UNIVERSITY OF CRETE

SCHOOL OF MEDICINE

GRADUATE PROGRAMME IN NEUROSCIENCES

NEUROLOGY/NEUROGENETICS LABORATORY

DIPLOMA THESIS

Association study of variants in genes related to sleep disorders with actigraphy data in dementia patients and controls

Μελέτη συσχέτισης πολυμορφισμών γονιδίων που σχετίζονται με διαταραχές του ύπνου και δεδομένων ακτιγραφίας σε ασθενείς με άνοια και νοητικά υγιείς μάρτυρες

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ABSTRACT

Introduction: Sleep is a vital function for humans. Accordingly, sleep disturbances can be the symptom of a disorder or can cause disease by themselves. In previous research, several genes that regulate circadian rhythms have been identified, including *CLOCK, PER1, PER2, PER3, CRY1, CRY2, ARNTL, NPAS2, RORA, RORB, REV1, VDR, BHLHE40, BHLHE41, NR1D1, TNF, CHRM3, BDNF, SLC6A4, SLC4A3, HCRTR1, HCRTR2, MECP2, GABBR1* and *HLA-DQB1.*

Aims: Our aim here was to identify the effect of variants in genes associated with circadian rhythms on selected sleep parameters as identified by actigraphy.

Methods: In our study, we analyzed variants (Single Nucleotide Polymorphisms: SNPs) in these genes for their association with sleep parameters, as identified through actigraphy in a large interdisciplinary cohort (Cretan Aging Cohort: CAC). For this, we associated WES data from CAC participants with the actigraphy scores in 4 sleep parameters (night TST, TST, night TiB, total TiB). Our study subsample included 164 participants (91 dementia, 73 controls). The first part of our study focused on assessing the effect of the presence or absence of SNPs in these 25 genes on the 4 sleep parameters scores. An outcome was considered statistically significant if resulting in a p value of <0.05. The second part of the study aimed to detect rare variants present in participants with extreme sleep phenotypes, as determined by the 4 sleep parameter scores.

Results: We found that the **rs1042098** (*SLC6A3* gene), the **rs8192440** (*CRY1* gene) and the **rs2304911** (*PER1* gene) variants showed a statistically significant difference comparing values of sleep parameters between SNP carriers and non-carriers, with the carriers of rs2304911 and rs1042098 having shorter objective sleep in all parameters and the carriers of rs8192440 having longer objective sleep in all parameters examined, comparing to the non-carriers group. Also, we identified several rare variants in this sample that had an association with an extreme sleep phenotype.

Conclusion: There is evidence that there may be an association of the genotype with the sleep phenotype, as determined by actigraphy. Further research is needed to better describe this association between shorter or longer sleep duration and the presence of the variants in the genes studied.

Keys words: sleep, clock genes, SNPs, actigraphy, sleep parameters, sleep disorders

ΠΕΡΙΛΗΨΗ

Εισαγωγή: Ο ύπνος είναι μια ζωτική λειτουργία για τον άνθρωπο. Επομένως, οι διαταραχές του ύπνου μπορεί να είναι το σύμπτωμα μιας διαταραχής ή η αιτία για να προκληθεί μια ασθένεια. Σε προηγούμενη έρευνα, έχουν εντοπιστεί αρκετά γονίδια που ρυθμίζουν τους κιρκάδιους ρυθμούς, συμπεριλαμβανομένων των *CLOCK, PER1, PER2, PER3, CRY1, CRY2, ARNTL, NPAS2, RORA, RORB, REV1, VDR, BHLHE40, BHLHE41, NR1D1, T3FN , SLC6A4, SLC4A3, HCRTR1, HCRTR2, MECP2, GABBR1* και *HLA-DQB1.*

Στόχοι: Ο στόχος μας εδώ ήταν προσδιορίσουμε την επίδραση των παραλλαγών στα γονίδια που σχετίζονται με τους κιρκάδιους ρυθμούς, σε επιλεγμένες παραμέτρους ύπνου όπως προσδιορίζονται από την ακτιγραφία.

Μέθοδολογία: Στη μελέτη μας, αναλύσαμε παραλλαγές (Single Nucleotide Polymorphisms: SNPs) σε αυτά τα γονίδια για να τα συσχετίσουμε με τις παραμέτρους ύπνου, όπως προσδιορίστηκαν μέσω της ακτιγραφίας σε ένα δείγμα, μέρος μια μεγάλη διεπιστημονικής μελέτης (Cretan Aging Cohort: CAC). Για να το πετύχουμε αυτό, συσχετίσαμε δεδομένα WES από συμμετέχοντες του δείγματος (Cretan Aging Cohort: CAC) με τις βαθμολογίες της ακτιγραφίας σε 4 παραμέτρους ύπνου (night TST, TST, night TiB, total TiB). Το τελικό μερος του δείγματος μας περιελάμβανε 164 συμμετέχοντες (91 ανοϊκοί, 73 μάρτυρες). Το πρώτο μέρος της μελέτης μας επικεντρώθηκε στην αξιολόγηση της επίδρασης της παρουσίας ή απουσίας των SNPs σε αυτά τα 25 γονίδια, στις 4 βαθμολογίες παραμέτρων ύπνου. Ένα αποτέλεσμα θεωρήθηκε στατιστικά σημαντικό εάν είχε ως αποτέλεσμα τιμή $p < 0,05$. Το δεύτερο μέρος της μελέτης είχε ως στόχο την ανίχνευση σπάνιων πολυμορφισμών που υπάρχουν σε συμμετέχοντες με ακραίους φαινοτύπους ύπνου, όπως προσδιορίζονται από τις 4 παραμέτρους ύπνου.

Αποτελέσματα: Εντοπίσαμε ότι οι παραλλαγές **rs1042098** (*SLC6A3* gene), **rs8192440** (*CRY1* gene) και **rs2304911** (*PER1* gene) έδειξαν πως υπάρχει μια στατιστικά σημαντική διαφορά συγκρίνοντας τις τιμές των παραμέτρων ύπνου μεταξύ συμμετεχόντων που φέρουν τα SNP και αυτών που δεν τα φέρουν, με τους συμμετέχοντες που φέρουν τις παραλλαγές rs1042098 και rs2304911 να εμφανίζουν μικρότερη διάρκεια ύπνου σε όλες τις παραμέτρους και τους φορείς της rs8192440 να εμφανίζουν μεγαλύτερη διάρκεια ύπνου σε όλες τις παραμέτρους που μελετήθηκαν, σε σχέση με την ομάδα που δεν έφερε τις παραλλαγές. Επίσης, εντοπίσαμε διάφορες

σπάνιες παραλλαγές σε αυτό το δείγμα που είχαν σχέση με έναν ακραίο φαινότυπο ύπνου.

Συμπέρασμα: Υπάρχουν ενδείξεις ότι μπορεί να υπάρχει συσχέτιση μεταξύ του γονότυπου με τον φαινότυπο ύπνου, όπως προσδιορίζεται από την ακτιγραφία. Περαιτέρω έρευνα απαιτείται για την καλύτερη περιγραφή αυτής της συσχέτισης ανάμεσα στην μικρή διάρκεια ύπνου ή στη μεγάλη διάρκεια ύπνου και την παρουσία των παραλλαγών στα γονίδια που μελετήθηκαν.

Λέξεις κλειδιά: ύπνος, γονίδια clock, SNPs, ακτιγραφία, παράμετροι ύπνου, διαταραχές ύπνου

CONTENTS

ABBREVIATIONS

5-HT: serotonin

A: adenine

Aβ: amyloid beta

Ach: acetylcholine

AD: Alzheimer disease

ADHD: attention-deficit/hyperactivity disorder

Ala (A): alanine

APOE: apolipoprotein *E*

APP: amyloid-beta precursor protein

Arg (R): arginine

ARNTL: Aryl Hydrocarbon Receptor Nuclear Translocator Like

ASD: autism spectrum disorder

Asn (N): asparagine

Asp (D): aspartic acid

ATP: adenosine triphosphate

AVP: arginine vasopressin

BDNF: brain-derived neurotrophic factor

BF: basal forebrain

BHLHE40: basic helix-loop-helix family member E40

BHLHE41: basic helix-loop-helix family member E41

C: cytosine

CAC: Cretan aging cohort

CHRM3: cholinergic receptor muscarinic 3

CRY1/CRY2: cryptosome1/cryptosome2

DA: dopamine

DR: dentate gyrus

DSPS: delayed sleep phase syndrome

EDTA: ethylenediaminetetraacetic acid

FASPS: familial advanced sleep phase syndrome

G: guanine

GABA: gamma-aminobutyric acid

GABBR1: gamma-aminobutyric acid type B receptor subunit 1

Gln (Q): glutamine

Glu (E): glutamic acid

Gly (G): glycine

GRP: calretinin, gastrin related peptide

GWAS: genome wide association studies

HA: histamine

HCRTR1/HCRTR2: hypocretin receptor1/hypocretin receptor 2

His (H): histidine

HLA-DQB1: major histocompatibility complex, class II, DQ beta 1

ipRGCs: intrinsically photoreceptive retinal ganglion cells

IL-1β: interleukin-1β

Ile (I): isoleucine

LDT/PPT: laterodorsal and pedunculopontine tegmental nuclei

LC: locus coeruleus

MCH: major histocompatibility complex

MCH: melanin-concentrating hormone

MECP2: methyl CpG binding protein 2

Met (M): methionine

MMSE: mini-mental state examination test

MNPO: median preoptic area

NE: norepinephrine (NE)

NPAS2: neuronal PAS domain protein 2

NR1D1: nuclear receptor subfamily 1 group D member 1

NREM: non rapid eye movement

OCD: obsessive-compulsive disorder

OXR1/OXR2: orexin 1 receptor/orexin 2 receptor

p: p-value

PER1/PER2/PER2: period1/period 2/period 3

PGD2: prostaglandin D2

Phe (F): phenylalanine

Pro (P): proline

PSEN1/PSEN2: presenilin1/presenilin2

REM: rapid eye movement

RORA/RORB: nuclear receptor ROR-alpha/RAR related orphan receptor B

SLC6A3/SLC6A4: solute carrier family 6 member 3/ solute carrier family 6 member 4

SCN: suprachiasmatic nucleus

Ser (S): serine

SLD: sublaterodorsal nucleus

SNP: single nucleotide polymorphism

T: thymine

Thr (T): threonine

TLS: translesion synthesis

TiB: total time in bed

TMN: tuberomammilary nucleus

TNF-α: tumor necrosis factor-α

Trp (W): tryptophan

TST: total sleep time

TTFL: transcriptional-translational feedback loop

Tyr (Y): tyrosine

Val (V): valine

VDR: vitamin D receptor

VIP: vasoactive intestinal peptide

VLPO: ventrolateral preoptic area

vPAG: ventral periaqueductal gray

VTA: ventral tegmental area

WES: whole exome sequencing

1. INTRODUCTION

Dr Allan Rechschaffen, who spent his whole academic career studying sleep and sleep deprivation at Sleep Laboratory in University of Chicago, stated that “If sleep doesn't serve an absolutely vital function, it is the greatest mistake evolution ever made.” Indeed, sleep seems to serve such a purpose, according to the discoveries that have been done so far. However, there is a great deal of information yet to be discovered. Below the basic knowledge regarding sleep’s mechanism is presented.

1.1 Definition of Sleep

Sleep occupies one third of our lives (Aminoff, Boller, & Swaab, 2011). It is such an important and vital activity, essential for functions such as memory consolidation, metabolism and immunity, that its long term deprivation causes death (Sanchez, Kalume, & de la Iglesia, 2022 ; Schwartz, & Klerman, 2019). While falling asleep, the body passes to an actively unconscious state and the brain, rests and reacts mostly to internal stimuli (Sanchez, Kalume, & de la Iglesia, 2022 ; Brinkman, Reddy, & Sharma, 2021). This state can be easily reversed with external stimuli. Although, very little is known for the mechanism that regulates sleep, it seems that several functions need to be completed so as to naturally pass to the conscious state (Sanchez, Kalume, & de la Iglesia, 2022 ; Schwartz, & Klerman, 2019).

1.2 Sleep architecture

There are two types of sleep states, the non-rapid eye movement (NREM) and the rapid eye movement one (REM) (Figure1). The states usually alternate cyclically 4 to 6 times during sleep time, with each stage lasting approximately 90-120 minutes (changes overnight). Both states present different characteristics in eye movement, muscle tone, brain wave activity which have been measured with the use of electroencephalogram (EEG). What is more, NREM sleep consists of 4 stages: N1, N2, N3 and N4. (Patel, Reddy, & Araujo, 2022 ; Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006).

1.2.1 NREM sleep

Most of the adult sleep time is spent on that state, more specifically 75-80% of the total sleep time. NREM sleep and sleep in general, begin with the N1 stage and

continue to N2, N3 and N4 until REM is reached. Each stage indicates sleep depth. NREM stage decreases as the night progresses while REM sleep increases. (Patel, Reddy, & Araujo, 2022 ; Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006).

N1

It signals the beginning of sleep and the beginning of a sleep cycle. Brain activity of low voltage mixed frequency waves indicates a “light” sleep state which lasts 1-7 minutes. Breathing continues regularly, while muscle tone is traced in the skeletal muscles. It can be disrupted by light external stimuli (Patel, Reddy, & Araujo, 2022 ; Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006).

N2

At this stage, sleep spindles and K-complexes (Figure2) are present in the electroencephalogram (EEG). It constitutes approximately 50% of total sleep time and lasts 10-25 minutes. It is suggested that memory consolidation occurs in N2 stage. As the heart and body temperature drop, it is more difficult to interrupt this sleep state (Patel, Reddy, & Araujo, 2022 ; Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006).

N3 & N4

This stages consist the deeper sleep or the slow wave sleep (SWS), indicated by the delta waves in brain activity. High voltage activity is present in both, but appears more increased in stage 4. Stage 4 lasts 20-40 minutes, when stage 3 lasts a few minutes. Tissue, bone and muscle growth and immune boosting procedures take place at this stage (Patel, Reddy, & Araujo, 2022 ; Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006).

1.2.2 REM sleep

The brain’s activity frequency is not stable and is characterized by low voltage waves and by high theta wave range at this state. There is asynchronous breathing rate and all muscles, besides the diaphragmatic breathing and eye muscles, are inactive. It starts by lasting 10 minutes and ends up lasting 1 hour at the later sleep cycles.

Dreaming occurs at the stage. It seems that dream recall results from waking up while going through the REM state (Patel, Reddy, & Araujo, 2022 ; Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006).

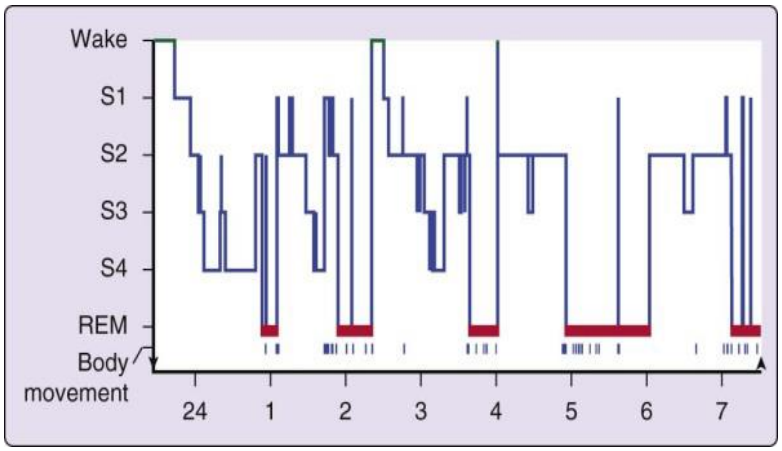


Figure1. The 4 stages on NREM sleep that alternate with REM sleep (Carskadon, & Dement, 2005).

1.3 Sleep physiology

In both NREM and REM states, a human brain functions differently. Every system adapts to the state of unconsciousness, serving a purpose which is beneficial overall for the proper functioning of the body (Table1).

1.3.1 Respiratory

It is suggested that, especially during REM sleep, there is a decrease in oxygen influx or an increase in carbon dioxide content of the blood or both, as a result of a lung hypoventilation. That may be caused, among other factors, by reduced pharyngeal muscle tone (Krieger, 2000 ; Simon, Landry, & Leifer, 2002). What is more, there is reduced tone in the intercostal and upper airway muscles, which results in low rib cage movement (Parker, & Dunbar, 2005). The cough reflex is absent as well (Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006).

1.3.2 Sympathetic and cardiovascular activity

There is an increase of sympathetic activity during the REM state compared to wakefulness. On the contrary, during the NREM state and as the sleep proceeds to

deeper states, it decreases. However, during the K-complexes of stage N2, heart rate and blood pressure increase and affect the sympathetic activity likewise. (Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006)

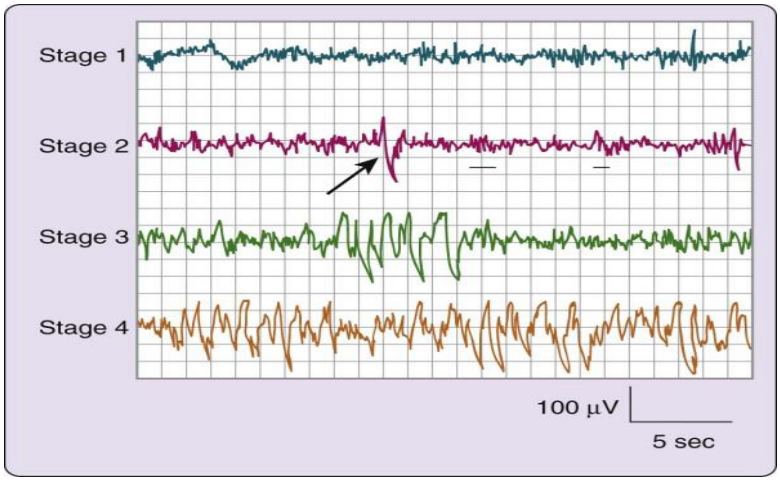


Figure2. Sleep states alteration and sleep cycles in a young adult's sleep overnight (Carskadon, & Dement, 2005). The arrow indicates the K-complex.

