EVALUATION OF ANTIHYPERTENSIVE DRUGS IN PATIENTS WITH OBSTRUCTIVE SLEEP APNEA AND IN RATS SUBMITTED TO CHRONIC INTERMITTENT HYPOXIA

LUCÍLIA CATARINA DAS NEVES DIOGO

Tese para obtenção do grau de Doutor em Ciências da Vida
na Especialidade de Farmacologia
na NOVA Medical School

January, 2015

EVALUATION OF ANTIHYPERTENSIVE DRUGS IN PATIENTS WITH OBSTRUCTIVE SLEEP APNEA AND IN RATS SUBMITTED TO CHRONIC INTERMITTENT HYPOXIA

Lucília Catarina das Neves Diogo

Orientadora:

Maria Emília Saraiva Monteiro, Professora Catedrática de Farmacologia

Co-orientadoras:

Ana Luísa Trigoso Papoila da Silva, Professora Auxiliar de Bioestatística

Cristina Bárbara Prista Caetano, Professora Associada de Pneumologia com Agregação

Tese para obtenção do grau de Doutor em Ciências da Vida na Especialidade de Farmacologia

January, 2015

The experimental work was performed at Chronic Diseases Research Center

(CEDOC), Nova Medical School/ Faculdade de Ciências Médicas

da Universidade Nova de Lisboa and in the

Department of Pneumology II, Centro Hospitalar Lisboa Norte, EPE

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA

The research described in this thesis was financially supported by

Fundação para a Ciência e Tecnologia (FCT - Portugal)

Grants: SFRH/BD/48335/2008 and PTDC/SAU-TOX/112264/2009

and CEDOC (Chronic Diseases Research Center)

The scientific content of the present thesis has been included in the publication of the following international scientific periodicals with referees:

Diogo LN, Pinto P, Bárbara C, Monteiro EC, Papoila AL. Neck circumference and body mass index as independent predictors of hypertension misclassification in patients suspected of obstructive sleep apnea. Blood Press Monit. 2015 Feb;20(1):8-15. doi: 10.1097/MBP.0000000000000080.

Diogo LN and Monteiro EC (2014). The efficacy of antihypertensive drugs in chronic intermittent hypoxia conditions. Front. Physiol. 5:361. doi: 10.3389/fphys2014.00361.

Diogo LN, Pinto P, Bárbara C, Papoila AL, Monteiro EC. The association between antihypertensive medication and blood pressure control in patients with obstructive sleep apnea (*Advances in Experimental Medicine and Biology*, *in press*).

Diogo LN, Faustino IV, Afonso RA, Pereira SA, Monteiro EC, Santos AI. Losartan voluntary oral administration – a less stressful approach in rats (*Journal of the American Association for Laboratory Animal Science*, *in press*).

Diogo LN, Pereira SA, Nunes AR, Faustino IV, Afonso RA, , Santos AI, Monteiro EC. Efficacy of carvedilol in reversing hypertension induced by chronic intermittent hypoxia (*in submission*).

Other manuscripts published during the author's doctoral studies not explicitly related with the content of the present thesis:

Sílvia V. Conde, Sacramento JF, Guarino MP, Gonzalez C, Obeso A, **Diogo LN**, Monteiro EC, and Ribeiro MJ. Carotid body, insulin and metabolic diseases: unraveling the links. Front. Physiol. 5:418. doi: 10.3389/fphys.2014.00418.

Inês Martins, Inês Matias, **Lucília N Diogo**, Emília C. Monteiro, Sérgio Dias. Chronic intermittent hypoxia affects hematopoiesis and modulates the bone marrow microenvironment (*in submission*).

Sacramento JF, Ribeiro MJ, Rodrigues T, Matafome P, **Diogo LN**, Guarino, MP, Seiça R, Monteiro EC and Conde SV. Alterations in insulin signaling pathways contribute to insulin resistance induced by chronic intermittent hypoxia. (*in submission*).

In equal parts, to Manuel,

to my parents,

to the memory of my grandfather.

Table of Contents

List of Tables

List of Figures

Section 2

Efficacy of Carvedilol in reversing HT induced by CIH

Voluntary oral administration as an alternative method to gavage

List of Acronyms and Abbreviations

ABPM	*Ambulatory Blood Pressure Monitoring*
ACEi	*Angiotension-Converting Enzyme Inhibitor*
AHDs	*Antihypertensive Drugs*
AHI	*Apnea-Hypopnea Index*
AP-1	*Activator Protein-1*
ARB	*Angiotensin II Receptor Blocker*
AT II	*Angiotensin II*
BMI	*Body Mass Index*
BP	*Blood Pressure*
CA	*Cathecolamines*
CB	*Carotid Body*
CCB	*Calcium Channel Blocker*
CHI	*Chronic Intermittent Hypoxia*
COPD	*Chronic Obstructive Pulmonary Disease*
CPAP	*Continuous Positive Airway Pressure*
CRP	*C- Reactive Protein*
CSA	*Central Sleep Apnea*
CVD	*Carvedilol*
DBP	*Diastolic Blood Pressure*
EDTA	*Ethylenediaminetetraacetic Acid*
ET-1	*Endothelin-1*
FiO_2	*Fraction of Inspired Oxygen*
GAMs	*Generalized Additive Models*
GAV	*Gavage*
HDL-C	*High-Density Lipoprotein-Cholesterol*
HIF-1α	*Hypoxia-Inducible Factor α*
HPLC	*High-Performance Liquid Chromatography*
HR	*Heart Rate*
HT	*Hypertension*
ICAM-1	*Intercellular Adhesion Molecule*
IH	*Intermittent Hypoxia*

IL	*Interleukin*
INN	*International Non-proprietary Name*
LDL-C	*Low-Density Lipoprotein- Cholesterol*
LST	*Losartan*
MAP	*Mean Arterial Blood Pressure*
MC	*Methylcellulose*
MCF	*(-)-menthyl chloroformate*
MNH	*Misclassified Non-Hypertensive Patients*
NADPH	*Nicotinamide Adenine Dinucleotide Phosphate*
NC	*Neck Circumference*
NH	*Non-Hypertensive Patients*
NO	*Nitric Oxide*
eNOS	*Endothelial Nitric Oxide Synthase*
NF-κB	*Nuclear Factor-κ-light Chain Enhancer of Activated B Cells*
NOX2	*NADPH oxidase 2*
NTS	*Nucleus of the Solitary Tract*
NUT	*Nut Paste*
Nx	*Normoxia*
OSA	*Obstructive Sleep Apnea*
$PaCO_2$	*Partial Pressure of Carbon Dioxide*
PB	*Peanut Butter*
PD	*Pulmonary Hypertension*
PRA	*Plasma Renin Activity*
PVN	*Paraventricular Nucleus Neurons*
RAAS	*Rennin-Angiotensin-Aldosterone System*
RCT	*Randomized Controlled Trials*
RERAs	*Respiratory Effort Related Arousals*
ROS	*Reactive Oxygen Species*
SaO_2	*Arterial Hemoglobin Oxygen Saturation*
SBP	*Systolic Blood Pressure*
SD	*Sugar Dough*
SDB	*Sleep-Disordered Breathing*
SEM	*Standard Error of the Mean*
SHR	*Spontaneously Hypertensive Rats*

SNA	*Sympathetic Nerve Activity*
SOD	*Superoxide Dismutase Mimetic*
SPSS	*Statistical Package for the Social Sciences*
TC	*Total Cholesterol*
TGL	*Triglycerides*
TNF-α	*Tumour Necrosis Factor α*
TSP	*Trigger Sleep Blood Pressure monitoring*
VEGF	*Vascular Endothelial Growth Factor*
WC	*Waist Circumference*

Acknowledgements

First of all, I would to express my heartfelt gratitude to my supervisor Professor Emília Monteiro, whose thoughtful consideration and guidance have been truly important. During the last six years you have been a constant source of creativity, strength and enthusiasm, being all that a mentor should be. Thank you for encouraging me and for allowing me to grow as a researcher. To work with you has been a great honour and a real pleasure to me!

I would also like to gratefully thank Professor Ana Luísa Papoila, my co- supervisor, for sharing with me her incredible statistical knowledge and for all those infinite hours that we spent "listening" to the data. Thank you also for your true generosity over the years and for just being unconditionally there for me in all the moments of discouragement and frustration. A simple "Never give up!" really meant a lot to me!

To Professor Cristina Bárbara, also co-supervisor of this thesis, I would like to thank her for her helpful advice during the study design and support during the recruitment. I learned so much, from you and your team, during the almost two years that I spent in Hospital Pulido Valente!

I would also like to extend my sincerest thanks to Professor Ana Isabel Moura Santos for her scientific advice and knowledge within the field of laboratory animal science, but foremost for her friendship and unconditional support throughout these last years. I am also very grateful to Professor Pedro Freire Costa for his many insightful comments and suggestions. "The quick brown fox jumped over the lazy dog's back" was really a useful tip!

To all my colleagues at NOVA Medical School and CEDOC, especially Teresa Monteiro, Sílvia Conde, Joana Batuca, Maria João Ribeiro, Joana Sacramento, Clara Dias, Nádia Grilo, Aline Marinho, Inês Faustino, Joana Gaspar and Fátima Martins, for all their help and support during this long journey. A special thanks to Rita Nunes for her true friendship, unconditional support and for all the hours we spent chatting and discussing experiments and results; Ricardo Afonso for being my "right hand" during the surgical procedures (our "four-hands" surgeries were a success!!!); Sofia Pereira, my dearest friend and great researcher, for all her support, interest and encouragement (Believe! The best is yet to come!) and also to Daniela Vasconcelos who started this beautiful adventure with me!

My gratitude also goes to Teresa Lopes, Sofia Ramos and Gracinda Menezes for their support and friendship, to Professor Teresa Gamboa for her advice during the setup of the Oxycycler System and also to Bruno Igreija and Nataniel Rosa for their helpful insights within the field of telemetry.

I would also like to thank physicians, in particular Professor Paula Pinto, and cardio-respiratory technicians from Centro Hospitalar Lisboa Norte, EPE (CHLN) Sleep Unit (Hospital Pulido Valente) for their valuable support during the patient recruitment period.

I will always be grateful to all my friends for sheltering me over the years. A special thanks to Raquel Salvaterra, my best friend for almost two decades, for being there to listen, party and comfort. You rock girl!

Lastly, I would like to extend my sincerest thanks and appreciation to my loving parents, Diogo and Madalena, who set a shining example from the first day of my life. Words cannot express how grateful I am for your emotional support no matter what path I choose. To my brother Zé, thank you for your infinite support throughout everything. To Paulo for being a true partner and for just being there for me with all his encouragement and support. To my "BIG" Manuel, the rainbow of my life, who is much more than any mother could ask for. Your smile and complicity have been my daily dose of inspiration. I hope that I have not lost too much of your "busy days" and I promise that our Disney trip will happen soon!! Finally, to my grandfather Oswaldo who unfortunately is no longer with me and whom I miss terribly, thank you for all the great moments we shared. This PhD thesis is a tribute to you!

I also would like to thank Fundação para a Ciência e Tecnologia (FCT), Chronic Diseases Research Center (CEDOC) and NOVA Medical School.

Abstract

Hypertension (HT) is a highly prevalent condition, although under diagnosed, in patients with obstructive sleep apnea (OSA). These conditions are closely related and 24-hour ambulatory blood pressure monitoring (ABPM) seems to be the most accurate measurement for diagnosing hypertension in OSA. However, this diagnostic tool is expensive and time-consuming and, therefore, not routinely used. On the other hand, although continuous positive airway pressure (CPAP) is considered the gold standard treatment for symptomatic OSA, its lowering effect on blood pressure (BP) seems to be modest and, therefore, concomitant antihypertensive therapy is still required. Data on antihypertensive drug regimens in patients with OSA are scarce and specific therapeutic guidelines for the pharmacological treatment of hypertension in these patients remain absent. The use of animal models of CIH, which mimic the HT observed in patients with OSA, is extremely important since it is imperative to identify preferred compounds for an adequate BP control in this group of patients. However, studies aimed at investigating the antihypertensive effect of antihypertensive drugs in this animal model are insufficient, and most reports on CIH animal models in which drugs have been tested were not designed to respond to pharmacological issues. Moreover, when testing antihypertensive drugs (AHDs) it becomes crucial to ensure the selection of a non-invasive and stress-free method for drug delivery. Although *gavage* is effective and a widely performed technique for daily dosing in laboratory rodents, it comprises a sequence of potentially stressful procedures for laboratory animals that may constitute bias for the experimental results.

The overall goal of the present translational research was to contribute to identify more effective AHDs for the treatment of hypertension in patients with OSA and investigate underlying mechanisms of systemic effects associated with OSA, as well as its modulation by AHDs. The specific aims were: first, to find new predictors based on anthropometric measures to identify patients that misclassify themselves as non-hypertensive, and thereby promote the selective use of ABPM; second, to investigate a hypothetical association between ongoing antihypertensive regimens and BP control rates in patients with OSA, before and after CPAP adaptation; third, to determine, in a rat model of CIH-induced hypertension, the efficacy of carvedilol (CVD), a nonselective beta-blocker with intrinsic anti-α1-adrenergic activity and antioxidant properties; fourth, to explore the effects of CIH on the pharmacokinetics profile of CVD and fifth, to investigate an alternative method to *gavage*, for chronic administration of AHDs to laboratory rats.

For that, in the first phase of this project, we used a sizeable sample of patients with OSA (n=369), that attended a first visit at Centro Hospitalar Lisboa Norte, EPE Sleep Unit, and underwent overnight polysomnography, 24-h ABPM and filled a questionnaire that included

ongoing antihypertensive medication profile registration. In the second phase, a rat experimental model of HT induced by a paradigm of CIH that simulates OSA was used.

The main findings of this work were: first, body mass index (BMI) and neck circumference (NC) were identified as independent predictors of hypertension misclassification in patients suspected of OSA; second, in patients with OSA, BP control is independent of both the antihypertensive regimen and the number of antihypertensive drugs, either before or after CPAP adaptation; third, although the doses of 10, 30 and 50 mg/Kg of CVD promoted a significant reduction in heart rate, no decrease in mean arterial pressure was observed; fourth, the S/(R+S) ratios of CVD enantiomers, between rats exposed to CIH and normoxic conditions, were different and fifth, voluntary ingestion proved to be an effective method for a controlled daily dose administration, with a define timetable, that is independent of handling and restraint procedures.

In conclusion, the clinical study showed that BP control in OSA patients is independent of both the antihypertensive regimen and the number of antihypertensive drugs. Additionally, our results highlight the lack of validity of self-reported hypertension and suggest that all patients suspected of OSA with undiagnosed hypertension and with a BMI and NC above 27 Kg/m^2 and 39 cm should be screened for hypertension, through ABPM. The results attained in the rat model of HT related to CIH suggest that the blockade of the sympathetic nervous system together with the putative pleiotropic effects of carvedilol is not able to revert hypertension induced by CIH and point out that the pharmacokinetic changes induced by CIH on S/(R+S) ratio are not apparently responsible for the lack of efficacy of carvedilol in reversing this particular type of hypertension. Finally, the results here presented support the use of voluntary oral administration as a viable alternative to *gavage* for chronic administration of a fixed dose of AHDs.

Key words: ambulatory blood pressure monitoring, antihypertensive drugs, carvedilol, chronic intermittent hypoxia, hypertension, obstructive sleep apnea, voluntary oral administration.

Resumo

A hipertensão arterial (HA) é uma patologia altamente prevalente, embora claramente subdiagnosticada, em doentes com síndrome de apneia obstrutiva do sono (SAOS). Estas duas patologias apresentam uma estreita relação e a monitorização ambulatória da pressão arterial (MAPA), por um período de 24 horas, parece ser o método mais preciso para o diagnóstico de hipertensão em doentes com SAOS. No entanto, esta ferramenta de diagnóstico para além de ser dispendiosa e envolver um número acrescido de meios técnicos e humanos, é mais morosa e, por conseguinte, não é utilizada por rotina no contexto do diagnóstico da SAOS. Por outro lado, apesar da aplicação de pressão positiva contínua nas vias aéreas (CPAP – *Continous Positive Airway Pressure*) ser considerada a terapêutica de eleição para os doentes com SAOS, o seu efeito no abaixamento da pressão arterial (PA) parece ser modesto, exigindo, por conseguinte, a implementação concomitante de terapêutica anti-hipertensora. Acontece que são escassos os dados relativos aos regimes de fármacos anti-hipertensores utilizados em doentes com SAOS e, acresce ainda que, as *guidelines* terapêuticas para o tratamento farmacológico da HA, neste grupo particular de doentes, permanecem, até ao momento, inexistentes. A utilização de modelos animais de hipóxia crónica intermitente (CIH), que mimetizam a HA observada em doentes com SAOS, revela-se extremamente importante, uma vez que se torna imperativo identificar fármacos que promovam um controle adequado da PA neste grupo de doentes. No entanto, estudos concebidos com o intuito de investigar o efeito anti-hipertensor dos fármacos neste modelo animal revelam-se insuficientes e, por outro lado, os escassos estudos que testaram fármacos anti-hipertensores neste modelo não foram desenhados para responder a questões de natureza farmacológica. Acresce ainda que se torna imprescindível garantir a escolha de um método para administração destes fármacos que seja não invasivo e que minimize o stress do animal. Embora a *gavagem* seja uma técnica indiscutivelmente eficaz e amplamente utilizada para a administração diária de fármacos a animais de laboratório, ela compreende uma sequência de procedimentos geradores de stress para os animais e, que podem por conseguinte, constituir um viés na interpretação dos resultados obtidos.

O objectivo global da presente investigação translacional foi contribuir para a identificação de fármacos anti-hipertensores mais efectivos para o tratamento da HT nos indivíduos com SAOS e investigar mecanismos subjacentes aos efeitos sistémicos associadas à SAOS bem como a sua modulação por fármacos anti-hipertensores. Os objectivos específicos foram: em primeiro lugar, encontrar novos critérios, baseados nas medidas antropométricas, que permitam a identificação de doentes com suspeita de SAOS, que erroneamente se auto-classifiquem como não-hipertensos, e desta forma promover um uso mais criterioso do MAPA; em segundo lugar, investigar a existência de uma hipotética associação entre os esquemas de fármacos anti-hipertensores e o controle da PA (antes e após a adaptação de CPAP) em doentes com SAOS;

em terceiro lugar, avaliar a eficácia do carvedilol (CVD), um fármaco bloqueador β-adrenérgico não selectivo com actividade antagonista α1 intrínseca e propriedades anti-oxidantes num modelo animal de hipertensão induzida pela CIH; em quarto lugar, explorar os efeitos da CIH sobre o perfil farmacocinético do CVD; e, em quinto lugar, investigar um método alternativo à *gavagem* para a administração crónica de fármacos anti-hipertensores a animais de laboratório.

Com este intuito, na primeira fase deste projecto, fizemos uso de uma amostra com um número apreciável de doentes com SAOS (n=369), que acorreram, pela primeira vez, à consulta de Patologia do Sono do CHLN e que foram submetidos a um estudo polissonográfico do sono, à MAPA e que preencheram um questionário que contemplava a obtenção de informação relativa ao perfil da medicação anti-hipertensora em curso. Numa segunda fase, utilizámos um modelo experimental de HT no rato induzida por um paradigma de CIH.

Do nosso trabalho resultaram os seguintes resultados principais: em primeiro lugar, o índice de massa corporal (IMC) e o perímetro do pescoço (PP) foram identificados como preditores independentes de "auto-classificação errónea" da HA em doentes com suspeita de SAOS; em segundo lugar, não encontramos qualquer associação com significado estatístico entre os vários esquemas de fármacos anti-hipertensores bem como o número de fármacos incluídos nesse esquemas, e o controle da PA (antes e depois da adaptação do CPAP); em terceiro lugar, apesar das doses de 10, 30 e 50 mg/kg de carvedilol terem promovido uma redução significativa da frequência cardíaca, não foi observado qualquer decréscimo na PA no nosso modelo animal; em quarto lugar, as razões S/(R+S) dos enantiómeros do CVD nos animais expostos à CIH e a condições de normóxia revelaram-se diferentes; e, em quinto lugar, a administração oral voluntária mostrou ser um método eficaz para a administração diária controlada de fármacos anti-hipertensores e que é independente da manipulação e contenção do animal.

Em conclusão, os resultados obtidos através do estudo clínico revelaram que o controle da PA, antes e após a adaptação do CPAP, em doentes com SAOS é independente, quer do esquema de fármacos anti-hipertensores, quer do número de fármacos incluídos num determinado esquema. Os nossos resultados salientam ainda a falta de validade da chamada *self-reported hypertension* e sugerem que em todos os doentes com suspeita de SAOS, com HA não diagnosticada e com um IMC e um PP acima de 27 kg/m^2 e 39 cm, respectivamente, a confirmação do diagnóstico de HA deverá ser realizada através da MAPA, ao invés de outros métodos que com maior frequência são utilizados com este propósito. Os resultados obtidos no modelo animal de HA induzida pela CIH sugerem que o bloqueio do sistema nervoso simpático, juntamente com os supostos efeitos pleiotrópicos do CVD, não parece ser a estratégia mais adequada para reverter este tipo particular de hipertensão e indicam que as alterações farmacocinéticas induzidas pela CIH no ratio S/(R+S) não justificam a falta de eficácia anti-hipertensora do CVD observada neste modelo animal. Por último, os resultados do presente trabalho suportam ainda a

viabilidade da utilização da administração oral voluntária, em alternativa à *gavagem*, para a administração crónica de uma dose fixa de fármacos anti-hipertensores.

Palavras-chave: Administração oral voluntária, apneia obstructiva do sono, carvedilol, fármacos anti-hipertensores, hipertensão, hipoxia crónica intermitente, medição ambulatória da pressão arterial.

Thesis Outline

The present thesis is divided into five main chapters, enumerated with Roman numbers, whose content is summarized below.

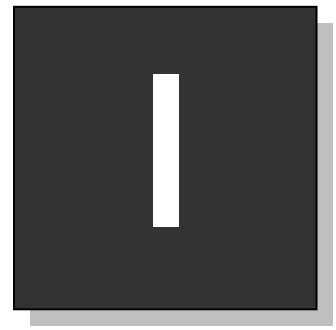
Chapter I is a general introduction to the thesis, summarizing the most relevant topics of literature in chronic intermittent hypoxia-related disorders, namely obstructive sleep apnea, and its close linkage with hypertension. Special focus is given to the efficacy of pharmacological interventions to revert hypertension related with chronic intermittent hypoxia conditions in both animals and humans. The content of this chapter has been included in the publication of a review article.

In **Chapter II**, we summarize the starting points for the establishment of the main goals of our research line. The general and specific research aims that will be addressed in this thesis are also listed in this chapter.

Chapter III contains the description of the general methods used during both clinical and experimental studies.

In **Chapter IV**, we present the results of this thesis, published or submitted to international peer-reviewed journals. Section 1 comprises two original articles regarding the results of the study performed in humans. Section 2 includes one original article concerning the results attained in the animal model of hypertension induced by chronic intermittent hypoxia and the other relating to an alternative method to *gavage*, for chronic administration of antihypertensive drugs to laboratory animals.

Chapter IV summarizes the main conclusions and limitations of our work and presents the future perspectives concerning the non-pharmacological and pharmacological management of hypertension related to obstructive sleep apnea.



INTRODUCTION

Chronic intermittent hypoxia-related disorders

Is is well established that intermittent hypoxia (IH) affects control of breathing, the autonomic nervous system and the cardiovascular system (Foster *et al.,* 2007). Chronic intermittent hypoxia (CIH) is a feature that is present in interstitial lung disease (Fletcher *et al.,* 1992a) and sleep-disordered breathing (SDB), and it has also been shown to occur in patients with hepatopulmonary syndrome (Tanné *et al.,* 2005; Ogata *et al.,* 2006; Palma *et al.,* 2008). Since several years ago, there has been growing interest concerning CIH due to the high relevance of the part assumed to be played by sleep-related breathing disorders in chronic diseases.

Sleep apnea/hypopnea disorders include centrally originated diseases and obstructive sleep apnea (OSA). Central sleep apnea (CSA) is characterized by a lack of drive to breathe during sleep, resulting in insufficient or absent ventilation and compromised gas exchange (Eckert *et al.,* 2007). In CSA, the cessation of respiration during sleep is not associated with ventilatory effort and there is sleep fragmentation due to arousals associated with reflexes activated by the ensuing hypoxemia (Paiva and Attarian, 2014). The major manifestations of CSA include high altitude-induced periodic breathing, idiopathic CSA, narcotic-induced central apnea, obesity hypoventilation syndrome, and *Cheyne-Stokes* breathing in heart failure (Eckert *et al.,* 2007). While the precipitating mechanisms involved in the several types of CSA may diverge, unstable ventilatory drive during sleep is the principal underlying feature (Eckert *et al.,* 2007). CSA is diagnosed in approximately 5% of the patients who undergo a polysomnographic study (Khan and Franco, 2014). On the other hand, OSA is briefly characterized by repetitive episodes of airflow cessation (apnea) or airflow reduction (hypopnea) caused by an obstructed or collapsed upper airway during sleep. Unlike CSA, obstruction occurs in OSA despite the central drive to breathe and inspiratory muscle activity (Levitzky, 2008). An appreciable number of factors are known to be linked to upper-airway collapse, namely reduced airway dilator muscle activity during sleep, upper-airway anatomy, obesity, decreased end-expiratory lung volume, ventilatory control instability, and rostral fluid shifts (Kapur, 2010). The repetitive episodes of apnea and hypopnea characteristic of OSA are closely associated with CIH, hypercapnia and an increase in intrathoracic pressure, leading to recurrent arousals and significant changes in sleep architecture. OSA is affecting a growing proportion of the common population, and the estimated prevalence in the 1990s was 9% for women and 24% for men among middle-aged adults (Young *et al.,* 1993). In addition, CSA can occur concomitantly with OSA. This last condition, recently labeled complex sleep syndrome, is observed in approximately 15% of the patients following treatment with continuous positive airway pressure (CPAP) (Paiva and Attarian, 2014). In a few words, complex sleep syndrome is a form of sleep-disordered breathing in which CSA persists or emerges when obstructive events have disappeared using a positive pressure device (Khan and Franco, 2014). In clinical practice, when a few central

apneas are observed in polysomnograms of patients with OSA, they are normally ignored because we do not presently understand their potencial clinical relevance.

Nowadays, it is well known that the outcomes of these sleep-related breathing disorders can lead to vascular diseases, contributing to a considerable increase in overall cardiovascular risk. The desaturation-reoxygenation sequence, a typical pattern coupled with the majority of respiratory events, is thought to be responsible for most of the associated cardiovascular morbidity (Lévy *et al.*, 2012). Although OSA has been associated with several cardiovascular conditions, it has been more closely etiologically connected to systemic HT (Kapa *et al.*, 2008), and the link between HT and OSA is now widely accepted and supported by different findings. Most episodes of OSA are coupled with sleep disruption, which *per se* increases sympathetic nerve activity and blood pressure (Morgan *et al.*, 1996). In addition, the occurrence of arousals appears to enhance the pressor effects of asphyxia during OSA (Morgan *et al.*, 1998), contributing synergistically to blood pressure increase. In any case, studies in both animals and humans underline the major role of hypoxia itself in promoting an increase in blood pressure (Brooks *et al.*, 1997b; Tamisier *et al.*, 2011).

Regarding CSA, this SDB, like OSA, is strongly linked to cardiac disease and cardiovascular outcomes (Brenner *et al.*, 2008). Indeed, the majority of patients with CSA have underlying cardiovascular disease, primarily heart failure, which is considered the most common risk factor for CSA, followed by atrial fibrillation (Bradley *et al.*, 1992). Moreover, like OSA, CSA has been implicated in heart failure pathophysiology (Mehra, 2014) and occurs in 30–50% of patients with left ventricular dysfunction and heart failure caused by HT, cardiomyopathy and ischemic heart disease (Bradley and Floras, 2003). Thus, CSA has significant co-morbidity with many cardiac conditions, which clearly contributes to an increase in the associated mortality and morbidity.

Besides systemic HT, chronic intermittent alveolar and systemic arterial hypoxia-hypercapnia can cause pulmonary HT (PH). SDB has also been found to be associated with PH, being considered one of the potential etiologies of PH (Galie *et al.*, 2009). During episodes of OSA, the subsequent oscillations in PaO_2 lead to a cyclical pattern of vasoconstrictions and relaxations in the pulmonary circulation responsible for the marked fluctuations observed in pulmonary arterial pressure (Dempsey, 2010). The perpetuation of this pattern leads to fixed elevations in pulmonary pressure (Dempsey, 2010). Some data suggest that even slight changes in pulmonary function, in the absence of lung disease, are able to induce PH in patients with OSA. Furthermore, it is important to bear in mind that PH could also be a cause of abnormal arterial blood gases during wakefulness (Dempsey, 2010) and that OSA itself can lead to PH (Sajkov and McEvoy, 2009). The major consequence of the increased pulmonary artery pressure, together with increased blood viscosity (a consequence of the renal release of

erythropoietin subsequent to hypoxemia), is the occurrence of right ventricle hypertrophy leading to *cor pulmonale* (Levitzky, 2008). The prevalence of this chronic cardiopulmonary condition among patients with SDB is estimated to range from 17% to 52% (Minic *et al.,* 2014), and 20–30% of untreated OSA patients suffer from PH (Dumitrascu *et al.,* 2013). Even if PH in this group of patients is typically not severe (Badesh *et al.,* 2010), OSA patients with PH have a higher mortality rate than OSA patients without PH (Minai *et al.,* 2009). A recent meta-analysis shows that CPAP is associated with a mild but statistically significant reduction in pulmonary artery pressure in OSA patients (Sun *et al.,* 2014). This decrease might translate into a better outcome in patients with PH secondary to OSA. However, more studies are needed to confirm this assumption.

OSA and Hypertension: How relevant is this linkage?

Since 2003, OSA has formally been recognized as a frequent and important secondary cause of HT and is one of the first causes to be screened mainly in patients with a suggestive phenotype, refractory HT and a non-dipping profile (Chobanian *et al.,* 2003; Mancia *et al.,* 2007). More recently, OSA has been identified as an independent risk factor for HT (Lavie *et al.,* 2000; Peppard *et al.,* 2000; Marin *et al.,* 2012), as one of the major clinical conditions that favors poorly controlled HT (Oliveras and Schmieder, 2013), and as the most common condition associated with resistant HT (Pedrosa *et al.,* 2011). OSA and HT are two prevailing risk factors for several cardiovascular events (Wang and Vasan, 2005; Baguet *et al.,* 2009). Due to their high prevalence and cardiovascular morbidity (Wolf *et al.,* 2007; Malhotra and Loscalzo, 2009), OSA and HT are now acknowledged as public health problems. Epidemiological data show that the estimated overall prevalence of HT among patients with OSA is approximately 50% and an estimated 30–40% of hypertensive patients are diagnosed with OSA (Calhoun, 2010), confirming the bidirectional relationship between OSA and HT. Moreover, OSA and HT are chronic diseases mostly diagnosed in active adults and because of the associations between OSA and obesity and advancing age, the public health burden of OSA related to cardiovascular disease is expected to rise in the coming years (Dempsey *et al.,* 2010). The use of both antihypertensive drugs (AHDs) and CPAP in these patients is for life and consequently treatment is associated with a high impact both in terms of costs and in patients' quality of life. Indeed, OSA generates an impressive economic burden, including medical costs, when compared to other equally relevant chronic diseases (Kapur, 2010; Badran *et al.,* 2014).

OSA and Hypertension: What is the problem?

CPAP is considered the gold standard treatment for mild, moderate and severe OSA due to its remarkable ability in providing pneumatic splitting of the upper airway and effectiveness in reducing the apnea-hypopnea index (AHI), symptoms, and cardiovascular morbidity and mortality (Hla *et al.,* 2002; Pepperell *et al.,* 2002; Wolf *et al.,* 2007; Epstein *et al.,* 2009; Mannarino *et al.,* 2012). Besides preventing hypoxemia, sleep disturbance and apnea episodes, CPAP reduces sympathetic activity, systemic inflammation and oxidative stress (Yorgun *et al.,* 2014). However, the results found for the effectiveness of CPAP on blood pressure (BP) control are still controversial. Table 1 summarizes the results of original studies in which the effect of CPAP on BP has been analysed.

Whereas some studies and meta-analyses (Bakker *et al.,* 2014; Varounis *et al.,* 2014) have reported modest effects for CPAP in lowering BP, others tend to support the beneficial effect of CPAP treatment on BP reduction and attenuating the risk of developing HT. In any case, although the lowering effect of CPAP on BP is relevant in terms of overall cardiovascular risk reduction, this effect is very limited when compared to the performance of AHDs in patients with essential HT (Pépin *et al.,* 2010). Thus, treating HT in patients with sleep apnea is proving to be a dificult task and there is consensus that the use of AHDs is mandatory. In spite of this, data on AHDs regimens in patients with OSA are scarce and there is a lack of specific therapeutic guidelines for the pharmacological treatment of HT in these patients. Furthermore, the effects of antihypertensive agents on OSA patients are not consistent (Parati *et al.,* 2012) and there are no data on the efficacy of specific AHDs regimens when associated with CPAP.

A new treatment for OSA patients is the oral appliance/mandibular advancement device (Guralnick and Bakris, 2012). Oral appliance therapy is an important alternative to CPAP for some patients with mild to moderate OSA (Iftikhar *et al.,* 2013). Despite a recent study (Andrén *et al.,* 2013) and a recent meta-analysis (Iftikhar *et al.,* 2013) which have shown some beneficial effects of this device in reducing blood pressure measurements, larger and longer randomized control trials are needed to confirm the effects of oral appliance therapy on BP control.

Clearly, more studies are required to identify first-line AHDs regimens for optimal BP control in this particular group of hypertensive patients (Tsioufis *et al.,* 2010; Parati *et al.,* 2013). Moreover, HT related to OSA needs to be managed as a specific entity and an earlier diagnosis of this type of HT seems to be as relevant as the selection of AHDs regimens.

Table 1. CPAP effect on blood pressure

Study design	n	Study duration	HT patients (%)	AHDs (Y/N)	Mean CPAP use (h/night)	BP outcome	Reference
RCT; parallel group; blinded endpoint	194	12 wks	100	Yes	5	↓ 3.1 mmHg MBP ↓ 3.2 mmHg DBP ↓ 3.1 mmHg SBP (NS)	Martinez-Garcia *et al.*, 2013
RCT; parallel group	118	4 wks	10	Yes	4.9	↓ 3.3 mmHg 24h MBP	Pepperell *et al.*, 2002
Case -controlled study	48	4 wks	79	Yes	5.1	↓ 5.2 mmHg DBP ↓ 3.8 mmHg SBP (NS)	Zhao *et al.*, 2012
Prospective randomized trial	32	9 wks	66	Yes	5.5	↓ ± 10 mmHg MBP ↓ ± 10 mmHg DBP ↓ ± 10 mmHg SBP (During both day and night-time)	Becker *et al.*, 2003
RCT; multicenter; parallel group	723	4 yrs	51.5	Yes	5.0	NS on new-onset HT	Barbé *et al.*, 2012
Prospective, single-center, long-term follow-up	91	5 yrs	100	Yes	*NA*	NS on 24h BP, SBP and DBP	Kasiakogias *et al.*, 2013
RCT; parallel group	40	6 mon	100	Yes	6.01	↓ Awake SBP (6.5 mmHg) and DBP (4.5 mmHg) NS nocturnal SBP and DBP	Pedrosa *et al.*, 2013
Retrospective chart review study	98	1 yr	100	Yes	6.3	↓ 5.6 mmHg MBP (resistant HT group) ↓ 0.8 mmHg MBP (controlled BP group)	Dernaika *et al.*, 2009
RCT; crossover	28	8 wks	100	Yes	4.8	↓ 2.1 mmHg 24h MBP (CPAP group) ↓ 9.1 mmHg 24h MBP (valsartan group)	Pépin *et al.*, 2010
Prospective cohort study	86	6 mon	55	Yes	4.8	↓ 4.92 mmHg 24h MBP	Robinson *et al.*, 2008
Observational study	24	12 wks	0	No	*NA*	↓ 5.3 mmHg 24h MBP	Yorgun *et al.*, 2014
Prospective cohort study	196	6 mon	85	Yes	*NA*	↓ 2.7 mmHg DBP ↓ 2.1 mmHg SBP	Börgel *et al.*, 2004
RCT; multicenter; double-blinded	340	12 wks	100	No	4.5	↓ 1.5 mmHg MBP ↓ 1.3 mmHg mean DBP ↓ 2.1 mmHg mean SBP	Durán-Cantolla *et al.*, 2010
RCT; multicenter; parallel group	44	6 wks	*NA*	Yes	5.0	NS on 24h SBP and DBP	Barbé *et al.*, 2001
RCT; crossover study; sham placebo	35	10 wks	100	Yes	5.2	NS on overall 24h MBP	Robinson *et al.*, 2006
Observational, monocentric; cohort study	495	3.4 yrs	40.4	Yes	*NA*	↓ Occurrence of systemic arterial HT	Bottini *et al.*, 2012
RCT; single-blinded	44	13.2 wks	100	Yes	5.1	Additional ↓ in office BP and ambulatory BP monitoring (CPAP+ 3 AHDs)	Litvin *et al.*, 2013
RCT, multicenter	359	1 yr	100	Yes	4.7	↓ 2.19 mmHg DBP NS ↓ 1.89 mmHg SBP NS	Barbé *et al.*, 2009
RCT	36	3 mon	*NA*	No	5.2	↓ 2 mmHg office DBP ↓ 5 mmHg office SBP ↓ 5 mmHg 24 h DBP ↓ 5 mmHg 24 h SBP	Drager *et al.*, 2011
RCT; parallel group	64	3 mon	100	Yes	> 5.8	↓ 6.98 mmHg 24h DBP ↓ 9.71 mmHg 24h SBP	Lozano *et al.*, 2010

AHDs, antihypertensive drugs; **BP**, blood pressure; **CPAP**, continuous positive airway pressure; **DBP**, diastolic blood pressure; **HT (%)**, percentage of hypertensive patients; **MBP**, mean blood pressure; **NA**, information not available; **NS**, no significant effect; **RCT**, randomized controlled trials; **SBP**, systolic blood pressure;↓, decrease.

What models are available to study Hypertension related to OSA?

Due to the high complexity and heterogeneity associated with OSA, considerable variability can be observed between reports addressed at the study of this disease. In addition, the scarcity of opportunities for patient investigation, in particular at the cellular level, has compromised progress in understanding the pathophysiology of OSA and the development of novel and specific treatments for this disorder. To overcome some of these limitations, several animal models and more recently, a model of OSA in healthy human volunteers (Tamisier *et al.*, 2009; Tamisier *et al.*, 2011) have been developed. Animal models, especially of IH, mimic OSA more easily than human models. The small size of rodents allows more rapid and intense changes in SaO_2 (arterial hemoglobin oxygen saturation) whereas humans require longer periods of hypoxia to induce arterial oxyhaemoglobin desaturation (Foster *et al.*, 2007). The combination of these two approaches is certain to contribute to the consolidation of prevention strategies and the development of more suitable treatments for OSA patients.

ANIMAL MODELS

The major advantage of the use of animal models is that they allow single components of the disease to be evaluated, accurately controlling the triggering events in terms of both severity and duration, and providing homogeneous populations (Lévy *et al.*, 2012). These models also provide an excellent opportunity to explore the underlying mechanistic pathways of HT related to OSA and their consequences under controlled conditions. Moreover, animal models have enabled the study of parameters that have proved difficult to assess in humans, particularly due to the need for organ harvesting to explore the mechanisms underlying the consequences of IH at the molecular level (Dematteis *et al.*, 2009). Thus, studies with animal models are good tools for overcoming some confounding factors present in human studies (e.g., the presence of comorbidities, disease duration, and behavioral and environmental variables) (Badran *et al.*, 2014), and for providing more specific information concerning the efficacy of drugs to be tested.

In 2009, Dematteis *et al.* used the terminology homologous (sharing the cause or pathophysiology of the human disease), predictive (responding to treatment similarly to the human disease) and isomorphic (displaying symptoms similar to those of the human disease although their cause and pathophysiology may differ) to categorize sleep apnea models (Dematteis *et al.*, 2009). According to these categories, most sleep apnea models are only partially isomorphic, focusing on a specific aspect of the human disease. As a matter of fact, none of the currently available animal models reproduce all aspects of human sleep apnea and they present some important limitations. Nonetheless, the animal models of sleep apnea have

brought out most of the available knowledge in this field and furthermore, almost all cardiovascular diseases known to be present in patients with OSA have been replicated in these models (Dumitrascu *et al.,* 2013).

The effective use of animals to study sleep apnea implies recognition of the natural similarities and differences between animals and humans to ensure the reliability of the experimental results. For instance, as rodents are nocturnal animals, the stimulus must be applied during the sleep-dominant phase of the diurnal cycle. Moreover, in humans the circadian distribution of sleep tends to be consolidated and normally monophasic, with a daily sleep duration of 7–8 hours, whereas it is polyphasic, relatively fragmented and with a duration of 12–15 hours in rodents (Toth and Bhargava, 2013). Another issue is related to the fact that rodents sleep in the prone position (Golbidi *et al.,* 2012); it is well known that supine OSA is the dominant phenotype of OSA syndrome and that the supine position favors upper airway collapse in humans (Joosten *et al.,* 2014). Furthermore, additional care must be taken to minimize external factors (*e.g.,* light exposure, photoperiod, noise, disruptions in the home environment, and post-surgical care in studies, for instance requiring implantation of telemetric devices) able to influence sleep in animals used in experimental research (reviewed in Toth and Bhargava, 2013).

The experimental animal models developed to mimic OSA have recently been reviewed (Dematteis *et al.,* 2009; Golbidi *et al.,* 2012; Davies and O'Donnell, 2013; Toth and Bhargava, 2013) and assembled taking into account the main injuries triggered by OSA. Despite attempts to use large animals (*e.g.,* dogs, lambs and pigs) to simulate upper airway obstruction, most research on the cardiovascular consequences of OSA has been performed in rodents. Alternative models (*e.g.,* cell cultures incubated in specific devices that perform oxygen fluctuations mimicking sleep apnea-related IH), mainly relevant to signaling investigation (Kumar *et al.,* 2003; Gozal *et al.,* 2005; Ryan *et al.,* 2005), represent a complementary approach to the most widely used sleep models. However, in spite of the recommendations to refine, reduce and replace (the 3Rs programme), these alternative models cannot replace animal models in the study of HT.

The natural models of sleep apnea include the English bulldog, the historic natural model of spontaneous obstruction (Hendricks *et al.,* 1987), the sleep-related central apnea models (*e.g.,* Sprague-Dawley rats (Carley *et al.,* 2000), spontaneously hypertensive (SH) rats (Carley *et al.,* 1998), C57BL/6J (Julien *et al.,* 2003; Liu *et al.,* 2010)), and the Zucker obese rat in which apnea is obesity-related (Ray *et al.,* 2007; Lee *et al.* 2008; Iwasaki *et al.,* 2012). The experimentally-induced models (*e.g.,* the sleep deprivation model, induced airway obstruction and the CIH model) are the most widely used. Due to model limitations and lack of extensive study, we only briefly describe the induced airway obstruction model and the sleep deprivation

model. Special focus will be given to the CIH model, based on the assumption that IH is the most effective paradigm to induce HT related to OSA and probably the most relevant stimulus regarding the cardiovascular sequelae of OSA.

Induced airway obstruction models

Briefly, the airway obstruction model involves surgical intervention (an endotracheal tube), which is an invasive procedure, or alternatively the use of a specific chamber with a latex neck collar that induces recurrent airway obstruction. This latter procedure, developed by Farré *et al.* (Farré *et al.,* 2007), is associated with high levels of stress due to the restriction of animal movement. In both approaches, the degree of obstruction is adjustable (Golbidi *et al.,* 2012) and in the case of induction of obstruction through endotracheal tube, the $PaCO_2$ can be adjusted to mimic human sleep apnea (Golbidi *et al.,* 2012). Many experiments using this method have not monitored the sleep state of the animals, but more recent studies have incorporated sophisticated apparatus that is able to detect sleep-awake states and allow close coordination between the initiation of airway obstruction and sleep onset (Schneider *et al.,* 2000).

This model allows the study of the potential consequences of strenuous breathing against an obstructed airway and can be used to study the cardiovascular consequences and risk factors of OSA (*e.g.,* systemic inflammation and coagulation), and to investigate the mechanisms that underlie OSA (Salejee *et al.,* 1993; Nacher *et al.,* 2007; Almendros *et al.,* 2008; Nacher *et al.,* 2009; Othman *et al.,* 2010; Almendros *et al.,* 2011). However, to the best of our knowledge, no study has yet shown that this obstruction model is able to mimic HT related to OSA. Furthermore, when testing AHDs, it became crucial to ensure the selection of a stress-free paradigm as it has been shown that any source of external stress on rodents can significantly increase heart rate and blood pressure (Brown *et al.,* 2000; Kramer *et al.,* 2000; Balcombe *et al.,* 2004; Bonnichsen *et al.,* 2005) and therefore contribute to confounding the experimental results. Finally, as the rat models of obstruction or asphyxia were developed in restrained or anesthetized rats, they are not good models for chronic administration of oral drugs, particularly AHDs.

Sleep deprivation models

In the last few years, several approaches have been used to trigger sleep deprivation in different animals, the rat being the animal of choice to date (Colavito *et al.,* 2013). In the "*multiple platform technique*", the animal is aroused from sleep when the characteristic loss of muscle tone that accompanies paradoxical sleep causes it to fall off the platform (Suchecki and Tufik, 2000). The "*gentle handling*" procedure, by far the most popular method, is based on direct

interaction with the experimenter, who actively keeps the animal awake through the use of external stimulation (*e.g.*, mild noises, tapping or gentle shaking of the cage, or by direct contact with the animal either using a soft brush or by hand), or by the introduction of novel objects or nesting material in the cages, which typically leads to active exploratory behavior (Colavito *et al.*, 2013).

These models are most often used to evaluate the neurophysiological aspects of OSA (Van Dongen *et al.*, 2003; Haack and Mullington, 2005; McKenna *et al.*, 2007; Ward *et al.*, 2009; Nair *et al.*, 2011) due to the high similarity between the structures of the nervous systems of rodents and humans (Badran *et al.*, 2014), and to illustrate some mechanistic pathways induced by this trigger (McGuire *et al.*, 2008; Tartar *et al.*, 2010; Liu *et al.*, 2011; Perry *et al.*, 2011). Nevertheless, some studies have also aimed to evaluate the cardiovascular outcomes induced by this OSA feature and have suggested that sleep fragmentation may have a far more important role in cardiovascular changes observed in OSA patients (Golbidi *et al.*, 2012). Even so, sleep deprivation studies have produced mixed results regarding BP outcomes.

In 1997, Brooks *et al.* suggested that sleep fragmentation, triggered by auditory stimulus, induced only acute changes in BP and did not affect daytime BP (Brooks *et al.*, 1997a; Brooks *et al.*, 1997b). In the same way, Bao *et al.*'s results showed that sleep fragmentation in rats, using acoustic stimuli for 35 days, did not elicit an increase in BP, probably due to some adaptation behavior (Bao *et al.*, 1997). However, more recent studies have shown that sleep deprivation leads to increased plasma concentrations of epinephrine and norepinephrine (Andersen *et al.*, 2005), ET-1/2 levels (Palma *et al.*, 2002), and increased heart rate and systolic blood pressure (Anderson *et al.*, 2004; Perry *et al.*, 2007). In addition, sleep fragmentation enhances plasma inflammatory cytokines (*e.g.*, TNF-α, IL-6, IL-1α, and IL-1β), leading to increased oxidative stress and inflammation (Yehuda *et al.*, 2009). These results add further evidence demonstrating that sleep deprivation may lead to serious cardiovascular consequences and may aggravate hypertensive features. However, despite the potential of the sleep deprivation model to induce HT related to OSA, it does not exactly mimic sleep fragmentation and presents one major shortcoming regarding the evaluation of AHDs efficacy that should be taken into account. Sleep deprivation is a stressful method and it is still unclear whether the method is itself a stressful stimulus (Palma *et al.*, 2002). Thus, in conclusion, sleep deprivation models are useful tools for unveiling various aspects of sleep function, studying the effects of sleep loss on subsequent brain function at the molecular, cellular and physiological levels, and evaluating cognitive impairment, but should be used with caution whenever stress can act as a confounding factor and compromise data interpretation.

CIH model

IH is now established as the dominant model of sleep apnea. Generally, this model makes use of specific ventilated chambers in which the animals are housed and cyclically exposed either to normoxia/hypoxia or room air to mimic the most relevant consequences of OSA. Hypoxic conditions can also be achieved by surgical intervention (an endotracheal tube) or by the use of a mask, which involves animal restraint and consequently high levels of stress (Golbidi *et al.*, 2012). In either case, animals breathe nitrogen-enriched air alternating with oxygen or normal air (Dematteis *et al.*, 2009). Thus, as with O_2, nitrogen plays an important role in this model as the flushing of the chambers with this gas allows the gradual lowering of O_2. The duration of the hypoxic and normoxic phases of the IH cycle, as well as the slopes of FiO_2 (fraction of inspired oxygen), decrease and increase, and are dependent on cage/chamber size and the gas flows and mixtures (Dematteis *et al.*, 2009).

The standard animal model of OSA was that described in the landmark study of Fletcher and Bao (Fletcher and Bao, 1996). Despite the presence of some drawbacks, this model has successfully been employed to study the changes in systemic arterial pressure and the impact of IH on a wide range of cardiovascular outcomes. One of the major limitations pointed to in this model is the absence of recurrent upper airway obstruction, abolishing the acute hemodynamic changes due to the negative intrathoracic pressure (Badran *et al.*, 2014). Marked negative intrathoracic pressure induces acute hemodynamic changes that are probably the starting point for chronic cardiovascular diseases (Bonsignore *et al.*, 1994). Despite the absence of upper airway occlusion, some respiratory efforts (intermittent tachypnea) occur, corresponding to a fluctuating hyperventilation that follows the IH cycles (Dematteis *el al*, 2009). However, this disadvantage allows the evaluation of CIH effects, namely chronic blood gas exchanges, without the interference of the mechanical aspects of OSA.

This model also fails to reproduce the transient hypercapnia, or at least eucapnia, which occurs in humans determined by airway occlusion. The first question concerning this issue should be: is $PaCO_2$ (partial pressure of carbon dioxide in the blood) relevant in humans? Hypercapnia is not a standard parameter analyzed in polysomnographic recordings in patients and therefore there is no consensus on the impact of $PaCO_2$ in arterial blood pressure in patients with OSA. In clinical studies of patients with moderate OSA, the changes in $PaCO_2$ have seemed to be irrelevant (Epstein *et al.*, 2001) or have shown a slight increase (Tilkian *et al.*, 1976) during the apneic events. However, a $PaCO_2$ increase may contribute to the severity of the cardiovascular consequences of OSA (Cooper *et al.*, 2005). The results shown by Fletcher *et al.* in rats suggest that the exposure to hypercapnia during IH is not a critical factor as the effect of IH on diurnal BP is similar, independently of the lower or higher levels of CO_2 (Fletcher *et al.*, 1995). Moreover, Bao *et al.* found that eucapnic IH in rats is a more powerful stimulus for inducing

acute BP increase than hypocapnic IH (Bao *et al.*, 1997). Similarly, Lesske *et al.* showed comparable changes in BP between two groups submitted to IH with or without hypercapnia (Lesske *et al.,* 1997). On the other hand, based on the results of different CIH experimental protocols in rodents, Kanagy concludes that the level of $PaCO_2$ influences the magnitude of an increase in BP (Kanagy, 2009). Concretely, eucapnic hypoxia induces a faster and greater increase than hypocapnic hypoxia (Kanagy, 2009), through mechanisms that presently remain unknown. Moreover, the greatest increases in BP have been observed in studies in which hypocapnia was prevented by CO_2 administration (Morgan, 2009). Likewise, Tamisier *et al.*, in a study performed in humans, reported that hypercapnic hypoxia leads to greater sympathetic activation than hypocapnic hypoxia (Tamisier *et al.*, 2009). In line with these findings, the presence of hypocapnic or eucapnic hypoxia conditions leads to an underestimated increase in BP that must be taken into account. In conclusion, although some data suggest that $PaCO_2$ may influence physiological responses to IH, further studies are needed to evaluate the combined effect of IH and hypercapnia. Another drawback that could be attributed to the IH paradigm is the fact that it is not accompanied by sleep fragmentation and does not incorporate monitoring of sleep.

Each group of researchers has applied its own specific paradigm and these discrepancies may compromise the straightforward comparison of the results. The several paradigms of CIH, which simulate the cyclical pattern of hypoxia experienced by patients with OSA, diverge in some respects, namely in the animal species involved, *e.g.*, Sprague-Dawley rats (Fletcher *et al.*, 1995; Kanagy *et al.*, 2001; Tahawi *et al.*, 2001; Allahdadi *et al.*, 2005; Chen *et al.*, 2005; Phillips *et al.*, 2005; Lai *et al.*, 2006), Wistar rats (Dunleavy *et al.*, 2005; Lefebvre *et al.*, 2006), C57BL/6J mice (Julien *et al.*, 2003), and CF-1 mice (Rosa *et al.*, 2011), the severity of hypoxia, the number of hypoxic episodes per hour of sleep, the number of days of hypoxic exposure (exposure duration), and CO_2 manipulation. Table 2 summarizes the variability observed in the CIH models.

These models typically create moderate to severe oxygen desaturation, thereby mimicking severe forms of OSA and may therefore not be applicable to mild and moderate clinical OSA (Dematteis *et al.*, 2009). CIH models with cycles of FiO_2 of 5% or less usually mimic severe forms of OSA in humans and produce maximal changes in BP and heart rate (Dematteis *et al.*, 2008). However, higher FiO_2 (8–10%) has been used in rodent models of CIH (Soukhova-O'Hare *et al.*, 2008; Knight *et al.*, 2011; Perry *et al.*, 2011; Bathina *et al.*, 2013).

Table 2. Reports on the effects of CIH on blood pressure

Species	Hypoxia cycle, Nadir FiO_2, duration and CO_2 manipulation (Y/N)	BP measurement	Effect on BP	Reference
Sprague-Dawley rats	20 cycles (90 s each) of 21–5% O2 and 0–5% CO2/hour; 7 h/day; 35 days; Yes	Tail-cuff method	↑ MAP (25-28 mmHg)	Allahdadi *et al.*, 2005
Sprague-Dawley rats	80 cycles (6 min each) 21–10% O2/ day; 8 h/day; 7 days; No	Telemetry	↑ MAP (7-10 mmHg)	Knight *et al.*, 2011
Sprague-Dawley rats	5% O2 12 times/h; 8 h/day; 7–21 days; No	Arterial catheterization	No changes in MAP	Iturriaga *et al.*, 2010
Wistar rats	10% O2 for 4 h/day and 21% O2 for 20 h/day; 56 days; Yes (PCO2 < 0.02%)	Arterial catheterization	No differences in systemic pressure	Kalaria *et al.*, 2004
Sprague-Dawley rats	2/3–20.9% O2 (3–6 s +15-18s; 2 cycles/min); 6–8 h/day; 35 days; No	Telemetry	↑ MAP (16 mmHg)	Tahawi *et al.*, 2001
C57BL/6J mice	21–5% O2 (60 s); 12 h/day; 5 weeks; No	Arterial catheterization	↑ Systemic BP (7.5 mmHg)	Campen *et al.*, 2005
SHR + Wistar rats	21–10% O2 (1 min cycles: 20 s + 40 s); 8 h/day; 14 days; No	Tail-cuff method + Arterial catheterization	Enhanced HT development in SHR + NS in Wistar rats	Belaidi *et al.*, 2009
Sprague-Dawley rats	2/3–20.9% O2 (3-6 s + 12 s; 2 cycles/min); 6–8 h/day; 35 days; No	Telemetry	↑ MAP (10 mmHg)	Fletcher *et al.*, 2000
LCR and HCR	21–10% O2 (3 min cycles); 8 h/day; 7 days; No	Telemetry	↑ MAP in both groups	Sharpe *et al.*, 2013
Sprague-Dawley rats	20 cycles (90 s each) of 21–5% O2 and 0–5% CO2/hour; 8 h/day; 11 days; Yes	Arterial catheterization	↑ MAP (30 mmHg)	Kanagy *et al.*, 2001
Sprague-Dawley rats	48 cycles (45 s + 30 s) 20.9–2/6% O2/ hour; 6 h/day; 30 days; No	Telemetry	↑ MAP (19.3 mmHg)	Lai *et al.*, 2006
C57BL/6J mice	21–5.7% O2 (alternating every 6 min); 12 h/day; 90 days; No	Arterial catheterization	↑ MAP (19.8 mmHg)	Lin *et al.*, 2007
Sprague-Dawley rats	21–10% O2 for 5 s every 90 s; 10 h/day; 4 weeks; No	Tail BP telemeter	↑ MAP (37 mmHg)	Liu *et al.*, 2013
SHR	21–10% O2 (alternating every 90 s); 12 h/day; 30 days; No	Tail-cuff method	↑ SBP and DBP (NA mmHg)	Soukhova-O'Hare *et al.*, 2008
Sprague-Dawley rats	21–4/6% O2 (every 60 s); 8 h/day; 5 days/week; 5 weeks; No	Tail-cuff method	↑ MAP (12 mmHg)	Chen *et al.*, 2005
Wistar rats	1 min cycles with 30 s of a 5% FiO2; 8 h/day; 14–21 days; No	Tail-cuff method + Arterial catheterization	Rapidly ↑ MAP (NA mmHg)	Totoson *et al.*, 2013
Sprague-Dawley rats	21–6% O2 (9 min cycles); 8 h/day; 14 days; No	Arterial catheterization	↑ MAP (9 mmHg)	Silva and Schreihofer, 2011
Wistar rats	20.8–6% O2 (9 min cycles: 5 min Nx); 8 h/day; 10 days; No	Arterial catheterization	↑ MAP (12 mmHg) ↑ SBP (9 mmHg) ↑ DBP (8 mmHg)	Zoccal *et al.*, 2009
Wistar-Hannover rats	21–10% O2 (2 min + 2 min); 1000–1600 h; Yes (PCO2 < 0.01%)	Arterial catheterization	No differences in MAP	Perry *et al.*, 2011
Sprague-Dawley rats	10 cycles (6 min each) of 21–6% O2 and 0–5% CO2/hour; 8 h/day; 28 days; Yes	Telemetry	↑ SBP (39 mmHg) ↑ DBP (33 mmHg)	Dyavanapalli *et al.*, 2014
C57BL/6J mice	21–7% O2 (120 s each cycle); 5 days/week; 8 h/day; 6 weeks; No	Telemetry	Significant ↑ MAP	Schulz *et al.*, 2014
Sprague-Dawley rats	21–10% O2 (cycle duration: NA); 8 h/day; 7 days; No	Telemetry	↑ MAP that persisted after CIH exposure	Bathina *et al.*, 2013

BP, blood pressure; **CIH**, chronic intermittent hypoxia; **DBP**, diastolic blood pressure; **h**, hour; **HCR**, high aerobic capacity rats; **HT**, hypertension; **LCR**, low aerobic capacity rats; **MAP**, mean arterial pressure; **NA**, information not available; **NS**, no significant effect; **Nx**, normoxia; **min**, minutes; **s**, seconds; **SBP**, systolic blood pressure; **SHR**, spontaneously hypertensive rats; ↑, increase.

The duration and frequency of hypoxic/normoxic periods are adjustable; usually, the higher the frequency the shorter the IH cycles (Golbidi *et al.*, 2012). There is a sizeable discrepancy regarding the duration of IH cycles, ranging from 120 cycles/h (30 second cycle; Fletcher *et al.*, 1992a; Julien *et al.*, 2003; Dematteis *et al.*, 2008), 80 cycles/h (6 min cycle; Knight *et al.*, 2011), 60 cycles/h (1 min cycle; Campen *et al.*, 2005), and when the chambers are larger, longer

cycles are often used, reducing the number of cycles/h (Zoccal *et al.*, 2007; Silva and Schreihofer, 2011) of daytime exposure, from 4 h/day (Kalaria *et al.*, 2004), 6 h/day (Lai *et al.*, 2006), 7 h/day (Fletcher *et al.*, 1992a), 8 h/day (Chen *et al.*, 2005; Belaidi *et al.*, 2009; Zoccal *et al.*, 2009; Knight *et al.*, 2011; Silva and Schreihofer, 2011; Dyavanapalli *et al.*, 2014; Schulz *et al.*, 2014), 10 h/day (Liu *et al.*, 2013) to 12 h/day (Lin *et al.*, 2007). The exposure duration of 8 h/day seems to be that on which there is the greatest consensus (see Table 2). The duration of exposure seems to affect the study outcomes more than the hypoxic *nadir* or the rate of hypoxic cycling (Davis *et al.*, 2013).

An advantage of CIH models is they allow exposures that can be extended over months, enabling the investigation of chronic consequences that might occur in humans (Toth and Bhargava, 2013). The number of days necessary to induce an increase in BP seems to be dependent on the CIH paradigm. Some authors suggest that the BP increase triggered by CIH represents a time-dependent effect (Prabhakar *et al.*, 2001; Hui *et al.*, 2003; Dematteis *et al.*, 2008; Zoccal *et al.*, 2009). Moreover, both the time and severity of hypoxia have been shown to play an important role in the cardiovascular response (Li *et al.*, 2007; Perry *et al.*, 2007). It has recently been shown that a period of 14 days is not long enough to induce structural changes in cardiovascular structures, but these are already apparent after 35 days of incubation (Dematteis *et al.*, 2008). Moreover, Iturriaga *et al.* report that the exposure of rats to CIH for 14 days enhanced the ventilatory response to hypoxia and produced a significant shift in heart rate variability, but these cardiorespiratory alterations occurred without noticeable changes in mean arterial BP until 21 days of CIH exposure (Iturriaga *et al.*, 2010). Whereas some short-term protocols (7–14 days) cause a significant increase in BP (Belaidi *et al.*, 2009; Knight *et al.*, 2011; Silva and Schreihofer, 2011; Bathina *et al.*, 2013), others show an increase in BP that occurs only after long-term exposure (35 days) to CIH (Prabhakar *et al.*, 2001; Chen *et al.*, 2005; Prabhakar *et al.*, 2005; Zoccal *et al.*, 2009) (see Table 2). Finally, most IH paradigms in rodents do not include CO_2 supplementation (Lin *et al.*, 2007; Fletcher *et al.*, 2009; Iturriaga *et al.*, 2010; Perry *et al.*, 2011; Bathina *et al.*, 2013). In fact, only some authors have manipulated the CO_2 levels (Ooi *et al.*, 2000; Kantores *et al.*, 2006; Dyavanapalli *et al.*, 2014) and fixed the values along the protocol (see Table 2).

Independently of the paradigm used to induce HT related to OSA, previous reviews are unanimous in reporting the development of mild HT, despite the divergent changes in arterial blood gases (Kanagy, 2009) (see Table 2). The exceptions found in this review (Kalaria *et al.*, 2004; Belaidi *et al.*, 2009; Iturriaga *et al.*, 2010; Perry *et al.*, 2011) are all related to the method used for BP measurement. It is apparent that arterial catheterization is not an accurate method of measuring BP in CIH models. The methods most often used for BP measurement in IH models (see Table 2) are the tail-cuff method (Allahdadi *et al.*, 2005; Chen *et al.*, 2005; Soukhova-

O'Hare *et al.,* 2008; Totoson *et al.,* 2008; Belaidi *et al.,* 2009), radiotelemetry (Fletcher *et al.,* 2000; Tahawi *et al.,* 2001; Lai *et al.,* 2006; Knight *et al.,* 2011; Bathina *et al.,* 2013; Sharpe *et al.,* 2013; Dyavanapalli *et al.,* 2014; Schulz *et al.,* 2014), and arterial catheterization (Kanagy *et al.,* 2001; Kalaria *et al.,* 2004; Campen *et al.,* 2005; Lin *et al.,* 2007; Belaidi *et al.,* 2009; Zoccal *et al.,* 2009; Iturriaga *et al.,* 2010; Perry *et al.,* 2011; Silva and Schreihofer, 2011; Totoson *et al.,* 2013).

The tail-cuff method is the most frequently used indirect method for monitoring BP (Kurtz *et al.,* 2005). In spite of this, in the context of chronic disease animal models, this method presents several disadvantages, such as the inability to assess the average level of BP throughout the day and night over the course of a study (Kurtz *et al.,* 2005). Moreover, this method induces significant stress, disturbing multiple aspects of the cardiovascular system (Kurtz *et al.,* 2005). Finally, its accuracy is dubious and most tail-cuff methods are not well suited to measuring diastolic pressure (Kurtz *et al.,* 2005).

Regarding radiotelemetry, the availability of this technique has represented an enormous advance in HT research. This method allow BP measurements to be taken in conscious freely moving laboratory animals and also provides the ability to measure BP at any time of the day or night and for extended periods of time (Kurtz *et al.,* 2005). The major limitations of radiotelemetry relate to the expense of acquisition and further maintenance of the telemetric devices, the need for surgical skills and training to perform the introduction of the catheter into the abdominal aorta or femoral artery, and also the risk of infection after the surgical intervention (Kurtz *et al.,* 2005).

Arterial catheterization allows direct measurement of BP with a fluid-filled external catheter. Like telemetry, it is accurate and reliable and permits assessment of BP lability and diurnal variations in BP (Kurtz *et al.,* 2005). This technique can be used effectively for acute studies in anesthetized animals or for long-term, continuous monitoring of arterial pressure in conscious animals (Kurtz *et al.,* 2005). However, the maintenance of catheters in chronic studies can be labor intensive and meticulous care of the catheters is required to avoid infections and to prevent failure during long-term experiments (Kurtz *et al.,* 2005).

Finally, the studies performed with CIH models have been very useful for elucidating mechanistic issues and also showing that in spontaneously hypertensive rats (SHR), IH accelerates the BP increase, with higher BP values after 14 days of exposure (Belaidi *et al.,* 2009). In contrast, in animals that are not prone to HT, IH has been found to trigger a rapid but moderate BP elevation (Campen *et al.,* 2005; Fletcher *et al.,* 1992a; Lai *et al.,* 2006; Lin *et al.,* 2007), even after several months of exposure (Lin *et al.,* 2007). According to Dematteis *et al.,*

BP increase in rats seems to be predominantly diastolic (Dematteis *et al.*, 2008). The same finding was also reported in the early study of Fletcher *et al.* (Fletcher *et al.*, 1992a).

