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Coordinator: Prof. Teresa Antonia Santantonio
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Hypoxia mediates cancer development and progression through HIF-1α and microRNA regulation

Supervisor:
Prof. Maurizio Margaglione
Prof. Maria Pia Foschino Barbaro

MAURIZIO MARGAGLIONE
21.07.2023 08:50:38
GMT+01:00

Co-supervisor:
Prof. Donato Lacedonia

PhD Candidate:
Giorgia Moriondo

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Thanks to the research.

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LIST OF PAPER

1. **Moriondo G**, Scioscia G, Soccio P, Tondo P, De Pace CC, Sabato R, Foschino Barbaro MP, Lacedonia D. Effect of Hypoxia-Induced Micro-RNAs Expression on Oncogenesis. Int J Mol Sci. 2022 Jun 4;23(11):6294. doi: 10.3390/ijms23116294.
2. Soccio P, **Moriondo G**, Lacedonia D, Tondo P, Quarato CMI, Foschino Barbaro MP, Scioscia G. EVs-miRNA: The New Molecular Markers for Chronic Respiratory Diseases. Life (Basel). 2022 Oct 5;12(10):1544. doi: 10.3390/life12101544.
3. **Moriondo G**, Soccio P, Tondo P, Scioscia G, Sabato R, Foschino Barbaro MP, Lacedonia D. Obstructive Sleep Apnea: A Look towards Micro-RNAs as Biomarkers of the Future. Biology (Basel). 2022 Dec 30;12(1):66. doi: 10.3390/biology12010066.
4. **Moriondo G**, Soccio P, Scioscia G, Tondo P, Leccisotti R, Colanardi M, Sabato R, Foschino Barbaro MP, Lacedonia D. MiRNAs under condition of continuous and intermittent hypoxia: possible link with oncogenesis? (ABSTRACT) European Respiratory Journal 2022 60: 2382; doi: 10.1183/13993003.congress-2022.2382

LIST OF ABBREVIATIONS

AASM: american academy of sleep medicine

ACF: acriflavine

AHI: apnea-hypopnea index

ANOVA: analysis of variance

ARNT: aryl-hydrocarbon receptor nuclear translocator

BAL: bronchoalveolar lavage

BMI: body mass index

CaCo2: colorectal adenocarcinoma cell lines

CH: continuous hypoxia

CO_2: carbon dioxide

COPD: chronic obstructive pulmonary disease

CPAP: continuous positive airway pressure

CRC: colorectal cancer

DMEM: dulbecco's modified eagle's medium

DMSO: dimethyl sulfoxide

ECG: electrocardiogram

EEG: electroencephalogram

EMG: electromyogram

EOG: electrooculogram

EPO: erythropietin

ESADA: european database on sleep apnea

ESS: epworth sleepiness scale

FBS: fetal bovine serum

FDA: food and drug administration

FOSQ: functional outcomes of sleep questionnaire

HIF-1: hypoxia inducible factor-1

HIF-1α: hypoxia inducible factor-1 alpha

HIF: hypoxia inducible factors

HRE: hypoxia response element

HRE: hypoxia response elements

HRM: hypoxia-regulated microRNA

HSAT: home sleep apnea test

IH: intermittent hypoxia

miRNA: microRNA

mRNA: messenger RNA

MSLT: multiple sleep latency test

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

NREM: non-rapid eye movement

O_2: oxygen

OD: optical density

ODI: oxygen desaturation index

oncomiR: oncogenic microRNA

OSA: obstructive sleep apnea

OSLER: oxford sleep resistance

PG: polygraphy

PSG: polysomnography

qRT-PCR: quantitative real-time polymerase chain reaction

REM: rapid eye movement

ROS: reactive oxygen species

SaO_2: partial pressure of oxygen in the arterial blood

SD: standard deviation

SO_2: partial pressure of oxygen

SpO_2: oxygen saturation

TNFα: tumor necrosis factor alpha

TS90: total sleep time with SpO_2 <90%

VEGF: vascular endothelial growth factor

ABSTRACT

Background: MicroRNAs (miRNAs) are small non coding RNAs which play a role in several cellular processes such as apoptosis, cell differentiation, proliferation and stress response. Thus, changes in miRNA expression are associated with the development and progression of human disease like cancer and many other systemic diseases. MiRNAs expression could be influenced by different stimuli such as oxidative stress, inflammatory response and hypoxia. Lately, some hypoxia-inducible miRNAs (HRMs, hypoxia-regulated miRNAs) have been identified. These HRMs are often activated in different types of cancers, suggesting their role in tumorigenesis.

Obstructive sleep apnea (OSA) is a breathing disorder characterized by recurrent obstructions of the upper airway associated with increased inspiratory efforts, intermittent hypoxemia and sleep fragmentation. Recent experiments suggest that cancer could be a disease associated with this sleep breathing disorder. The mechanisms that regulate gene expression during hypoxia are not fully understood but miRNAs expression seems to have an important role in various processes.

On these basis, the main aims of this experimental thesis are: i) to evaluate the roles of continuous hypoxia (CH) and miRNAs in cancer; ii) to evaluate how hypoxia could influence the expression of specific miRNAs in cells; iii) to assess the differences in the expression profile of specific miRNAs induced by continuous and intermittent hypoxia; iv) to evaluate changes in miRNAs expression in controls or patients affected by OSA and/or cancer and in colorectal cancer cells exposed to intermittent hypoxia (IH), and to evaluate their impact on tumor progression in vitro.

Materials and Methods: For this purpose, I used qRT-PCR to detect miRNAs expression both CaCo2 cells exposed to CH or IH, to 2 to 24/32h, with or without acriflavine (ACF), a HIF-1 inhibitor and in patients' sera. MTT and transwell invasion test were applied to investigate the proliferation and migration of CaCo2 exposed to IH and treated with miRNA inhibitors or ACF. HIF-1 activity was evaluated in CaCo2 cells after IH.

Results: The levels of almost all analyzed miRNAs (miR-21, miR-23b, miR-26a, miR-27b, and miR-145) were greater in continuous hypoxia versus normoxia. Furthermore, the expression of nearly all miRNAs was higher in IH than in CH.

In addition, the levels of miR-21, miR-26a and miR-210 increased in OSA and ONCO-OSA patients compared to controls. MiR-23b increased in ONCO-OSA patients, and miR-27b and miR-145 increased in OSA but not ONCO-OSA patients.

MiR-21, miR-26a, miR-23b and miR-210 increased in cells after IH. Proliferation and migration were increased by IH and reduced after either miRNA inhibition or ACF treatment. MiRNA inhibition

reduces HIF-1α gene expression. Conversely, the expression of these miRNAs was significantly decreased after ACF treatment.

Conclusions: We identified a series of miRNAs, induced by the hypoxic environment, in malignancies associated with hypoxia, especially OSA-associated intermittent hypoxia.

These data would demonstrate that there is a different response between the condition of intermittent versus chronic hypoxia, and the former appears to be much more harmful in terms of stimulation of the expression of some miRNAs. These miRNAs could be implicated in cancer development and progression through a regulatory loop involving HIF-1.

1. INTRODUCTION

Sleep disorders breathing are related to breathing alterations that determine an alteration of the physiological sleep. The most common of these disorders is Obstructive Sleep Apnea (OSA), a condition in which respiratory tracts, partially or completely, are repeatedly obstructed during sleep. This phenomenon can cause apnea or hypopnea episodes which lead to intermittent hypoxia, arterial oxygen desaturation and sleep fragmentation [1].

It has been recently shown that OSA is associated to the development and the progression of cancer [2-6]. Recent epidemiological studies have suggested that tumors incidence and mortality in patients affected by OSA are higher than those not affected by it. Moreover, polysomnography variables such as apnea-hypopnea index (AHI) and oxygen desaturation index (ODI) are correlated with tumor progression [7-10].

Mechanisms include intermittent hypoxemia and/or sleep fragmentation which affect sympathetic tone, angiogenesis, inflammatory processes and immunoregulatory cellular targets, leading to altered transcriptional regulation with oncogenic properties variations of tumors. As a consequence, tumors are more aggressive, invasive and resistant to therapies [4]. As a pathophysiologic feature of OSA, IH plays a major role in tumor development and progression [11-14].

However, potential dynamics of how IH, associated with OSA, may accelerate tumor progression is currently far from being completely understood. Proofs that may link OSA and human cancer are indeed contrasting and, because of low cancer incidence, the majority of studies conducted until today have gathered tumor subtypes to increase statistical power, thus missing the objective of demonstrating whether specific types of neoplastic cells may have different responses to intermittent hypoxemia and/or sleep fragmentation [15].

MicroRNAs (miRNAs) are small, non-coding RNAs which are involved in the regulation of several biological processes like cellular proliferation, apoptosis and cellular differentiation [16-19]. Numerous studies have demonstrated that an altered profile of expression of some microRNAs are often related to the outbreak and/or progression of several pathologies like cancer [20,21]. It is considered that miRNAs in cancer may show up as tumor or oncogenic suppressors, which restrict or promote tumor progression and angiogenesis [20]. Moreover, previous studies indicate that miRNAs can be diagnostic biomarkers, prognosis of tumor and eventual therapeutic targets [21]. Continuous hypoxia, as often found in tumors due to lack of oxygenation, can upregulate the expression of several miRNA [22,23], thus suggesting a role in tumorigenesis.

Intermittent hypoxia, as found in OSA patients, seem to influence tumor development and progression in different ways compared to chronic hypoxia [24]. However, many miRNAs are also regulated by IH in OSA patients [18, 22].

The hypoxia-inducible factor 1 (HIF-1) transcription factor, stabilized by hypoxia, activates a wide variety of genes which are fundamental to the adaptation of cells at low oxygen concentration by modulating cellular processes such as angiogenesis, glucose metabolism, survival, and cellular death.

In the last year studies have identified several miRNAs as HIF-1 targets regulated by hypoxia. We referred to them as HRM hypoxia-regulated microRNAs that, as aforesaid, are often activated in several tumor types such as breast and colorectal cancer [25].

As the cellular response to hypoxia involves the activation of several transcriptional regulators involved in inflammation, tumor invasion, angiogenesis, cell cycle block and apoptosis, so, we strongly believe that a better understanding of all these closely related mechanisms, as well as the identification of miRNAs sensitive to hypoxia, may prove fruitful in the search for new therapeutic targets and in the search for new and more effective anti-tumor therapies.

Based on the previous considerations, in this study firstly we evaluated the expression of different miRNAs in conditions of continuous hypoxia to study the possible relationship between hypoxia, miRNA and cancer and identify the miRNAs involved in carcinogenesis that are susceptible to hypoxia [23].

Secondly, we decide to better comprehend the regulation of the expression of these miRNAs associated with carcinogenesis by intermittent hypoxia, and to unravel the role of HIF-1 in this process.

In that respect, we analyzed the expression of 6 microRNAs, respectively miR-21, miR-23b, miR-26a, miR-27b, miR-145 e miR-210 in subjects affected by OSA and/or cancer, and in colorectal carcinomatous cell lines (CaCo2) which were exposed to IH to study cell response to hypoxia. HIF-1 and miRNA inhibitors were used to decipher their respective roles in cell proliferation and migration.

2. OBSTRUCTIVE SLEEP APNEA

2.1. BACKGROUND

Obstructive sleep apnea (OSA) is a sleep breathing disordered characterized by recurrent episodes of partial or complete collapse of the upper airway during sleep, resulting in reduced (hypopnea) or absent (apnea) airflow lasting for at least 10 seconds and associated with either cortical arousal or a fall in blood oxygen saturation [1,26].

The prevalence of this disorder is rising and is estimated to be 26%, and around 13% of men and 6% of women are diagnosed to have moderate to severe OSA [27]. However, recent studies have suggested that the prevalence of moderate to severe OSA is even higher at 50% in men and 23% in women [28]. OSA is becoming a major health concern due to its high prevalence, its association with cancer [2,14] and the higher risk it carries for cardiovascular disease, hypertension and mortality [29-32].

2.2. PATHOPHYSIOLOGY

OSA is characterized by repetitive partial or complete collapse of the upper airway during sleep, resulting in episodic reduction (hypopnea) or cessation (apnea) of airflow despite respiratory effort [1].

It is known that sleep is not just a passive phenomenon. In fact, during sleep, the central nervous system performs numerous activities, although it maintains a physiological state of loss of consciousness and poor responsiveness to stimuli.

In humans, normal sleep has its own rhythm composed of two distinct states NREM (non-rapid eye movement, also called slow wave sleep) and REM (rapid eye movement or paradoxical sleep), during which there is a cyclic sequence of frequencies waveforms that can be observed on the electro-encephalogram (EEG) during the polysomnographic examination. This composition of NREM + REM sleep is called the sleep cycle. In a healthy adult, four to five sleep cycles usually occur each night [33]. Conversely, in subjects with OSA, repeated episodes of partial or total upper airway obstruction can be observed during sleep, more than five per hour, leading to airway obstruction (apnea) or narrowing of the airways (hypopnea) while maintaining inspiratory efforts.

Apnea, by definition, is a complete interruption to the flow of air through the upper airways lasting at least 10 seconds; it is obstructive in nature when it is accompanied by closure of the upper airways associated with an increase in the thoraco-abdominal respiratory muscular effort aimed at restoring its patency. Hypopnea, on the other hand, can be defined both as a reduction in inspiratory flow of at least 50% compared to the normal value for a given subject, and as a 30% reduction in thoraco-

abdominal excursion, both lasting at least ten seconds and conditioning a reduction in oxygen saturation (SaO_2) greater than 3% [34].

OSA occurs mainly during REM sleep, a phase in which there is no muscle tone and it is easier to observe the occlusion of the upper airways. During REM sleep, muscle tone of the throat and neck, as well as that of the vast majority of skeletal muscles, are almost completely relaxed. This allows the tongue and soft palate/oropharynx to relax, reducing airway patency and potentially impeding or completely obstructing the flow of air into the lungs during inspiration, resulting in reduced respiratory ventilation. If reductions in ventilation are associated with sufficiently low blood-oxygen levels or with sufficiently high breathing efforts against an obstructed airway, neurological mechanisms may trigger a sudden interruption of sleep, called a neurological arousal. This arousal can cause an individual to gasp for air and awaken [35].

Most of the apnea events lead to the awakening of the subject and the return of muscle tone with consequent cessation of the airway obstruction generating a continuous fragmentation of sleep. The fundamental cause of OSA is a blocked upper airway, usually behind the tongue and epiglottis, whereby the otherwise patent airway, in an erect and awake patient, collapses when the patient is lying on his or her back and loses muscle tone upon entering deep sleep [35].

At the beginning of sleep, a patient is in light sleep and there is no tone loss of throat muscles. Airflow is laminar and soundless. As the upper airway collapse progresses, the obstruction becomes increasingly apparent by the initiation of noisy breathing as air turbulence increases, followed by gradually louder snoring as a Venturi effect forms through the ever-narrowing air passage.

The patient's blood-oxygen saturation gradually falls until cessation of sleep noises, signifying total airway obstruction of airflow, which may last for several minutes.

The reduction of alveolar ventilation involves a reduction in the partial pressure of oxygen and an increase in the partial pressure of CO_2 which stimulate the chemoreceptors and induce activation of the sympathetic nervous system; it is believed that it is this stimulus that determines the transition to a more superficial phase of sleep until actual awakening. With the end of the obstructive episode and the resumption of ventilation, this neuro-hormonal stimulus, associated with the alteration of ventilatory mechanics, determines the passage towards a more superficial phase of sleep or a short awakening, which involves a fragmentation of night sleep more or less evident and inconstantly perceived subjectively by patients [36].

2.3. MORBIDITY

Obstructive apneas and hypopneas result in large changes in intrathoracic pressure, intermittent hypoxemia, and arousal from sleep. These events initiate a cascade of interacting processes that contribute to adverse health outcomes [35,37].

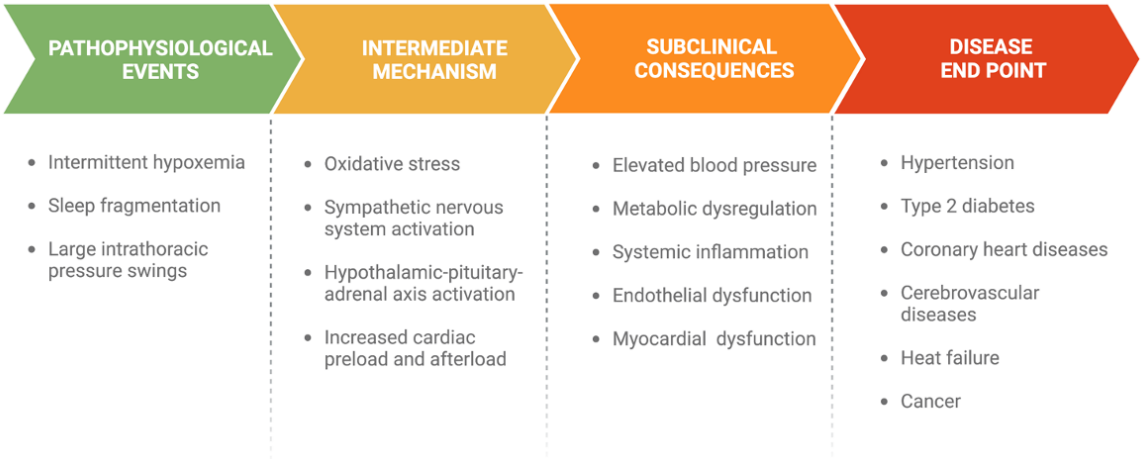
Although these arousals generally do not wake the patient, this sleep fragmentation is the primary cause of excessive sleepiness in individuals with OSA. Intermittent hypoxemia activates the sympathetic nervous system and is the major contributor to both acute and chronic elevation of blood pressure (**Figure 1**) [35].

Arousal from sleep, due to increased respiratory effort against an obstructed airway and to hypoxemia, also contributes to sympathetic activity and activation of the hypothalamic-pituitary-adrenal axis. Intermittent hypoxemia and reoxygenation result in production of reactive oxygen species [38]. Both sympathetic activity and oxidative stress contribute to blood pressure elevation, metabolic dysregulation, systemic inflammation, and endothelial dysfunction. Increased catecholamine levels decrease insulin sensitivity and promote pancreatic beta-cell apoptosis, suggesting a possible mechanism underlying the association of OSA with type 2 diabetes mellitus [39], which persists after adjustment for demographic factors and body mass index (BMI) [40].

Repetitive episodes of hypoxemia increase reactive oxygen species, which may further contribute to vascular disease, metabolic abnormalities, inflammation [36] and cancer [2,14].

Large intrathoracic pressure swings, which result from respiratory efforts against an obstructed upper airway, increase cardiac preload and afterload that, together with the effects of sympathetic activity, oxidative stress, inflammation, and gas exchange abnormalities, may contribute to heart failure and cardiac rhythm disturbances [35].

Figure 1. Possible explanations for the connection between OSA and negative health consequences



Several studies have shown that OSA is an independent risk factor for hypertension [41,42] and the prevalence of OSA is between 30% and 83% among patients with hypertension [43].

There is also a high prevalence of unrecognized OSA among patients with drug-resistant hypertension [44]. Furthermore, OSA increases the risk of cardiovascular diseases, such as congestive heart failure, fatal and non-fatal myocardial infarction, and stroke [33,45,46]. An increased risk for cancer, metabolic disorders, cognitive dysfunction, and all-cause mortality [47] as well as impaired quality of life and a higher risk for motor vehicle accidents [48,49] have also been reported.

The connection between OSA and these negative health consequences is complex, although, as said above, the intermittent hypoxemia is believed to promote oxidative stress, increased sympathetic activation, and systemic and vascular inflammation with endothelial dysfunction. These factors are believed to affect cardiovascular disease, metabolic dysfunction, cognitive impairment and cancer [2,14,35].

2.4. RISK FACTORS

The main risk factors for OSA in adults are obesity, gender and age; they also seem to constitute factors favoring genetic predisposition and the alteration of the muscle tone of the upper airways that occurs physiologically during sleep [50-53]. Poor upper airway muscle function, narrow upper airway due to craniofacial and soft tissues structures, low arousal threshold, small lung volume, and respiratory instability are all believed to be causative factors of OSA [54]. Other important risk factors are drinking alcohol, smoking and menopause [55-57].

In detail, among the most important factors which can contribute to the development of OSA are:

1) anatomical changes that can contribute to the reduction of the oropharyngeal space [58,59]. Therefore, in obese subjects, where the neck circumference increases (> 43 cm) and craniofacial alterations are often present, the risk of developing the disease increases considerably.