1.3.3 Cerebral blood flow

Following the same pattern, there is an increase in blood flow and metabolism during the REM state, compared to wakefulness, and on the contrary, a decrease in blood flow and metabolism during NREM state (Madsen et al.,1991).

1.3.4 Endocrine

Several hormones secretion is implicated during sleep. One of them is melatonin, whose function depends on the light-dark cycle as it assists with the transition from the wake state to the sleep state. What is more, growth hormone is secreted during slow wave sleep at the first part of total sleep duration (Parker, & Dunbar, 2005).

1.3.5 Renal

Another system to be influenced by sleep is renal. Reduction of the urine flow seems that is caused by limited presence of essential elements such as potassium, calcium etc. as well as changes in renal blood flow, hormone secretion, and sympathetic

neural stimulation among other factors (Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006)

Physiological Process	During NREM sleep	During REM sleep
brain activity	decreased related to wakefulness	increases in motor and sensory areas, while other areas are similar to NREM
heart rate	slower related to wakefulness	increases and varies compared with NREM
blood pressure	decreased related to wakefulness	increases (up to 30 percent) and varies from NREM
brain's blood flow	does not change from wakefulness in most regions	increases by 50 to 200 percent from NREM, depending on brain region
respiration	decreased related to wakefulness	increases and varies from NREM, but may show brief stoppages (apnea); coughing suppressed
airway resistance	increases related to wakefulness	increases and varies from wakefulness
body temperature	is regulated at lower set point than wakefulness; shivering initiated at lower temperature than during wakefulness	is not regulated - no shivering or sweating-temperature drifts toward that of the local environment
sexual arousal	occurs infrequently	increases from NREM (in both males and females)

Table1. Sleep physiology in NREM and REM sleep states (NHLBI, 2003)

1.4 Circadian rhythms

The circadian rhythm of mammals is usually described as a biological internal clock which modulates the body's essentials functions, based on the zeitgebers, which are environmental cues (Scammell, Arrigoni, & Lipton, 2017). Even though, it is affected by the earth rotation and the environmental cues during all 24 hours, it persists in the

absence of external cues (Cox, & Takahashi, 2019). This clock affects the sleep and wake cycles, with light playing an important role on the entrainment of the internal biological rhythms with environmental cycles (Sanchez, Kalume, & de la Iglesia, 2022). For the presence or absence of light (dark-light cycle), the clock is set to function accordingly and organize daily behavioral and physiological rhythms (such as sleep, locomotor activity). However, light is not the only environmental cue that participates in entrainment.

The entrainment, the alignment of the internal processes with the environmental stimuli, is crucial for survival (Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006 ; Schwartz, & Klerman, 2019). It regulates the sleep-wake cycle, physical activity and food consumption, body temperature, heart rate, muscle tone, and hormone secretion (Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006).

Chronotherapy, a therapy that aims to treat individuals by taking in consideration their circadian rhythms, has been widely used. Its principle suggests that by improving the sleep wake cycle, the treatment of other health issues could be enhanced. The medication for e.g cancer (chemotherapy) is administrated at the optimal time of the day of, which varies among patients and is based on their individual chronotype, so as to be more effective (Cardinali, Brown, & Pandi-Perumal. 2021).

1.4.1 Suprachiasmatic nucleus (SCN)

The suprachiasmatic nucleus (SCN) is the structure mainly responsible for controlling the circadian rhythms throughout the body (Figure3). It is located on the upper part of hypothalamus, above the optic chiasm. Both of its sides communicate with the third ventricle (Schwartz, & Klerman, 2019). It is also divided in the core, which is located ventrolaterally and is composed by neurons that express vasoactive intestinal peptide (VIP), calretinin, gastrin related peptide (GRP), or neurotensin, and the shell, which is located dorsomedially with its neurons expressing arginine vasopressin (AVP), angiotensin II, prokineticin-2, and met-enkephalin. (Scammell, Arrigoni, & Lipton, 2017 ; Morin, 2013).

Hence, each structure has region-specific effects on the ventral compared to dorsal SCN. Besides the neurons mentioned before, most of the neurons that comprise the SCN are GABAergic. (Scammell, Arrigoni, & Lipton, 2017).

The role of neurons expressing GABA in the SCN seems to depend on the state of the system each time, which means that they either have excitatory or inhibitory actions and they support synchrony or desynchrony (Albus, Vansteensel, Michel, Block, & Meijer, 2005 ; Aton, Huettner, Straume & Herzog, 2006 ; Mohawk, & Takahashi, 2011 ; Wagner, Castel, Gainer, & Yarom,1997).

Melatonin, a hormone which is released by the pineal gland, is regulated by the SCN and hence its release occurs with a certain rhythm and timing. Melatonin levels are high during the night and very low during the day (Sanchez, Kalume, & de la Iglesia, 2022). It is also known as the night hormone as light inhibits its secretion (Sanchez, Kalume, & de la Iglesia, 2022).

One of the major connections of SCN is the retinohypothalamic tract. The light stimulus is being detected by the photoreceptive retinal ganglion cells (ipRGCs) in the retina and via the retinohypothalamic tract it reaches the SCN. The SCN informs with the concomitant signals the structure of the body to ensure the entrainment. (Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006 ; Morin, 2013). Besides the retinohypothalamic pathway, there are two more afferent connections, the median raphe serotonergic pathway and the geniculohypothalamic (Morin, 2013).

Overall, the SCN, as a major circadian regulator, controls the wake-sleep system, the body temperature, the locomotor activity, the cycles of feeding and hormonal activity and is capable of adjusting all that under extreme conditions in order to ensure the organism's survival (Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006).

1.5 Sleep state vs Wake State

Sleep state and wake state occur every day, both driven by the circadian mechanism. Each one is characterized by different patterns and processes but both

influence each other. The interplay between two processes is what successfully regulates the sleep-wake system (Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006).

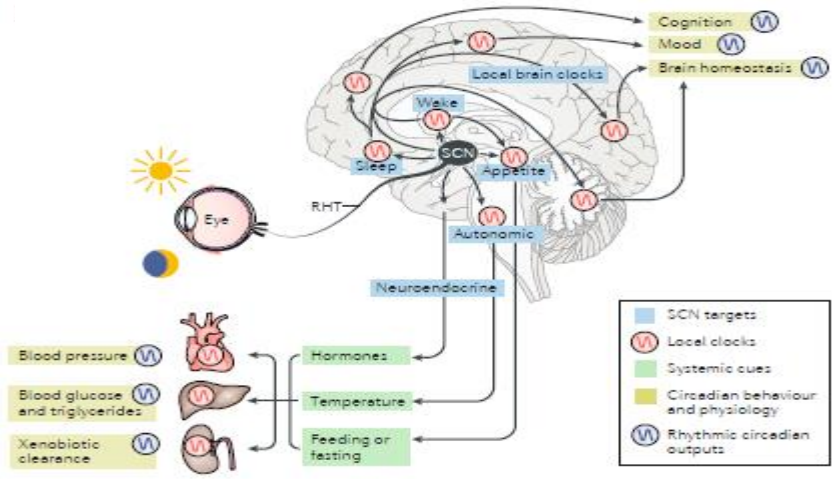


Figure3. The pathways implicated in SCN function, the brain projections and the synchronization of molecular clocks of peripheral organs (Hastings, Maywood, & Brancaccio, 2018).

1.5.1 Process S

Process S, or the homeostatic regulation of sleep, refers to the biological need for sleep. This drive for sleep builds up through the day and reaches its highest point at night, before bedtime (Sanchez, Kalume, & de la Iglesia, 2022). What is more, this sleep pressure increase, depends on the wake time of an individual. The more awake one stays, especially past bedtime, the more it mounts (Sanchez, Kalume, & de la Iglesia, 2022). During the night, as an individual's sleep progresses, it slowly disperses. In the morning, during wake time, it is at its lowest point, as a result of an adequate night sleep (Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006). It is suggested that there is an increase and decrease of Process S during REM and NREM sleep respectively (Schwartz, & Klerman, 2019).

1.5.2 Process C

Process C, or the circadian regulation of sleep, is regulated by the circadian system (Sanchez, Kalume, & de la Iglesia, 2022). Likewise Process S, Process C builds up

through the day, aiming to maintain wakefulness at certain periods during the day. It reaches its highest point in the morning and its lowest point at night before bedtime. (Gillette, & Abbott, 2005). It plays an important role at synchronizing the sleep-wake with the light-dark cycles of the environment and at maintaining each cycle clear and distinct (Gillette, & Abbott, 2005 ; Colten, Altevogt, & Institute of Medicine (US) Committee on Sleep Medicine and Research, 2006). Although the total sleep time is not affected in case Process C is absent, sleep unevenly and randomly is handed out during the day and night, resulting in the absence of distinct cycles (Gillette, & Abbott, 2005).

It is suggested that, besides their independent function, these two processes have an effect on each other, and mostly Process S has influence over Process C (Sanchez, Kalume, & de la Iglesia, 2022).

1.6 Sleep Biology

Several systems, neurotransmitters and pathways that are implicated in the sleep and wake stages have been studied. Below are separately presented: the circuitry that promotes wake, the circuitry that promotes sleep and the “flip flop switch” model.

1.6.1 Wake promoting systems

The reticular formation, an area that is spread from the core of the brainstem, to the medulla, up to the midbrain and then into the posterior hypothalamus, is implicated in the promotion of wakefulness via excitation through projections to the thalamus, hypothalamus, and basal forebrain. That is achieved by specific neurotransmitters, their role of which and their projections we discuss below (Figure4) (España, & Scammell, 2011).

Acetylcholine (ACh) is produced by the cholinergic neurons in the basal forebrain (BF), which is located in the front of hypothalamus, and in the brainstem. The most known functions in which it is implicated are learning, cognition, memory, wakefulness and REM sleep. The BF neurons project to the cortex and hippocampus

(Boucetta, & Jones, 2009). Cholinergic neurons are located in the pons, in the laterodorsal and pedunculopontine tegmental nuclei (LDT/PPT) and they activate the cortex by channeling Ach into the thalamus (Williams et al., 1994). Besides Ach, a great deal of γ-aminobutyric acid (GABA) is produced in the BF as well, which by its inhibitory action on the cortical interneurons, is likely activates the cortex (España, & Scammell, 2011).

Norepinephrine (NE), which is produced mainly in the locus coeruleus (LC), which is located below the fourth ventricle and in the brainstem, seems to promote arousal and wakefulness so as to respond to a challenge, an important stimuli or a stress condition (Scammell, Arrigoni, & Lipton, 2017; España, & Scammell, 2011).Furthermore, it seems that there is very low firing during NREM sleep and almost none during REM sleep from the LC neurons (España, & Scammell, 2011).

Histamine (HA) in the brain is mainly produced by the tuberomammilary nucleus (TMN), which is located at the base of the posterior hypothalamus and has connections with the forebrain and brainstem. Although HA is implicated in psychomotor functions, motivation and attention, (Van Ruitenbeek, Vermeeren, & Riedel, 2010 ; Passani, Blandina, & Torrealba, 2011), it seems that it plays an essential role in promoting wakefulness and more specific at initiating it. Adding to that, there is very low firing during NREM sleep, almost none during REM and high activation during wakefulness. However, yet little is known about which aspects of arousal it governs (Scammell, Arrigoni, & Lipton, 2017 ; España, & Scammell, 2011 ; Haas, Sergeeva, & Selbach, 2008).

Serotonin (5-HT) is mainly produced in the dorsal raphe nucleus and along the brainstem's midline with connections in the preoptic area, BF , hypothalamus, and thalamus. Among other functions, such as appetite and mood, it promotes wakefulness as well. Following the same pattern, the firing of 5-HT is high during arousal, lower during NREM sleep and almost non- existent during REM sleep (España, & Scammell, 2011).

Dopamine (DA) is produced in the substantia nigra, ventral tegmental area and ventral periaqueductal gray (vPAG) (Sanchez, Kalume, & de la Iglesia, 2022). Even though these areas fire during movement and reward, extracellular levels of DA are high during wakefulness and lower during NREM sleep. In agreement to that, it is

suggested that the role of DA is implicated in arousal when it is accompanied by high motivation or high physical activity levels (España, & Scammell, 2011).

Orexin/Hypocretin is a neuropeptide that is produced in the lateral and posterior hypothalamus. Orexin A and B (hypocretin 1 and 2) excite neurons via the OX1 and OX2 receptors and present high levels of firing during wakefulness and low levels during NREM and REM sleep. Their neurons project to all the arousal areas mentioned previously in this chapter, but mostly on the LC and TMN (España, & Scammell, 2011 ; Sakurai et al., 1998 ; Lee, Hassani, & Jones, 2005). Their role specifically seems to be important for enhancing arousal in a motivating context (such as seeking food) and maintaining long periods of wakefulness (Scammell, Arrigoni, & Lipton, 2017 ; España, & Scammell, 2011). What is more, there is some glutamate production from these neurons which excites certain neurons (Schöne et al., 2014).

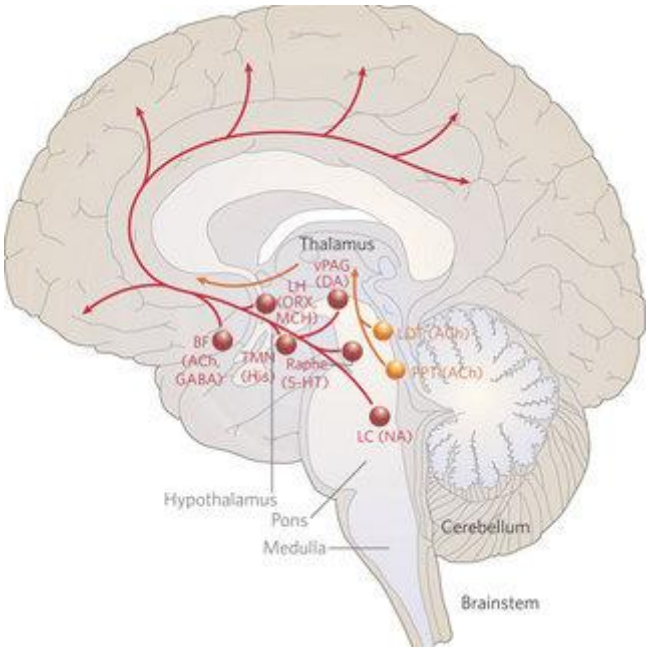


Figure4. The neurotransmitters, the regions and the projections implicated in wakefulness (Saper, Scammell, & Lu, 2005).

1.6.2 Sleep-Promoting Systems

NREM sleep is induced by the neurons of the the ventrolateral preoptic area (VLPO) and median preoptic area (MNPO), which are located in the anterior of

hypothalamus (Figure5). There, GABA neurotransmitter and neuropeptide galanin are produced, which project to all the arousal related areas such as LDT/PPT, LC, DR, TMN, and the orexin neurons. Therefore, it is suggested that sleep is promoted by the VLPO and MNPO by inhibiting all the areas mentioned above (España, & Scammell, 2011 ; Saper et al. 2010) with VLPO neurons firing during NREM sleep and the MNPO neurons firing on the onset of NREM sleep (España, & Scammell, 2011).

REM sleep seems to be induced by the neurons of the pons. It has been observed that some cholinergic neurons located in the LDT/PPT fire in both wakefulness and REM sleep or solely in REM sleep (España, & Scammell, 2011 ; Boissard et al.,2002). These neurons and Ach may assist in the generation of complex dreams during that sleep stage, by activating the cortex though the depolarization of thalamic neurons (España, & Scammell, 2011).

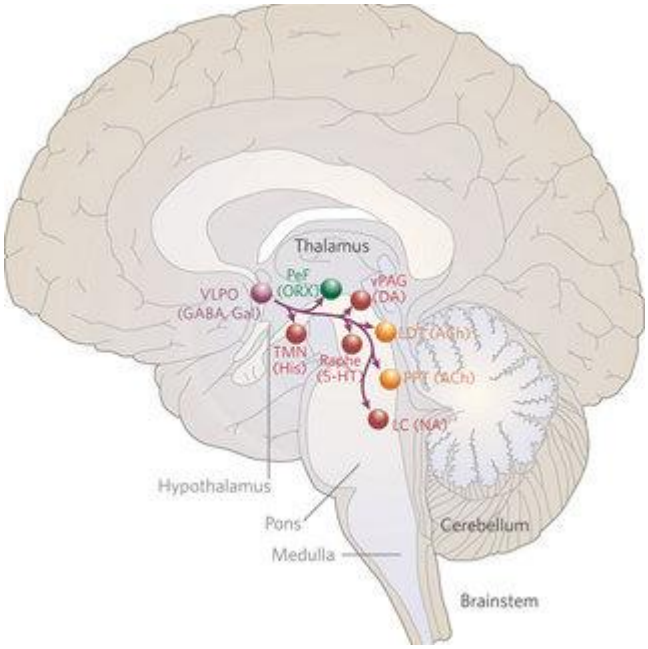


Figure5. The neurotransmitters, the regions and the projections implicated in sleep promotion (Saper, Scammell, & Lu, 2005).

GABA and glutamate, which are produced on the sublaterodorsal nucleus (SLD), are firing during REM sleep and may inhibit motor neurons. Moreover, these neurons could be inhibited by REM sleep-suppressing neurons in the mid-pons, which

provides an insight on how the transitions between REM and NREM sleep are regulated (Boissard et al., 2002).

Another explanation of how NREM and REM sleep alternate, lies in the interaction of monoamines, such as NE, 5-HT, and Ach. Monoamines are known for their inhibitory role on sleep. However, cholinergic neurons which fire during REM sleep are inhibited by 5-HT, NE and HA during the wake state and at some degree during NREM sleep (España, & Scammell, 2011 ; Leonard, & Llinás, 1994).Another inhibitory factor is Melanin-concentrating hormone (MCH), which is produced by neurons in the lateral hypothalamus, where orexin neurons are located as well, and have projections at the same areas as the orexin ones. MCH neurons are highly activated during REM sleep as a result of the arousal regions inhibition (España, & Scammell, 2011 ; Verret et al., 2003).

