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(54) Title: METHODS AND KITS FOR DIAGNOSING OBSTRUCTIVE SLEEP APNEA

(57) Abstract: The presently-disclosed subject matter provides methods and kits for diagnosing obstructive sleep apnea (OSA) in a subject, such as a human child, wherein a biological sample is provided from the subject and the amount of a Urocortin III peptide is determined from the sample. Further provided are methods for diagnosing OSA in a subject wherein the amount of a Urocortin III peptide and one or more peptides selected from a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide are determined in a biological sample from a subject.



WO 2010/036901 A1

METHODS AND KITS FOR DIAGNOSING OBSTRUCTIVE SLEEP APNEA

RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Serial No. 61/100,517, filed September 26, 2008, and U.S. Provisional Application Serial No. 61/242,640, filed September 15, 2009, the entire disclosures of which are incorporated herein by this reference.

GOVERNMENT INTEREST

[0002] Subject matter described herein was made with U.S. Government support under Grant Numbers T35DK072923-01, HL-065270, and HL-086662, awarded by the National Institutes of Health. The government has certain rights in the described subject matter.

TECHNICAL FIELD

[0003] The presently-disclosed subject matter relates to methods of diagnosing obstructive sleep apnea (OSA) and distinguishing OSA from primary snoring (PS). In particular, the presently-disclosed subject matter relates to diagnostic and prognostic methods that make use of biomarkers for OSA.

BACKGROUND

[0004] Obstructive Sleep Apnea (OSA) is a frequent condition affecting up to 3% of all pre-pubertal children and has now been recognized as imposing substantial physiological, neurological, and cognitive consequences (1-3). Indeed, if left untreated, OSA can lead to serious neurobehavioral and cardiovascular consequences. Among the first, daytime sleepiness, irritability, depression, attention deficit hyperactivity disorder (ADHD)-like behaviors, and poor cognitive function have all been reported in children suffering from OSA. In this regard, it has also been reported that OSA in children strongly and dose-dependently correlates with emotional problems, impaired school performance, hyperactivity, and aggressive and withdrawal behaviors. With respect to the cardiovascular consequences, untreated OSA been linked to hypertension, increased risk of myocardial infarction, and

stroke. Furthermore, severe and longstanding cases of OSA have been associated with pulmonary hypertension and, in some cases, sudden unexpected death. Despite the seriousness of these consequences of OSA, however, it has been shown that some of these adverse consequences can be reversed if the OSA is diagnosed and treated early.

[0005] In the clinical setting, history and physical examination have extremely poor predictive value in differentiating between primary snoring (PS) and OSA (12-15). As such, current diagnostic approaches for OSA require an overnight sleep study or polysomnography (PSG) in a sleep laboratory, which is both costly and inconvenient, as well as labor intensive. Furthermore, due to the relative unavailability of suitable sleep facilities, particularly, pediatric sleep facilities, long waiting periods and unnecessary delays in diagnosis and treatment are frequent. Accordingly, the development of non-invasive biomarkers capable of reliably distinguishing subjects with PS from those with OSA would greatly facilitate timely screening and diagnosis of OSA, especially in children, which is of great importance in ameliorating the overall outcome of OSA.

SUMMARY

[0006] This Summary describes several embodiments of the presently-disclosed subject matter, and in many cases lists variations and permutations of these embodiments. This Summary is merely exemplary of the numerous and varied embodiments. Mention of one or more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the presently-disclosed subject matter, whether listed in this Summary or not. To avoid excessive repetition, this Summary does not list or suggest all possible combinations of such features.

[0007] The presently-disclosed subject matter includes methods for diagnosing obstructive sleep apnea (OSA) in a subject by determining the amount of one or more biomarkers in a biological sample provided from the subject. In some embodiments, a method for diagnosing OSA is provided that comprises: providing a biological sample from the subject; determining an amount in the sample of a Urocortin (UCN) III peptide; and comparing the amount of the UCN III peptide in the sample, if present, to a control level of the UCN III peptide. In some embodiments, the subject is then diagnosed as having OSA, or a risk thereof, if there is a measurable difference in the amount of the UCN III peptide in the

sample as compared to the control level. In some embodiments, a treatment is then selected or modified based on the amount of UCN III that is determined in the sample.

[0008] Further provided, in some embodiments of the presently-disclosed subject matter, is a method for determining whether to initiate or continue prophylaxis or treatment of OSA in a subject, which comprises: providing a series of biological samples over a time period from a subject; analyzing the series of biological sample to determine an amount in each of the biological samples of a UCN III peptide; and comparing any measurable change in the amounts of the UCN III peptide in each of the biological samples to thereby determine whether to initiate or continue prophylaxis or therapy of the OSA. In some embodiments, the series of biological samples includes a first biological sample, which is collected prior to the initiation of the prophylaxis or treatment for the OSA, and a second biological sample, which is collected after the initiation of prophylaxis or treatment.

[0009] In some embodiments of the presently-disclosed methods, the amount of a UCN III peptide in a biological sample obtained from a subject can be determined in conjunction with one or more additional peptides to thereby diagnose OSA in a subject. In some embodiments, the amount of a UCN III peptide is determined along with the amount of one or more peptides selected from: a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide. In some embodiments, the amount of a UCN III peptide in a biological sample is determined along with the amounts of a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide in a biological sample.

[0010] In some embodiments, the amounts of a UCN III peptide, a Uromodulin peptide, an Orosomucoid peptide, or a Kallikrein 1 peptide are determined by using mass spectrometry analysis, immunoassay analysis, or both. In some embodiments, the mass spectrometry analysis can be selected from liquid-chromatography mass spectrometry (LC-MS), electrospray ionization mass spectrometry (ESI-MS), matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS), or surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). In some embodiments, the immunoassay analysis is performed by using an enzyme-linked immunosorbent assay (ELISA) or an immunoturbidimetric assay.

[0011] In some embodiments, methods for diagnosing OSA in a subject or methods for determining whether to initiate or continue prophylaxis or treatment of OSA in a subject are provided that further comprise providing an apparatus that is capable of detecting a UCN III peptide. In some embodiments, a probe for selectively binding the UCN III peptide is further provided.

[0012] Still further provided, in some embodiments of the presently-disclosed subject matter, are systems for diagnosing OSA in a subject. In some embodiments, a system for diagnosing OSA is provided that comprises: a means for obtaining a biological sample from a subject; a means for determining the amount in the sample of a UCN III peptide; and a means for comparing the amount of the UCN III peptide in the sample, if present, to a control level of the UCN III peptide such that the system is used to provide a diagnosis of OSA in a subject if there is a measurable difference in the amount of the UCN III peptide in the sample as compared to the control level.

[0013] In some embodiments, the biological sample is selected from: a urine sample, a saliva sample, a blood sample, a plasma sample, and a serum sample, which can all be readily obtained from a subject. In some embodiments, the subject is a human, such as, in some embodiments, a human child that is under that age of about 9 to about 2 years of age.

[0014] In some embodiments of the presently-disclosed subject matter, kits for diagnosing OSA in a subject are also provided. In some embodiments, a kit is provided that comprises an antibody capable of detecting a UCN III peptide and instructions for using the kit. In some embodiments, the kits further include one or more antibodies that are capable of detecting a Uromodulin peptide, an Orosomucoid 1 peptide, or a Kallikrein 1 peptide.

[0015] Advantages of the presently-disclosed subject matter will become evident to those of ordinary skill in the art after a study of the description, Figures, and non-limiting Examples in this document.

DESCRIPTION OF THE DRAWINGS

[0016] FIGS. 1A-1E are images showing two-dimensional differential in-gel electrophoretic (2D-DIGE) analysis of urinary proteins in children. FIG. 1A includes images of 2D-DIGE gels of urinary proteins of a child with primary snoring (PS), a child with obstructive sleep apnea (OSA), and a fluorescent overlay of those two images (OVERLAY). FIGS. 1B and 1C include gel images and graphs showing the differential intensity analysis for two protein spots showing increased expression in OSA. FIGS. 1D and 1E include gel images and graphs showing the differential intensity analysis for two proteins spots showing decreased expression in OSA.

[0017] FIG. 2 includes images of western blots for Tenascin, Tribbles homologue 2, and Zinc finger protein 81 from children with OSA, from with children PS, and from healthy controls (CO).

[0018] FIGS. 3A-3D include graphs showing receiver operator curves (ROC) for Kallikrein 1 (FIG. 3A), Urocortin III (FIG. 3B), Orosomucoid 1 (FIG. 3C), and Uromodulin (FIG. 3D) peptide levels, after correction for corresponding urinary creatinine concentrations.

[0019] FIG. 4 is a graph showing boxplots of urinary concentrations of Orosomucoid 1, after correction for corresponding urinary creatinine concentrations, in 30 control children (CO), 30 children with primary snoring (PS), and 60 children with obstructive sleep apnea (OSA).

[0020] FIG. 5 is a graph showing receiver operator curves for combinatorial analyses using one or more cut-off levels defined for Kallikrein 1, Urocortin III, and Orosomucoid 1, and Uromodulin urine levels, after correction for corresponding urinary creatinine concentrations.

[0021] FIG. 6 is a dot plot illustrating the discriminatory ability of using 3 or more cut off levels defined for Kallikrein 1, Urocortin III, Orosomucoid 1, and Uromodulin urine levels, after correction for corresponding urinary creatinine concentrations.

DESCRIPTION OF EXEMPLARY EMBODIMENTS

[0022] The details of one or more embodiments of the presently-disclosed subject matter are set forth in this document. Modifications to embodiments described in this document, and other embodiments, will be evident to those of ordinary skill in the art after a study of the information provided in this document. The information provided in this document, and particularly the specific details of the described exemplary embodiments, is provided primarily for clearness of understanding and no unnecessary limitations are to be understood therefrom. In case of conflict, the specification of this document, including definitions, will control.

[0023] Peptides described herein are described with reference to GENBANK[®] accession numbers and SWISSPROT identification numbers. The sequences cross-referenced in the GENBANK[®] and SWISSPROT databases are expressly incorporated by reference as are equivalent and related sequences present in GENBANK[®], SWISSPROT, or other public databases. Also expressly incorporated herein by reference are all annotations present in the GENBANK[®] and SWISSPROT databases associated with the sequences disclosed herein. Unless otherwise indicated or apparent the references to the GENBANK[®] database and the SWISSPROT database are references to the most recent version of the database as of the filing date of this Application.

[0024] While the terms used herein are believed to be well understood by one of ordinary skill in the art, definitions are set forth to facilitate explanation of the presently-disclosed subject matter.

[0025] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the presently-disclosed subject matter belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the presently-disclosed subject matter, representative methods, devices, and materials are now described.

[0026] Following long-standing patent law convention, the terms “a”, “an”, and “the” refer to “one or more” when used in this application, including the claims. Thus, for example, reference to “a peptide” includes a plurality of such peptides, and so forth.

