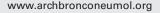# ARCHIVOS DE BRONCONEUMOLOGIA

Original Article

Variation in Protein Expression Depending on the Severity of Sleep Apnoea-Hypopnoea Syndrome

Bernabé Jurado Gámez [a],*, José Luis Gómez-Chaparro Moreno [b,c], María Muñoz Calero [d], Julia Ruiz Laguna [b], Luis Muñoz Cabrera [a], Andrés Cosano Povedano [a] and Juan López-Barea [b]

[a] *Servicio de Neumología, Unidad de Trastornos Respiratorios del Sueño, Hospital Universitario Reina Sofía, Córdoba, Spain*
[b] *Departamento de Bioquímica y Biología Molecular, Universidad de Córdoba, Córdoba, Spain*
[c] *Distrito Sanitario de Córdoba, Córdoba, Spain*
[d] *Servicio de Bioquímica Clínica, Hospital Universitario La Paz, Madrid, Spain*

ARTICLE INFO

ABSTRACT

Objective: A prospective study with a consecutive sample and a control group to determine whether protein expression in patients with sleep apnoea-hypopnoea syndrome (SAHS) is different from that of the control group (IAH ≤ 5).

Patients and methods: A total of 32 patients aged between 35 and 60 years who had a polysomnograph performed were included. Patients with an acute or chronic disease were excluded. The first dimension of the proteomic study was carried out on IPG strips (18 cm, pH4–7) and the second on SDS-PAGE gels in triplicate for each group. The gels were stained with SYPRO-Ruby (Bio-Rad®), the images obtained with an FX-Imager laser scanner and the spots were analysed using ProteomWeaver v.4.0 (Bio-Rad®) software. Significant changes between the gels were analysed by replicates and separately, being considered a significant change if the relative intensity of the spots was three times higher or lower than that of the control and if it was observed in 2 of the 3 replicates of each group, with a coefficient of variation of <20%.

Results: The patients were divided into 8 subjects per group (control, mild, moderate and severe). The comparison of the gels showed significant differences between the control group and the 3 clinical groups, with significant over-expression being observed in 3 spots, and under-expression in 7 spots in the control group.

Conclusion: There are significant changes in protein expression between a control group and patients in different stages of disease. The proteomic study can identify biomarkers associated with the diagnosis and severity of the SAHS.

© 2009 SEPAR. Published by Elsevier España, S.L. All rights reserved.

Variación de la expresión proteica en función de la gravedad del síndrome de apnea-hipopnea del sueño

RESUMEN

Objetivo: Estudio prospectivo con muestreo consecutivo y grupo control para determinar si la expresión proteica en pacientes con SAHS es diferente a la de un grupo control (IAH r5).

Pacientes y métodos: Fueron incluidos 32 pacientes, entre 35 y 60 años, a los que se les realizó una polisomnografía. Fueron excluidos los sujetos con enfermedad aguda o crónica. La primera dimensión del estudio proteómico se realizó en tiras IPG (18 cm, pH 4-7), y la segunda, en geles SDS-PAGE por triplicado para cada grupo. Los geles se tiñeron con SYPRO-Ruby (Bio-Rad®), se obtuvieron las imágenes con un escáner láser FX-Imager, y el análisis de los spots se realizó con el software Proteom Weaver v4.0 (Bio-Rad®). Se analiza-

* Corresponding author.
  E-mail address: bjg01co@hotmail.com (B. Jurado Gámez).

ron los cambios significativos entre los geles agrupados por réplicas y por separado, considerándose un cambio significativo si la intensidad relativa en los spots fue superior o inferior en 3 veces a la del control y se observó en 2 de las 3 réplicas de cada grupo con un coeficiente de variación o 20%.

Resultados: Los pacientes fueron divididos en 8 sujetos por grupo (control, leve, moderado y grave). La comparación de los geles constató diferencias significativas entre el grupo control y los 3 grupos clínicos, observándose 3 spots con sobreexpresión significativa y 7 spots subexpresados respecto al grupo control.

Conclusión: Existen cambios significativos en la expresión protéica entre un grupo control y pacientes en distintos estadios de enfermedad. El estudio proteómico puede identificar biomarcadores relacionados con el diagnóstico y gravedad del SAHS.