2) smoking can contribute to upper airway dysfunction as it promotes relaxation of the airway muscles and causes neural reflexes due to the nicotine [56,60,61];

3) sleeping in the supine position facilitates the onset of apnea [62];

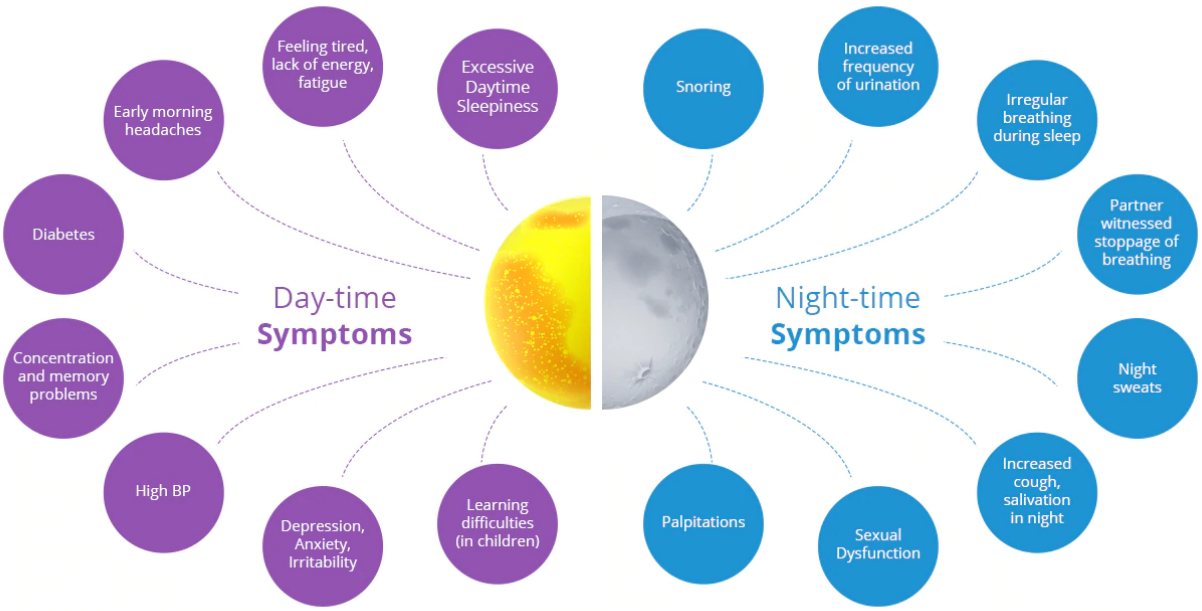
4) the presence of craniofacial anomalies (retrognathia and micrognathia, angulation of the skull base), nasal obstructions, tonsillar and/or adenoid hypertrophy, ogival palate, prolapse of the uvula, macroglossia, edema of the larynx [59];

5) the use of alcohol or other substances such as muscle relaxants or sedatives [55].

2.5. SYMPTOMS

OSA is a heterogeneous disease with a wide range of diurnal and nocturnal symptoms, which in most cases are underestimated by patients (**Figure 2**).

Figure 2. Main symptoms of OSA



The most frequent and characteristic nocturnal symptoms of OSA are snoring, breathlessness and episodes of choking or gasping [26].

However, it is necessary to specify that patients with OSA show different clinical presentations; there are symptomatic, asymptomatic or minimally symptomatic patients and these different phenotypic profiles can have a significant impact on clinical outcomes. In fact, the prognosis of symptomatic or asymptomatic patients with OSA is not necessarily the same even considering a similar severity of OSA [35].

The symptoms that people with OSA most frequently report include: excessive daytime sleepiness, reduced attention span and concentration, lower cognitive performance, tendency to doze off in unsuitable socio-work circumstances and during the performance of actions perceived as monotonous or repetitive or scarce in stimuli, such as driving a car along a route that does not require the subject's alertness. Nocturia and decreased libido are also reported [26].

2.6. DIAGNOSIS

In adults, both a clinical evaluation and an objective sleep study are required to diagnose OSA [63]. As said above, common symptoms of OSA are snoring, apneas, nocturnal choking, restless sleep, and excessive daytime sleepiness [62]. The most common symptom is excessive daytime sleepiness, but the severity of sleepiness does not seem to correlate to the severity of OSA [64].

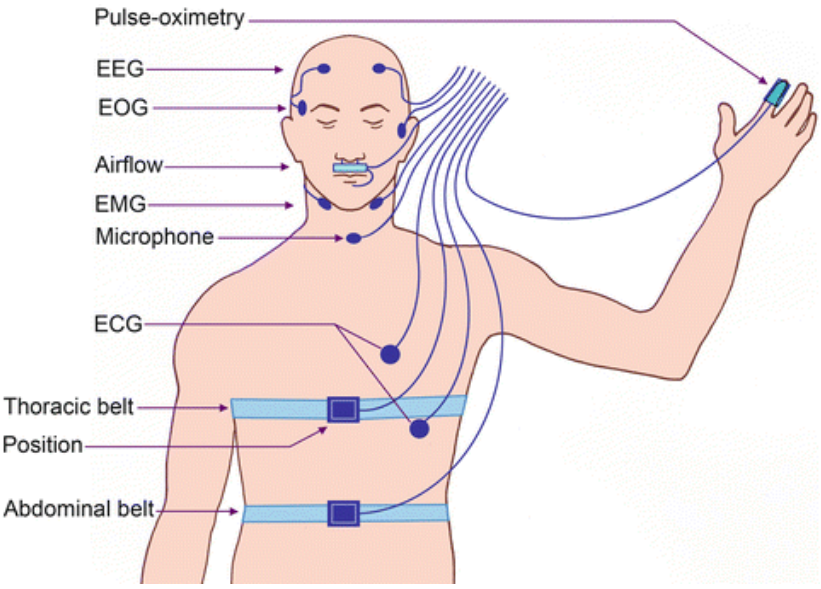
The most widely used questionnaire to assess sleepiness is the Epworth Sleepiness Scale (ESS) [65], and Functional Outcomes of Sleep Questionnaire (FOSQ) is also frequently used [66].

There are also objective tests to measure sleepiness, such as the multiple sleep latency test (MSLT) [67] and the Oxford sleep resistance (OSLER) [68] test. While questionnaires for screening for OSA have also been designed, such as the Stop-Bang Questionnaire, they are not sufficiently reliable to diagnose OSA [69].

Polysomnography (PSG) is the gold standard method to diagnose adult OSA [26]. PSG is performed overnight in a sleep laboratory and measures both sleep stages and respiratory functions. A standard PSG includes an electroencephalogram (EEG), electrooculogram (EOG), electrocardiogram (ECG), and electromyogram (EMG); monitors oronasal airflow, oxygen saturation, respiratory movements of the abdomen and thorax, body position, and transcutaneous carbon dioxide; and records audio and video during sleep (**Figure 3**). These data are used to generate several parameters, the most common of which is the apnea-hypopnea index (AHI), which measures the number of apneas and hypopneas per hour of sleep. The AHI is scored according to the manual of 2012 from American Academy of Sleep Medicine (AASM) [70].

Obstructive apnea syndrome can be classified into three degrees of severity based on the apnea/hypopnea index (AHI, apnea-hypopnea index), corresponding to the number of apnea and/or hypopnea episodes per hour of sleep. The pathology is mild if the AHI is between 5 and 15; moderate if the AHI is between 15 and 30, severe if the AHI is greater than 30 events per hour. Nonetheless, other factors such as oxygen desaturation index (ODI), which is the number of desaturations of more than 3% or 4% per hour, and the percentage of time in which desaturation persists during sleep may also influence the severity of the disease [71].

Figure 3. Common placement of electrodes and other recording equipment in PSG



Polysomnographic investigations are costly and time-consuming because they require adequate sleep laboratories ang highly specialized dedicated staff, and it is, therefore, not always available. Over time, devices with less channels have been developed that can be used at home. These devices, called home sleep apnea test (HSAT), do not include EEG, EOG and EMG and have been classified according to the number and type of sensors used (portable monitoring, out-of-center testing or oligosomnography) [72]. The AASM Task Force recommends the use of HSAT, in uncomplicated adult patients presenting with signs and symptoms indicative of a high risk of moderate to severe OSA. High risk is estimated by the presence of excessive daytime sleepiness and at least two of the following three criteria: severe and habitual snoring, partner-reported apnea or gasping or choking or diagnosis of hypertension [73].

Polygraphy (PG) is more widely available and allows the patient to perform the sleep study at home. However, the true sleep time, arousals, and sleep stages cannot be assessed with PG as it lacks EEG, EOG, and EMG. Consequently, true sleep time, some hypopneas, and sleep fragmentation are missed by PG, resulting in missed diagnosis of OSA or misdiagnosis of its severity. Consequently, it is important to perform PSG on patients that have excessive daytime sleepiness, a high clinical suspicion of OSA, and a normal PG [74].

In addition, among the HSAT approved by the US Food and Drug Administration (FDA) in the evaluation of the OSA, a good reliability of the peripheral arterial tonometry (PAT)-based technology was observed. In practice, this technology identifies respiratory events through the combined analysis of the amplitude of the PAT signal, which is reduced due to the vasoconstriction generated

by sympathetic activation at the cessation of the respiratory event, at the digital sensor level, the reduction in oxygen saturation, the increase in pulse rate and actigraphy.

PAT-based devices have been tested on different population classes, including those at low risk of OSA, with good results compared to both PSG and cardiorespiratory monitoring [75,76].

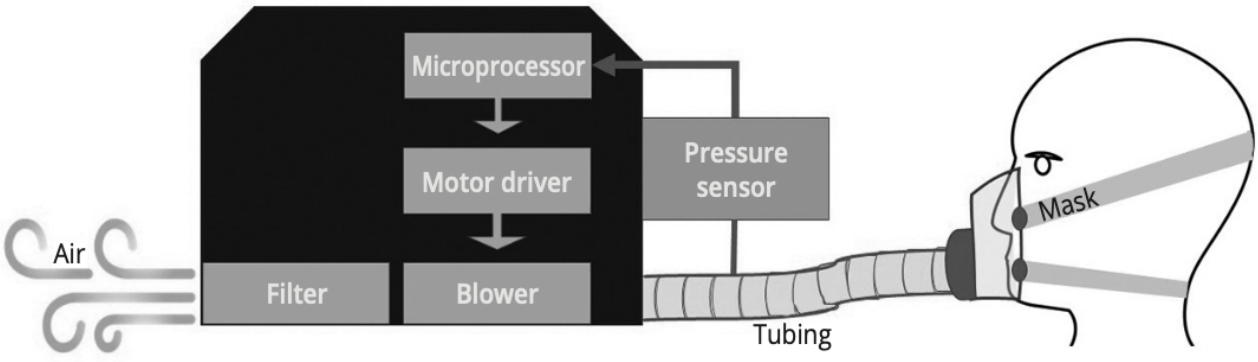
2.7. TREATMENT

The first-line treatment of OSA is nocturnal ventilation with a Positive Airway Pressure (PAP) device [77].

An AASM statement defined that the ventilation modalities available for the treatment of OSA are respectively, continuous positive airway pressure (CPAP); bilevel-PAP (bi-PAP); and auto-adjusting PAP (APAP).

CPAP is a treatment that uses air pressure to keep the airway open and is the primary treatment for adult OSA. CPAP is a ventilatory device consisting of a blower (compressor device that increase air pressure), a circuit or tube (connects the blower machine to the interface) and an interface that connects the device to the patients (**Figure 4**). Its action stabilizes the oropharyngeal walls and permeates the upper airway. Nevertheless, although studies have demonstrated the effectiveness of ventilation in reducing or resolving nocturnal respiratory abnormalities, the adherence rate to this treatment approach is low for a variety of reasons (ventilator-related, psychological or other) [78-80].

Figure 4. Schematic representation of a CPAP device.



Thus, therapeutic interventions in OSA can be varied, even combined, act on different disease targets and, above all, are personalized for each patient.

A mandibular retaining device (MRD) is also a common treatment option and it prevents the collapse of the upper airway by repositioning the lower jaw forwards. While studies have shown

improvements in respiratory parameters and sleepiness, this treatment is not as effective as CPAP and is recommended for less severe forms of OSA [81].

Before the introduction of CPAP and MRD, palate surgery, such as uvulopalatopharyngoplasty (UPPP), was the primary therapy for OSA. UPPP enlarges the oropharyngeal airway and reduces the collapsibility by adjusting the pharyngeal soft tissue (tonsils, uvula and soft palate) [82]. The efficacy and safety of UPPP have been questioned [83,84], but recent studies using modified UPPP in a selected group of patients have shown the surgery to be both safe and effective to improve respiratory parameters and sleepiness [85-89].

To select candidates for surgery it is important to assess the pharyngeal anatomy and patient obesity. The Friedman staging system is the most common clinical assessment method and is based on palate position, tonsil size and body mass index [90]. It has shown correlations between preoperative examination and surgical outcome [90,91], but low inter-examiner agreement might limit the value of this staging system [92]. Drug induced sleep endoscopy (DISE) is another tool for preoperative evaluation of adult patients with OSA and can be used to assess the level of obstruction. DISE evaluates the specific site and character of the upper airway obstruction during a pharmacological sleep. Although some studies have indicated a higher surgical success rate after DISE [93], there is insufficient evidence to claim that this approach leads to a better surgical outcome compared to a normal clinical evaluation of the upper airway in fully awake patients [94,95].

There are several alternative treatment options for OSA, including weight loss, bariatric surgery, sleep positioning, and upper airway stimulation. Obesity is a major risk factor for OSA, and meta-analyses have shown that weight loss through lifestyle changes or bariatric surgery can improve OSA parameters [96,97]. Furthermore, sleeping in the supine position often contributes to the collapse of the upper airway, so it is generally recommended that OSA patients sleep in a lateral position. However, even if patients have primarily positional OSA, studies have shown that positional therapy is inferior to CPAP [98]. Upper airway stimulation, through stimulation of the hypoglossal nerve, has shown promising results in patients with moderate to severe OSA and could become an alternative to CPAP [99,100].

3. OBSTRUCTIVE SLEEP APNEA AND INTERMITTENT HYPOXIA

In general hypoxia could be caused by environmental or pathological conditions in which the partial pressure for oxygen (PO_2) is reduced, either in the inspired air, in the arterial blood (PaO_2) or intracellular. Moreover, in a physiological context, the temporal aspect of hypoxia can differ in pattern, such as being continuous (CH) or intermittent (IH), and be of acute or chronic in duration [101]. IH is characterized by brief hypoxic exposures followed by re-oxygenation [101], and several physiological conditions, such as OSA, are characterized by IH.

The responses initiated in these different conditions of hypoxia might be different since the timing of hypoxic cycling and the degree and length of hypoxic exposure differs.

As previously explained, obstructive sleep apnea syndrome is a disease characterized by repeated episodes of apnea and hypopnea during sleep, which induce cyclical changes in oxyhemoglobin saturation/desaturation and sleep fragmentation. The desaturation-reoxygenation sequence is the typical pattern associated with most respiratory events, resulting in intermittent hypoxia. So, intermittent hypoxia is the main pathophysiological mechanism in OSA [102].

IH associated with OSA is characterized by cycles of hypoxia and reoxygenation that are markedly different from those of continuous hypoxia. The substantial difference between the two types of hypoxia is due to the oxygenation pattern (fluctuating or chronically low), the length and frequency of hypoxic periods. However, there are also differences in cellular and molecular response at both the systemic and local levels. These differences are critical for assessing the effects of downstream disease and for predicting response to specific therapies [14].

There are no consistent definitions in the literature to describe intermittent and chronic hypoxia. Nevertheless, we know that in conditions of intermittent hypoxia, the duration of the hypoxia/ reoxygenation cycles can vary by several orders of magnitude (from seconds to hours) and also the number of cycles (over a period of minutes, hours, days or weeks) can be different.

IH, associated with OSA, promotes oxidative stress by increasing the production of reactive oxygen species (ROS), increases angiogenesis, induces greater sympathetic activation with increased blood pressure and generates systemic and vascular inflammation with endothelial dysfunction that contributes to the various co-morbidities found in patients with OSA.

In hypoxic conditions, the body implements a series of coordinated responses aimed at restoring ideal levels of O_2 [103,104]. If the hypoxia is severe and prolonged, a series of factors are activated that can lead to cell death. On the other hand, if the hypoxia is moderate and intermittent, adaptation mechanisms are activated that last over time.

3.1. HYPOXIA INDUCIBLE FACTOR (HIF)

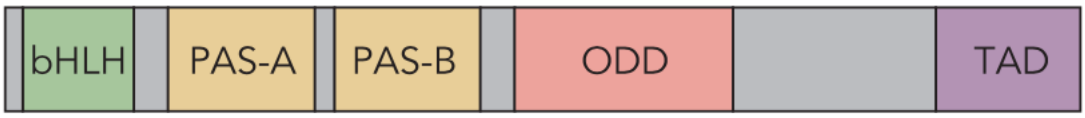
One of the responses at the molecular level consists in the activation of specific, or hypoxia-inducible, transcription factors called HIFs (Hypoxia Inducible Factors) [103] and their second messengers, erythropietin (EPO) and the growth factor for the vascular endothelium (VEGF).

HIF factors are hetero-dimeric complexes composed of an α subunit, whose stability is oxygen-dependent, and an oxygen-independent β-subunit. Three α subunits (1α, 2α and 3α) and one β subunit (also called ARNT, Aryl-hydrocarbon receptor nuclear translocator) have been identified, which however has different variants of splicing [103,104].

3.2. HIF-1α PROTEIN STRUCTURE

Human HIF-1α protein consists of 826 [105]. The α-subunit is composed of two N-terminal PAS domains, PAS-A and PAS-B, which together with the bHLH domain are required for heterodimer formation and DNA binding [106] (**Figure 5**). Two transactivation domains (TAD), N- terminal TAD (N-TAD) and C-terminal TAD (C-TAD) [107], are bridged by an inhibitory domain (ID), which is responsible for repression of TAD activity under normoxic conditions [108]. Overlapping the N-TAD is an oxygen dependent degradation domain (ODDD), which confers normoxic instability to the HIF-1α protein. HIF- 1α protein contains two PEST like motifs, rich in proline (P), glutamic acid (E), serine (S) and threonine (T), which is common for proteins with a short half-life [109].

Figure 5. Structural domains of HIF-1α protein



3.3. OXYGEN SENSING

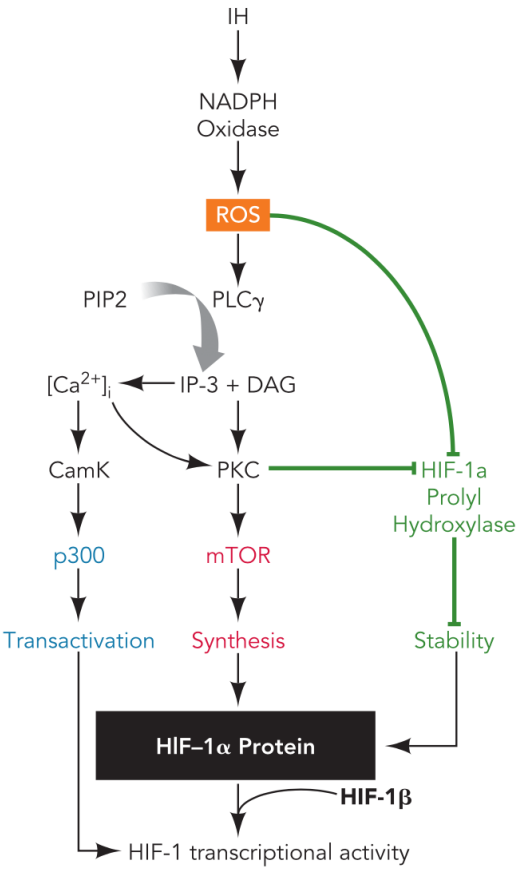
In the presence of normal quantities of oxygen, in the cytoplasm, the α subunits undergo two hydroxylation events catalyzed by the enzymes PHDs (prolyl hydroxylase domain proteins) and FIH (factor inhibiting HIF), which cause their degradation mediated by proteasome [103]. In hypoxic conditions, however, the PHDs and FIH enzymes are inactive due to the lack of oxygen necessary for hydroxylation reactions. In this way the α subunit is stabilized, and translocates in the nucleus to bind to the β subunit, forming a heterodimer (HIFα / HIFβ) which represents the active form of HIF, able to function as a transcription factor. The identified HIF factors (HIF1α / HIFβ, HIF2α / HIFβ, HIF3α / HIFβ) become active and therefore functional under hypoxic conditions. HIFα / HIFβ (HIF)

heterodimer binds to a region of DNA, from the identified consensus nucleotide sequence (5'-RCGTG-3 '), called HRE (Hypoxia Response Element, or HIF binding site), present in the promoters of hypoxia inducible genes [110].

Brief episodes of hypoxia and reoxygenation (intermittent hypoxia) are known to occur during obstructive sleep apnea. Despite the fact that intermittent hypoxia involves short (15–30 s) episodes of hypoxia followed by longer (e.g., 5 min) periods of reoxygenation, HIF-1 activity is induced, albeit by mechanisms that are distinct from those regulating its activity under conditions of chronic hypoxia [111].

The signal transduction pathways by which intermittent hypoxia activates HIF-1 have been delineated in the PC12 rat pheochromocytoma cell line, in which hypoxia was previously [112] shown to induce membrane depolarization and increased intracellular Ca^{2+}. When these cells were exposed to 60 cycles of 1.5% O_2 for 30 s followed by 20% O_2 for 5 min, HIF-1a protein and HIF-1 transcriptional activity were induced and increased further after 120 cycles [113]. In these cells, intermittent hypoxia triggered NADPH oxidase-dependent ROS production, which induced phospholipase Cg activity, leading to the generation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (**Figure 6**).

Figure 6. Signaling events following intermittent hypoxia (IH)



Binding of IP3 to its cognate receptor led to mobilization of intracellular Ca^{2+}, which activated calcium-calmodulin kinase (CamK) and, together with diacylglycerol, induced protein kinase C (PKC) activity. PKC stimulated mTOR-dependent HIF-1a synthesis and inhibited PHD2-dependent degradation of HIF-1a [111]. CamK phosphorylated the coactivator p300, thereby promoting its interaction with HIF-1a, leading to transcriptional activation [113]. In contrast to continuous hypoxia, in which HIF-1a is rapidly degraded ($t_{1/2}$ < 5 min) on reoxygenation [114], HIF-1a levels remain persistently elevated following intermittent hypoxia due to the persistent activation of mTOR [111], a finding that has significance in the context of obstructive sleep apnea, in which pathological cardiovascular and respiratory responses persist for hours after the termination of intermittent hypoxia [115].