[0027] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about”. Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification and claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently-disclosed subject matter.

[0028] As used herein, the term “about,” when referring to a value or to an amount of mass, weight, time, volume, concentration or percentage is meant to encompass variations of in some embodiments $\pm 20\%$, in some embodiments $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$, and in some embodiments $\pm 0.1\%$ from the specified amount, as such variations are appropriate to perform the disclosed method.

[0029] The terms “polypeptide”, “protein”, and “peptide”, which are used interchangeably herein, refer to a polymer of the 20 protein amino acids, including modified amino acids (e.g., phosphorylated, glycosylated, etc.) and amino acid analogs, regardless of size or function. Although “protein” is often used in reference to relatively large polypeptides, and “peptide” is often used in reference to small polypeptides, usage of these terms in the art overlaps and varies. The term “peptide” as used herein refers to peptides, polypeptides, proteins and fragments of proteins, unless otherwise noted. The terms “protein”, “polypeptide” and “peptide” are used interchangeably herein when referring to a gene product and fragments thereof. Thus, exemplary polypeptides include gene products,

naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, fragments, and analogs of the foregoing.

[0030] The terms “polypeptide fragment” or “fragment”, when used in reference to a polypeptide, refers to a polypeptide in which amino acid residues are absent as compared to the full-length polypeptide itself, but where the remaining amino acid sequence is usually identical to the corresponding positions in the reference polypeptide. Such deletions can occur at the amino-terminus or carboxy-terminus of the reference polypeptide, or alternatively both. A fragment can retain one or more of the biological activities of the reference polypeptide. In some embodiments, a fragment can comprise a domain or feature, and optionally additional amino acids on one or both sides of the domain or feature, which additional amino acids can number from 5, 10, 15, 20, 30, 40, 50, or up to 100 or more residues. Further, fragments can include a sub-fragment of a specific region, which sub-fragment retains a function of the region from which it is derived. When the term “peptide” is used herein, it is intended to include the full-length peptide as well as fragments of the peptide. Thus, an identified fragment of a peptide (*e.g.*, by mass spectrometry) is intended to encompass the fragment as well as the full-length peptide. As such, determining an amount of a biomarker in a sample can include determining an amount of the full-length biomarker polypeptide, modified variants, and/or fragments thereof.

[0031] The terms “Obstructive Sleep Apnea” and “Obstructive Sleep Apnea Syndrome,” are used interchangeably herein to refer to a condition that is characterized by a history of habitual snoring, and which is associated with repeated events of partial or complete obstruction of the upper airway during sleep. Obstructive Sleep Apnea (OSA) is a clinically complex syndrome affecting up to 3% of pre-pubertal children and causes subjects to experience both daytime and nighttime symptoms. As noted, the main nocturnal symptom is habitual snoring. However, subjects afflicted with OSA frequently experience further nocturnal symptoms including, but not limited to, respiratory pauses, noisy breathing sounds, paradoxical breathing movements, cyanosis, restless sleep, excessive diaphoresis, sleep enuresis, as well as recurring episodes gas exchange abnormalities, such as hypercapnia and hypoxemia, and frequent arousals (*i.e.*, sleep fragmentation). As such, OSA is typically ranked at the severe end of the clinical spectrum of sleep disordered breathing.

[0032] At the other end of the clinical spectrum is a less severe condition known as habitual or primary snoring (PS), which is typically considered to be a more benign form of upper airway resistance. PS occurs in the absence of gas exchange abnormalities or evidence

of snore-associated alterations in sleep architecture. Indeed, PS is a relatively frequent condition and is predicted to affect 10-12% of all school-aged children.

[0033] Currently, the ability to accurately diagnose OSA and distinguish between OSA and PS requires an overnight sleep study, or polysomnography, which is not readily available to many subjects due to the cost and the availability of a suitable sleep diagnostic facility. Thus, a subject may be afflicted with OSA for an extended period of time without an accurate diagnosis and subsequent treatment, which can thus cause the adverse consequences associated with OSA to be long-lasting or permanent. As such, the presently-disclosed subject matter provides methods and peptide biomarkers that can be utilized to diagnose OSA, and distinguish it from PS, without the need of an overnight sleep study, thereby providing earlier treatment opportunities.

[0034] Disclosed herein are methods for diagnosing OSA using peptide levels associated with OSA as biomarkers. In some embodiments, the presently-disclosed subject matter includes methods and kits for diagnosing OSA in a subject, and for determining whether to initiate or continue prophylaxis or treatment of OSA in a subject, by identifying at least one peptide biomarker associated with OSA in a biological sample from the subject.

[0035] In some embodiments, the at least one peptide biomarker that is associated with OSA is a Urocortin (UCN) III peptide. In some embodiments, the amount of a UCN III peptide in a biological sample from a subject is determined to thereby diagnose OSA in a subject. In some embodiments, a method for diagnosing OSA is provided in which the amount of a UCN III peptide is determined along with the amounts of one or more peptides selected from the group consisting of: a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide. In some embodiments, a method for diagnosing OSA is provided in which the amounts of a UCN III peptide, a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide are each determined in a biological sample obtained from a subject. A list of these exemplary peptide biomarkers of OSA, as well as exemplary GENBANK® Accession and SWISSPROT Identification Numbers for each of these peptide biomarkers, is provided in Table 1 below:

Table 1. Exemplary Biomarkers Associated with OSA.

Peptide Name	GENBANK [®] Accession Number	SWISSPROT Identification Number
Urocortin III	AAK67317	Q969E3
Uromodulin	AAL27853	P07911
Orosomucoid 1*	EAW87416	P02763
Kallikrein 1	AAH05313	P06870

*Orosomucoid 1 can also be referred to as Alpha-1-acid glycoprotein 1.

[0036] With regard to the presently-disclosed subject matter, the exemplary human peptides provided in Table 1 are not intended to limit the presently-disclosed subject matter to human polypeptide biomarkers. Rather, the present subject matter encompasses peptide biomarkers across animal species that are associated with OSA. In addition, standard gene/protein nomenclature guidelines generally stipulate human gene name abbreviations are capitalized and italicized, and protein name abbreviations are capitalized, but not italicized. Further, standard gene/protein nomenclature guidelines generally stipulate mouse, rat, and chicken gene name abbreviations italicized with the first letter only capitalized, and protein name abbreviations capitalized, but not italicized. In contrast, the gene/protein nomenclature used herein when referencing specific biomarkers uses first letter capitalized for the biomarker name, but is intended to be inclusive of genes (including mRNAs and cDNAs) and proteins across animal species.

[0037] A “biomarker” is a molecule useful as an indicator of a biologic state in a subject. With reference to the present subject matter, the biomarkers disclosed herein can be polypeptides (*e.g.*, UCN III, Uromodulin, Orosomucoid 1, or Kallikrein 1 peptides) that exhibit a change in expression or state, which can be correlated with the risk of developing, the presence of, or the progression of OSA in a subject. In addition, the peptide biomarkers disclosed herein are inclusive of messenger RNAs (mRNAs) encoding the peptide biomarkers associated with OSA, as measurement of a change in expression of an mRNA can be correlated with changes in expression of the polypeptide encoded by the mRNA. As such, determining an amount of a peptide biomarker associated with OSA in a biological sample is inclusive of determining an amount of the peptide biomarker, including fragments thereof, and/or an amount of an mRNA encoding the peptide biomarker either by direct or indirect

(*e.g.*, by measure of a complementary DNA (cDNA) synthesized from the mRNA) measurement of the mRNA.

[0038] As noted, in some embodiments of the presently-disclosed subject matter, a method for diagnosing OSA in a subject is provided. The terms “diagnosing” and “diagnosis,” as used herein, refer to methods by which the skilled artisan can estimate and even determine whether or not a subject is suffering from a given disease or condition. The skilled artisan often makes a diagnosis on the basis of one or more diagnostic indicators, such as for example a biomarker, the amount (including presence or absence) of which is indicative of the presence, severity, or absence of the condition.

[0039] Along with diagnosis, clinical disease prognosis is also an area of great concern and interest. If a more accurate prognosis can be made, appropriate therapy, and in some instances less severe therapy for the patient can be chosen. As such, “making a diagnosis” or “diagnosing”, as used herein, is further inclusive of determining a prognosis, which can provide for predicting a clinical outcome (with or without medical treatment), selecting an appropriate treatment (or whether treatment would be effective), or monitoring a current treatment and potentially changing the treatment, based on the measure of levels of diagnostic biomarkers disclosed herein.

[0040] The phrase “determining a prognosis” as used herein refers to methods by which the skilled artisan can predict the course or outcome of a condition in a subject. The term “prognosis” does not refer to the ability to predict the course or outcome of a condition with 100% accuracy, or even that a given course or outcome is predictably more or less likely to occur based on the presence, absence or levels of test biomarkers. Instead, the skilled artisan will understand that the term “prognosis” refers to an increased probability that a certain course or outcome will occur; that is, that a course or outcome is more likely to occur in a subject exhibiting a given condition, when compared to those individuals not exhibiting the condition. For example, in individuals not exhibiting the condition (*e.g.*, not expressing the biomarker or expressing it at a reduced level), the chance of a given outcome may be about 3%. In certain embodiments, a prognosis is about a 5% chance of a given outcome, about a 7% chance, about a 10% chance, about a 12% chance, about a 15% chance, about a 20% chance, about a 25% chance, about a 30% chance, about a 40% chance, about a 50% chance, about a 60% chance, about a 75% chance, about a 90% chance, or about a 95% chance.

[0041] The skilled artisan will also understand that associating a prognostic indicator with a predisposition to an adverse outcome is a statistical analysis. For example, a UCN III peptide level (*e.g.*, quantity of expression in a sample) of greater than a control level in some

embodiments can signal that a subject is more likely to suffer from OSA than subjects with a level less than or equal to the control level, as determined by a level of statistical significance. Alternatively, in some embodiments, a UCN III peptide level of less than or equal to a control level can signal that a subject does not suffer from OSA. Additionally, a change in UCN III peptide concentration from baseline levels can be reflective of subject prognosis, and the degree of change in marker level can be related to the severity of adverse events. Statistical significance is often determined by comparing two or more populations, and determining a confidence interval and/or a p value. *See, e.g.*, Dowdy and Wearden, *Statistics for Research*, John Wiley & Sons, New York, 1983, incorporated herein by reference in its entirety. Preferred confidence intervals of the present subject matter are 90%, 95%, 97.5%, 98%, 99%, 99.5%, 99.9% and 99.99%, while preferred p values are 0.1, 0.05, 0.025, 0.02, 0.01, 0.005, 0.001, and 0.0001.

