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Title: Serum Proteomic study of Women with Obsessive-Compulsive Disorder, Washing Subtype

**Authors:** 

Mona Zamanian-Azodi<sup>1,2</sup>, Mostafa Rezaei-Tavirani<sup>2,\*</sup>, Mohammad Mahboubi<sup>3</sup>, Mohsen Hamidpour<sup>2</sup>, Majid Rezaei Tavirani<sup>4</sup>, Mostafa Hamdieh<sup>5</sup>, Mohammad Rostami-Nejad<sup>6</sup>, Naser Nejadi<sup>7</sup>, Mohammad

Kamran Derakhshan⁵

1-National Elites Foundation, Tehran, Iran

2- Proteomics Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

3- Abadan School of Medical Sciences, Abadan, Iran

4-Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran.

5- Psychosomatic Department, Taleghani Hospital, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

6- Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

7- Department of Clinical Biochemistry, Faculty of Medical Science, Tarbiat Modares University,

Tehran, Iran

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#### **Abstract**

Many genetic studies are conducted for obsessive-compulsive disorder; however, there is not yet a throughout examination of proteome profile of this severe disorder. Here, the proteomic study of OCD patients' serum samples is conducted by the application of two-dimensional electrophoresis (2DE) followed by Mass Spectrometry (MALDI-TOF-TOF). A total of 240 protein spots were detected and among them, five significant differentially expressed protein spots with the fold change ≥ 1.5 were consider for further evaluations. These proteins include two isoforms of HP, IGKC, GC, and HPX. The first two shows down-regulation while the last ones indicate upregulation. Moreover, a validation study of overall HP levels in patients' serum via Nephelometric quantification confirmed the lower levels of this protein in the serum of OCD patients. Additionally, enrichment analysis and validation test revealed that inflammation is one of most dominant processes in OCD. It is suggested that these candidate proteins and their underlying processes (especially, inflammation) may be linked to OCD pathophysiology and may have a clinical utility after extensive validation studies.

\*Corresponding Author: Tavirany@yahoo.com

**Keywords:** Obsessive-Compulsive Disorder; Washing Subtype; Proteomics; Protein-protein Interaction Network Analysis

### Introduction

Obsessive-compulsive disorder (OCD) as a complex and debilitating mental condition, has about 2 to 3% lifetime prevalence around the world (1). The disorder is typical with unpleasant thoughts and compulsive behavior (2) that leads to dimensional life impairments (3). The etiology of OCD has been remained inconclusive over the past years. Treatment options for OCD are available; however, not promising for all cases (4) due to the heterogeneity of the disorder and limited pharmacotherapy approaches that are mostly designed for specific neurotransmitters (5). This fact implies on evaluation of other targets to better elucidate the underlying mechanisms of the disorder. In fact, OCD is considered about 40 to 45% heritable. That is, the first-degree family of the patients with OCD has the susceptibility of 4 to 10 times higher risk (6). It is known that a

complex combination of genetic and environmental factors is related to the disorder etiology (2). Interaction between these factors can result in different phenotypes knowing as subtypes (7). Molecular research can be beneficial in this regard; still, most of the studies of OCD are focused on genetic concept and the related polymorphisms as well as genome wide association studies(8, 9). Meanwhile, application of high throughput methods can be helpful to identify other molecular signatures especially proteins as the functional part of the organisms (10). Proteomics has been proved to be promising for psychiatric disorders. Many candidate biomarkers has been purposed for schizophrenia, depression, and bipolarity (11-13). One of the worthy human sources for proteome evaluation is serum. Many protein biomarkers can be detected through serum analysis, as it is easily provided and manageable. About thousands of secreted or leaked proteins from normal or damaged cells and tissues can be present in blood (14). On the other hand, understanding the disorder requires subtype profiling for individual genders (3). One of the frequent subtypes for women is the washing compulsion (15). Here, by examining the serum proteome of women with washing subtype of OCD, it is aimed to achieve a better understanding of its pathogenicity.

