Sleep Medicine 64 (2019) 92–100

Contents lists available at ScienceDirect

Sleep Medicine

journal homepage: www.elsevier.com/locate/sleep

Original Article

Urinary EPCR and dermcidin as potential novel biomarkers for severe adult OSA patients

Mikashmi Kohli [a], S.K. Sharma [b, *, 1], Vishwanath Upadhyay [b], Swati Varshney [c], Shantanu Sengupta [c], Trayambak Basak [d], V. Sreenivas [e]

[a] Department of Epidemiology, Biostatistics and Occupational Health, McGill University, Montreal, Canada
[b] Department of Molecular Medicine, Jamia Hamdard Institute of Molecular Medicine, Jamia Hamdard (Deemed-to-be University), New Delhi, India
[c] Genomics and Molecular Medicine Unit, CSIR-Institute of Genomics and Integrative Biology, Mathura Road, New Delhi, India
[d] Division of Nephrology and Hypertension, Vanderbilt Medical Center, Nashville, United States
[e] Department of Biostatistics, AIIMS, New Delhi, India

ARTICLE INFO

Article history:
Received 7 November 2018
Received in revised form
23 June 2019
Accepted 2 July 2019
Available online 8 July 2019

Keywords:
Obstructive sleep apnea
Urinary biomarkers
Non-invasive test
Endothelial protein C receptor
Dermcidin

ABSTRACT

Background: Due to low predictive values of obstructive sleep apnea (OSA) screening tools, there is a need for biomarker for screening of OSA patients at an early stage. The aim of the study was to evaluate differentially expressed proteins in blood and urine samples of OSA patients.
Methods: In this study, we used isobaric tagging for relative and absolute quantification (iTRAQ) based proteomics approach to identify differentially expressed proteins, which were subsequently verified and validated using enzyme-linked immunosorbent assay (ELISA) technique in adult OSA patients.
Results: Seventeen differentially expressed proteins were selected from iTRAQ data for verification, based on their clinical significance and reproducibility among different iTRAQ experiment sets. Five of these proteins (plasma = 2; urine = 3) were further validated in plasma (non-OSA- = 42; OSA = 198) and urine samples (non-OSA = 46; OSA = 197). ROC curve analysis for all OSA vs. non-OSA subjects ensured optimal diagnostic utility of two urinary proteins: Endothelial protein c receptor (EPCR) (AUC = 73%, cut-off: 35 pg/ml) and dermcidin (AUC = 74%, cut-off: 4.6 pg/ml). For severe OSA, diagnostic accuracy significantly improved with AUC as 88% and 82% for EPCR (cut-off: 46 pg/ml) and dermcidin (cut-off: 5.2 pg/ml) respectively. Sensitivity and specificity of combined performance of both urinary proteins for severe OSA were 94% and 91% respectively.
Conclusion: In this study, urinary EPCR and dermcidin emerged as novel biomarkers for screening severe OSA patients.

1. Introduction

Obstructive sleep apnea (OSA) is an emerging major public health problem worldwide and is characterized by repeated episodes of partial or complete upper airway obstruction, frequent gas exchange abnormalities, sleep fragmentation and arousals. According to a recent study, approximately 13% of men and 6% of women in the United States have moderate to severe OSA [1] and in India, the prevalence of OSA is reported to be 13.4% and 5.6% in men and women, respectively [2].

Obesity is considered to be an important risk factor for OSA and about 70% of OSA patients are obese [3]. Inflammation and oxidative stress in obese patients cause endothelial dysfunction [4]. Repetitive cycles of hypoxia and reoxygenation in OSA produce oxidative stress leading to increased systemic inflammation and impaired endothelial function [5]. The latter predisposes patients to various arterial diseases; moreover, OSA is associated with several adverse cardiovascular consequences including arterial hypertension, coronary artery disease (CAD) and stroke [6]. OSA patients frequently have several co-morbidities such as diabetes mellitus

* Corresponding author. Department of Internal Medicine, WHO Collaborating Centre for Research & Training in Tuberculosis, Centre of Excellence for EPTB [MoH & FW, GoI], All India Institute of Medical Sciences, New Delhi 110 029, India.
E-mail address: sksharma.aiims2@gmail.com (S.K. Sharma).
[1] Current Affiliations: Deptt of Molecular Medicine, Jamia Hamdard Institute of Molecular Medicine, Jamia Hamdard (Deemed-to-be University), Hamdard Nagar, Delhi 110 062.

https://doi.org/10.1016/j.sleep.2019.07.002
1389-9457/

and hypertension [6], which can result in endothelial dysfunction and arterial disease. A recent study has also reported endothelial dysfunction in both microvascular and macrovascular beds in OSA patients without co-morbidities [7].

Currently available screening tools for OSA such as the Epworth Sleepiness Scale (ESS) and Berlin questionnaire have low clinical predictive values [8]. Clinical practice guidelines for diagnosing adult OSA patients suggest that in-hospital; technician-supervised polysomnography (PSG) or home-based PSG is the gold standard for diagnosing OSA patients [9]. However, overnight PSG is technically cumbersome, costly, and has limited accessibility causing inconvenience to patients. These issues have spurred the quest for newer tools to screen OSA patients.

In view of the issues raised above, it becomes imperative to identify a new biomarker as a screening tool in OSA patients without comorbidities, which should be subsequently validated in OSA patients with comorbidities to assess its diagnostic performance in real world clinical practice.

Recently, several studies have explored potential of proteomics approach in identifying biomarkers in both pediatric and adult OSA patients [10–14]. However, no subsequent validation was carried out in these studies.

The present study aimed at identifying differentially expressed proteins in urine and plasma samples using isobaric tagging for relative and absolute quantification (iTRAQ) technique in OSA patients without co-morbidities, which were subsequently validated in a larger number of OSA patients with co-morbidities, hence evaluating them in the actual clinical scenario.