Introduction

Sleep apnoea-hypopnoea syndrome (SAHS) is a sleep breathing disorder with a prevalence of 2-4% among middle-aged adults.[1] The symptoms include snoring, observed apnoea, restless sleep and excessive daytime sleepiness.[2] These symptoms are due to frequent episodes of upper airway collapse, which affects ventilation, causes a sharp decrease in peripheral oxygen saturation (SaO_2) and results in microarousals and disordered sleep. This causes non-refreshing sleep and daytime sleepiness, aspects related to worse quality of life[3] and an increased risk of being involved in traffic accidents.[4-6] Furthermore, the episodes of hypoxia-reoxygenation are involved in the production of free radicals and oxidative stress,[7-9] and an increase in vascular mortality.[10-12] Therefore, early diagnosis of SAHS is fundamental, especially since *continuous positive airway pressure* (CPAP)[2] is an effective treatment, and delaying diagnosis and treatment leads to an increased demand on healthcare resources.

At present, polysomnography is the gold standard for the diagnosis of SAHS[2]. However, it is an expensive technique with very long waiting lists and is not available at all healthcare levels.[13,14] This makes diagnosis more difficult, and polysomnography is only performed on 10% of patients.[2,14]

Certain pathologies can modify protein expression. Modern proteomic analysis techniques make it possible to identify these changes and can greatly help diagnosis.[15] Although there are several strategies for analysing protein expression, the most common combination is bidimensional electrophoresis (2D-PAGE), followed by mass spectrometry.[16,17]

The identification of predictive serum markers of SAHS may be a useful tool in a global strategy for the disease, both from a diagnostic point of view and to obtain more knowledge about its physiopathology.

To date, the few proteomic studies into SAHS published have been performed in paediatric populations.[18] It is thought that in SAHS, protein expression can vary in the different stages of severity and it differs from that of another group of subjects without SAHS. As a result, based on the high impact of SAHS in healthcare, and the interest that proteomic studies may have for patients, our group designed a study to determine if protein expression in patients with SAHS differs from that in a control group without SAHS.

Patients and Methods

Design

This was a prospective study with a consecutive sample and control group. A complete medical history was taken for each patient, collecting information about sleep breathing disorders, paying special attention to comorbidities and any medication taken. Then a physical examination was performed, recording weight, size, blood pressure and SaO_2 (at rest and breathing room air), and haematological and plasma biochemistry parameters were taken.

Subjects were eligible for the study if they consulted the specific sleep breathing disorder clinic, and after an initial medical examination they underwent diagnostic polysomnography for suspected SAHS. The diagnosis was confirmed if the patients snored and had any of the following symptoms: observed apnoea, restless sleep and excessive daytime sleepiness, assessed with a scale which has been validated in Spanish (Epworth test>11).

The results of the patients diagnosed with SAHS (SAHS group) were compared with those obtained in the control group, which included subjects who had consulted the sleep disorder clinic (paired by age and BMI), and who were not diagnosed with SAHS after polysomnography.

The study was approved by the Ethics Committee for Clinical Research, and respected the subjects' fundamental rights and the ethical guidelines for biomedical research with human beings established in the Declaration of Helsinki (1964), the Council of Europe Convention on Human Rights and Biomedicine (1997), the Universal Declaration on the Human Genome of UNESCO, and the requirements established in Spanish legislation regarding biomedical research, personal data protection and bioethics (2007).

Patient Selection

To avoid changes attributable to gender, male patients aged between 35 and 60 years were included who were consecutively referred for diagnostic polysomnography due to suspected SAHS, and who gave their informed consent to participate in the study.

Exclusion criteria were the consumption of drugs, including tobacco and alcohol, and suffering from acute or chronic forms of: unstable ischemic heart disease, NYHA grade IV heart failure, chronic renal insufficiency, severe or uncontrolled systemic arterial hypertension, chronic liver disease, degenerative cerebrovascular disease, and severe lung disease (including needing nocturnal oxygen therapy).

