Cerebrospinal Fluid Protein Profiles in Alzheimer's Dementia Patients: A Bioinformatic Approach

Dear Editor,

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. A long asymptomatic phase and the absence of a definite confirmatory test for AD diagnosis results in about two-thirds of cases being undiagnosed early on.[1] Biomarkers may not only be important for early AD diagnosis but also in charting its pathogenesis. For a long time, the amyloid β and tau hypothesis has been considered the backbone of AD pathogenesis. AD is pathologically characterized by the deposition of extraneuronal amyloid β 42 (Aβ42) plaques and intraneuronal neurofibrillary tangles of phosphorylated tau protein. The validated cerebrospinal fluid (CSF) biomarkers of AD hence include low levels of Aβ42 and elevated total and phosphorylated tau.[2] However, the model has been questioned in recent times, and the role played by reactive oxygen species, mitochondrial dysfunction, metabolic syndrome-like alterations in the brain, and lipid dysmetabolism have been highlighted.[3] In the present study, we aimed to identify tentative CSF protein biomarkers of AD by liquid chromatography-tandem mass spectrometry (LC-MS/MS). We also planned a bioinformatics analysis of the data obtained to try to identify potential pathogenetic clues for AD.

We performed a case-control study with probable AD patients and age-sex–matched cognitively normal controls. Following written informed consent, 2 mL of CSF was collected from all patients

and 1 mL of CSF from each control. The study was performed between June 2015 and May 2017 in the Sir Sunderlal hospital of the Banaras Hindu University which is a large teaching and research medical institution of north India. AD patients were randomly enrolled from the outpatients' Geriatric services. Patients aged >60 years with cognitive issues were initially screened with the Hindi Mental State Examination (cutoff score ≤23) and then diagnosed as AD cases using the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5) criteria, supported by magnetic resonance imaging of the brain. Healthy controls were randomly chosen from patients undergoing operative procedures under spinal anesthesia. They underwent preoperative evaluation to rule out dementia. The study was initiated after ethical committee approval. AD patients on medications, those with illness requiring hospitalization, delirium, or any condition interfering with neuropsychological testing were excluded. Those with chronic illnesses were excluded from the control group.

Protein content in CSF samples was estimated by Bradford's reagent; then the samples were reduced and alkylated with 100 mM DTT at 60°C for 15 minutes and 200 mM iodoacetamide in the dark for 30 minutes, respectively. Further overnight digestion of the sample was carried out with trypsin in 1:25 ratio at 37°C. The peptide digest (3 µg) was separated by using Eksigent MicroLC 200 system (Eksigent, Dublin, CA) equipped with Eksigent C18-reverse phase column (100*0.3 mm, 3 µm, 120Å). The sample was loaded onto the column with 97% of mobile phase A (100% water, 0.1% Formic acid [FA]) and 3% of mobile phase B (100% Acetonitrile [ACN], 0.1% FA) at 7 µL/min flow rate. Peptides were eluted with a 120-minute linear gradient of 3% to 50% mobile phase B. 3 µg of peptide was loaded for each sample in column for Information Dependent Acquisition analysis and then was run for 2 hours. The column was washed with 3% ACN. Furthermore, 1 µl (~1-2 µg) of peptide was loaded for each sample in the column for SWATH analysis and then the sample was run for 2 hours followed by washing with 3% ACN. AB Sciex Triple Time-of-Flight 5,600 instrument and Information Dependent Acquisition protocol was used for the construction of fragment ion library. The spectral alignment and targeted data extraction of SWATH-MS (Sequential Window Acquisition of all Theoretical Mass Spectra) data were performed using Peakview software, Version 1.2.03 (AB Sciex, MA, USA). The peptide data were used for quantification of peptides of CSF using Markerview software, Version 1.2.1.1 (AB Sciex, MA, USA). The peptides with a *P* value ≤0.05 were considered significant with respect to differential expression in AD cases and controls (cutoff 1.5-fold for upregulated proteins; 0.75-fold for downregulated proteins). The peptide data from CSF samples of both AD and control groups were then compared with complete human proteome data available online. The differentially expressed proteins were subjected to bioinformatics analysis. STRING (version 11.0) was used to create a network map. Initially, medium confidence score was taken (0.4). Later outlier proteins were removed, and confidence score was increased to 0.9. Enrichment analysis was performed for the protein-protein interaction (PPI) network and patterns were identified.

