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Alexander Michael Ribeiro Santos

Transthyretin and Obstructive Sleep Apnea

Dissertação no âmbito do Mestrado em Química Medicinal,
orientada pela Professora Doutora Cláudia Cavadas e pela Doutora Ana Rita Álvaro e pelo Professor Doutor Rui Brito,
e apresentada ao Departamento de Química da Faculdade de Ciências e Tecnologia
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TRANSTHYRETIN AND OBSTRUCTIVE SLEEP APNEA

Dissertation under the scientific supervision of Ana Rita Álvaro (PhD), Professor Cláudia Cavadas (PhD), and Professor Rui Brito (PhD), presented to the Department of Chemistry of the Faculty of Sciences and Technology of the University of Coimbra, in fulfilment of the requirements for a Master degree in Medicinal Chemistry

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This research was performed in the Neuroendocrinology & Aging Group at the Center for Neuroscience and Cell Biology, University of Coimbra, Portugal, under the supervision of Ana Rita Costa Silva Álvaro, Professor Cláudia Margarida Gonçalves Cavadas, and Professor Rui Brito.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abeta	Amyloidβ
AD	Alzheimer's Disease
ACTH	Adrenocorticotropic Hormone
AHI	Apnea-Hypopnea Index
AL	Light-chain Amyloidosis
ANOVA	Analysis of Variance
APP	Amyloid Precursor Protein
ATTR	Transthyretin Amyloidosis
BBB	Blood-Brain Barrier
BMI	Body Mass Index
CA	Cardiac Amyloidosis
CPEC	Choroid Plexus Epithelial Cells
CPAP	Continuous Positive Airway Pressure
CRP	C-reactive Protein
CSF	Cerebral Spinal Fluid
DM2	Type-2 Diabetes Mellitus
DSM-V	Diagnostic Statistical Manual, Fifth Edition
EEG	Electroencephalogram
ELISA	Enzyme-Linked Immunosorbent Assay
EMG	Electromyogram
EOG	Electrooculogram
FAP	Familial Amyloidal Polyneuropathy
GC	Glucocorticoid
HbA1c	Hemoglobin A1c
HP	Hypothalamic-Pituitary
HPA	Hypothalamic-Pituitary-Adrenal
JAK	Janus-Kinase
JAK2	Janus-Kinase 2
N1	Non-Rapid Eye Movement Stage 1

N2	Non-Rapid Eye Movement Stage 2
N3	Non-Rapid Eye Movement Stage 3
NPY	Neuropeptide Y
NREM	Non-Rapid Eye Movement
OA	Osteoarthritis
OSA	Obstructive Sleep Apnea
PBS	Phosphate-Buffered Saline
PnPP	p-Nitrophenyl Phosphate
PSG	Polysomnography
PVN	Paraventricular Nucleus
RBP	Retinol-Binding Protein
RBP4	Human Retinol-Binding Protein
RCC	Retinol Circulating Complex
REM	Rapid Eye Movement
RNAi	Ribonucleic Acid interference
SCA	Senile Cardiac Amyloidosis
SCN	Superchiasmatic Nucleus
SEM	Standard Error of the Mean
SPZ	Subparaventricular Zone
SSA	Senile Systemic Amyloidosis
STAT	
STOP-BANG	Snoring, Tiredness, Observed apnea, high blood Pressure-Body mass index, Age, Neck circumference, Gender
STRA6	Stimulated by Retinoic Acid 6 ; Retinol-binding protein receptor
T4	Thyroxine
TBS	Tris-Buffered Saline
TTR	Transthyretin
WT-TTR	Wild-Type Transthyretin

Abstract

Obstructive Sleep Apnea (OSA) is characterized by recurring instances of upper airway obstruction during sleep, being most prevalent in men and in older age groups. Despite being one of the most common sleeping disorders, OSA is still highly undiagnosed, and when untreated is a risk factor for the development of several metabolic, cardiovascular, and age-related diseases. There is currently an urgent need to identify OSA biomarkers and therapeutic targets. Transthyretin (TTR) has implicated in the pathophysiology of several commonly comorbid conditions, and has previously been shown to be elevated in the blood serum of patients with OSA. In this context, the present work has three main aims: 1) to evaluate the impact of OSA and aging on TTR blood plasma concentrations throughout the day; 2) to evaluate the impact of CPAP therapy on TTR blood plasma concentrations in OSA patients throughout the day; 3) to investigate the potential utility of TTR as a biomarker for OSA.

The present study investigated diurnal patterns of plasma TTR concentrations in untreated OSA patients, healthy age-matched control subjects, and healthy young control subjects. This was accomplished by analyzing plasma at four timepoints throughout the day (8h, 11h, 16h30, and 22h30) through enzyme-linked immunosorbent assays (ELISAs). In the OSA cohort, this analysis was repeated following diagnosis after 4 months and 24 months of continuous positive airway pressure (CPAP) therapy.

Results indicated that healthy adults have distinct fluctuations in plasma TTR concentrations throughout the day, and that patients with OSA feature markedly flattened diurnal TTR curves in comparison. It was observed that average daily TTR concentrations, as well as TTR concentrations at 8h in the morning, decreased significantly in OSA patients after 24 months of adherent CPAP treatment. Additionally, in age-matched controls, diurnal fluctuations of TTR were phase shifted and daily TTR concentrations were significantly lower when compared to young controls.

With the aforementioned findings in consideration, plasma TTR could be utilized as a biomarker for OSA both as a diagnostic tool, and as a parameter to evaluate therapy outcomes. Further investigation may elucidate the specific role TTR plays in the underlying pathophysiology of OSA and process of aging.

Keywords: Obstructive Sleep Apnea, Transthyretin, ELISA, Biomarkers, CPAP

Resumo

A apneia obstrutiva do sono (AOS) é caracterizada por casos recorrentes de obstrução das vias aéreas superiores durante o sono, sendo mais prevalente em homens e grupos etários mais velhos. Apesar de ser um dos distúrbios do sono mais comuns, a AOS ainda é altamente sub-diagnosticada e, quando não tratada, é um fator de risco para o desenvolvimento de várias doenças metabólicas, cardiovasculares e relacionadas à idade. Atualmente, existe uma necessidade urgente de identificar biomarcadores de AOS e novos alvos terapêuticos para esta doença. A Transtirretina (TTR) é uma molécula envolvida na fisiopatologia de várias comorbidades e estudos recentes demonstraram estar elevada no soro sanguíneo de doentes com AOS. Neste contexto, o presente trabalho teve três objetivos principais: 1) avaliar o impacto da AOS e do envelhecimento nas concentrações plasmáticas de TTR ao longo do dia; 2) avaliar o impacto da terapia com CPAP nas concentrações plasmáticas de TTR no doente com AOS ao longo do dia; 3) investigar potencial da TTR como um biomarcador para AOS.

O presente estudo investigou padrões diurnos das concentrações plasmáticas de TTR em doentes com AOS não tratados, indivíduos saudáveis com controlo de idade e indivíduos saudáveis jovens. Esta análise foi realizada recolhendo o plasma em quatro momentos ao longo do dia (8h, 11h, 16h30 e 22h30) através de ensaios imunoabsorventes ligados a enzimas (ELISA). Na coorte da AOS, esta análise foi repetida após o diagnóstico, após 4 meses e 24 meses de terapia com continuous positive airway pressure (CPAP).

Os resultados indicaram que adultos saudáveis apresentam flutuações distintas nas concentrações plasmáticas de TTR ao longo do dia e que doentes com AOS apresentam curvas diurnas acentuadamente achatadas de TTR em comparação com controlos saudáveis. Observou-se que as concentrações médias diárias de TTR, bem como as concentrações de TTR às 8h da manhã, diminuíram significativamente em doentes com AOS após 24 meses de tratamento com CPAP. Além disso, em controlos da mesma idade, as flutuações diurnas do TTR sofreram um avanço de fase e as concentrações diárias de TTR foram significativamente menores quando comparadas com os controlos jovens.

Em suma, estes resultados sugerem que a TTR plasmática poderá constituir um potencial biomarcador para a AOS, bem como uma ferramenta de diagnóstico e/ou um parâmetro para avaliar a eficácia da terapêutica. No entanto, estudos adicionais serão necessários para elucidar o papel específico da TTR na fisiopatologia da AOS e no processo de envelhecimento fisiológico.

Palavras-chave: Obstructive Sleep Apnea, Transthyretin, ELISA, Biomarkers, CPAP

Chapter 1: Introduction

1.1. Circadian Rhythms

1.1.1. Circadian Rhythms: an overview

The large majority of humans define their daily schedules within 24-hour diurnal cycles, wherein periods of activity and inactivity are thought to be mostly modulated by time of day, and a correlative variation in light exposure (Maierova et al., 2016). While this is a common construct many are accustomed to, there is a evolutionarily conserved, and molecular basis to of this phenomena. Mammalia have evolved a biological clock localized to the anterior hypothalamus, referred to as superchiasmatic nuclei (SCN) (Hannibal, 2002). The SCN is largely referred to as a physiological 'master clock,' responsible for the synchronization and regulation of behavioral, homeostatic, and metabolic processes (Scheer, Morris & Shea, 2013).

Subordinate to the SCN, there exist site-specific cellular clocks localized in peripheral tissues and organs. These peripheral clocks are unresponsive to light/dark cycles, and rely on input from SCN to remain in synch with environmental and physiological demands. For the most part, the SCN communicates with subordinate peripheral clocks through the release of diffusible factors. These factors will interact with specialized areas throughout the ventricular system and lead to a rhythmic neuronal and hormonal signaling cascade (Oster et al., 2006). Most hormones have been reported to display distinct circadian profiles, and it is for this reason that the importance of peripheral clocks as pacemakers and modulators for hormonal homeostasis and metabolism should not be understated (Philippe & Dibner, 2015).

1.1.2. Circadian Endocrinology

A defining feature of circadian biology are endocrine factors, most of which exhibit time-of-day dependence, and circadian rhythmicity (Carili & Farabi, 2016). In this context, endocrine factors regulated by hypothalamic-pituitary (HP) axes are among the most thoroughly studied. The HP complex bridges the nervous and endocrine systems, and at its core exists a signaling cascade wherein signals from the hypothalamus will regulate the release of anterior pituitary hormones. This HP pathway is able to induce downstream signaling, often targeting peripheral tissues and organs such as the adrenal and thyroid glands. When brought into the fold, these peripheral organs

become part of an increasingly complex, time-integrated, feedback loop (Ikegami & Yoshimura, 2017). These reciprocating feedback loops, and their constitutional signaling cascades, exist along several distinct HP axes (Chrousos, 2000). Many such endocrine factors are key players in tightly regulated, temporally specific, feedback loops. Along one particular axis, the Hypothalamus-Pituitary-Adrenal (HPA) axis, modulatory factors include, though are not limited to, cortisol, Abeta, neuropeptide Y (NPY), retinoic acid, and insulin (Gamble et al., 2014; Hirsch & Zukowska, 2012; Pallet & Touyarot, 2015; LeSauter et al., 2009; Morgese et al., 2014).

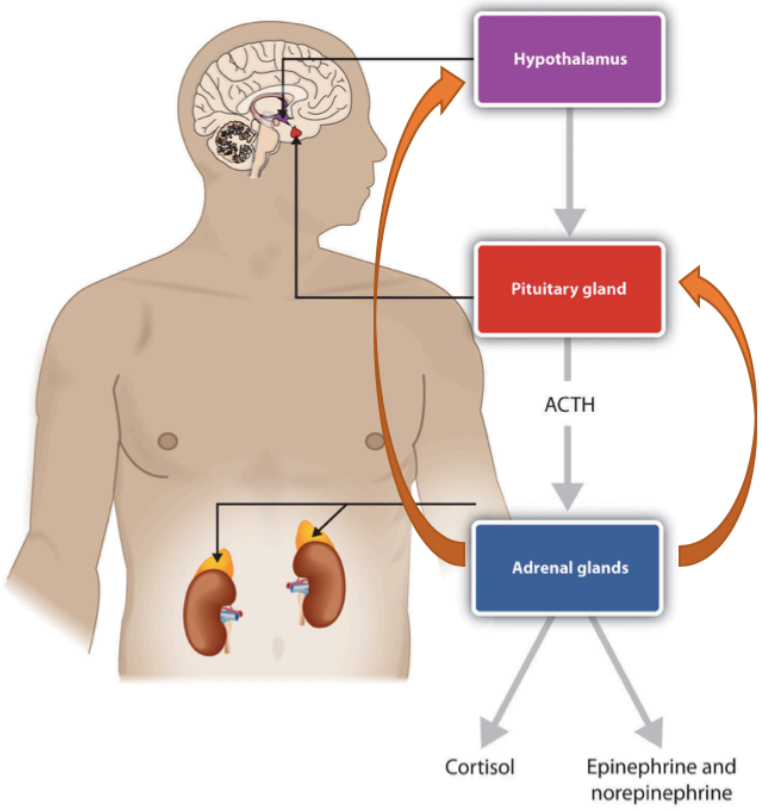


Figure 1. Schema depicting the HPA axis.
The HPA axis is comprised of three main components: the hypothalamus, the anterior pituitary, and the adrenal cortex. The HPA axis is largely dependent on the circadian rhythm, featuring diurnal variations marked by periods of higher and lower activity. The HPA axis is sensitive to input from each of its three components, and features a complex feedback loop along its signaling cascade (Chrousos, 2000). (Image adapted from BC Open Textbooks)

The HPA axis is a multifaceted neuroendocrine complex comprised of three units; the hypothalamus, the pituitary gland, and the adrenal gland. The HPA axis oscillates in time with the adrenal's circadian peripheral clock, featuring periods of higher and lower activity in a cyclical, diurnal manner (Chung et al., 2011). The HPA signal cascade initiates in the anterior pituitary, where corticotrophs will secrete adrenocorticotropic hormone (ACTH). Upon activation by

ACTH, melanocortin 2 receptors initiate a cascade of enzyme-mediated reactions resulting in the conversion of cholesterol into cortisol (Nader et al., 2010) (figure 1).

The primary end product of the HPA axis is the human glucocorticoid (GC) cortisol, and when assessing HPA axis activity we often measure cortisol concentrations in physiological media, such as serum, plasma, and saliva (Vining, McGinley, & 1983; Krsljak & Gosic, 2008). Cortisol is one of the most commonly used and researched biomarkers for stress (Joseph & Golden, 2018). It is important to consider that plasma levels of most hormones exhibit marked 24-hour rhythms, cortisol being no exception. This diurnal pattern of cortisol concentration appears to be as a result of circadian influences, though the HPA axis is also in-part modulated by the ultradian rhythm and stimuli (Carili & Farabi, 2016; Spencer & Deak, 2017). A variety of different stimuli are able entrain and modulate HPA activity. Perhaps the most prominent stimulus-induced effects displayed by the HPA are meal-induced spikes in cortisol. In an average healthy individual the diurnal cortisol cycle has been characterized by a rapid rise prior to awakening followed by a daily zenith shortly after awakening, a gradual decline throughout waking hours, and slight peaks in response to midday and evening meals (Lovallo, 2006; Vargas et la., 2018) (figure 2).