3.4. HIF-1α FUNCTIONS

HIF-1α is a small molecule, present in all cells, which regulates the presence of oxygen at the cellular level and stimulates the cell nucleus to produce factors that increase the presence of oxygen as a "countermeasure" to hypoxia.

The first major target of HIF-1α is EPO [116]. EPO is synthesized by cells in response to hypoxic conditions, increases the oxygen-carrying capacity of the blood by stimulating erythropoiesis in the bone marrow and represents one of the first indicative markers of hypoxia. Secondly, HIF-1α induces the expression of the vascular endothelial growth factor. VEGF is a potent mediator of angiogenesis that produces multiple effects, including those related to the development and physiology of the lungs [116].

Nitric oxide synthase (NOS) and heme oxygenase (HO) are also two HIF-1α target genes, both of which activate vasodilation, increasing local blood flow and lowering blood pressure.

Fourthly, anaerobic glycolysis becomes the predominant form of cellular ATP generation under conditions of limited oxygen supply. Therefore, glucose uptake and glycolysis are upregulated by HIF-1α.

In addition, HIF-1α, in hypoxic conditions, increases the expression of tyrosine hydroxylase, the rate-limiting enzyme for dopamine biosynthesis.

Finally, iron metabolism in humans is also stimulated in hypoxic circumstances, in particular in the presence of factors such as ceruloplasmin, transferrin and transferrin receptor [117].

Iron is a vital element in all living organisms and is required as an essential cofactor for oxygen-binding proteins. Extensive research has shown that HIF transcription factors function as central

mediators, allowing cells to adapt to critically low oxygen levels in both normal and compromised tissues.

HIF-1 therefore regulates the transcription of numerous genes, many of which encode proteins involved in the metabolic adaptation to hypoxia.

To date, more than 60 genes have been identified as presumed targets of HIF-1, among which we find genes involved in the development and progression of cancer, angiogenic factors, proliferation and survival factors, glucose transporters and glycolytic enzymes.

HIF-1α has been found to be expressed to a greater extent in many solid tumors. This feature can be explained precisely by its ability to induce the expression of a wide spectrum of genes that allow survival in hypoxic conditions. It is important to underline that HIF-1α is activated by many oncogenic pathways such as PI3K or RAS, the mechanisms of which are not well known. The central role of HIF-1α in tumor pathogenesis is thus evident [14,15].

4. OBSTRUCTIVE SLEEP APNEA AND CANCER

The fundamental pathophysiological mechanism that characterizes OSA is represented by chronic intermittent nocturnal hypoxia, which generates a chronic inflammatory cascade that causes diffused vascular epithelial damage [118]; the same chronic inflammatory mechanism could promote the development of cancer through the release of angiogenic factors.

In recent years, OSA has been found to be associated with higher rates of cancer and increased cancer mortality [6, 119 -122]. In particular, there is a strong association between cancer mortality and severity of nocturnal hypoxia [5]. A dose-dependent relationship between severity of hypoxemia and cancer incidence has also been identified [6]. Increasing severity of OSA also tends to increase the odds of other OSA-linked diseases such as stroke [123], coronary disease in women [124], and hypertension [41]. The likelihood of comorbidities increases with the severity of nocturnal hypoxemia/hypoxia, indicating a central role for intermittent hypoxia.

4.1. HYPOXIA AND CANCER

The potential link between cancer and OSA is particularly interesting given the well-known role of hypoxia in cancer progression. Hypoxia is a hallmark of solid tumors due to rapid cell proliferation and abnormal blood vessel formation. Tumor hypoxia induces cellular processes that lead to both short and long-term adaptation and survival, including angiogenesis, metastasis, and resistance to radio- and chemotherapy. Whereas chronic tumor hypoxia is inherent to tumors, intermittent hypoxia from OSA is a systemic source of fluctuating hypoxia. There are hypoxic gradients around the tumor, and cells located near the exterior and close to blood vessels will experience the oxygen fluctuations generated by OSA, whereas central regions likely remain more chronically hypoxic. The consequences of intermittent hypoxia on a tumor that may already have regions of chronic hypoxia are unknown [14].

Most past and current research aimed for the understanding of the role of chronic hypoxia in tumor progression and metastasis formation. However, the importance of IH is becoming more widely accepted and research on intermittent hypoxia increases. Chronic and intermittent hypoxia cause distinct effects, but IH has been shown to promote angiogenesis, resistance to anti-cancer treatment, intratumoral inflammation and tumor metastasis, to a higher extent than chronic hypoxia [125-130].

The molecular mechanisms causing these phenotypic changes are not well elucidated, but main factors might be changes in the regulation of transcription factors and changes in gene expression.

As above mentioned, cells adapt to the hypoxic microenvironment by up-regulating pro-survival mechanisms, the majority of which are coordinated by the transcription factor hypoxia inducible factor-1α (HIF-1α) [131]. Further, the oscillating changes in tissue oxygenation are widely considered as a source of reactive oxygen species (ROS) [132,133]. Several transcription factors have been reported to be activated by ROS. These are nuclear factor (erythroid-derived 2)-like 2 (Nrf2), activator protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).

4.2. INTERMITTENT HYPOXIA VS CHRONIC HYPOXIA

Conceptually, there is a difference between intermittent and chronic hypoxia due to the pattern of oxygenation (fluctuating or chronically low). The precise distinction between various forms of hypoxia is more complex due to several variables. This includes but is not limited to the length and frequency of the hypoxic periods. On a patient level, defining chronic and intermittent hypoxia is difficult due to individual patterns of change in inspired oxygen, oxyhemoglobin levels, and blood supply to specific organs or tissues. There are also differences in the cellular and molecular response to various patterns of change in oxygen at a systemic or local level. These changes have important downstream effects for hypoxia-related disease, and potentially with regard to specific therapies [14].

The definitions used in the literature to describe intermittent and chronic hypoxia are inconsistent and often lack physiological relevance. Particularly with intermittent hypoxia, the duration of hypoxia/reoxygenation cycles can vary by several orders of magnitude (seconds to hours). The number of cycles (over a period of minutes, hours, days, or weeks) varies widely. The severity of hypoxia used in models also has a wide range and is rarely justified based on in vivo measurements of tissue oxygen levels. To add to the confusion, intermittent hypoxia is often categorized using labels that lack consensus, such as fluctuating, acute, repetitive, transient, and cyclical, among others. The result is lack of clarity between where intermittent hypoxia ends and chronic hypoxia begins. The rapid cyclical intermittent hypoxia associated with OSA is distinct from what is usually understood as intermittent hypoxia in the cancer literature [14]. The effects of chronic tumor hypoxia from restricted blood supply and the effects of extended periods of intermittent hypoxia (hours to days before reoxygenation) resulting from erratic blood flow have been well studied. In contrast, the effect of rapid cyclical changes in oxygen observed in OSA (lasting seconds) is not well known, especially when it comes to cancer biology.

4.3. OSA, INTERMITTENT HYPOXIA AND CANCER

A number of epidemiological and clinical studies suggest that patients with OSA have higher rates of cancer.

A few years ago, a study showed, in a mouse model of OSA, the possibility of developing malignant tumors towards blood or skin cells (malignant melanoma) by triggering mechanisms of systemic inflammation, oxidative stress and immune dysregulation [134], factors that these have been associated with oncogenesis.

These data allowed us to hypothesize that exposure to chronic intermittent nocturnal hypoxia in humans could determine the origin of the malignant cell neoformation and increase the progression and distant metastatic spread [5,135].

In this regard, numerous clinical studies have been conducted in humans to evaluate the possible relationship between OSA and cancer.

Initially, an analysis conducted on 1,522 patients found that subjects with severe OSA were almost 5 times at risk of developing cancer [6].

Subsequently, a Spanish study conducted on about 5.000 patients with OSA has shown that the severity of the disease expressed by the intensity of nocturnal hypoxemia has a strong link with the development of malignant neoplasms in various parts of the body [136].

This relationship was also highlighted by a Canadian retrospective cohort study that showed that there is a close relationship between OSA and cancer [137].

In addition, according to a study conducted by Gozal D et al, disturbed and discontinuous sleep, troubled by frequent awakenings, could worsen the prognosis of tumors, increasing their aggressiveness and the ability to invade surrounding tissues. The mechanism would depend on the immune system, which, in the presence of irregular sleep, would not be able to effectively fight the disease [138]. The investigation was conducted using two groups of mice with tumors, half of which were placed in special cages capable of simulating the effects of restless sleep, and the other half in normal cages. After four weeks, tumors developed by guinea pigs housed in cages simulating sleep disturbances were twice as large as tumors from guinea pigs that slept continuously. Continuing in a new series of experiments, the researchers showed that these tumors are also much more aggressive, identifying a link of this phenomenon with certain cells of the immune system called tumor-associated macrophages. There are two types of these cells: TM1 that induce a strong immune response against cancer, and TM2 that instead create a fertile ground for the spread of the tumor, increasing its vascularization and - therefore - promoting its growth. Analyzing the tumors

developed in the presence of restless sleep, the researchers discovered the presence in very high quantities of TM2 associated with high levels of a molecule called TLR4 (Toll Like Receptor 4). At this point, the researchers replicated the experiment by injecting cancer cells into guinea pigs that were genetically modified to not express the TLR4 molecule; as a result of the new experiment, the differences in the development of neoplasms between animals with sleep disorders and those who slept regularly disappeared completely.

A further study by Torres M. et al hypothesized a link between OSA and the development of lung cancer. Observing the laboratory mice, the researchers have shown that the lack of oxygen intermittently during sleep accelerates the growth of lung cancer, but only in younger subjects; possibly due to a different response of macrophages and tumor-associated lymphocytes [139].

Recently a study conducted on 19.000 patients suffering from obstructive sleep apnea found a correlation between this disorder and the diagnoses of breast and prostate cancer, showing that the probability of a double diagnosis - OSA and cancer - is higher in women [140]. The researchers analyzed data from 19,556 people included in the European database on sleep apnea (ESADA) to explore the link between the severity of this disorder, low blood oxygen levels and cancer development. Participants included 5.789 women and 13.767 men, and for each participant, age, body mass index, smoking and alcohol consumption level were assessed, factors that could affect the risk of developing cancer. To assess the severity of OSA and the link to cancer development, the researchers looked at how many times the participants had partial or complete airway closure during sleep and how many times their blood oxygen levels during the night. they fell below 90%. The data obtained showed that the odds of cancer diagnosis were higher in women with OSA than in women without OSA and/or compared to men with OSA. Furthermore, breast cancer was most frequently encountered in women, while prostate cancer in men.

In addition to cancer incidence, OSA has also been linked to higher rate of cancer mortality.

In this regard, a study by the University of Foggia shows that there is a relationship between the outcomes of colorectal cancer and obstructive sleep apnea [141]. The study, published in the Journal of Oncology, shows that the presence of OSA is an independent prognostic factor of survival. Beyond the prognostic relevance, this finding could also have therapeutic implications. A prospective court of 52 patients with metastatic solid tumors, 29 of whom with metastatic colorectal cancer (mCRC), was analyzed. 34.6% of all patients and 34.5% of mCRC patients had OSA. In patients with mCRC, the presence of OSA correlated significantly with a reduced disease control rate (60% versus 94.7%). The authors argue that three possible main mechanisms may be involved in the relationship between OSA and colorectal cancer (CRC). Hypoxia could: i) increase the expression of a

transcription factor (HIF-1) involved in angiogenesis and resistance to therapy, ii) modulate cells of the immune system, hindering anti-tumor responses and promoting some pro - tumors, iii) sleep disturbance could alter circadian rhythms which are now known to be important for the expression of genes involved in cell proliferation and DNA repair.

All of these studies suggest that people who suffer from airway obstruction during sleep and whose blood oxygen saturation levels frequently drop below 90% are more likely to be diagnosed with cancer than people without OSA.

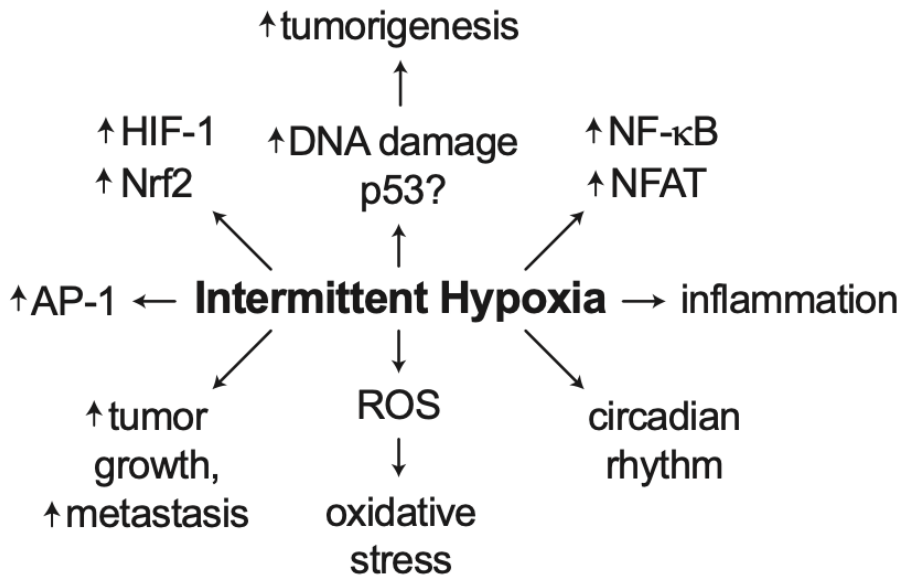
To date, no data have been published about the effect of CPAP therapy on cancer, and potentially innovative clinical trials are underway in this area. CPAP, in fact, thanks to its ability to attenuate hypoxemia and to modify the transcriptional profile of genes involved in cancer could represent an important ally in order to improve the therapeutic response in patients with cancer and OSA, however, further studies are necessary to support this hypothesis.

4.4. MOLECULAR MECHANISMS UNDERLYING IH AND CANCER

There are various postulated mechanisms by which intermittent hypoxia may affect tumor biology. In the setting of OSA, the rapidity of cyclical hypoxia may selectively activate or inhibit certain pathways in contrast to chronic hypoxia or what has been traditionally defined as “intermittent hypoxia.” The general mechanisms thought to play a role include oxygen-sensing pathways, oxidative stress, inflammation, endothelial function, clonal selection, and altered immune response (**Figure 7**). Transcription factors and their regulation (including post- translational modifications) are likely the fundamental link between rapid cyclical hypoxia and these downstream effects.

Intermittent hypoxia can act as a selective force in the clonal evolution of a tumor, selecting for populations with defects in DNA repair and resistance to apoptosis [142]. Furthermore, mutations in tumor suppressor genes may sensitize cells to the effects of intermittent hypoxia. For example, cells lacking functional p53 are more susceptible to genomic instability and potentially tumorigenesis if they experience cycles of hypoxia/reoxygenation [143].

Figure 7. Effects of IH in cancer development and progression



Intermittent hypoxia may also exert effects on the tumor by acting on cells in the tumor microenvironment, particularly endothelial cells. The vascular endothelium forms the interface between tissues and blood, and it regulates the passage of nutrients and oxygen while maintaining blood flow. Therefore, endothelial cells are particularly susceptible to the effects of fluctuating blood oxygen. Their behavior can be altered by the intermittent hypoxia seen in OSA, largely through changes in transcriptional regulation. This may have consequences for solid tumors, particularly since chronic hypoxia is known to cause endothelial dysfunction, which can increase tumor growth, angiogenesis, and metastasis [14]. In OSA, intermittent hypoxia can weaken the endothelial wall [144], creating gaps where tumor cells may escape more easily into the blood stream. A study using lung microvascular endothelial cells found that intermittent hypoxia led to reorganization of cytoskeleton and junction proteins causing endothelial barrier dysfunction [145]. In addition, the hypoxia-induced upregulation of secreted angiogenic growth factors in both tumor cells and endothelial cells can lead to vascular remodeling and angiogenesis [146]. All of these changes can alter tumor behavior and will be important to study when examining the effects of OSA on a tumor. The immune response is another important component of the tumor microenvironment, and the aggressive tumor phenotype observed in intermittent hypoxia may be dependent on changes to immune cells. There is some evidence that the host immune response is altered in mice in intermittent hypoxia via its effect on tumor-associated macrophages (TAMs). There is a reduction in tumor-inhibitory (M1) TAM phenotype and a shift toward the M2 tumor-promoting phenotype [147]. TAMs are viewed as an important component of the cancer microenvironment and can

contribute to tumor growth and invasion by releasing growth factors and proangiogenic cytokines. This response may be disease specific and is possibly altered by the local microenvironment or other factors. In this context, Murphy et al. found that intermittent hypoxia induced a shift toward M1 macrophage polarization in adipose tissue in a diabetes model using lean and diet-induced obese mice [148].

Disruption of circadian rhythm by OSA and/or associated intermittent hypoxia may also play a role in deregulated cell proliferation and cancer progression. The transcriptional regulation of circadian rhythm is disrupted by both OSA [149] and hypoxia [150,151], and deregulated circadian rhythm has been linked to cancer [152].

Given that inflammatory pathways are also linked to OSA, the systemic or local inflammatory response can influence tumor cell behavior. Oxidative stress and increased levels of reactive oxygen species are associated with OSA, and systemic increases in proinflammatory molecules such as interleukin 6 and 8 (IL-6/IL-8) and tumor necrosis factor-α (TNF-α) are seen [153]. It is also possible that the increase in sympathetic activity associated with the carotid body response to intermittent hypoxia may affect the tumor and its microenvironment [154]. The sympathetic response has also been linked to breast cancer metastasis [155]. Interestingly, the sympathetic response in OSA is partially driven by the transcriptional changes in the carotid body by the hypoxia-inducible factors [156]. The effects of IH from OSA augment both the immediate carotid body response to acute hypoxia [157,158] and longer activation of the carotid body that persists for several hours past the end of intermittent hypoxia, which is known as sensory long-term facilitation [159].

Potentially deleterious physiological processes, such as sympathetic activity and inflammation, are controlled largely through changes in gene expression and, therefore, protein expression and activity. This alters both the behavior of individual cells and more complex organ systems and the surrounding microenvironment. For example, IH from OSA activates HIF in the carotid body. Overactivation of HIF triggers the sympathetic response and subsequent systemic hypertension [156]. Therefore, transcription factors, which control gene expression, are a central node for controlling physiological changes that lead to long-term adaptations. Several transcription factors have been implicated in the pathological effects of OSA, in particular HIF and NF-κB, as well as the less studied (at least in intermittent hypoxia) activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT), and nuclear factor-like 2 (Nrf2).

Transcriptional activation leads to the expression of many proposed “biomarkers of OSA” and the associated systemic inflammatory and oxidative stress responses. This includes increased levels of IL-6, IL-8 and IL-10, TNF–α, C-reactive protein, and adhesion molecules (ICAM-1 and VCAM-1) [160].

Many of the genes encoding these biomarkers have promoter sites for HIF and NF-κB and, therefore, are subject to regulation by them.

To sum up, IH can activate a range of transcription factors, such as HIF-1, AP-1, NF-κB, NFAT and Nrf2, to express specific genes that are important for long-term adaptation [133].

In addition, intermittent hypoxia generates reactive oxygen species (ROS), which likely increases oxidative stress, inflammation, and DNA damage and could lead to increased tumorigenesis. Then, IH increases tumor growth and metastasis in animal models, mostly through poorly understood mechanisms.

5. MICRORNA

MicroRNAs (miRNAs) are a huge family of small (around 20-22 nucleotide-long) single-stranded non-coding RNAs, often evolutionary conserved among distant species, that have been classified as important post-transcriptional regulators of gene expression, binding to the 3' untranslated region (UTR) of a target mRNA and repressing their translation [16,17]. It is predicted that miRNAs represent 1-5% of the human genome and regulate at least 30% of protein-coding genes [161]. To this date has been estimated the discovery of ~ 2300 human mature microRNAs with 1115 being annotated in miRBASE V22 [162]. These small sequences have crucial function in several developmental and cellular processes. In eukaryotic species, such as vertebrate neural development [163], they play a key role in controlling genes implicating in neocortical development [164] as well as adult neural functions pathways. The first microRNA to be discovered was lin-4 that initially had been characterized as regulator of temporal development in C. elegans larvae [165]. Ambros and Ruvkun groups observed that a mutation in lin-4 would give an opposite effect on another gene, lin-14 [166]. Later, they revealed that lin-4, actually, is a small non-coding RNA and that its sequence is able to bind to lin-14 gene through its 3'UTR, thus, controlling and regulating lin-14 at posttranscriptional level [167,168]. Notably, lin-4 mutation causes a heterochronic development phenotype, suggesting since the beginning of their discovery that miRNAs are involved in regulating the timing of developmental process.

