Carbonic anhydrase activity in sleep apnea - a potential therapeutic mechanism for intervention

Davoud Eskandari

Doctoral thesis

Department of Internal Medicine and Clinical Nutrition
Institute of Medicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

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davoud.eskandari@lungall.gu.se

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This thesis is dedicated to my family, friends and colleagues that have supported me throughout the years..

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Department of Internal Medicine and Clinical Nutrition, Institute of Medicine
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Göteborg, Sweden

ABSTRACT

There is no pharmacological treatment for obstructive sleep apnea (OSA) in clinical practice. The overall aim of this thesis was to investigate the effect of carbonic anhydrase (CA) enzyme activity on sleep apnea severity and blood pressure (BP) regulation in OSA. We explored the association between arterial standard bicarbonate ($StHCO_3^-$), a proxy for CA activity, and apnea severity as well as hypertension status in a retrospective cohort of OSA patients (n=830, paper I). In a cross-sectional sleep clinic cohort (n=70), we explored the association between whole blood CA enzyme activity and OSA severity (paper II). Furthermore, we designed a randomized, placebo-controlled study to investigate the effect of pharmacological CA inhibition after zonisamide (ZNS) on sleep disordered breathing in overweight/obese OSA patients (n=42, paper III). Finally, the effect of CA inhibitor acetazolamide (AZT), continuous positive airway pressure (CPAP) or the combination thereof on sleep apnea and BP was investigated in a three-way cross-over study in 13 male hypertensive OSA patients (paper IV). Sleep disordered breathing was quantified by polysomnographic/polygraphic recording. Office systolic/diastolic BP (SBP/DBP) and vascular stiffness were assessed. Arterial/venous $StHCO_3^-$ was collected. In paper I, we found that arterial $StHCO_3^-$ was independently associated with apnea-hypopnea index (AHI) as the measure of OSA severity ($p<0.001$). In addition, arterial $StHCO_3^-$ was positively associated with both a hypertension diagnosis and DBP ($p=0.007$ and 0.048, respectively). In paper II, CA activity was associated with AHI, nocturnal hypoxemia as well as DBP ($p=0.007$, 0.011 and 0.046, respectively). In paper III and IV, therapeutic intervention using ZNS and AZT, significantly reduced AHI by 33(39) % (placebo-adjusted) and 42(27) % ($p=0.02$ and 0.001, respectively). AZT reduced office BP in parallel with improvement of vascular stiffness compared to CPAP. In conclusion, our studies suggest an independent association between CA activity and OSA. High CA activity may represent a novel mechanism for development of hypertension in OSA. Drugs with CA inhibitory properties may provide a promising target for disease modifying treatment in OSA and its related comorbidities.

Keywords: bicarbonate, blood pressure, carbonic anhydrase, hypertension, obesity, obstructive sleep apnea, vascular function

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1 Sammanfattning på svenska

Obstruktiv sömnapné (OSA) förekommer med en prevalens av 9 % respektive 24 % hos kvinnor och män i åldersintervallet 30-60 år. OSA är associerad med hjärt-kärlsjukdom, huvudsakligen hypertoni (HT), ischemisk hjärtsjukdom och stroke. OSA leder även till uttalad dagtidssömnighet, vilket har implikationer för trötthetsrelaterade olyckor och reducerad livskvalitet. Karbanhydraser (CA) är en familj av enzymer som katalyserar omvandlingen av CO_2 till bikarbonat och protoner. CA har associerats med en rad fysiologiska och patologiska tillstånd som glukoneogenes, tumerogenes samt tillväxt och virulens av patogener. CA utgör därmed ett fundamentalt fysiologiskt reglersystem för upprätthållande av stabil syra-bas balans i olika vävnader. Denna avhandling studerar sambandet mellan CA aktivitet och svårighetsgraden av OSA och blodtryck.

Delarbeten i avhandlingen omfattar epidemiologiska studier kring associationen mellan standard bikarbonat ($StHCO_3^-$), som en surrogatmarkör för CA aktivitet, och svårighetsgraden av OSA och hypertoni. Vidare undersöktes sambandet mellan CA enzym aktivitet i helblod och OSA i en tvärsnittstudie hos 70 kliniska OSA patienter. I en randomiserad placebo-kontrollerad studie studerades effekten av läkemedel med CA hämmande egenskaper avseende sömnapné hos OSA patienter (n= 42). Slutligen, analyserades effekten av CA hämning med läkemedlet acetazolamid på OSA såväl som blodtryck hos 13 manliga OSA patienter med etablerad hypertension. Metoder som implementerades i avhandlingen omfattade polysomnografi/polygrafi, blodtrycksmätning samt icke-invasiv arteriografi.

Studierna visar att en ökad aktivitet av CA i helblod var associerad med en högre intensitet av OSA. Vidare kunde vi fastställa ett samband mellan standard bikarbonat ($StHCO_3^-$) och såväl OSA som HT i en stor retrospektiv kohort (n=830). Vi kunde också visa att två läkemedel med CA hämmande egenskaper, zonisamid och acetazolamid, reducerade mängden OSA med 19-42 % hos patienter med måttlig till uttalad sjukdom. Behandlingseffekten, definierat som minst 20 % reduktion av AHI, uppnåddes av 70 % av behandlade patienter. Efter justering för följsamhet till behandling var effekten på AHI efter CPAP och acetazolamid inte signifikant skild. Patienter med hypertoni reducerade sitt blodtryck efter såväl zonisamid som acetazolamid. Trycksänkningen var direkt relaterad till en kärldilatation, uppmätt med icke-invasiv arteriografi.

Vi har i dessa studier identifierat en ny möjlig farmakologisk princip för behandling av åtminstone en subgrupp av patienter med OSA. Denna nya behandling kan innebära en sjukdomsmodifierande dimension framför nu tillgänglig konventionell mekanisk terapi som exempelvis CPAP eller intra-oral skena. Framtida studier får utvisa i vilken omfattning denna nya behandlingsform kan användas vid sömnapnébehandling.

2 List of original papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Eskandari D, Zou D, Grote L, Schneider H, Penzel T, Hedner J.

Independent associations between arterial bicarbonate, apnea severity and hypertension in a sleep apnea cohort

Submitted

II. Wang T, Eskandari D, Zou D, Grote L, Hedner J.

Increased Carbonic Anhydrase Activity is Associated with Sleep Apnea Severity and Related Hypoxemia.

SLEEP 2015; 38(7): 1067-1073

III. Eskandari D, Zou D, Karimi M, Stenlöf K, Grote L, Hedner J.

Zonisamide reduced obstructive sleep apnea: a randomised placebo-controlled study.

European Respiratory Journal 2014; 44(1): 140-149

IV. Eskandari D, Zou D, Grote L, Hoff E, Hedner J

Acetazolamide reduces blood pressure and sleep disordered breathing in hypertensive OSA patients

Submitted

3 Table of Contents

4 Abbreviations

AASM	American Academy of Sleep Medicine
AHI	Apnea-hypopnea index
AF	Atrial fibrillation
AZT	Acetazolamide
BMI	Body mass index
BP	Blood pressure
CA	Carbonic anhydrase
CAD	Coronary artery disease
CO_2	Carbon dioxide
CSA	Central sleep apnea
CSR	Cheyne-stokes respiration
CPAP	Continuous positive airway pressure
CV	Cardiovascular
DM	Diabetes mellitus
EDS	Excessive daytime sleepiness
ESADA	European Sleep Apnea Database
ESS	Epworth sleepiness scale
HF	Heart failure
HR	Heart rate
HT	Hypertension
LG	Loop gain
MSLT	Multiple sleep latency test
MWT	Maintenance of wakefulness test
ODI	Oxygen desaturation index

OHS	Obesity hypoventilation syndrome
OSA	Obstructive sleep apnea
PCO_2	Partial pressure of carbon dioxide
Pcrit	Pharyngeal critical pressure
PG	Polygraphy
PSG	Polysomnography
MAP	Mean arterial pressure
RCT	Randomized controlled trial
SA	Sleep apnea
SAA%	Sleep apnea alleviation
SDB	Sleep disordered breathing
$StHCO_3^-$	Standard bicarbonate
T2DM	Type II diabetes mellitus
UA	Upper airway
ZNS	Zonisamide

5 Introduction

Obstructive sleep apnea (OSA) is a highly prevalent breathing disorder in the adult population with a variable degree of symptoms and comorbidities[1]. OSA tends to occur in different forms whereby some patients may suffer from dominant hypersomnia or symptoms of insomnia others may have no- or very discrete symptoms. The detailed pathophysiology of OSA is not fully understood and several different underlying mechanisms have been proposed. It is likely that some of these mechanisms represent separate phenotypic traits in the clinical population. For instance, recent attempts to sub-classify causes of upper airway (UA) collapsibility in OSA have provided models that include both structural and functional physiological characteristics[2]. OSA, or particular components of the disorder, is widely accepted to contribute to comorbidities such as cognitive dysfunction[3, 4], metabolic disorders[5-7] and cardiovascular (CV) disease[8, 9]. Longitudinal population studies and registry data from sleep clinic cohorts have demonstrated a considerable increase in mortality[10, 11].

Conventional therapies such as continuous positive airway pressure (CPAP) effectively reduce both OSA and symptoms related to the condition. However, CPAP treatment is frequently hampered by poor compliance which limits the therapeutic potential of the therapy[12-14]. Limitations also apply for other therapies such as intraoral devices and surgical interventions applied in OSA. Taken together, there is a need for new forms of therapy in OSA. Such therapies should address the improved understanding of the underlying pathophysiology in OSA which recently has emerged. An additional target for novel forms of therapy in OSA should not only address the mere breathing disorder in OSA but also comorbidities such as hypertension (HT), metabolic disease and/or obesity.

5.1 Sleep apnea - definitions and criteria

OSA is defined on the basis of recurrent events of partial (hypopneas) or complete (apneas) cessation of breathing in spite of respiratory efforts during sleep. The most frequently applied definitions and criteria are those published by the American Academy of Sleep Medicine (AASM)[15]. Apneic events are defined as complete (≥90%) reduction of the amplitude of the breathing signal whereas hypopneas are defined as a reduction of amplitude by ≥30%. Hypopnea event should be associated with a ≥3 % decrease of oxygen desaturation or an arousal[15]. The duration of events should be at least 10 seconds. The apnea-hypopnea index (AHI) reflects the number of events per hour of sleep and this metric is conventionally used to assess the occurrence and severity of the disease. According to current convention the OSA diagnosis is based on a combination of an AHI ≥5 n/hr plus symptoms (e.g. excessive daytime sleepiness (EDS)) or a recognized comorbidity (e.g. HT or diabetes). Alternatively OSA may be diagnosed based on the respiratory disorder alone (AHI≥15 n/hr). It should be noted that the AASM does not explicitly list other potential markers of OSA such as hypoxic events or arousals from sleep as tools for diagnostic assessment. However, several research studies have applied various measures of oxygenation (e.g. oxygen desaturation index (ODI) 3 or 4%, mean overnight saturation or minimum overnight saturation) to quantify severity of sleep disordered breathing (SDB)[16, 17].

A frequently adopted clinical convention for classification of the OSA severity labels the condition as mild (5≤AHI<15), moderate (15≤AHI<30) or severe (AHI≥30). Diagnostic conventions such as those issued by the AASM also list both clinical conditions and symptoms that should alert health professionals on a possible sleep and breathing related conditions (Table 1).

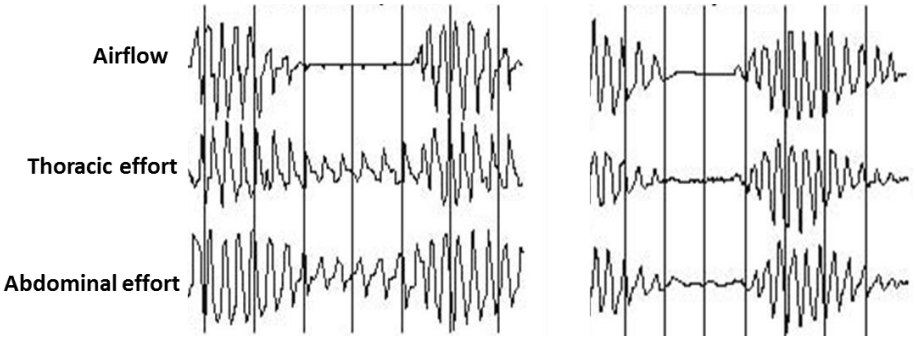
Table 1. Evaluation of clinical conditions and symptoms for the assessment of OSA (Adapted from the AASM)

Patients with high risk associated with OSA	Routine questions of clinical importance	Symptoms associated with OSA
• Obesity (BMI>30 kg/m^2) • Atrial fibrillation • Congestive heart failure • Type 2 diabetes • Stroke • Pulmonary hypertension • Treatment refractory hypertension • Nocturnal dysrhythmia • High-risk driving population • Pre operative for bariatric surgery	• Is the patient obese? • History of hypertension? • Complaints of daytime sleepiness? • Snorer? • Retrognathic features?	• Snorer • Witnessed apneas • Gasping or choking at night • Excessive daytime sleepiness not explained by other factors • Short sleep time • Sleep fragmentation • Non-refreshing sleep • Nocturia • Morning headaches • Decreased ability to concentrate • Memory loss • Decreased libido • Irritability

Central sleep apnea (CSA)

Central sleep apnea (CSA) is a form of periodic breathing where apneas/hypopneas are followed by periods of hyperventilation. In contrast to OSA, central apneas (airflow cessation of ≥10s with ≥90% reduction in airflow amplitude) occur without an increase of intrathoracic pressure or apparent respiratory effort (Figure 1). Central hypopneas are scored according to criteria similar to those used for obstructive hypopneas but without considering any evident snoring, paradoxical breathing, flatting of the nasal pressure flow or regard to the positive airway pressure flow signal[15]. Most forms of CSA appears to be associated with an impaired ventilatory drive in response to changes in the partial pressure of carbon dioxide (PCO_2)[18, 19].

Figure 1. Respiratory pattern of obstructive sleep apnea (right) and central sleep apnea (left)



Cheyne-Stokes respiration

Cheyne stokes respiration (CSR) is a specific form of central respiratory events which is characterized by a crescendo‑decrescendo pattern of the breathing signal. CSR occurs primarily during the non-rapid eye movement sleep and the cycle time (approximately 30-60 seconds) is generally longer than that conventionally seen in various forms of OSA and CSA[20, 21]. CSR is especially likely to occur during certain physiological situations such as high altitude sojourns and specifically during sleep at high altitude. Hence, unstable breathing may be elicited by alteration of the blood gas environment in combination with functional state. CSR appears to be more common in men compared with women and clinical studies have shown that CSR may be prevalent in 30-40% of patients with congestive heart failure[22, 23]. As with CSA, the mechanism by which hyperventilation leads to in fall in PCO_2 is also reflected in CSR patients. In CSR, hyperventilation during sleep induces a reduction of the PCO_2 (hypocapnia). Once this value reaches a level below the apneic threshold there is no stimulus to breathe and consequently a cessation of the respiratory drive and airflow[24]. Accordingly, patients with CSR appear to exhibit both daytime and night-time hypocapnia[18] as well as increased central and peripheral chemosensory responsiveness (increased controller gain)[25, 26,27]. An additional contribution to the appearance of CSR in patients with cardiac failure may be provided by the reduced functional lung-volume and delay in circulation time which signifies this patient group. Fluctuations in the chemosensory stimulus caused by the fluctuating blood gases leads to ventilatory instability[21].

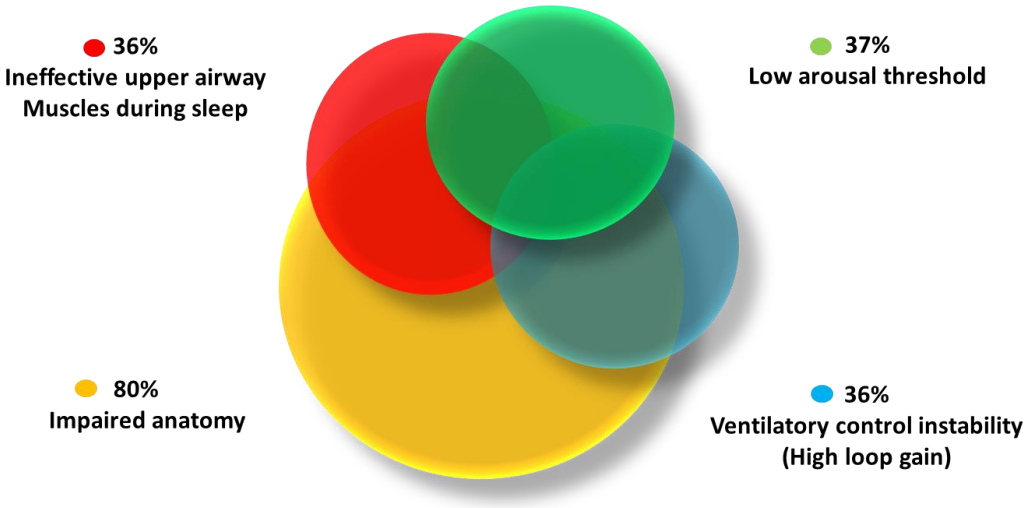
5.2 Pathophysiology of OSA - cause of airway obstruction

Airway obstruction in OSA generally occurs at the level of the soft palate or at the level of the tongue[28]. Static, as well as anatomical and dynamic neuromuscular factors appear to contribute. Static factors include components such as airway surface adhesion, posture of the neck and jaw and gravity. OSA will worsen in the supine sleeping position in most patients and many patients have strictly position dependent OSA[29]. Anatomic factors including tonsillar hypertrophy, large tongue volume, prominent soft palate or lateral pharyngeal walls[30], abnormal positioning of the mandible may, due to reduced volume of the UA, predispose to airway collapse[31]. In particular parapharyngeal fat pads may contribute to obesity related OSA. The dynamic component is provided by neuromuscular activity in the UA which is known to physiologically decrease during sleep[32, 33]. The aperture of the UA is determined by the transmural pressure, which results from the pressure in the airway lumen and the pressure in the surrounding tissue[34]. A reduction of transmural pressure will bring the airway to a critical point referred to as the pharyngeal critical pressure (Pcrit). For instance, a recently introduced method for intermittent hypoglossal nerve stimulation during sleep is known to evoke a genioglossal muscle contraction which results in a reduction of UA collapsibility[35].

