

# Serum biomarkers discovery of steroid-induced femoral head osteonecrosis in Chinese female by comparative proteome

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## Research

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# Abstract

**Background** Steroid-induced osteonecrosis of the femoral head (SONFH) is a disabling, aseptic and ischemic disease due to excessive glucocorticoids (GCs) usage. Patients with SONFH are commonly asymptomatic, which makes its early diagnosis is challenge, the pathological mechanisms of SONFH are not well-known, the purpose of the present study was to screen diagnostic biomarkers for SONFH.

**Methods** The differential expression of serum proteins from SONFH, traumatic osteonecrosis of the femoral head (TONFH) patients and healthy volunteers (CK) in Chinese females was compared using iTRAQ, and potential diagnostic biomarkers were verified by western blotting.

**Results** Gene Ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG), Domain and Clusters of Orthologous Groups (COG) analyses revealed key groups of proteins, pathways and domains differentially regulated among SONFH, TONFH and healthy volunteers in Chinese female, the results showed that peptidase S1, fibrinogen, transferrin, lipid transport domains and hematopoietic cell lineage, fat digestion, absorption, peroxisome proliferator-activated receptor (PPAR) pathways were associated with the development of SONFH. Finally, C-reactive protein (CRP), serum amyloid A protein (SAA1), alpha-1-acid glycoprotein 1 (ORM1) and dopamine beta-hydroxylase were selected for verification of differential expression using western blotting.

**Conclusions** Our data suggest that dysfunction of hematopoietic cell lineage, adhesion, fat digestion and absorption, PPAR pathways may be involved in the pathogenesis of SONFH, serum proteins SAA1, ORM1 could be used as new potential diagnostic biomarkers for SONFH.

## Background

Steroid-induced osteonecrosis of the femoral head cases are on the rise owing to long-term or mass glucocorticoids application in patients[1], which resulting in microcirculation disturbance, osteocyte death, articular surface collapse, if not treated properly, the rapid destruction of the hip joint seriously influences the patient's quality of life[2–6].

In the last few years, SONFH has attracted extensive attention, various hypotheses have been put forward for the etiological factors involved in corticosteroid-induced osteonecrosis, and it is now considered multiple factors contribute to the development of SONFH, including blood coagulation disorders, abnormal lipid metabolisms[7, 8], disorders of the vascular endothelium[9], fat embolism[10], oxidative stress[11–13], apoptosis[8], genetic variants, thrombosis, alcohol abuse, long-term corticosteroid use[14], systemic lupus erythematosus, inflammatory bowel disease, and also for immunosuppression after renal transplants[15–17]. Though there are many clinical and basis studies about femoral head necrosis in previous studies, it is still unable to clarify the specific pathophysiological mechanism of the process by which corticosteroid administration induces the development of osteonecrosis [18, 19].

SONFH affects about 5–7.5 million people in the world and its incidence is increasing in China[20], Patients with SONFH are commonly asymptomatic, hence, take into account these unfavorable characteristics of SONFH, early perception of susceptible signs in patients on steroid medication is needed for prevention and early intervention. To address this problem, it is crucial and urgent for medical community to study the pathogenesis, identify diagnostic protein biomarkers for population seems to be the key point making prevention more efficient and to develop proper therapies to disrupt the progress of SONFH.

The beginning of proteomic technology applying and screen biomarker in femoral head necrosis is relatively late and there are rare reports[12, 13, 21, 22]. So, in this study, differentially expressed protein in serum among SONFH, TONFH patients and healthy volunteers in Chinese females was compared using iTRAQ, the proteomics study of femoral head necrosis will be helpful to screen potential diagnostic biomarkers for SONFH.

## Materials And Methods

### Patients

The study included a total 15 fresh serum specimens, were divided into three groups, SONFH, TONFH and CK group, respectively, each group contains 5 independent individuals. All the female patients and healthy volunteers were derived from clinical laboratory between April 2014 and June 2016 from the first Affiliated Hospital of Guangxi University of Chinese Medicine (Nanning, China). The systemic lupus erythematosus combined with SONFH and TONFH patients were selected according to the definite diagnosis by at least three orthopedists (Supplementary Table 1). Serum was distinguished from blood moderately drawn from bodies and then stored at -80 °C until detection.