Polysomnography

Polysomnography was performed (Somnoscreen®, Somnomedic™, Germany). Monitoring was carried out of two electroencephalogram channels (C4/A1 and C3/A2), an electroculogram, and tibial and submental electromyograms. Air flow was studied with thermosensors and pressure signals were used as the main signal for the analysis. Likewise, snoring, electrocardiographic derivation (V2) and SaO_2 were recorded, as was chest and abdominal effort using impedance plethysmography. All the studies were scored manually in accordance with the Rechtschaffen and Kales criteria. Apnoea was diagnosed if there was a significant decrease (> 90%) in the air flow signal for ≥ 10 sec, and hypopnoea when there was an evident decrease in air flow (>60% and <90%) together with a drop in $SaO_2 \geq 3\%$ and/or one microarousal.

During polysomnography the following breathing variables were monitored: the apnoea-hypopnoea index (AHI) or the sum of apnoeas

and hypopnoeas per hour of sleep, SaO$_2$ awake, minimum SaO$_2$ during sleep, desaturation index (DI3), defined by the number of times SaO$_2$ decreased by ≥3% per hour of sleep, and finally, sleep time with SaO$_2$ <90% (T90).

Polysomnography was considered valid for the diagnosis if subjects had a minimum of 180 minutes sleep.

To avoid classification bias with AHI figures close to the norm, in this study patients were considered to have SAHS if the polysomnography showed an AHI ≥10, while in the control group subjects were included with an IAH ≤ 5. The severity of the SAHS was established depending on the number of respiratory events: mild SAHS (AHI ≥10 and ≤15), moderate (AHI>15 and ≤ 30) and severe (AHI>30).

Proteomic Analysis

After polysomnography, at 7.00 h blood samples (n=32) were collected using venipuncture in an SST Vacutainer tube (BD), following our hospital's usual protocol. The samples were left to coagulate in the cold for 30 min, and then centrifuged at 1,500 × g for 10 min at 4° C. The resulting serum was divided into aliquots and frozen at –80° C until being analysed. The whole process lasted less then 60 min after extraction.

Serum mixtures were prepared for each of the groups in the study (control, mild SAHS, moderate SAHS and severe SAHS). The Bradford assay[19] was used to quantify proteins for each subject so that each one provided the same amount of protein to the total mixture of the groups (control and mild, moderate and severe SAHS).

The most abundant serum proteins were equalised with the Proteominer kit (Bio-Rad®) following the manufacturer's instructions, and the samples were cleaned with the 2D Clean-up Kit (GE Healthcare) following the manufacturer's specifications.

The first dimension or isoelectrofocusing was performed with the Protean IEF Cell system (Bio-Rad®), using 18cm IPG strips with a pH 4-7 (GE Healthcare Immobiline Drystrip). The strips were loaded with 50 μg of protein and resuspended in 350μl urea buffer (6M urea; 0.5% Ampholine pH 3.5-10; 2% CHAPS; trace of bromophenol blue). Three replicas were made for each group in the study. Isoelectrofocusing was performed at 20° C, always following the same procedure until reaching a total of 52,000 V/h for the whole process.

The Protean Plus Dodeca cell system (Bio-Rad®) was used for the second dimension. After isoelectrofocusing, the IPG strips were equilibrated in two consecutive 30 min steps in equilibration buffer (50mM Tris-HCl, pH 8.8; 6M urea; 30% glycerol; 2% SDS; trace of bromophenol blue) complemented in the first step with 10mg/ml DTT. In a second step, 45mg/ml of iodoacetamide was added to the buffer to block the thiol groups. The strips were placed on 1mm thick, 12.5% polyacrylamide gels together with markers of molecular weights, and the separation was done in 2 steps at 20° C: 2.5 W/gel, 5 min and 15 W/gel, 6 h.

After the second dimension, the gels were set with 50% ethanol and 3% acetic acid and stained with SYPRO-Ruby (Bio-Rad®) following the manufacturers specifications.

The gels were digitalised with an imager FX laser scanner (Bio-Rad®) and the images were analysed with ProteomWeaver v4.0 (Bio-Rad®). The following detection parameters were used for the analysis: minimum radius: 4; minimum intensity: 2,000; minimum contrast: 10. The control group was used as the reference value and all the significant differences between the gels of the four groups were identified, both when grouped by replicates and separately.