The study involved six cases (five males, one female; mean age 68.5 ± 8.11 years) and six controls (four males, two females; mean age 67.08 ± 5.31 years). The median symptom duration was 2.5 years and mean Hindi Mental State Examination score in the case group was 17.5 (± 3.93). The Clinical Dementia Rating global score of all patients was 2. Thirteen proteins were upregulated in the CSF of AD patients [Supplementary Figure 1], while 30 proteins were downregulated [Supplementary Figure 2]. On STRING analysis, 20 proteins were found to be participating in a closed PPI network [Supplementary Figure 3]. After removing outliers, a simplified PPI network (confidence score 0.9) was generated [Supplementary Figure 4].

Functional enrichment analysis was also performed. The largest enrichment effect (strength >2) for biological processes was observed for behavioral fear response regulation (Apolipoprotein E [APOE] and Proenkephalin [PENK]) [Supplementary Figure 5]. APOE, Apolipoprotein H (APOH), prothrombin (F2), and PENK were found to be involved in multiple biological processes. The interactions of each of PENK, Osteopontin (SPP1), and Alpha 2-HS glycoprotein (AHSG), with APOE, a known pathogenetic marker of AD, may be relevant.

Six hierarchical clusters were significantly enriched. Regulation of insulin-like growth factor transport and uptake was a component of three of the six clusters, and included AHSG, APOE, insulin-like growth factor-2 (IGF-2) and F2 [Supplementary Figure 6]. There are interactions between insulin and amyloid-β precursor protein (AβPP or APP) but the mechanisms are uncertain.[4] Brain insulin resistance has been correlated with the pathogenesis of AD.[5] IGF-2 binds and activates insulin receptors.[6] Alterations in IGF-2 signaling result in decreased levels of insulin receptor substrate (IRS) mRNA and PI3K/phospho-Akt. The resulting increase in glycogen synthase kinase-3β activity increases amyloid precursor protein mRNA expression.[7] A decrease in prothrombin levels may indirectly signify greater conversion to thrombin. Thrombin stimulates inflammatory cytokines including IL-6 and monocyte chemoattractant protein-1, thus reducing IRS/Akt phosphorylation and producing insulin resistance.[8] AHSG also has an action on insulin-signaling. Both insulin-stimulated insulin receptor autophosphorylation and insulin-induced tyrosine phosphorylation of IRS-1 are inhibited by it.[9] However, at present, there is no clear evidence for the role of CSF AHSG in insulin resistance limited to the brain. Proenkephalin may be hypothesized to be involved in interactions of the insulin and opioid pathways in the brain. Insulin-receptor binding in the nucleus accumbens has been shown to activate opioid receptor-mediated release of presynaptic glutamate. This is involved in cognition.[10] The evidence linking APOE and cerebral insulin is already significant.

A molecular function of interest for us was glycosaminoglycan (GAG) binding. Five proteins were annotated with GAG binding (APOE, APOH, F2, serum amyloid A1 or SAA1, and CD44) [Supplementary Figure 7]. Aβ plaques are known to bind to GAGs. Aβ fibril formation is enhanced by these GAGs, leading to the extracellular accumulation of Aβ.[11] The APOE C-terminal domain binds with cell surface GAGs with APOE4 having a physiologically significant greater binding ability.[12]

Multiple CSF proteomic studies have been performed on AD patients. A study from the National Institute of Mental Health and Neuro Sciences (Bengaluru) reported differential expression of the proteins: microtubule associated protein tau, Neuronal pentraxin-2, Neurosecretory protein VGF, Glial fibrillary acidic protein, and Neural cell adhesion molecule 1 in the CSF of AD patients as compared to non-AD controls.[13] This study used high-resolution mass spectrometry. In a recent systematic review, authors generated a database of CSF proteins altered in AD patients, from 47 validated proteomics studies. The authors identified 27 proteins and 21 peptides with statistically significant alterations in AD.[14] However, previous data need to be supported by findings from different geographical zones. There is a paucity of data from north India in this regard which our study tried to address. This may also add new dimensions to the knowledge about AD pathogenesis. The major limitation of our study was a small sample size which may affect the external and internal validity of the results. However, despite this, we included a broader group of proteins in bioinformatic analysis by using 1.5-fold–change and 0.75-fold–change thresholds. We are planning to test the identified proteins in a larger sample size using enzyme-linked immunosorbent assay as part of the validation phase of this study. The identification of markers such as F2 and their close interaction with APOE is of interest. There is a need to explore the role of the insulin regulatory pathway and GAG binding in AD pathogenesis. STRING is an attractive software for creating PPI networks for analysis of LC-MS/MS data and may be useful in larger similar studies in the future. The findings from our study would need validation in larger groups of patients.