**Table 1. Sample information of experiments**

Group	Number of females		Age (years, Median[range])	
	DDA	WB	DDA	WB
SONFH	5	10	44.6 (39-53)	43.5 (23~65)
TONFH	5	5	45.4 (30~61)	45.4 (30~61)
CK	5	8	43.0 (32~46)	42.0 (28~46)

### Protein extraction and digestion

For protein extraction, 100 µl the serum samples (each for 5 adult females) were employed for high abundant protein removal based on the ProteoPrep Blue Albumin and IgG Depletion Kit[13]. The protein eluent was precipitated with cold acetone for 3 h at -20 °C. After centrifugation at 4 °C at 12000 g for 10

min, the protein deposit was redissolved in lysis buffer (8 M urea, 100 mM triethylammonium bicarbonate (TEAB)). The protein concentration was determined using a Modified Bradford Protein Assay Kit according to the manufacturer's instructions. For digestion, 100 µg protein of each sample was first reduced with 10 mM DTT at 37 °C for 60 min and then alkylated with 25 mM iodoacetamide (IAM) at room temperature for 45 min in darkness. The urea concentration of protein sample was diluted to less than 2 M by adding 100 mM TEAB. The protein pool of each sample was digested with Sequencing Grade Modified Trypsin with the ratio of protein: trypsin = 50 : 1 mass ratio at 37 °C overnight and 100:1 for a continuous digestion for 4 h.

### **Protein isobaric labelling and sample cleanup**

After trypsin digestion, peptides were desalted by Strata X SPE column and vacuum-dried. Peptides were reconstituted in 100 µL 100 mM TEAB and processed according to the manufacturer's protocol for 6-plex tandem mass tags (TMT) kit[23]. Briefly, one unit of TMT reagent were all added to peptide solution after thawed and dissolved in 41 µL isopropanol. The peptide mixtures were incubated for 1 h at room temperature, then pooled and dried by vacuum centrifugation. The dried and labeled peptides were reconstituted with HPLC solution A (2% ACN, pH 10) and then fractionated into fractions by high-pH reverse-phase HPLC using Waters Bridge Peptide BEH C18 (130 Å, 3.5 µm, 4.6×250 mm). Briefly, peptides were first separated with a gradient of 2% to 98% acetonitrile in pH 10 at a speed of 0.5 ml/min over 88 min into 60 fractions. Until then, the peptides were combined into 20 fractions and dried by vacuum centrifugation. After which, the peptide fractions were desalted using Ziptip C18 according to manufacturer's instructions and finally dried under vacuum before kept at -20 °C until MS analyses were performed.

### **High-resolution LC-MS/MS analysis**

The mass spectrometry survey was performed by Nano LC 1000 LC-MS/MS using a Proxeon EASY-nLC 1000 coupled to an LTQ-Orbitrap Elite. Trypsin digestion fractions were reconstituted in 0.1% formic acid (FA) and directly loaded onto a reversed-phase pre-column (Acclaim PepMap®100 C18, 3µm, 100Å, 75µm× 2cm) delivering at 5 µL/min in 100% solvent A (0.1 M acetic acid in water). After that, peptides eluted from the trap column were loaded onto a reversed-phase analytical column (Acclaim PepMap® RSLC C18, 2 µm, 100Å, 50 µm × 15 cm) which gradient was comprised of an increase from 10% to 35% solvent B (0.1% FA in 98% ACN) over 60 min, 35% to 50% in 10 min and climbing to 100% in 5 min at a constant flow rate of 250 nl/min on an EASY-nLC 1000 system. The eluent was sprayed via NSI source at the 1.8 kV electrospray voltage and then analyzed by MS/MS in LTQ-Orbitrap Elite. The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS. Full-scan MS spectra (from m/z 350 to 1800) were acquired in the Orbitrap with a resolution of 60,000. Ion fragments were detected in the Orbitrap at a resolution of 15,000, and the 20 most intense precursors were selected for subsequent decision tree-based ion trap higher energy collision induced dissociation (HCD) fragmentation at the collision energy of 38% above a threshold ion count of 300 in the MS survey

scan with 30.0s dynamic exclusion. Full width at half maximum (FHMW) at 400 m/z using an automatic gain control (AGC) setting of 1e6 ions. Fixed first mass was set as 100 m/z.

