Serum, urine, and breath-related biomarkers in the diagnosis of obstructive sleep apnea in children: is it for real?

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Purpose of review
The scarcity of pediatric sleep laboratories has thus far precluded timely diagnosis and treatment of pediatric obstructive sleep apnea (OSA), thereby increasing the risk for residual OSA-associated morbidities. Recent developments in transcriptomics, proteomics, and exhaled condensate biomarker discovery will be reviewed in the context of exploring the validity of such methods towards development of reliable and validated diagnostic approaches for pediatric OSA.

Recent findings
Gene expression arrays have revealed significant and reproducible changes in a restricted number of genes that should enable discriminatory ability in the recognition of OSA in children. Similarly, a number of urinary proteins have been identified that display outstanding receiver–operator properties towards the diagnosis of pediatric OSA. The technological improvements in both exhaled breath online high-pressure fast chromatography and biosensor surfaces with affinity for volatile compounds should also permit noninvasive diagnosis of pediatric OSA when combined and integrated with computational methods.

Summary
It is likely that the modest efforts thus far realized in the context of biomarker discovery for the diagnosis and clinical monitoring of OSA in children will experience major acceleration in the upcoming years and lead to a completely novel paradigm in the screening and diagnosis of this disease.

Keywords
artificial nose, biomarkers, exhaled breath condensate, pediatric sleep apnea, proteomics, transcriptomics

INTRODUCTION

The relative complexity and costs associated with overnight polysomnography as the gold standard tool employed for diagnosing the vast majority of sleep disorders has spurred the quest for alternative diagnostic methods, particularly in children. Among these, special interest has centered on the discovery of biomarkers. Here, the rationale for the quest for diagnostic biomarkers and the cumulative experience garnered over the last few years in the process of identifying biomarker candidates in pediatric sleep apnea will be described, along with some cautionary measures and predictions for future directions in this field.

Obstructive sleep apnea in children: diagnostic issues

The hallmark symptom of heightened airway resistance during sleep is the presence of habitual snoring, which may affect up to 27% of all children, with a median of 10–12% [1]. Snoring is the primary symptom of sleep apnea (OSA), which manifests as the occurrence of repeated events of partial or complete upper airway obstruction during sleep, and which results in disruption of normal ventilation, hypoxemia, and sleep fragmentation. Although the pathophysiology of pediatric OSA is multifactorial, enlarged adenotonsillar tissues have emerged as the major pathophysiological

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KEY POINTS

- Pediatric obstructive sleep apnea (OSA) is currently underdiagnosed and undertreated due to relative scarcity of pediatric sleep laboratories and the high cost associated with such diagnostic methods, thereby prompting the need for high-fidelity screening and diagnostic methods.
- Unique technological advances have occurred in multiple fields such as genomics and proteomics, along with development of powerful bioinformatic and statistical analytical tools, thereby enabling unbiased exploration of biomarker platforms for diagnostic purposes.
- Transcriptomic analyses of peripheral white blood cells show initial promise for elucidation of pathogenetic mechanisms that contribute to the development of pediatric OSA and its associated morbidities.
- Exploration of the deep urinary proteome in children should open the way for development of diagnostic kits using combinatorial biomarker approaches of utility in the clinical evaluation of the habitually snoring child.
- The transformational progress accomplished in recent years in the assessment of exhaled breath should allow for pragmatic implementation of noninvasive screening modalities using this approach in snoring children.

mechanism underlying OSA in children, with obesity rapidly emerging as the second most important contributor [2]. Pediatric OSA has been associated with increased risk for behavioral and mood disturbances and learning deficits, cardiovascular complications such as pulmonary hypertension, systemic hypertension, endothelial dysfunction, metabolic disturbances including insulin resistance and serum lipid alterations, enuresis, and compromised somatic growth [3]. The majority of habitually snoring children have primary snoring, defined as habitual snoring without disruption in sleep architecture, or alterations in alveolar ventilation and oxygenation. On the other hand, OSA is currently viewed as a disease in which symptoms associated with recurrent episodes of gas exchange abnormalities and repeated arousals are present, and increase the risk for associated morbidities. Although agreement is still lacking on the cut-off criteria for what constitutes the demarcation between primary snoring, that is, no disease, and OSA, that is, disease, it has been estimated that OSA affects 2–4% of all 2–8 year-old children [1]. Furthermore, the prevalence in infants and older children remains virtually unexplored. Thus, of every 100 habitually snoring children, one in four to six children will have OSA, and the major question then becomes, which one of these snoring children has OSA and which does not.