Financial support and sponsorship

a. National Program for Health Care of the Elderly, Ministry of Health and Family Welfare, Government of India.
b. Indian Council of Medical Research – Senior Research Fellowship grant 3/1/2/(23)/GER/2019-NCD-II.

Conflicts of interest

There are no conflicts of interest.

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References

1. Ho L, Fivecoat H, Wang J, Pasinetti GM. Alzheimer's disease biomarker discovery in symptomatic and asymptomatic patients: Experimental approaches and future clinical applications. Exp Gerontol 2010;45:15-22.
2. Jack Jr CR, Albert M, Knopman DS, McKhann GM, Sperling RA, Carillo M, *et al*. Introduction to revised criteria for the diagnosis of Alzheimer's disease: National Institute on Aging and the Alzheimer Association Workgroups. Alzheimer's Dement J Alzheimer's Assoc 2011;7:257-62.
3. Chakrabarti S, Khemka VK, Banerjee A, Chatterjee G, Ganguly A, Biswas A. Metabolic risk factors of sporadic Alzheimer's disease: Implications in the pathology, pathogenesis and treatment. Aging Dis 2015;6:282-99.
4. Shieh JC, Huang PT, Lin YF. Alzheimer's disease and diabetes: Insulin signaling as the bridge linking two pathologies. Mol Neurobiol 2020;57:1966-77.
5. Watson GS, Craft S. The role of insulin resistance in the pathogenesis of Alzheimer's disease. CNS Drugs 2003;17:27-45.
6. Lara Fernández E, Valverde N, de Luque V, Boraldi F, Santin LJ, Pavía J, *et al*. IGF-II as a neuroprotective and neuroplastic factor in an oxidative damage model induced by glucocorticoids. RiUMA 2018. Available from https://hdl.handle.net/10630/16601. [Last accessed on 2023 May].
7. Steen E, Terry BM, Rivera EJ, Cannon JL, Neely TR, Tavares R, *et al*. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease–is this type 3 diabetes? J Alzheimer's Dis 2005;7:63-80.
8. Mihara M, Aihara K, Ikeda Y, Yoshida S, Kinouchi M, Kurahashi K, *et al*. Inhibition of thrombin action ameliorates insulin resistance in type 2 diabetic db/db mice. Endocrinology 2010;151:513-9.
9. Andersen G, Burgdorf KS, Sparsø T, Borch-Johnsen K, Jørgensen T, Hansen T, *et al*. AHSG tag single nucleotide polymorphisms associate with type 2 diabetes and dyslipidemia: Studies of metabolic traits in 7,683 white Danish subjects. Diabetes 2008;57:1427-32.
10. Oginsky MF, Santana-Rodriguez Z, Ferrario CR. Insulin enhances presynaptic glutamate release in the nucleus accumbens via opioid receptor-mediated disinhibition. BioRxiv 2019;517797.
11. Ariga T, Miyatake T, Yu RK. Role of proteoglycans and glycosaminoglycans in the pathogenesis of Alzheimer's disease and related disorders: Amyloidogenesis and therapeutic strategies — A review. J Neurosci Res 2010;88:2303-15.
12. Yamauchi Y, Deguchi N, Takagi C, Tanaka M, Dhanasekaran P, Nakano M, *et al*. Role of the N-and C-terminal domains in binding of apolipoprotein E isoforms to heparan sulfate and dermatan sulfate: A surface plasmon resonance study. Biochemistry 2008;47:6702-10.
13. Sathe G, Na CH, Renuse S, Madugundu AK, Albert M, Moghekar A, *et al*. Quantitative proteomic profiling of cerebrospinal fluid to identify candidate biomarkers for Alzheimer's disease. Proteomics Clin Appl 2019;13:e1800105. doi: 10.1002/prca.201800105.
14. Pedrero-Prieto CM, García-Carpintero S, Frontiñán-Rubio J, Llanos-González E, Aguilera García C, Alcaín FJ, *et al*. A comprehensive systematic review of CSF proteins and peptides that define Alzheimer's disease. Clin Proteomics 2020;17:1-24. doi: 10.1186/s12014-020-09276-9.