## Data processing

The resulting MS/MS raw data were converted to mgf format profile with the software Proteome Discoverer (version 1.3, Thermo Scientific), generated peak lists were searched against the Homo Sapiens database (Taxon identifier: 9606, include 154527 protein sequences) consist of reviewed Swissprot database combined with unreviewed TrEMBL database, supplemented with frequently observed contaminants using Mascot 2.3.0. Trypsin/P was chosen as enzyme and two missed cleavages were allowed. Carbamidomethylation on cysteine was set as a fixed modification, and oxidation on methionine, acetylation in N-Term were set as variable modification. The searches were performed with using peptide mass tolerance of 20 ppm and product ion tolerance of 0.02 Da, resulting in 1% false discovery rate (FDR). For quantitation, proteins were required to contain at least two quantitated unique peptides. Proteins with fold changes  $> 1.2$  or  $< 0.83$  and significance t-test p-value  $< 0.05$  between two compared groups were considered to be differentially expressed.

Then proteins were classified by Gene Ontology annotation based on three categories: biological process, cellular component and molecular function. Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (<http://www.ebi.ac.uk/GOA/>). KEGG pathway is part of the KEGG database[24-26], which is a reference database for pathway mapping. KEGG Pathway analyses of identified proteins were extracted using the Search pathway tool in the KEGG Mapper platform (<http://www.genome.jp/kegg/mapper.html>). InterProScan, a sequence analysis application was also used for protein domain annotation based on protein sequence alignment algorithm and the InterPro domain database[27].

A two-tailed Fisher's exact test was employed to test the GO, KEGG pathway and Domain enrichment of the differential expression protein for all identified proteins. Correction for multiple hypothesis testing was carried out under standard FDR control methods, and pathways with a corrected p value  $< 0.05$  were considered the most significant.

Expression-based and functional enrichment-based clustering for different protein groups was used to explore potential relationships between different protein groups at special protein function. Firstly, all the protein groups obtained after functional enrichment analysis along with their P values were collected. Secondly, those categories enriched were sorted in at least one of the protein groups with a P value  $< 0.05$ . This filtered P value matrix was transformed by the function  $x = -\log_{10}(P \text{ value})$ . Thirdly, z-transformed applies on x values for each functional category and z scores were clustered by one-way hierarchical clustering (Euclidean distance, average linkage clustering). Finally, the expression pattern of the protein was completed using the timeclust function in the R package 'TCseq', a heatmap was generated by the software Heml 1.0.3 [28]. We also present the STRING network of some known/novel protein-protein interactions as an evidence view by using the String 9.0 (Search Tool for the Retrieval of Interacting Genes/Proteins) database of physical and functional interactions (<http://string-db.org/>) [29].

## Western blotting

Equal amounts of proteins (30 µg) were separated by 12% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). Membranes were blocked for 4 h at 4 °C with 5 % non-fat milk in 1 × TBST buffer and rinsed 3 times (10 min each) with 1 × TBST buffer. Next, the membranes were incubated with primary antibodies: Anti-C reactive protein (1:1000, Abcam, ab32412), Anti-alpha-1-acid glycoprotein 1 (1:1000, Abcam, ab134160), Anti-SAA1 (1:1000, Abcam, ab201660), Anti-dopamine beta-hydroxylase (1:2000, Abcam, ab96615) at 4 °C overnight and then washed with 1 × TBST buffer for 3 times followed by the secondary antibodies: Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) (1:20000, Jackson, 115-035-003) and Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L) (1:20000, Jackson, 111-035-003) for 4 h at 4 °C. After washing 3 times, the membranes were developed with using Western blotting ECL Substrate (Bio-Rad) detection methods (Solarbio, PE0010) and scanned with the ChemiDoc MP imaging system using Image Lab software (Bio-Rad).

## Results

### Protein identification and expression of different protein (EDPs) screening

A total of 950 proteins were identified in 5 replicates LC-MS/MS experiments at the high confidence of peptide selection (FDR=0.01), among which quantitative information was obtained for 529 proteins (Supplementary Table 2). Volcano diagram shows the EDPs of each comparison groups, with up-regulated proteins marked by red points distributing over the top right of the graph and down-regulated proteins marked by blue points distributing over the top left of the graph (Fig. 1). There were 17 proteins show a significant differential expression (fold change > 1.2 and p value < 0.05) between the SONFH and CK groups with 8 up-regulated and 9 down-regulated proteins (Table 2), meanwhile table 2 lists the KEGG pathway annotation and COG category of the EDPs between the SONFH and the Control groups.

