The role of inflammation in sciatica: the contradictory effect of macrophages

Djuric, N.

Chapter 6

Influence of different endplate pathologies on the inflammation profile of herniated discs: a proteomic approach

N. Djuric[1,2], S. Rajasekaran[3], C. Thangavel[1], M. Raveendran[4], D.C.R. Soundararajan [3], S.M. Nayagam[1], M.S. Matchado[1], K.S. Sri Vijay Anand[3]

[1]Ganga Research Centre, No 91, Mettuppalayam Road, Coimbatore, 641030, India. [2]Department of Neurosurgery, Leiden University Medical Center, Leiden.

[3]Department of Spine Surgery, Ganga Hospital, 313, Mettuppalayam Road, Coimbatore, 641043, India. [4]Department of Plant Biotechnology, Tamil Nadu Agricultural University, Coimbatore, 641003, India.

Submitted

"He who has a why to live for can bear almost any how"
(Friedrich Nietzsche)

Abstract

Purpose

The aim of this observational radiographic and proteomic study is to explore the influence of both MC and EPA on the inflammation profile of herniated discs using a proteomic and bioinformatics approach.

Methods

15 nuclei pulposi (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 AFT, 6 proteins were significantly altered in EPA: Catalase, Fibrogen 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, presence of MC resulted in a significant alteration of 9 proteins compared to patients without MC. Patients with MC showed a significantly higher expression of Clusterin and Lumigan, and lower expression of Catalase, Complement Factor B, Fibrogen beta chain, Protein disulfide-isomerase, Periostin, Alpha-1-antitrypsin and Pigment epithelium-derived factor. Together these alternated protein expressions resulted in a downregulation of pathways involved in detoxification of ROS, complement system and immune system.

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 that the pathological inflammatory state is dependent on the presence of MC.

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 focused on inflammation of the intervertebral disc.

Disc inflammation seems to function as a double-edged 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 end, 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 essential to develop a non-invasive tool that identifies different stages of inflammation.

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 focused 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, towards 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.

Materials & Methods

Patient population

IRB approval and informed consent was obtained. 15 nucleus pulposus samples were harvested from patients undergoing discectomy for radicular pain symptoms due to an extruded herniated disc.

Sample Collection

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 EPA 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]. 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.

Sample Processing

Around 100 mg Nucleus pulposus tissue from the 15 discs was subjected for extraction of total proteins and subjected to ESI-LC-MS/MS with conditions as described in our earlier report: Rajasekaran S et al., 2017 [12].

Bioinformatics analysis

A detailed description of the bioinformatic analysis and normalisation by spectral count was published earlier: Rajasekaran et al., 2020 [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, 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 8 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 multiple regression, for this analysis, protein expression (normalized PSM) was Log10 transformed, Assumptions of normalized residuals, influential cases (cook's distance >1), and homogeneity of variance had to be met. Two-tailed alfa 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.

Results

Patient characteristics

Out of the 15 included patients, 6 patients were characterized as AFT on MRI (Mean age 45.2±19.2 SD, 33% male) and 9 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, 8 patients did not show any MC on MRI (Mean age 41.5±17.9 SD, 50% male), and 7 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 Pfirmann et al [15]. All disc herniations were characterized as the extruded type according to Fardon et al (2014) [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 (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, 5 proteins were significantly affected by EPA status. EPA patients had 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) (Figure 1A, Table 2).

Furthermore, compared to those without MC; MC patients showed 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) (Figure 1B). At last, SERPINF1 (p=0.029), CAT (p=0.035) and FGB (p<0.001), which were all upregulated in EPA patients, were downregulated in MC patients (Figure 1B, Table 2).

Interaction analysis

Out of the 6 proteins that were significantly up/downregulated in EPA: CAT, P4HB, FGB and SERPINC1 revealed an interaction. 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 5 proteins (Figure 2A).

Out of the 7 proteins significantly up- or downregulated 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 (Figure 2B). An overview of the relevant pathways and their involved proteins can be found in Table 3.

