regulated in patients with MC compared to those without. Lines between the proteins illustrate a direct functional interaction between the two proteins, with either a stimulating or inhibiting effect, which results in two interaction cascades, one with six and one with three out the nine proteins. KRT1 was not included in any of the interaction cascades of EPA or MC and was only downregulated in MC+ patients in the AFT group. Arrows indicate up or downregulation in MC compared to no MC

Table 3 Up/downregulation of pathways in EPA versus AFT and MC vs no MC

A			
EPA pathways interaction analysis	<i>P</i> value	Matching proteins	Change in EPA
Common Pathway of Fibrin Clot Formation	0.0012	FGB,SERPINC1	Upregulated
Detoxification of Reactive Oxygen Species	0.0015	CAT,P4HB	Upregulated
Immune System	0.0114	CAT,FGB,P4HB,TNFRSF11B	Inconclusive
B			
MC pathways interaction analysis	<i>P</i> value	Matching proteins	Change in MC
Platelet degranulation	0.0018	CLU, FGB, SERPINA1	Downregulated
Detoxification of Reactive Oxygen Species	0.0020	CAT, P4HB	Downregulated
Regulation of Complement cascade	0.0025	CFB, CLU	Downregulated
Immune System	0.0018	CAT, CFB, CLU, FGB, P4HB, SERPINA1	Downregulated

This Table shows the Reactome pathways in which the significant up or downregulated proteins are involved. 3A shows the pathway results of the six proteins that were altered in EPA, 3B shows the pathway results of the nine proteins that were altered in MC. The first column describes the name of the pathway, the second the p value of the enrichment of the pathway provided by reactome, the third column lists the proteins that were picked up in the respective pathway, and the last column shows whether the pathway is up or downregulated in EPA(3A) / MC(3B). The up- or downregulation of the pathway was based on the up/downregulation of the involved proteins combined with their specific role in the pathway (stimulating or inhibiting the pathway). Change in a pathway is scored inconclusive when both stimulatory and inhibitory proteins are upregulated, and thus, no clear up or downregulation could be identified

Fig. 3 Verification by immunohistochemistry. **A** shows an example of the CD68+ cells in the herniated disc tissue, 100×enlarged. **B** shows the median expression of CD68+ cells per cm². Centre lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; data points are plotted as open circles. *n* = 8, 2, 4, 6 sample points

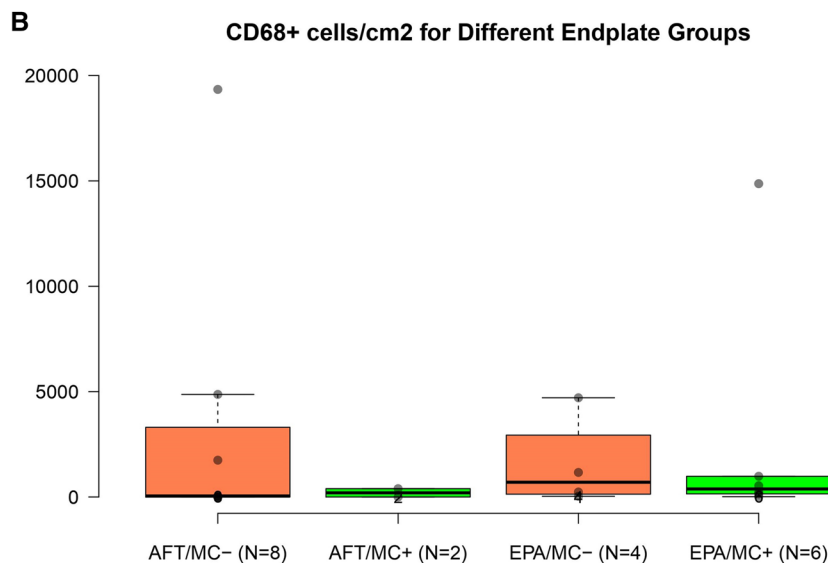
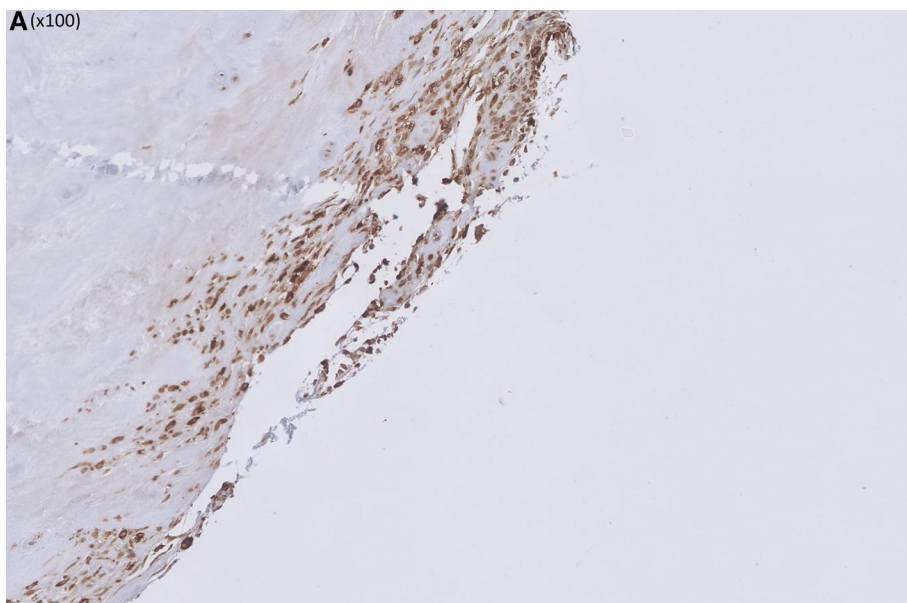