5.1. MICRORNAs BIOGENESIS AND REGULATION

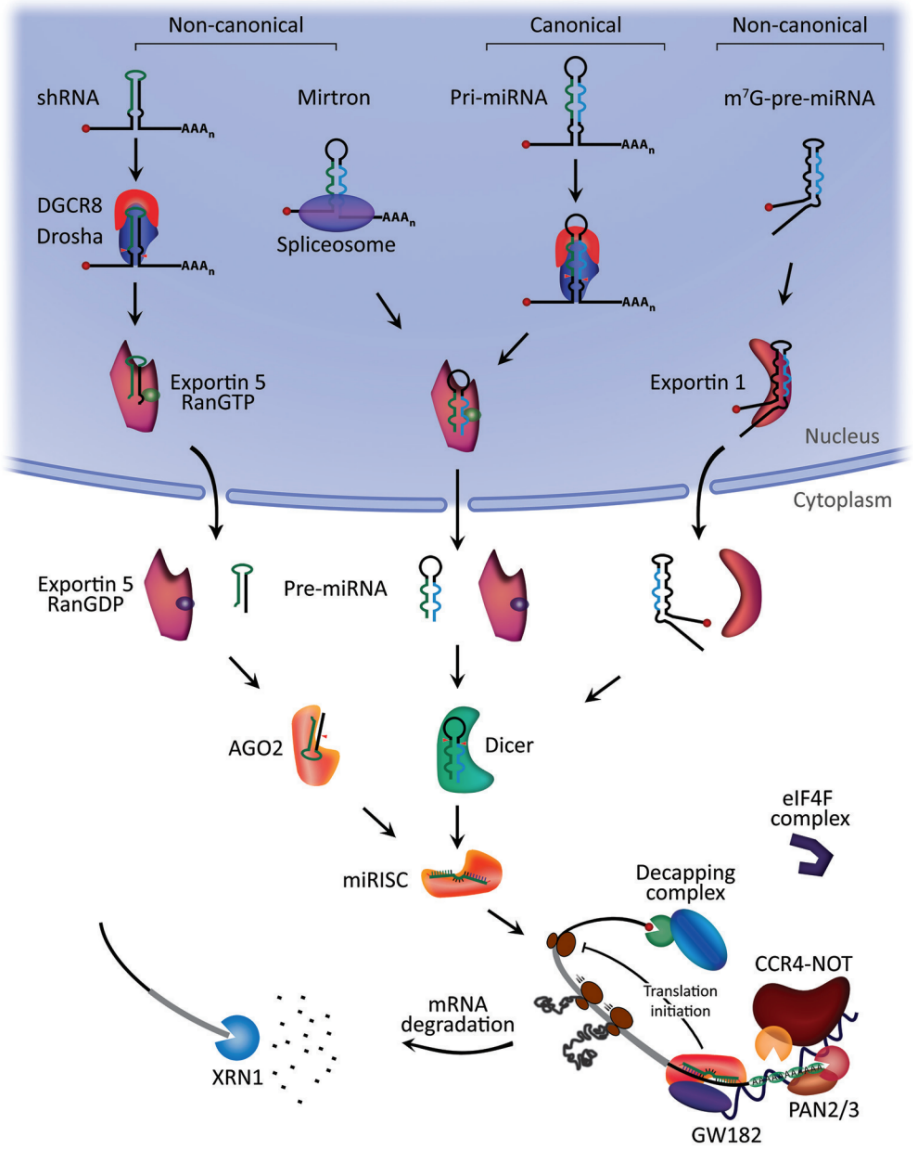
The biogenesis of miRNAs is a complex process that takes place in the nucleus and in the cytoplasm (**Figure 8**). MiRNA biogenesis starts with the processing of RNA polymerase II/III transcripts post- or co-transcriptionally [17]. About half of all currently identified miRNAs are intragenic and processed mostly from introns and relatively few exons of protein coding genes, while the remaining are intergenic, transcribed independently of a host gene and regulated by their own promoters [169]. Sometimes miRNAs are transcribed as one long transcript called clusters, which may have similar seed regions, and in which case they are considered a family [169]. The biogenesis of miRNA is classified into canonical and noncanonical pathways.

The canonical biogenesis pathway is the dominant pathway by which miRNAs are processed. In this pathway, pri-miRNAs are transcribed from their genes and then processed into pre-miRNAs by the microprocessor complex, consisting of an RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and a ribonuclease III enzyme, Drosha [170]. DGCR8 recognizes an N6-methyladenylated GGAC and other motifs within the pri-miRNA [171], while Drosha cleaves the pri-miRNA duplex at

the base of the characteristic hairpin structure of pri-miRNA. This results in the formation of a 2 nt 3′ overhang on pre-miRNA [172]. Once pre-miRNAs are generated, they are exported to the cytoplasm by an exportin 5 (XPO5)/RanGTP complex and then processed by the RNase III endonuclease Dicer [170,173]. This processing involves the removal of the terminal loop, resulting in a mature miRNA duplex [174]. The directionality of the miRNA strand determines the name of the mature miRNA form. The 5p strand arises from the 5′ end of the pre-miRNA hairpin while the 3p strand originates from the 3′ end. Both strands derived from the mature miRNA duplex can be loaded into the Argonaute (AGO) family of proteins (AGO1-4 in humans) in an ATP-dependent manner [175]. For any given miRNA, the proportion of AGO-loaded 5p or 3p strand varies greatly depending on the cell type or cellular environment, ranging from near equal proportions to predominantly one or the other [176]. The selection of the 5p or 3p strand is based in part on the thermodynamic stability at the 5′ ends of the miRNA duplex or a 5′ U at nucleotide position 1 [177]. Generally, the strand with lower 5′ stability or 5′ uracil is preferentially loaded into AGO, and is deemed the guide strand. The unloaded strand is called the passenger strand, which will be unwound from the guide strand through various mechanisms based on the degree of complementarity. The passenger strands of miRNA that contain no mismatches are cleaved by AGO2 and degraded by cellular machinery which can produce a strong strand bias. Otherwise, miRNA duplexes with central mismatches or non-AGO2 loaded miRNA are passively unwound and degraded [178].

To date, multiple non-canonical miRNA biogenesis pathways have been elucidated (**Figure 8**). These pathways make use of different combinations of the proteins involved in the canonical pathway, mainly Drosha, Dicer, exportin 5, and AGO2. In general, the non-canonical miRNA biogenesis can be grouped into Drosha/DGCR8-independent and Dicer-independent pathways. Pre-miRNAs produced by the Drosha/DGCR8- independent pathway resemble Dicer substrates. An example of such pre-miRNAs is mirtrons, which are produced from the introns of mRNA during splicing [179]. Another example is the 7-methylguanosine (m7G)-capped pre-miRNA. These nascent RNAs are directly exported to the cytoplasm through exportin 1 without the need for Drosha cleavage.

Figure 8. MicroRNA biogenesis



There is a strong 3p strand bias most likely due to the m7G cap preventing 5p strand loading into Argonaute [180]. On the other hand, Dicer-independent miRNAs are processed by Drosha from endogenous short hairpin RNA (shRNA) transcripts [181]. These pre-miRNAs require AGO2 to complete their maturation within the cytoplasm because they are of insufficient length to be Dicer-substrates [181]. This in turn promotes loading of the entire pre-miRNA into AGO2 and AGO2-dependent slicing of the 3p strand. The 3' -5' trimming of the 5p strand completes their maturation [182].

Target recognition and regulation by miRNAs is by itself a complex process [169]. The small size of miRNAs and the pairing between a miRNA and a target site that does not need to be perfect, offers a wide selection of genes that can be subject to regulation. Indeed, one miRNA can regulate multiple

mRNAs. However, the property that makes miRNAs versatile also hampers the prediction of putative targets. Although there are some exceptions, in animals, miRISC form partial duplexes with their targets and usually in the 3' UTR. The ordinary pairing between miRISC and mRNA is perfect between nucleotides 2 and 8 at the 5' end of the miRNA, known as the 'seed' region, and the target site. Due to the small size of the 'seed' region one miRNA can pair with mRNA in more than one location and with different conformations of the bulge. These different conformations can alter miRNA repression efficiency. The mechanism of miRNAs action has been under scrutiny, since it can occur by mRNA destabilization, translational repression and contrary to what is assumed, activates gene expression. Perfect pairing of the miRNA–mRNA duplex leads to endonucleolytic cleavage of the mRNA by Argonaute. This mechanism is more common in plants, but nevertheless can occur also in animals. Destabilization of the target mRNA through other mechanisms is a common outcome of miRNA regulation in animals. The miRISC complex usually associates with GW182 proteins. Depending on what proteins GW182 recruits next, the process of destabilization is different. Thus, if GW182 recruits CCR4-NOT complex, the mRNA becomes susceptible to exonucleolytic degradation of its poly(A) tail. If GW182 recruits CCR4-NOT complex and PABPC protein, mRNA repression will be prior to translation initiation, if it does not recruit PABPC it will be after translation initiation. Finally, miRISC can associates with fragile X mental retardation protein 1 (FMR1), stimulating gene expression [183]. This is a much rarer destabilization mechanism. This mode of action makes miRNAs extremely versatile and relevant to many cell mechanisms. Thus, their expression deregulation easily becomes involved in many diseases. Indeed, miRNAs have already been described as being relevant in viral diseases [184], bacterial infections [185], multiple sclerosis [186], type 2 diabetes [187], Parkinson's disease [188], Alzheimer's disease [189] and cancer [190-192], among others.

Above, I will focus on how miRNAs influence cancer and how cancer influences miRNAs expression.

5.2. MICRORNA IN CANCER

Due to their characteristics and their broad influence in cell homeostasis, soon after their discovery, miRNAs were associated with cancer [193,194]. In the past years it became evident that miRNAs expression levels differ between normal and tumor cells, have tissue-specific expression signatures and promote or suppress tumor development and progression, thereby influencing all the hallmarks of cancer postulated by Hanahan and Weinberg [195].

Cancer development involves multiple-step alterations in oncogenes and tumor suppressor genes over a period of time. Abundant data have already been published in respects to how important the

role of miRNAs is among many pathways involved in the pathogenesis of cancer. MiRNAs can function as oncogenes or tumor suppressors in the majority of cancers. Tumor suppressor miRNAs act by repressing oncogenes. These are usually down-regulated in cancer and the majority of the miRNAs are considered tumor suppressors. However, only few miRNAs have been already described as truly tumor suppressors, with functional data published. Curiously, some miRNAs can act as both suppressors and oncogenes, depending on the microenvironment. Oncogenic miRNAs, also known as oncomiRs, are much less frequent and tend to up-regulate oncogenes or suppress tumor suppressor genes [20].

To summarize, it is clear that miRNA expression is dysregulated in human malignancies. The underling mechanisms include chromosomal abnormalities, transcriptional control changes, epigenetic changes and defects in the miRNA biogenesis machinery [196].

Firstly, abnormal miRNA expression in malignant cells compared with normal cells are often attributed to alterations in genomic miRNA copy numbers and gene locations (amplification, deletion or translocation).

Secondly, miRNA expression is tightly controlled by different transcription factors, so abnormal expression of miRNA in cancer could be due to dysregulation of some key transcription factors, such as c-Myc and p53.

In addition, the epigenetic alteration is a well-known feature in cancer, including global genomic DNA hypomethylation, aberrant DNA hypermethylation of tumor suppressor genes and disruption of the histone modification patterns. It is believed that miRNAs, similar to protein-coding genes, are also susceptible to epigenetic modulation.46,47

Finally, as described above, miRNA biogenesis is elaborately controlled by several enzymes and regulatory proteins, such as Drosha, Dicer, DGCR8, Argonaute proteins and exportin 5, allowing correct miRNA maturation from primary miRNA precursors. Therefore, mutation or aberrant expression of any component of the miRNA biogenesis machinery could lead to abnormal expression of miRNAs [196].

In conclusion, many studies have demonstrated the expression of miRNAs is dysregulated in different tumors. Such dysregulation could be caused by multiple mechanisms, including amplification or deletion of miRNA genes, abnormal transcriptional control of miRNAs, dysregulated epigenetic changes and defects in the miRNA biogenesis machinery. Cancer cells with abnormal miRNA expression evolve the capability to sustain proliferative signaling, evade growth suppressors, resist cell death, activate invasion and metastasis and induce angiogenesis. MiRNA may function as

either tumor suppressor or oncogene under certain circumstances. Although miRNAs have multiple targets, their function in tumorigenesis could be due to their regulation of a few specific targets. Therefore, a future challenge will be to identify the critical targets of the miRNAs involved in cancer and establish their contribution to malignant transformation. Genome-wide profiling demonstrates that miRNA expression signatures are associated with tumor type, tumor grade and clinical outcomes, so miRNAs could be potential candidates for diagnostic biomarkers, prognostic biomarkers, therapeutic targets or tools. However, more efforts are still needed to screen miRNA candidates by deep sequencing and validate them as diagnostic and prognostic biomarkers in a large cohort of patient samples.

5.3. HYPOXIA-REGULATED MICRORNAs (HRMs)

In recent years, some hypoxia-inducible microRNAs (HRMs, hypoxia-regulated microRNAs) have been identified [197]. These HRMs are often activated in different types of cancers, such as breast and colon, suggesting their role in tumorigenesis. Evaluating changes in miRNA expression during hypoxia is critical to understanding the role of miRNAs in many diseases, such as cancer, and in numerous inflammatory processes [23].

To date, the mechanisms that regulate gene expression during hypoxia are not entirely clear, however, we know that many miRNAs are involved in the development of cancer.

In general, it is known that miRNAs are directly involved in the formation of tumors and we also know that about 6% of miRNAs have putative hypoxia response elements (HRE) sites in their DNA, indicating these miRNAs as possible targets of HIF and suggesting the possibility of their role associated with hypoxia [23].

As mentioned above, previous studies have discovered a number of miRNAs that are differentially expressed in response to hypoxia. For instance, miR-210, miR-155, miR-372, miR-373 and miR-10b were found to be up-regulated [198-202], whereas miR-20b and miR-200b were found to be down-regulated, in response to hypoxia [203,204] (**Table 1**).

Table 1. Differentially expressed miRNAs in response to hypoxia

UP-REGULATED miRNAs	DOWN-REGULATED miRNAs
miR-10b, miR-103, miR-107, miR-125a, miR-152, miR-155, miR-181b, miR-188, miR-191, miR-193b, miR-203, miR-205, miR-206, miR-21, miR-210, miR-213, miR-224, miR-23, miR-24, miR-26, miR27, miR-30a-5p, miR-30c, miR-30d, miR322, miR-333, miR-335, miR-339, miR373, miR-451, miR-491, miR-497, miR512-5p, miR-562, miR-93	let-7f, miR-128b, miR-150, miR-159, miR-17-92, miR181d, miR-196a, miR-196b, miR-199a, miR-199b, miR200a, miR-200b, miR-20b, miR-22, miR-25, miR-30, miR-424, miR-449, miR-489

Hypoxia response elements (HRE) contained in the promoter regions of HRM genes can be bound by the α and β subunits of HIF-1, and hypoxia can improve the affinity of such a complex thereby promoting the transcription of HRMs. Many HRMs, such as miR-210, -155, and -373, have been demonstrated to contain HREs by which HIF-1 regulates the expression of these HRMs [198,199, 205]. Many transcription factors, such as TWIST, PPARγ, and GATA1, can be regulated by HIF-1 at the transcriptional level [206-208]. As a result, HIF-1 can be involved in the regulation of miRNA expression by influencing these transcription factors.

For example, when HIF-1 is stabilized by insufficient oxygen levels, TWIST can be induced to up-regulate miR10b [209]. MiR-10b is a well-documented oncogenic miRNA that mediate the metastasis of various human cancers [210,211]. Therefore, HRM expression induced by HIF-1 may account for the mechanism by which hypoxia facilities tumor progression. Intriguingly, studies also reported that HIF-1 had been associated with down-regulation of miRNAs.

In addition to HIF-1, other genes and signaling pathways may also contribute to the adaptation of tumor cells to hypoxia. For example, oxygen deprivation could promote the induction of miR-21 via an Akt2-dependent process. The hypoxia-generated signals transduced by Akt2 have been reported to increase the activity of NF-κB and CREB, which were able to transcriptionally up-regulate the expression of miR-21 [212]. Hypoxia was also involved in the biogenesis of miRNA. The protein Ago2 is a crucial component of the RNA induced silencing complex (RISC), and the hydroxylation of Ago2 is a key step for the assembly of Ago2 to heat shock protein 90 (Hsp90) in RISC [213].

Thanks to the signature of regulating hundreds of target genes simultaneously, miRNAs are capable of repressing the expression of genes associated with hypoxia.

Therefore, when low O_2 induces the expression of HRMs, genes targeted by these miRNAs stabilize HIF-1 by forming positive-feedback loops. Moreover, some HRMs are also involved in the

destabilization of HIF-1. The down-regulation of miR-20b, miR-199, and miR-17-92 by hypoxia stabilized HIF-1 because these HRMs were able to repress the expression of HIF-1 through direct targeting [214,215].

Even though a number of studies on hypoxia and human cancer have been published, the physiological and pathophysiological regulation of hypoxia is still poorly understood [216].

As the cellular response to hypoxia involves the activation of several transcriptional regulators involved in inflammation, tumor invasion, angiogenesis, cell cycle block and apoptosis, I strongly believe that a better understanding of all these closely related mechanisms, as well as the identification of miRNAs sensitive to hypoxia, may prove fruitful in the search for new therapeutic targets and in the search for new and more effective anti-tumor therapies [23].

5.4. MICRORNA AND OSA

Recent studies revealed that expression patterns of specific microRNAs are associated with OSA development and progress [18].

In particular, a study by Santamaria-Martos et al. showed that patients with OSA exhibit a dysregulated miRNA profile, compared to non-OSA patients [217]. In this regard, it appears that intermittent hypoxia, associated with OSA, may lead to differential expression of some hypoxia-induced miRNAs. This evidence suggests the importance of miRNAs and the pathways regulated by them. An alteration in their expression could result in a deregulation of key genes and pathways that contribute to the development and progression of OSA.

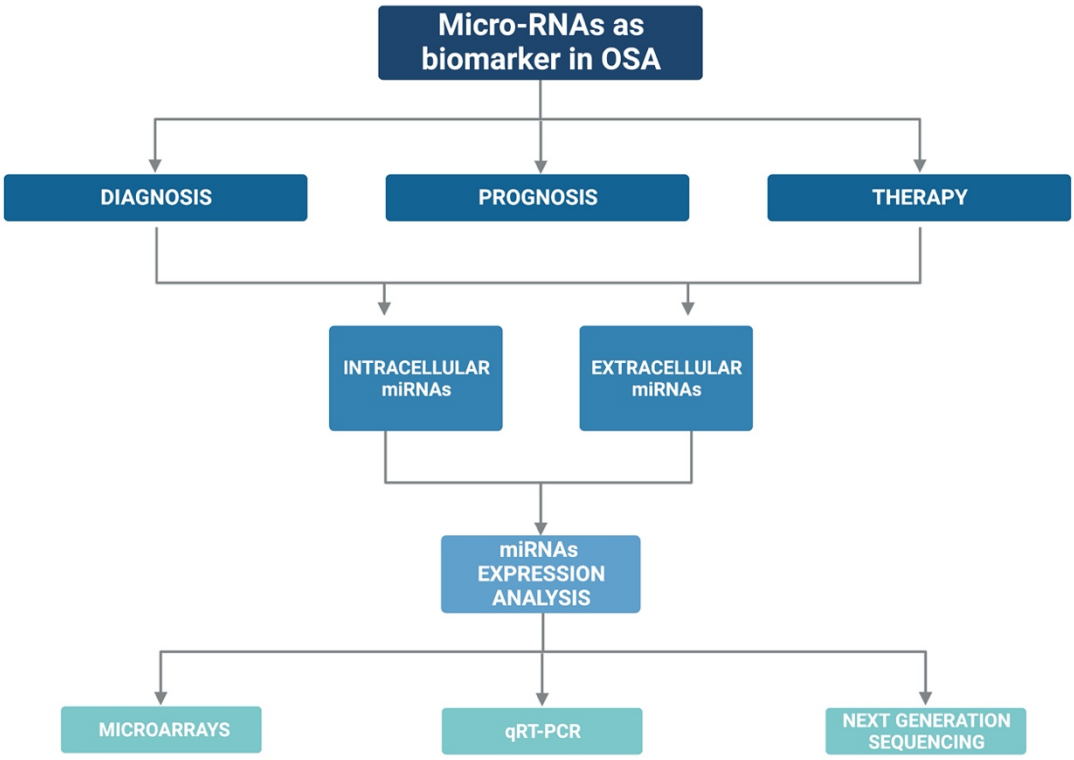
To date we known that microRNAs can be found in various biological fluids such as saliva, urine, bronchoalveolar lavage (BAL), plasma, serum and sputum and can play a significant role as potential biomarkers especially for diagnosing disease, for monitoring the therapeutic effect of a drug or for predicting tumor recurrence in patients treated with chemotherapy.

In particular, micro-RNAs seems to have many of the main features of a good biomarker: i) they are stable in the circulation, ii) they are resistant to digestion by RNase, iii) they are able to withstand extreme pH, high temperatures, long-term storage and multiple freeze-defreeze cycles [218].

Currently, there are several assumptions regarding their stability in the circulation: i) miRNAs may have unique modifications, such as methylation, adenylation, and uridylation that increase their stability and thus protect them against RNases [219]; ii) miRNAs could be protected by encapsulation in cell-derived microvesicles [220] or by specific RNA-binding proteins [221,222]. In addition, the expression of miRNAs can be easily assessed with various laboratory methods such as

quantitative q-PCR, microarray and sequencing [223,224]. **Figure 9** graphically represents the above mentioned.

Figure 9. Graphic representation on the possible role of miRNAs as biomarkers in OSA



Changes in miRNA expression levels are often associated with various diseases or some biological / pathological phases, such as OSA. So, all these aspects make miRNAs potential diagnostic, prognostic and therapeutic biomarkers for OSA.