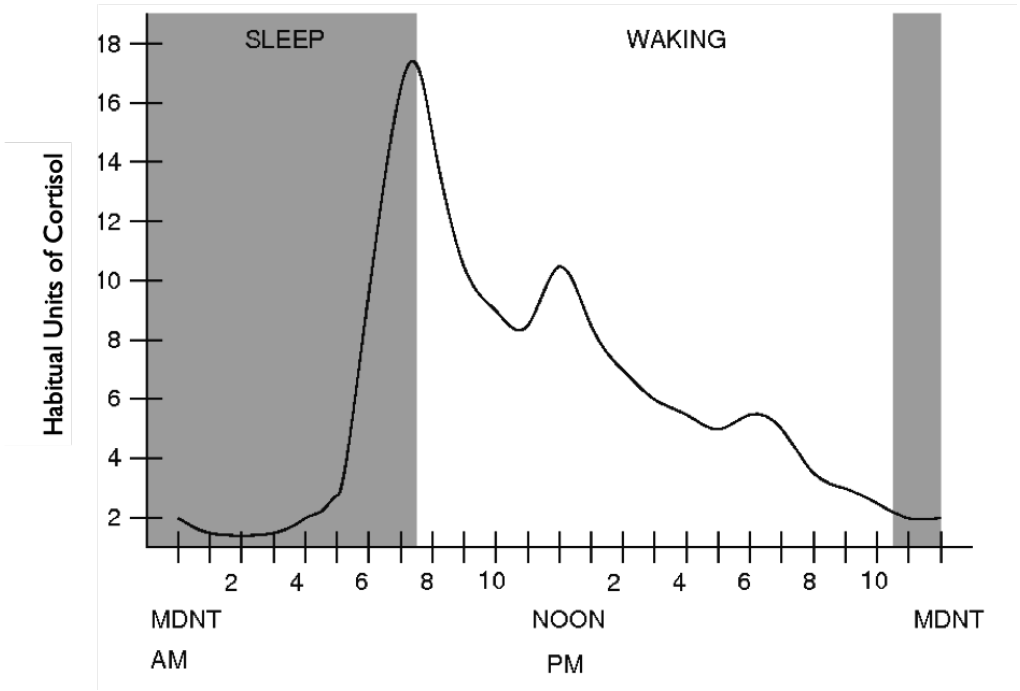


Figure 2. Depiction of normal diurnal blood cortisol fluctuations throughout the day.
Daily cycle of cortisol release is characterized by a rapid rise prior to awakening and a peak shortly after awakening, a gradual decline throughout waking hours, and slight peaks induced by midday and evening meals. (Image adapted from Lovallo, 2006)

Dysregulation or alterations in the HPA axis and its diurnal cortisol curve has been known to be characteristic in several pathological states such as obesity, depression and anxiety, obesity and type 2 diabetes mellitus (DM2). For example, those with DM2 often feature a flattening of the diurnal cortisol curve (Tu et al., 2013; Doane et al., 2013; Hek et al., 2013; Hajat et al., 2013; Patterson et al., 2013; Schrepf et al., 2014; Hackett, Steptoe & Kumari, 2014). Patients suffering from depression also display flattened diurnal cortisol curves, which is also accompanied by a blunting of the cortisol awakening response (Joseph & Golden, 2018). All this considered, it is important to reiterate that hierarchically superior to these processes the SCN, and thus the circadian rhythm, have direct and indirect roles in diurnal glucose metabolism, insulin sensitivity, glucose tolerance, and sleep-wake behavior (Stenvers et al., 2019; Carmo-Silva & Cavadas, 2017).

1.1.3. Sleep

Though often thought of as a period of inactivity and time lost, sleep is an active physiological process which can be characterized by distinct hormonal patterns, and subsequent influences in metabolic function and glucose homeostasis (Steiger, 2003; Morselli, 2012). Though the full spectrum of functions for which sleep is responsible for is yet to be understood, sleep may be clinically monitored through the use of several apparatuses, namely, the electroencephalogram (EEG), the electrocardiogram (EKG), the electrooculogram (EOG), and the Polysomnography (PSG) (Burns et al., 2007). When studying sleep, several variables can be quantified in order to characterize sleeping, such as, but not limited to, latency to sleep onset, the duration of the various stages of sleep, the relative contribution of sleep stages to total sleep time, and the transition between sleep stages (Dijk & Landolt, 2019). From a clinical perspective, the nature of sleep can be broadly differentiated between two periods of ocular activity, referred to simply as rapid eye movement (REM) sleep and non-rapid eye movement (NREM) sleep.

Throughout a normal night of sleep, humans will typically advance through four increasingly deep stages of sleep. The first three stages of sleep are considered subsets of NREM, and have been defined as sleep stages N1, N2, and N3. Following the first three NREM stages, those will healthy sleep patterns will then continue into the fourth and final stage: REM sleep. The sequential nature

of this defined sleeping pattern has been well characterized by EEG, and a progressive decrease in frequency can be seen even before the initiation of the lightest phase of sleep, N1, all the way through to the REM phase (figure 3).

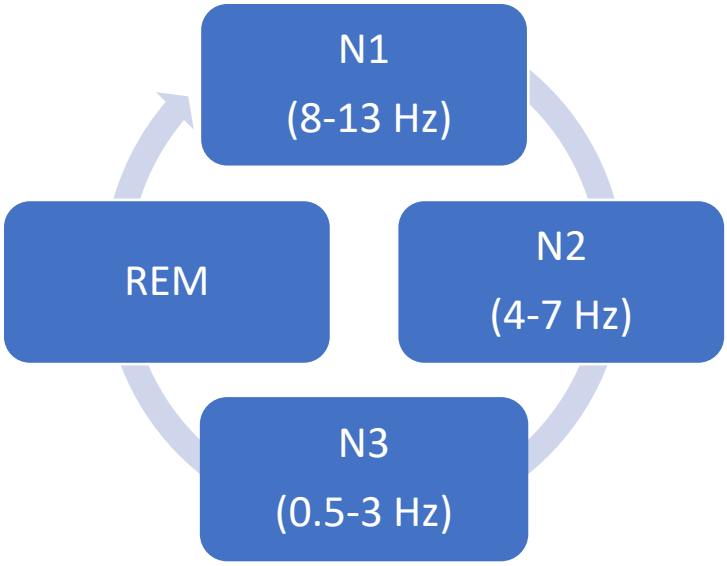


Figure 3. Schema of normal sleep architecture.
The sequential nature of this defined sleeping pattern has been well characterized by EEG, and a progressive decrease in frequency can be seen even before the initiation of the lightest phase of sleep, N1, all the way through N3. REM sleep does not follow the same pattern of descending frequency depicting by the N1-N3 NREM sleep sequence. Instead, REM sleep is associated with sharp theta waves, referred to as sawtooth waves (Carley & Farabi, 2016).

It appears that stress, sleep disturbance, and circadian disruption share many common molecular signaling and anatomic pathways (Phan & Malkani, 2019). While a thorough understanding of sleep and its purposes are unknown, the phenomenon has been found to be regulated through distinct homeostatic, allostatic, and circadian processes. Regulation of sleep by the circadian stems from the SCN. In healthy individuals the SCN is regulated during the day by photic input received by retinal ganglion cells and their secretion of melanopsin, and during the night via melatonin secretion from the pineal gland. While the SCN does feature modest projections to the ventrolateral preoptic nucleus, the majority of output from the SCN is directed towards the subparaventricular zone (SPZ). From the SPZ, there have been four defined channels of circadian output, specifically the dorsolateral, ventrolateral, dorsomedial, and ventromedial quadrants of the SPZ (Vujovic et al., 2015).

There exist more than 80 distinct sleep disorders as classified by the International Classification of Sleep disorders. Of them all, Obstructive Sleep Apnea is among the most common (Ong & Crawford, 2014).

1.2. Obstructive Sleep Apnea

1.2.1. Obstructive Sleep Apnea: Overview and Epidemiology

According to the Diagnostic Statistical Manual, Fifth Edition (DSM-V), breathing-related sleep disorders are classified as one of ten distinct sleeping disorders. Breathing-related sleep disorders can be further classified into four main categories: obstructive sleep apnea (OSA), central sleep apnea, sleep-related hypoventilation disorders, and sleep-related hypoxemia disorders. Of these four disorders, OSA is the most common, and can be clinically differentiated between adult and pediatric subtypes (Thorpy, 2017). The clinical significance of sleep stages in OSA has been acknowledged, and increasingly well-characterized. In OSA, sleep is fragmented by frequent arousal during N1 sleep, and a decrease in N3 and REM sleep (Basunia et al., 2016). It should be noted that though increased sympathetic activity is widely accepted to mediate the relationship between OSA and cardiovascular comorbidities, out of all stages of sleep, REM features the highest sympathetic activity (Alzoubaidi & Mokhlesi, 2017; Kohler & Stradling, 2012)

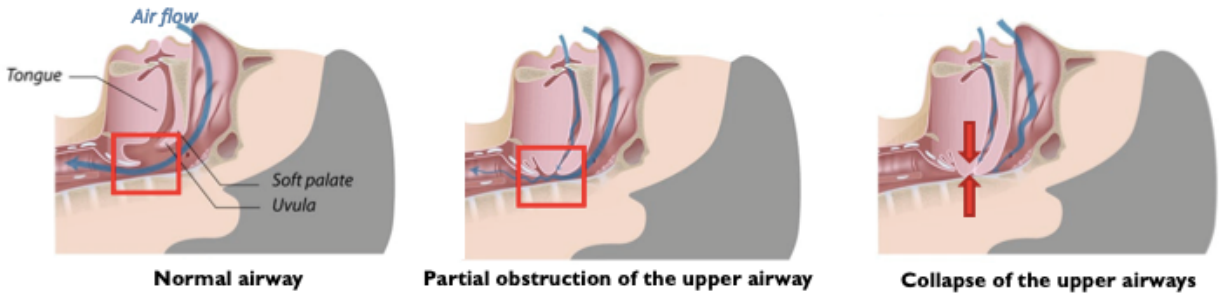


Figure 4. Anatomical depictions of healthy (unobstructed), partially obstructed airways, and completely obstructed airways.
OSA patients may present with partially occluded airways (hypopnea) which restricts airflow, or entirely occluded airways (apnea) which prevents airflow. Both instances of hypopnea and apnea affects normal respiratory function while sleeping. (image adapted from Shutterstock.com)

OSA is characterized by recurring instances of upper airway obstruction during sleep (Pham & Schwartz, 2015) (figure 4). These obstructive episodes may partially or entirely occlude the upper

airways and can occur throughout any stage of sleep, though recent findings have elucidated that the most severe instances of obstructive episodes occur during REM sleep stage (Kim et al., 2019). Skeletal muscle activity, though reduced when compared to wakeful states, are more active during N1 phase when compared to N2 or N3. The propensity for obstructive episodes during the REM stage may be in part due to the near complete loss of skeletal muscles tone, or atonia (McCarte, St. Louis, & Boeve, 2013; Alzoubaidi & Mokhlesi, 2017). Hypopnea is characterized as a reduction in airflow by at least 30 % for at least ten seconds with decrease in oxygen saturation, while apnea is defined as a cessation of airflow for at least ten seconds. The length of apneic or hypopneic events may last anywhere from ten seconds to over a minute, and the frequency of these episodes vary greatly from case to case (Jonas et al., 2017). These instances of hypopnea and apnea may result in episodic hypoxemia, nocturnal sympathetic nervous system activation, increased blood pressure, and elevated levels of oxidative stress, inflammation, and hypercoagulation (McEvoy et al., 2016).

OSA also negatively affects psychological functioning, as patients often report depression, anxiety, impaired cognitive capacity, and headaches (Krysta et al., 2016). Due to over-activity in the sympathetic nervous system, sleep fragmentation is a common feature of OSA, and patients can be highly debilitated by daytime fatigue and sleepiness as a result (Garvey et al., 2015). Remarkably, it has been found that OSA patients are about two to ten times more likely to be involved in a motor vehicle crash when compared to drivers without OSA (Ellen et al., 2006; Ayas et al., 2006). Furthermore, these OSA-related motor vehicle accidents have been estimated to involve 800,000 drivers and accumulate expenditures of up to $15.9 billion every year (Sassani et al., 2004). These findings not only illustrate the extent to which OSA may impair daily functioning in patients, but also how this disease may affect the general population.

Prevalence estimations for OSA vary greatly, as up to 90 % of individuals with OSA remain largely underdiagnosed and untreated, however, there is evidence that around one billion adults may be afflicted by OSA globally (Fleming et al., 2018; Benjafield et al., 2018). While the true prevalence of this disease may be currently elusive, several noticeable epidemiological trends have further characterized the nature of OSA. For example, adult men are twice as likely to suffer from OSA as adult women, and the likelihood of suffering from OSA has been known to increase with age,

being most prevalent between the ages of 45 and 64 years (Lin et al., 2008; Ayalon, Ancoli-Israel, & Drummond, 2010; Bixler et al., 1998).

1.2.2. Obstructive Sleep Apnea: Diagnosis and Treatment

Polysomnography (PSG) exams are the standard diagnostic tool used to diagnose sleep disorders, OSA included. PSG exams are typically performed at inpatient facilities or at a specialized sleep centers, and are usually performed at night to record night time sleeping patterns. This test will monitor brain activity, blood oxygen levels, heart rate, breathing rate, and eye and leg movements in order to best characterize not only if there is sleep disturbance, but also the nature of the disturbance (Madani et al., 2007; Roebuck et al., 2014). From the PSG exams, the severity of OSA can be determined by utilizing the Apnea-Hypopnea Index (AHI) and oxygen desaturation as parameters. The AHI is based on a integer scale, ranging in values from zero to greater than 30. The AHI measures the incidence of apneas and hypopneas, and provides an integer in order to diagnostically gauge the extent of OSA, and further define cases as either mild ($5 \leq AHI < 15$), moderate ($15 \leq AHI < 30$), or severe ($AHI \geq 30$) (Jonas et al., 2017).

While a PSG exam is critical for identifying cases of OSA, a comprehensive diagnosis requires the integration of several different screening tools. Questionnaires have proven to have of great utility in this respect, and continue to contribute greatly to our understanding of this disease. In particular, the STOP-BANG questionnaire has proven to have the highest methodological quality (Abrishami, Khajehdehi, & Chung, 2010). The STOP-BANG takes into consideration Snoring, Tiredness, Observed apnea, and high blood Pressure (STOP), as well as Body mass index, Age, Neck circumference, and Gender (BANG). Other screening tools may compliment the STOP-BANG, and have great clinical utility considering the variability in a patient's presentation of the disease. These may include, but are not limited to, the Epworth Sleepiness Scale, The Pittsburg Sleep Quality Index, and the Munich Chronotype Questionnaire (Mondal et al., 2013; Kim et al., 2015).

While revolutionary in diagnosing and characterizing OSA, the PSG comes with its own set of barriers. Several barriers include issues sleeping in strange and unfamiliar environments, unfamiliarity with study protocols, among others (Shaw et al., 2012). Additionally, while an

overnight PSG is able to obtain a variety of important neurophysiological signals, most data collected is ignored, and treatment decisions are largely informed by the AHI, which have their own limitations. For example, a patient with long respiratory events may experience severe hypoxemia but be defined by low AHI scores, while another patient with more frequent events, and higher AHI scores, may only be afflicted by comparably mild hypoxemia (Osman et al., 2018).

Once diagnosed, treatment approaches for OSA may include surgical intervention depending on the anatomical abnormalities unique to each respective case, though Continuous Positive Airway Pressure (CPAP) is the first-line treatment for most cases of OSA (Pavwoski & Shelgikar, 2017). The CPAP approach involves the ejection of air into the upper airway, in turn preventing pharyngeal collapse, and improving airway patency while sleeping (figure 5). Through preventing airway occlusions and providing a constant flow of air, CPAP reduces incidents of hypoxia, sleep fragmentation, and normalizes nocturnal blood-oxygen saturation, thereby improving the maladaptive effects of OSA (Ignacio-Alcantara, Espiritu-Picar, & Ledesma, 2013). CPAP is currently the most therapeutically- and cost-effective treatment for this disease, and CPAP therapy for OSA patients may potentially to reduce health care costs and recurrent hospitalizations (Donovan & Billings, 2018).

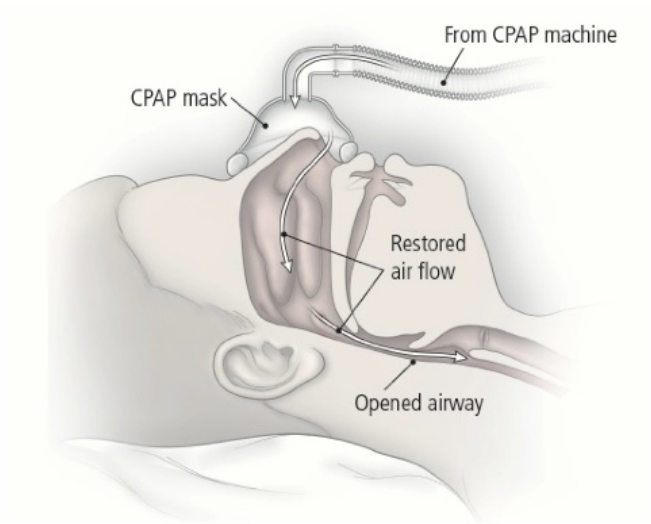


Figure 5. Depiction of CPAP apparatus and affected anatomy.
CPAP devices work by injecting a continuous flow of air into the upper airway of patients during sleep. Positive pressure prevents the upper airway from occluding during all stages of sleep. While effective in preventing instances of hypopnea and apnea, CPAP does not cure the underlying pathophysiological mechanisms of OSA, and requires nightly use. It is recommended that CPAPs are used for at least four hours daily (image adapted from Harvard Health Publishing).