Submitted: 09-Mar-2023 **Revised:** 09-May-2023 **Accepted:** 10-May-2023
Published: 06-Jul-2023

DOI: 10.4103/aian.aian_206_23

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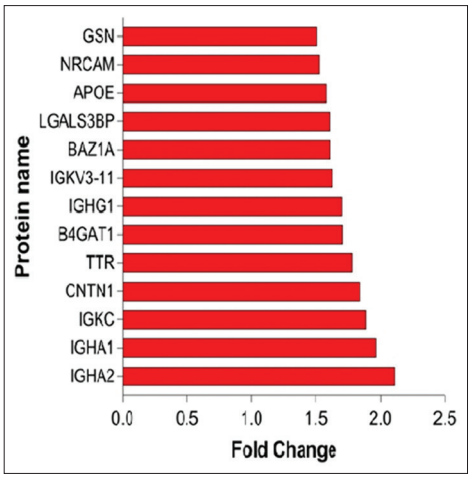
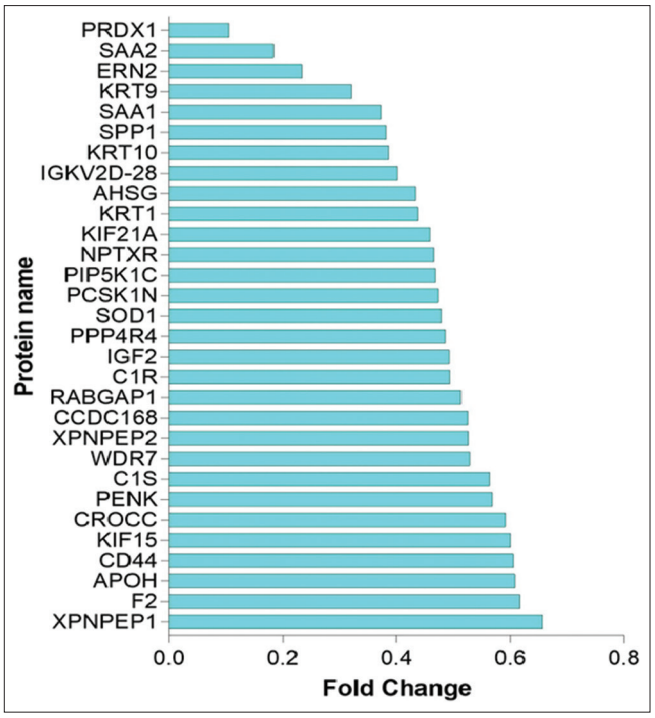
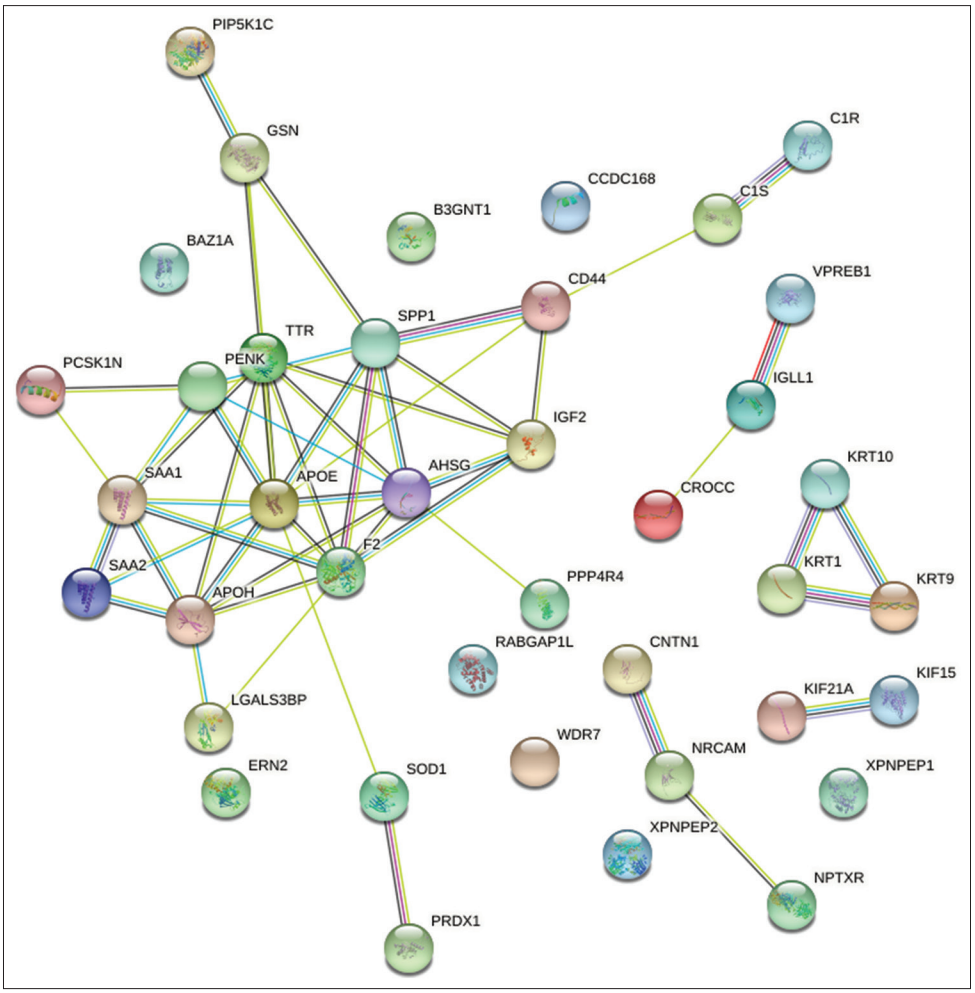