HUMAN MODELS

The variety of models of IH in healthy human subjects is much less impressive than that observed for animal models of sleep apnea. In terms of the exposure time, these models are usually divided into short-term and chronic (Foster *et al.*, 2007). In short-term IH models, generally the exposure time (20–60 min) and the duration of the hypoxia or voluntary apnea period (30 s) are very limited. The protocols of Cutler *et al.* and Tamisier *et al.* are good examples of short-term models (Cutler *et al.*, 2004; Tamisier *et al.*, 2009). In contrast, Foster *et al.* made use of a chronic model, exposing healthy human volunteers to an hour of IH (5 min hypoxia alternating with 5 min normoxia) daily for two weeks (Foster *et al.*, 2005). As in the animal models of IH, only some studies have controlled the level of CO_2 (Foster *et al.*, 2005), whereas others have not (Tamisier *et al.*, 2009). Regardless of the protocol followed, exposing humans to CIH implies careful supervision.

In 2001, Xie *et al.* exposed nine healthy human subjects during wakefulness to 20 min of isocapnic hypoxia (arterial O_2 saturation, 77–87%) and 20 minutes of normoxic hypercapnia (*end-tidal* PCO_2, 15.3–8.6 *Torr above eupnea*) on two separate days. The subjects breathed through a leak-free nasal mask and the neurocirculatory and ventilatory responses to these two stimuli were further evaluated (Xie *et al.*, 2001). These authors found that hypoxia induced a sympathetic activation that outlasted the chemical stimulus, whereas hypercapnia evoked a short-lived sympathetic activation (Xie *et al.*, 2001). Years later, in a study performed with a larger sample (n=31), Cutler *et al.* used a model of IH induced by voluntary apnea (30 s of hypoxic apnea every 1 min – simulating an AHI of 60/h – for 20 min) to determine if the cessation of breathing is important in prolonged sympathetic activation (Cutler *et al.*, 2004). This study also included two other groups that were exposed to intermittent hypercapnic hypoxia and to intermittent isocapnic/hypoxia, respectively (Cutler *et al.*, 2004). Their results support the hypothesis that short-term exposure to intermittent hypoxic apnea results in sustained elevation of postganglionic muscle sympathetic nerve activity and that hypoxia is the primary mediator of this response (Cutler *et al.*, 2004). The data reported by Leuenberger *et al.* one year later were in line with these results. They also found, in a study that enrolled 26 patients, a sustained sympathetic activation and also a transient elevation of BP following 30 min of voluntary end-expiratory apneas primed with a hypoxic gas mixture and lasting for 20 s in each minute (Leuenberger *et al.*, 2005).

Foster *et al.* carried out three main studies in healthy human volunteers. The first aimed to determine the ventilatory, cardiovascular and cerebral tissue oxygen response to two protocols

of IH (Foster *et al.*, 2005). This study involved 18 patients randomly assigned to short-duration IH (1 hour of 12% O_2 separated by 5 min of normoxia) or long-duration IH (30 min of 12% O_2). Both groups had 10 exposures over 12 days. Their findings show a rise in mean arterial blood pressure (MAP) that occurs throughout the daily exposure to short-duration IH but not during exposure to long-duration IH; moreover, they demonstrate that the vascular processes required to control blood flow and O_2 supply to cerebral tissue in a healthy human are delayed following exposure to 12 days of isocapnic IH (Foster *et al.*, 2005). In 2009, the same group reinforced the enrollment of IH on the pathogenesis of cardiovascular and cerebrovascular disease in patients with OSA (Foster *et al.*, 2009). They exposed 10 healthy subjects to IH (2 min of hypoxia: *nadir* PET,O_2 = 45.0 mmHg, alternating with 2 min of normoxia: *peak* PET,O_2 = 88.0 mmHg for 6 h) for 4 consecutive days and concluded that IH alters BP (MAP increased by 4 mmHg) and induces an increase in cerebral vascular resistance (Foster *et al.*, 2009). More recently, Foster *et al.* have assessed the role of the type I angiotensin II receptor in mediating an increase in arterial pressure associated with a single 6-hour IH exposure (Foster *et al.*, 2010). For that, they exposed nine healthy subjects to sham IH, IH with placebo medication, and IH with the type I angiotensin II receptor antagonist (losartan). Their findings demonstrate a significant increase in arterial pressure after exposure to isocapnic IH (Foster *et al.*, 2010). Furthermore, since this increase is prevented by the blockade of AT1 receptors, these results suggest an important role for the rennin-angiotensin-aldosterone system (RAAS) in the pathophysiology of HT related to OSA (Foster *et al.*, 2010).

Tamisier *et al.* have developed a novel model of nocturnal CIH in healthy humans, which represents an important step forward in the field, designed to overcome some of drawbacks and confounding factors that are present in studies of both animals and OSA patients (Tamisier *et al.*, 2009). To investigate the effects of CIH on sleep, BP and ventilatory control, these authors make use of altitude tents to mimic the cyclical arterial oxygen desaturations-resaturations of sleep apnea. They delivered O_2 for 15 s every 2 min during sleep while subjects breathed 13% O_2 in a hypoxic tent to create 30 cycles/h of cyclic desaturation-reoxygenation (SpO_2 range: 95–85%), and exposed subjects overnight for 8–9 h/day for two or four weeks (Tamisier *et al.*, 2009). Among other results, they show that waking normoxic arterial pressure increased significantly at two weeks for systolic and for diastolic at four weeks, that patients developed a sustained BP increase during the day and exhibited a steeper BP decrease at night compared to baseline BP values, and finally, that this model produces clinically relevant fluctuations in SaO_2 (Tamisier *et al.*, 2009). Although undoubtedly relevant, the authors recognize the presence of several respects in which their model does not mimic sleep apnea, *e.g.*, no negative intrathoracic pressure development, higher percentage of sleep time at < 90% SaO_2 and poikilocapnia (Tamisier *et al.*, 2009). However, some of these limitations can be overcome to achieve a

pattern of IH more akin to OSA features. The same group further used this model in 2011 to shed light on the profile of the BP increase previously described to determine if it is sustained and to explore potential underlying physiological mechanisms. The authors found that only 2 weeks of severe IH exposure produces a sustained daytime BP increase in the setting of sympathetic activation and blunted vascular sympathic baroreflex gain in healthy volunteers (Tamisier *et al.,* 2011).

In conclusion, to date, only a small number of studies have been conducted using healthy human models of IH and these have primarily been aimed at elucidating the role of IH in sustained sympathetic activation and cerebrovascular regulation. Only a few studies have evaluated BP outcomes (Foster *et al.,* 2009; Tamisier *et al.,* 2009; Foster el al, 2010; Tamisier *et al.,* 2011) and none of these models have truly been used to assess the efficacy of AHDs in the treatment of HT related to OSA. In fact, in the later work of Foster *et al.,* losartan (the angiotensin II AT1 receptor antagonist) was used only to demonstrate a mechanistic pathway rather than to evaluate its efficacy (Foster *et al.,* 2010). Thus, future research in this field is clearly needed.

What are the mechanisms involved in the pathogenesis of Hypertension related to OSA?

Fletcher *et al.* were pioneers in demonstrating the hypertensive effect of CIH (Fletcher *et al.,* 1992a) and the role of the sympathetic nervous system, peripheral receptors and rennin-angiotensin system in this response (Fletcher *et al.,* 1992b, 1999, 2000, 2002). This group also showed that surgical denervation of peripheral chemoreceptors, adrenal demedullation and chemical denervation of the peripheral nervous system prevented the increase in BP in response to CIH stimulus (Fletcher *et al.,* 1992b; Bao *et al.,* 1997). After Fletcher *et al.*'s first work, many reports enabled confirmation of the relationship between IH and BP increases and contributed to elucidating the underlying mechanisms. Kanagy *et al.* reported increased plasma endothelin-1 levels in rats exposed for 11 days to CIH, which also demonstrated an appreciable increase in MAP (Kanagy *et al.,* 2001). In 2006, Lai *et al.* suggested that chronic IH-induced sustained HT was associated with the facilitation of cardiovascular sympathetic outflow followed by decreases in baroreflex sensitivity in conscious rats (Lai *et al.,* 2006). Along the same line, the work undertaken by Zoccal *et al.* provided strong evidence to support the idea that rats submitted to CIH show an increase in sympathetic activity, which seems to be essential in the maintenance of high BP values in the CIH model (Zoccal *et al.,* 2007). Another group revealed that although elevated sympathetic nerve activity (SNA) may contribute to CIH-induced HT, reduced adrenergic vascular reactivity buffers the cardiovascular impact of exaggerated acute raises in SNA (Silva and Schreihofer, 2011). Data attained by Knight *et al.*

indicated that CIH induces an increase in FosB/ΔFosB in autonomic nuclei and suggested that activator protein-1 (AP-1) transcriptional regulation may contribute to stable adaptative changes that support chronically elevated BP (Knight *et al.*, 2011). Also in 2011, Liu *et al.* demonstrated that CIH activates the HIF-1α/endothelin system, through CIH-NADPH oxidase-mediated ROS production, and this enhances the development of resistant vasoconstriction and elevates BP in rats (Liu *et al.*, 2011). The study undertaken by Bathina *et al.* revealed that the knockdown of tyrosine hydroxylase in the nucleus of the solitary tract (NTS) reduces the CIH-induced persistent increase in MAP, suggesting that noradrenergic A_2 neurons in NTS play a role in the cardiovascular responses to CIH (Bathina *et al.*, 2013). More recently, Schulz *et al.* have shown that NADPH oxidase 2 (NOX2) knockout blocks the development of HT induced by CIH, suggesting that this type of HT is mediated by reactive oxygen species (ROS) derived from the activation of NOX2 within cells located outside the cardiovascular system (Schulz *et al.*, 2014).
The mechanisms involved in the genesis of HT related to OSA have recently been reviewed (Lavie and Lavie, 2009; Bosc *et al.*, 2010; Sunderram and Androulakis, 2012; Zhang and Si, 2012; Lévy *et al.*, 2013) and broadly include the following: sympathetic nervous system stimulation mediated mainly by the activation of carotid body chemoreflexes, decreased vascular responses to nitric oxide, increased plasma concentrations of endothelin, and elevation of proinflammatory cytokines (TNF-α, IL-6, VEGF). While for some of these mechanisms (*e.g.*, activation of the RAAS, endothelial dysfunction, systemic inflammation, metabolic anomalies, and genetic contribution) the relationship with OSA and subsequent cardiovascular morbidity remain partially unclear and there is a need to gather more evidence, for others (*e.g.*, the increase in sympathetic activity and acute effects of negative intrathoracic pressure), there seems to be more agreement on the linkage and it is well-documented (Parati *et al.*, 2013). In fact, based on data attained from patients with OSA, it is widely accepted that sympathetic activation, inflammation and oxidative stress play major roles in the pathophysiology of this particular type of HT. In addition, the use of animal models has revealed that CIH is the critical stimulus underlying sympathetic activity and HT, and that this effect requires the presence of functional arterial chemoreceptors (Fletcher, 2000). However, it should be also mentioned that HT related to OSA probably results not only from increased carotid chemoreflex but also from decreased baroreceptor activity (Dumitrascu *et al.*, 2013). It is also important to highlight the potential role of obesity as an intermediate factor in the pathway of HT related to OSA (O'Connor *et al.*, 2009; Young *et al.*, 2007).

The mechanisms involved in the pathogenesis of HT can be summarized in relation to two main pathways: sympathetic nervous system stimulation mediated mainly by activation of carotid body (CB) chemoreflexes and the systemic effects of chronic intermittent hypoxia (CIH), mainly due to the activation of NOX2 and subsequent ROS production. Figure 1 illustrates the

hypothesized pathways by which intermittent hypoxia leads to HT. Briefly, repetitive obstructive apneas or hypopneas lead to increased intrathoracic pressure, sleep fragmentation, recurrent hypercapnia, and IH. This last phenomenon plays a pivotal role in triggering several intermediary mechanisms and molecular pathways that contribute to the initiation and progression of cardiac and vascular pathology. First, IH enhances sympathetic nervous system activity, leading to vasoconstriction and systemic hypertension through RAAS activation, and an increase in catecholamine secretion and plasma level of vasoconstrictive ET-1. Episodic hypoxia also favors the stabilization of HIF-1α and the production of ROS, which is followed by increased expression of NF-κB and decreased NO bioavailability, the most important vasodilatory molecule synthesized by the endothelium. Angiotensin II (AT II) and ET-1 both seem to be implicated in vascular remodeling and ROS formation, which is increased through the activation of vascular NADPH oxidase and xanthine oxidase. ROS molecules induce a cascade of inflammatory pathways linked to an overexpression of adhesion molecules and pro-inflammatory cytokines, and oxidative stress may trigger sympathetic hyperactivation and vice versa. ROS production is required for HIF-1α induction and HIF-1α induction is required for ROS production. In addition, HIF-1α promotes the expression of ET-1 and transcriptional activation of VEGF and other growth factors. Activation of NF-κB also seems to be central in inflammation induced by IH due to its regulatory role in the production of pro-inflammatory mediators (*e.g.*, TNF-α, IL-6, IL-8, ICAM-1, and CRP). These signaling pathway proteins, combined with RAAS, decreased expression of eNOS, and increased ROS production and stabilization of HIF-1, participate in the molecular mechanisms underlying the endothelial dysfunction induced by IH. Together, these mechanisms progress to fluid retention, changes in cardiac output and vascular tone, and vascular remodeling, leading to systemic HT, one of the major consequences of OSA.

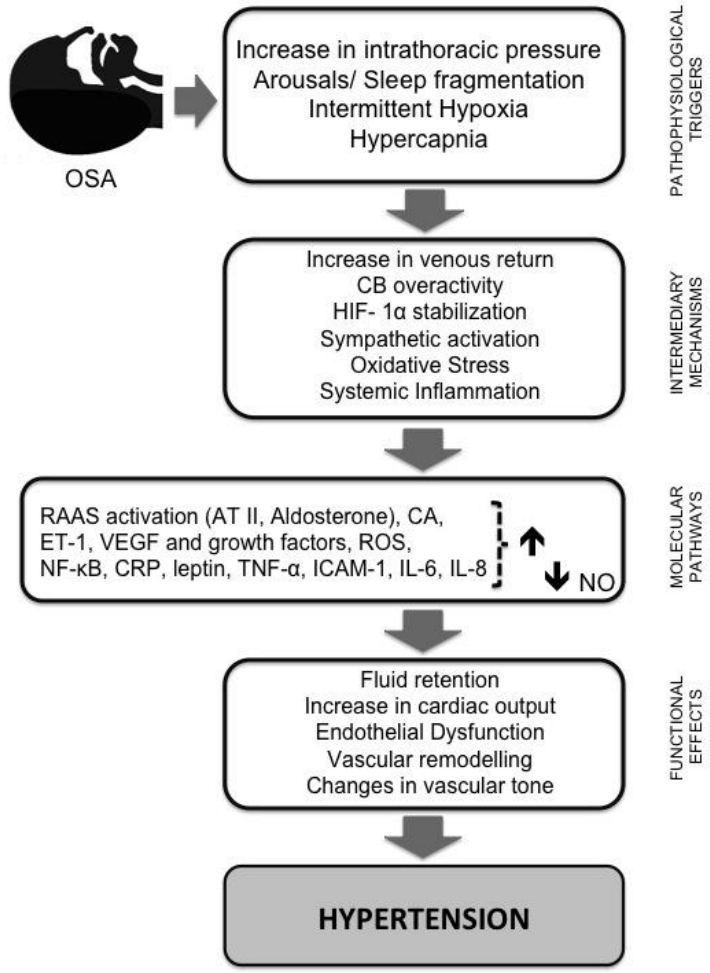


Figure 1: Schematic diagram summarizing the pathways by which intermittent hypoxia leads to hypertension.

AT II, angiotensin II; **CA**, catecholamine levels; **CRP**, C- reactive protein; **CB**, carotid body; **ET-1**, endothelin 1; **HIF-1α**, hypoxia-inducible factor α; **IH**, intermittent hypoxia; **IL**, interleukin; **ICAM-1**, intercellular adhesion molecule; **NADPH**, nicotinamide adenine dinucleotide phosphate; **NO**, nitric oxide; e**NOS**, endothelial nitric oxide synthase; **NF-κB**, nuclear factor-κ-light chain enhancer of activated B cells; **RAAS**, renin-angiotensin-aldosterone system**; ROS**, reactive oxygen species; **TNF- α**, tumour necrosis factor α; **VEGF**, vascular endothelial growth factor.

What is already known concerning the efficacy of AHDs?

HUMANS

Despite the considerable number of studies involving OSA patients, only a few have investigated the efficacy of different AHDs and in general, they tend to be individual drug studies. Moreover, most of the studies only take into account the number of drugs taken by patients to adjust this variable and are difficult to interpret as most of the patients were already under AHDs regimens. This lack of information could be attributed to the large number of possible different AHDs regimens observed in OSA patients. Table 3 summarizes the most relevant studies that have investigated the efficacy of AHDs in OSA patients.

Table 3. Studies of the efficacy of AHDs in OSA patients

Study design	n	CPAP (Y/N)	AHDs; dosage (mg/day)	BP measurement	BP outcome	Ref.
RCT; double-blinded; balanced incomplete block design (6 w each drug + 3 w washout)	40	No	Atenolol (50); amlodipine (5); enalapril (20); hydrochlorothiazide (25); losartan (50)	Office BP 24 h ABPM	↓ in office SBP and daytime ABPM NS for all drugs; Atenolol ↓ night-time 24h SBP and DBP more effectively than amlodipine, enalapril or losartan	Kcraiczi *et al.*, 2000
RCT; double-blinded; crossover schedule (8 w each drug + 2–3 w washout	15	No	Atenolol (50); isradipine (2.5): hydrochlorothiazide (25); spirapril (6)	Office BP	Slight ↓ BP for all drugs; Only atenolol affected BP variability	Salo *et al.*, 1999
RCT; double-blinded; crossover (8 w each drug + 2–3 w washout	18	NA	Atenolol (50); isradipine (2.5); hydrochlorothiazide (25); spirapril (6)	24 h ABPM	↓ mean 24h SBP (except for HCTZ) ↓ mean 24h DBP (for all drugs) NS ↓ mean night-time SBP and DBP (for all drugs)	Pelttari *et al.*, 1998
RCT (3 months each treatment)	75	Yes	Treatment with at least 3 drugs at adequate doses, including a diuretic	24 h ABPM	CPAP + AHDs regimen: ↓ 4.9 mmHg 24h DBP; AHDs regimen alone: NS	Lozano *et al.*, 2010
RCT; single-blinded (3 w each regimen)	44	Yes	Valsartan (160) + amlodipine (5–10) + hydrochlorothiazide (25)	Office BP 24 h ABPM	AHDs alone: ↓ office and 24 h SBP and DBP Additional ↓ in office BP and ambulatory BP monitoring (CPAP+ 3 AHDs)	Litvin *et al.*, 2013
RCT; crossover (8 w each treatment + 4 w washout)	23	Yes	Valsartan (160)	Office BP 24 h ABPM	CPAP: ↓ 2.1 mmHg 24h MBP and ↓ 1.3 mmHg night-time MBP (NS) VAL: ↓ 9.1 mmHg 24h MBP and ↓ 6.1 mmHg night-time MBP	Pepin *et al.*, 2010
RCT (8 w)	12	No	Spironolactone (25–50) added to current medication (mean number of AHDs: 4.3 (SD=1.1)	Office BP 24 h ABPM	↓ 17 mmHg 24h SBP ↓ 10 mmHg 24h DBP	Gaddam *et al.*, 2010
RCT; double-blinded (8 days)	12	NA	Metoprolol (100); cilazapril (2.5)	Office BP 24 h ABPM	MET: ↓ 13 mmHg 24h SBP and ↓ 5 mmHg 24h DBP CIL: ↓ 13 mmHg 24h SBP and ↓ 17 mmHg 24h DBP	Mayer *et al.*, 1990
RCT; double-blinded; crossover (2 w each treatment + 3 w washout)	16	No	Doxazosin (4–8); enalapril (10–20)	24 h ABPM	DOX: ↓ 4.1 mmHg 24h SBP and ↓ 5.1 mmHg 24h DBP EN: ↓ 12.6 mmHg 24h SBP and ↓ 8.9 mmHg 24h DBP 24h MBP: no differences between groups	Zou *et al.*, 2010
RCT; double-blinded; parallel group; single center (6 w)	31	No	Nebivolol (5); valsartan (80)	Office BP	NEB: ↓ 14.6 mmHg SBP and ↓ 8.6 mmHg DBP VAL: ↓ 11.6 mmHg SBP and ↓ 8.9 mmHg DBP No differences between treatments	Heitmann *et al.*, 2010
RCT; prospective; crossover; parallel group (2 single doses of each drug + 2 w washout)	11	No	Nifedipine slow-release (40); carvedilol (20)	Office BP TSP method	NIF: ↓ 24.2 mmHg mean SBP and ↓ 18.7 mmHg mean DBP CAR: ↓ 16 mmHg mean SBP and ↓ mean 8.6 mmHg DBP	Kario *et al.*, 2014
RCT; double-blinded; placebo-controlled (8 days)	23	NA	Cilazapril (2.5)	Invasive arterial BP (arteria brachialis)	↓ 10 mmHg MBP (vs. ↓ 4.3 mmHg MBP for placebo)	Grote *et al.*, 1994

ABPM, ambulatory blood pressure monitoring; **AHDs**, antihypertensive drugs; **BP**, blood pressure; **CAR**, carvedilol; **CIL**, cilazapril; **CPAP**, continuous positive airway pressure; **DBP**, diastolic blood pressure; **DOX**, doxazosin; **EN**, enalapril; **HCTZ**, hydrochlorothiazide; **MBP**, mean blood pressure; **MET**, metoprolol; **NA**, information not available; **NEB**, nebivolol; **NIF**, nifedipine; **NS**, no significant effect; **RCT**, randomized controlled trials; **SBP**, systolic blood pressure; **SD**, standard deviation; **TSP**, trigger sleep BP monitoring; **VAL**, valsartan; **w**, week; **↓**, decrease.

In a study undertaken by Pelttari *et al.*, the AH effects of four different AHDs (atenolol: a beta-blocker; isradipine: a calcium channel blocker; hydrochlorothiazide: a diuretic; spirapril: an angiotension-converting enzyme inhibitor) in obese patients with OSA and HT were compared using ambulatory blood pressure monitoring (ABPM) (Pelttari *et al.*, 1998). This study revealed that although daytime HT was quite easily controlled by the single use of these drugs (especially with atenolol and isradipine; diuretics did not significantly lower BP) none of the AHDs were able to produce a significant decrease in nocturnal BP (Pelttari *et al.*, 1998). Mayer *et al.* carried out another comparative study between cilazapril (an angiotension-converting enzyme inhibitor) and metoprolol (a beta-blocker) (Mayer *et al.*, 1990). Their findings showed that despite the short period of therapy (one week), both metoprolol and cilazapril lowered nighttime BP in OSA patients (Mayer *et al.*, 1990).

A multiple crossover study examined the BP-lowering effect of the five major AHDs classes (atenolol: beta-blocker; amlodipine: calcium channel blocker; enalapril: angiotension-converting enzyme inhibitor; hydrochlorothiazide: diuretic; losartan: angiotensin II receptor blocker) and showed that atenolol induced the most pronounced effect in lowering BP (Kcraiczi *et al.*, 2000). Atenolol was more efficient in reducing mean nighttime diastolic and systolic BP (measured by ABPM) compared to amlodipine, enalapril, hydrochlorothiazide, and losartan (Kcraiczi *et al.*, 2000). Salo *et al.* investigated the effects of four AHDs (atenolol; isradipine: a calcium channel blocker; hydrochlorothiazide; spirapril: an angiotension-converting enzyme inhibitor) on cardiovascular autonomic control and reactivity in HT OSA patients (Salo *et al.*, 1999). This group reported that of the four drugs, only atenolol effected BP variability (Salo *et al.*, 1999). Thus, the results of these two pilot studies are in line with those arguing the involvement of the sympathetic system in the pathophysiology of HT related to OSA, suggesting that beta-blockers, in particular atenolol, may have beneficial effects beyond BP reduction in patients with OSA. However, both studies presented low levels of causation, which could have limited the ability to detect differences between classes.

Nevertheless, it has been advanced that angiotension-converting enzyme inhibitors (ACEi) treatment could exacerbate OSA by inducing upper airway inflammation (Cicolin *et al.*, 2006). The comparison between chronic treatments of ACEi and angiotensin AT1 receptor antagonists in terms of AH efficacy and levels of inflammatory markers has never been performed either in humans with OSA or in animal models. More recently, other study compared the effect of doxazosin (an α1- adrenergic receptor antagonist) and enalapril (an angiotensin-converting enzyme inhibitor) on nocturnal BP control and concluded that the former has a proportionally poorer effect than the latter (Zou *et al.*, 2010). In 1994, Grote *et al.* performed a study aimed at assessing the effectiveness of cilazapril (an angiotension-converting enzyme inhibitor) in managing high BP in patients with OSA. Although the study comprised a small sample size, the

results suggested that cilazapril is effective in reducing BP in all sleep stages (Grote *et al.*, 1994). In another small study, Heitmann *et al.* evaluated the effect of nebivolol (a third generation beta-blocker) on BP reduction and sleep apnea activity in HT patients with mild to moderate OSA in comparison with valsartan (an angiotensin II receptor blocker) and concluded that the effect of these AHDs were similar (Heitmann *et al.*, 2010). Despite the same limitations, these studies highlight the role of the RAAS in the pathophysiology of HT related to OSA

In two past studies (Lozano *et al.*, 2010; Litvin *et al.*, 2013), patients either received CPAP in combination with AHDs or alternatively, the pharmacological treatment alone, allowing the evaluation of the effects of CPAP and AHDs independently or in conjunction. In the study undertaken by Lozano *et al.*, patients were under an AHDs regimen with at least three drugs at adequate doses, including a diuretic (Lozano *et al.*, 2010). The authors noted a significant decrease in the mean 24-h diastolic BP in patients who received CPAP in addition to conventional treatment, suggesting that resistant HT treated with both CPAP and AHDs provides greater BP reduction than AHDs alone (Lozano *et al.*, 2010). However, in patients who used CPAP less than the average (5.6±1.52 h/night) and for those treated with conventional treatment alone, there was no significant difference in the 24-h ambulatory BP values (Lozano *et al.*, 2010). These findings are in line with those reported by Litvin *et al.*, attained with patients who received stepped dose titration of AHDs treatment (valsartan 160 mg + amlodipine 5–10 mg + hydrochlorothiazide 25 mg) for three months before CPAP was added (Litvin *et al.*, 2013). These findings seem to suggest that the best strategy to treat HT related to OSA involves the combination of OSA treatment with CPAP and the use of AHDs. This combination is likely to be more effective in lowering both daytime and nighttime BP than either treatment alone (Phillips and O'Driscoll, 2013). In addition, Pepin *et al.* explored RAAS inhibition using losartan in a crossover randomized control trial. In this study, the authors compared the efficacy of CPAP and valsartan in reducing BP in HT patients with OSA never treated for either condition (Pepin *et al.*, 2009). They reported that although the BP decrease was significant with CPAP treatment, valsartan induced a fourfold higher decrease in mean 24-h BP than CPAP in this specific sample (Pepin *et al.*, 2009).

In an earlier report, 74 of the 393 OSA patients using AH medications on a regular basis for more than six months were deemed to have been treated "ineffectively" (Lavie and Hoffstein, 2001), but the characterization of these medications was not reported. The same limitation is found in the study of Deleanu *et al.*, which aimed to study the effect of medication-controlled HT on OSA patients (Deleanu *et al.*, 2014). The authors suggested that controlled BP abates sleepiness and reduces remaining symptoms (*e.g.*, headaches, impotence and morning fatigue).

These findings could be much more interesting if the regimens responsible for these effects were revealed.

In a recent study, Kario *et al.* aimed to evaluate the effects of bedtime dosing of vasodilating (nifedipine, a calcium channel blocker) vs sympatholytic (carvedilol, a nonselective β-blocker/α1-blocker) AH agents on the sleep BP profile in HT OSA patients (Kario *et al.*, 2014). For this, they made use of a new BP monitoring method, the trigger sleep BP monitoring (TSP) method, which is based on the automated fixed-interval measurement function with an additional oxygen-triggered function that initiates BP measurement when oxygen desaturation falls below a set variable threshold continuously monitored by pulseoximetry (Kario *et al.*, 2014). The BP lowering effects of nifedipine on the mean and minimum sleep systolic BP were stronger than those of carvedilol; moreover, sleep systolic BP surge (the difference between the hypoxia peak systolic BP – SBP – measured by the oxygen-triggered function and SBP within 30 minutes before and after the peak SBP) was only significantly reduced by carvedilol (Kario *et al.*, 2014). Thus, both drugs are effective in decreasing sleep BP (Kario *et al.*, 2014) but the effect of carvedilol seems to be related more specifically to the hypoxia stimuli than nifedipine.

Finally, Cichelero *et al.* recently published the protocol of their randomized double-blind clinical trial, which seeks to compare the efficacy of chlorthalidone (a diuretic) with amiloride (also a diuretic) versus amlodipine (a calcium channel blocker) as a first drug option in patients older than 40 years of age with stage I HT and moderate OSA (Cichelero *et al.*, 2014). The findings of this study have not yet been reported.

In summary, individual drug studies find that the blockade of β1-adrenergic receptors (*e.g.*, atenolol and nebivolol) and the renin-angiotensin-aldosterone (RAA) pathway, including both ACEi and angiotensin AT1 receptor antagonists, might be helpful. Spironolactone (a mineralocorticoid receptor antagonist) has been proposed has a very useful tool in cases of resistant HT (Ziegler *et al.*, 2011a, 2011b), a very prevalent condition in OSA patients (Oliveras and Schmieder, 2013; Solini and Ruilope, 2013) in which aldosterone levels are generally elevated, as well as for severe OSA patients (Ziegler *et al.*, 2011a). Moreover, a study performed by Gaddam *et al.* (Gaddam *et al.*, 2010) has provided preliminary evidence that treatment with this drug substantially reduces the severity of OSA and improves BP in patients with both OSA and resistant HT (Gaddam *et al.*, 2010). These results seem promising but need to be confirmed in further larger studies. In contrast, despite volume overload appears to play a large role in the development of OSA (Owen and Reisin, 2013), diuretics, namely thiazide, have not been very effective AH agents in OSA patients without fluid retention (Ziegler *et al.*, 2011b). Calcium channel blockers, although effective in lowering BP, seem to present an effect less related to hypoxia stimuli. Moreover, Nerbass *et al.* reported that the use of these drugs might impact negatively on sleep duration in HT patients with OSA (Nerbass *et al.*, 2011). They

reported that the use of calcium channel blockers was associated with significant reduction in total sleep time and lower sleep efficiency (Nerbass *et al.,* 2011). Thus, their prescription can be questionable in these patients.

Despite the findings of these studies, they present some limitations and important data are missing. The major limitations comprise the following: the variability of subjects included in the studies as most of them were performed in non-AHDs naïve patients; the severity and chronicity of HT, which were not taken into account and consenquently the clinical relevance of BP reduction is questionable; the drug effectiveness in reducing nocturnal BP, which was not assessed in some studies; the confounding risk factors for HT that might be present in OSA patients (*e.g.*, obesity) and were not properly addressed in most studies. Furthermore, we can point out several questions that are still unanswered, *e.g.*: how many OSA patients are controlled under monotherapy with beta- blockers, angiotensin-converting enzyme inhibitors (ACEis), and angiotensin II receptor blockers (ARBs)? Beta-blockers or RAAS blockers are apparently effective, but should they be used alone or in combination? How many OSA patients remain uncontrolled despite the use of two or more AHDs? How do different AHDs behave when included in an AHDs regimen? In addition, the impact of these studies in clinical practice is unknown because epidemiological studies designed to investigate the AH medication profile in OSA patients are lacking. In addition, the more recent recommendations for the management of patients with OSA and HT are inconclusive regarding the use of AHDs and recognize the lack of strong evidence for the establishment of a first-line AHDs regimen for these patients (Parati *et al.,* 2013). Other authors support the idea that as there is no clear evidence for prefering a specific class of AHDs, the selection should primarily be guided by the patient's cardiometabolic profile and associated comorbidities (*e.g.*, obesity, metabolic syndrome, diabetes mellitus, and cardiovascular diseases) (Tsioufis *et al.,* 2010). Moreover, these authors recommend that due to the lack of relevant trials focused on the use of associations of AHDs in OSA patients, the choice should rely on current HT guidelines and the adverse effects of AHDs also need to be considered (Tsioufis *et al.,* 2010). The limited evidence base restricts the ability to make informed treatment choices. Thus, larger scale observational and clinical studies are needed to address these and possibly other limitations and bring new insights to the field.

Another problem concerning the studies carried out in humans is that HT is frequently not recognized in patients with OSA (Baguet *et al.,* 2009), and it is important to highlight that patients with elevated BP who do not carry the diagnosis of HT may be misclassified as non-HT (Wang and Vasan, 2005). Consequently, aggressive control of BP must be warranted in OSA patients and an accurate method for BP measurement should be used in the early diagnosis of clinically suspected OSA patients. Taking into account the advantages and limitations of the several methods of BP measurement, 24-h ABPM seems to be superior to office BP

measurement and home BP monitoring in diagnosing HT in patients with suspected OSA (Parati *et al.*, 2012).

ANIMALS

As previously stated, a rather wide variety of animal models has been used to evaluate the cardiovascular consequences of OSA and to study the cause-effect mechanisms in OSA. As CIH causes a moderate increase in BP, drugs can be tested further to modulate this effect. However, studies aimed at investigating the AH effect of drugs on animal models are scarce. Table 4 summarizes the studies that have evaluated the effects of AHDs on BP in animal models of CIH.

In a study undertaken to clarify the role of renal sympathetic nerve activity and plasma renin activity (PRA) in the diurnal BP response to chronic IH, Fletcher *et al.* demonstrated that the pharmacological blockade of the RAAS with losartan prevented the rise in BP induced by CIH (Fletcher *et al.*, 1999; Fletcher *et al.*, 2002). Losartan and other angiotensin antagonists (A-779, an Ang-(1–7) antagonist; ZD7155, an AT1 antagonist; PD123319, an AT2 receptor antagonist) were further used by da Silva *et al.* (da Silva *et al.*, 2011) to investigate the role of endogenous angiotensin peptides within the hypothalamic paraventricular nucleus (PVN) neurons to control BP in a rat model of CIH-induced HT. These authors concluded that endogenous angiotensin peptides acting in the PVN contribute to IH-induced increases in MAP observed in this rat model. In 2013, losartan was used once again to test the role of the brain RAAS in CIH HT (Knight *et al.*, 2013). The work of this group provided evidence that brain RAAS contributes to HT related to CIH and that brain RAAS appears to be critical for the development and maintenance of the sustained HT during normoxia (Knight *et al.*, 2013).

Other groups have found that the systemic administration of endothelin (ET) receptor antagonists in rodents prevents the increase in BP during CIH exposure (Kanagy *et al.*, 2001; Allahdadi *et al.*, 2008; Belaidi *et al.*, 2009). The data provided by Allahdadi *et al.* showed that an endothelin receptor antagonist (ETA: BQ-123) acutely decreased the MAP dose dependently in rats exposed to IH but not sham rats, suggesting that targeting ETA receptors may be a selective and effective treatment of HT related to OSA (Allahdadi *et al.*, 2008). Belaidi *et al.* used SH rats and bosentan, a mixed endothelin receptor antagonist (Belaidi *et al.*, 2009). Their results showed that the administration of bosentan during chronic IH prevented the increase in BP and reinforced the idea that endothelin antagonists could be useful therapeutic tools in HT related to OSA (Belaidi *et al.*, 2009). The same effects were reported by Kanagy *et al.* for PD145065, a nonselective endothelin receptor antagonist (Kanagy *et al.*, 2001).

Table 4. Studies evaluating the effects of AHDs on BP in animal models of CIH

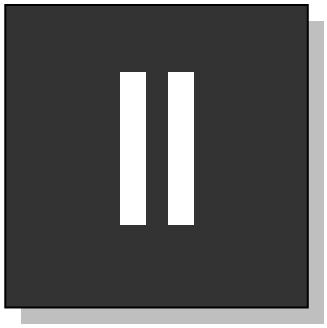
Species	CIH experimental protocol	Drugs/ intervention	BP measurement	Drug effect on BP	Reference
Sprague-Dawley rats	2/3- 20.9% O_2 (3-6 s +15-18s; 2 cycles/min); 6-8 h/day; 35 days	Losartan (15 mg/kg); gavage; 35 days	Telemetry	Significant ↓ MAP (98.2 ± 61.7 to 85.9 ± 62.7 mm Hg)	Fletcher *et al.*, 1999
Sprague-Dawley rats	5-21% O_2+ 5-0%CO_2 (20 cycles/h); 7 h/day; 14 days	A-779 (Ang-(1–7) antagonist); Losartan (2nmol/h) and ZD7155 (AT1 antagonists); PD123319 (AT2 receptor antagonist); osmotic minipumps delivered into PVN; 14 days.	Telemetry	↓ MAP: A-779: 5±1mm Hg, Losartan: 9±4mmHg, ZD7155: 11±4mmHg PD123319: 4±3 mmHg	da Silva *et al.*, 2011
Sprague-Dawley rats	20.9-10% (180 s cycles); 10h/day; 35 days	Losartan (15 mg/kg); p.o. (syringe technique); 35 days	*NA* (arterial catheterization?)	↓ SBP (10 mmHg)	Fenik *et al.*, 2012
Sprague-Dawley rats	80 cycles (6 min each) 21-10% O_2/ day; 8h/day; 7 days	Losartan (1µg/h); intracerebroventricular (miniosmotic pumps); 7 days	Telemetry	↓ MAP during both CIH exposure and normoxic period	Knight *et al.*, 2013
Sprague-Dawley rats	20 cycles (90s each) of 21-5% O_2 and 0-5% CO_2/hour; 7h/day; 14 days	BQ-123 (10 to 1,000 nmol/kg in bolus or 100 nmol/kg/day for chronic administration); iv or sc; 14 days	Tail-cuff method and telemetry	Acute administration: dose dependent ↓ MAP Chronic administration: prevented ↑MAP	Allahdadi *et al.*, 2008
SHR + Wistar rats	21-10% O_2 (1 min cycles: 20s+40s); 8h/day; 14 days	Bosentan (100 mg/Kg/dia); mixed in chow; 14 days	Tail-cuff method + Arterial catheterization	Prevented ↑MAP	Belaidi *et al.*, 2009
SHR	21-10% O_2 (every 90 seconds); 12 h/day; 30 days	Nifedipine (5 mg/Kg) and SOD mimetic (MnTMPyP; 10 mg/Kg); s.c.; 30 days	Tail-cuff method	Nifedipine: attenuate SBP and DBP SOD mimetic: ↓ SBP and DBP	Soukhova-O'Hare *et al.*, 2008
Sprague-Dawley rats	21-5% O_2 (every 60 s); 8h/day; 14-21 days	Melatonin (10 mg/Kg); i.p.; 14 or 21 days (30 min before hypoxic exposure)	Tail-cuff method	↓ SBP (21 mmHg)	Hung *et al.*, 2013
Sprague-Dawley rats	20 cycles (90s each) of 21-5% O_2 and 0-5% CO_2/hour; 7h/day; 14 days;	Tempol (1 mM); drinking water; 14 days	Telemetry	↓ MAP (17 mmHg)	Troncoso-Brindeiro *et al.*, 2007
Sprague-Dawley rats	12 cycles (300s each) of 21-5% O_2/h; 8h/day; 21 days	Ascorbic acid (1.25 g/L); drinking tap water; 21 days	Arterial catheterization	↓ MAP (29 mmHg)	Del Rio *et al.*, 2010
Sprague-Dawley rats	9 cycles (5 min+ 15s) of 21-5% O_2/hour; 8h/day; 10 days	SOD mimetic (MnTMPyP; 5 mg/Kg/day); i.p; 10 days	Arterial catheterization	↓↓ MAP	Kumar, 2006
Sprague-Dawley rats	20 cycles (90s each) of 21-5% O_2 and 0-5% CO_2/h; 8h/day; 11 days	PD145065 (ET receptor antagonist in cumulative drugs: 0.3, 3.0, 30, 300, 1000 nmol/Kg); bolus; 11 days	Arterial catheterization	Dose dependent ↓ MAP	Kanagy *et al.*, 2001
Sprague-Dawley rats	21-5% O_2 (12 times/h); 8 h/day; 14 days	Ebselen (specific ONOO- scavenger; 10 mg/kg/day); osmotic mini-pumps; 7 days	Telemetry	↓ elevated BP	Moya *et al.*, 2014

AHDs, antihypertensive drugs; **BP**, blood pressure; **CIH**, chronic intermittent hypoxia; **DBP**, diastolic blood pressure; **ET**, endothelin; **h**, hour; **HT**, hypertension; **i.p.**, intraperitoneal; **MAP**, mean arterial pressure; **NA**, information not available; **min**, minutes; **p.o.**, per os; **PVN**, hypothalamic paraventricular nucleus; **s**, seconds; **SBP**, systolic blood pressure; **SHR**, spontaneously hypertensive rats; **s.c.**, subcutaneous; **SOD**, superoxide dismutase mimetic; ↓, decrease; ↑, increase.

Soukhova-O'Hare *et al.*, designed a study based on the assumption that reactive oxygen species and altered L-Ca^{2+} channel activity may underlie the postnatal programing of exaggerated BP and cardiac remodeling (Soukhova-O'Hare *et al.*, 2008). To test this hypothesis, these authors used nifedipine, an L-calcium channel blocker, and a superoxide dismutase mimetic (MnTMPyP pentachloride); both attenuated BP (Soukhova-O'Hare *et al.*, 2008). Their results suggested that Ca^{2+} and reactive oxygen species-mediated signaling during IH are critical mechanisms underlying postnatal programing of an increased severity of HT in SHR. Kumar *et al.* reported similar results for the same superoxide dismutase mimetic (Kumar *et al.*, 2006). A year before, Troncoso Brindeiro *et al.* used another superoxide dismutase mimetic, tempol, and showed that scavenging superoxide prevents both the increase in ET-1 production and vascular ROS levels induced by CIH exposure (Troncoso Brindeiro *et al.*, 2007). The later work of Del Rio *et al.*, using the antioxidant ascorbic acid, showed that this substance prevented the increased plasma peroxidation and nitrotyrosine formation within the carotid body, as well as HT (Del Rio *et al.*, 2010), supporting the essential role of oxidative stress in the generation of carotid body chemosensory potentiation and systemic cardiorespiratory alterations induced by IH (Del Rio *et al.*, 2010).

More recently, Hung *et al.* tested the hypothesis that melatonin, previously shown to ameliorate oxidative injury and inflammation, could have a protective effect against IH-induced HT and endothelial dysfunction. This assumption was confirmed as melatonin promoted a decrease in systolic BP and prevented endothelial dysfunction with ameliorated levels of nitric oxide, endothelial-dependent relaxation, and expressions of eNOS and antioxidant enzymes (Hung *et al.*, 2013).

Based on the studies described, we can conclude that most reports on CIH animal models in which drugs have been tested were not designed to respond to pharmacological issues: they have been used solely as pharmacological tools to address physiological mechanisms. The experiments evaluate prevention but not the effectiveness of treatment. They must be planned first to induce HT and then evaluate the efficacy of cumulative doses of drugs because the translation of the results to humans obtained with simultaneous induction of HT and drug administration is not relevant. Other limitations of the pharmacological approaches included in these works are the absence of dose-response curves and comparison of the effectiviness of different drugs in the same animal model. Thus, other studies must be designed to overcome these drawbacks.



GENERAL AND SPECIFIC AIMS

Which were the starting points for the design of the present study?

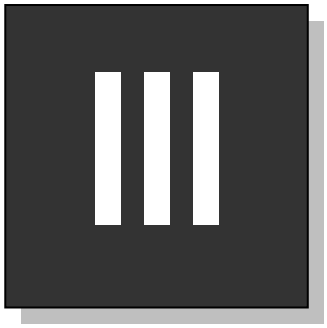
1) Close linkage between obstructive sleep apnea (OSA) and hypertension, highlighted by the extremely high prevalence of hypertension (HT) among patients with OSA.
2) Scarce effect of *continuous positive airway pressure* (CPAP), the gold standard treatment, on sustaining blood pressure (BP) control, which implies that the use of antihypertensive drugs is still required for patients with both OSA and HT.
3) Lack of specific guidelines for the pharmacological treatment of HT in patients with OSA.
4) Limited data on antihypertensive drug (AHDs) regimens in these patients.
5) Inconsistent effects of antihypertensive drug in patients with OSA.
6) Higher clinical prevalence of CIH conditions than acute or chronic sustained hypoxia.
7) Less explored mechanisms and consequences of chronic intermittent hypoxia (CIH) than for chronic sustained hypoxia.
8) Lack of experiments specifically planned and performed to evaluate the efficacy of cumulative doses of antihypertensive drugs in CIH conditions.

Facing these facts, the **general aim** of this thesis is to contribute to identify more effective AHDs for the treatment of hypertension in patients with OSA and investigate underlying mechanisms of systemic effects associated with OSA, as well as its modulation by AHDs. For that, we will use a sizeable sample of patients with OSA and, in order to surpass some confounding factors that might be present in the human study and to attain more specific information concerning drugs efficacy, a rat experimental model of hypertension induced by a paradigm of CIH that simulates OSA.

The **specific aims** of the present work are the following:

1) To highlight the relevance of Ambulatory Blood Pressure Monitoring (ABPM) in diagnosis and therapeutic decision-making for patients suspected of having OSA.
2) To find new predictors based on anthropometric measures to identify patients that misclassify themselves as non-hypertensive, and thereby promote the selective use of ABPM.
3) To characterize the phenotypic characteristics and, specifically, the patterns of AHDs used in patients with OSA.
4) To investigate a hypothetical association between ongoing antihypertensive regimens and BP control rates in patients with OSA, before and after CPAP adaptation.
5) To determine, in a rat model of CIH-induced hypertension, the efficacy of carvedilol, a nonselective beta-blocker with intrinsic anti-α_1-adrenergic activity and antioxidant properties.

6) To investigate an alternative method to gavage, for chronic administration of AHDs to laboratory rats, since when testing these drugs it becomes crucial to ensure the selection of a non-invasive and stress-free method for drug delivery.
7) To explore the effects of CIH on the pharmacokinetics profile of carvedilol.



GENERAL METHODS

CLINICAL STUDIES

Ethics

All patients were fully informed about the study and gave written consent (attachment #1) in accordance with the Declaration of Helsinki. The study protocol was previously approved by Centro Hospitalar Lisboa Norte's Ethics Committee (Ethical approval: 11st November 2009) and registered at ClinicalTrials.gov (NCT01803815).

Subjects and Study Design

Three hundred and sixty-nine consecutive patients clinically suspected of having OSA, aged above 18 years, who were attending their first visit at the Centro Hospitalar Lisboa Norte, EPE (CHLN) Sleep Unit, following referral by their general practitioner or other specialist, were assessed for eligibility. Patient inclusion started in April 2010 and was completed in July 2012. Exclusion criteria included severe psychiatric disease or an inability to understand the information required for informed consent.

At baseline (first visit) all patients were invited to undergo an overnight polysomnography, 24-h ambulatory blood pressure monitoring (ABPM) and completed a data collection form that included ongoing medication profile registration.

Predictors of HT misclassification

Patients with ABPM, sleep data and no prior diagnosis of hypertension were included in the **cross-sectional study** designed to evaluate the validity of self-reported hypertension and to find new criteria to identify patients that misclassify themselves as non-hypertensive (Figure 1).

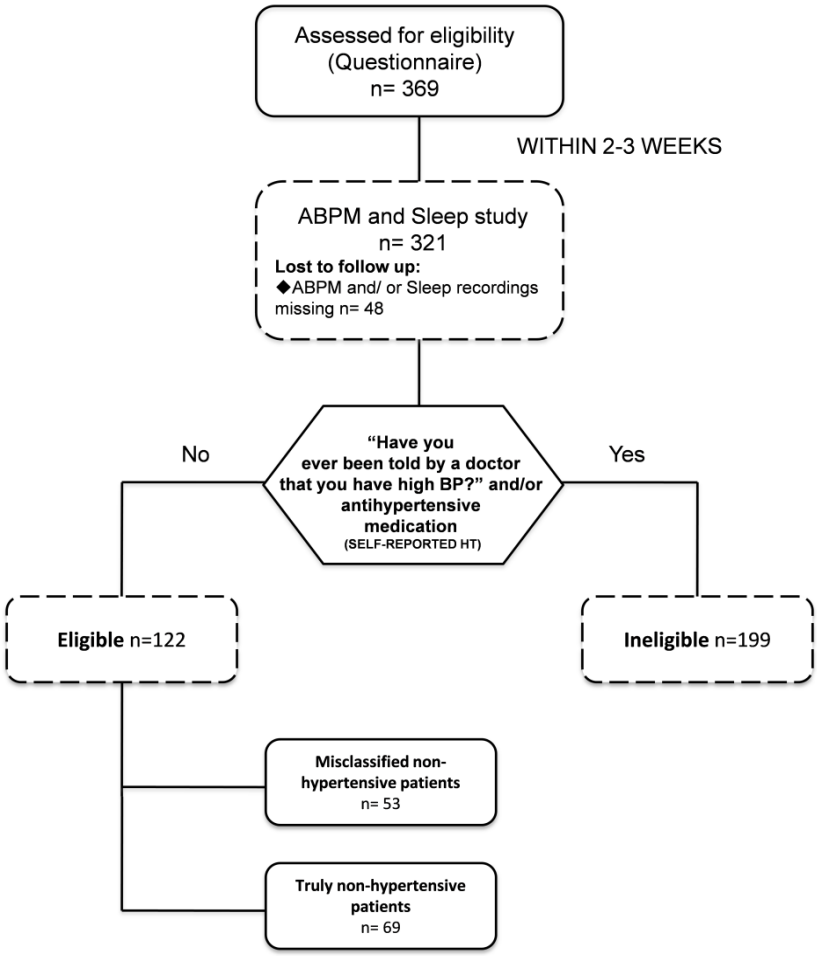


Figure 1: Predictors of HT misclassification: patient eligibility and follow-up.

ABPM: Ambulatory Blood Pressure Monitoring; **HT:** hypertension.

Of the 369 patients consecutively included, ABPM and/or sleep recordings were missing for 48, due to technical problems or because they did not attend the ABPM or sleep study. One hundred and twenty-two patients who were not taking antihypertensive medication answered "No" to the question "Have you ever been told by a doctor that you have high blood pressure?" and the remaining 199 answered yes or were under antihypertensive medication. Of the 369 only 122 patients were considered eligible for the study (misclassified non-hypertensive patients n=53; non-hypertensive patients n=69).

Association between antihypertensive medication and BP control

Once the diagnosis of OSA and hypertension was established, the criteria for treatment with CPAP confirmed and baseline ABPM measured (second visit), patients were scheduled for CPAP titration. Four to five weeks after CPAP adaptation (third visit), the device data were checked and patients with a CPAP mean daily use of at least 4 hours were scheduled for repeated 24-h ABPM. At this time, particular attention was paid to ensure that patients had not

changed their antihypertensive medication since the first evaluation. If any changes in either type of medication or dose had occurred, the patient was excluded from the study.

For this **prospective cohort study**, additional inclusion criteria were subsequently defined. Only patients with an apnea-hypopnea index > 5 events/hour, diagnosis of hypertension, a CPAP mean daily use of at least 4 hours and ambulatory 24-h ABPM recordings, performed at baseline and 1-3 months after checking CPAP adherence, were considered eligible for the study of the association between antihypertensive regimens and BP control, before and after CPAP adaptation (Figure 2).

Briefly, of the 369 patients consecutively included, 68 were patients with no diagnosis of OSA and 48 with no diagnosis of hypertension. The ABPM and/or sleep recordings were missing for 48 patients due to technical problems or because they did not attend the ABPM (n=41) or sleep study (n=7). Forty had no indication for CPAP therapy. One hundred and sixty-five were scheduled for CPAP adaptation; however, 7 refused CPAP, CPAP data were missing in 6 patients and 51 had no compliance. Of the 369, only the data from 90 patients were used to investigate the association between ongoing AH regimen and BP control rates after CPAP adaptation.

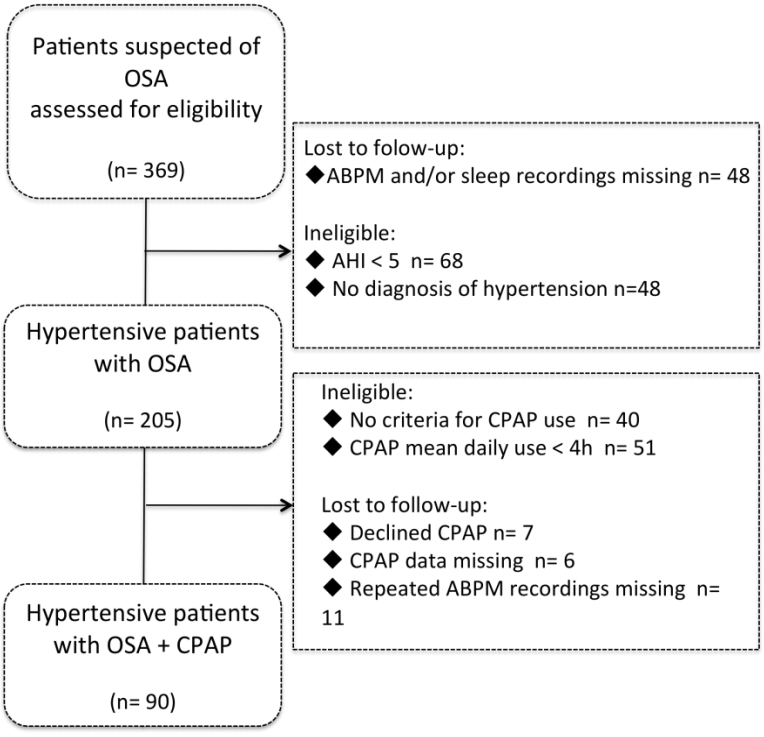


Figure 2: Association between antihypertensive medication and BP control: patient eligibility and follow-up.

AHI: Apnea-Hypopnea Index; **ABPM:** Ambulatory Blood Pressure Monitoring; **BP:** blood pressure; **CPAP:** Continuous Positive Airway Pressure; **OSA:** obstructive sleep apnea.

Data collection form and clinical assessment

Socio-demographic and anthropometric data, as well as smoking habits, comorbidities and ongoing medication profiles, were registered (attachment #2 and #3).

All patients were assessed for weight, height, neck circumference (NC) and waist circumference (WC) using standardised protocols. Weight was measured, with lightweight clothing and without shoes, to the nearest 100 g and height was assessed, with no shoes, to the nearest 0.5 cm, in both cases using calibrated instruments. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in metres (kg/m^2). Neck circumference, measured in the middle of the neck, at the level of the cricothyroid membrane (in men with a laryngeal prominence it was measured just below the prominence), to within 0.1 cm, with the patient's head positioned in the Frankfort horizontal plane, and WC, assessed at the level of the umbilicus to within 0.1 cm, were also recorded using a flexible non-stretchable measuring tape.

Regarding smoking habits, patients were classified as non-smoker, ex-smoker, and smoker (defined as those who smoke at least one cigarette per day over the previous year).

Comorbidities, with a focus on hypertension, were assessed. Hypertension was defined as self-reported hypertension (answer "yes" to the question "Have you ever been told by a doctor that you have high blood pressure?") or use of antihypertensive medication. Information about diabetes mellitus, dyslipidaemia, arrhythmias, asthma, chronic obstructive pulmonary disease (COPD), depression and anxiety, history of cardiovascular disease and hypothyroidism was also collected.

The ongoing medication profile (drug name (International Non-proprietary Name: INN) and dose) was recorded using the "Brown-Bag" medication review (a method in which patients are asked to bring all of their medications to each visit) (Caskie *et al.*, 2006). We analysed the use of antihypertensive drugs by regimen: 1-with angiotensin-converting enzyme inhibitors (ACEi); 2-with angiotensin II receptor blockers (ARAs); 3-with β-blockers; and 4-others (including diuretics and calcium channel blockers), and the number of antihypertensive drugs (1-one, monotherapy; 2-two or more, polytherapy) included in each patient regimen. In all patients, antihypertensive drugs were taken for at least six months without changing the medication profile until the end of the follow-up.

Sleep Evaluation

Patients underwent an overnight in-laboratory polysomnography or, as an alternative for the diagnosis of OSA in patients with a high pretest probability of moderate to severe OSA, a cardiorespiratory sleep study (Collop *et al.*, 2007). In-laboratory polysomnographic studies were performed using a multichannel polygraph (Model Embla S7000, Embla Systems Inc,

Broomfield, CO, USA) and ambulatory recordings were made using a validated portable digital recording unit (type 3) with sensors for the registration of airflow, saturation, respiratory movements of the chest, body position and snoring sounds (Embletta PDS device; Embla Systems Inc, Broomfield, CO, USA). Sleep recordings were manually scored, based on standard criteria (Collop *et al.*, 2007), by trained technicians. Apnea was defined as a complete cessation of airflow for at least 10 s, associated with oxygen desaturation of 3%. Hypopnea was defined as a discernible reduction in airflow of at least 50% for 10 s or more, with an accompanying desaturation of at least 3% or an arousal. The number of apneas and hypopneas per hour of sleep was calculated and, for both in-laboratory and ambulatory recordings, the apnea-hypopnea index (AHI) was considered. Respiratory effort related arousals (RERAs), scored in laboratory polisomnography sleep staging, were not considered in order to minimize the differences between the results of the two diagnostic approaches. OSA was categorized according to current AHI cut-offs of < 5 (non-diagnostic), $5 \geq$ and < 15 (mild), $15 \geq$ and < 30 (moderate) and ≥ 30 (severe) (Berry *et al.*, 2012; Collop *et al.*, 2007).

Twenty-four-hour ambulatory blood pressure monitoring (ABPM)

Twenty-four hour ABPM was performed at baseline, on a different day to that of the sleep evaluation, and 1-3 months after CPAP adaptation. All participants were instructed to continue their usual daily activities. Screening BP measurements were performed in accordance with the recommendations of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC) guidelines (Mancia *et al.*, 2007), using appropriately-sized arm cuff, on the non- dominant arm, with a non-invasive portable validated BP recorder (Spacelabs Model 90217; Spacelabs Healthcare, Redmond, WA, USA).

The equipment was programmed for cuff inflation every 20 minutes during the daytime period and every 30 minutes during the night-time. Daytime and night-time were individually predetermined depending on the participants' usual awake and sleep schedule. The data were considered valid when a minimum of 70% of the measurements was recorded without errors. BP recordings were analysed for the overall 24-hour period, daytime and night-time periods. Additionally, 24 h BP profile was assessed and patients were classified as showing a dipping or a non-dipping profile. A dipping profile was defined as a reduction in the average systolic and diastolic BP at night that was higher than 10% compared to daytime values. The data were analysed by an experienced cardio-respiratory technician.

Uncontrolled BP was defined according to ESC and ESH guidelines (Mancia *et al.*, 2013). BP was considered uncontrolled for systolic BP (SBP) values of 135 mm Hg or above, diastolic BP (DBP) values of 85 mm Hg or above, or both during daytime and systolic BP (SBP) values of 120 mm Hg or above, diastolic BP (DBP) values of 70 mm Hg or above, or both during night

time (Mancia *et al.*, 2013). Isolated nocturnal hypertension was defined, in accordance with the established BP thresholds for ABPM, as night-time SBP values of 120 mm Hg or DBP of 70 mm Hg or above, and isolated daytime hypertension as diurnal SBP of 135 mm Hg or DBP of 85 mm Hg or above. When both conditions were present, subjects were classified as having combined day-night hypertension (Mancia *et al.*, 2013).

Continuous Positive Airway Pressure (CPAP) therapy

CPAP titration was performed using self-adjusting CPAP devices (autoCPAP) (REMstar PR1 Auto, S8 AutoSet Spirit- Resmed Ltd), AutoSet Spirit S9 - Resmed Ltd). These devices automatically titrates the amount of pressure delivered to the patient to the minimum required to ensure an unobstructed airway, allowing optimal pressure settings. During the initial setup of the machine, the minimum (Pmin: 4 cm H_2O) and maximum (Pmax: 16 cm H_2O) pressures were set. All patients were followed-up 4-5 weeks after CPAP adaptation, in order to check for any side effects or problems with the treatment and, if required, for CPAP pressure adjustment.

CPAP adherence, as well as residual AHI, leak and pressure data, were assessed from the device counter. Optimal pressure was determined visually from the raw data by analyzing the pressure curve that included the periods with a leak lower than 0.4 L (90^{th} percentile leak). Data were analysed by experienced technicians using appropriate software (attachment #3).

Statistical Analysis

An exploratory analysis was carried out for all variables. Categorical data were expressed as frequencies and percentages, and continuous variables as mean or median, standard deviation (SD) or inter-quartile range (25th percentile-75th percentile). Univariable analysis was performed using Student's t-test and nonparametric (Fisher's exact test, $\chi 2$ and Mann-Whitney U) tests as appropriate. Welch's t-test was used as an adaptation of Student's t-test whenever unequal variances were obtained. Wilcoxon test and Sign test were used to compare ABPM data measured at baseline and after CPAP adaptation. The McNemar test was applied to compare the proportion of uncontrolled patients before and after CPAP adaptation. Finally, the Spearman's rho rank correlation was used to investigate the association between the anthropometric variables.

For the **categorization of NC and BMI**, the minimum p-value approach was applied (Mazumdar and Glassman, 2000). Based on a systematic search for the "best" cut point, this method obtains the point from a grid of marker values that is associated with the minimum p-value which corresponds to the maximum chi-squared test value. Generalized Additive Models (GAMs) (Hastie and Tibshirani, 1990) for binary response were used to confirm the cut-off points obtained using the minimum p-value approach. A logistic regression model was fitted to

the data in order to determine the relevance of AHI and BMI in non-hypertensive misclassification. The outcome binary variable was "misclassified non-hypertension" (yes: for patients who classified themselves erroneously as non-hypertensive, no: for truly non-hypertensive patients). The previously obtained cut-off points were used to discretize the continuous NC and BMI and the new categorical variables were then used as independent variables in the logistic regression model. The predictive ability of the resulting model was analysed using the Hosmer-Lemeshow goodness-of-fit test (Hosmer and Lemeshow, 1989). This test was used to compare the observed and expected frequencies of patients that misclassified themselves as non-hypertensive, based on the values of the estimated probabilities obtained by the logistic regression model. A high p-value would indicate that the model was performing well. The area under the Receiver Operating Characteristic curve (AUC) was used to evaluate the discriminative ability of the model. A value of 0.50 would be obtained when a model discriminates no better than chance, and a value of 1.0 means perfect accuracy.

In order to identify **baseline predictors of BP control** after CPAP adaptation, a univariable analysis was performed. The outcome variable was "uncontrolled BP" (yes: for patients with uncontrolled BP and no: for patients with controlled BP) and the multiple logistic regression model was subsequently fitted to the data. The predictive ability of the resulting model was analysed by the Hosmer-Lemeshow goodness-of-fit test (Hosmer and Lemeshow, 1989). A high p-value indicates that the model is performing well. The area under the Receiver Operating Characteristic curve (AUC) was used in order to evaluate the discriminative ability of the model. A value of 0.50 is obtained when a model discriminates no better than chance, and a value of 1.0 means perfect accuracy.

Results were considered significant when $\alpha=0.05$. Confidence intervals are presented when appropriate (95% CI). Statistical analysis was performed using the IBM SPSS Statistics (IBM Corp., Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

EXPERIMENTAL STUDIES

Ethics

The applicable institutional and governmental regulations concerning the ethical use of animals were followed, according to the NIH Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985), the European guidelines for the protection of animals used for scientific purposes (European Union Directive 2010/63/EU) and the Portuguese Law nº 113/2013. Experimental procedures were previously approved by the Institutional Ethics Committee of the NOVA Medical School for animal care and use in research (Ethics approval: 21st May 2011 (study in animals submitted to CIH conditions) and 21/2013/CEFCM (study for the refinement of oral dosing of antihypertensive drugs in rats).

Animals

Experiments were performed in male Wistar rats (*Ratus norvegicus*), aged 2-3 months, and obtained from the NOVA Medical School animal facility. This Wistar in house colony has been started with animals acquired from Charles River Laboratories (Crl:WI). Animals were housed individually in polycarbonate cages with wire lids (Tecniplast, Buguggiate, Varese, Italy), under 12 h light/dark cycles (8 am - 8 pm), at a room temperature 22 ± 2.0 °C and relative humidity 60± 10%. Rats were maintained on a standard laboratory diet (SDS diets RM1) and reverse osmosis water, given ad libitum. Corncob bedding (Probiológica, Lisbon, Portugal) was used and changed once a week. Animals were Specific Pathogen Free (SPF) according to FELASA recommendations (Nicklas *et al.*, 2002).

Experimental protocols

Efficacy of Carvedilol in reversing HT induced by CIH

Drugs and chemicals

Carvedilol (CVD) was kindly provided by Tecnimede (Sintra, Portugal; manufacturer batch: 5334-11-015) and methylcellulose (MC) was purchased from Sigma-Aldrich (Sintra, Portugal). R-(+)-CVD and S-(-)-CVD were obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). The enantiomerically pure chiral agent, (-)-menthyl chloroformate (MCF) was obtained from Sigma-Aldrich (Sintra, Portugal). HPLC grade solvents used in the extraction procedure and for the mobile phase of the chromatographic system were obtained from VWR (Lisbon, Portugal).

Experimental design

Experiments were performed in thirty-one male Wistar rats, aged 2-3 months and with mean body weight 308.4±45.36 g.

Three different doses were administered to investigate the long-term effects (25 days) of carvedilol (CVD) in an experimental model of hypertension related to CIH. Rats were randomly assigned and divided into five groups: Group 1 (CVD 10 mg/kg/day - CIH; n=5); Group 2 (CVD 30 mg/kg/day - CIH; n=7); Group 3 (CVD 50 mg/kg/day - CIH; n=8); Group 4 (control group administered vehicle (0.5% methylcellulose (MC)); n=5); Group 5 (CVD 50 mg/Kg/day – Normoxia (Nx); n=6). MC 0.5% (2 ml) was used to facilitate the dissolution and absorption of CVD, since it is a very lipid soluble drug (Rodriguez-Perez *et al.,* 1997).