CPAP therapy has its own barriers and inconveniences, however, especially as it relates to therapy toleration and adherence. For example, most patients with comorbid dementia are unable to

tolerate nocturnal CPAP treatment, a finding which is especially concerning considering 40-70 % of patients with Alzheimer's Disease (AD) experience five or more incidents of apnea-hypopnea per hour of sleep (Peter-Derex et al., 2014). This issue with adherence in CPAP can be seen across other demographic groups as well. In one study following women with OSA, it was found that only half of these patients continued their daily CPAP regimens two years after diagnosis and prescription of CPAP therapy (Libman et al., 2017). Nonadherence to CPAP therapy may be in part due to tolerability, as apparatuses can be uncomfortable and inconvenient.

1.2.3. Obstructive Sleep Apnea & Comorbid Conditions

Patients suffering from OSA, are often afflicted by various comorbid conditions. Among the most prevalent conditions being obesity, hypertension, Diabetes Mellitus, hyperlipidemia, and psychological disorders such as anxiety and depression (Sunwoo et la., 2018; Pinto et al., 2016). OSA's relationship with cardiovascular diseases is profound, as the disease has been associated with increased incidence of stroke, heart failure, atrial fibrillation, coronary artery disease, and increased all-cause mortality rate (Javaheri et al., 2017). There is robust evidence indicating that OSA has an independent role in hypertension. For example, one study indicated that patients with OSA eligible for CPAP treatment were about three times more likely to be diagnosed with hypertension over a four-year follow-up period (Marin et al., 2015). These associations are further supported by a multinational study which found that OSA severity indices, such as oxyhemoglobin desaturation index, were strong independent predictors of hypertension (Tkacova et al., 2014). These statistics allude to a relationship between OSA and oxidative stress, a hypothesis further supported by findings that CPAP treatment was able to ameliorate oxidative stress in OSA patients (Lavie, 2009).

There is accumulating evidence that OSA is independently associated with alterations in glucose metabolism, insulin resistance and risk for Type 2 Diabetes Mellitus (DM2) (Ip et al., 2002). Prevalence estimates linking OSA with DM2 may vary due to factors such as different diagnostic methodologies, though one study encompassing about 1,200 DM2 patients have found the prevalence of OSA to be about 71 % (Sudhakaran et al., 2015). It should be noted that obesity and visceral adiposity still confounds this relationship between OSA, insulin resistance and glucose tolerance (Tasali, 2008)

As previously mentioned, OSA has also been found to be associated with AD. There is an accumulating amount of evidence suggesting that OSA potentiates the neuropathological and clinical progression of AD through a combination of mechanisms such as disruption of sleep architecture, intermittent hypoxia and hemodynamic changes (Andrade et al., 2019). Both of these diseases are highly prevalent in older populations, and one study found that AD patients are five times more likely to present with OSA when compared with age-matched controls. This same study further found that about 50 % of patients experience OSA after being diagnosed with AD (Emamian et al., 2016). OSA patients have been found to exhibit significantly higher serum Abeta levels, a peptide known to be one of the most promising blood biomarkers for AD. Remarkably, it was found that this increase of Abeta in OSA patients was positively correlated with the AHI index and oxygen desaturation index (Zetterberg & Burnham, 2019; Bu et al., 2015).

1.2.4. Potential Biomarkers for OSA

Given the timely, costly, and variable methods currently employed by physicians to diagnose OSA, the search for reliable biomarkers specific to this disease is of upmost importance. Biomarkers are by definition objective, quantifiable characteristics of biological processes (Strimbu & Tavel, 2011). Biomarkers are especially important in medicine for diagnostic purposes, but they can also be used before diagnosis to gauge risk factors, and following diagnosis to measure therapy outcomes and prognosis (Califf, 2018).

Most biomarkers of interest have been associated with diseases comorbid with OSA, and may therefore present confounding factors. Hemoglobin A1c (HbA1c) is commonly used in clinical practice as a biomarker for prediabetes, and diabetes, while C-reactive protein (CRP) is a well-known biomarker for cardiovascular diseases, and systemic inflammation (Dorcely et al., 2017; Umeno et la., 2015; Huang et la., 2017). Though these biomarkers are also associated with comorbidities, there is great evidence that CRP may be independently associated with OSA. For example, one study found that CRP levels were found to be significantly higher in OSA, in a AHI-dependent manner, when compared to control samples (Hall et al., 2015). Previous studies have corroborated this, finding that CRP levels are decreased after three months and six months of CPAP therapy (Zhao et al., 2011; Mermigkis et al., 2012). There has also been compelling evidence

that HbA1c levels may prove to be a useful diagnostic tool in OSA. It has been found that among nondiabetic men, severity of OSA was associated with elevated HbA1c and increased fasting glucose levels (Papanas et al., 2009). Recently these findings were supported by a separate study, which observed that the severity of OSA was associated with elevated HbA1c levels independently of BMI in nondiabetic patients (Kurosawa et al., 2018).

There is mounting evidence pointing to a relationship between Abeta, sleep, and OSA. It has been found that concentrations of total serum Abeta, as well as Abeta isoforms 40 and 42, in OSA patients are significantly elevated when compared to controls, and positively correlated with the AHI index (Bu et al., 2015). Abeta's connection with sleep is also becoming better understood, as it has been found that Abeta isoforms 40 and 42 were higher in cerebral spinal fluid (CSF) during wakefulness, and lower during sleep (Lucey & Bateman, 2014). Additionally, it has been found that after one night of sleep deprivation, there was a significant increase in Abeta burden in the right hippocampus and thalamus (Shokri-Kojori et al., 2018).

Neuropeptide-Y (NPY) is an orexigenic neuropeptide serving as an important neuroendocrine factor which plays important roles in regulating food intake, energy homeostasis, anxiety, mood, and stress resilience (Holzer, Reichmann & Farzi, 2012). One study found NPY has been found to be elevated in OSA patients independently of obesity, and that CPAP treatment was able to lower these levels (Barcelo et al., 2004). A recent study supported these findings, indicating that serum NPY levels can reflect the severity of OSA (Tang et al., 2018).

1.3. Transthyretin

1.3.1. Transthyretin: an overview

Transthyretin (TTR) is a homotetrameric plasma protein that might be best known for its role as a Thyroxine (T4) transporter, and a carrier protein for Retinol Binding Protein (RBP), however the full spectrum of the protein's physiological roles are not yet fully understood (Gomes et al., 2018). In humans transthyretin has been found to be synthesized in various tissues, however it is prominently produced by the liver for circulation in the blood, in the choroid plexus for circulation in CSF, and also in pancreas, the light-sensitive retinal pigment tissue behind the retina (Johnson

et al., 2018; Tangthavewattana, Leelawatwattana, & Prapunpoj, 2019). Though usually found in its tetrameric form, TTR may also be found endogenously in its monomeric form (Newcomer & Ong, 2013).

TTR has a biological half-life of 1-2 days in humans, and is predominately found circulating in blood plasma and CSF (Ingenbleek, 2019; Batista, Sena-Esteves, & Saraiva, 2013). TTR is secreted by the liver in a diurnal manner, and in murine models has been found to feature circadian patterns in expression throughout the day (Martino & Tata, 2007). In wild-type mice, these findings were further corroborated showing that distinct fluctuations in serum TTR throughout the day (Gliniak, 2017). TTR levels in plasma also change with age, being lowest as newborns and continue augmented into adulthood until finally declining steadily after fifty years of age (Vieira & Saraiva, 2014).

1.3.2. TTR: Functions

Thyroxine is the most abundantly secreted thyroid hormone by the thyroid gland. It will circulate in plasma, and bind to its three carriers: thyroxine-binding globulin, TTR, and albumin. In blood circulation, 75 % of thyroid hormones are transported by TBG, up to 15 % by TTR, and 10 % by albumin. However, it has been found that the movement of T4 from the blood into the CSF is dependent on the concentration of free T4 in the serum and on T4 binding to choroid-plexus derived TTR (Richardson et al., 2015; Vieira & Saraiva, 2014). The true significance of thyroid hormone transport in the brain by TTR in choroid plexus epithelial cells (CPEC) is still not fully understood, but deterioration of CPECs, and their secretion of important proteins such as growth factors and TTR, is likely to have a role in Abeta removal and AD (Johnson et al., 2018).

TTR also plays a role in the uptake of retinol through its interaction with RBP (named RBP4 in humans). Approximately 40 % of plasma TTR will bind to RBP4, and in doing so creates a larger Retinol Circulating Complex (RCC) (Marchi et al. 2003). This larger RCC prevents the globular filtration of RBP4 by the kidneys, and thus increases the amount of circulating RBP4 made available for binding and circulation of poorly soluble retinol (Ingenbleek, 2018). Through these mechanisms, it appears TTR may have some regulatory role in the uptake of retinol by RBP4, and subsequent signaling cascades. RBP4 is a natural ligand for the cytosolic domain of membrane

receptor STRA6, an association which results in the recruitment and activation of janus kinase JAK2 (Berry et al., 2013). JAK activation initiates trans-phosphorylation of specific tyrosine residues, which generates docking sites for the recruitment of latent cytoplasmic transcription factors known as STATs. This JAK/STAT pathway is essential in the transference of signals from cell-membrane receptors to the nucleus, and have been found essential for a wide range of functions such as insulin action and lipid and glucose metabolism in adipocytes (Seif et al., 2017; Richard & Stephens, 2011).

TTR has been found to have multiple effects on Abeta, and though complex, the important relationship between this protein and peptide is becoming increasingly well-characterized. In a study conducted by Costa and colleagues, TTR was found to cleave Abeta, a process which lowers the amyloidogenicity of the peptide (Costa et al., 2008; Ribeiro et al., 2012). However, same study found that this proteolytic activity was inhibited by the presence of a particular form of alpha-APP peptides, specifically, those containing a Kunitz Protease Inhibitor domain (Costa et al., 2008). It has also become clear that the structural confirmation (namely between monomeric and tetrameric forms) of TTR has distinct effects on the Abeta peptide. For example, it has been found that TTR tetramers will bind more strongly with Abeta aggregates rather than with Abeta monomers, and that TTR tetramers may interact preferentially towards aggregates of Abeta rather than the monomeric form of the peptide (Du & Murphy, 2011). These findings were later corroborated by Garai and colleagues, where it was found that monomeric TTR in sub-stoichiometric amounts will suppress Abeta fibril formation. Additionally, this same study elucidated TTRs potentially protective role, finding that Abeta will co-aggregate with monomeric TTR to promote the formation of inert, non-fibrillar amorphous deposits (Garai et al., 2018). While classically known as a transporter for RBP4 and T4, TTR may also serve as an important transport protein for Abeta. In a study conducted by Alemi and colleagues, it was found that TTR was able to interact with the blood-brain-barrier (BBB), stimulating the efflux of Abeta in a one-way direction out of the brain (Alemi et al., 2016). Alemi and colleagues confirmed this in vivo, further finding that Abeta was internalized in hepatic cells, suggesting that TTR may serve as an Abeta carrier from the brain, through the BBB, and to the liver (Alemi et al., 2016).

1.3.3. TTR in disease pathology

TTR has been identified as a protein of interest in an increasing number of different diseases, in some cases playing in integral part in distinct pathologies. One of the most well-known areas of TTR research is concerned with amyloid formation. TTR amyloidosis exists in two main forms, Familial Amyloidal Polyneuropathy (FAP), and senile systemic amyloidosis (SSA), also referred to as Senile Cardiac Amyloidosis (SCA). FAP is a fatal autosomal-dominate genetic disorder characterized by amyloidal deposition and subsequent nerve lesions caused predominately by mutated and misfolded transthyretin (Ruberk & Berk, 2012). In SSA, amyloid fibrils contain wild-type forms of TTR (WT-TTR), and is largely an age-dependent disease. In fact, amyloidal deposition of WT-TTR occurs in the heart of 10-25 % of humans aged 80 years or older (Cascella et al., 2013). While FAP is the result of an inherited mutation in TTR, ageing has proven to be a clinically crucial factor since this disease can be clinically differentiated between early- and late-onset forms, with the mean age of onset being 61 years (Plante-Bordeneuve & Said, 2011). Systemic amyloidosis can involve a variety of organs and peripheral tissues, though there is accumulating evidence that the heart is one of the most affected organs (Blancas-Mejia & Ramirez-Alvarado, 2014; Witteles, 2016). Cardiac Amyloidosis (CA) is characterized by cardiac involvement of amyloidosis, the vast majority of which are caused by light-chain amyloidosis (AL) and transthyretin amyloidosis (ATTR) (Kyriakou et al., 2018). In wild-type ATTR, the heart is the most predominately affected organ and are most commonly seen in men between the ages of 65 and 95 years (Witteles, 2016).

TTR has also been implicated in AD, a neurodegenerative disease in large part characterized by the deposition of amyloid beta (Abeta) in the cerebrovascular structures and parenchyma of the brain (Rostagno et al., 2010). As previously mentioned, TTR has been found to sequester, bind to, and proteolytically cleave Abeta, thus ameliorating the toxicity of the peptide (Li et al., 2011). A number of studies support the view that increased expression of the *TTR* gene and/or TTR protein levels is neuroprotective in AD, though this effect may not be limited to TTRs involvement with Abeta (Sousa & Palha, 2009). Two ligands transported by TTR, thyroid hormones and retinol, have been linked to AD. Thyroid hormones have been reported to decrease Amyloid-Precursor-Protein (APP) expression, while retinoic acid and retinoid transport have been found to regulate APP expression and be involved in AD disease states (Sousa & Palha, 2009).

Recently, an interaction between diabetes, insulin resistance, and TTR has been elucidated. A study conducted by Gliniak, Brown and Noy found that the RBP receptor STRA6 regulates diurnal insulin responses in mice in a diurnal manner (Gliniak, Brown, & Noy, 2017). Interestingly, an earlier study found that STRA6 may only function under physiological conditions in which plasma RBP levels exceed that of TTR, highlighting TTRs potentially protective role against RBP-induced insulin resistance (Berry et al., 2012). One recent study conducted by Kwanbunjan and colleagues found that RBP4 and TTR levels were significantly elevated among subjects with high triglyceride levels, and that the there was a statistically significant correlation between levels of blood glucose and TTR (Kwanbunjan et al., 2018). It was also found that the islets of Langerhans in DM2 patients had proportionally more TTR-reactive alpha (glucagon) and beta (insulin) islet cells, despite TTR expression typically being found in the alpha cells (Westermark & Westermark, 2008). These findings, along with the fact that triglyceride levels are a common biomarker for insulin resistance, indicate that TTR may play an important role in the pathophysiology of insulin resistance, DM2, and their associated comorbid conditions (Li et al., 2013).

Indeed, many of the diseases connected to TTR have also been associated with aging, and current research only strengthens this association. For example, a recent study found that TTR amyloid deposition contributes to cellular and extracellular matrix damage in the articular cartilage of human osteoarthritis (OA) patients (Asasaki et al., 2016). In a separate in vivo study Matsuzaki and colleagues also found that TTR deposition increases disease severity in murine models of OA (Matsuzaki et al., 2017). Interestingly, Zhao, Buxbaum, & Reixach found that age-related oxidative modifications can make WT-TTR less thermodynamically stable compared to non-oxidized forms of the protein (Zhao, Buxbaum, & Reixach, 2014). These findings, coupled with the fact age is a common risk factor among the aforementioned TTR-associated diseases, have great implications for TTR's role in pathological states, particularly as it relates to age-related disease.

1.3.4. TTR as a Biomarker for OSA

TTR is frequently cited in research, but in most countries is rarely utilized in clinical practice (Delliere et al., 2018). However, TTR fulfills many of classical criteria defining a good biomarker such as having a small pool-size (10 mg/kg BW), having a short half-life of 1-2 days, and being

mainly synthesized by the liver (Igenbleek, 2019). TTR is also considered to be an acute phase protein, as its concentrations are known to increase or decrease by at least 25 % during inflammatory states (Kushner, 1982; Ahmed et al., 2012). It has been previously found that TTR was overexpressed in the blood serum of OSA patients (Jurado-Gamez et al., 2011). However, the relationship between TTR and OSA may be best appreciated through the protein's potential as a biomarker for several of OSA's comorbidities such as, obesity, insulin resistance and DM2, and AD.