As part of the OSA, a study that demonstrates how the analysis of microRNAs could allow the characterization of OSA and could help provide a more accurate diagnosis of the disease was recently published by Santamaria-Martos et al [217]. In this regard, in this study the expression profile of circulating miRNAs in patients with OSA and in non-OSA patients was examined to study the differences. The authors conducted an observational study of 230 male and female adults suspected of having OSA. All subjects underwent full PSG. The expression profile of plasma miRNAs was performed by array and validated with RT-qPCR. This analysis revealed that 6 miRNAs were significantly down regulated in male patients with OSA. The 6 miRNAs, that from this first analysis were found to be differently expressed in patients with OSA, were also evaluated after 6 months of

treatment with CPAP. The results showed that miRNA levels were increased in patients treated with OSA compared with patients without OSA. However, only for miR-345 did they demonstrate a significant difference. This study allowed to identify a cluster of miRNAs useful for differentiating non-OSA patients and patients with OSA and to associate CPAP treatment with a change in the profile of circulating miRNAs. They demonstrated that CPAP treatment can induce changes in the miRNA profile and therefore, microRNAs may be useful for treatment monitoring.

The same research group also published a study focusing on plasma miRNAs in which they explored the use of a miRNA panel in patients with resistant hypertension and OSA to study their role in predicting therapeutic response to continuous positive airway pressure (CPAP) [225]. They examined a pool of miRNAs in patients with OSA and resistant hypertension, a condition often linked to the disorder, and evaluated the effect of 3 months of CPAP treatment on their blood pressure. Albeit this investigation has provided evidence of miRNAs as markers in patient-guided therapy, most publications have focused on the potential role of miRNAs as diagnostic markers of disease.

One of the first researches conducted on the subject was that of Li et al [226]. In this work, starting from serum samples and combining sequencing and a bioinformatics approach, the authors were able to identify 104 miRNAs as potential markers of pathology. To verify the results, they conducted RT-PCR analyzes which revealed that miR-107, 199-3p and miR-485-5p were found to be downregulated, while miR-574-5p appeared upregulated, in patients with OSA compared to healthy controls. Numerous studies have been published on this topic in recent years.

Freitas et al. [227], by means of microarray analysis, examined the expression of about 2500 miRNAs in overweight adult males divided, after a PSG, into non-OSA, mild OSA, moderate OSA and severe OSA. The results were validated by RT-PCR and showed that miR-320e and miR-1254 exhibited a gradual increase in expression in relation to the severity of OSA.

Shao H et al. studied the expression profile of a series of miRNAs in the serum of patients with obstructive sleep apnea-hypopnea syndrome providing a theoretical basis for the search for molecular targets for the clinical diagnosis and treatment of this pathology [228].

Khalyfa A et al. have conducted various studies on the use of microRNAs as potential biomarkers for patients with OSA; one of these was conducted on a population of children with OSA and obesity and led to the identification of a subset of plasma miRNAs involved in endothelial function [229-231].

Since, as described above, OSA is associated with multiple comorbid conditions in the scientific community, interest is growing in the exploration of biomarkers to understand the mechanisms related to the disease and improve the stratification of OSA risk.

With regard to cardiovascular complications, a recent study has brought to light the role of miR-126a-3p in patients with OSA-associated hypertension, suggesting it as a new potential therapeutic target for the treatment of OSA-associated hypertension [232].

Yang X et al. likewise examined the serum miRNAs of patients with OSA, non-hypertensive and hypertensive and in "control" subjects [233]. The miRNA profile was evaluated by microarray and validated by RT-qPCR. One group of miRNAs showed a significant difference in OSA expression compared to controls. Let-7d-5p, miR-145-5p and miR-320b were less expressed in the non-hypertensive OSA group than in controls, while miR-26a-5p and miR-107 were less expressed in patients with hypertensive OSA than in controls. Let-7d-5p was up regulated in hypertensive patients with OSA compared to controls. These results show how the combination of let-7d-5p and miR-145-5p could identify patients with non-hypertensive OSA, while miR-26a-5p, miR-107 and miR-126-3p could allow the identification of hypertensive OSA.

Slouka D et al., in order to improve OSA diagnostics, also conducted a study using miRNAs as potential biomarkers circulating in the blood in the diagnosis and risk assessment of cardiovascular complications [234]. Their results demonstrate that miR-499 is involved in the regulation of gene expression during hypoxia and that it could be a new diagnostic biomarker for OSA.

Turning to neurological disorders, Targa A et al. studied the profile of circulating miRNAs associated with OSA in patients with Alzheimer's. They observed a subset of 15 miRNAs expressed differently in Alzheimer's patients with OSA and without OSA suggesting a plasma miRNA signature associated with the presence of OSA in Alzheimer's patients [235].

Li et al. described the effect of atherosclerosis on the miRNA profile of patients with OSA [236]. The goal was to identify specific serum miRNAs useful as indicators of atherosclerotic disease in patients with OSA. For this purpose, they analyzed a total of 116 male and female adult patients divided according to the presence of OSA and the carotid intima-media thickness.

The miRNA profiles were first evaluated by sequencing and subsequently validated by RT-qPCR. The expression of miR-664a-3p was downregulated in OSA patients compared to controls (in all 3 OSA groups subdivided according to normal carotid intima-media thickness). The authors suggested the potential of miR-664-3p as a non-invasive biomarker of atherosclerosis in OSA.

Finally, Chen YC et al. investigated the anti-inflammatory role of miR-21 and miR-23 via the TLR / TNF-α signaling pathway [237]. They observed that both miRNAs were down-regulated in patients with OSA compared to patients with primary light snoring, while TNF-α gene expression was increased. These results indicate that miR-21-5p overexpression could be a future new therapy for OSA.

All the studies listed demonstrate how microRNAs may have enormous potential to function as clinical diagnostic biomarkers for many diseases. They play a fundamental role in the pathogenesis of OSA. However, more in-depth research and confirmatory experiments are needed to establish the role of these miRNAs as biomarkers.

6. AIMS

Hypoxia plays an important role in the tumor microenvironment by allowing the development and maintenance of cancer cells, but the regulatory mechanisms by which tumor cells adapt to hypoxic conditions are not yet well understood. MiRNAs are recognized as a new class of master regulators that control gene expression and are responsible for many normal and pathological processes. Studies have shown that HIF-1 regulates a panel of miRNAs, whereas some of miRNAs target HIF-1. The interaction between miRNAs and HIF-1 can account for many vital events relevant to tumorigenesis, such as angiogenesis, metabolism, apoptosis, cell cycle regulation, proliferation, metastasis and resistance to anticancer therapy.

The aims of this thesis are:

- *Evaluate the roles of hypoxia and miRNAs in cancer and evaluate how hypoxia could influence the expression of specific miRNAs in cells.*

Recent studies have shown that hypoxia induces the expression of different miRNAs, many of which are directly involved in the development of cardiovascular diseases and also in cancer formation and progression. These has shown that there is a different response between the condition of intermittent hypoxia compared to the chronic one, and the first one seems to be, in some cases, much more harmful in terms of stimulating the expression of some miRNA.

So, the first aim of this thesis is to evaluate in colorectal cancer cells the expression of a group of miRNAs, respectively miR-21, miR-23b, miR-26a, miR-27b, and miR-145, which are all induced by a hypoxic environment, to better understand how continuous hypoxia could change their signature and to identify their eventual role in colorectal cancer (CRC), which is one of the most frequent tumors in women and men [141].

- *To evaluate changes in miRNAs expression in controls or patients affected by OSA and/or cancer and in colorectal cancer cells exposed to intermittent hypoxia, and to evaluate their impact on tumor progression*

It is well known that hypoxia and inflammation coincide at several points in the biology of cancer. However, intermittent hypoxia triggers transcriptional responses which differ from ones elicited by continuous hypoxia, and the duration of the hypoxic and reoxygenation periods could modulate hypoxic preconditioning. The differential effects of continuous and intermittent hypoxia, which have been described at the molecular, cellular and systemic levels, highlight the interest of investigating the effects of a high-rate intermittent hypoxia mimicking OSA on tumor growth. A hypoxic environment triggers some adaptive cellular mechanisms that largely rely on the transcription of

hypoxia-inducible factor (HIF-1α), which can also be modulated by interaction with inflammatory molecules. HIF-1α acts as a regulator of vascular endothelial growth factor (VEGF) in cancer, promoting angiogenesis and contributing to tumor growth. The newly formed vascular network could, however, present structural and functional abnormalities, which lead to a reduction in both perfusion and delivery of oxygen to the tumor tissue, thereby, promoting tumor necrosis.

Based on the above, the second aim of the project, is to comprehend the regulation of the expression of the same miRNAs (previously studied for continuous hypoxia) associated with carcinogenesis by intermittent hypoxia, and to unravel the role of HIF-1 in this process. In that respect, I analyzed the expression of miR-21, miR-23b, miR-26a, miR-27b, miR-145 e miR-210 in subjects affected by OSA and/or cancer, and in colorectal carcinomatous cell lines (CaCo2) which were exposed to IH to study cell response to hypoxia. HIF-1 and miRNA inhibitors were used to decipher their respective roles in cell proliferation and migration.

7. MATERIALS AND METHODS

7.1. POPULATION

All this study is carried out in collaboration into the lab of respiratory diseases, University of Foggia and the HP2 lab. Four groups of patients and healthy controls have been evaluated.

Written informed consent was obtained from all subjects and an ethical approval was obtained from both institutional review board (Ethics Committee, Policlinico Riuniti of Foggia, Italy and Comité de Protection des Personnes Sud-Est V, Grenoble, France). Protocols conformed to the principles of the Declaration of Helsinki.

A total of 78 patients and healthy subjects were recruited. The groups were divided as follows: healthy controls (n=13); cancer patients (n=26); OSA patients (n=26); patients with OSA and cancer (ONCO-OSA, n=13).

Patients' baseline characteristics are reported in **Table 2**.

All subjects were selected according to age and BMI, in order to achieve similar age and BMI between groups. All the patients underwent clinical assessment, respiratory functional tests and blood sampling. Human fasted sera were collected and stored at −80°C until use.

Main data collected were as follows: comorbidities (hypertension, heart disease, diabetes, chronic obstructive pulmonary disease, asthma) and nocturnal respiratory data including apnea-hyponea index (AHI), average SpO_2, time with SaO_2 lower than 90% (TS90) and oxygen desaturation index (ODI). OSA was defined as AHI > 5.

Almost all ONCO-OSA and cancer patients had colorectal cancer (22 of 39 patients), the remainder had ovarian cancer (n=4), breast cancer (n=4), lung cancer (n=2), gallbladder cancer (n=2), pancreatic cancer (n=4) or prostate cancer (n=1). **Table 3** shows types of cancer affecting cancer and ONCO-OSA patients.

Table 2. Clinical data of patients

	TOTAL	**HEALTY CONTROLS (H)**	**CANCER PATIENTS (C)**	**OSA PATIENTS (O)**	**ONCO-OSA PATIENTS (OO)**	**P-VALUE**	**TUKEY POST-HOC**
	(n = 78)	(n = 13)	(n = 26)	(n = 26)	(n = 13)		
Sex, % male	54	62	38	62	62	0,2998	-
Age, years (range)	69 (41-84)	61 (51-77)	70 (42-84)	71(58-80)	68 (41-79)	0,0585	-
BMI, $kg \cdot m^{-2}$ (range)	25,1 (18,3-44,0)	25,0 (20,3-33,5)	24,2 (18,3-28,1)	25,3 (20,4-44,0)	28,6 (19,1-33,3)	0,1043	-
Comorbidities							
Hypertension, %	21	0	27	0	69	**<0,0001****	OO>C>O=H
Diabetes, %	17	15	23	12	15	0,7349	-
Heart disease, %	37	31	19	65	23	**0,0035***	OO>O=C=H
COPD, %	15	38	12	8	15	0,0818	-
Asthma, %	3	0	4	0	8	0,4798	-
Nocturnal respiratory data							
SpO_2 mean, % (range)	94,0 (84,4-97,0)	93,0 (86,0-96,0)	94,9 (89,8-97,0)	94,0 (86,5-96,0)	93,0 (84,4-94,9)	**0,0006****	C>H=OO=O
TS90, % (range)	0,3 (0,0-98,4)	0,0 (0,0-98,4)	0,2 (0,0-29,3)	0,1 (0,0-69,6)	9,8 (0,2-96,5)	**0,0011****	O>H=OO=C
ODI, $events \cdot h^{-1}$ (range)	7,7 (0,0-41,0)	5,7 (0,0-26,8)	2,0 (0,2-9,5)	16,9 (1,2-41,0)	11,7 (7,6-31,9)	**<0,0001****	OO=O>H=C
AHI, $events \cdot h^{-1}$ (range)	5,7 (0,0-35,9)	4,0 (0,0-5,7)	2,1 (0,5-5,0)	16,7 (5,6-35,9)	14,7 (7,0-33,0)	**<0,0001****	O=OO>C=H

Data are reported as median ± min/max and were analyzed using Kruskal-Wallis test followed by Tukey post-hoc test.

*p ≤0.05 and **p ≤0.001

BMI: body mass index; COPD: chronic obstructive pulmonary disease; SpO_2: oxygen saturation; TS90: total sleep time with spo2 <90%; ODI: oxygen desaturation index; AHI: apnoea–hypopnoea index.

Table 3. Characteristics of patients with cancer

Type of cancer	**TOTAL** (n = 39)	**CANCER PATIENTS** (n = 26)	**ONCO-OSA PATIENTS** (n = 13)	**P-VALUE**
Colorectum, %	57	54	69	**0,0001****
Ovary, %	11	8	15	0,157
Lung, %	5	4	8	0,477
Gallbladder, %	5	4	8	0,477
Breast, %	11	15	0	**0,036***
Pancreas, %	5	8	0	0,258
Prostate, %	3	4	0	0,581
Uterus, %	3	4	0	0,581

Data are reported as %.
*p ≤0.05 and **p ≤0.001

7.2. CELL CULTURE

Human colorectal adenocarcinoma cell lines (CaCo2) were purchased from American Type Culture Collection (ATCC). CaCo2 were maintained in Dulbecco's modified Eagle's medium (DMEM) 4500 mg/L glucose (Gibco™ – Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco™ – Sigma Aldrich, USA) at 37° C in a humidified atmosphere containing 5% CO_2 and submitted to different duration of normoxia, continuous hypoxia or intermittent hypoxia exposure.

7.3. CONTINUOUS HYPOXIA EXPOSURE

CaCo2 cells (1.0×10^6) were seeded in T25 flask (Corning® – Merck, Germany) with 5mL of growth medium. After 3 days, the cells had a confluence of about 70%. Just before exposing the cells to continuous hypoxia (CH), the culture medium was replaced with DMEM supplemented with 1% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cultured CaCo2 cells were exposed to continuous hypoxia in an incubator (GALAXY 48 R, Eppendorf s.r.l., Milan, Italy) with oxygen maintained at 2% for 2 h, 4 h, 8 h, and 24 h. Normoxic controls were maintained at 37 °C and 5% CO_2.

7.4. INTERMITTENT HYPOXIA EXPOSURE

CaCo2 cells (1.5×10^5) were seeded in 6 well semipermeable plates (Zell-Kontakt Imaging FC plates, Germany) precoated with type I collagen in complete culture medium. After 24 hours, the cells had a confluence of about 60/70%. Just before exposing the cells to N/IH, the culture medium was replaced with DMEM supplemented with 1% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cultured CaCo2 cells were exposed to intermittent hypoxia (IH) as described before [238], alternating cycles of 5 minutes of normoxia (16% PO_2) and 5 minutes of hypoxia (2% PO_2) using a custom-made plate holder connected to two gas blenders (Gas Blender 100, MCQ Instruments, Rome, Italy) and located inside a standard cell culture incubator (SANYO, MCO-15AC).

Shortly, this system involves the use of commercial gas permeable plates (ZellKontakt, Germany), which must be pre-coated with type I collagen and is made up of a custom-made plate holder (SMTEC, Nyon, Switzerland) connected to two gas blenders (Gas Blender 100, MCQ Instruments, Rome, Italy). Is located inside a standard cell culture incubator (SANYO, MCO-15AC) and allows simultaneous exposure of four multiwell plates (6/24 or 96 well). As happens during the night in patients with OSA, cycles of IH for 8h followed by 16h of normoxia (wakefulness in patients with OSA) were applied. Control cells were exposed to constant normoxia (N, 16% PO_2). PCO_2 was maintained at 5% throughout exposure. The cells were subjected to this intermittent hypoxia

system for up to 8h. A long exposure of 32h was also performed, consisting of 8h of IH followed by 16h of normoxia and another 8h of IH.

7.5. VIABILITY ASSAY

CaCo2 cells (3.0×10^4 cells/well) were seeded in 96-well plates and exposed to complete medium. Based on the various experiments conducted, either for normoxia, continuous hypoxia, and intermittent hypoxia (for 2, 4, 8, 24 and 32h), cell's viability was evaluated by MTT (1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan) according to the manufacturer's protocol (Sigma-Aldrich, Milan, Italy). The cell's viability was calculated as follows: % viability = [optical density $(OD)_{560}$ of cell/OD_{560} of control × 100].

7.6. RNA ISOLATION

For both sera and cells after N/CH or IH exposure, total RNA was extracted by using TRIzolTM Reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. Concentration and quality of the eluted RNA were measured using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific). RNA purity was evaluated with the absorbance ratio $A_{260/280}$.

7.7. qRT-PCR

A 2ng amount of total RNA was used for cDNA synthesis using TaqMan MicroRNA RT kit (Thermo Fisher Scientific), according to the manufacturer's protocol.

Quantitative real-time polymerase chain reaction (qRT-PCR) assays for the quantitative determination of miRNA expression were performed in duplicate using CFX96 Touch Real-Time PCR Detection System instrument (Bio-Rad Laboratories, Inc). RNU6B was used as endogenous control [239]. miRNA's expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method [240]. **Table 4** shows miRNA sequences used.

Table 4. miRNAs sequence

Assay Name:	*Assay ID*	*Species*	*miRNA Sequence*
RNU6B	001093	human	CGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTTT
hsa-miR-21-5p	000397	human	UAGCUUAUCAGACUGAUGUUGA
hsa-miR-23b-5p	002126	human	UGGGUUCCUGGCAUGCUGAUUU
hsa-miR-26a-5p	000405	human	UUCAAGUAAUCCAGGAUAGGCU
hsa-miR-27b-5p	002174	human	AGAGCUUAGCUGAUUGGUGAAC
hsa-miR-145-5p	002278	Human	GUCCAGUUUUCCCAGGAAUCCCU
hsa-miR-210-3p	000512	human	CUGUGCGUGUGACAGCGGCUGA

7.8. TRANSFECTION ASSAYS

CaCo2 cells were transfected with commercially available miRNA Inhibitors for miR-21, miR-23b, miR-26a and miR-210 (Anti-miR™ miRNA Inhibitor: hsa-miR-21-5p, hsa-miR-23b-5p, hsa-miR-26a-5p and hsa-miR-210-3p - Invitrogen™) using Lipofectamine™ 2000 Reagent (Invitrogen™, Carlsbad, CA, USA) according to manufacturer's instructions.

One day before transfection, CaCo2 cells (3.0×10^5) were seeded on a 6-well plate in 2ml of growth medium without antibiotics per well. After 24 hours, the cells had a confluence of about 80%.

Inhibitors and LipofectamineTM 2000 were diluted in Opti-MEM reduced serum medium. After incubation for 20 min at room temperature, the mixtures were applied in each well. Final 100 nM and 5µL were used for the miRNA inhibitors and Lipofectamine, respectively. Anti-miR™ miRNA Inhibitor Negative Control (Invitrogen™) was used as a negative control.

Complete fresh medium was replaced 6h later and the cells were incubated at 37 °C in 5 % CO_2 for 24h before RNA extraction or functional assays.

7.9. PROLIFERATION ASSAYS

Transfected CaCo2 cells (5.0×10^3) were seeded in 96-well semipermeable plates pre-coated with type I collagen and submitted to 32h of N/IH exposure. Cell viability was then assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) staining as follows: 20 µL of MTT reagent was added to each well at a final concentration of 0.5 mg/ml and incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO_2. The medium was then aspirated and crystals were dissolved in 100 µl DMSO/well. After 20 minutes at room temperature, the optical density at 560 nm (OD560) was determined using CLARIOstar® Plus plate reader (BMG LABTECH, Germany). DMSO 0.1% was used as a control. Cell viability was calculated using the following formula: Cell viability=OD_{560}(sample)/OD_{560} (control) x100.

7.10. TRANSWELL MIGRATION ASSAYS

After transfection, CaCo2 cells (5.0×10^5) were seeded in the top chamber of Transwell 8.0 µm pore polycarbonate membrane cell culture inserts (Corning®, CLS3422-48EA, Sigma Aldrich, USA) coated with type I collagen, in serum-free DMEM medium. The bottom part of the chamber was filled with 600 µL of complete DMEM medium supplemented with 10% FBS as chemoattractant. After 48 hours of exposure to N/IH, the culture medium was aspired, the insert was washed with 150 µL of PBS and the cells on the top chamber were gently removed using an absorbent paper pad. Cells attached to the lower layer of the membrane were then fixed with 600 µL of absolute ethanol for 10 minutes, stained with 600 µL of 0.2% crystal violet for 10 minutes at room temperature and imaged with an

optical microscope coupled to a camera with magnification × 10 (Olympus CK2, Olympus, USA). Migration ability was estimated by measuring optical density at 595nm (OD_{595}) after solubilization in 20% acetic acid.