The duration of the obstructive breathing events is determined by the time that the Pcrit is exceeded and Pcrit has been used clinically as a composite measure of airway collapsibility in patients with sleep apnea (SA)[36-38]. The arousal that occurs at the end of the apnea/hypopnea is an important determinant for the re-opening of the UA when other restoring mechanisms have failed[33]. However, not all apneas/hypopneas are terminated by

an identifiable arousal and in such cases there may be other compensatory mechanisms such as increased genioglossus activity[39,40]. Eckert et al. (Figure 2) have proposed that approximately one third of OSA patients have a low threshold which may contribute to an oscillation in ventilator drive which acts to self-sustain apneic events[2]. This mechanism may be particularly important in various forms of CSA, but also in OSA. Hence, patients with a low arousal threshold may benefit from an elevation of threshold and this has been described after administration of a benzodiazepine in patients with OSA[41].

Figure 2. Different pathophysiological overlapping mechanisms considered in OSA (adapted from Eckert et al.[2])



The central nervous control of breathing appears to play an important role in all forms of apnea and instability may lead not only to central apnea but also to the development of OSA. This may be exemplified by experiments demonstrating that hypercapnia may prevent OSA events. Hyperventilation-induced hypocapnia, as seen at resolution of apneic event, leads to reduced pharyngeal dilator activity and limitation of inspiratory flow[34]. Sudden arousal from sleep (which occurs at the end of the apneic episode) increases the likelihood of a relative hyperventilation. The degree of hyperventilation is determined by the gain of the respiratory system in response to chemosensory stimulation (loop gain, LG). Prevailing theories suggest that a steeper LG will contribute to a higher risk of rendering the ventilatory system in a state of sustained oscillation[42-44]. LG has therefore recently emerged as a possible factor that reflects the (in) stability of the ventilatory system. An elevated LG could, according to some researchers be present in approximately one-third OSA patients[2]. Accordingly, a high LG has been associated with increased OSA severity and low LG is associated with stable breathing[44-47]. Supplemental oxygen and certain pharmacological interventions (e.g. acetazolamide (AZT)) reduced LG and AHI in small clinical trials with pre-selected high LG OSA patients[48-50].

The proneness of airway collapse during various static conditions, in combination with the complex interplay between anatomical and neuromuscular will determine the actual clinical phenotype of the OSA patient. Importantly, existing data and pathophysiological models do

not fully explain why factors such as sex, age, and ethnicity influence the prevalence of this disorder[51]. The apparent combination of various factors in OSA has been addressed in a recent model of SA which propose that different degrees of overlap between mechanism will occur (Figure 2)[2]. From this model it is evident that overlap is very common and that anatomical factors alone may be present in as many as 80% of patients with OSA. Other mechanism including ineffective UA muscle capacity, low arousal threshold and high LG may contribute to variable extent.

5.3 Epidemiology of OSA

OSA (defined as AHI≥5) is a common finding in the general adult population. The reported prevalence ranges between 17 and 24% in men and between 5 and 17% in women[1,52-56]. A recently published study by Heinzer and co-workers reported an even higher prevalence of OSA in men (84%) and women (61%)[57]. These high prevalence numbers have sparked a discussion on the validity of current thresholds for OSA diagnosis and prevalence. In fact, the most liberal diagnostic criteria for OSA do not appear to reflect any association with comorbid symptoms or outcome in OSA. Instead, longitudinal registry data suggests that the prevalence of for instance CV comorbidity and outcome appears to increase more distinctly at an AHI threshold of approximately 15[58]. The prevalence numbers are also affected if symptom criteria are introduced. The most common symptom is daytime sleepiness and the prevalence of symptomatic OSA (AHI≥5 combined with EDS) is reported to vary between 3 and 18 % in men and between 1 and 17 % in women[59].

5.4 Risk factors for OSA

Age

Several studies have shown that the prevalence of OSA linearly increases with age[52,56]. However, the age association appears to plateau around the age of 60 years[51,53]. The mechanism behind this non-linear association may be explained by altered anatomical and physiological features in parapharyngeal structures[60]. The prevalence of OSA appears to decrease after 65-70 years of age in both men and women[56].

Gender

OSA appears to be overrepresented in men with a 2:1 ratio in the general population[1,52-56]. However, recent data from a large European study suggests that this ratio may be in the order of 3:1 in clinical cohorts[61]. There are several potential explanations for the different OSA prevalence in men and women including for instance differences in UA structure and anatomy, hormonal status and body fat distribution[62-68]. However, the lower proportion of women in the clinical setting suggests that OSA may be underdiagnosed among women[69,70]. In fact, the vast majority of sleep research conducted up to mid-1990 were primarily conducted on the male population most likely due to neglect[71].

Obesity

Obesity is considered to be one of the strongest risk factors for OSA[1,31,35]. Approximately 50% of patients seen at European sleep centres had a body mass index (BMI) above 30 in the European Sleep Apnea Database study[7]. Longitudinal data shows that even moderate weight gain, in the order of 10% or 10 kg, may induce a moderate to severe OSA in patients without OSA at baseline. Weight gain may also worsen pre-existing OSA[72,73].
The detailed mechanisms behind OSA development in obesity are still largely unknown. A reduction of pharyngeal size and aperture due to local fat distribution[77] provides an evident explanation but other mechanisms related to breathing dynamics in the supine position[29], redistribution of body fluid to the UA tissue[74] and chemosensory attenuation associated with reduced leptin sensitivity[75] have also been proposed. However, in spite of this strong association between obesity and OSA it should be noted that approximately one third of patients with OSA are not obese. Hence, pathophysiological mechanisms other than those related to anatomical changes appear to be operational in many cases.

Nasal obstruction and craniofacial anatomy

Small experimental studies in healthy normal subjects have demonstrated an increase of apneas and arousals following nasal occlusion[76,77]. Other larger studies have confirmed nasal obstruction as an independent risk factor for snoring and OSA[78,79]. Moreover, OSA patients with chronical rhinitis were twice as likely to have moderate to severe OSA in comparison to patients without nasal symptoms[80].

Narrow UA and increased UA collapsibility as consequence of specific craniofacial features and characteristics have been associated with OSA development[81]. Increased mandibular body length and thickened lateral pharyngeal muscle wall appear as the strongest craniofacial predictors for OSA[28,82].

Genetics

Family members of OSA patients are more susceptible to the disorder[83] with the genetic risk factors being attributed to abnormalities in the control of breathing, obesity as well as craniofacial features[84-87]. Genome-wide scan analyses based on data from the Cleveland Family Study have identified candidate genes (chromosomes 1p, 2p, 12p, 19p) that are associated with AHI in OSA patients[86]. In addition, data from the same cohort found associations between single-nucleotide polymorphisms on chromosomes 8p21.3 and 8q24.1[88]. However, the association between the candidate genes and AHI was weakened in both studies after introducing BMI in the model. In fact, the heterogeneity and complexity of OSA and its symptoms make it difficult to find one unison genetic factor. Hence, genetic analysis in large cohorts of well characterized OSA patients and controls are necessary to identify the potential genetic background of OSA. Less frequent risk factors and causes for OSA have been reported including acromegaly[89], gastro-oesophageal reflux[90] and hypothyroid function[91].

5.5 Diagnostic tools for OSA

The Berlin questionnaire has been used as a validated screening tool with high sensitivity (0.80) and specificity (0.76) for the identification of patients at risk for OSA[92-94]. The questionnaire assesses snoring, daytime sleepiness and HT as well as anthropometric information including age, gender, height, weight and neck size. Validation studies using polysomnography (PSG) suggest that the Berlin questionnaire has a moderate sensitivity and low specificity at identifying OSA patients with respiratory disturbance index >5 n/hr[95].

Other validated screening tools include the STOP and the STOP-bang questionnaires. The STOP questionnaire assesses four domains including snoring, tiredness, witnessed apnea and high blood pressure (BP). STOP has a moderate sensitivity and specificity (0.66 and 0.60, respectively) for OSA (defined as AHI≥5)[96]. The STOP-bang questionnaire has added four domains including age, gender, BMI and neck size to the domains assessed in the STOP-questionnaire. The STOP-bang is accompanied by a high sensitivity (pooled 0.90) and low specificity (0.49) for detection of OSA (defined as AHI≥5) in various sleep clinic populations[97].

The Epworth sleepiness scale (ESS) and the Functional Outcome of Sleep Questionnaire (FOSQ) are frequently used questionnaires to assess subjective daytime sleepiness or the impact of sleepiness on multiple activities of everyday living, respectively[98,99]. The ESS reflects the propensity of falling asleep during a wide range of daily activities and a total ESS score ≥10 is generally considered to signal EDS. The FOSQ questionnaire is subcategorized into five different domains including general productivity, intimate and sexual activity, social outcomes, vigilance and activity level.

The Multiple sleep latency test (MSLT) and the Maintenance of wakefulness test (MWT) are used in the clinical setting to objectively assess EDS or associated alertness in OSA[100, 101]. In addition to assessment of symptoms in EDS, the MWT and MSLT are sometimes also used to evaluate the effectiveness of ongoing OSA therapy. The MSLT is performed in a dark room in which the patients are asked to try to go to sleep during five 20-minutes naps with two hour intervals. The test measures the patient's propensity to fall asleep. The MWT on the other hand measures the patient's ability to stay awake during monotonous conditions (i.e. in a dark and silent room). The test consists of four sessions of 40 minutes with each session separated by a two hour period. According to recent guidelines a mean sleep latency of <8 minutes during the MSLT is used to define EDS in SA patients[102]. Both the MSLT and MWT are considered to be very time consuming for the patient as well as for the sleep technician.

The current AASM classification of instruments used for diagnosis of SDB ranks diagnostic instruments into level I (Standard technician-attended PSG in the laboratory setting), level II (Comprehensive portable PSG), level III (Modified portable polygraphy (PG)) and level IV (Continuous single or dual channel) devices. The level I and II versions of PSG include electroencephalography, electrooculogram, electromyography (chin and leg), airflow, oxygen saturation, respiratory effort belts, and electrocardiography (heart rate (HR))[103]. Supervised level I PSG is considered to be time-consuming for the clinicians and patients and hence accompanied by less availability and higher cost. Level II PSG in the home-setting (ambulatory PSG) provides a reliable evaluation although the patient cannot be continuously supervised during the recording[104]. Data from a large scale population study suggested that reliable sleep scoring could be obtained from more than 98.6% of the recordings[105].

PG (level III and IV devices) is currently the most frequently used device class for the routine evaluation of suspected SDB, particularly in the Nordic countries. Signals recorded in the level III devices include airflow (nasal cannula), respiratory effort and oxygenation (pulse oximetry)[103]. Data from the European Sleep Apnea Database (ESADA) cohort suggest an approximately 1:1 usage ratio between PG and PSG in European patients investigated for OSA at academic centers[106]. The ESADA cohort also concluded that the AHI score, as evaluated by PG, was approximately 30% lower than that obtained in patients investigated by PSG[106]. As PG uses total recording time as the time basis, rather than actual sleep time, for the calculation of the AHI there is a dilution of the index.

The AASM has reviewed and produced standards for diagnostic recordings in OSA. The current recommendations reflect a global trend away from complex methodologies towards simpler and frequently portable techniques[103]. This development reflects the fact that less intrusive methods provide a recording that better resembles the regular sleeping conditions and that home recordings may better mimic the habitual sleep environment. It is also recognised that less demanding methods provide a more cost effective evaluation in conventional SA patients. However, a less detailed investigation might lead to a less well characterised condition and a difficulty for differential sleep related diagnoses such as motor disorders or parasomnias.

The objective and subjective measurements applied for the diagnosis of OSA is accompanied by the physician's evaluation of medical history, laboratory evaluation and anthropometric data. There are currently no systematically used biomarkers for the diagnosis or severity assessment of OSA. Such tools, particularly if they are associated with high sensitivity and specificity for one or more key components of the sleep and breathing disorder, would significantly influence the methods and practices of sleep medicine[107].

5.6 Clinical symptoms and consequences of OSA

Excessive daytime sleepiness

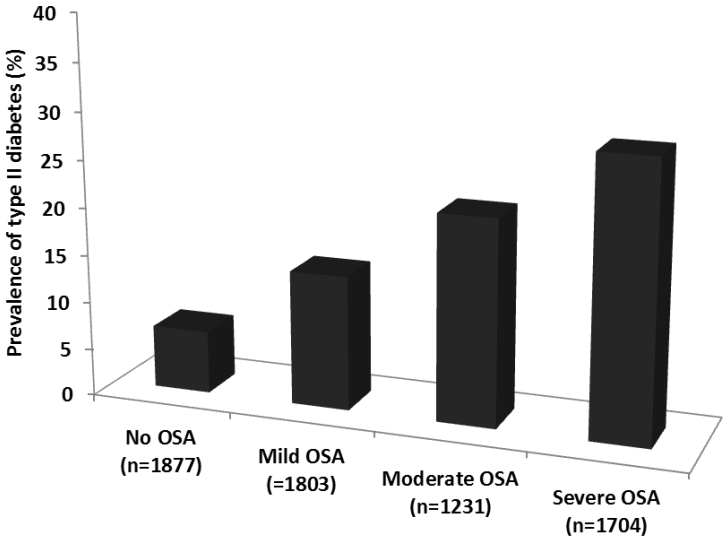
EDS is a hallmark symptom in OSA. The development of EDS in OSA is conventionally linked to the phenomena of respiratory arousals leading to sleep fragmentation and nocturnal hypoxemia associated with the apneic events[108,109]. However, this relationship has not been fully supported by recent larger studies[110-112]. Daytime sleepiness has been independently associated with AHI, EDS increases with OSA severity as well as it is more frequently observed in the female population[1,110]. Several studies have shown that CPAP is efficient in reducing EDS, particularly in patients with moderate to severe OSA[113-116].

Metabolic disease

Several recent epidemiological and clinical studies support an association between either insulin resistance or Type 2 diabetes mellitus (T2DM) and OSA and this association is, in several studies, partly independent of conventional risk factors such as obesity[5,117-119]. An analysis of data from the ESADA cohort suggested an increased risk of concomitant T2DM in

OSA patients[7] (Figure 3) supporting a contributing role of OSA in the development of T2DM[120].

Figure 3. Prevalence of type II diabetes across OSA severity classes in the ESADA cohort (Adapted from Kent et al.[7]).



Other studies focusing on glycaemic control have demonstrated an independent association between OSA and impaired insulin or glucose metabolism[5,118,121]. The association was linked to both the intensity of OSA as well as the extent of nocturnal hypoxemia[5,122-124]. Intermittent hypoxia, increased oxidative stress as well as sleep fragmentation have therefore been proposed as pivotal mechanistic links behind the associations between OSA and impaired glycaemic control[125-127]. OSA therefore appears to constitute a risk factor for T2DM as well as an exceptionally frequent comorbidity with adverse effects on glycemic control[128-130].

Several studies have addressed the effects of CPAP on insulin resistance and glycaemic control in non-diabetic and diabetic OSA patients[131,132]. While some studies showed weak effects on the homeostasis model assessment index in non-diabetic or diabetic patients others did not[129,133-135]. There are several potential explanations for the variable effects of therapy. A more recent study suggested that the extent of glycaemic control prior the CPAP may predict the results after treatment[136]. In addition the effect of CPAP may be more evident in lean compared to obese diabetes mellitus (DM) patients[132]. In this context it is interesting to note that CPAP therapy may have negative effects on body weight presumably due to reduction of energy metabolism[137]. Hence, future intervention studies on OSA need to incorporate simultaneous diet counselling. It is also possible that poor CPAP compliance (e.g. less than 4 hours per night) may explain at least in part the weak effects on glucose metabolism in some of these OSA treatment studies.

Hypertension

The relationship between OSA and HT has been well established in several population-based cohorts[53,138-140]. In fact, recent Joint National Committee (JNC-8) guidelines have positioned OSA as common cause of behind HT development[141]. Longitudinal data from the Wisconsin sleep cohort reported a three-fold increased likelihood of HT prevalence when comparing OSA to non-OSA patients[9]. Moreover, there is a high prevalence (between 35-70%) of HT in OSA patients and HT increases independently across OSA severity classes[105,142,143]. HT appears to be overrepresented in middle-aged OSA patients when the cut-off is set at <60 years[56,144]. Epidemiological data have shown an increased risk of HT in OSA patients with self-reported EDS[145,146]. Furthermore, OSA is highly prevalent in patients with drug resistant HT where the prevalence has been reported to be as high as 70%[147-149].

The pathophysiological mechanisms behind HT development in OSA appear to be multifaceted. The occurrence of repetitive apnea events induces an increased night/daytime sympathetic activity during night and the autonomic activation is associated with elevated BP in OSA[150-152]. Some studies suggest that hypoxia during apneas may be a particularly strong contributing factor to the sympathetic response[153,154]. This is also in line with findings from the large ESADA cohort suggesting that HT prevalence may be more strongly associated with intermittent hypoxia than apnea events per se[155]. The repetitive cycles of hypoxia/re-oxygenation, as seen in OSA, may also lead to impaired endothelial function as evidenced by studies demonstrating reduced levels of nitric oxide and vasodilation[156-160]. However, sleep fragmentation[161], increased intrathoracic pressure[162,163], a dysfunctional renin-angiotensin system and a reduction of the baroreflex threshold[164] have also been proposed as potential mechanisms behind BP elevation and subsequent HT development in OSA.