# MATERIALS AND METHODS

# **Human Subjects**

In this study, three groups of human serum samples including 20 healthy, 12 OCD (washing subtype), and 12 treated with Fluoxetine were analyzed and compared in terms of protein expression. In fact, among these 35 women with OCD washing type, 12 of them (sensitive to Fluoxetine treatment) were admitted in the study.

According to inclusion criteria in this study, healthy women were demographically matched to our patient cases. In addition, these healthy cases were without any previous history mental condition in both in their family and themselves, nor were prescript with any types of psychiatric medicines. Similarly, for OCD samples, no history of diagnosis of other mental conditions, comorbidities nor

were prescript with any types of psychiatric treatments as well as not receiving any specific medication for any other kinds of diseases. The patients were aged between 20-30 years old.

For this purpose, Yale-brown questionnaire was applied for each group (healthy and OCD samples) assessments. The enrollment of moderate OCD samples was based on DSM-V in Taleghani Hospital, Tehran, Iran. Their serum samples were provided prior to Fluoxetine prescription. Prior to the sampling, the patients were provided written informed consents. The ethical code for our study is IR.SBMU.REC.1393.299.

# Sample Preparation

Sample collection was by venipuncture rout and usage of needle with gauge  $2^{\circ}$ C. Serum samples were stored at  $-80^{\circ}$ C after separation twice via centrifuge at  $4^{\circ}$ C with 2000 g and 10 min duration prior to 30 min clotting in the room temperature.

### Proteomic Analysis

were All materials from GE 2D-electrophoresis HealthCare Life Sciences (http://www.gelifesciences.com) and SERVA Company (http://www.serva.de). Pooling was performed for the two groups individually and the protein extraction was by the use of 2-DE Clean-Up Kit (GE Healthcare). 2DE procedure was performed with three-time replications for the samples following each group assessment for protein concentration using 2-DE Quant Kit (GE Healthcare). Prior to the first dimension, Isoelectric Focusing (IEF), passive rehydration was applied for 8 hours. The separation based on pI, was as follow: Bio-Rad PROTEAN IEF Cell, 11 cm nonlinear IPG with pH range 4-7 for 7.5 hours at 20 °C according to Bio-Rad Protocol. Before the second dimension, a perpetration step is required to equilibrate the IPG strips for 30 min at room temperature for the SDS PAGE. The HPE FlatTop Tower (horizontal electrophoresis) using 2D HPE<sup>TM</sup> Double-Gel 12.5 % Kit (Serva Company) performed separation based on MW for about 3.5 hours. After electrophoresis, the gels were stained by application of SERVA HPE<sup>™</sup>Coomassie<sup>®</sup> Staining Kit according to the protocol and then scanned using a calibrated GS-800 densitometer (Bio-Rad) scanner (16). Protein expression of two samples were quantified and qualified by Progenesis SameSpots Software as an image analyzer. For expression changes, a value of 1.5-fold was considered and the differentially expressed protein spots were introduced considering ( $p \le 0.05$ ) using one-way ANOVA analysis. Finally, MALDI-TOF-TOF MS analyzed the candidate spots according to the relevant protocol. At the end, evaluation of the extracted peptides was handled by MS and the spectra were submitted to MASCOT (<a href="http://www.matrixscience.com">http://www.matrixscience.com</a>) for the purpose of protein identification.

#### Validation Test

Nephelometric quantification (Hitachi Auto Analyzer) of Haptoglobin in three OCD samples and six healthy samples was handled following the proteomics as a validation test. Additionally, Creactive protein (CRP) as one of the markers of inflammation in different diseases (17, 18), was assessed to provide more information related to our findings. The used method for assessing the concentration of this marker in our patients was turbidometry, (Hitachi Auto Analyzer), in which three samples of healthy and OCD cases were used, individually.

## Network Analysis

A network of introduced proteins was constructed by Cytoscape *v*, *3.5.1*. software (19) and String database (20). The interaction confidence cut off for the inquired proteins was set to 0.4 and the number of neighbor proteins was selected as 50. The centrality analysis was handled via NetworkAnalyzer which is installed in Cytoscape (21). The two important centrality criteria including degree and betweenness centrality were assessed for network analysis in this study. Cut off for hub and bottleneck was set as the amount above 10% of the total nodes with highest degree and betweenness centrality values. Furthermore, the enrichment analysis was handled by DAVID Bioinformatics Resources 6.8 (http://david.niaid.nih.gov) (22).

