Serum proteins differentially expressed in early- and late-onset preeclampsia assessed using iTRAQ proteomics and bioinformatics analyses

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Background. Preeclampsia remains a serious disorder that puts at risk the lives of perinatal mothers and infants worldwide. This study assessed potential pathogenic mechanisms underlying preeclampsia by investigating differentially expressed proteins (DEPs) in the serum of patients with early-onset preeclampsia (EOPE) and late-onset preeclampsia (LOPE) compared with healthy pregnant women.

Methods. Blood samples were collected from four women with EOPE, four women with LOPE, and eight women with normal pregnancies, with four women providing control samples for each preeclampsia group . Serum proteins were identified by isobaric tags for relative and absolute quantitation combined with liquid chromatography–tandem mass spectrometry. Serum proteins with differences in their levels compared with control groups of at least an absolute $\log_{10}$ fold change (i.e., >1.2 difference) and that were also statistically significantly different between the groups at $P < 0.05$ were further analyzed. Bioinformatics analyses, including gene ontology and Kyoto Encyclopedia of Genes and Genomes signaling pathway analyses, were used to determine the key proteins and signaling pathways associated with the development of PE and to determine those DEPs that differed between women with EOPE and those with LOPE. Key protein identified by mass spectrometry was verified by enzyme linked immunosorbent assay (ELISA).

Results. Compared with serum samples from healthy pregnant women, those from women with EOPE displayed 70 proteins that were differentially expressed with significance. Among them, 51 proteins were significantly upregulated and 19 proteins were significantly downregulated. In serum samples from women with LOPE, 24 DEPs were identified , with 10 proteins significantly upregulated and 14 proteins significantly downregulated compared with healthy pregnant women. Bioinformatics analyses indicated that DEPs in both the EOPE and LOPE groups were associated with abnormalities in the activation of the coagulation cascade and complement system as well as with lipid metabolism . In addition, 19 DEPs in the EOPE group were closely related to placental development or invasion of tumor cells. The downregulated of pregnancy-specific beta-1-glycoprotein 9 (PSG9) in the LOPE group was confirmed by ELISA.

Conclusion. The pathogenesis of EOPE and LOPE appeared to be associated with coagulation cascade activation, lipid metabolism, and complement activation. However, the pathogenesis of EOPE also involved processes associated with greater placental injury. This study provided several new proteins in

the serum which may be valuable for clinical diagnosis of EOPE and LOPE, and offered potential mechanisms underpinning the development of these disorders.

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Abstract

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Keywords: preeclampsia, early-onset preeclampsia, late-onset preeclampsia, proteomics, LC–MS/MS, iTRAQ

Introduction

Preeclampsia (PE), a hypertensive disorder complicating pregnancy, is the main cause of increased perinatal mortality among mothers and infants worldwide. The rate of PE in the United States is approximately 3.4% (Ghulmiyyah et al. 2012), and it is higher in developing countries (Ananth et al. 2013). PE is marked by new-onset hypertension occurring after 20 weeks of gestation, accompanied by either new-onset proteinuria or systemic multiple organ damage (Committee on Practice Bulletins—Obstetrics 2019). Severe PE may lead to convulsions, coma, cerebral hemorrhage, heart failure, placental abruption, disseminated intravascular coagulation, and even death (Bibbins-Domingo et al. 2017). Fetal delivery is the most effective treatment for PE, with other treatments of relieving symptoms used only as an attempt to gain time for enabling further maturity of the fetus. As a result, PE is the leading cause of premature birth and low birth weight, especially severe PE (Ananth et al. 2006).

The exact pathogenesis of PE remains unclear. However, PE is considered a placenta-derived disease because the syndrome resolves once the placenta is removed. There are two subtypes of PE: early- and late-onset preeclampsia. Their pathogeneses are not identical. Early-onset preeclampsia (EOPE) has a higher degree of placental damage, whereas late-onset preeclampsia (LOPE) may focus on the interaction between the normal senescence of the placenta and a maternal genetic susceptibility to cardiovascular and metabolic disease (Burton et al. 2019). However, pathophysiological changes of the placenta further lead to endothelial dysfunction and systemic inflammatory response, which are their common pathogenesis link. (Young et al. 2010).

The placenta is in direct contact with the maternal circulation. Therefore, any changes in the protein expression of placental tissue are reflected in maternal serum proteins (R 2007). If the types and levels of differentially expressed proteins (DEPs) in the serum of patients with PE can be researched quantitatively and holistically, specific serum biomarkers may be found for predicting PE and for further study of its pathogenesis. Compared with other proteomics methods, isobaric tags for relative and absolute quantitation (iTRAQ) combined with liquid chromatography–tandem mass spectrometry (LC-MS/MS) is considered more effective for searching for serum or plasma biomarkers (Moulder et al. 2018). Therefore, in the present study, we used iTRAQ and LC-MS/MS methods to identify serum proteins differentially expressed between EOPE and LOPE and between women with PE and healthy pregnant women. We then used bioinformatics analyses to determine the key proteins and related signaling pathways associated with the development of EOPE and LOPE.

Materials & Methods

Participants and clinical samples

The Medical Research Ethics Committee of Anhui Medical University reviewed and approved our research protocol and an informed patient consent form (Anhui Medical Ethics approval No. 20150192). All patients signed the approved written informed consent form prior to being included in the study.

Blood samples were collected from December 2018 to May 2019 from 16 pregnant women, 8 of whom had received a diagnosis of PE, and were in the Maternal and Child Health Hospital Affiliated with Anhui Medical University. A PE diagnosis was made consistent with the 2019

American College of Obstetricians and Gynecologists pregnancy hypertension guidance (Committee on Practice Bulletins—Obstetrics 2019). All included pregnant women were primipara and without a disease that may have affected their serum protein levels, such as infection, multiple pregnancy, or gestational diabetes mellitus. Four serum samples were obtained from and allocated to each of the following four groups: EOPE, the EOPE control, LOPE, and the LOPE control groups.

Protein extraction and quality testing

High-abundance proteins were removed from the serum samples using Pierce Top 12 Abundant Protein Depletion Spin Columns (Thermo Fisher). The protein concentration was determined by the Bradford method. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the proteins and evaluate the quality of samples.

Trypsin enzymatic hydrolysis and peptide iTRAQ isobaric labeling

After protein quantification, a centrifuge tube containing 60 μg of protein solution was mixed with 5 μL 1 M dithiothreitol at 37°C for 1 h. Then 20 μL of 1 M iodoacetamide was added and the solution allowed to react for 1 h at room temperature. All samples were pipetted into ultrafiltration tubes, and the filtrate was discarded after centrifugation. UA buffer (8 M urea, 100 mM Tris-HCl, pH 8.0, 100 μL) was added and the sample centrifuged at 14,000 *g* for 10 min; this step was repeated twice. Then, 50 mM NH_4HCO_3 (50 μL) was added and the filtrate discarded after centrifugation; this step was repeated three times. Trypsin buffer (40 μL) was added and mixed, and the samples were centrifuged at 600 rpm for 1 min. The samples were then subjected to enzymatic hydrolysis at 37°C for 12–18 h. All the remaining samples were used.

After labeling, the same volume of each sample was mixed together and desalted using a C18 cartridge.

LC-MS/MS analysis

The labeled samples were redissolved in 40 μL of 0.1% formic acid aqueous solution. The peptides were loaded onto a C18-reversed phase column (3 μm C18 resin, 75 μm × 15 cm). The mobile phases consisted of 2% methyl cyanide/0.1% formic acid/98% water and 80% methyl cyanide/0.08% formic acid/20% water. The gradient for the B phase increased linearly at 0–68 min from 7% to 36% and at 68–75 min, from 36% to 100%. Each sample was separated by capillary high-performance liquid chromatography and was analyzed by an Orbitrap Fusion Lumos mass spectrometer (Thermo Science). After the data were collected, they were processed according to the flowchart shown in Fig. 1. Protein identification and quantification were done by using the ProteinPilot Software, version 4.2 (Sciex). Human proteome databases containing UniProt sequences were used to perform peptide identification. Serum proteins with fold changes in their levels compared with control groups of at least 1.2 (in two of two replicates) and that were also statistically significantly different between the groups at $P < 0.05$ were further analyzed.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analyses

Metascape, a web-based resource (http://metascape.org) for gene and protein annotation, visualization, and integration discovery (Soonthornvacharin et al. 2017, Fang et al. 2019) was used to perform GO analyses. The KOBAS online analysis database

(http://kobas.cbi.pku.edu.cn/) was used to performed KEGG pathway analyses (Kanehisa et al. 2000). A two-sided $P < 0.05$ was regarded significantly different.