CPAP has been shown to modify and attenuate several of the above mentioned potential pathophysiological mechanisms in OSA. However, the effect of CPAP on BP in unselected patients with OSA is proportionally weak[165]. A recent large randomized controlled trial (RCT) reported a weak, however significant, effect on DBP -0.7 (95% CI=-1.4 to 0.0) when comparing CPAP treated (n=1346) to non CPAP treated (n=1341) OSA patients[13]. The effect size is approximately 2-3 mmHg according to meta-analysis and the strongest effects of CPAP with regards to BP reduction are observed in OSA patients with pre-existing antihypertensive medication and in patients with resistant HT[166-168].

Coronary artery disease

Coronary artery disease (CAD), defined as angina pectoris or myocardial infarction, have been reported as frequent as 40% in patients with OSA[8,16,169,170]. The reciprocal association has also been shown in a controlled study[171]. Cross-sectional data from the Sleep Heart Health Study reported a 1.27 fold increased risk of CAD among OSA[8] patients. A subsequent prospective study found an almost five times higher incidence of CAD in OSA patients when compared with OSA-free individuals[172]. CPAP treatment has been associated with a risk reduction of CAD in some but not all studies[58,172]. Indeed, the mechanisms that potentially might link OSA with CAD remain to be elucidated. Several routes potentially linking periodic hypoxia and vascular inflammation has been described[173]. A recent interesting study suggests that serum from SA patients may induce inflammation in coronary endothelial cells

when a novel endothelial biosensor approach (the serum cumulative inflammatory potential assay) was used[174].

Arrhythmias

Atrial fibrillation (AF) is a common arrhythmia in clinical practice and associated with increased CV morbidity and mortality[175,176]. Several of the conventionally considered risk factors for AF such as age, HT, DM, obesity, CAD and congestive heart failure overlap with those considered in OSA[177]. In this context it is also evident that consequences of OSA, including periodic hypoxia and hypercapnia, shifts of intrathoracic pressure, increased autonomic nervous system activity and sudden BP swings may increase the risk of AF in patients with OSA[178].

Recent studies have reported an increased prevalence of OSA among patients with AF and the strength of the association was related to the severity of OSA[179-181]. The odds ratio for AF in these studies ranged between 2.19 and 17.9. AF is independently associated with OSA and the risk is increased with by a factor of four when comparing severe to non-OSA patients[182]. Other studies suggested an increased incidence of AF in OSA[183]. Meta-analysis data in patients following catheter ablation due to recurrent AF show that untreated OSA increased the risk for recurrence of AF by 25% when compared with CPAP treated patients [184]. Furthermore, additional data from meta-analysis that included 8 studies that CPAP treatment was associated a 44% reduced risk of AF recurrence[185]. There is increasing consensus among cardiologists that screening and early detection of OSA may have important health beneficial effects. In fact, recent study by Abe et al. showed that CPAP treatment significantly reduced occurrence the OSA-associated arrhythmia risk amongst a sub-population of 316 Japanese OSA patients[186].

OSA has also been considered in ventricular arrhythmias. Early data have suggested that ventricular tachycardia and premature ventricular contractions in addition to sinus arrest, bradycardia, atrioventricular cardiac block were associated with SDB and occurred primarily during sleep[187]. A recent meta-analysis on ventricular arrhythmias has concluded there is insufficient reliable data to explore the association between OSA and ventricular arrhythmias [188].

Stroke

Epidemiological studies suggested an independent association between OSA and stroke[8,10, 189]. Conversely, there is a high prevalence of OSA (44-72%) in stroke patients[190]. More severe OSA (baseline AHI cut-offs AHI ≥20 and ≥30) increased the risk of stroke substantially, independent of potential confounding factors[10,191,192]. Valham et al. reported that the incidence of stroke increased in a OSA severity-dependent manner with a four-fold risk increase in odds-ratio when comparing severe OSA to non-OSA[193]. A recent meta-analysis based on data from six prospective studies reported a two-fold increase of stroke when comparing severe OSA (AHI>30) to non-OSA (AHI<5) patients[194]. In addition, CSA has been associated with an increased risk of stroke in SA patients[195,196]. There is inconsistent data when it comes to a potentially beneficial effect of CPAP on stroke risk in OSA patients. Whereas population based cohort studies have pointed to a reduced risk after CPAP most

RCT's have failed to replicate this finding[13,197,198]. However, an ad-hoc analysis of the SAVE-study suggested a possible beneficial effect of CPAP on stroke incidence[13]. Despite this, guidelines for stroke prevention recommend CPAP treatment as acute treatment following stroke, especially in patients with comorbid SA[199].

Congestive heart failure

The association between heart failure (HF) and SDB have been established by several studies[200-202]. Shahar et al. reported a strong association between HF and SDB in cross-sectional data from Sleep Heart Health Study[8]. The relationship these two disorders appear to be stronger in patients with HF-CSA[203]. Moreover, OSA as well as CSA has been reported independent contributing factors behind an increased mortality risk amongst HF patients[204, 205]. Both short/long term studies have suggested that CPAP therapy improves systolic BP, cardiac function as well as reduces the risk of mortality in OSA patients with HF[206-208]. Whether CPAP therapy may exhibit similar positive treatment effects in HF patients with CSA remains inconclusive[209,210]. A more recent controlled study in CSA-HF patients (n=1325) reported an increased risk of CV death (hazard ratio 1.34 [95% CI 1.09, 1.65]) in patients treated with adaptive servo-ventilation when compared to controls[211].

Mortality

Early studies addressing long term outcome and mortality in OSA suggested an independent association between OSA and increased mortality. Levy et al. reported that SDB was an independent predictor for mortality amongst 1622 patients[212]. More recent evaluation of the influence of OSA on mortality in large cohorts suggested a 2.87 fold increase in mortality during a 12-year follow-up in those with untreated severe OSA (AHI≥30 events/hr)[58]. CPAP was associated with a normalization of mortality rate. This study was corrected for potential confounders. A subsequent study with a similar design was the prospective Sleep Heart Health study which showed a two-fold increase (hazard ratio 2.09 [95% CI 1.31–3.33]) in middle-aged men with moderate to severe untreated OSA patients when compared to non-OSA patents[213]. The lower absolute difference in mortality between those with and without OSA in this study has been explained by a higher mean age. An even higher influence of OSA on mortality was reported in the Wisconsin Sleep Cohort with a (adjusted) all-cause mortality (hazard ratio 3.8 [95% CI 1.6 - 9.0]) in severe vs non-OSA patients and this increase was dose-dependent[11]. Several of these studies suggest that comorbid vascular disease (e.g. stroke, acute myocardial infarction, acute and chronic ischemic heart disease, AF, sudden cardiac arrest, cardiac dysrhythmias, cardiomyopathy, and pulmonary HT) may further increase mortality although this possibility needs to be confirmed in controlled trials. In fact, the presence of OSA increases the mortality rate in patients suffering from CV disease such as stroke or CAD[10,17 58,189,214].

All studies cited above are non-randomized. In fact there may be a possible ethical concern with regard to long term RCTs in OSA, particularly as comorbidities including EDS could increase the risk for motor vehicle accidents in those randomized to usual care[215,216]. Although CPAP has been shown to attenuate the mortality risk in OSA, recent data from the randomized controlled SAVE-study could not confirm this association as there was no significant difference between CPAP and non-CPAP users with regard to hard CV endpoints[13].

However, the study was compromised by a number of factors including a low use of CPAP in the treatment group, which makes the overall generalization of the study more difficult.

5.7 Current mainstay of OSA therapy

Continuous positive airway pressure (CPAP)

CPAP is widely considered as the first line therapy in OSA (Figure 4)[217,218]. CPAP provides a constant or variable positive airflow, which acts to splint the UA thereby preventing UA collapse. CPAP treatment has proven to be efficient in the reduction of OSA symptoms including objective and subjective daytime somnolence, as well as the improvement of cognitive function and quality of life[13,116,219]. The relative efficacy of CPAP seems to be more pronounced in patients with moderate to severe OSA. The overall efficiency of CPAP is in many cases limited by adherence to therapy which has been reported to be as low as 50% [12, 14, 220].

CPAP appears to have a relatively modest effect on BP in unselected OSA patients[221]. Meta-analysis in the area has suggested that CPAP significantly reduces BP and risk of CV events in OSA patients with moderate-to-severe disorder and comorbid arterial HT[58, 221-223]. The effect of CPAP on BP seems to be more pronounced in those with severe disorder and more severe HT[224]. Two recent randomized control studies could not confirm any beneficial effect of CPAP on CV endpoints including stroke, ischemic heart disease or death in patients with OSA and HT[13, 225].

Figure 4. Continuous positive airway pressure device (left) and mandibular device (right).

Mandibular devices

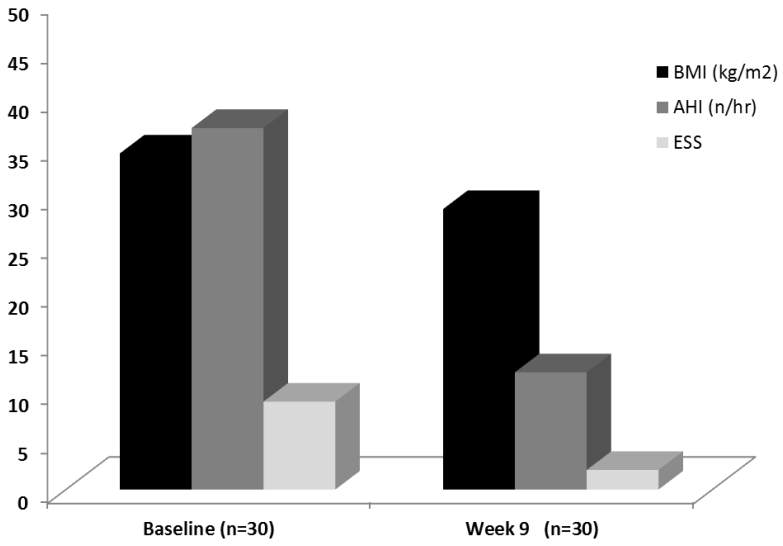
Mandibular advancement devices (Figure 4) are currently used for treatment of OSA[226]. They have been considered in most countries as useful treatment alternatives for patients with mild-moderate OSA and patients not tolerating CPAP. This particular group is considerable as a consequence of impaired long-term compliance and tolerability with CPAP[227]. Mandibular devices increase the UA cross sectional diameter and patency thereby reducing UA collapsibility[228]. Several studies have shown that these devices reduce OSA as well as

subjective and objective sleepiness[229-231]. Furthermore, mandibular advancement devices have been shown to reduce BP in prospective uncontrolled studies[230,232,233]. The overall effect on OSA, in terms of AHI reduction, of these devices is less pronounced than that obtained by CPAP but acceptance rates are usually higher[234].

Dietary weight counselling and bariatric surgery

Given the strong association between obesity and OSA, as shown in numerous studies[1,8,235], dietary and weight counselling is currently an under-utilized treatment modality in OSA[236]. Weight loss programs that include dietary counselling have been shown to lead to improvement of OSA, and to reduce daytime sleepiness and BP[237,238]. A controlled study by Johansson et al. using low caloric diet found that weight-loss was associated with significant improvements in OSA severity and EDS in obese patients with moderate to severe OSA[238] (Figure 5).

Figure 5. Effects of low-caloric diet and weight loss in moderate to severe OSA patients (Adapted by Johansson et al.[238])



Bariatric surgery is considered to be an effective method for management of obesity and obesity related comorbidities (e.g. HT and DM) compared to non-surgical interventions[239]. Prospective data from the Swedish Obesity Study concluded that bariatric surgery was associated with marked reductions in reported apneas and symptoms of OSA (e.g. EDS)[240]. In addition, meta-analysis data from 12 studies, assessing OSA by PSG, reported an overall mean BMI reduction by -18 kg/m^2 units and accompanied by mean AHI reduction of -38 events/hr[241]. Bariatric surgery may be considered in obese individuals with a BMI in excess of 35 kg/m^2 who failed to reduce weight by conventional methods[242,243].

Other surgical treatment

Most surgical procedures for OSA aim to modify the anatomy of the UA. Uvulopalatopharyngoplasty (UPPP) is conventionally considered as an established method for OSA treatment[244]. The overall aim with UPPP is to enlarge the UA diameter. However, in unselected cases the procedure is accompanied by a high failure rate (approximately 60%)[245]. A recent controlled study which used stringent criteria for patient selection reported a considerably higher success rate suggesting that this method may be considered in carefully selected cases[246]. Other proposed surgical treatment methods include nasal surgery. This method has been considered particularly useful in CPAP failure patients, particularly those cases where CPAP use is restricted by obstruction of the nasal airway. Nasal surgery may thereby increase adherence to pre-existing CPAP treatment[247, 248]. However, as the case with UPPP, nasal surgery has limited effect on OSA intensity[249]. Finally, a recently introduced method for intermittent stimulation of the hypoglossal nerve during sleep evokes a genioglossal muscle contraction which prevents UA collapsibility during sleep. Clinical data suggest that this method induces a reduction of the AHI in the order of 50% in preselected groups of patients with symptomatic OSA[35,250]. These effects persist even over time, but selection of patients is essential and those criteria are not very well defined yet.

Pharmacological treatment

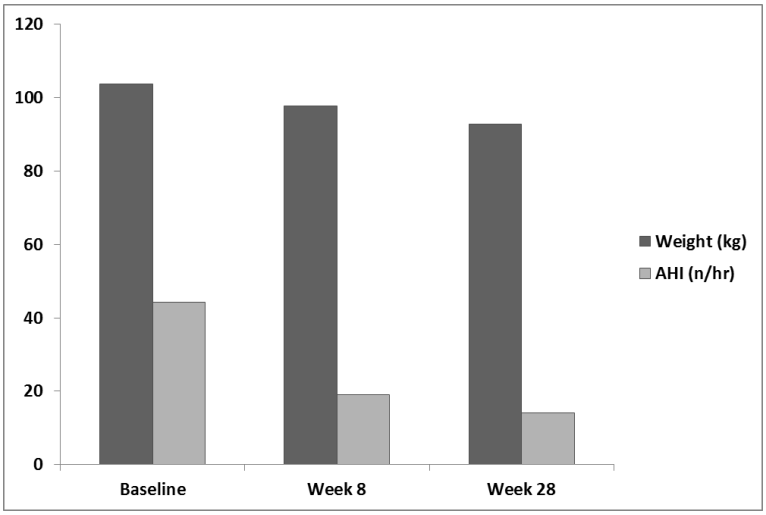
Given the limitations associated with several of the treatment modalities used in OSA, there is a need for a pharmacological form of therapy. This is also evident in view of the considerable pathophysiological heterogeneity that characterises unselected patients referred in the sleep medicine clinics[251]. Several smaller trials have investigated the effects of postulated pharmacological remedies in OSA but were in general accompanied by small effect sizes and no conclusive results in terms of OSA improvement[251,252]. Such trials include pharmacological agents such as gamma-aminobutyric acid and glutamate agonists, sex hormones, benzodiazepines glutamate, compounds modulating serotonin activity and theophyllines[251].

However, some more convincing results have been produced for pharmacologically induced weight reduction. For instance, an uncontrolled study of sibutramine, used for weight loss, in combination with weight counselling resulted in a mean BMI loss of 2.4 kg/m^2 and a 36% reduction of AHI[253]. This combination of effects may be particularly beneficial in overweight OSA patients with a comorbid metabolic disorder[253,254]. Whilst sibutramine was withdrawn from the market due to severe CV adverse events there are other interesting drugs that may be considered[255].

A formulation containing the combination of the appetite-suppressant drugs phentermine and topiramate induced a significant weight reductions (-10%) and there was an associated substantial decreases in AHI (approximately -70%) in obese patients with moderate severe OSA in a more recent randomized placebo controlled trial[256] (Figure 6). In addition, significant improvements were also found in nocturnal oxygen saturationSpO_2 and office BP. Long-term data on his particular formulation have demonstrated that a dose-dependent sustained weight loss may be achieved and there was an associated improvement of CV and metabolic variables in the obese patients in the study[257]. Another recent development is the GLP-1-analog (glucagon-like peptide) liraglutide which is indicated predominantly in T2DM. The clinical development program provided support for a reduction of body weight as well

as moderate improvement of SA resulting in the first label for obesity treatment in obese OSA patients defined as an BMI cut off of ≥27 kg/m^2 [258].

Figure 6. The effects on weight and AHI following phentermine/topiramate treatment (adapted from Winslow et al. [256])



Other recent studies have dealt with compounds that inhibit the enzyme acetylcholine esterase. A reference inhibitor of acetylcholine esterase, physostigmine, was associated with a 21.4% reduction of AHI and an improvement in minimum nocturnal SpO_2 by 8.7% in a placebo controlled single night study[259]. Another available inhibitor of ACE, donepezil, was also shown to significantly improve AHI, mean and minimum SpO_2 and EDS in patients with moderate to severe OSA in a short term RCT[260]. The effects were particularly pronounced during REM sleep. However, more recent controlled trials failed to confirm the beneficial effects of donepezil on OSA[261] (Hedner et al, unpublished data).

5.8 Carbonic anhydrase - background and physiology

Carbonic anhydrase in health and disease

The carbonic anhydrase (CA) enzyme exists in at least 16 different isoforms (CA I-XVI) and is classified into 4 groups based on the localization (cytosolic, mitochondrial, secreted, membrane linked) in mammals[262]. The structure and function of this enzyme has been extensively studied following its initial discovery in red blood cells in 1932[263, 264].

Figure 7. Main chemical reactions catalysed by carbonic anhydrase

$$H_2O + CO_2 \overset{CA}{\Leftrightarrow} H_2CO_3 \overset{CA}{\Leftrightarrow} H^+ + HCO_3^-$$

CA catalyses the conversion of CO_2 to bicarbonate and protons (Figure 7). This reaction is slow but the presence of CA accelerates the process by a factor of 10^6. Hence, the enzyme plays a pivotal role not only for the regulation and maintenance of the acid-base homeostasis (pH), but also for the transport of CO_2 (mainly via red blood cells) between tissues as well as in response to respiration[263, 265]. Bicarbonate production, pH regulation, as well as water balance controlled by CA, is vital to the function of several tissues. Dysfunctional CA activity has been associated with conditions like low gastric acid secretion, renal failure and glaucoma[262,266]. The enzyme is also involved in reactions that require bicarbonate as a substrate such as glukoneogenesis, lipogenesis, and ureagenesis[267]. Other processes where CA is involved include cancerogenesis (or tumorigenesis) as well as growth and virulence of pathogens[268,269].