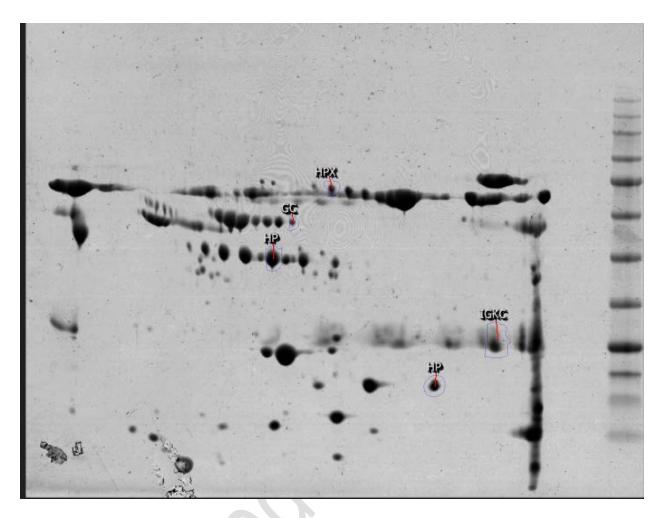
#### **Results**

People with washing subtype of OCD suffer from obsessive thoughts about contamination that compel them to perform ritual manners to temporary alleviate their anxiety (23). Not only this unreasonable behavior is time-consuming, but also it can result in physical injuries. (See figure 1)



**Figure 1.** Excessive hand washing in one of our OCD patients caused in red and chapped skin with some bleeding

A number of 240 protein spots were detected and five significantly expressed ones including HP, HPX, GC, and IGKC were identified. These proteins are labeled after mass spectroscopy identification on patient sample 2DE Gel in figure 2 and the analysis details are presented in table 1.



**Figure2.** Five identified protein spots via MS are assigned on the OCD patient gel. pH range is between 4-7 and the molecular weight is marked with the related ladder from 11-245 kDa.

**Table 1.** The list of five identified protein spots expression changes information. These proteins are ranked based on fold change values. Spot number (S-NO), Average Normalized Values (ANV)

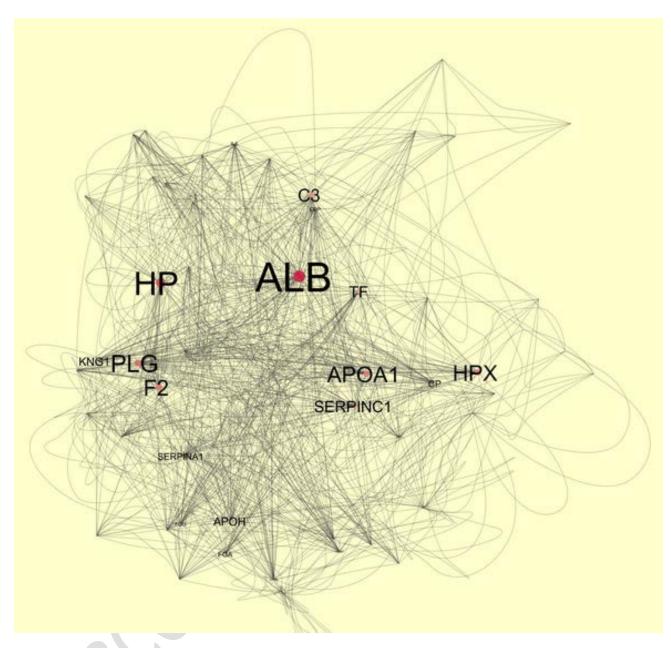
NO	S-NO	Name	MW	PI	Anova (p)	Fold	ANV(Control)	ANV(OCD)	Condition
1	236	HPX	74202	5.65	0.005	1.6	2815.000	4491.952	Up-
							2813.000	4491.932	regulated
2	85	HP	48010	5.32	4.827e-	1.6	2.071e+004	1.301e+004	Down-
					004		2.0716+004	1.3016+004	regulated
3	118	IGKC	25214	6.71	0.004	2.1	2.1877e+004	1.0471+004	Down-
								1.04/1+004	regulated
4	133	HP	17042	6.30	7.008e-	2.1	1.276e+004	6221.119	Down-
					004		1.2700+004	0221.119	regulated
5	59	GC	54526	5.38	0.003	3.5	491.000	1735.079	Up-
					0.003		491.000	1755.079	regulated