### Protein expression patterns and function clustering

To investigate the protein profile change under the two pathological situations compared to healthy volunteers, the protein Domain and KEGG pathway annotation were elaborated from InterProScan platform and KAAS online automatic annotation server (<http://www.genome.jp/tools/kaas/>). Then the confidence of function enrichment was evaluated by the fisher exact test, all quantified proteins were classified into 8 clusters based on the expression level (Fig. 2). Domain enrich-based clustering of each expression cluster shows that IgG enriched expressed highly in clusters 2, 4 and 6 in SONFH group, while the peptidase S1 and fibrinogen, transferrin presents present low expression enriched level in Clusters 3 and 5 in SONFH group, in Cluster 8 with enriched higher expression in lipid transport and vitellinogen proteins of the TONFH group. KEGG pathway enrich-based function clustering analysis exhibit remarkably enriched expressed proteins in hematopoietic cell lineage, adhesion and several steroid derived signal pathways of Cluster 4 and 6 in SONFH groups. Cluster 8 reveals high expression proteins in TONFH group distinctly enriched in fat digestion and absorption pathways. Interestingly, PPAR signal pathway was also find lower expression level in SONFH and TONFH groups.

## PPI network analysis

STRING was then used to further analyze the functional correlation of differentially expressed proteins between each group. The Fig. 3A announced that alpha-1-acid glycoprotein 1 (P02763) and haptoglobin (P00738, HP) connected the complement pathway and the coagulation pathway that may take potential interactions. And the interaction network EDPs between SONFH and TONFH indicated that beta-2-glycoprotein 1 (P02749, APOH) was employed an important role between these two pathological states (Fig. 3B).

## Western blot validations

Based on proteomic results, C-reactive protein, serum amyloid A protein, serum alpha-1-acid glycoprotein 1 and dopamine beta-hydroxylase were selectively examined by using western blot. As shown in Fig. 4, in SONFH versus TONFH and CK, significantly increased level of C-reactive protein, serum amyloid A protein, serum alpha-1-acid glycoprotein 1, which is consistent to iTRAQ (based on LC-MS/MS identification and quantification). Meanwhile, western blotting also verified the decreased expression of dopamine beta-hydroxylase (DBH) in TONFH versus SONFH and CK, respectively. Western blotting was consistent with the results of the mass spectrometry analysis, all these data added confidence to the results obtained from iTRAQ.

## Discussion

Currently, steroid-related SONFH is a frequently occurring disease that can lead to necrosis of bone tissue and arthritis of the hip joint in patients who receive high-dose corticosteroid therapy. Though lots of studies have been paid to clarify their pathogenesis, the pathogenesis of SONFH is still unclear, and very little proteomic research has been done to high-throughput investigate the simultaneous expression of serum proteins and diagnostic biomarkers in adult SONFH patients.

The present study investigated proteomic on SONFH, TONFH patients and healthy volunteers of Chinese female, the results show: (1) 17 proteins differentiated between SONFH and CK groups, in which there were 8 proteins up-regulated (ratio > 1.2, p-value < 0.05) and 9 proteins down-regulated (ratio < 1/1.2, p-value < 0.05). (2) In screening protein clusters, IgG enriched was up-regulated in SONFH group, the peptidase S1 and fibrinogen, transferrin presents down-regulated in SONFH group. (3) Hematopoietic cell lineage, adhesion and several steroid derived signal pathways were up-regulated in SONFH, and the fat digestion and absorption pathways with highly expression in TONFH group. PPAR signal pathway was also found lower expression level in SONFH, TONFH versus CK groups. (4) Several other proteins such as C-reactive protein, serum amyloid A protein, alpha-1-acid glycoprotein 1 and dopamine beta-hydroxylase displayed similar expression in SONFH, TONFH versus CK groups.

PPAR $\gamma$  are believed to be the master regulator of adipogenesis and also show well described anti-osteoblastogenic effects, which can stimulate adipogenesis in bone marrow cells and inhibit osteogenesis[30], PPAR $\gamma$ -deficient embryonic stem cells differentiated into osteoblasts[31], and several

drugs functioning on PPAR $\gamma$  inhibition have been confirmed efficient for SONFH[31–34]. In our study, the PPAR expression pattern in the femoral head osteonecrosis in both SONFH and TONFH groups was significantly lower than CK group, which is in line with previous studies showing the transcription factors PPAR $\gamma$  was clarified taking a crucial role in TONFH pathological process.