Enzyme linked immunosorbent assay (ELISA)

PSG9, the downregulated protein in the LOPE group, was verified by ELISA kit. The experiment was performed according to the kit instructions. Blood samples were collected from women with LOPE or normal pregnancies.

Statistical analysis

Two-tailed Mann-Whitney U test was performed with SigmaPlot software. Values are expressed as means ± SEM. A value of $P < 0.05$ was considered statistically significant.

Results

Participants

The mean and standard deviation (SD) age of the patients in the EOPE group was 31.8 ± 5.0 years, and they were at a mean (SD) gestation of 31.9 ± 2.9 weeks; the mean (SD) age of the participants in the EOPE control group was 30.0 ± 1.0 years, with a mean (SD) gestation of 31.5 ± 1.4 weeks. No significant difference was found in age ($P = 0.57$) or gestational weeks ($P = 0.84$) between these two groups. The mean (SD) age of the patients in the LOPE group was 24.8 ± 1.5 years, with 37.8 ± 1.5 weeks' gestation; the mean (SD) age of the participants in the LOPE control group was 27.0 ± 1.3 years, with a mean (SD) of 38.9 ± 1.3 weeks' gestation. No significant difference was found in age ($P = 0.15$) or gestation ($P = 0.37$) between these two groups.

173

174 ***SDS-PAGE***

175 The total proteins in the molecular weight range of 10–220 kDa from 16 samples were
176 effectively separated by SDS-PAGE. The proteins were not degraded, and the high-abundance
177 proteins were obtained (Fig. 2).

178

179 ***MS/MS spectrum analysis and Identification of DEPs***

180 LC-MS/MS is a powerful tool for identifying proteins in serum samples. We identified 413
181 serum proteins in the EOPE and EOPE healthy control groups, of which 70 were significantly
182 and differentially expressed between the two groups, with 51 upregulated and 19 downregulated
183 (Table 1, Fig. 3). We also identified 470 proteins in the LOPE and LOPE control groups, of
184 which 24 proteins were significantly and differentially expressed between the two groups, with
185 10 upregulated and 14 downregulated (Table 2, Fig. 3). Clustergrams generated describing the
186 expression of these DEPs indicated that the expression patterns between the patient EOPE or
187 LOPE groups obviously differed from their control groups, but the two patient groups, EOPE
188 and LOPE, clustered together (Fig. 3B and 3D).

189

190 ***GO functional annotation and enrichment analysis***

191 GO analysis is an important method and tool in the field of bioinformatics. It includes three
192 categories: cellular component, molecular function, and biological process. GO functional
193 annotation analysis results show the number of DEPs under each item in the three categories. GO
194 functional enrichment analysis provides significant GO functional terms associated with the
195 DEPs, that is, those biological functions significantly correlated with the DEP. In vivo, different

196 proteins coordinate with one another to generate a biological behavior, and a pathway-based
197 analysis helps to further understand those biological functions. A significant pathway enrichment
198 analysis can determine the most important biochemical and metabolic pathways and signal
199 transduction pathways associated with the DEPs.
200 The GO functional annotation analysis results for the EOPE group are shown in Fig. 4. For the
201 biological process category, the highest percentages of the proteins were associated with the term
202 *biological regulation* (n = 43 proteins; with the top 3 upregulated proteins in this process being
203 CHL1, LRP1, and CNTN1). For the cellular component category, the highest percentages of the
204 proteins were associated with the term *extracellular region* (n = 49 proteins; with the top 2
205 upregulated proteins in this process being MMP2 and CHL1). For the molecular function
206 category, the highest percentages of the proteins were associated with the term *binding* (n = 45
207 proteins; with the top 3 upregulated proteins in this process being MMP2, CHL1, and PEPD).
208 For the LOPE group vs. their controls, the highest percentages of the proteins in the biological
209 process category were associated with the term *biological regulation* (n = 17; with the top 3
210 upregulated proteins in this process being PAPPA2, F7, and vWF). The highest percentages of
211 the proteins in the cellular component category were associated with term *extracellular region* (n
212 = 18; with the top 3 upregulated proteins in this process being PAPPA2, CETP, and F7). The
213 highest percentages of the proteins in the molecular function category were associated with the
214 term *binding* (n = 13; with the top 3 upregulated proteins in this process being APPA2, CETP,
215 and F7). The terms in the GO functional enrichment analysis for the EOPE group vs. their
216 controls mainly included response to stress, defense response, and negative regulation of
217 catalytic activity, whereas the terms in the GO functional enrichment analysis for the LOPE

group vs. their controls included enzyme inhibitor activity and serine-type endopeptidase inhibitor activity (Fig. 5).

KEGG signaling pathway analysis

The KOBAS online analysis tool was used to identify the functions associated with the DEPs and the KEGG signaling pathways. Results of the KEGG pathway enrichment analysis showed that complement and coagulation cascades, proteoglycans in cancer, and metabolic pathways were the main signaling pathways associated with the EOPE group, whereas complement and coagulation cascades were the main signaling pathways associated with the LOPE group (Fig. 6). Thus, the results indicated that a functional change in coagulation was the main finding for both EOPE and LOPE.

PSG9 protein level in serum

To verify the PSG9 protein level in serum, we used ELISA to identify again. The data showed a significant difference between LOPE group and control group (4.54 ± 1.22 vs. 6.32 ± 1.73, Fig. 7). The result is consistent with the study of mass spectrometry.

Discussion

Proteomics methods have provided some important information regarding PE. For example, Blumenstein et al. (2009) used a differential in gel electrophoresis–based approach to identify changes in the plasma proteome of pregnant women who subsequently developed PE. They found that those DEPs are mainly involved in lipoprotein metabolism, the blood coagulation system, and the complement system. The results of the present study were consistent with those

of Blumenstein et al. (2009), but we also made some new discoveries (discussed below). These results suggest that the pathogenesis of PE is complex and is associated with multiple proteins and signaling pathways.

To maintain pregnancy and fetal growth progress within normal reference ranges, healthy pregnant women develop physiological hyperlipidemia. However, they do not experience angiopathy because high-density lipoprotein (a vascular protection factor) and low-density lipoprotein (LDL, an atherogenic factor) also increase to protect the vascular endothelium from injury (Jin et al. 2016, Wang et al. 2017). Compared with healthy pregnant women, pregnant women with PE display significantly increased serum triglycerides and LDLs, which may enhance oxidative stress and ultimately lead to vascular endothelial cell injury (Huda et al. 2009, Pohanka 2013). Our study found that there were six DEPs associated with lipid regulation increased in the PE groups, including ApoB, LRP1, ApoL3, ApoC-I, CETP, and ApoM. ApoB, the main carrier protein of LDL, is an atherogenic risk factor. Much evidence supports that ApoB is significantly upregulated in the plasma of patients with PE (LIN et al. 2019). CETP plays an important role in high-density lipoprotein metabolism and reverse cholesterol transport and is upregulated in the third trimester of pregnancy. Another study has reported that the TaqIB polymorphism of the *CETP* gene is significantly correlated with the triglyceride and total cholesterol levels in patients with severe PE (Belo et al. 2004). Here, we reported for the first time, to our knowledge, that LRP1, ApoC1, and ApoL3 are increased in the EOPE group. LRP1 and ApoC1 accelerate the development of atherosclerosis through different pathways (Westerterp et al. 2007, Mueller et al. 2018). Endothelial function decreases during PE, and endothelial dysfunction is a characteristic of atherosclerosis. ApoL3, as a regulator of MAPK

and FAK signaling in endothelial cells, has been shown to be involved in angiogenesis in vitro (Khalil et al. 2018). Increased ApoL3 in PE may be a compensatory response to endothelial dysfunction. Therefore, the current evidence suggests that dyslipidemia may be related to the development of PE, but the specific pathogenesis remains to be explored in future studies.