Statistical Analysis

The data are expressed as means, standard deviation, minimums and maximums for continuous variables and frequencies and percentages for the categories. The Mann-Whitney U test was used to compare continuous variables between the group diagnosed with SAHS and the control group, with a confidence interval of 95%. Two way comparisons were made, and a p value<0.05 was taken to be statistically significant.

Changes in the spots of the different gels were considered to be statistically significant if their relative intensity was 3 times higher or lower than that of the control, and if they appeared in 2 of the 3 replicas of each group with a coefficient of variation <20% and p<0.05.

For the statistical analysis of the data we used the Statistical Package for Social Sciences (SPSS 14) for Windows (SPSS, Chicago, IL, USA).

Results

Thirty-two male patients were studied aged 45±10.6 years and with a BMI of 31±5.4. 8 subjects had an AHI<5 (control group) and the other 24 formed the SAHS group, classified into three subgroups of mild, moderate and severe SAHS, with 8 subjects in each. No differences in age and BMI were observed between the two groups (table 1). Neither were there significant differences in systolic and diastolic blood pressure, nor in the usual biochemical parameters, except the Epworth scale score, which was significantly higher in the SAHS group. Logically, a higher number of respiratory events (AHI) were observed in the SAHS group, as well as a significant difference in nocturnal SaO$_2$ (DI3, T90, minimum SaO$_2$, mean SaO$_2$).

As stated above, the study centred only on the spots in the three gels of each SAHS group with intensities 3 times higher or lower than the control value, and applying a coefficient of variability of <20% and a confidence interval of 95%. Figure 1 shows the spots meeting these criteria: 3 spots were significantly overexpressed and 7 spots showed a significantly lower intensity than the control. Figure 2 shows the 3 overexpressed spots with the different intensity levels in each of the groups (control, mild, moderate and severe). They can all be of interest for the diagnosis of the disease, in particular the spots marked with the number 3,142 and 2,984 as there is a significant increase in expression in the three stages of the disease with regard to the control group. Likewise, the 7 spots revealing underexpression with regard to the control group are also of great interest (fig. 3). Several of these revealed a marked reduction in expression in the three stages of the disease.

Table 1

General and respiratory characteristics of the patients with sleep apnoea-hypopnoea syndrome and the control subjects. Data shown as mean (SD) and n (%)

Variable	Control group	SAHS group	P
Age (years)	44 ± 9.6	45 ± 11.1	U
BMI (kg/m^2)	29 ± 1.7	32 ± 6	U
EDS (Epworth)	7 ± 4.9	13 ± 3.6	< 0,01
SaO$_2$ awake (%)	96 ± 1.8	95 ± 1.8	U
SBP (mmHg)	123 ± 11	123 ± 13.3	U
DBP (mmHg)	77 ± 1.3	72 ± 10.7	U
Glycaemia (mg/dl)	98 ± 15.7	99 ± 12.1	U
Creatinine (mg/dl)	0.86 ± 0.091	0.87 ± 0.138	U
Total cholesterol (mg/dl)	187 ± 22	190 ± 32.8	U
HDL cholesterol (mg/dl)	48 ± 11.1	50 ± 26	U
Triglyceride (mg/dl)	121 ± 69.8	124 ± 38.8	U
AHI (number/hour of sleep)	3 ± 1.1	41 ± 35.7	< 0,001
DI3 (number/hour of sleep)	7 ± 3.4	39 ± 31.2	< 0,001
T90 (%)	0.2 ± 0.32	11 ± 13.2	< 0,001
Minimum SaO$_2$ (%)	88 ± 5.1	78 ± 10.5	0,007
Mean SaO$_2$ (%)	94 ± 0.51	91 ± 4.4	0,044

EDS Epworth: scale of excessive daytime sleepiness; AHI: sum of number of apnoeas and hypopnoeas per hour of sleep; DI3: number of times SaO$_2$ falls by >3% per hour of sleep; BMI: body mass index; T90 sleep time with SaO$_2$ <90%; DBP: diastolic blood pressure; SBP: Systolic blood pressure.

