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Circulating Exosomes in Obstructive Sleep Apnea as Phenotypic Biomarkers and Mechanistic Messengers of End-Organ Morbidity

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Abstract

Obstructive sleep apnea (OSA), the most severe form of sleep disordered breathing, is characterized by intermittent hypoxia during sleep (IH), sleep fragmentation, and episodic hypercapnia. OSA is associated with increased risk for morbidity and mortality affecting cardiovascular, metabolic, and neurocognitive systems, and more recently with non-alcoholic fatty liver disease (NAFLD) and cancer-related deaths. Substantial variability in OSA outcomes suggests that genetically-determined and environmental and lifestyle factors affect the phenotypic susceptibility to OSA. Furthermore, OSA and obesity often co-exist and manifest activation of shared molecular end-organ injury mechanisms that if properly identified may represent potential therapeutic targets. A challenge in the development of non-invasive diagnostic assays in body fluids is the ability to identify clinically relevant biomarkers. Circulating extracellular vesicles (EVs) include a heterogeneous population of vesicular structures including exosomes, prostasomes, microvesicles (MVs), ectosomes and oncosomes, and are classified based on their size, shape and membrane surface composition. Of these, exosomes (30–100 nm) are very small membrane vesicles derived from multi-vesicular bodies or from the plasma membrane and play important roles in mediating cell-cell communication via cargo that includes lipids, proteins, mRNAs, miRNAs and DNA. We have recently identified a unique cluster of exosomal miRNAs in both humans and rodents exposed to intermittent hypoxia as well as in patients with OSA with divergent morbid phenotypes. Here we summarize such recent findings, and will focus on exosomal miRNAs in both adult and children which mediate intercellular communication relevant to OSA and endothelial dysfunction, and their potential value as diagnostic and prognostic biomarkers.

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Sleep Disordered Breathing

Sleep-disordered breathing (SDB) is a general term referring to the presence of a spectrum of conditions in which partial or complete cessation of airflow along with central or obstructed breathing occurs many times throughout the night. In this setting, obstructive sleep apnea (OSA) is caused by the increased upper airway collapsibility along with insufficiency or loss of upper airway dilating muscle capacity promoting repeated pharyngeal narrowing (hypopnea) or closure (apnea), thereby leading to declines in oxyhemoglobin saturation and increases in the partial pressure of carbon dioxide in arterial blood (Jordan et al., 2014). To restore pharyngeal patency, patients have recurrent arousals from sleep, resulting in sleep fragmentation which in turn leads to daytime sleepiness and reduced quality of life. Moreover, the chronic cycles of hypoxia-re-oxygenation, i.e., intermittent hypoxia (IH), induce oxidative stress (ROS) and promote activation of inflammatory pathways (Lavie, 2003). In addition to fluctuations in oxygenation (IH), sleep fragmentation (SF), and episodic oscillations in carbon dioxide tension, the intermittent changes in upper airway resistance during sleep in OSA patients foster other physiological disturbances including increased intrathoracic pressure swings, altered acid-base status, and disruptions in autonomic function and behavioral state (Dempsey et al., 2010).

Obstructive sleep apnea (OSA), is considered by far the most common form of sleep-disordered breathing, is highly prevalent across the lifespan, and is associated with a myriad of adverse health consequences independent of other traditional cardio-metabolic risk factors and obesity, including an increased risk of death (Al Lawati et al., 2009; Caples et al., 2007; Tahrani et al., 2013; Young et al., 2008). SDB and more specifically OSA have been associated with a large spectrum of neurocognitive, behavioral, cardiovascular, and metabolic adverse consequences, which appear to be particularly prominent in obese patients (Amadeo et al., 2017; Floras, 2014; Luz Alonso-Alvarez et al., 2011; Marcus et al., 2012; Sforza and Roche, 2016), while also differentially affecting both genders and varying in severity according to chronological age (Mokhlesi et al., 2016).

A large body of evidence has clearly demonstrated that chronic IH is critically involved in the short-term and long-term cardiovascular consequences of OSA, including systemic hypertension, left ventricular hypertrophy, and endothelial dysfunction (Alchanatis et al., 2002; Ameli et al., 2007; Amin et al., 2002; Brooks et al., 1997; Lesske et al., 1997; Varadharaj et al., 2015) (Marin et al., 2005; Mason et al., 2012). Indeed, recent studies have shown clear and robust associations of OSA with the development of hypertension (Nieto et al., 2000; Peppard et al., 2000; Phillips and O'Driscoll, 2013; Ren et al., 2016), type II diabetes (Lai et al., 2016; Plihalova et al., 2016; Reichmuth et al., 2005), stroke (Arzt et al., 2005; Campos-Rodriguez et al., 2014; Ifergane et al., 2016), congestive heart failure (Cowie, 2016; Pearse and Cowie, 2016; Shahar et al., 2001), coronary artery disease (Loo et al., 2014; Marin et al., 2012; Selim et al., 2010), cardiac arrhythmias (Vizzardi et al., 2014), and cancer (Campos-Rodriguez et al., 2013).

Despite the high prevalence and important morbidity load (Figure 1), OSA remains significantly under recognized, and it is estimated that only 40% of those with OSA have been diagnosed (Banno et al., 2009), with the medical costs of untreated OSA in the United

States being estimated at US\$ 4 billion/year (Kapur et al., 1999; Young et al., 1997). The total economic impact of OSA is even greater than direct medical costs, in light of the sleepiness-associated work and motor vehicle accident risks, increased job productivity losses, and social disability needs (Hillman et al., 2006).

OSA in children is also a highly prevalent disorder that can result in significant morbidity, and is primarily associated with adenotonsillar hypertrophy (Capdevila et al., 2008; Lumeng and Chervin, 2008). The prominent increases in childhood overweight and obesity rates in the globe even among youngest of children have further translated into parallel increases in the prevalence of OSA, and such trends are undoubtedly associated with deleterious global health outcomes and life expectancy (Bixler et al., 2009; Kheirandish-Gozal and Gozal, 2012; Li et al., 2010; Marcus et al., 2012).