Table 4 Effect of AFT/EPA and MC status on CD68+ cells

Two-way ANOVA	MC- versus MC+	AFT versus EPA	Interaction EPA*MC
Log10(CD68+ /cm2)	$F(1)=0.424, p=0.52$	$F(1)=1.76, p=0.20$	$F(1)<0.01, p=0.97$

This Table displays the results of a Two-way ANOVA in which EPA and MC status were used as predictor for the Log transformed CD68+ positive cells per cm². Interaction effects between the two predictors were also included. *F* values with degrees of freedom and *p*-values are given. Column two and three showed the result of the ANOVA for the effect of EPA and MC respectively. The last column shows whether there was a significant interaction effect between EPA and MC

Discussion

This study explored the influence of EPA type herniation and MC on the inflammatory signature of the disc. The most important findings of this study are the different

effects that the two endplate pathologies have on the inflammation profile. EPA patients showed an upregulation coagulation and detoxification of ROS compared to AFT. By contrast, the detoxification of ROS, complement system and immune system were all downregulated in MC compared to patients without MC.

Coagulation

The increase in coagulation in EPA as compared to AFT was illustrated by a significant but limited increase in FGB, which is one of the fibrin components necessary for clot formation [17], and a significant decrease in SERPINC1, a protein that inhibits thrombin activity [18]. The increase in this pathway compared to AFT could be very well explained by the endplate being heavily vascularised, which requires increased coagulation to heal the wound after avulsion. In contrast, in the AFT type, no or little blood vessels are ruptured, and thus, upregulation of proteins involved in coagulation is less required. Interestingly, the protein alterations in MC patients suggested a downregulation of coagulation, which was illustrated by a significant but limited decrease in both FGB and SERPINA1, which has some inhibiting effects on thrombin activity [19]. However, this was accompanied by a significant but again limited increase in CLU, a protein excreted by platelets, of which the exact role remains to be elucidated. Based on this discrepancy and the limited changes in expression, the current evidence seems insufficient to conclude whether coagulation is downregulated in MC.

Detoxification of reactive oxygen species

Moreover, an EPA type herniation was correlated with an upregulation of detoxification of ROS. This upregulation was illustrated by a significant increase in P4HB, which functions as a chaperone at high concentrations [20], and a significant increase in CAT, a protein often excreted by macrophages with anti-oxidative and anti-inflammatory effects while preserving phagocytic and digestive capacities [21, 22]. In contrast, the presence of MC resulted in a significant downregulation of P4HB and limited but also significant downregulation of CAT, and consequently in a downregulation of detoxification of ROS.

From a clinical perspective, the upregulation of ROS detoxification illustrates an increase in ‘beneficial inflammation’ in avulsed endplate herniations as compared to the annular tear type. This increase in beneficial inflammation could be explained by the increased exposure to neovascularisation from the highly vascularised endplate [23], which can subsequently lead to infiltration of macrophages. This is in line with the results of the verification with immunohistochemistry, in which we found an insignificant trend towards higher numbers of macrophages in EPA discs. Unfortunately, not all herniated material can be absorbed in equal efficiency. This depends on the amount of cartilage pieces, and the quantity and functionality of the immune cells [9, 22]. Such an inadequately absorbed herniation may stimulate nucleus pulposus cells to induce a chronic inflammation process [24, 25], characterized by an increase in

pro-inflammatory cytokines, reactive oxygen and fibrotic changes [6, 26]. This chronic inflammation process can be identified on the MRI as MC [6], and can explain the reduced recovery rate.