Physiological hypercoagulability in healthy pregnant women can prevent intrapartum and postpartum hemorrhage. However, abnormalities in coagulation, the anti-coagulation system, and the fibrinolytic system lead to pathological hypercoagulability in PE (Dusse et al. 2011). In the LOPE group of the present study, coagulation factors VII, vWF, and SRPIND1 were upregulated group, whereas AAT and ATIII were downregulated compared with healthy controls. In the EOPE group, coagulation factor V, plasminogen, and ADAMTS13 were upregulated. These results are consistent with those of many previous trials (discussed below). The level of plasma FVII in patients with severe PE is significantly higher than that in healthy pregnant women; therefore plasma FVI levels may show high sensitivity and specificity in differentiating between PE and normal pregnancy (Dusse et al. 2016). The coagulation factor vWF is a specific marker that reflects damage of endothelial cells; thus, damaged microvascular endothelial cells in PE promote the expression of vWF. Owing to the activation of the intrinsic and exogenous coagulation pathways by damaged endothelial cells, a large number of coagulation factors are activated. This leads to a massive generation of thrombin, which consumes a large amount of anti-thrombin III, resulting in a significant decrease in its level (Pottecher et al. 2009, Demir et al. 2010). In response to abnormalities in the coagulation mechanism of PE, evidence-based medical study has also shown that oral administration of low-

dose aspirin during early pregnancy can significantly reduce the incidence rate of PE (Rolnik et al. 2017).

A growing number of studies have shown that abnormal expression with the complement system is associated with PE (Agostinis et al. 2010, Derzsy et al. 2010). Our results add to this evidence, showing that expression levels of CFD, CIQC, CIRL, C9, C1S, and C1R were significantly increased, while expression levels of C4B, FCN2, and FCN3 were significantly decreased in the EOPE group compared with their control group. Moreover, the expression levels of complement C1RL and C4B were decreased in the LOPE group compared with their controls.

Downregulation of PSG1 and PSG9 expressed in the PE groups is an interesting finding in our study. PSG is a pregnancy-specific glycoprotein that is synthesized and secreted into the blood by placental syncytiotrophoblast cells. Rong et al. (2017) found that PSG9 significantly promotes the angiogenesis of human umbilical vein endothelial cells. Chang et al. (2016) found a significant increase in the number of cases with deletions in the PSG gene locus among patients with PE. We hypothesize that downregulation of the PSG protein family may be involved in the pathogenesis of PE by affecting the proliferation of endothelial cells although no specific experimental evidence currently supports or disproves this hypothesis.

PE is considered a placenta-derived disease. In the classic two-stage model, placental stress leads to dysfunction of maternal peripheral endothelial cells, systemic inflammatory response, and the clinical syndrome of PE (Staff 2019). This model is also reflected in our mass spectrometry results. In both the EOPE and LOPE groups, abnormalities of coagulation cascade activation,

lipid metabolism, and complement activation were found. However, the main stressor associated with EOPE is placental hypoperfusion secondary to impaired spiral artery remodeling; by contrast, in LOPE, the cause is more likely attributable to a mismatch between normal maternal perfusion and the metabolic demands of the placenta and fetus (Burton et al. 2019). Spiral artery remodeling is completed by the placental trophoblast cells continuously invading and destroying the uterine spiral arterial wall and gradually replacing the endothelial cells. Many studies have focused on the control of this invasion (Wagner et al. 2011, Wu et al. 2016, Pollheimer et al. 2018).

Unexpectedly, we also found 19 DEPs in the EOPE group that were closely related to placental development or invasion of tumor cells. This result was not detected in the LOPE group, which showed an increase only in PAPPA2, which affects the invasion and metastasis of placental trophoblasts and leads to a gradual decline in placental function (Wagner et al. 2011). LYVE1, CST3, and NCAM1 are also reportedly involved in placental vascular remodeling (Song et al. 2010, Pawlak et al. 2019, Zhang et al. 2019). GRN, CD5L, ENO1, CHL1, CRISP3, VASN, and TNIK promote the invasive ability of tumor cells (Buhusi et al. 2003, Song et al. 2014, Chon et al. 2016, Aran et al. 2018, Bhandari et al. 2019, Voshtani et al. 2019, Wang et al. 2019). By contrast, SEMA4B, KRT1, KRT9, FBLN1, and PTGDS are involved in the anti-invasive activity of tumor cells (Blanckaert et al. 2015, Jian et al. 2015, Marano et al. 2018, Zhang et al. 2018). MMP2 plays an important role in trophoblast invasion and is generally thought to be downregulated in PE (Wu et al. 2016). We found the opposite result, that is, the expression of MMP2 was upregulated in EOPE; however, expression of PI16, which inhibits MMP2 activity (Hazell et al. 2016), was also upregulated. PEPD is a hydrolase that affects collagen

biosynthesis, cell proliferation, and matrix remodeling. Pehlivan et al. (2017) have shown that PEPD activity in the plasma, umbilical cord, and placental tissue of women with pregnancy-induced hypertension is higher than that of healthy pregnant women. Our results also indicated an upregulation of PEPD in the EOPE group. The dysregulation of the expression of these proteins may be an important cause of placental dysfunction in EOPE.

Furthermore, a study shows that soluble form of placenta-derived endoglin (sENG) was upregulated in the serum of EOPE, which involved in endothelial dysfunction in coordination with soluble fms-like tyrosine kinase (Venkatesha et al. 2006). Our mass spectrometry results was similar to that previous finding showing that endoglin was upregulated in EOPE but not in LOPE group. This finding may also indicate more severe endothelial dysfunction in EOPE.

Conclusions

In summary, the use of iTRAQ combined with LC-MS/MS was effective for screening serum for DEPs in PE. We used bioinformatics to analyze the DEPs that showed significant changes in their expression levels to provide potential indicators for detecting PE and ideas for the study of pathogenesis in PE. In conclusion, the pathogenesis of EOPE and LOPE appeared to be related to dysfunctions in coagulation cascade activation, lipid metabolism, and complement activation. However, compared with that in LOPE, the pathogenesis of EOPE was associated with greater placental injury. This study provides several new proteins in the serum which may potentially have value in clinical diagnosis of EOPE and LOPE and offers potential mechanisms for the development of EOPE and LOPE, contributing to the future research on prediction, prevention, and pathogenesis of PE.

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508

509

510 **Figure Legends**

511

512 **Figure 1: Flowchart of the data analysis procedure.**

513

514 **Figure 2. Removal of high-abundance proteins.** (A) Lane E is the original sample from the
515 early-onset preeclampsia (EOPE) group. Lane M contains markers. Lanes 1–4 are from serum
516 samples obtained from the EOPE group after removing the highly abundant proteins, whereas
517 lanes 5–8 are from the EOPE control group after removing the highly abundant proteins. (B)
518 Lane L is the original sample from the late-onset preeclampsia (LOPE) group. Lane M contains
519 markers. Lanes −1 to −4 are from the LOPE group after removing the highly abundant proteins,
520 whereas lanes −5 to −8 are from the LOPE control group after removing the high-abundance
521 proteins. The bands are clear and uniform and without protein degradation.

522

523 **Figure 3. Differential protein expression.** (A and C) Volcano plots with red dots on the right-
524 hand side indicating upregulation, green dots on the left-hand side indicating downregulation,
525 and black dots indicating no significant change in protein expression levels based on the criteria
526 of an absolute $\log_{10}$ fold change (FC; i.e., >1.2 difference) and $P < 0.05$ between early-onset
527 preeclampsia and its control group (A), and between late-onset preeclampsia and its control
528 group (C). (B and D) Clustergram for the expression of the DEPs between early-onset
529 preeclampsia and control (B) and between late-onset preeclampsia and control (D).

