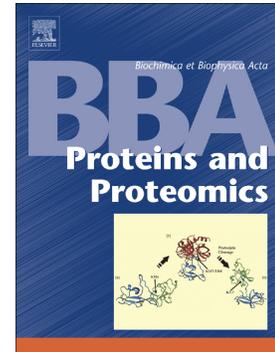


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Identification of serum biomarkers for premature ovarian failure

Da-Hye Lee¹, Chang-Zhu Pei¹, Jae-Yun Song², Kyung-Ju Lee², Bo-Seong Yun³, Kyu-Bum Kwack¹, Eun-Il Lee⁴, and Kwang-Hyun Baek^{1,*}

¹Department of Biomedical Science, CHA University,
Gyeonggi-Do 13488, Republic of Korea

²Department of Obstetrics and Gynecology, Korea University Anam Hospital, Korea
University College of Medicine, Seoul 02841, Republic of Korea

³Department of Obstetrics and Gynecology, Gangnam CHA Hospital,
College of Medicine, CHA University, Seoul 06135, Republic of Korea

⁴Department of Preventive Medicine, Korea University College of Medicine,
Seoul 02841, Republic of Korea

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Corresponding author:

Kwang-Hyun Baek
Department of Biomedical Science, CHA University,
335 Pangyo-Ro, Bundang-Gu, Seongnam-Si,
Gyeonggi-Do 13488, Republic of Korea

Tel: +82-31-881-7134
Fax: +82-31-881-7249
E-mail: baek@cha.ac.kr

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Abstract

Premature ovarian failure (POF) is defined when a female achieves menopause before the age of 40. Although many conditions are known to be causative for POF, the most common one is idiopathic. This study was undertaken to investigate the pathogenesis of POF using proteomic tools. Two-dimensional electrophoresis (2-DE) analysis was performed to screen for proteins differentially expressed in patients with POF. Using liquid chromatography-mass spectrometry / mass spectrometry (LC-MS / MS), we identified 11 significant proteins differentially expressed in the serum of POF patients: 5 proteins with expression increased more than two folds, 5 proteins with expression decreased more than two folds, and 1 protein expressed specifically in the serum of patients with POF. The results of the 2-DE analysis were further validated by Western blotting and ELISA analyses, which 5 reproductive system-related proteins (Ceruloplasmin, Complement C3, Fibrinogen α , Fibrinogen β , and SHBG) were selected. The different expression levels for these proteins were confirmed and demonstrated the possibility of using them as biomarkers to screen POF. These pre-clinical data provide plausible translational implications for targeting the pathogenesis of POF for each protein.

1. Introduction

Premature ovarian failure (POF) is a female specific disease that is defined when a woman achieves menopause before the age of 40, ultimately resulting in infertility. Symptoms include amenorrhea and hypo-estrogenemia such as hot flush, sleep disturbance, and irritability [1]. Assessment by the hypergonadotropic hypogonadic hormone test further categorizes POF, where a positive result is defined as having low levels of estradiol (E2), anti-Müllerian hormone (AMH), and inhibin B, and a high level of follicle-stimulating hormone (FSH, > 40 IU/L) [2]. A patient is generally diagnosed with POF when FSH levels measured at least twice (once initially and again after 4 to 6 weeks) are > 40 IU/L. However, the diagnosis of POF is not dependent upon FSH level alone; it also takes the mental status into consideration [1, 3]. Decreased E2 turns on the feedback mechanism to stimulate the anterior pituitary gland, resulting in increased FSH. AMH is produced by the preantral and antral follicles, and shows a decreasing level of expression as one ages, thereby making it a good marker for declining fertility. AMH level is very low in POF patients and is used as a diagnostic marker for POF [1, 4, 5].

Inhibin B is produced from early antral follicles of the follicular phase during the menstrual cycle and is a good marker for evaluating ovaries. Inhibin B is also decreased in POF patients [1]. There are many known causes of POF, which include, but not limited to, genetics, autoimmune disorders, infections, enzymatic disorders, ovariectomy, etc. However, many causes of POF are as yet far from understanding.

The study of POF currently in progress is being mainly carried out in the field of genomics [6]. Many studies on POF-related genes are focused on FMR1, FOXL2, GDF9, FSHR, etc [7-

10]. Various researches are also being performed to identify the cellular and molecular mechanisms of POF. The mechanisms studied include the PI3K / PTEN / Akt and TSC/mTOR signaling pathways, which may regulate follicular maturation. Abnormal regulation of these pathways can result in POF due to premature follicular maturation [11-13]. Although there are numerous studies on POF, protein biomarkers specific for POF diagnosis have not been established so far.

For the fundamental units of human bioactivity, proteins are repeatedly degraded and synthesized to maintain a balance, which, if broken, may result in a disease [14]. A biomarker can be a protein, a DNA, an RNA or a metabolite, that detects and indicates changes in the organism. Proteomics, the discipline of collectively studying all proteins within a cell or an organism, is widely used to identify protein biomarkers of diseases. Proteomics analysis mainly excavates biomarkers through two-dimensional electrophoresis-liquid chromatography-mass spectrometry / mass spectrometry (2-DE-MS / MS) [15-17]. 2-DE was first used to detect proteins specific to the POF patients, after which MS was employed to identify these proteins. Although proteomics is currently used extensively to identify biomarkers of numerous reproductive diseases [15, 16, 18], no biomarkers for POF have been identified to date. This study was therefore undertaken to identify biomarkers of POF by analyzing the differential expression of proteins in the serum of patients with POF.

2. Materials and methods

2.1. Patients

All patient blood samples were provided from the Korea University Anam Hospital (Seoul, Korea) between October 2016 and December 2017. The patients were classified as patients with POF (n=12), risk group of POF (n=23), and normal controls (n=136). Diagnosis of POF was based on hormone level tests of AMH, FSH, and estrogen. All samples were included with the criteria of AMH level being lower than 0.01 ng/ml. Briefly, 2 ml blood sample was collected from each patient and separated into plasma and blood cells by centrifugation at 1,500 x g for 15 min. The plasma was stored at -80°C till further experiments.

2.2. 2-DE analysis

The 2-DE analysis was performed using the plasma of POF patients (n=3). A super control, the mixture of random three normal samples, was also used as a control. Removal of the abundant proteins present in the plasma was accomplished by filtering through Multiple Affinity Removal Column (MARC) (Agilent, Wilmington, DE, USA). The isoelectric focusing (IEF) was performed with 3-10 Nonlinear Immobilized pH Gradient (NLIPG) at 80,000 V/hr, and the results were visualized using 9-17% gradient slab gel.

