Endocrine and neurophysiological examination of sleep disorders in Williams syndrome

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ABSTRACT

Background

A high rate of sleep disturbances have been reported in individuals with Williams syndrome (WS), but the underlying aetiology has yet to be identified. Melatonin and cortisol levels are known to affect and regulate sleep/wake patterns. We investigated the changing levels of these hormones in order to explore any relationship with sleep disturbances in children with WS.

Methods

Twenty seven children with WS and 27 typically developing (TD) children were recruited. Sleep was monitored using actigraphy and pulse oximetry. Parents completed Children's Sleep Habit Questionnaire (CSHQ). Saliva and first void morning urine samples were collected from the children. Saliva was collected at three time points: 4-6pm, before bedtime and first thing after awakening. Levels of salivary melatonin and cortisol were analysed by enzyme linked immunoassays. For determination of melatonin, cortisol and their metabolites in urine samples, specific Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS) method was developed.

Results

CSHQ and actigraphy indicated that children with WS were significantly affected by several types of sleep disturbances, including: abnormally high sleep latency and excessive night waking. Children in WS group had shallower falls in salivary cortisol levels and less pronounced rises in salivary melatonin at bedtime compared to TD controls ($p < 0.01$ and $p = 0.04$ respectively). Furthermore, it was found that children with WS also had significantly higher levels of bedtime cortisol compared to TD controls ($p = 0.03$). Using UHPLC-MS/MS analysis it was shown that

children with WS secrete less melatonin during the night compared to healthy controls ($p < 0.01$). Also, levels of cortisone, a metabolite of cortisol were significantly higher in the WS group ($p = 0.05$).

Conclusions

We found that children with WS had significant sleep disturbances which may be associated with their increased bedtime cortisol and lower evening melatonin. Both hormones play a significant role in the circadian rhythm and sleep/wake cycle, therefore it was necessary to look closely at these endocrine markers in individuals suffering from sleep disorders. Sleep problems in children with WS may adversely affect daytime activity and the quality of life, as well as social, emotional, health and economic functioning of the entire family. Hence, finding their cause is of great importance for affected children and their families.

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADHD	Attention Deficit Hyperactivity Disorder
ANCOVA	Analysis of covariance
APCI	Atmospheric pressure chemical ionisation
ASD	Autism Spectrum Disorder
AVP	Arginine vasopressin
BMI	Body- mass index
CNS	Central nervous system
CSHQ	Children's Sleep Habit Questionnaire
CRH	Corticotropin releasing hormone
CV%	Coefficient of variance
DS	Down's Syndrome
EEG	Electroencephalography
EIA	Enzyme immunoassay
ELISA	Enzyme- linked immunosorbent assay
ELN	ELASTIN gene
EMG	Electromyogram
EOG	Electro- oculogram
ESI	Electrospray ionisation
FISH	Fluorescence in situ hybridization
FLD	Fluorescence detection
fMRI	Functional magnetic resonance imaging
FSH	Follicle- stimulating hormone
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry

GH	Growth hormone
HLA	Human leukocyte antigen
HPA	Hypothalamus- pituitary- adrenal
HPLC	High performance liquid chromatography
HSD	11β-hydroxysteroid dehydrogenase
IQR	Interquartile range
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography- tandem mass spectrometry
LCR	Low-copy-repeat sequences
MCR	Minimal critical region
MS/MS	Tandem mass spectrometry
MT6s	6-sulfatoxymelatonin
m/z	Mass to charge ratio
NREM	Non rapid eye movement
OSAS	Obstructive sleep apnoea syndrome
QC	Quality control
PBMCs	Peripheral blood mononuclear cells
PSG	Polysomnography
PVN	Paraventricular nucleus
REM	Rapid eye movement
RIA	Radioimmunoassay
RT	Retention time
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SDB	Sleep-disordered breathing
SVAS	Supravalvular aortic stenosis
SWS	Slow-wave sleep
TD	Typically developing

TMB	Tetramethylbenzidine
TV	Television
UHPLC	Ultra-high performance liquid chromatograph
UHPLC-MS/MS	Ultra-high performance liquid chromatography-tandem mass spectrometry
UV	Ultra violet
WS	Williams syndrome
WSCR	Williams syndrome critical region

STATEMENT OF ORIGINALITY

My contribution to knowledge is the identification of the biomedical markers of sleep disturbances in Williams syndrome (WS) using saliva and urine samples; examination of associations between these markers and sleep parameters; as well as the development of a novel, fast ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for simultaneous analysis of endocrine markers of sleep, namely 6-sulfatoxymelatonin, cortisol and cortisone in urine. Studies were approved by Middlesex Natural Sciences Ethics sub-Committee (Approval Letter 524) and the Williams Syndrome Foundation, UK. All work was carried out independently.

THESIS OVERVIEW

The main aim of this work was to examine sleep problems observed in children with Williams syndrome (WS) in relation to secretion of the endocrine markers-melatonin and cortisol. The first chapter of the thesis focuses on sleep and circadian rhythm and includes an overview of WS as well as techniques used for studying sleep. The second part (Chapter 2) describes the methods used to study sleep behaviour at participants' homes, namely actigraphy and questionnaires. The results of this investigation are also presented (Chapter 3). The next part consists of description of laboratory methods used for analysis of biochemical markers of sleep, namely melatonin and cortisol (Chapter 4). This part is followed by the results section (Chapter 5). The final chapter of the thesis focuses on discussion, conclusions and possible future work (Chapter 6).

1. INTRODUCTION

This chapter provides an introduction of sleep and its functions in adults and typically developing children. Endocrine markers of sleep, in particular melatonin and cortisol are also outlined. Due to the fact that sleep research on children is still in its infancy, in particular in developmental disorders, it is then necessary to focus on adult studies rather than children. The second part of this chapter addresses studies on WS. The final part includes techniques used for the measurement of sleep as well as its endocrine markers.

1.1. Sleep physiology and circadian rhythm

The accepted view of sleep is generally defined as: *"Sleep has survived ubiquitously throughout all of mammalian evolution; some experiments have shown that animals cannot survive without sleep; and animals have made numerous behavioural and physiological accommodations to permit the survival of sleep in different habitats and life styles. Sleep persists in predators and prey; in carnivores and vegetarians; on the land and in the water (marine mammals); in most mammals as they lie down relaxed, in ruminants while they stand, in birds while they perch, and in dolphins which constantly swim; in hot and cold climates; in elephants and shrews; in sloths that hardly move and mice that hardly sit still; in the smartest and the dumbest of all mammalian species. These facts suggest a primary, essential, functional core to sleep…"* (Rechtschaffen, 1998)

1.1.1. Sleep

Sleep involves specific activity of the brain, controlled by elaborate and precise mechanisms (Hobson, 1995). Sleep is a physiological process controlled by two different biological clocks: the circadian rhythm – daily sleep- wake cycle, and the ultradian rhythm, which refer to processes with a period shorter than a 24h cycle (e.g. sleep stages) (Steriade & McCarley, 2005; Goldson & Reynolds, 2006). The circadian clock is a self- sustaining oscillator controlling many physiological and behavioral systems. The period of this oscillator lasts approximately 24 h (Albrecht, 2002).

1.1.1.1. Sleep stages

In adults as well as children, sleep can be divided into a sequence of five recurring stages of sleep (Loomis *et al.*, 1937) - four non-rapid eye movement stages (NREM) and a rapid eye movement stage (REM) (Hobson, 1995). After sleep onset, electroencephalography (EEG) recording changes progressively from a pattern of low voltage and high frequency to one of high voltage and low frequency. Thus, initially while falling asleep, the frequency spectrum of the EEG is shifted toward lower values and the amplitude of the cortical waves increases slightly. This period is called stage I sleep. In stage II, the frequency of EEG waves decreases and their amplitude increases (Loomis *et al.*, 1938; Purves *et al.*, 2008). In this stage, the first definitive EEG sign of sleep can be observed, and a complex wave sequences called sleep spindles are seen in the EEG recording (Hobson, 1995). Stage II is also characterised by reduced eye movements, respirations and heart rate. Stages III and

IV are the deepest non-REM stages (Goldson & Reynolds, 2006). In stage III sleep, the number of high- frequency spike clusters decreases, while the amplitude of low-frequency waves increases. The EEG activity of the stage IV sleep (slow- wave sleep, SWS) consists of low frequency (1–4 Hz), high-amplitude fluctuations called delta waves (Purves *et al.*, 2008). In the deepest stages of sleep the body is relaxed, breathing is slow and shallow, and the heart rate is low (Goldson & Reynolds, 2006).

In REM sleep, muscle tone is relaxed and the eyes move rapidly beneath the lids and the EEG recordings are highly similar to that of the awake state as the brain cycles back to the non-REM sleep stages (Purves *et al.*, 2008). It is this phase of sleep when dreams occur (Hobson, 1995) (Figure.1.1).

Thus, the full adult sleep cycle includes four NREM sleep stages and REM sleep and lasts approximately 90 to 100 min (Hobson, 1995). The time of one sleep cycle is relatively constant for the same person and for individuals of the same age. However, the proportion of REM sleep in each cycle is variable. This proportion is usually quite small in the first cycle, but increases through the night. Thus, half of the total REM sleep occurs in the last third of the night. The NREM sleep has an opposite distribution, occurring more in the first and second cycles than later during the sleeping period (Hobson, 1995).

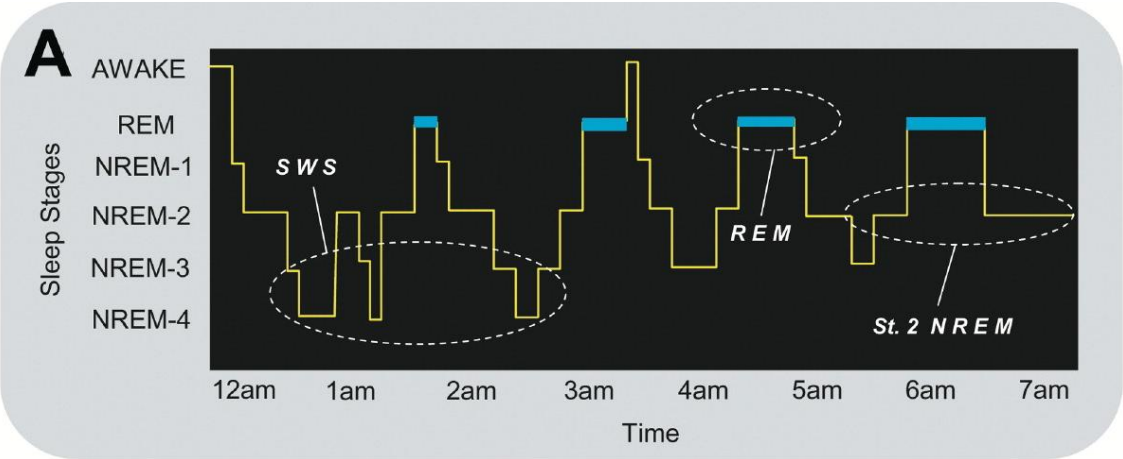


Figure 1.1. Adult sleep stage changes through the night (adapted from Walker & Stickgold, 2004). Figure demonstrate distribution of particular sleep stages during the night time sleep. It can be seen that the slow wave sleep (SWS) occurs during the first half of sleep time, while the proportion of REM sleep increases with the sleep time. NREM-1 to NREM-4 indicates four stages of non-rapid eye movement sleep.

During sleep cycles aminergic and cholinergic neurons secreting specific neurotransmitters play an important role. The aminergic neurons secrete serotonin and noradrenaline, while acetylocholine is secreted by cholinergic neurons. In contrast to action of serotonin or noradrenaline which is often inhibitory, acetylocholine is excitatory. In 1966, Jouvet proposed that the brainstem exerted its control of NREM sleep via serotonin and of REM sleep via noradrenaline (Jouvet, 1966). Taking into account this finding, Hobson and colleagues (1975), using the microelectrode technique, found that group of cells (cholinergic) were turned on and others (noradrenergic and serotonergic) turned off during REM sleep. The REM-on cells did not release serotonin or noradrenaline but possibly released acetylocholine. This hypothesis was called the reciprocal interaction model: when the aminergic (REM-off) cells were on (in waking), the cholinergic (REM-on) cells were inhibited and *vice versa*. In support of this hypothesis, McCarley and Hobson (1975) reported that the induction of REM sleep is possible either by increasing cholinergic excitation or reducing the aminergic inhibition of REM-generating neurons. Thus, in

summary, the brainstem contains two populations of neurons: one population becomes active during REM sleep (REM-on cells-cholinergic and excitatory) (Marrosu *et al.*, 1995; Lee *et al.*, 2003), while the other becomes inactive (REM-off cells-noradrenergic, serotonergic and inhibitory) (Portas *et al.*, 1998).

1.1.1.2. Sleep functions

Sleep is essential for optimal functioning in humans. Sleep functions include energy conservation (Sheldon, 2005a), body restoration and recovery (Adam & Oswald, 1997), and learning and memory consolidation in both, adults and children (Born *et al.*, 2006). Moreover it has a very important role in foetal development and structural development of brain in early life (Hobson, 1995). It has been reported that prolonged sleep deprivation in rodents, drosophila and possibly humans is fatal, which supports a "life sustaining" function of sleep (Rechtschaffen 1998; Cortelli *et al.* 1999; Shaw *et al.* 2002).

One of the functions of sleep is to conserve energy. Humans consume less energy when during sleep. Muscular tension is reduced, heart rate is lowered, blood pressure and body temperature are reduced, and respiration is slower especially in SWS. The metabolic rate during sleep is reduced by 8-10% compared to relaxed wakefulness (Rosenzweig *et al.*, 2005; Sheldon, 2005b). It has been reported that function of NREM sleep is restoration of body tissue and REM sleep of brain tissue (Adam & Oswald, 1997). The timing of growth hormone (GH) secretion which occurs during SWS also supports these findings (Takahashi *et al.*, 1968). Another important function of sleep is its role in learning and memory consolidation (Born *et al.*, 2006).

Walker and Stickgold (2004) in their review on sleep depended learning and memory consolidation, reported that the amount of REM sleep increases after training. Also, deprivation of REM, SWS or stage 2 NREM decreases subsequent memory performance and the overnight improvement correlates with these stages of sleep (Walker & Stickgold, 2004).

1.1.1.3. Neurophysiological changes during sleep

Several physiological changes can be observed during sleep and these include heart rate fluctuations, changes in respiratory rate and muscle tone. See Table 1.1. for detailed physiological functions amongst others during SWS and REM sleep.

Property	**Slow-wave sleep**	**REM sleep**
AUTONOMIC ACTIVITIES		
Heart rate	Slow decline	Variable with high bursts
Respiration	Slow decline	Variable with high bursts
Thermoregulation	Maintained	Impaired
Brain temperature	Decreased	Increased
Cerebral blood flow	Reduced	High
SKELETAL MUSCULAR SYSTEM		
Postural tension	Progressively reduced	Eliminated
Knee jerk reflex	Normal	Suppressed
Phasic twitches	Reduced	Increased
Eye movements	Infrequent, slow, uncoordinated	Rapid, coordinated
COGNITIVE STATE	Vague thoughts	Vivid dreams, well organized

HORMONE SECRETION		
Growth hormone secretion	High	Low
NEURAL FIRING RATES		
Cerebral cortex (sustained) activity	Many cells reduced and more phasic	Increased firing rates; tonic
EVENT-RELATED POTENTIALS		
Sensory-evoked	Large	Reduced

Table 1.1. Properties of slow wave sleep (SWS) and rapid eye movement (REM) sleep (Rosenzweig *et al.*, 2005). Table presents changes in autonomic activities, skeletal muscular system, cognitive state, hormone secretion, natural firing rates and event-related potentials between SWS and REM sleep.

1.1.1.4. Factors modifying/influencing sleep

There are several factors which modify sleep and one of those is chronological age (Figure 1.2.). The developmental characteristics of sleep are described in Section 1.1.3. SWS stage decreases through life whereas sleep arousals tend to increase with age. In contrast to adults, infants enter sleep directly through REM sleep before NREM (Pegg, 2006). REM latency increases and the percentage of time spent in REM or active sleep gradually decreases throughout infancy and childhood to 20-25% in adulthood (Kahn *et al.*, 1996; Montgomery-Downs *et al.*, 2006).

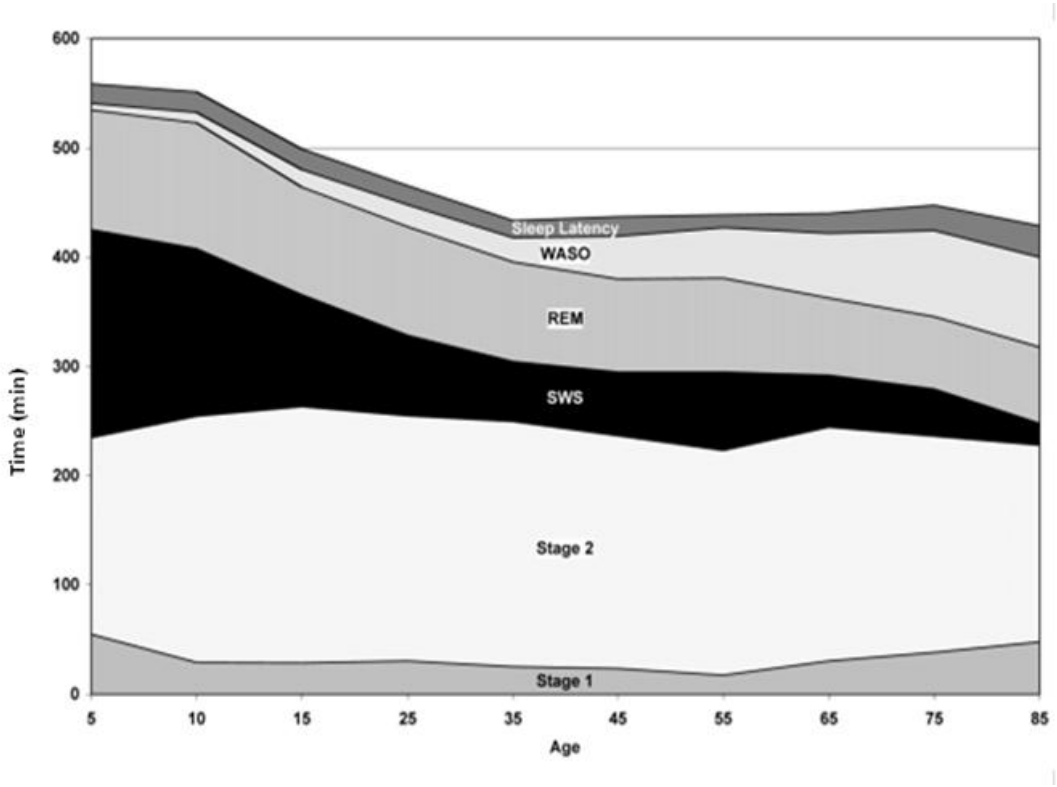


Figure 1.2. Age-related changes of sleep stages. Figure presents changes in time (minutes) for sleep latency, wake time after sleep onset (WASO), rapid eye movement (REM) sleep, slow wave sleep (SWS) and non-rapid eye movement sleep (NREM) sleep from 5 to 85 years of age (adapted from Ohayon *et al.*, 2004). It can be seen that time of SWS stage decreases through life whereas the occurrence of wake after sleep onset increases with age.

Studies on adult sleep have shown that prior sleep history may also affect sleep, as it controls the homeostatic need for sleep (Franken, 2013). Also, an individual who has experienced sleep loss on one or more nights will show a sleep pattern that favors SWS during recovery in first nights, whereas REM sleep tends to recover on the second or subsequent recovery nights (Carskadon & Dement, 2005). The distribution of sleep stages can also be affected by circadian phase at which sleep occurs. It can be observed in the case of shift change working or travelling across many time zones. Thus, for instance, if sleep is delayed to early morning, when REM sleep has its peak, then REM may occur at the onset of sleep and will predominate in the entire sleep cycle (Carskadon & Dement, 2005).

Temperature is another factor that affects sleep, an environment that is either too hot or cold can cause sleep disruptions (Jhaveri *et al.*, 2007). Bed-sharing may also affect sleep. It has been reported that bed-sharing children have less regular bed times, difficulty with sleep onset, more night-waking and seek out the parents following awakening during the night (Hayes *et al.*, 2001). Another environmental factor affecting sleep is exposure to television (TV) prior to going to bed. Paavonen and colleagues (2006) examined 5-6 years old children (n=321) and reported that active TV viewing as well as passive TV exposure, particularly viewing adult-targeted TV programs were strongly related to sleep disturbances in children. Similar findings were reported by BaHammam *et al.* (2006), showing significantly shorter sleep duration in elementary school age children (5-12 years old, n=1012) watching TV after 8:00 pm. Traffic noise at night may also cause sleep disturbances. The exposure-effect relationship between road traffic noise and sleep quality was found in both adults and children (Öhrström *et al.*, 2006).