530

531 **Figure 4. Gene ontology functional annotation.** The upregulated and downregulated proteins
532 in the three categories of biological process, cellular component, and molecular function in (A)

533 the early-onset preeclampsia group vs. its control group and (B) the late-onset preeclampsia
534 group vs. its control group.
535
536 **Figure 5: Gene ontology (GO) functional enrichment.** Enriched GO terms for the upregulated
537 and downregulated proteins and the associated protein numbers in (A) early-onset preeclampsia
538 vs. its control and (B) late-onset preeclampsia vs. its control.
539
540 **Figure 6. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment.** KEGG
541 pathways enriched for the upregulated and downregulated proteins and the associated protein
542 numbers in (A) early-onset preeclampsia vs. its control and (B) late-onset preeclampsia vs. its
543 control.
544
545 **Figure 7. Protein level of pregnancy-specific beta-1-glycoprotein 9 (PSG9) in serum**.
546 Concentration of PSG9 in the patient serum in control and LOPE groups. Values are shown as
547 the mean ± SEM (n = 10); $*P < 0.05$ for Control vs. LOPE.

Table 1(on next page)

List of differentially expressed proteins in the early-onset preeclampsia group.

1 Table 1: List of differentially expressed proteins in the early-onset preeclampsia group.

2

Regulated type	Protein accession	GENE NAME	PROTEIN DESCRIPTION	FC
UP	A0A024R6R4	MMP2	Matrix metallopeptidase 2	21.48
UP	A8K2X4	-	cDNA FLJ75401	12.87
UP	O00533	CHL1	Neural cell adhesion molecule L1-like protein	7.69
UP	J3K000	PEPD	PEPD protein	6.6
UP	A8K3I0	-	cDNA FLJ78437	6.31
UP	Q6UXB8	PI16	Peptidase inhibitor 16	5.76
UP	Q07954	LRP1	Prolow-density lipoprotein receptor-related protein 1	4.82
UP	Q12860	CNTN1	Contactin-1	4.64
UP	V9HWB4	HEL-S-89n	Epididymis secretory sperm binding protein Li 89n	4.63
UP	P35527	KRT9	Keratin, type I cytoskeletal 9	4.51
UP	P14543	NID1	Nidogen-1	3.86
UP	O95236	APOL3	Apolipoprotein L3	3.4
UP	A6XNE2	-	Complement factor D preproprotein	3.34
UP	P41222	PTGDS	Prostaglandin-H2	3.17
UP	V9H1C1	-	Gelsolin exon 4 (Fragment)	2.95
UP	Q9Y5Y7	LYVE1	Lymphatic vessel endothelial hyaluronic acid receptor 1	2.95
UP	Q13201	MMRN1	Multimerin-1	2.83
UP	B3KQF4	-	cDNA FLJ90373	2.74
UP	Q6MZL2	DKFZp686M 0562	Uncharacterized protein DKFZp686M0562 (Fragment)	2.71
UP	J3KPA1	CRISP3	Cysteine-rich secretory protein 3	2.65
UP	P01034	CST3	Cystatin-C	2.64
UP	A0A0S2Z4F1	EFEMP1	EGF Containing Fibulin Extracellular Matrix Protein 1	2.62
UP	P23142	FBLN1	Fibulin-1	2.57
UP	A0A384N669	-	Epididymis secretory sperm binding protein	2.48
UP	J3KNB4	CAMP	Cathelicidin antimicrobial peptide	2.47
UP	B7Z544	-	cDNA FLJ51742	2.43
UP	A8K061	-	cDNA FLJ77880	2.42
UP	A0A087WV75	NCAM1	Neural cell adhesion molecule 1	2.37
UP	Q9NQ79	CRTAC1	Cartilage acidic protein 1	2.24
UP	B2RBW9	-	cDNA, FLJ95746	2.23
UP	A0A024RAA7	C1QC	Adiponectin B	2.15
UP	P16070	CD44	CD44 antigen	2.06 2.01
UP	Q76LX8	ADAMTS13	Von Willebrand Factor-Cleaving Protease	2.01
UP	Q6EMK4	VASN	Vasorin	2

UP	D6RE86	CP	Ceruloplasmin (Fragment)	1.88
UP	H6VRG1	KRT1	Keratin 1	1.87
UP	Q86U17	SERPINA11	Serpin A11	1.81
UP	Q9UKE5	TNIK	TRAF2 and NCK-interacting protein kinase	1.75
UP	P61626	LYZ	Lysozyme C	1.74
UP	P02743	APCS	Serum amyloid P-component	1.74
UP	Q16853	AOC3	Membrane primary amine oxidase	1.73
UP	Q9NZP8	C1RL	Complement C1r subcomponent-like protein	1.73
UP	A0A024R035	C9	Complement C9	1.71
UP	A0A0A0MRJ7	F5	Coagulation factor V	1.66
UP	P09871	C1S	Complement C1s subcomponent	1.65
UP	Q6PIL8	IGK@	IGK@ protein	1.63
UP	P49908	SELENOP	Selenoprotein P	1.55
UP	P02654	APOC1	Apolipoprotein C-I	1.55
UP	D3DRR6	ITIH2	Inter-alpha (Globulin) inhibitor H2	1.54
UP	B4DPQ0	C1R	Complement C1r subcomponent	1.52
UP	J3KNP4	SEMA4B	Semaphorin-4B	1.51
DOWN	Q6GMX6	IGH@	IGH@ protein	0.56
DOWN	A0A0X9TD47	-	MS-D1 light chain variable region (Fragment)	0.43
DOWN	Q9UNU2	C4B	Complement protein C4B frameshift mutant (Fragment)	0.43
DOWN	P01591	JCHAIN	Immunoglobulin J chain	0.34
DOWN	Q14213	EBI3	Interleukin-27 subunit beta	0.34
DOWN	P28799	GRN	Granulins	0.33
DOWN	B2R7N9	-	cDNA, FLJ93532	0.32
DOWN	P00709	LALBA	Alpha-lactalbumin	0.29
DOWN	G5E9F7	PSG1	Pregnancy specific beta-1-glycoprotein 1	0.25
DOWN	A0A1B0GUU9	IGHM	Immunoglobulin heavy constant mu (Fragment)	0.23
DOWN	A0A075B6R9	IGKV2D-24	Immunoglobulin kappa variable 2D-24	0.23
DOWN	O75636	FCN3	Ficolin-3	0.22
DOWN	O43866	CD5L	CD5 antigen-like	0.2
DOWN	P06733	ENO1	Alpha-enolase	0.16
DOWN	P09172	DBH	Dopamine beta-hydroxylase	0.16
DOWN	Q15485	FCN2	Ficolin-2	0.13
DOWN	Q86TT1		Full-length cDNA clone CS0DD006YL02 of Neuroblastoma	0.08
DOWN	P00739	HPR	Haptoglobin-related protein	0.06
DOWN	P00738	HP	Haptoglobin	0.01

Table 2(on next page)

List of differentially expressed proteins in the late-onset preeclampsia group.

Table 2: List of differentially expressed proteins in the late-onset preeclampsia group.