7.11. HIF-1α GENE EXPRESSION

To determine the effect of N/CH exposure on HIF-1, HIF-1α expression was evaluated by qRT-PCR using SsoAdvanced ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), as specified by the manufacturer. Real-time reactions were set up in duplicate for each sample in 96-well plates in a reaction volume of 20 µL containing, respectively, 1X SsoAdvanced ™ SYBR® Green Supermix, 250 nM of specific primers, and 100 ng of cDNA.

The sequences of the primers used for amplification through qRT-PCR were listed in **Table 5**.

The reaction was carried out on the ABI-PRISM 7300 instrument according to the manufacturer's instructions. Gene expression was analyzed according to $2^{-\Delta\Delta Ct}$ relative quantification method using β-actin as internal control [241].

Following miRNA inhibition, total RNA was extracted as described above and reverse-transcribed by using iScript™ Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol.

Then, the expression of HIF-1α gene was evaluated by qRT-PCR using SsoAdvanced ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), as specified by the manufacturer.

Real-time reactions were set up in duplicate for each sample in 96-well plates in a reaction volume of 20 µL containing, respectively, 1X SsoAdvanced ™ SYBR® Green Supermix, 250 nM of specific primers, and 100 ng of cDNA.

The sequences of the primers used for amplification through qRT-PCR are listed in **Table 5**.

The reaction was carried out on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc) instrument, according to the manufacturer's instructions. Gene expression was analyzed according to $2^{-\Delta\Delta Ct}$ relative quantification method using β-actin as internal control [241].

Table 5. Primer sequences

Gene	*Species*		*Primer Sequence*
HIF-1α	human	FORWARD	5'-AAAATCTCATCCAAGAAGCC-3'
		REVERSE	5'-AATGTTCCAATTCCTACTGC-3'
β-actin	human	FORWARD	5'-GACGACATGGAGAAAATCTG-3'
		REVERS	5'-ATGATCTGGGTCATCTTCTC-3'

7.12. HIF-1α ACTIVITY

To determine the effect of N/IH exposure and acriflavine 0,5 μM on HIF-1α transcriptional activity, nuclear extract lysates were obtained from CaCo2 cells by using a Nuclear Extraction Kit (ab221978, Abcam). The protein concentrations of nuclear fractions were determined by Bradford assay using CLARIOstar® Plus plate reader (BMG LABTECH, Germany).

The activities of HIF1α in nuclear extract lysates were detected using the HIF-1α Transcription Factor Assay Kit (ab133104, Abcam) according to the manufacturer's protocols.

7.13. STATISTICAL ANALYSIS

Comparisons between groups were performed by ANOVA or Kruskal-Wallis test depending on whether the data were normally distributed or not. The data are presented as mean ± standard deviations (SD) or median ± range, depending on the normality of values.

Results were considered significant when p values were < 0.05.

All the statistical analyses were performed using GraphPad Prism software (version 9.0, GraphPad Software).

Spearman's correlation was used to assess relationships between miRNAs expression levels and main nocturnal respiratory data in OSA and ONCO-OSA patients.

Cluster analysis was performed on miRNAs' expression value in order to identify their relationships. A p-value below 0.05 has been considered statistically significant. GraphPad Software (version 9.0, GraphPad Software) and Orange (version 3.0, University of Ljubljana, Slovenia) were used for the analysis.

8. RESULTS

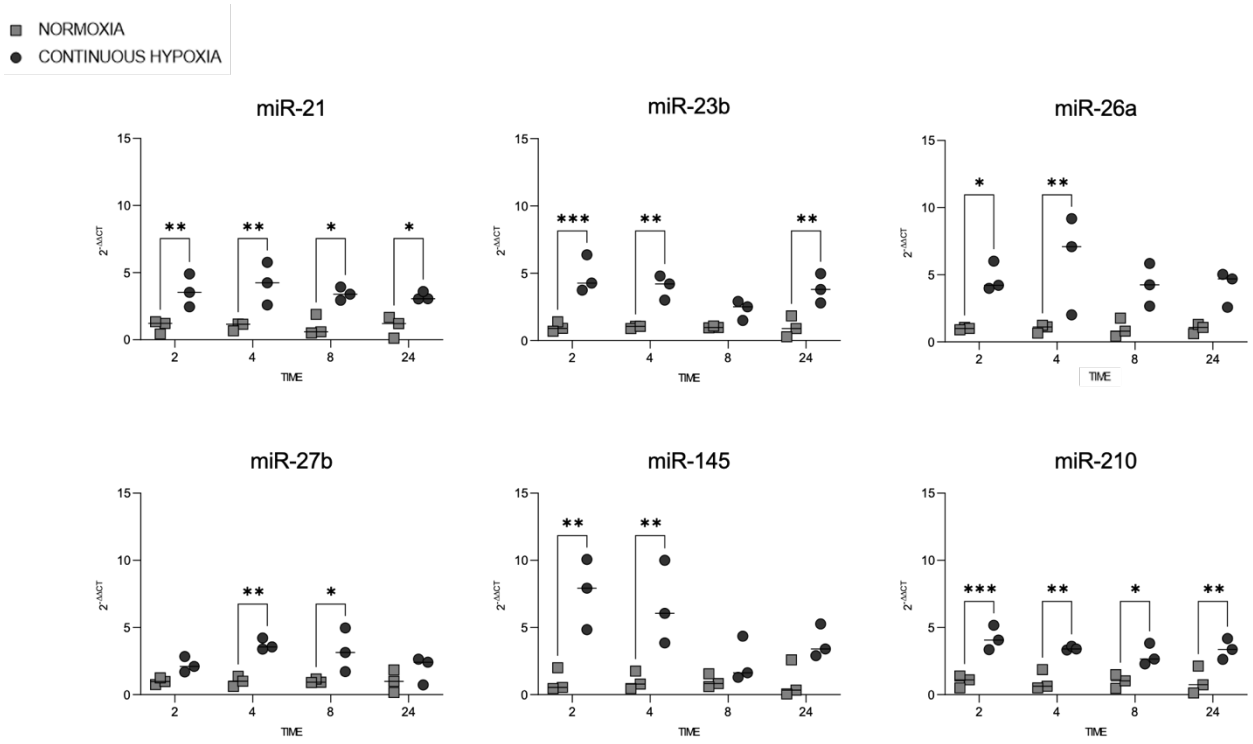
8.1. CONTINUOUS HYPOXIA EXPOSURE: miRNA EXPRESSION

The MTT assay showed that at 2, 4, 8, and 24 h of CH exposure CaCo2 viability did not change as compared with the normoxic control. The survival rate was the same under all tested conditions (data not shown).

Through qRT-PCR we tested the expression of miR-21, miR-23b, miR-26a, miR-27b, miR-145 and miR-210 in CaCo2 cells exposed to CH for 2, 4, 8 and 24 hours.

The expression of miRNAs was different in continuous hypoxia and normoxia (**Figure 10**).

Figure 10. Expression of different miRNAs in normoxia and continuous hypoxia (2% O_2)



Quantitative real-time PCR analysis of differentially expressed microRNAs in CaCo2 cells in condition of continuous hypoxia and normoxia. RNU-6B was used as endogenous control. The x axis shows the different times of exposure to hypoxia while the y axis shows the expression of each miRNA. Individual data and median are plotted. * p ≤0.05, **p ≤0.01, ***p ≤0.001.

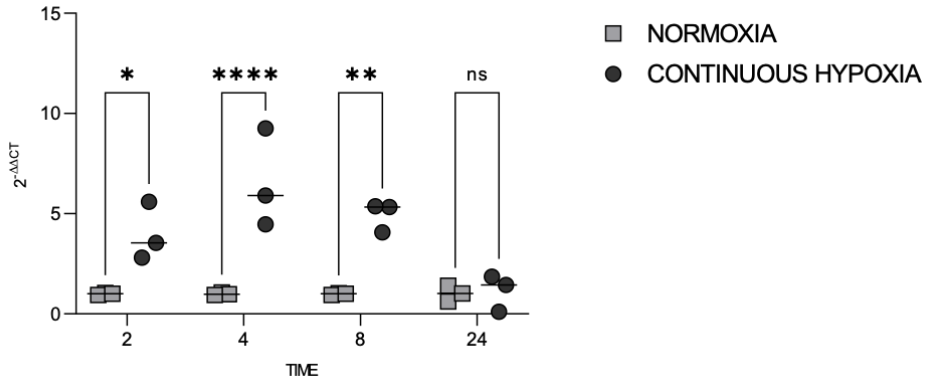
The levels of nearly all analyzed miRNAs were greater in continuous hypoxia compared to normoxia. Notably, miR-21 and miR-210 showed higher levels of expression in CH than in normoxia at all all-analyzed times (2, 4, 8, and 24 h), while for miR-26a and miR-145, we proved this difference only at 2 and 4 hours. We also detected an up-regulation among continuous hypoxia and normoxia at 2, 4 and 24 hours of miR-23b. Moreover, for miR-27b, there was an up-regulation at 4 and 8 hours.

However, for all the miRNAs analyzed, a higher expression under conditions of continuous hypoxia appears evident, although sometimes this difference is not statistically significant. Therefore, we can state the expression of nearly all analyzed miRNAs was greater in continuous hypoxia versus normoxia.

8.2. CONTINUOUS HYPOXIA EXPOSURE: HIF-1α GENE EXPRESSION

The mRNA expression of HIF-1α either in conditions of normoxia and chronic hypoxia was assessed by qRT-PCR. As shown in **Figure 11**, the results showed, in agreement with the expression of nearly all analyzed miRNAs, a higher expression of HIF-1α in conditions of continuous hypoxia when compared with normoxia. This is true for 2, 4, and 8 h of CH exposure; however, there is no significant expression difference at 24 h. This result could be explained by the fact that cells, under stressful conditions, are able to implement a series of strategies to react to external stimuli.

Figure 11. Relative mRNA expression of HIF-1α



Total RNA was extracted and qRT-PCR was performed in order to quantify HIF-1α. β-actin was used as internal normalizer. Individual data and median are plotted (n=3). * p ≤0.05, **p ≤0.01, ****p ≤0.0001. Ns means no significative.

8.3. OSA AND CANCER: MIRNA LEVELS IN PATIENTS

Through quantitative real-time PCR analysis (qRT-PCR) we tested the expression of 6 microRNA, respectively miR-21, miR-23b, miR-26a, miR-27b, miR-145 and miR-210 in cancer, OSA and ONCO-OSA patients compared to healthy control.

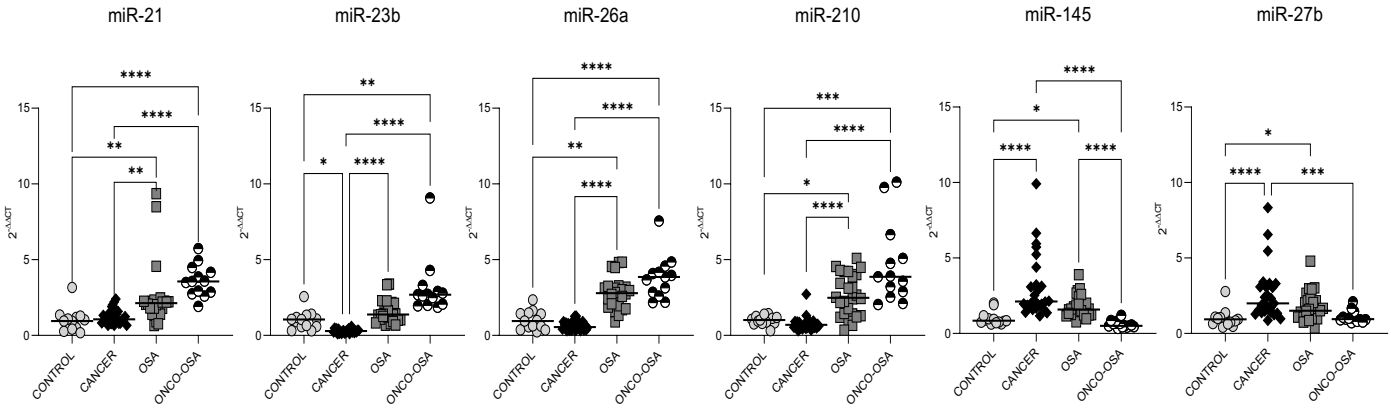
The expression of miR-21, miR-26a and miR-210 was significantly higher in OSA and in ONCO-OSA patients compared to control and cancer patients (**Figure 12**). miR-23b instead, was significantly lower in cancer patients than in controls, and higher in ONCO-OSA than in cancer and control patients.

For miR-27b and miR-145 we demonstrated a significant increase in both cancer and OSA groups compared to controls, but surprisingly, their expression was reduced in ONCO-OSA patients compared to cancer patients (**Figure 12A**).

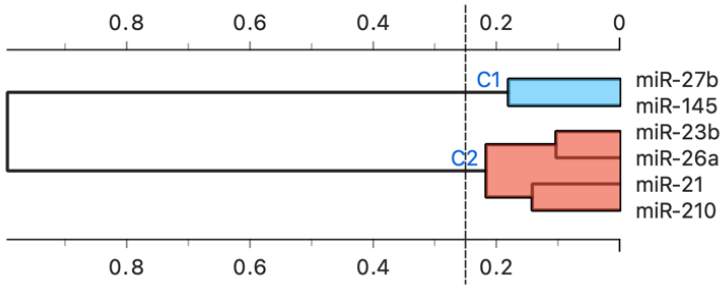
Clustering analysis of each miRNA average level showed that miR-21, miR-23b, miR-26a and miR-210 had a globally similar behavior (**Figure 12B**). On the other hand, miR-27b and miR-145 were clustered together and behave differently.

Figure 12. miRNA expression in patients

A.



B.



A. miRNA's expression in patients. Quantitative real-time PCR analysis of differentially expressed microRNAs in healthy subjects compared to patients with cancer, OSA and both cancer and OSA (ONCO-OSA). RNU-6B was used as endogenous control. Comparisons between groups were performed by Kruskal-Wallis test. Individual data and median are plotted. * p ≤0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001

B. Clustering of miRNAs. The results from the four patients groups were considered together for this analysis. The strength of correlation is inversely indicated by black line's length. Ward's method was applied in cluster analysis. Height ratio: 25% is shown.

In addition, we examined the correlation between the level of miRNAs and the main nocturnal respiratory data (**Table 6**). When considering all the groups, SpO2 correlated negatively with miR-21, miR-23, miR-26a and miR-210, and negatively with miR-27b and miR-145. AHI and ODI correlated

positively with miR-21, miR-23, miR-26a and miR-210, and negatively with miR-145. Finally, TS90 correlated positively with miR-21 and miR-23.

We also examined the correlations in OSA and ONCO-OSA groups separately. We found only a significant positive correlation between miR-27b and AHI in OSA patients. No significative correlation emerged in ONCO-OSA group.

Table 6. Correlations between miRNAs studied and main nocturnal respiratory data

Correlations	***SpO_2, %***	***TS90, %***	***ODI***	***AHI***
OSA				
miR-21	-0.147	0.106	0.108	0.169
miR-23b	-0.072	-0.015	0.337	0.227
miR-26a	0.075	-0.187	0.147	0.082
miR-27b	0.179	-0.271	0.166	**0.395***
miR-145	0.054	0.005	0.165	0.046
miR-210	-0.034	-0.128	0.279	-0.078
ONCO-OSA				
miR-21	0.264	-0.269	-0.104	-0.264
miR-23b	0.341	-0.286	-0.302	-0.198
miR-26°	0.154	-0.258	0.258	0.253
miR-27b	-0.137	0.247	-0.379	-0.088
miR-145	0.429	-0.440	-0.341	-0.418
miR-210	-0.418	0.357	0.544	0.374
ALL GROUPS				
miR-21	**-0.287***	**0.310****	**0.521*****	**0.558*****
miR-23b	**-0.377*****	**0.247***	**0.674*****	**0.711*****
miR-26°	**-0.283***	0.206	**0.694*****	**0.772*****
miR-27b	**0.338****	-0.128	-0.154	-0.167
miR-145	**0.386*****	-0.214	-**0.286***	**-0.357****
miR-210	**-0.281***	0.210	**0.630*****	**0.662*****

p value < 0.05 is marked in bold. *p≤0.05; **p≤0.001; ***p≤0.0001

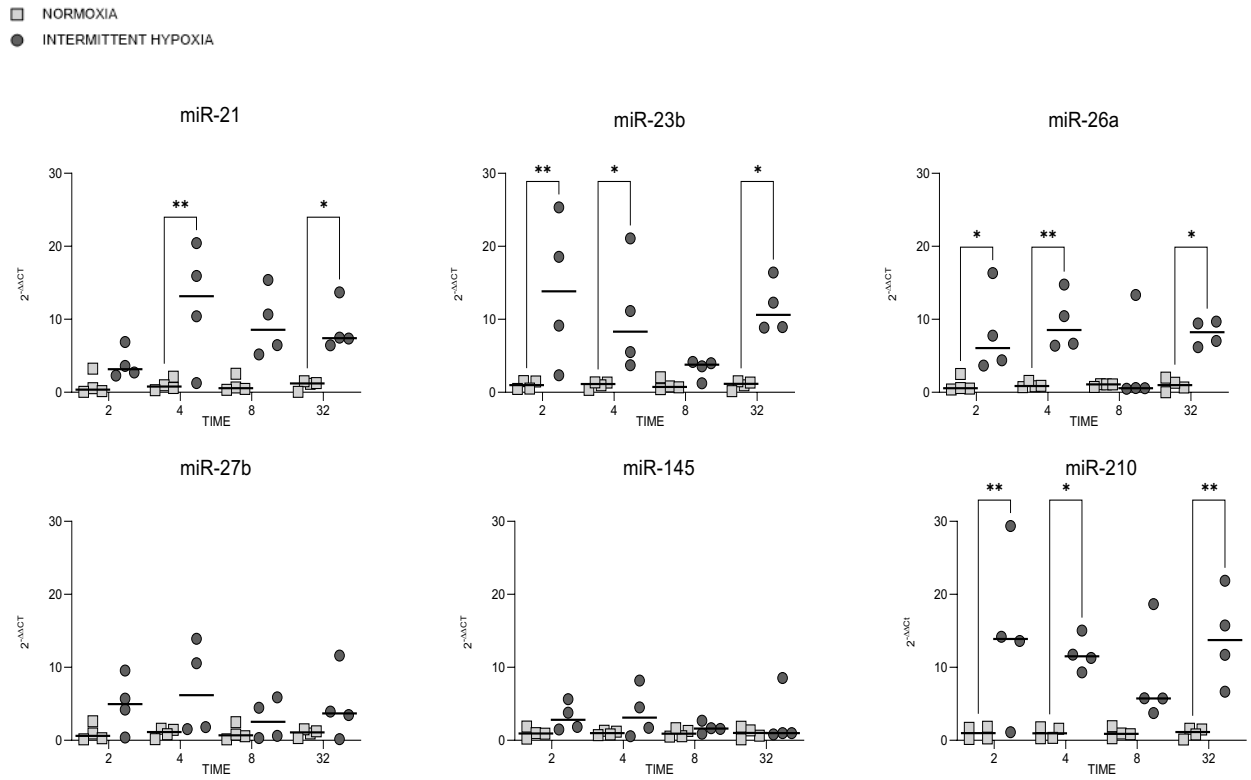
SpO_2: oxygen saturation; TS90: total sleep time with SpO_2 < 90%; ODI: oxygen desaturation index; AHI: apnoea–hypopnoea index.

8.4. INTERMITTENT HYPOXIA EXPOSURE: miRNA EXPRESSION

MiRNAs expression were also tested in colorectal cancer cell line (CaCo2) subjected to both normoxia and intermittent hypoxia for 2, 4, 8 and 32 hours.

The expression of miR-23b, miR-26a and miR-210 at 2, 4 and 32 hours was highest after IH exposure (**Figure 13**). MiR-21 increased in cells exposed to IH for 4 and 32 hours compared to normoxia. Unexpectedly, no statistically relevant differences were found for these above-mentioned miRNAs after 8h of IH exposure. MiR-27b and miR-145 showed no significant variation, whatever the exposure time tested (**Figure 13**).

Figure 13. Cellular expression of different microRNAs in normoxia and IH.



Quantitative real-time PCR analysis of differentially expressed microRNAs in CaCo2 cells in normoxia vs intermittent hypoxia. Comparisons between groups were performed by two-way ANOVA. Individual data and median are plotted (n=4). * p ≤0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001.