1.6.3 Sleep and wakefulness transitions

Sleep and wake states alternate in a ‘flip flop switch” circuit (Figure6). The alteration is compared to that type of circuit, as there are no or very fast transitional states. When one state occurs, the other is utterly inactive and both states are inhibitory to one another. (Saper, Scammell, & Lu, 2005). To promote the wake state, monoamines and cholinergic neurons disinhibit their own action by inhibiting the action of VLPO. To promote sleep, the VLPO neurons disinhibit their own action by inhibiting the arousal areas. The result of this mutual inhibition is a stable sleep and wake state (España, & Scammell, 2011).

Part of that circuitry is the orexins. Although orexins promote wakefulness, they do not directly inhibit VLPO, but they excite other neurons such as the monoaminergic ones that inhibit REM sleep (Saper, Scammell, & Lu, 2005 ; Bourgin et al., 2000).

Besides all the information presented above, there are more factors that affect and influence the sleep state. Adenosine, which is phosphorylated to ATP, a cell’s source of energy, has increased levels when a cell is fatigued and no longer phosphorylates it to ATP. This increase acts inhibitory to neurons implicated with arousal and disinhibitory to VLPO neurons. The peptides, which are released by the immune system cells called cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), promote sleep and most specific sleep onset (España, &

Scammell, 2011). Prostaglandin D2 (PGD2) levels in cerebrospinal fluid seems to be increased during sleep. This lipid, which is produced in the basal meninges, facilitates NREM sleep (Huang, Urade, & Hayaishi, 2007).

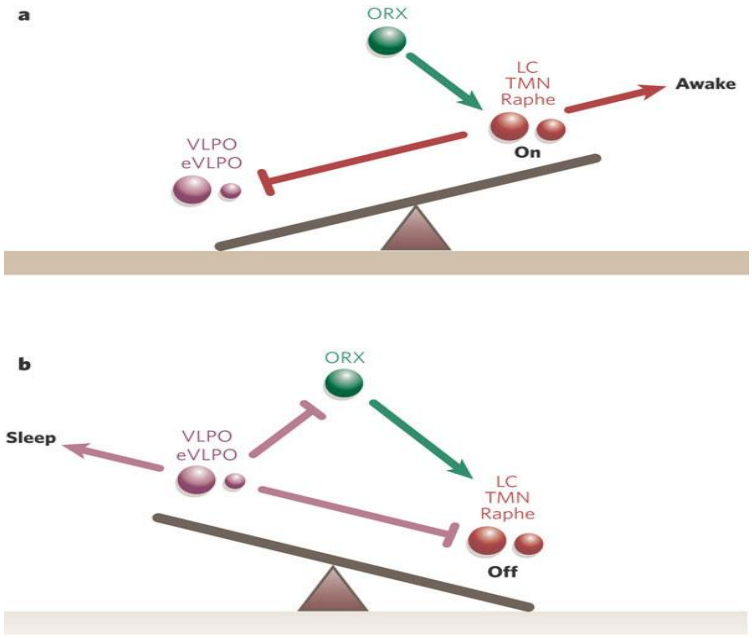


Figure6. The "flip-flop switch" model. **a.** wakefulness promotion **b.** sleep promotion (Saper, Scammell, & Lu, 2005).

1.7 Sleep genetics

There is strong evidence that several genes are involved in the circadian rhythm regulation, including the clock genes. Ongoing research identifies more and more genes that seem to be related to sleep mechanisms. Here we describe the most important genes implicated in the molecular clock underlying sleep architecture.

CLOCK gene is located in 4q12, encodes the clock protein and is implicated in circadian regulation (NCBI, 2004). ***ARNTL*** or ***BMAL1*** is located in 11p15.3, encodes a protein that forms a heterodimer with CLOCK and promotes the transcription of *PERIOD* and *CRYPTOSOME* genes. ***NPAS2*** is located in 2q11.2 and plays a great role in the molecular clock by forming heterodimer with *ARNTL* (NCBI, 2004). The ***PERIOD*** genes are circadian pacemakers and regulate locomotor activity, metabolism, and behavior. *PER1* is located in 17p13.1, *PER2* in 2q37.3 and

is implicated in familial advanced sleep phase syndrome 1 and *PER3* is located in 1p36.23 and is associated with familial advanced sleep phase syndrome 3. The ***CRYPTOSOME*** genes are regulators of the clock mechanism. *CRY1* is located in 11p11.2 and is associated with sleep-wake schedule disorder and delayed phase type and *CRY2* is located in 11p11.2 and seems to alter sleep patterns (NCBI, 2004).

These genes comprise the molecular clock and function by a transcriptional-translational feedback loop (TTFL) (Figure7) (Sanchez, Kalume, & de la Iglesia, 2022). As mentioned above, *CLOCK* binds with *ARNTL* and together they form a heterodimer which activates the transcription of *CRYPTOSOME* and *PERIOD* genes (Sanchez, Kalume, & de la Iglesia, 2022 ; Cox, & Takahashi, 2019). *CRYPTOSOME* and *PERIOD* genes bind and form a heterodimer and they inhibit their own promoter activity, *CLOCK-ARNTL* heterodimer (Sanchez, Kalume, & de la Iglesia, 2022). Thus, they repress their own gene transcription (Cox, & Takahashi, 2019).

BHLHE40 and ***BHLHE41*** genes are located in 3p26.1 and 12p12.1 respectively and they encode a basic helix-loop-helix protein. They are implicated in the molecular clock's function by binding in E-Box site of *PER1* promoter so as to repress *CLOCK/ARNTL's* transactivation of *PER1* (NCBI, 2004).

RORA and ***RORB*** genes encode the nuclear receptor ROR alpha and retinoid-related orphan receptor beta and are located in 15q22.2 and in 9q21.13. ***NR1D1*** is located in 17q21.1 and encodes the nuclear receptor REV-Erbα (NCBI, 2004).

These genes form a secondary feedback loop, in which REV-Erbα and ROR receptors claim for the binding sites on *ARNTL,* so as to inhibit or activate its expression respectively (Sanchez, Kalume, & de la Iglesia, 2022 ; Cox, & Takahashi, 2019 ; Jetten, 2009).

CHRM3 is the gene that encodes the cholinergic receptor muscarinic 3 and is located on the 1q43 position. It affects the function of Ach in the central and peripheral nervous system (NCBI, 2004). Following the previous research of the Ach implication on sleep and wake states, it has been showed that CHRM3 is essential for REM sleep and plays a role in NREM state as well (Niwa et al.,2018).

HCRTR1 and ***HCRTR2*** are the genes that encode the G-coupled hypocretin receptor 1 and 2, are located in 1p35.2 and 6p12.1 and bind the neuropeptides orexin 1 and 2 respectively (NCBI, 2004). They are primarily implicated in the feeding behavior (NCBI, 2004), but there is evidence suggesting that orexin deficiency in the brain is associated with a sleep disorder called narcolepsy (Shaw, Tafti, & Thorpy, 2013 ; Irukayama-Tomobe et al., 2017).

SLC6A3 gene is located in 5p15.33 and encodes a dopamine transporter which transfers the dopamine from the synaptic cleft back in the cytosol (NCBI, 2004). The primary role of this gene is the generation of a dopamine transporter (DAT). It is considered a risk factor for ADHD, ASD, and alcohol use disorder and there is little evidence that associates it with sleep architecture (Abel et al., 2020).

SCL6A4 gene is located in 17q11.2 and encodes a serotonin transporter which transfers the 5-HT from the synaptic cleft back in the presynaptic neuron (NCBI, 2004). Primarily associated with anxiety and OCD, there has been a link with sleep bruxism, increased complaint of sleep onset problems, shorter sleep duration and SLC6a4 gene (Abel et al., 2020).

The ***HLA-DQB1*** gene belongs to the human leukocyte antigen (HLA) system (the major histocompatibility complex [MHC] in humans) and consists of a beta chain (DQB) (NCBI, 2004). It is implicated in the function of the immune system and is located in the 6p21.32 (NCBI, 2004). There is evidence for its involvement in the pathogenicity of narcolepsy and in the obstructive sleep apnea (Shaw, Tafti, & Thorpy, 2013 ; Ollila, 2020 ; Momany et al., 2017).

MECP2 is located in Xq28 and encodes the MECP2 protein which is essential for development (NCBI, 2004). It is associated with neurodevelopmental disorders such as Rett Syndrome and there is a few past research that links the protein with disrupted sleep (Abel et al., 2020 ; Wither et a., 2012).

BDNF encodes a growth factor which is involved in the nerve development. It is located in 11p14.1 and its deficiency is implicated in neurodegenerative diseases such as AD and Parkinson (NCBI, 2004). It is suggested that it regulates sleep slow wave oscillations (Abel et al., 2020).

TNF gene is located in 6p21.33 and encodes a proinflammatory cytokine which belongs to the tumor necrosis factor family (NCBI, 2004). It plays a role in brain development, cell survival/apoptosis, neurogenesis and cell differentiation while it is implication in sleep disturbances has been studied (Da Silveira et al., 2021).

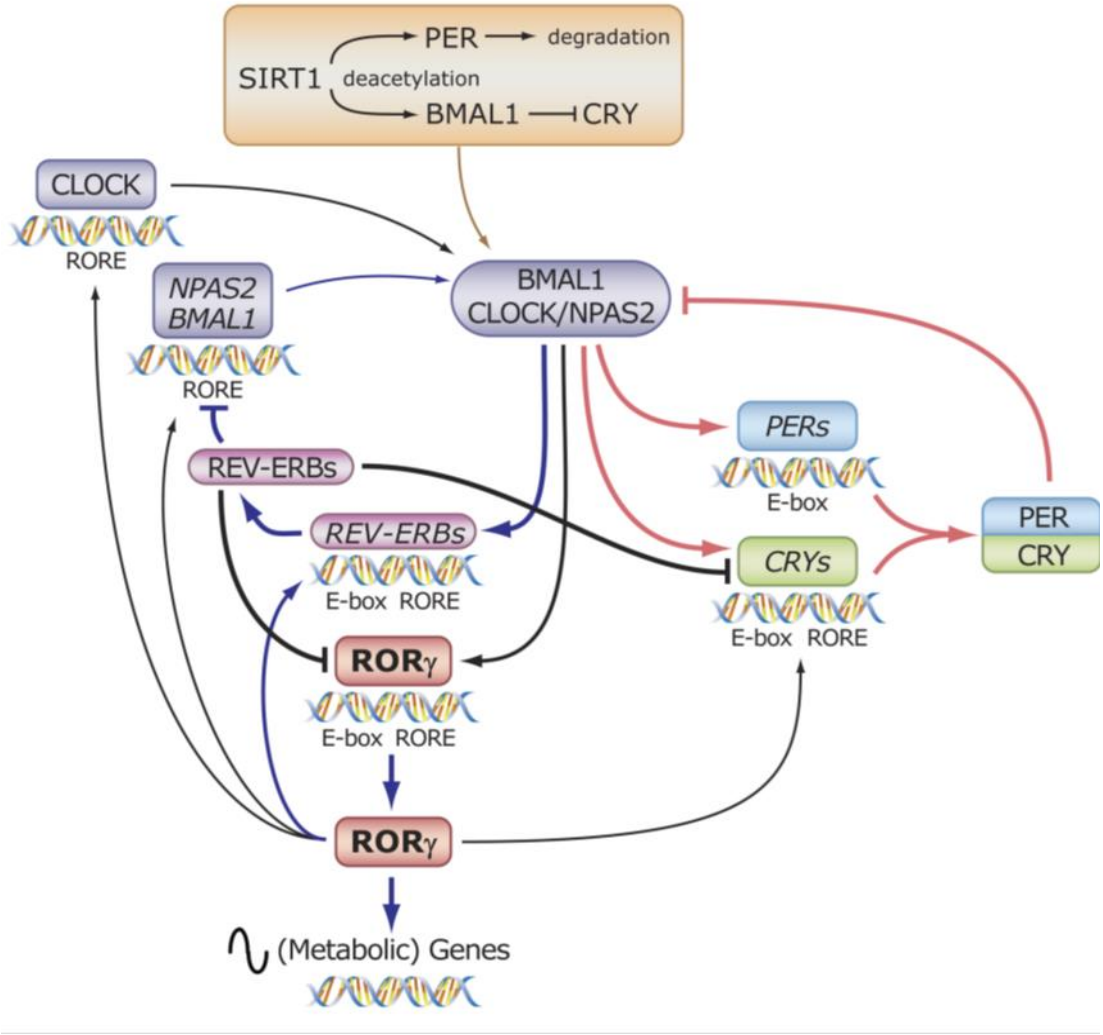


Figure7. The basic transcriptional-translational feedback loops (TTFL) regulated by the clock genes (Jetten, 2009).

GABBR1 gene encodes the GABA (B) receptor 1 and is located in 6p22.1. It is implicated in schizophrenia and epilepsy and it has been proposed that there may be an association of GABBR1 with obstructive sleep apnea (NCBI, 2004 ; Veatch et al., 2020).

REV1 gene is located in 2q11.2 and encodes a DNA repair protein which participates in the research of DNA polymerases for the repair (translesion

synthesis- TLS) of damaged DNA (NCBI, 2004). During replication, a damaged DNA can cause mutations and *REV1* gene product plays a mutagenic role in thatprocedure (Lin et al., 2019). Along with this role, it has been mentioned as a circadian gene in the literature (Christou et al., 2019, Jim et al., 2015).

VDR gene, located in 12q13.11, encodes the vitamin D3 receptor. It participates in immune responses and in the balance of several minerals (NCBI, 2004). There is a possible implication of this gene in the dysfunction of circadian mechanisms in Cretan population (Konstantara, 2016).

1.8 Sleep disorders

According to the latest ICD-11 version, sleep disorders or sleep-wake disorders are characterized by "difficulty initiating or maintaining sleep (insomnia disorders), excessive sleepiness (hypersomnolence disorders), respiratory disturbance during sleep (sleep-related breathing disorders), and disorders of the sleep-wake schedule (circadian rhythm sleep-wake disorders), abnormal movements during sleep (sleep-related movement disorders), or problematic behavioral or physiological events that occur while falling asleep, during sleep, or upon arousal from sleep (parasomnia disorders)". As a multifactor activity, sleep can be disturbed, shortened or prolonged due to health issues of all kinds, to medication or work and lifestyle (Gaine, Chatterjee, & Abel, 2018). As a consequence, deviation from normal sleep has been associated and seems to be implicated as a risk factor for diseases such as Alzheimer's and Parkinson's disease (51). Cognitive deficits and memory impair may occur as well (Gaine, M. E., Chatterjee, S., & Abel, T. (2018 ; Bishir et al., 2020). Below are presented the most common sleep disorders.

Narcolepsy is characterized by excessive daytime sleepiness and cataplexy. Cataplexy is the sudden loss of bilateral muscle control and facial muscle control in response of a strong emotion for a very short period of time, as long as the trigger of the emotion is present. Along with these two basic symptoms, hallucinations, sleep paralysis and disturbed sleep can be present as well as motor, cognitive, psychiatric, metabolic and autonomic deficits. The main factor that forms the neuropathology of this disorder is the loss of orexin neurons in the lateral hypothalamus. What is more, the positivity in *HLA-DQB1*06:02* seems to enhance the risk of narcolepsy. It can be

treated via symptomatic pharmacology and with non-pharmacological treatments. (Bassetti et al., 2019 ; Mahoney, Cogswell, Koralnik, & Scammell, 2019).

Insomnia is a sleep disorder characterized by long term difficulty falling asleep, maintaining the asleep state, early morning awakening along with impact on the daytime functionality. It could be either a primary disorder or a symptom or a risk factor to other diseases. *PER3* and *CLOCK* variants have been implicated in the disorder. The *HLA-DQB1*0602* seems to be involved in the genetic factors associated with insomnia (Shaw, Tafti, & Thorpy, 2013 ; Bollu, & Kaur, 2019).

In **Obstructive sleep apnea,** sleep is disturbed, as the upper nasal airway cannot function properly and causes a significant decrease of oxygen flow in the brain. Loud snoring, witnessed apneas during sleep, and excessive daytime sleepiness could be present as well and lead to significant health issues in all aspects of life (Slowik, & Collen, 2022). It is also a contributing factor to brain aging, a risk factor for neurodegenerative diseases such as AD (Weihs, 2021). It could be caused by several reasons, such as anatomic structure and other co-existent health issues (Slowik, & Collen, 2022). Although the genetic basis of obstructive sleep apnea is yet to be determined, there has been evidence that associates it with the presence of *HLA-DQB1*0602* allele (Momany et al., 2017). The treatment involves mostly invasive medical procedures (Slowik, & Collen, 2022).

Sleep disorders include disorders that affect the sleep-wake cycle, which is regulated by the circadian rhythms. Two of the most common circadian rhythm sleep disorders are the **Delayed Sleep Phase Syndrome (DSPS)** and the **Familial Advanced Sleep Phase Syndrome (FASPS).** The DSPS is characterized by a persistent delay of the sleep onset and offset and FASPS by a persistent advance of the sleep onset and offset (Ebisawa , 2007). Although sleep cycles alterations in DSPS are normal, people diagnosed with this syndrome have difficulty waking up early without the use of external stimuli such as alarm clock. As a result, they tend to feel tired and their cognitive skills and mood are being affected (Micic et al., 2016). On the contrary, individuals diagnosed with FASPS, due their early awakening time they tend to be punctual or even ahead to their daily scheduled activities (Tafti, Dauvilliers, & Overeem, 2007). *PER2, PER3* genes are implicated in the FASPS and *CRY1* gene potentially plays a role in the DSPS (NCBI, 2004 ; Ebisawa , 2007 ; Patke et al.,

2017). For the treatment of those disorders, chronotherapy, phototherapy, melatonin administration, hypnotics and cognitive behavioral therapy are being applied (Culnan, McCullough, & Wyatt, 2019).

1.9 Alzheimer Disease and Circadian Rhythms

Alzheimer disease is a neurodegenerative disease which causes the majority of dementia cases and affects the hippocampus. The main symptoms are memory loss, cognitive deficits, difficulty to deliver activities of daily living and mood disorders. The symptoms emerge gradually and they are mild at the early stages of the disease. The most severe symptoms and the total body dysfunction occur at the last stage, where the death does not occur by the disease itself but by e.g. an infection or another factor. A risk factor for the disease is aging, and as a consequence is mostly being diagnosed to individuals over the age of 65 years (Scheltens et al., 2021).