As previously mentioned, the association between TTR and blood glucose levels was found to be statistically significant in nondiabetic patients with a high-risk for DM2. Additionally, both TTR and its natural ligand, RBP4, has been found to be significantly elevated in nondiabetic patients with high triglyceride levels, furthering the protein's case as a potential biomarker for increased insulin resistance, obesity, and DM2 (Kraft et al., 2013; Kwanbunjan et al., 2018). Research has shown that an increase in systemic RBP4 levels are associated with DM2 and obesity in patients (Yang et al., 2005). TTR plays a key role in the circulation of RBP4 made available for free retinol to the STRA6 receptor, as up to 40% of circulating TTR binds to RBP4 (Marchi et al. 2003). However, it has also been found that TTR blocks the ability of retinol-bound RBP to associate with STRA6 (Berry et al., 2012). It appears that TTR not only affects the amount of RBP made available for free retinol, but also mediates the RBP-STRA6 interaction, and subsequent retinol uptake and JAK/STAT signaling, measurable by the RBP:TTR ratio (Berry et al., 2014). Abrupt declines in plasma TTR and RBP4 concentrations in stressful disorders has major thyroid and retinoid implications, many of which have been largely unrecognized by the scientific community (Igenbleek & Bernstein, 2015). However, the true importance of the coordination between these two molecules in disease pathology is becoming better understood. For example, the true utility of the TTR:RBP ratio in these molecular mechanisms has been highlighted by a recent study which found that STRA6 regulates diurnal insulin responses and JAK/STAT signaling (Gliniak, Brown, & Noy, 2017).

TTR has also been labeled as a potential biomarker for neurodegenerative diseases such as AD. Oxidative stress plays a central role in Abeta deposition, which is one of the defining pathological features associated with AD (Butterfield et al., 2001). Plasma TTR concentration are significantly lower in AD patients when compared to non-demented age-matched controls, and TTR levels

were even lower for those with rapid cognitive decline and severe cognitive impairment (Velayudhan et al., 2012). Additionally, it has been found that production of Abeta, its precursor, or its related peptides induces neuronal TTR transcription and synthesis (Li et al., 2012). It has also been established that AD is associated with reduced NPY and NPY receptor expression, and that the neuropeptide may be effective in reversing the Abeta accumulation characteristic to AD (Spencer et al., 2015). As previously touched upon, OSA patients, independent of BMI, have been found to have elevated NPY levels. Moreover, there is evidence that serum NPY levels may reflect the severity of OSA, and that CPAP therapy results in a decrease in NPY levels (Barcelo et al., 2004; Tang et al., 2018). Though TTR has a lesser understood relationship with NPY, there is evidence that much like its relationship Abeta, TTR is able to proteolytically cleave NPY (Tangthavewattana, Leelawatwattana, & Prapunpoj, 2019). Interestingly, TTR knockout mice were found to have increased levels of NPY. This same study found that intracerebroventricular administration of TTR in healthy growing rats resulted in a decrease of NPY in the dorsomedial hypothalamus and the PVN (Zheng et al., 2016). These findings allude to an interplay between TTR and neuropeptides Abeta and NPY, as well as the potential utility of TTR as an indicator of oxidative states.

It has been established that TTR has a close association with levels of oxidative stress, and that the rat TTR gene contains a GC-response element which has been evolutionarily conserved in humans (Sharma et al., 2019). It has also been found that TTR expression is directly regulated by stress and GCs (Li et al., 2011; Martinho et al., 2012). As previously mentioned the commonly used biomarker for stress is cortisol, a diurnal GC tightly regulated by the HPA axis. OSA is associated with an over-activation of the HPA axis, and significantly higher daily cortisol levels (Chrousos, 2016). However, there are mixed findings on whether or not CPAP therapy significantly decreases cortisol levels, in part due to variability in the time of day in which cortisol was measured (Kritkou et al., 2016; Tomfohr, Edwards & Dimsdale, 2012).

Time of day is an especially important parameter since cortisol has a short half-life (66-120 minutes), and restricting cortisol assessment to one time period per-day may be a clinical barrier (McKay & Cidlowski, 2003). Additionally, the underlying pathological events defining OSA occur mainly once per day, so biomarkers with half-lives greater than or equal to one day may better represent disease states throughout diagnosis and treatment. TTR may prove to be a potential

candidate as an OSA biomarker for several reasons. First, there is accumulating evidence that TTR has strong association with, and may be regulated by, stress, GCs, and oxidative states. Secondly, TTR has a 1-2 day half-life, a feature that may better represent chronic conditions such as OSA. Thirdly, TTR has already been identified as a potential biomarker for several of OSA's comorbidities, and has known relationships with other associated biological markers such as Abeta and NPY. And lastly, it has been found that serum TTR levels are elevated in patients with OSA (Jurado-Gamez et al., 2011)

Chapter 2: Objectives

Over the past several decades, our understanding of the diurnal plasma protein TTR has evolved tremendously. More than a plasma and CSF transport protein for RBP4 and T4, TTR appears to have intimate roles in the mediation of Abeta and NPY, two peptides which have been increasingly associated in age-related pathophysiological mechanisms. All of the aforementioned biological markers have been implicated in OSA. As an increasingly prevalent disease, the search for reliable diagnostic biomarkers and therapeutic targets is of great importance. Bearing this in mind, this project has the following specific aims:

1. Evaluate the impact of CPAP therapy on the diurnal TTR blood plasma concentrations of OSA patients;
2. Evaluate the impact of OSA and aging on the diurnal TTR blood plasma concentrations;
3. Investigate the potential of TTR as a potential biomarker for OSA.

At the completion of this project we expect to better understand the role of TTR in relation to OSA, and evaluate whether TTR may serve as a useful tool for diagnostic, prognostic, and therapeutic measures.

Chapter 3: Materials & Methods

3.1. Study Design

This study was carried out in collaboration with the "Centro de Medicina do Sono of Centro Hospitalar e Universitário de Coimbra" (CHUC), in Coimbra, Portugal. All aspects of this study have been approved by the ethical committee of the University of Coimbra's Faculty of Medicine and CHUC, and was completed in close cooperation with a medical team of physicians and nurses. In coordination with the medical team, male patients between the ages of 37 and 75 with high clinical suspicion of OSA were selected. Selected patients were informed of the study, and if interested in participation, were asked to sign an informed consent form.

During their first hospital stay (t_0) selected subjects were provided a questionnaire battery meant to address clinically relevant parameters such as, but not limited to, daily routines, sleep habits, physical and psychological states, medication use, physical exercise, dietary habits, and work and travel schedules. At t_0 these patients underwent PSG exams in the CHUC sleep clinic, where peripheral blood samples were collected at four separate time points (8h, 11h, 4h30, and 22h30). Following the PSG exam, the medical team generated a report detailing each patient's demographic data, medical history, as well as data from the PSG such as respiration, oxygenation, and movement throughout the overnight exam.

Following a diagnosis of OSA by the medical team, CPAP therapy was suggested for qualified patients. After four months (t_{4m}) and two years (t_{24m}) of CPAP therapy patients were scheduled for follow-up visits to the sleep center in order to assess therapy outcomes. During both follow-up visits blood samples were collected at the same four aforementioned time points throughout the day. Blood samples were also collected from age-matched controls, and young controls. Adherence to CPAP therapy was assessed using CPAP chip technology. The CPAP chip records the percentage of days in which CPAP machines are used for more than 4 hours a day. The chip also assesses the number of apnea and hypopnea episodes per hour of use.

Two separate groups of male control subjects were recruited for this study. Age-matched controls were patients who were initially recruited as research subjects due to high clinical suspicion for OSA, but were not diagnosed with OSA. Age-matched controls, as well other participants, are between the ages of 37 and 75. Young controls were also chosen on the basis of

age, being between the ages of 20 and 30. Additionally, young controls were in generally good health, and had not been previously diagnosed with OSA (Figure 6)

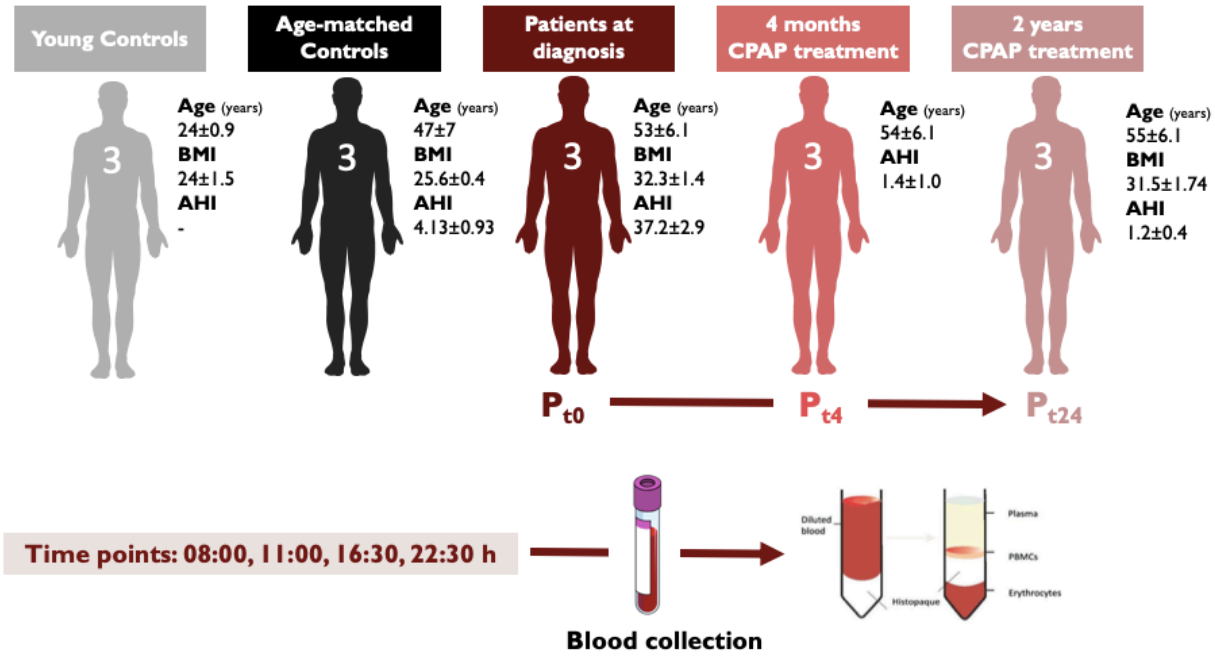


Figure 6. Schematic depicting research subject cohorts and protocol for blood sample collection.
Research design includes three subjects from each cohort; young healthy controls, age-matched healthy controls, and OSA patients. OSA patients were re-assessed at four months (t_{4m}) and 24 months (t_{24m}) following initial OSA diagnosis (t_0). Each subject pool was subjected to blood collections at four timepoints throughout the day (*8h, 11h, 16h30, 22h30) (Image from Barbara Santos).*

3.2. Development and optimization of a new Indirect-ELISA Assay for TTR

3.2.1. Reagents

Homemade ELISAs may be developed and optimized in order to detect immunoglobulins of particular proteins. Untreated, flat-bottom, clear 96-multiwell plates were obtained from Frilabo (Portugal). Monoclonal primary antibodies were obtained from Abnova (Tebubio, USA) and polyclonal alkaline phosphatase (AP)-conjugated secondary antibodies were obtained from Cedar Lane Labs (USA). p-Nitrophenyl Phosphate (PnPP) Enzyme substrate was obtained from Sigma-Aldrich (USA). Buffers and diluents were all homemade and created utilizing materials from the Center for Neurosciences and Cell Biology (CNC) and the Faculdade de Ciências e Technologia da Universidade de Coimbra (FCTUC), Department of Chemistry. A number of different coating buffers, blocking buffers, wash solutions, and PnPP solutions and concentrations were created and utilized (See Appendix-A for details on buffer and solution recipes).

3.2.2. General Indirect ELISA Procedure

Indirect ELISAs utilize unlabeled primary antibodies and labeled secondary antibodies in order to quantify target antigens. Indirect ELISAs have four main steps: coating, primary antibody addition, secondary antibodies addition, and enzyme substrate addition (Figure 7). Between these main steps additional blocking and washing steps are included intermittently depending on the nature

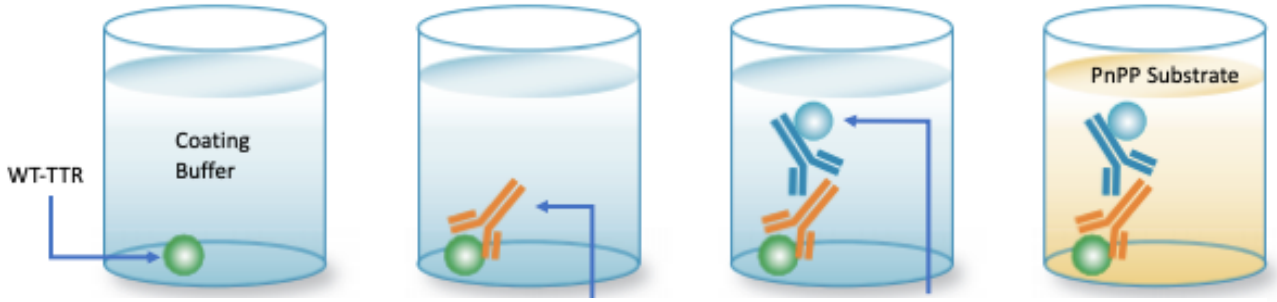


Figure 7. General scheme representing an indirect ELISA procedure.
There are four main steps characteristic to an indirect ELISA procedure; coating, primary antibody addition, secondary antibody condition, and enzyme substrate addition. Secondary antibody was conjugated with AP. The enzyme substrate utilized was PnPP. (image adapted from Bio-Rad).

of the ELISA and research aims. Both primary and secondary antibodies are typically diluted in blocking buffer in order to prevent non-specific binding (Yang & Ma, 2009).

In the first step of any indirect ELISA, antigens are first passively adsorbed or "coated" through hydrophobic interactions between microplate surfaces and non-polar protein residues. Incubation times for both the coating stage and antibody additions largely vary depending on materials used, and target antigens. Coating steps are typically followed by a washing step in order to remove unbound reagents and decrease background. Washing solutions are often composed of PBS or TBS and varying percentages of surfactants such as Tween20. Washing steps are usually repeated throughout the ELISA following antibody additions. After antigen coating and washing, it is common to perform a blocking step. Blocking buffers are often composed of PBS or TBS, and contain either proteins such as bovine serum albumin or non-fat dried milk or detergents such as Tween20 or Triton X-100. In this context, blocking buffers are utilized to further saturate any sites on the surface of microplates that may not contain target antigens, therefore preventing non-specific interactions (For details regarding the composition of blocking and washing buffers used in homemade-ELISA development, refer to the Appendix- A).

The second main step, addition of primary antibodies, is performed following coating, washing, and blocking in order to ensure primary antibodies bind specifically to coated antigen. Monoclonal antibodies are most commonly used, as they have higher specificity for particular epitopes on target antigens. For the purposes of this homemade ELISA optimization, mouse monoclonal antibodies raised against human TTR were utilized. Following the addition and incubation of primary antibodies, a second wash step is common in order remove excess or unbound antibodies. The addition of secondary antibodies follows the addition of primary antibodies, and this third main step usually requires a shorter incubation compared to the addition of primary antibodies. For the purposes of this homemade ELISA optimization polyclonal AP- conjugated secondary antibodies were utilized. These secondary antibodies were produced in a rabbit expression system, but are sensitive to mouse immunoglobins. By being expressed in a rabbit expression system, and raised against mouse immunoglobins, there is minimal cross-reactivity with human immunoglobins. A washing step follows the addition and incubation of secondary antibodies in order to remove unbound antibody enzymes.

The fourth and final step, addition of PnPP enzyme substrate, which will react with AP, is crucial for the detection of tagged antigen. Secondary antibodies contain enzymes which when catalyzed will produce color. This final step will continue indefinitely, and for this reason stop solutions are utilized to prevent reactions from producing erroneous results or progressing during quantification in spectrophotometers.

Though washing steps are necessary, it is possible that over-washing microwells may remove coated antigens in-turn decreasing sensitivity. Likewise, it is also possible that inadequate wash steps may produce high background signals during spectrophotometric readings. For these reasons variations of washing procedures were used. For example, throughout optimization each wash cycle featured between 2 and 5 cycles per wash step. Additionally, removal of wash buffer from microwells were performed either by aspiration, or decanting.