Discussion

The expression of certain proteins can be affected in response to environmental changes such as stress, physiological state and disease. This happens by increasing or decreasing the expression of protein-coding genes or due to changes in post translational modification. The 2D-PAGE technique is the first approach for the differential analysis of the levels of serum protein expression. This technique enables numerous proteins to be separated and, as this study shows, by applying restrictive selection criteria, 3 spots were identified with significantly higher intensities and 7 with significantly lower values. As a result, our study confirms that the 2D-PAGE technique shows that there are differences in protein expression between patients with different degrees of severity of SAHS and subjects in a control group. As a result, Proteomics opens new pathways for better understanding the physiopathological mechanisms associated with the illness and for the potential study of markers related to its diagnosis.

The prevalence of SAHS is high and only a small percentage of patients are diagnosed.[2,14] resulting in their quality of life being affected and increases in mortality from traffic accidents and cardiovascular events, which are related to different aetiopathogenic mechanisms. Proteomics can be a very useful tool in the diagnosis of SAHS and can extend our knowledge of the underlying physiopathological mechanisms of the disease. As figure 2 shows, in patients with SAHS there are various spots showing significant increases in intensity with regard to the control group, and are thus useful for making a diagnosis. Furthermore, the progressive increase in their values in the three stages of the disease can be related to its greater severity and can add prognostic value. Just as important is the protein underexpression observed in SAHS patients (fig. 3), which might mean that the reduction or disappearance of proteins could provide interesting information about the physiopathology of the disease.

The subsequent identification of these spots is the next step in order to isolate possible biomarkers, for which mass spectrometry has been used.[16,17] It is worth pointing out, however, that discovering signs of protein expression characteristic of a particular medical condition has diagnostic value in itself, even if the changed proteins are not identified, as Bradley.[20] established in his pioneering work,
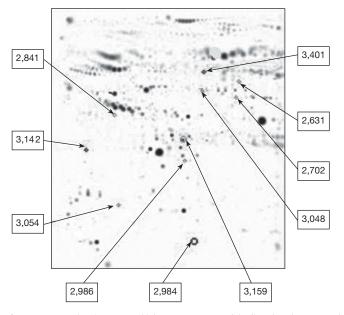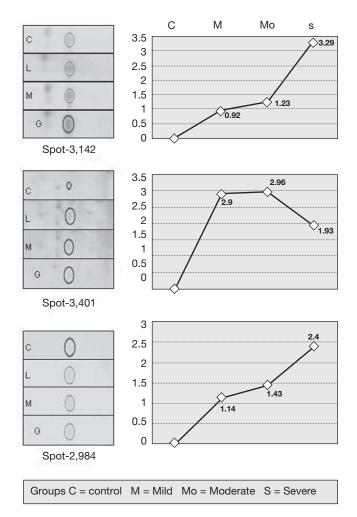
Figure 2. Magnified areas of gels with overexpressed spots. On the right, graph showing (red line) the degree of differential overexpression for each spot.

and has been confirmed by recent studies into environmental contamination.[21] At present, proteomic techniques are being applied in the field of medicine to discover new diagnostic markers of cancer,[22,23] and they are very useful tools in several pathologies.[24-28] However, very few studies have been performed into SAHS to date.[18] our study being the first to determine the proteomic profile of adult patients with SAHS at different stages of severity.