Animals from Groups 1, 2, 3 and 4 were gentled for 10 minutes daily for one week prior surgery, to minimise the discomfort related to experimental manipulation and reduce data variability, and were instrumented with indwelling blood pressure telemeters. Rats were allowed to recover for 10 days, after transmitter implantation surgery, before any measurements were recorded. A period of 5/6 days was then given for chamber acclimatization under normoxic conditions (21 O_2% + 79% N_2) and baseline cardiovascular data was recorded. Animals were then exposed to 60 days of CIH, during their sleep period, and CVD or vehicle administration, by gavage, started at Day 36 and lasted 25 days. Animals from Group 5 (normoxic rats) were exposed for 60 days to normal air (21% O_2 and 79% N_2), in the same room as the CIH animals in order to experience similar conditions. Similarly, CVD administration by gavage started at Day 36 and lasted 25 days for the other groups. Since these rats were specifically used for pharmacokinetic studies, the implantation of telemetry transmitters was not performed.

At the end of the experiments, 2-3 hours after drug or vehicle delivery, the rats were anaesthetised, by intraperitoneal injection with medetomidine (0.5mg/kg body weight; Domitor®, Pfizer Animal Health, Auckland, New Zealand) and ketamine (75mg/kg body weight; Imalgene 1000®, Mérial, Lyon, France), and cardiac puncture was performed without thoracotomy, with a 20 G needle with a 10 mL syringe, to collect blood for further quantifications. The animals were then euthanized with an intracardiac overdose of sodium pentobarbital (Eutasil®, Ceva Animal Health, Libourne, France), and the death was confirmed by cervical dislocation.

Rats were weighed at baseline and once a week during the entire study. The amount of carvedilol was adjusted weekly to ensure the doses of 10, 30 and 50 mg/Kg/day (p.o.). CVD was weighed daily, immediately before administration, dissolved in the vehicle and labelled individually for each rat. CVD dissolved in 0.5% MC (2 mL) or vehicle alone was given daily

to the animals on approximately the same schedule (9:00 to 9:30 am), always after BP measurement and before the exposure to CIH conditions. Control animals received equivalent daily oral gavage volumes (2 mL). All rats underwent a 7 day handling acclimatization period and were handled daily for a period of 2 minutes each by the same individual and accustomed to the gavage position, in a different animal room. Gavage was performed using a sterile polypropylene feeding tube (gauge 15; tip diameter: 3 mm; length: 78 mm; Instech Laboratories, Inc., USA) in order to reduce the risk of trauma, perforation and cross contamination (Morton *et al.*, 2001).

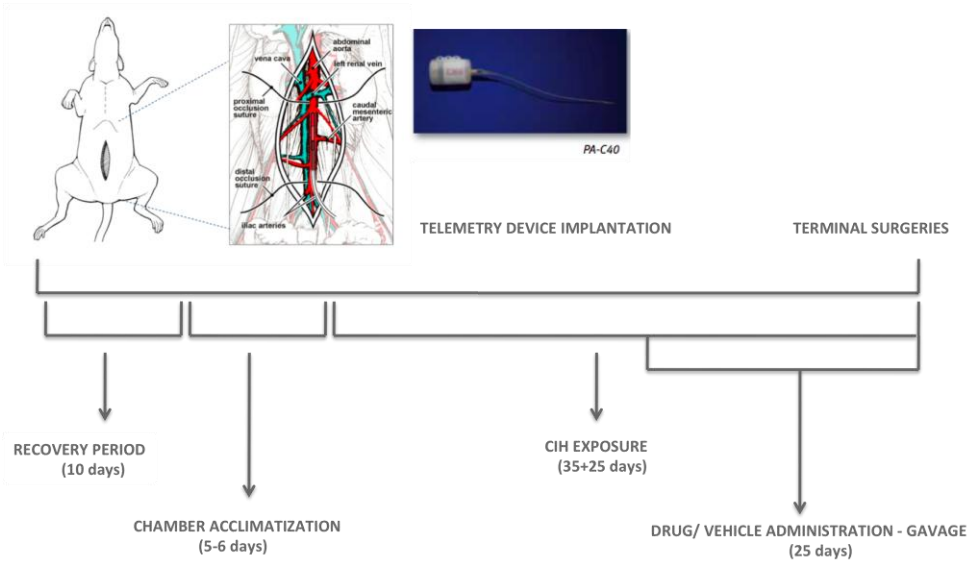


Figure 3: Experimental design of the study performed to evaluate the effects of carvedilol on HT related to CIH. CIH: Chronic intermittent hypoxia; **HT:** hypertension.

Radiotelemetry

Since hemodynamic measures are particulary prone to confounding factors (*e.g.* experimental stress due to the need for physical restraint of the animals, which leads to higher BP and heart rate (HR) recordings), we decided to use radiotelemetry in our experiments. This method allows daily recording of mean arterial BP, systolic and diastolic BP and HR, under conscious physiological condition, in unrestrained laboratory animals.

In our protocol, the animals were implanted with cylindrical shaped **transmitters**: model TA11PA-C40 (Data Sciences Corporation, St. Paul, MN, US). Output from the telemetry transmitters were recorded via radio frequency signals obtained through **Data Acquisition System** (Data Sciences Corporation, St. Paul, MN, US). The **DSI Data Exchange Matrix** is able to detect and forward the model and serial number of **receivers** (Figure 4) and the **Ambient Pressure Reference Monitor** (measures atmospheric pressure and provides dynamic

corrections via a digital signal to the computer, ensuring accurate pressure measurements in small animals when using DSI transmitters) to the **Dataquest® A.R.T. system**, which collects and maintains digital data from the receiver to the computer to optimize data integrity throughout the system.

BP and HR measurements, obtained during a 10s sampling period, were averaged and recorded during 15 minutes daily. Baseline recordings of BP and HR were performed for 3/4 days prior to CIH exposure.

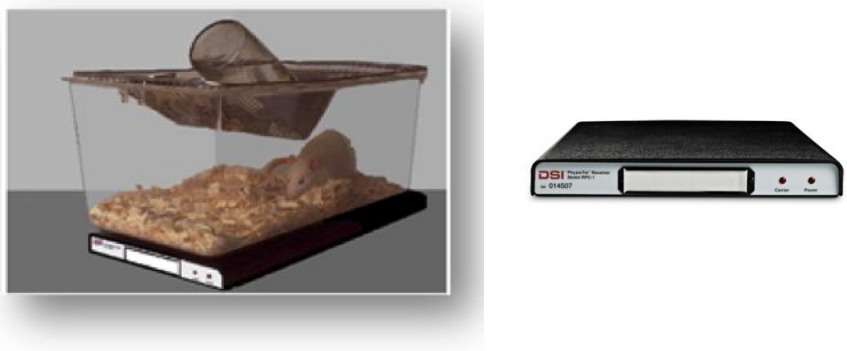


Figure 4: DSI PhysioTel® Receivers – RPC-1 Model for Small Animals (Data Sciences Corporation, St. Paul, MN, US). The RPC-1 Receiver is used for monitoring animals housed in plastic cages that can be placed on top of the receiver.

Device description

The PA-C40, weighs 7.8g and has an initial pressure accuracy of ±3 mmHg. This device consists of two major components: the device body and the pressure catheter (Figure 5).

Figure 5: The PA-C40 Transmitter: (A) Device Body and **(B)** Pressure Catheter (DSI, St. Paul, MN, US).

The **Device Body** contains:

(1) *Pressure sensor*: receives pressure fluctuations from the fluid-filled catheter and sends the signals to the electronics module;

(2) *Reusable electronic module*: translates the pressure fluctuations into digitized signals and transmits them to a receiver and it also contains a magnetically activated switch that allows the device to be switched on or off);
(3) *Battery*: provide the power supply for the electronics module;
(4) *Suture rib*: allows the surgeon to suture the device securely in place at the implant site).

The **Pressure Catheter** is polyurethane tubing that extends out of the device body (8 cm) and includes:

(1) *Non-compressible fluid*: relays pressure fluctuations to the sensor);
(2) *Thin-walled section*: tip of the catheter farthest from the device body that senses the dynamic portion of the pressure wave. It is designed to be completely inserted into the vessel where the desired pressure can be sensed. It contains biocompatible gel at the very tip, which prevents the non-compressible fluid from leaving the catheter and blood from clotting in the catheter tip;
(3) *Tip cover*: removal section of silicone tubing that protects the catheter tip until it is actually inserted into the desired vessel.

Surgical instrumentation

Animals were instrumented under medetomidine (0.5mg/kg body weight (i.p.); Domitor®, Pfizer Animal Health) and ketamine (75mg/kg body weight (i.p.); Imalgene 1000®, Mérial, Lyon, France) anesthesia with indwelling blood pressure telemeters (model TA11PA-C40, Data Sciences Corporation, St. Paul, MN, US).

The animals were provided with preoperative analgesia, using the opioid analgesic drug butorfanol (1mg/Kg/mL (s.c.); Dolorex®, Intervet International GmbH, Unterschleissheim, Germany), in order to ensure pain relief during and after surgery. The animal's body temperature was maintained throughout the experimental procedure using a heating pad. To minimise corneal desiccation, the eyes were lubricated with sterile saline. In preparation for abdominal incision, this area was shaved with a clipper, scrubbed with a povidone-iodine solution (Betadine®, Mundipharma AG, Basel, Switzerland) and 70% isopropyl alcohol, and covered with a sterile surgical drape. The aseptic conditions of the microsurgical instruments were ensured by autoclaving and maintained at all times with the use of a hot bead sterilizer (Fine Science Tools GmbH, Heidelberg, Germany).

The radiotelemetry transmitter was implanted aseptically into the abdominal aorta, exposed via a ventral midline incision in the abdominal cavity. The aorta was occluded using bulldog type clamps (Fine Science Tools GmbH, Heidelberg, Germany) placed immediately after renal artery bifurcation and immediately before femoral artery bifurcation (Figure 6).

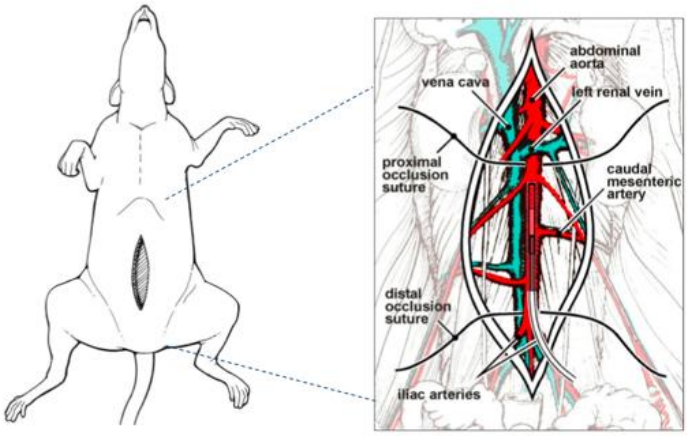


Figure 6: Surgical procedure for abdominal aorta cannulation with intraperitoneal cavity device placement (DSI, St. Paul, MN, US).

A bent needle (20G, B.Braun Medical, Melsugen, Germany) was used as a catheter introducer and the pressure tip of the telemeter was guided beneath the needle and introduced into the aorta. The puncture site and the surrounding tissue were dried and a small drop of tissue adhesive (Vetbound, 3M Company, St Paul, MN, USA) was applied to seal the insertion point. The vessel clamps were carefully removed and, if there was no bleeding, the intestines were replaced in their original position and the abdominal cavity was flushed with warmed sterile saline to avoid tissue adhesion. The body of the transmitter was then placed on top of the intestines and secured to the abdominal muscle by closing the abdominal incision and incorporating the suture rib on the device into the closure, using nonabsorbable sutures (Surgisilk 4/0, Sutures Ltd, Wrexham Wales UK) in a simple interrupted pattern (Figure 7). Finally, the skin incision was closed using nonabsorbable suture (Surgisilk 4/0, Sutures Ltd UK).

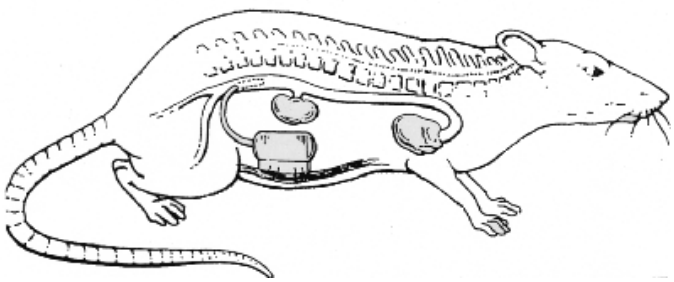


Figure 7: Intraperitoneal placement of the transmitter (DSI, St. Paul, MN, US).

Thirty minutes after surgery, the anaesthesia was reversed with atipamezole (0.25mg/Kg/2mL (i.p.); Antisedan®, Orion Pharma, Espoo, Finland). All animals were individually housed, with

environmental enrichment, to protect the wound sites and minimise stress. A heating pad on a low setting was provided to allow the animals to self-regulate temperature in the first hours after surgery. Postsurgical recovery was monitored by daily visual examination (return of normal postures and behaviours) and daily food and water intake. Rats were treated postoperatively for 2-3 days with the nonsteroidal anti-inflammatory drug carprofen (5 mg/Kg/1mL (s.c.), Rimadyl®, Vericore Limited, Dundee, UK).

CIH exposure

Animals were kept in a eucapnic atmosphere, inside medium A-chambers (76 x 51 x 51 cm, A-60274-P, Biospherix Ltd, NY, USA; Figure 8) with *ad libitum* food and water access. The chambers were equipped with gas injectors and sensors for oxygen (O_2) and carbon dioxide (CO_2) levels in order to ensure the accuracy of CIH cycles. Accumulation of CO_2 was prevented by the continuous flow of the gas mixtures, by the circulation of the gases inside de chambers through vent holes and by the presence in the chamber of self-indicating soda lime (AnalaR Normapur®, VWR International BVBA, Leuven, Belgium), which absorbs the expired CO_2. The CO_2 levels inside the chambers never exceeded 1%. A silica gel (Chameleon® C 2-6 mm, VWR International BVBA, Leuven, Belgium) container was also placed inside the chambers in order to absorb water. Oxygen concentration inside the chambers was controlled using 100% nitrogen (N_2) and 100% O_2 via electronically regulated solenoid switches in a three-channel gas mixer, which gradually lowered the oxygen in the chamber from 21% to 5% O_2 (OxyCycler AT series, Biospherix Ltd, NY, USA; Figure 8). O_2 was purchased as regular gas bottles (Gasin, Portugal), while N_2 was generated from the air using pressure swing adsorption technology via a high output nitrogen generator (Nitrogen 15 Plus, PSA Technology, Sysadvance, Maia, Portugal; Figure 8).

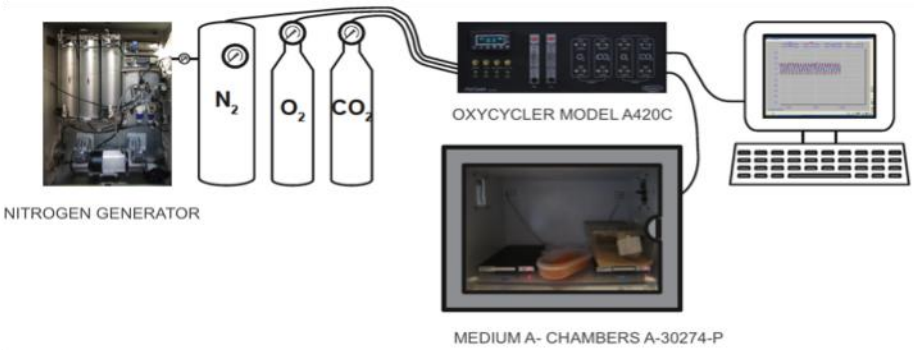


Figure 8: Typical Oxycycler AT Series System (Biospherix Ltd, NY, USA) setup.

The chambers were infused with 100% N_2 for 3.5 min to briefly reduce the O_2 concentration to 5%, and then with 100% O_2 for 7 min to restore oxygen to ambient levels of 21%, until the start

of the next CIH cycle. Each CIH cycle lasted 10.5 min and the rats were exposed during their sleep period (light period of light/dark cycle) to 5.6 CIH cycles/h, 10.5 h/day, for 60 days. During the remaining hours of the day, the chambers were ventilated with a constant flow of room air to keep oxygen levels at 21% (Figure 9).

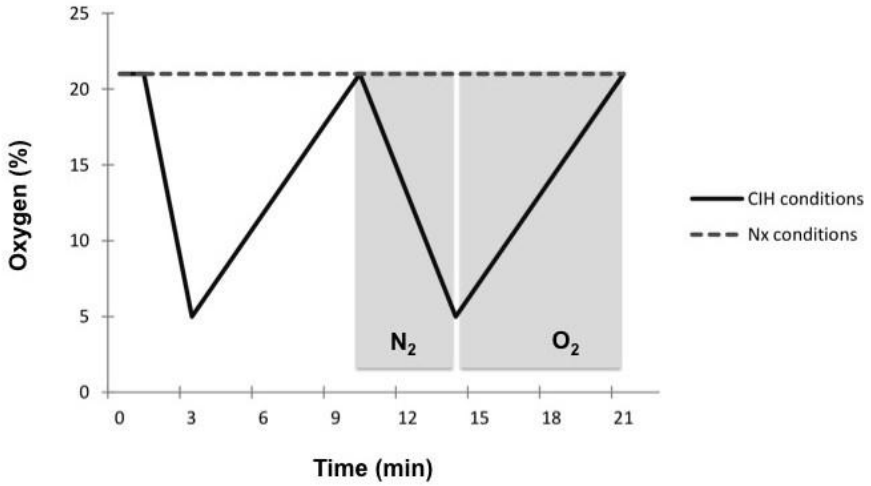


Figure 9: Graphical representation of oxygen levels inside the CIH chambers. Boxes represent the periods of chambers infusion with 100% N_2 or O_2 and correspond to one CIH cycle (10.5 min). Exposure to CIH occurred 10.5 h/day, for 60 days during rats sleep period. **CIH:** Chronic intermittent hypoxia; **Nx:** normoxia.

Blood sampling

Blood was collected to vacutainer tubes containing EDTA (ethylenediaminetetraacetic acid), for plasma sampling, kept on ice and immediately centrifuged at 3000 rpm (4 °C) for 10 min. Plasma samples were stored at −80 °C until analysed.

High-performance liquid chromatography (HPLC) analysis

R-(+)-CVD and S-(-)-CVD plasma concentrations were determined by HPLC, using a minor modification of the method described by Peccinini and colleagues (Peccinini *et al.*., 2008). Briefly, R-(+)-CVD and S-(-)-CVD were dissolved in methanol yielding stock solutions of 1 mg/mL. Calibration samples were prepared by adding known amounts of the diluted stock solution to rat plasma, and covered a range of 10 - 300 ng/mL. Subsequently, calibration and plasma samples (250 µL) were spiked with 12.5 µL of an aqueous solution of 0.1M sodium hydroxide and extracted with 3 mL chloroform, for 20 minutes, in a vertical shaker. After centrifugation (1800 g, 4 ºC, 4 min), the organic phase was evaporated to dryness at 60 ºC in a Speed-Vac concentrator (Labconco, Kansas City, MO, USA). The resulting residue was reconstituted in 50 µL of 0.1 M sodium hydroxide, and 50 µL of the chiral reagent MCF, at 1% concentration in dichloromethane (v/v), was added. The mixture was shaken in a vortex for 2 min. After adding 250 µL of water, carvedilol diastereoisomers were extracted with 3 mL

chloroform for 10 min in a vertical shaker. A new centrifugation was performed and the organic phase was evaporated to dryness. Finally, the residue obtained was reconstituted in 50 μL of the mobile phase and 30 μL was injected into the HPLC. Plasma samples were diluted in the mobile phase whenever appropriate.

HPLC (Schimadzu, Kyoto, Japan) was performed using a solvent delivery pump (model LC 9-A), autosampler (model 7725i; fluorescence detector: model RF 10AXL), and a column oven (model CTO-10AS VP). The stationary phase was a column (250 x 4 mm; particle size: 5 μm; LiChrospher 100 RP-18; Merck, New Jersey, USA) protected by a guard-column (4 x 4mm; particle size: 5 μm; LiChrospher 100 RP-18e; Merck, New Jersey, USA). The mobile phase consisted of a mixture of 0.25N acetate buffer (pH 3, adjusted with acetic acid) and methanol (27:73 v/v) at a flow rate of 1mL/min, at 25º C. Both solutions were degassed for 15 min by sonication (VWR, Carnaxide, Portugal). The excitation wavelength was 285 nm and the emission wavelength 680 nm. Data acquisition and processing were performed using Shimadzu Class VP 7.X software.

Voluntary oral administration as an alternative method to gavage

Drugs and chemicals

Carvedilol at 10 mg/kg kindly provided by Tecnimede (Sintra, Portugal; manufacturer batch: 5334-11-015) and losartan at 10 mg/kg, from Cinfa, S.A (generic, Pamplona, Navarra, Spain), were mixed with the three vehicles and administered to the animals. Analytical standards of carvedilol and losartan, for HPLC analysis, and methylcellulose (MC) were purchased from Sigma-Aldrich (Sintra, Portugal).

Experimental design

Experiments were performed in forty-one male Wistar rats, aged 2-3 months and with mean body weight 283 ±5.0 g.

Three different vehicles were tested for long-term oral administration (14 days) of two antihypertensive drugs (carvedilol: non-cardioselective beta blocker; losartan: angiotensin II receptor (type AT1) antagonist). Based on their consistency, palatability and previous reports (Abelson *et al.,* 2012; Goldkuhl *et al.,* 2010; Jacobsen *et al.,* 2011; Kalliokoski *et al.,* 2011; Isaksson *et al.,* 2011; Corbett *et al.,* 2012), nut paste (NUT, Nutella ®, Ferrero Ibérica SA, Llobregat, Spain), peanut butter (PB, Skippy ®, Unilever, London, UK) and sugar dough (SD, SweetArt ®, Entertraining Lda, Lisboa, Portugal) were used. NUT ingredients include sugar, vegetable oil, hazelnuts (13%), skim milk powder (8.7%), fat-reduced cocoa powder (7.4%), soy lecithin (emulsifier) and vanillin (flavouring). The caloric content is 21.75 kJ/g; protein

content is 7.1%; fat content 30.3% and sugars content is 54.7%. PB is composed of roasted peanuts, sugar, hydrogenated vegetable oils and salt. The caloric content is 26.10 kJ/g; protein content is 22.2%; fat content 49.4% and carbohydrate content is 23.8%. Finally, SD presents as ingredients sugar, cornstarch, glucose, vegetable fat, vegetable gums, stabilizer E420, preservatives E202, dye E102 and E131. The caloric content is 16.50 kJ/g; protein content is 3.5%; fat content 6.6% and carbohydrate content is 86.6%.

Rats were randomized following weaning into six groups of six rats each: group 1 (NUT - 28 days + NUT with 10 mg/kg/day carvedilol - 14 days); group 2 (PB - 28 days + PB with 10 mg/kg/day carvedilol - 14 days); group 3 (SD - 28 days + SD with 10 mg/kg/day carvedilol - 14 days); group 4 (NUT with 10 mg/kg/day losartan - 14 days); group 5 (PB with 10 mg/kg/day losartan - 14 days); group 6 (SD with 10 mg/kg/day losartan - 14 days) and two groups of five rats each: group 7 (10 mg/kg/day losartan administered by oral gavage - 14 days) and group 8 (10 mg/kg/day carvedilol administered by oral gavage – 14 days).

All rats used to test voluntary administration (groups 1 to 6) underwent a 2-day acclimatization period, during which they were fed 0.5 g of the respective vehicle once daily, in order to minimize neophobia (Abelson *et al.*, 2012) and avoid incomplete ingestion incidents. Drug or vehicles deliveries would be considered incomplete when any trace of vehicle was found in either Petri dish, side of the cage or in bedding, after the first hour.

In the first set of experiments (groups 1, 2 and 3) the vehicles were administered without drug for 28 days (in addition to the acclimatization period) in order to determine whether rats would voluntarily ingest them for such period of time and also to test the vehicles effect on blood glucose level and serum lipid profile. These animals were further used to test voluntary oral administration of carvedilol for additional 14 days. The vehicles were weighed, mixed with the respective amount of drug and offered to the animals in 92x16 mm polystyrene Petri dishes (Sarstedt AG & Co, Germany). The Petri dishes were placed vertically, attached with adhesive tape to the inner cage wall (Figure 10).

The vehicles, alone or mixed with the drug, were daily given to the animals at approximately the some schedule (11:00 to 12:00 am). The administration schedule as well as the ingestion profile (1- complete; 2- incomplete) and the time between vehicle and vehicle/drug mixture delivery and consumption (1- within 5 minutes or less; 2-between 5 minutes and 1 hour; 3- > 1 hour) were recorded.

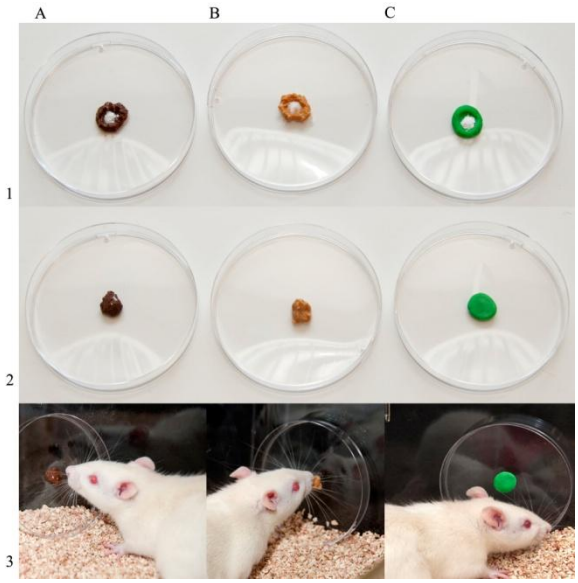


Figure 10: Method for voluntary ingestion of carvedilol and losartan with three different vehicles: nut paste **(A);** peanut butter **(B)** and sugar-dough **(C)**. **(1)** Carvedilol or losartan powder weighted directly over vehicle. **(2)** Vehicle-drug mixture shaped into a ball ready for delivery. **(3)** Rats ingesting vehicle-drug mixture from the polystyrene Petri dish placed vertically in the cage.

The animals of groups 7 and 8 underwent a 7 days handling acclimatization period. Rats were handled daily for a period of 2 minutes each by the same individual and accustomed to the gavage position, in a different animal facility procedures room. Handling training and gavage were performed at the same schedule (11:00 to 12:00 am) as for voluntary administration. Gavage was performed using a stainless steel gavage feeding needle, curved and with round tip (gauge 16; tip diameter: 3 mm; length: 75 mm; Fine Science Tools, California, USA).

Rats were weighed at baseline and once a week during the entire study. The amounts of carvedilol and losartan were adjusted weekly to ensure a daily dose of 10 mg/kg. Drugs were prepared daily, immediately before administration, when to be given either by gavage or along with vehicle. For gavage, Losartan was dissolved in reverse osmosis water (2 mL) and carvedilol in MC 0.5% (2 mL). When drugs were administered along with vehicle, the required amount of drug for each rat was mixed with 0.5 g of the respective vehicle. In the first set of experiments (groups 1, 2 and 3), water intake was measured weekly and blood glucose level and serum lipid profile were monitored, 2-3 hours after vehicle administration, at baseline, day 14 and day 28.

At the end of the experiments, 2-3 hours after drug delivery, rats were anaesthetised, by intraperitoneal injection with medetomidine (0.5mg/kg body weight; Domitor®, Pfizer Animal Health) and ketamine (75mg/kg body weight; Imalgene 1000®, Mérial, Lyon, France), and cardiac puncture was performed to collect blood for further drug quantification. The animals

were then euthanized with an intracardiac overdose of sodium pentobarbital, and the death was confirmed by cervical dislocation.

Blood sampling

Tail vein sampling: In groups 1, 2 and 3, blood samples were collected from the tail vein of conscious animals at baseline, day 14 and day 28. Rats were placed in a conventional plastic restrainer (n° 554-BSRR, Plas-Labs, Lansing, Michigan, USA), at a room temperature 24-27ºC. A scalpel blade was used to make an incision in the lateral tail vein. Around 100 µL of blood was collected from each animal, using a capillary tube (Hirschmann Laborgerate, Eberstadt, Germany), to eppendorfs and kept on ice. This procedure was also used for collecting two blood drops for glucose quantification. After collection, bleeding was stopped by compression for a few seconds and cleaned with iodopovidone. Serum samples were centrifuged in a microfuge at 3000 rpm (4º C) for 10 minutes, immediately decanted and stored at −80 °C until analysis. According to prior recommendations, the restrainer was washed and dried after each use to prevent pheromonally induced stress (Parasuraman *et al.,* 2010).

Cardiac puncture: Blood collection was performed without thoracotomy with a 20 G needle with a 10 mL syringe. For plasma sampling, approximately 3 mL of blood was collected to vacutainer tubes containing EDTA (ethylenediaminetetraacetic acid), kept on ice and immediately centrifuged at 3000 rpm (4 °C) for 10 min. Plasma samples were decanted prior to storage at −80 °C until analysis.

Measurement of blood glucose level and serum lipid profile

Blood glucose level was determined in mg/dl using a digital glucometer (Precision Xceed®, Abbott diabetes care Ltd, Oxon, UK). The serum levels of triglyceride (TGL), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein- cholesterol (LDL-C) were determined spectrophotometrically (Rx Daytona analyser, Randox Laboratories Ltd, UK), using enzymatic colorimetric assay kits (Randox Laboratories Ltd, UK). The results were expressed in mg/dL.

High-performance liquid chromatography (HPLC) analysis

Since all rats refused to ingest the mixture of carvedilol with the three different vehicles, we only set up a method for the quantification of losartan. Losartan plasma concentrations were determined by HPLC, using a minor modification of the method described by Yeung *et al.* (Yeung *et al.,* 2000). Briefly, losartan was dissolved in methanol yielding a stock solution of 1 mg/mL. Calibration samples were prepared by adding known amounts of the diluted stock solution to rat plasma and covered a range of 1 - 10 µg/mL. Subsequently, calibration samples

(200 µL) were extracted with 3 mL of ethyl acetate. After centrifugation (3000 rpm, 4 ºC, 10 min), the organic phase was evaporated to dryness at 60 ºC in a Speed-Vac concentrator (Labconco, Kansas City, MO, USA). The resulting residue was reconstituted in 150 µL of mobile phase and 100 µL was injected into HPLC.

HPLC was accomplished by using a solvent delivery pump (model LC 9-A; Schimadzu, Kyoto, Japan), autosampler (model 7725i; Schimadzu, Kyoto, Japan, UV-VIS spectrophotometric detector (model SPD-6 AV; Schimadzu, Kyoto, Japan.), column (250 x 4 mm; particle size: 5 µm; LiChrospher 100 RP-18; Merck, New Jersey, USA) protected by a guard-column (4 x 4mm; particle size: 5 µm; LiChrospher 100 RP-18e; Merck, New Jersey, USA) and a column oven (model CTO-10AS VP; Schimadzu, Kyoto, Japan). Data acquisition and processing were performed using Shimadzu Class VP 7.X software. The mobile phase consisted of solution A - methanol: acetonitrile: sodium dihydrogen phosphate monohydrate buffer (10mM; pH 3; 50:10:40; v/v/v) and solution B - methanol. All HPLC solvents were purchased from VWR International (Carnaxide, Portugal). Prior analysis, both solutions were degassed for 15 min by sonication (VWR, Carnaxide, Portugal). The elution program consisted of an isocratic flow of A solution for (9.5 min), followed by a linear gradient A:B (20:80 (v/v); 2.5 min), an isocratic step A:B (20:80; 4 min) and finally return to initial conditions in 2 min. The analytical run was performed with a mobile phase flow rate 0.8ml/min, at 25º C and detection wavelength of 230 nm.

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM). Unpaired t-test and Kruskal-Wallis test with Dunn´s multiple comparisons test were used, whenever appropriate, to evaluate the effect of CIH, CVD or MC 0.5% on the cardiovascular parameters. A comparison of CVD S/(R+S) plasma ratios between normoxic and CIH groups was performed using unpaired Student t-test. One-way ANOVA with Bonferroni's multiple comparison test and one-way analysis of variance with Tukey's multiple comparison tests were used, whenever appropriate, to evaluate the vehicles effect on glycaemia and lipid profile. The two-way repeated-measures ANOVA with post hoc comparison test was used to compare the vehicle effect on mean animal body weights and water intake, along the 28 days of experiments. Comparison in losartan plasma concentrations between vehicle groups was performed using one-way ANOVA with Dunnett's multiple comparison post hoc tests, using gavage as control.

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., version 5.01, San Diego, CA). Statistical significance for all tests was set at the level of $p<0.05$.

IV

RESULTS

SECTION 1

Section 1 presents the key results from the clinical studies carried out in the Department of Pneumology II, Centro Hospitalar Lisboa Norte (Hospital Pulido Valente), EPE.

The results obtained in this section originated one publication in *Blood Pressure Monitoring* and a manuscript that is currently accepted for publication in *Advances in Experimental Medicine and Biology*. The main findings of these works are listed bellow:

Neck circumference and body mass index as independent predictors of hypertension misclassification in patients suspected of having obstructive sleep apnea.
Diogo LN, Pinto P, Bárbara C, Monteiro EC, Papoila AL. *Blood Press Monit. 2015 Feb;20(1):8-15. doi: 10.1097/MBP.0000000000000080.*

1) 43.4% (53/122) of the patients misclassified themselves as non-hypertensive (*see Table 1, page 64*).
2) Patients that misclassified themselves as non-hypertensive presented significantly higher values of ABPM variables and anthropometric characteristics, compared to truly non-hypertensive patients (*see Table 1 and 2, pages 64 and 65*).
3) The cut points that best discriminate these two groups of patients were 27 Kg/m^2 and 39 cm for BMI and NC, respectively (*see Figure 3, page 66*).
4) BMI and NC were identified as independent predictors of hypertension misclassification in patients suspected of OSA (*see Table 3, page 66*).

The association between antihypertensive medication and blood pressure control in patients with obstructive sleep apnea.
Diogo LN, Pinto P, Bárbara C, Papoila AL, Monteiro EC. *Adv Exp Med Biol (in press).*

1) The prevalence of new diagnoses of OSA among those patients that attended the outpatient consultation of the CHLN sleep unit was 78.2% (283/362) (*see Figure 1, page 72*).
2) 63.9% (205/321) of the patients included were diagnosed with both OSA and hypertension (*see Figure 1, page 72*).
3) One hundred and fifty-five (155/205) patients were under antihypertensive medication and 31 different antihypertensive regimens were found (*see Table 3, page 76*).
4) 57.5% (95/155) of patients under antihypertensive medication had uncontrolled BP.
5) BP control is independent of both the antihypertensive regimen and the number of antihypertensive drugs (*see Table 4, page 77*).

6) The lack of association between antihypertensive regimens and the number of antihypertensive drugs and BP control remained after CPAP adaptation (*see Table 4, page 77*).
7) A decrease in median night-time systolic and diastolic BP was observed after CPAP adaptation (*see Table 5, page 78*). However, this decline was not enough to allow reclassification from uncontrolled to controlled BP in a substantial number of patients under AHDs.
8) Male gender, moderate/severe and non-dipper patients were independently associated with uncontrolled BP after CPAP adaptation (*see Table 6, page 78*).

Neck circumference and body mass index as independent predictors of hypertension misclassification in patients suspected of having obstructive sleep apnea

Lucília N. Diogo[a], Paula Pinto[b], Cristina Bárbara[b], Emília C. Monteiro[a] and Ana L. Papoila[a,c]

Objective(s) **Twenty-four-hour ambulatory blood pressure monitoring (ABPM) seems to be the most accurate way of diagnosing hypertension in patients with obstructive sleep apnea (OSA). However, this diagnostic tool is expensive and time-consuming and is therefore not used routinely. We aimed to find baseline predictors that enable the identification of patients who misclassify themselves as nonhypertensive to optimize the use of ABPM.**

Methods **Clinically suspected OSA patients ($n = 369$) were enrolled and underwent overnight polysomnography and 24-h ABPM, and completed a data collection form. Anthropometric measurements were assessed. Generalized additive models, the minimum *P*-value approach, and logistic regression models were used for data analysis. Results were considered significant when α is equal to 0.05.**

Results **One hundred and twenty-two patients who were not on antihypertensive medication did not report hypertension, but according to ABPM data, 43.4% ($n = 53$) of them had misclassified themselves as nonhypertensive. These patients had a significantly higher apnea–hypopnea index ($P < 0.001$), ABPM variables ($P < 0.001$), BMI ($P = 0.002$), and neck circumference (NC) ($P = 0.002$) than nonhypertensive patients ($n = 69$). BMI and NC emerged as independent predictors of hypertension misclassification. The cut-off points that best discriminated the two groups of patients were 27 kg/m^2 and 39 cm for BMI and NC, respectively. The resulting binary BMI and NC remained independent predictors of hypertension misclassification in the final model (odds ratio: 3.2, $P = 0.010$; odds ratio: 2.4, $P = 0.038$).**

Conclusion **Our findings emphasize the importance of ABPM for the diagnosis of hypertension in patients suspected of having OSA with a BMI and NC above 27 kg/m^2 and 39 cm, respectively. *Blood Press Monit* 00:000–000**

Blood Pressure Monitoring 2014, **00**:000–000

Keywords: ambulatory blood pressure monitoring, body mass index, hypertension, neck circumference, obstructive sleep apnea

[a]CEDOC, NOVA Medical School, NOVA University, [b]Pneumology Service II, Centro Hospitalar Lisboa Norte, EPE - Hospital Pulido Valente and [c]CEAUL, Lisbon, Portugal

Correspondence to Lucília N. Diogo, PharmD, MSc, NOVA Medical School, NOVA University, Campo dos Mártires da Pátria, 130 1169-056 Lisbon, Portugal Tel: +351 218803035; fax: +351 218803083; e-mail: lucilia.diogo@fcm.unl.pt

Received 31 March 2014 **Revised** 20 June 2014 **Accepted** 11 July 2014

Introduction

Hypertension and obstructive sleep apnea (OSA) are two prevailing risk factors for several cardiovascular events [1]. Although OSA has been associated with several cardiovascular conditions, it has been linked more etiologically to hypertension [2]. The link between hypertension and OSA is now widely accepted, although the underlying mechanisms remain unclear and several questions remain unanswered [3]. The estimated overall prevalence of hypertension among patients with OSA is ~50% and an estimated 30–40% of hypertensive patients are diagnosed with OSA [4]. Moreover, OSA has been identified as an independent risk factor for hypertension [5] and clinical prediction rules that include features such as hypertension, as well as anthropometric measurements, showed moderate to high sensitivity and specificity in identifying individuals at risk of moderate to severe OSA [6].

Continuous positive airway pressure (CPAP) is considered the gold standard treatment for mild, moderate, and severe OSA [7]. CPAP treatment is indicated for cases of moderate to severe OSA and also for patients with mild OSA accompanied by symptoms of excessive daytime sleepiness, impaired cognition, mood disorders, insomnia, or documented cardiovascular diseases, namely, hypertension [7]. Thus, during the diagnosis of clinically suspected OSA patients, screening for the presence of hypertension should be mandatory. In fact, many more patients with mild sleep apnea would be prescribed CPAP if ambulatory blood pressure monitoring (ABPM) was used to define hypertension in clinical practice.

Nevertheless, hypertension is frequently unrecognized in patients with OSA [1] and it is important to highlight that patients with elevated blood pressure (BP) who do not carry the diagnosis of hypertension may have been

1359-5237 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/MBP.0000000000000080

misclassified as nonhypertensive. Consequently, aggressive control of BP is warranted in OSA patients and an accurate method for BP measurement should be used in the early diagnosis of clinically suspected OSA patients. Twenty-four-hour ABPM seems to be superior to office BP measurement and home BP monitoring (HBPM) for the diagnosis of hypertension in patients with suspected OSA [3]. However, ABPM is a time-consuming, labor intensive, and expensive diagnostic tool and is thus not used routinely.

We hypothesized that anthropometric measures are associated with hypertension misclassification in patients suspected of OSA. Therefore, this cross-sectional study aimed, first, to find new predictors on the basis of anthropometric measures to identify patients who misclassify themselves as nonhypertensive, and thereby promote the selective use of ABPM. Second, the study aimed to highlight the relevance of ABPM in diagnosis and therapeutic decision-making for patients suspected of having OSA.

Methods

Patients

Three hundred and sixty-nine consecutive patients who were attending their first visit to the Centro Hospitalar Lisboa Norte, EPE Sleep Unit, following referral by their general practitioner or other specialist, were enrolled. Patient inclusion started in April 2010 and was completed in July 2012. All patients older than 18 years of age were considered eligible for the study and underwent overnight ambulatory polysomnography and 24-h ABPM, and completed a data collection form. Exclusion criteria included severe psychiatric disease or inability to understand the information required for an informed consent and previous diagnosis of hypertension (Fig. 1). All patients were fully informed about the study and provided written consent in accordance with the Declaration of Helsinki. The study protocol was approved previously by the ethics committee of CHLN, EPE.

Data collection form and clinical assessment

Sociodemographic and anthropometric data, as well as comorbidities and ongoing medication profile, recorded using the 'Brown-Bag' medication review (a method in which patients are asked to bring all their medications to each visit) [8], were recorded.

All patients were assessed for weight, height, neck circumference (NC), and waist circumference (WC) using standardized protocols. Weight was measured, with lightweight clothing and without shoes, to the nearest 100 g and height was assessed, with no shoes, to the nearest 0.5 cm, in both cases using calibrated instruments. BMI was calculated as body weight in kilograms divided by the square of height in meters (kg/m^2). NC, measured in the middle of the neck, at the level of the cricothyroid membrane (in men with a laryngeal prominence, it was measured just below the prominence), to within 0.1 cm, with the patient's head positioned in the Frankfort horizontal plane, and WC, assessed at the level of the umbilicus to within 0.1 cm, were also recorded using a flexible nonstretchable measuring tape.

Comorbidities, with a focus on hypertension, were assessed. Hypertension was defined as self-reported hypertension (answer 'yes' to the question 'Have you ever been told by a doctor that you have high blood pressure?') or use of antihypertensive medication.

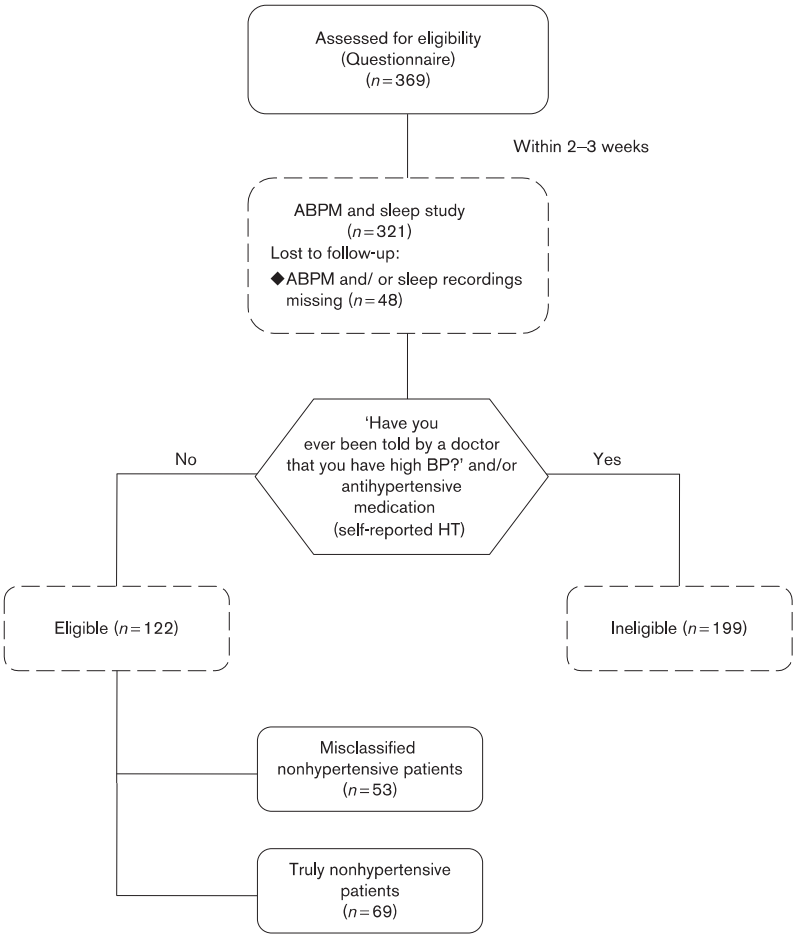
Sleep evaluation

Patients underwent an overnight in-laboratory polysomnography (fully diagnostic study) or, as an alternative for the diagnosis of OSA in patients with a high pretest probability of moderate to severe OSA, a cardiorespiratory sleep study at home [9]. All patients with dubious results attained from home studies were further subjected to a confirmation by a fully diagnostic polysomnography. In-laboratory polysomnographic studies were carried out using a multichannel polygraph (Model Embla S7000; Embla Systems Inc., Broomfield, Colorado, USA) and ambulatory recordings were made using a validated portable digital recording unit (type 3) with sensors for the registration of airflow, saturation, respiratory movements of the chest, body position, and snoring sounds (Embletta PDS device; Embla Systems Inc.). Sleep recordings were scored manually on the basis of standard criteria [9] by trained technicians. Apnea was defined as a complete cessation of airflow for at least 10 s, associated with oxygen desaturation of 3%. Hypopnea was defined as a discernible reduction in airflow of at least 50% for 10 s or more, with an accompanying desaturation of at least 3% or an arousal. The number of apneas and hypopneas per hour of sleep was calculated and, for both in-laboratory and ambulatory recordings, the apnea–hypopnea index (AHI) was considered. Respiratory effort-related arousals, scored using in-laboratory polysomnography sleep staging, were not considered to minimize the differences between the results of the two diagnostic approaches. OSA was categorized according to current AHI cut-offs of less than 5 (nondiagnostic), $5 \geq$ and < 15 (mild), $15 \geq$ and < 30 (moderate), and at least 30 (severe) [9].

Twenty-four-hour ambulatory blood pressure monitoring

Twenty-four-hour ABPM was performed at baseline on a different day to that of the sleep evaluation. Screening BP measurements were performed in accordance with the recommendations of the European Society of Hypertension and the European Society of Cardiology guidelines (2007) [10] using a noninvasive portable validated BP recorder (Spacelabs Model 90217; Spacelabs Healthcare, Redmond, Washington, USA). The equipment was programmed for cuff inflation every 20 min

Fig. 1



Flowchart of the study protocol. Of the 369 patients consecutively included, ABPM and/or sleep recordings were missing for 48 because of technical problems or because they did not attend the ABPM or the sleep study. One hundred and twenty-two patients who were not taking antihypertensive medication answered 'No' to the question 'Have you ever been told by a doctor that you have high blood pressure?' and the remaining 199 answered yes or were under antihypertensive medication. Of the 369 patients, only 122 were considered eligible for the study (misclassified nonhypertensive patients $n=53$; nonhypertensive patients $n=69$). ABPM, ambulatory blood pressure monitoring.

during the daytime period and every 30 min at night-time. Daytime and night-time were predetermined individually depending on the participants' usual awake and sleep schedule. The data were considered valid when a minimum of 70% of the measurements were recorded without errors. BP recordings were analyzed for the overall 24-h period, plus the daytime and night-time periods. The 24-h BP profile was also assessed and patients were classified as showing a dipping or a non-dipping profile. A dipping profile was defined as a reduction in the average systolic BP (SBP) and diastolic BP (DBP) at night of more than 10% compared with daytime values. The data were analyzed by an experienced cardiorespiratory technician. Uncontrolled BP was defined according to ESC and ESH guidelines [10]. BP was considered uncontrolled for SBP values of 135 mmHg or above, DBP values of 85 mmHg or above, or both during daytime, or SBP values of 120 mmHg or above, DBP values of 70 mmHg or above, or both during night-time [10].

Statistical analysis

An exploratory analysis was carried out for all variables. Categorical data were expressed as frequencies and

percentages, and continuous variables as mean or median, SD, or interquartile range (25th percentile–75th percentile). Univariable analysis was carried out using Student's *t*-test and nonparametric (Fisher's exact test, χ^2 and Mann–Whitney *U*-test) tests as appropriate. Spearman's ρ rank correlation was used to investigate the association between the anthropometric variables.

For the categorization of NC and BMI, the minimum *P*-value approach was applied [11]. On the basis of a systematic search for the 'best' cut-off point, this method obtains the point from a grid of marker values that is associated with the minimum *P*-value, which corresponds to the maximum χ^2-test value. Generalized additive models [12] for binary response were used to confirm the cut-off points obtained using the minimum *P*-value approach.

A logistic regression model was fitted to the data to determine the relevance of AHI and BMI in nonhypertensive misclassification. The outcome binary variable was 'misclassified non-hypertension' (yes: for patients who classified themselves erroneously as nonhypertensive, no: for truly nonhypertensive patients). The previously obtained cut-off points were used to discretize the continuous NC and BMI, and the new categorical variables were then used as independent variables in the logistic regression model. The predictive ability of the resulting model was analyzed using the Hosmer–Lemeshow goodness-of-fit test [13]. This test was used to compare the observed and expected frequencies of patients who misclassified themselves as nonhypertensive on the basis of the values of the estimated probabilities obtained by the logistic regression model. A high *P*-value would indicate that the model was performing well. The area under the receiver operating characteristic curve was used to evaluate the discriminative ability of the model. A value of 0.50 would be obtained when a model discriminates no better than chance, and a value of 1.0 indicates perfect accuracy.

Results were considered significant when α is equal to 0.05. Confidence intervals are presented when appropriate (95% CI). Statistical analysis was carried out using the IBM SPSS Statistics, version 19.0 (IBM Corp., Armonk, New York, USA), and R software (R Foundation for Statistical Computing, Vienna, Austria) [14].

Results

Patient characteristics and ABPM and sleep data

Patients were mainly men (65.9%), White (95.7%), mean age 55.9 (SD = 13.1) years. One hundred and twenty-two patients under no antihypertensive medication answered 'No' to the question 'Have you ever been told by a doctor that you have high blood pressure?'. According to ABPM measurements, 53 (43.4%) of these patients had misclassified themselves as nonhypertensive and the remaining 69 (56.6%) patients were actually nonhypertensive. Table 1 presents key patient characteristics at baseline summarized by group [misclassified nonhypertensive patients (MNH): $n = 53$; nonhypertensive patients (NH): $n = 69$]. Significant differences were found for all ABPM variables (mean 24-h BP, daytime SBP, and DBP and night-time SBP and DBP) and AHI, such that MNH patients showed, as expected, higher values for each variable.

Table 1 Baseline characteristics

Variable	MNH patients ($n = 53$)	NH patients ($n = 69$)	*P*
Age (years)	50.3 (11.8)	48.8 (13.1)	0.530*
Sex (male) [*n* (%)]	39 (73.6)	43 (62.3)	0.189†
Race, White [*n* (%)]	48 (90.6)	68 (98.6)	0.084‡
24-h ABPM (mmHg)			
Mean 24-h BP	96.0 (93.0, 98.0)	85.0 (80.5, 88.0)	< 0.001*
Daytime systolic BP	132.0 (126.5, 139.0)	118.0 (111.5, 125.0)	< 0.001*
Daytime diastolic BP	84.0 (81.5, 88.0)	75.0 (70.0, 78.0)	< 0.001*
Night-time systolic BP	116.0 (110.0, 125.5)	104.0 (97.0, 107.0)	< 0.001*
Night-time diastolic BP	72.0 (68.5, 76.5)	61.0 (58.0, 65.0)	< 0.001*
Sleep data			
AHI (events/h)	16.6 (5.7, 34.5)	8.4 (2.7, 13.9)	< 0.001*

Data are presented as mean (SD), median (P_{25}, P_{75}), median (minimum–maximum), or number (%).
ABPM, ambulatory blood pressure monitoring; AHI, apnea–hypopnea index; BP, blood pressure; MNH, misclassified nonhypertensive patients; NH, nonhypertensive patients.
*Mann–Whitney test.
†χ^2-test.
‡Fisher's exact test.

Among MNH patients, 49.1% showed a nondipper systolic profile, 32.1% showed a nondipper diastolic profile, 20.8% showed isolated daytime hypertension, 35.8% showed isolated night-time hypertension, and 43.4% showed both daytime and night-time hypertension. In the MNH (in-laboratory polysomnography $n = 21$; cardiorespiratory sleep study $n = 32$) and NH (in-laboratory polysomnography $n = 36$; cardiorespiratory sleep study $n = 33$) groups, 83.0 and 63.8% were diagnosed with OSA, respectively ($P = 0.019$). In terms of OSA severity, 38.6, 20.5, and 40.9% and 65.9, 22.7, and 11.4% of the MNH and NH patients, respectively, presented mild, moderate, and severe OSA ($P = 0.005$).

Comorbidities were similar in both groups. The most prevalent was dyslipidemia (34.0 vs. 42.0%; $P = 0.364$), followed by diabetes (7.5 vs. 7.2%; $P = 1.000$).

Anthropometric measurements

The mean BMI, NC, and WC were 30.6 (SD = 5.5) kg/m^2, 41.3 (SD = 3.8) cm, and 106.3 (SD = 13.4) cm for the MNH group and 27.7 (SD = 4.4) kg/m^2, 39.0 (SD = 3.9) cm, and 99.4 (SD = 13.0) cm for NH patients. All anthropometric variables in MNH patients were significantly higher than those in NH patients ($P = 0.002$; 0.002; and 0.005, respectively). These variables were also analyzed according to sex

(Table 2). Differences between sexes were found in both groups for NC and only in NH patients for WC.

Cut-off points and baseline predictors of hypertension misclassification

A preliminary analysis was carried out to identify which baseline variables (age, sex, BMI, NC, WC, smoking habits, and comorbidities) were associated with the outcome 'misclassified non-hypertension'. Only BMI, NC, and WC were associated with the outcome variable ($P = 0.003$, 0.003, 0.007, respectively). However, as WC was highly correlated with BMI (Spearman's $\rho = 0.868$, $P < 0.001$; Fig. 2), this variable was no longer considered.

Given the importance of cut-off points in daily clinical practice, a discretization of the continuous BMI and NC was performed. As no differences were found in BMI between sexes ($P = 0.669$), the corresponding cut-off point was calculated considering the entire sample. For NC, this analysis was carried out separately for each sex because women presented a thinner neck than men (36.6 cm, SD = 3.1 vs. 41.7, SD = 3.3; $P < 0.001$).

According to the minimum *P*-value approach and generalized additive models, the BMI (Fig. 3a and b) and NC cut-off points that best discriminated MNH from NH patients were 27 kg/m^2 and 39 cm, respectively (Fig. 3c and d). The NC cut-off point was the same for both sexes.

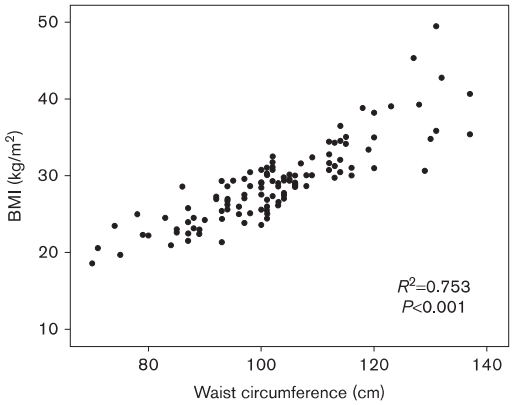
A new multivariable model was then fitted considering the outcome 'misclassified non-hypertension' and, as independent variables, the binary BMI and NC variables. The results showed that these two variables were associated independently with 'misclassified non-hypertension'. In fact, patients with a BMI higher than 27 kg/m^2 had a three-fold higher odds of being misclassified (odds ratio: 3.2, 95% CI: 1.32–7.62, $P = 0.010$) and patients with NC higher than 39 cm had approximately a two-fold higher odds of being misclassified (odds ratio: 2.4, 95% CI: 1.05–5.34, $P = 0.038$) than patients with a lower BMI or NC (Table 3). The model showed a good predictive (Hosmer–Lemeshow *P*-value = 0.311) and acceptable discriminative performance (area under the curve = 0.71; 95% CI: 0.61–0.80).

Table 2 Anthropometric data by sex

Variable	MNH patients ($n = 53$)	*P**	NH patients ($n = 69$)	*P**
BMI (kg/m^2)		0.951		0.702
Female	30.7 (6.4)		27.4 (4.6)	
Male	30.6 (5.3)		27.9 (4.3)	
NC (cm)		< 0.001		< 0.001
Female	37.7 (3.0)		36.0 (3.0)	
Male	42.4 (3.2)		40.9 (3.2)	
WC (cm)		0.641		0.042
Female	104.9 (16.4)		95.3 (13.3)	
Male	106.8 (12.3)		101.8 (12.4)	

Data are presented as mean (SD).
MNH, misclassified nonhypertensive patients; NC, neck circumference; NH, nonhypertensive patients; WC, waist circumference.
*Student's *t*-test.

Fig. 2



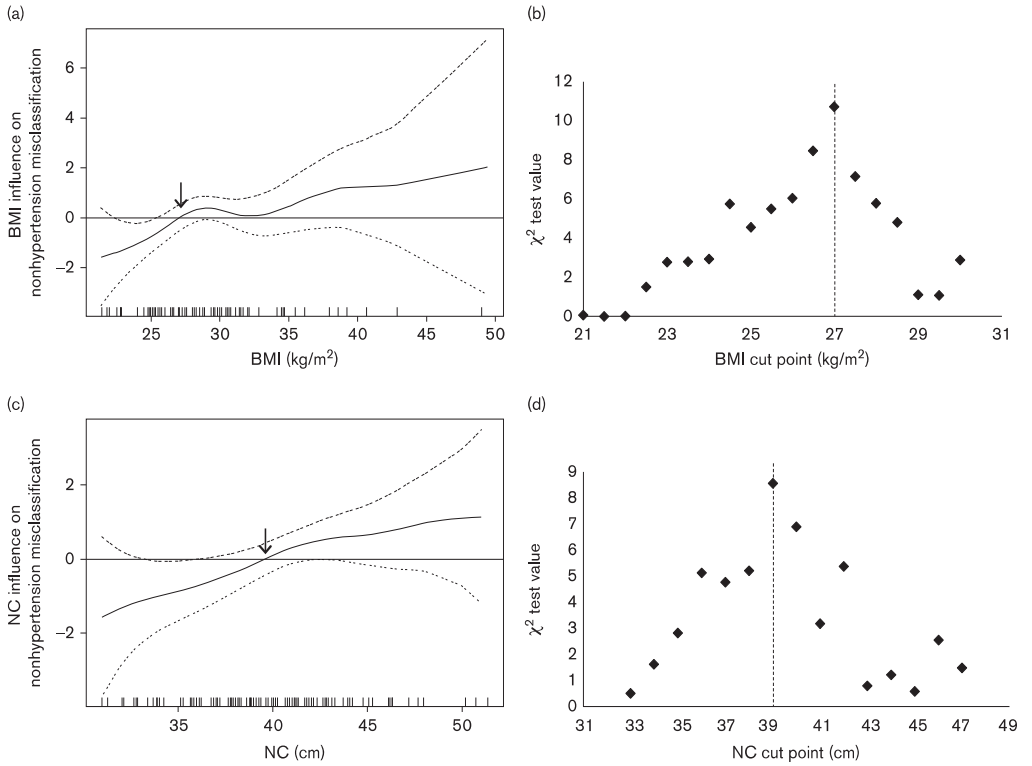
Correlation between waist circumference (WC) and BMI. R^2 indicates: square of Spearman's ρ rank correlation.

Discussion

The present study reports 24-h ambulatory BP data compared with self-reported hypertension in patients suspected of having OSA, and showed that, in our sample, a considerable number of patients misclassified themselves as nonhypertensive (43.4%). Although self-reported data are often more economically feasible and readily available compared with BP monitoring, our findings show that these reports may not provide truthful information. Such an inaccuracy will lead to an underestimation of hypertension prevalence if ABPM recordings are not taken into account. In OSA patients, this imprecision may have serious consequences as therapeutic decision-making is based, in part, on the presence of cardiovascular disease, namely, hypertension. For this reason, the validity of self-reported hypertension is highly questionable and should not replace any type of BP monitoring. However, the conclusions on this issue are far from unanimous and the number of variables that have been found to be associated with the accuracy of self-reported high BP is considerable [15].

Twenty-four-hour ABPM data showed that the frequency of a systolic or a diastolic nondipping pattern is significant in MHN patients. It is known that the lack of pressure fall at night, one of the major features of the 24-h BP profile in OSA patients [16], is itself associated with a poor cardiovascular prognosis [17]. Furthermore, Loredo *et al.* [18] showed that an absence of nocturnal dip in BP correlated inversely with the indices of sleep fragmentation, and changes in sleep architecture may be responsible for daytime hypersomnolence, the prime

Fig. 3



Influence of BMI (a) and neck circumference (NC) (c) on hypertension misclassification resulting from fitting a GAM to the data: lower values of BMI and AHI (negative values of the plotted function) have lower odds of hypertension misclassification. Minimum *P*-value approach: optimal BMI (b) and NC (d) cut-off points for misclassified nonhypertension. AHI, apnea–hypopnea index; GAM, generalized additive model.

Table 3 Multivariable analysis in which BMI and NC were identified as independent predictors of hypertension misclassification ($n = 122$)

		95% CI for OR		
	OR	Lower	Upper	*P*-value
BMI ≥ 27 kg/m^2	3.2	1.32	7.62	0.010
NC ≥ 39 cm	2.4	1.05	5.34	0.038

CI, confidence interval; NC, neck circumference; OR, odds ratio.

feature of OSA [1]. Thus, clinical observation of dipping or nondipping profiles is highly relevant, mainly for patients clinically suspected of having OSA.

ABPM also showed that most MNH patients presented isolated night-time hypertension or both daytime and night-time hypertension. In a study carried out by Burr *et al.* in 2008 [19], night-time BP was identified as the strongest predictor of cardiovascular mortality. Thus, the early recognition of nocturnal features, mainly recognized through the use of ABPM, is unquestionably relevant to improve the long-term outcomes of OSA patients.

Currently, BP measured in a clinical setting is considered the reference method for the general population, including patients with OSA [1]. Nevertheless, office BP measurement is affected by major problems such as the white-coat effect, observer bias, limited reproducibility, and the intrinsic inaccuracy of the auscultatory technique. Moreover, this method does not allow collection of information on physiological BP variability and nocturnal BP [3]. Consequently, office readings frequently lead to both overestimation and underestimation of hypertension as BP could be apparently normal in the office, but elevated outside the office, especially during the night-time period [3]. HBPM has been proposed as an alternative to office BP measurements or ABPM. Like ABPM, this method allows the registration of BP values during a patient's normal activities, avoiding the white-coat effect; however, such recordings are limited to short

periods of time. In addition, HBPM cannot provide detailed information on BP during night-time, precisely when OSA episodes occur. These limitations are overcome when ABPM is used. This technique offers several advantages over the previous two. The foremost is the possibility of collecting a large number of BP measurements as real BP is reflected more accurately by repeated measurements. In addition, ABPM allows the evaluation of BP profile over 24 h and can therefore detect the variability in circadian rhythms and identify patients with abnormal patterns of nocturnal BP [3]. Furthermore, the findings of a previous study have shown that the use of 24-h ABPM allowed the diagnosis of twice as much hypertension than did clinical measurement and therefore ABPM might be of particular significance in the hypertension diagnosis of OSA patients [20]. Moreover, there is strong evidence that the predictive value for major cardiovascular events is better with ABPM than with clinical measurement [19]. Another study supports the idea that ABPM should be mandatory for the routine diagnosis of hypertension for those at risk for masked hypertension [21]. This newly recognized cardiovascular entity is associated closely with OSA and adverse outcomes [22]. In brief, because of the specific features of OSA-related hypertension, ABPM seems to be the preferred method for BP measurement in patients suspected of having OSA.

Although ABPM should ideally be performed in all patients with suspected OSA, this type of BP measurement is time and labor intensive as well as an expensive diagnostic tool and is thus not used routinely. Given these limitations, we attempted to find an alternative tool that would allow the identification of patients who misclassify themselves as nonhypertensive. As anthropometric measurements (BMI, NC, and WC) have been shown to predict OSA [23,24] and other cardiovascular diseases [25,26], we decided to investigate the ability of these features to predict the misclassification of hypertension.

Our results showed that patients with undiagnosed hypertension and a BMI above 27 kg/m^2 and an NC higher than 39 cm had three-fold and two-fold higher odds, respectively, of misclassifying themselves as nonhypertensive than patients with a lower BMI or AHI. To the best of our knowledge, our study is the first to identify cut-off points for BMI and NC that can help discriminate between MNH and truly NH patients. The optimal BMI cut-off point that was found to predict the misclassification of hypertension was lower than the recommended cut-off point to predict OSA (30 kg/m^2) [27] and in the range of that recommended for global cardiovascular risk (25–29.9 kg/m^2) [28]. For NC, the same cut-off point was found at 39 cm for both sexes, and this value was similar to the ones described for OSA and cardiovascular disease risk [25,29].

We are aware that our sample should ideally have been larger and recruited from more than one center to avoid referral bias. However, our data were collected following a rigorous protocol and, in our opinion, the sample is representative of a typical population of patients suspected of having OSA. Nevertheless, the cut-off points that were found should be further validated in a larger prospective study. Another limitation of the study was the low casuistry of women in our sample. However, this imbalance was expected as OSA is more prevalent among men. In any case, sex was considered during the statistical analysis.

In conclusion, our results confirm the lack of validity of self-reported hypertension and emphasize the importance of ABPM in the minimization of underestimated hypertension among patients suspected of having OSA. In the present study, BMI and NC emerged as baseline clinical predictors of hypertension misclassification in patients suspected of having OSA. These findings suggest that all undiagnosed hypertensive patients referred to a sleep disorder center for symptoms suggesting OSA with a BMI and NC above 27 kg/m^2 and 39 cm, respectively (patients with a high odds of misclassifying themselves as nonhypertensive), should be screened for hypertension through ABPM. In our opinion, these results could facilitate the more selective use of ABPM, a high-cost, and time-consuming diagnostic tool, and also reduce the prevalence of undiagnosed hypertension among patients suspected of having OSA.