2. Methods

2.1. Study design and participants

The present study was a prospective cross-sectional study and was conducted in three phases: (i) identification phase, (ii) verification phase and, (iii) the validation phase during the period of 2013–2016. Patients were screened in the Sleep Clinic of the Department of Medicine, All India Institute of Medical Sciences (AIIMS), New Delhi which caters to the semi-urban population of Delhi and nearby areas. Overweight subjects with BMI $\geq$25 kg/m^2 and habitual snorers (snoring for more than three nights/week) were screened for OSA**.** Subjects included in identification and verification phases did not have any co-morbidities like hypertension, diabetes, dyslipidemia, cardiovascular disorder, chronic renal disorder, urinary tract infection, impaired neurocognitive function and were naïve to continuous positive air pressure (CPAP) therapy. Patients who were on drugs like non-steroidal anti-inflammatory drugs (NSAIDs) and angiotensin converting enzyme (ACE) inhibitors were also excluded. However, in the validation phase, for generalization of results, patients with comorbidities (except hypothyroidism or any anatomical deformities responsible for OSA) were included. The institutional ethics committee of AIIMS, New Delhi, approved the study (IEC/NP-389/2012&RP-20/2012) and all the experiments were performed in accordance with relevant guidelines and regulations. A written informed consent was taken from every recruited subject in the study.

A detailed anthropometry was performed on all subjects. For screening of the patients, two standard questionnaires were used; modified Berlin questionnaire (MBQ) [15] and ESS [16]. After screening, in-hospital, technician-supervised overnight PSG was done at the Sleep Laboratory, Department of Medicine, AIIMS, New Delhi. Enrolled patients underwent blood investigations including fasting blood glucose, HbA_1C levels, lipid profile, thyroid function tests, liver and renal functions tests. Urinary albumin and creatinine were also measured and their ratio was calculated. Subjects with AHI <5 events/hr were taken as non-OSA subjects and patients having AHI $\geq$5 events/hr were taken as OSA subjects. Patients with 5 $\leq$ AHI<15, 15 $\leq$ AHI<30 and AHI$\geq$30 events/hr were categorized as mild, moderate and severe OSA cases respectively.

2.1.1. Experiment design

In the identification phase, the protein profile in plasma and urine samples were analyzed using the iTRAQ technique. The verification phase involved segregation of the relevant and the consistently up-regulated or down-regulated proteins detected in the identification phase and verifying them using enzyme-linked immunosorbent assay (ELISA) technique. The proteins with statistically significant differences in levels between OSA and non-OSA groups from the verification phase were further selected for validation in a larger number of patients. Fig. 1 describes the experimental design of the study.

The study consisted of three phases: Identification, Verification and Validation phases. The first two phases consisted of OSA patients without any co-morbidities. In the validation phase, OSA patients with co-morbidities (except hypothyroidism and any anatomical abnormality).

2.1.2. Identification phase

2.1.2.1. Sample collection and processing. First-voided morning urine sample in a sterile vial and blood samples in EDTA vials were collected from the subjects. Plasma was harvested from peripheral blood samples. Plasma and urine samples were stored at −80 °C until further analysis. MARS-6 (Agilent, USA) column was used for immunodepletion of plasma samples and ProteoPrep immunoaffinity column (Sigma–Aldrich, St. Louis, USA) was used for urine samples as per manufacturer's protocol. The immunodepleted samples were further concentrated, buffer exchanged, digested by trypsin, iTRAQ labeled and strong cation exchange was done as described earlier [17,18]. These eluted fractions were vacuum dried and processed further for mass spectrometry.

2.1.2.2. Reverse phase separation and mass spectrometry. The peptide fractions were further analyzed by ESI-LC-MS/MS using quadropole TOF 5600 (AB Sciex) as described previously [19]. Data in the constituted MS and MS/MS spectra scan were obtained from quadropole TOF 5600 in the form of '.wiff' files. These '.wiff' files from each iTRAQ experiment were submitted for protein identification to ProteinPilot™ software (v. 4.5 Applied Biosystems/MDS Sciex, Foster City, CA) using a Paragon search method against the Homo sapiens SwissProt database and proteins were identified with global protein false discovery rate (FDR) of 1%.

2.1.3. Verification phase

The proteins, which were consistently up regulated (case: control ratio >1.2) or down regulated (case: control ratio< 0.8) among the various sets, with high peptide scores (for each protein, unique peptides were more than 2) and with clinical relevance were

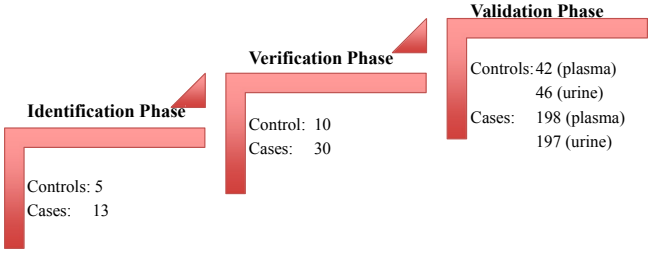

Fig. 1. Experiment design.

selected for verification phase using ELISA. Pre-coated ELISA plates were used for all selected molecules and the assay was performed as per the manufacturer's protocol (see List S1 in supplementary material).

2.1.4. Validation phase

The proteins, which were significantly different ($p < 0.05$) between OSA and non-OSA groups in the verification phase, were picked up for the validation phase. There were five proteins, which were selected for validation; two in plasma samples and three in urine samples. Tetranectin and vascular cell adhesion molecule-1 (VCAM-1) were validated in plasma samples. Alpha-1 microglobulin/bikunin precursor (AMBP), EPCR and dermcidin were validated in urine samples. ELISA kits used in the validation phase were same as those used in the verification phase.

2.2. Statistical analysis

Data were analyzed using Stata 12.1 (Texas, USA). The protein levels were transformed on log scale to normalize the data and were expressed as mean ± standard deviation (SD). For univariate analysis, Student's t-test was done to compare the two groups. For more than two groups, one-way analysis of variance (ANOVA) test, followed by Bonferroni correction was done. Receiver-operator characteristic (ROC) curves were plotted for assessing diagnostic accuracy. Crude odds ratios were computed through univariate logistic regression analysis. To adjust for possible confounding factors, multivariable logistic regression analysis was used.

3. Results

The results have been presented separately for each phase:

A **Identification phase:** For the identification phase, 15 subjects were enrolled as OSA subjects [mild: three; moderate: five; severe: seven] and six were enrolled as non-OSA subjects in the study. The demographic details, anthropometric data, biochemical parameters and PSG characteristics for the enrolled subjects have been provided in supplemental material (Tables S2-S4) suggesting that these subjects did not have any co-morbidities. From iTRAQ list of proteins for plasma samples, a total of 233, 250, 255, 254 proteins at 1% false discovery rate (FDR) were identified in four sets of iTRAQ experiment respectively. Similarly in urine samples, a total of 496, 505, 441 proteins were identified at 1% FDR in three sets of iTRAQ experiment respectively (see tables S5-S7 in supplementary material). Fig. 2 is a Venn diagram representing the number of proteins identified in each category in plasma and urine.