MALDI-TOF-TOF MS and MASCOT (<a href="http://www.matrixscience.com">http://www.matrixscience.com</a>) analyzed and identified the candidate protein spots. (See table 2)

Table 2. The list of identified protein spots with the detailed information obtained with MASCOT (P<0.05)

Protein Name	Uniprot	Protein Seq	Peptide	Matching	
	Code	Coverage	Matches	Score	
Ig kappa chain C region	P01834	48%	4	380	
Hemopexin	P02790	18%	7	416	
Haptoglobin	P00738	4%	7	597	
Haptoglobin	P00738	11%	6	561	
Vitamin D-binding protein	P02774	12%	7	434	

To get a better resolution of proteins' role in a whole interacting system, a network of identified proteins with the close surrounding proteins was mapped.( See figure 2)



**Figure 3.** The protein-protein interaction network analysis of the five characterized proteins with addition of 50 neighbor nodes and 577 edges via Cytoscape and String Plug-in. The proteins with the shown labels are the central nodes in the analyzed network. The bigger the labels, the more central nodes are. The designated cut off for interaction between nodes was set to 0.4.

**Table 3.** The centrality analysis of the constructed network of designated proteins. The proteins are ranked based on degree value. The two important centrality parameters including degree (D) and betweenness centrality (BC) are presented. The threshold for hub and bottleneck centralities is assigned as above the 10% of the highest common ranked proteins.

Row	Protein Name	Degree	<b>Betweenness Centrality</b>
1	ALB	48	0.05
2	HP	44	0.07
3	PLG	40	0.02
4	HPX	39	0.07
5	APOA1	39	0.02

To understand biology processes related to OCD, enrichment analysis was handled. The associated biological processes of our identified proteins are searched through DAVID Bioinformatics v6.8 as it is shown in table 4 and 5.

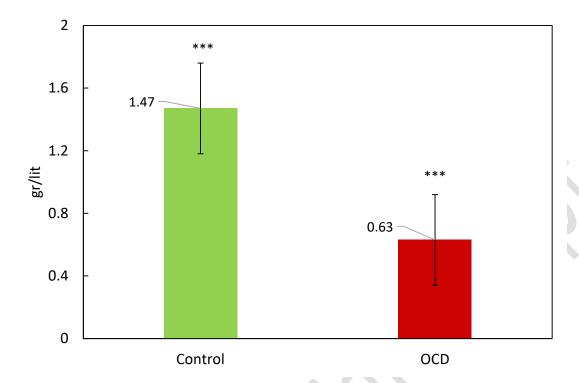
**Table 4.** The list of biological processes corresponded to each candidate proteins obtained from DAVID Bioinformatics v6.8.