2.3. LC-MS/MS for peptide analysis of Q-TOF

Nano LC-MS/MS analysis was performed using the nano HPLC system (Agilent, Wilmington, DE, USA). A nano chip column (150 mm × 0.075 mm, Agilent, Wilmington, DE, USA) was used for peptide separation. The mobile phase A for LC separation was 0.1% formic acid in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile.

The chromatography gradient was designed for a linear increase from 3% B to 45% B in 70 min, 45% B to 95% B in 1 min, 95% B in 9 min, and 3% B in 10 min, with the flow rate, maintained at 300 nL/min. Product ion spectra were collected in the information-dependent acquisition (IDA) mode and analyzed by Agilent 6530 Accurate-Mass Q-TOF using continuous cycles of one full scan TOF MS from 300-2000 m/z (1.0 sec) plus three product ion scans from 150-2000 m/z (1.5 sec each). Precursor m/z values were selected starting with the most intense ion, using a selection quadrupole resolution of 3 Da. The rolling collision energy feature determined the collision energy based on the precursor value and charge state. The dynamic exclusion time for precursor ion m/z values was 60 sec.

2.4. Database searching

The mascot algorithm (<http://www.matrixscience.com>, Matrixscience, Boston, MA, USA) identified the peptide sequences present in a protein sequence database. The criteria for the search were: 1. taxonomy, 2. mykiss fixed modification, 3. carboxyamidomethylated at cysteine residues, 4. variable modification, 5. oxidized at methionine residues, 6. maximum allowed missed cleavage: 2, 7. MS tolerance: 100 ppm, and 8. MS/MS tolerance: 0.1 Da. Only the peptides that resulted from digestion by trypsin were considered.

2.5. Western blotting

Plasma separated from the whole blood was boiled and loaded onto the SDS-PAGE gel. Proteins were resolved and transferred onto polyvinylidene fluoride (PVDF) microporous

membranes (Millipore, Billerica, MA, USA). Primary antibodies were incubated overnight with the membrane at 4 °C, followed by washing, exposing to secondary antibodies, and incubating for 1 hr at room temperature. Blots were detected by ECL reagent solution (Young In Frontier, Seoul, Korea).

2.6. Antibodies

Mouse anti-Complement C3 (C-4) monoclonal antibody (sc-25298), rabbit anti-Ceruloplasmin (H-60) polyclonal antibody (sc-20957), goat anti-Fibrinogen α (sc-18026), and anti-Fibrinogen β (sc-18029) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-SHBG polyclonal antibody (CSB-PA12289A0Rb) was purchased from CUSABIO (Wuhan, China).

2.7. Enzyme-linked immunosorbent assay (ELISA)

The concentration of proteins in the plasma of controls, POF risk group, and POF patients was measured using human Complement C3 ELISA kit (Abcam, Cambridge, MA, USA, ab108822), human Ceruloplasmin ELISA kit (ASSAYPRO, St. Charles, MO, USA, EC4201-1), human SHBG Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA, DSHBG0B), and human Fibrinogen ELISA kit (Abcam, Cambridge, MA, USA, ab108841) according to each manufacturer's protocol.

2.8. Statistical analysis

Densitometric analysis was performed for bands scanned from Western blotting analysis using Image J (National Institutes of Health, Bethesda, MD, USA), a *t*-test was performed by GraphPad Prism (GraphPad Software-ver. 5, La Jolla, CA, USA), and ANOVA was performed by one-way analysis of variance with Turkey's multiple comparison test for indicating the significant difference.

2.9. Ethics statement

The protocol of the present study was confirmed and approved by the Institutional Review Board of Korea University Anam Hospital (approval No.2016AN0251). Written informed consent was obtained from all subjects on enrollment.

3. Results

3.1. Classification of recruited blood samples

Proteomics analysis was employed in order to investigate proteins associated with POF. Since menopause is diagnosed by measuring hormone levels in the blood, all patients suspected of POF were screened for hormone levels. Menopause is biochemically characterized by a high level of FSH and a low level of AMH. In this study, the enrolled subjects were divided into following groups: control group (greater than the intermediate value expected at the age of examinee), risk group (greater than 10% of the intermediate value), and patient group (less than 10% of the intermediate value). A total of 136 controls, 23 at risk, and 12 patients were

recruited (Tables 1 and 2). Blood was collected and separated into serum and blood cells by centrifugation at 1,500 x g for 15 min.

3.2. 2-DE analysis of plasma proteins in patients with POF

To identify proteins that are differentially expressed in POF patients, the 2-DE analysis was performed using the separated plasma. To compare the expression level of proteins between normal and POF patients, plasma samples of 3 POF patients and 1 mixed normal plasma sample (n=3) were used. Excessive proteins were removed using MARC as previously described [15, 16]. 2-DE separation was performed in the pH range of 3 to 10 IEF strip. As a result, 301 spots from normal samples and 340 spots, 375 spots, and 339 spots from POF patients were detected (Fig. 1). Using MALD-TOF-MS/MS analysis, 30 spots were separated for unpaired spots and paired spots with control spots, each of which was increased or decreased more than two folds (Fig. 1). As a result, we identified 11 proteins that might be significant: 5 proteins (Complement component C3, Plasminogen, Fibrinogen α , Fibrinogen β , and α -2-macroglobulin precursor) with increased expression more than two folds, 5 proteins (Ceruloplasmin, Vitamin D-binding protein, Apolipoprotein L-1, α -globin, and β -globin) with decreased expression more than two folds, and 1 protein (SHBG) that was expressed specifically in patients with POF only (Table 3).

3.3. Validation of proteins in patients with POF

Next, Western blotting analysis was performed to validate the result of 2-DE analysis (Fig. 2). We selected 5 proteins (Ceruloplasmin, Complement C3, Fibrinogen α , Fibrinogen β , and SHBG), which are known to be involved in the reproductive system (Fig. 2A). As shown in Figure 2D, Ceruloplasmin was expressed more in patients with POF than the control group. This was contrary to the 2-DE result wherein it was expressed less in patients than that in the control group. The other proteins (Complement C3, Fibrinogen α , and Fibrinogen β) showed higher expressions in Western blotting analysis as well as 2-DE results. SHBG was confirmed to be specifically expressed in the POF patient group. Overall, our results indicate that Complement C3, Fibrinogen α , Fibrinogen β , and SHBG are highly expressed in more than 50% of patients as compared to the control group; therefore, it is possible to use these proteins as biomarkers for screening POF.

3.4. Validation of proteins in patients with the POF risk group

For the POF risk group, Western blotting analysis was performed to confirm the specific expression of proteins as shown in these patients (Fig. 3). As shown in Figure 3C, Complement C3 and SHBG were highly expressed in the risk group compared to the control group. Similar results were observed in the POF patient group.