AD is characterized by amyloid-beta peptide's accumulation (Aβ) in the medial temporal lobe and neocortical structures which causes amyloid plaques. Also, hyperphosphorylated tau accumulation leads to the formation of neurofibrillary tangles (Scheltens et al., 2021 ; Breijyeh, & Karaman, 2020). There are also genetic risk factors associated with the disease. Variants in the *APP* gene (encoding for the amyloid-beta precursor protein) and *PSEN1* and *PSEN2* genes (encoding for the presenilin 1 and 2, respectively) can increase the risk for autosomal dominant AD. What is more, *APOE* ε4 allele is a strong genetic factor for Alzheimer disease (Scheltens et al., 2021 ; Breijyeh, Z., & Karaman, R, 2020).

Besides genetics and aging, there are several other factors that add to the risk of AD such as lifestyle and diet. These factors are the aim of non-pharmacological interventions. Medication is commonly used so as to treat the cognitive and the neuropsychiatric symptoms caused by the disease (Scheltens et al., 2021 ; Breijyeh, Z., & Karaman, R, 2020).

Sleep disturbances have been associated with amyloid-beta and hyperphosphorylated tau accumulation. During normal sleep, these neurotoxic proteins are being cleared (Bishir, Bhat et al., 2020), a process that is impaired in sleep disorders. This could of importance for AD pathophysiology, as sleep disorders are common among individuals with AD and sleep deprivation seems to be a risk

factor for AD as well (Saeed, & Abbott, 2017 ; Wu, Dunnett, Ho, & Chang, 2019). These data highlight the possible importance of sleep in disease prevention and management.

2. SPECIFIC AIMS

2.1 First Aim

To study the possible association between single nucleotide variants in 25 circadian-rhythm associated genes and 4 sleep parameters measured by actigraphy (night TST, TST, night TiB, total TiB).

2.2 Second Aim

To investigate the presence of rare variants in the main 5 circadian sleep-associated genes in individuals showing extreme scores in the 4 sleep parameters (night TST, TST, night TiB, total TiB).

3. METHODOLOGY

3.1 Cohort/Sample

3.1.1 Sample collection

The sample used for this study consisted of 201 participants, 120 of which were individuals diagnosed with dementia and 81 healthy control individuals. The participants included in the study were a subsample of the Cretan Aging Cohort, which studied community-dwelling elders, from the area of Heraklion, Crete, Greece in relation to cognitive impairment diagnosis and risk factors (Zaganas et al., 2019). This study was completed in 2 phases. Phase 1 included the selection of the 3200 consented community-dwelling elders (60-100years old), as well as the gathering of information about them from a trained nurse with a structured questionnaire (medical history, socio-demographic information, medication). After having administered the Greek version of the Mini-Mental State Examination test (MMSE) to evaluate their cognition, 3140 were selected to continue to the Phase 2 (23/24 points cut off/<6 years education). During phase 2, a more specific and detailed neuropsychiatric and neuropsychological assessment took place (Zaganas et al., 2019).

The DNA and WES data were retrieved from “Thalis-University of Crete-Interdisciplinary Network for the Study of Alzheimer's Disease” project (Principal Investigator A. Vgontzas, Principal Investigator for the Genetic Substudy I. Zaganas). The genetic analysis in that study aimed to create a data base, combined with the information of the heredity history of individuals diagnosed with AD. This data base library would be an easy accessible source to process genetic data so as to study other diseases as well.

3.1.2. Blood collection from sample

The blood collection process took place in Medical Centers of each area or at the participant’s residence when their transportation was not possible The blood collection kit included:

- EDTA vacutainer tubes 3ml
- Vacutainer tubes without EDTA 3m

- Syringes 10ml with needles (BD Emerald)
- Butterfly needle 0,60x19mm
- Adhesive tape
- Alcohol
- Medical tourniquet
- Cotton
- Sharp disposal containers
- Examination gloves
- Medical trays

Four blood tubes were collected for each participant, 2 for plasma isolation, 1 for serum isolation and 1 DNA extraction and were stored at -20°C.

3.1.3. DNA Extraction from sample

The DNA extraction from whole blood sample was carried out with the QIAamp DNA blood mini kit (250) of QIAGEN (USA). As a result, the protocol proposed in the kit's handbook was followed (QIAamp® DNA Mini and Blood Mini Handbook, 2016).

The procedure followed, as described in the QIAGEN handbook, is presented below:

Preparing for the experiment

- All centrifugation steps are carried out at room temperature (15–25°C).
- 200 µl of whole blood yields 3–12 µg of DNA.
- Equilibrate samples to room temperature (15–25°C).
- Heat a water bath or heating block to 56°C.
- Equilibrate Buffer AE or distilled water to room temperature for elution.

Performing the experiment (for every sample)

- Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.

- Add 200 µl sample to the microcentrifuge tube. To avoid the presence of RNA at the final extraction, we add 4 µl of an RNase A stock solution (100 mg/ml) and we properly mix (vortex).
- Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15s.
- Incubate at 56°C for 10 min.
- Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
- Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
- Carefully apply the mixture to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g(8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
- Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.
- Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g;14,000 rpm) for 3 min.
- Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
- Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

After having successfully extracted the DNA, the purity of the yield was evaluated. According to QIAGEN kit's handbook, the maximum DNA concentration for 200µl

should be 34.0 ng/μl. Using the Jenway Genova Nano Spectrophotmeter, the DNA concentration and the absorption ratio A260/A280 of each sample were measured. The mean final DNA concentration of the samples was as high as 42.5 ng/μl, while the mean absorption ratio A260/A280, indicating DNA purity, was 1.793.

Finally, every sample was labeled with a special unique code to ensure anonymity and was split in 2 aliquots. One of those was added in the genetic bank, which was created for all the participants, and was stored in -20°C. The other aliquot was transferred to the Institute for Research and Technology (FORTH, Crete) for the whole exome sequencing procedure.

3.1.4 Whole Exome Sequencing

Whole exome sequencing (WES) is a New Generation Sequencing tool which sequences all protein-coding regions in the human genome (the exome) only so as to detect both exonic (coding) as well as splice-site variants by using only approximately 2% of sequencing "load". It has been shown that most of the disease associated variants are to be found in the exome, a fact that renders this method less time consuming and effective. On the contrary, genetic association studies (GWAS), a common method to identify gene variants and associate them as causative genes for a disease, enables the sequencing of the whole genome includes, both the coding and non-coding regions, and makes it more time consuming and harder to interpret all that information and result in the causality (Petersen et al., 2017)

WES was performed at the Unit of Genomics Analysis of Minotech at the Institute for Research and Technology (FORTH, Crete). Platform Ion Torrent PROTON (specifications: Ion PI chip v3) was used, after creating the corresponding library and preparing the sequencing reaction with Ion Ampliseq Exome™ kit (MA, USA) and Ion PI Hi-Q OT2 200™ kit (MA, USA), respectively. The bioinformatics processing and data storage was available by the software Ion Torrent Suite™ (MA, USA). The variant called by the software Ion Reporter v.5.0, following comparative analysis of the sample with the reference genome hg19**.**

3.2 Bioinformatics

All the information obtained from the Whole Exome Sequencing Analysis for all 201 participants was stored to Ingenuity Variant Analysis software, Qiagen (CA, USA). The data for 25 genes (Table5) (Table6), that were previously related to circadian mechanism and sleep disorders in the literature, were manually collected from the Ingenuity Variant Analysis software and organized at excel files. The files included information that characterized each SNP of the selected genes, as well as the number and codes of the participants (patients/controls) carrying those SNPs.
Eventually 164 had actigraphy scores and were used in the current study. All data used to describe variants and provide information about the participants were retrieved from the ingenuity database and from the first phase of Thalis study.

3.3 Actigraphy Study

An actigraphy study was performed with the use of an actigraphy (Actilife v6.9.5, GT3XP model, Pensacola, FL, USA), an actigraphy watch, that measures the sleep wake patterns via accerelometers that detect movement. It is a low cost method, can occur for a long period of time and allows the measurement to take place at the personal space of the participant (Schoch et al., 2021). Along with the actigraphy measurements, participants reported “bedtime” and “out of bed time” on a daily basis. All participants have undergone actigraphy study for 3 24-hours during weekdays (Study performed by A. Vgontzas, M. Basta, I. Koutentaki in the context of the Thalis-MNSAD program) (Basta et al., 2019). The actigraphy parameters that were chosen to be studied in the current study, following the methodology that previous studies have followed, were: total sleep time (TST), night TST, night time spent in bed (night TiB) and total time spent in bed (total TiB) (Basta et al., 2019, Basta et al., 2020, Basta et al., 2021).

Total time in bed is the time that participants spend in bed, even if they are awake. Night total sleep time is the total sleep time from the moment an individual starts to sleep until they wake up. Night time in bed is the time when participants go to bed. The total sleep time refers to the total amount of sleep time scored during the total recording time during a day(24hours) (Basta et al., 2019, Basta et al., 2020, Basta et al., 2021).

3.4 Statistical Analysis

All data were analyzed using SPSS (version 28.0, SPSS Inc., Chicago, Ill., USA). Firstly, an SPSS file for each gene (25 files in total) was created, including all the SNPs detected the cohort, where each participant was represented by a code (201 codes). Consequently, the score of each sleep parameter as well as the presence of every SNP were registered. The presence or absence of the SNPs was registered by using values: value 1 was used when the SNP was present and value 0 was used when the SNP was absent. Before starting with the main analysis, some participants were excluded, as there had no actigraphy data. The final participants were 164: 73 controls and 91 cases.

The bioinformatics analysis stored in excel files was used to present the information for each participant, which variant it carries or not and the scores in each sleep parameter. The results of the current study were divided in 2 analyses.

3.4.1 Phenotype / genotype analysis

We applied independent t-test to compare the sleep parameters scores of individuals carrying a SNP with the scores of individuals without this SNP. We used the box plot graphs to visualize the results. Each SNP of each gene was studied in relation with all 4 sleep parameters. From the results we excluded any associations that had $p \geq 0.05$, and hence they were not statistically significant. Also, we excluded any SNPs appearing in 4 or less ($N \leq 4$) partcipants at least in one of the 2 groups studied (individual with the variant-individual without the variant).

3.4.2 Extreme actigraphy scores in correlation with rare variants analysis

As mentioned previously, the actigraphy recordings spanned for 72hours. The aim was to detect the most extreme actigraphy scores (short-long objective sleep). The scores of all participants for each parameter were placed in ascending order. The participants with the shortest objective sleep 5% and the longest objective sleep 5% of all values were studied further as a subpopulation that expresses the most divergent scores in the sample. The 5% of the total 164 participants is 8.2, so the first and the last 8 participants were used for that part of the research.

Furthermore, we created tables that present the longest objective sleep and shortest objective sleep and the rare SNPs that each participant is carrying in the most studied clock genes: *PER1, PER2, PER3, CRY1, CRY2*. As rare SNPs, we designated the ones that were present to 4 or less participants (N≤4). Finally, a graph was created for each parameter to visualize, in ascending order, the scores of each participant and point out the extreme ones.

4. RESULTS

4.1. Phenotype / genotype analysis

In analyzing the association of the variants in 25 genes (Table_5_6) with the 4 sleep parameters, we identified several important associations. Specifically, variants in the *BDNF, CRY1, GABBR1, PER1, PER2, PER3, REV1* and *SLC6A3* genes, were found to be associated with differences in the 4 sleep parameters (night TST, TST, night TiB, total TiB). More specific, SNPs carriers present shorter objective sleep duration than the non- carriers, in the variables examined. However, carriers of the PER3 gene presented longer sleep duration in comparison with the non-carriers. Results are presented below in Table 2.

Gene	SNP	Night TST (Mean, SD, p)			TST (Mean, SD, p)			Night TiB (Mean, SD, p)			Total TiB (Mean, SD, p)		
		+rs	*-rs*	*p-value*	*+rs*	*-rs*	*p-value*	*+rs*	*-rs*	*p-value*	*+rs*	*-rs*	*p-value*
BDNF	rs412744440 rs145503674	-	-	-	-	-	-	469.44, 79.66	540.06, 93.01	0.03	-	-	-
CRY1	rs8192440	-	-	-	493.17, 125.70	453.27, 96.94	0.025	555.56, 89.52	517.73, 94.02	**0.009**	630.76 130.53	571.68, 122.75	**0.003**
GABBR1	rs29230, rs5875197	417.09, 79.71	449.42, 101.66	0.024	449.96, 90.43	492.40, 127.16	0.014	-	-	-	-	-	-
GABBR1	rs29267	408.47, 70.67	446.15, 99.94	0.07	442.97, 87.62	486.16, 121.16	0.011	-	-	-	-	-	-
PER1	rs2304911	400.66, 61.66	439.67, 96.37	0.016	429.96, 68.79	479.50, 117.47	**0.007**	-	-	-	-	-	-
PER2	rs35333999	-	-	-	-	-	-	-	-	-	524.10, 108.66	607.08, 129.55	0.020
PER3	rs10462021	464.70, 89.15	425.62, 92.94	0.028	-	-	-	-	-	-	-	-	-
REV1	rs1324957632	337.73, 75.76	437.48, 92.33	0.040	-	-	-	-	-	-			
REV1	rs717454	-	-	-	-	-	-	-	-	-	583.76, 124.39	643.44, 134.46	0.011
REV1	rs3087386	-	-	-	-	-	-	-	-	-	587.69, 129.19	641.55, 124.18	0.022
REV1	rs28369942	-	-	-	-	-	-	-	-	-	579.09, 139.49	621.39, 116.44	0.037
SLC6A3	rs1042098	407.17, 72.21	444.75, 98.41	**0.009**	444.79, 93.84	483.30, 118.52	0.032	505, 81.73	547.98, 95.28	**0.005**	567.61, 119.87	612.94, 131.56	0.038

Table2. The mean scores and the SD for each sleep parameter, as well as the p-value for the comparison between individuals with a SNP to those without the SNP are being presented. +rs: SNP carriers, –rs SNP non carriers.

***BDNF* variants**

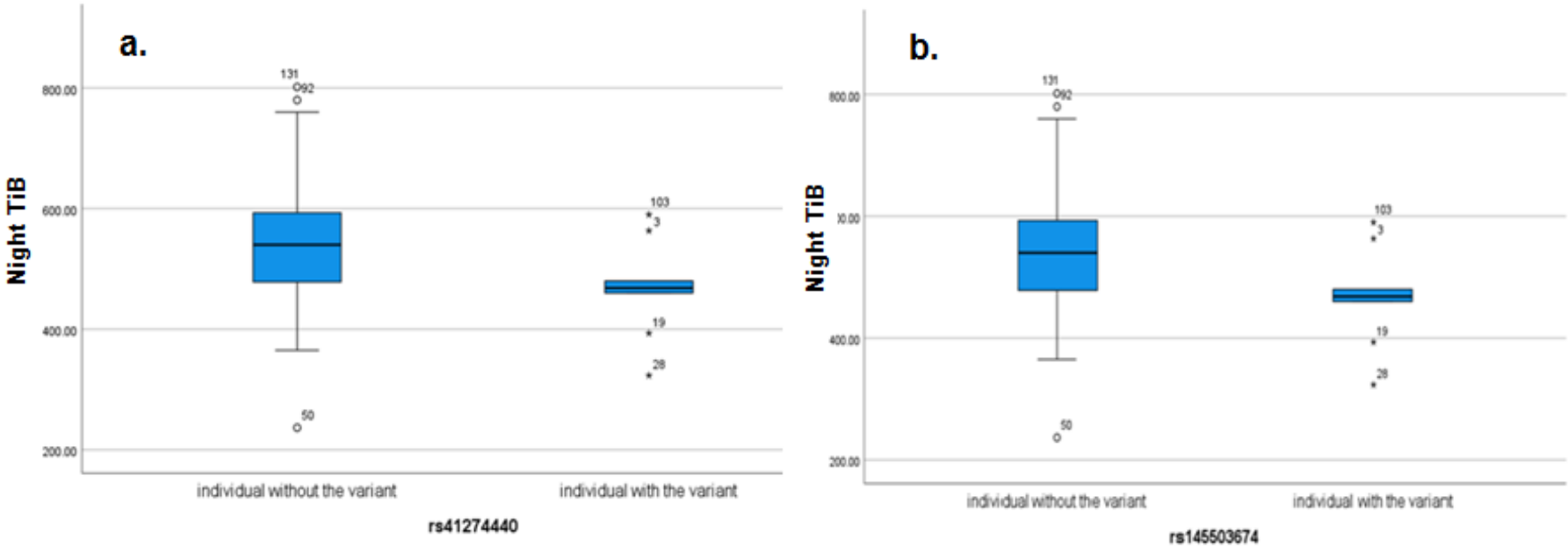


Figure8. a. The Night TiB in individuals with or without the *BDNF* rs41274440 variant. **b.** The Night TiB in individuals with or without the *BDNF* rs145503674 variant.

The variant of *BDNF* gene, **rs145503674,** is present to the individuals who tend to stay in bed at night (N=9, mean night TiB=469.4mins) less time than the ones without the variant (N=155, night TiB=540.06mins) (Figure8b). There is significance in this correlation with p=0.03. The exact same results came up from the association of another *BDNF* variant, **rs41274440** with night TiB (Figure8a).

***CRY1* variant**

The absence of the *CRY1* variant, **rs8192440,** is related to less sleep and time in bed (N=84, mean TST=453.27mins, mean total TiB= 571.68mins, mean night TiB=517.73mins) (Figure9a, 9b, 9c) compared to the participants carrying it (N=80, mean TST=493.17mins, mean total TiB=630.76mins, mean night TiB=555.56mins). The significance for TST is p=0.025, for total TiB is p=0.003 and for night TiB is p=0.009.