3.2.3. Antigen Standard Preparation

Human WT-TTR was produced in an *Escherichia coli* expression system at the Faculdade de Ciências e Technologia da Universidade de Coimbra (FCTUC), Department of Chemistry. Human WT-TTR was subsequently purified, and stored at -20 ℃ in phosphate buffered saline (PBS[1]).

3.2.4. Dialysis of PBS-Antigen Solution

Alkaline Phosphatase (AP) is sensitive to phosphatase solutions. In order to prevent cross-reactivity between AP-conjugated secondary antibodies and PBS, WT-TTR antigen required purification before application. WT-TTR was purified via dialysis utilizing Slide-A-Lyzer Dialysis Cassette (0.5-3 ml capacity) obtained from ThermoScientfic (USA). Dialysis cassettes were suspended in 1.5 L of tris-buffered saline (TBS[2]), and left to equilibrate while mixing overnight at 4 ℃ (figure 8)

[1] 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, KH_2PO_4, 1L H_2O, adjusted to pH 7.4 with HCl

[2] 12.0 g NaCl, 0.3g KCl, 4.5 g Tris-HCl, 1.5 L H_2O, adjusted to pH 7.5 with NaOH

Figure 8. Slide-A-Lyzer Dialysis method.
Dialysis cassettes were utilized remove PBS from TTR standard. PBS-TTR solutions were first injected into cassettes before being suspended in 1.5 L of TBS. Dialysis was left to equilibrate overnight at 4 °C. Following dialysis, TTR was removed from the cassette and subsequently quantified via UV spectroscopy. (Image adapted from ThermoFisher)

3.2.5. UV Spectrometry of Dialyzed WT-TTR

Following overnight dialysis, the equilibrated WT-TTR of an unknown concentration was removed from the dialysis cassette. In order to determine the concentration of dialyzed WT-TTR ultraviolet (UV) spectrometry was performed using a Spectronic Unicam UV-500 Spectrometer.

WT-TTR has a known molar absorptivity at wavelength 280 nm (77,600). UV spectroscopy readings were performed between wavelengths 200 nm and 350 nm. Between said wavelengths WT-TTR may be characterized by a peak at 220 nm and 280 nm depicting peptide bonds and aromatic amino acid residues, respectively. In UV spectroscopy WT-TTR should not display absorbance values at 320 nm, so absorbance values obtained from 320 were subtracted from 280 for internal validity (Figure 9).

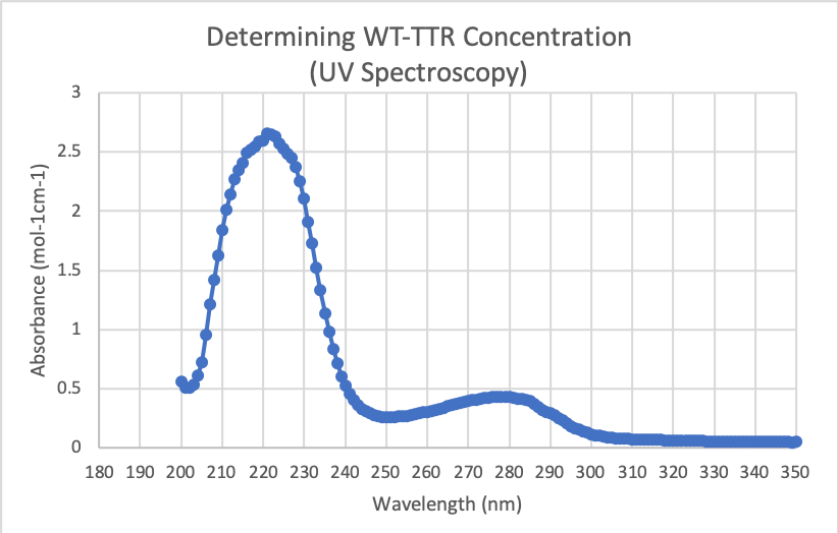


Figure 9. UV spectroscopy readout of dialyzed WT-TTR suspended in TBS.
WT-TTR may be characterized via UV spectroscopy due to known absorbance peaks at 220 nm depicting peptide bonds, and at 280 nm depicting aromatic amino acid residues.

The molar concentration of WT-TTR was determined using the Beer-Lambert Equation (Figure 10). The Beer-Lambert equation takes into account measured absorbance values, path-length of cuvettes, and known molar absorptivity values of proteins in order to calculate concentration.

Beer Lambert Equation

$$A = C \times L \times \varepsilon$$

A = Absorbance ($mol^{-1}cm^{-1}$)

C = Concentration (mol^{-1})

L = Path-length of cuvette (cm)

ε = Molar Absorptivity

Figure 10. Beer Lambert equation.
The Beer-Lambert Equation was utilized to determine WT-TTR concentrations following dialysis. In order to do so variables such as absorbance, path-length of cuvette, and molar absorptivity must be considered. TTR has a known molar absorptivity value (ε = 77,600), and the path-length of the cuvette in experiments was 1 cm.

3.2.6. Determining Optimal Antigen Concentration & Incubation Conditions

Previously dialyzed WT-TTR was aliquoted into concentrations between 3.6 μM and 4.7 μM, as endogenous plasma TTR has been found to be circulating at ranges between 3.4 μM and 5 μM (Rappley et al., 2014). Serial dilutions were performed on TTR in order to determine optimal concentrations for antigen coating. The main purpose of this exercise was to create a standard curve within a defined set of dilution ranges. In doing this, human plasma TTR may be compared to said standard curves, and subsequently quantified. Serial dilutions were conducted between the ranges of 10x and 200,000x. At times, target antigens in ELISAs require distinct incubation conditions. For this reason, antigen-coating incubations were conducted at room temperature for periods of time ranging from 30 minutes and 2 hours. Antigen-coating incubation were also performed at 4 ℃ overnight for periods of time ranging between 14 and 18 hours.

3.2.7. Determining Optimal Antibody Concentration & Incubation Conditions

Dilutions factors were also considered for both primary and secondary antibodies concomitantly through a checkerboard titration technique. This was accomplished by performing serial dilutions

on primary antibodies along microplate rows, while also serially diluting secondary antibodies down microplate columns. Primary antibody was provided by the manufacturer at a concentration of 0.37 μg/μL, and diluted between 500 x and 5000 x. Secondary antibody was provided by the manufacturer at a concentration of 1.5 μg/μL, and diluted between 200 x and 2000 x. Primary antibodies are particularly sensitive to incubation conditions, and for this reason several incubation conditions were tested. Incubations for primary antibodies were conducted at both room temperature for periods of time ranging from 30 minutes and 2 hours, and overnight at 4 °C overnight for periods of time ranging between 14 and 18 hours. Secondary antibody incubation conditions were also considered, and performed at room temperature for periods of time ranging between 30 minutes and 2 hours.

3.2.8. Determining Optimal Washing Procedure

Washing procedures can also have profound effects on the antigen and antibody binding. For this reason, several different washing techniques were performed. Washes were repeated between 2 and 5 times following both the antigen coating phase, and the primary antibody addition phase. Between repeated washes, wash buffer was removed through two main methods; aspiration, and decanting. During both phases, several different volumes of wash buffer per microwell were also tested, ranging between 100 μL and 250 μL.

3.3. Plasma TTR measurement by Commercial Indirect-ELISA Assay

Commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits (Abnova, USA) were utilized to measure plasma TTR.

3.3.1. Sample Collection

At the four aforementioned daily timepoints, peripheral blood (16-20 mL) was collected from superficial veins of the patient via venipuncture. Blood samples were immediately transferred into K2EDTA-coated collection tubes in order to prevent coagulation, and stored at 4°C until further processing.

3.3.2. Plasma Isolation from Peripheral Blood

In order to prevent conformational changes to TTR, peripheral blood was processed as soon as possible after collection. All available blood was diluted in 25 mL of PBS 1x and gradually added on top of 10 mL of histopaque. In order to separate blood cells from plasma, this mixture was centrifuged at 800 g at 20 °C for 20 minutes with no brake. Once separated into distinct layers, 4.5 mL of plasma was stored at -80 °C for future use (Figure 11).

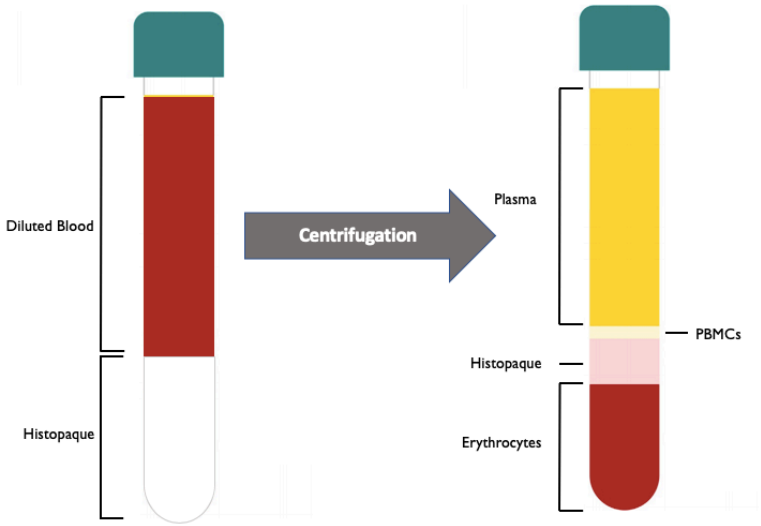


Figure 11. Schematic illustrating protocol for plasma isolation from peripheral blood. Blood was first diluted with PBS, and then carefully added to a tube containing histopaque. Blood-histopaque tubes were subsequently centrifuged at 800 g for 20 minutes without a brake at 20 °C. (Image adapted from Advances in biomarker detection: Alternative approaches for blood-based biomarker detection Miguel Rosadoa,b, Rafael Silvaa , Mariana G. Bexigac,d, John G. Jonesa , Bruno Manadasa,† , Sandra I. Anjoa,*,†)

3.3.3. Sample Preparation

TTR is particularly prone to undergo conformational changes due to changes in temperature, pH, and agitation. For these reasons freeze-thaw samples were kept to a minimum. During sample preparation, TTR was gradually thawed to ambient temperatures concomitantly with other required reagents. During aliquot preparation, excessive agitation and mixing was avoided to prevent fibrilization of TTR.

3.3.4. Optimization

The manufacturer recommended plasma sample dilution between a range of 40,000 x and 160,000 x in mix diluent. Optimization assays were conducted in order to determine which dilution ranges were best suited for our patient demographic, as it is known that TTR levels vary greatly depending on age and state of health. Initial optimization tests indicated that dilutions in the range of 20,000 x optimal for our research aims.

3.3.5. Immunoassay

TTR was measured utilizing a TTR sandwich-ELISA assay. With the exception of antigen dilutions, immunoassays were performed per manufacturers recommendations. All components, with the exception of the spectrophotometer, deionized and distilled water, and pipettes, were provided by the manufacturer (Abnova, USA). 96 well microplates were coated with a polyclonal antibody against human TTR. 50 μL of human plasma samples from OSA patients and control subjects were diluted 20,000x and then added to microwells. The plate was carefully tapped in order to thoroughly coat well surfaces and prevent the formation of bubbles. Wells were then covered with sealing tape and left to incubate for 2 hours. The microplate wells were then manually washed by adding 200 μL of wash buffer to each well, subsequently decanted by inversion, and then hit 4-5 times on absorbent material in order to completely remove the liquid. This process was repeated five times. 50 μL of biotinylated human TTR antibody was then added to each well. The microplate was tapped gently in order to thoroughly coat well surfaces and prevent the formation of bubbles. Again, the microplate was covered with sealing tape and left to incubate for 1 hour. Another washing step was performed following incubation exactly as described above.

50 μL of streptavidin-peroxidase (SP) conjugate was then added to each microwell. The plate was again tapped to thoroughly coat wells and prevent the formation of bubbles, and then covered with sealing tape before being left to incubate for 30 minutes. Another washing step was then performed exactly as described above. 50 μL of chromogen substrate was added to each well, followed by gentle tapping. The microwells were then left to incubate for 25 minutes. 50 μL of stop solution was lastly added to each well, and then immediately analyzed at a wavelength of 450 nm utilizing a Spectramax Plus 284 spectrophotometer.

3.3.6. Statistical Analysis

Results are presented as mean $\pm$ standard error of the mean (SEM). Comparisons between controls and OSA patients were analysis using Student's unpaired t-test with two-tailed p value. Comparisons between OSA patients over time throughout CPAP therapy were analyzed through one-way and two-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test in order to determine differences at distinct timepoints throughout the day. For all statistical analysis, a value of $p<0.05$ was considered as significant. The software Graphpad Prism 8.0 was utilized for all statistical analysis.

Chapter 4: Results

4.1. Control Cohort Characterization

4.1.1. Age-Matched Controls

The age-matched control cohort was comprised of three male patients between the ages of 36 and 60, with an average age of 47 ± 7.0 years. As defined by the BMI two patients were overweight (BMI= 26.2; 25.7), and one patient was of normal weight (BMI= 24.8). On average the age-matched controls had a BMI of 25.6 ± 0.4 (table 1). One of the three age-matched controls suffered from depression, while the other two had no known disorders or diseases.

The PSG exam found the average AHI of age-matched controls to be 4.13 ± 0.93 (table 1). Regarding oxygenation, controls were found to have a SpO_2 mean of 94.7 ± 0.7 and an average of 0.43 ± 0.3 desaturation events per hour (table 1). The EKG data indicated that waking heart rates of age-matched controls averaged 62 ± 9.1 bpm, while heart rates while sleeping averaged 61 ± 2.3 bpm (table 1).

Table 1. Clinical characterization of age-matched control subjects.

Medical history	Age (years)	47 ± 7.0
	BMI	25.6 ± 0.4
Sleep disruption events	Arousal events/h	45.4 ± 33.3
	Awakening events/h	3.7 ± 1.4
Respiration Summary	AHI	4.13 ± 0.93
Oxygenation Summary	SpO2 mean (%)	94.7 ± 0.7
	Desaturation events/h	0.43 ±0.3
EKG statistics	Heart rate, wake (bpm)	62 ± 9.1
	Heart rate, steady sleep (bpm)	61 ± 2.3

Parameters assessed in age-matched control subjects. Data depicting age and BMI of each patient were obtained by the medical team. Data concerning sleep disruption events (arousals and awakenings per hour), respiration (apnea and hypopnea index), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when in a steady sleep state) were measured in each patient during PSG test. All data is presented as mean ± SEM of 3 patients.

4.1.2. Young Controls

The young control cohort was comprised of three male patients between the age of 22 and 26, with an average age of 25 ± 0.9 years (table 2). As defined by the BMI one young control subject was overweight (BMI= 27.0), while the other two were of normal weight (BMI= 20.2; 23.2). The

average BMI between all young controls was 23.5 ± 2.0 (table 2). All young controls were in good health, having no pre-existing conditions or disorders, and no medication regimen for sleep-aid or otherwise.

Table 2. Clinical characterization assessed in young control subjects.

Medical history	Age (years)	25 ± 0.9
	BMI	23.5 ± 2.0

Parameters assessed in age-matched control subjects. Data depicting age and BMI of each patient were obtained by the medical team. All data is presented as mean ± SEM of 3 patients.

TTR is a protein present in human plasma that shows a transient expression along the day. This being the case, we collected blood in 4 different time points – 8h, 11h, 16h30 and 22h30 – to measure the levels of TTR throughout the day, in healthy subjects with different ages, young and age-matched, to OSA patients. OSA patients were assessed at 3 different instances along therapy: at the time of diagnosis (t_0), after 4 months of CPAP therapy (t_{4m}) and after 2 years of CPAP therapy (t_{24m}).

4.2. OSA Cohort Characterization

The OSA cohort was comprised of three male patients between 41 and 61 years old, with an average age of 53 ± 6.1 years (table 3-A). As defined by the BMI, one patient was overweight (BMI=29.8), while the other two patients were classified as obese class 1 (BMI= 32.4; 34.7). The average BMI between all three patients was 32.2 ±1.4 (table 3-A). Two of the three patients had comorbid disorders, one being afflicted by diabetes and the other by hypertension.

During initial PSG exams (t_0), patients were monitored to yield data on the incidence of apnea/hypopnea, respiration, oxygenation, heart rate, and sleep disruption events. Each patient was classified as having severe OSA as defined by the AHI (AHI≥30), and together had an average AHI of 37.2 ± 2.9 at the time of diagnosis (table 3-A).