Sleep can also be affected by drug and alcohol ingestion, as studies on adult sleep have shown. Several drugs, such as benzodiazepine suppress SWS, tricyclic antidepressants or some selective serotonin reuptake inhibitors suppress REM and can disrupt the distribution of sleep stages. Alcohol can also increase SWS and decrease REM early in the night and chronic ingestion of tetrahydrocannibol (marijuana) produces long term suppression of SWS (Freemon, 1982).

1.1.2. Clinical sleep disorders

A distinction can be drawn here between sleep problems and sleep disorders. Sleep problems are often minor, transient annoyances that everyone experiences from time to time. It is important to note that sleep problems may be culturally defined and are often reported by parents when there is a mismatch between the child's behaviour and the parents' expectations, or when the child's behaviour causes significant disruption to the parents' sleep (Mindell & Owens, 2003). In contrast, sleep disorders are defined by the International Classification of Sleep Disorders (American Academy of Sleep Medicine, 2005) which tend to be more serious and may interfere with daily life. Eight categories of sleep disorders are defined: insomnias, sleep-related breathing disorders, hypersomnias of central origin, circadian rhythm disorders, parasomnias, sleep-related movement disorders, isolated symptoms, and other sleep disorders. Some of the most common childhood disorders will be discussed here in more detail.

1.1.2.1. Insomnia

The most commonly experienced sleep disorder is insomnia. It has been reported that the behavioural insomnia affects 10-30% of the child population (American Academy of Sleep Medicine, 2005). Individuals suffering from this disorder have difficulty initiating or maintaining sleep (Hobson, 1995; Wilson & Nutt, 2005). Most affected individuals present with one or more of the three forms of insomnia: difficulty with falling asleep, difficulty getting back to sleep during the night, or inability to return to sleep, even for a short while, after waking during the night.

Episodes of insomnia can last several days or weeks, or develop chronic condition lasting months, years or even decades. The causes of insomnia include many common life events such as noisy neighbours, stressful family or work events, depression, serious health problems and conditioned alertness (Rothenberg, 1997). Insomnia is associated with feelings of restlessness, irritability, anxiety, daytime fatigue and tiredness (American Academy of Sleep Medicine, 2001). The most suitable treatment for insomnia is dependant on an accurate assessment of its aetiology (Gillin & Byerley, 1990).

Children can suffer from behavioural insomnia, which is characterised by bedtime struggles, trouble settling down and difficulty initiating and/or maintaining sleep. The child may refuse and resists going to bed and staying in bed. He/she will be unable to self-soothe to sleep and will awaken repeatedly throughout the night requiring parental attention, and/or require special conditions to fall asleep, such as certain toys, blankets, music, or the parent being in the room. This is generally accompanied by distress and impaired daytime functioning (Hill, 2011).

1.1.2.2. Parasomnias

Parasomnias are sleep disorders characterised by the occurrence of the diverse set of behavioural and physiological events, which intrude sleep. Parasomnias are manifestations of central nervous system (CNS) activation and may involve respiratory system, motor system or the cognitive system (Hobson, 1995; Rothenberg, 1997; American Academy of Sleep Medicine, 2001). These disorders are divided into four groups: arousal disorders (confusional arousals, sleep walking, sleep terror), sleep-wake transition disorders (sleep talking, night leg cramps),

parasomnias usually associated with REM sleep (nightmares, sleep paralysis) and other parasomnias (i.e. bruxism, sleep enuresis) (American Academy of Sleep Medicine, 2001). As parasomnias are reflection of CNS immaturity, they are more common in children than in adults and are generally outgrown with time (Thiedke, 2001). It has been also found that parasomnias are associated with a higher prevalence of other sleep disturbances in children (n=480) aged 6 to 11 years (Goodwin *et al.*, 2004). The authors also demonstrated that pre-adolescent school-aged children with sleep-disordered breathing (SDB) experienced more parasomnias than those without SDB.

1.1.2.3. Hypersomnia

Hypersomnia has been described as uncontrollable and pathologically deep and prolonged sleep in both, children and adults (Kleitman, 1963). The most common hypersomnia is narcolepsy. It is an autosomal dominant genetic disorder of the neural control mechanism that regulate sleep and waking (Aldrich, 1992; Rothenberg, 1997). It is genetically complex disorder. Studies suggest that it results from a combination of genetic and environmental factors. The tight association was found between human leukocyte antigen (HLA) allele *DQB1*0602* as well as *DRB1*1501* (Chabas *et al.*, 2003; Migawa *et al.*, 2008) and the narcolepsy, particularly in people who also have cataplexy and loss of hypocretins. The HLA complex helps the immune system distinguish the body's own proteins from proteins made by foreign invaders (such as viruses), thus, it has been suggest that narcolepsy has an autoimmune aetiology. Furthermore, it was also found that polymorphisms in

two immune related genes- *TCRA* and *P2RY11* are associated with narcolepsy (Han *et al.*, 2012).

Narcolepsy is characterised by irresistible sleepiness and repeated episodes of naps and lapses into sleep of short duration (usually less than 1 hour) (Hobson, 1995; American Academy of Sleep Medicine, 2001). The excessive sleepiness observed in this disorder is associated with cataplexy, hypnagogic or hypnopompic hallucinations and sleep paralysis- so called narcoleptic tetrad. An affected individual may demonstrate only one of the symptoms or the full syndrome. Cataplexy is a sudden decrease in muscle tone while awake, which varies from mild weakness to inability to move. Hypnagogic and hypnopompic hallucinations are vivid visual images or sounds, which occur while the narcoleptic patient is awake, thus may be misperceived as real. Sleep paralysis occur while the person is awakening and for a brief period of time cannot move or speak due to the fact that the normal inhibition of muscle tone during REM sleep fails to end. Each of the symptoms from narcoleptic tetrad is caused by the failure of the neural mechanisms which keep REM state separate from the wake state (Rothenberg, 1997).

1.1.2.4. Sleep-Disordered Breathing (SDB)

SDB is a disorder varying from mildly disordered to severely disordered sleep (Strollo & Rogers, 1996). A partial decrease in airflow for 10 seconds or more is called hypopnoea, while complete cessation of airflow for the same period of time – apnoea. These events are called obstructive when the muscles and soft tissue of the throat collapse and impede breathing. Obstructive apnoea and hypopnea are the most

common sleep disorders affecting breathing (Rothenberg, 1997). Obstructive sleep apnoea syndrome (OSAS) is characterised by repetitive episodes of upper airway obstruction occurring during sleep and usually associated with a reduction in blood oxygen saturation. This syndrome is associated with characteristic snoring pattern, consisting of loud snores and brief gasps that alternate with the episodes of silence (American Academy of Sleep Medicine, 2001). The key feature of this disorder is excessive daytime sleepiness observed in affected individuals (Rothenberg, 1997). Prevalence of SDB estimates vary depending on the type of diagnostic tools used, but it is likely that snoring occurs in around 11% of children aged 4 to 11 years (Ali *et al.*, 1993; Ali *et al.*, 1994; Goodwin *et al.*, 2003; Gozal, 2008), declining to around 6% in adolescence (Johnson & Roth, 2006). OSAS is thought to affect around 1 to 3% of children (Brunetti, 2001; Sogut *et al.*, 2005).

1.1.2.5. Restless Leg Syndrome

Restless leg syndrome is a sleep disorder characterised by unpleasant leg sensations usually between knees and ankles that cause an almost irresistible urge to move legs (Ekbom, 1960; American Academy of Sleep Medicine, 2001). This disagreeable feeling can be temporarily relieved by movement. The prevalence of restless leg syndrome is estimated to be around 2% in children and 5-10% in adults (Allen *et al.*, 2005; Picchietti *et al.*, 2007). This prevalence is much higher in children with Attention Deficit Hyperactivity Disorder (ADHD). It has been reported that up to 44% of subjects with ADHD have been found to have restless leg syndrome, while up to 26% of subjects with restless leg syndrome have been found to have ADHD or ADHD symptoms (Cortese *et al.*, 2005). The severity of this disorder range from

merely annoying to severely affecting sleep and quality of life (Allen *et al.*, 2005). Affected individuals have difficulties falling asleep or falling back to sleep during the night. The aetiology of this disorder is unknown, however, dopamine agonist are effective in reducing its symptoms (Rothenberg, 1997).

1.1.2.6. Periodic Limb Movement

Periodic limb movement is another common sleep disorder of unknown aetiology. It is characterised by periodic episodes of repetitive limb movements occurring at 20- to 40-second intervals or continually throughout the night (Rothenberg, 1997; American Academy of Sleep Medicine, 2001). This disorder becomes increasingly common with age (Ancoli-Israel *et al.*, 1991). The prevalence of periodic limb movement syndrome is estimated to be 4-11% in adults (Hornyak *et al.*, 2006) and according to different reports 1.2-11% in children (Crabtree *et al.*, 2003; Kirk & Bohn, 2004). Medical conditions, such as sleep apnoea syndrome or neuropsychiatric disorders may however lead to high rates of periodic limb movement syndrome observed in children (Hornyak *et al.*, 2006). The most common symptoms of this disorder, ranging from mild to severe, include difficulty falling asleep and maintaining sleep, fragmented sleep architecture, daytime sleepiness and frequent EEG arousals. Moreover, these syndromes tend to increase with stress. As with restless leg syndrome, the aetiology of this disorder is unknown, however, benzodiazepines and dopaminergic agents have been shown to suppress muscle contractions and regulate muscle movements during sleep (Spriggs, 2010).

1.1.2.7. Daytime sleepiness

Excessive sleepiness among children and adolescents has been identified as a major societal concern (Fallone *et al.*, 2002). It has been suggested that children with increased levels of daytime sleepiness are likely to experience impairments in behavioural, mood and performance domains (Fallone *et al.*, 2002). Unlike other sleep disorders described in the current section (Section 1.1.2), excessive daytime sleepiness is a secondary effect of various sleep-related problems and in infants and children may occur as a result of disorders leading to a sufficient loss of night-time sleep, such as insomnia, or persistent night waking (Stores, 2001; Kryger, 2005). Second most common cause of daytime sleepiness are breathing-related sleep disorders (i.e. sleep apnoea), because respiratory difficulties cause frequent arousals and awakening from sleep. In teenagers, daytime sleepiness may result from circadian rhythm disorders, sleep apnoea as well as narcolepsy. Some major psychiatric disorders, such as depression, as well as drugs used to treat them can also cause daytime sleepiness (Stores, 2001; Kryger, 2005). Moreover, the associations of excessive daytime sleepiness with obesity and anxiety have also been reported in school-aged children (Calhoun *et al.*, 2011).

1.1.3. Sleep in children

1.1.3.1. Sleep in typically developing children

Sleep and circadian rhythms are different in newborns than in older infants and children. Circadian rhythm is not fully established at birth, thus sleep can occur at any time of the day and night. The infants' pattern of sleep and wakefulness is irregular and is often coordinated by feeding times (Adair & Bauchner, 1993). Infants and young children spend majority of their time asleep, hence indicating that sleep is essential for a developing brain and body as well as maintaining optimal health (Fin Davis *et al.*, 2004). With an increased developmental age in infancy, awake periods during the day become longer, daytime sleep organise into naps and sleep starts to consolidate during night hours (Anders *et al.*, 1995). In general, infants older than 12 months show a dramatic change in sleep patterns. Total daily sleep tends to decrease to about 13 hours by age of 2 years, 12 hours by 3 to 4 years old and 11 hours by 5 years old. However, there is still considerable variability (Roffwarg *et al.*, 1966). There is less remarked and more gradual decrease in nocturnal sleep which continues until late adolescence (Iglowstein *et al.*, 2003). The gradual shift to later bedtime and sleep onset time that begins in middle childhood and accelerates in early to mid adolescence has also been reported (Owens, 2005; Ashworth *et al.*, 2013). The characteristic of particular stages of sleep in children is the same as those observed in adults (see Section 1.1.1) (Fin Davis *et al.*, 2004), however, the distribution of sleep stages differs. In the first year of life sleep starts from REM sleep (called active sleep in newborns) and the cycle is shorter than in adults, lasting approximately 50 to 60 min. Sleep stages develop as the brain matures

during first year of life. Active sleep constitutes approximately 50% of total sleep at birth and declines from infancy and childhood to 20-25% in adulthood (Kahn *et al.*, 1996; Montgomery-Downs *et al.*, 2006).

It has been reported that approximately 25% of all children experience some type of sleep problems at some point during their childhood (Owens, 2005). The type, relative prevalence, chronicity and severity of sleep problems can be affected by the number of variables associated with the child and/or parents as well as environmental variables. Child variables affecting sleep include temperament and behavioural style, individual variations in circadian preference, cognitive and language delays and the presence of coexisting medical and psychiatric conditions. Parental variables that impact sleep include parenting and discipline styles, parents' level of education and knowledge of child development, quality and quantity of sleep of parents and all family members as well as family stress. Finally, environmental variables include the physical environment (space, noise, room and bed-sharing), family composition (i.e. number, ages) and lifestyle issues (i.e. parental work status). A number of studies have examined the prevalence of parent- and child- reported sleep complaints in children and adolescents. Sleep problems observed in childhood range from short-term difficulties in falling asleep and night waking to more serious sleep disorders such as obstructive sleep apnoea (Owens, 2005). It has been reported that prevalence of parent-defined sleep problems in children 4 to 10 years of age ranges from 3.7% (sleep disordered breathing) to 15.1% (bedtime resistance), with 37% of the overall sample having significant sleep problems in at least one sleep domain (such as sleep anxiety, night waking, bedtime resistance) (Owens *et al.*, 2000b). Another study of over 14,000 school-aged children reported sleep problems in 20% of 5 year olds and 6% of 11 year olds (Rona *et al.*, 1998). It is however

important to note that parental reports may over- or underestimate the prevalence of sleep problems in their children, as the very definition of a sleep problem is often highly subjective (Owens *et al.*, 2000b).

1.1.3.2. Sleep in children with developmental disabilities

Sleep problems are far more common in children and adolescents with chronic medical or psychiatric conditions (Owens, 2005). It has been estimated that 30% to 80% of children with severe learning difficulties and at least half of children with less severe cognitive impairment are affected by significant sleep disturbances (Stores & Wiggs, 2003). Significant sleep disturbances have been reported in neurodevelopmental disabilities such as Asperger's syndrome (Allik *et al.*, 2006), and Attention Deficit Hyperactivity Disorder (ADHD) (Corkum *et al.*, 2001; Konofal *et al.*, 2001; Scott *et al.*, 2013); as well as in the syndromes of genetic aetiology- Smith-Magenis syndrome (Potocki *et al.*, 2000) and Williams syndrome (WS) (Bódizs *et al.*, 2009; Goldman *et al.*, 2009; Mason *et al.*, 2009; Annaz et *al.*, 2011; Ashworth *et al.*, 2013). These include sleep problems with initiation and maintenance of sleep, reduced length of sleep, irregular sleeping patterns and early morning waking (Owens, 2005).

Sleep disturbances have been widely described in children with Autism Spectrum Disorder (ASD) which is characterised by impairment in communication, social interaction and difficulty to adapt to novel experiences (American Psychiatric Association, 1994). The prevalence of sleep problems in this population is estimated to be in range 44-83% (Richdale, 1999) and increases with the severity of the autistic spectrum disorder (Mayes & Calhoun, 2009). The most frequent sleep problems

observed in this group are difficulty falling asleep, restlessness during sleep (Mayes & Calhoun, 2009), early morning awakening and multiple night arousals (Hering *et al.*, 1999) as well as frequent night waking (Williams *et al.*, 2004). Another developmental disorder affected by frequent sleep disturbances is ADHD. This disorder is one of the most prevalent child psychiatric conditions affecting 3-7.5% school-aged children. It is characterised by impulsivity/ hyperactivity and inattention (American Psychiatric Association, 1994; Centers for Disease Control and Prevention, 2001). Parents of children with ADHD commonly report disturbed and restless sleep in their children (Corkum *et al.*, 2001). Sleep problems observed in this population include high level of nocturnal activity (Konofal *et al.*, 2001), decreased sleep time (Scott *et al.*, 2013), restless leg syndrome (Cortese *et al.*, 2005), increased bedtime resistance (Corkum *et al.*, 2001), significantly shorter REM sleep and higher scores on insufficient sleep and sleep anxiety factors than children in the control group (Gruber *et al.*, 2009).

Sleep disorders are also common in other developmental disorders that have genetic aetiology, such as Down's syndrome (DS) and Prader-Willi syndrome. Cotton and Richdale (2006) reported the sleep maintenance problems in DS and excessive daytime sleepiness in children with Prader-Willi syndrome. The study of Turner and Sloper (1996) also revealed that as much as 43% of children with DS aged 7 to 14 years have problems settling at bedtime, 51% awaken at night and 38% suffer from night bed-wetting. Similar results were found by Carter *et al.* (2009) who reported increased bedtime resistance, sleep anxiety, night waking, parasomnias, SDB and daytime sleepiness in children with DS. Recently children with WS have been identified with sleep problems and are of interest in the current thesis (see details in Section 1.3.4.).

1.1.4. Circadian rhythm

Internal clocks that control biological rhythms are called circadian, from the Latin *circa* (about) and *dies* (day). These clocks have periods of about 24 h (Hobson, 1995). The circadian rhythm is generated in the suprachiasmatic nucleus (SCN) of the hypothalamus (Figure 1.3.), it regulates the timing and amplitude of several endocrinological functions, such as secretion of growth hormone, thyroid-stimulating hormone and prolactin, via neural or neurohumoral circuits (Zeitzer *et al.*, 2000). The endogenous clock located in the SCN generates other circadian rhythms in mammals as well, such as drinking, feeding, sleep-wake cycle, and temperature (Claustrat *et al.*, 2005).

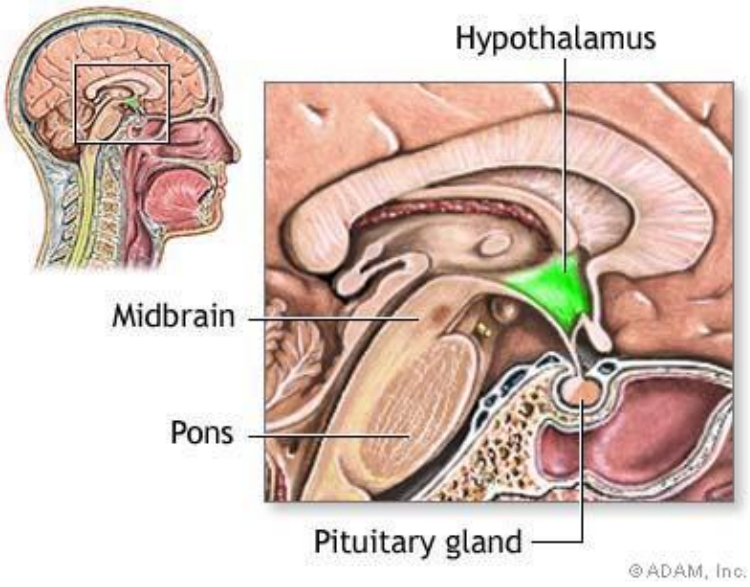


Figure 1.3. Location of the hypothalamus in human brain (The New York Times health Guide- Narcolepsy).

Markers of the circadian rhythm are the wake/sleep cycle, rest/activity cycle, temperature and hormones such as melatonin, cortisol and GH secretion as well as clock genes activity (Haffen, 2009) (Figure 1.4.).