3.5. Higher expression of proteins in POF patients

To further validate the results, the concentration of proteins in the plasma of controls, POF risk group, and POF patients was measured by ELISA. Concentrations of Ceruloplasmin,

Complement C3, Fibrinogen, and SHBG were higher in the plasma of POF patients compared with those in controls and POF risk group (Fig. 4). These results suggest that increased concentrations of these proteins are related to POF.

4. Discussion

There has been an increase in the prevalence of premature menopause in recent years, with 1 in 100 women in their 30's experiencing premature menopause. However, the mechanism of premature menopause has not been clearly elucidated yet. Furthermore, biomarkers that could diagnose POF have not been identified. Therefore, we decided to investigate proteins that are differentially expressed in POF patients to identify biomarkers that may help diagnose premature menopause. Female blood samples used in the study were provided by the Korea University Anam Hospital. These samples were classified as the control group, risk group, and patient group, based on the AMH levels in the sample by measuring hormone levels. The level of other hormones such as E2, FSH, and progesterone was also measured. Other tests including the prolactin level test were also conducted to rule out the known causes of POF. The patients were classified by comparing their AMH level with the average AMH level of their age group.

After obtaining the 2-DE results, 30 spots were selected due to the different size between the control group and the patient group. LC / MS analysis was performed on these spots to identify the proteins. Western blotting analysis and ELISA were then performed on Ceruloplasmin, Complement C3, Fibrinogen α , Fibrinogen β , and SHBG to validate the 2-DE data.

The result of the 2-DE analysis showed that expression of Ceruloplasmin in POF patients was decreased, while the expression of Complement C3, Fibrinogen α , and Fibrinogen β was increased compared with the control group. It was of interest that SHBG was specifically expressed in POF patients. However, Western blotting analysis showed the increased expression of Ceruloplasmin in POF patients and SHBG expressed in controls, which were inconsistent with 2-DE results. However, results of Western blotting analysis with the same samples used for 2-DE analysis were consistent with results of 2-DE analysis (Supplementary Figure 1). Since we randomly selected 3 people from 31 controls and 12 POF patients each for 2-DE analysis, it may be possible that this inconsistency might come from a limited expression of Ceruloplasmin in those samples.

Ceruloplasmin is known to play an important role in transporting copper through the body and affects pregnancy [19-21]. A previous study revealed that Ceruloplasmin levels are high in infertile female serum [22, 23]. However, the level remains unchanged with menopause, although it does increase with estrogen before and after menopause [19]. In this study, patients with POF were more likely to have higher Ceruloplasmin levels than normal women. Since alterations in Ceruloplasmin are likely to affect estrogen changes, it is proposed that there is possibility in its use as a diagnostic marker for POF.

Western blotting analysis in our study confirmed that Complement C3 was expressed more in patients than in the control group, which was consistent with 2-DE results. Complement C3 plays a central role in the activation of the complement system [24, 25]. The complement system is a part of the immune system that induces phagocytosis and inflammation to clear pathogens [24]. A previous study showed that Complement C3 is expressed more in infertile women [26]. Antiestrogens induce Complement C3 synthesis whereas progesterone blocks

Complement C3. Previous studies reported that the level of Complement C3 is regulated by E2 and increases after menopause [26, 27]. Our results indicate that Complement C3 is expressed more in patients with POF than in normal controls. Considering that POF could be caused by immune-mediated responses, Complement C3 could potentially be a biomarker for POF.

Fibrinogen functions primarily to occlude blood vessels and thereby stop excessive bleeding. Previous reports revealed that the level of fibrinogen increases after menopause, thereby increasing thrombus formation and the incidence of arteriosclerosis [28, 29]. The result of our study indicates that Fibrinogen α and Fibrinogen β were expressed more in POF patients than in normal controls.

SHBG is a glycoprotein attached to androgen and estrogen, and decreases in bioavailability. SHBG is more abundant in females than males. Although the level of estrogen and SHBG decrease during menopause, a report demonstrates that the concentration of SHBG is high in the serum of postmenopausal women [30]. Our results support the results of other studies that SHBG expression is increased in early menopausal patients [31]. Overall, these results support the claim that SHBG expression increases in patients with POF.

In conclusion, POF patients classified on the basis of AMH hormone were screened for differentially expressed proteins using 2-DE-LC-MS / MS. As a result, we selected Ceruloplasmin, Complement C3, Fibrinogen α , Fibrinogen β , and SHBG, and performed both Western blotting analysis and ELISA to verify the result of the 2-DE analysis. We demonstrated that more than half of the patients expressed increased protein levels than the control group, suggesting the possibility of using them as biomarkers to identify putative POF.

Further studies are required since the investigation on the relationship between the excavated proteins and POF has not been conducted yet. It is therefore necessary to investigate the biological functions of these proteins to demonstrate their possibility as biomarkers. This study will be a precedent for future studies on the relationship between the biological functions and POF.

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References

- [1] K. Jankowska, Premature ovarian failure, *Prz Menopauzalny*, 16 (2017) 51-56. <https://doi.org/10.5114/pm.2017.68592>
- [2] Ayesha, V. Jha, D. Goswami, Premature ovarian failure: An association with autoimmune diseases, *J Clin Diagn Res*, 10 (2016) QC10-QC12. <https://doi.org/10.7860/JCDR/2016/22027.8671>
- [3] M.B. Bhongade, S. Prasad, R.C. Jiloha, P.C. Ray, S. Mohapatra, B.C. Koner, Effect of psychological stress on fertility hormones and seminal quality in male partners of infertile couples, *Andrologia*, 47 (2015) 336-342. <https://doi.org/10.1111/and.12268>

- [4] A. Kruszynska, J. Slowinska-Srzednicka, Anti-mullerian hormone (AMH) as a good predictor of time of menopause, *Prz Menopauzalny*, 16 (2017) 47-50. <https://doi.org/10.5114/pm.2017.68591>
- [5] Y. Tokura, O. Yoshino, S. Ogura-Nose, H. Motoyama, M. Harada, Y. Osuga, Y. Shimizu, M. Ohara, T. Yorimitsu, O. Nishii, S. Kozuma, T. Kawamura, The significance of serum anti-mullerian hormone (AMH) levels in patients over age 40 in first IVF treatment, *J Assist Reprod Genet*, 30 (2013) 821-825. <https://doi.org/10.1007/s10815-013-9991-3>
- [6] C. Chapman, L. Cree, A.N. Shelling, The genetics of premature ovarian failure: Current perspectives, *Int J Womens Health*, 7 (2015) 799-810. <https://doi.org/10.2147/IJWH.S64024>
- [7] J. Rehnitz, D.D. Alcoba, I.S. Brum, J.E. Dietrich, B. Youness, K. Hinderhofer, B. Messmer, A. Freis, T. Strowitzki, A. Germeyer, Fmr1 expression in human granulosa cells increases with exon 1 CGG repeat length depending on ovarian reserve, *Reprod Biol Endocrinol*, 16 (2018) 65. <https://doi.org/10.1186/s12958-018-0383-5>.
- [8] F.T. Kuo, I.K. Bentsi-Barnes, G.M. Barlow, M.D. Pisarska, Mutant forkhead l2 (FOXL2) proteins associated with premature ovarian failure (POF) dimerize with wild-type FOXL2, leading to altered regulation of genes associated with granulosa cell differentiation, *Endocrinology*, 152 (2011) 3917-3929. <https://doi.org/10.1210/en.2010-0989>
- [9] H. Dixit, L.K. Rao, V. Padmalatha, M. Kanakavalli, M. Deenadayal, N. Gupta, B. Chakravarty, L. Singh, Mutational screening of the coding region of growth differentiation factor 9 gene in Indian women with ovarian failure, *Menopause*, 12 (2005) 749-754. <https://doi.org/10.1097/01.gme.0000184424.96437.7a>