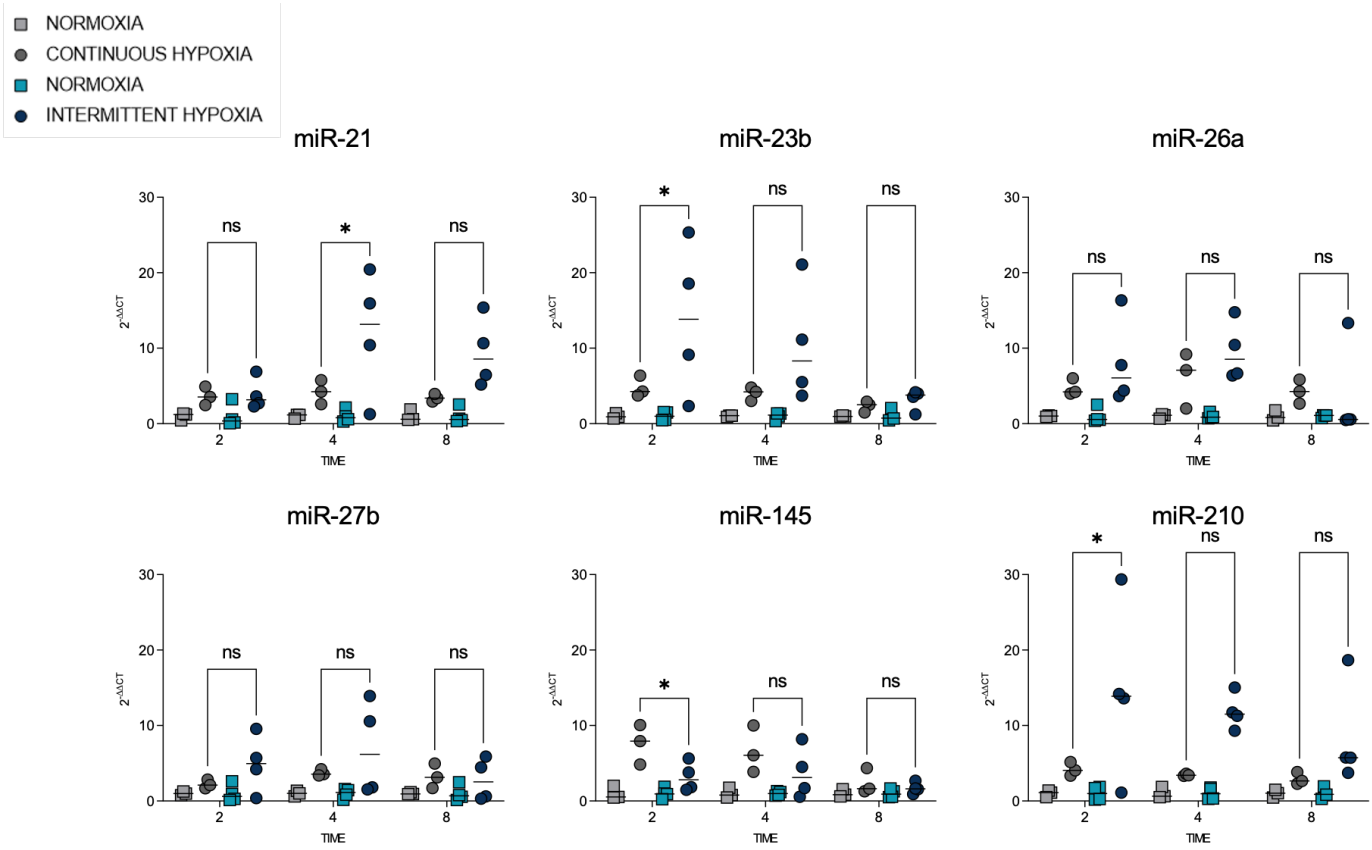
8.5. CH VERSUS IH: miRNA EXPRESSION

For the time intervals 2, 4 and 8 hours, the difference in the expression of the different microRNAs between continuous and intermittent hypoxia was tested. **Figure 14** shows only the differences between CH and IH.

In detail, miR-21 was higher at 4 hours in IH than in CH. MiR-23b, miR-145 and miR-210, on the other hand, showed a significant difference in expression only at 2 hours. The other miRNAs, specifically

miR-26a and miR-27b, showed no significant differences. For almost all analyzed miRNAs, however, even if without significant differences, a higher expression trend is evident under IH conditions.

Figure 14. MiRNA expression differences between continuous and intermittent hypoxia



qRT-PCR analysis of differentially expressed microRNAs in CaCo2 cells in normoxia vs continuous vs intermittent hypoxia. Comparisons between groups were performed by two-way ANOVA. Only the differences in expression between continuous and intermittent hypoxia are shown. Individual data and median are plotted. * p ≤0.05.

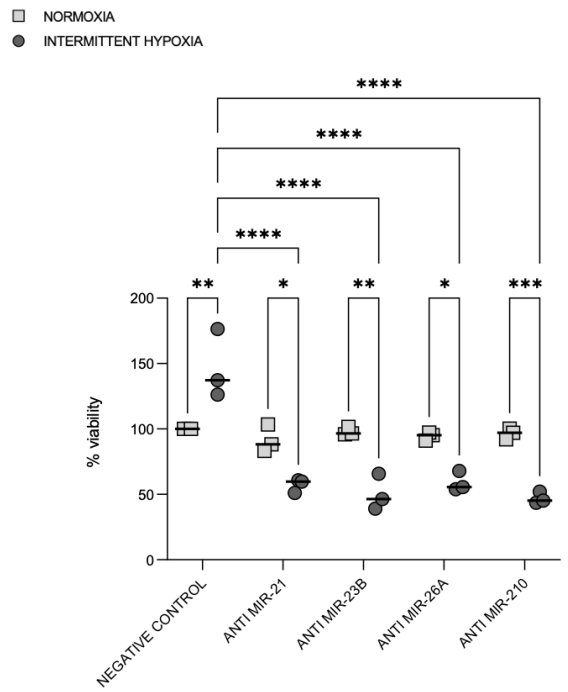
8.6. MIRNA INHIBITION REVERSES IH-INDUCED PROLIFERATION AND MIGRATION

We tested the impact of IH on cell proliferation and migration. We observed that after 32 to 48h, IH stimulated both cell proliferation and migration (**Figure 15A-C**).

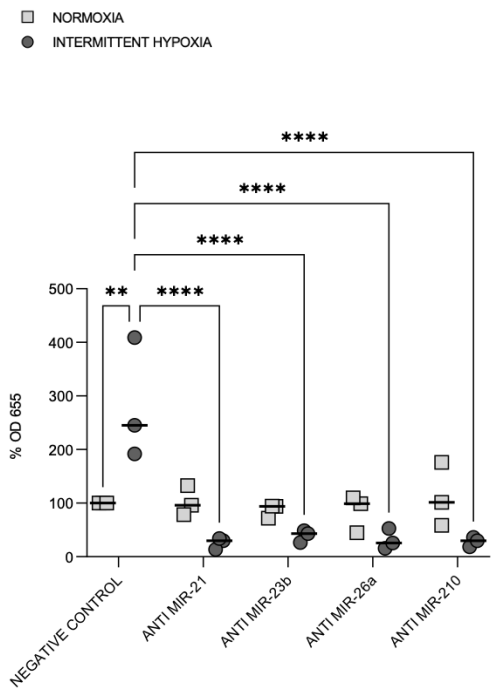
Then, in order to investigate the functional role of miRNAs, we used transient transfection of antisense inhibitors of miR-21, miR-23b, miR-26a and miR-210, respectively, to silence their expression in CaCo2 cells. We confirmed that all these four miRNA expressions can be significantly suppressed (**Figure 16**) by their respective inhibitors. Inhibition of each microRNA reduced proliferation (**Figure 15A**) and migration (**Figure 15B and 15C**) compared to IH negative control (p≤0.00001) and to normoxia.

Figure 15. Proliferation and migration test in CaCo2 cells both in normoxia and IH after miRNA inhibition.

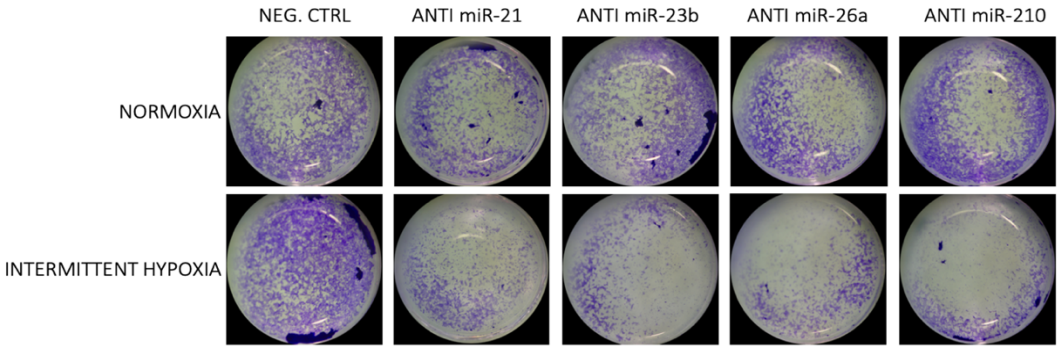
A.



B.



C.

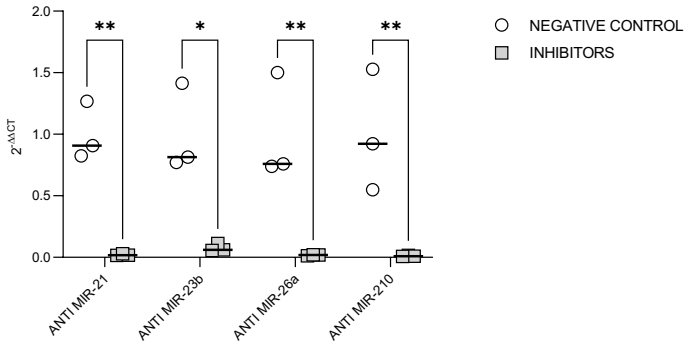


A. Proliferation test. CaCo2 cells were transfected with various miRNA inhibitor and then incubated in normoxia or IH for 32h. Cells were then assayed for viability by the MTT assay. Cells in normoxia transfected with negative control represented 100% of viability. Individual data and median are plotted (n=3). * p ≤0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001.

B. Transwell migration assay. Transwell experiments were performed to analyze the cell migration and invasion in CaCo2 cancer cells exposed to normoxia or intermittent hypoxia for 48h and transfected with miRNA inhibitors. Individual data and median are plotted (n=3). * p ≤0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001.

C. Pictures of the lower side of transwells after migration test and crystal violet staining, taken under a light microscope coupled to a camera at ×10 magnification.

Figure 16. Verification of microRNA inhibition by the miR-inhibitors



qRT-PCR in CaCo2 cells after transfection with miRNA inhibitors. RNU-6B was used as endogenous control. Comparisons between groups were performed by Kruskal-Wallis test. Individual data and medians are plotted (n=3). * p ≤0.05, **p ≤0.01.

8.7. HIF-1α EXPRESSION IS REDUCED BY MICRORNA INHIBITION

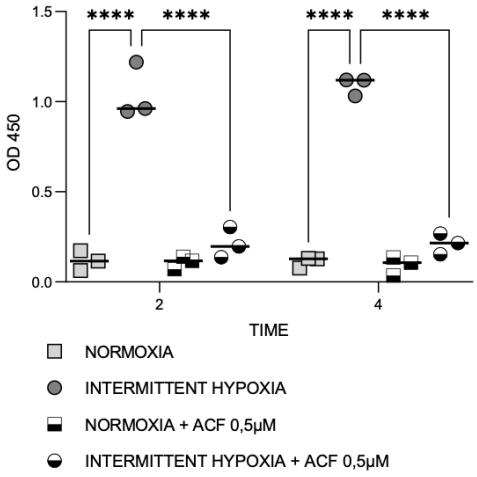
Then, we wanted to evaluate the links between IH, HIF-1 and miRNA. First, we observed that HIF-1α activity was increased by up to 9-fold after 4 hours of IH exposure (p≤0.0001). As expected, this HIF-1 activity was abolished after treatment with 0.5μM ACF (p≤0.0001) (**Figure 17A**).

An MTT assay was used to verify that a concentration of 0.5μM ACF had no cytotoxic effects on CaCo2 cell lines (data not shown).

Then, HIF-1α gene expression was tested following miRNA inhibition. HIF-1α gene expression was 2 to 3-fold lower with all inhibitors used (**Figure 17B**), suggesting that miRNAs are involved in the regulation of HIF-1α gene expression.

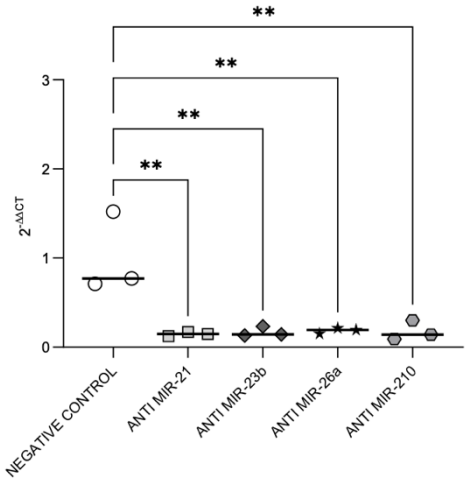
Figure 17.

A. Treatment with ACF abolished IH-induced HIF-1α activity.



Measurement of HIF-1α activity in CaCo2 cells exposed to normoxia or intermittent hypoxia for 2 and 4h, in presence or absence of 0.5 µM ACF. Individual data and median are plotted (n=3). **** p≤0.0001

B. miRNA inhibition suppresses HIF-1α gene expression

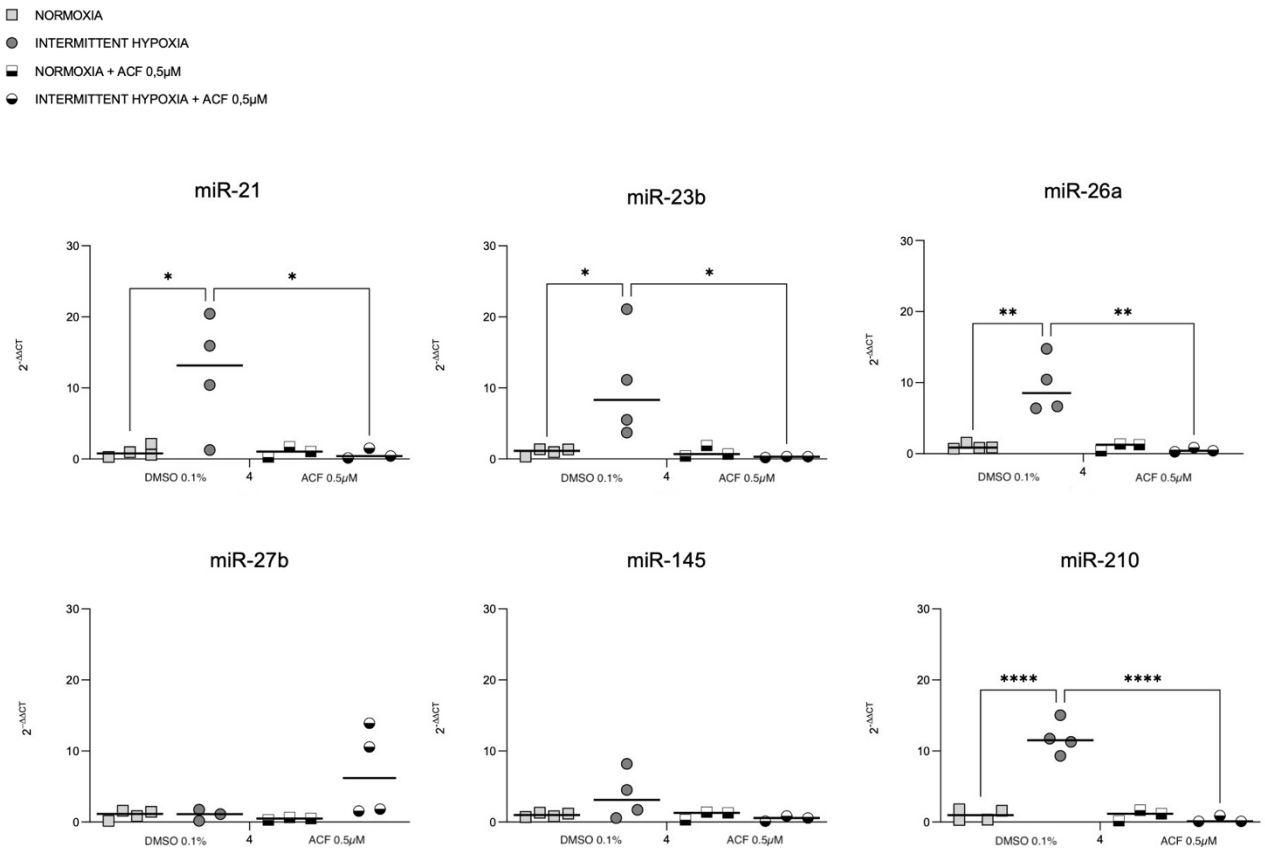


qRT-PCR of HIF-1α gene expression in CaCo2 cells after transfection with miRNA inhibitors. Comparisons between groups were performed by Kruskal-Wallis test. Individual data and median are plotted (n=3). * p ≤0.05, **p ≤0.01.

8.8. HIF-1 INHIBITION REVERSES IH-INDUCED MIRNA EXPRESSION

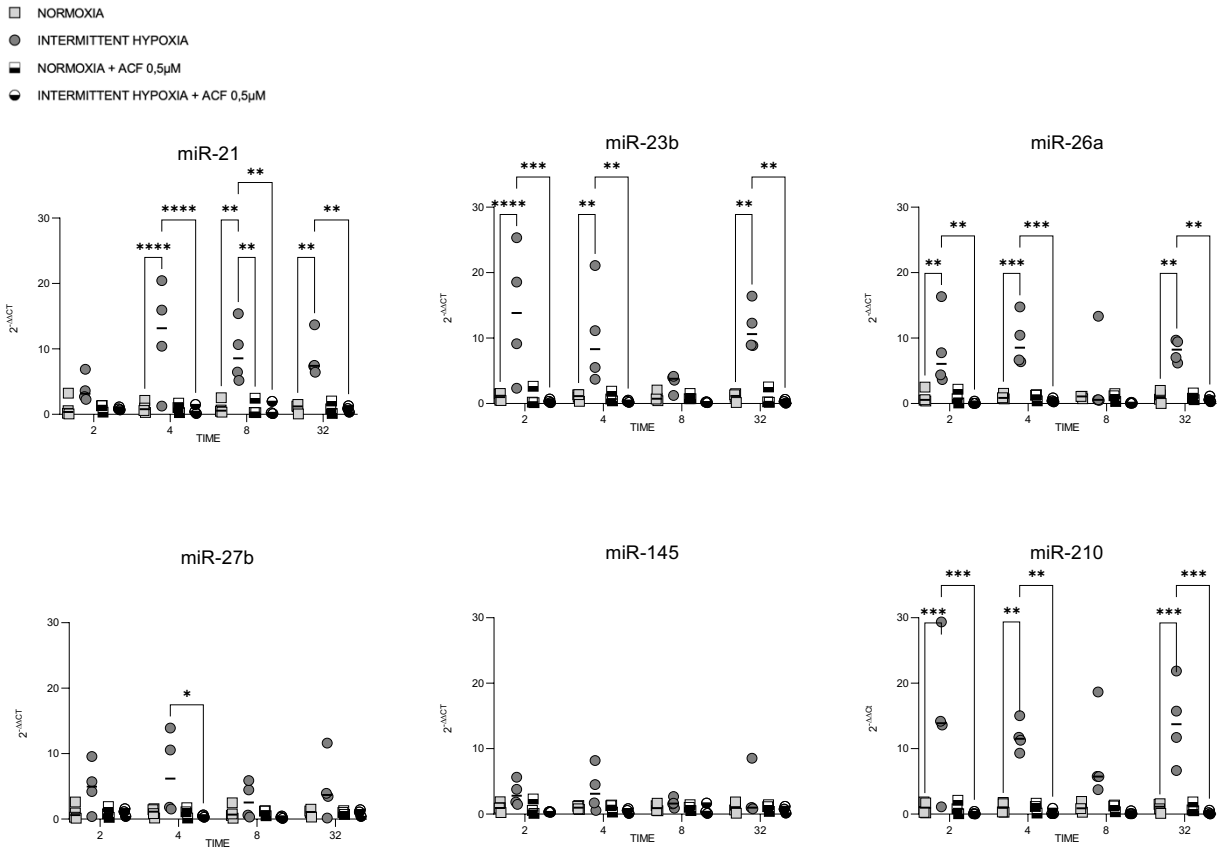
On the other hand, we tested if HIF-1 is mediating the regulation of miRNAs by IH. The increase of miR-21, miR-23b, miR-26a and miR-210 expression in CaCo2 after 4h of IH was significantly reversed following 0.5µM ACF treatment (**Figure 18**). Similar effects were observed at other time points (2, 8 and 32h) (**Figure 19**). No differences were found between normoxic conditions with and without ACF. MiR-27b and miR-145 expression did not show any significant difference following treatment with ACF.

Figure 18. IH-induced expression of miRNAs is reversed by ACF treatment in CaCo2 cells.



Cells were incubated in presence or absence of 0.5 µM ACF for 4h in condition of normoxia or IH. Individual data and median are plotted (n=4). *p≤0.05, **p≤0.01, ****p ≤0.0001.

Figure 19. ACF reduces microRNAs expression in condition of intermittent hypoxia in CaCo2 cells.