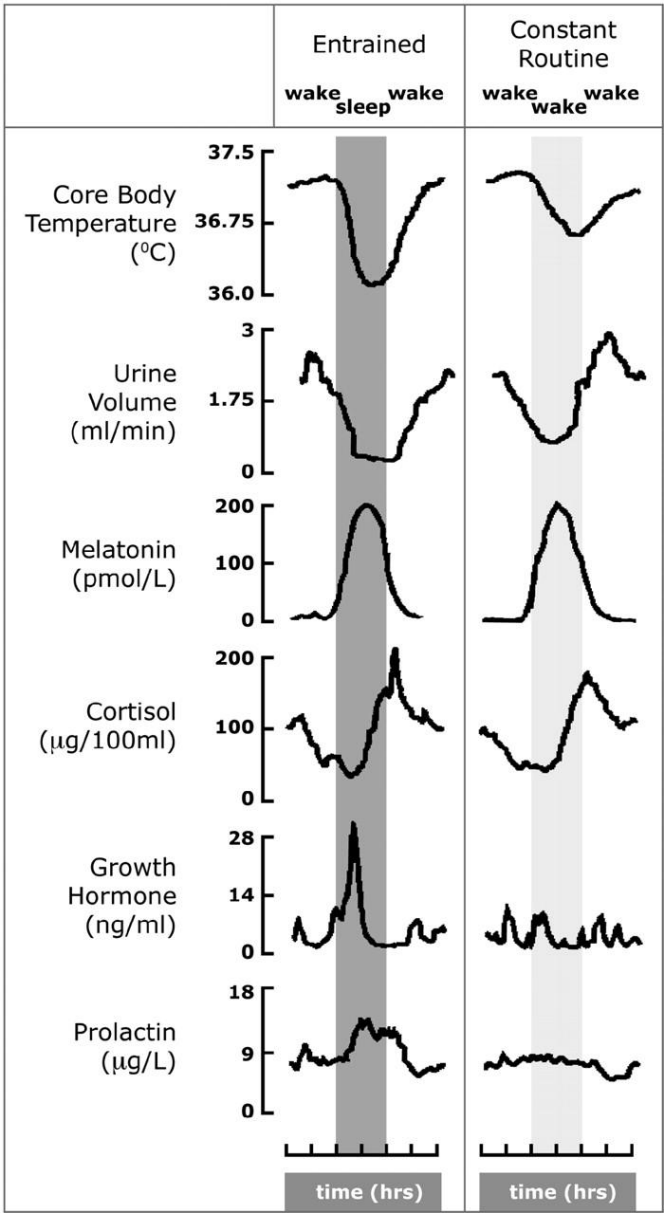


Figure 1.4. The biological rhythms controlled by circadian system. Figure presents daily cycles of physiology (core body temperature and urine volume) and hormone levels (melatonin, cortisol, growth hormone and prolactin) in human subjects with regular sleep/wake cycles entrained to light/dark cycles (on the left) as well as exposed to a constant routine (on the right). Progressive changes in physiological and endocrine status can be observed across day and night under normal sleep/wake routine. When exposed to constant routine in continuing wakefulness, physiological rhythms are maintained with slightly reduced amplitude. Endocrine rhythms of melatonin and cortisol secretion proved to be strongly circadian, while rhythm of growth hormone and prolactin are diminished due to their strict dependence on sleep (Czeisler & Klerman, 1999; Maywood *et al.*, 2007).

Sleep has a modulatory effect on many components of the endocrine system, and reciprocally, hormones such as melatonin and cortisol affect sleep architecture and display circadian rhythmicity. The diurnal pattern of melatonin and cortisol secretion proved to be independent of sleep. Furthermore, these hormones can also affect human daily cycle including sleep (Steiger, 2002; Reiter *et al.*, 2010). Melatonin and cortisol tend to run opposite to each other, cortisol approaches its low point when melatonin reaches its peak (Herman, 2005).

1.1.5. Melatonin

Melatonin, also known chemically as *N*-acetyl-5-methoxytryptamine, was first isolated from bovine pineal gland by Aaron Lerner (1958). It is produced mostly in pineal gland (Arendt, 2005) but also by the retina, lens, gastrointestinal tract, bone marrow and other tissues. It is also the main hormone secreted by pineal gland (Claustrat *et al.*, 2005).

1.1.5.1. Functions of melatonin

Melatonin decreases core temperature which plays a role in the onset and duration of sleep (Strassmann *et al.*, 1991; Arendt, 2005). Melatonin receptors can be found in peripheral blood vessels, thus the decrease of central temperature may be the result of peripheral vasolidation due to melatonin receptor stimulation (Van Der Helm-van Mil *et al.*, 2003).

Melatonin displays antioxidative properties. It scavenges the oxygen centered radicals including highly toxic hydroxyl radical. It also demonstrates activity on several antioxidative enzymes (such as superoxide dismutase, glutathione peroxidase and glutathione reductase) by increasing their levels and inhibits pro-oxidative enzyme nitric oxide synthase (Claustrat *et al.*, 2005; Berra & Rizzo, 2009).

Melatonin also displays possible activity on the immune system, by scavenging NO or free radicals in lymphoid cells (Claustrat *et al.*, 2005). Melatonin receptors were found in several lymphoid organs and in lymphocytes (Macchi & Bruce, 2004). Based on these findings, Poon and Pang (1996) suggested possible mechanisms of melatonin action on the immune system, including stimulation of opioid peptides by T lymphocytes and lymphokines, and inhibition of the immunorepressive action of corticosteroids.

Melatonin is a naturally occurring oncostatic neurohormone for the prevention of neoplastic growth. Numerous studies demonstrate its anti-tumor activity, including antiproliferative actions, immunostimulatory effects on host anticancer defences and antioxidant activity (Hill *et al.*, 1992; Lissoni *et al.*, 2003).

Melatonin has also a possible effect on circadian rhythm of blood pressure. It has been reported that administration of melatonin for 3 weeks in patients with essential hypertension reduces blood pressure without alternation of heart rate (Scheer *et al.*, 2004).

1.1.5.2. The circadian regulation of melatonin synthesis

The synthesis and release of melatonin by the pineal gland is under the influence of the suprachiasmatic nuclei of the hypothalamus which receives information from the retina about the daily pattern of light and darkness (Ganong, 2005). Neuronal pathways from SCN distribute circadian rhythm information to different areas of the brain, including the pineal gland (Cardinali & Pevet, 1998). The main neurotransmitter regulating the pineal gland is noradrenaline, which is released at night in response to stimulatory signals (photic information from retina) originating in the SCN (Cagnacci, 1996) (Figure 1.5.). The release of melatonin is entrained to the dark period. The photic information is transmitted to the central pacemaker via retino-hypothalamic fibers (Isobe *et al.*, 2001; Wilkinson, 2008), which originates from a small subset of retinal ganglion cells (Meijer & Rietveld, 1989). In the presence of light, the output from the retino-hypothalamic tract inhibits melatonin synthesis. Artificial light of sufficient intensity can also suppress melatonin production at night. This suppression is dose-dependent and varies with the spectral characteristics of the light stimulus (Lewy *et al.*, 1980).

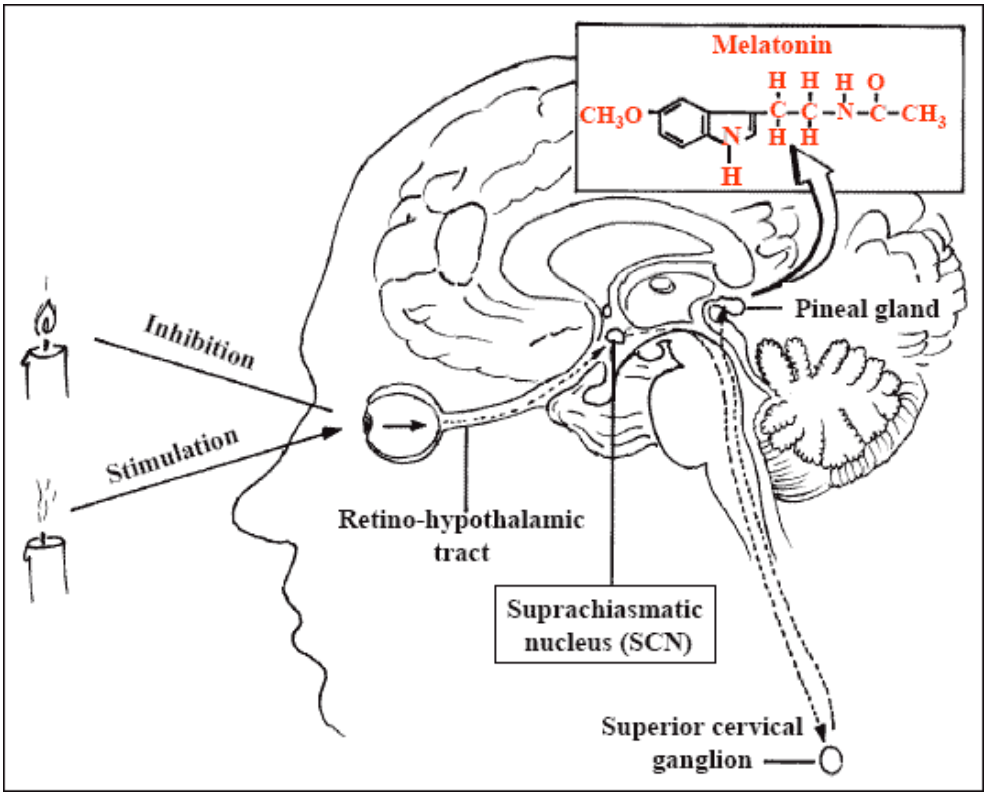


Figure 1.5. Schematic presentation of the stimulation (darkness) and inhibition (light) of melatonin secretion from pineal gland involving the neural pathway originating in retina and passing through suprachiasmatic nucleus (SCN), the main "circadian oscillator" in the brain, to reach pinealocytes via adrenergic nerves and adrenergic receptors at the cell membrane (Konturek *et al.*, 2007).

1.1.5.3. Biosynthesis of melatonin

Melatonin is synthesised indirectly from L-tryptophan taken from the blood by pinealocytes. Hydroxylation and decarboxylation of this amino acid gives serotonin. The latter is then converted to N-acetyl-5-hydroxytryptamine (N-acetyl-serotonin) by serotonin-N-acetyl transferase (arylalkylamine N-acetyl transferase- NAT), which in turn is methylated to form melatonin by way of a reaction catalysed by hydroxyindole-O-methyl transferase (Macchi & Bruce, 2004; Vela-Bueno *et al.*,

2007) (Figure 1.6.) The mRNAs encoding these enzymes are expressed with a day/night rhythm in the pineal gland (Bernard *et al.*, 1999).

The initiation of melatonin synthesis is triggered by the binding of noradrenaline to β-1 adrenergic receptors, which leads to the activation of pineal adenylate cyclase, increase in cyclic AMP (cAMP) and *de novo* synthesis of NAT or its activation (Figure 1.6.). The mechanism that limits the production of melatonin at night is represented by the cAMP-induced gene transcription repressor, which is activated in conjunction with NAT ((Stehle *et al.*, 1993).

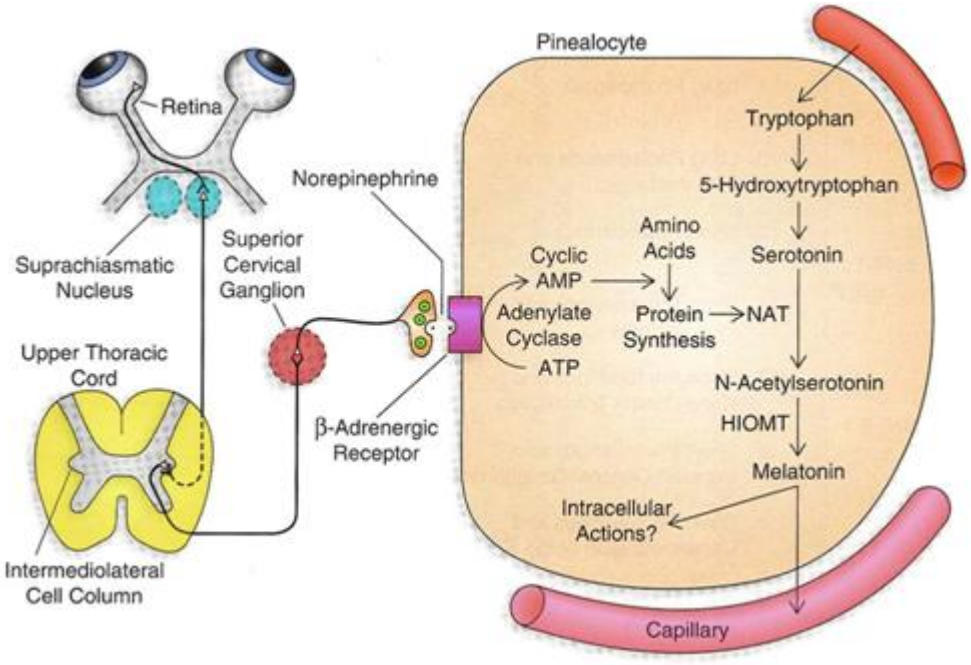


Figure 1.6. Central nervous system pathway and mechanism controlling melatonin secretion in mammals. The suprachiasmatic nuclei (SCN) of the hypothalamus which receives information from the retina about the daily pattern of light and darkness. The information from SCN goes through upper thoracic cord and superior cervical ganglion to pineal gland. The main neurotransmitter regulating the pineal gland is noradrenaline. Melatonin is synthesised indirectly from tryptophan taken from the blood by pinealocytes (cell of pineal gland) and then released into capillaries (Trinity University, http://www.trinity.edu).

Melatonin levels begin to increase before sleep, reach a maximum in plasma around 3.00–4.00 am (Claustrat *et al.*, 2005) and decrease before habitual wake up time to keep at a low level during the day (Ganong, 2005). Peak values of salivary melatonin range between 2-84 pg/ml in adults (Burgess & Fogg, 2008). Melatonin enters the saliva mainly via passive diffusion through the cells of the salivary glands, thus the saliva flow rate does not affect melatonin levels (Miles *et al.*, 1989; Radon *et al.*, 2001). In the passive diffusion, the transport of the analyte from plasma to saliva is related to the plasma/saliva concentration gradient (Khurana, 2006). The hormone moves from a region of higher concentration to one of lower concentration and the cell does not expend energy. Thus, melatonin diffuses readily through the lipo-protein membranes of the secretory cells in the salivary glands (Kaufman & Lamster, 2002). In addition, as melatonin is bound to albumin in serum, its salivary levels represent the free hormone levels. This provides clinically useful information, since free serum melatonin levels are the biologically active fraction of this hormone in serum (Kaufman & Lamster, 2002). The saliva level of melatonin is parallel to that observed in plasma, and is approximately three fold lower (McIntyre *et al.*, 1987; Voultsios *et al.*, 1997; Burgess & Fogg, 2008). The ability to synthesize melatonin differs among individuals. There are low and high secretors of melatonin. Normal daytime serum levels in healthy adults range from 0-20 pg/ml, whereas the night levels, when melatonin reaches its peak, vary between 50-200 pg/ml (Wetterberg, 1978; Waldhauser & Dietzel, 1985). However, within the same individual melatonin rhythm and its amplitude remains constant representing one of the most robust circadian rhythms (Arato, 1985; Bojkowski *et al.*, 1987). It should be noted that levels of melatonin change with age (Figure 1.7). The circadian rhythm of melatonin appears at the end of the neonatal period (Ardura *et al.* 2003) and melatonin reaches

highest nocturnal levels between 1 to 3 years of age (Waldhauser *et al.*, 1993). During childhood melatonin levels drop by approximately 80% until adult levels are reached (Figure 1.7.). Kennaway and collegues (1999) have shown that changes in melatonin with age were essentially complete before the age of 30 and further analyses of melatonin levels in 5-year age spans did not show any significance.

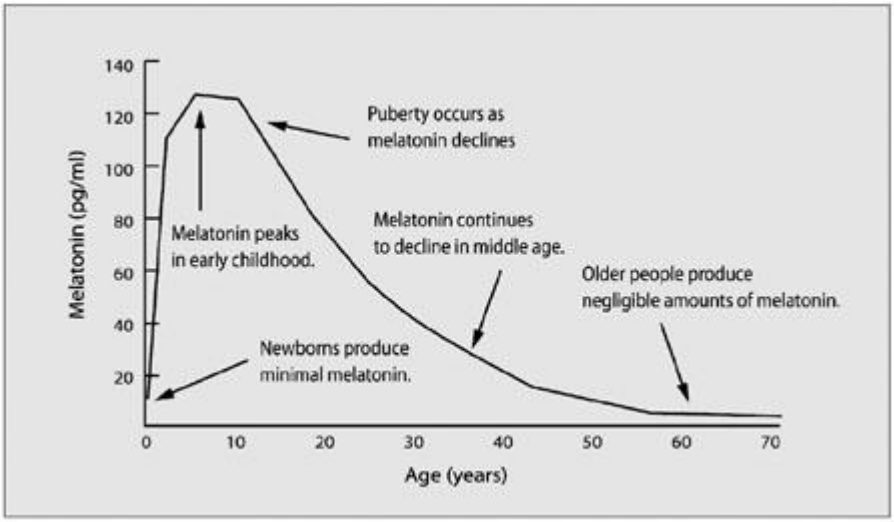


Figure 1.7 . Age variations of melatonin levels. Peak of melatonin secretion occur in early childhood and it gradually decreases from puberty through the life (Grivas & Savvidou, 2007).

Melatonin is highly lypophylic and somewhat hydrophilic as well, which facilitates passage across cell membranes. As no pineal storage of melatonin is available, upon biosynthesis it is released into capillaries, where up to 70% is bound to albumin in order to be transported through the blood and eventually reach the liver (Cardinali *et al.*, 1972; Pardridge & Mietus, 1980). Albumin is the most abundant protein in plasma and binds melatonin non-selectively (Borer, 2003). Transport of hormones in bound form extends their period of availability and action by preventing their rapid clearance from plasma. There is a dynamic equilibrium between the bound and free

fraction of melatonin (Nussey & Whitehead, 2001; Borer, 2003). The fraction bound to albumin is called weakly bound hormone (Kovacs & Ojeda, 2012). Melatonin can readily bind and dissociate from albumin. The free fraction of melatonin is biologically active and it is free to cross capillary walls and bind selectively with target cell receptors to exert an effect (Sherwood, 2010). Melatonin can be found in various fluids, tissues and cellular compartments (saliva, urine, cerebrospinal fluid, preovulatory follicle, semen, amniotic fluid and milk) (Claustrat *et al.*, 2005).

1.1.5.4. Catabolism of melatonin

The biological half-life of melatonin is short and according to different sources it ranges between 30 min (Brown *et al.*, 1997) and 1 h (Claustrat *et al.*, 1986; Mallo *et al.*, 1990). Melatonin is metabolised primarily in the liver, but also to much lesser extent in the kidneys. In the first step it is hydroxylated and then conjugated with sulphate and glucuronide. In humans the main metabolite of melatonin is 6-sulfatoxymelatonin (MT6s). Its urinary concentrations account for up to 90% of melatonin and closely parallels the plasma melatonin profile, as seen in administered melatonin studies (Arendt *et al.*, 1985; Macchi & Bruce, 2004). Liver and renal pathologies can alter clearance rates (Lane & Moss, 1985; Viljoen *et al.*, 1992) (See Section 1.1.6.5. for more details).

1.1.5.5. Melatonin and sleep

It is generally accepted that melatonin can serve as an endogenous synchroniser able to stabilize and reinforce circadian rhythms and maintain their mutual phase-relationship (Armstrong, 1989). Melatonin reinforces nocturnal decrease of core body temperature, which in turn facilitates sleep propensity (Strassmann *et al.*, 1991; Arendt, 2005). Melatonin excretion coincides with sleepiness and temperature nadir (Claustrat *et al.*, 2005). There is a clear relationship between the duration of sleep and melatonin secretion. Long-sleepers have a longer biological night than the short-sleepers. Long sleepers have longer nocturnal intervals of high plasma melatonin levels, increasing cortisol levels, low body temperature and increasing sleepiness (Aeschbach *et al.*, 2003). A study by Van Den Heuvel and colleagues (1997) examined the physiological role of melatonin in relation to sleep. Nocturnal sleep was recorded in 8 young men over 4 nights (aged 21.3 ± 0.9 years). Participants received Atenolol (medication suppressing melatonin secretion) on one of the last 2 nights and showed significantly increased total wake time and wakefulness after sleep onset, as well as decreased REM sleep and SWS. When melatonin (total 5 mg) was given after Atenolol on the next night, the changes in these parameters were reversed. These studies indicate that high levels of melatonin through the night promote sleep consolidation and that low melatonin levels are casual factor in some types of insomnia (Van Den Heuvel *et al.*, 1997; Macchi & Bruce, 2004). Another important fact is that the imbalance in sleep distribution between night and day in infants becomes more distinguished around 3-4 months of life, which correlates with the maturation of melatonin rhythm (Rivkees, 2003).

As described previously, the evening rise of melatonin secretion correlated with a fall in body temperature, facilitates sleep. From the other side, cortisol, which secretion increases before dawn, prepares us for physical and mental demands of awaking, by increasing heart rate, blood pressure and releasing glucose to our blood stream. If these mechanisms are disturbed, sleep disorders may occur.

1.1.6. Cortisol

Cortisol (hydrocortisone) is one of the major glucocorticoid hormones secreted by the adrenal cortex (Aron *et al.*, 2007).

1.1.6.1. Functions of cortisol

Cortisol is often referred to as a stress hormone, as it is involved in response to stress and anxiety. It supplies our body in fuel and energy, by such actions as increasing heart rate and releasing glucose to the blood stream (Seaward, 2006). Thus, the main actions of glucocorticoids include increasing the circulating levels of energy providing compounds such as glucose, through gluconeogenesis and glycogenolysis; free fatty acids, by selective lipolysis, with loss of body fat in the limbs and its accumulation over the lower face and trunk; as well as free amino acids, by increased breakdown of proteins (Loriaux, 2001). The other important function of cortisol is increasing cardiac output and response to catecholamines, thus increasing heart rate and blood pressure (Loriaux, 2001; Seifter *et al.*, 2005).