- [10] K.J. Woad, D. Prendergast, I.M. Winship, A.N. Shelling, FSH receptor gene variants are rarely associated with premature ovarian failure, *Reprod Biomed Online*, 26 (2013) 396-399. <https://doi.org/10.1016/j.rbmo.2013.01.004>
- [11] Y. Shimizu, F. Kimura, K. Takebayashi, M. Fujiwara, K. Takakura, K. Takahashi, Mutational analysis of the PTEN gene in women with premature ovarian failure, *Acta Obstet Gynecol Scand*, 88 (2009) 824-825. <https://doi.org/10.1080/00016340902971458>
- [12] D. Adhikari, W. Zheng, Y. Shen, N. Gorre, T. Hamalainen, A.J. Cooney, I. Huhtaniemi, Z.J. Lan, K. Liu, Tsc/mTORC1 signaling in oocytes governs the quiescence and activation of primordial follicles, *Hum Mol Genet*, 19 (2010) 397-410. <https://doi.org/10.1093/hmg/ddp483>
- [13] N. Yin, Y. Wang, X. Lu, R. Liu, L. Zhang, W. Zhao, W. Yuan, Q. Luo, H. Wu, X. Luan, H. Zhang, hPMSC transplantation restoring ovarian function in premature ovarian failure mice is associated with change of Th17/Tc17 and Th17/Treg cell ratios through the PI3K/Akt signal pathway, *Stem Cell Res Ther*, 9 (2018) 37. <https://doi.org/10.1186/s13287-018-0772-x>
- [14] P. Narayan, S. Ehsani, S. Lindquist, Combating neurodegenerative disease with chemical probes and model systems, *Nat Chem Biol*, 10 (2014) 911-920. <https://doi.org/10.1038/nchembio.1663>
- [15] Y.S. Kim, M.S. Kim, S.H. Lee, B.C. Choi, J.M. Lim, K.Y. Cha, K.H. Baek, Proteomic analysis of recurrent spontaneous abortion: Identification of an inadequately expressed set of proteins in human follicular fluid, *Proteomics*, 6 (2006) 3445-3454. <https://doi.org/10.1002/pm.ic.200500775>

- [16] M.S. Kim, B.H. Gu, S. Song, B.C. Choi, D.H. Cha, K.H. Baek, ITI-H4, as a biomarker in the serum of recurrent pregnancy loss (RPL) patients, *Mol Biosyst*, 7 (2011) 1430-1440. <https://doi.org/10.1039/c0mb00219d>
- [17] C.I. Phillips, M. Bogyo, Proteomics meets microbiology: Technical advances in the global mapping of protein expression and function, *Cell Microbiol*, 7 (2005) 1061-1076. <https://doi.org/10.1111/j.1462-5822.2005.00554.x>
- [18] S. Angelucci, D. Ciavardelli, F. Di Giuseppe, E. Eleuterio, M. Sulpizio, G.M. Tiboni, F. Giampietro, P. Palumbo, C. Di Ilio, Proteome analysis of human follicular fluid, *Biochim Biophys Acta*, 1764 (2006) 1775-1785. <https://doi.org/10.1016/j.bbapap.2006.09.001>
- [19] J.T. Dabek, M. Hyvonen-Dabek, M. Harkonen, H. Adlercreutz, Evidence for increased non-ceruloplasmin copper in early-stage human breast cancer serum, *Nutr Cancer*, 17 (1992) 195-201. <https://doi.org/10.1080/01635589209514187>
- [20] P.D. Pigatto, A. Soldarini, G. Guzzi, Copper metabolism and green serum during pregnancy, *Clin Biochem*, 49 (2016) 839-840. <https://doi.org/10.1016/j.clinbiochem.2016.04.015>
- [21] R.S. Bedwal, A. Bahuguna, Zinc, copper and selenium in reproduction, *Experientia*, 50 (1994) 626-640. PMID: 8033970
- [22] R.A.M. Hussein, R.M.H. Al-Salih, S.A.R. Ali, A study of prolactin, thyroid stimulating hormones, malondialdehyde and ceruloplasmin levels in infertile women, in Thi-Qar governorate/Iraq, *Thi-Qar Medical Journal*, 14 (2017) 14-21.

- [23] C. Clemente, F. Russo, M.G. Caruso, M. Giangrande, G. Fanizza, A. Di Leo, Ceruloplasmin serum level in post-menopausal women treated with oral estrogens administered at different times, *Horm Metab Res*, 24 (1992) 191-193. <https://doi.org/10.1055/s-2007-1003291>
- [24] J.V. Sarma, P.A. Ward, The complement system, *Cell Tissue Res*, 343 (2011) 227-235. <https://doi.org/10.1007/s00441-010-1034-0>
- [25] D. Ricklin, E.S. Reis, D.C. Mastellos, P. Gros, J.D. Lambris, Complement component C3 - the "Swiss Army Knife" of innate immunity and host defense, *Immunol Rev*, 274 (2016) 33-58. <https://doi.org/10.1111/imr.12500>
- [26] S.R. El Khoudary, K.J. Shields, H.Y. Chen, K.A. Matthews, Menopause, complement, and hemostatic markers in women at midlife: The study of women's health across the nation, *Atherosclerosis*, 231 (2013) 54-58. <https://doi.org/10.1016/j.atherosclerosis.2013.08.039>
- [27] P.G. Tepper, J.F. Randolph, Jr., D.S. McConnell, S.L. Crawford, S.R. El Khoudary, H. Joffe, E.B. Gold, H. Zheng, J.T. Bromberger, K. Sutton-Tyrrell, Trajectory clustering of estradiol and follicle-stimulating hormone during the menopausal transition among women in the Study of Women's Health across the Nation (SWAN), *J Clin Endocrinol Metab*, 97 (2012) 2872-2880. <https://doi.org/10.1210/jc.2012-1422>
- [28] B. Zegura, B. Guzic-Salobir, M. Sebestjen, I. Keber, The effect of various menopausal hormone therapies on markers of inflammation, coagulation, fibrinolysis, lipids, and lipoproteins in healthy postmenopausal women, *Menopause*, 13 (2006) 643-650. <https://doi.org/10.1097/01.gme.0000198485.70703.7a>