Acknowledgements

The authors thank all the cardiorespiratory technicians from CHLN, EPE for technical support.

L.N.D. is supported by the Portuguese Fundação para a Ciência e a Tecnologia (FCT) fellowship (SFRH/BD/48335/2008; PTDC/SAU-TOX/112264/2009) and CEDOC (Chronic Diseases Research Centre, Lisbon, Portugal). A.L.P.'s work was partially supported by the FCT, Portugal (PEst-OE/MAT/UI0006/2011). For the remaining authors, none were declared.

Conflicts of interest

There are no conflicts of interest.

References

1 Baguet JP, Barone-Rochette G, Pépin JL. Hypertension and obstructive sleep apnoea syndrome: current perspectives. *J Hum Hypertens* 2009; **23**:431–443.

2 Kapa S, Sert Kuniyoshi FH, Somers VK. Sleep apnea and hypertension: interactions and implications for management. *Hypertension* 2008; **51**:605–608.

3 Parati G, Lombardi C, Hedner J, Bonsignore MR, Grote L, Tkacova R, *et al.* European Respiratory Society; EU COST ACTION B26 members. Position paper on the management of patients with obstructive sleep apnea and hypertension: joint recommendations by the European Society of Hypertension, by the European Respiratory Society and by the members of European COST (COoperation in Scientific and Technological research) ACTION B26 on obstructive sleep apnea. *J Hypertens* 2012; **30**:633–646.

4 Calhoun DA. Obstructive sleep apnea and hypertension. *Curr Hypertens Rep* 2010; **12**:189–195.
5 Marin JM, Agusti A, Villar I, Forner M, Nieto D, Carrizo SJ, *et al*. Association between treated and untreated obstructive sleep apnea and risk of hypertension. *JAMA* 2012; **307**:2169–2176.
6 Kapur VK. Obstructive sleep apnea: diagnosis, epidemiology, and economics. *Respir Care* 2010; **55**:1155–1167.
7 Epstein LJ, Kristo D, Strollo PJ Jr, Friedman N, Malhotra A, Patil SP, *et al*. Adult Obstructive Sleep Apnea Task Force of the American Academy of Sleep Medicine. Clinical guideline for the evaluation, management and long-term care of obstructive sleep apnea in adults. *J Clin Sleep Med* 2009; **5**:263–276.
8 Caskie GI, Willis SL, Warner Schaie K, Zanjani FA. Congruence of medication information from a brown bag data collection and pharmacy records: findings from the Seattle longitudinal study. *Exp Aging Res* 2006; **32**:79–103.
9 Collop NA, Anderson WM, Boehlecke B, Claman D, Goldberg R, Gottlieb DJ, *et al*. Portable Monitoring Task Force of the American Academy of Sleep Medicine. Clinical guidelines for the use of unattended portable monitors in the diagnosis of obstructive sleep apnea in adult patients. Portable Monitoring Task Force of the American Academy of Sleep Medicine. *J Clin Sleep Med* 2007; **3**:737–747.
10 Mancia G, Fagard R, Narkiewicz K, Redon J, Zanchetti A, Böhm M, *et al*. Guidelines for the management of arterial hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). *Eur Heart J* 2007; **28**:1462–1536.
11 Mazumdar M, Glassman JR. Categorizing a prognostic variable: review of methods, code for easy implementation and applications to decision-making about cancer treatments. *Stat Med* 2000; **19**:113–132.
12 Hastie T, Tibshirani R. *Generalized additive models*. New York: Chapman & Hall; 1990.
13 Hosmer DW, Lemeshow S. *Applied logistic regression*. New York: John Wiley & Sons; 1989.
14 R Development Core Team. *R: A language and environment for statistical computing*. Vienna, Austria: R foundation for Statistical Computing; 2008.
15 Mentz G, Schulz AJ, Mukherjee B, Ragunathan TE, Perkins DW, Israel BA. Hypertension: development of a prediction model to adjust self-reported hypertension prevalence at the community level. *BMC Health Serv Res* 2012; **12**:312.
16 Hla KM, Young T, Finn L, Peppard PE, Szklo-Coxe M, Stubbs M. Longitudinal association of sleep-disordered breathing and nondipping of nocturnal blood pressure in the Wisconsin Sleep Cohort Study. *Sleep* 2008; **31**:795–800.
17 Dolan E, Stanton AV, Thom S, Caulfield M, Atkins N, McInnes G, *et al*. ASCOT Investigators. Ambulatory blood pressure monitoring predicts cardiovascular events in treated hypertensive patients – an Anglo-Scandinavian cardiac outcomes trial substudy. *J Hypertens* 2009; **27**:876–885.
18 Loredo JS, Nelesen R, Ancoli-Israel S, Dimsdale JE. Sleep quality and blood pressure dipping in normal adults. *Sleep* 2004; **27**:1097–1103.
19 Burr ML, Dolan E, O'Brien EW, O'Brien ET, McCormack P. The value of ambulatory blood pressure in older adults: the Dublin outcome study. *Age Ageing* 2008; **37**:201–206.
20 Baguet JP, Hammer L, Lévy P, Pierre H, Rossini E, Mouret S, *et al*. Night-time and diastolic hypertension are common and underestimated conditions in newly diagnosed apnoeic patients. *J Hypertens* 2005; **23**:521–527.
21 Phillips RA. Controversies in blood pressure goal guidelines and masked hypertension. *Ann N Y Acad Sci* 2012; **1254**:115–122.
22 Baguet JP, Lévy P, Barone-Rochette G, Tamisier R, Pierre H, Peeters M, *et al*. Masked hypertension in obstructive sleep apnea syndrome. *J Hypertens* 2008; **26**:885–892.
23 Davies RJ, Ali NJ, Stradling JR. Neck circumference and other clinical features in the diagnosis of the obstructive sleep apnoea syndrome. *Thorax* 1992; **47**:101–105.
24 Proimos E, Kiagiadaki D, Kaprana A, Chimona TS, Maroudias NJ, Papadakis CE. Clinical application of subjective measurements for OSAS assessment: predictive factors of syndrome severity. *ORL J Otorhinolaryngol Relat Spec* 2012; **74**:240–245.
25 Preis SR, Massaro JM, Hoffmann U, D'Agostino RB Sr, Levy D, Robins SJ, *et al*. Neck circumference as a novel measure of cardiometabolic risk: the Framingham Heart study. *J Clin Endocrinol Metab* 2010; **95**:3701–3710.
26 Vallianou NG, Evangelopoulos AA, Bountziouka V, Vogiatzakis ED, Bonou MS, Barbetseas J, *et al*. Neck circumference is correlated with triglycerides and inversely related with HDL cholesterol beyond BMI and waist circumference. *Diabetes Metab Res Rev* 2013; **29**:90–97.
27 Friedman M, Tanyeri H, La Rosa M, Landsberg R, Vaidyanathan K, Pieri S, Caldarelli D. Clinical predictors of obstructive sleep apnea. *Laryngoscope* 1999; **109**:1901–1907.
28 [No authors listed]. Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: executive summary. Expert panel on the identification, evaluation, and treatment of overweight in adults. *Am J Clin Nutr* 1998; **68**:899–917.
29 Soylu AC, Levent E, Sarıman N, Yurtlu S, Alparslan S, Saygı A. Obstructive sleep apnea syndrome and anthropometric obesity indexes. *Sleep Breath* 2012; **16**:1151–1158.

RESEARCH PAPER

THE ASSOCIATION BETWEEN ANTIHYPERTENSIVE MEDICATION AND BLOOD PRESSURE CONTROL IN PATIENTS WITH OBSTRUCTIVE SLEEP APNEA

LUCÍLIA N DIOGO[1*], PAULA PINTO[2], CRISTINA BÁRBARA[2], ANA L PAPOILA[1, 3] AND EMÍLIA C MONTEIRO[1]

[1]CEDOC, NOVA Medical School / Faculdade de Ciências Médicas, Universidade Nova de Lisboa, 1169-056 Lisboa, Portugal. [2]Serviço de Pneumologia II, Centro Hospitalar Lisboa Norte (CHLN)-Hospital Pulido Valente, 1600-190 Lisboa, Portugal [3] CEAUL, 1749-016 Lisboa, Portugal.

Background: Obstructive sleep apnea and hypertension are closely related diseases. The lowering effect of continuous positive airway pressure (CPAP) on blood pressure (BP) control is modest and concomitant antihypertensive therapy is still required. However, the best antihypertensive regimen for BP control in patients with OSA remains unknown. We aimed to investigate a hypothetical association between ongoing antihypertensive medication and blood pressure control (BP) rates in patients with OSA, in order to identify the most effective antihypertensive regimen prescribed in current treatment practice. **Methods:** We conducted a prospective observational study in a cohort of 205 patients with OSA and hypertension who underwent a sleep study and 24-h ambulatory blood pressure monitoring (ABPM). Ongoing antihypertensive medication profile was recorded. Logistic regression models were used to investigate the association between antihypertensive regimen and BP control, before (n=205) and, when applicable, after continuous positive airway pressure (CPAP) adaptation (n=90). **Results:** According to current guidelines and antihypertensive medication and/or 24h ABPM, 63.9% (205/321) of the patients were diagnosed with OSA and hypertension. One hundred and fifty-five (155/205) were under antihypertensive medication and 31 different antihypertensive regimens were found. However, the antihypertensive regimens and the number of antihypertensive drugs were not associated with BP control (p=0.847;p=0.991). After CPAP adaptation, a decrease in median night-time systolic and diastolic BP was observed (p=0.001;p=0.006). Nevertheless, the lack of association between antihypertensive regimens and the number of antihypertensive drugs and BP control remained (p=0.864;p=0.800). **Conclusions:** Our findings confirm that although CPAP improves nocturnal BP, this improvement is not enough to warrant a suitable 24h-BP control. This study shows, for the first time, that BP control is independent of both antihypertensive regimen and number of antihypertensive drugs and that none of the currently available antihypertensive drugs have been shown to be effective for the control of BP in patients with OSA. **Trial registration:** ClinicalTrials.gov: NCT01803815.

Keywords: ambulatory blood pressure monitoring; antihypertensive drugs; blood pressure; continuous positive airway pressure; hypertension and obstructive sleep apnea.

[*]**Corresponding author:** Lucilia Neves Diogo, PharmD, MSc, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo Mártires da Pátria, 130, 1169-056 Lisboa, Portugal, lucilia.diogo@fcm.unl.pt.

BACKGROUND

Obstructive sleep apnea is a highly prevalent sleep-related breathing disorder, briefly characterized by repetitive episodes of airflow cessation (apnea) or airflow reduction (hypopnea) in the upper airways during sleep. Although OSA has been associated with several cardiovascular conditions, it has been more etiologically connected to hypertension [1] and the link between OSA and hypertension is now well established and supported by different findings [2, 3]. Epidemiological data have shown that the prevalence of hypertension among patients with OSA is undoubtedly high and estimated to be around 50%. Likewise, it is also reported that 30-40% of hypertensive patients have OSA [4]. Moreover, OSA is renowned as a frequent secondary cause of hypertension [5, 6] and one of the major clinical conditions that favours poorly controlled hypertension [7]. More recently, OSA has been identified as an independent risk factor for hypertension [8] and as the most common condition associated with resistant hypertension [9]. However, the underlying mechanisms of hypertension-related OSA remain partially unclear and the best targets for the control of BP in these patients have not yet been established.

Continuous Positive Airway Pressure (CPAP) is considered the gold standard treatment for symptomatic OSA patients, due to its effectiveness in reducing the apnea-hypopnea index (AHI), sleepiness, cardiovascular morbidity and mortality and in improving the quality of life [10]. The effectiveness of CPAP on blood pressure (BP) control is still controversial [4, 8, 11-16] and CPAP seems to be insufficient to sustain BP control by itself; therefore, the use of antihypertensive drugs (AHD) is unavoidable. In fact, the findings from a recent study revealed that, in non-sleepy, hypertensive, OSA patients, the long-term use of CPAP is not associated with lower BP values or a need for less AHD for BP control [17].

In spite of this, data on antihypertensive drug regimens in patients with OSA are scarce and specific therapeutic guidelines for the pharmacological treatment of hypertension in these patients remain absent. Moreover, the effects of antihypertensive agents on OSA patients are not consistent [18] and there are limited data on whether a specific AHD regimen has any positive effect on the control of OSA [19]. Thus, more studies are requested in order to identify first-line AHD regimens for optimal BP control in this particular group of hypertensive patients [19, 20].

The main goal of this prospective cohort study was to investigate a hypothetical association between ongoing antihypertensive (AH) regimens and BP control rates in patients with OSA, before and after CPAP adaptation. We were also interested in identifying other variables that could provide information about BP control after CPAP adaptation. Additionally, we intended to investigate the phenotypic characteristics and, specifically, the patterns of antihypertensive drugs used in patients with OSA.

METHODS

Subjects

Three hundred and sixty-nine consecutive patients clinically suspected of having OSA, aged above 18 years, who were attending their first visit at the Centro Hospitalar Lisboa Norte, EPE (CHLN) Sleep Unit, following referral by their general practitioner or other specialist,

were assessed for eligibility. Patient inclusion started in April 2010 and was completed in July 2012. Exclusion criteria included severe psychiatric disease or an inability to understand the information required for informed consent, apnea-hypopnea index <5 events/hour and no diagnosis of hypertension. In order to fully accomplish the goal of the study, additional inclusion criteria were subsequently defined. Only patients with a CPAP mean daily use of at least 4 hours and ambulatory 24-h ABPM recordings, performed at baseline and 1-3 months after checking CPAP adherence, were considered eligible for the study of the association between antihypertensive regimens and BP control after CPAP adaptation (Figure 1).

All patients were fully informed about the study and gave written consent in accordance with the Declaration of Helsinki. The study protocol was previously approved by Centro Hospitalar Lisboa Norte's Ethics Committee.

Study Design

At baseline (first visit) all patients were invited to undergo an overnight polysomnography, 24-h ambulatory blood pressure monitoring (ABPM) and completed a data collection form that included ongoing medication profile registration.

Once the diagnosis of OSA and hypertension was established, the criteria for treatment with CPAP confirmed and baseline ABPM measured (second visit), patients were scheduled for CPAP titration.

Four to five weeks after CPAP adaptation (third visit), the device data were checked and patients with a CPAP mean daily use of at least 4 hours were scheduled for repeated 24-h ABPM. At this time, particular attention was paid to ensure that patients had not changed their antihypertensive medication since the first evaluation. If any changes in either type of medication or dose had occurred, the patient was excluded from the study.

The main outcome variables were blood pressure (BP) control rates (controlled and uncontrolled) in patients undergoing antihypertensive medication.

Data collection form and clinical assessment

Socio-demographic and anthropometric data, as well as smoking habits, comorbidities and ongoing medication profiles, were registered.

All patients were assessed for weight, height, neck circumference (NC) and waist circumference (WC) using standardised protocols. Body mass index (BMI) was calculated as body weight in kilograms divided by the square of height in metres: kg/m^2. NC was measured at the level of the cricothyroid membrane, with the patients' head positioned in the Frankfort horizontal plane and WC was assessed at the level of umbilicus; both were recorded using a flexible non-stretchable measuring tape.

Regarding smoking habits, patients were classified as non-smoker, ex-smoker, and smoker (defined as those who smoke at least one cigarette per day over the previous year).

Comorbidities, with special focus on hypertension, were assessed. Hypertension was defined as self-reported hypertension (answer "yes" to the question "Have you ever been told by a doctor that you have high blood pressure?") or the use of antihypertensive medication. Information about diabetes mellitus, dyslipidaemia, arrhythmias, asthma, chronic obstructive pulmonary disease (COPD),

depression and anxiety, history of cardiovascular disease and hypothyroidism was also collected.

The ongoing medication profile (drug name (International Non-proprietary Name: INN) and dose) was recorded using the "Brown-Bag" medication review (a method in which patients are asked to bring all of their medications to each visit) [21]. We analysed the use of antihypertensive drugs by regimen: 1-with angiotensin-converting enzyme inhibitors (ACEi); 2-with angiotensin II receptor blockers (ARAs); 3-with β-blockers; and 4-others (including diuretics and calcium channel blockers), and the number of antihypertensive drugs (1-one, monotherapy; 2-two or more, polytherapy) included in each patient regimen. In all patients, antihypertensive drugs were taken for at least six months without changing the medication profile until the end of the follow-up.

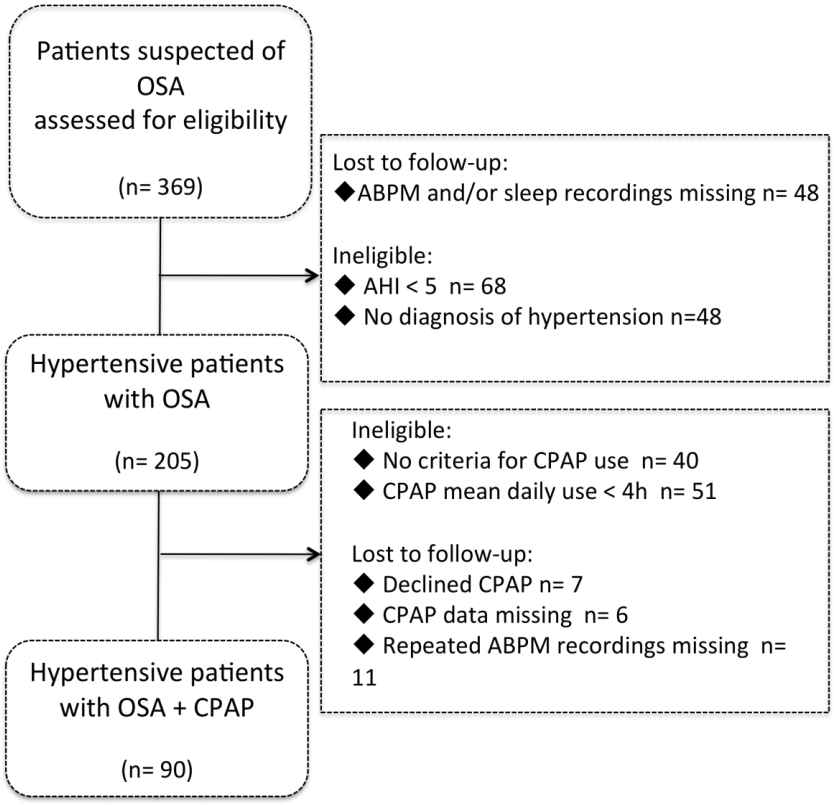


Figure 1: **Flowchart of study protocol.**

Of the 369 patients consecutively included, 68 were patients with no diagnosis of OSA and 48 with no diagnosis of hypertension. The ABPM and/or sleep recordings were missing for 48 patients due to technical problems or because they did not attend the ABPM (n=41) or sleep study (n=7). Forty had no indication for CPAP therapy. One hundred and sixty-five were scheduled for CPAP adaptation; however, 7 refused CPAP, CPAP data were missing in 6 patients and 51 had no compliance. Of the 369, only the data from 90 patients were used to investigate the association between ongoing AH regimen and blood pressure control rates after CPAP adaptation. **AHI**: Apnea-Hypopnea Index; **ABPM:** Ambulatory Blood Pressure Monitoring; **CPAP:** Continuous Positive Airway Pressure; **OSA**: obstructive sleep apnea.

Sleep Evaluation

Patients underwent an overnight in-laboratory polysomnography (PSG) or, as an alternative for the diagnosis of OSA in patients with a high pre-test probability of moderate to severe OSA, a cardiorespiratory sleep study [22]. In-laboratory polysomnographic studies were performed using a multichannel polygraph (Model Embla S7000, Embla Systems Inc., Broomfield, CO, USA) and ambulatory recordings were made using a validated portable digital recording unit (type 3) with sensors for the registration of airflow, saturation, respiratory movements of the chest, body position and snoring sounds (Embletta PDS device; Embla Systems Inc., Broomfield, CO, USA). Sleep recordings were manually scored, based on standard criteria [22], by trained technicians. Apnea was defined as a complete upper airway obstruction (reduction in airflow >80%) for at least 10s and hypopnea as a discernible reduction of airflow between 70% and 20% of the preceding period of stable breathing, for 10s or more. The number of apneas and hypopneas per hour of sleep was calculated and, for both in-laboratory and ambulatory recordings, the apnea-hypopnea index (AHI) was calculated. Respiratory effort related arousals (RERAs), scored in laboratory polysomnography sleep staging, were not considered in order to minimise the differences in results provided by the two diagnostic approaches. OSA was categorised according to current AHI cut-offs of <5 (non-diagnostic), 5 ≥ and < 15 (mild), 15 ≥ and < 30 (moderate) and ≥ 30 (severe) [22, 23].

Twenty-four-hour ambulatory blood pressure monitoring (ABPM)

Twenty-four hour ABPM was performed at baseline, on a different day to that of the sleep evaluation, and 1-3 months after CPAP adaptation. All participants were instructed to continue their usual daily activities. Screening BP measurements were performed in accordance with the recommendations of the European Society of Hypertension (ESH) guidelines [5], using appropriately-sized arm cuff, on the non- dominant arm, with a non-invasive portable validated BP recorder (Spacelabs Model 90217; Spacelabs Healthcare, Redmond, WA, USA). The equipment was programmed for cuff inflation every 20 minutes during the daytime period and every 30 minutes during the night-time. Daytime and night-time were individually predetermined depending on the participants' usual awake and sleep schedule. The data were considered valid when a minimum of 70% of the measurements were recorded without errors. BP recordings were analysed for the overall 24-hour period, daytime and night-time periods. Additionally, 24 h BP profile was assessed and patients were classified as showing a dipping or a non-dipping profile. A dipping profile was defined as a reduction in the average systolic and diastolic BP at night that was higher than 10% compared to daytime values. The data were analysed by an experienced cardio-respiratory technician.

Uncontrolled BP was defined according to ESC and ESH guidelines [6]. BP was considered uncontrolled for systolic BP (SBP) values of 135 mm Hg or above, diastolic BP (DBP) values of 85 mm Hg or above, or both during daytime and systolic BP (SBP) values of 120 mm Hg or above, diastolic BP (DBP) values of 70 mm Hg or above, or both during night time [6]. Isolated nocturnal hypertension was defined, in accordance with the established BP

thresholds for ABPM, as night-time SBP values of 120 mm Hg or DBP of 70 mm Hg or above, and isolated daytime hypertension as diurnal SBP of 135 mm Hg or DBP of 85 mm Hg or above. When both conditions were present, subjects were classified as having combined day-night hypertension [6].

Continuous Positive Airway Pressure (CPAP) therapy

CPAP titration was performed using self-adjusting CPAP devices (autoCPAP) (REMstar PR1 Auto, S8 AutoSet Spirit- Resmed Ltd), AutoSet Spirit S9 - Resmed Ltd). During the initial setup of the machine, the minimum (Pmin: 4 cm H_2O) and maximum (Pmax: 16 cm H_2O) pressures were set. All patients were followed-up 4-5 weeks after CPAP adaptation, in order to check for any side effects or problems with the treatment and, if required, for CPAP pressure adjustment. CPAP adherence was assessed from the device counter. Data were analysed by experienced technicians using appropriate software.

Statistical Analysis

An exploratory analysis was carried out for all variables. Categorical data were expressed as frequencies and percentages, and continuous variables as mean or median, standard deviation (SD) or inter-quartile range (25th percentile-75th percentile). Welch's t-test was used as an adaptation of Student's t-test whenever unequal variances were obtained. Wilcoxon test and Sign test were used to compare ABPM data measured at baseline and after CPAP adaptation. The McNemar test was applied to compare the proportion of uncontrolled patients before and after CPAP adaptation.

In order to identify baseline predictors of BP control after CPAP adaptation, a univariable analysis was performed. The outcome variable was "uncontrolled BP" (yes: for patients with uncontrolled BP and no: for patients with controlled BP) and the multiple logistic regression model was subsequently fitted to the data. The predictive ability of the resulting model was analysed by the Hosmer-Lemeshow goodness-of-fit test [24]. A high p-value indicates that the model is performing well. The area under the Receiver Operating Characteristic curve (AUC) was used in order to evaluate the discriminative ability of the model. A value of 0.50 is obtained when a model discriminates no better than chance, and a value of 1.0 means perfect accuracy.

Results were considered significant when α=0.05. Confidence intervals are presented when appropriate (95% CI). Statistical analysis was performed using the IBM SPSS Statistics (IBM Corp., Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

RESULTS

Patients Characteristics and ABPM data

Patients (n=369) were mainly male (65.9%), Caucasian (95.7%), with a mean age of 55.9 (SD=13.1) years. From all of the enrolled patients, 321 presented complete ABPM and sleep data (n=48 missing data). According to current guidelines and to antihypertensive medication and/or 24h ABPM, 63.9% (205/321) of them were diagnosed with OSA and hypertension (Figure 1).

In these patients, median apnea-hypopnea index was 20.0/h (P_{25}=10.2/h, P_{75}=32.6/h) and concerning OSA severity, 39.0% (80/205),

30.2% (62/205) and 30.7% (63/205) presented mild, moderate and severe OSA, respectively.

Table 1 shows key patient characteristics summarized according to BP control (controlled n=60/205, 29.3% and uncontrolled n=145/205, 70.7%). No differences were found between these two groups regarding anthropometric variables and smoking habits. The most prevalent self-reported comorbidities for hypertensive OSA patients were: dyslipidaemia (63.4%), depression/anxiety (23.9%), diabetes (22.0%) and hyperuricaemia (10.7%).

ABPM data, attained at baseline, are described in Table 2. From the 145 uncontrolled patients, 20 (13.8%) presented isolated diurnal hypertension, 52 (35.9%) isolated nocturnal hypertension and 73 (50.3%) both diurnal and nocturnal hypertension.

Table 1: Hypertensive OSA patient characteristics at baseline

Variable	All patients (n= 205)	Controlled (n=60)	Uncontrolled (n=145)	*p*
Age, years	59.0 (11.6)	62.2 (10.4)	57.7 (11.8)	0.012 $^{\Psi}$
Gender, male (%)	149 (72.7)	40.0 (66.7)	109 (75.2)	0.214 $^{\&}$
Race, Caucasian (%)	196 (95.6)	60 (100.0)	136 (93.8)	0.061 *
BMI, kg/m^2	31.0 (5.0)	31.5 (6.2)	30.8 (4.5)	0.420 $^{\#}$
Neck circumference, cm	42.2 (3.8)	42.1 (4.1)	42.2 (3.8)	0.822 $^{\Psi}$
Waist circumference, cm	109.6 (12.1)	110.1 (13.5)	109.4 (11.5)	0.727 $^{\Psi}$
Smoking habits				0.305 $^{\&}$
Non-smokers (%)	111 (54.1)	31 (51.7)	80 (55.2)	
Former smokers (%)	76 (37.1)	26 (43.3)	50 (34.5)	
Current smokers (%)	18 (8.8)	3 (5.0)	15 (10.3)	
Number of comorbidities >2 (%)$^{\Phi}$	114 (55.6)	39 (65.0)	75 (51.7)	0.082 $^{\&}$
Self-reported hypertension (%)	159 (77.6)	59 (98.3)	100 (69.0)	<0.001$^{\&}$

Data are presented as mean (SD: standard deviation), median (interquartile range: P_{25}, P_{75}) or number (%). $^{\Phi}$ including self-reported hypertension $^{\Psi}$ Student´s t-test $^{\#}$ Welch's t test $^{\&}$ Chi-squared test * Fisher's exact test; **OSA**: obstructive sleep apnea; **BMI**: body mass index; **AH**: antihypertensive drugs; **AHI**: apnea-hypopnea index.

Table 2: ABPM data at baseline

Variable	All patients (n=205)	Controlled (n=60)	Uncontrolled (n=145)	*p*
24-h ABPM (mmHg)**:**				
Mean 24-h BP	94.0 (86.0, 98.0)	83.0 (80.0, 85.8)	96.0 (93.0, 101.0)	<0.001$^{\Psi}$
Daytime systolic BP	129.0 (121.0, 139.0)	119.5 (111.3, 124.0)	135.0 (127.5, 144.0)	<0.001$^{\Psi}$
Daytime diastolic BP	79.0 (72.0, 85.0)	71.5 (67.0, 75.0)	83.0 (78.0, 88.0)	<0.001$^{\Psi}$
Night-time systolic BP	118.0 (107.5, 128.0)	105.5 (97.3, 109.0)	124.0 (115.5, 131.5)	<0.001$^{\Psi}$
Night-time diastolic BP	69.0 (62.0, 75.0)	61.0 (57.0, 64.0)	72.0 (67.0, 77.0)	<0.001$^{\Psi}$
24h-BP profile:				
Non-dipper systolic (%)	108 (52.7)	21 (35.0)	87 (60.0)	0.001 $^{\&}$
Non-dipper diastolic (%)	79 (38.5)	12 (20.0)	67 (46.2)	<0.001$^{\&}$

Data are presented as median (interquartile range: P_{25}, P_{75}) or number (%). $^{\Psi}$ Mann-Whitney test $^{\&}$ Chi-squared test ; **OSA**: obstructive sleep apnea; **ABPM**: ambulatory blood pressure monitoring; **BP**: blood pressure; **HT**: hypertension.

Patterns of antihypertensive drugs use

Of the 205 hypertensive OSA patients, 155 were under antihypertensive medication. The antihypertensive medication profile found in controlled and uncontrolled groups, respectively, was: 19 (31.7%) and 30 (20.7%) patients under monotherapy and 41 (68.3%) and 65 (44.8%) patients under regimens that included two or more antihypertensive drugs.

Thirty-one different antihypertensive regimens were found for hypertensive OSA patients (Table 3).

Table 3: Antihypertensive regimens in patients with OSA

Antihypertensive Regimen	Hypertensive OSA patients n=155
Combination of ARBs + diuretics	28 (18.1)
Monotherapy with ACEi	21 (13.5)
Monotherapy with ARBs	14 (9.0)
Combination of ACEi + diuretics	10 (6.5)
Combination of CCB + diuretics + ARBs	8 (5.2)
Combination of ARBs + CCB	8 (5.2)
Monotherapy with CCB	7 (4.5)
Combination of BB + diuretics + ARBs	6 (3.9)
Monotherapy with BB	5 (3.2)
Combination of BB + ACEi	4 (2.6)
Combination of CCB + ACEi	4 (2.6)
Combination of CCB + diuretics + ACEi	4 (2.6)
Monotherapy with diuretic	3 (1.9)
Combination of BB + diuretics	3 (1.9)
Combination of BB + diuretics + ACEi	3 (1.9)
Combination of BB + diuretics + ARBs + ACEi	3 (1.9)
Combination of CCB + BB	3 (1.9)
Combination of CCB + BB + diuretics + ACEi	3 (1.9)
Combination of ARBs + diuretics + ACEi	2 (1.3)
Combination of CCB + diuretics + ARBs + ACEi	2 (1.3)
Combination of CCB + diuretics + ACEi	2 (1.3)
Combination of CCB + BB + ARBs	2 (1.3)
Combination of CCB + diuretics + BB	2 (1.3)
Combination of BB + ARBs	1 (0.6)
Combination of two BB	1 (0.6)
Combination of CCB + diuretics	1 (0.6)
Combination of CCB + two diuretics + ACEi	1 (0.6)
Combination of CCB + diuretics + ARBs + BB	1 (0.6)
Combination of CCB + two diuretics + ACEi + BB	1 (0.6)
Combination of two CCB + ACEi + BB	1 (0.6)

Data are presented as number (%). **OSA**: obstructive sleep apnea; **AH**: antihypertensive drugs; **ACEi**: angiotensin-converting enzyme inhibitors; **ARBs**: angiotensin II receptor blockers; **BB**: beta-blockers; **CCB:** calcium channel blockers

Association between AH regimens and BP control

In this section, we performed a univariable analysis in order to investigate whether there is an AH regimen associated with a better BP control. No association was found between the number of antihypertensive drugs and the antihypertensive regimens and BP control (p=0.991 and p=0.847, respectively) (Table 4).

Besides AH regimens and number of AHD, the analysed variables were age (p=0.012), gender (p=0.214), BMI (p=0.420), neck circumference (p=0.822), waist circumference (p=0.727), smoking habits (p=0.305), number of comorbidities (p=0.082) and OSA severity (p=0.325). Age and the number of comorbidities

were selected for the multivariable analysis. However, none of these variables remained in the multivariable model.

Aware of the importance assigned by the clinicians to OSA severity and obesity in the management of OSA patients, analysis of the association between the number of AHD and BP control was additionally performed in severe OSA patients with a BMI ≥30 kg/m^2 and in mild/moderate OSA patients with a BMI <30 kg/m^2. In the subgroup of severe OSA patients with BMI ≥30 kg/m^2 (n=28), we found no statistical differences in BP control between patients undergoing polytherapy and monotherapy (p= 0.689): 44.4% (8/18) of patients under polytherapy and 30.0% (3/10) under monotherapy, respectively, presented controlled BP. In the subgroup of mild/moderate OSA patients with a BMI <30 kg/m^2 (n=51), there is an indication that the number of patients with controlled BP is higher (p=0.064) in those under monotherapy (69.2% (9/13)) than in those under polytherapy (39.5%(15/38)).

Table 4: Association between anti-hypertensive regimens/ number of anti-hypertensive drugs and BP Control at baseline and after CPAP adaptation

	Before CPAP			After CPAP		
Variable	Controlled (n=60)	Uncontrolled (n=95)	p	Controlled (n=33)	Uncontrolled (n=41)	p
Number of AH			0.991[&]			0.800[&]
One	19/49 (38.8)	30/49 (61.2)		9/22 (40.9)	13/22 (59.1)	
Two or more	41/106 (38.7)	65/106 (61.3)		24/52 (46.2)	28/52 (53.8)	
AH regimens			0.847[&]			0.864*
With ACEi	16/39 (41.0)	23/39 (59.0)		8/21 (38.1)	13/21 (61.9)	
With ARBs	24/58 (41.4)	34/58 (58.6)		11/25 (44.0)	14/25 (56.0)	
With BB	5/13 (38.5)	8/13 (61.5)		1/2 (50.0)	1/2 (50.0)	
Others	15/45 (33.3)	30/45 (66.7)		13/26 (50.0)	13/26 (50.0)	

Data are presented as number (%). [&] Chi-squared test * Fisher's exact test

CPAP: continuous positive airway pressure; **AH**: antihypertensive drugs; **ACEi**: angiotensin-converting enzyme inhibitors; **ARBs**: angiotensin II receptor blockers; **BB**: beta-blockers

Effect of combined CPAP use and antihypertensive medication on BP control

From the 369 patients that were initially included, only 90 fulfilled the inclusion criteria and were eligible to investigate the association between ongoing AH regimen and hypertension control rates after CPAP adaptation. Regarding ABPM data measured at baseline and after CPAP adaptation (Table 5), no significant differences were found between daytime systolic and diastolic BP (p=0.156 and p=0.132, respectively).

As expected, a decrease in median night-time systolic (median=4.5 mmHg) and diastolic BP (median=3.5 mmHg) was observed (p=0.001 and p=0.006) after CPAP adaptation. Nevertheless, this decline was not enough to allow reclassification from uncontrolled to controlled BP in a substantial number of patients under AHD (p=0.332). In fact, of the 46/74 (62.2%) uncontrolled patients at baseline, only 11/74 (14.9%) became controlled after CPAP

adaptation. For patients under no AHD (n=16/90), an improvement in BP control was observed in 8 patients (p=0.008).

After CPAP, the lack of association between antihypertensive regimens and the number of antihypertensive drugs and BP control remained (p=0.864 and p=0.800) (Table 4).

Table 5: ABPM data at baseline and after CPAP adaptation of patients with OSA, hypertension and CPAP mean daily use ≥ 4 hours (n=90)

Variable	Baseline	After CPAP adaptation	p^{Ψ}
Daytime systolic BP	128.5 (121.5, 138.0)	127.5 (119.0, 135.0)	0.156[#]
Daytime diastolic BP	79.0 (70.8, 84.0)	76.5 (71.0, 83.0)	0.132[Ψ]
Night-time systolic BP	118.5 (107.0, 129.0)	114.0 (106.8, 123.5)	0.001[#]
Night-time diastolic BP	69.0 (61.0, 74.0)	65.5 (60.0, 72.0)	0.006[Ψ]

Data are presented as median (interquartile range: P_{25}, P_{75}) [Ψ] Wilcoxon test [#] Sign Test **CPAP**: continuous positive airway pressure; **ABPM**: ambulatory blood pressure monitoring; **BP**: blood pressure.

Baseline predictors of uncontrolled BP after CPAP adaptation

Since no association was found between AHD regimens and BP control in OSA patients, we looked for baseline variables that could predict BP control after CPAP adaptation. A multivariable model was fitted considering the outcome "uncontrolled BP" after a previous univariable study. For the final model, OSA severity and BP dipper/non-dipper profile and gender were considered. Regarding OSA severity, the odds ratio was calculated after the aggregation of moderate and severe categories. Mild severity, dipper profile and female gender were considered the reference categories. The results showed that at baseline, male gender, moderate/severe and non-dipper patients were independently associated with uncontrolled BP after CPAP adaptation (OR: 2.5, 95% CI: 0.9-7.2, p=0.087; OR: 3.8, 95% CI: 1.2-12.4, p=0.028; OR: 3.5, 95% CI: 1.4-8.9, p=0.007) (Table 6). The model demonstrated a good predictive (Hosmer-Lemeshow p-value=0.776) and acceptable discriminative (AUC=0.74; 95% CI: 0.64-0.85) performance.

Table 6: Multivariable analysis in which gender, OSA severity and 24-h BP profile at baseline were identified as independent predictors of BP control after CPAP adaptation (n=90)

	OR	95% C.I. for OR		p-value
		Lower	Upper	
OSA severity **Reference category**= mild	3.8	1.2	12.4	0.028
24h BP profile **Reference category**= dipper	3.5	1.4	8.9	0.007
Gender **Reference category**= female	2.5	0.9	7.2	0.087

OR: odds ratio; C.I.: confidence interval; **OSA**: obstructive sleep apnea; **BP:** blood pressure

DISCUSSION

The prevalence of new diagnoses of OSA among those patients that attended the outpatient consultation of CHLN sleep unit was 78.2% (283/362). According to current guidelines and to antihypertensive medication and/or 24h ABPM, 63.9% (205/321) of them were diagnosed with both OSA and hypertension. At baseline, 31 different antihypertensive regimens were found for OSA patients. Regarding BP control, 57.5% of patients under antihypertensive medication had uncontrolled BP (95/155). The antihypertensive regimens and the number of antihypertensive drugs were not associated with BP control. After CPAP adaptation, the lack of association between antihypertensive regimens and the number of antihypertensive drugs and BP control remained, but we found that CPAP significantly improved BP control in patients under no AH medication. In a multivariable study, gender, OSA severity and 24h-BP profile were independently associated with uncontrolled BP after CPAP adaptation. Therefore, it can be stated that the risk of being uncontrolled is higher for males with moderate/severe OSA and a non-dipper BP profile, confirming the relevance of OSA in the pathophysiology of hypertension. In summary, these results provided evidence that this type of hypertension needs to be managed as a specific entity and that none of the currently available AH regimens have been shown to be particularly effective to control BP in patients with OSA.

We performed a real life study and, to the best of our knowledge, this is the first that has described the pattern of AH medication and evaluated the hypothetical association between ongoing antihypertensive regimen and BP control rates in patients with OSA.

In general, our sample matches a typical population of OSA patients. Actually, the high prevalence of new diagnoses of OSA, indicating that patients are being well-referred, as well as the distribution of OSA severity found in CHLN sleep consultation, is similar to those reported by other authors [25, 26]. The anthropometric baseline characteristics that were found are in line with those that have been previously described in the literature [27]. Additionally, these patients tend to present a higher number of comorbidities, mostly a high prevalence of hypertension, when compared to non-OSA patients [8, 25, 28]. The prevalence of hypertension among patients with OSA is estimated to be around 50% [4]. Our study revealed a higher prevalence of hypertension in OSA patients, probably due to the use of ABPM for the diagnosis of this disease. In addition to the high prevalence of hypertension, ABPM data revealed that, regarding 24h-BP profile, OSA patients showed a non-dipper systolic profile, which has already been reported [29-31]. The high prevalence of uncontrolled BP (70.7%; 145/205) and underdiagnosed hypertension (24.4%; 50/205) among these patients has also been similarly reported [2, 32, 33]. In a past study that included 59 patients who were not known to be hypertensive, hypertension was found in 42% of patients using clinical measurement and 76% using ABPM [34].

As far as we know, this is the first study to describe the pattern of AH medication in OSA patients. The high number of different AH regimens found (n=31) is symptomatic of the difficulty controlling BP in these patients, and of the lack of consensus and guidelines in this

matter, and also underlines the importance of the main question addressed in the present work. The number of patients with regimens that included two or more AH drugs (n=106) is impressive, and emphasises the difficulty in achieving BP control and the close relationship between OSA and resistant hypertension [35-38].

Despite the large number of studies involving OSA patients, only a few have investigated the efficacy of different AHD and, in general, are individual drug studies. Most of them have focused on evaluating the lowering-BP effect of AHD and did not assess whether this decrease leads to an augmentation of the number of controlled hypertensive patients. Moreover, the validity of some of these results is limited due to the very low casuistry of the samples. Nevertheless, their findings allow some conclusions. First, drugs that block the sympathetic stimulation of β1-adrenergic receptors seem to be the most effective AHD, since the β1-specific antagonist atenolol was the most effective AHD tested (in comparison with isradipine, hydrochlorothiazide, spirapril, amlodipine, enalapril and losartan) in two comparative studies [39, 40], and nebivolol [41] was also effective in patients with OSA. On the other hand, the angiotensin-converting enzyme inhibitors (e.g. cilazapril and enalapril) lowered BP effectively [42], but not as significantly as beta-blockers [40, 43]. The angiotensin II receptor blocker valsartan had a similar anti-hypertensive effect to nebivolol [41]. Spironolactone (competitive aldosterone antagonist) has been remarkably effective in treating resistant hypertension [44]; however, its effectiveness has not been evaluated in patients with OSA to date. Moreover, since only severe OSA has been associated with increased angiotensin II and aldosterone (moderate OSA was usually associated with normal levels of these agonists), rennin-angotensin–aldosterone (RAA) blockers had a modest anti-hypertensive effect in mild/moderate OSA [45]. Finally, thiazide diuretics have not been very effective in OSA patients without fluid retention [40]. Also, important data are missing, e.g. how many OSA patients are controlled under monotherapy with beta-blockers (BB), angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARB's)? How many OSA patients remained uncontrolled despite the use of two or more AH drugs? What should be the second drug added to BB or RAA blockers? How do individual AHD behave when included in an AH regimen? In addition, the impact of these studies in clinical practice is unknown, as epidemiological studies designed to investigate the AH medication profile in OSA patients are missing. In addition, the more recent recommendations for the management of patients with OSA and hypertension are inconclusive regarding the use of AHD and recognise the lack of strong evidence for the establishment of a first-line AH regimen for these patients [20]. Other authors sustain the idea that since there is no clear evidence for preference for a specific class of AHD, the selection should primarily be guided by the patient's cardiometabolic profile and associated comorbidities (e.g. obesity, metabolic syndrome, diabetes mellitus and cardiovascular diseases) [19]. Moreover, these authors recommend that, due to the lack of relevant trials focused on the use of associations of AHD in OSA patients, the choice should rely on current hypertension guidelines and the adverse effects of AHD need to be contemplated as well [19]. Our study was designed assuming that

clinicians based their therapeutic decisions on these assumptions.

Surprisingly, in our population, the most commonly prescribed AHD were diuretics and RAA blockers. However, the number of OSA patients using these AHD with uncontrolled BP was significantly high. In fact, 61.2% (30/49) and 61.3% (65/106) of patients under a monotherapy and polytherapy regimens, respectively, were uncontrolled. This evidence suggests that the increase in the number of antihypertensive drugs is not apparently relevant in OSA patients. If the low efficacy of polytherapy in controlling BP is linked to OSA severity, the difference between the efficacy of monotherapy and polytherapy in severe patients would be higher in the subgroup of severe patients with BMI $\geq$30 kg/m^2, which was not the case.

On the other hand, before CPAP adaptation, no associations were found between AH regimens and number of AHD and BP control. These findings suggest that none of the current AHD, either alone or in association, have been shown to be effective enough to control BP. Additionally, the number of AHD included in a specific regimen seems not to be relevant to the control of BP in patients with OSA.

In the study performed after CPAP adaptation, we decided to include only patients who were adherent with CPAP therapy for at least 4 hours per night on average, as this is a threshold that is often used to define minimum acceptable CPAP use. According to the suggestion of a past study, patients in these conditions of CPAP use had an appreciable decrease in the incidence of hypertension [16]. Additionally, the beneficial effect of nasal CPAP therapy on BP seems to be closely associated with the presence of untreated or resistant hypertension, with OSA severity (more pronounced in severe OSA) and with patient compliance to CPAP [2]. Therefore, it would be expected that the combined effect of CPAP and AHD on BP control was more pronounced than the isolated effect of AHD. However, the lack of association between AH regimens and BP control remained after CPAP adaptation, which suggests the slight effect of CPAP in controlling BP. These findings are consistent with the results of several past studies [4, 11-13]. Börgel et al. [33] revealed that the absence of antihypertensive drugs is an independent predictor for the lowering effect of CPAP therapy on systolic and diastolic BP. Furthermore, Dernaika et al. reported that the effects of CPAP therapy on BP regulation appear to be less evident in hypertensive patients under a drug regimen [46]. In our patient population, eight of the sixteen patients who were not on AH medication, after CPAP adaptation there BP became controlled. Both results suggest that an earlier diagnosis of hypertension related to OSA is more important than the AH regimen selection.

Moreover, the effect of CPAP on lowering BP was clearly more significant in nocturnal BP, mainly in nocturnal SBP. Anyway, however important, this effect was not enough to reach the intended purpose for these patients, since it did not allow the reclassification from uncontrolled to controlled BP. In fact, despite some authors [47] having reported the beneficial effect of CPAP in preventing the bursts of sympathetic activity present at the end of each respiratory event, in our case, the hypothetical reduction in sympathetic activity was not associated with an appreciable decrease in diurnal BP. Consequently, in patients with OSA, more important than studying the effect of CPAP alone on BP reduction is evaluating the

combined effect of CPAP and AH medication. The study performed by Dernaika et al. [46] evaluated the long-term effects of CPAP therapy on BP in patients with OSA and resistant hypertension. The results revealed that CPAP permitted the de-escalation of antihypertensive treatment in 71% of patients with resistant hypertension, but did not significantly alter the antihypertensive regimen in the controlled group. However, in our opinion, the study of Dernaika et al. [46] presents two main drawbacks. First, the study did not characterize the different AH associations (it only referred to the number of patients that were undergoing a specific AHD class) and second, it did not evaluate the efficacy of each class on BP control. The same limitations were found in the study of Börgel et al. [33], which was performed to evaluate the interaction between BP-lowering effects of CPAP and AHD. Additionally, Pépin et al. [48] designed a study to assess the respective efficacy of CPAP and valsartan (ARBs) in reducing BP hypertensive patients with OSA that had never been treated for either condition and speculated that the combined effect of both therapies on BP control might be additive in patients in whom hypertension is still uncontrolled by specific AH drugs. However, they did not quantify the combined effect of CPAP and valsartan. They found that valsartan induced a four-fold higher decrease in mean 24h BP than CPAP [48]. Thus, the results of our pilot study revealed, for the first time, that none of the current AH regimens are superior to the others, either alone or when combined with CPAP, in promoting BP control, and underlines that new AHD drugs for this particular population are definitely needed.

Finally, gender, OSA severity and 24 h BP profile at baseline were identified as independent predictors of BP control after CPAP adaptation. Our results show that male patients with an AHI higher than 15 events/hour of sleep and patients with a non-dipper profile had a three-fold increased odds of presenting uncontrolled BP after CPAP adaption. Although Robinson et al. [49] did not identify OSA severity as a predictor of the fall in 24h mean BP with CPAP treatment, the impact of this decrease in BP control remains unknown. In a more recent small study, male gender, Epworth sleepiness scale, BMI, smoking, alcohol use and baseline 24h mean BP were identified as independent predictors of a decrease in 24h mean BP after CPAP adaptation [50]. The apparent controversy of whether or not OSA severity is a predictor of the beneficial effect of CPAP in lowering BP could be explained by the differences in the outcome variable analysed. In our perspective, uncontrolled BP (present study) is a stronger and more relevant clinical outcome than the decrease in 24h-mean BP values.

Limitations

We are conscious about some limitations of our study. First, our sample should ideally be larger and provided from more than a single centre, in order to avoid referral bias. However, our centre could be considered representative of the general OSA population because we found similar characteristics to those described in the literature. The huge variability in AHD regimen found in the present work together with the sample size, does not allow us to identify the best AHD regimen for these patients. Additionally, the results of this exploratory study strongly suggest that none of the available AHD are clearly able to achieve adequate BP

control in OSA patients. Therefore, the impact of a much larger epidemiological study could be also limited and, in our opinion, the search for new therapeutic strategies will be more useful.

Additionally, despite the use of a "Brown-Bag" medication review in order to record patients ongoing medication profile, data regarding adherence to AH drug treatment (before and after inclusion) were checked only based on self-reported adherence and were not confirmed by a "pill-count" method or other. However, this variable does not invalidate the study, since it is common to all patients. Since the differences between efficacies within each class are not well documented, the evaluation of each drug *per se* was not considered relevant. Therefore, the analysis was conducted taking into account the regimens of AH drugs with higher prevalence. Nevertheless, the added value of the present study is the evaluation of the efficacy of AH regimens commonly used in these patients instead of analysing the effect of a specific AHD. Despite these drawbacks, our results provide new insights into the need for innovative AHD to treat OSA patients.

CONCLUSIONS

In conclusion, the findings of the present work suggest, for the first time, that none of the currently available antihypertensive drugs, either alone or in association, have been shown to be effective enough to control BP in these patients, either before or after CPAP. BP control is independent of both the antihypertensive regimen and the number of antihypertensive drugs and the increase in the number of AHD does not appear to be relevant to control BP in patients with OSA. Our results also confirm that although CPAP improves 24h-BP profile, it is not enough to warrant a suitable BP control in patients with OSA. Moreover, the finding that there is a significant improvement in BP control in patients under no AHD, after CPAP adaptation, suggest that an earlier diagnosis of hypertension related to OSA is more relevant than the AH regimen selection, probably because the available AH regimens are useful to prevent but not able to completely revert hypertension. Finally, male, moderate/severe and non-dipper patients, at baseline, had three-fold increased odds of being uncontrolled after CPAP adaptation.

COMPETING INTERESTS

All authors declare that they have no conflict of interest to the publication of this manuscript. No financial support has been involved.

AUTHORS' CONTRIBUTIONS

LND, CB, EM and ALP designed the study. LND, CB and PP collected the data. LND and ALP analyzed data. All authors participated in the interpretation of the results. LND, ALP and EM wrote the manuscript. All authors have read and approved the final manuscript.

ACKNOWLEDGMENTS

The authors would like to thank all of the cardio-respiratory technicians from Centro Hospitalar Lisboa Norte, EPE for their technical support.

The present work was supported by Fundação para a Ciência e Tecnologia (FCT) grant (PTDC/SAU-TOX/ 112264/2009) and CEDOC (Portugal). Lucília N. Diogo holds a FCT PhD grant (SFRH / BD / 48335 / 2008).

REFERENCES

1. Kapa S, Sert Kuniyoshi FH, Somers VK: Sleep apnea and hypertensioninteractions and implications for management. Hypertension 2008, 51: 605-8.
2. Baguet JP, Barone-Rochette G, Pépin JL: Hypertension and obstructive sleep apnea syndrome: current perspectives. J Hum Hypertens 2009, 23: 431.
3. Wolf J, Lewicka J, Narkiewicz K: Obstructive sleep apnea: an update on mechanisms and cardiovascular consequences. Nutr Metab Cardiovasc Dis 2007, 17: 233-40.
4. Calhoun DA: Obstructive sleep apnea and hypertension. Curr Hypertens Rep 2010, 12: 189-95.
5. Mancia G, De Backer G, Dominiczak A, Cifkova R, Fagard R, Germano G, et al: 2007 Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). Eur Heart J 2007, 28: 1462-536.
6. Mancia G, Fagard R, Narkiewicz K, Redon J, Zanchetti A, Böhm M, et al: 2013 ESH/ESC guidelines for the management of arterial hypertension: the Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). Eur Heart J 2013, 34: 2159-219.
7. Oliveras A, Schmieder RE: Clinical situations associated with difficult-to-control hypertension. J Hypertens 2013, 31 (Suppl. 1): S3-8.
8. Marin JM, Agusti A, Villar I, Forner M, Nieto D, Carrizo SJ, et al: Association between treated and untreated obstructive sleep apnea and risk of hypertension. JAMA 2012, 307: 2169-76.
9. Pedrosa RP, Drager LF, Gonzaga CC, Sousa MG, de Paula LK, Amaro AC, et al: Obstructive sleep apnea: the most common secondary cause of hypertension associated with resistant hypertension. Hypertension 2011, 58: 811-7.
10. Epstein LJ, Kristo D, Strollo PJ Jr, Friedman N, Malhotra A, Patil SP, et al; Adult Obstructive Sleep Apnea Task Force of the American Academy of Sleep Medicine: Clinical Guideline for the Evaluation, Management and Long-term Care of Obstructive Sleep Apnea in Adults. J Clin Sleep Med 2009, 5: 263-76.
11. Alajmi M, Mulgrew AT, Fox J, Davidson W, Schulzer M, Mak E, et al: Impact of continuous positive airway pressure therapy on blood pressure in patients with obstructive sleep apnea hypopnea: a meta-analysis of randomized controlled trials. Lung 2007, 185: 67-72.
12. Bazzano LA, Khan Z, Reynolds K, He J: Effect of nocturnal nasal continuous positive airway pressure on blood pressure in obstructive sleep apnea. Hypertension 2007, 50: 417-23.
13. Ziegler MG, Milic M, Elayan H: Cardiovascular regulation in obstructive sleep apnea. Drug Discov Today Dis Models 2011, 8: 155-160.
14. Pepperell JC, Ramdassingh-Dow S, Crosthwaite N, Mullins R, Jenkinson C, Stradling JR, Davies RJ: Ambulatory blood pressure after therapeutic and sub-therapeutic nasal continuous positive airway pressure for obstructive sleep apnea: a randomised parallel trial. Lancet 2002, 359: 204-10.
15. Becker HF, Jerrentrup A, Ploch T, Grote L, Penzel T, Sullivan CE, Peter JH: Effect of nasal continuous positive airway pressure treatment on blood pressure in patients with obstructive sleep apnea. Circulation 2003, 107: 68-73.
16. Barbé F, Durán-Cantolla J, Sánchez-de-la-Torre M, Martínez-Alonso M, Carmona C, Barceló A, Chiner E, et al; Spanish Sleep And Breathing Network: Effect of continuous positive airway pressure on the incidence of hypertension and cardiovascular events in non-sleepy patients with obstructive sleep apnea: a randomized controlled trial. JAMA 2012, 307: 2161-8.
17. Kasiakogias A, Tsioufis C, Thomopoulos C, Aragiannis D, Alchanatis M, Tousoulis D, Papademetriou V, Floras JS, Stefanadis C: Effects of continuous positive airway

pressure on blood pressure in hypertensive patients with obstructive sleep apnea: a 3-year follow-up. J Hypertens 2013, 31: 352-60.

18. Parati G, Lombardi C, Hedner J, et al.; European Respiratory Society; EU COST ACTION B26 members: Position paper on the management of patients with obstructive sleep apnea and hypertension: joint recommendations by the European Society of Hypertension, by the European Respiratory Society and by the members of European COST (COoperation in Scientific and Technological research) ACTION B26 on obstructive sleep apnea. J Hypertens 2012, 30: 633-46.
19. Tsioufis C, Kasiakogias A, Thomopoulos C, Manolis A, Stefanadis C: Managing hypertension in obstructive sleep apnea: the interplay of continuous positive airway pressure, medication and chronotherapy. J Hypertens 2010, 28: 875-82.
20. Parati G, Lombardi C, Hedner J, Bonsignore MR, Grote L, Tkacova R, et al; EU COST Action B26 members: Recommendations for the management of patients with obstructive sleep apnea and hypertension. Eur Respir J 2013, 41: 523-38.
21. Caskie GI, Willis SL, Warner Schaie K, Zanjani FA: Congruence of medication information from a brown bag data collection and pharmacy records: findings from the Seattle longitudinal study. Exp Aging Res 2006, 32: 79-103.
22. Collop NA, Anderson WM, Boehlecke B, Claman D, Goldberg R, Gottlieb DJ, Hudgel D, Sateia M, Schwab R; Portable Monitoring Task Force of the American Academy of Sleep Medicine: Clinical guidelines for the use of unattended portable monitors in the diagnosis of obstructive sleep apnea in adult patients. Portable Monitoring Task Force of the American Academy of Sleep Medicine. J Clin Sleep Med 2007, 3: 737-47.
23. Berry RB, Budhiraja R, Gottlieb DJ, Gozal D, Iber C, Kapur VK, et al: Rules for scoring respiratory events in sleep: update of the 2007 AASM Manual for the Scoring of Sleep and Associated Events. Deliberations of the Sleep Apnea Definitions Task Force of the American Academy of Sleep Medicine. J Clin Sleep Med 2012, 8: 597-619.
24. Hosmer DW, Lemeshow S: Applied Logistic Regression. New York: John Wiley & Sons; 1989.
25. Lavie P, Herer P, Hoffstein V: Obstructive sleep apnea syndrome as a risk factor for hypertension: population study. BMJ 2000, 320: 479-82.
26. Guralnick AS, Pant M, Minhaj M, Sweitzer BJ, Mokhlesi B: CPAP adherence in patients with newly diagnosed obstructive sleep apnea prior to elective surgery. J Clin Sleep Med 2012, 8: 501-6.
27. Friedman M, Tanyeri H, La Rosa M, Landsberg R, Vaidyanathan K, Pieri S, Caldarelli D: Clinical predictors of obstructive sleep apnea. Laryngoscope 1999, 109: 1901-7.
28. Fletcher EC, DeBehnke RD, Lovoi MS, Gorin AB: Undiagnosed sleep apnea in patients with essential hypertension. Ann Intern Med 1985, 103: 190-5.
29. Davies CW, Crosby JH, Mullins RL, Barbour C, Davies RJ, Stradling JR: Case-control study of 24 hour ambulatory blood pressure in patients with obstructive sleep apnea and normal matched control subjects. Thorax 2000, 55: 736-40.
30. Baguet JP, Lévy P, Barone-Rochette G, Tamisier R, Pierre H, Peeters M, Mallion JM, Pépin JL: Masked hypertension in obstructive sleep apnea syndrome. J Hypertens 2008, 26: 885-92.
31. Hla KM, Young T, Finn L, Peppard PE, Szklo-Coxe M, Stubbs M: Longitudinal association of sleep-disordered breathing and non-dipping of nocturnal blood pressure in the Wisconsin Sleep Cohort Study. Sleep 2008, 31: 795-800.
32. Lavie P, Hoffstein V: Sleep apnea syndrome: a possible contributing factor to resistant. Sleep 2001, 24: 721-5.
33. Börgel J, Sanner BM, Keskin F, Bittlinsky A, Bartels NK, Büchner N, Huesing A, Rump LC, Mügge A: Obstructive sleep apnea and blood pressure. Interaction between the blood pressure-lowering effects of positive airway pressure therapy and

antihypertensive drugs. Am J Hypertens 2004, 17: 1081-7.

34. Baguet JP, Hammer L, Lévy P, Pierre H, Rossini E, Mouret S, et al. Night-time and diastolic hypertension are common and underestimated conditions in newly diagnosed apnoeic patients. J Hypertens 2005; 23: 521-7.
35. Pedrosa RP, Drager LF, Gonzaga CC, Sousa MG, de Paula LK, Amaro AC, et al: Obstructive sleep apnea: the most common secondary cause of hypertension associated with resistant hypertension. Hypertension 2011, 58: 811-7.
36. Lloberes P, Lozano L, Sampol G, Romero O, Jurado MJ, Ríos J, Untoria MD, Tovar JL: Obstructive sleep apnea and 24-h blood pressure in patients with resistant hypertension. J Sleep Res 2010, 19: 597-602.
37. Pedrosa RP, Drager LF, G de Paula LK, Amaro AC, Bortolotto LA, Lorenzi-Filho G: Effects of obstructive sleep apnea treatment on blood pressure in patients with resistant hypertension: A randomized trial. Chest. 2013 Apr 18. doi: 10.1378/chest.13-0085.
38. Pimenta E, Stowasser M, Gordon RD, Harding SM, Batlouni M, Zhang B, Oparil S, Calhoun DA: Increased dietary sodium is related to severity of obstructive sleep apnea in patients with resistant hypertension and hyperaldosteronism. Chest 2013, 143: 978-83.
39. Pelttari LH, Hietanen EK, Salo TT, Kataja MJ, Kantola IM: Little effect of ordinary antihypertensive therapy on nocturnal high blood pressure in patients with sleep disordered breathing. Am J Hypertens 1998, 11: 272-9.
40. Kraiczi H, Hedner J, Peker Y, Grote L: Comparison of atenolol, amlodipine, enalapril, hydrochlorothiazide, and losartan for antihypertensive treatment in patients with obstructive sleep apnea. Am J Respir Crit Care Med 2000, 161: 1423-8.
41. Heitmann J, Greulich T, Reinke C, Koehler U, Vogelmeier C, Becker HF, Schmidt AC, Canisius S: Comparison of the effects of nebivolol and valsartan on BP reduction and sleep apnea activity in patients with essential hypertension and OSA. Curr Med Res Opin 2010, 26: 1925-32.
42. Zou D, Grote L, Eder DN, Radlinski J, Hedner J: A double-blind, crossover study of Doxazosin and Enalapril on peripheral vascular tone and nocturnal blood pressure in sleep apnea patients. Sleep Med 2010, 11: 325-8.
43. Mayer J, Weichler U, Herres-Mayer B, Schneider H, Marx U, Peter JH: Influence of metoprolol and cilazapril on blood pressure and on sleep apnea activity. J Cardiovasc Pharmacol 1990, 16: 952-61.
44. Pimenta E, Gaddam KK, Oparil S: Mechanisms and treatment of resistant hypertension. J Clin Hypertens (Greenwich) 2008, 10:239-44.
45. Ziegler MG, Milic M, Sun P: Antihypertensive therapy for patients with obstructive sleep apnea. Curr Opin Nephrol Hypertens. 2011, 20:50-5.
46. Dernaika TA, Kinasewitz GT, Tawk MM: Effects of nocturnal continuous positive airway pressure therapy in patients with resistant hypertension and obstructive sleep apnea. J Clin Sleep Med 2009, 5: 103-7.
47. Somers VK, Dyken ME, Clary MP, Abboud FM: Sympathetic neural mechanisms in obstructive sleep apnea. J Clin Invest 1995, 96: 1897-904.
48. Pépin JL, Tamisier R, Barone-Rochette G, Launois SH, Lévy P, Baguet JP: Comparison of continuous positive airway pressure and valsartan in hypertensive patients with sleep apnea. Am J Respir Crit Care Med 2010, 182: 954-60.
49. Robinson GV, Langford BA, Smith DM, Stradling JR: Predictors of blood pressure fall with continuous positive airway pressure (CPAP) treatment of obstructive sleep apnea (OSA). Thorax 2008, 63: 855-9.
50. Yorgun H, Kabakçi G, Canpolat U, Kirmizigül E, Sahiner L, Ates AH, et al: Predictors of blood pressure reduction with nocturnal continuous positive airway pressure therapy in patients with obstructive sleep apnea and prehypertension. Angiology 2013, 00(0): 1-6.

SECTION 2

Section 2 presents the key results from the experimental studies carried out at Nova Medical School (animal facility labs) and Chronic Diseases Research Center (CEDOC).
Please note that although the second study has been designed to test the viability of oral voluntary administration, for long-term delivery of both carvedilol and losartan, the animals systematically refused to ingest the mixture of carvedilol with the three different vehicles. For that reason, we decided to submit for publication only the results of losartan. Moreover, carvedilol had to be delivered by *gavage* in the first study since carvedilol voluntary oral administration systematically failed. Data regarding carvedilol voluntary oral administration will be discussed later in the Discussion Chapter.

The results obtained in this section originated two manuscripts that are currently *in submission* and accepted for publication in *JAALAS*, respectively. The main findings of these works are listed bellow:

Efficacy of carvedilol in reversing hypertension induced by chronic intermittent hypoxia in rats.
Diogo LN, Pereira SA, Faustino I, Nunes AR, Afonso RA, Santos AI, Monteiro EC *(in submission).*

1) CIH significantly increased MAP, diastolic and systolic BP in our animal model. No effect was observed for HR *(see Figure 3, page 97).*
2) No reduction in mean arterial pressure was observed with the doses of 10, 30 and 50 mg/Kg of CVD *(see Figure 5, page 99).*
3) All doses of CVD promoted a significant decrease in HR *(see Figure 6, page 100).*
4) The ratios S/(R+S) of CVD enantiomers between rats exposed to CIH and normoxic conditions were different *(see Figure 8, page 101).*
5) Animals exposed to CIH and treated with 50 mg/kg/day of CVD presented higher R-(+)-CVD and S-(-)-CVD plasma concentrations than normoxic animals treated with the same dose, although this difference did not reach statistical significance.

Losartan voluntary oral administration – a less stressful approach in rats
Diogo LN, Faustino IV, Afonso RA, Monteiro EC, Santos AI. *JAALAS* (*in press*)

1) Blood glucose levels were reduced by NUT administration and PB induces increased levels of triglycerides and total cholesterol *(see Figures 2, 3 and 4, pages 118, 119 and 120).*

2) SD group presented a higher concentration of losartan when compared with gavage group, without changing lipid and glucose profiles *(see Figure 5, page 121).*

3) The three vehicles are viable for daily single dose voluntary ingestion of losartan, from which SD proved to be the best alternative.

4) SD is better suited than gavage for losartan oral daily administration.

5) Voluntary ingestion proved to be an effective method for a controlled daily dose administration, with a defined timetable that is independent of handling and restraint procedures.

RESEARCH PAPER

EFFICACY OF CARVEDILOL IN REVERSING HYPERTENSION INDUCED BY CHRONIC INTERMITTENT HYPOXIA IN RATS

Lucília N Diogo[a]*, Sofia A Pereira[a], Ana R Nunes[a], Inês V Faustino[a], Ricardo A Afonso[a,b], Ana I Santos[b] and Emília C Monteiro[a]

[a]Centro de Estudos de Doenças Crónicas, CEDOC, NOVA Medical School / Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo dos Mártires da Pátria, 130, 1169-056 Lisboa, Portugal [b] Department of Physiology, NOVA Medical School / Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo dos Mártires da Pátria, 130, 1169-056 Lisboa, Portugal.