Immune system

In line with the immune-modulating effects of CAT, SERPINA1 is also known for its immune-modulating capacity. The expression of this protein is increased by immune cells during an inflammatory response to balance the pro-inflammatory cytokines and oxidative stress [27]. In addition, it has been shown to switch the type of microglia activity away from oxidative stress and pro-inflammatory cytokines towards tissue remodelling and phagocytosis [28, 29]. The finding that MC-type herniations associated with limited but significantly lower levels of SERPINA1 suggests an alteration in the type of infiltrating immune cells.

Also, in line with the altered immune cell infiltration in MC patients, is the downregulation of the complement system in MC, illustrated by a significant decrease in CFB, and an almost significant decrease in C3. This was accompanied by a limited but significant increase in CLU, which is an inhibitor of the complex system cascade [30]. Moreover, together with the decrease in the detoxification of ROS, a downregulation of immune response may together indicate a malfunctioning immune response. This is in line with the immunohistochemistry findings that showed no difference in number of macrophages, which illustrate that there is no downregulation in immune response, making a malfunctioning more likely. Such a malfunctioning immune response may create opportunities for subclinical infections with anaerobic bacteria, which is in line with the emerging evidence that MC is associated with bacterial infections [31, 32].

Tissue resorption

In addition to the inflammatory pathways, the alterations in protein expression also illustrated differences in tissue resorption. MC-type hernias illustrated a limited but significantly lower expression of SERPINF1, which could illustrate a deficiency in cartilage clearance [33], and is again in line with the reduced recovery rate associated with MC [8]. This cartilage clearance was also confirmed by the significant and considerable decrease in MC of POSTN, a protein participating in post-injury tissue regeneration processes, during which, it stimulated degradation of ECM through upregulation of matrix metalloproteases [34]. LUM belongs to the family of small-leucine-rich proteins, which could get accumulated as a part of healing response as its increased expression has been documented in fibrotic lesions previously secondary to stimulation

from inflammatory molecules such as TNF- α . Lumican has also been shown to modulate host response and play an important role activation of an innate immune mechanisms in response to bacterial lipopolysaccharides (LPS) and other pathogen-associated molecular patterns [35]. Further LUM has been documented to have an important role in inflammatory bowel diseases such as colitis and is believed to promote intestinal homeostasis by aiding innate immune and inflammatory responses [36]. The limited but significant accumulation of LUM in MC in this study adds evidence to a pro-inflammatory status in these discs which get activated possibly due to infective aetiology [37].

As this was the first study to compare protein expression between AFT and EPA, no comparisons with the previous literature could be made. Regarding MC, even though our study found a great variety of proteins involved in inflammation, none of the proteins reported by Dudli et al. were found in our analysis [6]. This can be explained by proteomics being less sensitive than a gene expression method, which prevents it from detecting proteins that are expressed in low quantities reported in previous studies such as MMP's, IL's and ADAMT's [6, 7, 38–40]. Nevertheless, similar to our results, Dudli et al. also showed that only a limited number of proteins were altered, thereby indicating that the differences are rather subtle [6]. Another limitation of this study is the limited sample size and the absence of correction for multiple testing. These results should therefore be interpreted as high-grade evidence, but instead as a starting point for more extensive research on the newly identified proteins and pathways outlined in this paper.

In summary, the proteomic inflammatory signature of AFT and EPA patients differed significantly, with EPA illustrating an increase in a beneficial inflammatory response. With regard to MC, those with MC showed a shift away from beneficial and likely towards detrimental inflammatory response. Taken together, the evidence presented in this paper portrays that endplate pathology does not necessarily lead to reduced recovery, but that the presence of MC illustrates a shift in the inflammatory proteome that makes spontaneous resorption less likely. Future studies should focus on validating these findings in a large study cohort and preferably integrate a cytokine assay and immune cell staining analysis.

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Availability of data and material Data will be available upon request.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Approval was obtained by ethical committee: Ganga hospital Ganga Medical Centre and Hospital Pvt Ltd Tamil Nadu.

Consent to participate and publish Informed consent was signed by each participant.

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