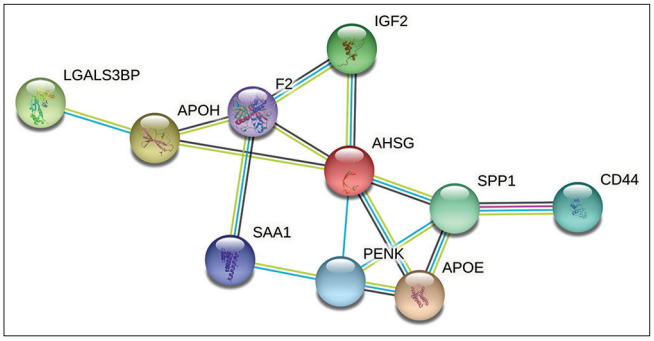
Supplementary Figure 1: A graph of upregulated 13 proteins in CSF of AD patients as compared to controls (analyzed by GraphPad PRISM 7.00.159 Tool). Fold change >1.5 is considered as threshold level for upregulation. **(**GSN, Gelsolin; NRCAM, Neuronal Cell Adhesion Molecule; APOE, Apolipoprotein E; LGALS3BP, Galectin-3-binding protein; BAZ1A, Bromodomain adjacent to zinc finger domain protein 1A; IGKV3-11, Immunoglobulin kappa variable 3-11; IGHG1, Immunoglobulin heavy constant gamma 1; B4GAT1, Beta-1,4-glucuronyltransferase 1; TTR, Transthyretin; CNTN1, Contactin-1; IGKC, Immunoglobulin Kappa Constant; IGHA1, Immunoglobulin Heavy Constant Alpha 1; IGHA2, Immunoglobulin Heavy Constant Alpha 2**)**



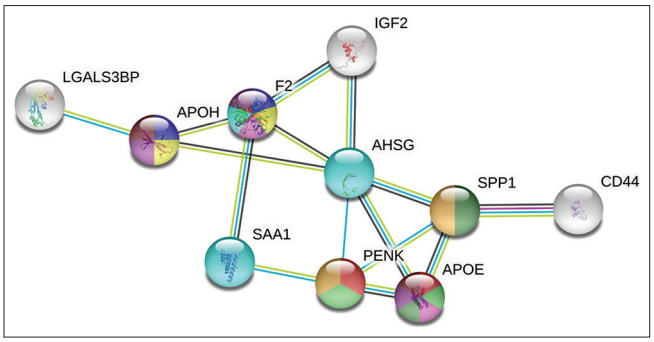
Supplementary Figure 2: A graph of down regulated 30 proteins in CSF of AD patients as compared to controls (analyzed by GraphPad PRISM 7.00.159 Tool). Fold change <0.75 is considered as threshold level for downregulation. (PRDX1, Peroxiredoxin-1; SOD1, Superoxide dismutase [Cu-Zn]; SPP1, secreted phosphoprotein 1/Osteopontin; CD44, CD44 antigen; AHSG, Alpha-2-HS-glycoprotein; KIF21A, Kinesin-like protein KIF21A; KIF15, Kinesin-like protein KIF15; PIP5K1C, Phosphatidylinositol 4-phosphate 5-kinase type-1 gamma; SAA1, Serum amyloid A-1 protein; SAA2, Serum amyloid A-2 protein; KRT1, Keratin type II cytoskeletal 1; KRT9, Keratin type I cytoskeletal 9; KRT10, Keratin type I cytoskeletal 10; C1R, Complement C1r subcomponent; C1S, Complement C1s subcomponent; IGKV2D-28, Immunoglobulin kappa variable 2D-28; ERN2, endoribonuclease IRE2; NPTXR, Neuronal pentraxin receptor; CCDC168, Coiled-coil domain-containing protein 168; PCSK1N, ProSAAS; F2, Prothrombin; PENK, Proenkephalin-A; PPP4R4, Serine/threonine-protein phosphatase 4 regulatory subunits 4; APOH, Beta-2-glycoprotein 1; IGF2, Insulin-like growth factor II; WDR7, WD repeat-containing protein 7; RABGAP1L, Rab GTPase-activating protein 1-like; CROCC, Rootletin; XPNPEP1, Xaa-Pro aminopeptidase 1; XPNPEP2, Xaa-Pro aminopeptidase 2)