Two Wnt signal pathway related proteins pigment epithelium-derived factor (P36955, PEDF) and Kallistatin (P29622) were identified down-regulated in SONFH group. And Wnt signaling pathway has been confirmed involved in pathogenesis of SONFH [35, 36], Prarastatin and Huogu I formula has been reported to prevent SONFH in rat through the affection on Wnt signal pathway[31, 34].

An immune response is involved in the pathogenesis of many diseases, including femoral head necrosis in adult patients [37–39]. In function clustering analysis, IgG domain containing proteins were observed dramatically elevated in steroid-induced SONFH Chinese women, were both significantly reduced abundance expression in TONFH and CK groups, revealing that an immune response may be involved in this disease.

Abnormal expression of lipoproteins and apolipoproteins would lead to the imbalance of lipid metabolism, the lipid metabolism imbalance has been either reported playing important role in SONFH pathogenesis [40–43].The TONFH patients showed higher abundance of lipid transport domain in this present study, which are involved in fat digestion and absorption pathway.

Recent reports have shown that key proteins involved in signal transduction and regulation[44], important proteins including tissue-type plasminogen activator, plasminogen activator inhibitor type 1, Crosslaps, and anti-p53 antibody were suggested as the diagnosis serum markers of nontraumatic femoral head necrosis[21]. A number of proteins, C-reactive protein, serum amyloid A protein, alpha-1-acid glycoprotein 1 and dopamine beta-hydroxylase in patients with SONFH are always significantly different from those in patients with TONFH and CK groups. Furthermore, four difference expressed proteins (CRP, SAA1, ORM1 and DBH) were verified by western blot and showed consistent results with iTRAQ. High sensitivity C-reactive protein has a drastic change under inflammation, which increased in osteoporotic/osteopenia women compared to obese normal[45], it has been well known as a diagnostic marker for osteopenia, atherosclerotic cardiovascular disease and some other diseases[46].

## Conclusions

In this study, the results suggest that dysfunction of hematopoietic cell lineage, adhesion, fat digestion and absorption, PPAR pathways may be involved in the pathogenesis of SONFH, serum proteins SAA1, ORM1 may be used as new potential diagnostic biomarkers for SONFH. Though we did verify four differentially expressed proteins, this study has some limitations, the proteomic was performed analysis at only one time point on three quite small study populations, further analysis of these results in larger study populations from different ethnic groups would provide more weight to the results. In summary, these findings would be helpful to advance knowledge in this area of study.

# Abbreviations

C9: Complete component C9, CP: Carboxypeptidase, GSN: Gelsolin, IGFBP3: Insulin-like growth factor-binding protein 3, CFP: Properdin, KRTDAP: Keratinocyte differentiation-associated protein, SERPINF1: Pigment epithelium-derived factor, F12: Coagulation factor XII, QSOX1: Sulfhydryl oxidase 1, SERPINA4: Kallistatin, F13A1: Coagulation factor XIII A, C8B: Complement component C8 beta chain, C5: Complement C5, CPB2: Carboxypeptidase B2, CD14: Monocyte differentiation antigen CD14, IGFBP3: Insulin-like growth factor-binding protein 3.

# Declarations

## Ethics approval and consent to participate

The study was approved by the Institutional Review Board of The First Affiliated Hospital of Guangxi University of Chinese Medicine, and informed consents were obtained from each patient and healthy volunteers.

## Consent for publication

Not applicable.

## Availability of data and materials

All data generated or analyzed during this study were included in this published article and its additional files.

## Competing Interests

The authors declare no financial and non-financial competing interests.

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## Authors' Contributions

ZP and ZCX conceived and designed the experiments. ZYQ, LJY and LT contributed reagents/materials/analytic tools. ZYQ, LJY, PDD and LT performed the experiments. LCR collected and analysed the data. LX, CJL and FSQ analysed the data. ZP and ZCX wrote the paper. LX helped review and edit the final paper. All authors read and approved the final manuscript.