As mentioned above, OSA in both adults and children is not only a prevalent condition but one that is clearly associated with multisystem involvement. However, evidence of morbidity is not a universal finding in patients with OSA. It also remains unclear whether those patients with evidence of one end-organ morbidity are at increased risk for developing another (Mokhlesi and Gozal, 2010). Chronic IH can lead to increased pro-inflammatory cytokine production, endothelial dysfunction, oxidative stress, metabolic dysregulation and insulin resistance (Christou et al., 2003; Ciftci et al., 2004). Several oxidative stress markers are increased in OSAS, and could amplify inflammatory cascades, induce endothelial dysfunction and development of atherosclerosis and promote central nervous system dysfunction (Lavie, 2003; Lavie, 2015; Wang et al., 2010; Zhang et al., 2012; Zhou et al., 2016). However the determinants of injury in any given patient with OSA are not well delineated, and in addition to disease severity and concurrent presence of other underlying conditions such as obesity, they are likely to involve both genetic and environmental contributions (Bhattacharjee et al., 2011; Kheirandish-Gozal and Gozal, 2013; Tan et al., 2014).

Obesity

Obesity has rapidly evolved into a global pandemic, and poses a significant healthcare and socioeconomic burden in both developed and developing countries. Obesity is a multifactorial disease that is often accompanied by other health problems, such as hypertension, dyslipidemia, fatty liver disease, diabetes, polycystic ovary syndrome, sleep-disordered breathing (SDB), psychosocial problems and other associated issues (Estrada et al., 2014; Haslam and James, 2005; James et al., 2004). Obesity affects one of every three individuals in the US with the incremental cost of healthcare related to obesity alone being expected to increase by over US \$500 billion by the year 2030 if the current trends are sustained (Finkelstein et al., 2012). According to the National Institutes of Diabetes and Digestive and Kidney Diseases, approximately two-thirds of American adults are either overweight or obese. Obesity in children is a primary risk factor for high blood pressure (BP) and hypertension, and these conditions associate in 13% of cases; moreover, children with hypertension have an increased risk of becoming hypertensive adults (Freedman et al., 2001). Furthermore, when comparing obese with non-obese children who were followed up

for a 22-year period, the presence of obesity alone independently predicted the long-term risks of diabetes or adulthood obesity (Liang et al., 2015).

It is well established that obesity plays an important role in the development of OSA in both adults and children (Crummy et al., 2008; Dayyat et al., 2009; Romero-Corral et al., 2010), such that interactions between these 2 conditions facilitate and amplify the risk for end-organ morbidities associated with OSA (Bahrami et al., 2008; Bhattacharjee et al., 2012; Bhattacharjee et al., 2011; Spruyt and Gozal, 2012), with multiple pathways being potentially involved.

Experimental Human Intermittent hypoxia

To investigate the separate contributions of IH and SF to end-organ dysfunction, several experimental IH protocols have been developed (Brugniaux et al., 2011; Champod et al., 2013; Gilmartin et al., 2010; Mateika et al., 2015; Navarrete-Opazo and Mitchell, 2014; Querido et al., 2012; Tamsier et al., 2011; Taylor et al., 2014; Tremblay et al., 2016; Weiss et al., 2015; Xing et al., 2014; Zhang et al., 2014). Studies in healthy human subjects have shown that sleep disruption and intermittent hypoxia can each decrease insulin sensitivity and worsen glucose tolerance. For example, healthy adult human subjects exposed to IH for relatively short durations manifest increase in their circulating plasma glucose levels (Louis and Punjabi, 2009; Newhouse et al., 2017). Similarly, Beaudin et al. (2014) exposed young healthy adults to IH for 4 days, and showed the presence of IH-induced perturbations in endothelial function that are reversible upon termination of the IH exposures (Beaudin et al., 2014; Champod et al., 2013; Foster et al., 2009; Gajos-Michniewicz et al., 2014). Longer exposures up to 4 weeks have resulted in the emergence of systemic elevations in blood pressure (Gilmartin et al., 2010; Tamsier et al., 2011).

Intermittent Hypoxia Exposures in Animals

Experimental IH patterns utilized in most of the animal-based studies have aimed to replicate OSA cyclical changes in oxygenation. Nevertheless, each laboratory has adapted a slightly different IH protocol such that cycle frequency (from seconds to few minutes), overall duration, $F_{I}O_2$ kinetics and magnitude, and restriction of IH to either the sleep or rest periods have led to huge variability and precluded direct comparisons across studies (Almendros et al., 2014b; Quintero et al., 2013). Notwithstanding the wide repertoire of IH-exposure paradigms, the overall findings have recapitulated the majority of the previously uncovered associations between OSA and end-organ morbidities. Indeed, animal experiments support for example the notion that IH exposures entail reductions in systemic and adipose tissue insulin sensitivity (Carreras et al., 2015; Gozal et al., 2017; Iiyori et al., 2007), facilitate emergence of dyslipidemia with elevations in serum total cholesterol and triglyceride levels that correlates with the degree of hypoxemia (Li et al., 2007), and promote the development and progression of atherosclerosis via multiple mechanistic pathways (Arnaud et al., 2011; Castro-Grattoni et al., 2016; Drager et al., 2012; Drager et al., 2013; Fang et al., 2012; Gautier-Veyret et al., 2013; Gemel et al., 2017; Gileles-Hillel et al., 2014; Jun et al., 2010; Li et al., 2011; Poulain et al., 2014; Tuleta et al., 2014; Wei et al., 2016). Furthermore, longer exposures to IH which reflect the marked delays that normally

occur in patients with OSA between onset of their disease and diagnosis and treatment may result in partially irreversible morbidities (Cortese et al., 2017; Gileles-Hillel et al., 2016). The impact of age at which IH exposures occur has also been preliminarily explored. For example, neonatal mice exposed to IH for 4 weeks had increased risk for cardiovascular disease during early adulthood, manifesting as systolic hypertension, altered baroreflex responses, and reduced heart rate variability indicative of altered balance in sympathetic-parasympathetic autonomic nervous system control, implicating epigenetic re-programming (Chu et al., 2015). Similarly, early life exposures to IH result in persistent cognitive deficits and behavioral perturbations (Cai et al., 2012; Kheirandish et al., 2005).