Regulated type	Protein accession	GENE NAME	PROTEIN DESCRIPTION	FC
UP	Q9BXP8	PAPPA2	Pappalysin-2	5.27
UP	A0A0S2Z3F6	CETP	Cholesteryl ester transfer protein plasma isoform 1	3.51
UP	P08709	F7	Coagulation factor VII	2.8
UP	Q6NS95	IGL@	IGL@ protein	2.67
UP	P04275	VWF	von Willebrand factor	2.46
UP	C0JYY2	APOB	Apolipoprotein B	1.93
UP	A0A140VK24	-	Testicular secretory protein Li 24 protein 1	1.74
UP	P05546	SERPIND1	Heparin cofactor 2	1.52
UP	O95445	APOM	Apolipoprotein M	1.34
UP	P80108	GPLD1	Phosphatidylinositol-glycan-specific phospholipase D	1.26
DOWN	P05543	SERPINA7	Thyroxine-binding globulin	0.82
DOWN	Q9NZP8	C1RL	Complement C1r subcomponent-like protein	0.63
DOWN	A8K2T7	-	Receptor protein-tyrosine kinase	0.61
DOWN	Q00887	PSG9	Pregnancy-specific beta-1-glycoprotein 9	0.6
DOWN	P07333	CSF1R	Macrophage colony-stimulating factor 1 receptor	0.56
DOWN	Q2L9S7	AAT	Alpha-1-antitrypsin MBrescia variant receptor 1	0.53
DOWN	A0A2S0BDD1	ATIII-R2	Antithrombin III isoform	0.49
DOWN	Q8WW79	SELL	L-selectin	0.48
DOWN	P40189	IL6ST	Interleukin-6 receptor subunit beta (Fragment)	0.47
DOWN	B2R7Y0	-	cDNA, FLJ93654	0.41
DOWN	Q12860	CNTN1	member 2 (SERPINB2)	0.36
DOWN	Q8WWZ8	OIT3	Oncoprotein-induced transcript 3 protein	0.31
DOWN	A0A140TA29	C4B	Complement C4-B	0.21
DOWN	B2R950	-	cDNA, FLJ94213	0.18