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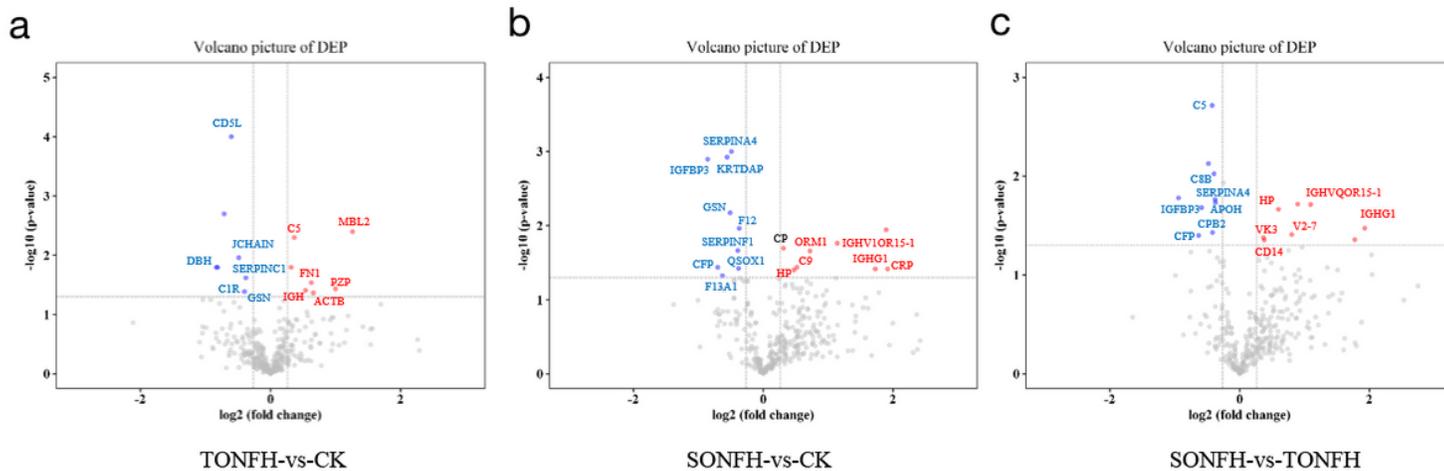
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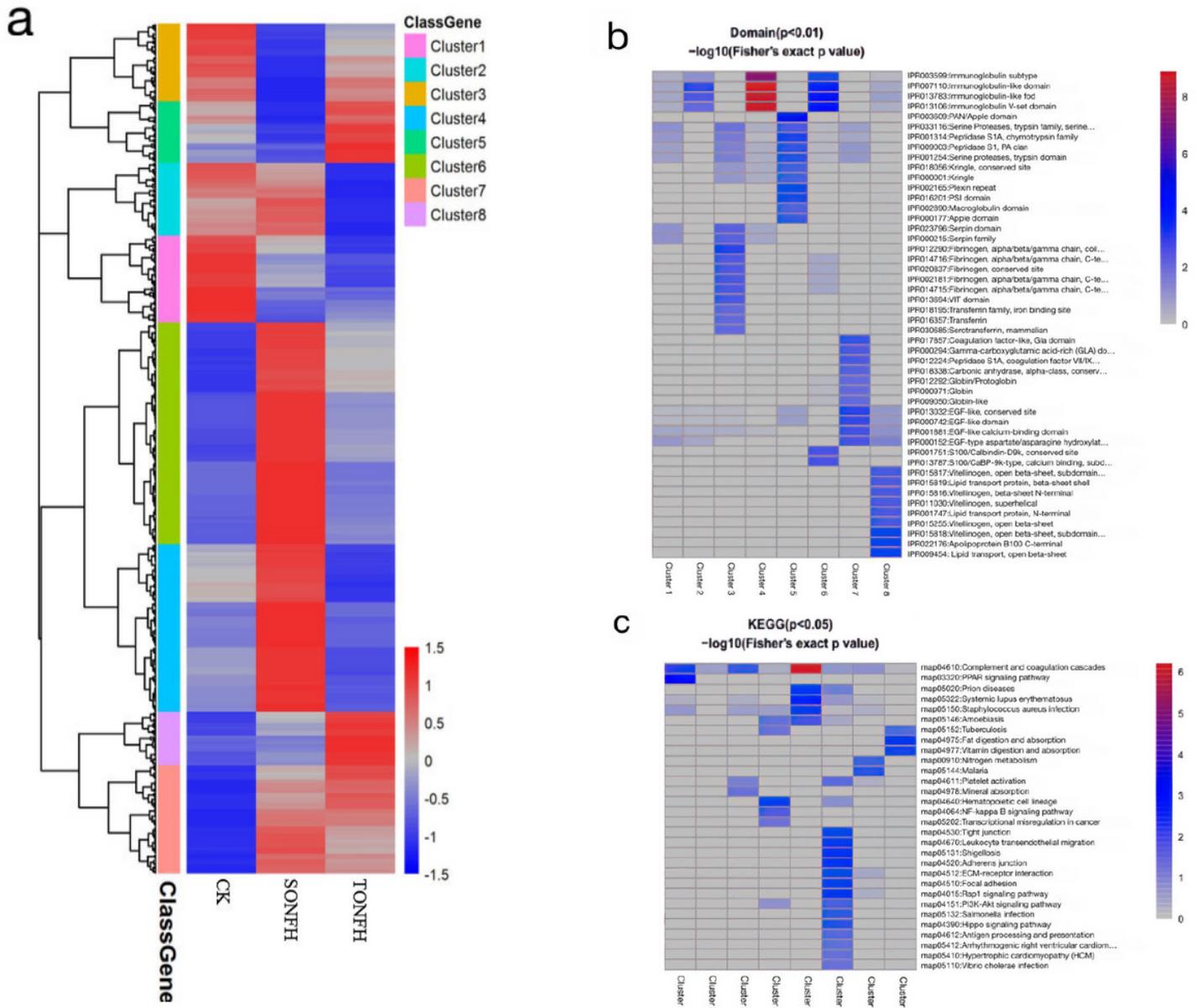
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## Figures