Gestational Intermittent Hypoxia

The Developmental Origins of Health and Disease theory suggests that there are critical periods during perinatal life in which maternal nutrition and other environmental factors can program changes that will affect offspring, and influence its susceptibility to metabolic disease during adulthood (Blackmore and Ozanne, 2013). However, the mechanisms by which early environmental insults can impose long-term effects on offspring through epigenetic modifications remain poorly defined (Reynolds et al., 2015). Although several studies have evaluated the association between OSA symptoms in pregnancy and adverse fetal outcomes, all of these studies were specifically circumscribed to the immediate postnatal period, and did not explore long-term consequences of OSA during gestation on the offspring (Bourjeily et al., 2013; Xu et al., 2014). Pregnancy has been associated with several alterations in sleep patterns and a high incidence of sleep disturbances (Facco et al., 2012; Pien and Schwab, 2004).

IH during late gestation appears to adversely impact the exposed offspring and manifest as metabolic syndrome during adulthood (Khalyfa et al., 2017). Indeed, we found that adult male, but not female offspring of pregnant mice that were exposed to late gestational IH developed body weight and food consumption, along with reduced daily energy expenditure, increased adiposity index, increased insulin resistance and systemic elevations of lipid levels, as well as altered macrophage populations and changes in DNA methylation patterns in visceral adipose tissues. Accordingly, gestational IH leads to a comprehensive array of adverse epigenetic and metabolic consequences associated with OSA during pregnancy in the offspring, and further provide a strong argument to identify the disease during pregnancy and also detect infants at risk for future morbidities.

Intercellular Interactions in Intermittent Hypoxia

As discussed above, there is consistent evidence from animal models and clinical studies suggesting that OSA enhances oxidative and inflammatory processes and leads to CNS, metabolic, autonomic, vascular, and cardiac dysfunction. The existence of a complex network of well-organized interactions between different cell populations that play a pivotal roles in several pathophysiological processes prompts the formulation of a conceptual framework whereby, similar to other disease states, direct cell-to-cell contact, cell-matrix interactions, and extracellular electric, chemical, or biological signals should promote the amplification and expansion of pathological consequences of IH (Sheikh et al., 2009; Tirziu

et al., 2010). In this context, discovery of integrative signature responses facilitating the detection of populations at risk of morbidities associated with OSA or identifying new therapeutic targets would be of great interest, particularly considering the wide heterogeneity in the expression of morbid phenotypes as induced by IH. Thus, the study of novel circulating markers as potential candidates would emerge as a promising venue, and among those one of the widely and actively explored include extracellular vesicles (EVs).

Extracellular Vesicle and Intercellular Communications

Cells are known to secrete a large variety of vesicles into the extracellular space. Cell-to-cell communication is necessary for proper coordination during development and among different cell types within adult tissues to allow for seamless and integrated coordination of cellular functions, a critical feature in the development and environmental adaptation of multicellular organisms. On the other hand, dysregulated pathways of communication appear to drive oncogenesis and cancer progression. Cell communication often involves soluble factors, such as cytokines, chemokines, growth factors and neurotransmitters, and their specific recognition by cell-surface receptors. Although there are different ways how intercellular communications can occur, direct cell-to-cell contact including the adhesion junction, release of soluble signaling molecules or exchange of cellular fragments such as EVs are the dominant forms of intercellular interactions (Ahmed and Xiang, 2011; Ogorevc et al., 2013).

Human body fluids contain a multitude of cell-derived vesicles, which are secreted by most cell types, and are commonly referred to as EVs, which can then travel long distances until they are up taken by receptor target cells (Lee et al., 2011). EVs have emerged in recent years as potent vehicles for cell-to-cell communication, particularly since the discovery that they contain functional mRNA, miRNA and DNA molecules that can be taken up by target (acceptor) cells (Baj-Krzyworzeka et al., 2006; Mittelbrunn and Sanchez-Madrid, 2012; Montecalvo et al., 2012; Valadi et al., 2007). A number of different EVs subpopulations have been described, and their classification is usually dependent upon their size and specific biogenesis (Thery et al., 2009). EVs are classified into 3 main classes: (a) microvesicles/microparticles/ectosomes with an approximate size of 100–1000 nm; (b) exosomes that are formed within the endosomal network and released upon fusion of multi-vesicular bodies with the plasma membrane and size ranging from 30–100 nm; and (c) apoptotic bodies with sizes between 100–5000 nm (Thery et al., 2009; Zhang and Grizzle, 2014). This latter subpopulation of EVs, which arises from cells undergoing apoptosis, is rapidly engulfed by phagocytic cells. EVs in general and especially exosomes have generated substantial excitement in view of their potential as disease biomarkers or as precision carriers for drug delivery.

Exosomes

Exosomes were first discovered in the mid-1980s by Johnstone and colleagues, who found that the transferrin receptor and some other membrane associated elements are selectively released in multi-vesicular body (MVB) derived circulating vesicles (Johnstone et al., 1987; Pan et al., 1985; Trams et al., 1981). There are two steps for exosome biogenesis: (a) the

inward budding of membranous vesicles of endosomes, (b) their release into a structure known as a multi-vesicular body (MVB). The formation of MVBs occurs during the maturation of early endosomes into late endosomes with the accumulation of intraluminal vesicles (Hanson and Cashikar, 2012). After maturation, MVBs are directed for fusion with either the lysosome, where their cargo will undergo lysosomal degradation, or to the plasma membrane, where their contents will be released into the extracellular space. When MVBs undergo this process, transmembrane proteins are incorporated into the membrane, maintaining a topological orientation similar to that of the plasma membrane (Escrevente et al., 2011).