B **Verification phase:** For the verification phase, 59 subjects were enrolled as OSA subjects [mild: 10; moderate: 17; severe: 32] and 13 patients were enrolled as non-OSA subjects in the study. Ten samples for each category of OSA and non-OSA were recruited in the verification phase. Demographic details, anthropometric data, biochemical parameters and the PSG characteristics of the subjects enrolled in verification phase have been provided in supplemental material (Tables S8-S10). Proteins in the verification phase were evaluated using ELISA. Table 1 represents the levels of these proteins in plasma samples and Table 2 represents the protein levels in urine samples. Plasma levels of tetranectin ($p = 0.005$) and VCAM-1 ($p = 0.02$) were significantly increased in OSA subjects with respect to non-OSA subjects. In urine samples, levels of AMBP ($p = 0.05$), EPCR ($p = 0.03$) and dermcidin ($p = 0.02$) were significantly differentially expressed in OSA subjects. These proteins from both plasma and urine samples were then selected for the validation phase.

C **Validation phase:** For the validation phase, 198 subjects were enrolled as OSA subjects for plasma and 197 subjects for urine samples (urine specimen for one patient leaked from the container and thus excluded). A total of 42 subjects were enrolled as non-OSA subjects for plasma and 46 for urine samples (four subjects did not give consent to draw blood sample for the study). Demographic details, anthropometric data, biochemical parameters and the PSG characteristics have been given in supplementary data tables (Tables S11-S16). Statistically significant proteins from the verification phase were then further evaluated in a larger number of samples using ELISA for validation. Table 3 represents the protein levels from plasma and urine samples validated in a large number of patients. Levels of tetranectin protein in plasma samples were significantly elevated in OSA subjects **($p = <0.001$)** and levels of EPCR and dermcidin proteins were increased in urine samples of OSA subjects **($p = <0.001$)**.

4. Discussion

Although PSG remains to be the current gold standard for OSA diagnosis, it may be costly and inconvenient to patients. Several questionnaires are available for screening OSA patients, however,

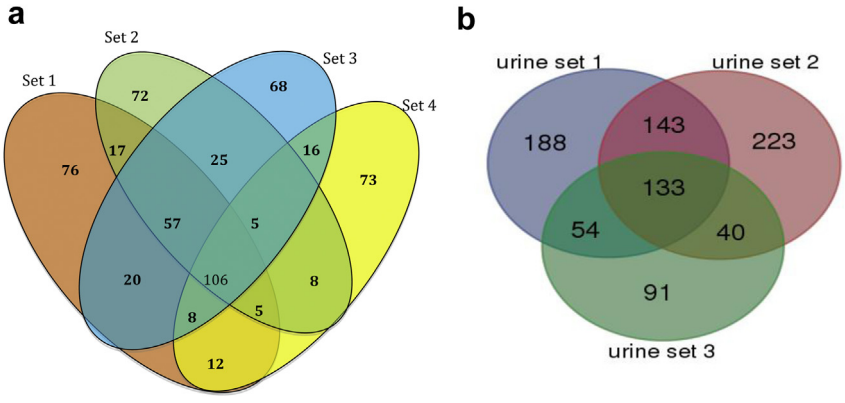

Fig. 2. a: Venn diagram for plasma samples. b: Venn diagram for urine samples.