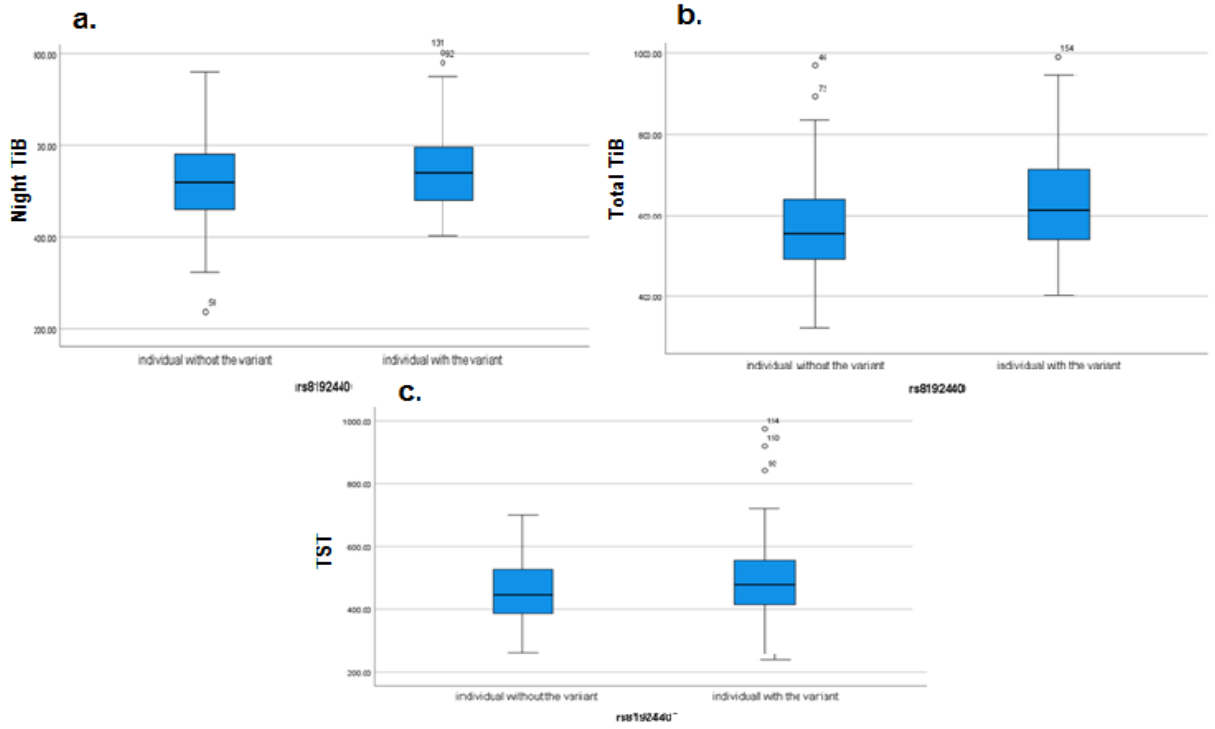


Figure9. a. The Night TiB in individuals with or without the *CRY1* rs8192440 variant. **b.** The Total TiB in individuals with or without the *CRY1* rs8192440 variant. **c.** The TST in individuals with or without the *CRY1* rs8192440 variant.

***GABBR1* variants**

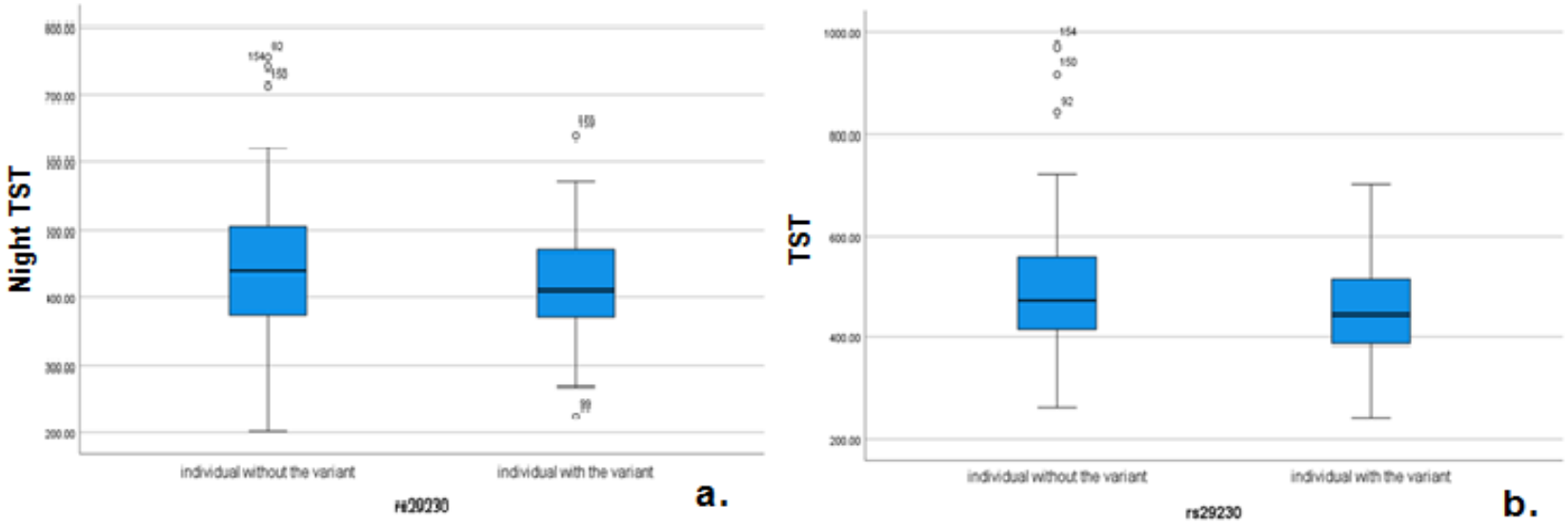
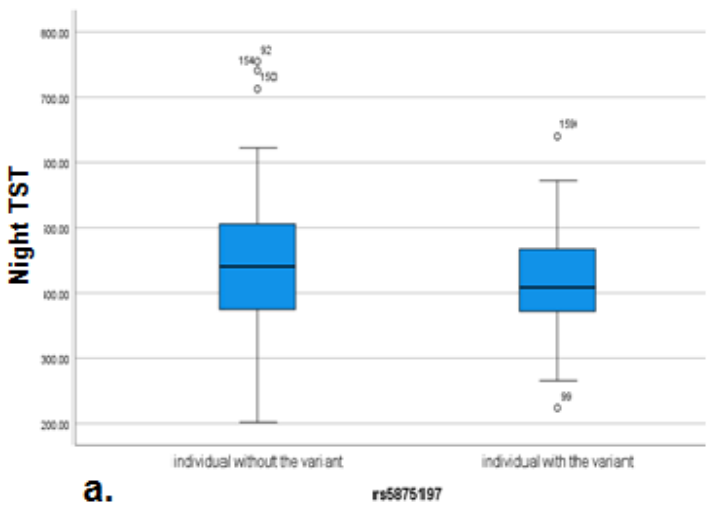


Figure10. a. The Night TST in individuals with or without the *GABBR1* rs29230 variant. **b.** The TST in individuals with or without the *GABBR1* rs29230 variant.

The *GABBR1* variants **rs29230, rs5875197** have shown statistical significance when associated with night TST and TST (p=0.024 and p=0.014 respectively). Regarding night TST, individuals carrying the variants (N=88) tend to sleep less at night (mean night TST=449.42mins) than the non-carriers (N=76, mean night TST=417.09mins) (Figure10a, 10b) (Fugure11a, 11b). The same applies to the TST, with the individuals carrying the variants sleeping 449.96mins in comparison with the non-carriers group, who scored 492.40mins. The **rs29267** variant demonstrates the same results as the other *GABBR1* variants: the variant carriers (N=51) sleep less (mean night TST=408.47mins, mean TST=442.97mins) (Figure12a, 12b) than the non- carriers (N=113, mean night TST=446.15mins, mean TST=486.16mins). The significance for night TST is p=0.007 and for TST is p=0.011.



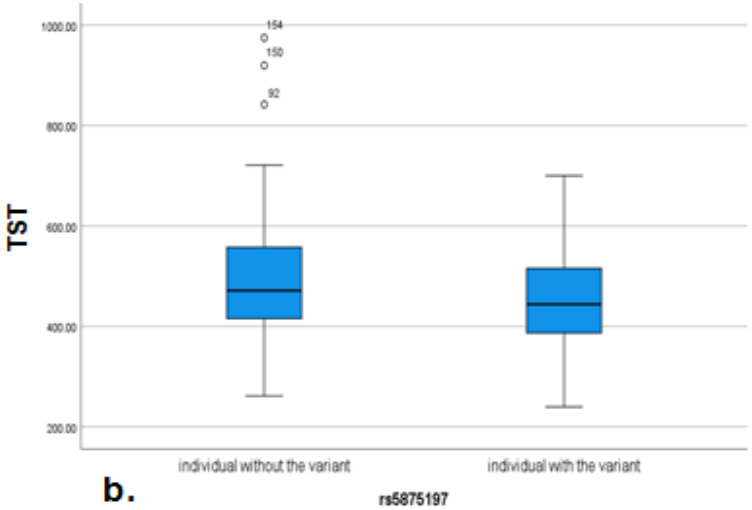
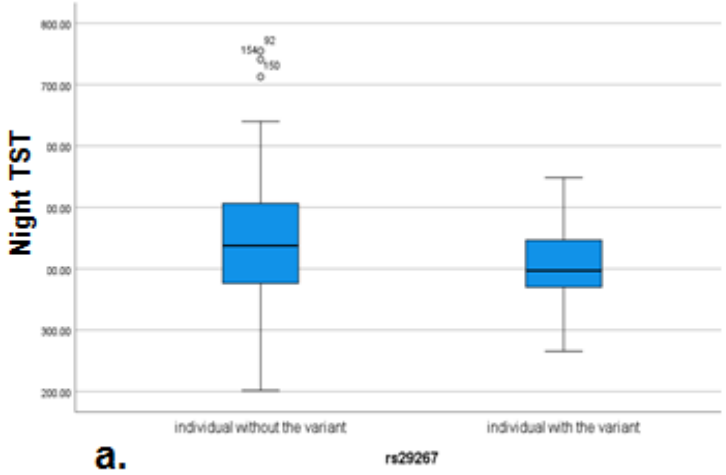


Figure11. a. The Night TST in individuals with or without the *GABBR1* rs5875197. **b.** The TST in individuals with or without the *GABBR1* rs5875197.



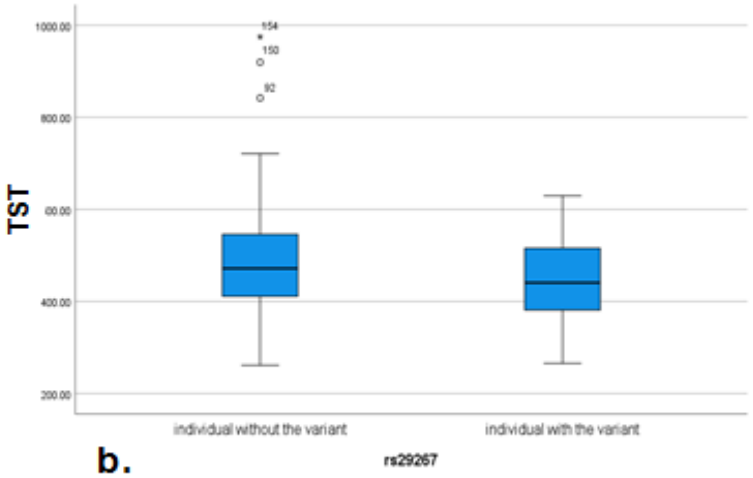


Figure12. a. The Night TST in individuals with or without the *GABBR1* rs29267 variant. **b.** The TST in individuals with or without the *GABBR1* rs29267 variant

***PER1* variant**

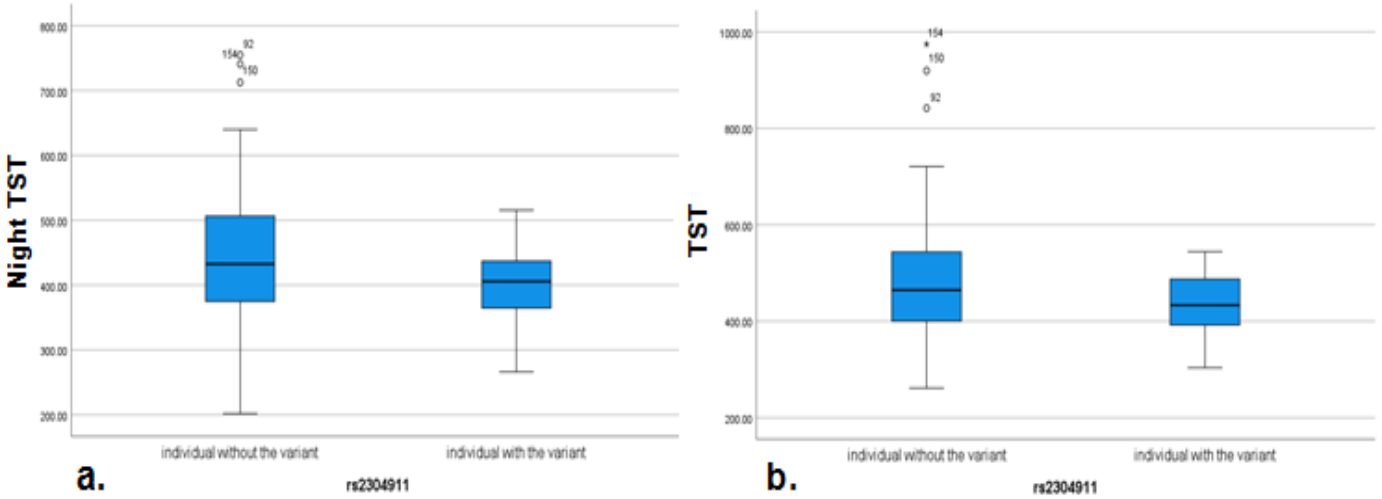


Figure13. a. The Night TST in individuals with or without the *PER1* rs2304911 variant. **b.** The TST in individuals with or without the *PER1* rs2304911 variant

The participants carrying the *PER1*'s, **rs2304911** variant (N=22) are prone to less sleep (mean TST= 429.06mins, mean night TST=400.66mins) (Figure13a, 13b) than the non-carriers, who had longer objective sleep(N=140, mean TST=479.50mins, mean night TST=439.67mins). The significance for night TST is p=0.016 and for TST is p=0.007.

***PER2* variant**

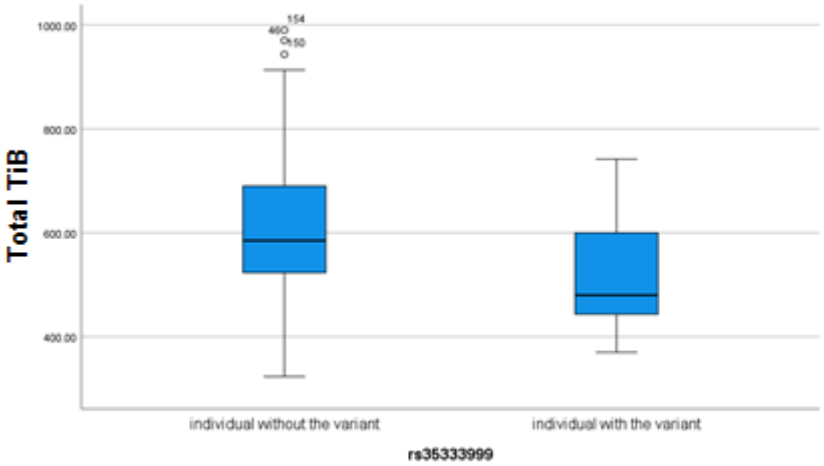


Figure14. The Total TiB in individuals with or without the *PER2* rs35333999 variant.

The *PER2* gene's, **rs35333999** is related with less time spent in bed (N=13, mean total TiB=524.10mins) (Figure14) in comparison to the group where it is absent (N=151, mean total TiB=607.08mins) with significance p=0.02.

***PER3* variant**

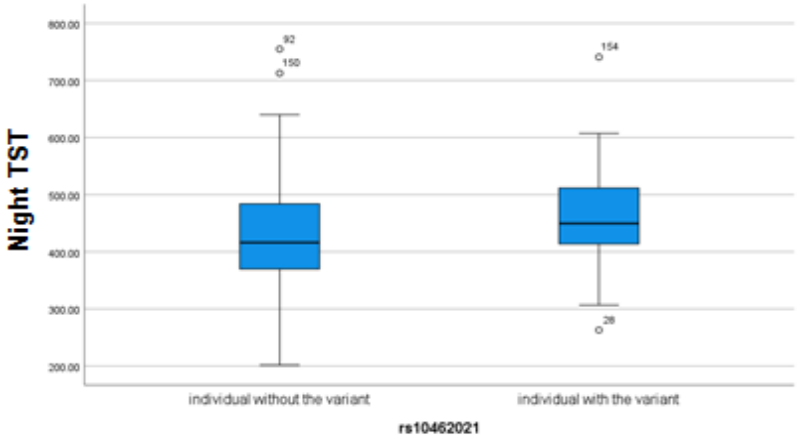


Figure15. Night TST (total sleep time) in individuals with or without the *PER3* rs10462021 variant.

Individuals that carry (N=37) the **rs10462021,** *PER3* variant, have longer objective sleep night TST (mean night TST=464.70mins) (Figure15) than the non-carriers (mean night TST= 425.62, p=0.028).

***REV1* variants**

Three *REV1* variants were found to be statistically important and their presence to be related to shorter total TiB in the sample. The participants (N=118) with **rs717454** had a score of 583.76mins (Figure16a) in mean total TiB, shorter than the non-carriers (N=46, mean total TiB=643.44mins, p=0.01). The presence (N=125) of **rs3087386** (Figure18b) resulted in a score of 587.69mins in mean total TiB and its absence in a score of 642.55mins in mean total TiB, with p=0.022.

Lastly, the **rs28369942** (Figure18c) variant is related with a mean total TiB of 579.09mins whereas the non-carriers had a score of 621.39mins for mean total TiB, with p=0.037.