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 of coagulation in EPA as compared to AFT was illustrated by an increase in FGB, which is one of the fibril components necessary for clot formation [17], and a 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 decrease in FGB, and SERPINA1, which has some inhibiting effects on thrombin activity [19]. However, this was accompanied by an increase in CLU, a protein excreted by platelets, of which the exact role remains to be elucidated. Therefore, 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 an increase in P4HB, which functions as a chaperone at high concentrations [20], and an 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, when comparing patients with MC to those without MC: CAT and P4HB were down regulated, and consequently detoxification of ROS was also downregulated.

From a clinical perspective, the upregulation of ROS detoxification illustrates an increase of 'beneficial inflammation' in avulsed endplate herniations as compared to the annular tear type. This increase of beneficial inflammation could be explained by the increased exposure to neovascularisation from the highly vascularised endplate [23]. 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 pulpous 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 lower expression of SEPRINA1 in MC patients compared to those without MC thus suggest 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 an 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 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. In MC patients, SERPINF1 was downregulated, illustrating a deficiency in cartilage clearance, which is, again, in line with the reduced recovery rate associated with MC [8]. This cartilage clearance was also confirmed by the 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 [33]. 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-A. 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 [34]. 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 [35]. The accumulation of LUM in MC in this study adds evidence to a pro-inflammatory status in these discs which get activated probably due to infective aetiology.

As this was the first study to compare protein expression between AFT and EPA, no comparisons with 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. (2017) 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 [6, 7, 36, 37]. Nevertheless, similar to our results, Dudli et al (2017) also showed that only a limited amount of proteins were altered, thereby indicating that the differences are rather subtle [6]. Another limitation of this study is the limited sample size and 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 essay and immune cell staining analysis.

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Appendix

Table 1. Patients characteristics

Case ID	Age	Sex	Pfirmann	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.