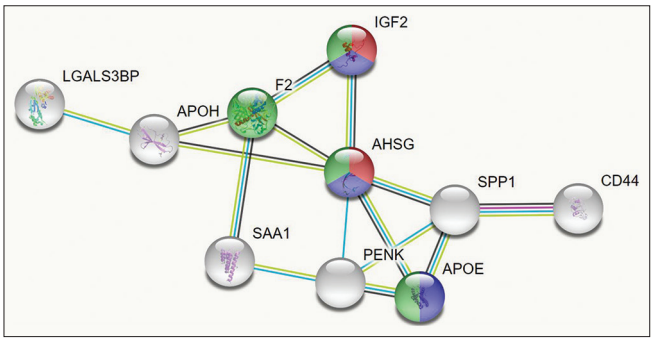
Supplementary Figure 3: Protein-protein interaction (PPI) network of upregulated and downregulated proteins in the cerebrospinal fluid of AD patients (STRING 11.0; confidence level 0.40)



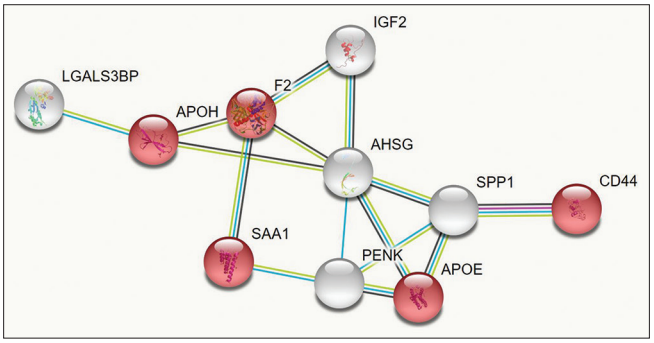
Supplementary Figure 4: Simplified PPI network after removing outliers (STRING 11.0; confidence level 0.90). A network of 10 closely interacting proteins was obtained: PENK, F2, APOH, AHSG, SAA1, SPP1, CD44, IGF2 are downregulated proteins while LGALS3BP and APOE are upregulated



Supplementary Figure 5: Enrichment analysis of STRING PPI network- Biological process. Proteins which are annotated with a particular process have been color-coded accordingly. Red- Regulation of behavioral fear response, Deep blue- Negative regulation of fibrinolysis, Light green- Locomotory exploration behavior, Yellow- Blood coagulation, intrinsic pathway, Pink- Negative regulation of platelets activation, Dark green- Steroids catabolic process, Light blue- Acute-phase response, Amber- Response to vitamin D, Violet- Negative regulation of blood coagulation, Brown- Negative regulation of endothelial cell proliferation



Supplementary Figure 6: Enrichment analysis of STRING PPI network- Local network clusters. Proteins which are part of a particular cluster have been color-coded accordingly. Red- Regulation of Insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding protein (IGFBPs), Deep blue- Regulation of insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding protein (IGFBPs) and plasma lipoprotein particle, Light green- Regulation of insulin-like growth factor (IGF) transport and uptake by insulin-like growth factor binding protein (IGFBPs) and formation of fibrin clot (clotting Cascade)



Supplementary Figure 7: Enrichment analysis of STRING PPI network- Molecular function. Proteins involved in glycosaminoglycan binding have been labeled with red