P02774	GC, vitamin D binding protein(GC)	Related Genes	Homo sapiens						
GOTERM_BP_DIRECT	vitamin D metabolic process, vitamin transport,								
P00738	haptoglobin(HP)	Related Genes	Homo sapiens						
GOTERM_BP_DIRECT		mmune system process, receptor-mediated endocytosis, defense response, acute-phase response, positive regulation of cell death, response to hydrogen peroxide, defense response to bacterium, negative regulation of oxidoreductase activity, cellular oxidant detoxification, negative regulation of hydrogen peroxide catabolic process,							
P02790	hemopexin(HPX)	Related Genes	Homo sapiens						
GOTERM_BP_DIRECT	positive regulation of immunoglobulin production, positive regulation of humoral immune response mediated by circulating immunoglobulin, cellular iron ion homeostasis, receptor-mediated endocytosis, heme transport, viral process, hemoglobin metabolic process, heme metabolic process, positive regulation of tyrosine phosphorylation of Stat1 protein, positive regulation of interferongamma-mediated signaling pathway,								
P01834	immunoglobulin kappa constant(IGKC)	Related Genes	<u>Homo sapiens</u>						
GOTERM_BP_DIRECT	P_DIRECT  retina homeostasis, proteolysis, receptor-mediated endocytosis, phagocytosis, recognition, phagocytosis, enqulfment, immune response complement activation, complement activation, classical pathway, Fc-epsilon receptor signaling pathway, Fc-qamma receptor signaling pathway involved in phagocytosis, defense response to bacterium, innate immune response, requiation of immune response, B cell receptor signaling pathway, positive regulation of B cell activation,								

**Table 5.** The functional chart of linked biological processes to the identified proteins. Among four queried proteins, HP, HPX, IGKC are common in receptor-,medicated endocytosis and HP and IGKC in defense response to bacterium. Based on designated statistical criteria including Threshold Count Protein per Terms:1 and EASE Score: 0.1 as the default, and the correction method: Benjamini, GC protein did not resulted in the output. The obtained Ease Score for GC =1, The EASE Score ranges from 0 to1.

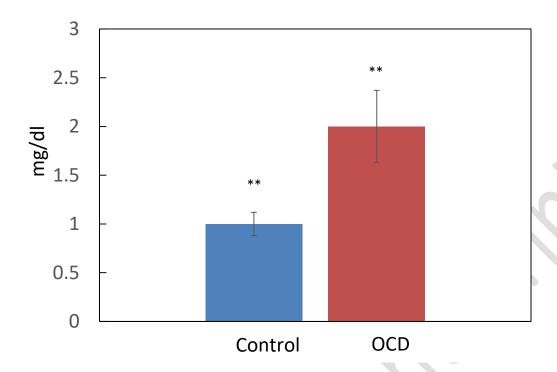
Sublist	<u>Category</u>	‡ <u>Term</u>		Genes	Count (	<u>%</u>	P-Value	Benjamini 🛊
	GOTERM_BP_DIRECT	receptor-mediated endocytosis	<u>RT</u>		3	75.0	3.6E-4	1.2E-2
	GOTERM_BP_DIRECT	defense response to bacterium	<u>RT</u>		2	50.0	2.6E-2	3.6E-1
1 gene(s) from your list are not in the output.								

The pattern of HP Levels changes of healthy and OCD samples via nephelometric test is compared as a bar chart in figure 4.



**Figure4.** Levels of HP in Control and OCD groups is compared obtained from validation test: nephelometric quantification. The green and red bars denote the average of HP Levels in Control and OCD samples. The error bars signifies standard deviation. The level of HP is decreased in OCD samples. P-value≤ 0.001 assigned with asterisks

Further evaluation was handled to get a better view of OCD mechanisms, based on obtained findings, CRP as one of known inflammation markers was screened in our samples. (See figure 5)



**Figure5.** Levels of CRP in Control and OCD groups is compared obtained from validation test: nephelometric quantification. The blue and red bars indicate the average of CRP Levels in Control and OCD samples. The error bars signifies standard deviation. The level of CRP is increased in OCD samples. P-value  $\leq 0.01$ , assigned with asterisks

# **Discussion**

Obsessive Compulsive Disorder is a complex neuropsychiatric disorder causing distress in human daily life (23). Figure 1 from one of our studied OCD patients, indicates the hardship of OCD washers' life and what they have to cope with in every single day. Molecular agents that can cause such behavior worth pursuing. In fact, the molecular aspects of OCD remained to be evaluated despite many pervious genetic and genome-wide studies (24). In this sense, proteomics has proved to be a novel prerequisite towards understanding expressional modification in a disease state (25). In a way that, through elucidating fundamental proteins in OCD pathogenicity, the underlying pathways can be identified. Here, sera proteome of healthy and OCD samples were analyzed and