Due to the widespread expression of the enzyme in human organs and tissue the biological principle has become target for various compounds with CA activating or inhibitory properties. For instance, the CA inhibitor dorzolamide has been used as an anti-glaucoma agent which reduces the intra-ocular pressure as a consequence of decreased bicarbonate level and aqueous humour production[266]. CA inhibitors have also been considered as anticancer/anti-metastatic treatment as various CA iso-enzymes are overexpressed in hypoxic tumour cells. Blocking the CA enzyme capacity to produce protons leads to a less acidified environment in which tumour growth and proliferation rate is reduced[270].

CA has also been targeted in obesity research[271]. RCT´s using CA inhibitors like zonisamide (ZNS) and topiramate have shown prominent weight reduction in obese patients[256,272]. The mechanisms behind the weight loss effect may in part include a modulation of de-novo lipogenesis[267]. Moreover, the expression of CAIII in subcutaneous adipocytes from obese patients was reduced (Jernås et al. unpublished data). It was hypothesized that CAIII maintains a scavenger role by a reduction of the amounts of free radicals in the fat cell.

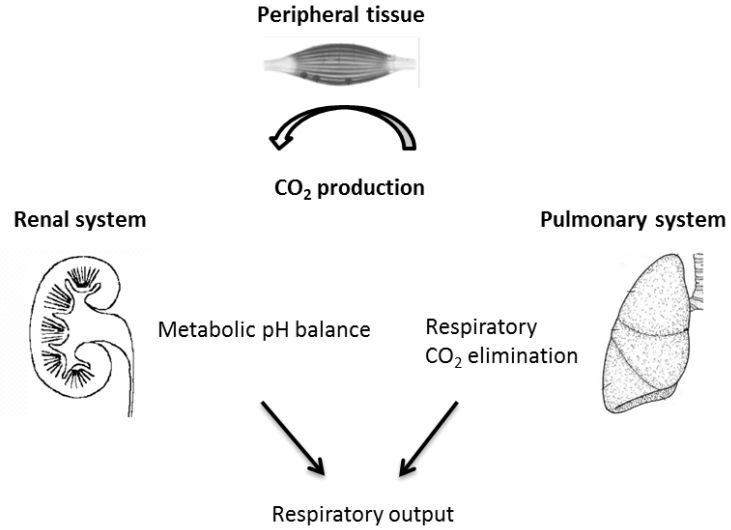
Carbonic anhydrase in sleep apnea

CA enzymes are, as previously mentioned, widely expressed in the human body. With respect to relevance for ventilatory control and respiration, the CA enzymes are expressed in the lung, kidney, and red blood cells as well as in CNS regions corresponding to the central and peripheral chemosensors[273]. The occurrence of repeated apneas/hyponeas during sleep exposes the patient to cycles of hypoxia/hyperoxia and hypo/hypercapnia. In that sense OSA may resemble other situations with relative hypoxia including strenuous physical activity, chronic hypoxia or complex hyperventilation states that are known to alter the CA activity[274-276]. Previous studies have also suggested that hypoxia influences the CA activity in human and chick embryonic development[277-280]. There are several mechanisms which could increase CA activity in the human body such as tissue hypoxemia as well as high altitude hypoxic exposure[279,281]. Moreover, an increased expression and activity of the CA enzyme may occur due to oxidative metabolism or reduced oxygen tension occurring at the molecular level[274, 282].

The ventilatory effects of CA inhibition have been extensively studied in the past. Pharmacological agents with CA inhibitory properties (e.g. AZT) have been used for various respiratory conditions or diseases such as respiratory failure, high altitude periodic breathing and central apnea of type CSR as well as in OSA[282-286]. In fact, small controlled studies have

reported that pharmacological CA inhibition reduces AHI with30 to 50% and improves nocturnal oxygenation in patients with OSA[49,282,283,287]. Furthermore, RCTs have reported a suppression of both obstructive and central breathing events by CA inhibition in OSA patients traveling to high-altitude[288,289]. An improvement on SDB and SDB related symptoms (e.g. EDS) in response to treatment with AZT has also been reported in patients with CSA, more specifically in heart failure patients with CSR[290,291]. CA inhibitors have commonly been proposed as prophylaxis in healthy subjects in situations where periodic breathing may develop, such as acute-mountain sickness[292,293].

Figure 8. Overview the principle mechanism behind increased ventilation by carbonic anhydrase inhibition



The most important mechanism by which CA inhibition can promote ventilation is considered to be via the renal system (Figure 8)[263]. Inhibition of CA in proximal and distal tubules of the renal system generates a loss of bicarbonate in urine with metabolic acidosis as consequence and this will further promote ventilation via chemosensory mechanism[294]. The response of the induced respiratory alkalosis is to reduce PCO_2 and hydrogen ion (raising pH) concentration by an increase in ventilation[295]. In addition, the stimulatory effects of CA inhibition on ventilation may also be attributed to the inhibition of the enzyme in red blood cells and vascular endothelium[285,296,297]. CA inhibition increases tissue CO_2 retention in the vicinity of peripheral and central chemoreceptors which, in addition to the metabolic acidosis, will provide a further stimulus to ventilate. CA inhibition reduces the rate of response to CO_2 while increasing the hypoxic ventilatory response[273,298]. More recent studies have shown that CA inhibitors (e.g. AZT) improve ventilatory stability by reducing LG in CRS and OSA patients[49,290]. In addition, as reported by Edwards et al. AZT reduces the ventilatory overshoot in response to an arousal, an effect which further improves the ventilatory stability in selected OSA patients[299].

Carbonic anhydrase and cardiovascular function/disease

In addition to effects on respiration, CA enzymes appear to be expressed in various tissues where their activity may relate to CV regulation (e.g. HT, Table 2). Most of the insights in this area have been obtained in experiments using inhibition of the CA system. It is possible that the hemodynamic effects induced by CA inhibitors in some cases may involve mechanisms unrelated to CA[300]. However, in the context of OSA with comorbid vascular disease such as HT it may be speculated that a possible dual effect on respiration and hemodynamic control would be of particular value.

As shown in Table 2, CA is present in vascular endothelium, a tissue which is known to produce multiple substances (e.g. nitric oxide, endothelin 1) directly implied in the regulation of vascular tone[301-303]. The enzyme is also present in vascular smooth muscle, blood lines (red, white and platelets) and in the heart (low concentrations)[304-306]. The expression of CA enzymes in these tissues is likely to contribute to regulation of physiological processes that include vascular resistance, cardiac contractility, diuresis and fluid shift[307-310]. In addition, with regard to CV regulation, the CA enzyme is expressed in central nervous system around peripheral and central chemoreceptors[273,311,312]. Indeed, there are numerous sites and pathways by which CA inhibition can affect the CV regulation in OSA patients.

Table 2. Location and expression levels for different CA isoenzymes in the cardiovascular system (adapted from Swenson, 2014[300])

Carbonic anhydrase enzyme	Tissue expression	Activity level
I	Red cells, vascular smooth muscles	Low
II	Red cell, kidney ,lung, endothelium, heart, vascular smooth muscle, brain vascular smooth muscle	High
III	Vascular smooth muscle, skeletal muscle	Very Low
IV	Kidney, lung, brain, heart Endothelial cells	High
V	Mitochondria	High
IX	GI tract, cancer cells, hypoxic cells	High
XII	Kidney, brain, heart	High
XIV	Kidney, brain, heart	High

Experimental studies have shown that CA inhibition contributes to vasodilation via direct or indirect mechanisms. In a forearm flow model CA inhibition modulated a calcium-activated potassium channel (decreasing cytosolic Ca^{2+} concentration) and induced vasodilation[307].

Moreover, CA inhibitors, in very high concentrations, may block voltage gated calcium channels and thereby alter the vascular tone[313]. Experimental in-vivo and in-vitro studies have shown that the hypotensive effect of calcium-channel blocker to an extent is associated with their ability to inhibit CA in the vascular smooth muscles and erythrocytes[314, 315]. CA inhibitors may also induce vasodilation by modulation of nitric oxide metabolism in the vascular endothelium[316,317]. Other mechanisms by which CA inhibition may cause vasodilation include their ability to reduce the CO_2 carrying capacity of red blood cells. The increase of tissue pCO2 will cause hypercapnia with a subsequent vasodilatory response to reduce CO_2 levels[318,319].

Several physiologic effects of CA inhibition are more apparent during hypoxic conditions (e.g. high-altitude). Experimental studies on hypoxia exposed animals showed a decrease of pulmonary vascular resistance and vasoconstriction following treatment with AZT[320,321]. The effects of AZT on hypoxic pulmonary vasoconstriction were also demonstrated in RCT´s that included healthy subjects traveling to high-altitude[286,322]. However, this effect may not have been related to CA inhibition[323,324]. An effect on systemic HT was demonstrated by Parati et al. who reported that elevated brachial BP following acute exposure to high-altitude and hypobaric hypoxia in healthy volunteers was counteracted by AZT[325]. A similar effect, linked to reduction of nocturnal transcutaneous PCO_2, was reported in OSA patients traveling to high- altitude[289].

It should be noted that several conventional CA inhibitors (e.g. AZT or ZNS), not accounting for thiazides and loop diuretics, act as diuretics and have shown to reduce extracellular fluid volume (5-10%)[326]. In the context of the current thesis it is also plausible that diuretic effect related to CA inhibition potentially may account for BP lowering effects. However, studies have shown that CA inhibition by AZT has not resulted in any significant BP reductions in normal subjects with essential HT[327,328].

6 Aims of the thesis

The overall aim of this project was to investigate the association between obstructive sleep apnea (OSA) and activity of the carbonic anhydrase (CA) system. In addition, we aimed to explore the influence of CA inhibition on blood pressure (BP) control and sleep apnea severity in OSA patients. The thesis is based on the following studies;

1. Paper I
   Aimed to establish an association between arterial standard $StHCO_3^-$, as a proxy for CA activity, OSA severity as well as hypertension.

2. Paper II
   Explored the association between the whole blood CA enzyme activity and OSA severity. In addition, the association of CA activity and blood pressure (BP) was investigated.

3. Paper III
   Investigated the therapeutic effects of a pharmacological CA inhibitor, zonisamide, on SDB in overweight/obese OSA patients.

4. Paper IV
   Explored the combined therapeutically effectiveness of AZT (CA inhibitor) in direct comparison with CPAP therapy on sleep disordered breathing and blood pressure/vascular function in patients with OSA.

7 Methods

7.1 Study population and design

Four different groups of patients were studied in this thesis. All four study populations in paper I through IV had been referred for investigation of suspected SDB. The study populations were predominantly middle-aged males. Obesity was common as was HT and sleepiness. The population in paper IV was selected based on an existing HT diagnosis.

Table 3. Baseline characteristics of patients in Papers I through IV.

	Paper I	**Paper II**	**Paper III**	**Paper IV**
Cohort	OSA patients	OSA patients	OSA patients	OSA patients
Population, n	830	70	42	13
Age (yrs)	51 (10)	54 (13)	51 (12)	64 (7)
Gender(% Males)	93	69	93	100
BMI (kg/m^2)	30 (5)	30 (6)	31 (2)	29 (4)
SBP (mmHg)	146 (20)	138 (20)	135 (17)	157 (11)
DBP (mmHg)	94 (13)	84 (11)	84 (9)	86 (11)
HR (bpm)	73 (11)	69 (11)	59 (8)	62 (9)
AHI (n/hr)	32 (24)	27 (23)	47 (24)	37 (22)
ESS	-	11 (5)	13 (4)	9 (4)
HT prevalence (%)	53	46	32	100*

*= *Population based on hypertensive OSA patients*.

7.2 Study design and procedure

Epidemiological sleep clinic cohort (Paper I)

Paper I was based on a retrospective population cohort containing patient referrals (n=1665) to the Marburg Sleep Disorders Centre (between the years 1989 and 1992)[329]. An AHI cut-off of ≥5 events/hr was implemented for inclusion. Exclusion criteria included respiratory failure (n=163), obesity hypoventilation syndrome (OHS) (n=38) and COPD (n=163). An additional 363 patients were excluded due to missing data (arterial blood gases, pulmonary function, and BP). A total of 830 patients met the above mentioned criteria and were included in the

final data analysis which investigated the association between arterial standard bicarbonate ($StHCO_3^-$), apnea severity and HT.

Experimental study (Paper II)

Patients (n=74) included in this experimental study were randomly recruited among those referred for suspected OSA to the Department of Sleep Medicine at the Sahlgrenska University Hospital. Exclusion criteria included an established history of COPD or OHS (n=4). The final analysis was made on data from 70 patients that underwent a full night cardiorespiratory polygraphy recording at the sleep clinic. Blood samples for assessment of CA-activity, office BP and HR were collected and recorded in the morning immediately following to the sleep recording.

Interventional studies (Paper III and IV)

The papers III and IV included patients with a recognized diagnosis of OSA and considered for therapy with CPAP. The patients were recruited at the Department of Sleep Medicine, Sahlgrenska University Hospital. The detailed inclusion and exclusion criteria applied in these studies are given in Table 4.

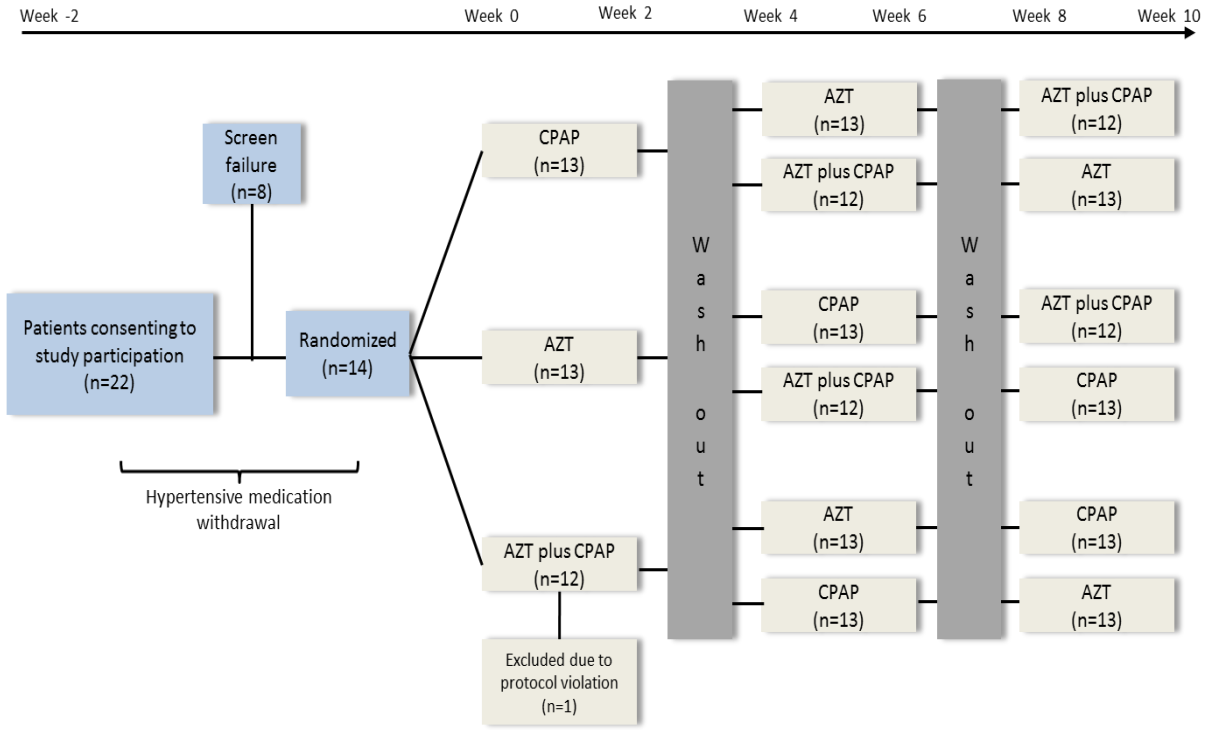
Table 4. Inclusion and exclusion criteria for interventional studies paper III and IV.

	Paper III	Paper IV
Inclusion criteria's	• Males/females 18 to 75 years, • AHI>15 n/h and an ESS>6, • BMI between >27 and <35 kg/m2 • Clinically normal physical findings and laboratory values, as judged by the investigator	• Males 18 to 75 years • AHI of >15 n/h and ESS >6 • BMI ≤ 35 kg/m2 • Established hypertension defines SBP/DBP >= 160/95, either systolic or diastolic accounted for) or ongoing hypertensive treatment
Exclusion criteria's	• Hypersensitivity to sulphonamides or zonisamide • Seizure disorders • Unstable cardiovascular-, pulmonary- or gastrointestinal disease • Depression or any form of alcohol and substance abuse interfering with the study protocol. • Patients with an occupational hazard resulting from sleepiness • Patients with severe nocturnal hypoxemia (>10 episodes with SpO_2 with desaturation below 50% or poor resaturation capacity	• Hypersensitivity to sulphonamides or acetazolamide • Clinically verified central sleep apnea • Seizure disorders • Therapy resistant HT • Unstable cardiovascular-, pulmonary- or gastrointestinal disease, • Impaired renal or hepatic function, • Depression or any form of alcohol and substance abuse interfering with the study protocol. • Patients with an occupational hazard resulting from sleepiness • Patients with severe nocturnal hypoxemia (>10 episodes with SpO_2 with desaturation below 50% or poor resaturation capacity

Paper III was based on an interventional study using randomized, placebo-controlled, double-blind, and parallel group design. In addition to a placebo-controlled phase (a total of 4 weeks), the study also contained an open-label phase (5 months) that compared the effect of ZNS and CPAP on SDB. A total of 50 patients were screened and subsequently randomized (n=47) to ZNS (n=16), placebo (n=16) or CPAP treatment (n=15). Three patients withdrew consent prior to randomization. Following the short-term treatment phase, study subjects from the placebo group were allocated to ZNS treatment. Assessments of SDB, office BP, blood samples and biochemistry, anthropometrics, questionnaire data were conducted at baseline, 4 weeks and 24 weeks. Baseline PSG included a habituation night.