[0042] In other embodiments, a threshold degree of change in the level of a prognostic or diagnostic biomarker can be established, and the degree of change in the level of the indicator in a biological sample can simply be compared to the threshold degree of change in the level. A preferred threshold change in the level for markers of the presently-disclosed subject matter is about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 50%, about 75%, about 100%, about 150%, or more. In yet other embodiments, a “nomogram” can be established, by which a level of a prognostic or diagnostic indicator can be directly related to an associated disposition towards a given outcome. The skilled artisan is acquainted with the use of such nomograms to relate two numeric values with the understanding that the uncertainty in this measurement is the same as the uncertainty in the marker concentration because individual sample measurements are referenced, not population averages.

[0043] In some embodiments of the presently-disclosed subject matter, multiple determinations of one or more diagnostic or prognostic peptides associated with OSA, such as UCN III, can be made, and a temporal change in the biomarker peptides can be used to monitor the progression of disease and/or efficacy of appropriate therapies directed against the disease. In such an embodiment, for example, one might expect to see a decrease or an increase in the UCN III peptide(s) over time during the course of effective therapy. Thus, the presently disclosed subject matter provides, in some embodiments, a method for determining whether to initiate or continue treatment of OSA in a subject. In some embodiments, the method comprises determining an amount of a UCN III peptide in biological samples collected from the subject at a plurality of different time points and comparing any

measurable changes in the amounts of the UCN III peptide in the samples collected at different time points. For example, a first time point can be selected prior to initiation of a treatment and a second time point can be selected at some time after initiation of the treatment. UCN III peptide levels can be measured in each of the samples taken from different time points and qualitative and/or quantitative differences noted. A change in the amounts of the UCN III peptide levels from the first and second samples can be correlated with determining treatment efficacy and/or progression of the disease in the subject.

[0044] In some embodiments, a method for determining whether to initiate or continue prophylaxis or treatment of OSA in a subject is provided that further comprises determining an amount in a biological sample of a UCN III peptide in conjunction with one or more peptides selected from a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide at different time points such that measurable changes in the various peptides over time can then be compared. For example, in some embodiments, the amount of a UCN III peptide is determined along with a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide at different time points and qualitative and/or quantitative differences in the levels of each peptide are noted. Similar to the methods described above with respect to identifying UCN III independently of other peptide biomarkers, a change in the amounts of the UCN III, Uromodulin, Orosomucoid 1 peptide, and/or Kallikrein 1 peptide levels in the samples that are collected at different time points can be correlated with determining treatment efficacy and/or progression of the disease in the subject.

[0045] The terms “correlated” and “correlating,” as used herein in reference to the use of diagnostic and prognostic biomarkers, refers to comparing the presence or quantity of the biomarker in a subject to its presence or quantity in subjects known to suffer from a given condition (*e.g.*, OSA); or in subjects known to be free of a given condition, *i.e.* “normal individuals.” For example, a UCN III peptide level in a biological sample can be compared to a level known to be associated with OSA. The sample’s UCN III peptide level is said to have been correlated with a diagnosis; that is, the skilled artisan can use the UCN III peptide level to determine whether the subject suffers from OSA, and respond accordingly. Alternatively, the sample’s UCN III peptide level can be compared to a control UCN III peptide level known to be associated with a good outcome (*e.g.*, the absence of OSA), such as an average level found in a population of normal subjects.

[0046] In certain embodiments, a diagnostic biomarker is correlated to a condition or disease by merely its presence or absence. In other embodiments, a threshold level of a diagnostic biomarker can be established, and the level of the indicator in a subject sample can

simply be compared to the threshold level. In yet other embodiments, multiple determinations of one or more diagnostic biomarkers can be made, and a temporal change in the marker can be used to determine a diagnosis, including a prognosis.

[0047] The skilled artisan will understand that, while in certain embodiments comparative measurements can be made of the same diagnostic marker at multiple time points, one can also measure a given marker at one time point, and a second marker at a second time point, and a comparison of these markers can provide diagnostic information.

[0048] In some embodiments, a method for diagnosing OSA in a subject comprises providing a biological sample from the subject; determining an amount in the sample of a UCN III peptide; and comparing the amount of the UCN III peptide in the sample, if present, to a control level of the UCN III peptide. If there is a measurable difference in the amount of the UCN III peptide in the sample as compared to a control level, the subject can then be diagnosed as having OSA or a risk thereof.

[0049] Disclosed herein are data demonstrating that UCN III peptide levels in a biological sample obtained from a subject can be utilized as a biomarker for diagnosing subjects with OSA. Further disclosed herein, however, are additional data and information demonstrating that while UCN III peptide levels can be used to accurately diagnose OSA, the determination of an amount of a UCN III peptide level in a biological sample along with the amount of a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide in the same biological sample can allow for a diagnosis of OSA with increased sensitivity and specificity. As such, in some embodiments, a method for diagnosing OSA in a subject is provided that further comprises determining an amount in the sample of one or more peptides selected from: a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide. In some embodiments, the amounts of a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide in a biological sample are determined along with the amount of a UCN III peptide to thereby diagnose a particular subject as having OSA.

[0050] With regard to the step of providing a biological sample from the subject, the term “biological sample” as used herein refers to any body fluid or tissue potentially comprising a peptide biomarker associated with OSA, such as those disclosed herein. In some embodiments, for example, the biological sample can be a urine sample, a saliva sample, a blood sample, a serum sample, a plasma sample, or sub-fractions thereof.

[0051] Turning now to the step of determining an amount of a peptide biomarker associated with OSA in the biological sample, various methods known to those skilled in the art can be used to identify a peptide biomarker in the provided biological sample. In some

embodiments, determining the amount of a peptide biomarker in samples comprises using an RNA measuring assay to measure mRNA encoding peptide biomarkers associated with OSA in the sample and/or using a protein measuring assay to measure amounts of the peptides biomarkers in the sample.

[0052] In certain embodiments, the amounts of the peptide biomarkers can be determined by probing for mRNA of the peptide biomarker (*e.g.*, UCN III, Uromodulin, Orosomucoid 1, and/or Kallikrein 1) in the sample using any RNA identification assay known to those skilled in the art. Briefly, RNA can be extracted from the sample, amplified, converted to cDNA, labeled, and allowed to hybridize with probes of a known sequence, such as known RNA hybridization probes (selective for mRNAs encoding the peptide biomarkers) immobilized on a substrate, *e.g.*, array, or microarray, or quantitated by real time PCR (*e.g.*, quantitative real-time PCR, such as available from Bio-Rad Laboratories, Hercules, California, U.S.A.). Because the probes to which the nucleic acid molecules of the sample are bound are known, the molecules in the sample can be identified. In this regard, DNA probes for one or more of the mRNAs encoding the biomarker peptides of the presently-disclosed subject matter can be immobilized on a substrate and provided for use in practicing a method in accordance with the present subject matter.

[0053] With regard to determining amounts of the biomarker peptides associated with OSA disclosed herein in samples, mass spectrometry and/or immunoassay devices and methods can be used to measure the peptides in samples, although other methods are well known to those skilled in the art as well. *See, e.g.*, U.S. Pat. Nos. 6,143,576; 6,113,855; 6,019,944; 5,985,579; 5,947,124; 5,939,272; 5,922,615; 5,885,527; 5,851,776; 5,824,799; 5,679,526; 5,525,524; and 5,480,792, each of which is hereby incorporated by reference in its entirety. Immunoassay devices and methods can utilize labeled molecules in various sandwich, competitive, or non-competitive assay formats, to generate a signal that is related to the presence or amount of an analyte of interest. Additionally, certain methods and devices, such as biosensors and optical immunoassays, can be employed to determine the presence or amount of analytes without the need for a labeled molecule. *See, e.g.*, U.S. Pat. Nos. 5,631,171; and 5,955,377, each of which is hereby incorporated by reference in its entirety.

[0054] Thus, in certain embodiments of the presently-disclosed subject matter, the peptide biomarkers of the presently-disclosed subject matter are analyzed using an immunoassay. The presence or amount of a peptide biomarker (*e.g.*, UCN III) can be determined using antibodies or fragments thereof specific for each marker and detecting

specific binding. For example, in some embodiments, an antibody specifically binds UCN III, which is inclusive of antibodies that bind the full-length peptide or a fragment thereof.

[0055] Any suitable immunoassay can be utilized, for example, enzyme-linked immunoassays (ELISA), radioimmunoassays (RIAs), competitive binding assays, immunoturbidimetric assays, and the like. Specific immunological binding of the antibody to the marker can be detected directly or indirectly. Direct labels include fluorescent or luminescent tags, metals, dyes, radionucleotides, and the like, attached to the antibody. Indirect labels include various enzymes well known in the art, such as alkaline phosphatase, horseradish peroxidase and the like.

[0056] The use of immobilized antibodies or fragments thereof specific for the peptides biomarkers of the presently disclosed subject matter (*e.g.*, UCN III, Uromodulin, Orosomucoid 1, and/or Kallikrein 1) is also contemplated by the presently-disclosed subject matter. The antibodies can be immobilized onto a variety of solid supports, such as magnetic or chromatographic matrix particles, the surface of an assay plate (such as microtiter wells), pieces of a solid substrate material (such as plastic, nylon, paper), and the like. An assay strip can be prepared by coating the antibody or a plurality of antibodies in an array on a solid support. This strip can then be dipped into the test biological sample and then processed quickly through washes and detection steps to generate a measurable signal, such as for example a colored spot.

[0057] In some embodiments, mass spectrometry (MS) analysis can be used alone or in combination with other methods (*e.g.*, immunoassays) to determine the presence and/or quantity of the peptide biomarker of interest (*e.g.*, UCN III, Uromodulin, Orosomucoid 1, or Kallikrein 1) in a biological sample. In some embodiments, the MS analysis comprises liquid chromatography-mass spectrometry (LC-MS). In some embodiments, the MS analysis comprises matrix-assisted laser desorption/ionization time-of-flight MS analysis (MALDI-TOF-MS), such as for example direct-spot MALDI-TOF or liquid chromatography MALDI-TOF mass spectrometry analysis. In some embodiments, the MS analysis comprises electrospray ionization MS (ESI-MS), such as for example liquid chromatography (LC) ESI-MS. Mass spectrometry analysis can be accomplished using commercially-available spectrometers, such as for example triple quadrupole mass spectrometers. In some embodiments, the MS analysis comprises surface enhanced laser desorption/ionization time-of-flight mass spectrometry analysis (SELDI-TOF-MS). Methods for utilizing MS analysis to detect the presence and quantity of biomarker peptides in biological samples are known in the art. *See*, for example, U.S. Patents 6,925,389; 6,989,100; and 6,890,763 for further

guidance, as well as Slominski, *et al.*, *Journal of Clinical Endocrinology and Metabolism*, 2000 Feb.; 85(2): p. 815-823, each of which are incorporated herein by this reference.