Unfortunately, complaints of snoring, fatigue and somnolence, diminished cognitive performance, behavioral problems, or headaches by nonapneic habitually snoring children will not reliably discriminate them from those children in whom OSA is present [4–8]. Consequently, efforts to diagnose OSA while relying on clinical symptoms and physical examination have consistently underperformed, and led to the conclusion that such approaches are indeed unable to reliably identify pediatric patients with clinically relevant OSA [9,10]. Accordingly, despite its multiple flaws and limitations, the overnight polysomnogram-derived apnea hypopnea index (AHI) has been selected and has thus far served as the key discriminator between primary snoring and OSA in both research and clinical settings, even when overarching agreement as to the OSA-defining AHI is lacking. Consequently, it should not come as a surprise that only a minority of children being evaluated for habitual snoring in Ear Nose and Throat clinics undergo overnight polysomnography [11].

In 2002, the American Academy of Pediatrics published clinical practice guidelines for the diagnosis and management of pediatric OSA [12], and since then overnight polysomnographic (PSG) assessment has been viewed as mandatory for the definitive diagnosis of OSA in children, particularly since clinical history and physical examination are not only insufficient to confirm its presence, but are also markedly inept at determining OSA severity [9,13]. Furthermore, the attribution of 'gold standard' status to the PSG was further consolidated when comparisons with abbreviated tests such as overnight oximetry, ECG recordings, nocturnal videotaping, or respiratory multichannel recordings have been found as having substantial limitations as either screening or diagnostic methods [14–25]. Unfortunately, overnight sleep studies are onerous, labor-intensive, impose substantial discomfort on the children and their families, and are relatively inaccessible to children, such that waiting times between referral for evaluation to diagnosis commonly take 5–6 months across the United States and around the world. Therefore, development of simple, cheap, and reliable diagnostic tools that permit more expanded screening of at-risk populations, and enable accurate identification of the children with definitive disease or with definitive absence of disease would revolutionize the field and provide timely access to clinical care to a large sector of the pediatric population, thereby reducing the health burden of OSA.

BIOMARKERS

A working definition of biomarkers in this context is essential before addressing the question of whether alternative diagnostic methods that utilize these indicators are possible for pediatric OSA. I will here define a biomarker as a 'biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease'. A biomarker may therefore be used to determine whether a disease is present or absent, or to delineate how well the patient responds to a treatment for a disease or condition. Of note, a biomarker may also be termed as 'molecular marker and signature molecule'.

Five phases have been proposed by the National Institutes of Health (NIH) 'Early Detection Research Network' in the process of evaluating biomarkers [26,27]. Phase 1 includes exploratory studies in which biomarkers are discovered through knowledge-based profiling (genes or proteins) to distinguish between diseased and normal samples. The analysis of this phase is usually characterized by ranking and selection, or by finding suitable ways to combine biomarkers. Phase II has two important components, namely development of an assay and validation for reproducibility and portability to other laboratories. In Phase III, the sensitivity and specificity of the test are appraised in a cohort of individuals who have not yet been diagnosed, a time consuming and usually onerous process, as blinded phenotyping of the cohort is needed in order to properly evaluate the biomarker panel. Phase IV should then evaluate the sensitivity and specificity of the test in a prospective cohort, in order to ascertain the false referral rate for treatment based on the tested biomarkers. Finally, Phase V evaluates the overall benefits and risks of the new diagnostic test on the screened population. In general, an ideal set of biomarkers should be safe, easy, inexpensive to measure, should track the success or failure of therapy, and should be consistent across sexes and ethnic groups. Furthermore, it should be sensitive and specific and have a high predictive value, which can be assessed using the diagnostic odds ratio (DOR) [DOR = (sensitivity/1 – specificity)/(1 – sensitivity/specificity)], as well as by the negative and positive likelihood ratios. It is important to emphasize that most biological markers have wide ranges of values that overlap between persons with and without disease, such that cut-off points must be critically appraised for their ability to accurately distinguish disease [28].

Approaches to diagnostic biomarker discovery using readily accessible human samples include blood, urine, and exhaled condensates. The main technologies aiming to uncover such biomarkers include transcriptomics, proteomics, and metabolomics. Although, a thorough description of each of these methodologies is beyond the scope of this article, I will provide brief summaries of the available published work on the first two of these potentially fruitful areas of investigation.