Another function of cortisol is direct inhibition of natural killer cells activity, reduction of the sytnthesis of prostacyclins, thus suppression of inflammation and immune system, and also sequestration of eosinophils (Zhou *et al.*, 1997). The abnormal pattern of cortisol secretion, such as flattened diurnal cortisol, has been linked to a poor prognosis for women with breast cancer, thus cortisol can be a predictor of breast cancer survival (Sephton *et al.*, 2000).

Cortisol acts on calcium metabolism and bone formation by decreasing serum levels of this ion and accelerating osteoporosis.

There are also several studies indicating that cortisol modulates hippocampus-dependent declarative memory functions. It affects cognitive, as well as emotional networks of the CNS (Het *et al.*, 2005).

Another function of this hormone is increasing free water clearance and inhibition of vasopressin (Loriaux, 2001).

In the case of the glucocorticoid deficiency, effects such as increased sensitivity to insulin, thus tendency to hypoglycaemia, and reduced gluconeogenesis can be observed (Silbernagl, 2000). Overexposure/abnormally high levels of cortisol can lead to health problems such as sleep disorders, hyperglycaemia (De Feo *et al.*, 1989) and memory impairment (Newcomer *et al.*, 1999; Lupien *et al.*, 1997).

1.1.6.2. Circadian regulation of cortisol synthesis

Cortisol, in addition to that seen in the stress response, is secreted in a pulsatile fashion that displays a circadian rhythm. This distinct 24-h rhythm of cortisol secretion occurs as a result of the hypothalamus-pituitary-adrenal (HPA) axis regulation by circadian oscillators (Gallagher *et al.*, 1973). It has been reported that

the SCN influences HPA activity via projections to the paraventricular nucleus (PVN) as well as by direct autonomic innervation of the zona fasciculate (Buijs *et al.*, 1999). At the time of awakening, direct innervations by the SCN acts as a zeitgeber (synchroniser) to the adrenal cortex (Scheer & Buijs, 1999). Similarly to melatonin production, information about the light/dark cycle is transmitted by the retina to the SCN in the hypothalamus. This occurs in spite of the negative feedback effects of cortisol on the hypothalamus and pituitary. The first few hours of sleep have an inhibitory effect on cortisol secretion (Weitzman *et al.*, 1983) and morning exposition to bright light induces elevation of cortisol level (Leproult *et al.*, 2001). Cortisol levels are the highest in early morning (McVicar *et al.*, 2007), they increase dynamically in the first 45 min after awakening, decline throughout afternoon and the evening (Plat *et al.*, 1996; Van Cauter, 2000; Clow *et al.*, 2010) with nadir early in the sleep period (New, e*t al.*, 1989). This pattern of cortisol secretion proved to be stable across days and weeks among individuals with wide age range (Pruessner *et al.*, 1997). Salivary cortisol levels correlate well with the amount of free cortisol in blood ($r \geq 0.90$). This agreement is caused by the fact that, as in the case of melatonin (Section 1.1.5.3.), cortisol enters the cell and the oral cavity mainly by passive diffusion, thus its salivary levels are independent of saliva flow rate (Kirschbaum & Hellhammer, 2000). In blood, the majority of cortisol is bind to cortisol binding globulin (CBG). The unbound fraction of steroid is considered the free, bioavailable fraction, which enters the salivary gland and salivary duct by passive diffusion along the gradient without expenditure of any energy (Zava, 2004; Khurana, 2006). Hence, saliva provides a convenient diagnostic fluid from which to monitor non-invasively, the bioavailable fraction of steroid hormones circulating in the blood stream and entering the tissues (Zava, 2004). The normal values of salivary

cortisol range from 10 to 27 nmol/l at 8.00 am and 2 to 4 nmol/l at 8.00 pm (Laudat *et al.,* 1988). Small elevations of daytime levels can be associated with high-protein meals (especially at lunch time), drinks containing caffeine and stressful events (New, e*t al.,* 1989; Gibson *et al.,* 1999). It is suggested that high levels of cortisol in the morning are linked with a stress-related preparation in regard to the upcoming day (Wilhelm *et al.,* 2007, Clow *et al.,* 2010). It has also been reported that the rhythmicity of cortisol secretion is unrelated to such factors as age, weight, smoking status and alcohol consumption during the previous night, as well as duration of sleep, physical activity and morning routines (Edwards *et al.,* 2001). There is also significant individual variation, although a given person tends to have consistent rhythms (Stone *et al.,* 2001).

1.1.6.3. Biosynthesis and metabolism of cortisol

Cortisol is synthesized from cholesterol under the control of adrenocorticotropic hormone (ACTH) secreted by the anterior pituitary gland in the zona fasciculate, the second of three layers comprising the outer adrenal cortex (New e*t al.,* 1989). Cholesterol is imported to mitochondria and converted to pregnenolone by cholesterol desmolase. Pregnenolone in turn, is converted to 17-hydroxypregnolone by 17α- hydroxylase after its transfer from mitochondria to endoplasmic reticulum. 17-hydroxypregnolone undergoes conversion to 17-hydroxyprogesteron by 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomarase enzyme complex. There is also an alternative pathway in zona fasciculate and reticularis, which leads from pregnenolone to 17α-hydroxyprogesteron via conversion to progesterone. This pathway however, is of less importance. In the next step, which is microsomal, 17α-

hydroxyprogesteron is hydroxylated to 11-deoxycortisol by 21α-hydroxylase. The final step takes place in mitochondria and involves hydroxylation of 11-deoxycortisol by 11β-hydroxylase, what forms cortisol (Loriaux, 2001; Yadav, 2004) (Figure 1.8.).

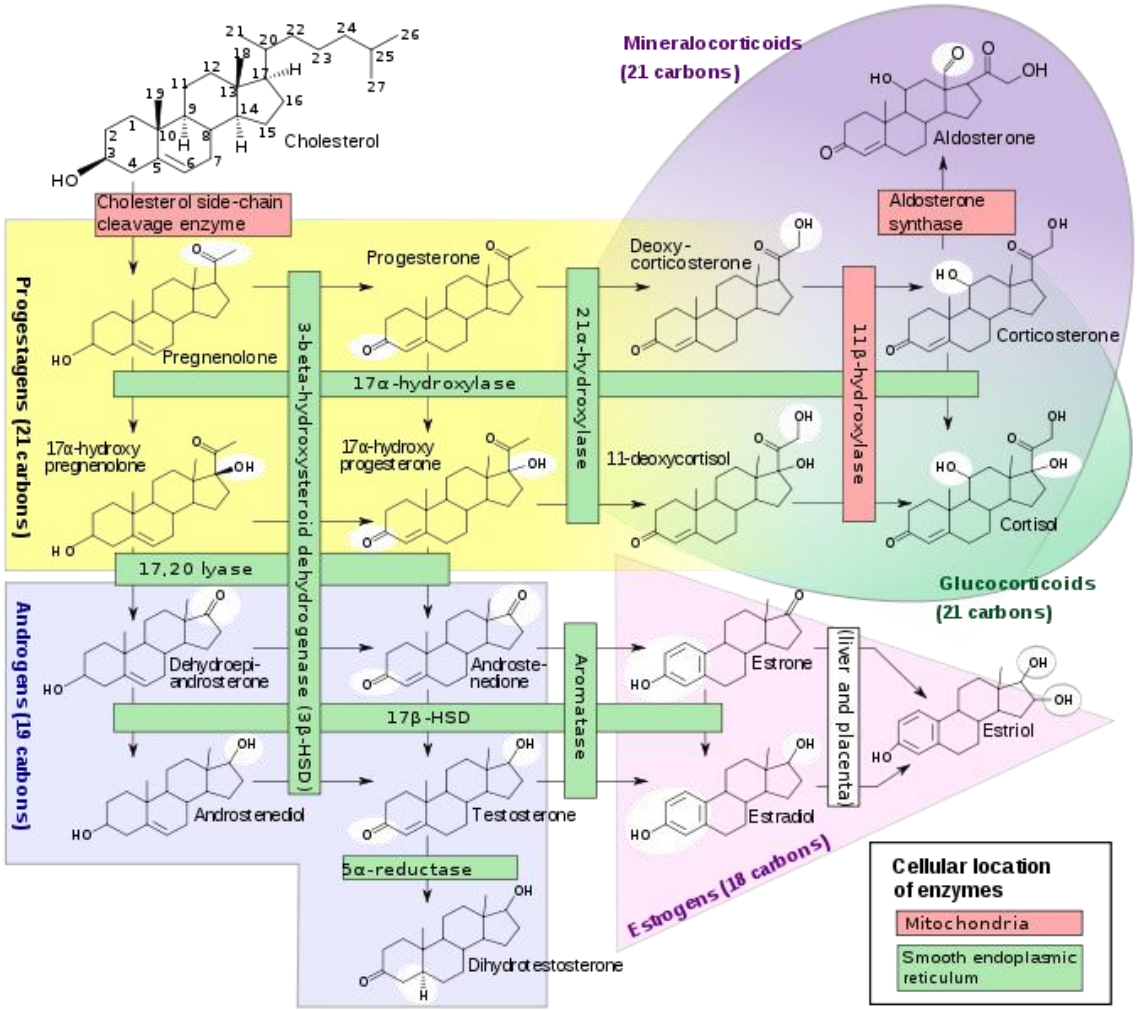


Figure 1.8. Major human steroidogenic pathways. Key enzymes are shown near arrows indicating chemical reactions. The red/green colour of the boxes represents cellular location of enzymes with red indicating mitochondria and green- smooth endoplasmic reticulum. (www.citizendia.org).

1.1.6.4. Negative feedback loop regulation of cortisol synthesis

The secretion of cortisol is ultimately controlled by the CNS which stimulates hypothalamus to release corticotropin releasing hormone (CRH) into the portal vein. CRH from the hypothalamus stimulates the secretion of ACTH in the pituitary gland by binding the receptors on corticotrophs in the anterior pituitary (New *et al.*, 1989; Rinne *et al.*, 2002). ACTH in turn acts on the adrenal cortex causing the synthesis of cortisol. High levels of this glucocorticoid travel through the blood stream back to the hypothalamus and pituitary suppressing CRH and ACTH production and also target tissues (liver, skeletal muscle and adipose tissue) through the body to mediate stress response (Figure 1.9.) (Besser & Thorner, 1994; Holt & Hanley, 2007; MacLaren & Morton, 2012). ACTH probably controls cortisol by controlling the movement of calcium into the cortisol-secreting target cells (Davies *et al.*, 1985). It has been reported that ACTH can activate calcium ions, that leads to their entry through calcium channels. This mechanism is essential for ACTH-induced cortisol production (Enyeart *et al.*, 1993; Omura *et al.*, 2007).

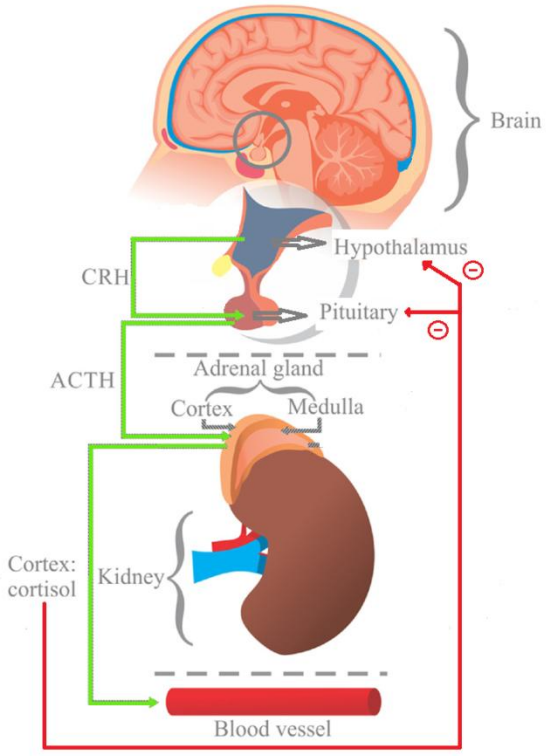


Figure 1.9. **Negative feedback loop of cortisol secretion**. In response to stimuli from central nervous system (e.g. stress) the hypothalamus releases corticotropin releasing hormone (CRH), that stimulates pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH in turn acts on the adrenal cortex causing the synthesis of cortisol, which is then released into the blood circulation. High levels of this glucocorticoid travel through the blood stream back to the hypothalamus and pituitary suppressing CRH and ACTH production (adapted and modified from Campos-Rodriguez *et al.*, 2013).

Arginine vasopressin (AVP) also exerts an important influence on ACTH secretion by augmenting the response to CRH (Goodman, 2003). It also appears that there is positive reciprocal interaction between CRH and AVP at the level of hypothalamus, with each neuropeptide stimulating the secretion of the other (Bernardini *et al.*, 1994). The most important function of AVP in the human organism is however inhibition of diuresis and thus, regulation of body's water retention (Schrier & Abraham, 2007).

Thus, as it has been described, cortisol acts in negative feedback loop on the HPA axis (McVicar *et al.*, 2007), which is one of the primary biological pathways linking

psychological factors and the immune system (Kirschbaum & Hellhammer, 1994). Cortisol suppression of CRH and ACTH prevents overproduction of cortisol. Breakdown of this feedback system leads to severe problems due to uncontrolled production this major glucocorticoid (Aron *et al.*, 2007).

1.1.6.5. Metabolism and excretion of cortisol

The primary site of cortisol metabolism is liver, whereas kidneys are the main site of the excretion of this glucocorticoid. The main task of hepatic metabolism is to decrease the biologic activity of the hormone and to increase its water solubility through the conversion to hydrophilic form which can easily be excreted in the urine. Cortisol is bound in plasma with very high affinity. Approximately 5-10% is bound to albumin, and the majority is bound to CBG, that protects this hormone from degradation. CBG provides plasma storage of bound steroid, while the free fraction of cortisol passes freely across cell membranes to initiate hormone action by binding to the glucocorticoid receptor within the cytoplasm of the target cells (Murphy 2000; Sherwood, 2010). The binding to CBG is rapidly reversible, thus, cortisol may quickly be transferred from the bound to the free state. The free fraction of cortisol is in constant equilibrium with bound form. It has also been found that during inflammatory process and due to exposure to serine protease from granulocytes, CBG is cleaved and releases all or most of its bound steroid. This process enhances the delivery of cortisol to the sites of inflammation (Murphy 2000). The major metabolic reactions of cortisol include the irreversible reduction of $C_{4\text{-}5}$ bond producing dihydrocortisol, which is metabolised to tetrahydrocortisol by the reduction of the C_3 ketone group. Tetrahydrocortisol is further conjugated with

glucuronic acid or sulphuric acid that yields a highly water soluble form and can also undergo reduction to cortol (Brownie, 1992; Yadav, 2004) (Figure 1.10.).

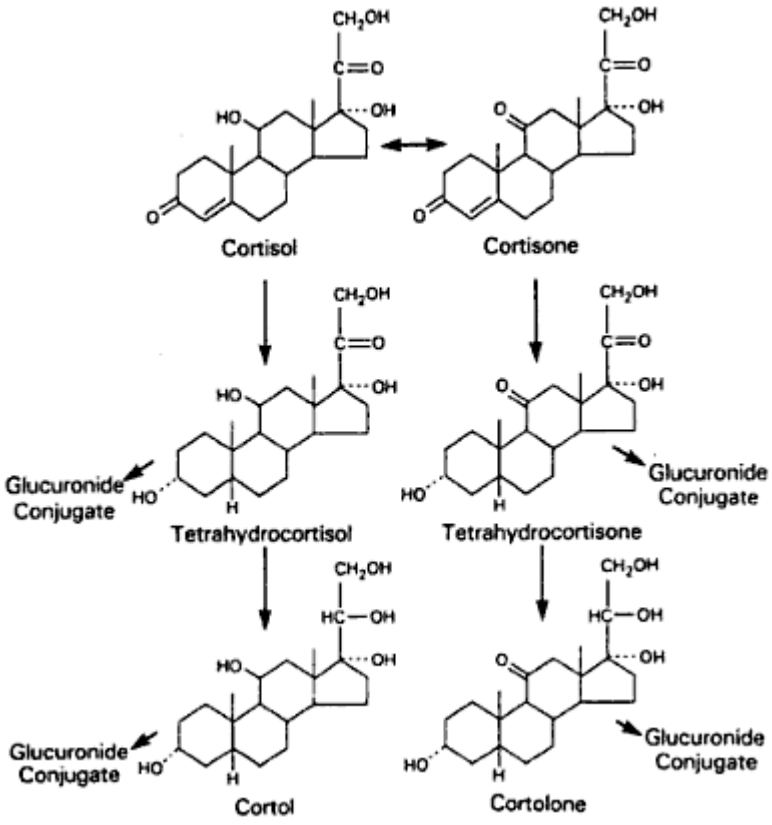


Figure 1.10. Major metabolic pathways for cortisol. Reduction of the double bond at C_4 produces urinary metabolites with both the 5β and 5α orientations (Loriaux, 2001).

Another pathway of cortisol metabolism that can be distinguished includes the oxidation of hydroxyl group on carbon 11 of active cortisol to form inactive cortisone, that lowers the affinity for adrenal steroid hormone receptors. Cortisol differs from cortisone only by the presence of a hydroxyl group at carbon 17. Cortisone circulates in blood at about one-fifth the concentration of cortisol. Cortisol (hydrocortisone) can be converted to cortisone in mineralocorticoid target cells and cortisone can be reduced to cortisol in the liver and other glucocorticoid target cells. These reactions are carried out by the 11β-hydroxysteroid dehydrogenase system

(HSD I and HSD II) at extraadrenal sites. HSD I can catalyze the reaction in both directions, thus may activate or inactivate the hormone. HSD II catalyzes only the conversion of cortisol to cortisone (Figure 1.11.) (Goodman, 2003).

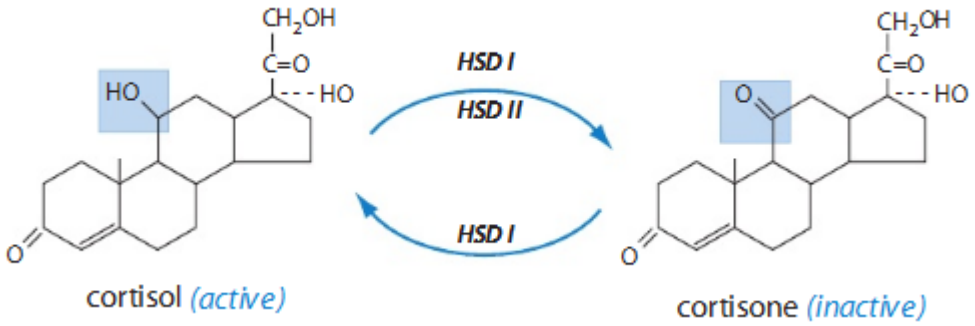


Figure 1.11. The cortisol/cortisone shuttle. HSD I can catalyze the reaction in both directions, thus may activate or inactivate the hormone. HSD II catalyzes only the conversion of cortisol to cortisone (Goodman, 2003).

Cortisone may be then metabolised following the same steps as in the cortisol metabolic pathway producing tetrahydrocortisone, cortolone and conjugated products of each (Figure 1.10). The half-life of cortisol is relatively long compared to other steroids and it equals 60 to 100 min (Loriaux, 2001). The majority of unconjugated cortisol that is filtered through the kidney is reabsorbed. Most of the cortisol which is excreted in urine is in the form of conjugates with sulphate and glucuronide. However, due to the fact that urinary free cortisol is derived from the unbound or bioactive cortisol by glomerular filtration, and its concentration increases rapidly as the binding capacity of CBG is exceeded, the urinary free cortisol levels directly reflects the bioactive plasma concentration (Brownie, 1992; Loriaux, 2001).

As stated in the Section 1.1.5.4. liver pathologies can alter clearance rates of hormones (Lane & Moss, 1985; Viljoen *et al.*, 1992). The reduced rate of hormonal metabolism has been reported in liver diseases (e.g. hepatitis, cirrhosis), what might

be related to a decrease in hepatic blood flow (Marshall & Bangert, 2008; McCornick, 2011). The changes in urinary excretion pattern may be subsequently observed. In addition, any failure in hormone metabolism results in a rise in blood hormone levels, what affects the balance between secretion rates of hormones and their utilisation. However, there is a feedback mechanism between plasma hormone levels and hormone secretion, what prevents any but temporary increase in circulating levels (McCornick, 2011). It has also been reported that there seems to be little difficulty in the hormonal conjugation process, even in the presence of hepatocellular disease. In addition, liver synthesises many plasma proteins including albumin and CBG (Murphy, 2000; Farkas & Farkas, 2009). Thus, liver dysfunction may result in reduced levels of these carrier proteins, which in turn may affect transport of cortisol and melatonin through the circulation and cause the increase of levels of free, biologically active fractions of these hormones (Farkas & Farkas, 2009; Jones, 2013).