Figure 1

Flowchart of the data analysis procedure

Flowchart of the data analysis procedure

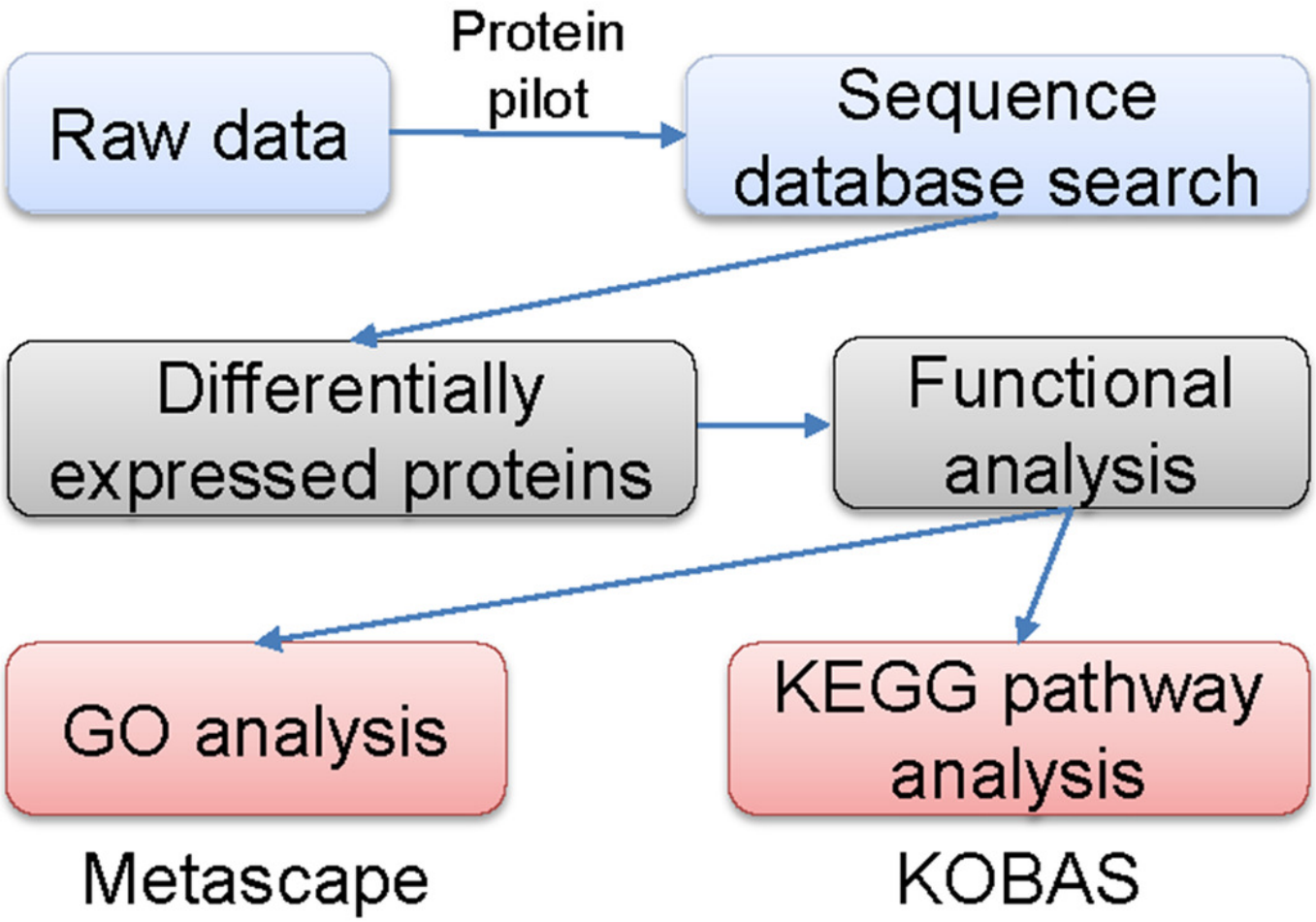


Figure 2

Removal of high-abundance proteins

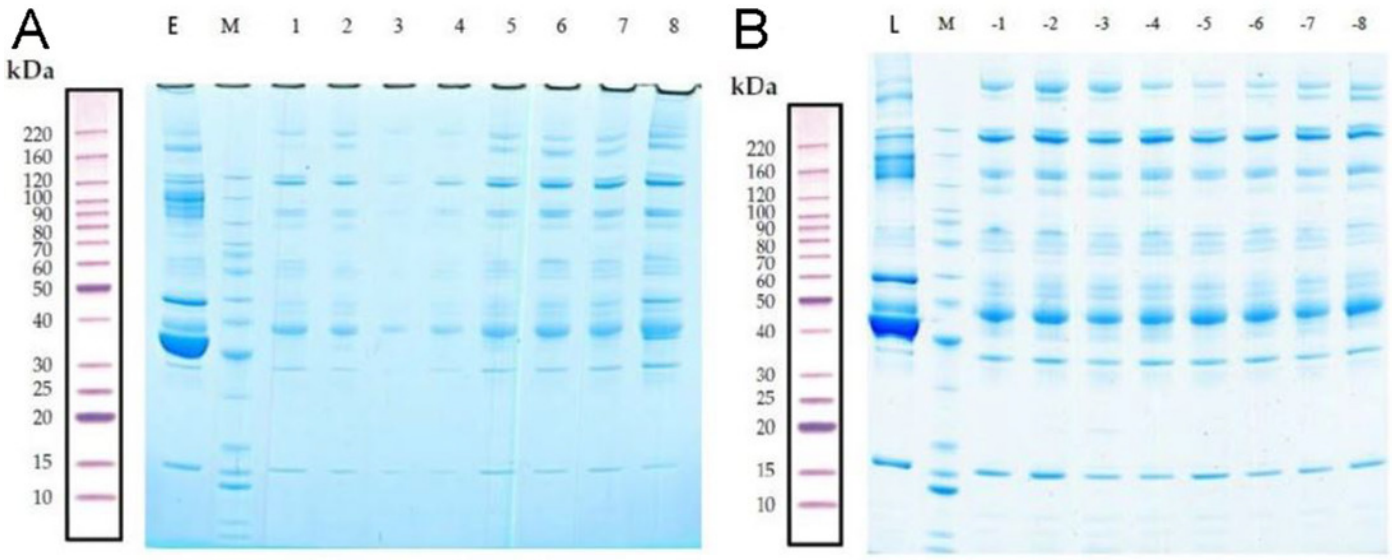


Figure 3

Differential protein expression a

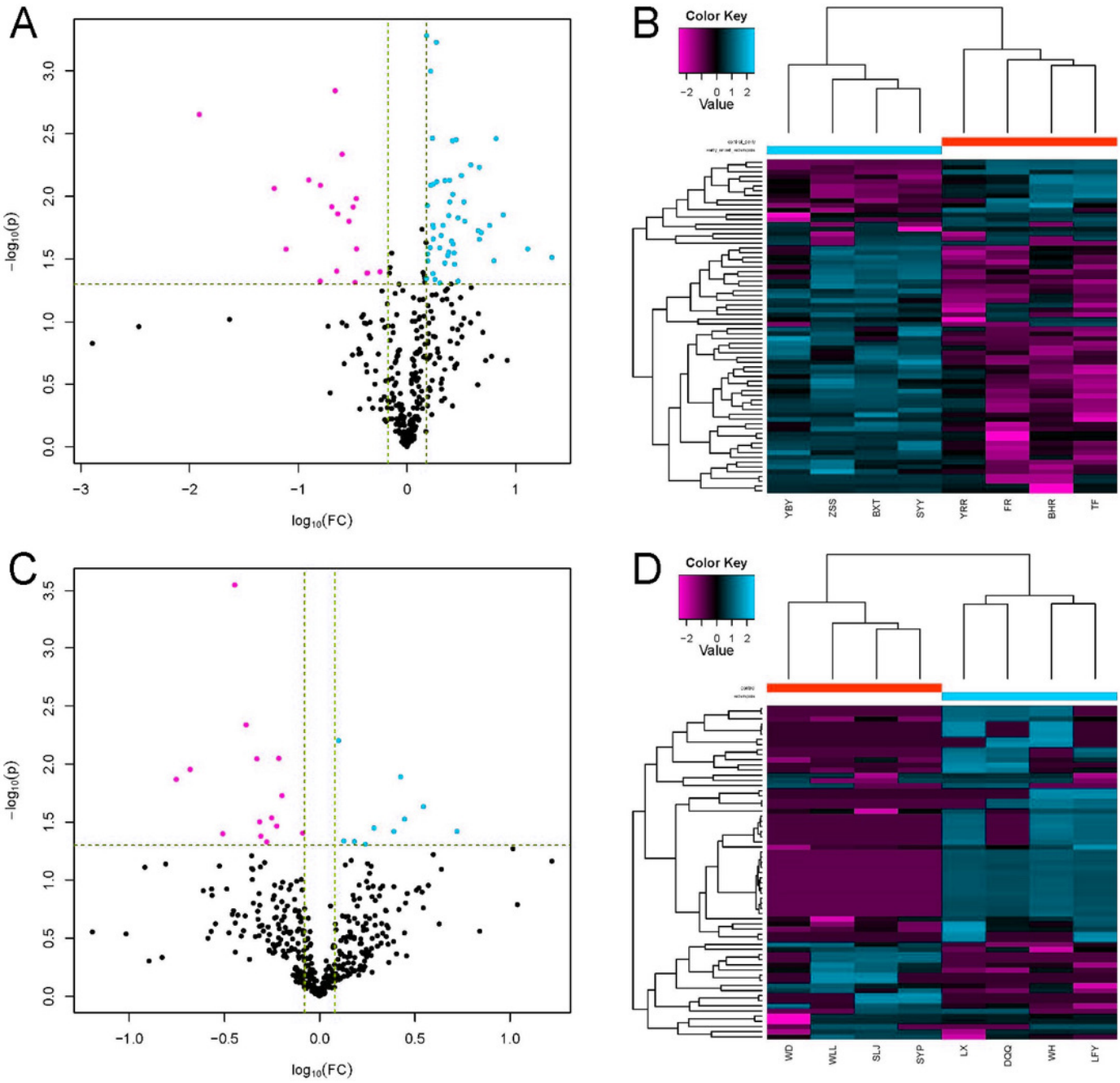


Figure 4

Gene ontology functional annotation

The upregulated and downregulated proteins in the three categories of biological process, cellular component, and molecular function in (A) the early-onset preeclampsia group vs. its control group and (B) the late-onset preeclampsia group vs. its control group.