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

Protein Gene symbol	N (AFT/EPA)	Beta (P-value) AFT vs EPA	N (no MC/MC)	Beta (P-value) no MC vs MC
A1BG	5/5	-0.021 ±0.171 (0.907)	6/4	-0.216 ±0.175(0.258)
A2M	6/9	0.119 ±0.296 (0.696)	8/7	-0.366 ±0.291 (0.232)
ACTB	2/7	-0.077 ±0.221 (0.739)	5/4	-216 ±0.185 (0.285)
AGT	3/8	0.094 ±0.197 (0.647)	6/5	-0.214 ±0.176(0.258)
ANXA2	6/8	0.089 ±0.157 (0.584)	8/6	-0.306 ±0.157 (0.077)
APOA1	5/7	-0.184 ±0.209 (0.401)	6/6	0.373 ±0.206 (0.104)
C3	6/9	-0.077 ±0.131 (0.565)	8/7	-0.269 ±0.129 (0.058)
C5	4/5	-0.427 ±0.193 (0.077)	6/3	0.164 ±0.215(0.480)
CA1	6/6	-0.081 ±0.146 (0.591)	6/6	0.269 ±0.144 (0.094)
CA2	6/2	0.236 ±0.292 (0.456)	2/6	0.064 ±0.337 (0.856)
CAT	5/7	0.393 ±0.105 (0.005)**	6/6	-0.257 ±0.103 (0.035)*
CFB	6/7	0.235 ±0.156 (0.163)	8/5	-0.434 ±0.160 (0.022)*
CLU	6/9	0.031 ±0.067 (0.649)	8/7	0.177 ±0.066 (0.019)*
COL2A1	6/8	0.124 ±0.296 (0.684)	7/7	0.038 ±0.289(0.897)
FGB	5/6	0.385 ±0.097 (0.007)**	5/6	-0.72 ±0.097 (<0.001)**
FGG	6/8	0.163 ±0.211 (0.455)	8/6	-.347 ±0.211 (0.128)
FN1	6/9	0.144 ±0.093 (0.146)	8/7	-0.084 ±0.091 (0.373)
GAPDH	6/9	0.048 ±0.129 (0.717)	8/7	-0.100 ±0.126 (0.446)
GSN	6/9	0.065 ±0.194 (0.742)	8/7	-0.292 ±0.190 (0.151)
HBB	6/9	-0.016 ±0.132 (0.907)	8/7	0.098 ±0.130 (0.467)
HP	6/9	-0.058 ±0.169 (0.736)	8/7	-0.82 ±0.629 (0.629)
HPX	6/8	-0.038 ± 0.150 (0.807)	8/6	-0.120 ±0.150 (0.441)
HSPG2	5/3	0.051 ±0.322 (0.880)	4/4	0.087 ±0.312 (0.792)
HTRA1	6/9	-0.186 ±0.151 (0.243)	8/7	0.178 ±0.148 (0.253)
KRT1	6/9	0.167 ±0.105 (0.138)	8/7	-0.077 ±0.103 (0.469)
KRT16	3/7	0.19 ±0.151 (0.249)	4/6	-0.101 ±0.141 (0.498)
KRT6A	2/6	0.162 ±0.168 (0.381)	4/4	0.025 ±.146 (0.868)
LUM	6/9	0.061 ±0.075 (0.431)	8/7	0.183 ±0.074 (0.029)*
LYZ	6/5	-0.207 ±0.156 (0.220)	5/6	0.031 ±0.156 (0.848)
P4HB	5/5	0.507 ±0.189 (0.031)*	6/4	-0.439 ±0.193 (0.038)*
PKM	4/4	0.098 ±0.268 (0.730)	3/5	-0.571 ±0.277 (0.094)
POSTN	6/6	-0.291 ±0.205 (0.120)	7/5	-0.650 ±0.208 (0.012)*
PRG4	4/9	-0.286 ±212(0.207)	7/6	0.138 ±0.197 (0.500)
SERPINA1	6/9	-0.014 ±0.088 (0.875)	8/7	-0.192 ±0.087 (0.047)*
SERPINC1	5/6	-0.629 ±0.135 (0.002)**	6/5	0.105 ±0.135 (0.460)
SERPINF1	6/8	0.527 ±0.200 (0.023)*	7/7	-0.499 ±0.198 (0.029)*
SERPING1	6/8	0.035 ±0.159(0.831)	8/6	-0.274 ±0.159 (0.112)
THBS1	5/7	-0.081 ±0.225 (0.726)	7/5	-0.118 ±0.225(0.614)

Protein Gene symbol	N (AFT/EPA)	Beta (P-value) AFT vs EPA	N (no MC/MC)	Beta (P-value) no MC vs MC
TNFRSF11B	5/4	0.289 ±0.084 (0.014)*	5/4	-0.044 ±0.084 (0.620)
VIM	5/5	-0.117 ±0.256 (0.660)	6/4	-0.446 ±0.261 (0.117)
VTN	3/9	-0.215 ±0.260 (0.430)	6/6	-0.056 ±0.225 (0.810)

Table 2 displays the results of the multiple regression analysis in which EPA and MC status were used as predictor for the listed protein expression. Beta ±SE and p-values are given, * indicates P<0.05, ** indicates P<0.01.

Table 3. Up/down regulation of pathways in EPA vs AFT and MC vs no MC

A			
EPA pathways interaction analysis	**P-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	**P-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

Table 3 shows the reactome pathways in which the significant up or downregulated proteins are involved. **3A** shows the pathway results of the 6 proteins that were altered in EPA, **3B** shows the pathway results of the 9 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 down regulation 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.