[0058] Although certain embodiments of the methods disclosed herein only call for a qualitative assessment of the presence or absence of the one or more biomarkers in the biological sample, other embodiments of the methods call for a quantitative assessment of the amount of each of the one or more markers in the biological sample. Such quantitative assessments can be made, for example, using one of the above mentioned methods, as will be understood by those skilled in the art.

[0059] In certain embodiments of the presently-disclosed methods, a subject is diagnosed as having OSA upon determining, in a biological sample obtained from the subject, an amount of a UCN III peptide, either alone or in combination with the amounts of one or more peptides selected from a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide. In other embodiments of the method, the determination of an amount of a Urocortin III peptide, either alone or in combination with one or more peptides selected from a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide, in a biological sample obtained from the subject results in the subject being identified as having a risk of OSA.

[0060] In certain embodiments of the method, it can be desirable to include a control sample that is analyzed concurrently with the biological sample, such that the results obtained from the biological sample can be compared to the results obtained from the control sample. Additionally, it is contemplated that standard curves can be provided, with which assay results for the biological sample can be compared. Such standard curves present levels of protein markers as a function of assay units, *i.e.*, fluorescent signal intensity, if a fluorescent signal is used. Using samples taken from multiple donors, standard curves can be provided for control levels of the one or more biomarkers in normal tissue.

[0061] The analysis of biomarkers can be carried out separately or simultaneously with additional biomarkers within one test sample. For example, several biomarkers (*e.g.*, UCN III, Uromodulin, Orosomucoid 1, and Kallikrein peptides) can be combined into one test for efficient processing of multiple samples and for potentially providing greater diagnostic and/or prognostic accuracy. In addition, one skilled in the art would recognize the value of testing multiple samples (for example, at successive time points) from the same subject. Such testing of serial samples can allow the identification of changes in marker levels over time. Increases or decreases in marker levels, as well as the absence of change in marker levels, can provide useful information about the disease status that includes, but is not limited

to identifying the approximate time from onset of the event, the appropriateness of drug therapies, the effectiveness of various therapies, and identification of the subject's outcome, including risk of future events.

[0062] As noted, depending on the embodiment of the method, identification of the peptides biomarkers associated with OSA can be a qualitative determination of the presence or absence of the biomarkers, or it can be a quantitative determination of the concentration of the peptide biomarkers. In this regard, in some embodiments, the step of identifying the subject as having OSA or a risk thereof requires that certain threshold measurements are made, *i.e.*, the levels of a peptide biomarker in the biological sample exceeds or is below the control level. In certain embodiments of the method, the control level is any detectable level of the peptide biomarkers. In other embodiments of the method where a control sample is tested concurrently with the biological sample, the control level is the level of detection in the control sample. In other embodiments of the method, the control level is based upon and/or identified by a standard curve. In other embodiments of the method, the control level is a specifically identified concentration, or concentration range. As such, the control level can be chosen, within acceptable limits that will be apparent to those skilled in the art, based in part on the embodiment of the method being practiced and the desired specificity, etc.

[0063] Further provided, in some embodiments of the presently-disclosed subject matter, are systems for diagnosing OSA in a subject. In some embodiments, a system for diagnosing OSA in a subject is provided that comprises a means for obtaining a biological sample from a subject; a means for determining the amount in the sample of a UCN III peptide; and a means for comparing the amount of a UCN III peptide in a sample, if present, to a control level of the UCN III peptide such that the subject can be diagnosed as having OSA if there is a measurable difference in the amount of the UCN III peptide in the sample as compared to a control level. In other embodiments, various means for determining the amount, as well as means for comparing the amount, of a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide can be provided in an exemplary system such that the amounts of one or more of the foregoing peptides can also be determined.

[0064] The means for obtaining a biological sample from a subject in accordance with the presently-disclosed systems can comprise any means known to those of ordinary skill in the art which can be used for obtaining a particular biological sample from a subject. For example, to obtain a blood sample from a subject, a hypodermic needle or other such suitable device can be used to collect a blood sample from an artery or vein of a subject. As another example, in embodiments where a urine or saliva sample is obtained from a subject, the

means for obtaining the sample can comprise a cup or other suitable container into which the urine or saliva sample can be deposited.

[0065] With respect to the means for determining an amount of a peptide biomarker associated with OSA in a subject, in some embodiments, the means for determining the amount of the peptide biomarker can comprise an apparatus, such as a mass spectrometry apparatus, or a probe for selectively binding a peptide biomarker associated with OSA. For example, in some embodiments, the probe can be an antibody that selectively binds to the peptide biomarker. As another example, in other embodiments where the amount of a peptide biomarker is determined by measuring the amount of mRNA of the peptide biomarker, the means for determining the amount of the peptide biomarker can be an RNA hybridization probe that is selective for the mRNA encoding the peptide biomarker.

[0066] Still further provided, in some embodiments of the presently-disclosed subject matter, are kits for the analysis of biomarkers that comprise antibodies having specificity for one or more biomarkers associated with OSA. Such a kit can comprise devices and reagents for the analysis of at least one test sample. In some embodiments, the kits further comprise instructions for using the kit. Optionally, in some embodiments, the kits can also contain one or more reagents or devices for converting a biomarker level to a diagnosis or prognosis of the subject.

[0067] In some embodiments, a kit is provided that comprises an antibody capable of detecting a UCN III peptide in a subject and instructions for using the kit. In other embodiments, a kit is provided that further comprises one or more antibodies capable of detecting one or more peptides selected from a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide. In some embodiments, a kit is provided that includes antibodies capable of detecting a UCN III peptide, a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide such that the levels of each of the peptides can be detected in a biological sample obtained from a subject.

[0068] With respect to the presently-disclosed subject matter, the subject can be a vertebrate subject. A vertebrate subject can be warm-blooded; a warm-blooded vertebrate subject can be a mammal. A mammalian subject can be a human. In some embodiments, a human subject can be a child. A child can be a human that is under the age of about 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1. In some embodiments, the subject is a human child that is under the age of about 9 to about 2 years of age.

[0069] As used herein, the term “subject” includes both human and animal subjects. Thus, veterinary therapeutic uses are provided in accordance with the presently-disclosed

subject matter. As such, the presently-disclosed subject matter provides for the diagnosis of mammals such as humans, as well as those mammals of importance due to being endangered, such as Siberian tigers; of economic importance, such as animals raised on farms for consumption by humans; and/or animals of social importance to humans, such as animals kept as pets or in zoos. Examples of such animals include but are not limited to: carnivores such as cats and dogs; swine, including pigs, hogs, and wild boars; ruminants and/or ungulates such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels; and horses. Also provided is the treatment of birds, including the treatment of those kinds of birds that are endangered and/or kept in zoos, as well as fowl, and more particularly domesticated fowl, *i.e.*, poultry, such as turkeys, chickens, ducks, geese, guinea fowl, and the like, as they are also of economic importance to humans. Thus, also provided is the treatment of livestock, including, but not limited to, domesticated swine, ruminants, ungulates, horses (including race horses), poultry, and the like.

[0070] The practice of the presently-disclosed subject matter can employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. *See, e.g.*, Molecular Cloning A Laboratory Manual (1989), 2nd Ed., ed. by Sambrook, Fritsch and Maniatis, eds., Cold Spring Harbor Laboratory Press, Chapters 16 and 17; U.S. Pat. No. 4,683,195; DNA Cloning, Volumes I and II, Glover, ed., 1985; Oligonucleotide Synthesis, M. J. Gait, ed., 1984; Nucleic Acid Hybridization, D. Hames & S. J. Higgins, eds., 1984; Transcription and Translation, B. D. Hames & S. J. Higgins, eds., 1984; Culture Of Animal Cells, R. I. Freshney, Alan R. Liss, Inc., 1987; Immobilized Cells And Enzymes, IRL Press, 1986; Perbal (1984), A Practical Guide To Molecular Cloning; See Methods In Enzymology (Academic Press, Inc., N.Y.); Gene Transfer Vectors For Mammalian Cells, J. H. Miller and M. P. Calos, eds., Cold Spring Harbor Laboratory, 1987; Methods In Enzymology, Vols. 154 and 155, Wu et al., eds., Academic Press Inc., N.Y.; Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987; Handbook Of Experimental Immunology, Volumes I-IV, D. M. Weir and C. C. Blackwell, eds., 1986.

[0071] The presently-disclosed subject matter is further illustrated by the following specific but non-limiting examples.

EXAMPLES

[0072] Example 1 – Subject Selection and Diagnosis of Obstructive Sleep Apnea by Overnight Polysomnography

[0073] Children who were referred for evaluation of habitual snoring and suspected sleep disordered breathing were recruited. As controls, children from the community who had no history of any chronic or acute disorder, and who did not snore, were also invited to participate. Exclusion criteria for all subjects included the presence of genetic or systemic disorders, significant neuromuscular diseases, renal disease, or any acute infectious processes. All parents completed a detailed intake clinical questionnaire. Height and weight and vital signs were recorded for each child, and body mass index (BMI) z score was calculated using CDC 2000 growth standards and associated online software. A BMI z-score >1.65 ($>95^{\text{th}}$ percentile) was considered as fulfilling obese criteria.

[0074] All subjects underwent overnight polysomnography (PSG) using standard techniques (18). Children were studied for up to 12 hours in a quiet, darkened room maintained at an ambient temperature of 24° . No medication was used to induce sleep. During that time, the following parameters were measured: chest and abdominal wall movement by inductance plethysmography; heart rate by electrocardiography; and air flow by a side-stream end-tidal capnograph that also provides breath-by-breath assessment of end-tidal carbon dioxide levels (BCI SC-300, Menomonee Falls, WI), a nasal pressure cannula, and an oronasal thermistor. Arterial pulse oxygen saturation (SpO_2) was assessed by pulse oximetry (Nellcor N 100 (Nellcor Inc, Hayward, CA)), with simultaneous recording of the pulse waveform. The bilateral electro-oculogram, 8 channels of electroencephalogram (2 frontal, 2 occipital, 2 temporal, and 2 central leads), chin and anterior tibial electromyograms, and analog output from a body position sensor were also monitored. All measures were digitized using a commercially available system (Sandman, Nellcor Puritan Bennett, Kanata, ON, Canada, or Stellate Instruments, Montreal, QC, Canada). Tracheal sound was monitored with a microphone sensor, and a digital time-synchronized video recording was performed. A sleep technician followed patient behavior and confirmed sleep position by an infrared camera located inside the room. All of the studies were initially scored by a certified technician and were then reviewed by a physician who was experienced in pediatric PSG and underwent training in an accredited fellowship program.