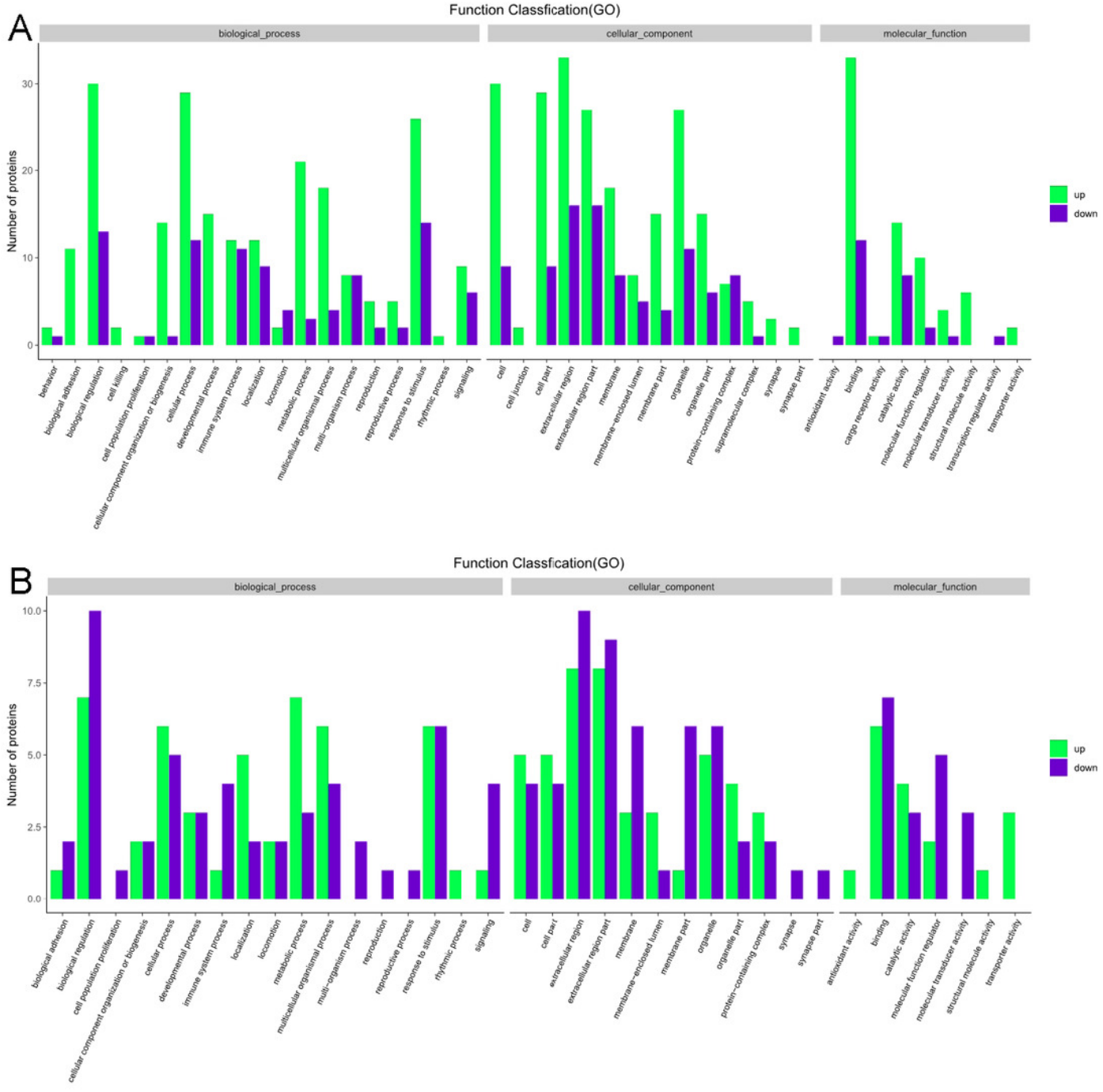
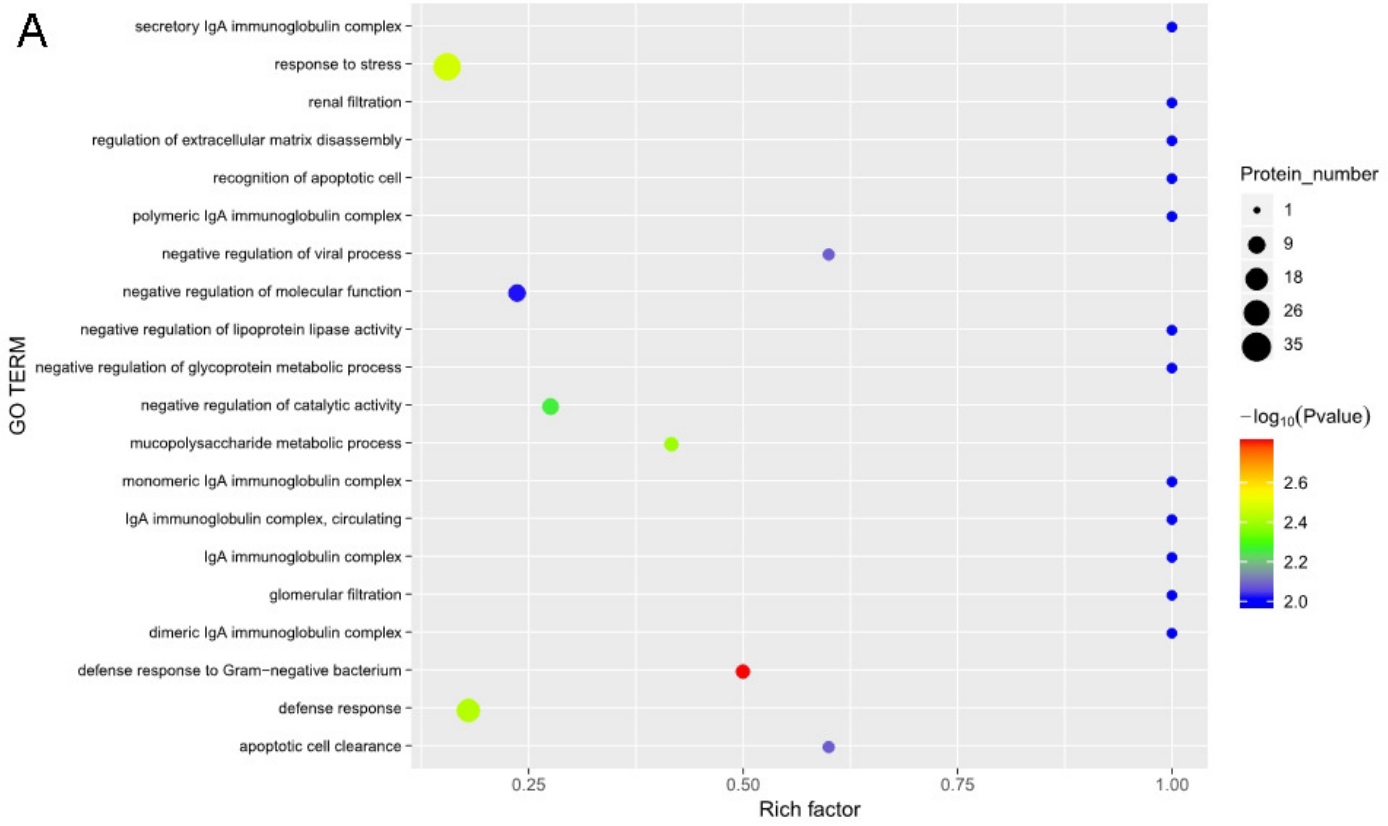

A
Function Classfication(GO)
biological_process
cellular_component
molecular_function
Number of proteins
up
down
behavior
biological adhesion
biological regulation
cell killing
cell population proliferation
cellular component organization or biogenesis
cellular process
developmental process
immune system process
localization
locomotion
metabolic process
multicellular organismal process
multi-organism process
reproduction
reproductive process
response to stimulus
rhythmic process
signaling
cell
cell junction
cell part
extracellular region
extracellular region part
membrane
membrane-enclosed lumen
membrane part
organelle
organelle part
protein-containing complex
supramolecular complex
synapse
synapse part
antioxidant activity
binding
cargo receptor activity
catalytic activity
molecular function regulator
molecular transducer activity
structural molecule activity
transcription regulator activity
transporter activity
B
Function Classfication(GO)
biological_process
cellular_component
molecular_function
Number of proteins
up
down

Figure 5

Gene ontology (GO) functional enrichment

Enriched GO terms for the upregulated and downregulated proteins and the associated protein numbers in (A) early-onset preeclampsia vs. its control and (B) late-onset preeclampsia vs. its control.


A
secretory IgA immunoglobulin complex
response to stress
renal filtration
regulation of extracellular matrix disassembly
recognition of apoptotic cell
polymeric IgA immunoglobulin complex
negative regulation of viral process
negative regulation of molecular function
negative regulation of lipoprotein lipase activity
negative regulation of glycoprotein metabolic process
negative regulation of catalytic activity
mucopolysaccharide metabolic process
monomeric IgA immunoglobulin complex
IgA immunoglobulin complex, circulating
IgA immunoglobulin complex
glomerular filtration
dimeric IgA immunoglobulin complex
defense response to Gram−negative bacterium
defense response
apoptotic cell clearance
GO TERM
0.25
0.50
0.75
1.00
Rich factor
Protein_number
1
9
18
26
35
−log10(Pvalue)
2.6
2.4
2.2
2.0