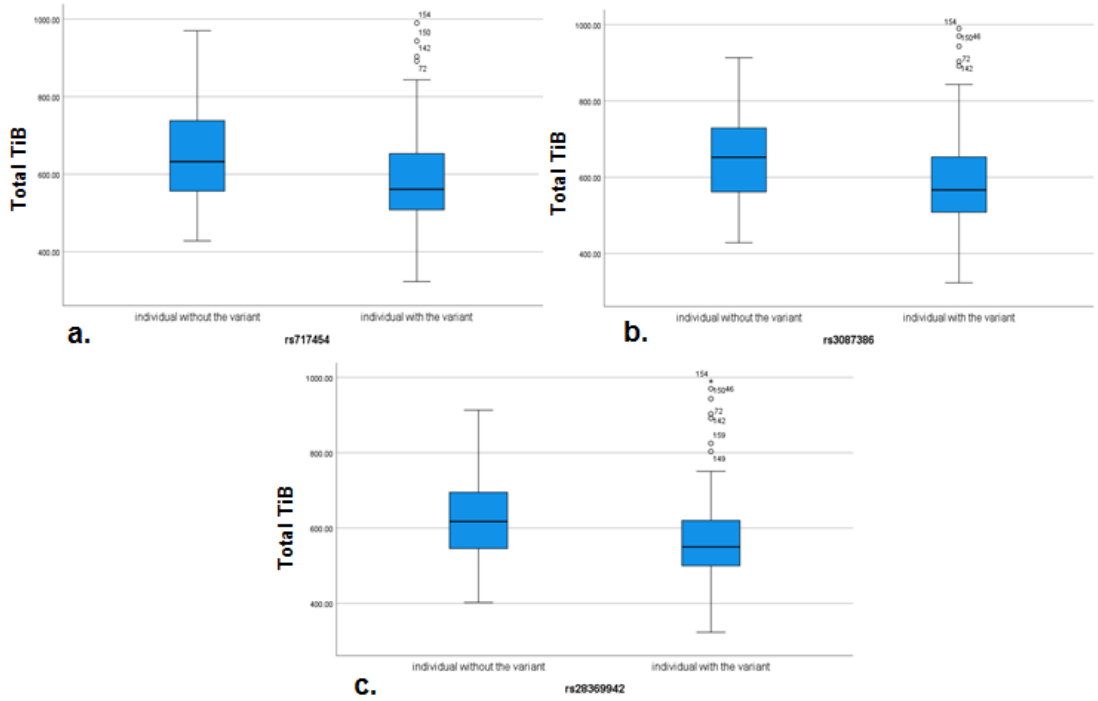


Figure16. a. Total TiB in individuals with or without the *REV1* rs717454 in variant. **b.** Total TiB in individuals with or without the *REV1* rs3087386. **c.** Total TiB in individuals with or without the *REV1* rs28369942 variant.

The association of **rs1324957632** with night TST was significant, with p=0.040. Mean night TST for carriers (N=5) was 337.73mins while for non-carriers was 437.48mins (N=159) (Figure17).

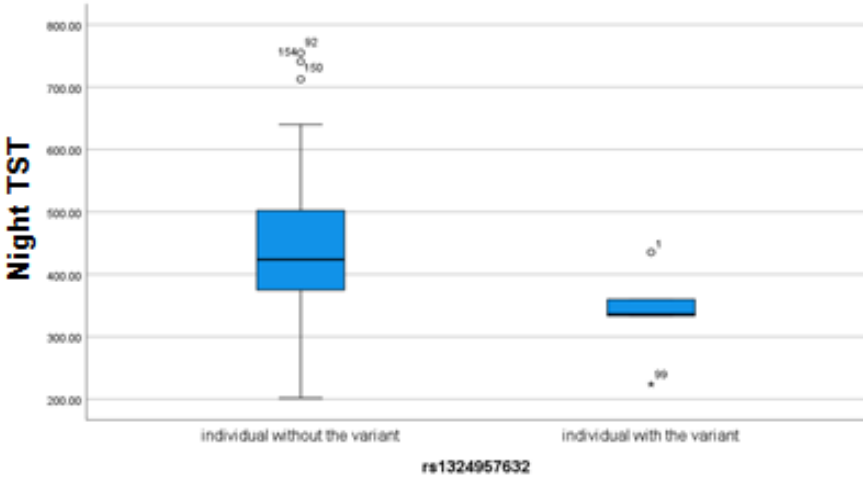


Figure17. Night TST in individuals with or without the *REV1* rs1324957632 variant.

SLC6A3 variant

The **rs1042098's** association with all the parameters was found to be statistical important. As far as it concerned night TST, the group that carried the variant (N=45) had a shorter objective sleep (mean night TST=407.17mins) than the one that did not carry it (N=119, mean night TST= 444.75mins, p=0.009) (Figure18a). Individuals with the variant (N=45) tend to sleep less (mean TST=444.79mins) than individuals that do not carry the variant (N=119, mean TST=483.30mins, p=0.032) (Figure18b). Night TiB (p=0.005) and total TiB (p=0.038) objective sleep was shorter (N=45, mean night TiB=505mins, mean total TiB=567.61mins) (Figure18c, 18d) when the variant was present and longer when absent (N=119, mean night TiB=547.98mins, mean total TiB=612.94mins).

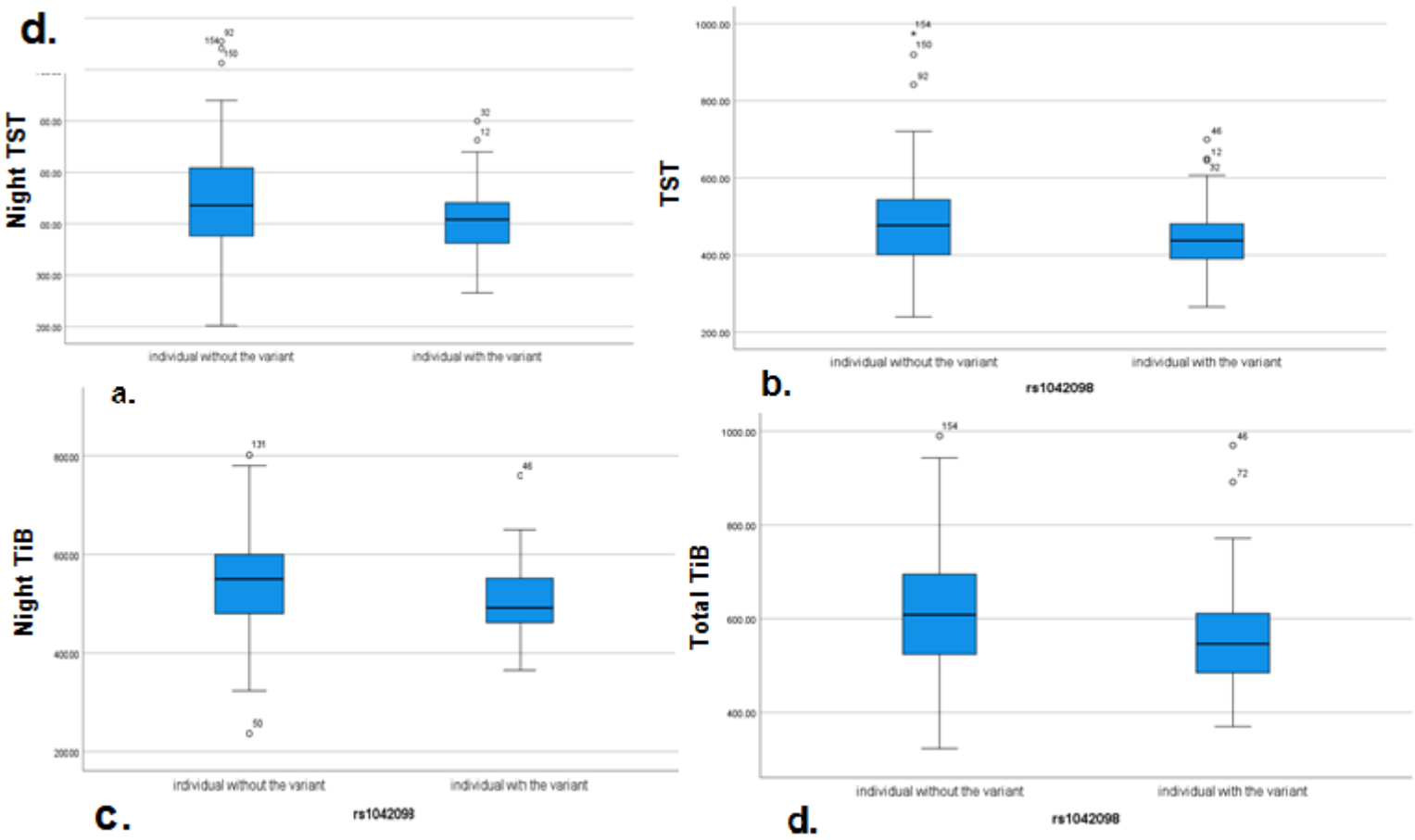


Figure18. **a.** Night TST in individuals with or without the *SLC6A3* rs 1042098 variant. **b.** TST in individuals with or without the *SLC6A3* rs 1042098 variant. **c.** Night TiB in individuals with or without the *SLC6A3* rs 1042098 variant. **d.** Total TiB in individuals with or without the *SLC6A3* rs 1042098 variants.

4.2 Extreme actigraphy scores in correlation with rare variants

Shorter Objective SleepScores									
Participant	Night TST(mins)	TST (mins)	Night TiB(mins)	Total TiB(mins)	*PER1*	*PER2*	*PER3*	*CRY1*	*CRY2*
KB7796	202	261.63	236.67	351.67	-	rs1301193813	-	-	-
PX0846	224	239.67	-	-	New41	-	-	-	-
AL0191	263	263	323.33	323.33	-	-	rs140974114	-	-
KG0590	265.67	265.67	370	370	-	-	-	-	-
LO0744	266.33	-	-	-	-	New45	rs371822849	-	-
AM0662	280	-	381.67	-	New34, rs754411014	-	-	-	-
ThK2997	288.33	-	-	-	New39	-	-	-	-
ME0544	292.5	314.50	-	-	-	-	-	-	-
TP1374	-	-	365	-	New39	New45	rs12121492	-	-
XG0539	-	302.33	370	370	-	-	New49	rs772775637	
KP0524	-	-	381.67	381.67	-	-	-	-	-
XP3022	-	-	388.33	388.33	-	New44	-	-	-
LX0247	-	-	-	402.67	New37	-	-	-	-
TS1923	-	-	-	410	-	-	-	-	-
TG0358	-	303.67	-	-	-	-	-	-	-
SN2411	-	306.33	-	-	-	-	-	-	-

Table3. The 5% of the participants with the shorter objective sleep scores in sleep parameters and the rare variants they carry in *PER1, PER2, PER3, CRY1, CRY2* genes.

Longest Objective Sleep Scores									
Participant	Night TST(mins)	TST (mins)	Night TiB(mins)	Total TiB(mins)	*PER1*	*PER2*	*PER3*	*CRY1*	*CRY2*
BM0182	755	842	780	885	-	-	-	-	-
FM5555	741	974.67	750	990	rs918239431	-	rs139315125 rs150812083	-	-
KS4444	712.67	919.67	720	943.33	-	-	-	-	-
BX0708	640	700	-	-	-	rs751755416	-	rs888780235	-
ME0816	622.33	-	707.67	-	-	rs76355956	-	-	-
SA0085	615.33	721	-	903.33	-	-	-	-	-
ED1703	607.33	-	-	-	rs148204955	-	-	-	-
TP0508b	605.33	-	-	-	-	-	-	-	-
MA1164	-	-	801.67	-	rs55655060, New28	-	rs146454363	-	-
BE0698	-	699.67	760	970	-	-	-	-	-
AM2217	-	-	730	-	-	-	-	-	-
MM1731	-	-	705	-	-	-	-	-	-
SE2322	-	-	-	913.33	-	rs560008620	-	-	-
ST1125	-	-	-	891.67	-	-	-	-	-
KG1522	-	-	-	843.33	-	-	-	-	-
KZ2731	-	659.33	-	-	New24	rs1301193813	-	rs17038934	-
PI1553b	-	650	-	-	-	-	rs143936373	-	-

Table4. The 5% of the participants with the longest objective sleep scores in sleep parameters and the rare variants they carry in *PER1, PER2, PER3, CRY1, CRY2* genes

Night TST

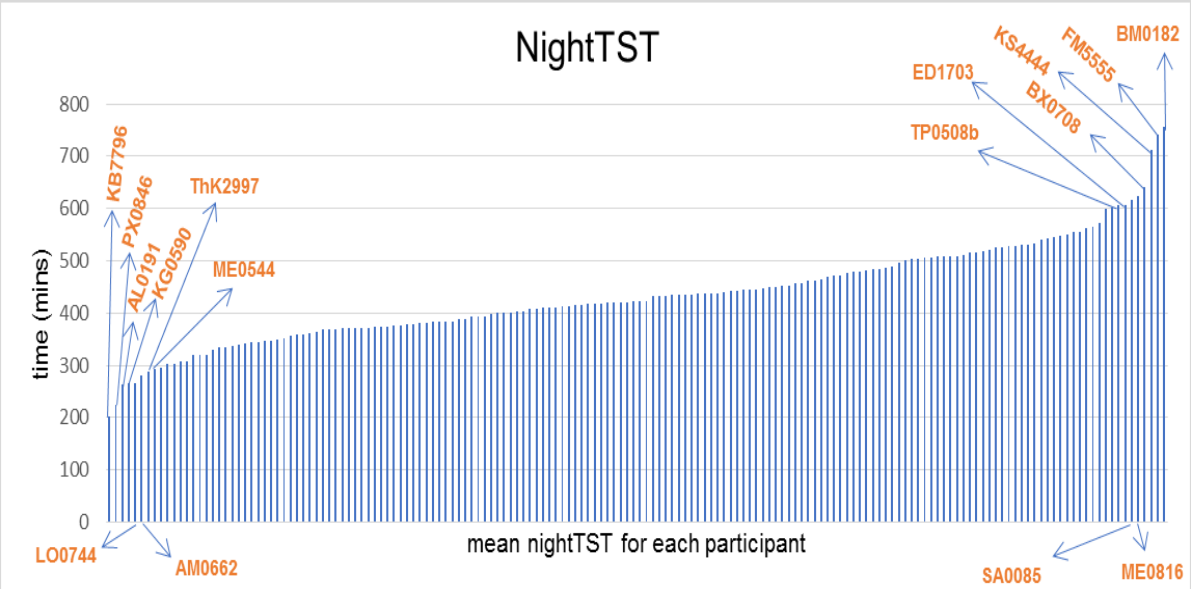


Figure19. Visual presentation of the mean night total sleep time (night TST) for each participant with ascending order. The first and last 8 participants have been noted on the graph.

The first parameter measured was night total sleep time (night TST) (Figure19) (Table3). The **shortest** score of objective sleep time was detected was 202mins and belongs to the participant KB7796 who carries the *PER2* **rs1301193813**. This variant is classified as missense and there is uncertain significance linked to its pathogenicity (Adzhubei et al., 2010), where alteration at position 718 causes the replacement of G with A and glutamic acid (E) 1075 is replaced with aspartic acid (D). Besides KB7796, one more participant carries the variant, KZ2731, who scores 555.33mins in night TST. Further research is required so as to specify whether this disrupted sleep pattern is associated with shorter or longer objective sleep.

Participant PX0846, who scored 224mins and 239.67mins in TST , solely carries a novel *PER1* variant which was named **new41**. It causes a frame shift mutation where the deletion of G at the 3328 position, results in protein loss of alanine (Ala) 110. This data could imply a possible association.

Participant Al0191, with 263mins score, is the only that carries the *PER3* variant**,** **rs140974114**, which causes a missense mutation, likely benign. G is being replaced with A at 2249 position and causes a serine (Ser) 750 asparagine (Asn) protein

alteration. Although PolyPhen2 (Adzhubei et al., 2010) score predicts that this variant is a benign one, it is worth mentioning that AL0191 participant, where the variant is present, has extremely short objective sleep in all the parameters night TiB (323.33mins), total TiB(323.33mins) and TST (263mins).

The LO0744 participant scores 266.33mins and carries **new45**, a novel *PER2* variant, and **rs371822849**, a *PER3* variant. Regarding new45 variant, in total 4 participants carry it, with participant TP1374 scoring 365mins in night TiB. The third participant scores 432.33mins in night TST and TST and has the long objective sleep of 540mins in night TiB and total TiB. The fourth participant has long objective sleep in all parameters (night TST, TST: 519.33mins, night TiB, total TiB: 556.67mins). New45 variant is not characterized yet or linked to any protein alteration or deletion. No clear conclusion can be drawn based on the data presented above about a possible linkage. The r371822849 is present only once in the sample, has a synonymous impact which does not alter the encoded protein or cause any damage and therefore it would not be further analyzed in this study.

The score of AM0662 participant carrying the *PER1* variants, **rs754411014** and the novel **new34**, is 280mins.The rs754411014 is present only once in the sample and has an in-frame impact, where the CCT codon in areas 1967, 1969 is deleted and as a result there is a serine 656 and a serine 640 deletion. Although this mutation can cause symptoms, according to PolyPhen2 (Adzhubei et al., 2010) prediction both protein alterations are benign. The new 34 is present twice and in the second participant had long objective sleep (night TST: 463.67mins, TST: 597mins, night TiB: 600mins, total TiB: 840mins). AM0662 objective sleep fluctuates though, from very short to long (TST: 371mins, night TiB: 381.67mins, total TiB: 508.33mins). New34 has a missense function where G is replaced in C in the 2646 position and as result glutamine (Gln) 882 is replace with histidine (His), an alteration characterized benign by PolyPhen2 (Adzhubei et al., 2010).

The novel *PER1* variant **new39** is present in 3 participants and among them are ThK2997 and TP1374. TP1374 scores 365mins in Night TiB and ThK2997 scores 288.33mins in night TST. The third participant was not a part of the first 5% of the sample with the shorter objective sleep. New39 variant is not characterized yet or linked to any protein alteration or deletion.