[0075] Sleep architecture was assessed by standard techniques (19). Central, obstructive, and mixed apneic events were counted. Obstructive apnea was defined as the absence of

airflow with continued chest wall and abdominal movement for duration of at least 2 breaths (18). Hypopneas were defined as a decrease in oronasal flow of $\geq 50\%$ with a corresponding decrease in SpO_2 of 3% or more and/or an arousal (18). The obstructive apnea hypopnea index (OAHl) was defined as the number of obstructive apneas and hypopneas per hour of total sleep time (TST). Arousals were identified as defined by the American Sleep Disorders Association Task Force report (20).

[0076] Subjects were then divided into 3 groups according to whether they had habitual or primary snoring (PS) or not and their sleep study findings. Criteria for obstructive sleep apnea (OSA) included an obstructive apnea index (AI) greater than 1/h TST, and/or an obstructive apnea-hypopnea index (AHI) greater than 2/h TST with a nadir oxygen saturation value less than 92%. PS children were defined as children with habitual snoring and an AHI less than or equal to 2/h TST, with no evidence of any alterations in arousal indices and normal gas exchange during sleep. Control children were non-snoring children that showed similar sleep finding criteria as those with PS.

[0077] A total of 120 subjects between the ages of 2 and 9 years were studied. Their demographic characteristics (**Table 2**) revealed that there were no significant differences in age, sex, ethnicity, or BMI distribution among the 3 groups, and that the frequency of asthma and allergies was also similar among the 3 groups.

Table 2. Demographic characteristics of 60 children with OSA, 30 matched children with primary snoring (PS), and 30 matched controls.

	OSA (n=60)	PS (n=30)	CO (n=30)
Mean age (years)	6.6 ± 0.7	6.7 ± 0.4	6.6 ± 0.4
Male gender (n)	32	16	16
BMI z score	1.18 ± 0.03	1.01 ± 0.04	0.98 ± 0.04
Systolic Blood Pressure (mmHg)	106.2 ± 4.1	103.2 ± 3.9	102.8 ± 3.8
Diastolic Blood pressure (mmHg)	68.9 ± 2.7	66.8 ± 2.6	65.9 ± 3.1
Ethnicity			
African American	20	10	10
White Non-Hispanic	40	20	20
Maternal educational attainment			
College or higher	38	19	20
High School or lower	22	11	10
Maternal smoking			
Yes	23	11	9
No	37	19	11
Asthma	7	4	3
Allergies	12	6	6

[0078] Furthermore, as expected from the study design, there were significant differences in several PSG measures in the OSA group, compared to the other 2 groups, but no differences between PS and CO (Table 3).

Table 3. Polysomnographic findings in 60 children with OSA, 30 matched children with primary snoring (PS), and 30 matched controls.

	OSA (n=60)	PS (n=30)	CO (n=30)
Sleep efficiency (%)	88.6 ± 6.0	91.6 ± 7.9	90.7 ± 8.8
Sleep latency (min)	17.3 ± 14.8*	24.7 ± 22.8	27.4 ± 19.9
REM latency (min)	112.7 ± 49.1	121.1 ± 38.4	124.1 ± 47.7
Stage 1 (%TST)	10.6 ± 9.2	8.3 ± 4.9	7.8 ± 5.9
Stage 2 (%TST)	48.9 ± 6.6	42.4 ± 9.6	41.3 ± 9.6
SWS (%TST)	19.6 ± 7.0*	23.7 ± 6.4	25.2 ± 7.1
REM (%TST)	22.4 ± 6.1	24.6 ± 7.1	26.6 ± 7.8
Spontaneous arousal index (/hrTST)	4.4 ± 3.4**	8.2 ± 2.8	7.5 ± 2.2
Respiratory arousal index (/hrTST)	10.4 ± 10.1***	1.8 ± 0.4	0.7 ± 0.5
AHI (/hr TST)	12.4 ± 5.6***	0.8 ± 0.4	0.4 ± 0.3
Mean SpO ₂ (%)	95.7 ± 1.9**	97.8 ± 0.7	98.6 ± 0.4
SpO ₂ nadir (%)	78.6 ± 6.3***	93.8 ± 1.9	94.8 ± 1.4

[0079] Example 2 – Differential In-Gel Electrophoresis and Mass Spectrometry Analysis of Biomarkers in Obstructive Sleep Apnea

[0080] To identify and assess peptide biomarkers for OSA, the first morning void was collected on the day following the sleep study. Urine samples (20 ml) were collected in 1 ml of protease-inhibitor cocktail (0.1 mg/ml leupeptin, 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF) and 1 mM sodium azide in 1M Tris, pH 6.8) and were stored at –80°C until analysis.

[0081] To analyze the urine samples using two-dimensional differential in-gel electrophoreses (2D-DIGE), the samples were first centrifuged at 1000 × g for 5 minutes and passed through 0.34 mm Whatman chromatography paper in order to remove cell debris and nuclei. The supernatants were then subjected to centrifugation (12,000 RPM for five minutes), and the resulting pellets were resuspended in 200 µl 2-D cell lysis buffer of the following composition: 30 mmol/L Tris-HCl, pH 8.8, containing 7 mol/L urea, 2 mol/L thiourea, and 4% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate [CHAPS]). These procedures were performed at 4°C. The protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA).

[0082] To label proteins (analytical gel), 30 µg of each of the protein samples were incubated with 0.7 µl of diluted CYDYE™ (1:5 diluted in dimethylformamide from 1 nmol/µstock, GE Health care, Piscataway, NJ) at 4°C for 30 minutes. The labeling was stopped by adding 0.7 µl of 10 mmol/L L-Lysine and incubating at 4°C for 15 minutes. The labeled samples were then mixed together, and an equal volume of 2 x 2-D sample buffer (8 mol/L urea, 4% CHAPS, 20 mg/ml dithiothreitol [DTT], 2% pharmalytes and trace amount of bromophenol blue) and 100 µl of de-streak solution (GE Healthcare) were added. The total sample volume was adjusted to 260 µl by adding rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes, and trace amount of bromophenol blue).

[0083] The samples were then incubated at room temperature for 10 minutes on a shaker and centrifuged for 10 minutes at 16,000 x g. After centrifugation, the supernatants were then loaded onto a 13 cm immobilized pH gradient strip holder (GE Healthcare). Thirteen cm immobilized pH gradient strips (pH 3 to 10) were put on the loaded samples and 1 ml of mineral oil was added. Isoelectric focusing were done following the protocol provided by the manufacturer (GE Healthcare). On completion of the isoelectric focusing, the strips were equilibrated in buffer 1 (50 mmol/L Tris-HCl, pH 8.8, containing 6 mol/L urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue, and 10 mg/ml DTT) for 15 minutes and buffer 2 (50 mmol/L Tris-HCl, pH 8.8, containing 6 mol/L urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue, and 45 mg/ml DTT) for 10 minutes with gentle agitation. The immobilized pH gradient strips were then rinsed in the SDS-gel running buffer, transferred to a SDS-polyacrylamide gel (10.5% SDS-gel prepared using low florescent glass plates) and sealed with 0.5% (w/v) agarose solution (in SDS-gel running buffer). The electrophoresis was performed at room temperature until the dye fronts ran out of the gels. Image scans were then performed immediately following the gel electrophoresis using Typhoon TRIO (Amersham BioSciences, Piscataway, NJ). The scanned images were then analyzed by Image Quant software (version 5.0, Amersham BioSciences), and subjected to in-gel analysis and cross-gel analysis using DeCyder software version 6.0 (Amersham BioSciences). The ratio change of the protein differential expression was obtained from in-gel DeCyder software analysis.

[0084] The analytical gels for the 2D-DIGE were run in duplicate with a total of 150 µg of proteins being loaded on each gel. Further, in these experiments, a total of 150 2D-DIGE gel experiments were performed so as to pair the 60 samples from OSA with either PS or CO, and also to pair CO and PS samples. In addition, 38 additional 2D-DIGE gels were performed to ascertain reproducibility and consistency of the findings.

[0085] Upon analysis of the results from these experiments, the overall number of detectable protein spots ranged from 713 to 789 in all urine samples (**FIG. 1**). In particular, analysis of differentially expressed species revealed the presence of 28 prominently differentially expressed spots, of which 23 were consistently present across all gels.

[0086] To further analyze and obtain the identity of these 23 consistent protein spots on the gels, preparatory gels loaded with a total of 500 µg of proteins along with labeled proteins were run to pick spots. Protein spots were selected based on a cut-off of a 1.5 fold change on the 2D-gels, and were picked by an Ettan Spot Picker (Amersham BioSciences) and subjected to in-gel trypsin digestion, peptide extraction, and desalting before matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MS; ABI 4700, Applied Biosystems, Foster City, CA). A GPS EXPLORER™ (Applied Biosystems) workstation was used to search the database to match both MS and MS/MS data to proteins in database. Spectral data were also converted into “.dta” files (DTA's) and searched against the IPI human non-redundant database using the Open Mass Spectrometry Search Algorithm (OMSSA; from NCBI); *e*-value cutoff was 0.01. All matched sequences were manually validated.

[0087] The analysis of the 23 consistent spots was conducted on at least 8 separate gels for each spot. Upon completion of these experiments, 12 non-redundant proteins were able to be consistently identified. In addition to these 12 non-redundant proteins, 4 additional proteins, namely gelsolin, perlecan, albumin, and immunoglobulin were also confirmed as being differentially expressed, as previously reported (16). A list of the 12 novel non-redundant proteins that were identified with high level of confidence on the basis of amino acid sequencing of 12 to 26 peptides per digested spot is presented in **Table 4**.

Table 4. Urinary Proteins Altered in OSA

Increased:	Decreased:
Uromodulin	Kallikrein 1
Urocortin III	Zinc finger protein 81
Bikunin	Zinc finger protein 36/1
Tenascin	
Human Tribbles homologue 2	
Orosomucoid 1	
Alpha 1 microglobulin	
PCAF histone acetylase	
Prolyl hydroxylases domain	

[0088] Based on the 2D-DIGE quantification approach using DECYDER™ software, 3 of these 12 proteins were significantly decreased in OSA, and the expression of the other 9 proteins was increased in OSA, as depicted in **Table 4**. There were no identifiable differences between PS and CO for these or other proteins.

[0089] Example 3 – Immunoassay Analysis of Biomarkers in Obstructive Sleep Apnea

[0090] To confirm the identity of the candidate proteins that were differentially expressed in OSA urine samples, immunoblotting, enzyme-linked immunosorbent assay, or immunoturbidity approaches were utilized, mostly using commercially available antibodies.