Exosomes have been found in essentially all body fluids under both healthy and morbid conditions (Gallo et al., 2012; Jenjaroenpun et al., 2013; Khalyfa A et al., 2016; Simpson et al., 2009). Exosomes have been successfully isolated from cell culture conditioned medium (Balaj et al., 2011) and different body fluids including plasma (Ashcroft et al., 2012), serum (Dalton, 1975), saliva (Keller et al., 2011), amniotic fluid (Keller et al., 2011), breast milk (Hata et al., 2010), and urine (Wiggins et al., 1987). The gold standard and most commonly used protocol for exosome isolation/purification is differential centrifugation, which involves several centrifugation and ultracentrifugation steps. However, such protocols vary across users, leading to inconsistencies in the recovery of exosomes, mainly because of different biofluid viscosity properties (Momen-Heravi et al., 2012; Yuana et al., 2011). Recently, several alternative methods were introduced and successfully implemented for isolation and purification of exosomes, including antibody-coated magnetic beads, microfluidic devices, precipitation technologies (ExoQuick™), and filtration technologies. A consensus for characterization of exosomes has been recently adopted and should be therefore followed to enable comparative studies across different laboratories (Lotvall et al., 2014). The composition of exosomes obviously differs from cell type to cell type. According to the most recent version of the exosome content database, exosomes from various organisms and various cell types in the current version of ExoCarta show 41,860 proteins, >7,540 RNA and 1,116 lipid molecules (Keerthikumar et al., 2016; Mathivanan et al., 2012).

Exosomes were initially thought to serve simply as “garbage bags” for cells to get rid of unwanted constituents. However, an increasing body of evidence has demonstrated that exosomes play an important role in cell-to-cell communication and influence both physiological and pathological processes. For example, exosomes were recognized as being closely involved in the function of the immune system. In addition, exosomes were found to function as “communication shuttles” between cells and transduce signals in recent years, and that such interactions could re-encode genes of target cells and play a part in the development, invasion, metastasis and drug resistance of cancer (Azmi et al., 2013).

The general composition of exosomes is illustrated in Figure 2. For example, exosomes are enriched in particular cellular proteins, including the tetraspanins CD63, CD9, and CD81, endosomal sorting complexes required for transport (ESCRT)-related proteins Alix and Tsg101, MHC I, and heat shock proteins (Vlassov et al., 2012). Exosomes derived from immune cells are also enriched in MHC II and co-stimulatory molecules (Thery et al., 2009). Exosomes also package cellular RNAs and protect them from degradation (Valadi et al., 2007), such that exosomes isolated from serum contain RNA that represents a subset of the

RNA present in the exosome-producing cells (Skog et al., 2008). Thus, exosomes and their contents are potentially promising as biomarkers, particularly for diseases wherein the diseased cells produce many exosomes. For these reasons, diagnostic analysis of exosomal proteins and RNA has received much attention and even been commercialized (Vlassov et al., 2012). In general, exosomes naturally deliver mRNA, miRNA, various noncoding RNA, mitochondrial DNA, genomic DNA, and proteins (Guescini et al., 2010; Valadi et al., 2007; Waldenstrom et al., 2012). Exosome-transferred miRNAs were also suggested to be functional in target cells, as miRNAs from T-cell exosomes caused inhibition of target genes in dendritic cells (Mittelbrunn et al., 2011), and miRNAs contained in exosomes from Epstein-Barr virus infected B-cells affected the expression of target genes in monocytes (Pegtel et al., 2010). Moreover, it remains not clear whether a set of mRNAs is targeted to all exosomes despite their cell of origin, in addition to cell-type-specific mRNAs. On the miRNAs side, it is still unclear whether specific miRNA sequences, rather than the whole set of intracellular miRNAs, are targeted to exosomes.

Exosomal RNAs are not only stable and accessible biomarkers, but can also be functionally transferred from parent cells to recipient cells (Valadi et al., 2007). For instance, mRNAs released in exosomes by glioblastoma cells are translated by recipient brain microvascular endothelial cells (Skog et al., 2008). Exosomes secreted by macrophages can deliver miRNAs into breast cancer cells to promote invasion (Yang et al., 2011). Exosomes secreted by human colorectal cancer cells contain mRNAs, miRNAs and natural antisense RNAs that can be transferred into the human hepatoma and lung cancer cell lines (Chiba et al., 2012). Interestingly, breast cancer cells secrete exosomes with specific capacity for cell-independent miRNA biogenesis, while normal cell-derived exosomes lack this ability; these cancer exosomes stimulate non-tumorigenic epithelial cells to form tumors by altering their transcriptome (Melo et al., 2014). Besides immunity and cancer, exosomes have been shown to play important roles in cardiovascular, metabolic and neurological disease, (Danielson and Das, 2014; Khalyfa and Gozal, 2014; Khalyfa et al., 2016b; Yelamanchili et al., 2015). Due to the restricted available evidence on exosome biology and IH, we will focus only the rest of this paper on miRNAs and mRNA. As shown in Figure 3, there are several tools to evaluate exosomes.