Participants KG0590 and ME0544 have very short objective sleep, however they do not carry any of the rare variants of this sample.

The FM5555 participant has **long** objective sleep in all sleep parameters studied (night TST: 741mins, TST: 974.67mins, night TiB: 750mins, total TiB: 990mins) (Figure19) (Table 4). The *PER1* **rs918239431** is present in that participant only, is not characterized yet or linked to any protein alteration or deletion and there is no reference of it in the existing literature. The *PER3* **rs139315125** and **rs150812083** are also found in FM5555 only and both have a missense effect and are related to familial advanced sleep phase syndrome. The rs139315125 variant replaces C with G in the 1247 position which alters the protein by replacing histidine (His) 416 with Arginine (Arg). According to PolyPhen2 (Adzhubei et al., 2010), this mutation is predicted as probably damaging (0.989/1.0). The rs150812083 variant replaces C with G at the 1240 position which alters the protein by replacing proline (Pro) 414 with alanine (Ala). Following the same pattern, this mutation is also characterized probably damaging (1.0/1.0). It is important to mention that this participant has started medication 2 years prior the examination. The Cipraplex is a selective serotonin reuptake inhibitor-SSRI which is mainly used to treat depression and anxiety related disorders with somnolence to be one of the common side effects (Galinos,n.d.).

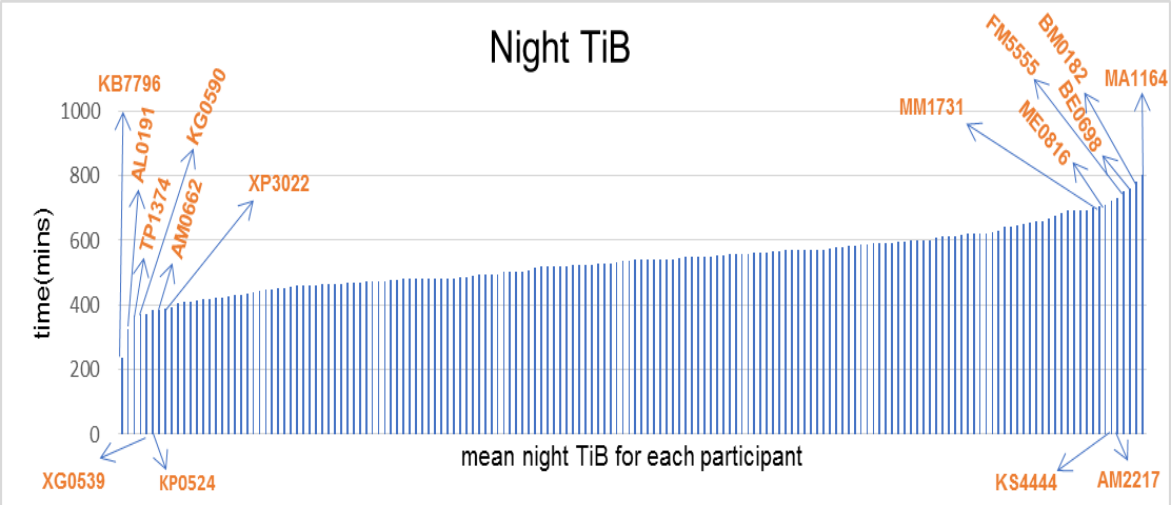
Only the BX0708 participant carries the *CRY1* **rs888780235** and *PER2* **rs751755416** variants. The *CRY1* is not characterized yet or linked to any protein alteration or deletion and the *PER2* one has a missense function which is responslible for replacing G with A at 2429 position and the serine (Ser) 810 is replaced with asparagine (Asn). According to PolyPhen2 (Adzhubei et al., 2010), it is probably damaging (1.0/1.0). Adding to that, the scores of BX0708 (night TST: 640mins, TST: 700mins, night TiB: 690mins, total TiB: 825mins) and the rest of the participant's medical history, which seems not to affect with the participant's sleep patterns (vertigo-medication with no sleep related side effects), indicate that there might be an association between phenotype and genotype in this case.

The *PER2* **rs76355956** variant is being carried by two participants. ME0816 (night TST, TST: 622.33mins, night TiB, total TiB: 706.67mins) has long objective sleep in all parameters while the other participant was not a part of the first 5% of the sample

with the longest objective sleep. The variant is a missense mutation which replaces G with A in 3613 position and alters the protein by replacing valine (Val) 1205 with methionine (Met) and is characterized as probably damaging by PolyPhen2 (Adzhubei et al., 2010) (0.997/1.0). This variant has been previously associated with delayed sleep-wake phase disorder (Miyagawa et al., 2019). However, we should consider that both participants receive heavy antidepressant and anxiolytic medication. This could possibly affect their sleeping habits and thus we cannot clearly imply an association.

The participant ED1703 carries the *PER1* **rs148204955** is not characterized yet or linked to any protein alteration or deletion.

Although, participants BM0182, KS4444, SA0085 and TP0805b have long objective sleep, they do not carry any of the rare variants of this sample.



Night TiB

Figure20. Visual presentation of the mean night time in bed for each participant with ascending order. The first and last 8 participants are being noted on the graph.

The second parameter that was measured and correlated with phenotypes was night time spent in bed (night TiB) (Figure20). The **shortest objective sleep** was observed as expected to the ones that had short objective night TST as well: KB7796, AL0191 and AM0662 (Table 3). Participant's TP1374 variants were analyzed before, except *PER3* **rs12121492** which is a synonymous mutation and

does not alter the encoded protein or cause any damage and therefore it would not be further analyzed in this study.

The *CRY1* **rs772775637** and the novel *PER3* variant **new 49** are being carried by participant XG0539. XG0539 has shorter objective sleep in night TST and TST (302.33mins) than in night TiB and total TiB (370mins). The *CRY1* variant has a stop gain impact by altering C with T in position 199 which results in a non- functional protein (p.R67*). The new 49 variant has a splicing impact which has not been clarified whether is damaging or not.

Night TST and TST sleep of XP3022 participant is shorter (320mins) whereas night TiB and total TiB sleep is longer (388.33mins). XP3022 and another participant (not a part of the first 5% with the shortest objective sleep) carry the novel *PER2* variant **new 44,** which has a missense effect (C replaces T in 361 position) and replaces histidine (His) 121 with tyrosine (Tyr). PolyPhen2's prediction (Adzhubei et al., 2010) is characterized probably damaging (1.0/1.0).

Participants KP0524 and KG0590 have short objective sleep. However, they do not carry any of the rare SNPs of this sample.

The **longest objective sleep** score in this parameter belongs to MA1164 participant, who carries 3 variants (Figure20) (Table 4). For *PER1* **rs55655060,** 3 participants carry it, with two of them not being a part of the 5% with the shortest objective sleep whereas MA1164 (night TST, TST: 600.33mins, night TiB, total TiB: 801.67) has longer objective sleep. However, this variant has not been characterized yet or linked to any protein alteration or deletion.

The novel *PER1* **new28**, has a frame shift impact, inserts C in 2291 and 2292 positions which causes the loss of alanine (Ala) 765. The *PER2* **rs146454363** has not been characterized yet or linked to any protein alteration or deletion. An association between the long objective sleep scores and the new28 could be presumed, while taking in consideration the dementia and diabetes diagnosis and the multiple medications that the participant receives daily. Although, participants BM0182, KS4444, BE0698, AM2217, KS4444 and ME1731 have long objective sleep, they do not carry any of the rare variants of this sample.

Total TiB

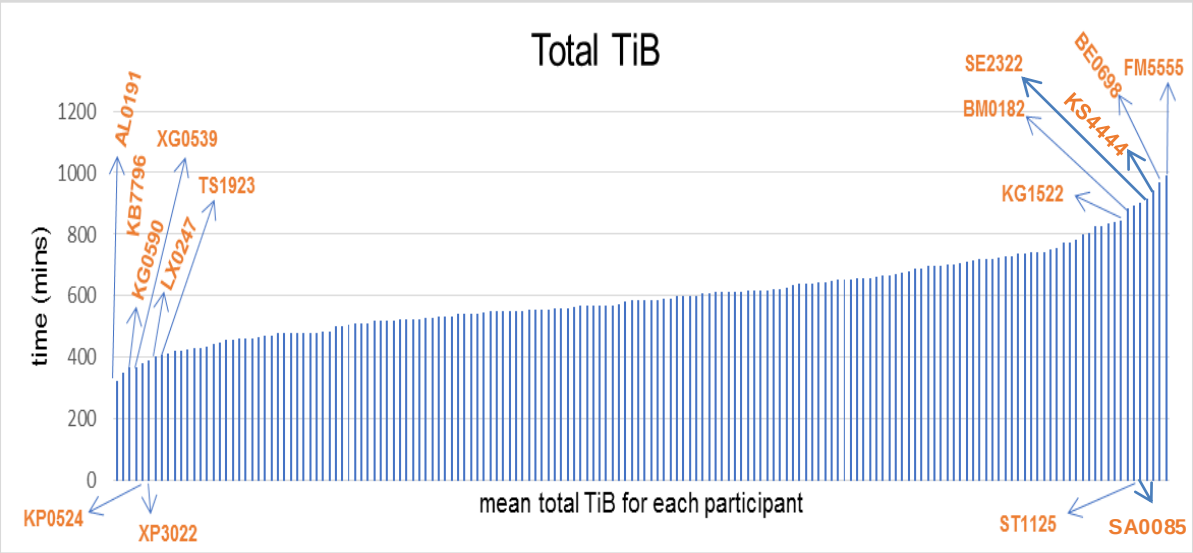


Figure21. Visual presentation of the mean total time in bed for each participant with ascending order. The first and last 8 participants are being noted on the graph.

The third parameter measured and correlated with phenotypes was total time spent in bed (total TiB). The **shorter objective sleep** was observed as expected to the ones that had shorter night TST and night TiB as well: KB7796, AL0191, XG0539 and XP3022 (Figure21) (Table3).

As mentioned before, KP0524 and KG0590 do not carry any of the rare SNPs of this sample along with participant TS1923.

Participant LX0247 (Total TiB=402.67mins) carry the novel *PER1* variant **new37,** which has a missense impact, replaces G with C at the 3025 position and the protein is altered by the replacement of glycine (Gly) 1009 with arginine (Arg). The PolyPhen2's prediction (Adzhubei et al., 2010) is benign.

The longest objective sleep once again belongs to FM5555 (Figure21) (Table4). Many participants that hold the longest objective sleep scores in this parameter such as BE0698, KS4444, SA0085, ST1125, BM0182 and KG1522 do not carry any of the rare variants identified in this sample.

The *PER2* variant **rs560008620** is a missense alteration where C is replaced with T in the 2896 position and arginine (Arg) 966 is replaced with tryptophan (Trp). It is a probably damaging one (Adzhubei et al., 2010) (0.958/1.0) and a possible linkage could be assumed given the scores of SE2322 (night TST: 484.67mins, TST: 547.67mins, night TiB: 675mins, total TiB: 913.33mins) while taking in consideration the dementia diagnosis and the dementia and anxiolytic medication.

TST

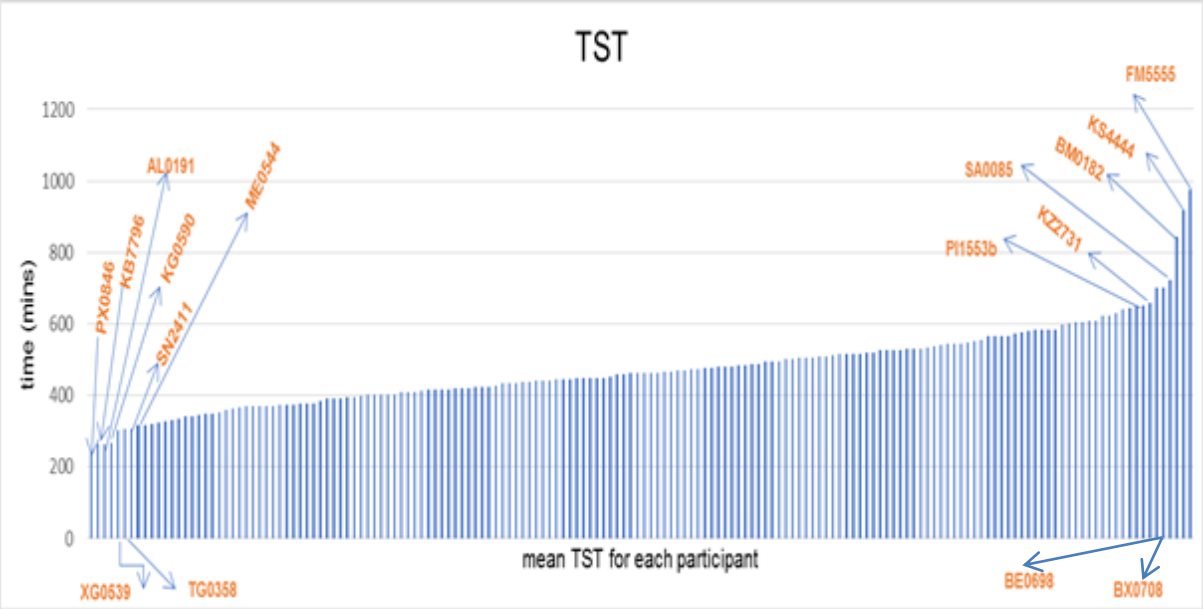


Figure22. Visual presentation of the mean total sleep time for each participant with ascending order. The first and last 8 participants are being noted on the graph.

Most of the scores for total sleep time and the participants baring the rare variants were mentioned in the previous analysis. PX0846, KB7796, AL0191 and XG0539 keep the **shortest** objective sleep whereas the rest of the participants such as KG059, TG0358, SN2411 and ME0544 do not carry any rare variants (Figure22) (Table3).

Besides FM5555 and BX0708, KZ2731 has long objective sleep scores and carries 3 variants (Figure22) (Table4). *CRY1* **rs17038934** has not been characterized yet or linked to any protein alteration or deletion. *PER1*'s novel variant **new24** has frame shift impact where C in position 2980 is deleted but there is no evidence about the

protein's alteration. *PER2* **rs1301193813** was described in night TST's part and there was no conclusion leading to any kind of phenotype genotype association.

Participant PI1553b (night TST: 600mins, TST, night TiB: 650mins, total TiB: 700mins) carries the *PER3* **rs143936373** variant which has missense impact, replaces G with A in the 541 position and replaces alanine (Ala) 181 with threonine (Thr). PolyPhen2 prediction is benign (Adzhubei et al., 2010).

Following the same pattern with the previous analyses, KS4444, BM0182, SA0085 and BE0698 have long objective sleep but the carry no rare variants.

5. DISCUSSION

Our study is unique, in the sense that it combines actigraphy and WES (whole exome sequencing), two robust methods for analyzing sleep and genetic background of individuals. In the 25 genes studied in this cohort of 164 individuals (*CLOCK, ARNTL, NPAS2, PER1, PER2, PER3, CRY1, CRY2, RORA, RORB, REV1, VDR, BHLHE40, BHLHE41, NR1D1, TNF, CHRM3, BDNF, SLC6A4, SLC4A3, HCRTR1, HCRTR2, MECP2, GABBR1, HLA-DQB1*), we have identified 472 SNPs, of which 74 were novel (they do not have a dbSNP code yet). In this study, we present the variants that showed a statistically significant difference in comparing values of sleep parameters between SNP carriers and non-carriers. Also, we present the rare variants in this sample that had an association with an extreme sleep phenotype.

In detail, the **rs1042098** *SLC6A3* variant was associated with decreased night sleep time and time in bed in our study. Variants in the *SLC6A3* gene (OMIM 126455) have been shown to be the cause of infantile Parkinsonism-dystonia type 1 (OMIM 613135), a disease that is inherited in an autosomal recessive manner and starts in infancy. This disease presents with parkinsonism and other movement disorders (Hamosh et al., 2005). The dopamine transporter encoded by the *SLC6A3* gene is a key player in dopaminergic neurotransmission, as it mediates dopamine reuptake from the dopaminergic synapses (NCBI, 2004). The rs1042098 *SLC6A3* variant found in our study has not been linked to disturbed sleep patterns in previous literature. Nonetheless, it has been previously associated with ADHD symptoms in children (Waldie et al., 2017), with childhood depression (D'Souza et al., 2016) and in neurobehavioral recovery following pediatric TBI (Treble- Barna et al., 2017). It is currently unclear how this rs1042098 *SLC6A3* variant mediates decreased sleep, as shown in our study. However, there is evidence that dopaminergic system is implicated in the sleep-wake mechanism (see Introduction, chapter 1.6).

The *CRY1* variant, **rs8192440** was related with long objective sleep scores in all parameters. *CRY1* (OMIM 601933) gene is a major circadian regulator and it is associated with Delayed sleep phase syndrome (OMIM 614163). This circadian rhythm sleep disorder causes disrupted sleep patterns related by delayed sleep onset and offset and is inherited in a autosomal dominant manner (Patke et al., 2017) (see Introduction, chapter 1.8). Even though *CRY1* is a basic circadian clock

gene, this synonymous SNP has not been associated with sleep disturbances in the literature and has been characterized as a benign one by Polyphen2 (Adzhubei et al., 2010). It has been previously associated with a positive response in the lithium administration to bipolar patients (McCarthy et al., 2011).

The *PER1* variant**, rs2304911**, is associated with shorter objective sleep in comparison to the non-carriers group. *PER1* gene (OMIM 602260) is a major clock gene which regulates the circadian pacemaker via the TTFL (see Introduction, chapter 1.7) and is implicated in the tumorgenesis of endocrine cancers (Angelousi et al., 2019). This specific variant that had a statistically significant result in our analysis but there is no reference of it in the existing literature regarding its clinical significance.