Paper IV was based on a single center, open (blinded for data analysis), randomized three-way cross over trial. Details of the study design are given in Figure 9. A total of 22 male patients were identified as eligible but only 14 met all inclusion/exclusion criteria. All ongoing antihypertensive treatment was washed-out prior to randomization. One patient was excluded during withdrawal of antihypertensive medication due to high BP. The remaining 13 patients were randomized to receive AZT, CPAP or the combination of the two (n=12). The subsequent allocation to the treatment modalities followed a blinded randomization code administered by personnel not involved in the study. Each of the six study visits applied an ambulatory PG recording during sleep as well as assessment of office BP, blood samples and biochemistry, anthropometrics and a program of daytime functional hemodynamic tests performed in conjunction to the sleep recording. Treatment periods were separated by a two weeks wash-out period.

Figure 9. Overview of study flow chart for Paper IV



7.3 Ethical considerations

Paper I was approved by the Ethics committee at Marburg University, Marburg, Germany. Protocols for papers II-VI were approved by the Ethics committee at University of Gothenburg. Written and oral informed consent was obtained from all participants prior to the study entry. Paper III and IV were registered on online clinical registries sites clinicaltrials.gov and European Clinical Trials Database (EudraCT).

7.4 Anthropometric, clinical and questionnaire data

Anthropometric and clinical data were collected throughout all studies. Bodyweight and height were determined to the nearest 0.1 kilograms and centimetres, respectively. BMI (kg/m^2) was calculated as bodyweight (kilograms) divided by the height squared (meters). Sagittal diameter, waist circumference and hip girth, all expressed in centimetres, were assessed in Paper III.

Information regarding comorbidities (e.g. DM and CV disease), concomitant medication, smoking and alcohol consumption was based on self-reported information or a physician´s diagnosis and recorded and documented for all subjects. Potential suicidal risk or behaviour as well as anxiety (Papers III and IV) were assessed by relevant validated questionnaires including the Columbia suicide severity rating scale and the Zung self-rated Depression and Anxiety scales[330-332]. The Clinical Global Impression scale, severity and improvement scales, was used to determine symptom severity, treatment responses and efficacy. Side effects and safety were monitored prior to study start and monitored throughout the entire study period(s). The Epworth sleepiness scale (ESS, Paper II-IV), the Functional Outcomes of Sleep Questionnaire and the Fatigue impact scale (Paper III) were used to determine subjective EDS as well as sleep and fatigue-related effects on quality of life[98,99,333].

7.5 Biochemistry assessment

In paper I, arterial blood gases were obtained at 12 a.m. following the sleep assessment and analysed using a Radiometer gas analyser (Radiometer, Copenhagen, Denmark). Biochemistry and venous blood sample data in Paper II-IV were collected in the morning subsequent to the sleep recording visits. All assessments were conducted during fasting conditions. Samples were analysed according to clinical routine procedures at the Department of Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg.

7.6 Blood pressure, heart rate and hemodynamic assessment

Office BP and HR measurements were assessed according to international standard[334-336]. Systolic/diastolic BP (SBP/DBP) and HR were determined with the patient in a sitting position following a minimum of 10 minutes rest and expressed as the mean of two subsequent recordings in Paper I. In paper II-IV BP and HR were assessed with an automated Omron M6 recorder (Omron Healthcare, Kyoto, Japan). Office SBP/DBP and HR in paper II-III and IV were expressed as the mean of two or three subsequent recordings (1 minute intervals) in the supine position after a 10-minute rest, respectively. Office BP was expressed in mmHg and HR in terms of mean beats per minute (bpm). Mean arterial pressure (MAP) was calculated according to the formula MAP= (1/3*SBP) + (2/3*DBP).

Definition of hypertension (paper I)

Patients with a previous history of clinically diagnosed HT and/or on ongoing hypertensive medication were defined as hypertensives. In addition, patients with no previous hypertensive medical history and/or no ongoing anti-hypertensive treatment were defined as normotensives.

Non-invasive Arteriography (Paper IV)

Arterial stiffness and central hemodynamic parameters were determined by a validated oscillometric method using a sensor placed at the skin surface over the radial artery (Arteriograph, TensioMed®, Hungary, version 1.10.1.11)[337,338]. Arterial stiffness and central hemodynamic parameters included aortic and brachial augmentation index, central aortic SBP/DBP and pulse pressure. All non-invasive arteriography recordings were measured during fasting conditions between 8-10 a.m. and conducted in a semi-recumbent position following a minimum of 10 minutes rest in the supine position. All data were automatically analyzed according to the built-in device algorithm.

7.7 Sleep studies

Polygraphic recordings

Paper I

The ambulatory PG recordings in paper 1 were performed using the MESAM 4 polygraphy device (MAP®, Munich, Germany). This device has been validated for assessment of SDB in clinical and epidemiological studies[339,340]. The PG montage included finger pulse oximetry, an electrical-miniature microphone placed over the larynx to assess snoring, ECG, and a circular sensor below the sternum to assess body position. Apnoic and hypopneic events were visually determined using a ≥4% oxygen desaturation criterion. A more detailed description of the scoring procedure has previously been described elsewhere[340]. Information derived from sleep diaries (lights on/off, periods of sleep interruption, time of going to bed/awakening) was used to estimate sleep time. The AHI was defined as the number of total apneas and hypopneas divided by sleep time.

Paper II and IV

Ambulatory (Paper IV) or sleep laboratory attended (Paper II) cardiorespiratory PG recording were conducted using the Embletta® X10 Portable Digital System device (Embla, CO, USA). The recording montage consisted of a nasal cannula, thorax/abdominal respiratory effort belts by means of inductive plethysmography, and finger pulse oximetry. Apnea events were defined as a cessation of airflow (≥90%). Hypopnea events were defined as a decrease of airflow (≥ 30%) associated with a ≥ 3% or 4% (paper II) oxygen desaturation. For an event classification duration of at least 10 sec was required. Apnea-hypopnea index (AHI) was calculated as the total number of apnea/hypopnea events divided by analysis time (lights off/lights on period during recording session). The ODI was calculated as the total number of ≥4% desaturations divided by analysis time. Mean nocturnal oxygen saturation (SpO_2) was calculated as the mean oxygen saturation measured by finger pulse oximetry during analysis time. Data was scored and analyzed according to the 2007 AASM criteria[102]. OSA severity

(Paper I and II) was defined according to a grading scale as mild (5≤AHI<15 n/h), moderate (15≤AHI<30 n/h) or severe (AHI≥30 n/h).

Ambulatory PSG (Paper III)

All study participants underwent ambulatory polysomnography (PSG) studies using the Embla A10 system (Embla, Flaga, Reykjavik, Iceland). The ambulatory PSG recording montage (Paper III) included electroencephalograms (C3-A2, C4-A1, FZ-A2, OZ-A1), 2-channel electrooculograms, submental (chin) and bilateral tibialis electromyograms and 2-channel electrocardiogram. In addition, information from a nasal cannula and an oro-nasal thermistor, thoracic and abdominal respiratory effort belts, body position sensor and finger pulse oximetry was used for the assessment of SDB. The AHI was calculated as the total number of apneas/hypopneas divided by total sleep time. Apneic events were defined as an almost complete cessation (≥90%) of the airflow persisting at least ≥10 seconds. Hypopneic events were defined as a decrease in airflow signal (≥50%) persisting ≥10 seconds and associated with a ≥3% decrease in oxygen desaturation or an arousal. The ODI was calculated as the total number of ≥4% desaturation divided by total sleep time. Furthermore, the mean, minimum and time spent below 90% oxygen saturation were recorded. All data were analyzed and scored according to the 2007 AASM criteria by an experienced and blinded (for treatment allocation) PSG technician. Study participants underwent two full consecutive PSG nights at baseline[102]. Data from the first PSG was considered as obtained from a habituation night and was not included in the analysis.

Subjective sleep time

In paper III, subjective sleep time (habitual sleep time) for assessment of the overall CPAP treatment effect (concept of OSA alleviation was assessed by sleep diaries initiated two weeks prior to each PSG-visit[12]. The habitual sleep time was calculated as the mean of all subjectively reported sleep time in the CPAP and ZNS groups at 24 weeks. Habitual sleep time in Paper IV was calculated as the mean of the lights-off to lights-on times assessed by PG in accordance with each study visit (a total of six visits).

7.8 CPAP initiation, compliance and adherence (Paper III and IV)

CPAP treatment was conducted by standard auto adjusting positive airway pressure devices (S8 Autoset Spirit II or S9 Autoset, ResMed Ltd, Sydney, Australia). CPAP pressure range was set to vary between a minimum of 5 and a maximum of 15 cmH_2O. CPAP introduction and training was conducted according to standard hospital clinical routines. Mask pressure, leakage, usage hours (mean/total) and residual AHI were documented by the built-in device memory cards at each follow-up visit. Patients were encouraged to contact the study personal between the scheduled visits if any problem with the device occurred. Data was acquired and analysed by ResScan™ software (version 3.14, ResMed Ltd, Sydney, Australia).

7.9 Study drug , titration and compliance

Paper III

ZNS (100mg, Zonegran®, Eisai Ltd, UK) or placebo tablets were titrated in a stepwise manner starting with a daily dosage of 100 mg and an option to escalate dosing on a weekly basis to

reach a maximum daily administered dosage of 300 mg. Recommended time for intake of medication was set at 8-10 pm corresponding to 1-2 hours prior to sleep. Compliance and mean daily dosage with the study drug and placebo were determined and calculated by tablet count. In the event of a side-effect the daily dosage could be reduced (100 mg steps). The total duration of the drug treatment was 20 weeks or 24 weeks pending on the randomisation schedule.

Paper IV

AZT (250mg, Diamox®, Mercury Pharmaceuticals Ltd, London, UK) was administered according to incremental dosing steps starting at 250mg/day and set to reach a maximum dosage of 750mg/day. Recommended medication intake was at two hours prior to bedtime. Compliance and mean daily dosage with the study drug and placebo was determined and calculated by tablet count. The total duration of drug treatment was 2x2 weeks. In the event of a side-effect the daily dosage could be reduced (250 mg steps)

7.10 Therapy compliance adjustment (Paper III and IV)

SDB (e.g. AHI and ODI) was adjusted for CPAP and drug compliance in order to compare therapeutic efficacy of the two treatment modalities. Drug compliance (%) was calculated as actual total dosage/day divided by maximum daily dosage, as determined by tablet count. Furthermore, the obtained value was multiplied with treatment effect, e.g. absolute change in AHI/ODI (%). CPAP efficacy was adjusted and corrected for therapy (hrs/night) and habitual subjective sleep length (hrs/night corresponding to overall apnea exposure) in order to reflect the proportional sleep time with CPAP use (%).

Sleep apnea alleviation (SAA%, paper III) was computed and expressed as the therapeutic effect of CPAP after adjustment for absolute user time, relative efficacy of the therapy (corrected AHI or residual AHI) and the mean habitual sleep time. SAA% enables a comparison between mechanical and pharmacological treatment that adjusts for the incomplete compliance with CPAP. The details of the SAA% assessment has previously been described elsewhere[12, 287]. In brief, compliance data from the built-in CPAP meter was adjusted for subjective habitual sleep time. Furthermore, the CPAP treatment effects on AHI/ODI relative to the baseline were calculated. SAA% was expressed as the multiplied percentage of efficacy and actual user time of CPAP.

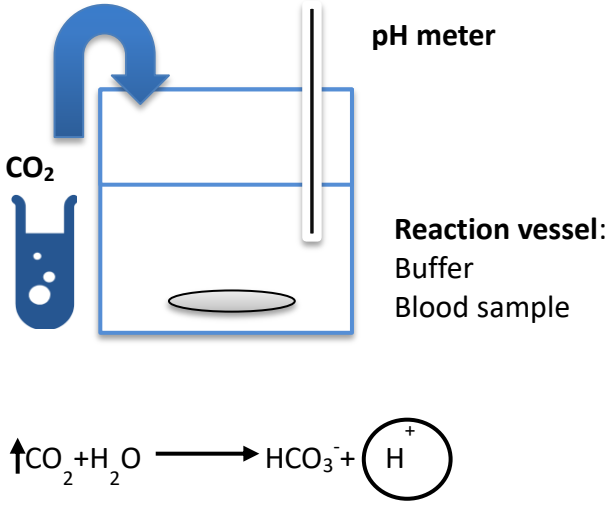
7.11 Assessment of carbonic anhydrase activity (Paper II)

The method used to determine CA activity from blood samples has previously been described[341,342]. CA enzyme activity assessments were conducted by repeated measurements and duplicate samples in the analysis in order adjust for possible inter- and intra-assay variability. A pH meter (Docu-pH+, Sartorius, Sweden) was used to continuously monitor pH. Venous blood samples were obtained and stored at -70 ^{o}C. A procedure of thawing, re-freezing and thawing was performed for hemolysation of the sample. A buffer (pH range 6-8) comprised of MES (2-(N-morpholino) ethanesulfonic acid), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sodium chloride and potassium chloride was prepared. All subsequent analyses were performed on ice in order to maintain temperatures between 0-1 ^{o}C. A separate solution consisting of distilled water was continuously flushed

with CO_2 (100%) for a minimum of 30 min. The venous blood sample (diluted (1:2000) in 0.9% saline) was added to the buffer solution in a reaction vessel with continuously monitored pH (see Figure 10). After a baseline pH (8.00 ±0.03) had been established, the CO_2 saturated solution was added into the reaction vessel (Figure 10).

The pH was monitored during 120 seconds. Self-developed software was used for plotting and analyzing the pH curves. Area under the curve (AUC, range 802-837) was calculated and defined as the sum of pH assessments during the recording interval and assumed to correspond to the venous blood CA-activity (Figure 10). The CA-activity was defined as AUC (arbitrary units). Thus, a lower calculated AUC corresponds to higher CA-activity (i.e. a shorter reaction time).

Figure 10. Overview of the methodological procedure for the assessment of carbonic anhydrase activity and the catalyzed reaction by addition of carbon dioxide to the reaction vessel



7.12 Statistics

The statistical analysis in papers I-IV was conducted using IBM SPSS 19, 20 and 22 (SPSS Inc, Chicago, USA). A two-tailed p-value of <0.05 was considered as statistically significant. Data are presented as mean (standard deviation, (SD)), median and interquartile range (IQR, 25% to 75%), and mean change and 95% confidence interval (CI). The normality and distribution of data were determined by Kolmogorov-Smirnov test or Shapiro–Wilk t-test. Kruskal-Wallis test and one way analysis of variance (ANOVA) was used to determine across group differences. Data in the interventional studies (paper III and IV) were analysed as per-protocol. The arterial $StHCO_3^-$ as well as the AHI and ODI were logarithmized in paper I and II, respectively, in order to receive normally distributed data.

Paper I

Associations between arterial $StHCO_3^-$, AHI, SBP and DBP were studied by Spearman correlation. Group differences between different $StHCO_3^-$ quartiles were assessed by independent sample t-test. Independent association between arterial $StHCO_3^-$, AHI,

hypertensive status as well as office BP were studied using multivariate generalized linear models.

Paper II

Group comparisons were assessed by Mann-Whitney U-test. The association between CA activity, OSA and BP were addressed using Pearson and Spearman correlations. Independent associations between CA-activity, OSA severity and BP were assessed by generalized linear models, with logAHI/ logODI, and sitting diastolic BP respectively, as the dependent variables.

Paper III

Within group differences with regards to sleep-related, anthropometric and biochemistry variables at week 4 and 24 were studied by paired sample t-test. Comparison between treatment modalities was assessed by analysis of covariance (ANCOVA).

Paper IV

ANOVA or Kruskal-Wallis was used to compare and assess between group differences at baseline. Within group comparisons were computed by Paired sample t-test and Wilcoxon signed-rank. Associations between venous $StHCO_3^-$, respiratory and BP variables were assessed by Pearson and Spearman correlations. Between groups comparisons were studied by independent sample t-test or Mann-Whitney.

Sample size estimation

In paper III, the study sample size was determined based on data from in-house pharmacological studies in OSA patients. We assumed that the pharmacological treatment would reduce AHI by ≥7.5 (6.0) events per hour compared to placebo. A parallel group design with 30 patients on each treatment arm would have a power exceeding 90% for detecting a treatment group difference in primary efficacy variable(s) (using a two-sample t-test with a 5% significance level).

In paper IV, the sample size was determined based on data similar studies that had examined the effect of CPAP or a CA-inhibitory drug on MAP[287]. In detail, a cross-over design study would need 30 patients, based on that a reduction of MAP after AZT treatment would exceed that of CPAP by 5 (10) mmHg and a to obtain a power of 80% for detecting a treatment group difference (two tailed t-test with a 5% significance level).