1.1.6.6. Cortisol and sleep

Wilhelm and others (2007) suggested that since the HPA axis is a vital part of the human stress response system, cortisol responses to psychological or physiological stimuli can superimpose the circadian rhythm. The central circadian clock, a function of the SCN of the hypothalamus, regulates awakening at the end of normal nocturnal sleep, and is also responsible for circadian regulation of pituitary and adrenal activity (Czeisler *et al.*, 1980; Van Cauter & Turek, 1995). The human SCN has direct projections to PVN which secretes CRH. As it is well understood, CRH acts on anterior pituitary leading to release of ACTH, what results in secretion of cortisol by

the adrenal glands (Buijs *et al.*, 2003). It has been reported that not only circadian rhythmicity in ACTH and cortisol secretion is closely associated with patterns of sleep and wakefulness, but also circulating ACTH and cortisol are at lowest levels during SWS and secretory activity is markedly enhanced during REM sleep (Born & Fehm, 1998). As it has been already described (Section 1.1.1.), the REM sleep occurs more often during late sleep. It has been hypothesised that the increase in cortisol and ACTH levels occurring during the final cycles of REM sleep may play a role in the process of spontaneous awakening (Follenius *et al.*, 1985; Spath-Schwalbe *et al.*, 1991; Born *et al.*, 1999). The studies of Born and colleagues (1989) have shown that cortisol infusion during sleep influences sleep patterns, by increasing the time spent in wakeful episodes during stage I sleep and decreasing time spent in REM sleep. Another evidence for a close association between the SCN influence on sleep/wake cycle and HPA activity is the cortisol awakening response, when the concentration of cortisol is rapidly increasing (Clow *et al.*, 2010). As in the case of melatonin, there is a relationship between chronotype and the concentration of cortisol. Bailey and Heitkemper (1991) analysed early-morning cortisol in saliva of 20 healthy participants 23-39 years of age. Each participant collected seven saliva samples at 20-minutes intervals over the period of 2 h beginning with the wake up time. Cortisol levels were determined using radioimmunoassay (RIA) method. These studies demonstrated that morning-active individuals awaken early show more marked elevation of salivary free cortisol in the morning than evening-active individuals. It has also been reported that lower cortisol levels immediately after awakening were found to be related to poor sleep quality in patients with primary insomnia (Backhaus *et al.*, 2004).

1.1.7. Clock genes

Clock genes are transcriptional regulators underlying circadian rhythm generation at the cellular level (Franken & Dijk, 2009). The core feedback loop of these molecular oscillations contains positive and negative elements. The positive elements are basic helix-loop-helix transcription factors: CLOCK, BMAL1, NPAS2, which all contain PAS domains. BMAL1 can form active heterodimers either with CLOCK or NPAS2, that can enable the transcription of *Cryptochrome (Cry)* and *Period (Per)* genes. The translation outcome: *Cry* and *Per* suppresses CLOCK:BMAL1 and NPAS2:BMAL1 dimers leading to the production of negative elements in the feedback loop (Franken & Dijk, 2009). As reported, the clock gene expression has been observed in saliva, skin samples and peripheral blood mononuclear cells (PBMCs) (James *et al.*, 2007). Reinitiation of the cycle occurs when the proteins in the nucleus break down and the genes are released from inhibition to become active again. The whole cycle takes about 24 hours (Hastings, 1998). This molecular circadian cycle is not only active in SCN but it is expressed in most peripheral tissues. Previous examination demonstrates that light exposure affect the phase in SCN and also the functional activity of peripheral organs (Cailotto *et al.*, 2009). Thus, in conclusion light can be considered as the most important synchronizer for the SCN which causes the changes in melatonin and cortisol secretion as well as in clock genes expression.

Several studies suggest that human clock gene variants contribute to phenotypic differences observed in various behavioral and physiological processes and can have an impact on circadian rhythm (Albrecht, 2002; Viola *et al.*, 2007; Lee *et al.*, 2010; Franken, 2013; Zhang *et al.*, 2013). Table 1.2. presents type of clock genes and their functions. Oster *et al.* (2006) have found clock gene expression in the adrenal cortex,

prefiguring a role of the clock in regulating glucocorticoid and mineralocorticoid biogenesis. The authors reported that in *Per2/Cry1* double mutant mouse lacking a circadian clock, HPA axis regulating cortisol secretion was defective. In addition, it has been also reported that mutation in *Per2* is responsible for familial advanced sleep phase syndrome (Toh *et al.*, 2001; Ebisawa, 2007). Another investigation of clock gene mutation in mice have shown that sleep duration is reduced by 1-2 hrs in C*lock* mutant mice (Naylor *et al.*, 2000). In addition it has been reported that the *Per3* polymorphism correlates with diurnal preference. The longer allele was associated with diurnal preference for morning activity, while the shorter allele with evening diurnal preference (Archer *et al.*, 2003). Moreover, the authors have shown that the shorter allele was strongly associated with the delayed sleep phase syndrome.

It has also been reported that expression of clock genes (such as *Per1, Cry1, Npas4*) can be influenced by secretion of both, melatonin and cortisol (Yamamoto *et al.*, 2005; Imbesi *et al.*, 2009; Mavroudis *et al.*, 2012; Zeman & Herichová, 2013). Experiments in human PBMCs and fibroblasts established that glucocorticoids, after binding to glucocorticoid receptors, induce the expression of the clock gene *Per1* (Balsalobre, 2002; Burioka *et al.*, 2005). In line with these findings Yamamoto *et al.*, (2005) examined the expression of clock genes in response to corticosterone injection in mice and found the elevation of the expression of *Per1* in peripheral organs. On the other hand, studies by Imbesi *et al.* (2009) indicated that melatonin receptor 1 is involved in the regulatory action of melatonin on neuronal clock gene expression. Von Gall *et al.* (2006) reported that in the pars tuberalis (part of anterior lobe of the pituitary gland) of the melatonin receptor 1 knock-out mouse, the expression of *Per1, Cry1, Clock and Bmal1* was dramatically reduced. Thus,

melatonin receptor 1 may control clock gene expression and in turn generation of circadian rhythm.

Gene	Organism	Characteristics of protein product	Clock function
Input genes			
Cry	*D. melanogaster*	Similar to DNA photolyases	Conveys photic information to PER-TIM
Per1, Per2	Mammals	PAS domain	Induced by light in the SCN, *Per1* important for photic entrainment
OPT4	Mammals	Melanopsin	Photoreceptor, mediates light input signals to the SCN oscillator
Pdfr	*D. melanogaster*	G protein-coupled receptor	Neuronal clock synchroniser in the brain
VIP	Mammals	Neuropeptide	Neuronal clock synchroniser in the SCN
VIPR2	Mammals	G protein-coupled receptor	Neuronal clock synchroniser in the SCN
Nocte	*D. melanogaster*	Large glutamine-rich protein	Temperature synchroniser of behavioural activity
ID2	Mammals	bHLH-containing transcriptional repressor	Regulates photic entrainment
DEXRAS1	Mammals	RAS-like G protein	Potentiates photic and supresses non-photic entrainment
cGKII	Mammals	cGMP-dependent protein kinase II	Mediates light entrainment by regulating *Per1* and *Per2* expression
GR	Mammals	Glucocorticoid receptor	Resets circadian phase in peripheral tissues
Pacemakers			
Clock	Mammals, birds, fish, amphibians	bHLH-PAS factor	Dimerises with *Bmal1* and activates clock and clock-controlled gene expression
Per	Mammals (3genes), fish, insects	PAS domain	Negatively regulates CLOCK-BMAL1-driven transcription, positively regulates *Bmal* (mammals) or *Clock* (Drosophila) expression

Tim	Mammals, fish, *D. melanogaster*		In Drosophila dimerises with PER to express CLOCK- BMAL1-driven transcription and activate clock expression
Bmal1	Mammals, fish, *D. melanogaster*	bHLH-PAS factor	Dimerises with CLOCK and activates CLOCK and CLOCK controlled gene expression
Cry	Mammals, *D. melanogaster*	Similar to DNA photolyases	In Drosophila, binds TIM and inhibits PER-TIM dimer. In mammals, negatively regulates CLOCK-BMAL1-driven transcription; translocates PER to the nucleus
Npas2	Mammals	bHLH-PAS domain containing transcription factor	Paralogue of CLOCK, dimerises with BMAL1
Dec1, Dec2	Mammals	bHLH-containing transcription factor	Repress CLOCK-BMAL1-mediated transcription
Output genes			
Pdf	*D. melanogaster*	Neuropeptide	Links the molecular clock to behaviour
Avp	Mammals	Vasopressin peptide	Controls the activity of various output pathways
Takeout	*D. melanogaster*	Similarity to ligand-binding proteins	Involved in output pathways that link the clock to feeding
TGFA	Mammals	Cytokine	Regulates cell proliferation
EGFR	Mammals	Receptor Tyr kinase	Regulates cell proliferation
gprk2	*D. melanogaster*	G protein-couples receptor kinase	Regulates clock-dependent rhythmic olfactory responses

Table 1.2. The most important clock genes in animal and fungi, their protein products and function (adapted and modified from Cermakian & Sassone-Corsi, 2000 and Zhand & Kay, 2008). Abbreviations: bHLH- basic helix-loop-helix, SCN- suprachiasmatic nucleus.

1.1.8. Measurements of endocrine markers of sleep in children

Sleep architecture and/or patterns have been widely studied both in adults and children using such methods as questionnaires, actigraphy and polysomnography (PSG) measurements (see Section 1.2.1) (Owens *et al.*, 2000b; Sadeh & Acebo, 2002; Wiggs *et al.*, 2005). However, thus far, very few studies analysed endocrine sleep markers such as cortisol and melatonin and none of them included individuals with WS. Salti and others (2000) analysed nocturnal pattern of melatonin in blood of 16 healthy school-aged children (8 boys and 8 girls; 8.7 to 16.8 years of age) using RIA. Samples were collected in 30-minutes intervals between 7 pm and 7 am at the research ward. The results of these studies demonstrated significant circadian rhythmicity of melatonin with peak at approximately 2.30 am. In another study, Kohyama (2002) examined melatonin levels in saliva (n = 42) and urine (n = 14) of healthy 3-year old children, collected in the morning just after awakening. The aim of this study was to analyse the relationship between the nocturnal sleep habits and the melatonin levels in youngsters. The authors hypothesised that children who fall into sleep late in the night receive higher amounts of light, than those who fall into sleep early in the night, what may suppress melatonin secretion. In this study, the potential negative physiological consequence of light exposure at night time during early stage of life was confirmed by finding lower melatonin levels in late sleepers. Melatonin levels in saliva appeared to be significantly higher in children with sleep onset times of earlier than 10 pm (6.3 pg/ml) than in the samples of children with later sleep onset times (4.8 pg/ml). Respectively, the average urinary concentration of melatonin metabolite, was higher in samples with earlier sleep onset times (255.0ng/mg creatinine), than with later sleep onset (178.5 ng/mg creatinine). Both

of these studies included samples collected from healthy children. It has been reported that the morning levels of melatonin in plasma range between 0-20 pg/ml in children and adolescents (1-11 years of age) (Waldhauser *et al.* 1984), however, it has also been shown that melatonin reaches highest nocturnal levels between 1 to 3 years of age (Waldhauser *et al.*, 1993).

Several studies examined sleep problems and altered circadian rhythms in children with ASD as well as other developmental disabilities, such as DS, ADHD and Smith-Magenis syndrome (Potocki *et al.*, 2000; Corkum *et al.*, 2001; Tordjman *et al.*, 2005; Corbett *et al.*, 2006; Cotton & Richdale, 2006; Mayes & Calhoun, 2009). For example Tordjman and colleagues (2005) analysed nocturnal urinary excretion of MT6s by RIA in groups of children and adolescents with ASD (n = 49) and healthy control individuals, age and gender matched (n = 88). The findings of this study clearly indicate that nocturnal production of melatonin was significantly reduced in children with ASD and also significantly negatively correlated with severity of autism. Another study examining difference in melatonin levels were based on measurements of salivary melatonin in children with enuresis (n = 14) compared to healthy controls (n = 11). Saliva samples were collected in 4-hr intervals from 8 am to 4 am the next day and analysed using a commercial RIA. The highest reported values were always detected at 4 am, however, there were no significant difference overall in melatonin levels between children with enuresis and controls (Ardura-Fernandez *et al.*, 2007). Levels of melatonin has been analysed in other neurodevelopmental disabilities as well. Potocki and others (2000) measured MT6s in urine of 28 patients with Smith-Magenis syndrome. All but one patient demonstrated inverted circadian rhythm of this metabolite. The Smith-Magenis

syndrome is caused by an interstitial deletion of chromosome 17p11.2. It is characterised as a multisystem disorder with distinctive craniofacial appearance (deep-set eyes, square jaw, full and everted upper lip) as well as complex pattern of physical, developmental, and behavioral features including speech delay, high prevalence of individuals with hearing loss, learning difficulties, short statue, but also sleep disturbances, such as daytime sleepiness, difficulty falling asleep at night and nocturnal awakening (Smith *et al.*, 1998; Smith & Gropman, 2005).

Unlike melatonin, cortisol has not been studied as a sleep marker in children with developmental disabilities caused by various genetic disorders, such as WS or DS. However, there are studies examining this hormone in healthy children and children with autism. Kiess and colleagues (1995) examined salivary cortisol levels in samples from 138 healthy infants, children and adolescents, as well as 14 adults. Saliva samples were collected at home using cotton swabs at 8 am, 1 pm, and 6 pm before meals. Cortisol was measured using fluorescent immunoassay. The aim of this study was to examine the relationship between cortisol levels and age, pubertal stage, weight and gender. No sex difference was found, however, after the age of 6 years old, cortisol levels correlated positively with pubertal stages regardless of sampling time (morning, midday, evening). These findings were not however confirmed by other studies, reporting that cortisol levels were relatively stable across pubertal maturation (Matchock *et al.,* 2007) and no correlation between age (2-18 years old) and cortisol was found (Knutsson *et al.,* 2007). Several studies reported decrease in sleep time during adolescence (Karacan *et al.*, 1975; Carskadon, 1990; Iglowstein *et al.*, 2003), however, there is no evidence of association between cortisol and sleep time through puberty. The relationship between age and cortisol

levels was also studied by Price and others (1983). The focus of this study was to determine the age of appearance of circadian rhythmicity of cortisol. The group of participants consisted of 8 healthy infants. Their saliva was collected by mothers (4 samples in 24 h time) at monthly intervals for the first 6 months of life and analysed by RIA. This study concluded that circadian rhythmicity of cortisol appeared by the age of 3 months (Price *et al.*, 1983). One of the studies examining circadian rhythms of cortisol secretion in children with autism was performed by Corbett and colleagues (2006). Saliva samples were collected from boys aged 6 to 11 with (n = 12) and without autism (n = 10) in the afternoon, evening and morning and assayed using RIA. In this study, both groups showed circadian variation of cortisol, however, children with autism demonstrated statistically significant elevations of cortisol levels following exposure to novel, non-social stimulus. Another study, by Richdale and Prior (1992) investigated the basal urinary cortisol rhythm in a group of high-functioning children with autism (4-14 years of age) and matched controls. All participants were asked to collect 7 samples during the day, however, when the participant provided a urine sample during the night, it was included within morning sample. Urinary cortisol was analysed using a standard RIA kit. The circadian rhythm of cortisol in children with autism was normal, however, the tendency towards cortisol hypersecretion in this group was observed. This tendency may indicate an environmental stress response in children with autism. To date, there are no studies investigating cortisol as possible factor for sleep disorders in children, neither has this hormone been studied in genetic disorders. Cortisol plays a significant role in our circadian rhythm and sleep/wake cycle, therefore it is necessary to examine closely this endocrine marker in individuals with sleep problems.

1.2. Analyses of sleep and circadian rhythm

1.2.1. Methods of sleep analyses

There are several methods of analysing sleep properties. These include pulse oximetry, actigraphy and questionnaires, which are used in current studies as well as PSG.

1.2.1.1. Polysomnography (PSG)

PSG is a gold standard multi-parametric study of sleep consisting of continuous nocturnal monitoring of electrencephalogram (EEG), electro-oculogram (EOG), electromyogram (EMG), electrocardiogram (ECG), nasal/oral airflow by capnography and pressure transduction, end-tidal CO_2 trend by capnometry, chest and abdominal wall respiratory movement by piezocrystal belts, body position, sonography measuring upper respiratory tract sound, finger plethysomnography measuring pulse volume and pulse oximetry. After the test is completed, the records are scored and evaluated in usually 20 to 30-seconds epochs (Sheldon, 2005c; Snasel *et al.*, 2011). The weak points of this method are that it is expensive and labour-intensive and the measurement needs to be undertaken in an unfamiliar environment, which is a sleep laboratory (Portier *et al.*, 2000). It usually requires two nights stay, as the first night is necessary for acclimatisation (Kingshott & Douglas, 2000).

Electroencephalogram (*electro*- electrical, *encephalon*- brain, *gram*- record) is the recording of the electrical activity of the brain measured by conductors attached to the scalp (Webb, 1975; Niedermeyer & da Silva, 2004). The EEG machine amplifies

the small electrical potential changes and converts them to a mechanical write-out on continuously running paper belt. The EEG measurement of sleeping person allow to differentiate particular stages of sleep (Figure 1.12.) (Rosenzweig *et al.*, 2005). The main diagnostic applications of EEG are epilepsy, coma, encephalopathies and brain death (Niedermeyer & da Silva, 2004).

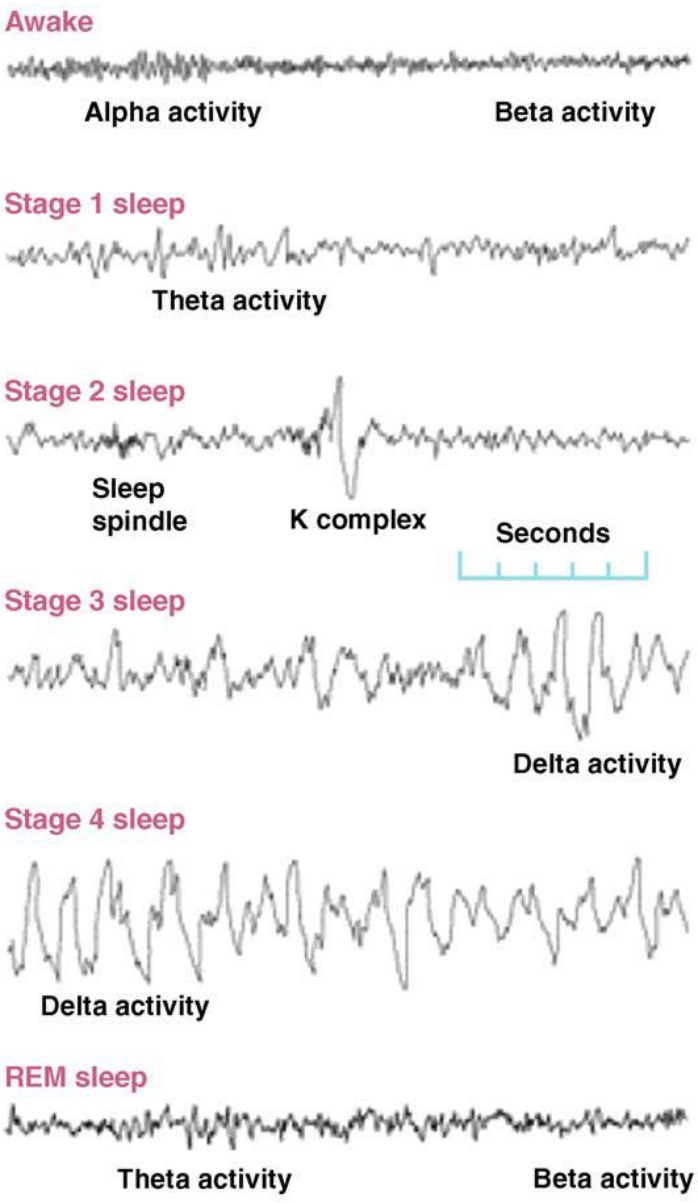


Figure 1.12. EEG pattern of different stages of human sleep. Awake: low voltage, fast alpha and beta activity. Stage 1: 4-7 Hz theta activity. Stage 2: 11-15 Hz sleep spindles and K complexes. Stage 3 and 4: High voltage delta activity. REM: low voltage fast theta activity similar to the awake state (Horne, 1988).

Electro-oculography and electromyography usually supplement the EEG recording of the brain. EOG is the recording of eye movements and EMG is the recording of electrical activity of the muscle (muscle tension). In order to get the EMG recording, electrodes are placed on the skin over a muscle. For the PSG measurements, EMG electrodes are placed on the chin and left and right anterior tibial muscle. These positions allow researchers to study different sleep disorders, such as bruxism, REM sleep behaviour disorder, which may present with unusual muscle activity, that can be recorded by EMG measured on the chin (Rosenzweig *et al.*, 2005; Sheldon, 2005c). The EOG recording can provide important information in regard to differentiation between particular stages of sleep, especially REM sleep. The electrodes are placed at the outer canthus of each eye (Sheldon, 2005c).