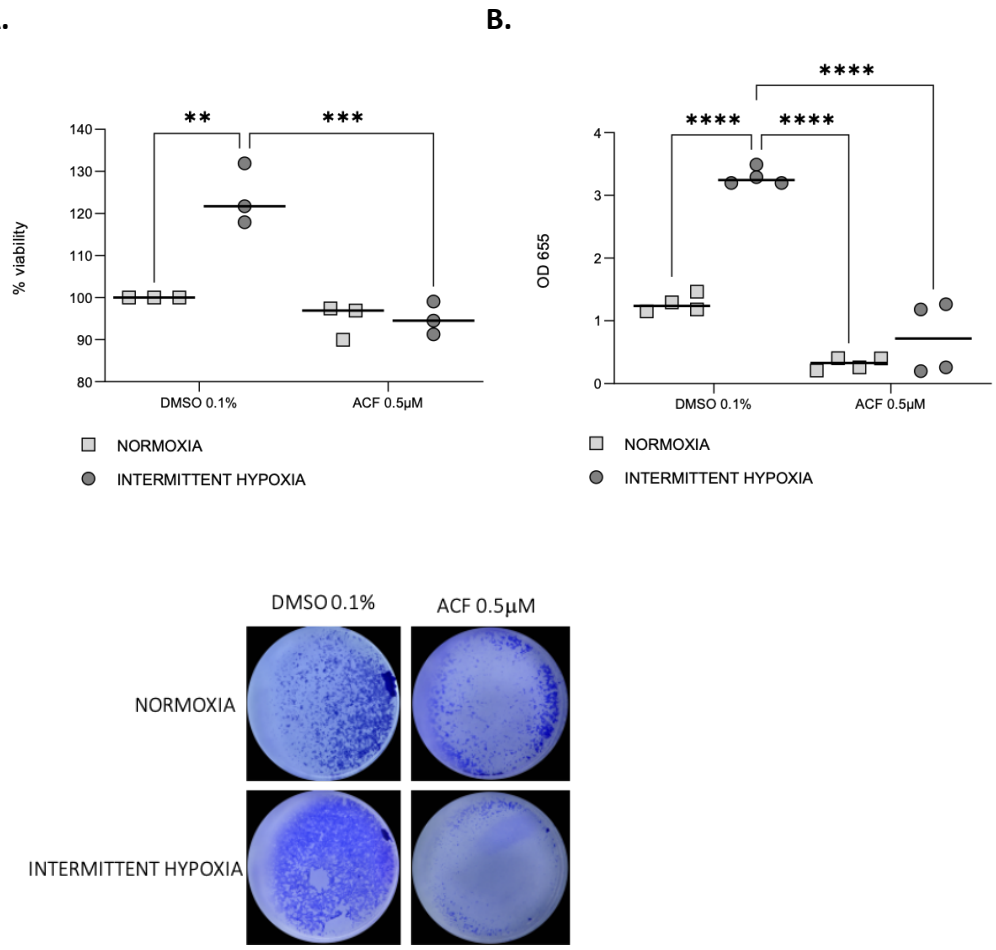


Cells were incubated in presence or absence of 0.5 µM ACF for 2, 4, 8 and 32h both in condition of normoxia and IH. Total RNA was extracted and qRT-PCR was performed in order to quantify microRNA. The x axis shows the different times of exposure to hypoxia while the y axis shows the expression of each miRNA. RNU-6B was used as endogenous control (n=4). *p≤0.05, **p≤0.01, ***p≤0.001, ****p ≤0.0001.

8.9. HIF-1 INHIBITION REVERSES IH-INDUCED PROLIFERATION AND MIGRATION

Proliferation (**Figure 20A**) and migration (**Figure 20B and 20C**) of CaCo2 cells were significantly increased in IH in cells treated with DMSO 0.1% as a control (p=0.0014 and p≤0.0001). This increase of proliferation and migration was abolished by 0.5µM ACF (p=0.0004 and p≤0.0001).

Figure 20. ACF reverses IH-induced proliferation and migration of CaCo2 cells.



A. Viability of CaCo2 cells assessed by MTT assay in condition of normoxia and intermittent hypoxia with 0.1% DMSO or 0.5 µM of acriflavine (ACF) for 32 h. Cells in normoxia treated with DMSO 0.1% represented 100% of viability. Individual data and median are plotted (n=3). **p≤0.01, ***p≤0.001.

B. Transwell migration assay were performed to analyze the cell migration after exposure to normoxia or intermittent hypoxia for 48h, in presence or in absence of 0.5 µM ACF. Individual data and median are plotted (n=4). ****p ≤0.0001.

C. Pictures of the lower side of transwells after migration test and crystal violet staining, taken under a light microscope coupled to a camera at ×10 magnification.

9. DISCUSSION

To our knowledge, this is the first study to combine microRNA analyses on patients and in vitro approaches to explore the effects of hypoxia, especially IH exposure mimicking OSA, on colorectal cancer development and progression.

We currently know that miRNAs are involved in the development and progression of cancer and that they are able to regulate the expression of many oncogenes and tumor suppressor genes involved in the pathogenesis of cancer [242]. However, it is difficult to fully understand the role of miRNAs in carcinogenesis as their function can vary depending on the target tissue.

To date, the molecular mechanisms by which miRNAs modulate cellular processes have yet to be fully elucidated, which is why the study of the specific functions of miRNAs in carcinogenesis could be useful to evaluate their therapeutic potential as diagnostic and prognostic markers of disease [243]. Recently, some studies have identified hypoxia-inducible miRNAs, HRMs, which are often activated in different types of tumors, suggesting their role in tumorigenesis [197].

This thesis aimed, first of all, to evaluate the expression of different miRNAs in conditions of continuous hypoxia to study the possible relationship between hypoxia, miRNA and cancer, and to identify the miRNAs involved in carcinogenesis that are susceptible to hypoxia.

Then, the second purpose was to analyze microRNA in patients with OSA, cancer and both OSA and cancer finding significant differences in miRNA expression between groups.

In addition, using devices specifically designed to mimic the IH pattern of OSA, we analyzed the same microRNA in colorectal cancer cells and we demonstrated that in vitro, intermittent hypoxia induces increased cell proliferation and migration mediated by these microRNAs.

Furthermore, we evaluated the role of HIF-1α and its involvement in microRNA pathway. We demonstrated that HIF-1α activity increased after IH and that blocking HIF-1 with acriflavine abolished the IH-induced increase in miRNA expression and cell proliferation and migration. This suggests that HIF-1 mediates cell proliferation and migration through miRNA upregulation. Finally, HIF-1 gene expression was decreased following the inhibition of some miRNAs, suggesting a feedback loop by which miRNAs can in turn regulate HIF-1 expression (**Figure 21**).

9.1. MIRNA REGULATION BY CONTINUOUS HYPOXIA

The main finding of this study is that nearly all of the miRNAs analyzed appear to have increased expression under conditions of continuous hypoxia; however, this statement is true at all studied time intervals for miR-21 and miR-210 only. The other miRNAs under study showed increased expression only in some of the time points analyzed.

Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that normally stimulate angiogenesis and that takes part in all those cellular mechanisms that restore normal oxygen supply to tissues following hypoxia. Tumors that over-express VEGF are able to grow and metastasize [244]. HIF-1α is a transcription factor that responds to hypoxia and stimulates the release of VEGF from parts of cells. VEGF binds to receptors on the endothelial cells and triggers a tyrosine kinase signaling pathway that leads to angiogenesis [245]. As previously mentioned, some miRNAs appear to be involved in the regulation of the HIF pathway by acting on specific signaling molecules that function as oncogenes or tumor suppressors. The miRNA most involved in this mechanism is certainly miR-210 [246].

All the miRNAs analyzed in this study are hypoxia-inducible miRNAs, however, there is evidence showing their altered expression in some types of tumors [247]. This, therefore, leads us to hypothesize that hypoxia, either chronic or intermittent, could lead to an alteration of the expression of specific miRNAs involved in the formation of cancer.

MiR-210 was often up-regulated in many types of tumors and its overexpression is able to promote the migration and invasion of tumor cells [198]. MiR-210 is moreover a miRNA induced by hypoxia and has a role in the regulation of cell proliferation, differentiation and apoptotic response. Our study shows that in colorectal adenocarcinoma cells the level of miR-210 during continuous hypoxia is higher than in normoxia at all times. This result seems to be in contrast with what was highlighted by Lacedonia et al. in a study conducted on three different types of hypoxia where miR-210 appeared down-regulated under conditions of CH [22]. Nevertheless, our results were in agreement with Gee et al., Tagcherer et al. and Qu et al., according to which miR-210 appeared up-regulated in colorectal cancer compared to controls and its involvement in hypoxia-induced metastases was observed [248-250]. In fact, we know that hypoxia is one of the main factors contributing to a poor prognosis in patients with cancer and high levels of miR-210 can influence the course of the disease but, to date, there are no further studies investigating the role of hypoxia in the expression of this miRNA.

MiR-23b is another hypoxia-regulated microRNA involved in apoptosis that appears to be up-regulated in some cancers such as those in the pancreas and colon [243]. In our study, miR-23b levels at 2, 4 and 24 hours were higher in conditions of hypoxia. This result is again, in contrast with what was previously demonstrated by Lacedonia et al [22].

However, aberrant expression of miR-23b has been demonstrated in the development of several cancers. Chen L. et al, for example, investigated the oncogenic significance and function of miR-23b

in glioma [251]. They observed that miR-23b expression was elevated in glioma cells and that miR-23b acted through the HIF-1a/VEGF signaling pathway.

MiRNA-21 and miRNA-26a are released from endothelial cells [252]. Previous work has shown that both miRNAs are expressed at the cellular level in response to hypoxic conditions, therefore hypoxia seems to be a factor capable of inducing the activation of endothelial cells and consequently the release of these two miRNAs [252]. Our work shows results consistent with what has just been said, even if miR-21 appears higher in chronic hypoxia at all time intervals while miR-26a seems to be more expressed in hypoxia just at 2 and 4 hours. These results could suggest the existence of two different endothelial responses in relation to the time of exposition to hypoxic condition.

A recent study by Blick C. et al showed that miR-145 plays an important role in hypoxia-dependent apoptosis in bladder cancer [253]. In this work, Blick C. and his collaborators demonstrated that miR-145 was significantly increased in response to hypoxia in bladder cancer cells and that this miRNA represents a target gene of HIF. Our work shows high levels of miR-145 in conditions of continuous hypoxia when compared to normoxia at 2 and 4 hours.

Several studies in the literature suggest that miR-145 is a miRNA that acts as a tumor suppressor by inhibiting tumor growth and angiogenesis and this miRNA appears to be down-regulated in various types of tumors. Yu Yin et al. observed that miR-145 was significantly downregulated in plasma and tumor tissues of colorectal cancer patients and that miR-145 overexpression inhibited cell proliferation, migration and invasion. In this previous work they also demonstrated that miR-145 blocks the activation of the AKT and ERK1/2 pathways and the expression of HIF-1 and VEGF [254]. Based on our results we can therefore think that the low levels of miR-145 found in IH may play a role in tumor development and progression, unlike when we observe for CH. Based on our results, we can therefore think that the miR-145 levels found in CH may somehow be a regulatory mechanism when hypoxia and cancer are combined. The same can be said for the miR-27b [255]. At present, many studies have reported that miR-27b plays important roles in cancer progression and have shown that miR-27b functions as a tumor suppressor in various types of cancers [256,257].

9.2. MIRNA REGULATION BY OSA/INTERMITTENT HYPOXIA

The results of this study elucidated that miR-21, miR-23b, miR-26a and miR-210 were higher in ONCO-OSA patients than in cancer patients and healthy control, and were upregulated in CaCo2 cells following IH. MiR-27b and miR-145 were lower in ONCO-OSA compared to cancer, and their expression did not change in vitro in CaCo2 cells submitted to IH. In addition, miR-21, miR-26a and

miR-210 had a higher expression in OSA compared to cancer and healthy controls. Interestingly, miRNAs expression correlated with the severity of sleep apnea as evaluated by AHI, ODI and SpO_2. Clustering analysis allows the identification of miRNAs with similar expression profiles, that are generally involved in the same cellular functions or the same regulatory pathway [258]. We showed that in patients, miR-21, miR-23b, miR-26a and miR-210 clustered together, while miR-27b and miR-145 were in a different cluster. These results are consistent with the fact that the 4 miRNAs in the first cluster positively correlate with sleep parameters (AHI, ODI, SpO_2) while the other 2 negatively correlate with the same parameters. It is also consistent with their regulation in CaCo2 cells, and with the finding that inhibiting miR-210, miR-21, miR-23b or miR-26a led to the reversion of IH-induced cell proliferation and migration.

It is known that miR-210 mediates important processes associated with tumorigenesis such as proliferation or angiogenesis. In fact, it targets many other miRNAs encoding proteins that play key roles in proliferation, DNA and RNA binding and repair, differentiation, development and apoptosis [259,260]. miR-210 was involved in colorectal cancer migration and invasion [261]. Our results about miR-210 expression seem to be in agreement with previous studies showing an upregulation of miR-210 in OSA patients [22,262].

Similarly, results for miR-21 and miR-23b are in line with those found in the literature. Indeed, several studies describe them as miRNAs upregulated in many tumors [251,263,264]. They were elevated in one study investigating their expression in OSA patients [22], and miR-21 was upregulated in mice models of OSA [265,266]. However, another study reported that miR-21 and miR-23 expression was downregulated in OSA patients [267].

MiR-26a is induced by hypoxia and its expression is upregulated during cell differentiation. Previous studies have demonstrated its involvement in different types of cancers and that its target genes are involved in cellular process such as proliferation, differentiation, apoptosis, invasion and metastasis [268]. Our results are in line with a study showing that miR-26a was associated with OSA severity in patients [268], although conflicting data showed that miR-26a was upregulated by chronic but not intermittent hypoxia [235].

Interestingly, although these 4 miRNAs are usually associated with cancer, in our cohort they were not elevated in cancer patients. By contrast, they were elevated in OSA patients (except for miR-23) and in ONCO-OSA patients, suggesting that OSA rather than cancer is responsible for the serum expression of these miRNAs.

On the other hand, miR-145 and miR-27b are generally downregulated in various types of cancer and considered as inhibitors of tumor cell proliferation and migration [245,269,270], although

conflicting data showed that miR-27 stimulated cell proliferation and invasion [271]. In our study, these 2 miRNAs were found elevated in cancer patients and OSA patients but not in ONCO-OSA patients and in CaCo2 cells exposed to IH, suggesting a regulatory mechanism when OSA and cancer are combined.

Surprisingly, while the miRNAs regulation was generally consistent at 2, 4 and 32h of exposure, no statistically significant differences were detected at 8 hours. These data could be explained assuming an adaptation of cells to hypoxia. In the early stages of exposure, the hypoxic environment induces an increased expression of these specific miRNAs. At 8 hours there may be an adaptation which, however, cannot be maintained following a further cycle of exposure to IH. Further experiments are needed to explain our observations.

9.3. DIFFERENCE BETWEEN CH AND IH

As mentioned above, there are two different types of hypoxia: intermittent hypoxia (IH) and continuous hypoxia (CH) occurring in malignant tumors and the pathogenetic mechanisms underlying these two types of hypoxia are completely different. They are both associated with the activation of hypoxia-inducible factor-1 (HIF-1) and nuclear factor-κB (NF-κB), which induce changes in gene expression. However, while the role is known of chronic hypoxia in solid tumors is well understood, little is known about intermittent hypoxia. Intermittent hypoxia indeed appears to influence tumor development and progression differently than chronic hypoxia. Actually, we only known that intermittent hypoxia acts, more than continuous hypoxia, on activation of many transcription factors including hypoxia-inducible factor-1 (HIF-1) and its second messengers, erythropoietin (EPO) and vascular endothelial growth factor (VEGF).

Consistent with what has been reported, our results show that almost all the analyzed miRNAs have a trend of higher expression in IH condition than in CH.

However, despite this trend being evident, we did not find a significant statistical difference for all miRNAs. In this regard, we strongly believe that further future studies are needed to better understand the impact of continuous hypoxia and intermittent hypoxia, associated with OSA, on many cancer-related aspects.

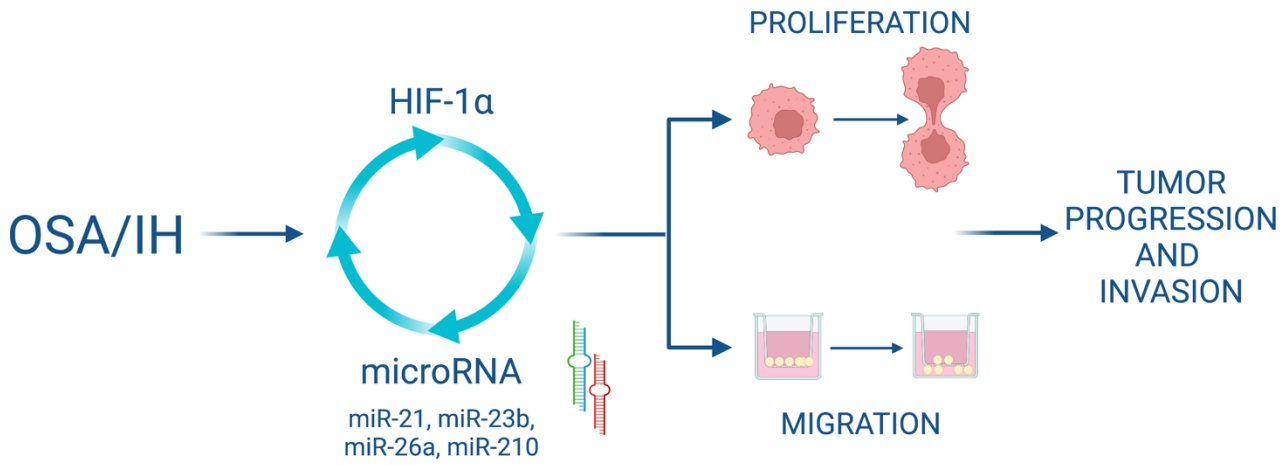
9.4. INTERCONNEXION OF HIF-1 AND MIRNA EXPRESSION

In this study, we wanted to investigate the involvement of HIF-1 in the expression of specific miRNAs and its role in proliferation and migration of cancer cells exposed to intermittent hypoxia. In this regard, we used acriflavine, an inhibitor of HIF-1 dimerization, which decreased HIF-1 transcriptional activity and showed anticancer efficacy in vivo [272]. We showed that inhibiting HIF-1 with

acriflavine abolished the IH-induced increase in miRNA expression. This suggests that HIF-1 is necessary for miRNA upregulation. This is consistent with data showing that miR-210 [261], miR-21 [273-275], miR-26a [276] are targets of HIF-1. On the other hand, we observed that inhibition of miR-21, miR-23b, miR-26a and miR-210 in turn led to significant reduction of HIF-1α gene expression. HIF-1 has indeed been described as a target of miR-21 [273-275], miR-23 [251] and miR-26 [276]. Our data thus support the hypothesis of a hypoxia-triggered feedback loop involving the expression of HIF-1 and several miRNAs (**Figure 21**).

We showed that acriflavine inhibited proliferation and migration of colorectal cancer cell exposed to IH acriflavine treatment. This is line with existing data showing the regulatory role of acriflavine in tumors [272,277] and with the known role of HIF-1 in tumor progression induced by IH [278]. Altogether, our results highlight a loop between HIF-1 and some miRNAs induced by IH/OSA and contributing to cancer development and progression. This hypothesis is recapitulated on **Figure 20**.

Figure 21. HIF-1 and miRNA are involved in a feedback loop mediating IH effects on cell proliferation and migration



9.5. LIMITATIONS OF THE STUDY

Due to the relative rarity of combination of OSA and cancer in patients, our clinical study relies on relatively small population and pairing is not perfect with some differences remaining (i.e.

comorbidities frequency). However, we evidenced statistically significant differences in miRNA expression, suggesting that the variations are strong enough to be detected in this small cohort. Moreover, due to the predominance of colorectal cancer in the cancer and ONCO-OSA groups, we chose colorectal cancer cells (CaCo2) to investigate the cellular mechanisms induced by IH. Further studies will be required to confirm these mechanisms in other colorectal cancer cell lines and other types of cancer.

10. CONCLUSION

In conclusion, with this thesis, we have identified some miRNAs involved in different ways in the response to different types of hypoxia, highlighting that the expression of these small molecules can vary under hypoxic conditions and that miRNAs could play a role in the development of several diseases, including cancer.

Our work demonstrates a different response between the condition of intermittent hypoxia and that of continuous hypoxia, bringing to light that the former appears to be, in some cases, much more dangerous in terms of stimulating the expression of some miRNAs.

Based on these preliminary results, we therefore believe that intermittent and continuous hypoxia can activate different molecular pathways; however, further studies are needed in this regard.

In addition, this study found an abundance of differentially expressed miRNAs both in patients affected by OSA, cancer and OSA plus cancer and in colorectal cancer cell exposed to IH. To our knowledge, this is the first time that expression of miRNAs was examined at the clinical and preclinical level and that their functional consequences on IH-induced tumor progression are demonstrated. We postulate that those miRNAs might play a pivotal role in the mechanism of OSA aggravating tumor development and progression.

Moreover, the present study confirms the fundamental role of HIF-1 as a master regulator of tumor cells response to hypoxia, and brings to light a loop between OSA-associated intermittent hypoxia, HIF-1 and microRNA that needs to be further investigated but which could provide some initial insights into the mechanism linking OSA and cancer.

Further studies are warranted to confirm our observations and investigate the specific signaling pathways of each microRNA. Targeting HIF-1 and/or the miRNA could represent new therapeutic strategies in OSA patients affected by cancer.

11. POTENTIAL APPLICATIONS AND IMPACTS OF THE RESULTS

My thesis project stems from the awareness that intermittent hypoxia, more than continuous hypoxia, is able to enhance cancer progression in patients with OSA.

Furthermore, OSA is known to increase the risk of several types of malignancy and enhance tumor growth. Therefore, based on the aforementioned knowledge, the project thesis has the following expected results:

- Improve our knowledge about possible role of OSA in progression and prognosis of patients with tumors;
- Better understand which are the mechanisms that links OSA and cancer, with particular attention to effects of intermitted and continuous hypoxia;
- Characterize pathway of miRNA as biomarkers able to extend the knowledge about the relationship between OSA and the genesis, progression of human cancer;
- Better knowledge of biomolecular mechanisms involved in carcinogenesis in subjects with OSA, could lead to identification of possible specific therapeutic targets and subsequent development of targeted drug therapies for different types of cancer suffering from OSA;
- To develop an “expert system” based on new methods of computational analysis which could be useful to define cancer evolution, therapy response and prognosis of patients.

12. REFERENCES

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