Figure 1. Significantly altered protein expression

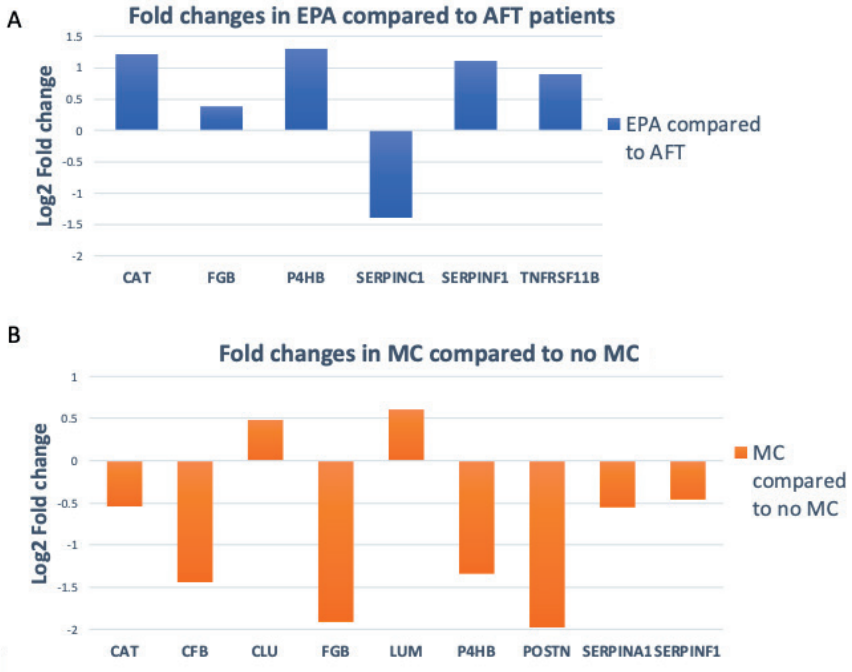

Figure 1 illustrates the significantly different expressed proteins between AFT vs EPA and no MC vs MC, as assessed by a multiple regression with EPA and MC status as predictor. Figure **1A** shows the 7 proteins that differed significantly between AFT and EPA. AFT is used as baseline to illustrate the Log2 fold changes in EPA as compared to AFT, all proteins symbols are shown on the X axis, Log2 fold changes in protein expression (nPSM) are shown on the Y axis. Figure **1B** displays the 9 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. all proteins symbols are shown on the X axis, Log2 fold changes in protein expression (nPSM) are shown on the Y axis.

Figure 2. Protein-protein interaction analysis by STRING database

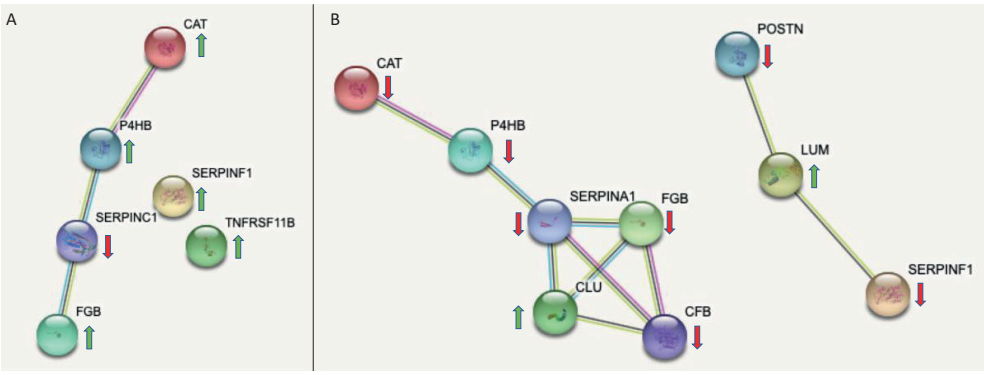

Figure 2 illustrates the protein-protein interactions revealed by STRING database between AFT vs EPA and no MC vs MC, as assessed by a multiple regression with EPA and MC status as predictor Figure **2A** shows the interactions between the proteins that were significantly up or down regulated in EPA compared to AFT. Lines between the proteins illustrate a direct functional interaction between the two proteins, with either a stimulating of inhibiting effect, which connects 4 out the 6 proteins in an interaction cascade. Arrows indicate up or downregulation in EPA compared to AFT. **2B** shows the interactions 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 of inhibiting effect, which results in 2 interaction cascades, one with 6 and one with 3 out the 9 proteins. Arrows indicate up or downregulation in MC compared to no MC.