8 Main Results and discussion

8.1 Association between CA-activity and OSA

Paper I

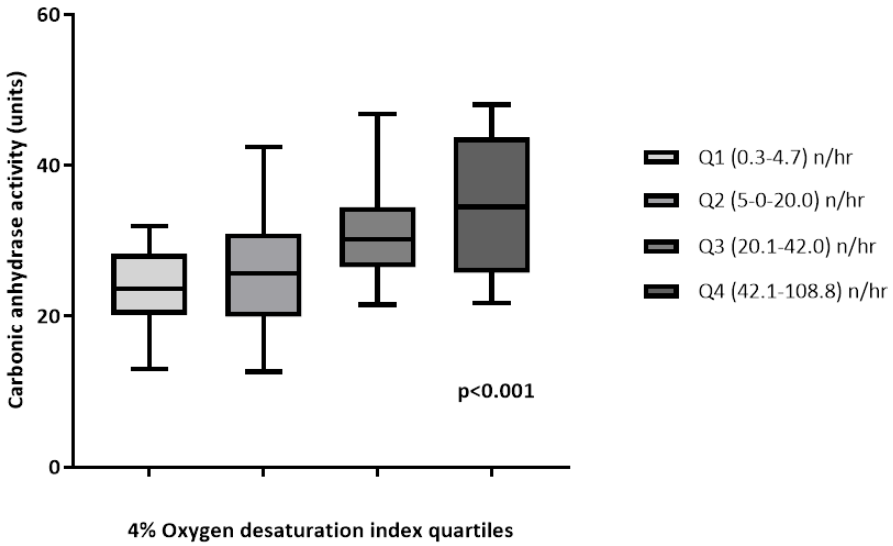
This study included 830 predominantly middle-aged overweight-obese males undergoing a diagnostic sleep study (93.3% men, age 51(10) years, BMI 30(5) kg/m^2, AHI 32(24) n/h, Table 3). Arterial blood gases had been performed in all patents and standard HCO_3^- ($StHCO_3^-$) was found to increase gradually across OSA severity classes although the magnitude of the mean change was moderate (ANOVA, p<0.001). AHI in the lowest quartile was 27(21) and in the highest quartile 39(26) suggesting that considerable SA was present in all quartiles. $StHCO_3^-$ was positively correlated with AHI (Spearman correlation r=0.16, p<0.001) and $StHCO_3^-$ was independently associated with AHI (Q1 vs. Q4 *β*=10.6, p<0.001) in a generalized linear model controlling for sex, age, BMI, smoking, alcohol consumption, pO_2, pCO_2 and HT status.

$StHCO_3^-$ was approximately almost one mmol/l higher in hypertensive (24.9±2.7) compared with normotensive (24.1±1.9) patients (p>000.1). Moreover, the association between OSA in the highest severity class (AHI≥30) and $StHCO_3^-$ was observed only in hypertensive OSA (and not normotensive) (p<0.007 and p<0.001 compared with the moderate (AHI 15-29.9) and mild (AHI 5-14.9), respectively. $StHCO_3^-$ expressed as $logStHCO_3^-$ was independently associated with a clinical diagnosis of HT in a generalized linear model controlling for sex, age, BMI, smoking, alcohol, PO_2, PCO_2 and apnea severity (Q1 vs. Q4 *β*=8.o, SE 3.0, 95%CI (2.1-13.8, p=0.007). The association between AHI and BP appeared to be stronger for diastolic than SBP.

Paper II

Baseline characteristics of 70 patients participating in this study are shown in Table 3. The CA activity in patients without OSA, (AHI<5), mild (5≤AHI<15), moderate (15≤AHI<30) or severe (AHI≥30) OSA was 23.7(5.5), 25.2(8.3), 27.3(6.0) and 33.9(8.5) units, respectively (Kruskal-Wallis test, p=0.001). CA activity increased in a dose-response manner along with 4% oxygen desaturation index (ODI4) quartiles (Kruskal-Wallis test, p<0.001, Figure 11) and peaked in the two upper compared with the lowest quartile (Mann-Whitney test, for Q3 p=0.001 and for Q4 p<0.001). There was a positive correlation between CA activity and AHI as well as ODI4 (Spearman correlation, r=0.44 and 0.47, respectively, both p<0.001). Mean nocturnal SpO_2 was negatively associated with CA activity (Pearson correlation, r^2= 0.091, p=0.011). In two separate generalized linear models controlling for age, sex, BMI, pre-sleep oxygen saturation, mean nocturnal SpO2, HT status and use of diuretic medication, CA activity was found to be significantly associated with logAHI and logODI4 (β=0.020 and 0.017, p=0.007 and p=0.011, respectively). This association remained significant after controlling for haemoglobin concentration.

Figure 11. CA activity in relation to quartiles of 4% oxygen desaturation index



Discussion and implications (papers I and II)

All patients in these studies were those investigated for SA in a routine sleep clinic setting. Hence, the recruitment is heavily skewed against subjects with established OSA and the contribution of healthy subjects is low. Moreover, the prevalence of comorbid medical conditions that may influence the results is high[59]. These limitations would tend to decrease the possibility to discriminate patterns of SDB or HT in relation to a tentative biomarker such as $StHCO_3^-$. Indeed, the distribution of $StHCO_3^-$ was also wide at any given AHI severity.

The HCO_3^- concentration in the body is regulated by multiple mechanisms, including renal excretion, which are likely to attenuate potential respiration (or OSA) induced changes in $StHCO_3^-$[263]. Moreover, $StHCO_3^-$ in the arterial blood stream may not be fully representative for the state of the acid-base balance in various compartments of the body. Particularly not those involved in chemosensory control of respiration. In our material $StHCO_3^-$ ranged from 22.0 in the lowest AHI quartile to 27.2 in the highest. Although this difference across severity groups was proportionally low it remains that $StHCO_3^-$ may reflect a clinically relevant difference in CA activity.

Further, it is important to recognize that $StHCO_3^-$ may be regarded as a surrogate marker for CA activity in the body. As such, assayed $StHCO_3^-$ might stem from multiple sources provided by various CA isoenzymes for instance in the lung, capillary epithelium, and kidney or muscle[262,273]. In paper II we attempted to directly assess CA activity in whole venous blood by means of a newly developed ex-vivo technique based on pH conversion speed. In consideration of the described localization of various CA isoenzymes it is likely that we in this model predominantly assess activity of CA I and II which represent the prevailing isoforms in the erythrocyte membrane or cytoplasm[262]. Much like the situation for $StHCO_3^-$ in the previous experiments the activity was increased across the AHI severity range. Moreover, in this experiment, which also assessed various measures of oxygen saturation in particular

nocturnal saturation, and hypoxemia, there was a similar severity related increase of CA activity. While the first cohort study used an early and less advanced diagnostic technique without assessment of oxygenation[339,340], a more conventional technique was applied in the second study. It is possible that relative inaccuracy in the sleep recording may have contributed to the relatively high variation in the scatter plots depicting the correlation between the AHI and the 4%ODI and CA activity. CA activity is conventionally linked to CO_2 elimination rather than hypoxemia and a potential mechanistic link between hypoxia and CA activity in the context to OSA remains to be clarified. However, animal experiments have suggested a role of CA in regulating the ventilatory responses to both O_2 and CO_2 in the carotid body[343-345]. It is also likely that respiratory events during sleep will lead to local tissue acidification and CO_2 retention in a manner that triggers an elevated activity of CA in several tissues[277,278 346]. In addition, other factors such as the ventilatory stability may be affected by local CA activity. The "loop gain", which may be regarded to reflect the ventilatory response to CO_2, determines ventilator stability and this integrity may be increased by pharmacological inhibition of the CA system[43, 44,49] .

When summarizing the two studies dealing with markers of CA activity in patients with OSA there appears to be an elevation of CA activity (or the surrogate biomarker $StHCO_3^-$) in subjects with more severe OSA. The causality of this association remains unknown but may be addressed by experiments that either assesses the CA activity before and after elimination of OSA or the effect of pharmacological inhibition of CA on the intensity of OSA. The current experiments do not provide solid support for a potential role of CA activity monitoring as a potential biomarker in OSA. However, there is a possibility that elevated CA activity in association with OSA represents a unique phenotype of the disorder. Candidate characteristics of such a phenotype may be speculated to include either high loop-gain or unstable breathing of elevated likelihood of HT development.

8.2 Influence of CA inhibition on OSA

Paper III

The CA inhibitor ZNS (n=13) was compared to placebo (n=15) in a randomized, controlled 4 week study addressing SDB and electrophysiological assessed sleep. The two study groups did not differ at baseline, expect for a marginally bigger waist to hip ratio in the ZNS group. Patients were middle aged, obese and had severe OSA (age 54(12) yrs, BMI 31(2) kg/m^2, AHI 46(23) n/hr and ESS 12(4), respectively). ZNS, compared to placebo, induced a potent reduction of both AHI and ODI (Table 5). The reduction of AHI in the ZNS group was primarily due to alleviation of hypopneas (-5.7 [-10.8 to 0.4], p=0.07). Furthermore, group comparisons showed significant improvements in the mean SpO_2 and the arousal index that favoured ZNS (p=0.04 and 0.02, respectively). BMI was marginally reduced by ZNS but the size of the weight reduction was unrelated to the change of the AHI or ODI. Furthermore, ZNS potently reduced the venous $StHCO_3^-$ concentration, but this change did not correlate with the improvement in AHI or ODI. No improvements were recorded for either EDS or fatigue.

Table 5. Baseline values and effects of 4 weeks treatment of placebo or ZNS on sleep-related variables

	Placebo [95% CI] n=15		Zonisamide [95% CI] n=13		
Variables	Baseline Mean (SD)	Mean change (95%CI)	Baseline Mean (SD)	Mean change (95%CI)	P-value
AHI (n/h)	49.6 (22.6)	4.0 [-5.6 to 13.7]	41.6 (23.9)	-8.7 [-16.5 to -0.9]	0.04
ODI (n/h)	42.7 (21.0)	-0.5 [-4.7 to -5.6]	35.2 (21.3)	-8.8 [-15.6 to -2.0]	0.03
Mean SpO_2 (%)	92.5 (2.3)	0.01 [-0.75 to 0.77]	93.2 (2.0)	1.27 [0.23 to 2.30]	0.04
Minimum SpO_2 (%)	73.9 (0.8)	0.7 [-2.1 to 3.6]	78.9 (6.3)	1.3 [-1.9 to 4.5]	n.s
ESS	11.7 (3.5)	-0.3 [-2.3 to 1.6]	13.0 (5.5)	1.4 [-0.6 to 3.4]	n.s
BMI (kg/m^2)	31.1 (1.9)	0.2 [0.0 to 0.4]	30.8 (2.8)	-1.2[-0.4 to 0.0]	<0.01
HCO_3^- (mmol/L)	25.8 (0.8)	0.3 [-0.2 to 0.8]	24.7 (1.4)	-2.7 [-3.5 to -1.9]	<0.01

Abbreviations: AHI= apnea hypopnea index; ODI=oxygen desaturation index; ESS= Epworth sleepiness scale; BMI= body mass index; HCO_3-= venous standard bicarbonate

CPAP (n=11) treatment was superior to ZNS (n=22) with regard to OSA reduction as well as improvement of OSA-related outcomes such as EDS and fatigue at 24 weeks. Study drug compliance was 96.4 (8.7) % at 24 weeks while the averaged CPAP usage over 24 weeks was 5.2 (1.4) hours/night. The significant difference in AHI/ODI reduction was maintained also after adjustment of CPAP and drug compliance (computation of a SAA%, Table 6). CPAP treatment did not induce any changes in $StHCO_3^-$. Secondary outcomes including nocturnal oxygenation were improved by ZNS but ZNS induced increased apnea duration at the 24 week assessment. Body composition, including neck circumference, BMI and sagittal diameter was significantly reduced by ZNS compared with CPAP but this change was not correlated with the reduction of AHI or ODI in the ZNS group. The effect on $StHCO_3^-$ induced by ZNS was maintained at 24 weeks but remained unrelated to the change in AHI and ODI (Spearman correlation=-0.02 and 0.17, p=0.94 and 0.45, respectively).

Table 6. Baseline values and effects of 24 weeks treatment of CPAP or ZNS on sleep-related variables

	CPAP [95% CI] n=11		Zonisamide [95% CI] n=22		
Variables	Baseline Mean (SD)	Mean change (95%CI)	Baseline Mean (SD)	Mean change (95%CI)	P-value
AHI (n/h)	47.9 (26.7)	-42.5 [-57.7 to -27.3]	47.1 (24.1)	-8.0 [-15.9 to -0.2]	<0.01
ODI (n/h)	44.9 (26.2)	40.4 [-55.2 to -25.5]	39.5 (22.4)	-8.1 [-15.1 to -0.2]	<0.01
Mean SpO_2 (%)	92.5 (2.4)	2.8 [0.9 to 4.7]	93.1 (2.0)	0.8 [0.2 to 1.4]	0.01
Minimum SpO_2 (%)	78.4 (8.4)	8.1 [5.1 to 11.2]	80.0 (7.8)	1.3 [-0.8 to 3.4]	<0.01
ESS	12.8 (3.8)	-4.4[-7.0 to -1.7]	12.6 (4.6)	-0.4 [-2.4 to 1.5]	0.02
BMI (kg/m^2)	31.0 (2.3)	0.7 [0.3 to 1.2]	30.7 (2.4)	-0.8 [-1.2 to -0.4]	<0.01
HCO_3^- (mmol/L)	25.5 (0.7)	0.7 [0.1 to 1.3]	25.1 (1.0)	-2.3 [-3.0 to -1.6]	<0.01
Compliance adjusted AHI (n/hr)		-27,3 [-38,3 to -16,6]		-7,7 [-15,3 to -0,2]	0.01
Compliance adjusted ODI(n/hr)		-26,9 [-37,5 to -16,3]		-7,8 [-14,5 to -1,1]	0.01
SAA%		61 [51 to 71]		13 [-4 to 30]	<0.01

Abbreviations: AHI= apnea hypopnea index; ODI=oxygen desaturation index; ESS= epworth sleepiness scale; BMI= body mass index; HCO_3^-= venous standard bicarbonate

Paper IV

This was an open, randomized, cross-over study comparing respiratory and hemodynamic effects of AZT, CPAP and AZT/CPAP in thirteen male OSA subjects (age 64 (7) years, BMI 29 (4) kg/m^2, AHI 37 (23) n/hr). The analyzed groups did not differ at baseline with respect to anthropometrics, SDB or BP. Mean study drug compliance was 94.6 (7.1)% and 94.5 (7.6)% in the AZT and AZT/CPAP group, respectively. Mean CPAP usage was 4.8 (2.2) and 5.0 (2.0) hrs/night for the CPAP and AZT/CPAP groups and habitual sleep time was 7.3 (1.2) hr/night. The main findings from paper IV are summarized in Table 7.

CPAP, AZT and AZT/CPAP all significantly reduced AHI and ODI (all $p<0.005$, Table 7). The reduction of the AHI and ODI corresponded to 42 (27) and 46 (25) %, respectively, compared with baseline in the AZT group. The change was mainly due to elimination of apneic events (-10.3 [-17.6 to -2.9] n/hr, data not shown). Measures of oxygenation including mean overnight and minimum SpO_2 were increased after AZT. However, the hypopnea duration was increased after AZT (7.2 [1.9 to 12.5] sec, p=0.01). The resolution of respiratory events was more pronounced after CPAP and AZT/CPAP than after AZT (all $p<0.05$, Table 7). CPAP and AZT/CPAP improved minimum SpO_2 more effectively than AZT but only AZT/CPAP treatment improved mean SpO_2 compared to AZT therapy ($p<0.01$). The differences between

therapeutic modalities in terms of AHI or ODI did not remain significant if an adjustment for CPAP and drug compliance was made (Figure 12).

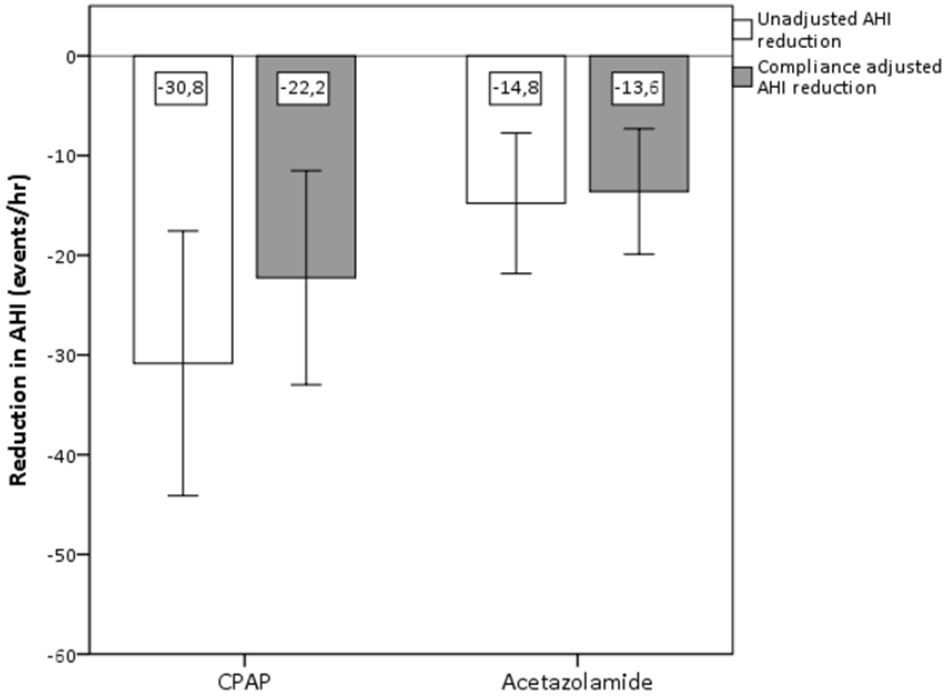
There were no recorded improvements in EDS as reflected by the ESS score in the treatment groups. BMI was reduced after AZT but the size of the change in BMI was not associated with the reduction in AHI or ODI (Spearman correlation, r^2=0.24 and -0.17, p=0.42 and 0.61, respectively). Venous $StHCO_3^-$ was reduced in the AZT and AZT/CPAP groups and the degree of reduction of $StHCO_3^-$ concentration was linearly associated with the reduction of AHI (Spearman correlation, r^2=0.66 p=0.013).