ECG is a measurement of patient's cardiac activity including heart rate and rhythm. Heart rate change through the sleep stages, declining from stage 1 to stage 4 NREM and increasing in REM sleep, however the general heart rate in sleep is about 4-8 beats per minute slower than during quiet wakefulness (Willis *et al.*, 1990). The polysomnographic measurements use the lead II ECG, which continuous recordings are assessed for rhythm, rate and abnormalities that may be associated with central or obstructive respiratory problems (Sheldon, 2005c).

1.2.1.2. Actigraphy

Actigraph is a computerised wristwatch-like device that monitors a persons' activity. This method can be used for sleep-wake identification, as well as calculating the sleep efficiency, sleep latency, fragmentation index, immobile and mobile minutes

during sleep. The actigraphs (actiwatches) have an analogue system detecting movement in two or three axis by piezoelectric beam. The detected movements are further translated into digital counts accumulated across certain intervals (e.g. 1 minute). The actigraphs have an internal memory and can record data continuously over certain period of time (e.g. 1 week) (Sadeh & Acebo, 2002; Sazonov *et al.*, 2004). Computer software, used to read the data from actigraphs, can calculate levels of activity/inactivity, and sleep/wake parameters (such as total sleep time, percentage of time spent asleep, number of awakenings) (Ancoli-Israel *et al.*, 2003). The actigraphy has been validated against PSG and it proved total sleep time, sleep efficiency and the number of awakenings to be in line with PSG both in adults and children (85 to 90% agreement between the two methods) (Corkum *et al.*, 2001; Kushida *et al.*, 2001; Gay *et al.*, 2004). Several other studies reported that wrist actigraphy can successfully differentiate between sleep and wake state, and monitor circadian sleep/wake disturbances (Broughton *et al.*, 1996), however, based on this method, one cannot characterise clinical sleep disorders nor sleep stages (Thorpy *et al.*, 1995). Also, actigraphy may not always be appropriate for certain groups, for example insomniac patients who may be awake but remain still for long periods of time. American Academy of Sleep Medicine Standards of Practice Committee developed guidelines on the clinical use of this technology. One of these guidelines indicate that actigraphy is particularly useful in studying infants and children, as well as in special paediatric population as PSG can be difficult to perform and/or interpret (Morgenthaler *et al.*, 2007). Actigraphy also demonstrates high accuracy, simplicity of use and low intrusiveness (Sazonov *et al.*, 2004).

1.2.1.3. Pulse oximetry

Pulse oximetry is non-invasive method monitoring the pulse rate and arterial oxygen saturation. The pulse oximetry sensor consisting of light source and a detector is placed on the both sides of any pulsating arterial vascular bed, such as earlobe, finger, toe or in the younger infants on the foot. The pulse oximetry method is based on the difference of the light absorbance between reduced and oxygenated haemoglobin, and the pulsating blood between the source and the detector (Tremper & Barker, 1989). Thus, the pulse of the vessel and the oxygen saturation of the arterial haemoglobin cause the changes of the light path length that modifies the amount of light detected. Pulse oximeter calculates the arterial haemoglobin oxygen saturation with no interference from surrounding venous blood, skin, connective tissue or bone (Yelderman & New, 1983; Sheldon, 2005c). Pulse oximeters are increasingly used for the evaluation of SDB (Netzer *et al.*, 2001). These monitors are easy to use in a home environment and provide accurate information on oxygen levels (Urschitz, 2003).

1.2.1.4. Questionnaires

The use of questionnaires is an efficient and cost-effective method for gathering a large amount of data. In the developmental literature a widely used questionnaire is the Children's Sleep Habits Questionnaire (CSHQ; Owens *et al.*, 2000a), a 33-item parent-report questionnaire covering the major clinical symptoms of childhood sleep problems. Its scores can consistently differentiate between children with sleep disorders and children without, though it has not yet been quantified for atypical

populations, such as children with developmental disorders. Although the CSHQ provides an excellent measure for assessing sleep, parent reporting is not always entirely accurate as parents are not always aware of their children's sleep problems. Large inconsistencies have been found between sleep diaries, questionnaires and actigraphy data. Generally, parents are able to be relatively accurate when reporting bedtime, wake time and assumed sleep time, but their reports are less consistent than actigraphy when recording actual sleep time, night wakings and sleep quality, especially if the child does not cry out for parental attention when they wake during the night. However, questionnaires are usually used as a first step to characterize sleep patterns in a child. Therefore it is recommended that objective methods such as PSG or actigraphy ought to be used in conjunction with parent reports to gain reliable information about children's sleep (Sadeh *et al.*, 2000; Corkum *et al.* 2001; Shott *et al.*, 2006; Werner *et al.* 2008; Holley *et al.*, 2010).

1.2.2. Laboratory measurements of endocrine markers of sleep

Several bioanalytical methods for determination of melatonin, cortisol and their metabolites have been reported using various laboratory techniques. The most common methods used for analysis of these hormones in children as well as for research studies on sleep disorders are radio- and enzyme based immunoassays using saliva, plasma and urine as matrices (Table 1.3.) (Salti *et al.*, 2000; Van Cauter *et al.*, 2000; Kunz *et al.*, 2004; Corbett *et al.*, 2006). More recently, however, these methods are being replaced by more sensitive and faster liquid chromatography - mass spectrometry (LC-MS) based methods (Taylor *et al.*, 2002; Eriksson *et al.*, 2003; Barrett *et al.*, 2005; Jensen *et al.*, 2011). General clinical analysis of melatonin and cortisol are

mostly based on the use of immunoassay based methods, mainly because of the convenience and limited number of clinical laboratories with liquid chromatography tandem mass spectrometry (LC-MS/MS) equipment (Inder *et al.*, 2012).

Hormone	Type of analysis	Medium	Method	References
Melatonin	Research studies on sleep disorders	Saliva	RIA	Ardura-Fernandez *et al.*, 2007
		Urine	RIA	Tordjman *et al.*, 2005; Potocki *et al.*, 2000
			ELISA	Kunz *et al.*, 2004
	Analysis in children	Plasma	RIA	Salti *et al.*, 2000
		Saliva	RIA	Kohyama *et al.*, 2002
		Urine	ELISA	Lee *et al.*, 2012
	Methods publications	Plasma	LC-MS	Hartter *et al.*, 2001
		Saliva	HPLC-FLD	Romsing *et al.*, 2006
			LC-MS/MS	Eriksson *et al.*, 2003; Jensen *et al.*, 2011
		Urine	HPLC-FLD	Minami *et al.*, 2008
Cortisol	Research studies on sleep disorders	Plasma	RIA	Van Cauter *et al.*, 2000
		Urine	RIA	Prinz *et al.*, 2000
	Analysis in children	Saliva	FIA	Kiess *et al.*, 1995
			RIA	Price *et al.*, 1983; Corbett *et al.*, 2006
			ELISA	Shimada *et al.*, 1995
		Urine	RIA	Richdale & Prior, 1992
	Methods publications	Saliva	LC-MS/MS	Jensen *et al.*, 2011
		Urine	LC-MS/MS	Machacek *et al.*, 2001; Nassar *et al.*, 2001; Taylor *et al.*, 2002; Barrett *et al.*, 2005

Table 1.3. The most common method of analysis of melatonin and cortisol used in the research studies on sleep disorders, the analysis in children as well as method development publications. It can be seen that immunoassay based methods

are the main methods used in research and analysis of melatonin and cortisol in children in plasma, saliva and urine. More recently, however, the number of publication regarding new methods using liquid chromatography and/or mass spectrometry based methods is increasing. Abbreviations: ELISA- enzyme-linked immunosorbent assay, HPLC-FLD- high performance liquid chromatography-fluorescence detection, LC-MS- liquid chromatography- mass spectrometry, LC-MS/MS- liquid chromatography tandem mass spectrometry, RIA- radioimmunoassay.

Radio- and enzyme based immunoassays are widely used for the determination of these compounds in plasma, saliva and urine (Zhiri *et al.*, 1986; Chegini *et al.*, 1995; Kovács *et al.*, 2000; Jerjes *et al.*, 2006; Lee *et al.*, 2012). Both approaches are based on the formation of antigen-antibody complex. The immunoassay techniques are divided into competitive and non-competitive. In competitive assays the microtiter plate is coated with a known amount of antibody. The antigen in the sample (e.g. melatonin) competes with the labelled antigen for a fixed number of antibody binding sites. Enzymes (enzyme immunoassays) or radioisotopes (RIAs) can be used as labels (Estridge *et al.*, 2000). The most common isotopes used for RIA analysis are ^{125}I, ^{3}H and ^{57}Co (Nuclear Energy Agency, 2004). Non-competitive immunoassays may be used to detect the presence of either antigen or antibody in a sample. In this type of assays, labelled antibody and antigen interact without deliberate introduction of competing molecules (Stanley, 2002). In enzyme based immunoassays an enzyme catalyses colourimetric reaction after adding the substrate. In RIA, the signal of radioactivity is measured (Moore, 2002). Intensity of colour/radioactivity is inversely (competitive assay) or directly (non-competitive assay) proportional to the amount of antigen in the sample (Elgert, 2009; Iles & Roitt, 2012).

Although RIA is a very sensitive method (detection limit of 10^{-9} to 10^{-12} mol/L) (Wybranska *et al.*, 2002; Saha, 2010), it is tedious, requires book-keeping and the use of radioactive isotopes is potentially hazardous and produces radioactive waste (Elgert, 2009). Another disadvantage of RIA is a short shelf life (Mahlen, 1998). To overcome these drawbacks, it has been replaced by enzyme linked immunosorbent assay (ELISA) in many clinical and basic science laboratories (Elgert, 2009). Theoretically sensitivity of ELISA is around 10^{-14} mol/L which meets the sensitivity requirement for many clinical analyses (Diamandis *et al.*, 1996; Moore, 2002). Another advantage of enzyme immunoassays over RIA is that the enzyme preparations are stable with long shelf life and they represent a minimal hazard to laboratory staff (Moore, 2002).

Despite the wide use of immunoassays, there are a number of limitations of these methods; (i) lack of an internal standard to monitor routine variation in the response of the measuring system/analyte; (ii) a limited number of samples can be analysed at a time; and (iii) antibody cross-reactivity with other structurally similar compounds that may be present in the sample (Nahoul *et al.*, 1982).

Other analytical techniques used for analysis of melatonin and cortisol are based on chromatographic methods. These include high performance- liquid chromatography (HPLC) with fluorescence detection (Romsing *et al.*, 2006) or ultra violet (UV) detection (Taylor *et al.*, 2002), gas chromatography-mass spectrometry (GC-MS) (Nùñez-Vergara *et al.*, 2001) and LC-MS/MS (Machacek *et al.*, 2001; Nassar *et al.*, 2001; Eriksson *et al.*, 2003; Barrett *et al.*, 2005). It has been reported that chromatographic methods provide a means of separating cortisol from any interferents in the matrix and allow quantification of cortisone (Taylor *et al.*, 2002).

It has also been shown that LC-MS/MS offer better specificity and accuracy than immunoassay based methods (Tiosano *et al.*, 2003).

Gas chromatography (GC) is an analytical technique used for the separation of volatile mixtures. Gas chromatographs consist of a gaseous mobile phase, an injection port, a separation column containing the stationary phase, a detector and a data recording system. Samples are vaporised and injected onto the head of the chromatographic column. The inert flowing mobile phase transports the sample to the column (Prasad, 2010). The compounds are separated due to differences in their partitioning behaviour between the mobile gas phase and the stationary phase in the column (Sheehan, 2009). In GC-MS, the mass spectrometer acts as a detector for the gas chromatograph. Mass spectrometry (MS) is one of the most sensitive detection technique of molecular analysis (Vogeser & Seger, 2008; Loos *et al.*, 2011) and will be described on the next page.

There are some disadvantages to using GC. It is limited to separation of volatile samples and not suitable for thermally labile samples. It is also fairly difficult to utilise for preparative purposes and requires elaborate instrumentation, such as a mass spectrometer to confirm identity of an analyte peak (McNair & Miller, 1997).

Liquid chromatography (LC) is another technique that is used for separation of range of molecules, it utilises high pressure for pumping liquids through columns packed with a stationary phase (Ahuja, 2003). An HPLC system consists of solvent reservoirs to hold the LC solvents necessary for continuous operation of the system; the pumps providing constant flow of the mobile phase through the system and allowing controlled mixing of different solvents from different solvent reservoirs. An injector is used to introduce an aliquot of a sample into the stream of mobile phase

prior to its entering the column (Sherwood, 2005; Kazakevich & LoBrutto, 2007). Separation is carried out using the LC column which comprises of a narrow stainless steel tube packed with fine, chemically modified silica or polymer based particles (Niessen, 2006). Compounds are separated on the basis of their relative interaction with the chemical coating on these particles and the solvent/mobile phase eluting through the column. In the next step, analytes are ionised. The two most common ionisation interfaces that are used for LC-MS are the atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) interfaces. In ESI interface, the eluent from the LC column is forced through a narrow capillary that has a high potential applied to it. This results in the generation of a very fine mist of charged droplets carrying ions surrounded by solvent molecules. Further, as the droplets move towards the entrance of mass analyser, the solvent evaporates and the charge concentration results in the droplets dividing into even finer droplets. The evaporation process occurs due to the presence of a nebulising gas, usually nitrogen and the interface being heated to temperatures of 200ºC or more. The process results in formation of isolated gas-phase ions (Niessen, 2006; Hacker *et al.*, 2009). APCI is also based on ionising sample at atmospheric pressure. However, this mode of ionisation is used for non-polar compounds that have some volatility (Loos *et al.*, 2011). Once the eluent stream is desolvated and gas phase ions have been formed, the beam of ions is introduced into the mass analyser. The basic function of a mass analyser is to measure the mass to charge ratio (*m/z*) of ions and this provides means of separating the ions. There are several types of mass analysers that can be used which include magnetic sector, quadrupole, ion trap, time-of-flight and fourier transform ion cyclotron resonance instruments (Kazakevich & LoBrutto, 2007). The quadrupole mass spectrometer was previously used for analysis of cortisol and

melatonin (Taylor & Singh, 2002; Kushnir *et al.*, 2003; Eriksson *et al.*, 2003; Jensen *et al.*, 2011). This type of mass analyser consists of four cylindrical or parabolicaly shaped rods in a parallel configuration (Figure 1.13.). Both positive and negative electric fields are applied to the rods and adjusted by a current voltage such that only the ion of given corresponding frequency (resonant ion) can successfully pass down the axis between the rods and reach the detector. This frequency is related to the *m/z* ratio of the ion; hence its measurement gives the molecular weight. Other ions have unstable trajectories and collide with the rods (Hacker *et al.*, 2009; Loos *et al.*, 2011).

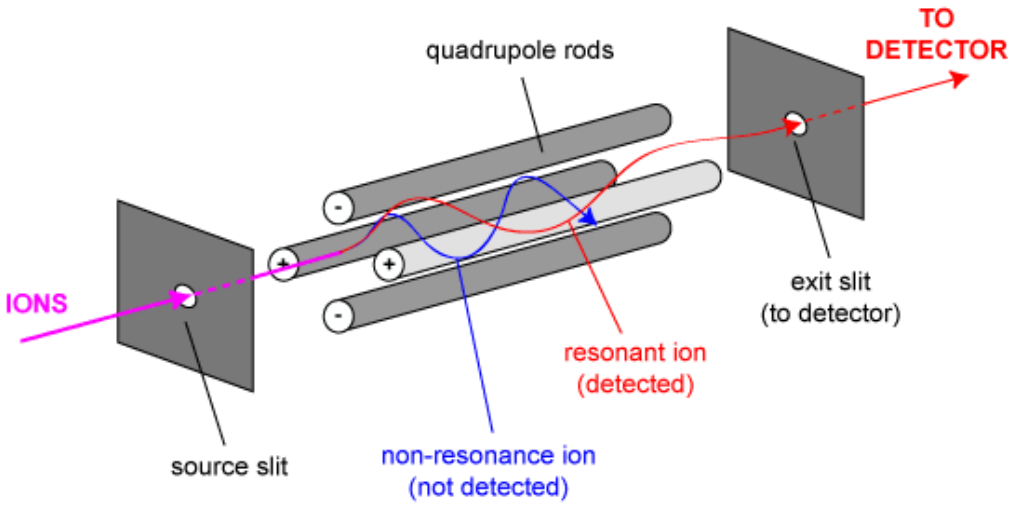


Figure 1.13. The mass analyser of the mass spectrometer (http://www.chm.bris.ac.uk/).

LC-MS/MS is the HPLC system coupled with MS detector. It is an analytical technique that combines separation of compounds using chromatography with mass analysis of a mass spectrometer. The liquid chromatography component is used to physically separate compounds and the mass spectrometer is used to detect the compounds on the basis of their *m/z* ratio. Tandem MS (MS/MS) as opposed to single-stage MS involves at least two stages of mass analysis. In the most common

MS/MS systems a first analyser is used to isolate a precursor ion, which then undergoes fragmentation to product ions measured by second mass spectrometer (de Hoffmann & Stroobant, 2007). Thus, the specificity and sensitivity is significantly improved (March & Todd, 2005). MS/MS analysis also offer enhanced speed in comparison to a single quadrupole instruments. There are several advantages to using this technique over GC-MS and LC-MS assays; (i) the sample preparation time is lower, which increases the speed of analysis; (ii) it enables direct screening of a complex mixture for a specific target compound; and (iii) it provides the highest degree of certainty in analyte identification (Bart, 2005). The sensitivity of MS/MS is typically in the sub-pg range (Bart, 2005).

Although MS is the most sensitive detection technique, several other detectors, such as, fluorescence and UV detectors may be used. Maximum sensitivity reached by fluorescence detection is 10^{-9} to 10^{-11} mg/ml, while by the UV detectors approximately 10^{-8} to 10^{-9} mg/ml (Kazakevich & LoBrutto, 2007). The drawback of fluorescence detection technique is that it requires compounds to have native fluorescence or to be chemically modified to induce fluorescence. Also, its response is only linear over small concentration range, usually two orders of magnitude (Sherwood, 2005). On the other hand, UV detectors can be only used for the compounds that absorb in the UV range of wavelengths of interest (Scott, 1977).

HPLC offers a number of advantages over GC. One of them is that polar compounds such as drug metabolites can be analysed directly (Kwong, 2002). Also, the majority of difficult separations are often more readily achieved using HPLC. There are several reasons for this. In HPLC systems, both phases participate in chromatographic separation process. This separation is achieved by the particular

interactions between sample molecules and the stationary and mobile phase(s), thus more selective interactions are possible with the sample molecule. This provides an additional variable for regulating and improving separation. As GC system consists of only one phase (flowing gas), these interactions are absent. Also, in HPLC, a wide range of selectivity is provided by a large variety of unique column packings (stationary phases). Another advantage of HPLC is the lower temperature used in this separation technique, what increases the effectiveness of intermolecular interaction and in turn improves chromatographic separation (Ahuja, 2003).

It has been reported that methods based on LC- UV detection for the measurement of urinary free cortisol and cortisone, requires an analysis time longer than 20 min in order to obtain good resolution of these analytes from interferents (Taylor *et al.*, 2002). The use of liquid chromatography- tandem mass spectrometry (LC-MS/MS) has overcome these limitations (Machacek *et al.*, 2001; Nassar *et al.*, 2001).

The latest separation technology is ultra-high performance liquid chromatography (UHPLC), however, to date it has not been used for the analysis of melatonin and cortisol. UHPLC retains the practicality and principles of HPLC, while increasing the attributes of speed, sensitivity and resolution (Swartz, 2005; Prathap *et al.*, 2013). It is using reversed phase columns with smaller particles and/or higher flow rates for increased speed with superior peak resolution and sensitivity. Due to hardware adjustments it is also possible to work at extreme pressures (approximately 12,000 psi) that gives marked improvement of chromatographic performance for complex mixture separation (Lindon, 2007; Hyotylainen & Kivilompolo, 2011). Compared to HPLC system, UHPLC gives more than doubling of peak capacity. Also, almost 10-fold increase in speed, as well as 3- to 5-fold increase in sensitivity can be obtained

in comparison with a conventional stationary phase depending on the detection technique (Swartz, 2005; Lindon, 2007). MS detection is significantly enhanced by UHPLC, as the increased peak concentrations with reduced chromatographic dispersion at lower flow rates promotes increased source ionization efficiencies (reduced ion suppression) (Swartz, 2005; Lindon, 2007).