Background and Aim: Animal models of chronic intermittent hypoxia (CIH) mimic the HT observed in patients with OSA. Antihypertensive drugs were used in these models to address the physiological mechanism but not to revert established hypertension. We aimed to investigate the efficacy of carvedilol, a nonselective beta-blocker with intrinsic anti- α1-adrenergic activity and antioxidant properties in a rat model of CIH-induced hypertension. The variability of carvedilol enantiomers in plasma concentrations was also evaluated. **Methods:** Male Wistar rats with indwelling blood pressure telemeters were exposed during their sleep period to 5.6 CIH cycles/h, 10.5 h/day, for 60 days. Carvedilol administration, by gavage, began on Day 36 of the CIH period and lasted 25 days. Animals specifically used for pharmacokinetic studies were exposed for 60 days to normal air (21% O_2 and 79% N_2), in the same room as the CIH animals. R-(+)-CVD and S-(-)-CVD plasma concentrations were determined by HPLC. Statistical analysis was performed using GraphPad Prism and statistical significance for all tests was set at the level of $p<0.05$. **Results:** CIH significantly increased diastolic and systolic blood pressure ($p<0.05$, for all variables) by 25.7 and 21.6 mmHg respectively, while no effect was observed for HR in our animal model. Despite the doses of 10, 30 and 50 mg/Kg of CVD promoted a significant reduction in HR ($p<0.05$, for all doses), no decrease in arterial pressure was observed. The S/(R+S) ratio of CVD enantiomers was lower ($p<0.05$) in rats exposed to CIH than in animals under normoxic conditions. **Conclusions:** Together our results suggest that the blockade of the sympathetic nervous system together with the putative pleiotropic effects of carvedilol was not able to revert hypertension induced by CIH. Despite finding that CIH induced pharmacokinetic changes in the R/(R+S) ratio, they are not apparently responsible for the lack of efficacy of carvedilol in reversing this particular type of hypertension.

Key words: antihypertensive drugs, beta-blockers, carvedilol, chronic intermittent hypoxia, telemetry.

***Corresponding author:** Lucilia Neves Diogo, PharmD, MSc, NOVA Medical School / Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo Mártires da Pátria, 130, 1169-056 Lisboa, Portugal, lucilia.diogo@fcm.unl.pt

INTRODUCTION

Chronic intermittent hypoxia (CIH) is a feature that is present in sleep-disordered breathing, namely in obstructive sleep apnea (OSA). Although OSA has been associated with numerous cardiovascular conditions, it has been more etiologically connected to systemic hypertension (Kapa *et al.,* 2008). The mechanisms involved in the pathogenesis of HT can be summarised in relation to two main pathways: sympathetic nervous system stimulation mediated mainly by activation of carotid body chemoreflexes and/or asphyxia, and the systemic effects of CIH (for a review see Diogo and Monteiro, 2014).

The effects of antihypertensive agents on OSA patients are not consistent and it is imperative to identify preferred compounds for adequate BP control in this group of patients (Parati *et al.,* 2013). Studies aimed at investigating the antihypertensive effect of antihypertensive drugs (AHDs) in animal models of CIH are scarce, however, and were not designed to respond to pharmacological issues (Allahdadi *et al.,* 2008; Belaidi *et al.,* 2009; da Silva *et al.,* 2011; Del Rio *et al.,* 2010; Fenik *et al.,* 2012; Fletcher *et al.,* 1999; Hung et al, 2013; Kanagy *et al.,* 2001; Knight *et al.,* 2013; Kumar *et al.,* 2006; Moya *et al.,* 2014; Soukhova O'Hare *et al.,* 2008; Troncoso Brindeiro *et al.,* 2007). AHDs have been used solely as pharmacological tools to address physiological mechanisms and to prevent HT (Diogo and Monteiro, 2014). Although individual drug studies, performed in humans, find that the blockade of β1- adrenergic receptors (e.g. atenolol, carvedilol, nebivolol and metoprolol) might be helpful (Heitmann *et al.,* 2010; Kario *et al.,* 2014; Kraiczi *et al.,* 2000; Mayer *et al.,* 1990; Pelttari *et al.,* 1998) in reversing HTA related to OSA, to the best of our knowledge, no experimental study has yet been designed to evaluate the long-term effects of carvedilol (CVD) or other beta-adrenergic blocking agents.

CVD is a non-selective beta-blocker with intrinsic anti-α 1-adrenergic activity and with recognised antioxidant properties. The broad range of its adrenergic inhibition seems to offer advantages over other beta-adrenergic blocking agents (DiNicolantonio and Hackam, 2012). This antihypertensive drug is administered orally as a racemic mixture of two enantiomers and its beta-blocking activity is the result of the S- enantiomer, while its alpha-blocking activity is the result of both R- and S-enantiomers (Peccinini *et al.,* 2008).

We aimed to investigate, in a rat model of CIH-induced hypertension, the efficacy of carvedilol in reducing both BP parameters and heart rate. Secondly, we sought to explore the effects of CIH on the pharmacokinetics profile of carvedilol, in order to test the hypothesis that IH changes carvedilol pharmacokinetics, compromising or enhancing their efficacy. Together these experiments might contribute to attaining more specific information concerning carvedilol use in HT related to OSA.

MATERIAL AND METHODS

Drugs and chemicals

CVD was kindly provided by Tecnimede (Sintra, Portugal; manufacturer batch: 5334-11-015) and MC was purchased from Sigma-Aldrich (Sintra, Portugal). R-(+)-CVD and S-(-)-CVD were obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany).

The enantiomerically pure chiral agent, (-)-menthyl chloroformate (MCF) was obtained from Sigma-Aldrich (Sintra, Portugal). HPLC grade solvents used in the extraction procedure and for the mobile phase of the chromatographic system were obtained from VWR (Lisbon, Portugal).

Animals

Experiments were performed in thirty-one male Wistar rats (Ratus norvegicus), aged 60-75 days, with mean body weight 308.4±45.36 g, obtained from the NOVA Medical School animal facility. Animals were housed individually in polycarbonate cages with wire lids (Tecniplast, Buguggiate, Varese, Italy), under 12 h light/dark cycles (8 am - 8 pm), at a room temperature 22 ± 2.0 °C and relative humidity 60± 10%. Rats were maintained on a standard laboratory diet (SDS diets RM1) and reverse osmosis water, given ad libitum. Corncob bedding (Probiológica, Lisbon, Portugal) was used and changed once a week. Animals were Specific Pathogen Free (SPF) according to FELASA recommendations (Nicklas *et al.*, 2002).

A growth curve for body weight gain in the first 180 days was obtained with daily weights from three hundred and fifty seven male Wistar rats born in the NOVA Medical School animal facility. Animals from the colony were randomly weighed, inside the animal rooms, for a period of one year until a sample of 25 (91 to 180 days) or 50 (1-90 days) weights per day was achieved. The experimental groups in the present report were not part of the colony growth chart.

The applicable institutional and governmental regulations concerning ethical use of animals were followed, according to the NIH Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985), the European guidelines for the protection of animals used for scientific purposes (European Union Directive 2010/63/EU) and Portuguese Law nº 113/2013. Experimental procedures were previously approved by the Institutional Ethics Committee of the NOVA Medical School for animal care and use in research (approval date: May 11th, 2011).

Experimental design

Three different doses were administered to investigate the long-term effects (25 days) of CVD in an experimental model of hypertension related to CIH. Rats were randomly assigned and divided into five groups: Group I (CVD 10 mg/kg/day - CIH; n=5); Group 2 (CVD 30 mg/kg/day - CIH; n=7); Group 3 (CVD 50 mg/kg/day - CIH; n=8); Group 4 (control group administered vehicle (0.5% methylcellulose (MC)); n=5); Group 5 (CVD 50 mg/Kg/day – Nx; n=6). MC 0.5% (2 mL) was used to facilitate the dissolution and absorption of CVD, since it is a very lipid soluble drug, (Rodriguez-Perez *et al.*, 1997).

Animals from Groups 1, 2, 3 and 4 were gentled for 10 minutes daily for one week prior surgery, to minimise the discomfort related to experimental manipulation and reduce data variability, and were instrumented with indwelling blood pressure telemeters. Rats were allowed to recover for 10 days, after transmitter implantation surgery, before any measurements were recorded. A period of 5/6 days was then given for chamber acclimatization under normoxic conditions (21 O_2% + 79% N_2) and baseline cardiovascular data was recorded.

Animals were then exposed to 60 days of CIH, during their sleep period, and CVD or vehicle administration, by gavage, started at day 36 and lasted 25 days. Animals from Group 5 (normoxic rats) were exposed for 60 days to normal air (21% O_2 and 79% N_2), in the same room as the CIH animals in order to experience similar conditions. Similarly, CVD administration by gavage started at Day 36 and lasted 25 days for the other groups. Since these rats were specifically used for pharmacokinetic studies, the implantation of telemetry transmitters was not performed.

At the end of the experiments, 2-3 hours after drug or vehicle delivery, the rats were anaesthetised, by intraperitoneal injection with medetomidine (0.5mg/kg body weight; Domitor®, Pfizer Animal Health, Auckland, New Zealand) and ketamine (75mg/kg body weight; Imalgene 1000®, Mérial, Lyon, France), and cardiac puncture was performed without thoracotomy, with a 20 G needle with a 10 mL syringe, to collect blood for further quantifications. The animals were then euthanized with an intracardiac overdose of sodium pentobarbital (Eutasil®, Ceva Animal Health, Libourne, France), and the death was confirmed by cervical dislocation.

Rats were weighed at baseline and once a week during the entire study. The amount of carvedilol was adjusted weekly to ensure the doses of 10, 30 and 50 mg/Kg/day (p.o). CVD was weighed daily, immediately before administration, dissolved in the vehicle and labelled individually for each rat. CVD dissolved in 0.5% MC (2 mL) or vehicle alone was given daily to the animals on approximately the same schedule (9:00 to 9:30 am), always after BP measurement and before exposure to CIH conditions. Control animals received equivalent daily oral gavage volumes (2 mL). All rats underwent a 7 day handling acclimatization period and were handled daily for a period of 2 minutes each by the same individual and accustomed to the gavage position, in a different animal room. Gavage was performed using a sterile polypropylene feeding tube (gauge 15; tip diameter: 3 mm; length: 78 mm; Instech Laboratories, Inc., USA) in order to reduce the risk of trauma, perforation and cross contamination (Morton *et al.*, 2001).

Surgical instrumentation

Animals were instrumented under medetomidine (0.5 mg/kg body weight (i.p.); Domitor®, Pfizer Animal Health) and ketamine (75 mg/kg body weight (i.p.); Imalgene 1000®, Mérial, Lyon, France) anaesthesia with indwelling blood pressure telemeters (model TA11PA-C40, Data Sciences Corporation, St. Paul, MN), allowing daily recording of mean arterial BP, systolic and diastolic BP and heart rate (HR). The animals were provided with preoperative analgesia, using the opioid analgesic drug butorfanol (1mg/Kg/mL (s.c.); Dolorex®, Intervet International GmbH, Unterschleissheim, Germany), in order to ensure pain relief during and after surgery. The animal's body temperature was maintained throughout the experimental procedure using a heating pad. To minimise corneal desiccation, the eyes were lubricated with sterile saline. In preparation for abdominal incision, this area was shaved with a clipper, scrubbed with a povidone-iodine solution (Betadine®, Mundipharma AG, Basel, Switzerland) and 70% isopropyl alcohol, and covered with a sterile surgical drape. The aseptic conditions of

the microsurgical instruments was ensured by autoclaving and maintained at all times with the use of a hot bead sterilizer (Fine Science Tools GmbH, Heidelberg, Germany).

The radiotelemetry transmitter was implanted aseptically into the abdominal aorta, exposed via a ventral midline incision in the abdominal cavity. The aorta was occluded using bulldog type clamps (Fine Science Tools GmbH, Heidelberg, Germany) placed immediately after renal artery bifurcation and immediately before femoral artery bifurcation. A bent needle (20G, B.Braun Medical, Melsugen, Germany) was used as a catheter introducer and the pressure tip of the telemeter was guided beneath the needle and introduced into the aorta. The puncture site and the surrounding tissue were dried and a small drop of tissue adhesive (Vetbound, 3M Company, St Paul, MN, USA) was applied to seal the insertion point. The vessel clamps were carefully removed and, if there was no bleeding, the intestines were replaced in their original position and the abdominal cavity was flushed with warmed sterile saline to avoid tissue adhesion. The body of the transmitter was then placed on top of the intestines and secured to the abdominal muscle by closing the abdominal incision and incorporating the suture rib on the device into the closure, using non-absorbable sutures (Surgisilk 4/0, Sutures Ltd, Wrexham Wales UK) in a simple interrupted pattern. Finally, the skin incision was closed using non-absorbable sutures (Surgisilk 4/0, Sutures Ltd UK).

Thirty minutes surgery, the anaesthesia was reversed with atipamezole (0.25 mg/Kg/2mL (i.p); Antisedan®, Orion Pharma, Espoo, Finland). All animals were individually housed, with environmental enrichment, to protect the wound sites and minimise stress. A heating pad on a low setting was provided to allow the animals to self-regulate temperature in the first hours after surgery. Postsurgical recovery was monitored by daily visual examination (return of normal postures and behaviours) and daily food and water intake. Rats were treated postoperatively for 2-3 days with the nonsteroidal anti-inflammatory drug carprofen (5 mg/Kg/1mL (s.c), Rimadyl®, Vericore Limited, Dundee, UK).

Chronic intermittent hypoxia (CIH) exposure

Animals were kept in a eucapnic atmosphere, inside medium A-chambers (76 x 51 x 51 cm, A-60274-P, Biospherix Ltd, NY, USA) with ad libitum food and water access. The chambers were equipped with gas injectors and sensors for oxygen (O_2) and carbon dioxide levels (CO_2) in order to ensure the accuracy of CIH cycles. Accumulation of CO_2 was prevented by the continuous flow of the gas mixtures, the circulation of the gases inside de chambers through vent holes and by the presence in the chamber of self-indicating soda lime (AnalaR Normapur®, VWR International BVBA, Leuven, Belgium), which absorbs the expired CO_2. The CO_2 levels inside the chambers never exceeded 1%. A silica gel (Chameleon® C 2-6 mm, VWR International BVBA, Leuven, Belgium) container was also placed inside the chambers in order to absorb water. Oxygen concentration inside the chambers was controlled using 100% nitrogen (N_2) and 100% O_2 via electronically regulated solenoid switches in a three-channel gas mixer, which gradually lowered the oxygen in the chamber from 21% to 5% O_2 (OxyCycler AT series,

Biospherix Ltd, NY, USA). The chambers were infused with 100% N_2 for 3.5 min to briefly reduce the O_2 concentration to 5%, and then with 100% O_2 for 7 min to restore oxygen to ambient levels of 21%, until the start of the next CIH cycle. Each CIH cycle lasted 10.5 min and the rats were exposed during their sleep period to 5.6 CIH cycles/h, 10.5 h/day, for 60 days. During the remaining hours of the day, the chambers were ventilated with a constant flow of room air to keep oxygen levels at 21%. O_2 was purchased as regular gas bottles (Gasin, Portugal), while N_2 was generated from the air using pressure swing adsorption technology via a high output nitrogen generator (Nitrogen 15 Plus, PSA Technology, Sysadvance, Maia, Portugal).

Collection of radiotelemetry data

Output from the telemetry transmitters was recorded via radio frequency signals obtained through the Data Acquisition System from Data Sciences International (DSI, St. Paul, Minn.). BP and HR measurements, obtained during 10 seconds sampling periods, were averaged and recorded for 15 minutes daily. Baseline recordings of BP and HR were performed for 5/6 days prior to CIH exposure.

Blood sampling

For plasma sampling, blood was collected to vacutainer tubes containing EDTA (ethylenediaminetetraacetic acid), kept on ice and immediately centrifuged at 3000 rpm (4 °C) for 10 min. Plasma samples were stored at −80 °C until analysed.

High-performance liquid chromatography (HPLC) analysis

R-(+)-CVD and S-(-)-CVD plasma concentrations were determined by HPLC, using a minor modification of the method described by Peccinini and colleagues (Peccinini *et al.*, 2008). Briefly, R-(+)-CVD and S-(-)-CVD were dissolved in methanol yielding stock solutions of 1 mg/mL. Calibration samples were prepared by adding known amounts of the diluted stock solution to rat plasma and covered a range of 10 - 300 ng/mL. Subsequently, calibration and plasma samples (250 μL) were spiked with 12.5 μL of an aqueous solution of 0.1M sodium hydroxide and extracted with 3 mL chloroform, for 20 minutes, in a vertical shaker. After centrifugation (1800 g, 4 ºC, 4 min), the organic phase was evaporated to dryness at 60 ºC in a Speed-Vac concentrator (Labconco, Kansas City, MO, USA). The resulting residue was reconstituted in 50 μL of 0.1 M sodium hydroxide and 50 μL of the chiral reagent MCF, at 1% concentration in dichloromethane (v/v), was added. The mixture was shaken in a vortex for 2 min. After adding 250 μL of water, carvedilol diastereoisomers were extracted with 3 mL chloroform for 10 min in a vertical shaker. A new centrifugation was performed and the organic phase was evaporated to dryness. Finally, the residue obtained was reconstituted in 50 μL of the mobile phase and 30 μL was injected into the HPLC. Plasma samples were diluted in the mobile phase whenever appropriate.

HPLC (Schimadzu, Kyoto, Japan) was accomplished by using a solvent delivery pump (model LC 9-A), autosampler (model 7725i; fluorescence detector: model RF 10AXL), and a

column oven (model CTO-10AS VP). The stationary phase was a column (250 x 4 mm; particle size: 5 µm; LiChrospher 100 RP-18; Merck, New Jersey, USA) protected by a guard-column (4 x 4mm; particle size: 5 µm; LiChrospher 100 RP-18e; Merck, New Jersey, USA). The mobile phase consisted of a mixture of 0.25N acetate buffer (pH 3, adjusted with acetic acid) and methanol (27:73 v/v) at a flow rate of 1mL/min, at 25º C. Both solutions were degassed for 15 min by sonication (VWR, Carnaxide, Portugal). The excitation wavelength was 285 nm and the emission wavelength 680 nm. Data acquisition and processing were performed using Shimadzu Class VP 7.X software.

Statistics

Data are presented as mean ± standard error of the mean (SEM). Unpaired t-test and Kruskal-Wallis test with Dunn´s multiple comparisons test were used, whenever appropriate, to evaluate the effect of CIH, CVD or MC 0.5% on the cardiovascular parameters. A comparison of CVD S/(R+S) plasma ratios between normoxic and CIH groups was performed using unpaired Student t-test. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., version 5.01, San Diego, CA). Statistical significance for all tests was set at the level of $p<0.05$.

RESULTS

Effect of CIH on body weight

To study the effect of CIH on body weight we started by characterising the NOVA Medical School colony growth curve for body weight gain. Male Wistar rats (n=357) were weighed daily, on a calibrated scale, and plotted as shown in Figure 1. From Day 1 to Day 90 each average point was obtained with 50 weights, and from Day 91 to Day 180 with 25 weights. This growth curve allowed the comparison between rats exposed to CIH and age-matched male Wistar rats obtained from the NOVA Medical School animal facility.

The mean body weight of the animals at baseline (60-75 day-old animals; n=20) was 325±9.9 g, which fits the expected body weight for animals of this age (338±7.6 g) in this population (Figure 1; line A; $p=0.4072$). Rats exposed to 35 days of CIH (95-110 day-old animals; n=20) weighed (351±9.1 g) significantly less ($p<0.0001$) compared with age-matched healthy male Wistar rats (424±1.8 g) from the NOVA Medical School animal facility (Figure 1; line B). This growth retardation was also observed at the end of the 25-day period of both CIH exposure and CVD administration (120-135 day-old animals; n=20; 358±9.4 g; Figure 1; line C), since the mean value of the colony was 452±3.4 g at the same age interval ($p<0.0001$).

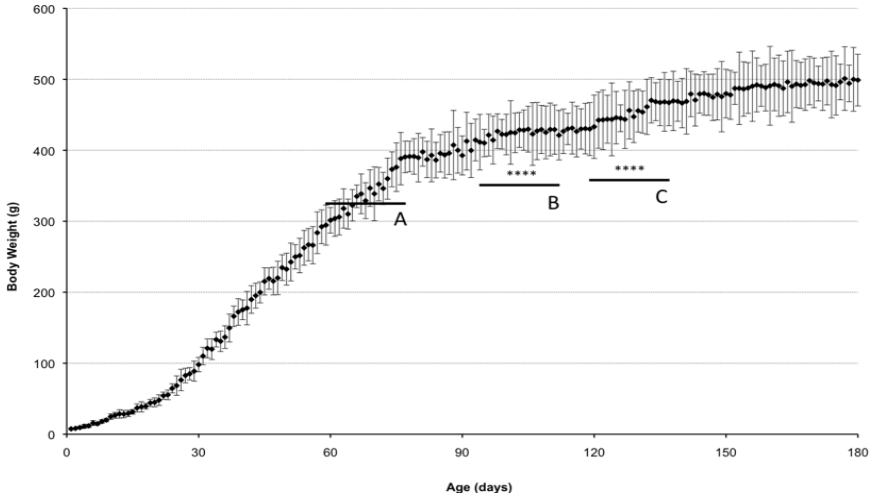


Figure 1: Effect of CIH on body weight in comparison with the growth curve for body weight gain of male Wistar rats (n=357) born in the NOVA Medical School animal facility. Filled lines indicate the mean body weight of the experimental animals: (A) at baseline (60-75 day-old animals; n=20); (B) exposed to 35 days of CIH (95-110 day-old animals; n=20) and (C) administered with CVD for 25 days and submitted to 60 days of CIH (n=20). Data are expressed as mean±SEM. **** p<0.0001 Unpaired t-test. **CIH:** chronic intermittent hypoxia; **CVD:** carvedilol.

Effect of CIH on blood pressure and heart rate

Blood pressure and heart rate were measured in the 6 days before (normoxic period) and throughout the 35 days of CIH exposure. Figure 2 shows raw BP and HR data obtained daily during the experimental protocol.

Since at the onset of CIH the BP values were not yet stabilised and hypertension induced by CIH was essentially observed after the second week of exposure (Figure 2A), we decided to exclude the data attained during the first seven days of CIH exposure from the statistical analysis, for both BP and HR.

Animals exposed to 35 days of CIH exhibited significantly higher mean arterial blood pressure (Nx: 100.9±2.195 mmHg vs. CIH: 126.1±4.231 mmHg; $p<0.001$), diastolic BP (87.4±2.37 mmHg vs. CIH: 113.1±4.913 mmHg; $p<0.001$) and systolic BP (115.7±2.523 mmHg vs. CIH: 137.3±2.216 mmHg; $p<0.0001$) relative to their own baseline values (normoxic period) (Figure 3A, B and C). No changes in mean HR were observed during CIH exposure (Figure 3D; Nx: 405.1±8.013 bpm vs. CIH: 396.4±5.012 bpm).

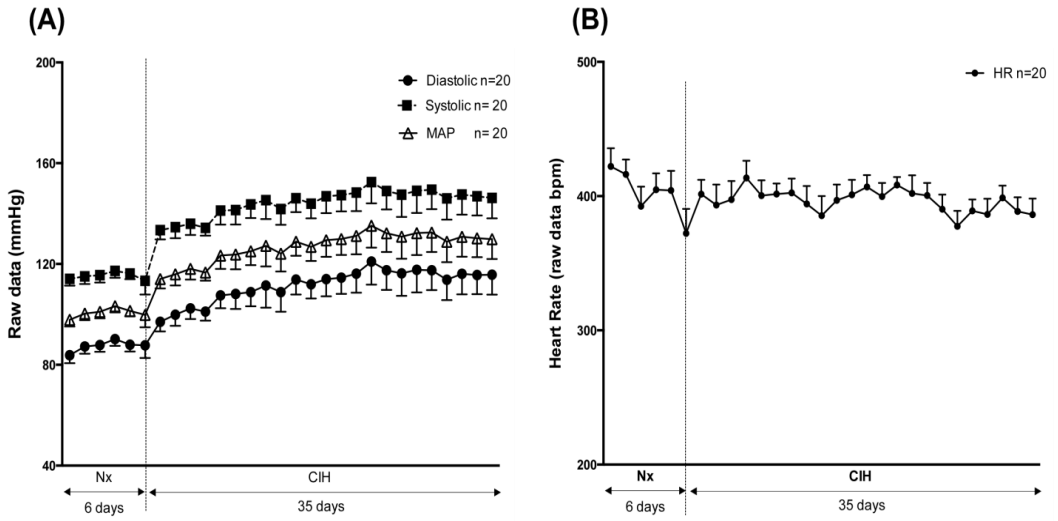


Figure 2: Grouped data showing the daily average recordings of (A) MAP, systolic BP, diastolic BP; and (B) heart rate of rats submitted to CIH for 35 days (n=20). **BP:** blood pressure; **bpm**: beats per minute; **CIH:** chronic intermittent hypoxia; **HR:** heart rate; **MAP:** mean arterial blood pressure; **Nx:** normoxia.

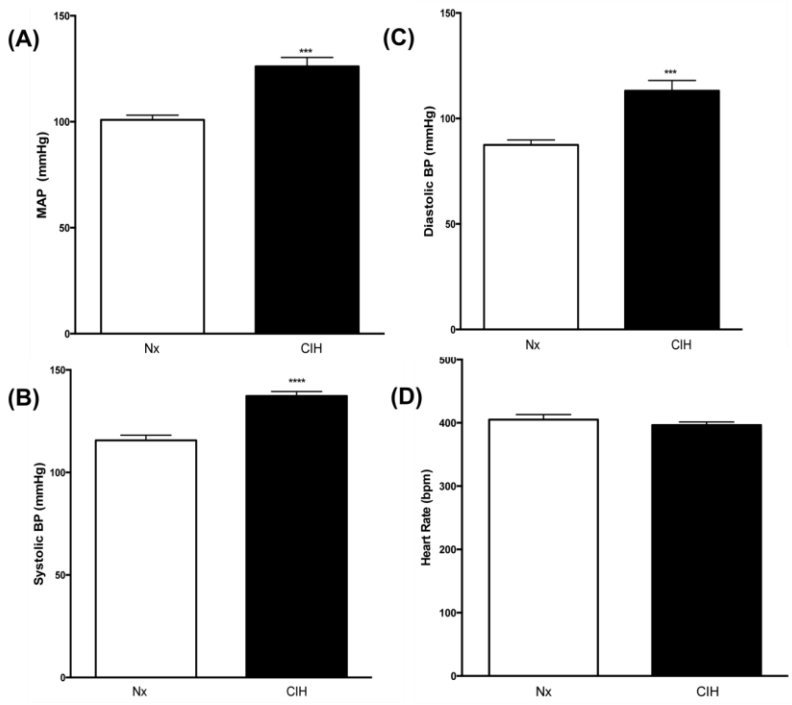


Figure 3: Effect of CIH on (A) mean arterial blood pressure; (B) systolic blood pressure; (C) diastolic blood pressure and (D) heart rate, in male Wistar rats (n=20). Data are expressed as mean±SEM. *** p<0.001 and **** p<0.0001 Unpaired t-test. **BP:** blood pressure; **bpm:** beats per minute; **CIH:** chronic intermittent hypoxia; **MAP:** mean arterial blood pressure; **Nx:** normoxia.

Effect of CVD on blood pressure and heart rate

Figure 4 summarises the mean arterial blood pressure and HR raw data, of rats administered

with MC 0.5% (control-vehicle group), obtained daily during the experimental protocol (n=5). In this group, no differences were found in mean arterial blood pressure (117.9±0.721 mmHg vs. 119±0.54 mmHg), systolic BP (132±0.894 mmHg vs. 133.6±0.585 mmHg), diastolic BP (104±0.456 mmHg vs. 105.5±0.576 mmHg) and HR (379.9±5.208 bpm vs. 370.1±4.343 bpm) between the 35 days of CIH exposure and 25 days of CIH plus MC 0.5% administration, respectively.

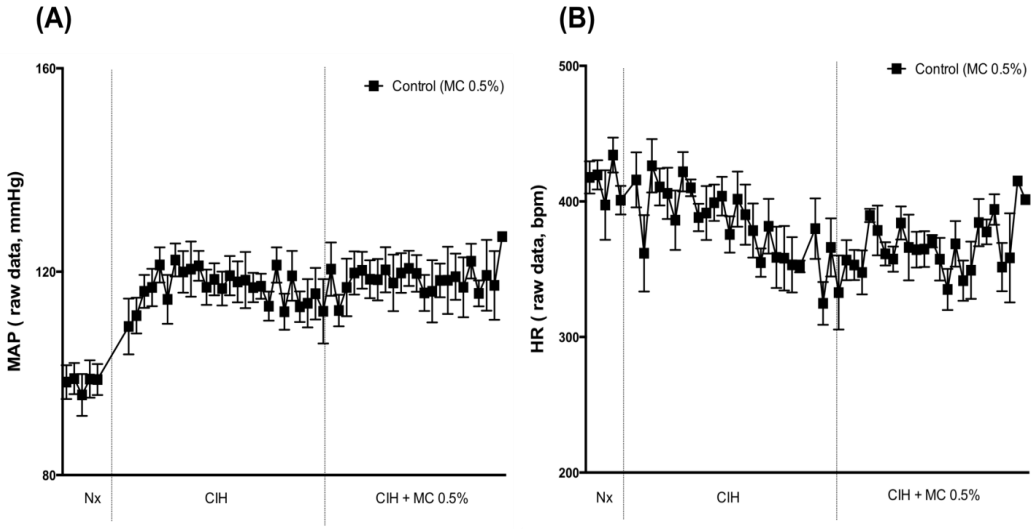


Figure 4: Grouped data showing the daily average recordings of (A) MAP; and (B) heart rate of rats administered with MC 0.5% for 25 days and submitted to 60 days of CIH (control-vehicle group; n=5). **CIH:** chronic intermittent hypoxia; **MAP:** mean arterial blood pressure; **MC:** methylcellulose; **Nx:** normoxia.

We tested three different doses of CVD (10, 30 and 50 mg/kg) and no decrease in mean arterial pressure was observed (Figure 5). Curiously, daily administration of CVD 30 mg/kg produced a slight increase in mean arterial pressure (Figure 5; 121.4±0.852 mmHg (CIH) vs. 125.4±0.841 mmHg (CIH+CVD 30); $p<0.05$), diastolic (104.5±0.762 mmHg (CIH) vs. 108.8±0.623 mmHg (CIH+CVD 30); $p<0.001$) and systolic BP 140.2±0.684 mmHg (CIH) vs. 144.7±0.735 mmHg (CIH+CVD 30); $p<0.001$). No significant changes in diastolic (121.0±1.928 mmHg (CIH) vs. 122.6±1.371 mmHg (CIH+CVD 50)) or systolic BP 157.0±1.404 mmHg (CIH) vs. 154.7±2.369 mmHg (CIH+CVD 50)) were observed for the dose of 50 mg/Kg. The dose of 10 mg/Kg evoked a slight increase in diastolic BP (119.2±0.9161 mmHg (CIH) vs. 129.3±1.679 mmHg (CIH+CVD 10); $p<0.001$) while no effect was observed for systolic BP 146.2±0.901 mmHg (CIH) vs. 143.6±1.285 mmHg (CIH+CVD 10)).

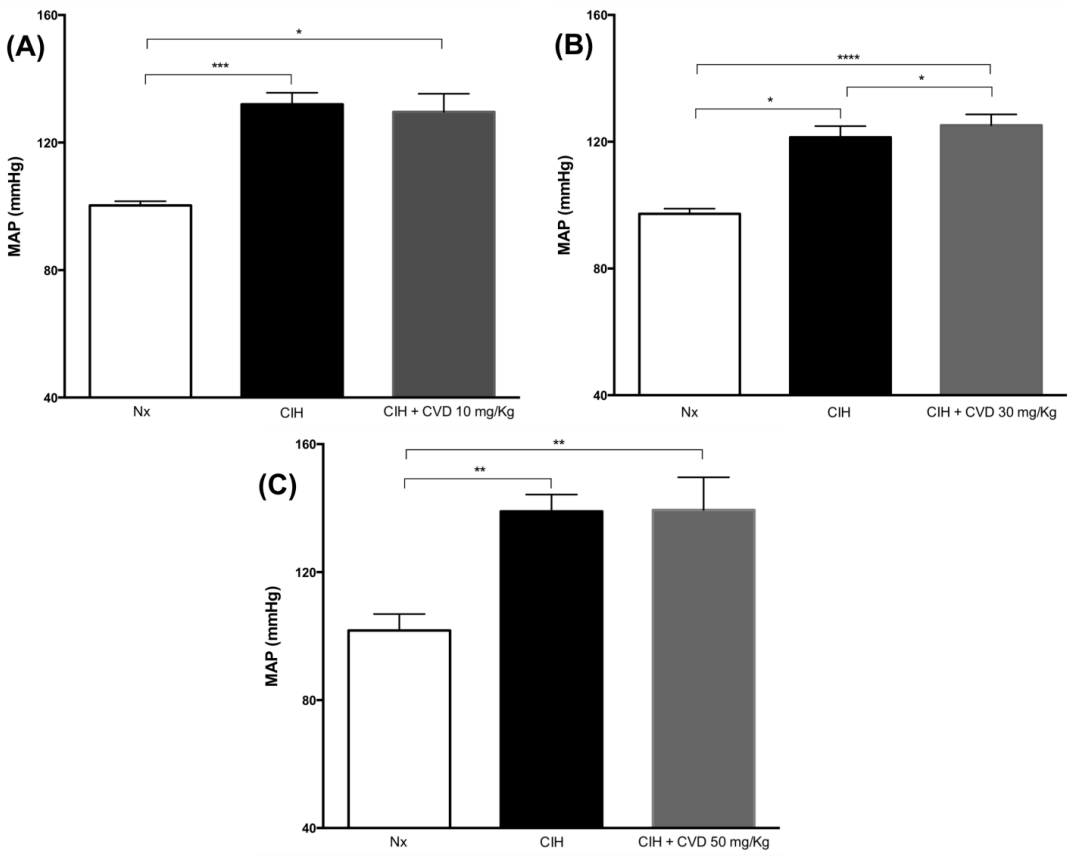


Figure 5: Effect of CVD (A) 10 mg/kg (n=5); (B) 30 mg/kg (n=7) and (C) 50 mg/kg (n=8) daily administration (25 days) on mean arterial blood pressure of rats submitted to 60 days of CIH. Data are expressed as mean±SEM. * p<0.05, ** p<0.01 and *** p<0.001 Kruskal-Wallis test with Dunn´s multiple comparisons test. **CIH:** chronic intermittent hypoxia; **CVD:** carvedilol; **MAP:** mean arterial blood pressure; **Nx:** normoxia.

In contrast, all doses of CVD promoted a significant decrease in mean HR (403.8±4.808 bpm (CIH) vs. 353.3±4.950 bpm (CIH+CVD 10), p<0.0001; 384.4±4.501 bpm (CIH) vs. 346.1±3.729 bpm (CIH+CVD 30), p<0.0001; 400.2±3.461 bpm (CIH) vs. 339.4±7.601 bpm (CIH+CVD 50), p<0.0001) compared with values obtained during the 35 days of CIH exposure, consistent with its beta-blocking activity (Figure 6A, B and C).

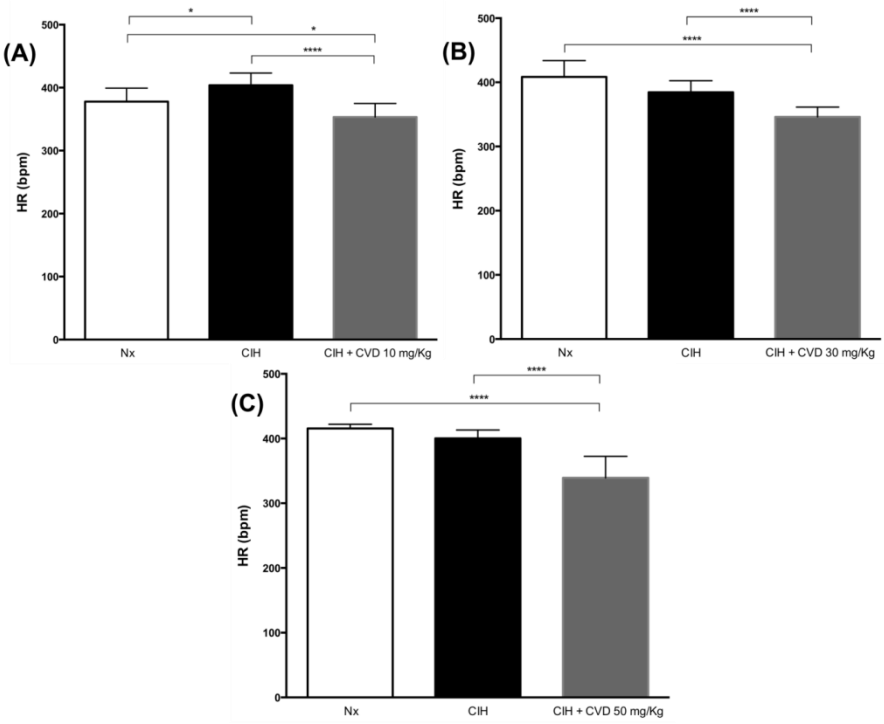


Figure 6: Effect of CVD (A) 10 mg/kg (n=5); (B) 30 mg/kg (n=7) and (C) 50 mg/kg (n=8) daily administration (25 days) on heart rate of rats submitted to 60 days of CIH. Data are expressed as mean±SEM. * p<0.05 and **** p<0.0001 Kruskal-wallis test with Dunn´s multiple comparisons test. **Bpm:** beats per minute; **CIH:** chronic intermittent hypoxia; **CVD:** carvedilol; **Nx:** normoxia.

R-(+)-carvedilol and S-(-)-carvedilol plasma concentrations

R-(+)-CVD and S-(-)-CVD plasma concentrations were determined in Groups 3 and 5. The chromatograms of R-(+)-CVD and S-(-)-CVD in rat plasma samples are presented in Figure 7.

In both groups, the level of R-(+)-CVD was higher than S-(-)-CVD. Animals exposed to CIH for 60 days and treated with 50 mg/kg/da-y of CVD (Group 3) presented higher R-(+)-CVD (941.9±284.4 ng/mL) and S-(-)-CVD (436.0±85.01 ng/mL) plasma concentrations than normoxic animals (531.6±54.52 ng/mL and 358.6±44.11 ng/mL, respectively), although this difference did not reach statistical significance.

The ratios S/(R+S) of CVD enantiomers between rats exposed to CIH (0.3214±0.0216) and normoxic conditions (0.3883±0.0144) were different (Figure 8; $p<0.05$), however, the levels of R-(+)-CVD + S-(-)-CVD obtained in CIH exposed rats (1378.0±328.9) were not statistically different from the ones measured in normoxic rats (890.0±97.83; $p>0.05$; unpaired t-test).

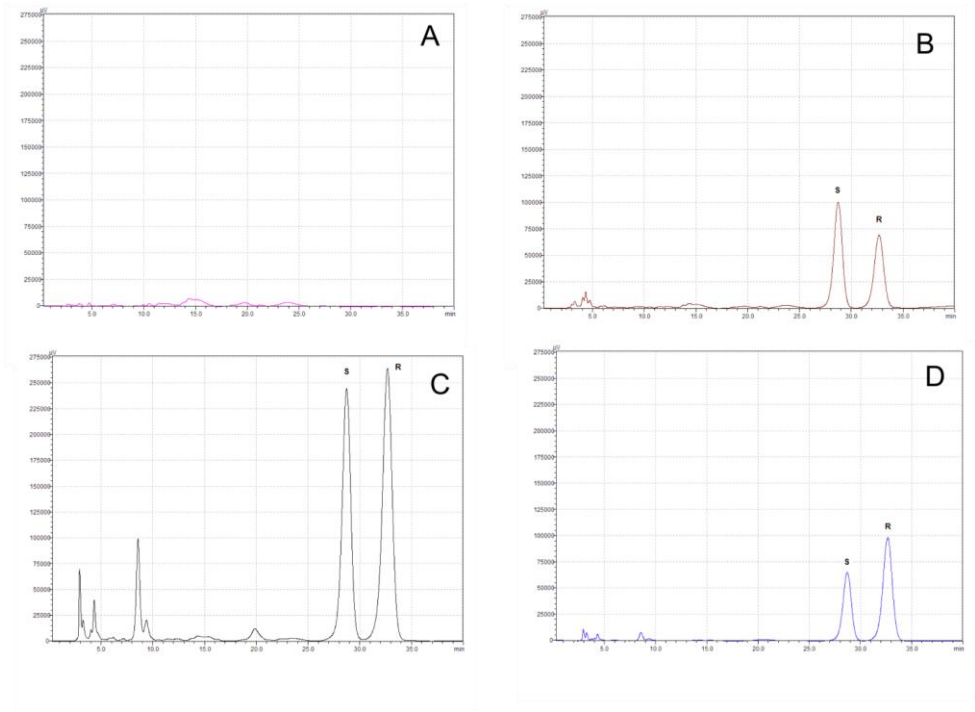


Figure 7: The HPLC chromatograms resulting from derivatization with MCF of (A) blank rat plasma; (B) calibration sample – rat plasma spiked with 100 ng/mL of R-(+)-CVD and S-(-)-CVD; (C) plasma sample of a rat exposed to chronic intermittent hypoxia treated with 50 mg/Kg/day of racemic CVD and (D) plasma sample of a normoxic rat treated with 50 mg/Kg/day of the racemic CVD.

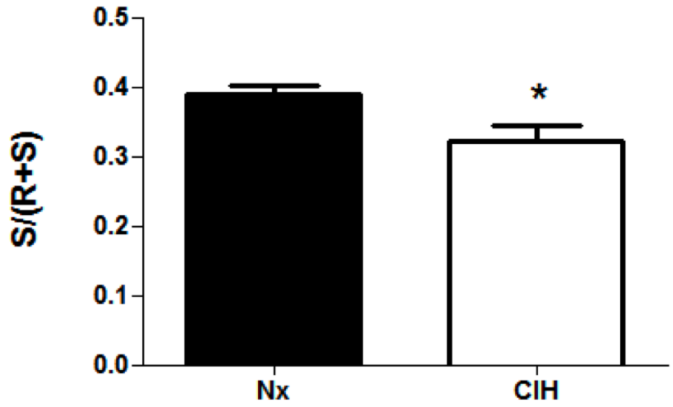


Figure 8: Ratio S(-)-carvedilol / (R(+)-carvedilol + S(-)-carvedilol) in rats exposed to normoxia (Nx n=6) and chronic intermittent hypoxia (CIH; n=8) treated with 50 mg/Kg/day of racemic carvedilol (CVD). Data are expressed as mean±SEM. Unpaired student t-test * p<0.05. **Nx:** normoxia; **CIH:** chronic intermittent hypoxia.

DISCUSSION

We hypothesised that CVD, an antihypertensive drug with α1- and β-adrenoceptor antagonist activity, would be effective in reducing both BP and HR, since it is broadly consensual that sympathetic activation and oxidative stress plays a relevant role in the pathophysiology of

HT related to OSA (Sunderram and Androulakis, 2012). We found that CIH significantly increased MAP, diastolic and systolic BP in our animal model, while no effect was observed for HR. Although doses of 10, 30 and 50 mg/Kg of CVD promoted a significant reduction in HR, no decrease in mean arterial pressure was observed. Interestingly, we also found that CIH changed the plasma concentrations of CVD enantiomers however, the lower ratios S/(R+S) of CVD enantiomers observed in rats exposed to CIH do not apparently explain the lack of efficacy of CVD in reversing this particular type of hypertension.

The animal model used in the present work mimics the intermittent hypoxic episodes that occur chronically in patients suffering from OSA. Obesity is a major cause of airway obstruction in these patients but it is also postulated that OSA can trigger reflex mechanisms that favour the development of metabolic syndrome in these patients (Mannarino *et al.,* 2012). Our model allows us to separate the mechanical and hypercapnic components of obstruction from the effect of intermittent hypoxia itself. Several authors have reported weight loss in CIH-exposed rats when compared with control rats (Silva and Schreihofer *et al.,* 2011; Soukhova-O'Hare *et al.,* 2008; Totoson *et al.,* 2013; Zoccal *et al.,* 2008). Singh and Sevamurthy suggested that the weight loss observed after CIH exposure is due to a reduction in food intake (Singh and Sevamurthy, 1993). More recently, other authors measured daily food intake and reported that the reduced body weight gain in CIH rats was not associated with reduced food consumption but related to a significantly higher metabolism and energy expenditure (Fenik *et al.,* 2012). In addition to reporting the weight loss, our results showed, for the first time, that CIH induces a deviation in the weight changes observed in age-matched Wistar rats, leading to a significant retardation of body growth. This finding suggests that intermittent hypoxia by itself does not contribute to maintaining an overweight status in obese patients with OSA. The effect of CIH on body weight deserves further study in obese animals.

CIH is now established as the dominant model of sleep apnea. Generally, this model makes use of specific ventilated chambers in which the animals are housed and cyclically exposed either to normoxia/hypoxia or room air to mimic the most relevant consequences of OSA (Diogo and Monteiro, 2014). In our protocol, rats were housed in cages placed inside medium A-chambers that were large enough to prevent air jet stress-induced changes in cardiovascular parameters and also to allow the rats to be housed in groups during the extended period of CIH exposure in order to decrease stress levels. Our paradigm of CIH in rats roughly corresponds to sleep apnea lasting for more than two years in humans, which matches the clinical practice of OSA diagnosis. In humans, an apnea-hypopnea index (AHI) bellow 5 events/hour of sleep is under the physiological range (Berry *et al.,* 2012). A paradigm of 5.6 cycles/h of exposure to CIH in rats, even with correction for higher metabolic activity in animals (Germack *et al.,* 2002), is below the AHI cut-offs for moderate OSA (>15 events/hour of sleep).

Animal models of CIH diverge in some aspects, such as the animal species involved, the severity of hypoxia, the number of hypoxic episodes per hour of sleep, the number of days of hypoxic

exposure (exposure duration), and CO_2 manipulation (for a review see Diogo and Monteiro, 2014). Anyway they are unanimous in reporting the development of mild hypertension (Allahdadi *et al.,* 2005; Bathina *et al.,* 2013; Campen *et al.,* 2005; Chen *et al.,* 2005; Dyavanapalli *et al.,* 2014; Fletcher *et al.,* 2000; Kanagy *et al.,* 2001; Knight *et al.,* 2011; Lai *et al.,* 2006; Lin *et al.,* 2007; Liu *et al.,* 2013; Sharpe *et al.,* 2013; Silva and Schreihofer, 2011; Schulz *et al.,* 2014; Soukhova-O'Hare *et al.,* 2008; Tahawi *et al.,* 2001; Totoson *et al.,* 2013; Zoccal *et al.,* 2009). In agreement with these reports, CIH significantly increased mean arterial pressure, diastolic and systolic BP in our animal model, highlighting once again the major role of CIH in the development of hypertension.

The effects of CIH on HR result from a balance between sympathetic nervous system overactivation and baroreflex control. Our results are in line with previous studies that described no changes in HR (Dyavanapalli *et al.,* 2014; Knight *et al.,* 2011; Soukhova-O'Hare *et al.,* 2008; Zoccal *et al.,* 2009) and match the lower rate of variability that has been described for patients with OSA (Trimer *et al.,* 2014). Apparently, CIH does not impair the baroreflex control of HR. Increases in HR were only observed in a study performed with anesthetised rats exposed to CIH for 90 days (Lin *et al.,* 2007). In humans, baroreflex control of HR was reported to be lower in obese than non-obese awake humans and improved after weight reduction (Grassi *et al.,* 1998). Since our animal model is lean this is also consistent with the absence of HR changes.

After validation of our rat model of hypertension related to OSA, we decided to test CVD since sympathetic activation and oxidative stress play an important role in the pathophysiology of hypertension related to OSA and, beta-blockers have not yet been tested in this animal model. Studies investigating the antihypertensive effect of drugs on animal models are scarce and drugs have been used solely as pharmacological tools to address physiological mechanisms (for a review see, for example, Diogo and Monteiro, 2014). The experiments evaluate prevention, but not the effectiveness of treatment. Other limitations of the pharmacological approaches included in these works are the absence of dose-response curves and comparison of the effectiveness of different drugs in the same animal model. Aware of these drawbacks, our study was planned to first induce hypertension and then evaluate the efficacy of cumulative doses of CVD because the translation of the results obtained with simultaneous induction of hypertension and drug administration to humans is not relevant.

In humans, β -blockers have been used empirically to treat HT in OSA patients. Several studies were designed to evaluate the efficacy of atenolol (Kraiczi *et al.,* 2000; Salo *et al.,* 1999; Pelttari *et al.,* 1998), metoprolol (Mayer *et al.,* 1990) and nebivolol (Heitmann *et al.,* 2010). To the best of our knowledge, only one study was performed to evaluate the effects of CVD in OSA patients (Kario *et al.,* 2014). This study compares a bedtime dose of nifedipine, a calcium channel blocker with CVD in the sleep BP profile (Kario *et al.,* 2014). It found that the BP lowering effects of nifedipine on the mean and minimum sleep systolic BP were stronger than those of carvedilol, but the effect of carvedilol seemed to be related more

specifically to the hypoxia stimuli than nifedipine. In general, these drug studies found that the blockade of β-adrenergic receptors during short periods (one to six weeks) might be helpful in reducing BP but none has established BP control as an endpoint. The evidence was not consistent, however, because some studied reported decreases in night-time BP while others only in daytime BP. These studies were performed in AHDs non-naïve patients with several comorbidities and do not allow us to distinguish between the beneficial effect of beta-blockers against the acute sympathetic overactivity triggered by obstruction and their effects on the mechanisms triggered by hypoxia itself (for a review see Diogo and Monteiro, 2014). Studies in humans are also limited by the inability to identify the chronicity of OSA and hypertension in each patient. This can interfere with drug efficacy, assuming that the mechanisms involved in hypertension induced by OSA might be time-dependent. For instance, it has been proposed that IH is able to induce a renal hormetic response: short-term IH induces a protective response against the renal oxidative damage, but long-term IH exposure induces a damaging effect on the kidneys (Sun *et al.,* 2012).

The doses of CVD tested (10, 30 and 50 mg/kg/day) were selected based on previous studies performed in different models of hypertension (Bertera *et al.,* 2012a; Bertera *et al.,* 2012b; Chen *et al.,* 2013; Di Verniero *et al.,* 2010; Rodriguez-Perez *et al.,* 1997). The lack of efficacy of CVD cannot be attributed to the doses because they were effective in reducing HR in our model, in reducing BP in other models of HT and are higher than the doses administered to humans (12.5-50 mg).

CVD was daily administered by gavage, the most widely used method for the precise oral dosing of rodents. This method can, however, elicit a stress response, and it has been shown that any source of external stress on rodents can significantly increase heart rate and blood pressure, effects that persist for around 3 hours (Brown *et al.,* 2000; Bonnichsen *et al.,* 2005; Kramer *et al.,* 2000; Balcombe *et al.,* 2004). Aware of this fact we administered CVD only after monitoring BP parameters and the measurements were made on the day after administration and before exposure to IH conditions.

Carvedilol is a lipophilic third generation β-blocking agent, and it is assumed that it easily crosses the blood-brain barrier. Several advantages have been claimed for third generation agents over the traditional β-blockers. First, drugs such as CVD exhibit a broad range of adrenergic inhibition, blocking postsynaptic β1, β2 and α1 receptors and presynaptic β2 receptors (DiNicolantonio and Hackam, 2012). Secondly, unlike traditional agents, CVD promotes vasodilatation through different mechanisms, which translate into a more favourable hemodynamic profile compared to non-vasodilating beta-blockers (Rath *et al.,* 2012). In addition to its alpha-adrenergic receptor blocking properties, it has been suggested that CVD-induced increased nitric oxide (NO) plasma levels (Afonso *et al.,* 2006; Vanhoutte and Gao, 2013) and antioxidant properties (Rath *et al.,* 2012; Feuerstein and Ruffolo, 1995) could also contribute to the vasorelaxation and subsequent reduction in vascular resistance evoked by CVD. CVD is also noted as a suppressor of the synthesis of endothelin-1 (ET-1) (Saijonmaa *et*

al., 1997), as a drug with antiproliferative actions (Cheng *et al.*, 2001) and with beneficial effects on vascular and cardiac structural remodelling (Bakris, 2009; Chen *et al.*, 2013), endothelial dysfunction (Feuerstein and Ruffolo, 1995) and progression of target organ damage (Bakris, 2009). Finally, CVD also has a calcium channel blocking effect by inhibiting voltage-dependent L-type Ca2+ in vascular smooth muscle cells (Nakajima *et al.*, 2003), inhibits the rennin-angiotensin system (Cheng *et al.*, 2001) by blocking β1 receptors and enhances the plasma concentration of atrial natriuretic peptide (Cheng *et al.*, 2001).

Since these pleiotropic effects of CVD match the pathophysiological mechanisms described for CIH (for a review see Diogo & Monteiro, 2014), the lack of efficacy of carvedilol in reversing hypertension induced by CIH was unexpected.

The finding that the three doses of CVD promoted a significant decrease in HR confirmed that the lack of antihypertensive efficacy couldn't be attributed to the absence of beta-blocking activity.

Another explanation could be that CIH might change the pharmacokinetic profile of CVD, compromising their antihypertensive efficacy. Carvedilol is clinically administered as a racemic mixture in which nonselective beta-adrenoreceptor blocking activity is present in the S-(-) enantiomer and alpha-adrenergic blocking activity is present in both R-(+) and S-(-) enantiomers at equal potency (Peccinini *et al.*, 2008). A decrease in HR without no effects on BP might thus be explained by a selective increase in the levels of S-(-)-CVD. There is some evidence that hypoxic conditions can broadly change drug clearance (Chen *et al.*, 2013; Gao *et al.*, 2013; Vij *et al.*, 2012) or drug effects (Nunes *et al.*, 2010).

In the present work, we found, for the first time, that CIH changes CVD pharmacokinetics by mechanisms that deserve further exploration, however, the lower ratio S/(R+S) observed in rats submitted to CIH could not easily explain the lack of hypotensive effects.

Another putative explanation for the lack of efficacy of carvedilol is that the blockade of the sympathetic nervous system along with all the pleiotropic properties of CVD, might not be enough to reverse hypertension induced by CIH.

From a broad perspective, we can speculate that the majority of CVD effects were produced in experiments that planned to address only one variable involved in the pathophysiology of hypertension and some in acute conditions. Few studies of hypertension with the chronic administration of CVD have been performed in rat models. In a model of hypertension induced by unilateral renal artery narrowing (Chen *et al.*, 2013), CVD (20 mg/kg/day) administered by gavage for 8 weeks, caused a significant and sustained reduction of SBP apparent in the first two weeks of treatment, but it was not able to completely reverse the hypertensive effect of the surgical procedure. In hypertension induced by renal ablation (5/6), only rats receiving doses of 10 and 20 mg/Kg/day of CVD (but not of 5 mg/Kg/day) exhibited significant decreases in SBP apparent at week five of treatment (Rodriguez-Perez *et al.*, 1997). These findings, together with the lack of efficacy of CVD found in our animal model, support the theory that mechanisms other than this type of renal damage are involved in hypertension induced by CIH.

Finally, it has been described that CIH leads to an increased stiffness and reduced vessel wall distensibility (Phillips et al., 2006) and induces significant renal inflammation and fibrosis. (Sun *et al.,* 2012). We can therefore postulate that CIH induces structural changes in vessels, renal tubules, etc., that are not targeted by CVD.

CONCLUSION

In conclusion, the lack of efficacy of CVD in reversing hypertension induced by CIH in our animal model suggests that the blockade of sympathetic nervous system alone does not seem to be the best strategy for reversing established hypertension in sleep apnea conditions. Sympathetic overactivity may be an early step in the mechanism leading to more permanent effects due to prolonged sympathetic overactivity or through local effects of recurrent IH that are not reversed by drugs such as CVD. This study also pointed out, for the first time, that CIH changes CVD pharmacokinetics through mechanisms that deserve further exploration. Drugs that have proved to be useful in essential hypertension treatment should thus be tested in studies specifically designed for secondary hypertension induced by CIH.

ACKNOWLEDGMENTS

The authors would like to thank Gracinda Menezes for her technical support and Professor Teresa Gamboa for her advice during the setup of the Oxycycler System. The present work was supported by Fundação para a Ciência e Tecnologia (FCT) grant (PTDC/SAU-TOX/ 112264/2009) and CEDOC (Portugal). Lucília N. Diogo holds a FCT PhD grant (SFRH / BD / 48335 / 2008).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Afonso RA, Patarrão RS, Macedo MP, Carmo MM., 2006. Carvedilol action is dependent on endogenous production of nitric oxide. Am J Hypertens. 19, 419-25.

Allahdadi, K.J., Cherng, T.W., Pai, H., Silva, A.Q., Walker, B.R., Nelin, L.D., et al. (2008). Endothelin type A receptor antagonist normalizes blood pressure in rats exposed to eucapnic intermittent hypoxia. Am. J. Physiol. Heart Circ. Physiol. 295, H434-40. doi: 10.1152/ajpheart.91477.2007.

Allahdadi, K.J., Walker, B.R., Kanagy, N.L. (2005). Augmented endothelin vasoconstriction in intermittent hypoxia-induced hypertension. Hypertension 45, 705–709.

Bakris G., 2014. An in-depth analysis of vasodilation in the management of hypertension: focus on adrenergic blockade. J Cardiovasc Pharmacol. 53, 379-87.

Balcombe, J.P., Barnard, N.D., Sandusky, C. (2004). Laboratory routines cause animal stress. Contemp. Top. Lab. Anim. Sci. 43, 42-51.

Bathina, C.S., Rajulapati, A., Franzke, M., Yamamoto, K., Cunningham, J.T., Mifflin S. (2013). Knockdown of tyrosine hydroxylase in the nucleus of the solitary tract reduces elevated blood pressure during chronic intermittent hypoxia. Am. J. Physiol. Regul. Integr. Comp. Physiol. 305, R1031-9. doi: 10.1152/ajpregu.00260.2013.

Belaidi, E., Joyeux-Faure, M., Ribuot, C., Launois, S.H., Levy, P., Godin-Ribuot, D. (2009). Major role for hypoxia inducible factor-1 and the endothelin system in promoting myocardial infarction and hypertension in an animal model of obstructive sleep apnea. J. Am. Coll. Cardiol. 53, 1309-17. doi: 10.1016/j.jacc.2008.12.050.

Berry RB, Budhiraja R, Gottlieb DJ, Gozal D, Iber C, Kapur VK, Marcus CL, Mehra R, Parthasarathy S, Quan SF, Redline S, Strohl KP, Davidson Ward SL, Tangredi MM. (2012). Rules for scoring respiratory events in sleep: update of the 2007 AASM Manual for the Scoring of Sleep and

Associated Events. Deliberations of the Sleep Apnea Definitions Task Force of the American Academy of Sleep Medicine. J Clin Sleep Med 8: 597-619. doi: 10.5664/jcsm.2172.

Bertera FM, Del Mauro JS, Chiappetta D, Polizio AH, Buontempo F, Taira CA, Höcht C. (2012a). Enantioselective pharmacokinetic and pharmacodynamic properties of carvedilol in spontaneously hypertensive rats: focus on blood pressure variability. Naunyn Schmiedebergs Arch Pharmacol. 385, 325-35. doi: 10.1007/s00210-011-0698-7.

Bertera F, Di Verniero CA, Mayer MA, Chiappetta D, Buontempo F, Polizio AH, Taira CA, Höcht C. (2012b). Pharmacokinetic and pharmacodynamic properties of carvedilol in fructose hypertensive rats. Xenobiotica. 42, 206-19. doi: 10.3109/00498254.2011.604746.

Bonnichsen, M., Dragsted, M., Hansen, A.K. (2005). The welfare impact of gavaging laboratory rates. Anim. Welfare. 14, 223–227.

Brown, A.P., Dinger, N., Levine, B.S. (2000). Stress produced by gavage administration in the rat. Contemp. Top. Lab. Anim. Sci. 39, 17-21.

Campen, M.J., Shimoda, L.A., O'Donnell, C.P. (2005). Acute and chronic cardiovascular effects of intermittent hypoxia in C57BL/6J mice. J. Appl. Physiol. 99, 2028-35.

Chen, L., Einbinder, E., Zhang, Q., Hasday, J., Balke, C.W., Scharf, S.M. (2005). Oxidative stress and left ventricular function with chronic intermittent hypoxia in rats. Am. J. Respir. Crit. Care Med. 172, 915–920.

Chen J, Huang C, Zhang B, Huang Q, Chen J, Xu L. (2013). The effects of carvedilol on cardiac structural remodeling: the role of endogenous nitric oxide in the activity of carvedilol. Mol Med Rep. 7, 1155-8. doi: 10.3892/mmr.2013.1329.

Chen XY, Zeng YM, Zhang YX, Wang WY, Wu RH. (2013). Effect of chronic intermittent hypoxia on theophylline metabolism in mouse liver. Chin Med J (Engl). 126, 118-23.

Cheng J, Kamiya K, Kodama I. (2001). Carvedilol: molecular and cellular basis for its multifaceted therapeutic potential. Cardiovasc Drug Rev. 19, 152-71.

da Silva, A.Q., Fontes, M.A., Kanagy, N.L. (2011). Chronic infusion of angiotensin receptor antagonists in the hypothalamic paraventricular nucleus prevents hypertension in a rat model of sleep apnea. Brain Res. 1368, 231-8. doi: 10.1016/j.brainres.2010.10.087.

Del Rio, R., Moya, E.A., Iturriaga, R. (2010). Carotid body and cardiorespiratory alterations in chronic intermittent hypoxia: the oxidative link. Eur. Respir. J. 36, 143-50. doi: 10.1183/09031936.00158109.

Diogo, L.N., Monteiro, E.C. (2014). The efficacy of antihypertensive drugs in chronic intermittent hypoxia conditions. Front. Physiol. 5:361. doi: 10.3389/fphys.2014.00361.

DiNicolantonio, J.J., Hackam, D.G. (2012). Carvedilol: a third-generation β-
blocker should be a first-choice β-blocker. Expert Rev. Cardiovasc. Ther. 10, 13-25. doi: 10.1586/erc.11.166.

Di Verniero CA, Bertera F, Buontempo F, Bernabeu E, Chiappetta D, Mayer MA, Bramuglia GF, Taira CA, Höcht C. (2010). Enantioselective pharmacokinetic-pharmacodynamic modelling of carvedilol in a N-nitro-l-arginine methyl ester rat model of secondary hypertension. J Pharm Pharmacol. 2010 Jul;62(7):890-900. doi: 10.1211/jpp.62.07.0010.

Dyavanapalli, J., Jameson, H., Dergacheva, O., Jain, V., Alhusayyen, M., Mendelowitz, D. (2014). Chronic intermittent hypoxia-hypercapnia blunts heart rate responses and alters neurotransmission to cardiac vagal neurons. J. Physiol. 16, 1-13. doi: 10.1113/jphysiol.2014.273482.

Fenik, V.B., Singletary, T., Branconi, J.L., Davies, R.O., Kubin, L. (2012). Glucoregulatory consequences and cardiorespiratory parameters in rats exposed to chronic-intermittent hypoxia: effects of the duration of exposure and losartan. Front. Neurol. 3:51. doi: 10.3389/fneur.2012.00051.

Feuerstein GZ, Ruffolo RR Jr. (1995). Carvedilol, a novel multiple action antihypertensive agent with antioxidant

activity and the potential for myocardial and vascular protection. Eur Heart J. 16, 38-42.

Fletcher, E.C., Bao, G., Li, R. (1999). Renin activity and blood pressure in response to chronic episodic hypoxia. Hypertension. 34, 309-14.

Fletcher, E.C. (2000). Effect of episodic hypoxia on sympathetic activity and blood pressure. Respir. Physiol. 119, 189-97.

Gao J, Ding XS, Zhang YM, Dai DZ, Liu M, Zhang C, Dai Y. (2013). Hypoxia/oxidative stress alters the pharmacokinetics of CPU86017-RS through mitochondrial dysfunction and NADPH oxidase activation. Acta Pharmacol Sin. 2013 Dec;34(12):1575-84. doi: 10.1038/aps.2013.94.

Germack R, Leon-Velarde F, Valdes De La Barra R, Farias J, Soto G, Richalet JP. (2002). Effect of intermittent hypoxia on cardiovascular function, adrenoceptors and muscarinic receptors in Wistar rats. Exp Physiol. 87, 453-60.

Grassi G, Seravalle G, Colombo M, Bolla G, Cattaneo BM, Cavagnini F, Mancia G (1998). Body weight reduction, sympathetic nerve traffic, and arterial baroreflex in obese normotensive humans. Circulation. 97, 2037–2042.

Heitmann, J., Greulich, T., Reinke, C., Koehler, U., Vogelmeier, C., Becker, H.F., et al. (2010). Comparison of the effects of nebivolol and valsartan on BP reduction and sleep apnoea activity in patients with essential hypertension and OSA. Curr. Med. Res. Opin. 26, 1925-32. doi: 10.1185/03007995.2010.497326.

Hung, M.W., Kravtsov, G.M., Lau, C.F., Poon, A.M., Tipoe, G.L., Fung, M.L. (2013). Melatonin ameliorates endothelial dysfunction, vascular inflammation, and systemic hypertension in rats with chronic intermittent hypoxia. J. Pineal Res. 55, 247-56. doi: 10.1111/jpi.12067.

Kanagy, N.L.,Walker, B.R., Nelin, L.D. (2001). Role of endothelin in intermittent hypoxia-induced hypertension. Hypertension. 37, 511–515.

Kapa, S., Sert Kuniyoshi, F.H., Somers, V.K. (2008). Sleep Apnea and Hypertension: interactions and implications for management. Hypertension. 51, 605-8. doi: 10.1161/HYPERTENSIONAHA.106.076190.

Kario, K., Kuwabara, M., Hoshide, S., Nagai, M., Shimpo, M. (2014). Effects of Nighttime Single-Dose Administration of Vasodilating vs Sympatholytic Antihypertensive Agents on Sleep Blood Pressure in Hypertensive Patients With Sleep Apnea Syndrome. J. Clin. Hypertens. 16, 459-66. doi: 10.1111/jch.12327.