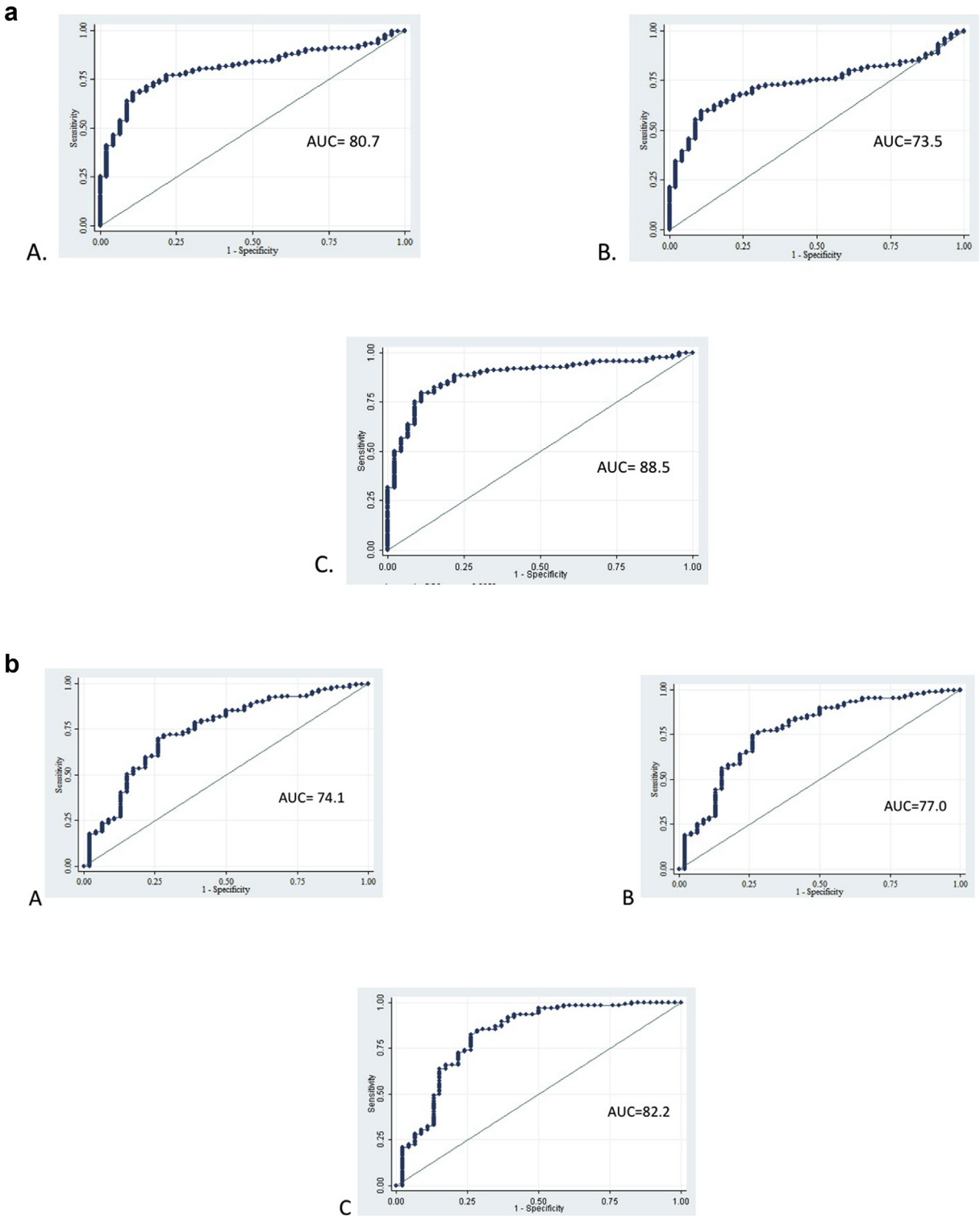

Fig. 3. a: Representative ROC for EPCR in (A) OSA cases versus non-OSA subjects; (B) moderately severe cases versus non-OSA subjects and (C) severe OSA cases versus non-OSA subjects. b: Representative ROC for dermcidin in (A) OSA cases versus non-OSA subjects; (B) moderately severe cases versus non-OSA subjects and (C) severe OSA cases versus non-OSA subjects.

their clinical predictive value is low. In this study, we planned to identify a biomarker in blood or urine of PSG confirmed OSA patients. We therefore, carefully selected OSA patients without comorbidities for the identification and verification phases. Since most OSA patients were obese, we selected non-OSA controls who had obesity as per the WHO definition of obesity for south Asian population [BMI$\geq$25 kg/m^2] [20] and who had negative PSG. These non-OSA subjects had habitual snoring, as snoring is often considered a marker for OSA. We deliberately excluded OSA patients with comorbidities in identification and verification phases

Table 1
Protein levels in plasma samples of subjects recruited in verification phase.

Plasma Proteins	Non-OSA (n = 10)	Mild OSA (n = 10)	Mod. OSA (n = 10)	Sev. OSA (n = 10)	p*	All Cases	P† Non-OSA vs. OSA
Tetranectin (ug/ml)							
Median	1.06	1.01	0.99	1.29	**0.005**	1.12	0.64
IQR	(1.01–1.14)	(0.89–1.16)	(0.93–1.06)	(1.17–1.55)		(0.95–1.32)	
VCAM-1 ‡ (pg/ml)							
Median	536.73	–	–	591.61	**0.02**	591.61	**0.02**
IQR	(523.35–561.10)			(559.26–638.78)		(559.26–638.78)	
Dermcidin ‡ (pg/ml)							
Median	881.11	–	–	871.96	0.52	871.96	0.52
IQR	(858.52–905.87)			(841.29–901.74)		(841.29–901.74)	
SAA4							
(ug/ml)	0.20	0.20	0.10	0.16	0.05	0.15	0.06
Median	(0.19–0.3)	(0.67–0.26)	(0.04–0.16)	(0.12–0.23)		(0.06–0.23)	
IQR							
Vit DBP							
(pg/ml)	9.05	11.95	6.70	5.50	0.39	9.0	0.94
Median	(7.57–12.77)	(9.87–14.30)	(4.32–13.12)	(1.90–11.05)		(4.70–13.0)	
IQR							

Data are expressed as median (interquartile range). VCAM-1- vascular cell-adhesion molecule-1, SAA4-serum amyloid A4, Vit DBP- vitamin D binding protein. *Based on ANOVA; † based on Student's t-test. ‡ These proteins were evaluated in both plasma and urine and only in non-OSA and severe OSA subjects.
Statistical significance was considered at p<0.05.

as these conditions themselves can be associated with altered endothelial function. The results of the present study report that two urinary proteins; EPCR and dermcidin showed acceptable optimal diagnostic accuracy in OSA patients.

Urinary EPCR (43 kDa) levels were found to be 2.6-fold higher in OSA subjects as compared to non-OSA subjects. There are two forms of EPCR, membrane bound (mEPCR) and soluble form (sEPCR). The soluble form of EPCR (sEPCR) binds to protein C (PC) and generates activated PC (APC), thereby regulating the

Table 2
Protein levels in urine samples of subjects recruited in verification phase.

Urine Proteins	Non-OSA (n = 10)	Mild OSA (n = 10)	Mod. OSA (n = 10)	Sev. OSA (n = 10)	p*	All Cases	P† Non-OSA vs. OSA
AMBP (pg/ml)							
Median	1324.2	1356.25	1160.15	1006.85	**0.05**	1197.6	**0.13**
IQR	(1307.4–1372.7)	(1220.4–1436.1)	(546.7–1328.6)	(731.17–1189.29)		(798.9–1331.7)	
EPCR (pg/ml)							
Median	36.3	49.29	84.19	125.75	**0.03**	73.9	**0.03**
IQR	(13.45–75.56)	(19.79–105.29)	(52.10–135.27)	(43.60–208.73)		(42.96–157.95)	
Dermcidin‡ (pg/ml)							
Median	4.02	–	–	8.06	**0.02**	8.06	**0.02**
IQR	(1.82–6.77)			(7.24–16.39)		(7.24–16.39)	
AGP (pg/ml)							
Median	31.92	31.67	32.41	32.0	**0.49**	31.9	**0.50**
IQR	(31.6–32.7)	(31.4–32.0)	(31.4–32.6)	(31.6–32.4)		(31.5–31.9)	
CADM4 (pg/ml)							
Median	7.7	6.98	7.11	5.70	**0.12**	6.8	**0.18**
IQR	(6.31–8.44)	(6.17–7.81)	(6.24–8.26)	(4.89–6.84)		(5.51–7.88)	
LDHB (pg/ml)							
Median	178.86	180.44	177.89	183.44	**0.83**	180.4	**0.70**
IQR	(170.95–180.61)	(169.56–182.40)	(163.6–185.4)	(167.9–190.3)		(167.1–187.1)	
Uromodulin (pg/ml)							
Median	6350.6	10645.8	13478.9	10291.4	**0.52**	10291.42	**0.32**
IQR	(3004.2–16.63.1)	(5062.5–17725.3)	(4361.8–26414.9)	(6516.2–16933.8)		(5042.3–21795.8)	
HSPG (pg/ml)							
Median	7.89	7.07	6.85	7.59	**0.44**	7.07	**0.97**
IQR	(6.96–8.55)	(5.97–7.47)	(6.41–7.25)	(6.88–9.00)		(6.15–7.88)	
PEBP4 (pg/ml)							
Median	8.32	7.29	6.32	8.70	**0.84**	6.88	**0.99**
IQR	(1.59–15.39)	(3.84–11.21)	(2.28–11.46)	(4.88–15.02)		(3.80–12.82)	
VCAM1‡ (pg/ml)							
Median	16.77	–	–	16.89	**0.45**	16.89	**0.45**
IQR	(12.14–36.55)			(9.05–30.77)		(9.05–30.77)	
Osteopontin (pg/ml)							
Median	19.38	19.82	37.29	70.08	**0.16**	42.15	**0.07**
IQR	(8.49–30.71)	(9.48–58.97)	(24.30–61.99)	(27.47–90.23)		(19.50–75.52)	