Exosomal miRNAs in OSA and Intermittent Hypoxia

Circulating miRNAs have been proposed as attractive candidates as both diagnostic and prognostic biomarkers in various diseases, including a spectrum of cardiovascular conditions, whereby dysregulation of specific miRNAs in response to genetic or environmental factors can contribute to aberrant gene expression patterns underlying vascular or metabolic dysfunction (Hamilton et al., 2013). miRNAs are small noncoding RNAs (approximately 22 nucleotides) that post-transcriptionally regulate gene expression by blocking translation or inducing degradation of the targeted mRNA (Bartel, 2009), and currently more than 2,000 human miRNAs are registered (miRBase Release). There are an estimated ~ 45,000 miRNA-targeting sites in the human genome, affecting the expression of ~ 60% of genes (Friedman et al., 2009). miRNA genes can be coded by the intronic regions of protein-coding genes (intronic miRNAs) or by sequences outside protein-coding genes (intergenic miRNAs) (Inui et al., 2010). Furthermore, miRNAs have been implicated in

almost every cardiovascular disorder in which they have been examined, including heart failure, cardiac hypertrophy, remodeling after myocardial infarction, arrhythmias, atherosclerosis, atrial fibrillation, and peripheral artery disease (Adachi et al., 2010; Bonauer et al., 2010; Kawashima and Shioi, 2011; Thai et al., 2010). Since miRNAs can be readily detected in various body fluids, they have been advanced as useful biomarkers for the diagnosis and characterization of systemic diseases including type 2 diabetes mellitus, hypertension, obesity, and cardiovascular disease (D'Alessandra et al., 2010; Ortega et al., 2013; Zampetaki et al., 2010; Zampetaki et al., 2012).

The biological processes of miRNA-regulated gene expression are various including cell proliferation, cell differentiation, apoptosis, signal transduction, immune response, stress resistance, fat metabolism, insulin secretion and hematopoiesis (Ambros, 2003; Bartel, 2004; Wang and Sen, 2011). In addition, miRNAs are involved in a number of metabolic processes, such as maintenance of cellular glucose, cholesterol, triglyceride, and fatty acid metabolism (Rottiers and Naar, 2012).

In the context of OSA, two studies focusing on plasma miRNAs have been recently published from our laboratory. One of such studies explored the potential use of a miRNA panel in patients with resistant hypertension and OSA to predict whether a favorable hypotensive response to CPAP would occur (Sanchez-de-la-Torre et al., 2015). In a subsequent study, we examined children with obesity who were also matched for demographic and anthropometric characteristics but differed in their endothelial functional phenotype. In these children, we identified a subset of miRNAs in their plasma that readily segregated those children with endothelial dysfunction (ED) from those with normal endothelial function (NEF) (Khalyfa et al., 2016a). In a subsequent study we expanded such initial findings to children with OSA or obesity, and examined exosomal miRNA cargo rather than plasma miRNAs (Khalyfa et al., 2016a). In this setting, divergent endothelial phenotypes led to identification of 5 differentially expressed exosomal miRNAs. Among them, miRNA-630 expression in exosomes was significantly reduced in children with ED, regardless of whether they had OSA, obesity, or both. We had transfected a specific mimic of miRNA-630 to exosomes from ED children, and also transfected a miRNA-630 inhibitor to exosomes from NEF children, and treated endothelial cells with these transfected exosomes. As controls, exosomes transfected with scrambled sequences of either the mimic or the inhibitor were used. These experiments showed that increases or decreases in miRNA 630 recapitulated the previously assessed endothelial functional phenotype in the children. Furthermore, we could inject exosomes from ED or NEF children and reproduce the endothelial functional phenotype in naïve healthy mice. Finally, we also performed mRNA transcriptomic analyses of the treated endothelial cells with exosomes treated with either mimic, inhibitor or scrambled controls to determine the gene targets of miRNA-630 in endothelial cells. These experiments revealed a total of 416 miRNA-630 gene targets in endothelial cells, corresponding to 10 major functional pathways many of which have been previously identified as playing important roles in endothelial function and homeostasis (Khalyfa et al., 2016a).

In parallel, we have conducted independent assessments of plasma-derived exosomes in healthy adults exposed to experimental IH for 4 days followed by a normoxic recovery

period of similar duration. Exosomes after IH exposures showed not only the ability to disrupt endothelial function in naïve endothelial cells *in vitro* and recruit the expression of adhesion molecules that promote monocyte adherence, but the presence of several differentially expressed miRNAs in their cargo and the widespread transcriptomic effects of such exosomes in endothelial cells. Moreover, after normoxic recovery, such cargo content and functional properties reverted to baseline (Khalyfa et al., 2016c).

Using an experimental model of IH in mice, we performed similar experiments with the intent to detect exosomal miRNAs that would promote changes in malignant properties of cancer cells (Almendros et al., 2016). Such experiments were a logical sequel to initial observations that IH mimicking OSA can induce changes in the biological characteristics of solid tumors *in vivo* (Almendros et al., 2014a). We identified 11 distinct miRNAs in IH-exposed mice, and further uncovered their gene targets in TC1 tumor cells (Almendros et al., 2016). These preliminary findings on exosomes in OSA or in human or murine models of OSA consisting of IH provide compelling justification to expand efforts to characterize how IH alters exosome generation and release as well as exosomal cargo and function.

MiRNAs actively participate in the modulation of important cell physiological processes and are involved in the pathogenesis of lung diseases such as lung cancer, pulmonary fibrosis, asthma and chronic obstructive pulmonary disease. Several studies have been indicated that there is association between miRNAs and other form of hypoxia (e.g. acute lung injury or pulmonary hypertension). For example, miR-29-3p was explored to play a role in the activation of pulmonary adventitial fibroblasts suggesting that miR-29a-3p regulates the activation and phenotype of pulmonary adventitial fibroblasts in hypoxia and has preventative and therapeutic potential in hypoxic pulmonary hypertension (Luo et al., 2015).