Knight, W.D., Little, J.T., Carreno, F.R., Toney, G.M., Mifflin, S.W., Cunningham, J.T. (2011). Chronic intermittent hypoxia increases blood pressure and expression of FosB/DeltaFosB in central autonomic regions. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, R131-9. doi: 10.1152/ajpregu.00830.2010.

Knight, W.D., Saxena, A., Shell, B., Nedungadi, T.P., Mifflin, S.W., Cunningham, J.T. (2013). Central losartan attenuates increases in arterial pressure and expression of FosB/ΔFosB along the autonomic axis associated with chronic intermittent hypoxia. Am. J. Physiol. Regul. Integr. Comp. Physiol. 305, R1051-8. doi: 10.1152/ajpregu.00541.2012.

Kraiczi, H., Hedner, J., Peker, Y., Grote, L. (2000). Comparison of atenolol, amlodipine, enalapril, hydrochlorothiazide, and losartan for antihypertensive treatment in patients with obstructive sleep apnea. Am. J. Respir. Crit. Care Med. 161, 1423-8.
Kramer, K., Voss, H.P., Grimbergen, J.A., Mills, P.A., Huetteman, D., Zwiers, L., Brockway, B., 2000. Telemetric monitoring of blood pressure in freely moving mice: a preliminary study. Lab. Anim. 34, 272–280.

Kumar, G.K., Rai, V., Sharma, S.D., Ramakrishnan, D.P., Peng, Y.J., Souvannakitti, D., et al. (2006). Chronic intermittent hypoxia induces hypoxia-evoked catecholamine efflux in adult rat adrenal medulla via oxidative stress. J. Physiol. 575, 229-39.

Lai, C.J., Yang, C.C., Hsu, Y.Y., Lin, Y.N., Kuo, T.B. (2006). Enhanced sympathetic outflow and decreased baroreflex sensitivity are associated with intermittent hypoxia-induced systemic hypertension in conscious rats. J. Appl. Physiol. 100, 1974–1982.

Lin, M., Liu, R., Gozal, D., Wead, W.B., Chapleau, M.W., Wurster, R., et al. (2007). Chronic intermittent hypoxia

impairs baroreflex control of heart rate but enhances heart rate responses to vagal efferent stimulation in anesthetized mice. Am. J. Physiol. Heart Circ. Physiol. 293, H997-1006.

Liu, X., Deng, Y., Shang, J., Yang, X.H., Liu, K., Liu, H.G., et al. (2013). Effect of NADPH oxidase inhibitor apocynin on the expression of hypoxia-induced factor-1α and endothelin-1 in rat carotid body exposed to chronic intermittent hypoxia. J. Huazhong Univ. Sci. Technolog. Med. Sci. 33, 178-84. doi: 10.1007/s11596-013-1093-z.

Mannarino MR, Di Filippo F, Pirro M. (2012). Obstructive sleep apnea syndrome. Eur J Intern Med. 23, 586-93. doi: 10.1016/j.ejim.2012.05.013.

Mayer, J., Weichler, U., Herres-Mayer, B., Schneider, H., Marx, U., Peter, J.H. (1990). Influence of metoprolol and cilazapril on blood pressure and on sleep apnea activity. J. Cardiovasc. Pharmacol.16, 952-61.

Morton, D.B., Jennings, M., Buckwell, A., Ewbank, R., Godfrey, C., Holgate, B., *et al.*, Joint Working Group on Refinement, (2001). Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. Lab. Anim. 35, 1-41.

Moya, E., Arias, P., Carlos, V., Oyarce, M., Iturriaga, R. (2014). Ebselen prevents the carotid body chemosensory potentiation and reverses the hypertension induced by intermittent hypoxia (873.1). The FASEB Journal, 28: 873.1.

Nakajima T, Ma J, Iida H, Iwasawa K, Jo T, Omata M, Nagai R. (2003). Inhibitory effects of carvedilol on calcium channels in vascular smooth muscle cells. Jpn Heart J. 44, 963-78.

Nicklas, W., Baneux, P., Boot, R., Decelle, T., Deeny, A.A., Fumanelli, M., Illgen-Wilcke, B.; FELASA (Federation of European Laboratory Animal Science Associations Working Group on Health Monitoring of Rodent and Rabbit Colonies), (2002). Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Lab. Anim. 36, 20-42.

Nunes AR, Batuca JR, Monteiro EC. (2010). Acute hypoxia modifies cAMP levels induced by inhibitors of phosphodiesterase-4 in rat carotid bodies, carotid arteries and superior cervical ganglia. Br J Pharmacol. 2010 Jan 1;159(2):353-61. doi: 10.1111/j.1476-5381.2009.00534.x.

Parati, G., Lombardi, C., Hedner, J., Bonsignore, M.R., Grote, L., Tkacova, R., et al. (2013). Recommendations for the management of patients with obstructive sleep apnoea and hypertension. Eur. Respir. J. 41, 523-38. doi: 10.1183/09031936.00226711.

Peccinini RG, Ximenes VF, Cesarino EJ, Lanchote VL (2008). Stereoselective analysis of carvedilol in human plasma and urine using HPLC after chiral derivatization. Biopharm Drug Dispos. 29, 280-8. doi: 10.1002/bdd.613.

Pelttari, L.H., Hietanen, E.K., Salo, T.T., Kataja, M.J., Kantola, I.M. (1998). Little effect of ordinary antihypertensive therapy on nocturnal high blood pressure in patients with sleep disordered breathing. Am. J. Hypertens. 11, 272-9.

Phillips SA, Olson EB, Lombard JH, Morgan BJ. (2006). Chronic intermittent hypoxia alters NE reactivity and mechanics of skeletal muscle resistance arteries. J Appl Physiol 100, 1117-23.

Rath G, Balligand JL, Dessy C. (2012). Vasodilatory mechanisms of beta receptor blockade. Curr Hypertens Rep. 14, 310-7. doi: 10.1007/s11906-012-0278-3.

Rodriguez-Perez JC, Losada A, Anabitarte A, Cabrera J, Llobet J, Palop L, Plaza C. (1997). Effects of the novel multiple-action agent carvedilol on severe nephrosclerosis in renal ablated rats. J Pharmacol Exp Ther. 283, 336-44.

Salo, T.M., Kantola, I., Voipio-Pulkki, L.M., Pelttari, L., Viikari, J.S. (1999). The effect of four different antihypertensive medications on cardiovascular regulation in hypertensive sleep apneic patients--assessment by spectral analysis of heart rate and blood pressure variability. Eur. J. Clin. Pharmacol. 55, 191-8.

Saijonmaa O, Metsärinne K, Fyhrquist F. (1997). Carvedilol and its metabolites suppress endothelin-1 production in human endothelial cell culture. Blood Press. 6, 24-8.

Schulz, R., Murzabekova, G., Egemnazarov, B., Kraut, S., Eisele, H.J., Dumitrascu, R., et al. (2014). Arterial hypertension in a murine model of sleep apnea: role of NADPH oxidase 2. J. Hypertens. 32, 300-5. doi: 10.1097/HJH.0000000000000016.

Sharpe, AL., Andrade, M.A., Herrera-Rosales, M., Britton, S.L., Koch, L.G., Toney, G.M. (2013). Rats selectively bred for differences in aerobic capacity have similar hypertensive responses to chronic intermittent hypoxia. Am. J. Physiol. Heart Circ. Physiol. 305, H403-9. doi: 10.1152/ajpheart.00317.2013.

Silva, A.Q., Schreihofer, A.M. (2011). Altered sympathetic reflexes and vascular reactivity in rats after exposure to chronic intermittent hypoxia. J. Physiol. 589, 1463-76. doi: 10.1113/jphysiol.2010.200691.

Singh SB, Selvamurthy W. (1993). Effect of intermittent chronic exposure to hypoxia on feeding behaviour of rats. Int J Biometeorol. 37, 200-2.

Soukhova-O'Hare, G.K., Ortines, R.V., Gu, Y., Nozdrachev, A.D., Prabhu, S.D., Gozal, D. (2008). Postnatal intermittent hypoxia and developmental programming of hypertension in spontaneously hypertensive rats: the role of reactive oxygen species and L-Ca2+ channels. Hypertension. 52,156-62.

Sun W, Yin X, Wang Y, Tan Y, Cai L, Wang B, Cai J, Fu Y. (2012). Intermittent hypoxia-induced renal antioxidants and oxidative damage in male mice: hormetic dose response. Dose Response. 2012 Oct 1;11(3):385-400. doi: 10.2203/dose-response.

Sunderram, J., Androulakis, I.P. (2012). Molecular mechanisms of chronic intermittent hypoxia and hypertension. Crit. Rev. Biomed. Eng. 40, 265-78.

Tahawi, Z., Orolinova, N., Joshua, I.G., Bader, M., Fletcher, E.C. (2001). Altered vascular reactivity in arterioles of chronic intermittent hypoxic rats. J. Appl. Physiol. 90, 2007–2013.

Totoson, P., Fhayli, W., Faury, G., Korichneva, I., Cachot, S., Baldazza, M., et al. (2013). Atorvastatin protects against deleterious cardiovascular consequences induced by chronic intermittent hypoxia. Exp. Biol. Med. (Maywood). 238, 223-32. doi: 10.1177/1535370212473696.

Trimer R, Mendes RG, Costa FS, Sampaio LM, Delfino A Jr, Arena R, Aletti F, Ferrario M, Borghi-Silva A. (2014). Is there a chronic sleep stage-dependent linear and nonlinear cardiac autonomic impairment in obstructive sleep apnea? Sleep Breath. 18, 403-9. doi: 10.1007/s11325-013-0900-x.

Troncoso Brindeiro, C.M., da Silva, A.Q., Allahdadi, K.J., Youngblood, V., Kanagy, N.L. (2007). Reactive oxygen species contribute to sleep apnea-induced hypertension in rats. Am. J. Physiol. Heart Circ. Physiol. 293, H2971-6.

Vanhoutte PM, Gao Y. (2013). Beta blockers, nitric oxide, and cardiovascular disease. Curr Opin Pharmacol. 13, 265-73. doi: 10.1016/j.coph.2012.12.002.

Vij AG, Kishore K, Dey J. (2012). Effect of intermittent hypobaric hypoxia on efficacy & clearance of drugs. Indian J Med Res. 2012;135:211-6.

Zoccal, D.B., Bonagamba, L.G., Paton, J.F., Machado, B.H. (2009). Sympathetic-mediated hypertension of awake juvenile rats submitted to chronic intermittent hypoxia is not linked to baroreflex dysfunction. Exp. Physiol. 94, 972-83.

Zoccal, D.B., Simms, A.E., Bonagamba, L.G., Braga, V.A., Pickering, A.E., Paton, J.F., et al. (2008). Increased sympathetic outflow in juvenile rats submitted to chronic intermittent hypoxia correlates with enhanced expiratory activity. J. Physiol. 586, 3253-65.

RESEARCH PAPER

LOSARTAN VOLUNTARY ORAL ADMINISTRATION - A LESS STRESSFUL APPROACH IN RATS

Lucília N Diogo[a*], Inês V Faustino[a], Ricardo A Afonso[a,b], Sofia A Pereira[a], Emília C Monteiro[a] and Ana I Santos[b]

[a]Centro de Estudos de Doenças Crónicas, CEDOC, NOVA Medical School / Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo dos Mártires da Pátria, 130, 1169-056 Lisboa, Portugal [b] Department of Physiology, NOVA Medical School / Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo dos Mártires da Pátria, 130, 1169-056 Lisboa, Portugal.

ABSTRACT

Gavage is a widely performed technique for daily dosing in laboratory rodents. Although it is effective, gavage comprises a sequence of potentially stressful procedures for laboratory animals that may constitute bias for the experimental results, especially when the drugs to be tested interfere with stress dependent parameters. We aimed to test suitable vehicles for drug delivery by voluntary ingestion. Male Wistar rats (2-3 months) were used to test nut paste (NUT), peanut butter (PB) and sugar dough (SD) as vehicles for long-term voluntary oral administration of losartan, an angiotensin II receptor blocker. Vehicles were administered 28 days without drug in order to assess the glucose level and serum lipid profile. Losartan was mixed with vehicles and offered to the animals or administered by gavage (14 days) for subsequent quantification of losartan plasma levels by HPLC. After a 2-day acclimatization period, all animals voluntarily ate the vehicles, either alone or mixed with losartan. Blood glucose levels were reduced by NUT administration ($p<0.01$). PB induces increased levels of triglycerides and total cholesterol ($p<0.05$). SD group presented a higher concentration of losartan when compared with gavage group ($p<0.01$), without changing lipid and glucose profiles. Our results showed that the three vehicles are viable for daily single dose voluntary ingestion of losartan, from which SD proved to be the best alternative. Drug bioavailability was not reduced, suggesting that voluntary ingestion is highly effective for chronic oral administration of losartan. The results presented herein point to a welfare-based refinement for drug administration in laboratory rodents.

Key words: gavage; voluntary oral administration; losartan; nut paste; sugar dough; peanut butter.

[*]**Corresponding author:** Lucilia Neves Diogo, PharmD, MSc, NOVA Medical School / Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Campo Mártires da Pátria, 130, 1169-056 Lisboa, Portugal, lucilia.diogo@fcm.unl.pt.

INTRODUCTION

Oral drug administration as a single daily dose in animal models is commonly done with water-diluted drugs, gavage or voluntary ingestion.

Gavage is widely performed for precise oral dosing in rodents. It is commonly used in efficacy and toxicity studies, as well as drug discovery. This technique, applicable in conscious animals, is a rapid and efficient mean of accurately delivering fixed doses of the drug to be tested (Turner *et al.,* 2012). However, gavage comprises a sequence of procedures potentially stressful for laboratory animals. Removing the animal from the cage, manually restraint and insertion of a flexible or rigid dosing cannula into the esophagus in direction to the stomach (Atcha *et al.,* 2010; Brown *et al.,* 2000), cause high levels of stress even in trained animals. Additionally, there are also welfare issues related to the use of gavage when it is done by inexperienced people and/or in long-term studies (Huang-Brown and Guhad, 2002). Indeed, gastro-esophageal aspiration and pulmonary injury represent recurrent complications of gavage dosing of rodents that may be triggered by technical gavage errors (Damsch *et al.,* 2011). Moreover, the use of gavage for different drugs and vehicles can elicit a significant stress response in a vehicle- and dose volume-dependent fashion (Brown *et al.,* 2000).

When testing antihypertensive drugs it becomes crucial to ensure the selection of a non-invasive and stress-free method for drug delivery, since it has been shown that any source of external stress on rodents can significantly increase heart rate and blood pressure (Brown *et al.,* 2000; Bonnichsen *et al.,* 2005; Kramer *et al.,* 2000; Balcombe *et al.,* 2004) and therefore contribute for confounding the experimental results.

Several alternatives to gavage, performed with less human interference, have been tested in the last years. Administration of a drug mixed with an attractive vehicle, by voluntary ingestion, seems to be an effective method for drug administration in a non-invasive way (Goldkuhl *et al.,* 2008; Jacobsen *et al.,* 2011). In previous attempts to refine the gavage procedure, nut paste has been successfully used for analgesic drugs delivery (Abelson *et al.,* 2012; Goldkuhl *et al.,* 2008; Jacobsen *et al.,* 2011; Kalliokoski *et al.,* 2011) and shown to be a promising option for estrogen administration (Isaksson *et al.,* 2011). Other alternatives included the use of transgenic dough diet-pill dosing method (Walker *et al.,* 2012), flavored gelatin preparation - "jello" (Flecknell *et al.,* 1998), sugar cookie dough (Corbett *et al.,* 2012), honey (Kuster *et al.,* 2012) and syringe-feeding technique (Atcha *et al.,* 2010).

In our study we used nut paste (NUT), peanut butter (PB) and sugar dough (SD) as drug vehicles due to their palatability, consistency and previously reported results. We aimed to investigate the suitability of these vehicles for voluntary oral administration and, additionally, if they can be used as an alternative method to gavage, for chronic administration of losartan to laboratory rats. We hypothesized that low amounts of these vehicles, even when administered to rats during a long period of time, will not induce relevant changes in blood glucose or lipid profiles, while efficiently delivering the tested drug, as assessed by losartan serum concentration. Accordingly, we further hypothesized that the serum concentrations of the drug delivered by

voluntary ingestion would be at least as high as with gavage.

MATERIAL AND METHODS

Animals

Experiments were performed in forty-one male Wistar rats (*Rattus norvegicus L.*), aged 2-3 months, with mean body weight 283 ±5.0 g, obtained from the NOVA Medical School animal facility. This Wistar in house colony has been started with animals acquired from Charles River Laboratories (Crl:WI). Animals were housed individually in polycarbonate cages with wire lids (Tecniplast, Buguggiate, Varese, Italy), under 12 h light/dark cycles (8 am - 8 pm), at a room temperature 22 ± 2.0 °C and relative humidity 60± 10%. Rats were maintained on standard laboratory diet (SDS diets RM1) and reverse osmosis water, given *ad libitum*. Corncob bedding (Probiológica, Lisbon, Portugal) was used and changed once a week. Animals were Specific Pathogen Free (SPF) according to FELASA recommendations (Nicklas *et al.*, 2002).

Applicable institutional and governmental regulations concerning ethical use of animals were followed, according to the NIH Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1985), the European guidelines for the protection of animals used for scientific purposes (European Union Directive 2010/63/EU) and the Portuguese Law nº 113/2013. Experimental procedures were previously approved (nr. 21/2013/CEFCM) by the Institutional Ethics Committee of the NOVA Medical School for animal care and use in research.

Experimental protocol

Three different vehicles were tested for long-term oral administration (14 days) of losartan, an antihypertensive drug (angiotensin II receptor (type AT1) antagonist). Based on their consistency, palatability and previous reports (Abelson *et al.*, 2012; Goldkuhl *et al.*, 2010; Jacobsen *et al.*, 2011; Kalliokoski *et al.*, 2011; Isaksson *et al.*, 2011; Corbett *et al.*, 2012), nut paste (NUT, Nutella ®, Ferrero Ibérica SA, Llobregat, Spain), peanut butter (PB, Skippy ®, Unilever, London, UK) and sugar dough (SD, SweetArt ®, Entertraining Lda, Lisboa, Portugal) were used. NUT ingredients include sugar, vegetable oil, hazelnuts (13%), skim milk powder (8.7%), fat-reduced cocoa powder (7.4%), soy lecithin (emulsifier) and vanillin (flavoring). The caloric content is 21.75 kJ/g; protein content is 7.1%; fat content 30.3% and sugars content is 54.7%. PB is composed of roasted peanuts, sugar, hydrogenated vegetable oils and salt. The caloric content is 26.10 kJ/g; protein content is 22.2%; fat content 49.4% and carbohydrate content is 23.8%. Finally, SD presents as ingredients sugar, cornstarch, glucose, vegetable fat, vegetable gums, stabilizer E420, preservatives E202, dye E102 and E131. The caloric content is 16.50 kJ/g; protein content is 3.5%; fat content 6.6% and carbohydrate content is 86.6%.

Rats were randomized following weaning into six groups of six rats each: group 1 (NUT - 28 days); group 2 (PB - 28 days); group 3 (SD - 28 days); group 4 (NUT with 10 mg/kg/day losartan - 14 days); group 5 (PB with 10 mg/kg/day losartan - 14 days); group 6 (SD with 10 mg/kg/day losartan - 14 days) and one

group of five rats: group 7 (10 mg/kg/day losartan administered by oral gavage - 14 days).

All rats used to test voluntary administration (groups 1 to 6) underwent a 2-day acclimatization period, during which they were fed 0.5 g of the respective vehicle once daily, in order to minimize neophobia (Abelson *et al.*, 2012) and avoid incomplete ingestion incidents. Drug or vehicles deliveries would be considered incomplete when any trace of vehicle was found in either Petri dish, side of the cage or in bedding, after the first hour.

In the first set of experiments (groups 1, 2 and 3) the vehicles were administered without drug for 28 days (in addition to the acclimatization period) in order to determine whether rats would voluntarily ingest them for such period of time and also to test the vehicles effect on blood glucose level and serum lipid profile. The vehicles were weighed, mixed with the respective amount of drug and offered to the animals in 92x16 mm polystyrene Petri dishes (Sarstedt AG & Co, Germany). The Petri dishes were placed vertically, attached with adhesive tape to the inner cage wall (Figure 1). The vehicles, alone or mixed with the drug, were daily given to the animals at approximately the some schedule (11:00 to 12:00 am). The administration schedule as well as the ingestion profile (1- complete; 2- incomplete) and the time between vehicle and vehicle/drug mixture delivery and consumption (1- within 5 minutes or less; 2-between 5 minutes and 1 hour; 3- > 1 hour) were recorded.

The animals of group 7 underwent a 7 days handling acclimatization period. Rats were handled daily for a period of 2 minutes each by the same individual and accustomed to the gavage position, in a different animal facility procedures room. Handling training and gavage were performed at the same schedule (11:00 to 12:00 am) as for voluntary administration. Gavage was performed using a stainless steel gavage feeding needle, curved and with round tip (gauge 16; tip diameter: 3 mm; length: 75 mm; Fine Science Tools, California, USA).

Rats were weighed at baseline and once a week during the entire study. The amounts of losartan were adjusted weekly to ensure a daily dose of 10 mg/kg. Losartan was prepared daily, immediately before administration, when to be given either by gavage or along with vehicle. For gavage, Losartan was dissolved in reverse osmosis water (2 mL). When Losartan was administered along with vehicle, the required amount of drug for each rat was mixed with 0.5 g of the respective vehicle. In the first set of experiments (groups 1, 2 and 3), water intake was measured weekly and blood glucose level and serum lipid profile were monitored, 2-3 hours after vehicle administration, at baseline, day 14 and day 28.

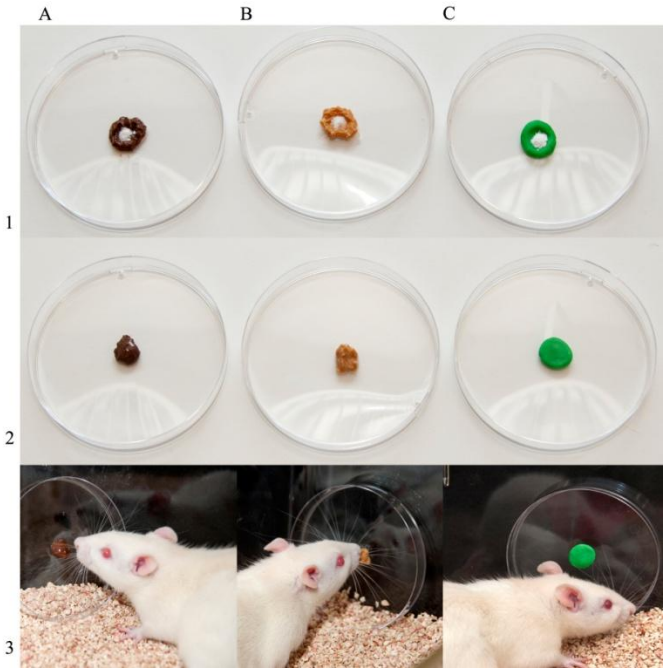


Figure 1: Method for voluntary ingestion of losartan with three different vehicles: nut paste (A); peanut butter (B) and sugar-dough (C). (1) Losartan powder weighted directly over vehicle. (2) Vehicle-drug mixture shaped into a ball ready for delivery. (3) Rats ingesting vehicle-drug mixture from the polystyrene Petri dish placed vertically in the cage.

At the end of the experiments, 2-3 hours after drug delivery, rats were anesthetized, by intraperitoneal injection with medetomidine (0.5mg/kg body weight; Domitor®, Pfizer Animal Health) and ketamine (75mg/kg body weight; Imalgene 1000®, Mérial, Lyon, France), and cardiac puncture was performed to collect blood for further drug quantification. The animals were then euthanized by an intracardiac overdose of sodium pentobarbital, and the death was confirmed by cervical dislocation.

Blood sampling

Tail vein sampling: In groups 1, 2 and 3, blood samples were collected from the tail vein of conscious animals at baseline, day 14 and day 28. Rats were placed in a conventional plastic restrainer (n° 554-BSRR, Plas-Labs, Lansing, Michigan, USA), at a room temperature 24-27ºC. A scalpel blade was used to make an incision in the lateral tail vein. Around 100 µL of blood was collected from each animal, using a capillary tube (Hirschmann Laborgerate, Eberstadt, Germany), to eppendorfs and kept on ice. This procedure was also used for collecting two blood drops for glucose quantification. After collection, bleeding was stopped by compression for a few seconds and cleaned with iodopovidone. Serum samples were centrifuged in a microfuge at 3000 rpm (4º C) for 10 minutes, immediately decanted and stored at −80 °C until analysis. According to prior recommendations, the restrainer was washed and dried after each use to prevent pheromonally induced stress (Parasuraman *et al.*, 2010).

Cardiac puncture: Blood collection was performed without thoracotomy with a 20 G needle with a 10 mL syringe. For plasma sampling, approximately 3 mL of blood was collected to vacutainer tubes containing EDTA (ethylenediaminetetraacetic acid), kept on ice and immediately centrifuged at 3000 rpm (4 °C) for 10 min. Plasma samples were decanted prior to storage at −80 °C until analysed.

Measurement of blood glucose level and serum lipid profile

Blood glucose level was determined in mg/dl using a digital glucometer (Precision Xceed®, Abbott diabetes care Ltd, Oxon, UK). The serum levels of triglyceride (TGL), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein- cholesterol (LDL-C) were determined spectrophotometrically (Rx Daytona analyser, Randox Laboratories Ltd, UK), using enzymatic colorimetric assay kits (Randox Laboratories Ltd, UK). The results were expressed in mg/dL.

HPLC analysis

Losartan plasma concentrations were determined by HPLC (High-performance liquid chromatography), using a minor modification of the method described by Yeung and colleagues (Yeung *et al.,* 2000). Briefly, losartan was dissolved in methanol yielding a stock solution of 1 mg/mL. Calibrations samples were prepared by adding known amounts of the diluted stock solution to rat plasma and covered a range of 1 - 10 µg/mL. Subsequently, calibration samples (200 µL) were extracted with 3 mL of ethyl acetate. After centrifugation (3000 rpm, 4 ºC, 10 min), the organic phase was evaporated to dryness at 60 ºC in a Speed-Vac concentrator (Labconco, Kansas City, MO, USA). The resulting residue was reconstituted in 150 µL of mobile phase and 100 µL was injected into HPLC.

HPLC was accomplished by using a solvent delivery pump (model LC 9-A; Schimadzu, Kyoto, Japan), autosampler (model 7725i; Schimadzu, Kyoto, Japan, UV-VIS spectrophotometric detector (model SPD-6 AV; Schimadzu, Kyoto, Japan.), column (250 x 4 mm; particle size: 5 µm; LiChrospher 100 RP-18; Merck, New Jersey, USA) protected by a guard-column (4 x 4mm; particle size: 5 µm; LiChrospher 100 RP-18e; Merck, New Jersey, USA) and a column oven (model CTO-10AS VP; Schimadzu, Kyoto, Japan). Data acquisition and processing were performed using Shimadzu Class VP 7.X software. The mobile phase consisted of solution A - methanol: acetonitrile: sodium dihydrogen phosphate monohydrate buffer (10mM; pH 3; 50:10:40; v/v/v) and solution B - methanol. All HPLC solvents were purchased from VWR International (Carnaxide, Portugal). Prior analysis, both solutions were degassed for 15 min by sonication (VWR, Carnaxide, Portugal). The elution program consisted of an isocratic flow of A solution for (9.5 min), followed by a linear gradient A:B (20:80 (v/v); 2.5 min), an isocratic step A:B (20:80; 4 min) and finally return to initial conditions in 2 min. The analytical run was performed with a mobile phase flow rate 0.8mL/min, at 25º C and detection wavelength of 230 nm.

Drugs

Losartan at 10 mg/kg, from Cinfa, S.A (generic, Pamplona, Navarra, Spain), was mixed with the

three vehicles and administered to the animals. Analytical standards of losartan, for HPLC analysis, were purchased from Sigma-Aldrich (Sintra, Portugal).

Statistical Analysis

All data are represented as mean ± standard error of the mean (SEM). One-way ANOVA with Bonferroni's multiple comparison test and one-way analysis of variance with Tukey's multiple comparison test were used, whenever appropriate, to evaluate the vehicles effect on glycaemia and lipid profile. The two-way repeated-measures ANOVA with post hoc comparison test was used to compare the vehicle effect on mean animal body weights and water intake, along the 28 days of experiments. Comparison in losartan plasma concentrations between vehicle groups was performed using one-way ANOVA with Dunnett's multiple comparison post hoc test, using gavage as control. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., version 5.01, San Diego, CA). Statistical significance for all tests was set at the level of $p<0.05$.

RESULTS

Vehicles and vehicles-drug mix ingestion

During the 2-day acclimatization period the vehicles were made available in the cages as shown in figure 1. Animals of groups 1 and 4 were offered nut paste (NUT), 2 and 5 peanut butter (PB) and 3 and 6 sugar dough (SD). The time between vehicle presentation and complete consumption was less than 1 hour in groups 1, 2, 4 and 5. Five animals from group 3 and four from group 6 took more than 1 hour in the first day but in the second day all animals took less than 1 hour to eat the SD. After the acclimatization period, all animals of groups 1, 2 and 3, voluntarily ingested the vehicles. The time between vehicle delivery and its consumption was five minutes or less, for all cases. No incomplete drug ingestion incidents were observed after the acclimatization period (groups 4, 5 and 6). Furthermore, ingestion of the vehicle-drug mixture was immediate (less than 5 minutes) for all animals.

Physiological data, blood glucose levels and lipid profile

There was no vehicle effect on mean animal body weights and water intake, throughout the 28 days of experiments ($p>0.05$). Vehicle ingestion did not induce any deviation to the weight variation observed in animals of this age in this population. Weight gain was in average 90 g for the 18 animals of groups 1, 2 and 3 and water intake increased in average 14.8 ml during the 28 days.

Interestingly, unlike PB and SD groups, a significant decrease in mean blood glucose levels was observed along the experimental period for the NUT group of animals (Figure 2 A). Moreover, at the 28-day time-point the mean glycaemia of NUT group was significantly lower than the one observed for the other two groups (Nut paste vs. PB: $p<0.05$; Nut paste vs. SD: $p<0.01$; Figure 2 D). The glycemic profile remained unchanged in PB and SD groups ($p>0.05$) (Figure 2 B and C). Regarding the lipid profile, serum levels of total cholesterol gradually increased in both NUT ($p<0.05$; baseline vs. 28 days) and PB ($p<0.05$; baseline vs. 28 days) groups (Figure 3 A and B). However, these values remained unaltered

(p>0.05) in SD group (Figure 3 C). Anyway, the mean total cholesterol levels of the three groups at both 14-day and 28-day time points were not significantly different (p>0.05) (Figure 3 D). In the SD group, a non-significant increase (p>0.05) in serum triglycerides concentrations was observed during the 28 days (Figure 4 C). The PB group showed the same tendency but this time the increase was statistically significant (p<0.05; baseline vs. 28 days; p<0.01 14 days vs. 28 days; Figure 4 B). Serum triglycerides values remained unchanged (p>0.05) in the NUT administration (Figure 4 A). Once again, the mean total triglycerides concentrations of the three groups at both 14-day and 28-day time points were not significantly different (p>0.05) (Figure 4 D). Decreased levels of HDL-C were observed at day 14 for both SD (p<0.01) and PB (p<0.001) groups. These values progressively increased and, at the end of the experiment, were similar to those observed at baseline (p>0.05: baseline vs. 28 days, for both groups). No differences in HDL-C values were found between vehicles at the three time points (p>0.05) and NUT did not affect the HDL-C levels (Figure 3 E). Finally, no significant changes on LDL-C values were observed either for NUT, PB or SD groups along the experimental period (Figure 3 F).

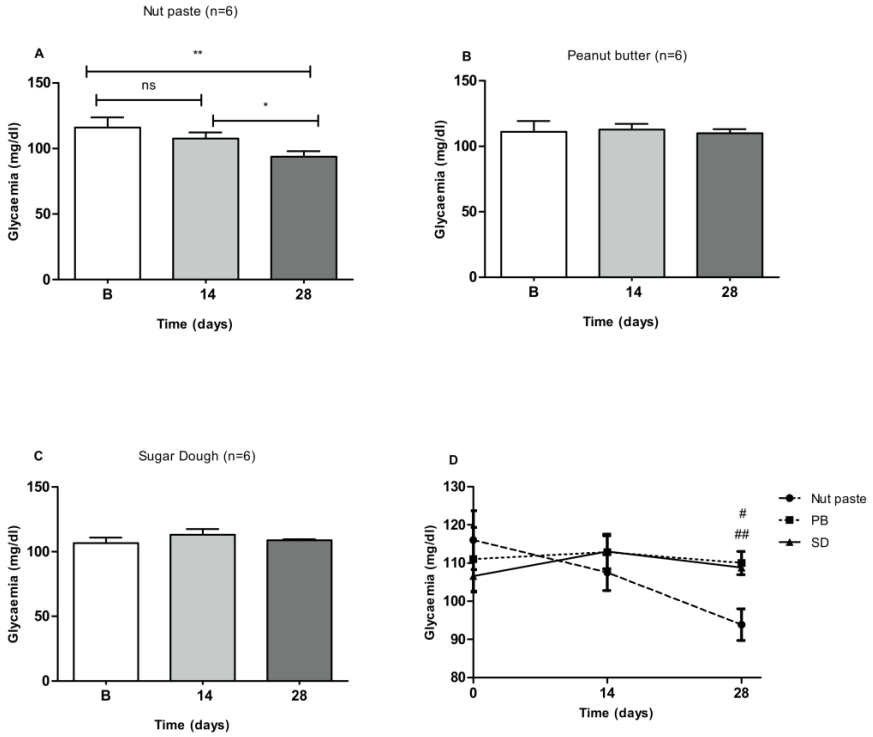


Figure 2: Mean glycaemia values (mg/dl) in the serum of Wistar rats fed with 0.5 g of (A) Nut paste; (B) Peanut butter and (C) Sugar Dough vs. time (D). Comparison of the three vehicles effects on glycaemia at 14 and 28-day time points. Data are expressed as mean±SEM; n=6 for all groups. * p<0.05; ** p<0.01 - One-way ANOVA with Bonferroni's multiple comparison test; # p<0.05 (nut paste vs. sugar dough); ## p<0.01 (nut paste vs. peanut butter) – One-way analysis of variance with Tukey's multiple comparison test.

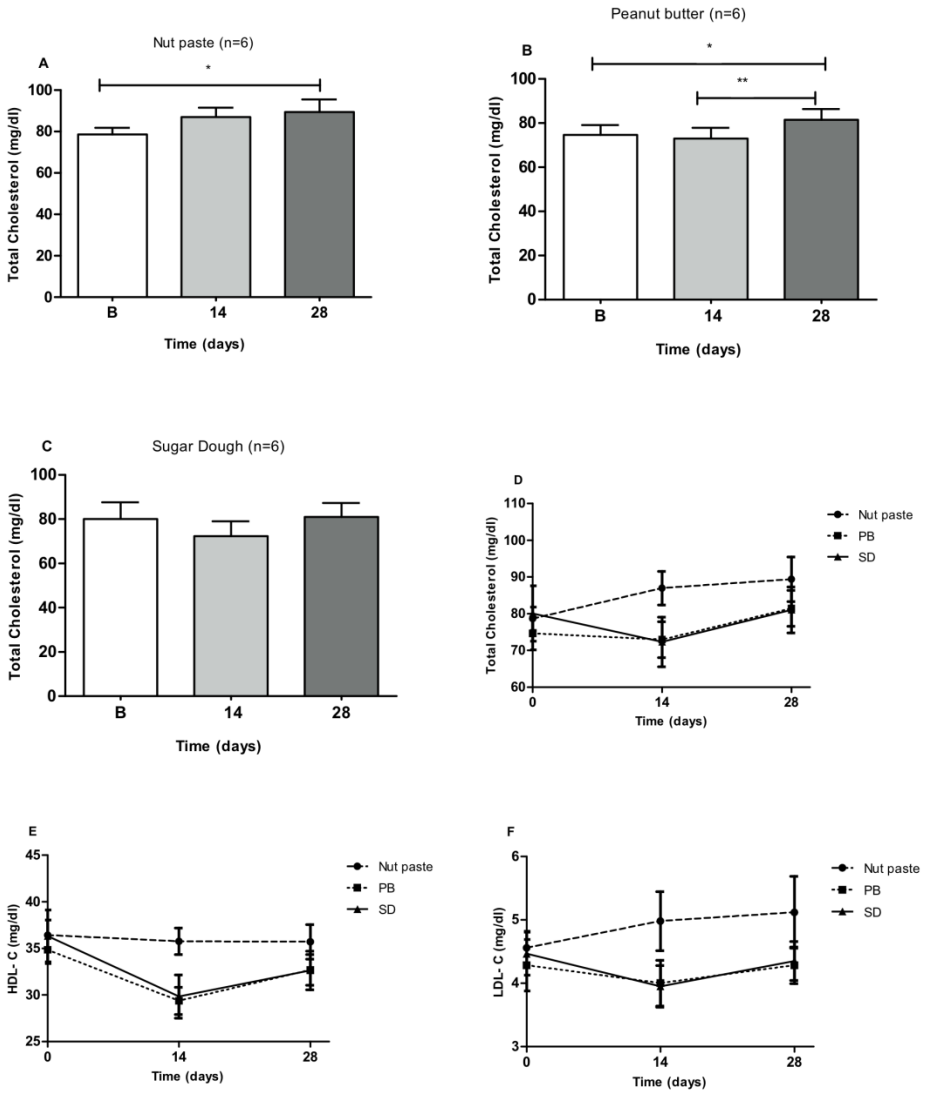


Figure 3: Mean total cholesterol concentrations (mg/dl) in the serum of Wistar rats fed with 0.5 g of (A) Nut paste; (B) Peanut butter and (C) Sugar Dough vs. time (* $p<0.05$; **$p<0.01$ - One-way ANOVA with Bonferroni's multiple comparison test). Comparison of the three vehicles effects on (D) total cholesterol, (E) HDL-C and (F) LDL-C levels at 14 and 28-day time points (One-way analysis of variance with Tukey's multiple comparison test). Data are expressed as mean±SEM (n=6 for all groups).

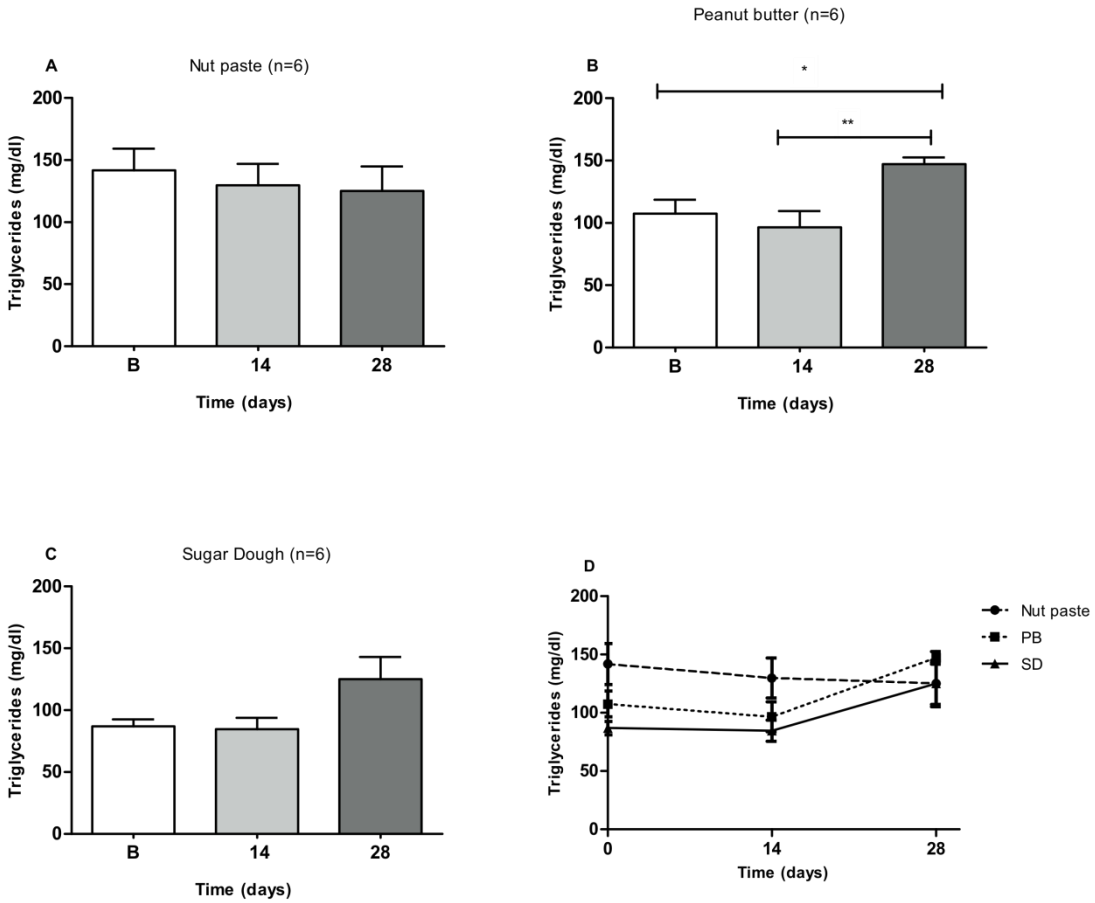


Figure 4: Mean triglycerides concentrations (mg/dl) in the serum of Wistar rats fed with 0.5 g of (A) Nut paste; (B) Peanut butter and (C) Sugar Dough vs. time (* $p<0.05$; ** $p<0.01$ - One-way ANOVA with Bonferroni's multiple comparison test). (D) Comparison of the three vehicles effects on triglycerides levels at 14 and 28-day time points. Data are expressed as mean±SEM (n=6 for all groups).

Losartan plasma concentrations

After 14 days of losartan daily administration by gavage the mean plasma concentration was 0.9±0.09 μg/mL. A slight but non-significant decrease of the plasma concentration of losartan was observed in the PB group (0.7±0.09 μg/mL). However, when losartan was given along with NUT, its plasma concentration tended to be higher than in the gavage group (1.3 ±0.15 μg/mL), although this difference did not reach statistical significance. When SD vehicle was used, a significantly higher losartan plasma concentration was achieved (1.7±0.14 μg/mL; $p<0.01$ vs. gavage). These results are presented in Figure 5.

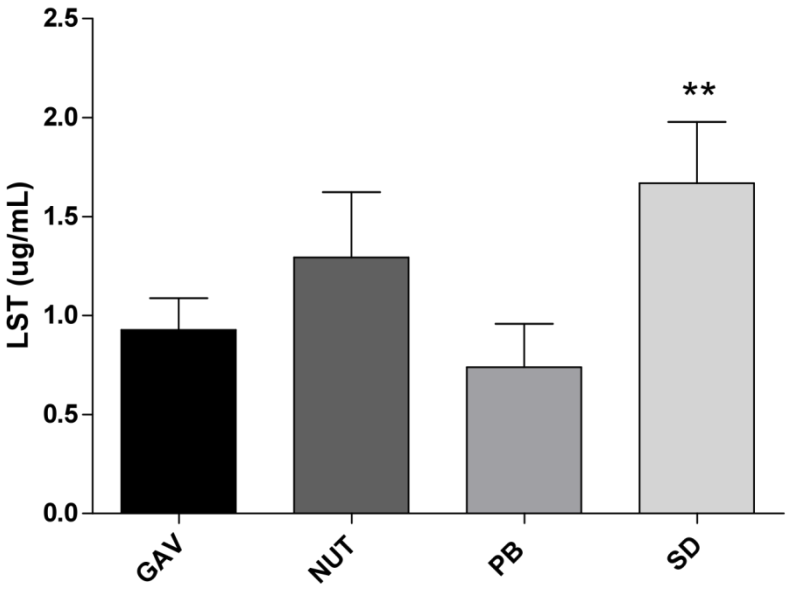


Figure 5: Losartan (LST) plasma concentrations (μg/mL), from blood collected by cardiac puncture after 14 days of daily ingestion, in gavage (GAV), nut paste (NUT), peanut butter (PB) and sugar dough (SD) rats. Data are expressed as mean±SEM (n=6 for all groups) ** p<0.01 compared to gavage - One-way ANOVA with Dunnett's multiple comparison test.

DISCUSSION

We have tested nut paste (NUT), peanut butter (PB) and sugar dough (SD) as potential vehicles for chronic losartan administration to rats in order to surpass the stress induced by orogastric forced administration (gavage). Voluntary ingestion proved to be an effective method for a controlled daily dose administration, with a define timetable, that is independent of handling and restraint procedures. Moreover, as gavage, this approach mimics the single-dose administration, widely used in human clinical practice.

Male Wistar rats, aged 2-3 months and housed individually were used to avoid hypothetical effects of estrogen on blood lipids (Ganong, 2002). Although Sharp and collaborators (Sharp *et al.,* 2002) recommend that animals should be ideally housed in group to decrease the stress levels, in our study, animals were single-housed during all protocol in order to ensure correct dosage and to confirm vehicle or vehicle plus drug complete ingestion. The palatability of NUT, PB and SD showed to be attractive for the animals, since the animals ingested them almost immediately and completely. Furthermore, the consistency of these vehicles facilitates the weighing and the mixing of powder, thereby ensuring the accuracy of the drug dose. The amount of vehicle given to the animals (0.5g) was shown to be enough to hold the weighed drug, whereas still appeared attractive for the animals and not too large to cause significant metabolic changes. Offering the mixture in a Petri dish placed vertically inside the cage proved to be an effective solution to ensure

complete ingestion. Before it has been reported that placing NUT in a container on the floor of the cage is not recommended, since neophobic animals tend to burry the novel item, and thus mix the vehicle with the bedding material (Abelson *et al.*, 2012).

Although some authors have recommended a period of up to five days for animal acclimatization to the vehicles (Isaksson *et al.*, 2011), in our study, two days proved to be enough time to ensure that there was no neophobic behavior after that. In order to reduce the stress levels associated with handling and restrained we decided to use a one-week period of acclimatization in the gavage group.

Oral gavage has been recently refined from metal gavage needle to a sterile flexible feeding tube which reduces the risk of trauma, perforation and cross contamination, (Morton *et al.*, 2001), the stress related to orogastric administration is not overcome by this approach.

Losartan is the first orally available angiotensin-receptor antagonist without agonist properties (Sica *et al.*, 2005). Following oral administration, losartan is rapidly absorbed, reaching maximum concentrations 1–2 hours post-administration (Sica *et al.*, 2005). This drug is freely soluble in water and the reported bioavailability of losartan with a 50 mg tablet is 32.6% (Sica *et al.*, 2005). Dose administration with meals slows the rate of absorption and reduces the area under the plasma concentration-time curve (AUC) of losartan and its metabolite by approximately 10% (Simpson *et al.*, 2000). Food can have a significant effect on drug absorption through several mechanisms, including delay in gastric emptying, increases in splanchnic blood flow, and changes in gastrointestinal secretions. These effects can alter tablet disintegration, drug dissolution, and drug transit through the gastrointestinal tract. Facing this, we decided to offer the vehicle- drug mixtures at the first hours of light in order to ensure that drug ingestion took place in a period with lower regular food intake. The vehicle amount used did not show to interfere with losartan bioavailability.

According to Chan and colleagues, blood sampling site can strongly influence a substantial number of routinely tested blood parameters such as glucose, TC, TGL and HDL-C and highlight the need to standardize sampling sites, especially when repeated blood sampling is required (Chan *et al.*, 2013). To avoid this untoward experimental bias, only blood collected from the tail vein, in three different days, was used for measurement of blood glucose level and serum lipid profile. Accordingly, no differences were observed among groups of animals when comparing basal levels, i.e., prior to gavage or vehicle administration. Moreover, previous studies in the literature showed that there are no significant age-related changes in glycemia (Ribeiro *et al.*, 2008) or lipid profiles (Uchida *et al.*, 1978) throughout the time period of our study. Therefore, any changes observed between different vehicles are caused by differences in the vehicles composition and not age-related.

Analysis of the results suggested that NUT, PB and SD are possible alternatives to gavage for chronic administration of losartan, since their ingestion was not associated with weight gain or

increased water ingestion and were all fully eaten by the animals. However, taken into account the glycemia and lipid profile, SD proved to be the most suitable vehicle for the administration of this antihypertensive drug for two main reasons. First, the mean plasma concentration of losartan was significantly higher than the one attained when gavage was used. Secondly, SD didn't induce any significant change on both glycaemia and lipid profile along the 28 days. On the other hand, the use of PB seems to be the least adequate due to the changes on the rat's lipid profile associated with a 22% reduction of losartan plasmatic concentration. Even though the NUT group showed a glycemia reduction and increased total cholesterol, the losartan plasma concentration was 29% higher than gavage. These results could suggest that SD maximizes losartan absorption, leading to a higher bioavailability.

In the present study, only Cmax concentrations were measured. It must be highlighted that losartan has an extensive first-pass effect with an oral bioavailability of 32%, showing that transcelullar intestinal absorption (P-glycoprotein) and biotransformation (CYP3A4) clearly impacts its bioavailability and allows drug-nutrients interactions.

In our opinion, the higher losartan concentrations attained in sugar dough group, compared with gavage, might be explained by three different mechanisms: an absorption delay, an increased absorption or a lower biotransformation. In fact, the high content of sugar present in sugar dough might increase the rate and/or extent of drug absorption since it has been described that sucrose fatty acid esters might improve the intestinal absorption of poorly absorbable drugs via a transcellular and a paracellular pathways (Yamamoto *et al.*, 2014). Additionally, it has been suggested that high carbohydrate content may lower CYP450 activity, particularly when high doses are administered (Sonawane *et al.*, 1988).

Substances that leave an unpleasant odor or flavor could discouraged voluntary ingestion and compromised the success of this method. Thus, we suggest that a pilot study should be carried out in order to ensure that all rats would successfully ingest the drug-vehicle mixture.

CONCLUSION

The results presented in this report support that the use of voluntary oral administration is a viable alternative for chronical administration of a fixed dose of losartan. In fact, our results suggest that SD is better suited than gavage for losartan oral daily administration. Furthermore, these data provide evidence that SD is the most adequate vehicle for losartan administration in rats in order to overcome the several shortcomings associated to gavage. The drug bioavailability was not reduced, suggesting that this approach is an effective oral dosing method for chronic administration of losartan. This refinement might be of interest for safety evaluation of drugs, particularly for those which toxic effects are associated with stress.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors would like to gratefully acknowledge Isabel Ribeiro da Silva for her technical assistan. The present work was supported by Fundação para a Ciência e Tecnologia (FCT) grant (PTDC/SAU-TOX/ 112264/2009) and CEDOC (Portugal). Lucília N. Diogo holds a FCT PhD grant (SFRH / BD / 48335 / 2008).

REFERENCES

Abelson, K.S., Jacobsen, K.R., Sundbom, R., Kalliokoski, O., Hau, J., 2012. Voluntary ingestion of nut paste for administration of buprenorphine in rats and mice. Lab Anim. 46, 349-51. doi: 10.1258/la.2012.012028.

Atcha, Z., Rourke, C., Neo, A.H., Goh, C.W., Lim, J.S., Aw, C.C., Browne, E.R., Pemberton, D.J., 2010. Alternative method of oral dosing for rats. J. Am. Assoc. Lab. Anim. Sci. 49, 335-43.

Balcombe, J.P., Barnard, N.D., Sandusky, C., 2004. Laboratory routines cause animal stress. Contemp. Top. Lab. Anim. Sci. 43, 42-51.

Bonnichsen, M., Dragsted, M., Hansen, A.K., 2005. The welfare impact of gavaging laboratory rates. Anim. Welfare. 14, 223–227.

Brown, A.P., Dinger, N., Levine, B.S., 2000. Stress produced by gavage administration in the rat. Contemp. Top. Lab. Anim. Sci. 39, 17-21.

Chan, Y.K., Davis, P.F., Poppitt, S.D., Sun, X., Greenhill, N.S., Krishnamurthi, R., Przepiorski, A., McGill, A.T., Krissansen, G.W., 2012. Influence of tail versus cardiac sampling on blood glucose and lipid profiles in mice. Lab. Anim. 46, 142-7. doi: 10.1258/la.2011.011136.

Corbett, A., McGowin, A., Sieber, S., Flannery, T., Sibbitt, B., 2012. A method for reliable voluntary oral administration of a fixed dosage (mg/kg) of chronic daily medication to rats. Lab. Anim. 46, 318-24. doi: 10.1258/la.2012.012018.

Damsch, S., Eichenbaum, G., Tonelli, A., Lammens, L., Van den Bulck, K., Feyen, B., et al 2011. Gavage-related reflux in rats: identification, pathogenesis, and toxicological implications (review). Toxicol. Pathol. 39, 348-60. doi: 10.1177/0192623310388431.

Flecknell, P.A., Roughan, J.V., Stewart, R., 1999. Use of oral buprenorphine ('buprenorphine jello') for postoperative analgesia in rats--a clinical trial. Lab. Anim. 33, 169-74.

Ganong, W.F., 2002. Handbook of medical physiology. EGC, Jakarta.

Goldkuhl, R., Hau, J., Abelson, K.S., 2010. Effects of voluntarily-ingested buprenorphine on plasma corticosterone levels, body weight, water intake, and behaviour in permanently catheterised rats. In Vivo. 24, 131-5.

Huang-Brown, K.M., Guhad, F.A., 2002. Chocolate, an effective means of oral drug delivery in rats. Lab Anim (NY). 31, 34-6.

Isaksson, I.M., Theodorsson, A., Theodorsson, E., Strom, J.O., 2011. Methods for 17β-oestradiol administration to rats. Scand. J. Clin. Lab. Invest. 71, 583-92. doi: 10.3109/00365513.2011.596944.

Jacobsen, K.R., Kalliokoski, O., Hau, J., Abelson, K.S.P. 2011. Voluntary ingestion of buprenorphine in mice. Anim. Welfare. 20, 591–596.

Kalliokoski, O., Abelson, K.S., Koch, J., Boschian, A., Thormose, S.F., Fauerby, N., et al. 2010. The effect of voluntarily ingested buprenorphine on rats subjected to surgically induced global cerebral ischaemia. In Vivo. 24, 641-6.

Kalliokoski, O., Jacobsen, K.R., Hau, J., Abelson, K.S., 2011. Serum concentrations of buprenorphine after oral and parenteral administration in male mice. Vet. J. 187, 251-4. doi: 10.1016/j.tvjl.2009.11.013.

Kramer, K., Voss, H.P., Grimbergen, J.A., Mills, P.A., Huetteman, D., Zwiers, L., Brockway, B., 2000. Telemetric monitoring of blood pressure in freely moving mice: a preliminary study. Lab. Anim. 34, 272–280.

Küster, T., Zumkehr, B., Hermann, C., et al. 2012. Voluntary ingestion of antiparasitic drugs emulsified in honey represents an alternative to gavage in mice. J. Am. Assoc. Lab. Anim. Sci. 51, 219-23.

Morton, D.B., Jennings, M., Buckwell, A., Ewbank, R., Godfrey, C., Holgate, B., *et al.,* Joint Working Group on Refinement, 2001. Refining procedures for the administration of substances. Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. Lab. Anim. 35, 1-41.

Nicklas, W., Baneux, P., Boot, R., Decelle, T., et al; FELASA Working Group on Health Monitoring of Rodent and Rabbit Colonies), 2002. Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Lab. Anim. 36, 20-42.

Parasuraman, S., Raveendran, R., Kesavan, R., 2010. Blood sample collection in small laboratory animals. J. Pharmacol. Pharmacother. 1, 87-93. doi: 10.4103/0976-500X.72350.

Ribeiro RT, Afonso RA, Guarino MP, Macedo MP. 2008. Loss of postprandial insulin sensitization during aging. The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences 63:560-5.

Sharp, J.L., Zammit, T.G., Azar, T.A., Lawson, D.M., 2002. Stress-like responses to common procedures in male rats housed alone or with other rats. Contemp. Top. Lab. Anim. Sci. 41, 8-14.

Sica, D.A., Gehr, T.W., Ghosh, S. 2005. Clinical pharmacokinetics of losartan. Clin. Pharmacokinet. 44, 797-81.
Simpson, K.L., McClellan, K.J. 2000. Losartan: a review of its use, with special focus on elderly patients. Drugs Aging. 16, 227-50.

Simpson KL, McClellan KJ. 2000. Losartan: a review of its use, with special focus on elderly patients. Drugs & Aging 16: 227-50.

Sonawane BR , Coates PM , Yaffe SJ , Koldovsky O. (1983). Influence of dietary carbohydrates (alpha-saccharides) on hepatic drug metabolism in male rats. Drug and Nutricional Interactions. 2:7-16.

Turner, P.V., Vaughn, E., Sunohara-Neilson, J., Ovari, J., Leri, F., 2012. Oral gavage in rats: animal welfare evaluation. J. Am. Assoc. Lab. Anim. Sci. 51, 25-30.

Uchida K, Nomura Y, Kadowaki M, Takase H, Takano K, Takeuchi N. 1978. Age-related changes in cholesterol and bile acid metabolism in rats. Journal of Lipid Research 19:544-52.

Walker, M.K., Boberg, J.R., Walsh, M.T., Wolf, V., Trujillo, A., Duke, M.S., Palme, R., Felton, L.A., 2012. A less stressful alternative to oral gavage for pharmacological and toxicological studies in mice. Toxicol. Appl. Pharmacol. 260, 65-9. doi: 10.1016/j.taap.2012.01.025.

Yamamoto A, Katsumi H, Kusamori K, Sakane T. 2014. Improvement of intestinal absorption of poorly absorbable drugs by various sugar esters. Yakugaku Zasshi. 134, 47-53.

Yeung, P.K., Jamieson, A., Smith, G.J., Fice, D., Pollak, P.T., 2000. Determination of plasma concentrations of losartan in patients by HPLC using solid phase extraction and UV detection. Int. J. Pharm. 204, 17-22.

V

DISCUSSION AND CONCLUSIONS

The general discussion is divided into five informal sections. The first one summarizes the relevant findings of both clinical and experimental studies. Then, we describe how the present work matches what is already known in the literature. So, it is inevitable that there is some content overlap between this section and chapter IV. The next sections outline the main limitations of our work and explore some future perspectives concerning the management of hypertension related to obstructive sleep apnea. Finally, we highlight the added value and the impact of the present work to the field.

Summary of relevant findings

CLINICAL STUDIES

The prevalence of new diagnoses of OSA among those patients that attended the outpatient consultation of CHLN sleep unit was 78.2% (283/362).

A considerable number of patients misclassified themselves as non-hypertensive (43.4%; 53/122), confirming the lack of validity of self-reported hypertension and emphasizing the importance of ABPM in the minimization of underestimated hypertension among patients suspected of OSA. These patients presented significantly higher values of ABPM variables and anthropometric characteristics, compared to truly non-hypertensive patients (69/122). The cut points that best discriminates these two groups of patients were 27 Kg/m^2 and 39 cm for BMI and NC, respectively. BMI and NC were identified as independent predictors of hypertension misclassification in patients suspected of OSA. So briefly, these findings suggest that all undiagnosed hypertensive patients referred to a sleep disorder centre for symptoms suggesting OSA with a BMI and NC above 27 Kg/m^2 and 39 cm, respectively (patients with a high odds of misclassifying themselves as non-hypertensive), should be screened for hypertension, through ABPM.

Furthermore, according to current guidelines and to antihypertensive medication and/or 24h ABPM, 63.9% (205/321) of the patients included were diagnosed with both OSA and hypertension. At baseline, 31 different antihypertensive regimens were found for OSA patients. Regarding BP control, 57.5% of patients under antihypertensive medication had uncontrolled BP (95/155). BP control is independent of both the antihypertensive regimen and the number of antihypertensive drugs and the increase in the number of AHD does not appear to be relevant to

control BP in patients with OSA. After CPAP adaptation, the lack of association between antihypertensive regimens and the number of antihypertensive drugs and BP control remained, but we found that CPAP significantly improved BP control in patients under no AH medication. This last finding suggest that an earlier diagnosis of hypertension related to OSA is more relevant than the AH regimen selection, probably because the available AH regimens are useful to prevent but not able to revert structural changes associated to hypertension. In a multivariable study, gender, OSA severity and 24h-BP profile were independently associated with uncontrolled BP after CPAP adaptation. Therefore, it can be stated that the risk of being uncontrolled is higher for males with moderate/severe

OSA and a non-dipper BP profile, confirming the relevance of OSA in the pathophysiology of hypertension. In summary, these results provided evidence that this type of hypertension needs to be managed as a specific entity and that none of the currently available AH drugs, either alone or in association, have been shown to be particularly effective to control BP in patients with OSA, either before or after CPAP adaptation.

EXPERIMENTAL STUDIES

The main purpose of our first experimental study was to investigate the efficacy of an antihypertensive drug, with α1- and β-adrenoceptor antagonist activity, recognized antioxidants properties and other pleiotropic effects, in reversing hypertension induced by CIH. We hypothesized that CVD would be effective in reducing both BP and HR, since it is broadly consensual that sympathetic activation and oxidative stress plays a relevant role in the pathophysiology of HT related to OSA (Sunderram and Androulakis, 2012). We found that CIH significantly increased MAP, diastolic and systolic BP in our animal model, while no effect was observed for HR. In addition, despite the doses of 10, 30 and 50 mg/Kg of CVD promoted a significant reduction in HR, no decrease in mean arterial pressure was observed. Interestingly, we also found that CIH changes the plasma concentrations of CVD enantiomers however, the lower ratios S/(R+S) of CVD enantiomers observed in rats exposed to CIH do not apparently explain the lack of efficacy of CVD in reversing this particular type of hypertension.
At the same time, we also have tested nut paste (NUT), peanut butter (PB) and sugar dough (SD) as potential vehicles for chronic antihypertensive drugs administration to rats in order to surpass the stress induced by orogastric forced administration (gavage). Blood glucose levels were reduced by NUT administration and PB induces increased levels of triglycerides and total cholesterol. SD group presented a higher concentration of losartan when compared with gavage group, without changing lipid and glucose profiles. So, our results showed that the three vehicles are viable for daily single dose voluntary ingestion of losartan, from which SD proved to be the best alternative. In fact, our data suggest that SD is better suited than gavage for losartan oral daily administration and provide evidence that SD is the most adequate vehicle for losartan administration in rats in order to overcome the several shortcomings associated to gavage. Since drug bioavailability was not reduced, voluntary ingestion proved to be an effective method for a controlled daily dose administration, with a defined timetable, that is independent of handling and restraint procedures. Moreover, as *gavage*, this approach mimics the single-dose administration, widely used in human clinical practice.

How the present work fits what is already known?

CLINICAL STUDIES

Our **first clinical study** reports 24-hour ambulatory BP data compared with self-reported hypertension

in patients suspected of OSA. Although self-reported data is often more economically feasible and readily available compared to BP monitoring, our findings show that these reports may not provide truthful information. Such inaccuracy will surely lead to an underestimation of hypertension prevalence if ABPM recordings are not taken into account. In OSA patients, this imprecision may have serious consequences since therapeutic decision-making (e.g. CPAP application) is based, in part, on the presence of cardiovascular disease, namely hypertension. For this reason, the validity of self-reported hypertension is highly questionable and should not replace any type of BP monitoring. However, the conclusions regarding this issue are far from unanimous and the number of variables that have been found to be associated with the accuracy of self-reported high BP is considerable (Mentz *et al.*, 2012).

Twenty-four-hour ABPM data showed that the frequency of systolic or diastolic non-dipping pattern is significant in misclassified non-hypertensive (MNH) patients. It is known that the lack of pressure fall at night, one of the major features of the 24-h BP profile in OSA patients (Hla *et al.,* 2008), is itself associated with a poor cardiovascular prognosis (Dolan *et al.,* 2009). Furthermore, Loredo *et al.* (2004) showed that an absence of nocturnal dip in BP correlated inversely with the indices of sleep fragmentation (Loredo *et al.,* 2004), and changes on sleep architecture may be responsible for daytime hypersomnolence, the prime feature of OSA (Baguet *et al.,* 2009). Thus, clinical observation of dipping or non-dipping profiles is extremely relevant, mainly for patients clinically suspected of having OSA.

ABPM also revealed that most MNH patients presented isolated nighttime HT or both daytime and night-time hypertension. In a study performed by Burr *et al.* (2006), night-time BP was identified as the strongest predictor of cardiovascular mortality (Burr *et al.,* 2006). Thus, the early recognition of nocturnal features, mainly recognized through the use of ABPM, is unquestionably relevant to improve the long-term outcomes of OSA patients.

Currently, BP measured in a clinical setting is considered the reference method for the general population, including patients with OSA (Baguet *et al.,* 2009). Nevertheless, office BP measurement is affected by major problems such as the white-coat effect, observer bias, limited reproducibility and the intrinsic inaccuracy of the auscultatory technique. Moreover, this method is unable to collect information on physiological BP variability and nocturnal BP (Parati *et al.,* 2012). Consequently, office readings frequently lead to both overestimation and underestimation of hypertension, since BP could be apparently normal in the office but elevated outside the office, especially during the night-time period (Parati *et al.,* 2012) or *vice versa*. Home BP monitoring (HBPM) has been proposed as an alternative to office BP measurements or ABPM. Like ABPM, this method allows the registration of BP values during a patient's normal activities, avoiding the white-coat effect; however such recordings are limited to short periods of time. Additionally, HBPM is unable to provide detailed information on BP during night-time, precisely when OSA episodes occur. These limitations are overcome when ABPM is used. This technique offers several advantages over the previous two. The foremost is the

possibility of collecting a large number of BP measurements, since real BP is reflected more accurately by repeated measurements. In addition, ABPM allows the evaluation of BP profile over 24 hours and therefore can detect the variability in circadian rhythms and identify patients with abnormal patterns of nocturnal BP (Parati *et al.,* 2012). Furthermore, the findings of a previous study have shown that the use of 24-h ABPM allowed the diagnosis of twice as much hypertension than did clinical measurement and therefore ABPM might be of particular significance in the hypertension diagnosis of OSA patients (Baguet *et al.,* 2005). Moreover, there is strong evidence that the predictive value for major cardiovascular events is better with ABPM than with clinical measurement (Burr *et al.,* 2008). Another study supports the idea that ABPM should be mandatory for the routine diagnosis of hypertension for those at risk for masked hypertension (Phillips *et al.,* 2012). This recognized cardiovascular entity is closely associated with OSA and adverse outcomes (Baguet *et al.,* 2008). In brief, due to the specific features of OSA-related hypertension, ABPM seems to be the preferred method for BP measurement in patients suspected of having OSA.