[29] C.E. Bonduki, D.M. Lourenco, E.L. Motta, J.M. Soares, Jr., M.A. Haidar, E.C. Baracat, Effect of estrogen-progestin hormonal replacement therapy on blood coagulation and fibrinolysis in postmenopausal women, *Clinics (Sao Paulo)*, 62 (2007) 553-560. PMID: 17952314

[30] G.L. Hammond, Diverse roles for sex hormone-binding globulin in reproduction, *Biol Reprod*, 85 (2011) 431-441. <https://doi.org/10.1095/biolreprod.111.092593>

[31] P. Skalba, M. Wojtowicz, J. Sikora, Androgen and SHBG serum concentrations in late post-menopause women, *Med Sci Monit*, 9 (2003) CR152-156. PMID: 12640346

Figure legend

Figure 1. 2-DE images of POF patients. The most dominant proteins in human serum derived from normal and POF patients were depleted by an immunoaffinity column (MARC) and then other proteins were separated by 2-DE. 2-DE images of serum after depletion: a cocktailed normal control (A) and 3 POF patients (B-D). 870 μ g of serum proteins were separated on pH 3-10 NLIPG strip and then on 9-17% gradient slab gel. The y-axis shows the molecular weights in kDa, and the x-axis represents the pH.

Figure 2. Expression of proteins in the POF group. Western blotting analysis of serum proteins from controls (n=31) and POF patients (n=12). (A) Five reproductive system-related proteins are indicated. The arrows in the panel indicate differentially expressed proteins in POF patients (right) compared with the control (left). (B) Western blotting analysis was performed using SDS-PAGE gel. (C) Western blotting analysis was performed using non SDS-PAGE gel. (D) Representative images of Western blotting analysis. Quantification of Ceruloplasmin, Fibrinogen α , Fibrinogen β , Fibrinogen γ , SHBG, and Complement C3 protein expression by the semiquantitative way using Image J. Significant difference was assessed by a Student's *t*-test, where *p*-value is **p* < 0.05 or ***p* < 0.01 or ****p* < 0.001.

Figure 3. Expression of proteins in the POF risk group. Western blotting analysis was performed to compare the expression level of each protein between controls (n=31) and POF risk group (n=23). (A) Western blotting analysis using SDS-PAGE gel. (B) Western blotting analysis using non SDS-PAGE gel. (C) Representative images of Western blotting analysis.

Quantification of Ceruloplasmin, Fibrinogen α , Fibrinogen β , Fibrinogen γ , SHBG, and Complement C3 protein expression by the semiquantitative way using Image J. Significant difference was assessed by a Student's *t*-test, where *p*-value is **p* < 0.05 or ***p* < 0.01 or ****p* < 0.001.

Figure 4. The protein levels in the plasma of POF patients. The concentration of Ceruloplasmin, Complement C3, Fibrinogen, and SHBG was measured by ELISA using plasma samples of normal controls (n=8), POF risk group (n=8), and POF patients (n=8). Significant difference was assessed by one-way analysis of variance, where *p*-value is **p* < 0.05 or ***p* < 0.01 or ****p* < 0.001.

Table 1

Information of analyzed POF risk group

POF risk group	Age	AMH (ng/mL)	FSH (mIU/mL)	Estradiol (E2) (pg/ml)	Progesterone (ng/ml)
1	29	0.87	5.46	6.6	0.7
2	24	1.96	2.35	20.5	3.8
3	37	0.82	3.71	26.2	0.2
4	36	0.46	0.17	7.9	0.4
5	37	0.89	1.68	101.4	4.4
6	39	0.82	1.21	81.4	17.4
7	36	0.78	1.13	196.6	11.9
8	30	1.82	4.68	171.3	0.9
9	31	1.71	3.34	110.0	7.5
10	36	0.01	21.00	171.0	0.6
11	32	1.77	3.07	75.7	4.8
12	36	0.72	4.36	91.8	0.5
13	34	0.83	4.32	28.2	0.2
14	30	1.33	4.60	31.9	1.7
15	39	0.52	4.56	78.4	0.3
16	32	2.01	6.90	47.0	0.07
17	37	0.42	4.20	48.4	<0.05
18	37	0.70	1.82	193.1	8.64
19	36	1.49	5.90	125.0	<0.05
20	29	1.47	7.10	97.4	<0.05
21	37	0.27	9.80	81.9	1.30
22	35	0.42	6.26	31.0	<0.05
23	37	1.24	3.60	65.6	<0.05

Table 2

Information of analyzed POF patients

POF patients	Age	AMH (ng/ml)	FSH (mIU/mL)	Estradiol (E2) (pg/ml)	Progesterone (ng/ml)
1	39	0.01	35.46	12.9	0.3
2	45	<0.01	7.86	85.5	<0.1
3	36	<0.01	41.31	15.6	0.3
4	29	<0.01	27.41	39.0	0.5
5	39	0.13	0.94	6.0	<0.1
6	35	0.03	36.32	38.8	1.1
7	36	<0.01	10.24	84.4	<0.1
8	44	<0.01	80.50	4.0	0.2
9	44	<0.01	36.46	42.7	0.2
10	41	0.01	54.51	4.0	0.4
11	36	0.01	99.27	4.0↓	<0.1
12	44	<0.01	1.50	46.7	<0.1

Table 3

LC-MS results of spot analysis selected through 2-DE analysis

Spot number	Access number	Protein name	Score	Peptide matched	emPAI	MW
70	gi 116117	Ceruloplasmin	44	NNEGTYYSPTYNPQSR	0.04	122,9
92	gi 179665	Complement component C3	71	SGSDEVQVGQQR	0.02	188,5
110	gi 130316	Plasminogen	50	LSSPAVITDK	0.05	93,2
256	gi 223918	Fibrinogen α	223	ADSGEGDFLAEGGGVR, GGSTSYGTGSETESPR, GSESGIFTNTK	0.31	49,7
268	gi 177870	α -2-macroglobulin precursor	47	AIGYLNTGYQR	0.03	164,0
276	gi 139641	Vitamin D-binding protein	99	TSALSAK, VMDKYTFELSR	0.15	54,5
295	gi 182430	β -fibrinogen precursor, partial	99	QDGSVDFGR, EDGGGWYNR	0.17	55,5
381	gi 12232634	Apolipoprotein L-I	64	VTEPISAESGEQVER	0.11	42,3
417	gi 36452	SHBG-related protein (288 AA)	100	TSSSFQVR, QAEISASAPTSR	0.16	31,9
494	gi 28547	α -globin	117	VLSPADKTNVK, VGAHAGEYGAEALER	0.74	15,3
499	gi 256028940	β -globin	302	VHLTPEEKSAVTALWGKVNVDDEVGGEALGR, SFGDLSTPDAVMGNPK, VVAGVANALAHKYH	3.93	16,1