Data are expressed as median (interquartile range). The p-values have been calculated from the normalization of data after logarithmic conversion of protein levels. VCAM-1- vascular cell-adhesion molecule-1, AMBP- alpha-1 microglobulin/bikunin precursor; EPCR-endothelial protein C receptor; AGP- alpha-1 glycoprotein; ACHAP-achetylcholinesterase associated protein; CADM4-cell adhesion molecule 4; LDHB- lactate dehydrogenase B; HSPG-heparansulphate proteoglycan; PEBP4- phosphatidylethanolamine binding protein 4. *Based on ANOVA; † based on Student's t-test. ‡ These proteins were evaluated in both plasma and urine and only in non-OSA and severe OSA subjects.
Statistical significance was considered at p<0.05.

Table 3
List of proteins and their levels by ELISA in plasma and urine samples in non-OSA and sub-categories of OSA subjects.

Plasma Proteins	Non-OSA (n = 42)	Mild OSA (n = 34)	Mod. OSA (n = 46)	Sev. OSA (n = 118)	p*	All Cases	Mod + Sev	p†		
								Non-OSA vs. OSA	Non-OSA vs. Mod + Sev OSA	Non-OSA vs. Sev OSA
Tetranectin‡										
Median	1.9	1.5	1.9	2.0	**<0.001**	2.0	2.0	0.11	<0.001	**0.02**
IQR	(1.3–2.3)	(1.1–2.5)	(1.4–2.3)	(1.8–2.8)		(1.6–2.6)	(1.7–2.7)			
VCAM-1§										
Median	81.8	122.6	158.9	130.1	0.07	134.1	136.1	**0.01**	**0.01**	0.10
IQR	(42.7–165.9)	(48.3–270.1)	(74.8–245.2)	(69.3–255.5)		(68.8–255.5)	(70.4–254.6)			
Urine Proteins	**Non-OSA (n = 46)**	**Mild (n = 33)**	**Mod. (n = 40)**	**Sev. (n = 124)**	**p**	**All Cases**	**Mod + Sev**	**p value**		
								Non-OSA vs. cases	**Non-OSA vs. Mod + Sev**	**Non-OSA vs. Sev**
AMBP‖										
Median	2.4	2.1	2.2	2.7	0.99	2.5	2.6	**< 0.001**	0.18	0.99
IQR	(1.5–2.9)	(0.8–2.5)	(1.1–3.0)	(2.0–4.3)		(1.7–3.6)	(1.8–4.1)			
EPCR§										
Median	26.8	12.9	31.5	100.9	**<0.001**	68.7	86.1	**< 0.001**	**<0.001**	**<0.001**
IQR	(13.6–38.7)	(8.2–38.0)	(13.1–72.6)	(60.0–160.0)		(27.8–132.3)	(41.3–147.7)			
Dermcidin§										
Median	2.6	3.6	4.2	7.8	**<0.001**	6.7	7.1	**< 0.001**	**<0.001**	**<0.001**
IQR	(1.5–5.4)	(1.8–6.5)	(2.0–8.4)	(5.3–11.5)		(3.8–10.5)	(4.5–11.4)			

Data are presented as median and inter-quartile range. The p-values have been calculated after normalization of data using logarithmic conversion. Mod: moderate; Sev: severe. *Based on ANOVA; † based on Student's t-test; ‡ Data in ug/mL; §Data in pg/mL; ‖Data presented in ng/mL.VCAM-1- vascular cell-adhesion molecule 1; alpha-1 microglobulin/bikunin precursor; EPCR-endothelial protein C receptor.
Furthermore, diagnostic accuracy of the proteins was evaluated using area under receiver operating characteristics curve (AUC). Table 4 gives the AUC for the significant proteins both in plasma and urine samples. AUC of the significant proteins; EPCR and dermcidin have been represented in Fig. 3a and b. To adjust for the possible confounding factors, a multivariate analysis was done in the validation phase (Table 5).
As PSG is the current gold standard for diagnosing OSA, we evaluated the combined diagnostic performance of these two proteins with PSG as the reference standard (Table 6). The combined sensitivity when both EPCR and dermcidin are positive is 81% for OSA and 94% for severe OSA. The likelihood ratio for positive test (LR+) was 4.4 for OSA and 10.3 for severe OSA suggesting that a subject with positive test for both these proteins have a higher probability of severe OSA.
Statistical significance was considered at $p<0.05$.

Table 4
Diagnostic performance of the plasma and urine proteins in OSA.

Proteins	Non-OSA vs. OSA				Moderate + severe OSA vs. non-OSA				Severe OSA vs. non-OSA			
	AUROC (%)	Cut off	Sn. (%)	Sp. (%)	AUROC (%)	Cut off	Sn. (%)	Sp. (%)	AUROC (%)	Cut off	Sn. (%)	Sp. (%)
Tetranectin	57.1 (47.9–66.3)	>1.94 ug/ml	54	54.7	60.3 (50.6–70.0)	>1.99 ug/ml	54.8	54.7	63.6 (53.6–73.6)	>1.99 ug/ml	58.4	59.5
VCAM-1	62.1 (52.8–71.5)	>106.8 pg/ml	57.8	57.1	62.7 (53.2–72.3)	>107.1 pg/ml	57.9	57.1	61.7 (51.8–71.6)	>108.2 pg/ml	57.6	57.1
AMBP	52.3 (43.6–61.1)	>2409 pg/ml	55.3	52.1	55.4 (46.5–64.4)	>2530 pg/ml	56.7	56.5	58.8 (49.5–68.2)	>2603 pg/ml	57	56.3
EPCR	73.5 (66.9–80.0)	>35.3 pg/ml	71	71.7	80.7 (54.5–86.9)	>39.8 pg/ml	76.8	76	88.5 (83.1–93.8)	>46.2 pg/ml	84.5	82.6
Dermicidin	74.1 (65.8–82.3)	>4.5 pg/ml	75	71.1	77.0 (68.7–85.2)	>4.6 pg/ml	73.7	73.9	82.2 (74.1–90.2)	>5.2 pg/ml	76	74

OSA-obstructive sleep apnea; VCAM-1- vascular endothelial cell adhesion molecule; EPCR-endothelial protein c receptor; AUROC- area under receiver operating characteristics curve; Sn-sensitivity; Sp- Specificity.