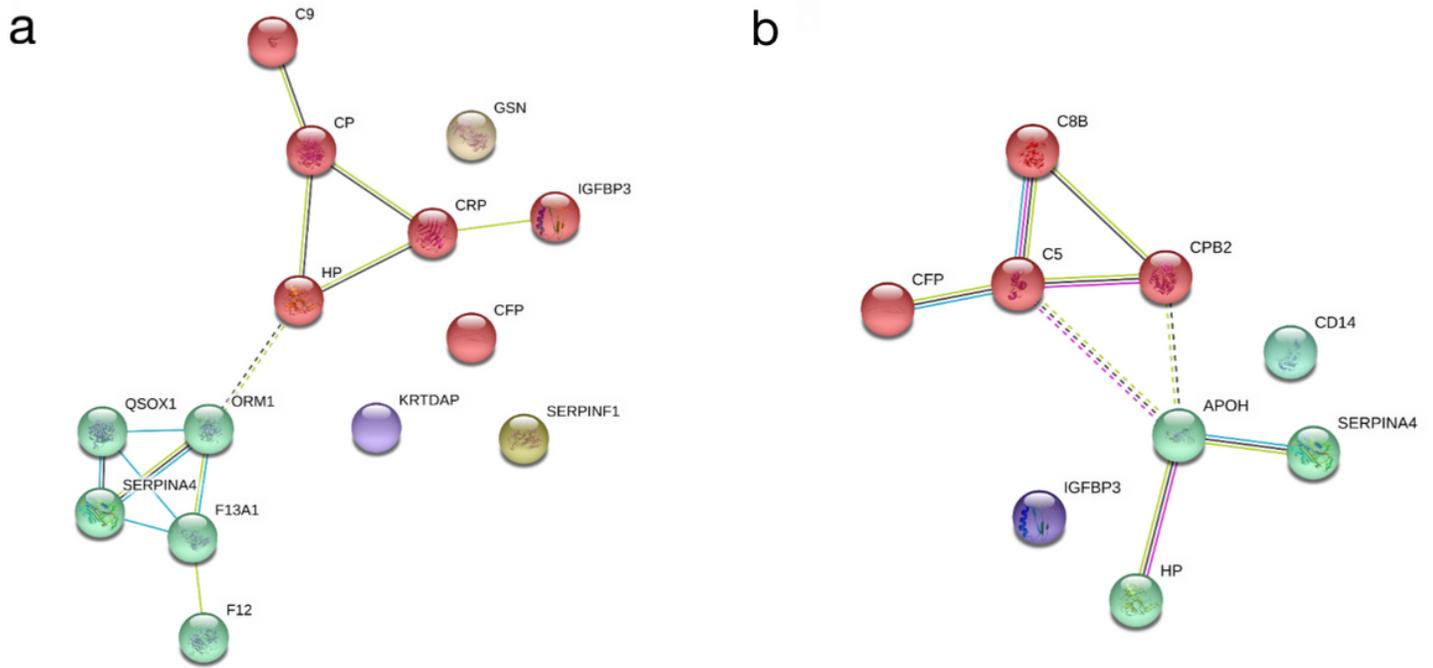
**Figure 1**

Volcano plots for EDPs screening. a EDPs in TONFH versus CK. b EDPs in SONFH versus CK. c DEPs in SONFH versus TONFH. The down- and up-regulated proteins were colored with blue plots in the upper left and red plots.