The PSG exam elucidated the effect OSA potentially had on the oxygenation of the patients. It was found that patients of this cohort had an average peripheral capillary oxygen saturation (SpO_2) of 91.3 ± 0.9 (table 3-A). Desaturation events, described as decrease in mean oxygen saturation by ≥ 4 % which last for at least 10 seconds over a period of 120 seconds, were also calculated.

Patients featured an average of 10 ± 0.9 (table 3-A) desaturation events per hour. Patients featured an average 21.6 ± 6.8 (table 3-A) arousal events per hour, and 6.2 ± 3.1 (table 3-A) awakening events per hour. Electrocardiogram (EKG) were performed concomitantly with the initial PSG exam. While awake, it was found that patients had an average heart rate of 84 ± 6.6 (table 3-A) beats per minute (bpm). During sleep, patients had an average heart rate of 74 ± 0.8 (table 3-A). After four months, patient adherence to CPAP therapy was assessed. On average, 94.7 % ± 5.3 of patients utilized their CPAP device for more than 4 hours per day (table 3-B). Adherence was also assessed after 24 months (t_{24m}) of therapy. At this time point was found that on average 86.1 % ± 6.9 of patients utilized their CPAP device for at least four hours a day. AHI as also assessed at t_{4m} and t_{24m} following the commencement of CPAP therapy. AHI significantly decreased between t_0 and t_{4m} ($p=0.005$) from an average of 37.2 ± 2.9 (table 3-A; figure 12) to 1.4 ± 1.0. AHI at t_{24m} was also significantly lower than t_0 (0.006 ± X), averaging 2.57 ± 1.77 (table 3-C; figure 12). BMI was also assessed at t_{4m}. BMI was found to decrease from an average of 32.2 ±1.4 to 31.5 ± 1.74, though the difference was not statistically significant (table 3-C).

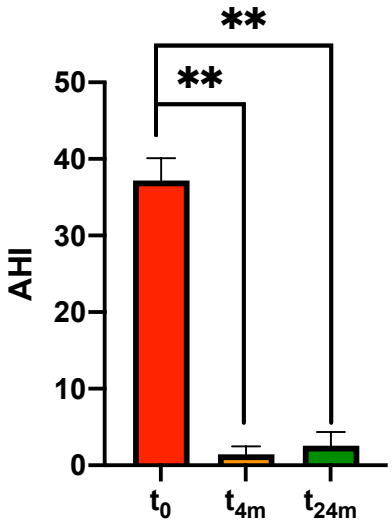


Figure 12. OSA patients AHI before treatment (t_0), after 4 months of CPAP therapy (t_{4m}), and after 24 months of CPAP therapy (t_{24m}).
This graph depicts the AHI of OSA patients at the time of diagnosis, four months following CPAP treatment, and 24 months following CPAP treatment. AHI scores significantly decreased after CPAP treatment at both t_{4m} and t_{24m}. All data is presented as mean ± SEM with n=3 at all time points. **p<0.01, one-way ANOVA

Table 3. Clinical characterization of OSA patients before treatment (t_0), after four months of CPAP therapy (t_{4m}), and after 24 months of CPAP therapy (t_{24m}).
(A) Parameters assessed in OSA patients before treatment. Data depicting age and BMI of each patient were obtained by the medical team. Data concerning sleep disruption events (arousals and awakenings per hour), respiration (apnea and hypopnea index), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when in a steady sleep state) were measured in each patient during PSG test. All data is presented as mean ± SEM of 3 patients. **(B)** Parameters assessed in OSA patients after four months of CPAP treatment. Data depicting age was obtained by research team. Adherence (percentage of days with more than 4h of CPAP use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. All data is presented as mean ± SEM of 3 patients. **(C)** Parameters assessed in OSA patients after 24 months of CPAP treatment. Data depicting age was obtained by research team. Data depicting BMI was obtained by medical team. Adherence (percentage of days with more than 4h of CPAP use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. All data is presented as mean ± SEM of 3 patients.

(A)

		$t0_m$
Medical history	Age (years)	53 ±6.1
	BMI	32.3 ± 1.4
Sleep disruption events	Arousal events/h	21.6 ± 6.8
	Awakening events/h	6.2 ± 3.1
Respiration Summary	AHI	37.2 ± 2.9
Oxygenation Summary	SpO2 mean (%)	91.3 ± 0.9
	Desaturation events/h	10 ± 9.9
EKG statistics	Heart rate, wake (bpm)	84 ± 6.6
	Heart rate, steady sleep (bpm)	74 ± 0.8

(B)

		t_{4m}
Medical History	Age	53 ± 6.1
Adherence	Days with more than 4h of use	94.7 ± 5.3
Respiration Summary	AHI	1.4 ± 1.0

(C)

		$t24_m$
Medical history	Age (years)	55 ± 6.1
	BMI	31.5 ± 1.74
Adherence	Days with more than 4h of use	86.1 ± 6.9
Respiration Summary	AHI	2.57 ± 1.77

4.3. Evaluation of TTR in OSA Patients

Averaged plasma TTR concentrations throughout the day were assessed by ELISA in both untreated OSA patients (t_0) and controls. In control subjects (young and age-matched) the TTR concentration (55.6 μg/mL ± 4.1) was compared to OSA patients (t_0). We found that daily plasma TTR concentrations significantly decreased in OSA patients (t_0; 44.5 μg/mL ±2.7; p=0.0289) (Figure 13-A). However, upon further investigation it appears averaged daily TTR values may not best characterize alterations to plasma TTR concentrations in OSA patients.

The diurnal rhythm of TTR was assessed in both control and OSA subjects by measuring plasma TTR concentrations at the four aforementioned timepoints throughout the day. When comparing OSA and control subjects, distinct differences in diurnal plasma TTR profiles can be seen (Figure 13-B). In controls subjects (young and age-matched) plasma TTR concentrations peak in the morning (70.8 μg/mL ± 9.43), and steadily decrease into the afternoon. At 16h30, plasma TTR levels begin to climb again into the following morning in this same cohort. In OSA subjects, this characteristic fluctuation is not observed, and TTR levels oscillate minimally without featuring any remarkable apexes in TTR concentration. Additionally, at 16h30 when control subjects TTR levels trend upwards, the TTR concentration in OSA patients appears to trend downwards (Figure 13-B).

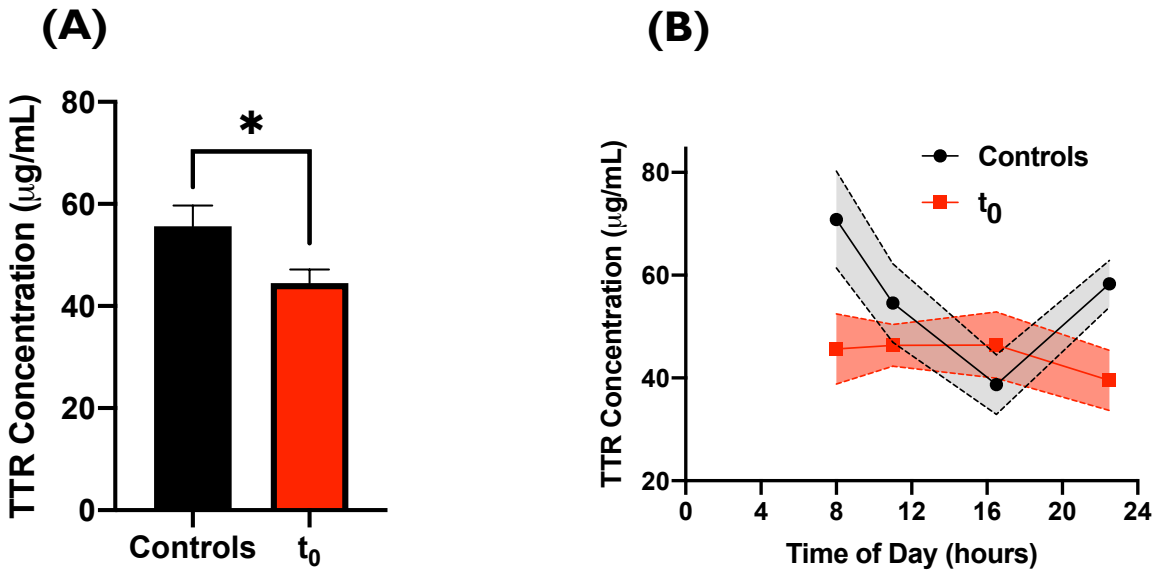


Figure 13. Averaged daily plasma TTR levels in control subjects and in untreated OSA patients (t_0). **(A)** This graph depicts averaged daily TTR plasma concentrations in controls (young and age-matched) and in untreated OSA patients (t_0). Averaged daily plasma TTR concentrations were significant lower in a OSA patients (t_0) when compared to controls. Data is presented as mean ± SEM with n=6 (controls) and n=3 (t_0). *p<0.05, Student's t test. **(B)** This graph depicts diurnal TTR profile in controls (young and age-matched) and in untreated OSA patients (t_0). There are marked differences in the diurnal TTR profile between control subjects and untreated OSA patients (t_0). The diurnal TTR profile in control subjects featured early morning peaks, which gradually decreased to an afternoon nadir before rising again into the following morning. The diurnal TTR profile in untreated OSA patients (t_0) did not feature any remarkable peaks throughout the day, and showed little oscillation. When control subjects' TTR concentrations trended upwards into the early morning, untreated OSA patients' (t_0) TTR values trended slightly downwards. All data is presented as mean ± SEM with n=6 (controls) and n=3 (t_0).

4.4. Effect of CPAP therapy on TTR in OSA Patients

Averaged plasma TTR concentrations throughout the day were assessed in OSA patients throughout CPAP therapy. This was accomplished by averaging daily plasma TTR levels throughout the day in controls (young and age-matched) and in patients at t_0, t_{4m} and t_{24m}. As described above, in control individuals, TTR levels are higher than detected in OSA patients. CPAP therapy was not found to decrease averaged daily TTR values in OSA patients at t_{4m} (44.2 μg/mL ± 3.7). However, at t_{24m} average daily TTR concentrations (32.4 μg/mL ± 2.5) were found to be significantly lower when compared to t_0 (44.5 μg/mL ± 2.7; p=0.0157) and t_{4m} (p=0.0163). When compared to controls (55.6 μg/mL ± 4.1) averaged TTR values at t_{24m} were found to be significantly lower (32.4 μg/mL ± 2.5; p=0.0005) (figure 14-A). These results indicate that CPAP treatment induces alterations in TTR average concentrations in plasma.

When examining averaged TTR values at distinct timepoints throughout the day in control and OSA subjects, there was a statistically significant decrease in TTR concentration (35.9 μg/mL ± 5.7) at 8h at t_{24m} when compared to t_0 (45.7 μg/mL ± 6.9; p=0.0157) and t_{4m} (45.2 μg/mL ± 6.6; P=0.0163) concentrations at the same timepoint (figure 14-B). When the diurnal TTR profile of

control subjects are juxtaposed with OSA patients, distinct differences can be noted. In controls subjects, plasma TTR concentrations peak in the morning (70.8 μg/mL ± 9.4), and steadily decrease into an afternoon nadir around 16h30 (38.7 μg/mL ± 5.8). Following this nadir, plasma TTR levels in control subjects appeared to climb into the following morning, as expected considering the aforementioned peak in plasma TTR at 8h (70.8 μg/mL ± 9.4). In OSA subjects however, this characteristic fluctuation is not observed and TTR levels oscillate minimally. In particular, when control subjects' TTR levels trend upwards at 16h30, the TTR concentration in OSA patients appears to trend slightly downwards. While CPAP therapy may have affected both averaged daily TTR values and TTR concentrations at distinct timepoints throughout the day, it did appear to mediate the flattening of diurnal TTR oscillations characteristic to healthy controls at t_{4m} or t_{24m} (figure 14-C, D, E).

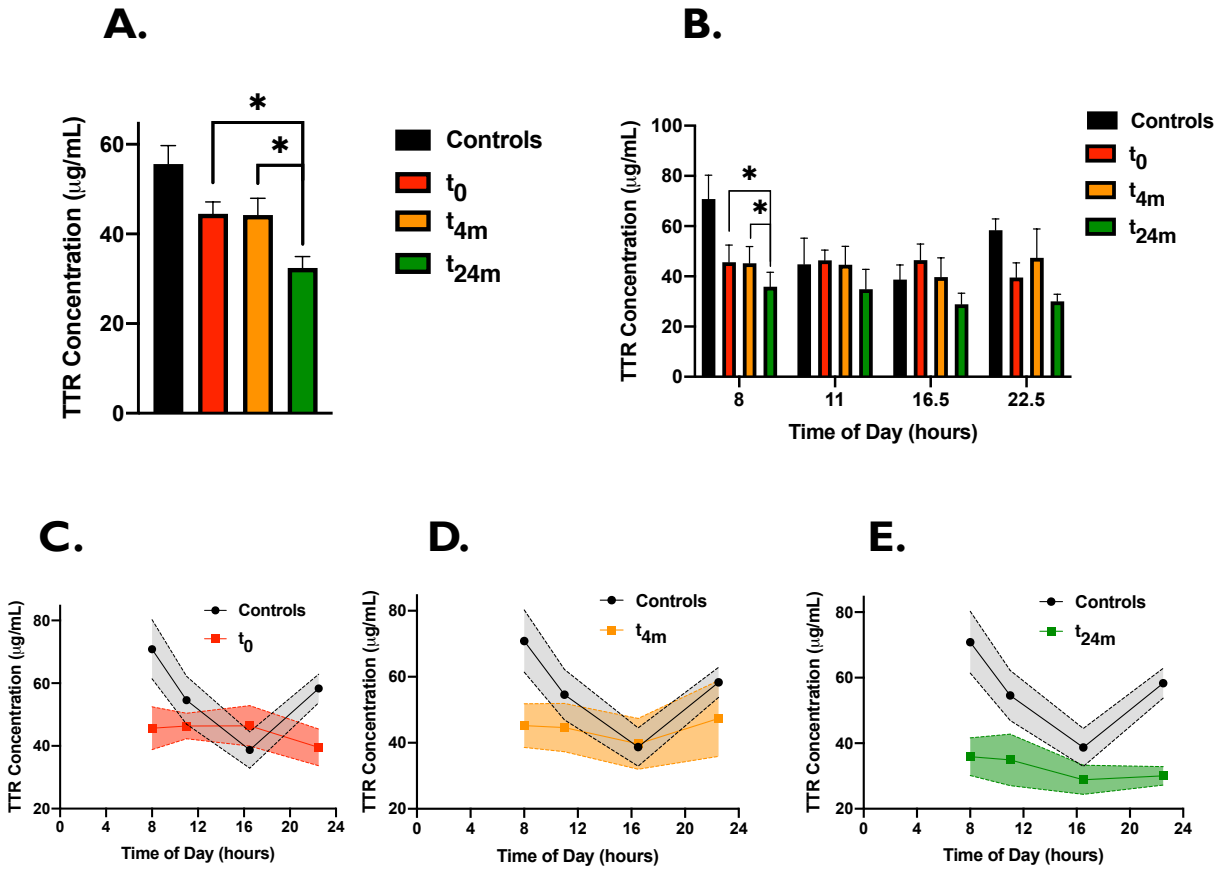


Figure 14. Averaged daily values and diurnal profile of plasma TTR in controls subjects and OSA patients throughout therapy.