Table 5a
Multivariate logistic regression for proteins.

Proteins	Non-OSA vs. OSA			Moderate *plus* severe OSA vs. non-OSA			Severe OSA vs. non-OSA		
	Cut off	OR	AOR	Cut off	OR	AOR	Cut off	OR	AOR
EPCR pg/ml	<35	1.00	1.00	<40	1.00	1.00	<46.2	1.00	1.00
	>35	6.2 (3.0–12.7)	3.9 (1.47–10.6)	>40	11.2 (5.1–24.5)	10.7 (3.8–29.9)	>46.2	24.7 (10.0–60.7)	25.7 (8.4–78.6)
Dermicidin pg/ml	<4.6	1.00	1.00	<4.6	1.00	1.00	<5.2	1.00	1.00
	>4.6	6.5 (3.1–13.3)	3.3 (1.36–8.16)	>4.6	8.23 (3.9–17.3)	3.7 (1.4–9.9)	>5.2	9.1 (4.2–20.1)	5.7 (2.2–14.9)

OSA- Obstructive sleep apnea; EPCR-endothelial protein c receptor; OR- Odds ratio; AOR- Adjusted odds ratio.

Table 5b
Multivariate logistic regression for combination of proteins.

Proteins	Case vs. Non-OSA subjects			Moderate *plus* severe vs. Non-OSA subjects			Severe vs. Non-OSA subjects		
	Odds ratio (95 CIs)	Adjusted odds ratio (95 CIs)	p value	Odds ratio (95 CIs)	Adjusted odds ratio (95 CIs)	p value	Odds ratio (95 CIs)	Adjusted odds ratio (95 CIs)	p value
EPCR - Dermcidin -	1.00	1.00	–	1.00	1.00	–	1.00	1.00	–
EPCR + Dermcidin -	5.8 (2.2–15.4)	4.8 (1.5–15.9)	**< 0.01**	8.4 (2.84–24.84)	6.8 (1.7–26.2)	**< 0.01**	32.4 (8.4–124.2)	28.9 (6.2–133.1)	**<0.01**
EPCR − Dermcidin +	6.2 (2.2–17.3)	3.4 (1.0–11.0)	**0.04**	5.83 (2.11–16.07)	2.9 (0.8–9.9)	0.08	11.25 (3.1–40.4)	6.4 (1.5–26.7)	**0.01**
EPCR + Dermcidin +	19.5 (7.2–52.5)	13.1 (4.0–42.9)	**<0.001**	45.26 (13.90–147.36)	33.0 (7.9–139.8)	**<0.01**	154 (34.6–684.9)	98.0 (19.6–489.9)	**<0.01**

EPCR-endothelial protein c receptor; CIs-confidence intervals; OSA-obstructive sleep apnea.
For cases vs. non-OSA subjects: EPCR(−): EPCR<35 pg/mL and EPCR(+): EPCR>35 pg/mL.Dermcidin(−): dermcidin<4.6 pg/mL; dermcidin(+): dermcidin>4.6 pg/mL.
For moderate + severe vs. non-OSA subjects: EPCR(−): EPCR<40 pg/mL and EPCR(+): EPCR>40 pg/mL.Dermcidin(−): dermcidin<4.6 pg/mL; dermcidin(+): dermcidin>4.6 pg/mL.
For severe vs. non-OSA subjects: EPCR(−): EPCR<46 pg/mL and EPCR(+): EPCR>46 pg/mL.Dermcidin(−): dermcidin<5.2 pg/mL; dermcidin(+): dermcidin>5.2 pg/mL.

Table 6
Diagnostic performance of the proteins with polysomnography as the reference standard.

Variables	OSA vs. Non-OSA				Moderate *plus* Severe OSA vs. Non-OSA				Severe OSA vs. non-OSA			
	Sn (%)	Sp (%)	PPV (%)	NPV(%)	Sn (%)	Sp (%)	PPV (%)	NPV(%)	Sn (%)	Sp (%)	PPV(%)	NPV(%)
EPCR + Dermcidin -	60 (36/60)	79 (27/34)	84 (36/43)	53 (27/51)	64 (27/42)	82 (28/34)	82 (27/33)	65 (28/43)	84.3 (27/32)	86 (30/35)	84 (27/32)	86 (30/35)
EPCR − Dermcidin +	58 (33/57)	82 (27/33)	85 (33/39)	53 (27/51)	62 (25/40)	78 (28/36)	76 (25/33)	65 (28/43)	75 (15/20)	79 (30/38)	65 (15/23)	86 (30/35)
EPCR + Dermcidin +	81 (104/128)	82 (27/33)	94 (104/110)	53 (27/51)	86 (97/112)	87 (28/32)	96 (97/101)	65 (28/43)	94 (77/82)	91 (30/33)	96 (77/80)	86 (30/35)

OSA-obstructive sleep apnea; EPCR-endothelial protein c receptor; Sn: sensitivity; Sp-specificity. Sens- Sensitivity; Spec-specificity; PPV- positive predictive value; NPV-negative predictive value.
For cases vs. non-OSA subjects: EPCR(−): EPCR<35 pg/mL and EPCR(+): EPCR>35 pg/mL.Dermcidin(−): dermcidin<4.6 pg/mL; dermcidin(+): dermcidin>4.6 pg/mL.
For moderate + severe vs. non-OSA subjects: EPCR(−): EPCR<40 pg/mL and EPCR(+): EPCR>40 pg/mL.Dermcidin(−): dermcidin<4.6 pg/mL; dermcidin(+): dermcidin>4.6 pg/mL.
For severe vs. non-OSA subjects: EPCR(−): EPCR<46 pg/mL and EPCR(+): EPCR>46 pg/mL.Dermcidin(−): dermcidin<5.2 pg/mL; dermcidin(+): dermcidin>5.2 pg/mL.

downstream coagulation, inflammatory and apoptotic pathways in antagonistic manner of the mEPCR [19–22]. The soluble form of EPCR (sEPCR) is a procoagulant, anti-apoptotic and pro-inflammatory protein, which may lead to increased risk of atherosclerosis, cancer and other inflammatory diseases respectively [19–22]. It favors a net pro-inflammatory state. It has been reported that OSA is an inflammatory condition [6], thus acquiescing with the elevated levels of sEPCR. Previous studies have also reported the shedding of EPCR as a potential marker for endothelial damage, vasculopathy and renal injury [23–25].