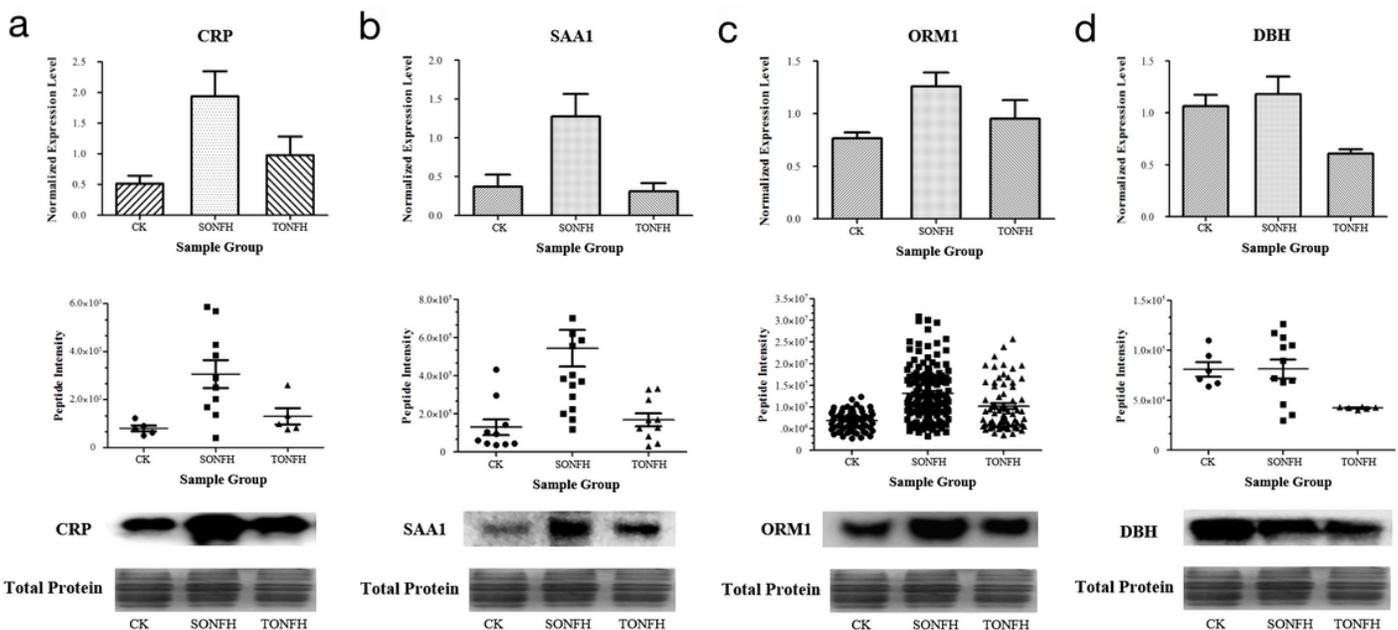
**Figure 2**

Clustering of protein expression patterns and functional enrichment. a Differential protein relative expression matrix heatmap. b,c Functional enrichment-based clustering for protein groups in Domain and KEGG. The color  $-\log_{10}$  (Fisher exact test p value) represents the credibility of enrichment.



**Figure 3**

Differentially expressed protein (alpha-1-acid glycoprotein 1(ORM1), haptoglobin(HP), and beta-2-glycoprotein 1 (APOH)) interaction network analysis. a SONFH versus CK. b SONFH versus TONFH. Colors represent the types of evidence for the association: green = neighbourhood, red = gene fusion, pink = experiments, light green = text mining, blue = cooccurrence, dark blue = coexpression, purple = homology.



**Figure 4**

Validation of 4 EDPs, protein expression level and unique peptide intensity of four proteins. a C-reactive protein(CRP). b Serum amyloid A protein(SAA1). c Alpha-1-acid glycoprotein 1(ORM1). d Dopamine beta-hydroxylase (DBH) between each group.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryTable1.xls](#)
- [SupplementaryTable2.xls](#)