(A) This graph depicts averaged daily plasma TTR concentrations in controls (young and age-matched), untreated OSA patients (t_0), OSA patients after 4 months of CPAP therapy t_{4m}, and OSA patients after 24 months of CPAP therapy t_{24m}. In OSA patients, average daily TTR concentration was significantly lower in t_{24m} when compared to both t_0 and t_4. Data is presented as mean ± SEM with n=6 (controls), n=3 (t_0), n=3 (t_{4m},), and n=3 (t_{24m}). *$p<0.05$, one-way ANOVA. **(B)** This graph depicts TTR concentrations at distinct times of day - 8h, 11h, 16h30, 22h20 - , in controls (young and age-matched), untreated OSA patients (t_0), OSA patients after 4 months of CPAP therapy t_{4m}, and OSA patients after 24 months of CPAP therapy t_{24m}. At time point 8h TTR concentration were significantly lower at t_{24} when compared to both t_0, and t_4. Data is presented as mean ± SEM with n=6 (controls), n=3 (t_0), n=3 (t_{4m}), and n=3 (t_{24m}). *$p<0.05$, two-way ANOVA. **(C)** This graph depicts diurnal TTR profile in controls (young and age-matched) and in untreated OSA patients (t_0). The diurnal TTR profile in control subjects are characterized by morning zeniths, a gradual decline into an afternoon nadir, followed by a gradual increase into the evening and early morning. The diurnal TTR profile in untreated OSA patients (t_0) did not feature any remarkable peaks throughout the day, and showed little oscillation. When control subjects' TTR concentrations trended upwards into the early morning, untreated OSA patients' (t_0) TTR values trended slightly downwards. Data is presented as mean ± SEM with n=6 (controls) and n=3 (t_0). **(D)** This graph depicts diurnal TTR profile in controls (young and age-matched) and in OSA patients after 4 months of CPAP therapy (t_{4m}). The diurnal TTR profile in control subjects are characterized by morning zeniths, a gradual decline into an afternoon nadir, followed by a gradual increase into the evening and early morning. The diurnal TTR profile in treated OSA patients (t_{4m}) did not feature any remarkable peaks throughout the day, and showed little oscillation. TTR values trended slightly downwards. Data is presented as mean ± SEM with n=6 (controls) and n=3 (t_{4m}). (E) This graph depicts diurnal TTR profile in controls (young and age-matched) and in OSA patients after 24 months of CPAP therapy (t_{24m}). The diurnal TTR profile in control subjects are characterized by morning zeniths, a gradual decline into an afternoon nadir, followed by a gradual increase into the evening and early morning. The diurnal TTR profile in treated OSA patients (t_{24m}) did not feature any remarkable peaks throughout the day, and showed little oscillation. Data is presented as mean ± SEM with n=6 (controls) and n=3 (t_{24m}).

4.5. The Effect of Aging on Plasma TTR

Initially we analyzed the healthy subjects all together, young and age-matched controls. However, there were marked differences in averaged plasma TTR concentration between age-matched (46.8 μg/mL ± 5.6) and young controls (64.4 μg/mL ± 4.9). Averaged daily TTR values were found to be significantly higher (p=0.045) in young controls when compared to age-matched controls (figure 15-A). The diurnal profile of TTR also appears to be affected by aging. Both cohorts of controls feature morning peaks, afternoon nadirs, and gradually increasing TTR concentrations into the evening. However, the afternoon nadirs in young controls (11h; 46.6 μg/mL ± 21.0) occur around midday, whereas in age-matched controls this daily low (26.9 μg/mL ± 0.59) occurs around 16h30 (figure 15-B). The significantly lower levels of TTR in age-matched controls, together with the phase shift in daily nadirs, suggest that aging may affect the expression profile of TTR.

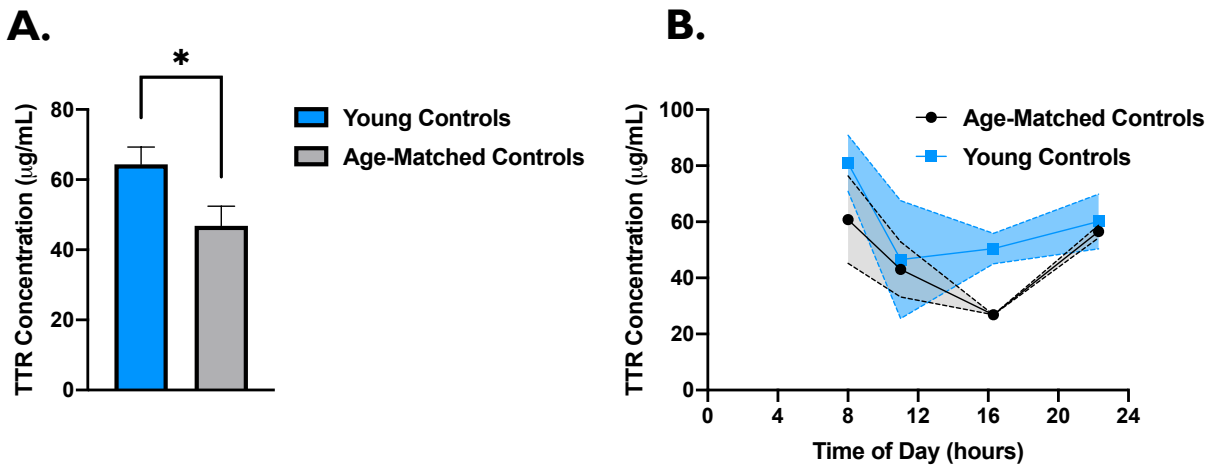


Figure 15. Averaged daily values and diurnal profile of plasma TTR in age-matched and young control subjects.
(A) This graph depicts averaged daily TTR concentrations in young controls and age-matched controls. The average daily TTR concentration in age-matched controls were significantly lower when compared to young controls. Data is presented as mean ± SEM with n=3 (age-matched controls) and n=3 (young controls). *p<0.05, unpaired t test **(B)** This graph depicts the diurnal plasma TTR profile in both age-matched and young controls. Both control cohorts' TTR concentrations are characterized by morning zeniths, a gradual decline into an afternoon nadir, followed by a gradual increase into the evening and early morning. The afternoon nadir in age-matched controls is phase-shifted when compared to young controls, occurring at in the afternoon around 16h30 rather than at midday.

Chapter 5: Discussion

Since its discovery in the 1950s our understanding of TTR, its functions, and related pathophysiological mechanisms, has evolved dramatically (Robbins, 2002). Much more than a thyroxine and RBP carrier, TTR has been shown to have complex homeostatic functions, an accumulating association with oxidative stress and age-related diseases, and diurnal rhythmicity (Zhao, Buxbaum & Reixach, 2017; Sharma et al., 2019, Zhao et al., 2013, Gliniak, 2017). Despite being frequently cited in research, TTR is seldom used in clinical practice (Delliere et al., 2018). However, TTR has recently been utilized successfully as a therapeutic target for the treatment of Familial Amyloidal Polyneuropathy and Cardiac Amyloidosis, alluding that protein's potential utility in clinical contexts (Maurer et al., 2018).

Though OSA has been defined as one of the most prevalent sleep disorders, up to 90 % of individuals with OSA may remain undiagnosed and untreated (Thorpy, 2017; Fleming et al., 2018). While the true burden of OSA on the global population may yet be elusive, the prevalence of this condition may increase due to an aging population, and concomitant increases in the prevalence of comorbid conditions such as DM2 and obesity (Miner & Kryger, 2017, Ingelfinger & Jarcho, 2017, Romero-Corral et al., 2010). By the same token, untreated OSA and its characteristic features such as HPA dysregulation, oxidative stress, and sleep fragmentation are risk factors for other diseases (Hirotsu, Tufik, & Anderson, 2015; Liguori et al., 2018).

An all too common barrier to diagnosis of OSA are the diagnostic measures themselves. Not only are diagnostic parameters variable among countries and institutions, the main diagnostic technique, PSGs, have their own set of limitations. PSGs require overnight stays in a sleep center, can be costly, and may not best represent an average night of sleep in patients (Osman et al., 2018; Shaw et al., 2012). For those who have been diagnosed with OSA, CPAP therapy has been shown to considerably mediate the disease's underlying pathophysiological mechanism, and improve prognosis and quality of life (Batool-Anwar et al., 2016; Ou et al., 2015). However, CPAP is preventative rather than curative, requires daily use, and adherence rates remain unacceptably low (Osman et al., 2018).

In this context, OSA, and its clinical management, has two urgent needs. First, alternative or complementary diagnostic biological markers able to reliably indicate OSA disease states and prognosis. And second, the identification of potential therapeutic targets able to complement or replace current approaches to therapy. TTR has already been defined as a potentially reliable

clinical biomarker due to it being mainly synthesized by the liver, having a small pool size, and a half-life of 1-2 days. Additionally, TTR is considered an acute phase protein and endogenous concentrations are known to increase or decrease by at least 25 % during inflammatory states (Ahmed et al., 2012). With this in mind, TTR may indeed be well suited as biomarker for OSA, as it is a chronic condition which mainly affects patients once per day during sleep.

In this research study we sought out to investigate TTR in relation OSA by assessing and comparing plasma TTR concentrations in both healthy controls and OSA patients. Since TTR has been characterized as a diurnal protein, sensitive to the circadian rhythm with differential expression throughout the day, plasma TTR concentrations were assessed in both control subjects and OSA patients at four time points throughout the day: 8h, 11h, 16h30, 22h30. In order to gauge TTR's potential relationship with the pathophysiological mechanisms characteristic to OSA, as well as the protein's utility in measuring therapy outcomes and prognosis, TTR was also assessed in patients at the time of diagnosis and throughout therapy after 4 months and 24 months of CPAP use.

In order to avoid differences and variability between subjects due to gender, only male subjects were selected for both control and OSA cohorts. Though OSA affects both males and females, males are considerably more likely to be afflicted by OSA when compared to women. This may in-part be due to OSA's effect on endocrinological processes, a caveat elucidated by the fact that women become more likely to develop OSA following post-menopausal hormonal alterations (Young, Skatrud, & Peppard, 2013). Though persons of any age my develop OSA, age has been identified as a risk factor for the development and severity of OSA, being most prevalent between the ages of 45 and 64 (Bixler et al., 1998; Deng et al., 2014). For this reason, OSA patients with an average age of 53 $\pm$ 6.1 years, and age-matched controls with an average age of 47 $\pm$ 7.0 years were selected for this study. In order to further investigate the role that aging may have on the pathophysiology of OSA, young patients were also recruited, having an average age of 25 $\pm$ 0.9 years. The selected OSA patients cohort may well represent the average OSA patient, not only due to being male and in the appropriate age-range, but also because all patients had excessive weight, and on average were considered to be obese class 1. Additionally, this cohort featured comorbid conditions commonly found in OSA patients, specifically, DM2 and hypertension (Young, Skatrud, & Peppard, 2013). That being said, age-matched controls were patients that while not diagnosed or treated for OSA, still had high clinical suspicion for the disease. Additionally,

age-matched controls were on average considered to be overweight according to the BMI, and one of the subjects suffered from depression.

Though it has been previously found that TTR was overexpressed in the blood serum of OSA patients (Jurado-Gamez et al., 2011), the results obtained in the present work indicated that average plasma TTR levels throughout the day were significantly lower in OSA patients when compared to non-OSA controls. However, the aforementioned study only measured TTR in the morning at 7h30 during a fasting state. While the present results did not indicate a significant difference between control subjects and OSA patients at 8h in the morning, this may have largely been due to our low sample size. Diurnal TTR concentration profiles of controls featured distinct morning zeniths, a characteristic not found in the diurnal profiles of OSA patients at the same timepoint. However, upon further investigation, CPAP treatment was found to significantly decrease plasma TTR concentrations in OSA patients in the morning at 8h after 24 months of adherent therapy. The discrepancy between the findings in aforementioned study and the results of this thesis may be in-part due to the differences in serum and plasma. In Jurado-Gamez and colleagues' research blood samples were subjected to cold clotting as part of their research design, while in blood samples obtained in this work clotting was prevented through the use of K2EDTA-coated collection tubes. Additionally, Jurado-Gamez and colleagues utilized isobaric tags for relative and absolute quantification (iTRAQ) followed by mass spectrometry analysis, in contrast to ELISAs followed by spectrometry utilized in the present research work.

As expected, AHI was significantly decreased in response to CPAP therapy after 4 months and 24 months of adherent therapy. However, significant decreases in both averaged daily plasma TTR concentrations and in plasma TTR at timepoint 8h were not noted until after 24 months of continuous CPAP therapy. This may indicate that decreases in TTR value may be indicative of overall recovery, rather than a direct response to palliative CPAP intervention.

There was a remarkable difference between the diurnal plasma TTR profile of OSA patients and controls. The diurnal profile in control subjects are characterized by morning zeniths, a gradual decline into an afternoon nadir, followed by a gradual increase into the evening and following morning. In OSA patients both before and following 4 and 24 months of continuous CPAP therapy, this oscillatory profile was not observed, and there was little overall fluctuation. However, the gradual trend upwards following afternoon nadirs characteristic to controls subjects seemed to

be inversed in patients before the commencement of CPAP therapy. This downward trend found in OSA patients before therapy was not found at 4 months or 24 months following CPAP therapy, and though insignificant when compared to control subjects, there is a slight upward trend following afternoon nadirs in treated OSA patients. In the composite, the obtained results make clear that OSA patients have significantly flattened diurnal plasma TTR profiles when compared to control subjects. This observation is of significant clinical importance, as alterations of diurnal profiles of certain endogenous molecules has been associated with various disease states, such as depression and anxiety, cardiovascular diseases, immune disorders, obesity and DM2 (Tu et al., 2013; Doane et al., 2013; Hek et al., 2013; Hajat et al., 2013; Patterson et al., 2013; Schrepf et al., 2014; Hackett, Steptoe & Kumari, 2014). In OSA specifically, patients display a flattening of the cortisol awakening response, and hypocortisolism upon awakening (Ghiciuc et al., 2013; Ghiciuc et al., 2016)

It has been well established that plasma TTR levels steadily decline after about fifty years of age (Vieira & Saraiva, 2014). With this in consideration, it was important to assess the differential effects aging may have on average TTR concentrations and diurnal profiles as it relates to OSA pathology. Young controls had significantly higher averaged TTR concentrations when compared to age-matched controls, a finding that was expected as the average age of age-matched controls was 47 $\pm$ 7.0 years. Regarding the diurnal profile of control patients, both age-matched controls and young controls featured characteristic morning peaks, a gradual decline into an afternoon nadir, followed by a gradual increase into the evening and following morning. However, in young controls the daily nadir occurred around midday, whereas in age-matched controls this daily lull in plasma TTR concentration occurred later in the afternoon, around 16h30. Phase shifts are commonly cited as adaptive mechanism, mediated by the circadian rhythm, in response to circumstances such as travel and shift work (Eastman et al., 2015). However, the process of aging has been known to produce phase shifts towards morning chronotypes, and recent evidence has indicated that aging may be accompanied by changes to molecular rhythms that could affect altered cognition and sleep (Chen et al., 2015). With this in mind, it is possible that a delayed phase of diurnal TTR production could be an adaptive mechanism to the natural aging process. In this context, it is also possible that blunted TTR profile characteristic to OSA patients enrolled in this study may also be an adaptive mechanism related to underlying OSA pathology.

All this considered, the results obtained in this thesis suggest that TTR may be suitable biomarker for the diagnosis, prognosis, and measurement of therapy outcomes in OSA. However, the utilization of TTR for such purposes requires the consideration of parameters such as age of patients, comorbid diseases, and time of day.

Chapter VI: Conclusion

Through the present study it was concluded that:

1. **Plasma TTR concentrations have distinct diurnal fluctuations in healthy adults.**

   These diurnal profiles are characterized by morning zeniths, a gradual decline into an afternoon nadir, and subsequent increase into the evening and following morning.

2. **In OSA diurnal plasma TTR curves are markedly flattened compared to healthy adults.**

3. **Continuous adherent CPAP therapy over a period of 24 months significantly decreases daily plasma TTR concentrations in OSA patients.**

4. **Continuous adherent CPAP therapy over a period of 24 months significantly decreases morning plasma TTR concentrations at 8h in OSA patients.**

5. **The aging process results in lower daily plasma TTR concentrations and a phase delay in the diurnal plasma TTR curve.**

Future Perspectives

Though the present study yielded significant results, findings were largely limited due to the small sample size. The findings in this research may be further built upon and corroborated through the utilization of larger cohorts of both OSA patients and controls. Additionally, in order to definitively gauge TTR's relationship with OSA, a similar study examining patients with varying severity of OSA, such as patients presenting with mild and moderate OSA, would be essential.

The present study could be complemented by juxtaposing the diurnal oscillations of other hormones, such as cortisol, with the oscillatory profile of TTR in patients with OSA. In a similar light, concomitantly examining molecules TTR has a known relationship with such as, RBP, T4, Abeta, and NPY, throughout the day may also help to elucidate TTRs role in OSA pathology. Examining TTR in coordination with these other parameters would be especially important for identifying TTR as a distinct biomarker for OSA considering the disease's many comorbidities.

Integral components of the circadian clock, clock genes, have been shown to be dysregulated by OSA (Burioka et al., 2008). Considering the changes found in the present study to the oscillatory profile of plasma TTR, and the fact the TTR gene contains a GC-response element, investigation into potential genetic alterations OSA may have on TTR expression may prove to be enlightening (Sharma et al., 2019).