SAHS is frequently associated with comorbidities, particularly obesity, hypertension, diabetes mellitus, etc. However, patients with severe organ pathology were not included in order to avoid associated comorbidities which could affect their proteomic profile and lead to bias in the study. However, this could be a limitation of our study since the sample is less representative of the SAHS population. Another potential limitation is that some proteins are not represented well using the 2D-PAGE technique, for example those with a low molecular weight (<20 kKDa) or with extreme isoelectric points (<4 and >9). On the other hand, the number of 8 patients in each group is big enough in a pilot study to show that there are significant changes in protein expression, although further research is necessary with larger sample sizes in order to study these aspects in greater depth. As mentioned in the Method, patients with an AHI >5 and <10 were excluded, so as to classify appropriately those subjects with SAHS and those without and to avoid classification bias. However, including these patients in the mild SAHS group could have changed the protein expression for it and theoretically reduce the differences found. Despite these limitations, the confidence level and probability
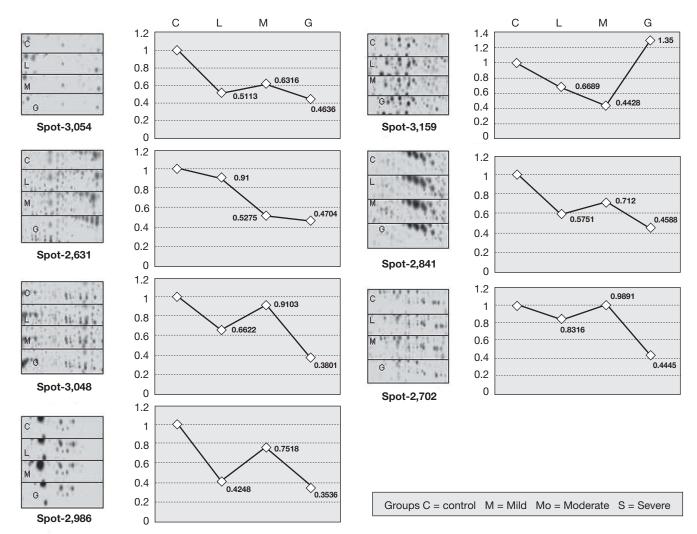
Figure 1. Image showing spots which are overexpressed (red) and underexpressed (green) compared to control.

Spot-3,054

Spot-2,631

Spot-3,048

Spot-2,986

Spot-3,159

Spot-2,841

Spot-2,702

Groups C = control   M = Mild   Mo = Moderate   S = Severe

Figure 3. Magnified areas of gels with underexpressed spots. The degree of differential underexpression for each spot is shown by the green line.

required in the statistical analysis make the results of the research conclusive.

This is the first study to apply proteomics to the study of adult patients with SAHS. Even with the limitations mentioned above, this study shows that, compared to subjects without SAHS, patients at different stages of the disease show significant changes in protein expression. This is a very interesting finding as it will make it possible to identify useful biological markers for the diagnosis of the disease and to improve our knowledge of some physiopathological mechanisms involved in SAHS.

Conflict of Interest

The authors affirm that they have no conflicts of interest.

References

1. Durán J, Esnaola S, Rubio R, Iztueta A. Obstructive sleep apnea-hypopnea and related clinical features in a population-based sample of subjetc aged 30 to 70yr. Am J Respir Crit Care Med. 2001;163:685-9.
2. Grupo Español de Sueño. Consenso Nacional sobre el síndrome de apneas-hipopneas del sueño. Arch Bronconeumol. 2005; p. 41.
3. Moore P, Bardwell WA, Ancoli-Israel S, Dimsdale JE. Association between polysomnographic sleep measures and healt-related quality of life in obstructive sleep apnea. Eur Sleep Res. 2001;10:303-8.
4. George CFP. Sleep apnea, alertness, and motor vehicle crashes. Am J Med Crit Care Med. 2007;176:954-6.
5. Terán-Santos J, Jiménez-Gómez A, Cordero-Guevara J. The association between sleep apnea and the risk of traffic accidents. Cooperative Group Burgos-Santander. N Engl J Med. 1999;340:847-51.
6. Masa JF, Rubio M, Findley LJ. Habitually sleepy drivers hare a high frequency of automobile crashes associated with respiratory disorders during sleep. Am J Respir Crit Care Med. 2000;162:1407-12.
7. Yamauchi M, Nakano H, Maekawa J, Okamoto Y, Ohnishi Y, Suzuki T, et al. Oxidative stress in obstructive sleep apnea. Chest. 2005;127:1674-9.
8. Hernández C, Durán-Cantolla J, Lloberes P, González M. Novedades en la epidemiología, la historia natural, el diagnóstico y el tratamiento del síndrome de apneas-hipopneas durante el sueño. Arch Bronconeumol. 2009;45:3-10.
9. Barceló A, Barbé F, de la Peña M, Vila M, Pérez G, Piérolas J, et al. Antioxidant status in patients with sleep apnoea and impact of continuous positive airway pressure treatment. Eur Respir J. 2006;756-60.
10. Martí S, Sampol G, Muñoz X, Torres F, Roca A, Lloberes P, et al. Mortality in severe sleep apnoea-hypopnoea syndrome patients: impact of treatment. Eur Respir J. 2002;20:1511-8.
11. Yaggi HK, Concato J, Kernan WN, Lichtman JH, Brass LM, Mohsenin V. Obstructive sleep apnea as a risk factor for stroke and death. N Engl J Med. 2005;353:2034-41.
12. Marín JM, Carrizo SJ, Vicente F, Agustí A. Long-term cardiovascular outcomes in men with obstructive sleep apnoea-hypopnoea with or without treatment with continuous positive airway pressure: an observational study. Lancet. 2005;365:1046-53.
13. Durán-Cantolla J, Mar J, de la Torre Muñecas G, Rubio Aramendi R, Guerra L. El síndrome de apneas-hipopneas durante el sueño en España. Disponibilidad de recursos para su diagnóstico y tratamiento en los hospitales del Estado español. Arch Bronconeumol. 2004;40:259-67.
14. Masa JF, Monserrat JM, Durán J, and the Spanish Group of Breathing Sleep Disorders. Diagnostic access for sleep apnea in Spain. Am J Respir Crit Care. 2004;170:195.
15. Rodríguez-Ortega MJ, Grosvik BE, Rodríguez-Ariza A, Goksoyr A, López-Barea J.