[0091] First, immunoblotting was used to confirm the identity of Tenascin, Tribbles homologue 2, and Zinc finger protein 81 in the urine samples. Briefly, to perform the immunoblotting assays, urine sample proteins were first centrifuged for 10 minutes at 13,000 rpm. After centrifugation, soluble protein content was measured using a protein assay kit. Thirty µg of protein from each sample were then diluted 1:1 in SDS-polyacrylamide gel electrophoresis loading buffer and electrophoresed on 12% SDS-polyacrylamide gels (Invitrogen). Proteins were then transferred to ImmobilonP membranes (Millipore) and blocked for 2 hours at room temperature with blocking buffer (4% nonfat milk powder) in TBST (20 mmol/L Tris base; 500 mmol/L NaCl; 0.05% Tween 20, pH 7.4). Protein bands were immunolocalized on transblots with commercially-available primary antibodies followed by horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibody

and developed by SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Quantification of protein bands was performed in triplicate for each sample by determination of the relative optical density (ImageJ).

[0092] As shown in FIG. 2, the western blots confirmed the increased expression in urinary proteins of Tenascin and Tribbles homologue 2, and further confirmed the decreased expression of Zinc finger protein 81 in children with OSA, as compared to healthy controls and those with PS.

[0093] Urine levels of Kallikrein 1, Urocortin III, Uromodulin and Orosomucoid 1, were then confirmed and analyzed using ELISA assays or immunoturbidimetric assays. Briefly, urine levels for Kallikrein 1 and Uromodulin were assayed using commercially available ELISA kits following the manufacturer's instructions (Human Kallikrein /PSA DuoSet, cat #DY1344, R&D Systems, Minneapolis, MN, which has a sensitivity of 0.03 ng/mL, exhibits linearity between 0.1-47.8 ng/ml, and has inter- and intra-assay coefficients of variability of 15.3.0% and 10.6%, respectively.; Human Uromodulin, MD Biosciences, cat #M036020, St Paul, MN, which has a sensitivity of 6.62 ng/mL, exhibits linearity between 6.5-625 ng/ml, and has inter- and intra-assay coefficients of variability of 12.0% and 7.6%, respectively.). For Urocortin III, an in-house ELISA assay was developed using custom-made monoclonal antibodies raised against the c-terminal epitope of the published protein sequence. In preliminary assays, the sensitivity was 0.5 pg/ml, and linearity was exhibited from 0.7-35 pg/ml, with inter- and intra-assay coefficients of variability of 14.7 and 9.6%, respectively. Finally, urinary levels of Orosomucoid 1 were analyzed using a particle-enhanced immunoturbidimetric assay as previously reported (21). Urinary creatinine level was measured for each sample, and urine levels for the various peptides were corrected for corresponding urine creatinine concentration.

[0094] Once the levels of Kallikrein 1, Urocortin III, Uromodulin, and Orosomucoid 1 were determined in the urine samples, the sensitivity and specificity for each of the biomarkers were calculated on the basis of tabulating the number of correctly classified samples. Confidence intervals (95% CI) based on exact binomial calculations were carried out in MedCalc (version 8.1.1.0, MedCalc Software, Mariakerke, Belgium). A receiver operating characteristic (ROC) plot was then obtained by plotting all sensitivity values on the y axis against their equivalent 1-specificity values on the x axis for all available thresholds (MedCalc Software). Further, the area under the curve (AUC) was evaluated, as it provided a single measure of overall accuracy that was not dependent on a particular threshold (22). The reported unadjusted *P*-values were then calculated using the natural logarithm-transformed

intensities and the Gaussian approximation to the *t*-distribution. Statistical adjustment for multiple testing was carried out by the method described by Reiner et al (23).

[0095] Upon analysis of the results from these experiments, it was observed that Kallikrein 1 urine levels were significantly reduced in the urine samples of children with OSA. Indeed, Kallikrein 1 levels in CO children were 3.32 ± 0.8 pg/ml/mg creatinine and were 3.02 ± 0.6 pg/ml/mg creatinine in children with PS, but were 0.85 ± 0.3 pg/ml/mg creatinine ($p < 0.001$) in children diagnosed with OSA. Furthermore, ROC analyses revealed that Kallikrein 1 levels of ≤ 1.54 pg/ml/mg creatinine achieved 96.7% sensitivity and 76.7% specificity to predict OSA (**FIG. 3A**).

[0096] In contrast to the levels of Kallikrein 1 peptides that were detected in children with OSA, Urocortin III urine levels were significantly increased in OSA (6.9 ± 0.4 pg/ml/mg creatinine) compared to either PS or CO (3.8 ± 0.2 pg/ml/mg creatinine; $p < 0.001$). Of note, custom developed ELISA assays for either Urocortin I or Urocortin II using commercially available antibodies showed similar levels for these proteins in the urine samples of the 3 groups, definitively confirming the high specificity of the 2D-DIGE-MS approach. Furthermore, ROC analyses revealed that Urocortin III levels of ≤ 4.4 pg/ml/mg creatinine achieved 93.7% sensitivity and 78.7% specificity to predict OSA (**FIG. 3B**).

[0097] Uromodulin peptide levels were also increased in OSA (156.4 ± 13.0 ng/ml/mg creatinine compared to 29.1 ± 6.9 ng/ml/mg creatinine in CO ($p < 0.0001$), and 37.8 ± 10.6 ng/ml/mg creatinine in PS ($p < 0.0001$ vs. OSA). Additionally, ROC analyses revealed that Uromodulin levels of > 44.6 pg/ml/mg creatinine achieved 88.3% sensitivity and 80.0% specificity to predict OSA (**FIG. 3D**).

[0098] Orosomucoid 1 levels were measured in all urine samples. PS and CO Orosomucoid 1 urine levels were similar and were therefore merged. Their mean values were 25.0 ± 1.8 pg/ml/mg creatinine compared to 67.1 ± 3.9 pg/ml/mg creatinine in individuals diagnosed with OSA ($p < 0.0001$; **FIG. 4**). In that regard, ROC analyses further revealed that Orosomucoid 1 levels of > 35 pg/ml/mg creatinine achieved 90% sensitivity and 83.3% specificity to predict OSA (**FIG. 3C**).

[0099] The ROC analyses described above revealed that the Kallikrein 1, Urocortin III, Uromodulin and Orosomucoid 1 peptides could be used independently as reliable markers for OSA. However, ROC analyses using those 4 peptides in combination revealed that if all 4 peptide biomarkers were employed, the presence of levels exceeding the cut-offs described above for two or more of the proteins yielded a 100% sensitivity and 96.5% specificity to predict OSA (**FIG. 5**). Moreover, the presence of levels exceeding the cut-offs described

above for 3 or more of the 4 peptides yielded a 95% sensitivity and 100% specificity to diagnose OSA (**FIG. 6**).

[00100] For those 4 proteins for which quantitative assessments were possible for the whole cohort, namely Kallikrein 1, Urocortin III, Uromodulin, and Orosomucoid 1, ROC analyses for each of the proteins were globally sensitive and specific in identification of OSA, and, furthermore, combinatorial approaches yielded highly predictive performances. Taken together these results thus indicate that a determination of the amounts of Kallikrein 1, Urocortin III, Uromodulin, and Orosomucoid 1 peptides, either alone or in combination, in a biological sample can be used as an effective diagnostic tool for OSA.

[00101] Throughout this document, various references are mentioned. All such references are incorporated herein by reference, including the references set forth in the following list:

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[00102] It will be understood that various details of the presently disclosed subject matter can be changed without departing from the scope of the subject matter disclosed herein. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

CLAIMS

What is claimed is:

1. A method for diagnosing obstructive sleep apnea in a subject, comprising:
 - (a) providing a biological sample from the subject;
 - (b) determining an amount in the sample of a Urocortin III peptide; and
 - (c) comparing the amount of the Urocortin III peptide in the sample, if present, to a control level of the Urocortin III peptide, wherein the subject is diagnosed as having obstructive sleep apnea if there is a measurable difference in the amount of the Urocortin III peptide in the sample as compared to the control level.
2. The method of claim 1, further comprising providing an apparatus capable of detecting the Urocortin III peptide.
3. The method of claim 1, further comprising providing a probe for selectively binding the Urocortin III peptide.
4. The method of claim 1, wherein the biological sample comprises urine, saliva, blood, plasma, or serum.
5. The method of claim 1, wherein the subject is human.
6. The method of claim 5, wherein the subject is human and under the age of about 9 to about 2 years of age.
7. The method of claim 1, wherein determining the amount in the sample of the Urocortin III peptide comprises determining the amount in the sample of the Urocortin III peptide using mass spectrometry (MS) analysis, immunoassay analysis, or both.
8. The method of claim 7, wherein the mass spectrometry (MS) analysis is selected from the group consisting of liquid-chromatography mass spectrometry (LC-MS), electrospray ionization mass spectrometry (ESI-MS), matrix-assisted laser desorption ionization time-of-

flight mass spectrometry (MALDI-TOF-MS), and surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS).

9. The method of claim 7, wherein the immunoassay analysis is selected from the group consisting of an enzyme-linked immunosorbent assay (ELISA) and an immunoturbidimetric assay.

10. The method of claim 1, further comprising determining an amount in the sample of one or more peptides selected from the group consisting of: a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide.

11. The method of claim 1, further comprising determining an amount in the sample of a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide.

12. The method of claim 1, further comprising selecting a treatment or modifying a treatment for the obstructive sleep apnea based on the determined amount of the Urocortin III peptide.

13. A method for determining whether to initiate or continue prophylaxis or treatment of obstructive sleep apnea in a subject, comprising:

- (a) providing a series of biological samples over a time period from the subject;
- (b) analyzing the series of biological samples to determine an amount in each of the biological samples of a Urocortin III peptide; and
- (c) comparing any measurable change in the amounts of the Urocortin III peptide in each of the biological samples to thereby determine whether to initiate or continue the prophylaxis or therapy of the obstructive sleep apnea.

14. The method of claim 13, wherein the biological sample comprises urine, saliva, blood, plasma, or serum.

15. The method of claim 13, wherein the subject is human.

16. The method of claim 15, wherein the subject is human and under the age of about 9 to about 2 years of age.

17. The method of claim 13, wherein determining the amount in the sample of the Urocortin III peptide comprises determining the amount in the sample of the Urocortin III peptide using mass spectrometry (MS) analysis, immunoassay analysis, or both.
18. The method of claim 17, wherein the mass spectrometry (MS) analysis is selected from the group consisting of liquid-chromatography mass spectrometry (LC-MS), electrospray ionization mass spectrometry (ESI-MS), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS).
19. The method of claim 17, wherein the immunoassay analysis is selected from the group consisting of an enzyme-linked immunosorbent assay (ELISA) and an immunoturbidimetric assay.
20. The method of claim 13, wherein the series of biological samples comprises a first biological sample collected prior to initiation of the prophylaxis or treatment for the obstructive sleep apnea and a second biological sample collected after initiation of the prophylaxis or treatment.
21. The method of claim 13, further comprising analyzing the series of biological samples to determine an amount in each of the biological samples of one or more peptides selected from the group consisting of: a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide.
22. The method of claim 13, further comprising analyzing the series of biological samples to determine an amount in each of the biological samples of a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide.
23. A kit for diagnosing obstructive sleep apnea in a subject, the kit comprising one or more antibodies capable of detecting a Urocortin III peptide in a biological sample obtained from a subject and instructions for using the kit.
24. The kit of claim 23, wherein the subject is human.