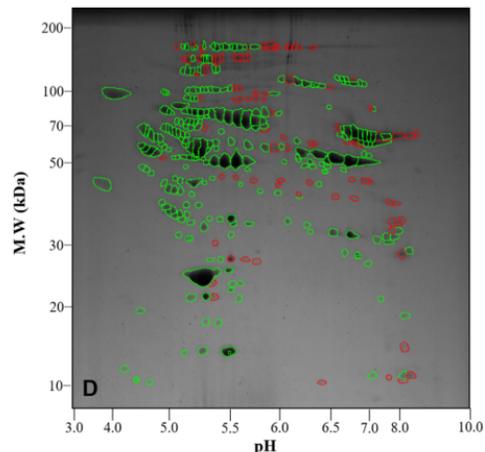
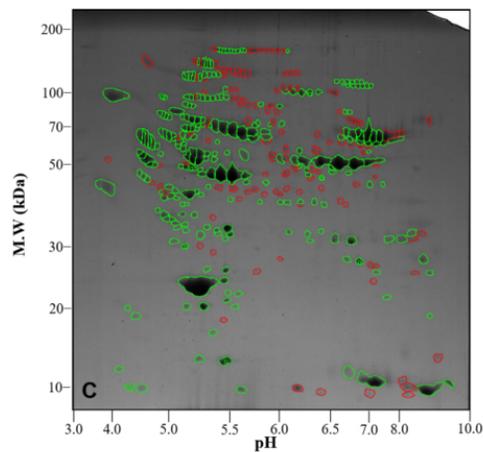
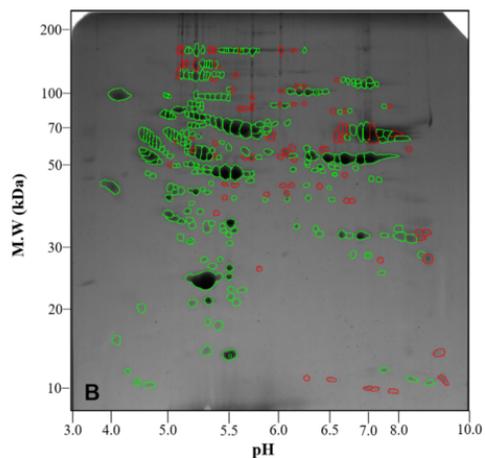
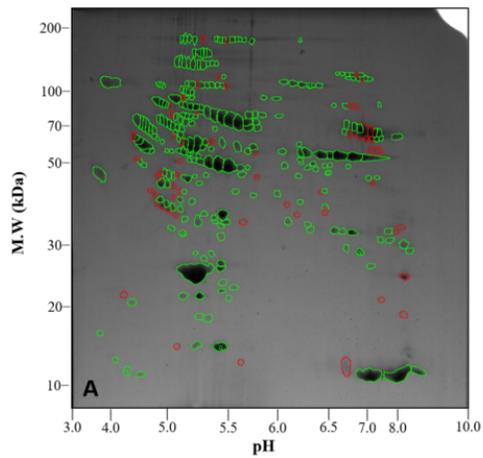
Ions score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event.

Individual ions scores > 31 indicate identity or extensive homology ($p < 0.05$).

Database: nr_Human Human_20160602_curated (1015462 sequences; 342936396 residues)

Highlight:

- 5 reproductive system-related proteins (Ceruloplasmin, Complement C3, Fibrinogen α , Fibrinogen β , and SHBG) were differentially expressed in POF patients.
- They can be used as provisional biomarkers for identifying POF patients.
- These proteins may be helpful in understanding the molecular mechanism of the causes of POF and will provide some insights into the pathogenesis and management of POF.
- This is the first identification of differentially expressed proteins in the serum of POF patients.