In this context, several questions have been raised regarding circulating miRNAs and exosomes: First, why are circulating miRNAs stable? In fact, the blood contains high levels of RNase activity that degrades exogenously added mRNA within seconds, indicating that miRNAs are unlikely to exist in a unprotected state (Tsui et al., 2002). Several studies indicated that serum or plasma miRNA levels do not appear to be affected by incubation of the serum or plasma samples at 4°C or room temperature, multiple freeze–thaw cycles, long-term storage or even boiling or treatment with acid or base (Chen et al., 2008; Gilad et al., 2008; Ho et al., 2010; Mitchell et al., 2008). Second, what are the functions of circulating RNAs? A number of studies have demonstrated the presence of mRNA in various microvesicles (Ratajczak et al., 2006; Skog et al., 2008) and when exosomal mRNAs were analyzed bioinformatically, their key functions included cellular development, protein synthesis and RNA post-transcriptional modifications. Third, which cells are targeted by circulating miRNAs? While many of these suggested roles for circulating miRNAs are plausible, elucidation of the functional role requires demonstration of a gene regulatory effect of miRNAs after being transferred to recipient target cells. As exosomes bear the same surface proteins and ligands as their parent cells, receptor mediated interactions could potentially occur with specific recipient cells. Valadi et al. showed that mast cell-derived exosomes transferred labeled RNA to other mast cells, and the mRNA component was translated. As this RNA transfer was to mast cells only, and did not occur to CD4+ cells, this study provides evidence that the exosome exchange represents a controlled and highly

specific communication pathway (Valadi et al., 2007). Several studies have since addressed this issue, showing effects of horizontally transferred miRNAs on recipient cell gene expression (Kosaka et al., 2010; Pegtel et al., 2010). Increasing evidences have shown that exosome composition contains both a common and a specific set of RNA, proteins and lipids (Kim et al., 2013; Mathivanan and Simpson, 2009). In addition to exosomes mRNAs and miRNAs cargos, there are other cargoes which including enzymatic cargo (Lozito and Tuan, 2012; Nojima et al., 2016), protein cargo (Rezeli et al., 2016; Simpson et al., 2008; Sinha et al., 2017), and bioactive lipids content (Choi et al., 2013; Frey and Gaipf, 2011) which may play an important function of EVs/exosomes.

Exosome Release

The release of exosomes is a specifically regulated cellular process and cells can release exosomes in response to certain physical, chemical and biological stimuli including low pH, thermos-and-oxidative stress and hypoxia (although the effects of IH have yet to be examined). Also, exosome release is sensitive to the changes in intracellular calcium in some cell types, and depolarization induced by K^+ appears to increase the secretion of neuronal exosomes (Eldh et al., 2010; Hedlund et al., 2011; Parolini et al., 2009). Numerous steps could affect the intracellular decision to degrade or release exosomes, including intracellular transport of multivesicular bodies (MVBs) along microtubules to the plasma membrane, creation of docking sites at the plasma membrane, or attachment protein receptor (SNARE) proteins that mediate fusion with either lysosomes or the plasma membrane. Molecular regulators implicated in exosome release include multiple molecules implicated in MVB docking, including the GTPases and Rabs family (Stenmark, 2009). Different Rab proteins play in regulating exosome release are cell-specific, depending on the differential expression and function of particular effectors. In fact, Rab4, Rab5, Rab11, Rab35, Rab27a and Rab27b have been implicated in different steps of exosome release in different cell types (Hsu et al., 2010; Koles et al., 2012; Savina et al., 2002). Other studies have described different mechanisms of exosome secretion that involve the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein YKT6 (Gross et al., 2012). Briefly, the cytoskeleton and the contractile machinery of the cell move, attracting the opposing membranes with the assistance of the SNARE complex before pinching off the membrane connection and releasing the vesicle into the extracellular space (Cocucci et al., 2007; Sudhof and Rothman, 2009). Interestingly, silencing Rab27a by RNA interference disrupted exosome-dependent and -independent mechanisms that modify the tumor microenvironment and can also reduce tumor growth and metastasis (Bobrie et al., 2012). Other Rab proteins such as Rab11 and Rab35 might serve as alternative targets for impairing the release of exosomes by inhibiting the docking of MVBs with the plasma membrane (Hsu et al., 2010; Savina et al., 2005). Furthermore, other processes such as acid sphingomyelinase activation in glial cells (Bianco et al., 2009) or endothelial cells (Serban et al., 2016); neutrophil sphingomyelinase-2 signaling (Kosaka et al., 2010); and higher order oligomerization (Buschow et al., 2009; Muntasell et al., 2007) have been shown to play an important role in EVs release under various hemostatic or stress conditions.

Exosome Uptake

Exosomes have emerged as one of the main players in intercellular communication, and exosomes can carry various cargos such as proteins, mRNAs and miRNAs to recipient cells. Uptake of exosomes and their cargo can induce and/or inhibit different cellular and molecular pathways that lead to the alteration of cell behavior. The most established mechanism by which exosomes can modulate the recipient cells is by direct interaction. There are multiple ways for uptake of exosomal cargo by recipient cells including: phagocytosis, clathrin- or caveolin-mediated endocytosis, lipid raft mediated endocytosis, in which caveolin or clathrin is also involved (Mulcahy et al., 2014). Other mechanisms exosomal uptake include micropinocytosis, where exosomes are enclosed in the so called macropinosomes or exosomes can be trapped in membrane ruffles (Nakase et al., 2015).

Exosomes can be internalized into recipient cells via a number of potential mechanisms: (i) by endocytosed or internalized into an endocytic compartment or MVB, from which they may undergo back-fusion, by fusing with the limiting membrane and releasing their cargo into the cytoplasm of the recipient cell (Parolini et al., 2009); (ii) by fusing with the plasma membrane, releasing the cargo directly into the cytoplasm of the recipient cell; and (iii) receptor ligand mediated interactions could result in either signal transduction or exosomal internalization (Fitzner et al., 2011; OrLoughlin et al., 2012). Mechanisms for interaction and uptake of exosomes to target cells appear to involve clathrin-dependent and independent pathways that are most likely specific to a given cellular source of exosomes and a given recipient cell type (Colombo et al., 2014). Interactions between exosomes and recipient/target cells can also be mediated through direct signaling interactions via surface-expressed molecules including integrins (Fedele et al., 2015; Hoshino et al., 2015). Several mechanisms of exosomes internalization are reported (Xu et al., 2016) including receptor-mediated endocytosis (LFA1, TIM1 and TIM4), phagocytosis, and direct plasma membrane fusion (Thery et al., 2009). Understanding exosome internalization and transfer of their cargo to target cells will be critically important for IH-related biological processes.