Table 7. Treatment effects of CPAP, AZT or AZT/CPAP on sleep disordered breathing

	CPAP [95% CI]	AZT [95% CI]	AZT plus CPAP [95% CI]
Variables	**N=12**	**N=11**	**N=12**
TRT (hrs)	-0.7 [-2.0 to 0.6]	-0.1 [-0.7 to 0.5]	-0.2 [-1.0 to 5.1]
AHI (n/h)	-30.8 [-44.1 to -17.6]	-14.8 [-21.8 to -7.7][ab]	-38.6 [-50.0 to -27.2]
ODI (n/h)	-33.8[-49.3 to -18.3]	-15.6 [-22.7 to -8.5][ab]	-35.5 [-46.4 to -24.5]
AI (n/h)	-19.9 [-32.3 To -7.6]	-10.3 [-17.6 to -2.9]	-20.8 [-32.5 to -9.2]
HI (n/h)	-10.9 [-17.0 to -4.8]	-4.5 [-8.3 to -0.7][b]	-17.8 [-24.1 to -11.5]
Hypopnea duration (s)	-10.3 [-19.9 to -0.8]	7.2 [1.9 to 12.5][a]	0.9 [-6.3 to 8.1]
Mean SpO_2 (%)	2.5 [1.2 to 3.9]	1.5 [0.5 to 2.5][b]	4.9 [2.8 to 7.0]
Minimum SpO_2 (%)	14.5 [9.1 to 19.9]·	3.0 [0.9 to 5.1][ab]	12.2 [7.9 to 16.4]
BMI (kg/m^2)	0.2 [-0.1 to 0.4]	-0.4 [-0.5 to-0.2][a]	-0.4 [-0.6 to-0.1][c]
ESS	0.1 [-1.7 to 1.8]	-1.1 [-2.8 to 0.6]	0.0 [-2.4 to 2.4]
HCO_3^- (mmol/L)	0.1 [-0.8 to 0.9]	-6.8 [-7.4 to -6.1][a]	-7.0 [-7.8 to -6.2][b]

Abbreviations: TRT= total recording time; AHI=apnea hypopnea index; ODI=oxygen desaturation index; AI=apnea index; HI=hypopnea index; SpO_2=oxygen saturation; ESS=Epworth sleepiness scale; BMI= body mass index; a= p<0.05 for AZT to CPAP comparisons; b= p<0.05 AZT to AZT/CPAP comparisons; c= p<0.05 for AZT/CPAP to CPAP comparisons.

Figure 12. Mean unadjusted and compliance adjusted reduction in apnea-hypopnea index (AHI) after treatment with CPAP or AZT



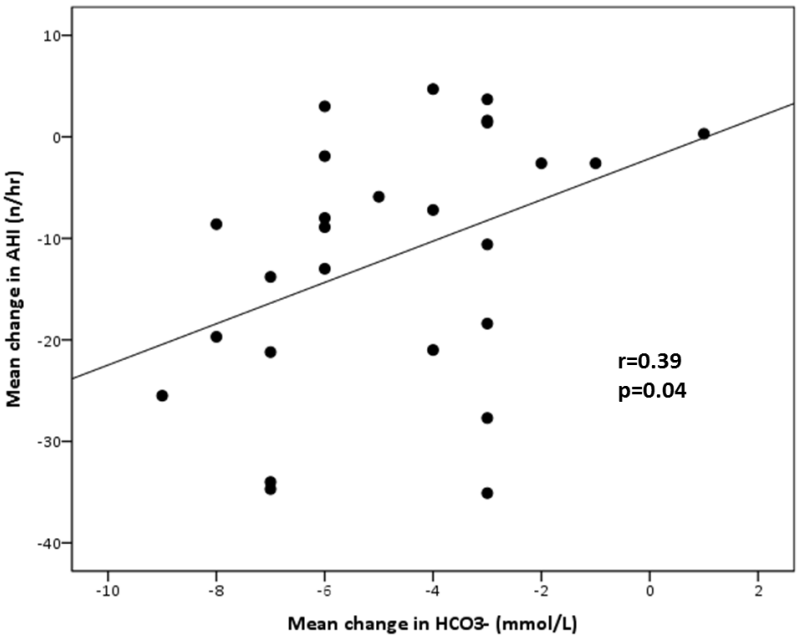
The effect of CA inhibition in OSA was applied and studied by two different compounds, ZNS and AZT, in papers III and IV, respectively. In order to better evaluate the overall effect of CA inhibition in OSA we subsequently added the data obtained in these two studies. As a general observation AZT appeared to more potently modify OSA although the observed differences may not necessarily reflect pharmacological differences but rather the influence of study design, study durations, dosing or blinding procedures which differed between the two studies. Hence, data from this combined analysis, which is summarized in Table 8, needs to be interpreted with caution. This combined analysis suggested that respiratory related markers of OSA were potently reduced (in the order of 30%), along with $StHCO_3^-$ (approximately 20%) and the BMI (approximately one unit). In this combined analysis 16/26 (69.6%) of patients treated with either ZNS or AZT could be labelled responders (≥20% reduction of AHI) and 8/26 (30.8%) reduced the AHI by at least 50%. The number of responders (≥20% reduction of AHI) in the respective AZT and ZNS treated groups were 76.7% and 46.2%, respectively. The reduction in AHI was positively correlated with the change in $StHCO_3^-$ (Spearman correlation r2=0.44 and p=0.04, Figure 13) when both compounds were taken into account. However, the effects on AHI did not remain significant when BMI was included as a covariate in the univariate model.

Table 8. Estimated therapeutic effect of CA inhibition on OSA as reflected by two different substances (ZNS and AZT) in Paper III and IV

	AZT plus ZNS (n=26) Baseline Mean (SD)	AZT plus ZNS (n=26) Follow-up Mean (SD)	P-value
AHI (n/hr)	39.9 (21.1)	28.1 (19.9)	<0.01
ODI (n/hr)	36.5 (19.8)	24.5 (17.5)	<0.01
Mean SpO_2 (%)	92.7 (2.7)	94.1 (2.0)	<0.01
Minimum SpO_2 (%)	78.0 (6.5)	80.1 (6.1)	<0.01
HCO_3^- (mmol/L)	25.5 (1.5)	20.2 (1.9)	<0.01
BMI (kg/m^2)	30.1 (3.4)	29.8 (3.3)	<0.01
	AZT plus ZNS (n=26) Mean (SD)		
% change AHI	-29.7 (29.7)		<0.01
% change ODI	-32.3 (28.5)		<0.01
% change HCO_3^-	-18.2 (9.0)		<0.01
% change BMI	-0.9 (1.0)		<0.01

Abbreviations: AHI= apnea hypopnea index; ODI=oxygen desaturation index; ESS= epworth sleepiness scale; BMI= body mass index; HCO_3-= venous standard bicarbonate

Figure 13. Relationship between the change in AHI and the change HCO_3^-



Discussion and implications (papers III and IV)

CA inhibitors, ZNS and AZT, reduced OSA severity in these randomized controlled studies and the effect was more pronounced after AZT. Both therapies appeared to reduce $StHCO_3^-$ and the BMI. The apparently lower efficacy of ZNS in terms of effects on AHI may be related to the different pharmacological properties of this substance. ZNS is known to have potent CA inhibitory properties but we cannot exclude that patients in our ZNS study were proportionally underdosed and did not receive equipotent dosages of the CA inhibitor[267]. There is a relative paucity of data in the literature regarding relative potency of ZNS as a CA inhibitor.

An alternative explanation for the relative difference in potency between ZNS and AZT may be attributed to selective differences in the inhibition of CA isoenzymes[300]. In the context of chemosensory modulation of ventilation only the inhibition of CA isoenzymes II, IV and XIII would be expected to directly influence ventilation. Moreover, blockage of CA isoenzyme I and II are known to modulate erythrocyte function[347,348]. In remains unknown to what extent differences in binding characteristic may contribute specifically to the size of the reduction of AHI and beneficial effect on oxygenation in OSA.

Table 9. Treatment effects of AZT on OSA or CSA reported in various studies

Author	Year	Type of SDB	n	Dosage (mg)	Duration (days)	Δ AHI	%-change AHI	Δ SpO2	%-increase SpO_2	High altitude
Tojima	1987	OSA	9	250	7	-10	-41.7	1	1.1	No
Whyte	1988	-	10	1000	14	-24	-48.0	-		No
Inoue	1999	OSA	75	365	40	-8	-29.6	-		No
Edwards	2012	OSA	12	1000	7	-26	-52.0	2	2.1	No
Latshang	2012	OSA	51	750	3	-1.2	-34.3	2	2.3	Yes
Nussbaumer	2012	OSA	45	1000	3	-3	-5.5	3	3.5	Yes
Ulrich	2014	OSA	18	500	3	-13	-21.3	4	4.5	Yes
Ulrich	2015	OSA	23	500	7	-11	-61.1	4	4.7	No
Eskandari	2016	OSA	13	750	14	-14.8	-42.4	1.5	1.6	No
Javaheri	2006	CSA-HF	12	300	3	-21	-47.7	1	1.0	No
Latshang	2012	CSA	51	750	3	-9	-69.2	2	2.3	Yes
Nussbaumer	2012	CSA	45	1000	3	-17	-73.9	3	3.5	Yes
Ulrich	2014	CSA	18	500	3	-22	-71.0	4	4.5	Yes

The therapeutic effects of CA inhibition on OSA/CSA by AZT have previously been demonstrated (Table 9)[49,282,283,288,289,349-352]. The effect on AZT on OSA in our study coincided with that reported elsewhere and it is noted that the number of clinical responders among randomly selected OSA patients in our study was rather high. In fact, all previous studies, including our own, are based on rather small patient populations. Moreover, OSA, as described above, is known to be a disorder including multiple phenotypes. Assuming that a "preferential phenotype" of OSA when CA inhibition is considered would be those with a modified loop-gain or a low arousal threshold (see Eckert et al.[2], Figure 2) it is possible that

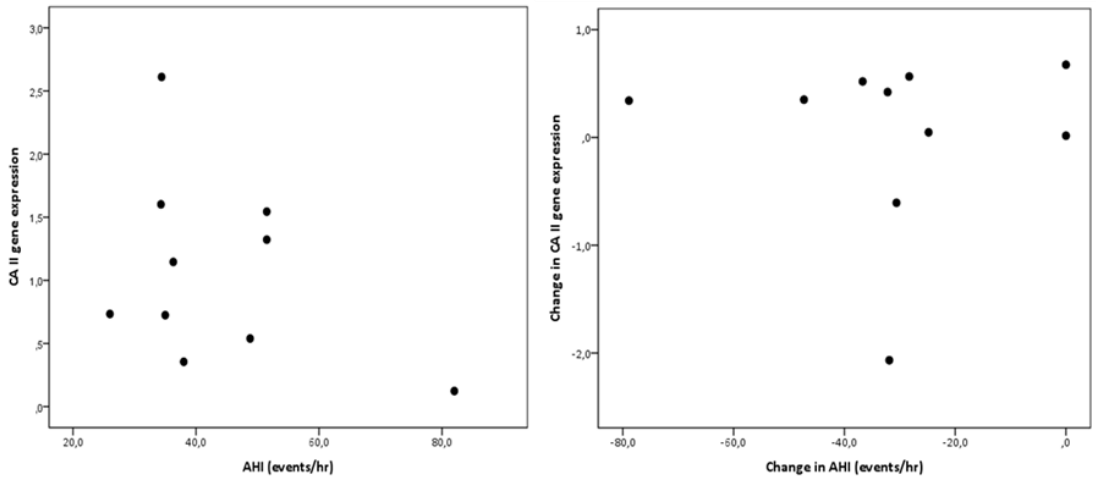
our study randomly ended up randomizing a higher proportion of this type of patients. Hence, it may be warranted that future studies are balanced with respect to clinical phenotype.

CA activity was not directly monitored in our controlled studies. However, we opted to assess $StHCO_3^-$ as a surrogate marker for CA activity[263]. Our ex-vivo data suggest that is associated with the degree of more extensive oxygen desaturation is associated with higher CA enzyme activity[342]. $StHCO_3^-$ was uniformly reduced after CA inhibition in our studies and appeared to be associated with the AHI reduction. It remains to be determined, in context of OSA, if there is a causal link whereby OSA induces an increase in CA activity or if, in fact, a proceeding increase in CA activity acts to increase OSA severity. Future studies to address this question may evaluate if CPAP therapy will modify CA activity (preferably assessed by direct measures of CA activity in whole blood). Preliminary data suggested that CPAP therapy had no effect on CA enzyme expression in human fat cells (a hypoxic compartment) from morbidly obese patients with severe OSA (unpublished data, Table 10 Figure 14). However, this area needs to be better explored in future studies in order to better understand issues related causality.

Table 10. The effects of CPAP treatment on OSA and CA (II) expression (unpublished data)

	Baseline N=10 Mean (SD)	Follow-up N=10 Mean (SD)	P-value
Age (years)	52 (6)		
Mean CPAP usage (hrs)	3.4 (2.7)		
AHI (n/hr)	43.8 (15.9)	12.7(20.5)	<0.01
ODI (n/hr)	36.0 (17.9)	12.4 (18.9)	<0.01
CA II expression	1.1 (0.7)	1.1 (0.5)	n.s

Figure 14. CA expression in relation to AHI at baseline (left) and change in CA expression in relation to the change in AHI following CPAP treatment (right)



Obesity is strongly associated with OSA and weight reduction is known to reduce OSA in most patients with moderate to severe obesity[7,72]. Previous studies on the CA inhibitors ZNS and topiramate have demonstrated notable weight reductions in obese patients during short to intermediate treatment periods[257,272]. A RCT by Winslow et al. demonstrated that a phentermine/topiramate combination reduced body weight along with AHI in obese patients with OSA[256]. The effects of ZNS on body composition are considered to be two-fold; it inhibits CA isoenzymes involved in processes related to de novo lipogenesis and modulates GABA and glutamatergic transmission, thus functioning as an appetite suppressant[267]. When the ZNS and AZT groups were pooled together we found that BMI decreased marginally but had a strong influence on the AHI reduction (data not shown) suggesting that body weight changes should be taken into account when the potential therapeutic effects of CA inhibitors in OSA are evaluated. In fact, added body weight reduction, in long-term studies, would be expected to add reduction of SDB in addition to that achieved in study III.

Finally, as previously described, several CA inhibitors such as AZT are known to exhibit diuretics properties and to reduce extracellular fluid volume with as much as -5 to 10%[326]. It is therefore possible that that the effect of CA inhibition on OSA may be related to a reduced caudal to rostral fluid displacement occurring during sleep and horizontal body positioning as demonstrated in several publications by a Toronto based researcher group[353,354]. This effect would contribute to a relative reduction of UA size and collapsibility with a resulting improvement of OSA

8.3 Association between CA activity and blood pressure

Paper I

Paper I addressed, among other aims, the association between a HT status as well as actual BP to $StHCO_3^-$ (applied as a surrogate marker for CA activity). HT was prevalent in 53.3% of the patients and prevalence increased with OSA severity class. $StHCO_3^-$ was higher in hypertensive (n=442) the normotensive (n=388) patients (24.9(2.7) and 24.1(1.9) mmol/l, p<0.001, respectively). The percentage of patients with a clinical HT diagnosis was 46.8, 49.8, 54.3 and 62.7%, respectively, in the $StHCO_3^-$ quartiles (Q1 to Q4) (p=0.007). Both OSA severity and higher $StHCO_3^-$ were associated with a higher prevalence of HT in this population (both p<0.01). In a generalized linear model controlling for sex, age, BMI, smoking, alcohol consumption, pO_2, pCO_2 and apnea severity, $LogStHCO_3^-$ was independently associated with a clinical diagnosis of HT (β=8.0, SE 3.0, 95%CI [2.1-13.8], p=0.007).

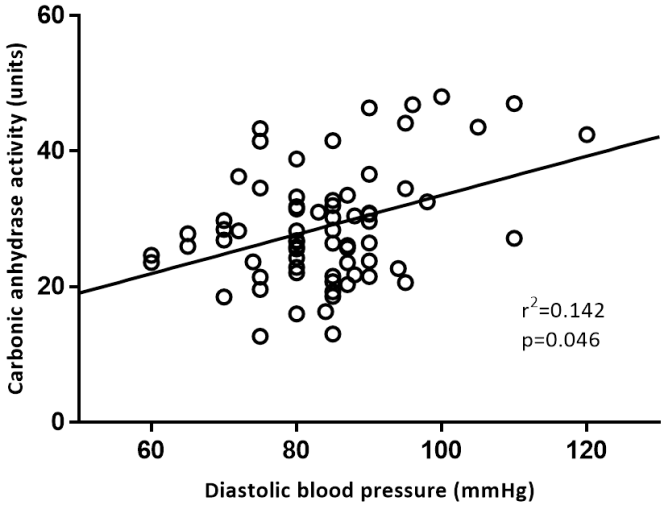
An additional analysis was performed to study the relationship between $StHCO_3^-$ and office BP. There was a weak but significant association between $StHCO_3^-$ and SBP and DBP (Spearman correlation, r=0.10 and 0.12, p=0.003 and <0.001, respectively). In generalized linear models with SBP and DBP, respectively, as dependent variables, a positive independent association was found between $logStHCO_3^-$ and DBP (β=27.6, SE 14.0, 95%CI [0.2-55.0], p=0.048) but not with SBP (β=17.1, SE 22.3, 95%CI [-26.7-60.9], p=0.45) after adjustment for sex, age, BMI, smoking, alcohol usage, pO_2, pCO_2 and apnea severity.

Paper II

The association between CA activity and BP was addressed in a post-hoc analysis in paper II. A history of HT, other CV disease (CAD/stroke) or DM was found in 46%, 19% and 4% of patients, respectively. Angiotensin converting enzyme inhibitors and angiotensin II blockers, beta-blockers, calcium-channel blockers and diuretics was used by 17.1, 5.7, 24.3, 11.4, and 18.6%, respectively, of the patients.

In an analysis which accounted for all patients, irrespective of their HT status, suggested that CA activity was positively associated with resting diastolic BP (Pearson correlation, r^2=0.142, p=0.001, Figure 15), but not systolic BP (p=0.098). Furthermore, CA activity was independently associated with DBP after adjusting for potential confounders such as sex, age, BMI, mean SpO_2 and AHI in a generalized linear model (β=0.32, p=0.046). There was no interaction between use of antihypertensive medication and CA activity in relation to DBP (p=0.096).