green=paired spots
red=non-paired spots

Figure 1

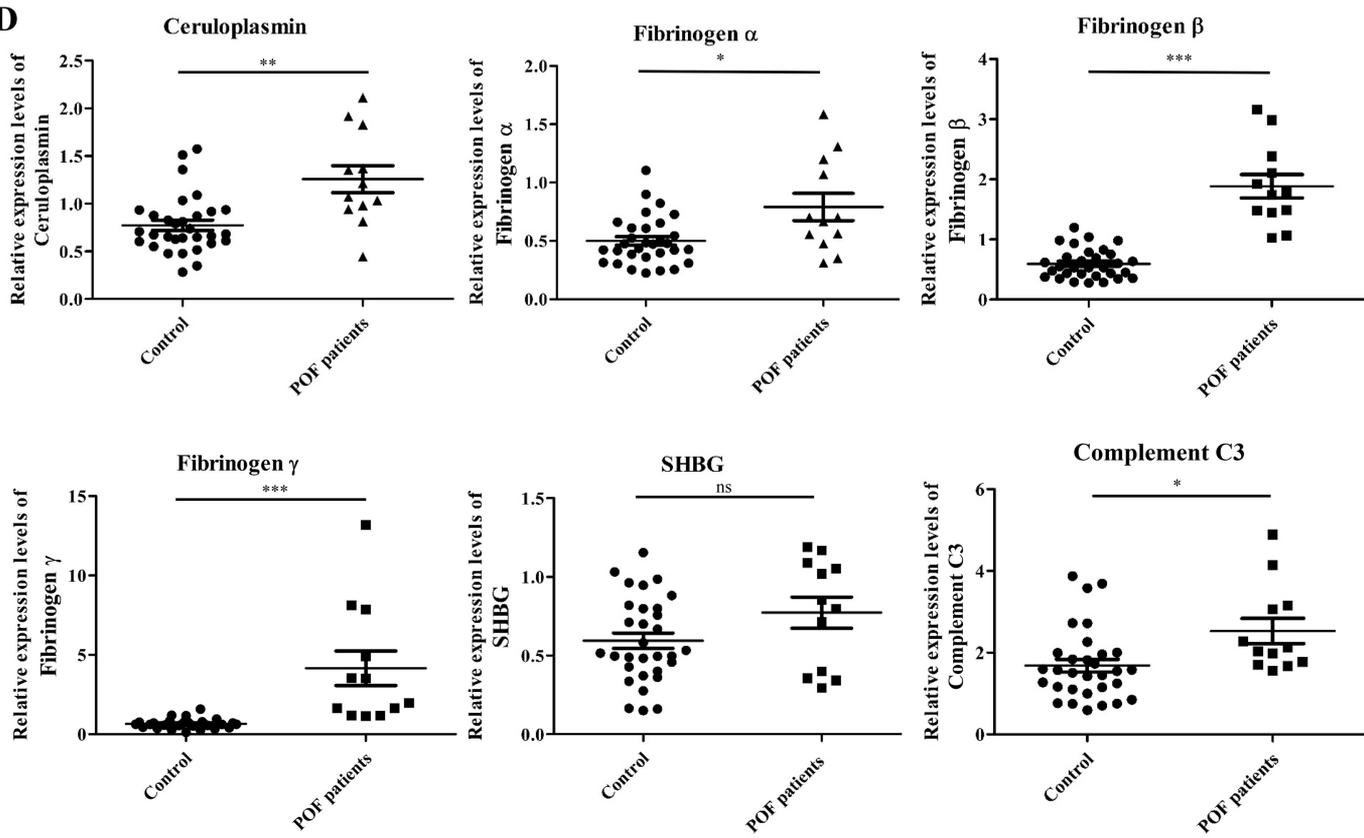
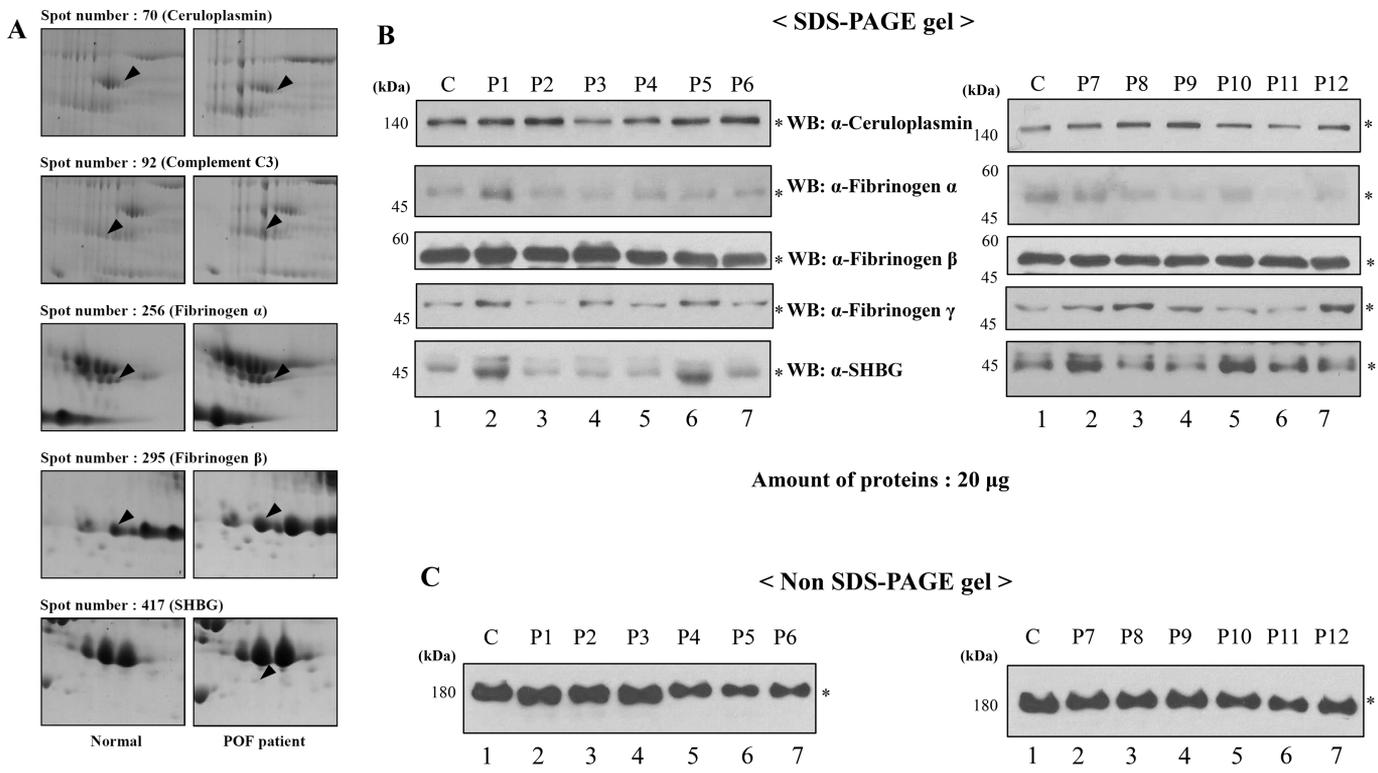
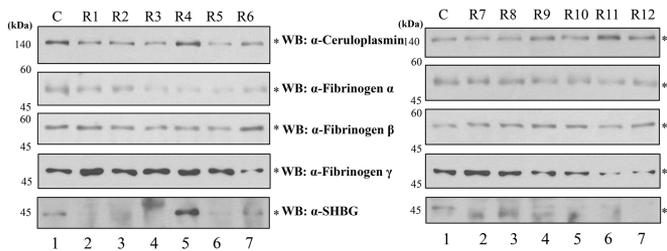