Although ABPM should ideally be performed in all patients with suspected OSA, this type of BP measurement is time- and labour-intensive as well as an expensive diagnostic tool and consequently is not routinely used. Given these limitations, we tried to find an alternative tool that would allow the identification of patients that misclassify themselves as non-hypertensive. Since anthropometric measurements (BMI, NC and WC) have been shown to predict OSA (Davies *et al.,* 1992; Proimos *et al.,* 2012) and other cardiovascular diseases (Preis *et al.,* 2010; Vallianou *et al.,* 2013), we decided to investigate the ability of these features to predict the misclassification of hypertension.

Our results showed that patients with undiagnosed hypertension and a BMI above 27 Kg/m^2 and an NC higher than 39 cm had three-fold and two-fold higher odds, respectively, of misclassifying themselves as non-hypertensive than patients with a lower BMI or AHI. The optimal BMI cut-off point that was found to predict the misclassification of hypertension was lower than the recommended cut-off point to predict OSA (30 Kg/m^2) (Friedman *et al.,* 1999) and in the range of that recommended for global cardiovascular risk (25–29.9 Kg/m^2) (Expert Panel on the Identification, Evaluation, and Treatment of Overweight in Adults, 1998). Concerning NC, the same cut-off point was found at 39 cm for both genders, and this value was similar to the ones described for OSA prediction and cardiovascular disease risk (Preis *et al.,* 2010; Soylu *et al.,* 2012).

Additionally, we performed a real life study in order to investigate a hypothetical association between ongoing antihypertensive regimens and BP control rates in patients with OSA, before and after CPAP adaptation.

In general, our sample matches a typical population of OSA patients. Actually, the high prevalence of new diagnoses of OSA, indicating that patients are being well referred, as well as the distribution of OSA severity found in CHLN sleep consultation, is similar to those reported by other authors (Guralnick *et al.,* 2012; Lavie *et al.,* 2000). The anthropometric baseline characteristics that were

found are in line with those that have been previously described in the literature (Friedman *et al.*, 1999). Additionally, these patients tend to present a higher number of comorbidities, mostly a high prevalence of hypertension, when compared to non-OSA patients (Fletcher *et al.*, 1985; Lavie *et al.*, 2000; Marin *et al.*, 2012). The prevalence of hypertension among patients with OSA is estimated to be around 50% (Calhoun, 2010). Our study revealed a higher prevalence of hypertension in OSA patients, probably due to the use of ABPM for the diagnosis of this disease. In addition to the high prevalence of hypertension, ABPM data revealed that, regarding 24h-BP profile, OSA patients showed a non-dipper systolic profile, which has already been reported (Davies *et al.*, 2000; Hla *et al.*, 2008). The high prevalence of uncontrolled BP (70.7%; 145/205) and underdiagnosed hypertension (24.4%; 50/205) among these patients has also been similarly reported (Börgel *et al.*, 2000; Baguet *et al.*, 2009; Lavie and Hoffstein, 2001). In a past study that included 59 patients who were not known to be hypertensive, hypertension was found in 42% of patients using clinical measurement and 76% using ABPM (Baguet *et al.*, 2005).

As far as we know, this is the first study to describe the pattern of AH medication in OSA patients. The high number of different AH regimens found (n=31) is symptomatic of the difficulty in controlling BP in these patients, and of the lack of consensus and guidelines in this matter, and also underlines the importance of the main question addressed in the present work. The number of patients with regimens that included two or more AH drugs is impressive, and emphasises the difficulty in achieving BP control and the close relationship between OSA and resistant hypertension (Pedrosa *et al.*, 2011; Pimenta *et al.*, 2013).

Despite the huge number of studies involving OSA patients, only a few have investigated the efficacy of different AHD and, in general, are individual drug studies. Most of them have focused on evaluating the lowering-BP effect of AHD and did not assess whether this decrease leads to an augmentation of the number of controlled hypertensive patients. Moreover, the validity of some of these results is limited due to the very low casuistry of the samples and by the incapacity to identify the chronicity of OSA and hypertension in each patient. This can interfere with the drug efficacy, assuming that the mechanisms involved in hypertension induced by OSA might be time-dependent. For instance, it has been proposed that IH is able to induce a renal hormetic response, i.e.: short-term IH induces a protective response against the renal oxidative damage, but long-term IH exposure induces a damage effect on the kidney (Sun *et al.*, 2013). Nevertheless, their findings allow some conclusions. First, drugs that block the sympathetic stimulation of β1-adrenergic receptors seem to be the most effective AHD, since the β1-specific antagonist atenolol was the most effective AHD tested (in comparison with isradipine, hydrochlorothiazide, spirapril, amlodipine, enalapril and losartan) in two comparative studies (Kraiczi *et al.*, 2000; Pelttari *et al.*, 1998), and nebivolol (Heitmann *et al.*, 2010) was also effective in patients with OSA. On the other hand, the angiotensin-converting enzyme inhibitors (e.g. cilazapril and enalapril) lowered BP effectively (Zou *et al.*, 2010), but not as

significantly as beta-blockers (Kraiczi *et al.,* 2000; Mayer *et al.,* 1990). The angiotensin II receptor blocker valsartan had a similar anti-hypertensive effect to nebivolol (Heitmann *et al.,* 2010). Spironolactone (competitive aldosterone antagonist) has been remarkably effective in treating resistant hypertension (Pimenta *et al.,* 2008); however, its effectiveness has not been evaluated in patients with OSA to date. Moreover, since only severe OSA has been associated with increased angiotensin II and aldosterone (moderate OSA was usually associated with normal levels of these agonists), rennin-angotensin–aldosterone (RAA) blockers had a modest anti-hypertensive effect in mild/moderate OSA (Ziegler *et al.,* 2011). Finally, thiazide diuretics have not been very effective in OSA patients without fluid retention (Kraiczi *et al.,* 2000). Also, important data are missing, e.g. how many OSA patients are controlled under monotherapy with beta-blockers (BB), angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARB's)? How many OSA patients remained uncontrolled despite the use of two or more AH drugs? What should the second drug added to BB or RAA blockers be? How do individual AHD behave when included in an AH regimen? In addition, the impact of these studies in clinical practice is unknown, as epidemiological studies designed to investigate the AH medication profile in OSA patients are missing. In addition, the more recent recommendations for the management of patients with OSA and hypertension are inconclusive regarding the use of AHD and recognise the lack of strong evidence for the establishment of a first-line AH regimen for these patients (Parati *et al.,* 2013). Other authors sustain the idea that since there is no clear evidence for preference for a specific class of AHD, the selection should primarily be guided by the patient's cardiometabolic profile and associated comorbidities (e.g. obesity, metabolic syndrome, diabetes mellitus and cardiovascular diseases) (Tsioufis *et al.,* 2010). Moreover, these authors recommend that, due to the lack of relevant trials focused on the use of associations of AHD in OSA patients, the choice should rely on current hypertension guidelines and the adverse effects of AHD need to be contemplated as well (Tsioufis *et al.,* 2010). Our study was designed assuming that clinicians based their therapeutic decisions on these assumptions.

Surprisingly, in our population, the most commonly prescribed AHD were diuretics and RAA blockers. However, the number of OSA patients using these AHD with uncontrolled BP was significantly higher. In fact, 61.2% (30/49) and 61.3% (65/106) of patients under a monotherapy and polytherapy regimens, respectively, were uncontrolled. This evidence suggests that the increase in the number of antihypertensive drugs is not apparently relevant in OSA patients. If the low efficacy of polytherapy in controlling BP was linked to OSA severity, the difference between the efficacy of monotherapy and polytherapy in severe patients would be higher in the subgroup of severe patients with BMI $\geq$30 kg/m^2, which was not the case.

On the other hand, before CPAP application, no associations were found between AH regimens and number of AHD and BP control. These findings suggest that none of the current AHD, either alone or in association, have been shown to be effective enough to control BP. Additionally, the number of

AHD included in a specific regimen seems not to be relevant to the control of BP in patients with OSA.

In the study performed after CPAP adaptation, we decided to include only patients who were adherent with CPAP therapy for at least 4 hours *per night* on average, as this is a threshold that is often used to define minimum acceptable CPAP use. According to the suggestion of a past study, patients in these conditions of CPAP use had an appreciable decrease in the incidence of hypertension (Barbé *et al.,* 2012). Additionally, the beneficial effect of nasal CPAP therapy on BP seems to be closely associated with the presence of untreated or resistant hypertension, with OSA severity (more pronounced in severe OSA) and with patient compliance to CPAP (Baguet *et al.,* 2009). Therefore, it would be expected that the combined effect of CPAP and AHD on BP control was more pronounced than the isolated effect of AHD. However, the lack of association between AH regimens and BP control remained after CPAP adaptation, which suggests the slight effect of CPAP in controlling BP. These findings are consistent with the results of several past studies (Alajmi *et al.*, 2007; Calhoun, 2010; Ziegler *et al.,* 2011). Börgel *et al.* (2004) revealed that the absence of antihypertensive drugs is an independent predictor for the lowering effect of CPAP therapy on systolic and diastolic BP (Börgel *et al.,* 2004). Furthermore, Dernaika *et al.* (2009) reported that the effects of CPAP therapy on BP regulation appear to be less evident in hypertensive patients under a drug regimen (Dernaika *et al.,* 2009). In our case, eight of the sixteen patients that were on no AH medication switched from uncontrolled to controlled after CPAP adaptation. Both results suggest that an earlier diagnosis of hypertension related to OSA is more important than the AH regimen selection.

Moreover, the effect of CPAP on lowering BP was clearly more significant in nocturnal BP, mainly in nocturnal SBP. Anyway, however important, this effect was not enough to reach the intended purpose for these patients, since it did not allow the reclassification from uncontrolled to controlled BP. In fact, despite some authors (Somers *et al.,* 1995) having reported the beneficial effect of CPAP in preventing the bursts of sympathetic activity present at the end of each respiratory event, in our case, the hypothetical reduction in sympathetic activity was not associated with an appreciable decrease in diurnal BP. Consequently, in patients with OSA, more important than studying the effect of CPAP alone on BP reduction is evaluating the combined effect of CPAP and AH medication. The study performed by Dernaika *et al.* (2009) evaluated the long-term effects of CPAP therapy on BP in patients with OSA and resistant hypertension (Dernaika *et al.,* 2009). The results revealed that CPAP permitted the de-escalation of antihypertensive treatment in 71% of patients with resistant hypertension, but did not significantly alter the antihypertensive regimen in the controlled group. However, in our opinion, the study of Dernaika *et al.* (Dernaika *et al.,* 2009) presents two main drawbacks. First, the study did not characterise the different AH associations (it only referred to the number of patients that were undergoing a specific AHD class) and second, it did not evaluate the efficacy of each class on BP control. The same limitations were found in the study of Börgel *et al.*

(2004), which was performed to evaluate the interaction between BP-lowering effects of CPAP and AHD (Börgel *et al.,* 2004). Additionally, Pépin *et al.* (2010) designed a study to assess the respective efficacy of CPAP and valsartan (ARBs) in reducing BP hypertensive patients with OSA that had never been treated for either condition and speculated that the combined effect of both therapies on BP control might be additive in patients in whom hypertension is still uncontrolled by specific AH drugs (Pépin *et al.,* 2010). However, they did not quantify the effect of the association; this study showed that valsartan induced a four-fold higher decrease in mean 24h BP than CPAP (Pépin *et al.,* 2010). Thus, the results of our pilot study revealed, for the first time, that none of the current AH regimens are superior to the others, either alone or when combined with CPAP, in promoting BP control, and underlines that new AHD drugs for this particular population are definitely needed.

Finally, gender, OSA severity and 24 h BP profile at baseline were identified as independent predictors of BP control after CPAP adaptation. Our results show that male patients with an AHI higher than 15 events/hour of sleep and patients with a non-dipper profile had a three-fold increased odds of presenting uncontrolled BP after CPAP adaption. Although Robinson *et al.* (2008) did not identify OSA severity as a predictor of the fall in 24h mean BP with CPAP treatment; the impact of this decrease in BP control remains unknown (Robinson *et al.,* 2008). In a more recent small study, male gender, Epworth sleepiness scale, BMI, smoking, alcohol use and baseline 24h mean BP were identified as independent predictors of a decrease in 24h mean BP after CPAP adaptation (Yorgun *et al.,* 2013). The apparent controversy of whether or not OSA severity is a predictor of the beneficial effect of CPAP in lowering BP could be explained by the differences in the outcome variable analysed. In our perspective, uncontrolled BP (present study) is a stronger and more relevant clinical outcome than the decrease in 24h-mean BP values.

EXPERIMENTAL STUDIES

The animal model used to investigate the efficacy of carvedilol in reversing hypertension induced by CIH mimics the intermittent hypoxic episodes that occur chronically in patients suffering from OSA. Obesity is a major cause of airway obstruction in these patients but it is also postulated that OSA can trigger reflex mechanisms that favour the development of metabolic syndrome in these patients (Mannarino *et al.,* 2012). Our model allows us to separate the mechanical component of obstruction from the effect of intermittent hypoxia itself. Several authors have reported weight loss in CIH-exposed rats when compared with control rats (Silva and Schreihofer *et al.,* 2011; Soukhova-O'Hare *et al.,* 2008; Totoson *et al.,* 2013; Zoccal *et al.,* 2008). Singh and Sevamurthy (1993) suggested that the weight loss observed after CIH exposure is due to a reduction in food intake (Singh and Sevamurthy, 1993). More recently, other authors measured daily food intake and reported that the reduced body weight gain in CIH rats was not associated with reduced food consumption but related to a significantly higher metabolism and energy expenditure (Fenik *et al.,* 2012). In addition to reporting

the weight loss, our results showed, for the first time, that CIH induces a deviation in the weight changes observed in age-matched Wistar rats, leading to a significant retardation of body growth. This finding suggests that intermittent hypoxia by itself does not contribute to maintaining an overweight status in obese patients with OSA. The effect of CIH on body weight deserves further study in obese animals.

CIH is now established as the dominant model of sleep apnea. Generally, this model makes use of specific ventilated chambers in which the animals are housed and cyclically exposed either to normoxia/hypoxia or room air to mimic the most relevant consequences of OSA (Diogo and Monteiro, 2014). In our protocol, rats were housed in cages placed inside medium A-chambers that were large enough to prevent air jet stress-induced changes in cardiovascular parameters and also to allow the rats to be housed in groups during the extended period of CIH exposure in order to decrease stress levels. Our paradigm of CIH in rats roughly corresponds to a sleep apnea lasting for more than two years in humans, which matches the time of OSA diagnosis in clinical practice. In humans, an apnea-hypopnea index (AHI) bellow 5 events/hour of sleep is under the physiological range (Berry *et al.*, 2012). A paradigm of 5.6 cycles/hour of exposure to CIH in rats, even with correction for higher metabolic activity in animals (Germack *et al.*, 2002), is below the AHI cut-offs for moderate OSA (>15 events/hour of sleep).

Animal models of CIH diverge in some aspects, such as the animal species involved, the severity of hypoxia, the number of hypoxic episodes per hour of sleep, the number of days of hypoxic exposure (exposure duration), and CO_2 manipulation (for a review see Diogo and Monteiro, 2014). Anyway they are unanimous in reporting the development of mild hypertension (Allahdadi *et al.*, 2005; Bathina *et al.*, 2013; Campen *et al.*, 2005; Chen *et al.*, 2005; Dyavanapalli *et al.*, 2014; Fletcher *et al.*, 2000; Kanagy *et al.*, 2001; Knight *et al.*, 2011; Lai *et al.*, 2006; Lin *et al.*, 2007; Liu *et al.*, 2013; Sharpe *et al.*, 2013; Silva and Schreihofer, 2011; Schulz *et al.*, 2014; Soukhova-O'Hare *et al.*, 2008; Tahawi *et al.*, 2001; Totoson *et al.*, 2013; Zoccal *et al.*, 2009). In agreement with these reports, CIH significantly increased mean arterial pressure, diastolic and systolic BP in our animal model, highlighting once again the major role of CIH in the development of hypertension.

The effects of CIH on HR result from a balance between sympathetic nervous system overactivation and baroreflex control. Our results are in line with previous studies that described no changes in HR (Dyavanapalli *et al.*, 2014; Knight *et al.*, 2010; Soukhova-O'Hare *et al.*, 2008; Yamamoto *et al.*, 2013; Zoccal *et al.*, 2009) and match the lower rate of variability that has been described for patients with OSA (Trimer *et al.*, 2014). Apparently, CIH does not impair the baroreflex control of HR. Increases in HR were only observed in a study performed with anesthetised rats exposed to CIH for 90 days (Lin *et al.*, 2007). Additionally, in humans, baroreflex control of HR was reported to be lower in obese than non-obese awake humans and improved after weight reduction (Grassi *et al.*, 1998). Since our animal model is lean this is also consistent with the absence of HR changes.

After validation of our rat model of hypertension related to OSA, we decided to test CVD, since sympathetic activation and oxidative stress play an important role in the pathophysiology of hypertension related to OSA. Furthermore, beta-blockers have not yet been tested in this animal model and in limited clinical studies they proved to be helpful to reduce BP in OSA patients. Studies investigating the antihypertensive effect of drugs on animal models are scarce and drugs have been used solely as pharmacological tools to address physiological mechanisms (for a review see, for example, Diogo and Monteiro, 2014). The experiments evaluate prevention but not the effectiveness of treatment. Other limitations of the pharmacological approaches included in these works are the absence of dose-response curves and comparison of the effectiveness of different drugs in the same animal model. Aware of these drawbacks, our study was planned to first induce hypertension and then evaluate the efficacy of cumulative doses of CVD because the translation of the results obtained with simultaneous induction of hypertension and drug administration to humans is not relevant.

The doses of CVD tested (10, 30 and 50 mg/kg/day) were selected based on previous studies performed in different models of hypertension (Bertera *et al.*, 2012a; Bertera *et al.*, 2012b; Chen *et al.*, 2013; Di Verniero *et al.*, 2010; Rodriguez-Perez *et al.*, 1997). The lack of efficacy of CVD cannot be attributed to the doses because they were effective in reducing HR in our model, in reducing BP in other models of HT, and are higher than the doses administered to humans (12.5-50 mg).

Carvedilol is a lipophilic third generation β-blocking agent and it is assumed that it easily crosses the blood-brain barrier. Several advantages have been claimed for third generation agents over the traditional β-blockers. First, drugs such as CVD exhibit a broad range of adrenergic inhibition, blocking postsynaptic β1, β2 and α1 receptors and presynaptic β2 receptors (DiNicolantonio and Hackam, 2012). Secondly, unlike traditional agents, CVD promotes vasodilatation through different mechanisms, which translates into a more favourable hemodynamic profile compared to non-vasodilating beta-blockers (Rath *et al.*, 2012). In addition to its alpha-adrenergic receptor blocking properties, it has been suggested that CVD-induced increased nitric oxide (NO) plasma levels (Afonso *et al.*, 2006; Vanhoutte and Gao, 2013) and antioxidant properties (Rath *et al.*, 2012; Feuerstein and Ruffolo, 1995) could also contribute to the vasorelaxation and subsequent reduction in vascular resistance evoked by CVD. CVD is also noted as a suppressor of the synthesis of endothelin-1 (ET-1) (Saijonmaa *et al.*, 1997), as a drug with antiproliferative actions (Chen *et al.*, 2001) and with beneficial effects on vascular and cardiac structural remodelling (Bakris, 2009; Chen *et al.*, 2013), endothelial dysfunction (Feuerstein and Ruffolo, 1995) and progression of target organ damage (Bakris, 2009). Finally, CVD also has a calcium channel blocking effect by inhibiting voltage-dependent L-type Ca2+ in vascular smooth muscle cells (Nakajima *et al.*, 2003), inhibits the rennin-angiotensin system (Cheng *et al.*, 2001) by blocking β1 receptors and enhances the plasma concentration of atrial natriuretic peptide (Cheng *et al.*, 2001).

Since these pleiotropic effects of CVD match the pathophysiological mechanisms described for CIH

(for a review see Diogo & Monteiro, 2014), the lack of efficacy of carvedilol in reversing hypertension induced by CIH was unexpected.

The finding that the three doses of CVD promoted a significant decrease in HR confirmed that the lack of antihypertensive efficacy couldn't be attributed to the absence of beta-blocking activity.

Another explanation could be that CIH might change the pharmacokinetic profile of CVD, compromising their antihypertensive efficacy. Carvedilol is clinically administered as a racemic mixture in which nonselective beta-adrenoreceptor blocking activity is present in the S-(-) enantiomer and alpha-adrenergic blocking activity is present in both R-(+) and S-(-) enantiomers at equal potency (Peccinini *et al.,* 2008). A decrease in HR without no effects on BP might thus be explained by a selective increase in the levels of S-(-)-CVD. There is some evidence that hypoxic conditions can broadly change drug clearance (Chen *et al.,* 2013; Gao *et al.,* 2013; Vij *et al.,* 2012) or drug effects (Nunes *et al.,* 2010).

In the present work, we found, for the first time, that CIH changes CVD pharmacokinetics by mechanisms that deserve further exploration, however, the lower ratio S/(R+S) observed in rats submitted to CIH could not easily explain the lack of hypotensive effects.

Another putative explanation for the lack of efficacy of carvedilol is that the blockade of the sympathetic nervous system, along with all the pleiotropic properties of CVD, might not be enough to reverse hypertension induced by CIH. From a broad perspective, we can speculate that the majority of CVD effects were produced in experiments that planned to address only one variable involved in the pathophysiology of hypertension, and some in acute conditions. Few studies of hypertension with the chronic administration of CVD have been performed in rat models. In a model of hypertension induced by unilateral renal artery narrowing (Chen *et al.,* 2013), CVD (20 mg/kg/day) administered by gavage for 8 weeks, causes a significant and sustained reduction of SBP apparent in the first two weeks of treatment, but it was not able to completely reverse the hypertensive effect of the surgical procedure. In hypertension induced by renal ablation (5/6), only rats receiving doses of 10 and 20 mg/Kg/day of CVD (but not of 5 mg/Kg/day) exhibited significant decreases in SBP apparent at week five of treatment (Rodriguez-Perez *et al.,* 1997). These findings, together with the lack of efficacy of CVD found in our animal model, support the theory that mechanisms other than this type of renal damage are involved in hypertension induced by CIH.

Finally, it has been reported that CIH leads to an increased stiffness and reduced vessel wall distensibility (Phillips *et al.,* 2006) and induces significant renal inflammation and fibrosis (Sun *et al.,* 2013). We can therefore postulate that CIH induces structural changes in vessels, renal tubules, etc., that are not targeted by CVD.

CVD was daily administered by *gavage*, since in the study that we simultaneously conducted in order to investigate an alternative method to *gavage* (oral voluntary administration) for chronic administration of AHDs (*e.g.* carvedilol and losartan), the animals systematically refused to ingest the

mixture of carvedilol with the three vehicles tested. This behaviour was probably due to the fact that CVD may leave a residual unpleasant odour or flavour that discourages voluntary consumption. The time between delivery and small amounts consumption was always superior to 1 hour, and even when the vehicle-CVD mixtures were left overnight the animals failed to completely eat them. Despite gavage is the most widely used method for the precise oral dosing of rodents, it can elicit a stress response, and it has been shown that any source of external stress on rodents can significantly increase heart rate and blood pressure, effects that persist for around 3 hours (Brown *et al.,* 2000; Bonnichsen *et al.,* 2005; Kramer *et al.,* 2000; Balcombe *et al.,* 2004). Aware of this fact we administered CVD only after monitoring BP parameters, and the measurements were made on the day after administration and before exposure to IH conditions.

In the study designed to test the viability of oral voluntary administration for long-term delivery of CVD and losartan (angiotensin II receptor blocker), male Wistar rats, aged 2-3 months and housed individually were used to avoid hypothetical effects of estrogen on blood lipids (Ganong, 2002). Although Sharp and colleagues recommend that animals should be ideally housed in group to decrease the stress levels (Sharp *et al.,* 2002), in our study, animals were single-housed during all protocol in order to ensure correct dosage and to confirm vehicle or vehicle plus drug complete ingestion. The palatability of NUT, PB and SD showed to be attractive for the animals, since all animals ingested them almost immediately and completely. Furthermore, the consistency of these vehicles facilitates the weighing and the mixing of powder, thereby ensuring the accuracy of the drug dose. The amount of vehicle given to the animals (0.5g) was shown to be enough to hold the weighed drug, whereas still appeared attractive for the animals and not too large to cause significant metabolic changes. Offering the mixture in a Petri dish placed vertically inside the cage proved to be an effective solution to ensure complete ingestion. Before it has been reported that placing NUT in a container on the floor of the cage is not recommended, since neophobic animals tend to burry the novel item, and thus mix the vehicle with the bedding material (Abelson *et al.,* 2012).

Although some authors have recommended a period of up to five days for animal acclimatization to the vehicles (Isaksson *et al.,* 2011), in our study, two days proved to be enough time to ensure that there was no neophobic behaviour after that. In order to reduce the stress levels associated with handling and restrained we decided to use a one-week period of acclimatization in the *gavage* group. Oral *gavage* has been recently refined from metal *gavage* needle to a sterile flexible feeding tube which reduce the risk of trauma, perforation and cross contamination (Morton *et al.,* 2001). However, the stress related to orogastric administration is not overcome by this approach.

Unlike what it was observed for carvedilol, all rats successfully ingested losartan mixed with either NUT, PB or SD and therefore we only completed the study for losartan.

Losartan is the first orally available angiotensin-receptor antagonist without agonist properties (Sica *et al.*, 2005). Following oral administration, losartan is rapidly absorbed, reaching maximum concentrations 1–2 hours post-administration (Sica *et al.*, 2005). This drug is freely soluble in water and the reported bioavailability of losartan with a 50 mg tablet is 32.6% (Sica *et al.*, 2005). Dose administration with meals slows the rate of absorption and reduces the area under the plasma concentration-time curve (AUC) of losartan and its metabolite by approximately 10% (Simpson and McClellan, 2000). Food can have a significant effect on drug absorption through several mechanisms, including delay in gastric emptying, increases in splanchnic blood flow, and changes in gastrointestinal secretions. These effects can alter tablet disintegration, drug dissolution, and drug transit through the gastrointestinal tract. Facing this, we decided to offer the vehicle- drug mixtures at the first hours of light in order to ensure that drug ingestion took place in a period with lower regular food intake. The vehicle amount used did not show to interfere with losartan bioavailability.

According to Chan *et al*. (2012), blood sampling site can strongly influence a substantial number of routinely tested blood parameters such as glucose, TC, TGL and HDL-C and highlight the need to standardize sampling sites, especially when repeated blood sampling is required (Chan *et al.*, 2012). To avoid this untoward experimental bias, only blood collected from the tail vein, in three different days, was used for measurement of blood glucose level and serum lipid profile. Accordingly, no differences were observed among groups of animals when comparing basal levels, i.e., prior to *gavage* or vehicle administration. Moreover, previous studies in the literature showed that there are no significant age-related changes in glycemia (Ribeiro *et al.*, 2008) or lipid profiles (Uchida *et al.*, 1978) throughout the time period of our study. Therefore, any changes observed between different vehicles are caused by differences in the vehicles composition and not age-related.

Analysis of the results suggested that NUT, PB and SD are possible alternatives to *gavage* for chronic administration of losartan, since their ingestion was not associated with weight gain or increased water ingestion and were all fully eaten by the animals. However, taken into account the glycaemia and lipid profile, SD proved to be the most suitable vehicle for the administration of this antihypertensive drug for two main reasons. First, the mean plasma concentration of losartan was significantly higher than the one attained when *gavage* was used. Secondly, SD didn't induce any significant change on both glycaemia and lipid profile along the 28 days. On the other hand, the use of PB seems to be the least adequate due to the changes on the rat's lipid profile associated with a 22% reduction of losartan plasmatic concentration. Even though the NUT group showed a glycaemia reduction and increased total cholesterol, the losartan plasma concentration was 29% higher than *gavage*. These results could suggest that SD maximizes losartan absorption, leading to a higher bioavailability.

In the present study, only Cmax concentrations were measured. It must be highlighted that losartan has an extensive first-pass effect with an oral bioavailability of 32%, showing that transcelullar intestinal

absorption (P-glycoprotein) and biotransformation (CYP3A4) clearly impacts its bioavailability and allows drug-nutrients interactions.

In our opinion, the higher losartan concentrations attained in sugar dough group, compared with *gavage*, might be explained by three different mechanisms: an absorption delay, an increased absorption or a lower biotransformation. In fact, the high content of sugar present in sugar dough might increase the rate and/or extent of drug absorption since it has been described that sucrose fatty acid esters might improve the intestinal absorption of poorly absorbable drugs via a transcellular and a paracellular pathways (Yamamoto *et al.*, 2014). Additionally, it has been suggested that high carbohydrate content may lower CYP450 activity, particularly when high doses are administered (Sonawane *et al.*, 1988).

Substances that leave an unpleasant odour or flavour could discouraged voluntary ingestion and compromised the success of this method. Thus, we suggest that a pilot study should be carried out in order to ensure that all rats would successfully ingest the drug-vehicle mixture.

What are the main limitations?

CLINICAL STUDIES

We are conscious about some limitations of our clinical studies. Our sample should ideally be larger and provided from more than a single centre, in order to avoid referral bias. However, our centre could be considered representative of the general OSA population because we found similar characteristics to those described in the literature. Another limitation of the study was the low casuistry of women in our sample. However, this unbalance was expected since OSA is more prevalent among males. Anyway, gender was considered during the statistical analysis. The huge variability in AHD regimen found in the present work together with the sample size, does not allow us to identify the best AHD regimen for these patients. Additionally, the results of this exploratory study strongly suggest that none of the available AHD are clearly able to achieve adequate BP control in OSA patients. Therefore, the esults of a much larger epidemiological study could be also frustrating and, in our opinion, the search for new therapeutic strategies will be more useful.

Additionally, despite the use of a “Brown-Bag” medication review in order to record patients ongoing medication profile, data regarding adherence to AH drug treatment (before and after inclusion) were checked only based on self-reported adherence and were not confirmed by a “pill-count” method or other. However, this variable does not invalidate the study, since it is common to all patients. Since the differences between efficacies within each class are not well documented, the evaluation of each drug per se was not considered relevant. Therefore, the analysis was conducted taking into account the regimens of AH drugs with higher prevalence. Nevertheless, the added value of the present study is the evaluation of the efficacy of AH regimens commonly used in these patients instead of analysing the effect of a specific AHD. Despite these drawbacks, our results provide new insights into the need

for innovative AHD to treat OSA patients.

EXPERIMENTAL STUDIES

Regarding the experimental studies, one of the limitations that can be pointed out is the different number of animals that was allocated to each experimental group. In the first study, due to the limited number of age-matched animals available, we decided to allocate more animals to the group of CVD 50 mg/kg (higher dose tested). The major arguments for this decision were: first, this group would be used for the pharmacokinetic study and, second, we had already noted the limited impact of the lower doses of CVD on BP. Furthermore, the use of only one antihypertensive drug, the absence, at the moment, of mechanistic data (we collected tissue samples for further analysis) to explain the negative results, along with the lack of catecholamines quantification and assessment of sympathetic activity can also be pointed as additional limitations.

In the second study, once again, the number of animals dictated that one of the groups would have one less animal. Considering that the most studied and more invasive method is the *gavage*, we opted to allow this group to have only 5 animals. Nevertheless, in both studies, the standard error of the mean (SEM) was acceptably low in all groups, suggesting that the number of animals was also acceptable and statistical analysis would not be compromised. For these reasons and in light of the 3Rs approach, it is our opinion that additional animals would not be required to draw conclusions from both studies.

Additionally, another drawback of the second study may have been the evaluation of single-point drug concentrations (Cmax) instead of using the area under the curve (AUC) approach. However, our HPLC method was validated for a volume of 200 μL of plasma, analyzed in duplicate. Thus tail puncture sampling for AUC achievement at the time point of 7 days would require the collection of at least 1 mL of blood that would represent approximately 6% of the total blood volume. Since such a volume would take one week to be recovered, we decided to perform only losartan quantification as a terminal single-point measure. The main argument for this decision was a simple attempt to not interfere with the losartan steady state and with major animal volemia during the experiments.

What is still unknown and should be addressed?

Theoretically, since OSA has been identified as an independent risk factor for hypertension, the first approach to manage HT related to OSA should be control the underlying condition *i.e.* avoid the repetitive episodes of apnea or hypopnea caused by an obstructed or collapsed upper airway during sleep. However, normalize the apnea-hypopnea index (AHI) is not an easy task for two main reasons. First, the cause of upper-airway collapse, as well as the etiology of OSA, is multifactorial. In fact, an appreciable number of factors is known to be linked to upper-airway collapse (*e.g.* reduced airway dilator muscle activity during sleep, upper-airway anatomy, obesity, decreased end-expiratory lung volume, ventilatory control instability, and rostral fluid shifts). Secondly, to date, the results of clinical trials designed to assess the effectiveness of several drugs (e.g. noradrenergic, serotoninergic and

nicotinic agents) in treating the obstruction associated to OSA have been disappointing (Veasey *et al.*, 2006). These pharmacological agents have not been successful in reducing significantly the AHI in patients with OSA (Veasey *et al.*, 2006). Furthermore, although CPAP is considered the gold standard treatment for OSA due to its ability in providing pneumatic splitting of the upper airway and effectiveness in reducing the apnea-hypopnea index, CPAP effect seems to be not enough to sustain by itself BP control in patients with OSA. Besides that, CPAP presents other disadvantages (e.g. CPAP device needs to be used every and complains of nightmask discomfort, nasal congestion, and nose and throat dryness) which are able to compromise patients compliance.

Consequently, there is consensus that HT related to OSA is gaining more relevance as an independent nosological condition that needs a systematic approach to identify the best therapeutic strategy. One major challenge is gaining an understanding of whether the blockade of the reflex pathways triggered by carotid body activation is sufficiently effective to control BP in itself in HT related to OSA. Eventually, other pathways directly stimulated by hypoxia at a cellular level should be explored in depth and manipulated to attain relevant clinical control of these patients.

Moreover, the use of some new non-pharmacological approaches should also be considered for the management of HT related to OSA. Taken in perspective, the pathophysiology of this type of HT, which involves an increase in sympathetic activity, renal denervation seems to be a logical approach since the signalling between the kidneys and the central sympathetic nervous system is bidirectional and occurs through the renal afferent and efferent nerves (Böhm *et al.*, 2013). Indeed, accumulating evidence indicates that the kidneys act both as a generator and a recipient of sympathetic activity: sympathetic afferents travel from the kidneys to the central nervous system and promote sympathetic nervous system overactivity in response to renal injury and, vice versa, efferent sympathetic nerves travel to the kidneys (Faselis *et al.*, 2014). Moreover, sympathetic overactivity results in enhanced renin release, increased sodium and water reabsorption, and reduced renal blood flow and glomerular filtration rate (Faselis *et al.*, 2014).

The beneficial role of renal denervation in the management of resistant HT and other cardiovascular diseases has been reported and reviewed extensively (Böhm *et al.*, 2013; Faselis *et al.*, 2014; Grassi *et al.*, 2012; Pimenta and Oparil, 2012; Tsioufis *et al.*, 2014; Ukena *et al.*, 2013; Urban *et al.*, 2013). Moreover, Shantha and Pancholy (Shantha and Pancholy, 2014) have recently undertaken a systematic review of the effect of renal sympathetic denervation on AHI in patients with OSA. Curiously, they concluded that this approach is associated with a significant reduction in mean AHI (Shantha and Pancholy, 2014). However, as the authors pointed out, these results need further validation due to the low causal basis of the studies included in the analysis and due to the fact that only one of these studies was performed fully in a specific population of OSA patients; in the remaining studies, the diagnosis of OSA was only established after inclusion (Shantha and Pancholy, 2014). In a recent pilot study, the effect of renal denervation on BP control in patients with OSA was explored (Witkowski *et*

al., 2011). Despite the low causal basis (n=10), their findings demonstrated a significant BP decrease within three months, which was further enhanced at six months, exhibiting a drop pattern similar to clinical studies in resistant HT (Witkowski *et al.*, 2011). Nontheless, further studies are needed to support this impressive effect of renal denervation and to ensure the safety of this technique for patients with HT related to OSA.

Like renal denervation, carotid baroreceptor stimulation has also been proposed as a novel antihypertensive therapy based on the recent evidence that baroreceptors might play an important role even in long-term BP regulation (Grassi *et al.*, 2012; Lovic *et al.*, 2014; Papademetriou *et al.*, 2011). The main similarities and differences between these two novel approaches have been reviewed extensively by a group of Italian researchers (Grassi *et al.*, 2014; Seravalle *et al.*, 2014). Although electrical baroreflex stimulation appears to be safe and effective, and might represent a useful tool for managing resistant HT (Lovic *et al.*, 2014), to the best of our knowledge, the effectiveness of this approach has not been yet tested in models of IH. Thus, further investigation in this specific field would be welcome.

In line with the pioneer study performed by Fletcher *et al.* (1992c) that established that carotid body (CB) ablation eliminated the hypertension related to CIH, McBryde *et al*. (2013) have shown that CB deafferentation, through bilateral carotid sinus nerve denervation, promotes an effective and lasting AH response in SHR and reduces the overactive sympathetic activity. They have also demonstrated that associated with renal denervation, carotid sinus nerve denervation remains effective and produces a cumulative response (McBryde *et al.*, 2013). In line with these findings, they propose carotid sinus nerve denervation as an effective AH treatment in patients with sympathetically mediated diseases (McBryde *et al.*, 2013).

More recently, Burchell et al. have reviewed the potential of a new device for the control of arterial HT (Burchell *et al.*, 2014). The *ROX coupler* device creates an anastomosis between the iliac artery and vein, diverting a calibrated amount of arterial blood into the venous system, reducing vascular resistance and increasing arterial compliance (Burchell *et al.*, 2014). This non-pharmacological approach seems to be a promising tool in the management of patients with resistant HT due to its ability to provide an immediate and sustained reduction in BP (Burchell *et al.*, 2014). The safety and efficacy of the *ROX coupler* in the treatment of this type of HT is now being evaluated in a European multicentre randomized study (Burchell *et al.*, 2014). Positive results in patients with drug-resistant HT leave open the possibility of the use of the *ROX coupler* device becoming a new strategy for the management of HT related to OSA.

Furthermore, drugs that have proved to be useful in essential HT treatment should be tested promptly in studies specifically designed for secondary HT induced by CIH. On the other hand, given the particularities of HT related to OSA, the recourse to tailored treatments should be considered as a possibility. Furthermore, it also appears to be imperative to look for new AHDs able to reverse HT

quickly and effectively in patients with OSA as BP control is still not achievable in a significant proportion of these patients.

Finally, the contribution of animal models to this approach is unquestionable in terms of avoiding the confounding risk factors for HT that tend to be present in OSA patients. In addition, drugs that have been used as pharmacological tools to understand pathophysiological mechanisms should now be investigated regarding their efficacy in reversing HT induced by CIH.

What are the added value and the impact of the present work to the field?

To the best of our knowledge, this is the first work that described the pattern of AH medication and evaluated the hypothetical association between ongoing antihypertensive regimen and BP control rates, in patients with OSA. Our findings suggest, for the first time, that none of the currently available antihypertensive drugs, either alone or in association, shown to be effective enough to control BP in these patients, either before or after CPAP. Moreover, the increase in the number of AHD seems to be not relevant to BP control in patients with OSA. Our study was also the first to identify cut-off points based on anthropometric measurements that can help the discrimination between misclassified non-hypertensive patients and truly non-hypertensive patients. These findings may contribute for a more selective use of ABPM, a high-cost and time- consuming diagnostic tool and therefore reduce the prevalence of undiagnosed hypertension among patients suspected of obstructive sleep apnea.

Concerning the experimental studies, our results showed the lack of efficacy of carvedilol in reversing hypertension induced by CIH in our animal model and suggest that the blockade of sympathetic nervous system alone does not seem to be the best strategy for reversing established hypertension in sleep apnea conditions. Sympathetic overactivity may be an early step in the mechanism leading to more permanent effects due to prolonged sympathetic overactivity or through local effects of recurrent IH that are not reversed by drugs such as CVD. This study also pointed out that CIH induces pharmacokinetic changes in CVD although are not responsible for its lack of efficacy in reversing this particular type of hypertension. Together these findings open up new doors for future research to better understand the mechanisms underlying HT related to OSA and to seek novel therapeutic targets and strategies to manage this type of HT. At last, our results support that the use of voluntary oral administration is a viable alternative, to *gavage*, for chronic administration of a fixed dose of an AHD and also point to a welfare-based refinement for drug administration in laboratory rodents. This refinement might be of interest for safety evaluation of drugs, particularly for those which adverse effects are associated with stress.

VI

REFERENCES

Abelson KS, Jacobsen KR, Sundbom R, Kalliokoski O, Hau J. (2012). Voluntary ingestion of nut paste for administration of buprenorphine in rats and mice. Laboratory Animals 46, 349-51. doi: 10.1258/la.2012.012028.

Afonso RA, Patarrão RS, Macedo MP, Carmo MM. (2006). Carvedilol action is dependent on endogenous production of nitric oxide. Am J Hypertens. 19, 419-25.

Alajmi M, Mulgrew AT, Fox J, Davidson W, Schulzer M, Mak E, Ryan CF, Fleetham J, Choi P, Ayas NT (2007). Impact of continuous positive airway pressure therapy on blood pressure in patients with obstructive sleep apnea hypopnea: a meta-analysis of randomized controlled trials. Lung, 185: 67-72.

Allahdadi, K.J., Cherng, T.W., Pai, H., Silva, A.Q., Walker, B.R., Nelin, L.D., et al. (2008). Endothelin type A receptor antagonist normalizes blood pressure in rats exposed to eucapnic intermittent hypoxia. Am. J. Physiol. Heart Circ. Physiol. 295, H434-40. doi: 10.1152/ajpheart.91477.2007.

Allahdadi, K.J., Walker, B.R., Kanagy, N.L. (2005). Augmented endothelin vasoconstriction in intermittent hypoxia-induced hypertension. Hypertension 45, 705–709.

Almendros, I., Carreras, A., Ramírez, J., Montserrat, J.M., Navajas, D., Farré, R. (2008). Upper airway collapse and reopening induce inflammation in a sleep apnoea model. Eur. Respir. J. 32, 399-404. doi: 10.1183/09031936.00161607.

Almendros, I., Farré, R., Planas, A.M., Torres, M., Bonsignore, M.R., Navajas, D., et al. (2011). Tissue oxygenation in brain, muscle, and fat in a rat model of sleep apnea: differential effect of obstructive apneas and intermittent hypoxia. Sleep. 34, 1127-33. doi: 10.5665/SLEEP.1176.

Andersen, M.L., Martins, P.J., D'Almeida, V., Bignotto, M., Tufik, S. (2005). Endocrinological and catecholaminergic alterations during sleep deprivation and recovery in male rats. J. Sleep Res. 14, 83-90.

Andersen, M.L., Martins. P.J., D'Almeida. V., Santos. R.F., Bignotto. M., Tufik. S. (2004). Effects of paradoxical sleep deprivation on blood parameters associated with cardiovascular risk in aged rats. Exp. Gerontol. 39, 817-24.

Andrén, A., Hedberg, P., Walker-Engström, M.L., Wahlén, P., Tegelberg, A. (2013). Effects of treatment with oral appliance on 24-h blood pressure in patients with obstructive sleep apnea and hypertension: a randomized clinical trial. Sleep Breath. 17, 705-12. doi: 10.1007/s11325-012-0746-7.

Atcha Z, Rourke C, Neo AH, Goh CW, Lim JS, Aw CC, Browne ER, Pemberton DJ. (2010). Alternative method of oral dosing for rats. Journal of the American Association for Laboratory Animal Science 49, 335-43.

Badesch, D.B., Raskob, G.E., Elliott, C.G., Krichman, A.M., Farber, H.W., Frost, A.E., et al. (2010). Pulmonary arterial hypertension: baseline characteristics from the REVEAL Registry. Chest. 137, 376-87. doi: 10.1378/chest.09-1140.

Badran, M., Ayas, N., Laher, I. (2014). Insights into obstructive sleep apnea research. Sleep Med.15, 485-495. doi: 10.1016/j.sleep.2014.01.009.

Baguet JP, Hammer L, Lévy P, Pierre H, Rossini E, Mouret S, et al (2005). Night-time and diastolic hypertension are common and underestimated conditions in newly diagnosed apnoeic patients. J Hypertens. 23, 521-7.

Baguet JP, Lévy P, Barone-Rochette G, Tamisier R, Pierre H, Peeters M et al. (2008). Masked hypertension in obstructive sleep apnea syndrome. J Hypertens. 26, 885-892. doi: 10.1097/HJH.0b013e3282f55049.

Baguet JP, Barone-Rochette G, Pépin JL (2009). Hypertension and obstructive sleep apnoea syndrome: current perspectives. J. Hum. Hypertens. 23, 431-43. doi: 10.1038/jhh.2008.147.

Bakker, J.P., Edwards, B.A., Gautam, S.P., Montesi, S.B., Durán-Cantolla, J., Barandiarán, F.A., et al. (2014). Blood pressure improvement with continuous positive airway pressure is independent of obstructive sleep apnea severity. J. Clin. Sleep. Med. 10, 365-9. doi: 10.5664/jcsm.3604.

Bakris G. (2014). An in-depth analysis of vasodilation in the management of hypertension: focus on adrenergic blockade. J Cardiovasc Pharmacol. 53, 379-87. doi: 10.1097/FJC.0b013e31819fd501.

Balcombe, J.P., Barnard, N.D., Sandusky, C. (2004). Laboratory routines cause animal stress. Contemp. Top. Lab. Anim. Sci. 43, 42-51.

Bao, G., Randhawa, P.M., Fletcher, E.C. (1997). Acute blood pressure elevation during repetitive hypocapnic and eucapnic hypoxia in rats. J. Appl. Physiol. 82, 1071-8.

Barbé, F., Durán-Cantolla, J., Capote, F., de la Peña, M., Chiner, E., Masa, J.F., et al. (2009). Long-term effect of continuous positive airway pressure in hypertensive patients with sleep apnea. Am. J. Respir. Crit. Care Med. 181, 718-26. doi: 10.1164/rccm.200901-0050OC.

Barbé, F., Durán-Cantolla, J., Sánchez-de-la-Torre, M., Martínez-Alonso, M., Carmona, C., Barceló, A., et al. (2012). Effect of continuous positive airway pressure on the incidence of hypertension and cardiovascular events in non-sleepy patients with obstructive sleep apnea: a randomized controlled trial. JAMA. 307: 2161-8. doi: 10.1001/jama.2012.4366.

Barbé, F., Mayoralas, L.R., Duran, J., Masa, J.F., Maimó, A., Montserrat, J.M., et al. (2001). Treatment with continuous positive airway pressure is not effective in patients with sleep apnea but no daytime sleepiness. a randomized, controlled trial. Ann. Intern. Med. 134, 1015-23.

Bathina, C.S., Rajulapati, A., Franzke, M., Yamamoto, K., Cunningham, J.T., Mifflin S. (2013). Knockdown of tyrosine hydroxylase in the nucleus of the solitary tract reduces elevated blood pressure during chronic intermittent hypoxia. Am. J. Physiol. Regul. Integr. Comp. Physiol. 305, R1031-9. doi: 10.1152/ajpregu.00260.2013.

Bazzano LA, Khan Z, Reynolds K, He J: Effect of nocturnal nasal continuous positive airway pressure on blood pressure in obstructive sleep apnea. Hypertension 2007, 50: 417-23.

Becker, H.F., Jerrentrup, A., Ploch, T., Grote, L., Penzel, T., Sullivan, C.E., et al. (2003). Effect of nasal continuous positive airway pressure treatment on blood pressure in patients with obstructive sleep apnea. Circulation. 107: 68-73.

Belaidi, E., Joyeux-Faure, M., Ribuot, C., Launois, S.H., Levy, P., Godin-Ribuot, D. (2009). Major role for hypoxia inducible factor-1 and the endothelin system in promoting myocardial infarction and hypertension in an animal model of obstructive sleep apnea. J. Am. Coll. Cardiol. 53, 1309-17. doi: 10.1016/j.jacc.2008.12.050.

Berry RB, Budhiraja R, Gottlieb DJ, Gozal D, Iber C, Kapur VK, Marcus CL, Mehra R, Parthasarathy S, Quan SF, Redline S, Strohl KP, Davidson Ward SL, Tangredi MM (2012). Rules for scoring respiratory events in sleep: update of the 2007 AASM Manual for the Scoring of Sleep and Associated Events. Deliberations of the Sleep Apnea Definitions Task Force of the American Academy of Sleep Medicine. J. Clin. Sleep Med. 8, 597-619. doi: 10.5664/jcsm.2172.

Bertera FM, Del Mauro JS, Chiappetta D, Polizio AH, Buontempo F, Taira CA, Höcht C. (2012a). Enantioselective pharmacokinetic and pharmacodynamic properties of carvedilol in spontaneously hypertensive rats: focus on blood pressure variability. Naunyn Schmiedebergs Arch Pharmacol. 385, 325-35. doi: 10.1007/s00210-011-0698-7.

Bertera F, Di Verniero CA, Mayer MA, Chiappetta D, Buontempo F, Polizio AH, Taira CA, Höcht C. (2012b). Pharmacokinetic and pharmacodynamic properties of carvedilol in fructose hypertensive rats. Xenobiotica. 2012 Feb;42(2):206-19. doi: 10.3109/00498254.2011.604746.

Böhm, M., Linz, D., Urban, D., Mahfoud, F., Ukena, C. (2013). Renal sympathetic denervation: applications in hypertension and beyond. Nat. Rev. Cardiol. 10, 465-76. doi: 10.1038/nrcardio.2013.89.

Bonnichsen, M., Dragsted, M., Hansen, A.K. (2005). The welfare impact of gavaging laboratory rates. Anim. Welfare. 14, 223–227.

Bonsignore, M.R., Marrone, O., Insalaco, G., Bonsignore, G. (1994). The cardiovascular effects of obstructive sleep apnoeas: analysis of pathogenic mechanisms. Eur. Respir. J. 7, 786-805.

Börgel J, Sanner BM, Keskin F, Bittlinsky A, Bartels NK, Büchner N, Huesing A, Rump LC, Mügge A. (2004). Obstructive sleep apnea and blood pressure. Interaction between the blood pressure-lowering effects of positive airway pressure therapy and antihypertensive drugs. Am. J. Hypertens. 17, 1081-7.

Bosc, L.V., Resta, T., Walker, B., Kanagy, N.L. (2010). Mechanisms of intermittent hypoxia induced hypertension. J. Cell. Mol. Med. 14, 3-17. doi: 10.1111/j.1582-4934.2009.00929.x.

Bottini, P., Taranto-Montemurro, L., Novali, M., Bettinzoli, M., Roca, E., Andreoli, C., et al. (2012). Effects of CPAP on systemic hypertension in OSAH:A monocentric, observational, cohort study. Respir. Med. 106,1329-34. doi: 10.1016/j.rmed.2012.05.007.

Bradley, T.D., Floras, J.S. (2003). Sleep apnea and heart failure: Part II: central sleep apnea. Circulation. 107, 1822-6.

Bradley, T.D., Phillipson, E.A. (1992). Central sleep apnea. Clin. Chest. Med. 13, 493-505.

Brenner, S., Angermann, C., Jany, B., Ertl, G., Störk, S. (2008). Sleep-disordered breathing and heart failure a dangerous liaison. Trends Cardiovasc. Med. 18, 240-7. doi: 10.1016/j.tcm.2008.11.006.

Brooks, D., Horner, R.L., Kimoff, R.J., Kozar, L.F., Render-Teixeira, C.L., Phillipson, E.A. (1997a). Effect of obstructive sleep apnea versus sleep fragmentation on responses to airway occlusion. Am. J. Respir. Crit. Care Med. 155, 1609-17.

Brooks, D., Horner, R.L., Kozar, L.F., Render-Teixeira, C.L., Phillipson, E.A. (1997b). Obstructive sleep apnea as a cause of systemic hypertension. Evidence from a canine model. J. Clin. Invest. 99, 106-9.

Brown, A.P., Dinger, N., Levine, B.S. (2000). Stress produced by gavage administration in the rat. Contemp. Top. Lab. Anim. Sci. 39, 17-21.

Burchell, A.E., Lobo, M.D., Sulke, N., Sobotka, PA, Paton, JF. (2014). Arteriovenous anastomosis: is this the way to control hypertension? Hypertension. 64:6-12. doi: 10.1161/HYPERTENSIONAHA.114.02925.

Burr ML, Dolan E, O'Brien EW, O'Brien ET, McCormack P (2008). The value of ambulatory blood pressure in older adults: the Dublin outcome study. Age and Ageing. 37, 201-6. doi: 10.1093/ageing/afm193.

Calhoun, D.A. (2010). Obstructive sleep apnea and hypertension. Curr. Hypertens. Rep. 12, 189-95. doi: 10.1007/s11906-010-0112-8.

Campen, M.J., Shimoda, L.A., O'Donnell, C.P. (2005). Acute and chronic cardiovascular effects of intermittent hypoxia in C57BL/6J mice. J. Appl. Physiol. 99, 2028-35.

Carley, D.W., Berecek, K., Videnovic, A., Radulovacki, M. (2000). Sleep-disordered respiration in phenotypically normotensive, genetically hypertensive rats. Am. J. Respir. Crit. Care Med. 162, 1474-9.

Carley, D.W., Trbovic, S.M., Radulovacki, M. (1998). Diazepam suppresses sleep apneas in rats. Am. J. Respir. Crit. Care Med. 157, 917-20.

Caskie GI, Willis SL, Warner Schaie K, Zanjani FA (2006). Congruence of medication information from a brown bag data collection and pharmacy records: findings from the Seattle longitudinal study. Exp. Aging Res. 32, 79-103.

Chan YK, Davis, PF, Poppitt, SD, Sun X, Greenhill NS, Krishnamurthi R, Przepiorski A, McGill AT, Krissansen GW. (2012). Influence of tail versus cardiac sampling on blood glucose and lipid profiles in mice. Laboratory Animals 46, 142-7. doi: 10.1258/la.2011.011136.

Chen, L., Einbinder, E., Zhang, Q., Hasday, J., Balke, C.W., Scharf, S.M. (2005). Oxidative stress and left ventricular function with chronic intermittent hypoxia in rats. Am. J. Respir. Crit. Care Med. 172, 915–920.

Chen J, Huang C, Zhang B, Huang Q, Chen J, Xu L. (2013). The effects of carvedilol on cardiac structural remodeling: the role of endogenous nitric oxide in the activity of carvedilol. Mol Med Rep. 7, 1155-8. doi: 10.3892/mmr.2013.1329.

Chen XY, Zeng YM, Zhang YX, Wang WY, Wu RH. (2013). Effect of chronic intermittent hypoxia on theophylline metabolism in mouse liver. Chin Med J (Engl). 126, 118-23.

Cheng J, Kamiya K, Kodama I. (2001). Carvedilol: molecular and cellular basis for its multifaceted therapeutic potential. Cardiovasc Drug Rev. 19, 152-71.

Chobanian, A.V., Bakris, G.L., Black, H.R., Cushman, W.C., Green, L.A., Izzo, J.L. Jr et al. (2003). Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. Hypertension. 42, 1206-52.

Cichelero, F.T., Martinez, D., Fuchs, S.C., Gus, M., Moreira, L.B., Fuchs, F.D. (2014). The effect of antihypertensive agents on sleep apnea: protocol for a randomized controlled trial. Trials. 2, 15-1. doi: 10.1186/1745-6215-15-1.

Cicolin, A., Mangiardi, L., Mutani, R., Bucca, C. (2006). Angiotensin-converting enzyme inhibitors and obstructive sleep apnea. Mayo Clin. Proc. 81, 53-5.

Colavito, V., Fabene, P.F., Grassi-Zucconi, G., Pifferi, F., Lamberty, Y., Bentivoglio, M., et al. (2013). Experimental sleep deprivation as a tool to test memory deficits in rodents. Front. Syst. Neurosci. 13, 7-106. doi: 10.3389/fnsys.2013.00106.

Collop NA, Anderson WM, Boehlecke B, Claman D, Goldberg R, Gottlieb DJ, Hudgel D, Sateia M, Schwab R; Portable Monitoring Task Force of the American Academy of Sleep Medicine (2007). Clinical guidelines for the use of unattended portable monitors in the diagnosis of obstructive sleep apnea in adult patients. Portable Monitoring Task Force of the American Academy of Sleep Medicine. J. Clin. Sleep Med. 3, 737-47.

Cooper, V.L., Pearson, S.B., Bowker, C.M., Elliott, M.W., Hainsworth, R. (2005). Interaction of chemoreceptor and baroreceptor reflexes by hypoxia and hypercapnia - a mechanism for promoting hypertension in obstructive sleep apnoea. J. Physiol. 568, 677-87.

Corbett A, McGowin A, Sieber S, Flannery T, Sibbitt, B. (2012). A method for reliable voluntary oral administration of a fixed dosage (mg/kg) of chronic daily medication to rats. Laboratory Animals 46, 318-24. doi: 10.1258/la.2012.012018.

Cutler, M.J., Swift, N.M-, Keller, D.M., Wasmund, W.L., Smith, M.L. (2004). Hypoxia-mediated prolonged elevation of sympathetic nerve activity after periods of intermittent hypoxic apnea. J. Appl. Physiol. 96, 754-61.

da Silva, A.Q., Fontes, M.A., Kanagy, N.L. (2011). Chronic infusion of angiotensin receptor antagonists in the hypothalamic paraventricular nucleus prevents hypertension in a rat model of sleep apnea. Brain Res. 1368, 231-8. doi: 10.1016/j.brainres.2010.10.087.

Damsch S, Eichenbaum G, Tonelli A, Lammens L, Van den Bulck K, Feyen B, Vandenberghe J, Megens A, Knight E, Kelley M. (2011). Gavage-related reflux in rats: identification, pathogenesis, and toxicological implications (review). Toxicologic Pathology 39, 348-60. doi: 10.1177/0192623310388431.

Davies CW, Crosby JH, Mullins RL, Barbour C, Davies RJ, Stradling JR (2000). Case-control study of 24 hour ambulatory blood pressure in patients with obstructive sleep apnea and normal matched control subjects. Thorax. 55, 736-40.

Davies RJ, Ali NJ, Stradling JR (1992). Neck circumference and other clinical features in the diagnosis of the obstructive sleep apnoea syndrome. Thorax. 47, 101-105.

Davis, E.M., O'Donnell, C.P. (2013). Rodent models of sleep apnea. Respir. Physiol. Neurobiol. 188, 355-61. doi: 10.1016/j.resp.2013.05.022.

Del Rio, R., Moya, E.A., Iturriaga, R. (2010). Carotid body and cardiorespiratory alterations in chronic intermittent hypoxia: the oxidative link. Eur. Respir. J. 36, 143-50. doi: 10.1183/09031936.00158109.

Deleanu, O.C., Mălăuț, A.E., Nebunoiu, A.M., Micheu, M.M., Mihălțan, F.D. (2014). Obstructive sleep apnea syndrome and arterial hypertension--a complicated relationship? The role of controlling blood pressure values in patients with OSAS. Pneumologia. 63, 36-43.

Dematteis, M., Godin-Ribuot, D., Arnaud, C., Ribuot, C., Stanke-Labesque, F., Pépin, J.L., et al. (2009). Cardiovascular consequences of sleep-disordered breathing: contribution of animal models to understanding the human disease. ILAR. 50, 262-81.

Dematteis, M., Julien, C., Guillermet, C., Sturm, N., Lantuejoul, S., Mallaret, M., et al. (2008). Intermittent hypoxia induces early functional cardiovascular remodeling in mice. Am. J. Respir. Crit. Care Med. 177, 227-35.

Dempsey, J.A., Veasey, S.C., Morgan, B.J., O'Donnell, C.P. (2010). Pathophysiology of sleep apnea. Physiol. Rev. 90, 47-112. doi: 10.1152/physrev.00043.2008.

Dernaika, T.A., Kinasewitz, G.T., Tawk, M.M. (2009). Effects of nocturnal continuous positive airway pressure therapy in patients with resistant hypertension and obstructive sleep apnea. J. Clin. Sleep Med. 5, 103-7.

Diogo, L.N., Monteiro, E.C. (2014). The efficacy of antihypertensive drugs in chronic intermittent hypoxia conditions. Front. Physiol. 5:361. doi: 10.3389/fphys.2014.00361.

DiNicolantonio, J.J., Hackam, D.G. (2012). Carvedilol: a third-generation β-blocker should be a first-choice β-blocker. Expert Rev. Cardiovasc. Ther. 10, 13-25. doi: 10.1586/erc.11.166.

Di Verniero CA, Bertera F, Buontempo F, Bernabeu E, Chiappetta D, Mayer MA, Bramuglia GF, Taira CA, Höcht C. (2010). Enantioselective pharmacokinetic-pharmacodynamic modelling of carvedilol in a N-nitro-l-arginine methyl ester rat model of secondary hypertension. J Pharm Pharmacol. 2010 Jul;62(7):890-900. doi: 10.1211/jpp.62.07.0010.

Dolan E, Stanton AV, Thom S, Caulfield M, Atkins N, McInnes G, et al (2009). ASCOT Investigators. Ambulatory blood pressure monitoring predicts cardiovascular events in treated hypertensive patients--an Anglo-Scandinavian cardiac outcomes trial substudy. J. Hypertens. 27, 876-85. doi: 10.1097/HJH.0b013e328322cd62.

Drager, L.F., Pedrosa, R.P., Diniz, P.M., Diegues-Silva, L., Marcondes, B., Couto, R.B., et al. (2011). The effects of continuous positive airway pressure on prehypertension and masked hypertension in men with severe obstructive sleep apnea. Hypertension. 57, 549-55. doi: 10.1161/HYPERTENSIONAHA.110.165969.

Dumitrascu, R., Heitmann, J., Seeger, W., Weissmann, N., Schulz, R. (2013). Obstructive Sleep Apnea, Oxidative Stress and Cardiovascular Disease: Lessons from Animal Studies. Oxid. Med. Cell. Longev. 2013, 234631. doi: 10.1155/2013/234631.

Dunleavy, M., Dooley, M., Cox, D., Bradford, A. (2005). Chronic intermittent asphyxia increases platelet reactivity in rats. Exp. Physiol. 90, 411–416.

Durán-Cantolla, J., Aizpuru, F., Montserrat, J.M., Ballester, E., Terán-Santos, J., Aguirregomoscorta, J.I., et al. (2010). Continuous positive airway pressure as treatment for systemic hypertension in people with obstructive sleep apnoea: randomised controlled trial. B.M.J. 24, 341:c5991. doi: 10.1136/bmj.c5991.

Dyavanapalli, J., Jameson, H., Dergacheva, O., Jain, V., Alhusayyen, M., Mendelowitz, D. (2014). Chronic intermittent hypoxia-hypercapnia blunts heart rate responses and alters neurotransmission to cardiac vagal neurons. J. Physiol. 16, 1-13. doi: 10.1113/jphysiol.2014.273482.

Eckert, D.J., Jordan, A.S., Merchia, P., Malhotra, A. (2007). Central sleep apnea: Pathophysiology and treatment. Chest. 131, 595-607.

Epstein, .LJ., Kristo, D., Strollo, P.J. Jr, et al. (2009). Clinical Guideline for the Evaluation, Management and Long-term Care of Obstructive Sleep Apnea in Adults. J. Clin. Sleep Med. 5, 263-76.

Epstein, L.J., Jervis, O.J. Jr, Henderson, J.H. 2nd, Sullivan, M., Mohsenifar, Z. (2001). Measurement of gastric $P(CO_2)$ as an index of tissue hypoxia during obstructive sleep apnea. Respiration. 68, 28-34.

Expert Panel on the Identification, Evaluation, and Treatment of Overweight in Adults (1998). Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults: executive summary. Am. J. Clin. Nutr. 68, 899-917.

Farré, R., Nácher, M., Serrano-Mollar, A., Gáldiz, J.B., Alvarez, F.J., Navajas, D., et al. (2007). Rat model of chronic recurrent airway obstructions to study the sleep apnea syndrome. Sleep. 30, 930-3.

Faselis, C., Doumas, M., Kokkinos, P., Tsioufis, C., Papademetriou, V. (2014). The Role of Renal Nerve Ablation for the Management of Resistant Hypertension and other Disease Conditions: Benefits and Concerns. Curr. Vasc. Pharmacol. 12, 38-46.

Fenik, V.B., Singletary, T., Branconi, J.L., Davies, R.O., Kubin, L. (2012). Glucoregulatory consequences and cardiorespiratory parameters in rats exposed to chronic-intermittent hypoxia: effects of the duration of exposure and losartan. Front. Neurol. 3:51. doi: 10.3389/fneur.2012.00051.

Feuerstein GZ, Ruffolo RR Jr. (1995). Carvedilol, a novel multiple action antihypertensive agent with antioxidant activity and the potential for myocardial and vascular protection. Eur Heart J. 16, 38-42.

Flecknell PA, Roughan JV, Stewart R. (1999). Use of oral buprenorphine ('buprenorphine jello') for postoperative analgesia in rats--a clinical trial. Laboratory Animals 33, 169-74.

Fletcher, E.C., Bao, G. (1996). The rat as a model of chronic recurrent episodic hypoxia and effect upon systemic blood pressure. Sleep. 19, S210-2.

Fletcher, E.C. (2000). Effect of episodic hypoxia on sympathetic activity and blood pressure. Respir. Physiol. 119, 189-97.

Fletcher, E.C., Bao, G., Li, R. (1999). Renin activity and blood pressure in response to chronic episodic hypoxia. Hypertension. 34, 309-14.

Fletcher, E.C., Bao, G., Miller, C.C. 3rd. (1995). Effect of recurrent episodic hypocapnic, eucapnic, and hypercapnic hypoxia on systemic blood pressure. J. Appl. Physiol. 78, 1516-21.

Fletcher EC, DeBehnke RD, Lovoi MS, Gorin AB (1985). Undiagnosed sleep apnea in patients with essential hypertension. Ann Intern Med. 103, 190-5.