TTR has been found to be modified by oxidative post-translational processes which subsequently affect its binding abilities (Henze et al., 2015). Moreover, it has recently been found that OSA patients feature evening and morning alterations to the red cell proteome, likely in response to oxidative stress (Feliciano et al., 2017). With this in mind, a closer look into TTR and potential post-translational alterations could help us better understand how TTR responses to oxidative stress, OSA, and related comorbidities.

TTR has already been clinically utilized as a therapeutic target for the treatment of Familial Amyloidal Polyneuropathy and Cardiac Amyloidosis (Maurer et al., 2018). However, these therapies act on TTR either through stabilization of native tetrameric forms, or through gene silencing via ribonucleic acid interference (RNAi) (Butler et al., 2016; Yang, 2019). As TTR's role

in the pathophysiology of OSA is still yet to be fully understood the clinical utility of these drugs in relation to OSA may not yet be serviceable. However, this study found that aging had a profound effect on daily TTR concentrations, and noticeably altered daily fluctuation of TTR. With this in mind, it is possible that TTR has a role in the process of aging. If this is the case, modulating levels of TTR expression via RNAi may be of interest in the currently thriving field of anti-aging research and medicine.

Appendices

Appendix A – Homemade ELISA Buffers and Solutions

(A)

	Na_2HCO_3	$NaHCO_2$	H_2O	HCl	NaOH	pH
Coating Buffer	5.98 mM	44.0 mM	100mL	Variable on pH	n/a	7.0
	5.98 mM	44.0 mM	100mL	Variable on pH	n/a	7.6
	5.98 mM	44.0 mM	100mL	Variable on pH	n/a	8.0
	14.0 mM	34.9 mM	100mL	n/a	Variable on pH	9.5
	14.9 mM	34.9 mM	100mL	n/a	Variable on pH	9.5

(B)

	TBS	Tween20	pH
Wash Solution	400mL	2.5mL (10%)	7.0
	400mL	2.5mL (5%)	7.0
	400mL	1.0mL (5%)	7.0
	400mL	n/a	7.0
	400mL	1.0 mL (10%)	8.0

(C)

	TBS	BSA	NFDM	pH
Blocking Buffer	100mL	1g	n/a	7.6
	100mL	1g	n/a	8.0
	100mL	1g	n/a	9.5
	100mL	n/a	0.1% w/v	8.0
	100mL	n/a	0.3% w/v	9.5
	100mL	n/a	0.1% w/v	9.5

Panel 1. Recipes of buffers and solutions utilized homemade ELISA optimization.
(A) Coating buffers utilized during homemade ELISA optimization.
(B) Blocking buffers utilized during homemade ELISA optimization
(C) Wash solutions utilized during homemade ELISA.

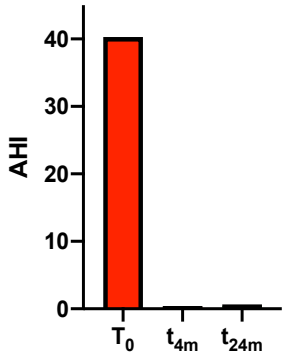
Appendix B – Individual Patient Data

Patient 1:

(A)

		t_0
Medical history	Age (years)	61
	BMI	29.8
Sleep disruption events	Arousal events/h	34.1
	Awakening events/h	4.3
Respiration Summary	AHI	40.3
Oxygenation Summary	SpO2 mean (%)	90
	Desaturation events/h	29.8
EKG statistics	Heart rate, wake (bpm)	75
	Heart rate, steady sleep (bpm)	73

(D)



(B)

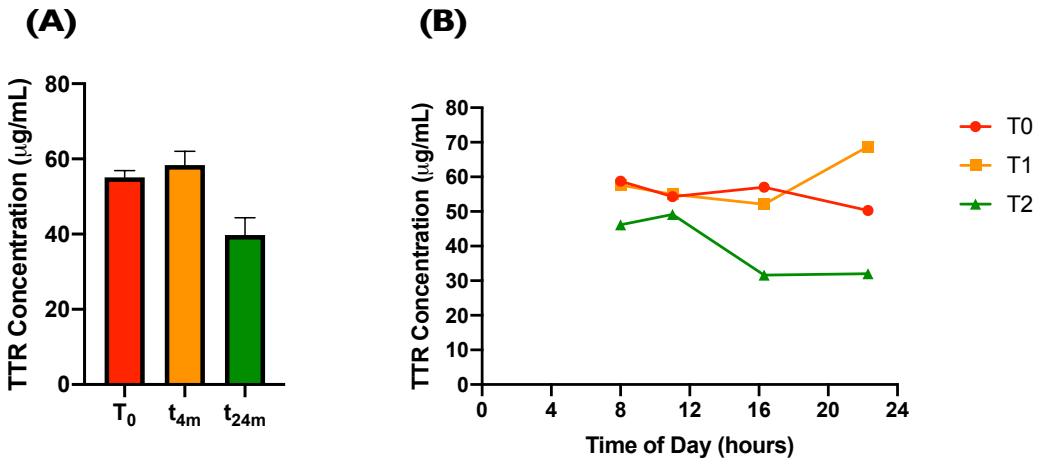
		t_{4m}
Medical History	Age	61
Adherence	Days with more than 4h of use	100
Respiration Summary	AHI	0.4

(C)

		t_{24m}
Medical history	Age (years)	63
	BMI	28.7
Adherence	Days with more than 4h of use	93
Respiration Summary	AHI	0.6

Panel 2. Main clinical parameters assessed in OSA patient 1 before treatment (t_0), after four months of CPAP therapy (t_{4m}), and after 24 months of CPAP therapy (t_{24m}).
(A) Parameters assessed in OSA patient 1 before treatment. Data depicting age and BMI of patient 1 were obtained by the medical team. Data concerning sleep disruption events (arousals and awakenings per hour), respiration (apnea and hypopnea index), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when in a steady sleep state) were measured in patient 1 during PSG test. **(B)** Parameters assessed in OSA patient 1 after four months of CPAP treatment. Data depicting age was obtained by research team. Adherence (percentage of days with more than 4h of CPAP use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. **(C)** Parameters assessed in OSA patient 1 after 24 months of CPAP treatment. Data depicting age was obtained by research team. Data depicting BMI was obtained by medical team. Adherence (percentage of days with more than 4h of CPAP use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. **(D)** This graph depicts the AHI of OSA patient 1 at the time of diagnosis, four months following CPAP treatment, and 24 months following CPAP treatment.

Patient 1:



Panel 3. Total plasma TTR & Diurnal plasma TTR curve OSA patient 1 before treatment (t_0), after four months of CPAP therapy (t_{4m}), and after 24 months of CPAP therapy (t_{24m}).

A) This graph depicts averaged daily TTR concentrations in OSA patient 1before therapy (t_0), after 4 months of CPAP therapy t_{4m}, and after 24 months of CPAP therapy t_{24m}. **(B)**This graph depicts diurnal TTR profile in OSA patient 1 before therapy (t_0), after 4 months of CPAP therapy t_{4m}, and after 24 months of CPAP therapy t_{24m}.

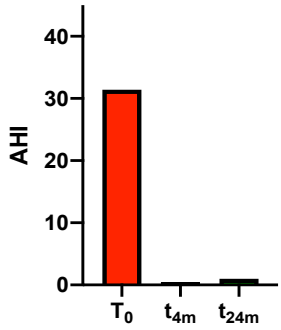
Patient 2:

Please note that desaturation data for patient 2 at t_0 was not available.

(A)

		t_0
Medical history	Age (years)	41
	BMI	32.4
Sleep disruption events	Arousal events/h	10.8
	Awakening events/h	2.1
Respiration Summary	AHI	31.4
Oxygenation Summary	SpO2 mean (%)	93
EKG statistics	Heart rate, wake (bpm)	81
	Heart rate, steady sleep (bpm)	75

(D)



(B)

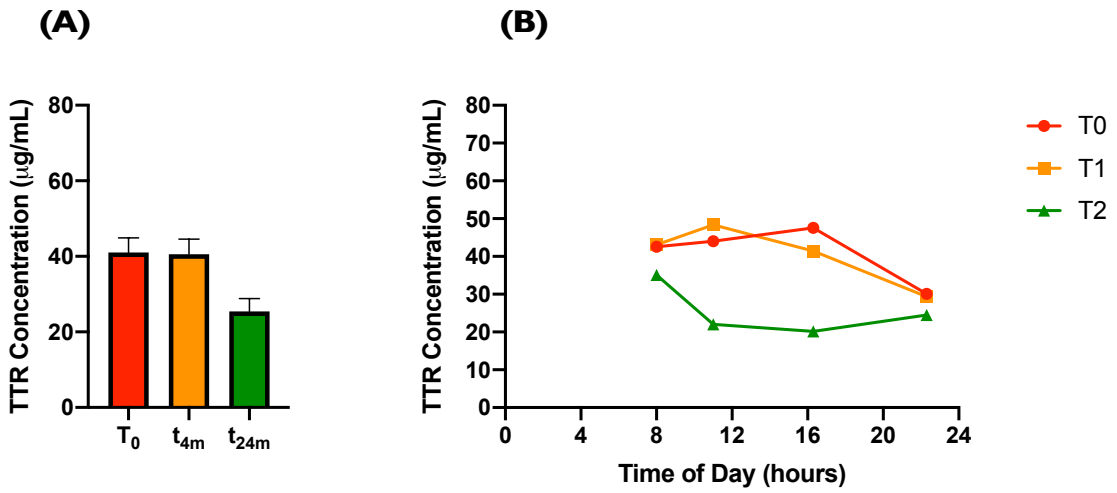
		t_{4m}
Medical History	Age	41
Adherence	Days with more than 4h of use	84
Respiration Summary	AHI	0.4

(C)

		t_{24m}
Medical history	Age (years)	43
	BMI	31.1
Adherence	Days with more than 4h of use	92
Respiration Summary	AHI	1

Panel 4. Main clinical parameters assessed in OSA patient 2 before treatment (t_0), after four months of CPAP therapy (t_{4m}), and after 24 months of CPAP therapy (t_{24m}).
(A) Parameters assessed in OSA patient 2 before treatment. Data depicting age and BMI of patient 2 were obtained by the medical team. Data concerning sleep disruption events (arousals and awakenings per hour), respiration (apnea and hypopnea index), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when in a steady sleep state) were measured in patient 2 during PSG test. **(B)** Parameters assessed in OSA patient 2 after four months of CPAP treatment. Data depicting age was obtained by research team. Adherence (percentage of days with more than 4h of CPAP use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. **(C)** Parameters assessed in OSA patient 2 after 24 months of CPAP treatment. Data depicting age was obtained by research team. Data depicting BMI was obtained by medical team. Adherence (percentage of days with more than 4h of CPAP use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. **(D)** This graph depicts the AHI of OSA patient 2 at the time of diagnosis, four months following CPAP treatment, and 24 months following CPAP treatment.

Patient 2:



Panel 5. Total plasma TTR & Diurnal plasma TTR curve OSA patient 2 before treatment (t_0), after four months of CPAP therapy (t_{4m}), and after 24 months of CPAP therapy (t_{24m}). A) This graph depicts averaged daily TTR concentrations in OSA patient 2 before therapy (t_0), after 4 months of CPAP therapy t_{4m}, and after 24 months of CPAP therapy t_{24m}. **(B)**This graph depicts diurnal TTR profile in OSA patient 2 before therapy (t_0), after 4 months of CPAP therapy t_{4m}, and after 24 months of CPAP therapy t_{24m}.

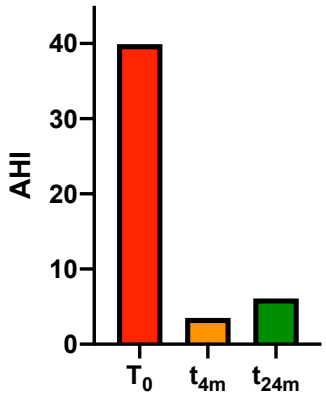
Patient 3:

Please note that BMI data for patient 3 at t_{24m} was not available.

(A)

		t_0
Medical history	Age (years)	57
	BMI	34.7
Sleep disruption events	Arousal events/h	19.8
	Awakening events/h	12.2
Respiration Summary	AHI	39.9
Oxygenation Summary	SpO2 mean (%)	91
	Desaturation events/h	0.2
EKG statistics	Heart rate, wake (bpm)	97
	Heart rate, steady sleep (bpm)	74

(D)



(B)

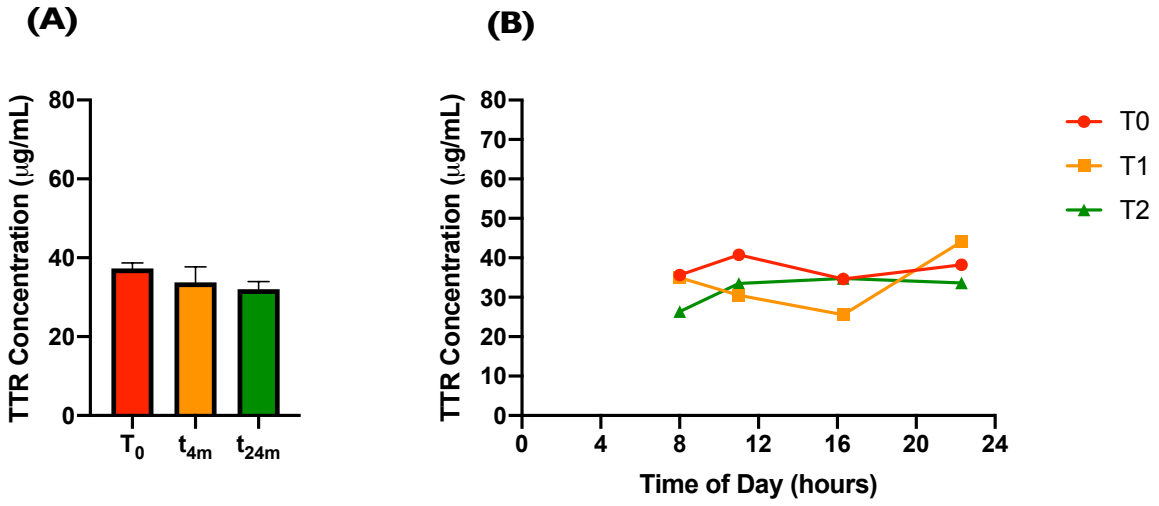
		t_{4m}
Medical History	Age	57
Adherence	Days with more than 4h of use	100
Respiration Summary	AHI	3.5

(C)

		t_{24m}
Medical history	Age (years)	59
Adherence	Days with more than 4h of use	72.1
Respiration Summary	AHI	6.1

Panel 6. Main clinical parameters assessed in OSA patient 3 before treatment (t_0), after four months of CPAP therapy (t_{4m}), and after 24 months of CPAP therapy (t_{24m}).
(A) Parameters assessed in OSA patient 3 before treatment. Data depicting age and BMI of patient 3 were obtained by the medical team. Data concerning sleep disruption events (arousals and awakenings per hour), respiration (apnea and hypopnea index), oxygenation (percentage of arterial oxygen saturation and desaturation events per hour) and EKG statistics (heart rate when awake and when in a steady sleep state) were measured in patient 3 during PSG test. **(B)** Parameters assessed in OSA patient 3 after four months of CPAP treatment. Data depicting age was obtained by research team. Adherence (percentage of days with more than 4h of CPAP use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. **(C)** Parameters assessed in OSA patient 3 after 24 months of CPAP treatment. Data depicting age was obtained by research team. Data depicting BMI was obtained by medical team. Adherence (percentage of days with more than 4h of CPAP use) and respiration (apnea and hypopnea events per hour) were recorded by the CPAP chip. **(D)** This graph depicts the AHI of OSA patient 3 at the time of diagnosis, four months following CPAP treatment, and 24 months following CPAP treatment.

Patient 3:



Panel 5. Total plasma TTR & Diurnal plasma TTR curve OSA patient 3 before treatment (t_0), after four months of CPAP therapy (t_{4m}), and after 24 months of CPAP therapy (t_{24m}).
A) This graph depicts averaged daily TTR concentrations in OSA patient 3 before therapy (t_0), after 4 months of CPAP therapy t_{4m}, and after 24 months of CPAP therapy t_{24m}. **(B)**This graph depicts diurnal TTR profile in OSA patient 3 before therapy (t_0), after 4 months of CPAP therapy t_{4m}, and after 24 months of CPAP therapy t_{24m}.

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