25. The kit of claim 24, wherein the subject is human and under the age of about 9 to about 2 years of age.

26. The kit of claim 23, further comprising one or more antibodies capable of detecting one or more peptides selected from the group consisting of: a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide.

27. The kit of claim 23, further comprising antibodies capable of detecting a Uromodulin peptide, an Orosomucoid 1 peptide, and a Kallikrein 1 peptide.

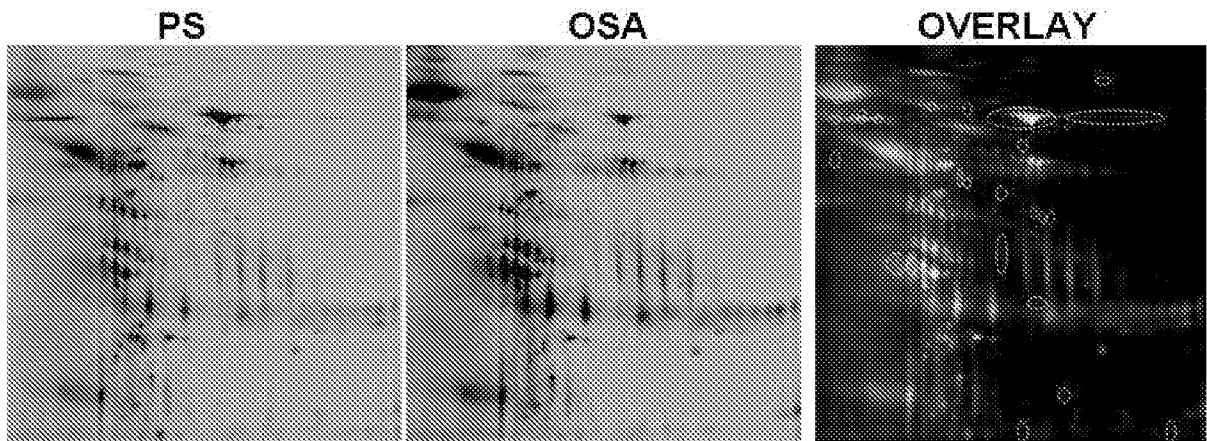


FIG. 1A

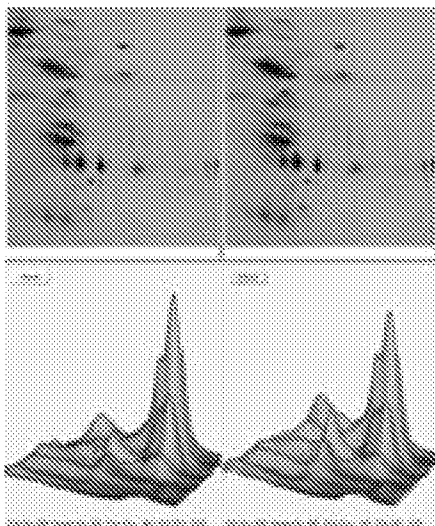


FIG. 1B

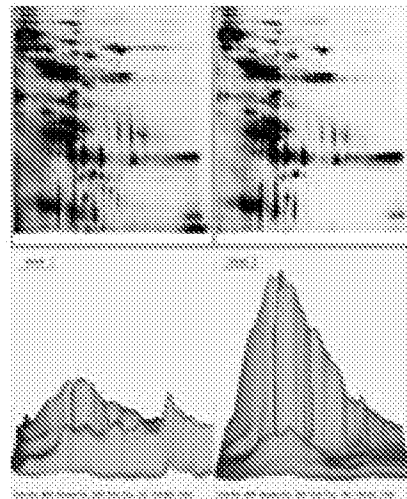


FIG. 1C

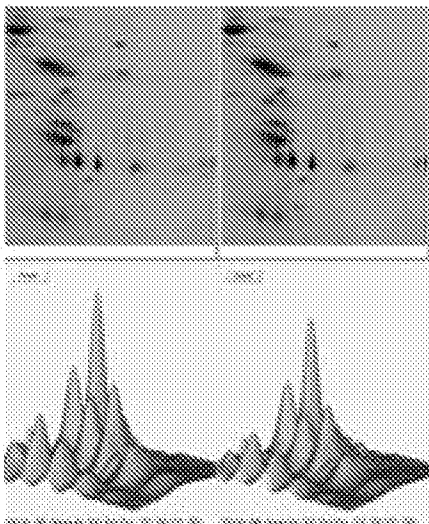


FIG. 1D

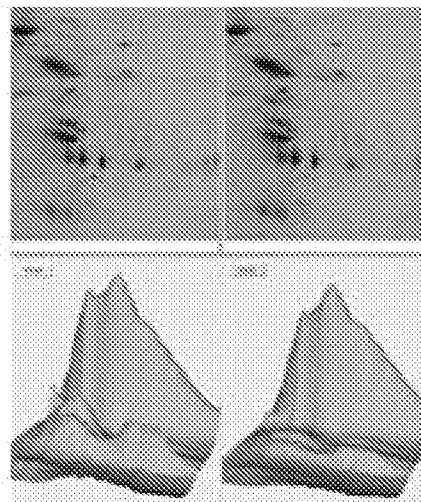


FIG. 1E

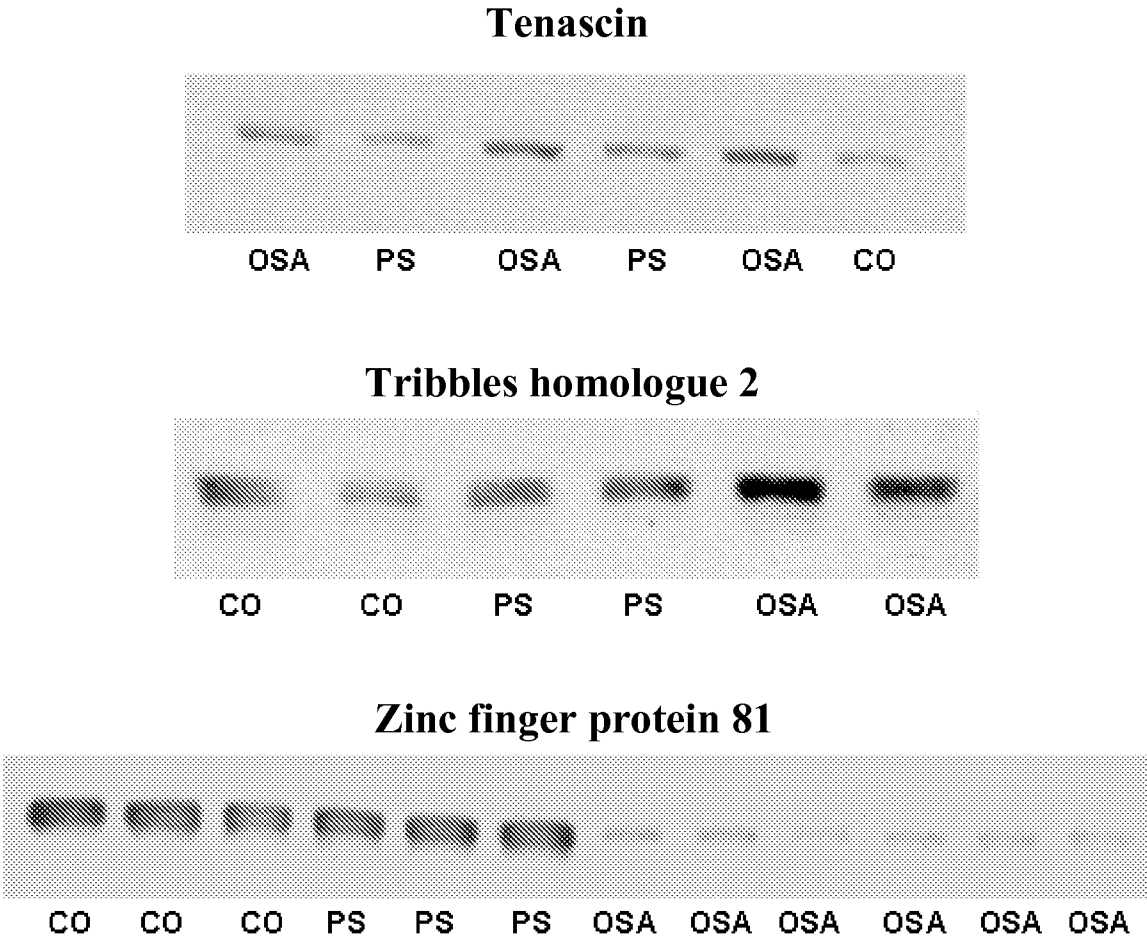
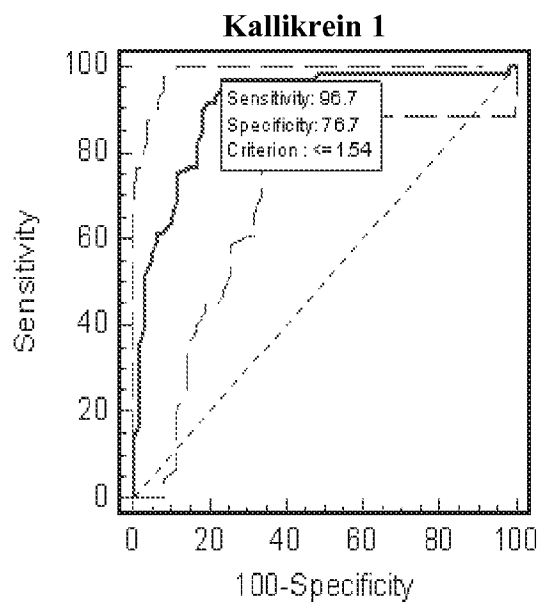
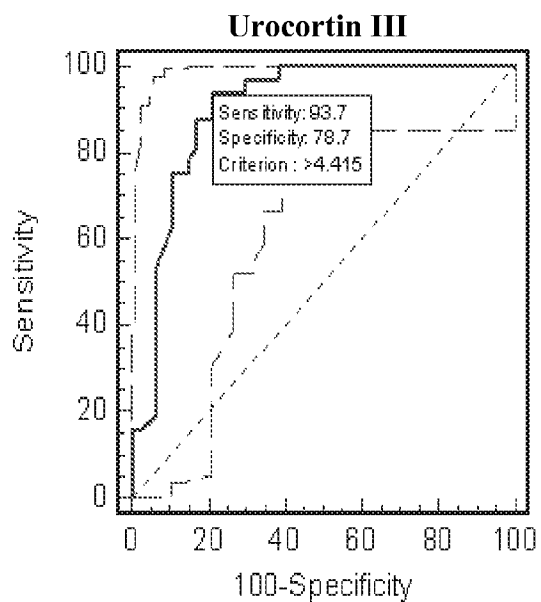
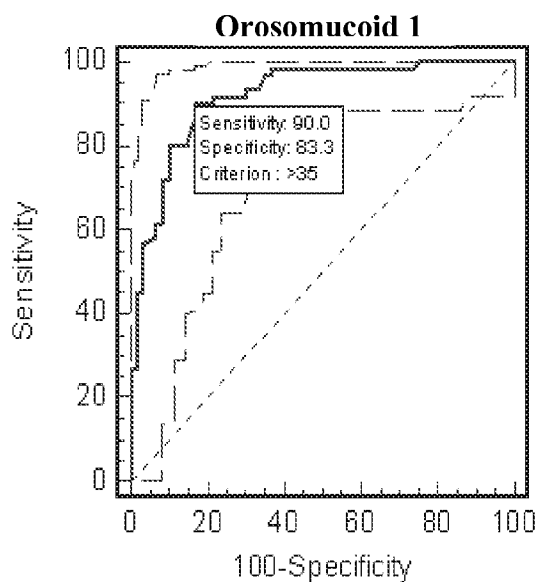
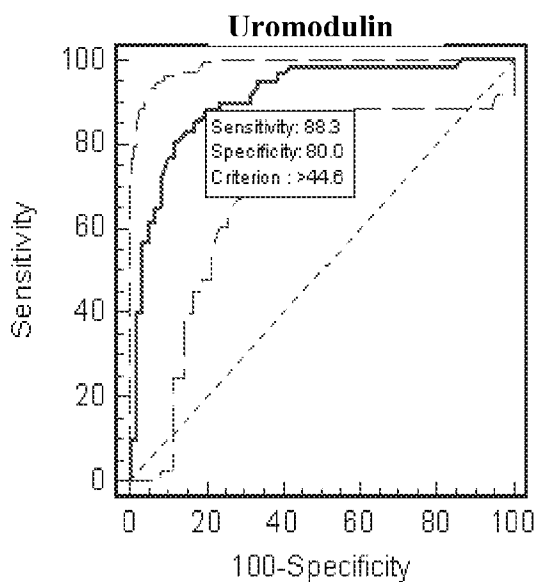