Fletcher, E.C., Lesske, J., Qian, W., Miller, C.C. 3rd, Unger, T. (1992a). Repetitive, episodic hypoxia causes diurnal elevation of blood pressure in rats. Hypertension. 19, 555-61.

Fletcher, E.C., Lesske, J., Culman, J., Miller, C.C., Unger, T. (1992b). Sympathetic denervation blocks blood pressure elevation in episodic hypoxia. Hypertension. 20, 612-9.

Fletcher, E.C., Lesske, J., Behm, R., Miller, C.C. 3rd, Stauss, H., Unger, T. (1992c). Carotid chemoreceptors, systemic blood pressure, and chronic episodic hypoxia mimicking sleep apnea. J. Appl. Physiol. 72, 1978-84.

Fletcher, E.C., Orolinova, N., Bader, M. (2002). Blood pressure response to chronic episodic hypoxia: the rennin-angiotensin system. J. Appl. Physiol. 92, 627-33.

Foster, G.E., Brugniaux, J.V., Pialoux, V., Duggan, C.T., Hanly, P.J., Ahmed, S.B., et al. (2009). Cardiovascular and cerebrovascular responses to acute hypoxia following exposure to intermittent hypoxia in healthy humans. J. Physiol. 587, 3287-99. doi: 10.1113/jphysiol.2009.171553.

Foster, G.E., Hanly, P.J., Ahmed, S.B., Beaudin, A.E., Pialoux, V., Poulin, M.J. (2010). Intermittent hypoxia increases arterial blood pressure in humans through a Renin-Angiotensin system-dependent mechanism. Hypertension. 56, 369-77. doi:10.1161/HYPERTENSIONAHA.110.152108.

Foster, G.E., McKenzie, D.C., Milsom, W.K., Sheel, A.W. (2005). Effects of two protocols of intermittent hypoxia on human ventilatory, cardiovascular and cerebral responses to hypoxia. J. Physiol. 567, 689–699.

Foster, G.E., Poulin, M.J., Hanly, P.J. (2007). Intermittent hypoxia and vascular function: implications for obstructive sleep apnoea. Exp. Physiol. 92, 51-65.

Friedman M, Tanyeri H, La Rosa M, Landsberg R, Vaidyanathan K, Pieri S, et al. (1999). Clinical predictors of obstructive sleep apnea. Laryngoscope. 109, 1901-7.

Friedman O, Bradley TD, Chan CT, Parkes R, Logan AG (2010). Relationship between overnight rostral fluid shift and obstructive sleep apnea in drug-resistant hypertension. Hypertension 56:1077-82.

Gaddam, K., Pimenta, E., Thomas, S.J., Cofield, S.S., Oparil, S., Harding, S.M., et al. (2010). Spironolactone reduces severity of obstructive sleep apnoea in patients with resistant hypertension: a preliminary report. J. Hum. Hypertens. 8, 532-7. doi: 10.1038/jhh.2009.96.

Galie, N., Hoeper, M.M., Humbert, M., Torbicki, A., Vachiery, J.L., Barbera, J.A., et al. (2009). Guidelines for the diagnosis and treatment of pulmonary hypertension. Eur. Respir. J. 34,1219-63. doi: 10.1183/09031936.00139009.

Gao J, Ding XS, Zhang YM, Dai DZ, Liu M, Zhang C, Dai Y. (2013). Hypoxia/oxidative stress alters the pharmacokinetics of CPU86017-RS through mitochondrial dysfunction and NADPH oxidase activation. Acta Pharmacol Sin. 2013 Dec;34(12):1575-84. doi: 10.1038/aps.2013.94.

Ganong WF. (2002). Handbook of medical physiology. EGC, Jakarta.

Germack R, Leon-Velarde F, Valdes De La Barra R, Farias J, Soto G, Richalet JP. (2002). Effect of intermittent hypoxia on cardiovascular function, adrenoceptors and muscarinic receptors in Wistar rats. Exp Physiol. 87, 453-60.

Golbidi, S., Badran, M., Ayas, N., Laher, I. (2012). Cardiovascular consequences of sleep apnea. Lung. 190, 113-32. doi: 10.1007/s00408-011-9340-1.

Goldkuhl R, Hau, J, Abelson KS. (2010). Effects of voluntarily-ingested buprenorphine on plasma corticosterone levels, body weight, water intake, and behaviour in permanently catheterised rats. In Vivo. 24, 131-5.

Gozal, E., Sachleben, L.R. Jr, Rane, M.J., Vega, C., Gozal, D. (2005). Mild sustained and intermittent hypoxia induce apoptosis in PC-12 cells via different mechanisms. Am J Physiol Cell Physiol. 288, C535-42.

Grassi, G., Seravalle, G., Brambilla, G., Bombelli, M., Dell'Oro, R., Gronda, E., et al. (2012). Novel antihypertensive therapies: renal sympathetic nerve ablation and carotid baroreceptor stimulation. Curr. Hypertens. Rep. 14, 567-72. doi: 10.1007/s11906-012-0312-5.

Grassi, G., Seravalle, G., Brambilla, G., Cesana, F., Giannattasio, C., Mancia, G. (2014). Similarities and differences between renal sympathetic denervation and carotid baroreceptor stimulation. Curr. Vasc. Pharmacol. 12, 63-8.

Grassi G, Seravalle G, Colombo M, Bolla G, Cattaneo BM, Cavagnini F, Mancia G (1998). Body weight reduction, sympathetic nerve traffic, and arterial baroreflex in obese normotensive humans. Circulation. 97, 2037–2042.

Grote, L., Heitmann, J., Schneider, H., Ploch, T., Penzel, T., Peter, J.H., et al. (1994). Twenty-four-hour blood pressure control: effect of cilazapril on continuous arterial blood pressure during sleep, and physical and mental load in patients with arterial hypertension and sleep apnea. J. Cardiovasc. Pharmacol. 3, S78-82.

Guralnick, A., Bakris, G.L. (2012). Approaches for targeting blood pressure control in sleep disorders. Curr. Opin. Nephrol. Hypertens. 21, 469-74. doi: 10.1097/MNH.0b013e32835623f5.

Haack, M., Mullington, J.M. (2005). Sustained sleep restriction reduces emotional and physical well-being. Pain.119, 56-64.

Hastie T, Tibshirani. (1990). Generalized Additive Models. New York: Chapman & Hall.

Heitmann, J., Greulich, T., Reinke, C., Koehler, U., Vogelmeier, C., Becker, H.F., et al. (2010). Comparison of the effects of nebivolol and valsartan on BP reduction and sleep apnoea activity in patients with essential hypertension and OSA. Curr. Med. Res. Opin. 26, 1925-32. doi: 10.1185/03007995.2010.497326.

Hendricks, J.C., Kline, L.R., Kovalski, R.J., O'Brien, J.A., Morrison, A.R., Pack, A.I. (1987). The English bulldog: a natural model of sleep-disordered breathing. J. Appl. Physiol. 63, 1344-50.

Hla, K.M., Skatrud, J.B., Finn, L., Palta, M., Young, T. (2002). The effect of correction of sleep-disordered breathing on BP in untreated hypertension. Chest. 122, 1125-32.

Hla KM, Young T, Finn L, Peppard PE, Szklo-Coxe M, Stubbs M (2008). Longitudinal association of sleep-disordered breathing and nondipping of nocturnal blood pressure in the Wisconsin Sleep Cohort Study. Sleep 31, 795-800.

Hosmer DW, Lemeshow S. (1990). Applied Logistic Regression. New York: John Wiley & Sons.

Hui, A.S., Striet, J.B., Gudelsky, G., Soukhova, G.K., Gozal, E., Beitner-Johnson, D., et al. (2003). Regulation of catecholamines by sustained and intermittent hypoxia in neuroendocrine cells and sympathetic neurons. Hypertension. 42, 1130-6.

Huang-Brown KM, Guhad FA. (2002). Chocolate, an effective means of oral drug delivery in rats. Laboratory Animals (NY) 31: 34-6.

Hung, M.W., Kravtsov, G.M., Lau, C.F., Poon, A.M., Tipoe, G.L., Fung, M.L. (2013). Melatonin ameliorates endothelial dysfunction, vascular inflammation, and systemic hypertension in rats with chronic intermittent hypoxia. J. Pineal Res. 55, 247-56. doi: 10.1111/jpi.12067.

Iftikhar, I.H., Hays, E.R., Iverson, M.A., Magalang, U.J., Maas, A.K. (2013). Effect of oral appliances on blood pressure in obstructive sleep apnea: a systematic review and meta-analysis. J. Clin. Sleep Med. 9, 165-74. doi: 10.5664/jcsm.2420.

Isaksson IM, Theodorsson A, Theodorsson, E, Strom JO. (2011). Methods for 17β-oestradiol administration to rats. Scandinavian Journal of Clinical & Laboratory Investigation 71, 583-92. doi: 10.3109/00365513.2011.596944.

Iturriaga, R., Moya, E.A., Del Rio, R. (2010). Cardiorespiratory alterations induced by intermittent hypoxia in a rat model of sleep apnea. Adv. Exp. Med. Biol. 669, 271-4. doi: 10.1007/978-1-4419-5692-7_55.

Iwasaki, Y.K., Shi, Y., Benito, B., Gillis, M.A., Mizuno, K., Tardif, J.C., et al. (2012). Determinants of atrial fibrillation in an animal model of obesity and acute obstructive sleep apnea. Heart Rhythm. 9,1409-16.e1. doi: 10.1016/j.hrthm.2012.03.024.

Jacobsen KR, Kalliokoski O, Hau J, Abelson KSP. (2011). Voluntary ingestion of buprenorphine in mice. Animal Welfare 20, 591–596.

Joosten, S.A., O'Driscoll, D.M., Berger, P.J., Hamilton, G.S. (2014). Supine position related obstructive sleep apnea in adults: pathogenesis and treatment. Sleep Med. Rev. 18, 7-17. doi: 10.1016/j.smrv.2013.01.005.

Julien, C., Bayat, S., Levy, P. (2003). Vascular reactivity to norepinephrine and acetylcholine after chronic intermittent hypoxia in mice. Respir. Physiol. Neurobiol. 139, 21–32.

Kalaria, R.N., Spoors, L., Laude, E.A., Emery, C.J., Thwaites-Bee D., Fairlie J., Oakley A.E., Barer D.H., Barer G.R. (2004). Hypoxia of sleep apnoea: cardiopulmonary and cerebral changes after intermittent hypoxia in rats. Respir. Physiol. Neurobiol. 140, 53-62.

Kalliokoski O, Abelson KS, Koch J, Boschian A, Thormose SF, Fauerby N, Rasmussen RS, Johansen FF, Hau J. (2010). The effect of voluntarily ingested buprenorphine on rats subjected to surgically induced global cerebral ischaemia. In Vivo 24, 641-6.

Kalliokoski O, Jacobsen KR, Hau J, Abelson KS. (2011). Serum concentrations of buprenorphine after oral and parenteral administration in male mice. The Veterinary Journal 187, 251-4. doi: 10.1016/j.tvjl.2009.11.013.

Kanagy, N.L. (2009). Vascular effects of intermittent hypoxia. ILAR J. 50, 282-8.

Kanagy, N.L.,Walker, B.R., Nelin, L.D. (2001). Role of endothelin in intermittent hypoxia-induced hypertension. Hypertension. 37, 511–515.

Kantores, C., McNamara, P.J., Teixeira, L., Engelberts, D., Murthy, P., Kavanagh, B.P., et al. (2006). Therapeutic hypercapnia prevents chronic hypoxia-induced pulmonary hypertension in the newborn rat. Am. J. Physiol. Lung Cell Mol. Physiol. 291, L912-22.

Kapa, S., Sert Kuniyoshi, F.H., Somers, V.K. (2008). Sleep Apnea and Hypertension: interactions and implications for management. Hypertension. 51, 605-8. doi: 10.1161/HYPERTENSIONAHA.106.076190.

Kapur, V.K. (2010). Obstructive sleep apnea: diagnosis, epidemiology, and economics. Respir. Care. 55,1155-67.

Kario, K., Kuwabara, M., Hoshide, S., Nagai, M., Shimpo, M. (2014). Effects of Nighttime Single-Dose Administration of Vasodilating vs Sympatholytic Antihypertensive Agents on Sleep Blood Pressure in Hypertensive Patients With Sleep Apnea Syndrome. J. Clin. Hypertens. 16, 459-66. doi: 10.1111/jch.12327.

Kasiakogias, A., Tsioufis, C., Thomopoulos, C., Aragiannis, D., Alchanatis, M., Tousoulis, D., et al. (2013). Effects of continuous positive airway pressure on blood pressure in hypertensive patients with obstructive sleep apnea: a 3-year follow-up. J. Hypertens. 31: 352-60. doi: 10.1097/HJH.0b013e32835bdcda.

Khan, M.T., Franco, R.A. (2014). Complex sleep apnea syndrome. Sleep Disord. 2014, 798487. doi: 10.1155/2014/798487.

Knight, W.D., Little, J.T., Carreno, F.R., Toney, G.M., Mifflin, S.W., Cunningham, J.T. (2011). Chronic intermittent hypoxia increases blood pressure and expression of FosB/DeltaFosB in central autonomic regions. Am. J. Physiol. Regul. Integr. Comp. Physiol. 301, R131-9. doi: 10.1152/ajpregu.00830.2010.

Knight, W.D., Saxena, A., Shell, B., Nedungadi, T.P., Mifflin, S.W., Cunningham, J.T. (2013). Central losartan attenuates increases in arterial pressure and expression of FosB/ΔFosB along the autonomic axis associated with chronic intermittent hypoxia. Am. J. Physiol. Regul. Integr. Comp. Physiol. 305, R1051-8. doi: 10.1152/ajpregu.00541.2012.

Kraiczi, H., Hedner, J., Peker, Y., Grote, L. (2000). Comparison of atenolol, amlodipine, enalapril, hydrochlorothiazide, and losartan for antihypertensive treatment in patients with obstructive sleep apnea. Am. J. Respir. Crit. Care Med. 161, 1423-8.

Kramer, K., Voss, H.P., Grimbergen, J.A., Mills, P.A., Huetteman, D., Zwiers, L., Brockway, B. (2000). Telemetric monitoring of blood pressure in freely moving mice: a preliminary study. Lab. Anim. 34, 272–280.

Kumar, G.K., Kim, D.K., Lee, M.S., Ramachandran, R., Prabhakar, N.R. (2003). Activation of tyrosine hydroxylase by intermittent hypoxia: involvement of serine phosphorylation. J. Appl. Physiol. 95, 536-44.

Kumar, G.K., Rai, V., Sharma, S.D., Ramakrishnan, D.P., Peng, Y.J., Souvannakitti D, Prabhakar NR. (2006). Chronic intermittent hypoxia induces hypoxia-evoked catecholamine efflux in adult rat adrenal medulla via oxidative stress. J. Physiol. 575, 229-39.

Kurtz, T.W., Griffin, K.A., Bidani, A.K., Robin, L. Davisson, R.L., Hall, J.E. (2005). Recommendations for Blood Pressure Measurement in Humans and Experimental Animals: Part 2: Blood Pressure Measurement in Experimental Animals. A Statement for Professionals From the Subcommittee of Professional Pressure Research and Public Education of the American Heart Association Council on High Blood. Hypertension. 45,142–161.

Küster T, Zumkehr B, Hermann C, Theurillat R, Thormann W, Gottstein B, Hemphill, A. (2012). Voluntary ingestion of antiparasitic drugs emulsified in honey represents an alternative to gavage in mice. Journal of the American Association for Laboratory Animal Science 51, 219-23.

Lai, C.J., Yang, C.C., Hsu, Y.Y., Lin, Y.N., Kuo, T.B. (2006). Enhanced sympathetic outflow and decreased baroreflex sensitivity are associated with intermittent hypoxia-induced systemic hypertension in conscious rats. J. Appl. Physiol. 100, 1974–1982.

Lavie, L., Lavie, P. (2009). Molecular mechanisms of cardiovascular disease in OSAHS: the oxidative stress link. Eur. Respir. J. 33, 1467-84. doi: 10.1183/09031936.00086608.

Lavie, P., Herer, P., Hoffstein, V. (2000). Obstructive Sleep Apnea as a risk factor for hypertension: population study. BMJ. 320, 479-82.

Lavie, P., Hoffstein, V. (2001). Sleep apnea syndrome: a possible contributing factor to resistant. Sleep. 24, 721-725.

Lee, S.D., Kuo, W.W., Bau, D.T., Ko, F.Y., Wu, F.L., Kuo, C.H., et al. (2008). The coexistence of nocturnal sustained hypoxia and obesity additively increases cardiac apoptosis. J. Appl. Physiol. 104, 1144-53. doi: 10.1152/japplphysiol.00152.2007.

Lefebvre, B., Godin-Ribuot, D., Joyeux-Faure, M., Caron, F., Bessard, G., Levy, P. et al. (2006). Functional assessment of vascular reactivity after chronic intermittent hypoxia in the rat. Respir. Physiol. Neurobiol. 150, 278–286.

Lesske, J., Fletcher, E.C., Bao, G., Unger, T. (1997). Hypertension caused by chronic intermittent hypoxia--influence of chemoreceptors and sympathetic nervous system. J. Hypertens.15, 1593-603.

Leuenberger, U.A., Brubaker, D., Quraishi, S., Hogeman, C.S., Imadojemu, V.A., Gray, K.S. (2005). Effects of intermittent hypoxia on sympathetic activity and blood pressure in humans. Auton. Neurosci. 121, 87-93.

Levitzky, M.G. (2008). Using the pathophysiology of obstructive sleep apnea to teach cardiopulmonary integration. Adv. Physiol. Educ. 32, 196-202. doi: 10.1152/advan.90137.2008.

Lévy, P., Ryan, S., Oldenburg, O., Parati, G. (2013). Sleep apnoea and the heart. Eur. Respir. Rev. 22, 333-52. doi: 10.1183/09059180.00004513.

Lévy, P., Tamisier, R., Arnaud, C., Monneret, D., Baguet, J.P., Stanke-Labesque, F., et al. (2012). Sleep deprivation, sleep apnea and cardiovascular diseases. Front. Biosci. 4, 2007-21.

Li, J., Savransky V., Nanayakkara, A., Smith, P.L., O'Donnell, C.P., Polotsky, V.Y. (2007). Hyperlipidemia and lipid peroxidation are dependent on the severity of chronic intermittent hypoxia. J. Appl. Physiol. 102, 557-63.

Lin, M., Liu, R., Gozal, D., Wead, W.B., Chapleau, M.W., Wurster, R., et al. (2007). Chronic intermittent hypoxia impairs baroreflex control of heart rate but enhances heart rate responses to vagal efferent stimulation in anesthetized mice. Am. J. Physiol. Heart Circ. Physiol. 293, H997-1006.

Litvin, A.Y., Sukmarova, Z.N., Elfimova, E.M., Aksenova, A.V., Galitsin, P.V., Rogoza, A.N., et al. (2013). Effects of CPAP on "vascular" risk factors in patients with obstructive sleep apnea and arterial hypertension. Vasc. Health Risk Manag. 9, 229-35. doi: 10.2147/VHRM.S40231.

Liu, C., Cao, Y., Malhotra, A., Ling, L. (2011). Sleep fragmentation attenuates the hypercapnic (but not hypoxic) ventilatory responses via adenosine A1 receptors in awake rats. Respir. Physiol. Neurobiol. 175, 29-36. doi: 10.1016/j.resp.2010.09.003.

Liu, J.N., Zhang, J.X., Lu, G., Qiu, Y., Yang, D., Yin, G.Y., et al. (2010). The effect of oxidative stress in myocardial cell injury in mice exposed to chronic intermittent hypoxia. Chin. Med. J. (Engl). 123, 74-8.

Liu, X., Deng, Y., Shang, J., Yang, X.H., Liu, K., Liu, H.G., et al. (2013). Effect of NADPH oxidase inhibitor apocynin on the expression of hypoxia-induced factor-1α and endothelin-1 in rat carotid body exposed to chronic intermittent hypoxia. J. Huazhong Univ. Sci. Technolog. Med. Sci. 33, 178-84. doi: 10.1007/s11596-013-1093-z.

Lloberes P, Lozano L, Sampol G, Romero O, Jurado MJ, Ríos J, Untoria MD, Tovar JL. (2010). Obstructive sleep apnea and 24-h blood pressure in patients with resistant hypertension. J. Sleep Res. 19, 597-602. doi: 10.1111/j.1365-2869.2010.00839.x.

Loredo JS, Nelesen R, Ancoli-Israel S, Dimsdale JE (2004). Sleep quality and blood pressure dipping in normal adults. Sleep. 27, 1097-103.

Lovic, D., Manolis, A.J., Lovic, B., Stojanov, V., Lovic, M., Pittaras, A., et al. (2014). The pathophysiological basis of carotid baroreceptor stimulation for the treatment of resistant hypertension. Curr. Vasc, Pharmacol. 12, 16-22.

Lozano, L., Tovar, J.L., Sampol, G., Romero, O., Jurado, M.J., Segarra, A., et al. (2010). Continuous positive airway pressure treatment in sleep apnea patients with resistant hypertension: a randomized, controlled trial. J. Hypertens. 28, 2161-8. doi: 10.1097/HJH.0b013e32833b9c63.

Malhotra, A., Loscalzo, J. (2009). Sleep and cardiovascular disease: an overview. Prog. Cardiovasc. Dis. 51:279-84. doi: 10.1016/j.pcad.2008.10.004.

Mancia, G., Fagard, R., Narkiewicz, K., Redon, J., Zanchetti, A., Böhm, M., et al. (2007). Guidelines for the Management of Arterial Hypertension: The Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). Eur. Heart J. 28, 1462-536. doi: 10.1093/eurheartj/eht151. 6.

Mancia G, Fagard R, Narkiewicz K, Redon J, Zanchetti A, Böhm M, et al. (2013). 2013 ESH/ESC guidelines for the management of arterial hypertension: the Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). Eur. Heart J. 34, 2159-219. doi: 10.1093/eurheartj/eht151.

Mannarino, M.R., Di Filippo, F., Pirro, M. (2012). Obstructive sleep apnea syndrome. Eur. J. Intern. Med. 23, 586-93. doi: 10.1016/j.ejim.2012.05.013.

Marin, J.M., Agusti, A., Villar, I., Forner, M., Nieto, D., Carrizo, S.J., et al. (2012). Association between treated and untreated obstructive sleep apnea and risk of hypertension. JAMA. 307, 2169-76. doi: 10.1001/jama.2012.3418.

Martínez-García, M.A., Capote, F., Campos-Rodríguez, F., Lloberes, P., Díaz de Atauri, M.J., Somoza, M., et al. (2013). Effect of CPAP on blood pressure in patients with obstructive sleep apnea and resistant hypertension: the HIPARCO randomized clinical trial. JAMA. 310, 2407-15. doi: 10.1001/jama.2013.281250.

Mayer, J., Weichler, U., Herres-Mayer, B., Schneider, H., Marx, U., Peter, J.H. (1990). Influence of metoprolol and cilazapril on blood pressure and on sleep apnea activity. J. Cardiovasc. Pharmacol.16, 952-61.

Mazumdar, M., Glassman, J.R. (2000). Tutorial in biostatistics - Categorizing a prognostic variable: Review of methods, code for easy implementation and applications to decision-making about cancer treatments. Statistics in Medicine. 19, 113-132.

McBryde, F.D., Abdala, A.P., Hendy, E.B., Pijacka, W., Marvar, P., Moraes, D.J., et al. (2013). The carotid body as a putative therapeutic target for the treatment of neurogenic hypertension. Nat. Commun. 4, 2395. doi: 10.1038/ncomms3395.

McGuire, M., Tartar, J.L., Cao, Y., McCarley, R.W., White, D.P., Strecker, R.E., et al. (2008). Sleep fragmentation impairs ventilatory long-term facilitation via adenosine A1 receptors. J. Physiol. 586, 5215-29. doi: 10.1113/jphysiol.2008.158121.

McKenna, J.T., Tartar, J.L., Ward, C.P., Thakkar, M.M., Cordeira, J.W., McCarley, R.W., et al. (2007). Sleep fragmentation elevates behavioral, electrographic and neurochemical measures of sleepiness. Neuroscience.146, 1462-73.

Mehra, R. (2014). Obstructive sleep apnea research: challenges and new horizons. Sleep. Med. 15, 483-4. doi: 10.1016/j.sleep.2014.03.004.

Mentz G, Schulz AJ, Mukherjee B, Ragunathan TE, Perkins DW, Israel BA (2012). Hypertension: development of a prediction model to adjust self-reported hypertension prevalence at the community level. BMC Health Serv. Res. 11, 12:312. doi: 10.1186/1472-6963-12-312.

Minai, O.A., Ricaurte, B., Kaw, R., Hammel, J., Mansour, M., McCarthy, K., et al. (2009). Frequency and impact of pulmonary hypertension in patients with obstructive sleep apnea syndrome. Am. J. Cardiol. 104, 1300–6. doi: 10.1016/j.amjcard.2009.06.048.

Minic, M., Granton, J.T., Ryan, C.M. (2014). Sleep disordered breathing in group 1 pulmonary arterial hypertension. J. Clin. Sleep. Med. 10, 277-83. doi: 10.5664/jcsm.3528.

Morgan, B.J. (2009). Intermittent hypoxia: keeping it real. J. Appl. Physiol. 107, 1-3. doi: 10.1152/japplphysiol.00304.2009.

Morgan, B.J., Crabtree, D.C., Puleo, D.S., Badr, M.S., Toiber, F., Skatrud, J.B. (1996). Neurocirculatory consequences of abrupt change in sleep state in humans. J. Appl. Physiol. 80, 1627-36.

Morgan, B.J., Dempsey, J.A., Pegelow, D.F., Jacques, A., Finn, L., Palta, M., et al. (1998). Blood pressure perturbations caused by subclinical sleep-disordered breathing. Sleep. 21, 737-46.

Morton DB, Jennings M, Buckwell A, Ewbank R, Godfrey C, Holgate B, Inglis I, James R, Page C, Sharman I, Verschoyle R, Westall L, Wilson AB. (2001). Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. British Veterinary Association Animal Welfare Foundation/Fund for the Replacement of Animals in Medical Experiments/Royal Society for the Prevention of Cruelty to Animals/Universities Federation for Animal Welfare. Laboratory Animals 35, 1-41.

Moya, E., Arias, P., Carlos, V., Oyarce, M., Iturriaga, R. (2014). Ebselen prevents the carotid body chemosensory potentiation and reverses the hypertension induced by intermittent hypoxia (873.1). The FASEB Journal, 28: 873.1.

Nácher, M., Farré, R., Montserrat, J.M., Torres, M., Navajas, D., Bulbena, O., et al. (2009). Biological consequences of oxygen desaturation and respiratory effort in an acute animal model of obstructive sleep apnea (OSA). Sleep Med. 10, 892-7. doi: 10.1016/j.sleep.2008.09.014.

Nácher, M., Serrano-Mollar, A., Farré, R., Panés, J., Seguí, J., Montserrat, J.M. (2007). Recurrent obstructive apneas trigger early systemic inflammation in a rat model of sleep apnea. Respir. Physiol. Neurobiol. 155, 93-6.

Nair, D., Zhang, S.X., Ramesh, V., Hakim, F., Kaushal, N., Wang, Y., et al. (2011). Sleep fragmentation induces cognitive deficits via nicotinamide adenine dinucleotide phosphate oxidase-dependent pathways in mouse. Am. J. Respir. Crit. Care Med.184,1305-12. doi: 10.1164/rccm.201107-1173OC.

Nakajima T, Ma J, Iida H, Iwasawa K, Jo T, Omata M, Nagai R. (2003). Inhibitory effects of carvedilol on calcium channels in vascular smooth muscle cells. Jpn Heart J. 44, 963-78.

Nerbass, F.B., Pedrosa, R.P., Genta, P.R., Drager, L.F., Lorenzi-Filho, G. (2011). Calcium channel blockers are independently associated with short sleep duration in hypertensive patients with obstructive sleep apnea. J. Hypertens. 29, 1236-41. doi: 10.1097/HJH.0b013e3283462e8b.

Nicklas W, Baneux P, Boot R, Decelle T, Deeny AA, Fumanelli M, Illgen-Wilcke B. (2002). FELASA (Federation of European Laboratory Animal Science Associations Working Group on Health Monitoring of Rodent and Rabbit Colonies), Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Laboratory Animals. 36, 20-42.

Nunes AR, Batuca JR, Monteiro EC. (2010). Acute hypoxia modifies cAMP levels induced by inhibitors of phosphodiesterase-4 in rat carotid bodies, carotid arteries and superior cervical ganglia. Br J Pharmacol. 2010 Jan 1;159(2):353-61. doi: 10.1111/j.1476-5381.2009.00534.x.

O'Connor, G.T., Caffo, B., Newman, A.B., Quan, S.F., Rapoport, D.M., Redline S., et al. (2009). Prospective study of sleep-disordered breathing and hypertension: the Sleep Heart Health Study. Am. J. Respir. Crit. Care Med. 179, 1159-64. doi: 10.1164/rccm.200712-1809OC.

Ogata, T., Nomura, M., Nakaya, Y., Ito, S. (2006). Evaluation of episodes of sleep apnea in patients with liver cirrhosis. J. Med. Invest. 53, 159-166.

Oliveras, A., Schmieder, R.E. (2013). Clinical situations associated with difficult-to-control hypertension. J. Hypertens. 31, S3-8. doi: 10.1097/HJH.0b013e32835d2af0.

Ooi, H., Cadogan, E., Sweeney, M., Howell, K., O'Regan, R.G., McLoughlin, P. (2000). Chronic hypercapnia inhibits hypoxic pulmonary vascular remodeling. Am. J. Physiol. Heart Circ. Physiol. 278, H331-8.

Othman, M., Gordon, S.P., Iscoe, S. (2010). Repeated inspiratory occlusions in anesthetized rats acutely increase blood coagulability as assessed by thromboelastography. Respir Physiol Neurobiol. 171, 61-6. doi: 10.1016/j.resp.2010.01.010.

Owen, J., Reisin, E. (2013). Obstructive sleep apnea and hypertension: is the primary link simply volume overload? Curr. Hypertens. Rep. 15, 131-3. doi: 10.1007/s11906-013-0345-4.

Paiva, T., Attarian, H. (2014). Obstructive sleep apnea and other sleep-related syndromes. Handb. Clin. Neurol. 119, 251-71. doi: 10.1016/B978-0-7020-4086-3.00018-7.

Palma, B.D., Gabriel, A. Jr, Bignotto, M., Tufik, S. (2002). Paradoxical sleep deprivation increases plasma endothelin levels. Braz. J. Med. Biol. Res. 35, 75-9.

Palma, D.T., Philips, G.M., Arguedas, M.R., Harding, S.M., Fallon, M.B. (2008). Oxygen desaturation during sleep in hepatopulmonary syndrome. Hepatology 47, 1257-1263. doi: 10.1002/hep.22143.

Papademetriou, V., Doumas, M., Faselis, C., Tsioufis, C., Douma, S., Gkaliagkousi, E., et al. (2011). Carotid baroreceptor stimulation for the treatment of resistant hypertension. Int. J. Hypertens. 2011, 964394. doi: 10.4061/2011/964394.

Parasuraman S, Raveendran R, Kesavan R. (2010). Blood sample collection in small laboratory animals. Journal of Pharmacology and Pharmacotherapeutics 1: 87-93. doi: 10.4103/0976-500X.72350.

Parati, G., Lombardi, C., Hedner, J., Bonsignore, M.R., Grote, L., Tkacova, R., et al. (2013). Recommendations for the management of patients with obstructive sleep apnoea and hypertension. Eur. Respir. J. 41, 523-38. doi: 10.1183/09031936.00226711.

Parati, G., Lombardi, C., Hedner, J., Bonsignore, M.R., Grote, L., Tkacova, R. et al. (2012). Position paper on the management of patients with obstructive sleep apnea and hypertension: joint recommendations by the European Society of Hypertension, by the European Respiratory Society and by the members of European COST (COoperation in Scientific and Technological research) ACTION B26 on obstructive sleep apnea. J. Hypertens. 30, 633-46. doi: 10.1097/HJH.0b013e328350e53b.

Peccinini RG, Ximenes VF, Cesarino EJ, Lanchote VL (2008). Stereoselective analysis of carvedilol in human plasma and urine using HPLC after chiral derivatization. Biopharm Drug Dispos. 29, 280-8. doi: 10.1002/bdd.613.

Pedrosa, R.P., Drager, L.F., G de Paula, L.K., Amaro, A.C., Bortolotto, L.A., Lorenzi-Filho, G. (2013). Effects of obstructive sleep apnea treatment on blood pressure in patients with resistant hypertension: A randomized trial. Chest. 144, 1487-94. doi: 10.1378/chest.13-0085.

Pedrosa, R.P., Drager, L.F., Gonzaga, C.C., Sousa, M.G., de Paula, L.K., Amaro, A.C., et al. (2011). Obstructive sleep apnea: the most common secondary cause of hypertension associated with resistant hypertension. Hypertension. 58, 811-7. doi: 10.1161/HYPERTENSIONAHA.111.179788.

Pelttari, L.H., Hietanen, E.K., Salo, T.T., Kataja, M.J., Kantola, I.M. (1998). Little effect of ordinary antihypertensive therapy on nocturnal high blood pressure in patients with sleep disordered breathing. Am. J. Hypertens. 11, 272-9.

Pépin, J.L., Tamisier, R., Barone-Rochette, G., Launois, S.H., Lévy, P., Baguet, J.P. (2009). Comparison of continuous positive airway pressure and valsartan in hypertensive patients with sleep apnea. Am. J. Respir. Crit. Care Med. 182, 954-60. doi: 10.1164/rccm.200912-1803OC.

Peppard, P.E., Young, T., Palta, M., Skatrud, J. (2000). Prospective study of the association between sleep-disordered breathing and hypertension. N. Engl. J. Med. 342, 1378-84.

Pepperell, J.C., Ramdassingh-Dow, S., Crosthwaite, N., Mullins, R., Jenkinson, C., Stradling J.R., Davies R.J. (2002) Ambulatory blood pressure after therapeutic and sub-therapeutic nasal continuous positive airway pressure for obstructive sleep apnea: a randomised parallel trial. Lancet. 359: 204-10.

Perry, J.C., Bergamaschi, C.T., Campos, R.R., Andersen, M.L., Casarini, D.E., Tufik, S. (2011). Differential sympathetic activation induced by intermittent hypoxia and sleep loss in rats: Action of angiotensin (1-7). Auton. Neurosci. 160, 32-6. doi: 10.1016/j.autneu.2010.11.006.

Perry, J.C., D'Almeida, V., Souza, F.G., Schoorlemmer, G.H., Colombari, E., Tufik, S. (2007). Consequences of subchronic and chronic exposure to intermittent hypoxia and sleep deprivation on cardiovascular risk factors in rats. Respir. Physiol. Neurobiol. 156, 250-8.

Phillips, C.L., O'Driscoll, D.M. (2013). Hypertension and obstructive sleep apnea. Nat. Sci. Sleep. 5, 43–52. doi: 10.2147/NSS.S34841.

Phillips RA (2012). Controversies in blood pressure goal guidelines and masked hypertension. Ann. N. Y. Acad. Sci. 1254, 115-122. doi: 10.1111/j.1749-6632.2012.06489.x.

Phillips, S.A., Olson, E.B., Lombard, J.H., Morgan, B.J. (2005). Chronic intermittent hypoxia alters norepinephrine reactivity and mechanics of skeletal muscle resistance arteries. J. Appl. Physiol. 100, 1117–1123.

Pimenta, E., Oparil. S. (2012). Renal Sympathetic Denervation for Treatment of Hypertension. Curr. Treat. Options Cardiovasc. Med. 14, 127-135.

Pimenta E, Gaddam KK, Oparil S. (2008). Mechanisms and treatment of resistant hypertension. J Clin Hypertens (Greenwich). 10, 239-44.

Pimenta E, Stowasser M, Gordon RD, Harding SM, Batlouni M, Zhang B, Oparil S, Calhoun DA. (2013). Increased dietary sodium is related to severity of obstructive sleep apnea in patients with resistant hypertension and hyperaldosteronism. Chest 2013.143, 978-83. doi: 10.1378/chest.12-0802.

Prabhakar, N.R., Fields, R.D., Baker, T., Fletcher, E.C. (2001). Intermittent hypoxia: cell to system. Am. J. Physiol. Lung Cell Mol. Physiol. 281, L524-8.

Prabhakar, N.R., Peng, Y.J., Jacono, F.J., Kumar, G.K., Dick, T.E. (2005). Cardiovascular alterations by chronic intermittent hypoxia: importance of carotid body chemoreflexes. Clin. Exp. Pharmacol. Physiol. 32, 447-9.

Preis SR, Massaro JM, Hoffmann U, D'Agostino RB Sr, Levy D, Robins SJ, et al. (2010). Neck circumference as a novel measure of cardiometabolic risk: the Framingham Heart study. J. Clin. Endocrinol. Metab. 95, 3701-10. doi: 10.1210/jc.2009-1779.

Proimos E, Kiagiadaki D, Kaprana A, Chimona TS, Maroudias NJ, Papadakis CE (2012). Clinical application of subjective measurements for OSAS assessment: predictive factors of syndrome severity. ORL J. Otorhinolaryngol. Relat. Spec. 74, 240-5. doi: 10.1159/000342278.

R Development Core Team. R: A language and environment for statistical computing. R foundation for Statistical Computing, Vienna, Austria; 2008. ISBN 3-900051-07-0, URL http://www.R-project.org. Accessed 25 July 2013

Rath G, Balligand JL, Dessy C. (2012). Vasodilatory mechanisms of beta receptor blockade. Curr Hypertens Rep. 14, 310-7. doi: 10.1007/s11906-012-0278-3.

Ray, A.D., Magalang, U.J., Michlin, C.P., Ogasa, T., Krasney, J.A., Gosselin, L.E., et al. (2007). Intermittent hypoxia reduces upper airway stability in lean but not obese Zucker rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293, R372-8.

Ribeiro RT, Afonso RA, Guarino MP, Macedo MP. (2008). Loss of postprandial insulin sensitization during aging. J Gerontol A Biol Sci Med Sci. 63, 560-5.

Robinson, G.V., Langford, B.A., Smith, D.M., Stradling, J.R. (2008). Predictors of blood pressure fall with continuous positive airway pressure (CPAP) treatment of obstructive sleep apnea (OSA). Thorax. 63: 855-9. doi: 10.1136/thx.2007.088096.

Robinson, G.V., Smith, D.M., Langford, B.A., Davies, R.J., Stradling, J.R. (2006). Continuous positive airway pressure does not reduce blood pressure in nonsleepy hypertensive OSA patients. Eur. Respir. J. 27, 1229-35.

Rodriguez-Perez JC, Losada A, Anabitarte A, Cabrera J, Llobet J, Palop L, Plaza C (1997). Effects of the novel multiple-action agent carvedilol on severe nephrosclerosis in renal ablated rats. J Pharmacol Exp Ther. 283, 336-44.

Rosa, D.P., Martinez, D., Picada, J.N., Semedo, J.G., Marroni, N.P. (2011). Hepatic oxidative stress in an animal model of sleep apnoea: effects of different duration of exposure. Comp. Hepatol. 10 :1. doi: 10.1186/1476-5926-10-1.

Ryan, S., Taylor, C.T., McNicholas, W.T. (2005). Selective activation of inflammatory pathways by intermittent hypoxia in obstructive sleep apnea syndrome. Circulation. 112, 2660-7.

Saijonmaa O, Metsärinne K, Fyhrquist F. (1997). Carvedilol and its metabolites suppress endothelin-1 production in human endothelial cell culture. Blood Press. 6, 24-8.

Sajkov, D., McEvoy, R. D. (2009). Obstructive sleep apnea and pulmonary hypertension. Prog Cardiovasc Dis. 51, 363-370. doi: 10.1016/j.pcad.2008.06.001.

Salejee, I., Tarasiuk, A., Reder, I., Scharf, S.M. (1993). Chronic upper airway obstruction produces right but not left ventricular hypertrophy in rats. Am. Rev. Respir. Dis. 148, 1346-50.

Salo, T.M., Kantola, I., Voipio-Pulkki, L.M., Pelttari, L., Viikari, J.S. (1999). The effect of four different antihypertensive medications on cardiovascular regulation in hypertensive sleep apneic patients--assessment by spectral analysis of heart rate and blood pressure variability. Eur. J. Clin. Pharmacol. 55, 191-8.

Schneider, H., Schaub, C.D., Chen, C.A., Andreoni, K.A., Schwartz, A.R., Smith, P.L., et al. (2000). Effects of arousal and sleep state on systemic and pulmonary hemodynamics in obstructive apnea. J. Appl. Physiol. 88, 1084-92.

Schulz, R., Murzabekova, G., Egemnazarov, B., Kraut, S., Eisele, H.J., Dumitrascu, R., et al. (2014). Arterial hypertension in a murine model of sleep apnea: role of NADPH oxidase 2. J. Hypertens. 32, 300-5. doi: 10.1097/HJH.0000000000000016.

Sharpe, AL., Andrade, M.A., Herrera-Rosales, M., Britton, S.L., Koch, L.G., Toney, G.M. (2013). Rats selectively bred for differences in aerobic capacity have similar hypertensive responses to chronic intermittent hypoxia. Am. J. Physiol. Heart Circ. Physiol. 305, H403-9. doi: 10.1152/ajpheart.00317.2013.

Seravalle, G., Mancia, G., Grassi, G. (2014). Role of the Sympathetic Nervous System in Hypertension and Hypertension-Related Cardiovascular Disease. High Blood Press. Cardiovasc. Prev. 21, 89-105. doi: 10.1007/s40292-014-0056-1.

Shantha, G.P., Pancholy, S.B. (2014). Effect of renal sympathetic denervation on apnea-hypopnea index in patients with obstructive sleep apnea: a systematic review and meta-analysis. Sleep Breath. May 17. doi: 10.1007/s11325-014-0991-z.

Sharpe, AL., Andrade, M.A., Herrera-Rosales, M., Britton, S.L., Koch, L.G., Toney, G.M. (2013). Rats selectively bred for differences in aerobic capacity have similar hypertensive responses to chronic intermittent hypoxia. Am. J. Physiol. Heart Circ. Physiol. 305, H403-9. doi: 10.1152/ajpheart.00317.2013.

Sharp JL, Zammit TG, Azar TA, Lawson, DM. (2002). Stress-like responses to common procedures in male rats housed alone or with other rats. Contemporary Topics in Laboratory Animal Science 41, 8-14.

Silva, A.Q., Schreihofer, A.M. (2011). Altered sympathetic reflexes and vascular reactivity in rats after exposure to chronic intermittent hypoxia. J. Physiol. 589, 1463-76. doi: 10.1113/jphysiol.2010.200691.

Simpson KL, McClellan KJ. (2000). Losartan: a review of its use, with special focus on elderly patients. Drugs & Aging 16, 227-50.

Singh SB, Selvamurthy W. (1993). Effect of intermittent chronic exposure to hypoxia on feeding behaviour of rats. Int J Biometeorol. 37, 200-2.

Solini, A., Ruilope, L.M. (2013). How can resistant hypertension be identified and prevented? Nat. Rev. Cardiol. 10, 293-6. doi: 10.1038/nrcardio.2013.23.

Somers VK, Dyken ME, Clary MP, Abboud FM. (1995). Sympathetic neural mechanisms in obstructive sleep apnea. J. Clin. Invest. 96, 1897-904.

Sonawane BR , Coates PM , Yaffe SJ , Koldovsky O. (1983). Influence of dietary carbohydrates (alpha-saccharides) on hepatic drug metabolism in male rats. Drug and Nutricional Interactions 2, 7-16.

Soukhova-O'Hare, G.K., Ortines, R.V., Gu, Y., Nozdrachev, A.D., Prabhu, S.D., Gozal, D. (2008). Postnatal intermittent hypoxia and developmental programming of hypertension in spontaneously hypertensive rats: the role of reactive oxygen species and L-Ca2+ channels. Hypertension. 52,156-62. doi: 10.1161/HYPERTENSIONAHA.108.110296.

Soylu AC, Levent E, Sarıman N, Yurtlu S, Alparslan S, Saygı A (2012). Obstructive sleep apnea syndrome and anthropometric obesity indexes. Sleep Breath. 16, 1151-8. doi: 10.1007/s11325-011-0623-9.

Suchecki, D., Tufik, S. (2000). Social stability attenuates the stress in the modified multiple platform method for paradoxical sleep deprivation in the rat. Physiol. Behav. 68, 309-16.

Sun W, Yin X, Wang Y, Tan Y, Cai L, Wang B, Cai J, Fu Y. (2012). Intermittent hypoxia-induced renal antioxidants and oxidative damage in male mice: hormetic dose response. Dose Response. 2012 Oct 1;11(3):385-400. doi: 10.2203/dose-response.

Sun, X., Luo, J., Xiao, Y. (2014). Continuous positive airway pressure is associated with a decrease in pulmonary artery pressure in patients with obstructive sleep apnoea: A meta-analysis. Respirology. doi: 10.1111/resp.12314.

Sunderram, J., Androulakis, I.P. (2012). Molecular mechanisms of chronic intermittent hypoxia and hypertension. Crit. Rev. Biomed. Eng. 40, 265-78.

Tahawi, Z., Orolinova, N., Joshua, I.G., Bader, M., Fletcher, E.C. (2001). Altered vascular reactivity in arterioles of chronic intermittent hypoxic rats. J. Appl. Physiol. 90, 2007–2013.

Tamisier, R., Gilmartin, G.S., Launois, S.H., Pépin, J.L., Nespoulet, H., Thomas, R., et al. (2009). A new model of chronic intermittent hypoxia in humans: effect on ventilation, sleep, and blood pressure. J. Appl. Physiol. 107, 17-24. doi: 10.1152/japplphysiol.91165.2008.

Tamisier, R., Pépin, J.L., Rémy, J., Baguet, J.P., Taylor, J.A., Weiss, J.W., et al. (2011). 14 nights of intermittent hypoxia elevate daytime blood pressure and sympathetic activity in healthy humans. Eur. Respir. J. 37, 119-28. doi: 10.1183/09031936.00204209.

Tanné, F., Gagnadoux, F., Chazouilleres, O., Fleury, B., Wendum, D., Lasnier, E., et al. (2005). Chronic liver injury during obstructive sleep apnea. Hepatology. 41,1290-1296.

Tartar, J.L., McKenna, J.T., Ward, C.P., McCarley, R.W., Strecker, R.E., Brown, R.E. (2010). Sleep fragmentation reduces hippocampal CA1 pyramidal cell excitability and response to adenosine. Neurosci. Lett. 469, 1-5. doi: 10.1016/j.neulet.2009.11.032.

Tilkian, A.G., Guilleminault, C., Schroeder, J.S., Lehrman, K.L., Simmons, F.B., Dement, W.C. (1976). Hemodynamics in sleep-induced apnea. Studies during wakefulness and sleep. Ann. Intern. Med. 85, 714-9.

Toth, L.A., Bhargava, P. (2013). Animal models of sleep disorders. Comp. Med. 63, 91-104.

Totoson, P., Fhayli, W., Faury, G., Korichneva, I., Cachot, S., Baldazza, M., et al. (2013). Atorvastatin protects against deleterious cardiovascular consequences induced by chronic intermittent hypoxia. Exp. Biol. Med. (Maywood). 238, 223-32. doi: 10.1177/1535370212473696.

Trimer R, Mendes RG, Costa FS, Sampaio LM, Delfino A Jr, Arena R, Aletti F, Ferrario M, Borghi-Silva A. (2014). Is there a chronic sleep stage-dependent linear and nonlinear cardiac autonomic impairment in obstructive sleep apnea? Sleep Breath. 18, 403-9. doi: 10.1007/s11325-013-0900-x.

Troncoso Brindeiro, C.M., da Silva, A.Q., Allahdadi, K.J., Youngblood, V., Kanagy, N.L. (2007). Reactive oxygen species contribute to sleep apnea-induced hypertension in rats. Am. J. Physiol. Heart Circ. Physiol. 293, H2971-6.

Tsioufis, C., Kasiakogias, A., Thomopoulos, C., Manolis, A., Stefanadis, C. (2010). Managing hypertension in obstructive sleep apnea: the interplay of continuous positive airway pressure, medication and chronotherapy. J. Hypertens. 28, 875-82. doi: 10.1097/HJH.0b013e328336ed85.

Tsioufis, C., Dimitriadis, K., Thomopoulos, C., Doumas, M., Papademetriou, V., Stefanadis, C. (2014). Renal and cardiac effects of renal sympathetic denervation and carotid baroreceptor stimulation. Curr. Vasc. Pharmacol. 12, 55-62.

Turner PV, Vaughn E, Sunohara-Neilson J, Ovari J, Leri, F. (2012). Oral gavage in rats: animal welfare evaluation. Journal of the American Association for Laboratory Animal Science 51, 25-30.

Uchida K, Nomura Y, Kadowaki M, Takase H, Takano K, Takeuchi N. (1978). Age-related changes in cholesterol and bile acid metabolism in rats. J Lipid Res. 19, 544-52.

Ukena, C., Mahfoud, F., Ewen, S., Cremers, B., Laufs, U., Böhm, M. (2013). Renal denervation in the treatment of hypertension. Curr. Hypertens. Rep. 15, 363-9. doi: 10.1007/s11906-013-0363-2.

Urban, D., Ewen, S., Ukena, C., Linz, D., Böhm, M., Mahfoud, F. (2013). Treating resistant hypertension: role of renal denervation. Integr. Blood Press. Control. 6, 119-128. doi: 10.2147/IBPC.S33958.

Vallianou NG, Evangelopoulos AA, Bountziouka V, et al (2013). Neck circumference is correlated with triglycerides and inversely related with HDL cholesterol beyond BMI and waist circumference. Diabetes Metab. Res. Rev. 29, 90-97. doi: 10.1002/dmrr.2369.

Van Dongen, H.P., Maislin, G., Mullington, J.M., Dinges, D.F. (2003). The cumulative cost of additional wakefulness: dose-response effects on neurobehavioral functions and sleep physiology from chronic sleep restriction and total sleep deprivation. Sleep. 26, 117-26.

Vanhoutte PM, Gao Y. (2013). Beta blockers, nitric oxide, and cardiovascular disease. Curr Opin Pharmacol. 13, 265-73. doi: 10.1016/j.coph.2012.12.002.

Varounis, C., Katsi, V., Kallikazaros, I.E., Tousoulis, D., Stefanadis, C., Parissis, J., et al. (2014). Effect of CPAP on blood pressure in patients with obstructive sleep apnea and resistant hypertension: A systematic review and meta-analysis. Int. J. Cardiol. 175, 95-8. doi: 10.1016/j.ijcard.2014.04.240.

Veasey SC, Guilleminault C, Strohl KP, Sanders MH, Ballard RD, Magalang UJ (2006). Medical therapy for obstructive sleep apnea: a review by the Medical Therapy for Obstructive Sleep Apnea Task Force of the Standards of Practice Committee of the American Academy of Sleep Medicine. Sleep. 29, 1036-44.

Vij AG, Kishore K, Dey J. (2012). Effect of intermittent hypobaric hypoxia on efficacy & clearance of drugs. Indian J Med Res. 2012;135:211-6.

Walker MK, Boberg JR, Walsh MT, Wolf V, Trujillo A, Duke MS, Palme R, Felton LA. (2012). A less stressful alternative to oral gavage for pharmacological and toxicological studies in mice. Toxicology and Applied Pharmacology 260, 65-9. doi: 10.1016/j.taap.2012.01.025.

Wang, T.J., Vasan, R.S. (2005). Epidemiology of uncontrolled hypertension in the United States. Circulation. 112, 1651-62.

Ward, C.P., McCoy, J.G., McKenna, J.T., Connolly, N.P., McCarley, R.W., Strecker, R.E. (2009). Spatial learning and memory deficits following exposure to 24 h of sleep fragmentation or intermittent hypoxia in a rat model of obstructive sleep apnea. Brain Res. 1294,128-37. doi: 10.1016/j.brainres.2009.07.064.

Witkowski, A., Prejbisz, A., Florczak, E., Kądziela, J., Śliwiński, P., Bieleń, P., et al. (2011). Effects of renal sympathetic denervation on blood pressure, sleep apnea course, and glycemic control in patients with resistant hypertension and sleep apnea. Hypertension. 58, 559-65. doi: 10.1161/HYPERTENSIONAHA.111.173799.

Wolf, J., Lewicka, J., Narkiewicz, K. (2007). Obstructive sleep apnea: an update on mechanisms and cardiovascular consequences. Nutr. Metab. Cardiovasc. Dis. 17:233-40.

Xie, A., Skatrud, J.B., Puleo, D.S., Morgan, B.J. (2001). Exposure to hypoxia produces long-lasting sympathetic activation in humans. J Appl Physiol. 91, 1555-62.

Yamamoto A, Katsumi H, Kusamori K, Sakane T. 2014. Improvement of intestinal absorption of poorly absorbable drugs by various sugar esters. Yakugaku Zasshi. 134, 47-53.

Yehuda, S., Sredni, B., Carasso, R.L., Kenigsbuch-Sredni, D. (2009). REM sleep deprivation in rats results in inflammation and interleukin-17 elevation. J. Interferon Cytokine Res. 29, 393-8. doi: 10.1089/jir.2008.0080.

Yeung PK, Jamieson A, Smith GJ, Fice D, Pollak PT. (2000). Determination of plasma concentrations of losartan in patients by HPLC using solid phase extraction and UV detection. International Journal of Pharmaceutics 204, 17-22.

Yorgun, H., Kabakçi, G., Canpolat, U., Kirmizigül, E., Sahiner, L., Ates, A.H., et al. (2014). Predictors of blood pressure reduction with nocturnal continuous positive airway pressure therapy in patients with obstructive sleep apnea and prehypertension. Angiology. 00, 1-6. doi: 10.1177/ 0003319713477908.

Young, T., Palta, M., Dempsey, J., Skatrud, J., Weber, S., Safwan, B. (1993). The occurrence of sleep-disordered breathing among middle-aged adults. N. Engl. J. Med. 328, 1230-5.

Young, T., Peppard, P., Palta, M., Hla, K.M., Finn, L., Morgan, B., et al. (2007). Population-based study of sleep-disordered breathing as a risk factor for hypertension. Arch. Intern. Med. 157, 1746-52.

Zhang, W., Si, L.Y. (2012). Obstructive sleep apnea syndrome (OSAS) and hypertension: pathogenic mechanisms and possible therapeutic approaches. Ups. J. Med. Sci. 117, 370-82. doi: 10.3109/03009734.2012.707253.

Zhao, Q., Liu, Z.H., Luo, Q., Zhao, Z.H., Zhang, H.L., Wang, Y. (2012) Effects of continuous positive airway pressure on blood pressure and daytime sleepiness in obstructive sleep apnea patients with coronary heart diseases under optimal medications. Sleep Breath. 16, 341-7. doi: 10.1007/s11325-011-0498-9.

Ziegler, M.G., Milic, M., Elayan, H. (2011a). Cardiovascular Regulation in Obstructive Sleep Apnea. Drug Discov. Today Dis. Models. 8, 155-160.

Ziegler, M.G., Milic, M., Sun, P. (2011b). Antihypertensive therapy for patients with obstructive sleep apnea. Curr. Opin. Nephrol. Hypertens. 20, 50-5. doi: 10.1097/MNH.0b013e3283402eb5.

Zoccal, D.B., Bonagamba, L.G., Oliveira, F.R., Antunes-Rodrigues, J., Machado, B.H. (2007). Increased sympathetic activity in rats submitted to chronic intermittent hypoxia. Exp. Physiol. 92, 79-85.

Zoccal, D.B., Bonagamba, L.G., Paton, J.F., Machado, B.H. (2009). Sympathetic-mediated hypertension of awake juvenile rats submitted to chronic intermittent hypoxia is not linked to baroreflex dysfunction. Exp. Physiol. 94, 972-83. doi: 10.1113/expphysiol.2009.048306.

Zoccal, D.B., Simms, A.E., Bonagamba, L.G., Braga, V.A., Pickering, A.E., Paton, J.F., et al. (2008). Increased sympathetic outflow in juvenile rats submitted to chronic intermittent hypoxia correlates with enhanced expiratory activity. J. Physiol. 586, 3253-65. doi: 10.1113/jphysiol.2008.154187.

Zou, D., Grote, L., Eder, D.N., Radlinski, J., Hedner, J. (2010). A double-blind, crossover study of Doxazosin and Enalapril on peripheral vascular tone and nocturnal blood pressure in sleep apnea patients. Sleep Med. 11, 325-8. doi: 10.1016/j.sleep.2009.10.004.

VII

ATTACHMENTS

ATTACHMENT #1

INFORMAÇÃO AO VOLUNTÁRIO DO ESTUDO

A. CONTEXTUALIZAÇÃO E OBJECTIVOS DO ESTUDO

Foi convidado a participar num estudo, da Faculdade de Ciências Médicas da Universidade Nova de Lisboa, intitulado: "Avaliação da terapêutica anti-hipertensora em doentes com Sindroma de Apneia Obstructiva do Sono". Neste documento ser-lhe-à facultada uma breve descrição do mesmo. Esta informação deverá ser lida cuidadosamente e caso tenha alguma dúvida adicional deverá esclarecê-la com o seu médico assistente.

A Sindroma de Apneia Obstructiva do Sono é uma morbilidade bastante frequente e que se encontra associada a outras patologias. É comum os doentes com apneia do sono evidenciarem hipertensão arterial diurna, daí que na sua maioria se encontrem medicados com fármacos anti-hipertensores. Neste sentido, o objectivo geral do estudo, em que o convidamos a participar, é justamente contribuir para a selecção, de entre os fármacos actualmente disponíveis, da terapêutica anti-hipertensora de eleição a implementar aos doentes com apneia do sono.

B. DESCRIÇÃO DOS PROCEDIMENTOS DO ESTUDO

O estudo irá decorrer nas consultas de Patologia do Sono efectuadas no Centro Hospitalar de Lisboa Norte (CHLN) e deverá envolver cerca de 100/150 participantes.

Numa primeira fase, ser-lhe-ão solicitados os seus dados sócio-demográficos (data de nascimento, sexo e raça), antropométricos (peso, altura, perímetro abdominal e perímetro do pescoço) e os dados relativos à presença de outras patologias, para além da apneia do sono. Nesta fase ser-lhe-á ainda solicitado a avaliação dos seus valores de pressão arterial. Esta avaliação será efectuada em sua casa por um período de 24 horas. O aparelho bem como todas as informações relativas ao seu funcionamento ser-lhe-ão transmitidas pelo seu médico ou por um técnico de saúde. O seu médico irá, igualmente, solicitar-lhe a realização de um estudo do sono, cujos dados obtidos serão também incluídos no estudo, e que traga todos os medicamentos que constam da sua medicação actual.

De acordo com os resultados obtidos através destes exames, o médico poderá prescrever-lhe a utilização de um aparelho denominado CPAP que utilizará à noite em sua casa, e a realização de nova medição dos valores de pressão arterial. Mais uma vez, estes dados serão recolhidos e incluídos no estudo.

É importante que saiba que, todas as avaliações a que vai ser sujeito são realizadas por rotina na consulta de patologia do sono da sua unidade de saúde, e, portanto, a sua participação no estudo não lhe exigirá qualquer vinda adicional ao hospital.

C. RISCOS E BENEFÍCIOS

O estudo não tem qualquer risco associado uma vez que apenas lhe pedimos que nos faculte a informação que atrás fizemos referência, e os exames a que vai ser sujeito não são invasivos ou dolorosos. O seu médico assistente está isento da responsabilidade por quaisquer danos relacionados com a sua deslocação às consultas de patologia do sono da sua unidade de saúde.

Relativamente aos benefícios, apesar de não haver um benefício directo ou a curto prazo para si, os resultados obtidos através deste estudo, poderão a longo prazo, contribuir para uma optimização da terapêutica anti-hipertensora em doentes com apneia do sono e, consequentemente, para um melhor controlo desta patologia. Pela sua participação neste estudo não lhe serão concedidos quaisquer benefícios financeiros.

D. CONFIDENCIALIDADE

A participação neste estudo vai ser mantida confidencial e o seu nome não será revelado a ninguém para além do seu médico assistente e do investigador envolvido no estudo. Todos os intervenientes no estudo estão sujeitos a confidencialidade. Se os resultados do estudo forem publicados em literatura médica, o seu nome não será em qualquer circunstância revelado.

E. BASE DE PARTICIPAÇÃO

A participação neste estudo é completamente voluntária, não sendo paga qualquer quantia pela sua contribuição no estudo. O seu médico assistente também não receberá nenhuma remuneração pela realização deste estudo. Pode recusar-se a participar ou desistir do estudo em qualquer altura, sem qualquer desvantagem ou perda de cuidados de saúde a que tenha direito. Qualquer dúvida que tenha poderá sempre esclarecê-la com o seu médico assistente.

DECLARAÇÃO DE CONSENTIMENTO INFORMADO

Declaro que li a informação anterior e compreendi o objectivo do estudo. Todas as minhas dúvidas à cerca do mesmo foram respondidas pelo meu médico assistente. Toda a informação verbal e escrita e as discussões sobre o estudo estão em Português, idioma em que sou fluente.

Concordo que os meus dados sejam registados e mais tarde possam vir a ser utilizados para publicação dos resultados do estudo. Sei que a minha identificação será mantida confidencial.

Após ter lido cuidadosamente, ter-me sido completamente explicada a informação anterior e ter reflectido, a minha assinatura, abaixo, indica que consinto voluntariamente participar neste estudo.

_____ Data : ___ /___ /___

Assinatura do Doente

_____ Data : ___ /___ /___

Assinatura do Clínico assistente

ATTACHMENT #2

Caderno de recolha de dados

Centro nº | 0 | 1 |

Data da consulta |__|__| |__|__| |__|__|
dia mês ano

Visita nº | 1 |

Etiqueta CHLN identificativa do doente

DADOS do DOENTE

1. Pessoais

Altura (m) |__|,|__|__|

Peso (kg) |__|__|__|

Raça: Caucasiana ☐ Negra ☐ outra_____

Perimetro do pescoço (cm) |__|__|

Perimetro abdominal (cm) |__|__|__|

Hábitos tabágicos? Não fumador ☐ Ex-fumador ☐ Fumador ☐ UMA |__|__|__|

Café? Não ☐ Sim ☐ nº cafés: |__|__|

Hipertensão? Não ☐ Sim ☐

2. Co-morbilidades

Diabetes _____ ☐ desde | | | | | |
mês ano

Dislipidemia _____ ☐ desde | | | | | |
mês ano

DPOC _____ ☐ desde | | | | | |
mês ano

Asma _____ ☐ desde | | | | | |
mês ano

Hipotiroidismo _____ ☐ desde | | | | | |
mês ano

Cardiopatia isquémica _____ ☐ desde | | | | | |
mês ano

Arritmias _____ ☐ desde | | | | | |
mês ano

Outras ☐ Qual? _____ desde | | | | | |
mês ano

Acidentes de viação por ter adormecido ☐ nº _____

Caderno de recolha de dados

Centro nº | 0 | 1 |

Data da consulta |__|__| |__|__| |__|__|
dia *mês* *ano*

Visita nº | 1 |

Etiqueta CHLN identificativa do doente

3. Folheto de Pedido de medicação

	Sim	Não
Foi entregue o folheto de pedido de medicação ao doente?	☐	☐

4. Consentimento informado

	Sim	Não
O doente assinou o consentimento informado?	☐	☐

5. Pedido de MAPA

	Sim	Não
Foi solicitado ao doente a realização de MAPA?	☐	☐

6. Pedido de Polissonografia

	Sim	Não
Foi solicitado ao doente a realização de Polissonografia?	☐	☐

ATTACHMENT #3

Caderno de recolha de dados

Centro nº | 0 | 1 |

Data da consulta |__|__||__|__||__|__|
dia mês ano

Visita nº | 2 |

Etiqueta CHLN identificativa do doente

DADOS do DOENTE

1. Pedido de medicação

	Sim	Não
O doente trouxe consigo a medicação que está a tomar?	☐	☐

2. Terapêutica actual

Fármaco (DCI)	mg por toma	Nº de tomas	Mais de 6 meses?
	|__|__|__|__|	|__|__|	☐
	|__|__|__|__|	|__|__|	☐
	|__|__|__|__|	|__|__|	☐
	|__|__|__|__|	|__|__|	☐
	|__|__|__|__|	|__|__|	☐
	|__|__|__|__|	|__|__|	☐
	|__|__|__|__|	|__|__|	☐
	|__|__|__|__|	|__|__|	☐
	|__|__|__|__|	|__|__|	☐
	|__|__|__|__|	|__|__|	☐
	|__|__|__|__|	|__|__|	☐
	|__|__|__|__|	|__|__|	☐

Caderno de recolha de dados

Centro nº | 0 | 1 |

Data da consulta |___|___| |___|___| |___|___|
dia *mês* *ano*

Visita nº | 2 |

Etiqueta CHLN identificativa do doente

Fármaco (DCI)	**mg por toma**	**Nº de tomas**	**Mais de 6 meses?**
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐
	|__|__|__|__|	|__|	☐

Avaliação da Terapêutica anti-hipertensora em doentes com OSA
Página 2 de 3

ATTACHMENTS

Caderno de recolha de dados

Centro nº | 0 | 1 |

Data da consulta |___|___| |___|___| |___|___|
dia *mês* *ano*

Visita nº | 2 |

Etiqueta CHLN identificativa do doente

3. Tipo de Doente

Sem diagnóstico de OSA ☐

Diagnóstico de OSA sem indicação para implementação de CPAP ☐

Diagnóstico de OSA com indicação para implementação de CPAP ☐

4. Estudo Polissonográfico do Sono

IAH (eventos/h): _____

Classificação da SAOS/ Grau: Ligeiro ☐ Moderado ☐ Grave ☐

ATTACHMENT #4

Caderno de recolha de dados

Centro nº | 0 | 1 |

Data da consulta |__|__| |__|__| |__|__|
dia *mês* *ano*

Visita nº | 3 |

Etiqueta CHLN identificativa do doente

DADOS do DOENTE

1. CPAP

Modelo utilizado: _____

Data de aplicação |__|__|__|__|__|__|
dia *mês* *ano*

Adesão (h): _____

Pressão 95%: _____

Fuga 95%: _____

Pressão media (cmH_2O): _____

IAH (eventos/h): _____

2. Pedido de MAPA

	Sim	Não
Foi solicitado ao doente a realização de MAPA?	☐	☐

3. Medicação

	Sim	Não
O doente mantém a medicação?	☐	☐