1.3. Williams syndrome (WS)

WS was first described in the early 1960s, by Williams and colleagues (1961) who reported the case of four patients having the same clinical symptoms: supravalvular aortic stenosis (SVAS), mental "subnormality" and unusual facial features. Shortly after, Beuren and others (1962) reported the syndrome independently including dental anomalies, peripheral pulmonary aortic stenosis and a friendly personality. As a result, this condition is called Williams syndrome or Williams-Beuren syndrome.

WS is an autosomal dominant genetic disorder caused by a microdeletion of 28 genes (Shubert, 2009) on chromosome 7q11.23 including the ELASTIN gene (*ELN*) (Chen, 2006; Ewart *et al.*, 1993). This ssyndrome affects boys and girls equally (American Academy Of Pediatrics, 2001) and occurs at approximately 1 in 7500 to 1 in 20000 live births in the UK and worldwide (Tassabehji *et al.*, 1999; Stromme *et al.*, 2002).

1.3.1. Clinical symptoms

Individuals with WS have characteristic "elfin" facial appearance, which can be easily recognised by an experienced observer due to their distinctiveness (Figure 1.14.) (Semel & Rosner, 2003; Smoot *et al.*, 2005; Morris, 2006). The extent of dysmorphology varies over the time in the same individual as well as among different individuals (Burn, 1986; Morris *et al.*, 1988). Characteristic facial features include: flat facial profile, broad forehead, prominent eyes, full cheeks, macrostomia (wide mouth), small jaw/small chin, short-upturned nose with full nasal tips, flat bridge of the nose, large ears, full lips, epicanthal folds, bitemporal narrowness and periorbital fullness. Distinctive features include as well malocclusion, small widely spaced teeth, long philtrum, malar hypoplasia, strabismus and stellate or lacy irides among blue-eyed individuals (Semel & Rosner, 2003; Smoot *et al.*, 2005; Morris, 2006).

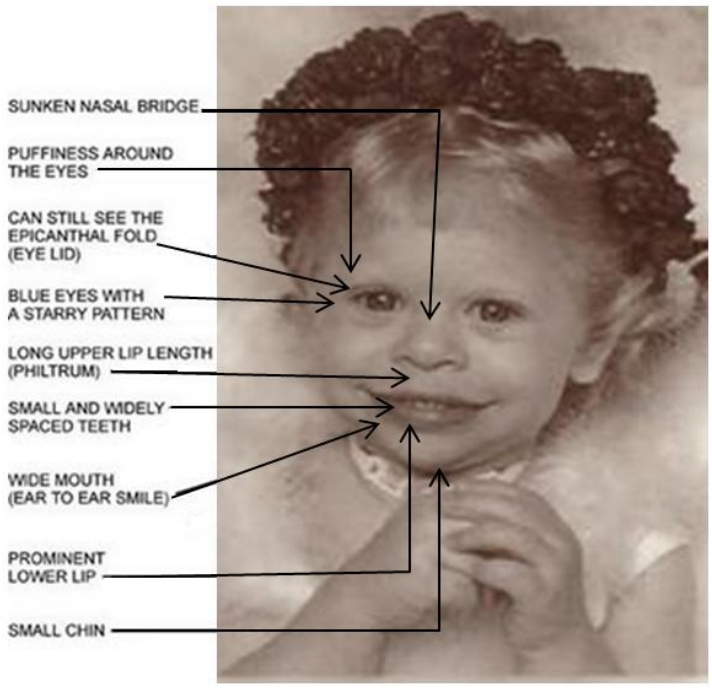


Figure 1.14. Characteristic facial features in WS (Semel & Rosner, 2003; http://dailypostal.com/2010/04/13/williams-syndrome-removes-racial-bias/).

WS has been described as a multisystem disorder with leading cardiovascular problems. The most common and the most significant clinically is SVAS, which may be present in isolation or in conjunction with other vascular stenoses and it occurs in approximately 75% of individuals (Morris, 2006; Ergul *et al.*, 2012). SVAS causes narrowing of the aorta and is a result of the deletion of one copy of *ELN* (haploinsufficiency for ELN) (Smoot *et al.*, 2005). This pathology often forms the basis for the diagnosis of WS particularly in infancy when other signs may be lacking (Smoot *et al.*, 2005). Other common cardiovascular findings are peripheral pulmonic stenosis, which occurs mostly in infancy (Morris, 2006) and hypertension affecting approximately 50% of individuals (Zalzstein *et al.*, 1991; Wessel *et al.*, 1997; Eronen, 2002). There is a likelihood that mild to moderate pulmonary stenoses can improve spontaneously with time, but the reason for that is not known (Giddins *et al.*, 1989). It is reported that approximately 22% of all individuals with WS require surgical intervention due to cardiovascular lesions (Smoot *et al.*, 2005).

Connective tissue abnormalities lead to several symptoms observed in the individuals with WS, such as: hoarse, low-pitched voice, inguinal/umbilical hernia, bowel/bladder diverticulae, rectal prolapse, joint limitation or laxity, and soft and lax skin (Morris, 2006).

Also, growth abnormalities are present in most individuals with WS (Morris *et al.*, 1988; Pankau *et al.*, 1992). The growth pattern is characterized by intrauterine growth retardation, failure to thrive in infancy (70%), poor weight gain and linear growth in the first four years. A rate of linear growth is 75% of normal in childhood with a brief pubertal growth spurt. However final adult height typically falls in the low range of normal adult height (Pankau *et al.*, 1992; Morris, 2006).

Renal malformations are quite common among individuals with WS. It is reported that 35-50% of these individuals have structural abnormalities of urinary tract, from which 15-20% are abnormalities of kidneys, such as solitary, duplicated, hypoplasic or scarred kidney (Pober *et al.*, 1993; Pankau *et al.*, 1996). Enuresis, weak bladder and frequent voiding affects as many as 50% of individuals with WS. These conditions may be caused by bladder diverticuli (40%) (Morris *et al.*, 1988) as well as reduced bladder capacity and detrusor overactivity, both observed in 60% of the WS population (Sammour *et al.*, 2006). All these abnormalities may contribute to recurrent urinary tract infections affecting approximately 30% of adults with WS (Morris *et al.*, 1990; Pober *et al.*, 1993). Another abnormality of the urinary tract is renal artery stenosis in 50% and nephrocalcinosis in less than 5% of individuals with WS (Pankau *et al.*, 1996; Sforzini *et al.*, 2002). Renal dysplasia, recurrent infections, renal artery stenosis and nephrocalcinosis, can all contribute to renal failure occasionally reported in WS (Steiger *et al.*, 1988; Ichinose *et al.*, 1996).

Endocrine problems in WS are mainly associated with calcium, glucose and thyroid abnormalities as well as early puberty (in 50% of WS individuals) (Morris, 2006; Pober & Morris, 2007).

The most common abnormalities of calcium metabolism are hypercalcaemia (15%) and hypercalciuria (30% of WS population) (Morris, 2006), but their aetiology is unknown (Kruse *et al.*, 1992). Hypercalcaemia occurs usually in infancy but may recur in adult life producing symptoms like poor feeding, vomiting, failure to thrive, constipation and irritability (Martin *et al.*, 1984; Morris *et al.*, 1990). Hypercalciuria can occur independently from hypercalcaemia and it increases the risk of nephrocalcinosis (Morris *et al.*, 1988).

The most common thyroid abnormality is hypothyroidism. Early onset of this condition has been reported in children with WS and the frequency of subclinical hypothyroidism increases in adulthood affecting approximately 20% of WS individuals (Pober *et al.*, 2000; Morris, 2006).

Abnormal glucose metabolism affects the majority of WS population (Pober *et al.*, 2001; Masserini *et al.*, 2013). Cherniske and others (2004) observed extremely high frequency of abnormal glucose tolerance in adults with WS compared to adults in the general population with similar body mass index's (BMIs). Diabetes mellitus affects 15% of adults with WS (Morris *et al.*, 1988; Morris *et al.*, 1990).

Several gastrointestinal problems occur both in children and adults with WS. The most common problems in infants are gastroesophageal reflux, constipation (both found in almost 50% of infants), disordered suck and swallow, textural aversion, vomiting and colic (Morris *et al.*, 1988). Prolonged colic (over 4 months) may be related to gastroesophageal reflux, chronic constipation and idiopathic hypercalcaemia (Morris, 2006). Chronic abdominal pain occurs commonly in children and adults with WS. The possible causes of this condition include gastroesophageal reflux, hiatal hernia, peptic ulcer disease, cholelithiasis, diverticulitis, ischemic bowel disease, chronic constipation, and somatisation of anxiety (Morris, 2006). It has been also reported that the occurrence of coeliac disease is increased in children with WS (9.6%) in comparison with the general population (0.5%) (Giannotti *et al.*, 2001).

Infants and young children with WS are often affected by hypotonia and lax joints, while older children and adults demonstrate hypertonia and hyperreflexia together

with fine motor disturbances leading to difficulty with tool use and handwriting at all ages (Chapman *et al.,* 1996; Morris, 2006). The gradual tightening of heel cords and hamstrings results in a stiff gait, lordosis and kyphosis by adolescence (Morris *et al.*, 1988; Kaplan *et al.*, 1989). Progressive joint limitation affects coordination and motor skills, thus the impairment of oculomotor control, dysmetria, limb and gait ataxia are frequently found in WS population (Pober & Szekely, 1999).

Another neurological abnormality among individuals with WS is Chiari malformation type I found in both children and adults. It is the malformation of the brain caused by displacement of the cerebellar tonsils below the level of the foramen magnum (Kaplan *et al.,* 1989; Pober & Filiano, 1995) and gives such symptoms as headache, neck and arm pain, upper extremity weakness, difficulty swallowing, nausea and impaired coordination (Jacob & Rhoton, 1997). In addition, it has been reported that sleep disordered breathing problems are highly prevelant in all age groups of patients suffring from Chiari malformation (Dauvilliers *et al.,* 2006; Murray *et al.,* 2006). Using high-resolution imaging, it has also been found that the corpus collosum, which is the largest inter-hemispheric commissure in the brain, as well as the whole brain size is reduced in individuals with WS (Schmitt *et al.,* 2001; Chiang *et al.,* 2007). There is a reduction in volume of both, white matter (by 18 %) and grey matter (11 %) with posterior regions showing greater reduction in comparison to frontal ones. The volume of cerebellum is however relatively preserved (Reiss *et al.,* 2000; Reiss *et al.,* 2004; Chiang *et al.,* 2007). In addition, altered neuronal packing density, as well as abnormal folding patterns of cerebral cortex have been reported (Galaburda *et al.,* 2002; Van Essen *et al.,* 2006).

Relationship between brain abnormalities and cognition in WS is discussed in Section 1.3.3)

WS is diagnosed clinically (Morris, 2006) and confirmed by the detection of hemizygosity at the elastin locus using fluorescence in situ hybridization (FISH) analysis (Figure 1.15.) or other molecular techniques, such as microsatellite analyses, microarrays and multiplex ligation-dependent probe amplification (Borg *et al.*, 1995; Smoot *et al.*, 2005, Rees *et al.*, 2012). These methods detect the deletion in 95-99% of patients with clinical diagnosis and allow an approximate estimation of the deletion size (Smoot et al., 2005; Morris, 2006; Shubert, 2009). Individuals with clinical features of WS, whose FISH test did not detect the deletion in WS critical region (WSCR) are referred for further diagnosis involving atypical deletion or other diagnoses (Smoot *et al.*, 2005).

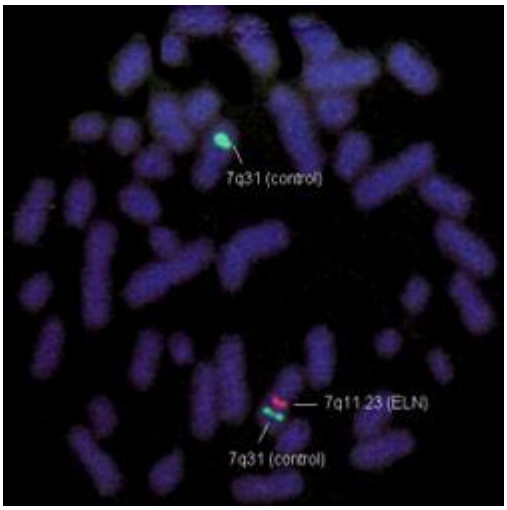


Figure 1.15. FISH test using elastin probe (red) from chromosome 7q11.23 and a control probe (green) from chromosome 7q31 showing the absence of elastin gene from the individual with WS (http://www.dnalabsindia.com/neonatologist_pediatrician.php).

1.3.2. Genetics

WS is an autosomal genetic disorder caused by microdeletion of 28 coding genes, which have been identified so far (Shubert, 2009) on the long arm of chromosome 7 (7q11.23) including *ELN* (Ewart *et al.*, 1993; Chen, 2006). The mutation is spontaneous, however, the transmission from parent to child can occasionally occur (Morris, 2006). The common deletion of 1.5 to 1.8 mega base pairs (Mb) of DNA is referred to as the WSCR and occurs with equal frequency on the maternally or paternally inherited chromosome 7 (Perez Jurado *et al.*, 1996; Chen, 2006). The WSCR includes a single copy gene region of approximately 1.2 Mb flanked by three large low-copy-repeat sequences (LCR), set up in LCR blocks A, B and C. These LCR blocks are arranged in different complexes located on centromeric (C-A-B cen), medial (C-B-A mid) and telomeric (B-A-C tel) part of WS locus. Each of these complexes has a size of approximately 320 kb and includes one of each block (Chen, 2006) (Figure 1.16.). As seen in the case of other microdeletion syndromes, the deletions in the WSCR develop due to high similarity of LCR blocks, especially block B. These highly homologous sequences predispose to unequal crossing-over and misalignment of gametes during meiosis (Francke, 1999; Smoot *et al.*, 2005; Chen, 2006).

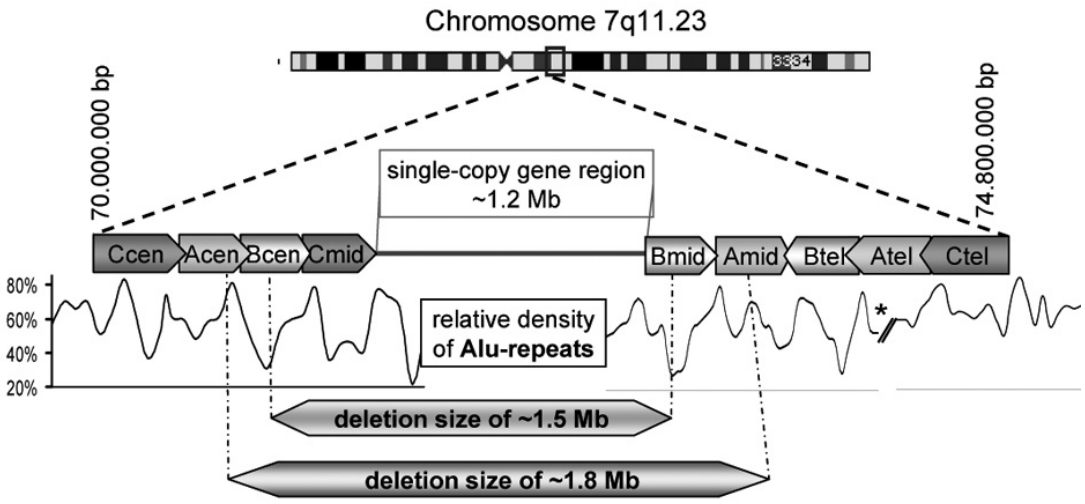


Figure 1.16. Graphical view of Williams syndrome critical region (WSCR) and the density of *Alu*-repeats in the section of low-copy repeat (LCR) blocks. *Alu*-repeats are thought to predispose regions for homologous recombination, causing genetic exchanges such as duplications, deletions and translocations. The horizontal arrows show the common deletion sizes of ~1.5 Mb and ~1.8Mb (Chen, 2006).

Another, much rarer consequence of these homologous duplications is an inversion of WSCR found in approximately 7% of the general population (Osborne *et al.*, 2001). In almost all cases the inversion carrier is clinically normal, however, the risk of conceiving a child with typical WS is increased by 4 (Morris *et al.*, 2004).

The minimal critical region (MCR) for WS, as well as genotype-phenotype associations have been defined studying individuals with atypical deletions (Botta *et al.*, 1999; Tassabehji *et al.*, 1999; Karmiloff-Smith *et al.*, 2003; Tassabehji *et al.*, 2005; Edelman *et al.*, 2007) (Figure 1.17.). The MCR defines nine genes from *ELN* to *GTF2I*. It has been reported that loss of one copy of the genes from MCR is both, sufficient and necessary to cause full WS (Wu *et al.*, 1998).

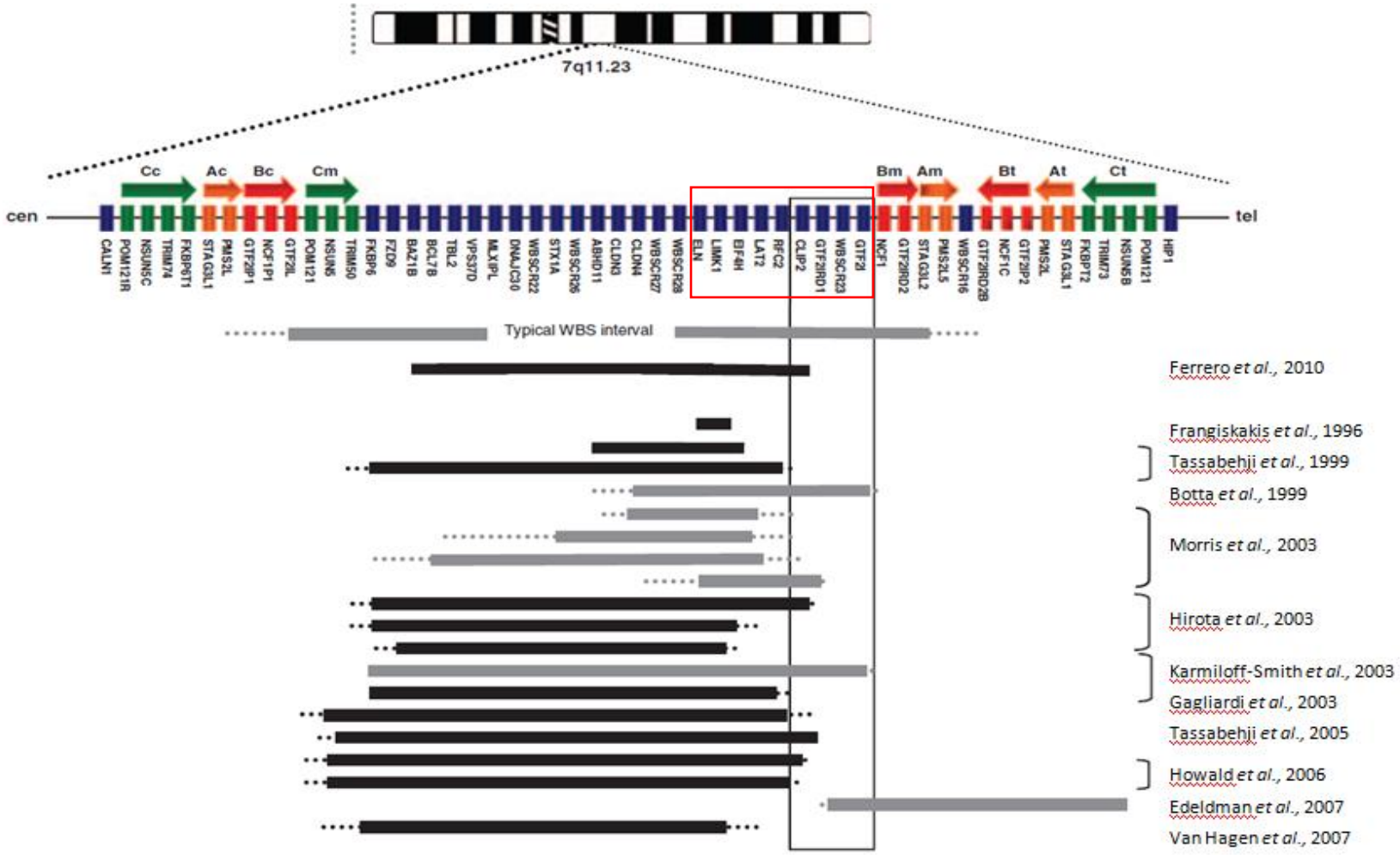


Figure 1.17. Summary of deletion mapping of WS classical and atypical patients. A schematic representation of the genes mapping at 7.11.23

critical region is shown below the human chromosome pictogram. The thick horizontal arrows represent the three large blocks of low-copy repeats, labeled as A, B and C, with centromeric (c), medial (m) and telomeric (t) sequence indicated. The typical 1.54 Mb WS deletion interval is depicted below as a solid like, whereas the dotted lines represents extended boundaries of the rarer 1.84 Mb deletion. The solid lines below show deletion mapping of WS atypical patients reported in the literature. Gray bars represent deletions of WS patients reported by authors with typical and well-documented WS cognitive profile, while black bars represent deletions of WS patients with absent or not well-defined WS cognitive profile. Deletion mapping of WS atypical cases defined a critical region for the visual-spatial construction, cognition and social behaviour in WS (depicted by a gray vertical rectangle). The minimal critical region for WS consisting of nine genes is depicted in a red rectangle. (Ferrero *et al., 2010).*

For a few genes, genotype/phenotype correlation have been established. Details of the genes in the WSCR as well as their proposed function and role in WS phenotype are summarized in Table 1.4. Numerous studies demonstrate that the deletion of *ELN* causes the WS cardiovascular phenotype (SVAS) (Metcalfe *et al.*, 2000; Micale *et al.* 2010). *ELN* encodes tropoelastin. Multiple molecules of this protein bind together forming mature elastin protein. Elastin is the major component of elastic fibers, that provide strength and flexibility to connective tissue (Brooke *et al.*, 2003; Harvey & Ferrier, 2011). In WS, production of tropoelastin is reduced by half, what reduces the amount of mature elastin protein and consequently disrupts the normal structure of elastic fibers in many connective tissues. As a result, elastic fibers that make up large blood vessels are thinner than normal. To compensate, the smooth muscle cells that line the large blood vessels, e.g. aorta, increase in number, making the vessel thicken, narrower and as a result less flexible and resilient than normal. These vessels can narrow, increasing the resistance to normal blood flow and leading to serious medical problems (Tassabehji at al., 1998; Metcalfe *et al.*, 2000; Harvey & Ferrier, 2011). Haploinsufficiency for another gene found in the WSCR- *LIMK1*, encoding LIM-kinase1 protein, was proposed to be responsible for hyperacusis and

progressive hearing loss (Matsumoto *et al.*, 2011). LIM-kinase1 protein is highly expressed in neurons and plays role in regulating cytoskeletal structure via actin filaments (Smoot *et al.*, 2005). Several findings report that the most telomeric genes in the WSCR (*CYLN2*, *GTF2IRD1*, *GTF2-I*) may be responsible for learning difficulties in WS (Francke, 1999; Hoogenraad *et al.*, 2002; Hakimi *et al.*, 2003). Individuals with WS, who have a deletion excluding some of these three genes or all of them, have borderline to normal intelligence and varying degrees of impairment of visuospatial functioning (Morris *et al.*, 2003). In addition, *GTF2-I* haploinsufficiency could be a contributor to the hypersociability in WS individuals (Sakurai *et al.*, 2011). Furthermore, there are studies suggesting association of *GTF2IRD1* and *BAZ1B* (Tassabehji *et al.*, 2005; Ashe *et al.*, 2008) with craniofacial features as well as *STX1A* with diabetes mellitus (Lam *et al.*, 2005). It has been also reported that the haploinsufficiency of *BAZ1B* and *TFII-I* may contribute to hypercalcaemia in WS (Osborne, 2010; Letavernier *et al.*, 2012).