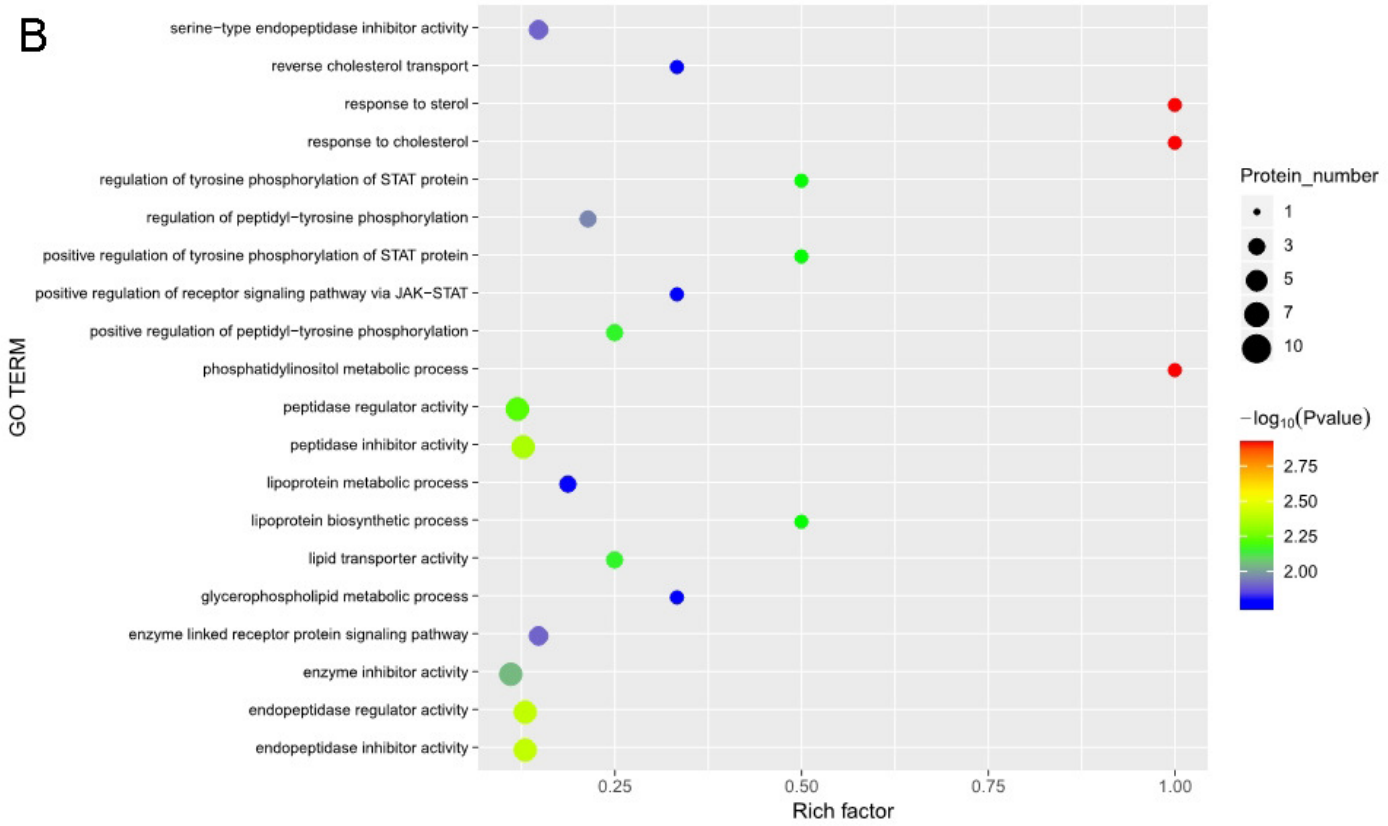

B
serine−type endopeptidase inhibitor activity
reverse cholesterol transport
response to sterol
response to cholesterol
regulation of tyrosine phosphorylation of STAT protein
regulation of peptidyl−tyrosine phosphorylation
positive regulation of tyrosine phosphorylation of STAT protein
positive regulation of receptor signaling pathway via JAK−STAT
positive regulation of peptidyl−tyrosine phosphorylation
phosphatidylinositol metabolic process
peptidase regulator activity
peptidase inhibitor activity
lipoprotein metabolic process
lipoprotein biosynthetic process
lipid transporter activity
glycerophospholipid metabolic process
enzyme linked receptor protein signaling pathway
enzyme inhibitor activity
endopeptidase regulator activity
endopeptidase inhibitor activity
GO TERM
0.25
0.50
0.75
1.00
Rich factor
Protein_number
1
3
5
7
10
−log10(Pvalue)
2.75
2.50
2.25
2.00

Figure 6

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment

KEGG pathways enriched for the upregulated and downregulated proteins and the associated protein numbers in (A) early-onset preeclampsia vs. its control and (B) late-onset preeclampsia vs. its control.

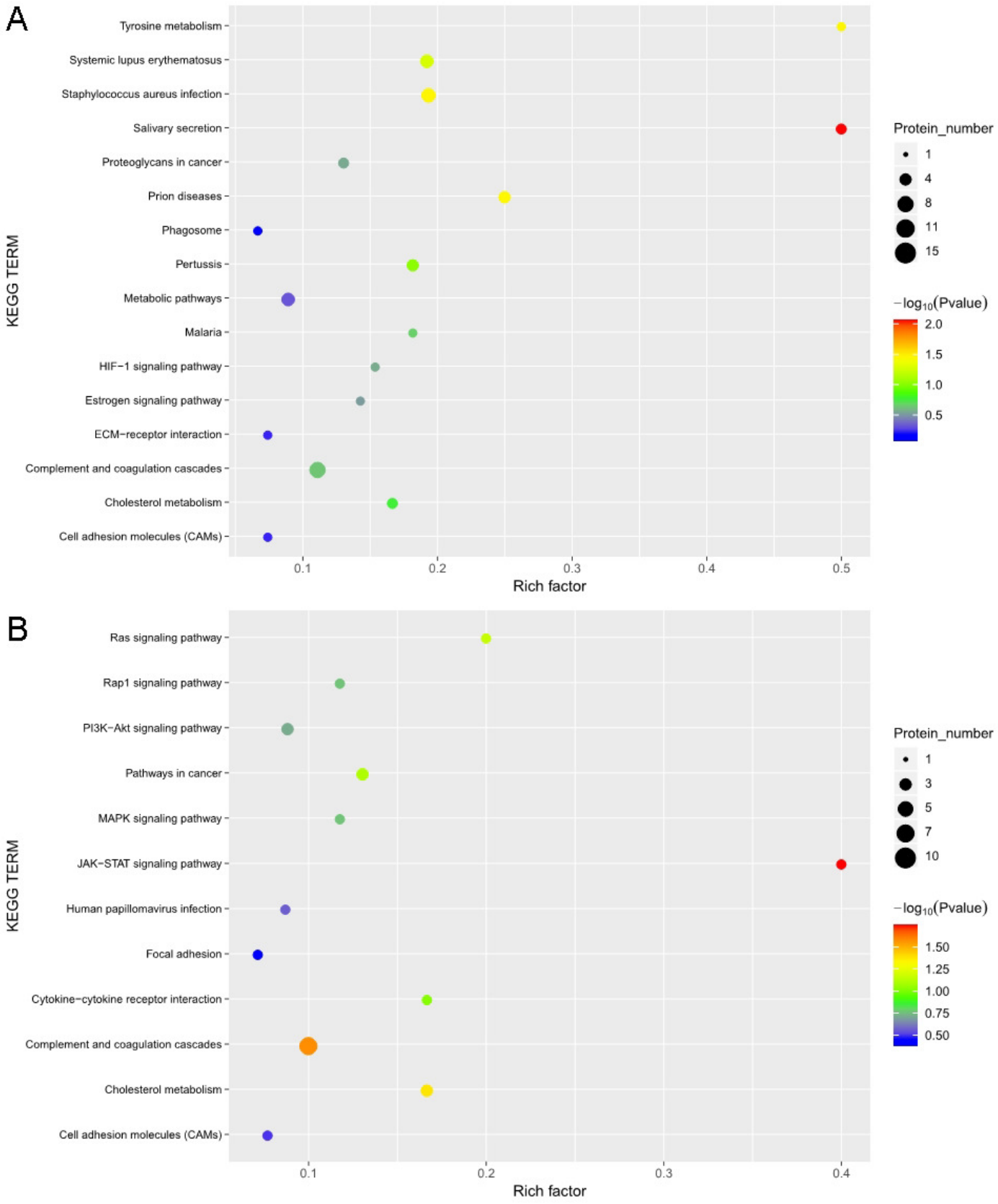

A
Tyrosine metabolism
Systemic lupus erythematosus
Staphylococcus aureus infection
Salivary secretion
Proteoglycans in cancer
Prion diseases
Phagosome
Pertussis
Metabolic pathways
Malaria
HIF−1 signaling pathway
Estrogen signaling pathway
ECM−receptor interaction
Complement and coagulation cascades
Cholesterol metabolism
Cell adhesion molecules (CAMs)
KEGG TERM
0.1
0.2
0.3
0.4
0.5
Rich factor
Protein_number
1
4
8
11
15
−log10(Pvalue)
2.0
1.5
1.0
0.5
B
Ras signaling pathway
Rap1 signaling pathway
PI3K−Akt signaling pathway
Pathways in cancer
MAPK signaling pathway
JAK−STAT signaling pathway
Human papillomavirus infection
Focal adhesion
Cytokine−cytokine receptor interaction
Complement and coagulation cascades
Cholesterol metabolism
Cell adhesion molecules (CAMs)
KEGG TERM
0.1
0.2
0.3
0.4
Rich factor
Protein_number
1
3
5
7
10
−log10(Pvalue)
1.50
1.25
1.00
0.75
0.50

Figure 7

Protein level of pregnancy-specific beta-1-glycoprotein 9 (PSG9) in serum

Concentration of PSG9 in the patient serum in control and LOPE groups. Values are shown as the mean ± SEM (n = 10); *$P < 0.05$ for Control vs. LOPE.