Besides the most statistically significant results presented above, more SNPs, with $p<0.05$ were also identified. The *PER2,* **rs35333999** variant carriers spent less time spent in bed*. PER2* gene (OMIM 603427) is also majorly implicated in the circadian regulation (see Introduction, chapter 1.7) via the TTFL and associated with familial advanced sleep phase syndrome 1 (OMIM 604348). It is an autosomal dominant inheritance disorder where sleep onset and offset, in comparison with DSPS, are very early (Jones et al. 1999). The rs35333999 is a missense variant where G is replaced with A in 2707 position and alters the protein by replacing valine (V) 903 with isoleucine (I). In the literature, it has been linked with later chronotype (Chang et al., 2019). The *PER3,* **rs10462021,** has shown longer objective night sleep time in the carriers group. *PER3* gene (OMIM 603427) is also a part of the TTFL that controls the circadian rhythm (see Introduction, chapter 1.7) and is associated with familial advanced sleep phase syndrome 3 (OMIM 616882). The syndrome is an autosomal dominant inheritance disorder, characterized by early sleep onset and offset (Zhang et al., 2016). The rs10462021 is a missense variant where A is replaced with G in the 3446 position and histidine (H) 1149 is replaced with arginine (R).

REV1 gene (OMIM 606134) has a mutagenic role in the DNA repair (See Introduction, chapter 1.7). The carriers of **rs717454, rs3087386** and **rs28369942** had shorter objective sleep in total TiB than the non-carriers, whereas **rs1324957632** carriers group sleep less time at night from the non-carriers. The rs717454 was

previously associated with mood disorders (Xiao et al., 2017). The rs3087386 , a missense variant, where the protein is altered with the replacement of phenylalanine (Phe) 257 with serine (Ser), may be possible related as a risk factor for lung cancer survival (Xu et al., 2013). The rs28369942 and rs1324957632 have not been linked to any clinical findings yet. Even though, there has not been found sleep related evidence in the literature, all variants of REV1 had shorter objective sleep in all parameters. It seems that through its role, REV1 affects the circadian regulation. However this hypothesis needs to be further investigated and clarified.

The *BDNF* gene (OMIM 113505) is a neurotrophic factor and its deficiency is implicated in neurodegenerative diseases (see Introduction, chapter 1.7). The **rs412744440** and **rs145503674** variants are related to shorter objective total TiB, comparing with the non-carriers but there is no evidence in literature regarding their clinical significance.

The *GABBR1* variants, **rs29230, rs5875197** and **rs29267** carriers had shorter objective sleep time than the non-carriers. *GABBR1* gene encodes the GABA (B) receptor 1 and is implicated in diseases such as schizophrenia and epilepsy (NCBI, 2004). There are no significant data regarding the synonymous rs29230 variant. The same applies for the rs5875197 and rs29267.

Regarding the rare polymorphisms study, our aim was to search through the sample for rare variants in combination with extreme phenotypes (eligible to the criteria set) in order to explore the possibility of an association and a causality.

Participant FM555 had extremely long objective sleep in all parameters and carried solely the missense *PER3* **rs139315125** and **rs150812083** which are related to familial advanced sleep phase syndrome (Zhang et al., 2016). As we take in consideration the medication the participant is receiving, we cannot but indicate the association between the long objective sleep scores and the presence of two variants already linked to the sleep disorder. Furthermore, some variants of *PER2* present very interesting correlations. *PER2* missense **rs751755416** variant was present to BX0708 participant only, who had long objective sleep and was labeled as probably damaging by Polyphen2. Despite this association between ‘’a rare variant and very long objective sleep” that we observed, there has not been any reference of rs751755416 in literature. The **rs76355956,** a missense and probably damaging

variant, is being carried by a participant who demonstrated long objective sleep scores in all parameters. Although, this *PER2* variant was previously associated with delayed sleep-wake phase disorder (Miyagawa et al., 2019), we have to take in consideration the effects of ME0816's medication on the regulation of the circadian rhythms before assuming the presence of a possible association.

Additionally, regarding the rare variants, two novel *PER1* variants, **new24** and **new28,** have been detected in participants with very long objective sleep. Both of them have a frame shift impact but there have not been mentioned in the literature. However, it seems as an important finding, amenable to further research, while taking in consideration the medication's effect that KZ2731 (new28 carrier) receives.

The novel *PER2* variant, **new44,** was associated with shorter objective sleep. As mentioned before, it has a missense impact and was characterized as probably damaging by Polyphen2. Considering all these, we could indicate a possible association that needs to be further analyzed and studied. Another novel variant of the *PER1* gene, **new41** causes a frame shift mutation and was associated with shorter objective sleep. These data could imply a possible association.

Regarding the limitations on this study, the sample consisted of individuals from Crete area. It needs to be done further research on samples from other regions of Greece or other places in the world so as to enhance the evidence. Along with that, it is important for the data already studied to be validated, so as to create stronger evidence where a possible causality between the genotype and the disorder's phenotype will be based on. Regarding the actigraphy data, the duration of the recordings was short and there was a lack of objective sleep apnea data as well. Furthermore, the medication the participants received was a factor that was taken in consideration to the outcome's interpretation. However, this factor is impossible to be eliminated in general, due to the age and the multiple health issues the participants experienced, and more thorough research is demanded to directly associate variants with sleep disorders. Last, in the second part of the study, only 5 out of 25 genes were used to identify rare variants in the participants with shorter or longer objective sleep.

This research aimed to enhance the knowledge that is building around sleep genetics based on another research carried out several years ago using the same

tools but less participants from the sample and the basic clock genes only (Konstantara, 2016) (Table6). Having it as a starting point, more genes related to circadian mechanism according to literature where collected to be analyzed. Moreover, this data could be used in future gene based analysis and polygenic risk score analysis, where the total effect of many SNPs can be associated with the disease (Baker et al., 2019) and the genotype of an individual along with GWAS data are used so as to estimate a possible causality of the individual's genotype with a disease or a trait (Choi et al., 2020), respectively. The genotyping of each gene studied in this research could be used for the evaluation of a disease (although it has not been clarified whether there is a causative connection or not between them). Last but not least, the genetic markers are important for the early diagnosis and prevention of a disease so as to inform the patient and act accordingly, aiming to improve its prognosis, hinder its progress or even cure it.

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SUPPLEMENTARY DATA

Table5. The genes associated with sleep disorders and studied in the current research. All SNPs detected in the 201 participant sample and the numbers of the carrier and non-carriers are being presented

Gene	SNP	Cases	Controls	Gene	SNP	Cases	Controls
TNF	rs4645843	5	4	**HCRTR2**	rs2653349	118	80
	rs577329144	0	2		rs12111299	1	0
	rs867435711	2	0		rs9475219	1	0
	rs375864945	1	0		rs9396073	53	40
	rs3093665	19	13		rs3122169	120	79
	rs3093662	27	24		rs62416819	53	40
	new	3	0		rs62416818	53	40
CHRM3	rs751548647	1	1		New10	1	0
	rs200553848	1	0	**MECP2**	rs587783092	13	9
	rs201489195	0	1		rs61752387	1	1
	rs140545853	0	1		rs782482746	0	1
	rs145865028	0	1		rs61748385	1	0
	rs201656302	0	1		rs782743949	1	1
	rs199549014	0	1		rs1057520135	3	2
	rs200122635	1	0		rs782739532	3	3
	rs2067481	9	8		rs61753975	0	1
	rs753116253	0	1		rs3850326	6	1
	New1	1	0		rs61749711	0	1
	New2	1	0		rs61750245	1	2
	New3	1	0		rs61753012	2`	1
	New4	1	0		rs3027927	1	0
BDNF	rs76656357	1	1		rs2071569	1	0
	rs145503674	2	7		New11	1	1
	rs41274440	2	7		New12	1	0
	rs748025933	0	1		New13	1	0
	rs376019229	1	1		New14	1	0
	rs79679324	0	2		New15	66	44
	rs8192466	1	0		New16	6	0
	rs139352447	1	0		New17	2	1
	rs66866077	9	8		New18	0	1
	rs909900837	2	0	**GABBR1**	rs56034620	6	2
	rs200573189	0	1		rs183265065	0	1
	rs6265	41	24		rs754019548	0	1
	rs2353512	120	81		rs559736409	0	1
SLC6A4	rs74845752	2	1		rs1805057	1	3
	rs1007216124	0	1		rs200280988	2	0
	rs28914832	1	0		rs190501135	0	1
	rs33919215	1	0		rs370044776	0	1
	rs200015551	1	1		rs76632246	2	2
	rs6355	1	0		rs200753831	0	1
	rs41274284	2	0		rs78592538	0	1
	rs41274280	2	0		rs1805056	19	11
	rs34876533	2	0		rs29243	3	1
	rs34083002	2	0		rs29220	54	32

	rs28914830	3	1		rs17854216	36	26
	New5	0	1		rs17854217	36	26
	New6	1	0		rs29225	36	26
SLC6A3	rs112801202	1	2		rs29253	36	26
	rs780852886	1	0		rs17177979	1	0
	rs747783826	0	1		rs29262	36	26
	rs768979705	3	0		rs29263	36	26
	rs147837176	2	0		rs29230	36	26
	rs942434534	2	1		rs29267	37	31
	rs369942690	1	0		rs740884	32	17
	rs201605046	2	1		rs5875197	52	41
	rs145114326	1	1		New19	1	0
	rs41282615	0	1	**HLA-DQB1**	rs760326072	1	0
	rs1049930802	0	1		rs386699586	18	14
	rs369805676	1	1		rs41543118	1	0
	rs8179035	0	1		rs76282264	2	1
	rs192044159	1	1		rs12722107	5	2
	rs756371809	1	0		rs9274407	116	77
	rs6350	14	13		rs1130375	67	49
	rs460000	44	37		rs1130368	63	41
	rs6347	54	37		rs3204373	5	2
	rs8179031	1	2		rs1049079	3	1
	rs8179029	45	32		rs1554169762	67	45
	rs429699	120	81		rs796500328	66	42
	rs6349	3	3		rs1049068	13	8
	rs28363149	2	0		rs281862065	71	43
	rs1042098	68	52		rs281874782	5	2
	New7	1	0		rs1063318	65	41
	New8	0	1		rs1049082	79	54
	New9	1	0		rs68027833	63	41
HCRTR1	rs143453875	1	0		rs1049100	63	41
	rs200961371	0	1		rs1063321	85	57
	rs200965303	1	0		rs1049107	63	41
	rs41501244	2	0		rs1063322	100	65
	rs766527549	0	1		rs41542812	26	22
	rs144603792	0	1		rs41544112	10	5
	rs1056564939	0	1		rs1049086	92	63
	rs35443818	2	0		rs1063323	85	57
	rs41263963	19	4		rs1049087	59	38
	rs2271933	101	64		rs1049088	63	41
	rs7516785	0	1		rs1049130	111	78
	rs1056526	89	62		rs1049133	119	81
	rs11806980	1	0		rs701564	67	51
HCRTR2	rs41403545	2	1		rs1049092	103	67
	rs202113562	0	1		rs1130398	85	57
	rs199945618	0	1		rs1130399	28	13
	rs41381449	1	1		rs28688207	21	9
	rs564227915	1	0		rs9273528	28	13
	rs41271312	1	0		New20	1	0

Table6. The clock genes studied in the previous and current research. All SNPs detected in the 201 participant sample and the numbers of the carrier and non-carriers are being presented.

Gene	SNP	Cases	Controls	Gene	SNP	Cases	Controls
CLOCK	New21	1	0		rs74795714	4	0
	rs766067893	0	1		rs937895139	1	0
	rs200566706	1	0		New25	0	1
	rs34812164	1	0		rs754411014	1	0
	rs780830912	0	1		rs918239431	1	0
	New22	1	0		rs148204955	1	0
	rs767458103	1	0		rs746932415	0	1
	rs188447644	2	0		New26	2	0
	rs34897046	5	4		rs778368752	0	1
	rs6855837	0	1		New27	5	0
	rs74643067	5	4		New28	1	0
	rs3736544	97	67		New29	40	14
	rs35793438	5	4		New30	1	0
ARNTL	rs145121921	0	1		rs748386886	1	0
	New23	0	2		New31	1	0
	rs200521706	1	1		rs150726488	1	0
	rs371906248	1	0		New32	1	0
	rs374112499	0	1		New33	0	2
	rs77363897	4	1		New34	2	0
	rs140130779	0	1		rs143964144	`0	1
	rs10832030	119	81		New35	1	0
	rs2290037	19	7		New36	1	0
NPAS2	rs113107029	0	2		New37	1	0
	rs201271037	2	0		New38	2	1
	rs183671025	1	0		New39	1	2
	rs80034641	1	0		New40	2	4
	rs138318020	1	0		rs146151077	0	1
	rs17025128	6	0		rs200162062	0	1
	rs200967952	1	1		New41	1	0
	rs146893880	1	0		rs968568350	1	0
	rs2305158	43	34		rs144699916	0	1
	rs9223	78	41		rs55720390	7	3
	rs13430345	1	1		rs55655060	4	1
	rs2278727	56	31		rs112854368	7	2
	rs11541353	39	35		rs112185134	5	2
	rs60866311	3	0		rs3027178	67	41
	rs2305160	108	75		rs10462024	72	44
	rs112862134	4	4		rs2304911	15	10
	rs1562313	40	16		rs2735611	101	69
	rs35503589	19	16		rs2253820	101	72
	rs2289950	19	16		rs2585405	111	76
	rs3841571	1	3		rs112474322	5	1
	rs41280595	38	36	**PER2**	New42	3	1
PER1	New24	1	0		New43	1	0

	rs753765505	1	0		rs144982607	1	0
	rs72845601	0	1		New44	1	1
	rs1249790523	1	0		rs780618280	1	0
	rs35826160	6	3		rs146454363	3	1
	New45	4	2		rs139315125	1	0
	New46	1	0		rs150812083	1	0
	rs751755416	1	0		New52	2	0
	rs35333999	5	10		rs143936373	0	1
	New47	1	0		rs12121492	0	1
	rs371760410	4	4		rs140974114	1	1
	rs560008620	1	0		rs228654	35	18
	rs1301193813	1	1		rs10462021	29	20
	rs376683523	1	0		rs1773135	103	67
	rs76355956	1	1		rs2640909	57	36
	rs116298301	3	3		rs12023156	101	73
	New48	1	0		rs2640908	46	31
	rs762946532	0	1		rs17031614	8	4
	rs140875436	0	1		rs228697	35	18
	rs77592057	0	1		rs228696	120	81
	rs80156481	0	1		rs2859387	61	46
	rs138121985	0	1		rs697693	46	31
	rs750332678	0	2		rs10462020	29	20
	rs77146655	0	1		rs228690	25	13
	rs75156302	0	1		rs79372391	8	4
	rs2304672	33	24		rs228669	118	81
	rs2304674	52	37		rs228668	120	80
	rs13033501	35	25		rs12566042	8	4
	rs3217472	35	21		rs149164395	8	4
	rs78907943	4	2		rs488728	109	73
	rs2304669	13	10		rs707465	63	45
	rs2304670	38	25		rs697682	16	7
	rs2304671	10	10		New53	2	0
	rs10195959	4	2	**CRY1**	rs772775637	0	1
	rs78832829	5	2		rs201634474	2	0
	rs35873326	4	2		rs888780235	1	0
	rs934945	51	37		rs184039278	0	2
PER3	rs200038116	1	1		New54	1	1
	rs371822849	2	0		rs17038934	3	1
	rs35072750	0	1		rs7306763	0	1
	rs144178755	1	1		rs8192440	103	72
	rs12750400	0	2	**CRY2**	rs150408838	3	0
	New49	0	1		rs369568207	1	0
	New50	62	47		rs181375232	0	1
	rs11121034	103	67		New55	1	0
	rs1776342	103	67		rs113851044	3	1
	rs11576985	10	13		rs773506901	1	0
	rs1558460360	0	1		rs2292912	111	76
	New51	1	0		rs2292913	119	76
	rs921226492	1	0		rs11394694	111	75

VDR	rs748166237	0	1		rs765172820	1	0
	rs746039894	1	0		rs199616498	0	3
	rs752590757	1	0		rs1022212450	1	0
	rs540774973	0	1		rs150952015	14	10
	rs150775215	0	1		rs150805444	1	0
	rs2228570	101	74		rs200227651	0	1
	rs2228572	4	0		rs117458658	2	1
	rs10783218	16	4		rs143635644	1	0
	rs1168267	40	19		rs73424068	3	2
	rs1168266	94	70		rs78039601	7	3
	rs731236	70	51		rs17237283	0	1
REV1	rs549798949	0	1		rs76532214	7	2
	New56	1	1		rs61740274	32	20
	New57	0	1		rs11071539	120	1
	rs558361733	6	0	**RORB**	rs144902615	1	0
	rs1324957632	6	1		New65	0	1
	New58	3	0		New66	0	1
	New59	3	0		rs1264695746	0	1
	New60	1	0		rs41307459	3	2
	rs3087383	2	0		rs3818559	75	53
	rs571039597	1	0		rs2273975	45	28
	rs3087385	9	2		rs139770757	39	17
	rs147615288	4	0	**BHLHE40**	New67	0	1
	rs777771050	2	1		rs182209585	0	3
	New61	0	1		New68	0	1
	rs754043439	0	1		rs111860035	1	0
	rs3087396	6	2		rs908078	40	36
	rs138292917	1	0		rs78035155	3	0
	rs147467855	0	1		rs2271566	35	31
	New62	9	2	**BHLHE41**	New69	0	1
	New63	7	2		rs368411883	1	1
	New64	0	1		rs76786372	11	3
	rs774097504	1	0		rs370994677	1	1
	rs28382881	0	1		rs200886903	1	0
	rs28382973	0	1		rs372622178	1	0
	rs28369942	45	53		rs1240463456	1	1
	rs3087403	69	42		New70	7	3
	rs3087386	89	61		rs61754129	0	3
	rs3087399	8	10		rs1871557	13	7
	rs10175852	8	10	**NR1D1**	rs17616365	1	0
	rs13409359	8	11		rs778452400	0	1
	rs717454	85	57		rs138791655	2	0
	rs3087401	2	1		New71	1	0
	rs2305354	87	60		rs201870231	58	23
RORA	rs201488277	1	0		New71	0	1

NR1D1	rs1237927783	0	1
	rs148075782	1	0
	rs140172310	0	1
	rs140575858	1	0
	New74	0	1
	rs61736536	2	0
	rs2102928	106	69
	rs2314339	29	24
	rs939346	117	75
	rs883871	33	24