Figure 15. Relation between CA activity and sitting diastolic blood pressure



Discussion and implications (papers I and II)

In paper I and II, we found that increased CA activity was significantly associated with BP and HT status, independent of OSA. These are the first studies to establish a link between CA activity and BP regulation in OSA patients. Although this association does not provide any information on the possible causal relationships, between these two phenomena, several CA isoenzymes have been shown to associate with mechanisms related to BP and volume regulation including diuresis, fluid shift, vascular resistance and cardiac contractility[307-310]. Intermittent hypoxia has been considered as an important mechanism for HT development in OSA. In paper II, we found that increased CA activity was associated with the degree of nocturnal hypoxemia. The conventional AHI/ODI measures mainly reflect the frequency of SDB. It is possible that CA enzyme activity serves as a sensitive marker for hypoxic load in OSA patients. Increased sympathetic activity is a known hallmark phenomena in OSA patients. Indeed, experimental data suggest that adrenergic agonists may activate CA

isoenzyme I and II in erythrocytes and vascular smooth muscle tissue[355]. The CA enzyme plays a fundamental role for the bicarbonate metabolism, thus the association between CA activity and BP can be mirrored by bicarbonate in study I. It may be argued that the significant association between HT status and bicarbonate is not clinically relevant. In fact, the majority of the patients in paper I was within clinically defined normal ranges of bicarbonate [22-27mmol/L]. Hence, our studies support the notion that $StHCO_3^-$ (e.g. CA activity) plays a role in BP regulation rather than constituting a clinical predictor for HT in patients with OSA.

8.4 Influence of CA inhibition on BP

Paper III and IV

Patients in paper III were included regardless of HT status and there were 6 patients with established HT (all on antihypertensive medication). There were no overall effects of ZNS on BP in the whole study group (n=13) but an ad-hoc analysis in the hypertensive subgroup at 4 weeks showed a reduction in MAP (-4.7[-9.3 to -0.2], $p<0.05$). This decrease in MAP could primarily be attributed to an effect on SBP (-10.3 [-18.0 to -2.6], $p=0.03$). There was no change in BP in the normotensive group (n=7) and BP was not significantly changed in ZNS treated patients in the open arm of the study at 24 weeks.

Paper IV included exclusively hypertensive patients with either a newly detected HT (resting SBP/DBP >140/90) or ongoing antihypertensive medication (1-3 medications). HT was generally well controlled in these patients and the mean supine SBP/DBP on previously prescribed antihypertensive medication was 146(14)/82(9) mmHg. Following a three week medication wash-out period BP had increased to 157(11)/86(11). The pressures reached at the end of the AZT and AZT/CPAP treatment periods were 145 (7)/81(10) and 144(7)/80(8) mmHg, respectively, and these pressures did not differ significantly from those obtained on the pre-study medication regimen.

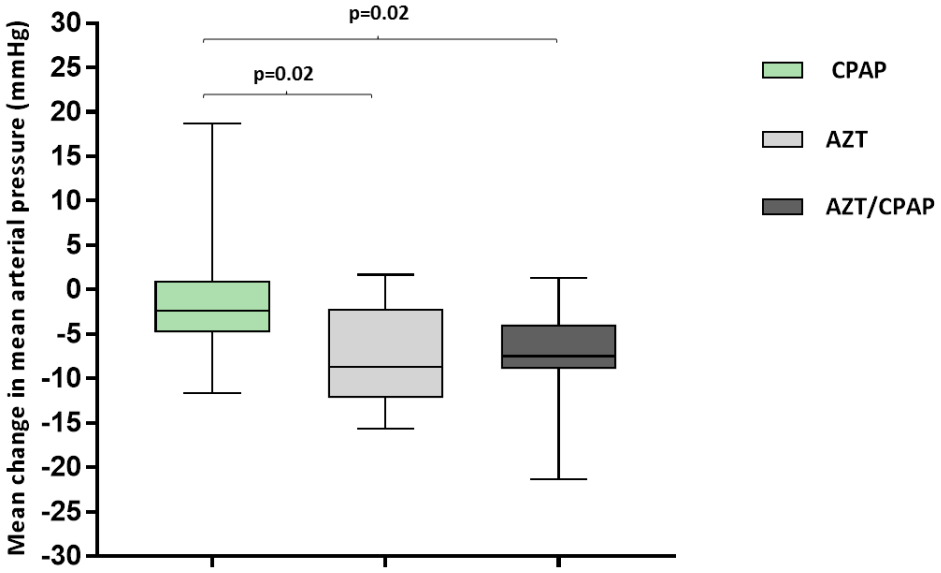
Both AZT and AZT/CPAP treatment significantly reduced SBP and DBP compared with baseline (all $p<0.01$) whereas SDP as well as DBP were only marginally (not significantly) reduced by CPAP alone (Table 11, Figure 16). There was a slight increase in supine HR in the AZT group but the value did not differ statistically from the other two treatment periods. The changes in SBP, DBP or MAP in the AZT or AZT/CPAP groups were not significantly associated with changes in either AHI or BMI (data not shown).

Table 11. Treatment effects of CPAP, AZT and AZT/CPAP on blood pressure

Variables	CPAP [95% CI] N=13	AZT [95% CI] N=13	AZT plus CPAP [95% CI] N=12
Supine SBP (mmHg)	-1.8 [-8.2 to 4.6]	-10.7 [-16.1 to -5.2][a]	-10.8 [-18.5 to -3.2]
Supine DBP (mmHg)	-0.3 [-5.2 to 4.6]	-5.3 [-9.2 to -1.5]	-5.4 [-8.4 to -2.4]
Supine HR (bpm)	-0.0[-3.8 to 3.8]	2.3 [0.5 to 4.1]	2.1[-0.1 to 4.3]
Supine MAP (mmHg)	-0.8 [-5.2 to 3.6]	-7.1 [-10.7 to -3.5][a]	-7.2 [-10.9 to -3.5][b]
SBPao (mmHg)	1.6 [-13.7 to 17.0]	-16.7 [-27.0 to -6.1][a]	-13.0 [-24.5 to -1.6]
PPao (mmHg)	-0.8 [-13.4 to 11.8]	-8.2 [-14.5 to -1.8]	-6.4 [-17.1 to -4.3]
AIXao (%)	2.7 [-4.1 to 9.5]	-7.3 [-12.5 to -2.1][a]	-6.7 [-12.2 to -1.7][b]
AIXbra (%)	5.3 [-8.2 to 18.8]	-7.7 [-27.9 to 12.4]	-13.3 [-24.1 to -2.5][b]

Abbreviations:SBP= systolic blood pressure;DBP= diastolic blood pressure; HR= heart rate; MAP= mean arterial blood pressure SBPao= aortic systolic blood pressure; PPao= aortic pulse pressure; AIXao= aortic augmentations index; AIXbra= augmentations index brachialis; a=p<0.05 AZT to CPAP comparison; b=p<0.05 AZT/CPAP to CPAP comparison

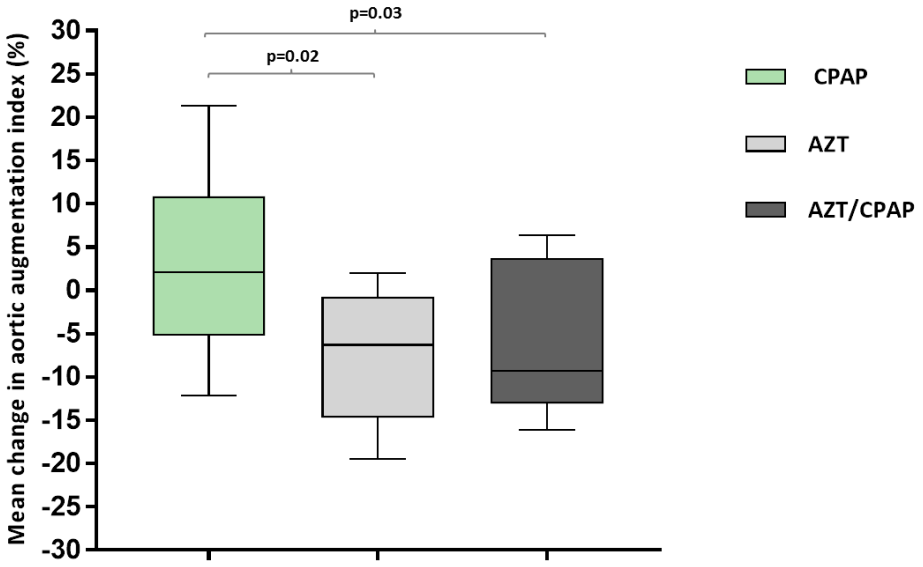
Figure 16. Treatment effects of CPAP, AZT and AZT/CPAP on mean arterial pressure



AZT alone, or in combination with CPAP, reduced the central hemodynamic parameters aortic SBP, aortic pulse pressure and aortic augmentation index (all p<0.05, Table 11 and Figure 17) as assessed by non-invasive radial tonometry. In addition, the AZT/CPAP

combination was found to reduce augmentation index in the brachial artery ($p<0.02$). CPAP treatment alone had no effect on measures of either central aortic BP or vascular stiffness.

Figure 17. Treatment effects of CPAP, AZT and AZT/CPAP on aortic augmentation index.



Discussion and implications (papers III and IV)

Both AZT and ZNS appear to reduce office BP in OSA patients with comorbid HT and these antihypertensive effects were considerably stronger than those obtained by CPAP. In addition, data from paper IV suggest that non-invasively obtained measures of central BP and vascular stiffness might be beneficially influenced by AZT. However, the current study design precludes firm statements on whether this effect on hemodynamics was specific to comorbid BP elevation in OSA. It may be that similar antihypertensive effects could be obtained also in HT unrelated to OSA. In fact, several controlled studies in normotensives or hypertensive patients have not been able to demonstrate any BP reducing effect after AZT[327,328,356,357]. Nevertheless it is worth noting that CA inhibitors have been widely considered for potential antihypertensive properties. However, many compounds have been dismissed due to drug related side-effects or lack of efficacy and inferiority to novel, better tolerated types of antihypertensive[141,]. One exception is the use of thiazides in hypertension as this group of diuretics is known to have CA inhibitory properties[141,300].

Several experimental studies and RCTs suggest that CA inhibition by AZT may modulate hemodynamics specifically during hypoxic and/or hypobaric conditions. Pulmonary vascular resistance and vasoconstriction were reduced after AZT in animal studies[320,321]. Moreover AZT reduced BP in healthy subjects during high-altitude sojourns[286,322]. AZT was also shown to counteract excessive brachial BP elevation subsequent to acute exposure to high-altitude and hypobaric hypoxia in healthy volunteers[325]. Analogous effects during similar conditions have been reported in OSA patients[289]. Hence, there is compelling evidence linking inhibition

of CA activity and hemodynamic modulation in OSA. However, it should be noted that OSA is a condition characterized by intermittent hypoxia and that data from hypobaric/hypoxic conditions not necessarily may apply.

Our non-invasive data suggested that the antihypertensive effect of AZT may include an attenuation of vascular stiffness. Other human studies using a forearm flow mode have demonstrated a vasodilatory response to local AZT infusion which may involve a modulation of calcium-activated potassium channels[307]. Yet other studies suggest that CA inhibition may induce vasodilation by modulation of nitric oxide metabolism in the vascular endothelium[316, 317]. Along with this mechanism there is a relative metabolic acidosis in local tissue generated by CA inhibition that might lead to vasodilation[318,319]. It has also been proposed that CA inhibitors induce a blockade of voltage gated calcium channels[313]. In fact, conventional calcium channel blockers (e.g. verapamil and amlodipine) have been shown to reduce the influx of intracellular calcium, as a result of CA inhibition in erythrocytes (human data) and vascular smooth muscle (animal data), in a manner that results in vasodilation[314,315]. It should also be mentioned that the BP lowering effect of AZT may have been due to a diuretic effect[326,358]. Diuresis may have reduced the extracellular fluid, blood plasma volume and cardiac output and thereby reducing BP[359].

8.5 Clinical implication

Conventionally applied therapy in OSA is, due to its mechanical character, often complex and cumbersome for the patient. Admittedly there is an advantage in mechanical therapies such as CPAP, which relates to a low number of side effects. However, compliance, as suggested by recent long-term protocols, may reach as low as 50% suggesting that there is a need of novel therapeutic strategies that not only specifically address pathogenic mechanisms in OSA, but also are widely tolerated by patients with the disorder.

Current mechanical therapies in OSA do not address all aspects of comorbidities linked to SDB. For instance, CPAP therapy is at best body weight neutral and in fact several studies suggest that there is a weight increase after initiation of CPAP. The mechanism behind this change may be a withdrawal of apnea-related work of breathing following CPAP. CA inhibition, as produced in the current study, is known to induce weight loss and this effect may introduce a disease modifying component into OSA therapy (particularly when pharmacological therapy is combined with CPAP in overweight OSA patients)

A similar reasoning may apply to the control of CV morbidities such as HT in OSA. The current results suggest that high CA activity may constitute a specific mechanism for BP elevation in OSA and that BP may be potently reduced by CA inhibition in these patients. It remains to be explored if various forms of HT are equally responsive for CA inhibition in OSA or if HT in response to elevated CA activity represents a specific phenotype in OSA. Nevertheless, CA inhibition introduces a possibility for a disease modifying therapy in OSA-related HT.

Yet another implication of the current research is the possibility to identify a novel and specific biomarker in OSA. The apparent dose-related association between OSA and CA activity suggests that a development of a biomarker may be feasible. Bicarbonate was used

as a surrogate marker of CA activity in the current study and has been considered as a state as well as a trait marker in patients with more severe hypoventilation states such as the OHS. Our studies suggested a considerable variability in the CA-activity marker at any given level of AHI. However, it is possible that markers of CA activity better reflect the state of various physiological functions in OSA than does the AHI or similar markers of SDB.

It may be concluded that the current studies outline one of the first systematically evaluated potential pharmacological therapies in OSA. Future studies in this area will need to focus on how this therapeutic principle may be optimized in terms of improved patient selection, adaptation to key pathophysiological mechanisms and selection of ideal drug candidates.

8.6 Study limitations

Paper I

SDB was assessed with a contemporary polygraphy recording device on two consecutive nights in order to reduce the potential effect of an inaccurate AHI value due to first night effect or night-to-night variability. Weaknesses include a lack of quantitative data on overnight hypoxic events like oxygen desaturation in the multivariate analyses. However, nocturnal hypoxic exposure was captured as 4% oxygen desaturation events that were used for computation of the AHI. Another weakness in this study is the lack of detailed information on the type of antihypertensive medication. Also, it cannot be excluded that prescribed antihypertensive medication, e.g. diuretics such as hydrochlorothiazide, may have influenced the association between $StHCO_3^-$ and BP although the influence of thiazides on $StHCO_3^-$ is likely to be very limited. Finally, the cross sectional design of our study did not allow any firm conclusions regarding the causality of the demonstrated associations.

Paper II

Our study population was a clinical cohort and a there was no control group in the data analysis. Blood gas assessments were not performed in the study and we could therefore not evaluate out CA-activity method in relation to a bicarbonate value. Furthermore, the cross-sectional design in this study did not allow us to draw any conclusions on the causality of the association between apnea severity and CA activity. Future studies that evaluate the elimination of OSA by CPAP are needed to better address this issue. It cannot be excluded that ongoing medication may have affected CA activity. However, an analysis controlling for the use of diuretics did not suggest that this was the case. Our analysis method only permitted assessment of total CA activity in whole blood. It remains unknown whether OSA was associated with alteration of specific CA isoenzymes or a change of CA activity in other compartments of the body. Finally, due to a change of clinical routines, haemoglobin data was only available in a subgroup of patients. However, our subgroup analysis suggested that the association between CA activity and apnea severity was independent of haemoglobin concentration.

Paper III

Study limitations include a failure to reach the pre-set patient number prior to the study drug expiry date and due to recruitment difficulties. The initial power calculation indicated a minimum of 90 patients but only 42 were finally included in the study. However, the study hypothesis was verified in spite of the lower number of participants. We set out to prove the effects of ZNS in unselected OSA patients and therefore no particular attempts were made to optimize weight reduction or to characterize specific phenotypic traits in the study population. Furthermore, another limitation in the study concerns the fact that CA-activity was not assessed. Despite an adequate randomization procedure the patients in the CPAP group were younger than the ZNS group at baseline. Twenty percent of the ZNS patients reported dysphoria as a side-effect at 24 weeks, although there was no difference at 4 weeks compared to placebo.

Paper IV

The major limitation of the study was that CA activity was not directly assessed. Instead, $StHCO_3^-$ was used as a surrogate marker and other physiological mechanisms involved in $StHCO_3^-$ metabolism may have influenced the result. The power calculation in this study was 30 patients. Hence, we managed to recruit only 14 patients and this caused several of the analyses to be performed in a potentially underpowered study. PSG was not performed and the severity of the breathing disorder may have been underestimated. This study included hypertensive patients with OSA but not those without. Hence, it is possible that the effects recorded in the study are generic to HT rather than specifically linked to HT in OSA. Finally, this was a cross-over study with the possibility of a carry-over effect between treatment periods. However, we were not able to identify such effects after statistical evaluation of the data and the treatment order was randomized.

9 Conclusion and future perspectives

The current studies suggest that mechanisms related to CA activity may have important implications for the development and maintenance not only of SDB but also comorbid conditions such as CV complications and obesity in OSA. In that sense our data identifies a novel potential mechanism for HT development in OSA. We also identify a potential pharmacological principle for treatment of at least a subgroup of patients with OSA. Future studies are needed to determine the relative importance of this mechanism in the treatment of OSA and if a CA activity related principle may be used to tailor an effective therapy. The prospect of such a therapy is that of a disease modifying principle and in this respect it would provide an improvement on top of currently available therapeutic alternatives.

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