The most common methods for detecting exosomes uptake are the use of fluorescent lipid membrane dyes to stain exosomes membranes. Examples of such dyes include PKH67, PKH26, rhodamine B (also known as R18), DiI and DiD which are lipophilic dyes (Atay et al., 2011; Fitzner et al., 2011; Franzen et al., 2014; Morelli et al., 2004; Tian et al., 2010). Subsequent entry of EVs into recipient cells can be measured using methods such as flow cytometry and confocal microscopy. Instead of labelling whole exosomes, we have shown more recently that exosomal RNA can be labeled by assessing RNA uptake in cell culture (Khalyfa A et al., 2016). Furthermore, exosome uptake into recipient cells appears to be phagocytic processes dependent on dynamin2 and PI3K (phosphatidyl inositol-3-kinase). The uptake process relies on a variety of specific cell surface molecules on the receptor cell (T-cell membrane protein 4 Tim4, but not Tim1, has been shown to be involved in exosomes uptake) as well as proteins like integrins, annexins, galectin, and ICAM1 (inter-cellular adhesion molecule 1) on the exosomal surface. Uptake of exosomes is possibly a non-random event and recent papers suggest a mechanistic basis to target cell selection by invoking tetraspanins as entry selection markers (Rana and Zoller, 2011). This is supported by a defined set of exosome associated tetraspanins that appear to direct targeting to

endothelial cells to promote angiogenesis and vasculogenesis. As mentioned above, how IH affects such processes remains virtually unexplored.

Exosomes and Cardiovascular and Metabolic Diseases

Under pathological conditions such as IH, exosomes could contribute to the establishment of a pro-inflammatory phenotype that leads to endothelial dysfunction (Agouni et al., 2008), and our aforementioned work seems to support this concept. In addition, two recent studies have reported the presence of a correlation between exosomes and metabolic dysfunction, and such work could inferentially be adapted to study the impact of OSA on metabolic function. One such study showed that *ob/ob* mice display elevated numbers of exosomes compared to wild-type mice (Phoonsawat et al., 2014). The second study demonstrated that exosome levels bearing cystatin C were positively related to metabolic complications of obesity in patients with clinically vascular diseases (Kranendonk et al., 2014). These findings uncover a potential role of exosomes in the pathogenesis of metabolic diseases. Several other studies have identified exosomes in the culture supernatants of mouse adipose tissues (Deng et al., 2009), rat primary adipocytes (Muller et al., 2009), and mouse adipocyte cell line 3T3-L1 (Sano et al., 2014) that exhibit biological activity. For example, exosomes isolated from the culture supernatant of visceral adipose tissue excised from mice showed that injection of the exosomes derived from diet-induced or genetically (leptin-deficient *ob/ob*) obese mice into wild-type lean mice results in macrophage activation and insulin resistance (Deng et al., 2009). Also, isolated exosomes from the supernatants of differentiated 3T3-L1 cells under hypoxic conditions are enriched in enzymes related to lipogenesis and promote lipid accumulation in recipient 3T3-L1 adipocytes (Sano et al., 2014).

Exosomes have pro- as well as anti-angiogenic properties (Yang et al., 2008). Endothelial cell (ECs) cultures release MVs containing metalloproteinase proteins MMP-2 and MMP-9 (Taraboletti et al., 2002). These endothelial-MVs (EMVs) promote matrix degradation and formation of new blood vessels. Also, MVs from platelets (PMVs) promote proliferation, survival, migration, and formation of capillary-like structures of ECs *in vitro* (Kim et al., 2004). In addition to pro-angiogenic features, EMVs also inhibit angiogenesis as they can stimulate the production of endothelial reactive oxygen species (ROS) (Burger and Kipps, 2006). Lymphocyte-derived MVs generated after actinomycin D treatment *in vitro* decrease nitric oxide (NO) and increase ROS production by stimulating phosphatidylinositol 3-kinase, xanthine oxidase and nicotinamide adenine dinucleotide phosphate oxidase pathways (Yang et al., 2008). Thus, reduced NO and increased ROS production mediated via exosome activity inhibits angiogenesis.

Exosomes and Neurodegenerative Diseases

The function of human nervous system is critically dependent on proper interneuronal communication. Many cell types including neurons, astrocytes, and glia, have the capacity to release a variety of membranous vesicles into the extracellular space. In the CNS, exosomes have been implicated in neuronal development and several pathologies (Schneider and Simons, 2013; Vella et al., 2008). Secretion of exosomes from neurons and astrocytes was

first demonstrated in cultures of primary cortical neurons prepared from rat embryos (Faure et al., 2006). Exosomes from multipotent MSCs mediate the miR-133b transfer to astrocytes and neurons, which regulate gene expression responsible for neurite remodeling and functional recovery after stroke (Xin et al., 2013). Exosomes were also found to be released from cultured cortical cells and hippocampal neurons, which contain receptor molecules that support glutamatergic synaptic activity (Lachenal et al., 2011). When activated, microglia serve as antigen presenting cells by secreting exosomes under pathological conditions (Kettenmann et al., 2011). The main physiological roles of exosomes include eliminating cellular waste, regulating immune response and communicating between neural cells (Gupta and Pulliam, 2014; Sharples et al., 2008). A number of proteins involved in diseases have been identified in their normal and pathogenic states to be associated with exosomes, and include PrP, tau, A β , α -synuclein and SOD1 (Quek et al., 2017). This suggests that exosomes may be involved in the spreading of these misfolded proteins in the brain. We are unaware of any published work on the impact of IH on exosome release and their biological properties in the CNS. Considering the extensive evidence indicating substantial structural and functional changes induced by chronic IH in brain regions cognitive, behavioral, mood, and autonomic functions (Burckhardt et al., 2008; Capone et al., 2012; Cheng et al., 2011; Darnall et al., 2017; Dayyat et al., 2012; Douglas et al., 2010; Goldbart et al., 2003; Gozal et al., 2001; Kanaan et al., 2006; Kim et al., 2015; Knight et al., 2011; Li et al., 2004; Lim et al., 2016; Nair et al., 2011; Row et al., 2007; Sapin et al., 2015; Shan et al., 2007; Xu et al., 2015; Xu et al., 2004; Yagishita et al., 2017; Zhan et al., 2005), it would be important to explore the potential contributions of exosomes in these contextual settings both as biomarkers for CNS morbidity as well as novel therapeutic targets. In addition, circulating EVs/exosomes have been associated with various diseases severity. For examples, these vesicles have been suggested for clinical biomarkers for OSA (Ayers et al., 2009; Kim et al., 2011; Yun et al., 2010), for pulmonary hypertension (Amabile et al., 2008; Bakouboula et al., 2008), for sepsis (Mostefai et al., 2008), and for COPD (Takahashi et al., 2012; Thomashow et al., 2013).