TRANSCRIPTOMICS

Unlike genome-wide association studies that aim at gaining insights into the genetic variability operating on and contributing to the expression of disease phenotypes, gene expression profiling can often be performed using relatively small cohorts, and may therefore be a viable approach for exploration of gene-related markers in pediatric sleep disorders. Thus far, the vast majority of the published transcriptomic studies on sleep-disordered breathing have been conducted in animal models, including gene expression patterns during sleep and sleep deprivation [29,30], and have also focused on the transcriptional effects of intermittent hypoxia or sleep fragmentation in heart, adipose tissues, and pluripotent stem cells [31–33,34▪,35▪▪]. Although these studies provide invaluable information regarding the integrated gene expression changes associated with specific perturbations that mimic selected components of OSA, the clinical relevance of such findings remains elusive with respect to human disease. As part of our efforts to translate these approaches to pediatric OSA, Khalyfa *et al.* [36] explored the genome-wide expression changes occurring in peripheral blood leukocytes of 20 nonobese children with OSA and 20 matched controls. Several differentially expressed genes and pathways emerged in children with OSA, and of particular relevance was the fact that a great proportion of the differentially expressed genes mapped to inflammatory processes. Consequently, these findings provide additional confirmation of the ubiquitous and dose-dependent activation of inflammatory markers present in the plasma of children with OSA as had been originally identified several years previously [37–41,42▪▪]. In an effort to further refine potential diagnostic cut-off values for the PSG-based AHI, we assessed the peripheral blood leukocyte transcriptome of children with primary snoring (i.e., AHI <2 and habitual snoring) versus matched control, nonsnoring children (AHI <1 and no snoring), and found subtle, albeit significant changes in transcriptional programs involved in metabolic pathways such as insulin signaling and adipocyte differentiation, which were further confirmed in an expanded cohort by measuring plasma-based evidence of insulin resistance [43]. The ability to employ

transcriptomic strategies in the development of a diagnostic algorithm was explored and deemed as a promising initial approach (Fig. 1; [44]). There is no doubt that advances in computational data mining and the discovery of disease-modifying biological targets may provide further refinements in the development of diagnostic or therapeutic algorithms for pediatric OSA. Indeed, we recently employed such approaches in a microarray-based study on the causes of adenotonsillar hypertrophy in children with OSA [45]. In this study, integration of whole-genome expression profiling of tonsillar tissues was combined with topographic analysis of genetic networks, thereby allowing the identification and validation of high-priority therapeutic targets that orchestrate the tonsillar and adenoidal hypertrophic response in OSA.

PROTEOMICS

Proteomics refers to the assessment of the comprehensive repertoire of proteins, including the various posttranslational modifications they undergo, as may be found in biological samples. The fundamental assumption posits that the effects of a disease process on the proteome may identify candidates with unique functional or pathophysiological relevance. It is noteworthy that the human proteome is comprised of hundreds of thousands of distinct molecules, as opposed to the 'relative' simplicity of the genome. Such proteomic complexity is the result of a vast array of posttranscriptional (e.g., RNA splicing) and posttranslational modifications (e.g., proteolytic cleavage, glycosylation, phosphorylation, sulfation, nitrosylation) that generate a multiplicity of distinct proteins that all map to a single gene.

The most important and versatile instrument currently used for large-scale proteomics is the mass spectrometer, an instrument that measures mass-to-charge ratio characteristics of proteins and peptides, leading to their correct identification [46]. In this context, the application of shotgun proteomics using several methodological strategies has fostered a unique explosion of our understanding of the deep proteome while enabling quantitative assessment of differential protein expression between biological samples [47,48]. Moreover, alternative proteomic