Supplementary Appendix

Table S1. Identification of Inflammation related pathways in the total NP proteome

Pathway	P-value	Proteins identified
leukocyte mediated immunity	2,76E-30	57
immune effector process	1,46E-26	39
immune response	5,48E-26	79
neutrophil degranulation	1,86E-22	52
myeloid leukocyte activation	4,99E-21	71
myeloid leukocyte activation	4,99E-21	56
leukocyte activation involved in immune response	1,97E-20	8
regulation of complement activation	2,85E-20	23
complement activation	1,73E-19	22
regulation of humoral immune response	3,96E-19	24
regulation of acute inflammatory response	6,42E-18	25
complement activation, classical pathway	7,18E-17	18
regulation of inflammatory response	1,13E-13	35
regulation of immune response	4,38E-13	55
activation of immune response	1,12E-12	36
positive regulation of immune system process	2,32E-11	52
regulation of immune effector process	3,04E-10	31
complement activation, alternative pathway	2,97E-09	9
innate immune response	9,44E-09	31
acute inflammatory response	1,23E-07	13
inflammatory response	1,31E-07	101
antimicrobial humoral response	1,36E-07	17
adaptive immune response	7,99E-07	22
interleukin-12-mediated signaling pathway	8,37E-05	40
toll-like receptor signaling pathway	0,00013	10
positive regulation of apoptotic cell clearance	0,00035	4
regulation of transforming growth factor beta receptor signaling pathway	0,00043	10
antimicrobial humoral immune response mediated by antimicrobial peptide	0,00055	10
negative regulation of immune system process	0,0088	18
positive regulation of cytokine production	0,0089	17
positive regulation of NF-kappaB transcription factor activity	0,0114	9

Table S1 lists all the pathways and gene ontology processes from DAVID and STRING database related to inflammation, which were found in the total NP proteome. Proteins involved in these pathways were subsequently included in the study analysis. The name of the pathway is listed in the first column, the second column provides the p-value of the pathway/process enrichment, the 3th column shows the nr. of proteins of that pathway that were present in the study sample.

Table S2. List of protein names and gene their corresponding gene symbols

Gene Symbol	Protein Name
A1BG	Alpha-1B-glycoprotein
A2M	Alpha-2-macroglobulin
ACTB	Actin, cytoplasmic 1
AGT	Angiotensinogen
ANXA2	Annexin A2
APOA1	Apolipoprotein A-I
C3	Complement C3
C5	Complement C5
CA1	Carbonic anhydrase 1
CA2	Carbonic anhydrase 2
CAT	Catalase
CFB	Complement factor B
CLU	Clusterin
COL2A1	Collagen alpha-1(II) chain
FGB	Fibrinogen beta chain
FGG	Fibrinogen gamma chain
FN1	Fibronectin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSN	Gelsolin
HBB	Hemoglobin subunit beta
HP	Haptoglobin
HPX	Hemopexin
HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein
HTRA1	Serine protease HTRA1
KRT1	Keratin, type II cytoskeletal 1
KRT16	Keratin, type I cytoskeletal 16
KRT6A	Keratin, type II cytoskeletal 6A
LUM	Lumican
LYZ	Lysozyme C
P4HB	Protein disulfide-isomerase
PKM	Pyruvate kinase
POSTN	Periostin
PRG4	Proteoglycan 4
SERPINA1	Alpha-1-antitrypsin
SERPINC1	Antithrombin-III
SERPINF1	Pigment epithelium-derived factor
SERPING1	Plasma protease C1 inhibitor
THBS1	Thrombospondin-1
TNFRSF11B	Tumor necrosis factor receptor superfamily member 11B; Osteoprotegerin
VIM	Vimentin
VTN	Vitronectin

Table S2 lists the proteins from the inflammation related pathways that were eligible for statistical analysis. Column 1 displays the gene symbols of the proteins, the protein names are listed in column 2.