Exosomes in Clinical Medicine

A potential challenge in the development of non-invasive diagnostic assays based on exosome analysis in body fluids is the ability to identify the clinically relevant cluster of exosomes from all the other numerous exosomes. Such approaches are slowly developing and should provide opportunities to explore novel mechanisms of disease pathogenesis that can also be harnessed as clinical tools. Besides the obvious questions of disease and tissue specificity, current techniques used in the isolation and characterization of exosomes remain laborious, and suffer from a lack of standardization, as well as from high variability, and such considerations will have to be resolved before the processing of exosomes from blood and other bodily fluids as “liquid biopsies” becomes economically viable, reproducible and validated. Advances in microfluidics and optical sensing methods should enable greater robustness and selectivity in exosome characterization.

Additionally, exosomes could be harnessed as delivery vehicles since they possess an intrinsic homing ability relative to other synthetic particles, thus avoiding their unwanted accumulation in organs other than the target tissues. They demonstrate no inherent toxicity,

are not associated with any long-term differentiation issues among engrafted cells or tumor generation (Thirabanasak et al., 2010), and carry no apparent risk of aneuploidy (Buyanovskaya et al., 2009) or immune rejection following in vivo allogeneic administration (Bruno et al., 2009). The pro-generative effects mediated by exosomes depend on their enrichment in bioactive lipids, the delivery of proteins that improve cell function, and the presence of both the desired restricted sets of mRNAs and miRNAs. In addition, it is possible to introduce drugs into exosomes and convert them to promising candidate drug-delivery vehicles with the ability to carry both hydrophobic and hydrophilic drugs which enable crossing of biological barriers such as the blood brain barrier (Alvarez-Erviti et al., 2011; Batrakova and Kim, 2015).

It has been indicated that biomarkers including DNA, RNA, proteins and metabolites that can reflect an individual's state of health or disease (Magni et al., 2010). The National Institutes of Health Biomarkers in 1998 defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Strimbu and Tavel, 2010). Based on their utility in practice, biomarkers can provide insights on diagnosis, prognosis, regression or response to treatment of a disease (Perera and Weinstein, 2000). Currently, invasive and non-invasive procedures are often employed to identify biomarkers and the sleep field in general, and that of OSA in particular is especially concerned about development and validation of such biomarkers (Mullington et al., 2016). Considering that virtually all bodily fluids contain exosomes, this creates unparalleled opportunities to exploit these vesicles as reservoirs of disease biomarkers in the context of precision medicine as it related to SDB and associated morbidities (Kalra et al., 2013; Pack, 2016; Properzi et al., 2013).

Conclusions

OSA and one of its major pathogenetic components, i.e., IH, have emerged as a most prevalent chronic disorder worldwide, and have been associated with increased risk for multiple organ system morbidities, albeit coupled with an expansive heterogeneity in the phenotypic expression of such adverse consequences. Exosomes contain a rich cargo of proteins, RNA, lipids and metabolites which are specifically sorted and often reflect the biological state of the cell type of origin. Thus, exosomes in body fluids provide a rich source of potential biomarkers. The capacity of exosomes to convey a cargo to distant recipient cells has led the scientific community to consider exosomes as important mechanistic determinants as well as therapeutic vehicles. There is little doubt that future studies focused on exosome biology in the context of IH and OSA should enable important advances in our understanding of the disease and its consequences while opening the door for biomarker discovery and therapeutics.

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Highlights

- Obstructive sleep apnea (OSA) is a prevalent disease across the lifespan.
- OSA is characterized by intermittent hypoxia during sleep (IH), sleep fragmentation (SF), and episodic hypercapnia.
- OSA is associated with increased risk for morbidity and mortality primarily affecting cardiovascular, metabolic, and neurocognitive systems.
- Exosomes are very small membrane vesicles derived from nearly every cell in the body.
- Exosomes contain a rich cargo of highly regulated proteins, RNA, DNA, lipids and metabolites.
- Exosomes play important roles in mediating cell-cell communication via selective cargo transfer to target cells.
- Unique subsets of exosomal miRNAs in both adult and children mediate intercellular communication relevant to OSA and endothelial dysfunction.
- Exosomes can be used as potential diagnostic and prognostic biomarkers.

Figure 2. Diagram schematically illustrating some of the processes involved in generation of exosomes, their major content and delivery to target cells.

Figure 3.

Diagram depicting an integrated bioinformatics analysis of exosomes contents and functional reporter assays. Isolated exosomes can be analyzed to profile the genomic, transcriptomic, proteomic, and lipidomic contents. With the generated datasets, multiple integrated analyses can be performed including Gene Ontology (biological process and molecular function) enrichment, protein interaction network, pathway and domain enrichment, and subcellular localization analysis. Administration of biological fluids-derived exosomes or specific cell source-derived exosomes to target cells or animals enables functional characterization. Selective modification of exosome cargo that is predicated upon bioinformatic predictions of effector genes/molecules and delivery to targeted functional reporter assays enables confirmation of mechanistic assumptions.