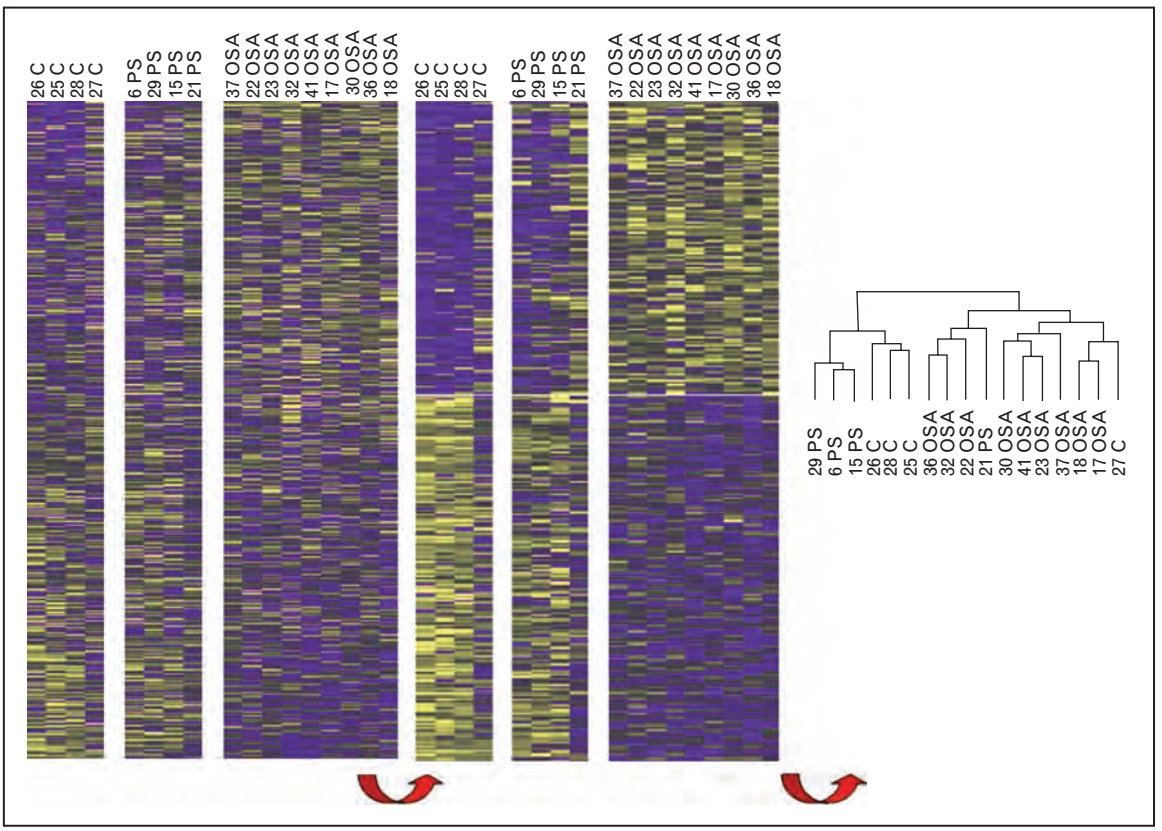

FIGURE 1. Infogram of whole genome gene expression arrays before (left panel) and after (mid panel) computationally driven rearrangements to illustrate differences in gene expression between children with obstructive sleep apnea as compared to either matched controls or children with primary snoring. Hierarchical clustering analyses (right panel) further permitted near-perfect assignment to the correct diagnostic category based on the gene expression patterns.

techniques that combine bidimensional electrophoresis (2D-PAGE) and mass spectrometry have already yielded important insights into disease processes. More recently, specific sets of practical guidelines towards exploration of specific human-derived biological samples have been developed [49].

Considering the technological and bioinformatic challenges posed by the application of proteomic analyses to the discovery of biomarkers in human disease, it should not come as a surprise, that only a very small number of studies have been published using proteomics to investigate sleep disorders. In the first attempt to explore the presence of diagnostic biomarkers in pediatric OSA, Shah *et al.* [50] identified three differentially expressed proteins that were associated with OSA. In this study, they compared the relative abundance of various proteins in sera obtained from 20 children with polysomnographically diagnosed OSA and from 20 children, who were habitual snorers, but in whom sleep studies did not find evidence of gas exchange alterations. However, although three proteins displayed relatively favorable predictive values, the earlier methodologies employed in this study did not allow for accurate identification of the proteins. Consequently, the utility of these unidentified putative biomarkers could not be confirmed using the subsequent phases of biomarker discovery described above. In a similar preliminary study by Jurado-Gamez *et al.*, [51▪] three proteins were identified that demonstrated increased expression in the serum of adult patients with OSA, whereas seven other proteins showed reduced levels when compared with controls. Taken together, these studies suggest that further efforts using proteomic approaches are justified. More recently, we employed iTRAQ techniques (isobaric tags for relative and absolute quantification) in serum samples from adults with varying severity of OSA, and found that protein expression is differentially regulated according to disease severity, and appears to involve lipid and vascular metabolic pathways [52▪▪]. Preliminary and encouraging findings in urinary samples obtained from a small sample of children [53] redirected some of our efforts at proteomic biomarker discovery to this easily obtained biological material. In a subsequent and more comprehensive study, Gozal *et al.* [54] examined the urine proteome of children with OSA ($n = 60$) versus patients with primary snoring ($n = 30$), and controls ($n = 30$) using two-dimensional gel electrophoresis. Despite the relatively important limitations associated with gel-based approaches, application of fluorescent dye labeling of proteins permitted semi-quantitative assessment of relative protein abundances between samples. This strategy improved the detection of the total number of proteins in the urine samples and identified 16 unique proteins that were differentially expressed in the OSA group. ELISA validation subsequently identified four of these proteins (kallikrein-1, urocortin-3, orosomucoid-1 and uromodulin). Using combinatorial biomarker discriminative approaches, the presence of OSA could be identified posthoc with a sensitivity of 95% and specificity of 100%. Clearly, these exciting findings must be validated in larger independent cohorts, and such efforts are currently underway. However, there is no doubt that proteomic-based technologies offer unique possibilities for the development of screening and diagnostic strategies that should be clinically applicable to the evaluation and treatment of adult and pediatric sleep apnea.