Another protein that showed optimal diagnostic accuracy was dermcidin. Levels of dermcidin (11 kDa) in urine samples were found to be about 2-fold higher in patients with OSA as compared to non-OSA subjects. Dermcidin is an anti-microbial protein, first reported to be secreted from the sweat glands [26]. However, recently this protein has been associated with several disease conditions, but its pathophysiological significance is still unraveled. Increased plasma levels of dermcidin have been observed in patients with hypertension, suggesting its possible role in hypertensive patients [27]. Studies have also shown dermcidin to be a potent inducer of platelet aggregation thereby implicating its role in CAD. This protein also inhibits insulin synthesis from pancreatic β-cells, thus leading to diabetes mellitus [27]. As this protein causes both hypertension and diabetes and has never been studied in OSA, it may help in explaining high association of these co-morbidities with OSA [5,6].

Repetitive hypoxia might lead to hypoxic injury to the kidney [28], leading to decreased tubular reabsorption of these low molecular proteins and thus increased protein levels in urine. Further studies are required to elucidate the source, function and exact role of dermcidin in OSA patients.

In the present study, these proteins were analyzed in combinations of either one of these being positive and when both are positive with PSG as the reference standard. In the overall scenario of OSA vs. non-OSA setting, when either EPCR or dermcidin was positive (levels of EPCR> 35 pg/ml or dermcidin <4.6 pg/ml), the sensitivity was close to 60%. However, when both these proteins were positive as per their respective cut-offs, the sensitivity rose to 81%. It was also observed that the positive predictive values (PPV) were high for each of these conditions in OSA vs. non-OSA subjects; ranging from 85% to 94%. Due to the high PPV, these proteins can be a good rule-in test for screening of OSA.

A multivariable analysis of the two important proteins was done to evaluate their performance as potential biomarkers. The crude odds ratios of EPCR and dermcidin, before adjusting for possible confounders like hypertension, diabetes and dyslipidemia, were 6.2 and 6.5 respectively, while the adjusted odds ratios for the proteins were 3.9 ($p = < 0.001$) and 3.3 ($p = <0.001$) respectively suggesting that even after adjusting for the possible confounders, the odds ratio for association of these proteins with OSA was high.

PSG is not easily accessible to every patient and may be expensive and inconvenient to the patient who might need this test in order to confirm the diagnosis of OSA. The present study suggests that these proteins or a combination of these proteins can be of great additive value if they are included in the existing battery of screening tests. After applying different cut-offs for the individual proteins or the combination of proteins, it can be determined whether or not a PSG is required for a specific patient. The diagnostic accuracy of these proteins in severe OSA vs. non-OSA subjects is exemplary with a sensitivity of 94%, and specificity 91% suggesting that these novel biomarkers can help screen severe OSA patients who require immediate diagnosis and therapeutic treatment intervention.

Our data suggest that EPCR and dermcidin can be used as a non-invasive urinary biomarkers for OSA, especially in severe OSA patients. In the past, most of the studies have reported protein biomarkers after creatinine normalization. However, in the current study, we followed a different approach in which we have presented protein concentrations without creatinine normalization. In support, a recent study suggested that normalizing biomarker concentrations would lead to under or over-estimation of the biomarker levels unless, the biomarker concentration is linear with creatinine concentrations or it behaves exactly like creatinine (during renal metabolism). Therefore, it has been suggested not to normalize biomarker concentration using creatinine concentrations [29].

The major strengths of the current study are (1) the study used an off-gel proteomics technique, iTRAQ which is more robust than the conventional gel-based method; (2) the study was carried out in three phases namely, identification phase, verification phase and validation phase with a large sample size for the validation phase, which is the ideal protocol to carry out any biomarker study; (3) patients included in the identification phase and verification phase were free from any co-morbidities which carefully excluded the possibility of any confounding conditions being responsible for up-regulation or down-regulation of proteins; and (4) the two urinary proteins identified to be differentially expressed in OSA are non-invasive potential biomarkers.

Limitations of the study include (1) there is a need to assess the levels of these proteins in patients with only renal dysfunction with respect to OSA patients with and without any overt renal co-morbidity; (2) there is a need to assess the levels of dermcidin in plasma samples of a large number of OSA subjects to understand the source of increased dermcidin levels in urine; and (3) other than excessive weight and snoring, we did not assess these biomarkers in patients with high apnea risk, such as heart failure.

Future studies are required to validate these findings in other populations as well as have a longitudinal study cohort, where OSA patients can be followed up to assess development and/or progression of any arterial diseases.

In conclusion, EPCR and dermcidin emerged as two potential biomarkers for the diagnosis of OSA patients, especially in severe OSA cases. Levels of these proteins were elevated in OSA patients. No previous study in the English literature has reported these proteins in OSA. Initial validation of these proteins in OSA patients indicates a good diagnostic performance and the combination of these two proteins can be of additive value to the currently used screening algorithm for OSA, which will help in judicious use of PSG testing as well as facilitate rapid diagnosis of severe OSA.

Patent: A patent has been filed for this work with the Government of India under the reference number 201711022357.

Authors' roles

Conception and Design: SKS, MK, SS, VU.
Analysis and Interpretation: MK, SV, TB, VS.
Drafting and revising the manuscript: MK, SKS, SV, VU, VS.

Funding

Department of Biotechnology, Government of India and JC Bose National Fellowship (SB/S2/JCB-04/2013) of Professor SK Sharma of the Department of Science & Technology, Ministry of Science and Technology, Government of India, New Delhi.

Acknowledgements

Authors thank Department of Biotechnology, Government of India for providing financial aid for the study. The authors would also like to thank the sleep laboratory staff, Ms. Tsering, Mr,

Sreejith, Mr. Shashikant, Mr. Mohan Babu for their help during the overnight polyomnography test of the patient. Authors also acknowledge the help of laboratory technician Mr. Deepak Kheraliya and medical social worker, Mr. Ajit for their help in this study. Professor SK Sharma was supported by the JC Bose National Fellowship (SB/S2/JCB-04/2013) of the Department of Science & Technology, Ministry of Science and Technology, Government of India, New Delhi.