FIG. 2

**FIG. 3A****FIG. 3B****FIG. 3C****FIG. 3D**

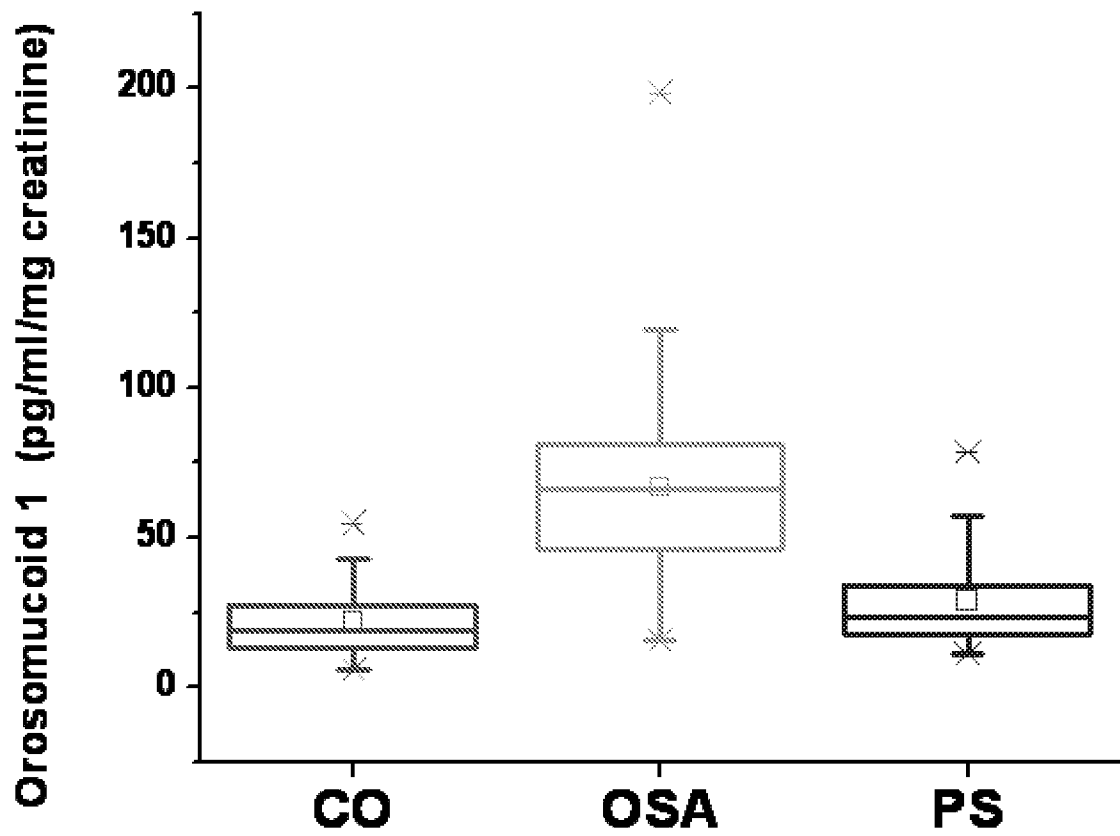


FIG. 4

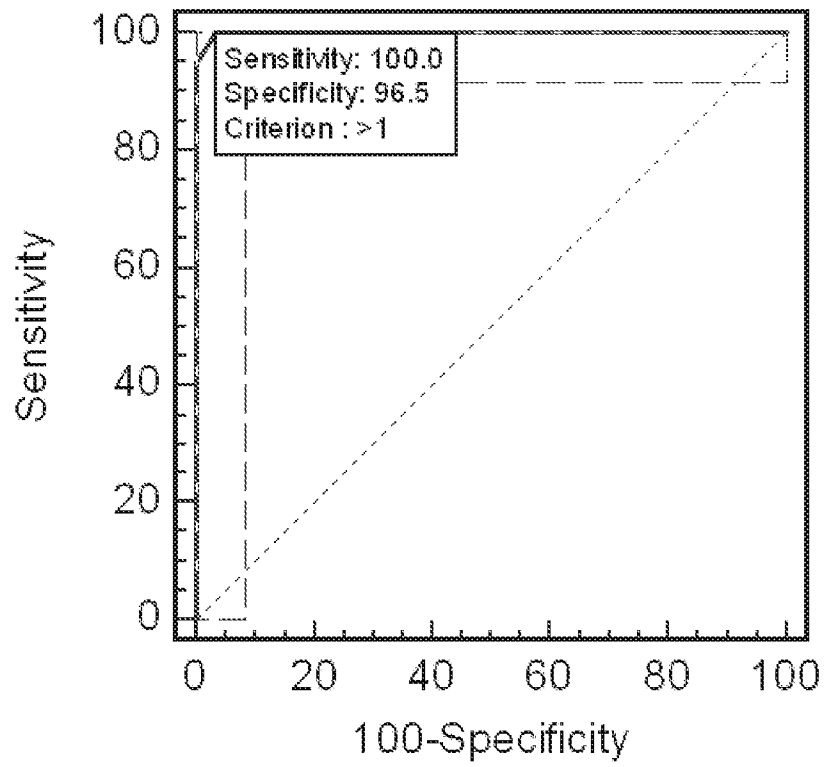


FIG. 5

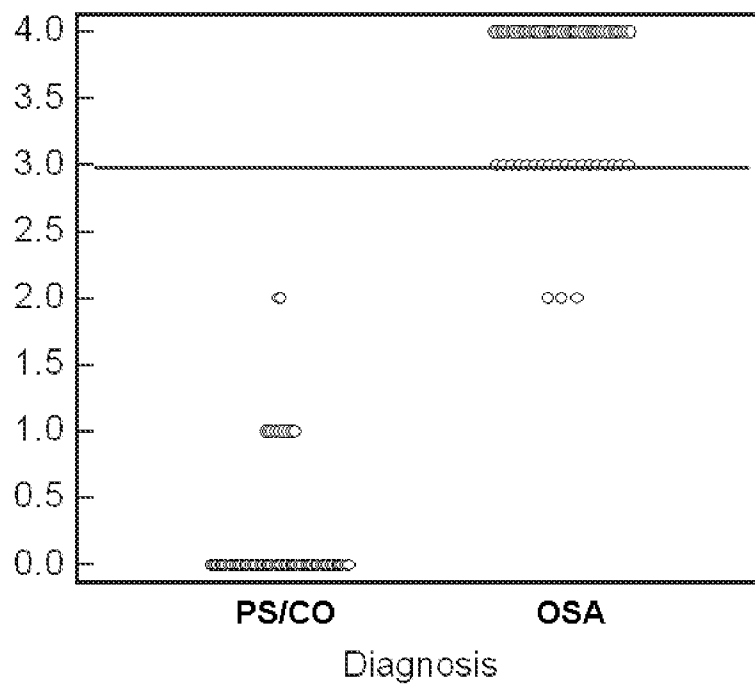


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 09/58392

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C07K 14/435; G01N 33/53 (2009.01)

USPC - 530/350, 435/7.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

USPC: 530/350, 435/7.1

IPC(8): C07K 14/435; G01N 33/53 (2009.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 436/63

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Electronic Databases Searched: PubWEST DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=NO; OP=ADJ, Google Scholar, Google Patent
Search Terms Used: apnea, sleep disorder, snor\$, urocortin, urocortin 3, urocortin III, UCN 3, UCN3, UCN-3, Kallikrein, Uromodulin, Orosomucoid, child\$, CRH, CRH receptor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 20060029980 A1 (Gozal et al.) 9 February 2006 (09.02.2006) entire documents, esp: abstract, paras [0005], [0010], [0011], [0020]-[0022], [0026], [0033], [0034], [0036] Fig. 3.	1-27
Y	US 20060293232 A1 (Levy et al.) 28 December 2006 (28.12.2006). entire document, esp: paras [0092]-[0093], [0165]-[0166], [0209], [0211], [0282], Table I.	1-27
P	Gozal et al. 2D-DIGE Proteomic Approaches Reveal Urine Candidate Biomarkers in Pediatric Obstructive Sleep Apnea. Am. J. Respir. Crit. Care Med. September 24, 2009 (abstract available at < http://ajrccm.atsjournals.org/cgi/content/abstract/200905-0765OCv1 >).	1-27
A	Lewis et al. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. April 3, 2001 PNAS 98 (13), 7570-7575 (2001).	1-27

☐ Further documents are listed in the continuation of Box C.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search

5 November 2009 (05.11.2009)

Date of mailing of the international search report

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Name and mailing address of the ISA/US

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