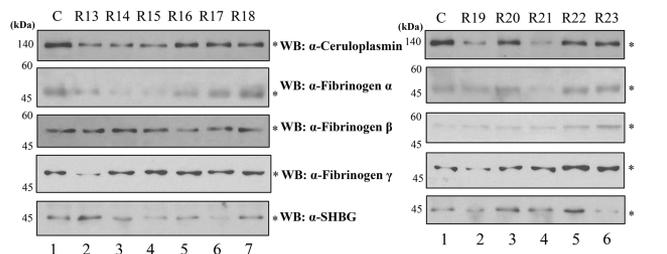
Figure 2

A

< SDS-PAGE gel >

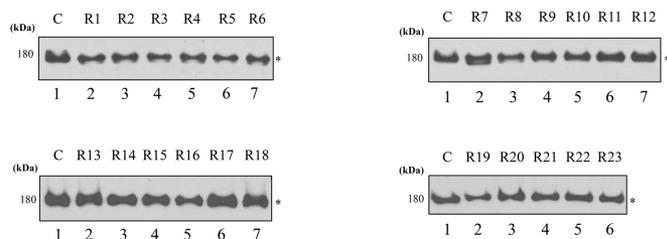
Amount of proteins : 20 μ g

< SDS-PAGE gel >

Amount of proteins : 20 μ g

B

< Non SDS-PAGE gel >

WB: α -Complement C3
Amount of proteins : 3 μ g

C

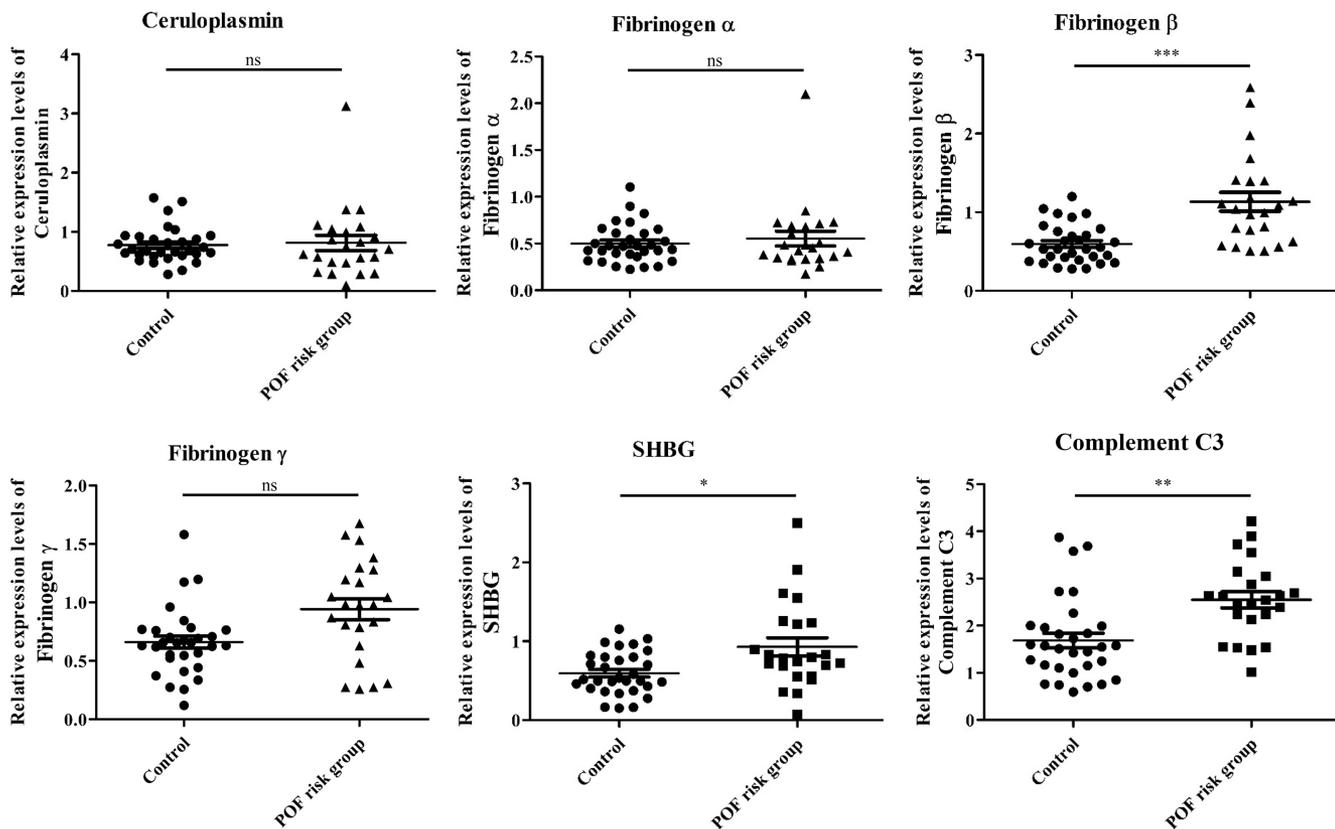


Figure 3

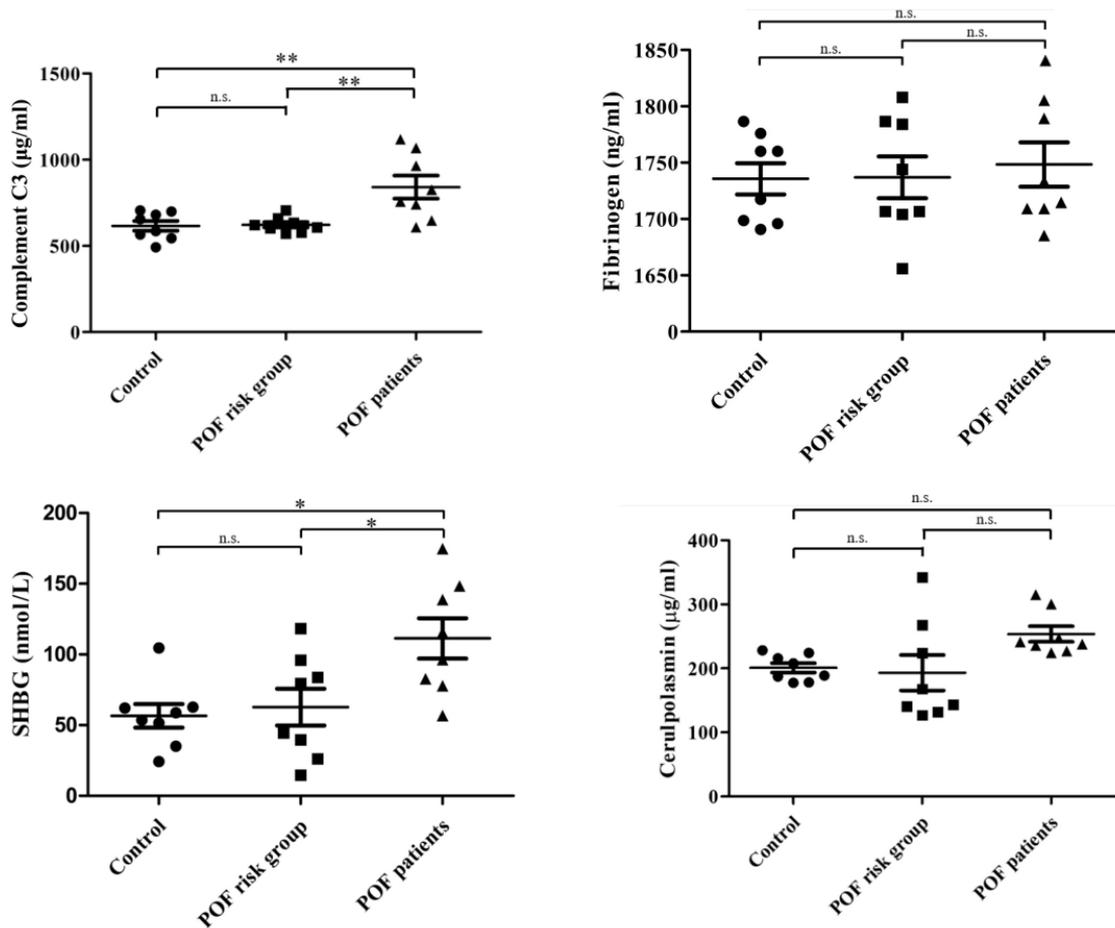


Figure 4