EXHALED CONDENSATE AND ARTIFICIAL NOSES

An artificial nose is defined as a device that simulates the human olfactory system so as to detect various volatile compounds. In the olfactory system, neurons specifically respond to odours by means of appropriate receptors. Any given single olfactory neuron will respond to several different odours, following which pattern recognition processes generated by combining the responses of multiple olfactory neurons will enable accurate identification of specific odours [55,56]. Similarly, electronic noses are based on the analysis of volatile compounds using a semi-selective array of electronic sensors, resulting in the recognition of specific odour patterns [57]. The combination of chemical sensor arrays, pattern recognition algorithms, and complex statistical modeling has created an electronic nose technology that results in signatures that can be reliably grouped based on the tightness of clustering. Electronic nose technology is therefore a potentially promising approach in clinical medicine, particularly since electronic nose devices are portable, testing is noninvasive, and results are obtained in almost real time.

In parallel with the development of artificial nose technologies, it became apparent that analysis of exhaled breath provides a noninvasive approach to investigate airway inflammation, which is particularly attractive in children. For example, use of the fractional exhaled nitric oxide concentration has now been extensively applied to inflammatory lung diseases such as asthma, and other pertinent markers have since been investigated [58▪▪]. Not surprisingly, similar approaches have been explored, albeit in a limited fashion, in the context

of pediatric and adult OSA. These investigations have revealed the presence of increased inflammatory markers in patients with OSA [59–63], and severity-dependent levels of inflammatory markers were reported in exhaled breath condensate samples of both children and adults with OSA [64–69, 70▪▪,71]. Furthermore, some of these markers have been proposed as reliable tools for either OSA diagnosis or for monitoring the effect of treatment [72,73]. Detection of volatile compounds in exhaled breath represents an exciting technique that may provide noninvasive and potentially cost effective means for screening, diagnosis and monitoring of treatment outcomes in OSA. The more recent development of on-line identification and quantitation of volatile compounds in exhaled breath through the use of ultra-fast gas chromatography further presents exciting prospects for the field of biomarker discovery in the context of sleep disorders.

CONCLUSION

The exponential acceleration in technological breakthroughs within the biomedical sciences has led to a revolution in the way we approach and think about diseases, including their diagnosis and treatment. The ability to comprehensively interrogate complex biological systems along with major advances in computing power and statistical methods have provided us with unique tools that enable the development of applications that can be translated to the bedside. I anticipate that the modest progress thus far accomplished in the context of sleep disorders in general, and of OSA in particular, will tremendously accelerate in the upcoming years while using these innovative and exciting techniques. Such efforts will undoubtedly lead to unprecedented resolution, accuracy, and ultimately to field validation and commercialization of OSA-related biomarkers. As we edge closer to achieving these goals, the prospects for understanding the pathophysiology of sleep disordered breathing, and for devising logical algorithms aimed at screening, diagnostic and therapeutic interventions appear very promising.

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Conflicts of interest

D.G. is also supported by an investigator-initiated unrestricted grant from ResMed on urine biomarkers in adult sleep apnea, and serves a scientific consultant for Gallleon Pharmaceuticals in issues related to respiratory control. The author has no additional conflicts of interest to declare.

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- ▪ of special interest
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