compared. The analysis shows that there are some isoforms of our protein spots that are altered and may have a role in the OCD risk. As indicated in figure2, a number of five matched differentially expressed protein spots are shown on the OCD gel, chosen for further analysis by mass spectrometry. The identified proteins are HP, HPX, IGKC, and GC. Among them IGKC, and HP isoforms are decreased while GC and HPX isoforms are increased in expression levels as tabulated in table 1. Further information linked to the protein spots identified in our sample are presented in table 2. To get a better resolution of the role of chosen proteins in an interactome profile, a network of them was constructed as it is shown in figure 3. The map analysis expressed that there are some proteins that are considered to be prominent in the network integrity and consequently and may play important contribution in the disorder. Based on centrality analysis in table 3, a number of six proteins are shown to have highest values of degree and betweenness, individually known as hub and bottleneck, respectively. Among them, five proteins were common that are assigned as hub-bottlenecks. HP and HPX as our the inquired proteins seems to have more central roles in the network integrity than the other candidate differentially altered proteins. Additionally, the comparison between the expression changes of our found isoforms in OCD sera and in other types of psychiatric disorder denote noteworthy information. In a way that, the common and differential mechanisms of these mental conditions can be better understood. All these proteins except IGKC, imply on expression changes in other psychiatric disorders (26-28). Hemopexin (HPX) as a type 2 acute phase reactant glycoprotein has antioxidant and iron homeostasis activities (29). Here, its isoform shows increased levels in our OCD sample that implies on presence of inflammation in OCD. It is also previously referred that HPX synthesis is induced in OCD condition (30). What is more, similar behavior of HPX has been reported in other mental disorders such as schizophrenia, mania, and depression (27). However, in here we only were able to determine one of its isoforms and other ones expressed no significant differential expression changes. Network analysis also implies on the importance of this protein in the network strength, which gives more credit to its major position in the disorder condition. Another significantly elevated protein in OCD sample is vitamin D binding protein. This protein has fundamental participation for binding to vitamin D (GC). The expression alteration of this protein may have associations with reduced vitamin D levels in OCD patients (31, 32). The increment of GC has been also evident in one recent study of bipolar patients (28). Immunoglobulin kappa chain C (IGKC), the protein that have been previously studied by our team (24), shows lower

expression levels in OCD patients. No correlation between this protein and other psychiatric condition has been yet reported to our knowledge. This protein is active in immune response (33). Two isoforms of haptoglobin were detected that their lower expression levels were determined in OCD patients. Furthermore, our validation study confirms the overall reduced levels of HP in OCD patients that can be inferred from figure 4. One of the main tasks of HP is an antioxidant activity (34), meanwhile it has been known that oxidative stress is one of the active processes in OCD (35, 36). Therefore, the reduced levels of HP in OCD serum may have correlation to this phenomenon. Also, as mentioned earlier, HP is one of the most crucial proteins in an interactome system. All these facts, supports the indispensable relation of HP in OCD risk. What is more, as it is clear from the table 4, and 5, enrichment investigation of our identified proteins except for GC, suggests the association of inflammation processes in OCD condition. Due to the possible correlation of inflammatory agents to our disorder, CRP as one of the important markers in the blood, was also screened as validation test in our patients, individually. The result in figure 5 shows that the CRP level is elevated in OCD patients' serum. In addition to this, CRP was searched in our constructed network in which was found moderately central. In which, offers more support to the fact that OCD may be suggested as an inflammatory disorder and thereby immunomodulatory therapies may be helpful in this regard (37). Overall, these candidates and their underlying processes may have a potential clinical interest in OCD future treatment.

#### Conclusion

In conclusion, our findings express that some of the blood vital proteins that are responsible in many essential processes in the body may be prominent in OCD underlying mechanisms. One of which, HP, validated in this study, may particularly serve as a novel biomarker. Furthermore, this study supports the role of inflammation in OCD risk. In view of this new insight in to OCD complexity, therapeutic strategies can be targeted. However, to validate this claim, more investigation is required.

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