Conflict of interest

None.

The ICMJE Uniform Disclosure Form for Potential Conflicts of Interest associated with this article can be viewed by clicking on the following link: https://doi.org/10.1016/j.sleep.2019.07.002.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sleep.2019.07.002.

References

[1] Peppard PE, Young T, Barnet JH, et al. Increased prevalence of sleep-disordered breathing in adults. Am J Epidemiol 2013;kws342.
[2] Sharma SK, Kumpawat S, Banga A, et al. Prevalence and risk factors of obstructive sleep apnea syndrome in a population of Delhi, India. Chest 2006;130:149–56.
[3] Strobel RJ, Rosen RC. Obesity and weight loss in obstructive sleep apnea: a critical review. Sleep 1996;19:104–15.
[4] Atkeson A, Jelic S. Mechanisms of endothelial dysfunction in obstructive sleep apnea. Vasc Health Risk Manag 2008;4:1327–35.
[5] Somers VK, White DP, Amin R, et al. Sleep apnea and cardiovascular disease. An American heart association/American college of cardiology foundation scientific statement from the American heart association council for high blood pressure research professional education committee, council on clinical cardiology, stroke council, and council on cardiovascular nursing in collaboration with the national heart, lung, and blood Institute national center on sleep disorders research (national institutes of health). Circulation 2008;118:1080–111.
[6] Peled N, Kassirer M, Shitrit D, et al. The association of OSA with insulin resistance, inflammation and metabolic syndrome. Respir Med 2007;101:1696–701.
[7] Farooqui FA, Sharma SK, Kumar A, et al. Endothelial function and carotid intima media thickness in obstructive sleep apnea without comorbidity. Sleep Breath 2016. https://doi.org/10.1007/s11325-016-1371-7.
[8] Jonas DE, Amick HR, Feltner C, et al. Screening for obstructive sleep apnea in adults: evidence report and systematic review for the US preventive services task force. JAMA 2017;317:415–33.
[9] Kapur VK, Auckley DH, Chowdhuri S, et al. Clinical practice guideline for diagnostic testing for adult obstructive sleep apnea: an American academy of sleep medicine clinical practice guideline. J Clin Sleep Med 2017;13:479–504.
[10] Kim J, Lee S, In K, et al. Increase in serum haptoglobin and apolipoprotein M in patients with obstructive sleep apnoea. J Sleep Res 2009;18:313–20.
[11] Jurado-Gamez B, Gomez-Chaparro JL, Muñoz-Calero M, et al. Serum proteomic changes in adults with obstructive sleep apnoea. J Sleep Res 2012;21:139–46.
[12] Seetho IW, Siwy J, Albalat A, et al. Urinary proteomics in obstructive sleep apnoea and obesity. Eur J Clin Invest 2014;44:1104–15.
[13] Gozal D, Jortani S, Snow AB, et al. Two-dimensional differential in-gel electrophoresis proteomic approaches reveal urine candidate biomarkers in pediatric obstructive sleep apnea. Am J Respir Crit Care Med 2009;180:1253–61.
[14] Shah ZA, Jortani SA, Tauman R, et al. Serum proteomic patterns associated with sleep-disordered breathing in children. Pediatr Res 2006;59:466–70.
[15] Sharma SK, Vasudev C, Sinha S, et al. Validation of the modified Berlin questionnaire to identify patients at risk for the obstructive sleep apnoea syndrome. Indian J Med Res 2006;124:281–90.
[16] Johns MW. A new method for measuring daytime sleepiness: the Epworth Sleepiness Scale. Sleep 1991;14:540–5.
[17] Varshney S, Bhardwaj N, Basak T, et al. Identification of differentially expressed proteins in vitamin B_{12}. J Pract Cardiovasc Sci 2015;1:45.
[18] Basak T, Tanwar VS, Bhardwaj G, et al. Plasma proteomic analysis of stable coronoary artery disease indicates impairment of reverse cholesterol pathway. Sci Rep 2016;6:28042.
[19] Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein C pathway. Blood 2007;109:3161–72.
[20] World Health Organization. Global database on body mass index. Available at: http://apps.who.int/bmi/inde[18]x.jsp?introPage=intro_3.html.
[21] Joyce DE, Gelbert L, Ciaccia A, et al. Gene expression profile of antithrombotic protein c defines new mechanisms modulating inflammation and apoptosis. J Biol Chem 2001;276:11199–203.
[22] Kurosawa S, Esmon CT, Stearns-Kurosawa DJ. The soluble endothelial protein C receptor binds to activated neutrophils: involvement of proteinase-3 and CD11b/CD18. J Immunol Baltim Md 1950 2000;165:4697–703.
[23] Saposnik B, Peynaud-debayle E, Stepanian A, et al. Elevated soluble endothelial cell protein C receptor (sEPCR) levels in women with preeclampsia: a marker of endothelial activation/damage? Thromb Res 2012;129:152–7.
[24] Zaghloul A, Al-Bukhari T a MA, Al-Pakistani HA, et al. Soluble endothelial protein C receptor and high sensitivity C reactive protein levels as markers of endothelial dysfunction in patients with type 1 and type 2 diabetes mellitus: their role in the prediction of vascular complications. Diabetes Res Clin Pract 2014;106:597–604.
[25] Sesin CA, Yin X, Esmon CT, et al. Shedding of endothelial protein C receptor contributes to vasculopathy and renal injury in lupus: in vivo and in vitro evidence. Kidney Int 2005;68:110–20.
[26] Schittek B, Hipfel R, Sauer B, et al. Dermcidin: a novel human antibiotic peptide secreted by sweat glands. Nat Immunol 2001;2:1133–7.
[27] Ghosh R, Maji UK, Bhattacharya R, et al. The role of dermcidin isoform 2: a two-faceted atherosclerotic risk factor for coronary artery disease and the effect of acetyl salicylic acid on it. Thromb Thromb 2012 2012;2012:e987932.
[28] Haase VK. Mechanisms of hypoxia responses in renal tissue. JASN 2013;24:537–41.
[29] Waikar SS, Sabbisetti VS, Bonventre JV. Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. Kidney Int 2010;78:486–94.