Changes in protein expression profiles in bivalve molluscs (Chamaelea gallina) exposed to four model environmental pollutants. Proteomics. 2003;3:1535-43.

16. Montes Nieto R, Fuentes-Almagro CA, Bonilla-Valverde D, Prieto Álamo MJ, Jurado J, Carrascal M, et al. Proteomics in free-living Mus spretus to monitor terrestrial ecosystems. Proteomics. 2007;7:4376-87.

17. Pütz S, Reinder J, Reinder Y, Sickmann A. Mass Spectrometry-Based Peptide Quantification: Applications and limitations. Expert Rev Proteomic. 2005;2:381-92.

18. Sahah ZA, Jortani SA, Taiman R, Valdes R, Gozal D. Serum proteomic patterns associated with sleep-disordered breathing in children. Pediatric Research. 2006;59:466-70.

19. Bradford MM. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Analytical Biochem. 1976;72:248-54.

20. Bradley BP, Shrader EA, Kimmel DG, Meiller JC. Protein expression signatures: an application of proteomics. Mar Environ Res. 2002;54:373-7.

21. Vioque-Fernández A, Alves de Almeida E, López-Barea J. Assessment of Doñana National Park contamination in Procambarus clarkii: Integration of conventional biomarkers and proteomic approaches. Sci Total Environ. 2009. p. 1784-97.

22. Diamandis EP, Van der Merwe DE. Plasma protein profiling by mass spectrometry for cancer diagnosis: opportunitis and limitations. Clin Cancer Research. 2005;11:963-5.

23. Engwegen JY, Gast MC, Schellens JH, Beijnen JH. Clinical proteomic: searching for better tumours markers wit SELDI-TOF mass spectrometry. Trends Pharmacol Sci. 2006;27:251-9.

24. Gray RD, MacGregor G, Noble D, Imrie M, Dejar M, Boyd AC, et al. Sputum proteomics in inflamatory and suppurative respiratory diseases. Am J Respir Crit Care Med. 2008;178:444-52.

25. Kipnis E, Hansen K, Sawa T, Moriyama K, Zurawel A, Ishizaka A, et al. Proteomic analysis of undiluted lung epithelial lining fluid. Chest. 2008;134:338-45.

26. Jacot W, Lhermitte L, Dossat N, Pujol JL, Molinari N, Daurès JP, et al. Serum proteomic profiling of lung cancer in high-risk groups and determination of clinical outcomes. J Torac Oncol. 2008;3:840-50.

27. Maeda J, Hirano T, Ogiwara A, Akimoto S, Kawakami T, Fukui Y, et al. Proteomic analysis of stage I primary lung adenocarcinoma aimed at individualisation of postoperative therapy. B J Cancer. 2008;98:596-603.

28. Lee EJ, In KH, Kim JH, Lee SY, Shin C, Shum JJ, et al. Proteomic analysis in lung tissue of smokers and COPD patients. Chest. 2009;135:344-52.