Direct linkage between structural brain abnormalities and neurobehavioral profile in children with WS has also been suggested (Wang *et al.*, 1992; Volterra *et al.*, 1996; Fahim *et al.*, 2012). In addition, some of the genes deleted in WS, such as *STX1A* and *LIMK1* are expressed in brain prenatally and/or postnatally (Frangiskakis *et al.*, 1996; Osborne *et al.*, 1997), while others, such as the gene for replication factor C subunit 2 (RFC2), may be involved in brain development (Peoples *et al.*, 1996). The protein product of RFC2 is a part of multimeric complex involved in DNA elongation during replication (Chen *et al.*, 1992). Thus, it has been suggested that a gene in the deletion region may contribute to the sleep disorders in individuals with WS (Arens *et al.*, 1998). However, none of the studies reported so far confirmed the

association between genes in WS deletion region and sleep problems as well as/or levels of endocrine markers of circadian rhythm and sleep.

Gene	Protein Function	Proposed role in WS phenotype	Reference
ELN	Plays an essential role in a structure of connective tissue	Cardiovascular and connective tissue abnormalities	Metcalfe *et al*., 2000; Micale *et al*., 2010
LIMK1	Involved in the normal development of the central nervous system, as well as regulation of cytoskeletal structure	Possible role in cognition	Frangiskakis *et al*., 1996; Tassabehji *et al*., 1996; Smoot *et al*., 2005
CYLN2	Role in attaching endosomes to microtubules	Possible role in learning difficulties, involved in normal cognitive and brain development	Francke, 1999; Hoogenraad *et al*., 2002
GTF2IRD1	Transcription factor involved regulation of tissue development and differentiation	Possible role in learning difficulties, involved in normal cognitive and brain development, also possible associations with craniofacial features as well as maintaining normal circadian locomotor activity and endocrinological response to stress	Francke, 1999; Franke *et al*.,1999; Tassabehji *et al*., 2005; Schneider *et al*., 2012
GTF2-I	Transcription factor involved regulation of tissue development and differentiation	Possible role in learning difficulties, involved in normal cognitive and brain development	Danoff *et al*., 2004; Dai *et al*., 2009
GTF2IRD2	Involved in development of facial features and tooth formation	Unknown	Tipney *et al*., 2004
BAZ1B	Part of protein complex involved in replication, transcription and chromatin maintaince	Abnormal vitamin D metabolism and hypercalcaemia, also possible associations with craniofacial features	Kitagawa *et al*., 2003; Ashe *et al*., 2008
STX1A	Plays a vital role in neurotransmitter release	Proposed role in normal cognitive development and intelligence. Associations with diabetes have also been found	Nakayama *et al*., 1998; Lam *et al*., 2005; Gao *et al*., 2010
EIF4H	Positive regulator of protein synthesis at the level of translation initiation	Unknown	Francke, 1999; Majka *et al*., 2004

LAT2	Membrane-associated adaptor molecule	Unknown	Martindale *et al.*, 2000
RFC2	Part of multimeric complex involved in DNA elongation during replication	Possible role in brain development	Peoples *et al.*, 1996; Majka *et al.*, 2004
NCF1	Plays a role in the immune system and electron transport	Role in hypertension	Del Campo *et al.*, 2006
ABHD11	Expressed ubiquitously in body although biological function unknown	Unknown	Merla *et al.*, 2002
BCL7B	Unknown	Unknown	Francke, 1999
CLDN3/ CLDN4	Involved in formation and function of tight-junctions of epithelial cells	Unknown	Francke, 1999; Bagherie-Lachidan *et al.*, 2009
FKBP6	Highly expressed in human testes, essential role in homologous pairing in meiosis and in male fertility	Unknown	Crackower *et al.*, 2003
MLXIPL	Transcription factor that activates expression of glucose-regulated and lipogenic genes	Unknown	Li *et al.*, 2010
NSUN5	Putative methyltransferase	Unknown	Shubert *et al.*, 2009
TBL2	Gene product unclear, may play a role in tumour suppression	Unknown	Perez-Jurado *et al.*, 1999
TRIM50	Possible role in ubiquitin-mediated proteasome pathway	Unknown	Micale *et al.*, 2008
VPS37D	Role in regulation of a vesicular trafficking process, it is a part of endosomal sorting complex required for transport	Unknown	Shubert *et al.*, 2009
WBSCR22	Strongly expressed in testes, protein function unknown	Unknown	Doll & Grzeschik, 2011

Table 1.4 Genes within the Williams syndrome critical region (WSCR) and their proposed protein function as well as proposed role in the WS phenotype.

1.3.2.1. Mouse models

The WS mouse models play an important role in defining genotype/phenotype correlations. Due to the fact that atypical WS patients are very rare, the statistical significance as well as conclusive evidence is difficult, often impossible to obtain. Creating animal models with targeted single or multiple gene knockouts and then carrying out testing in controlled environment helps to overcome these issues. Mice are ideal candidates to model human genetic disorder, due to the fact that 99 % of a mouse genome sequence has direct counterparts in humans, thus, genomic alternations should affect the same biological pathways throughout development and result in similar phenotypes (Gunter & Dhand, 2002) . Figure 1.18 presents the organisation of human and mouse WS regions.

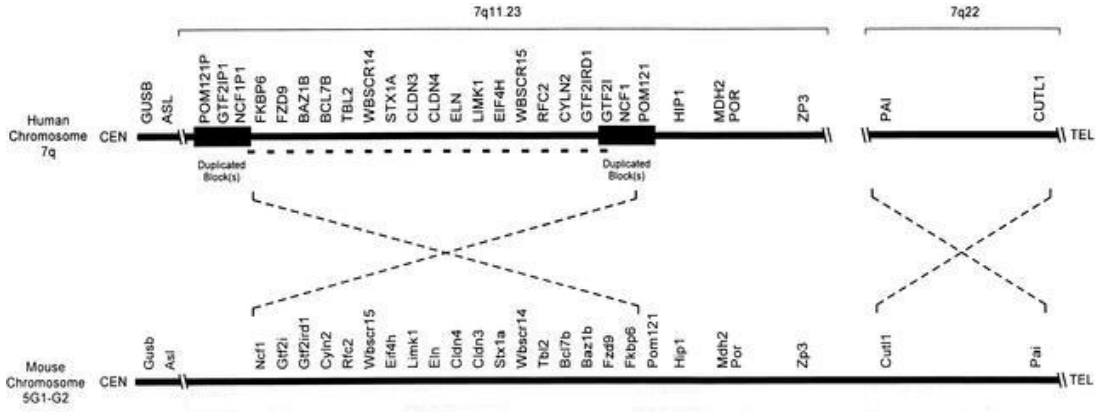


Figure 1.18. Long-range organisation of human and mouse Williams syndrome regions. Figure presents a physical map of the WS regions on human chromosome 7q and mouse chromosome 5G. The position of the known genes residing within and flanking the interval commonly deleted in WS is emphasised. Bold dashed lines indicate human WS region of ~1.6 Mb interval, which is flanked by duplicated blocks of DNA of near-identical sequence (estimated at ~300 kb in size; indicated by dark rectangles). The relative positions of the centromere (CEN) and telomere (TEL) are also indicated. The inverted orientation of the two discontiguous segments of human chromosome 7 relative to the single contiguous segment of mouse chromosome 5G are also shown (DeSilva *et al.,* 2002).

In order to determine how the genes within WSCR contribute to WS phenotype, Li *et al.* (2009) created knockout mouse models: proximal deletion mice lacking *Gtf2i* to *Limk1*, distal deletion mice lacking *Limk1* to *Fkbp6* and the double heterozygotes model with complete human deletion. It has been found that increased sociability, but also higher level anxiety as well as growth retardation was associated with proximal deletion, while cognitive defects and deficits in locomotor activity with distal deletion. Findings of this study have also suggested that genes within both, the proximal and distal deletion are involved in craniofacial and brain development. In line with these findings, Tassabehji *et al.,* (2005) reported craniofacial abnormalities in mouse model of *Gt2ird1* located in proximal deletion region, while Ashe *et al.* (2008) reported similar findings, but using *Baz1b* mouse model located in distal deletion region. Several other studies have created single-gene knockout mouse models of WS and these are summarised in Table 1.5. The study of Schneider *et al.,* (2012) is of particular interest for the current work. The authors examined *Gt2ird1* knock-out mice and reported that *Gt2ird1* null mice showed decreased circadian locomotor activity, increased anxiety and elevated endocrinological response to stress, while *Gt2ird1* heterozygous mice displayed decreased circadian activity.

Gene	Phenotype	Reference
FKBP6	Fertile with no phenotype observed	Crackower *et al.*, 2003
FZD9	Diminished seizure threshold, abnormal hippocampal structure	Zhao *et al.*, 2005; Ranheim *et al.*, 2005
BAZ1B	Mild craniofacial abnormalities, low frequency of cardiac malformations	Ashe *et al.*, 2008;
STX1A	no deficits in learning and memory, anxiety or locomotor activity observed (small number of mice tested)	Fujiwara *et al.*, 2006; McRory *et al.*, 2008
ELN	Hypertension, decreased aortic compliance and mild cardiac atrophy	Li *et al.*, 1998; Faury *et al.*, 2003; Goergen *et al.*, 2011
CLIP2	Mild growth deficiency, hyppocampal dysfunction, deficits in motor coordination	Hoogenrad *et al.*, 2002
GTF2IRD1	Mild growth deficiency, hypersociability, learning and memory deficits, craniofacial abnormalities, decreased circadian activity	Tassabehji *et al.*, 2005; Young *et al.*, 2008; Palmer *et al.*, 2010; Schneider *et al.*, 2012

Table 1.5. Phenotypic features of single gene heterozygous mouse models of WS (with haploinsufficiency of one copy of genes listed on the left hand side of the table) (adopted and modified from Osborne *et al.*, 2010).

1.3.3. Behavioural and cognitive profile

The behavioral phenotype for WS is characterised by a distinctive cognitive profile with a characteristic pattern of strengths and weaknesses and unique personality profile (Semel & Rosner, 2003). Most individuals with WS have mild to moderate learning difficulties with mean IQ between 50-60 (Karmiloff-Smith *et al.*, 2003; Smoot *et al.*, 2005). Some of the relative strength abilities in WS cognitive profile include language skills, face recognition, auditory rote memory and musical skills

(Morris & Mervis, 2000; Smoot *et al.*, 2005; Mervis & Velleman, 2011). Although speech is usually delayed with the first words at about 2 years and first sentences at about 3 years, older children and adolescents have a language rich in affect, prosody and narrative enrichment (Bellugi *et al.*, 1994). Several studies reported that children with WS have better auditory rote memory abilities than children with learning difficulties of mixed aetiologies, matched by chronological age (Bellugi *et al.*, 1994; Jarrold *et al.*, 1998; Klein & Mervis, 1999). Face recognition in WS is good albeit atypical mechanism of recognition has been noted (Karmiloff-Smith, 1998; Karmiloff-Smith *et al.*, 2004; Annaz *et al.*, 2009). Some significant weaknesses are within the domain of visuospatial functioning (Mervis & Klein-Tasman, 2000). Many individuals with WS have great problems with hand writing, perceptual planning and drawing objects in a disjointed manner (Bellugi *et al.*, 1988; Bellugi *et al.*, 1992; Wang *et al.*, 1995; Bertrand *et al.*, 1997; Klein & Mervis, 1999). As no correlation has been observed between visuospatial cognition and severity of ocular problems in WS, it was suggested that this deficit has its origins in the brain (van der Geest *et al.*, 2004). Abnormal frontoparietal circuitry is thought to underlie the visuospatial weekness (Galaburda *et al.*, 2002, Atkinson *et al.*, 2003; Meyer-Lindenberg & Zink, 2007). Meyer-Linderberg *et al.* (2004) have shown hypoactivation in the parietal portion of the dorsal stream, which is one of the sensory pathways of primary visual cortex, when participants focused on the spatial location of objects and faces. Moreover, the volume of grey matter in a region neighbouring the dorsal stream was also found to be reduced. Thus, the authors suggested that lack of neuronal integrity in this region may prevent information coming from primary visual cortex from further dorsal stream processing (Meyer-Linderberg *et al.*, 2004). It has aslo been found that adult WS patients have reduced

activation in the visual and parietal cortices in comparison to age and gender-matched healthy controls, during a global processing task (Mobbs *et al.,* 2007). Both studies were performed using functional magnetic resonance imaging (fMRI). Difficulties with reading, spelling and especially mathematics are also observed and WS individuals often reach only about 7 to 8 year level in these areas (Howlin *et al.*, 1998).

The behavioural phenotype for WS is characterised by their unusual personality profile. Hypersociability is the most noticeable behavioural feature (Carrasco *et al.*, 2005; Jones *et al.*, 2000) as people with WS are often described as overly friendly (Gosch & Pankau, 1997), outgoing (Beuren *et al.*, 1962), approachable (Tomc *et al.*, 1990), never going unnoticed in a group (Dykens & Rosner, 1999) and highly empathic (Gosch & Pankau, 1994), but also as lacking social judgment skills (Einfeld *et al.*, 1997). On the other hand, high level of anxiety, worries and fears has also been reported (Dykens, 2003), however, the degree and intensity of anxiety may vary over time (Smoot *et al.*, 2005). Dykens (2003) examined anxiety and phobia in 120 children and adults with WS (age 6 - 48 years) compared to 70 IQ, age and gender-matched individuals with learning difficulties of mixed or nonspecific aetiologies. It was found that 96 % of WS participants showed persistent anxiety producing fears for at least 6 months and 35 % met all criteria for clinical phobia with half of them having more than one subtype of phobia. Sub-clinical phobia was found in 84 % of WS individuals, meeting all criteria for phobia apart of impaired adaptive functioning. The natural environment subtype was seen in 94 % (72 % of these had fears of thunderstorms and 55 % of high places), 44 % showed other types (e.g. afraid of being alone), and 22 % showed the animal subtype. The least amount

of fears were found in boys and girls aged 6 to 12 years. Fears generally increased with advancing age, often peaking in the adult years. Longitudinal course of anxiety in children and adolescents with WS (n = 45, aged 4 – 13 years) was also examined for a period of at least 1 year (Woodruff-Borden *et al.,* 2010). Of the entire study sample, 51.1 % was diagnosed with anxiety. Developmental trajectories have shown persistence of anxiety over time and suggested that the odds of a positive diagnosis did not change with time. Moreover, chronic, persistent anxiety within the period of 5 years after initial diagnosis was shown in 62.2 % of those WS individuals with anxiety diagnosis. Another study has also examined anxiety in 132 children with WS (4 – 16 years old) (Leyfer *et al.,* 2009). Children with WS showed significantly greater prevalence of specific phobia, generalised anxiety disorder and separation anxiety in comparison to children in general population. Both studies showed that anxiety is highly prevalence in all ages in the WS population. Furthermore, Lense *et al.,* (2013) reported elevated cortisol levels in response to the novel settings (residential summer camp), what could be a result of increased anxiety.

The anxiety profile in WS may be explained by abnormal activity in the amygdala, that is a complex brain structure, which is activated when the senses perceive something of special significance. It plays a crucial role in processing, controlling and memorising the emotional reactions (Ono *et al.,* 1995; Talarovicova *et al.,* 2007) as well as in the development and expression of fear (Davis, 1992). It has been shown that lesions of the amygdala directly linked to its cortical regions, can cause disinhibition and impaired social function (Bauman *et al.,* 2004; Willis *et al.,* 2010). In 13 adults with WS, Meyer-Lindenberg *et al.* (2005) found significantly diminished amygdala activation when viewing images of threatening or angry faces, relative to age and gender-matched healthy control participants. This reduction in the

responsiveness to social danger may contribute to the social disinhibition and diminished fear of strangers that is characteristic of WS. Conversely, participants with WS showed abnormally increased activation when viewing threatening non-social scenes, suggesting a likely neural basis for the high levels of non-social anxiety in WS (Figure 1.19).

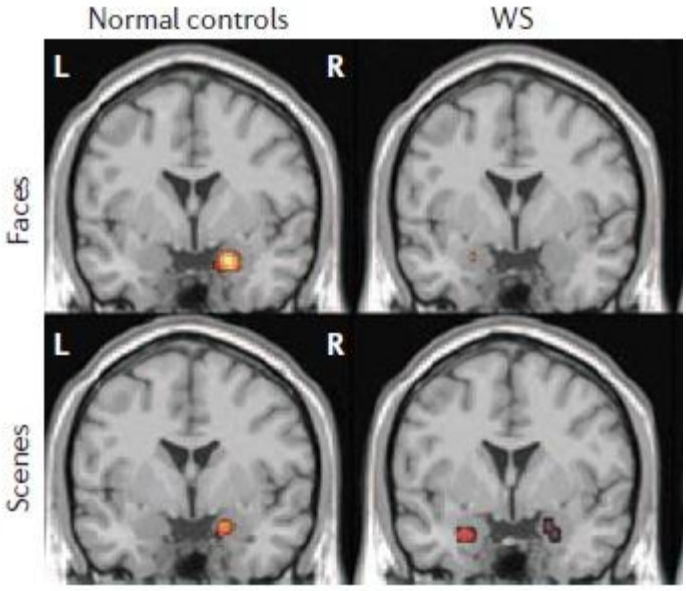


Figure 1.19. Abnormal regulation of the amygdala in participants with WS (right) compared to controls (left). The amygdala activates more for threatening scenes (bottom), but less for threatening faces(top) (Meyer-Lindenberg *et al.*, 2005).

There are also studies suggesting the link between activation of amygdala and the regulation of REM sleep (Benca *et al.*, 2000; Sanford *et al.*, 2002). Amygdala is heavily interconnected with brainstem nuclei known to be involved in sleep control, however, it has received little attention from sleep researchers (Morrison *et al.*, 2000; Jha *et al.*, 2005). It has been shown that electrical activation of central nucleus of the amygdala can increase the occurrence of REM sleep (Sanford *et al.*, 2002). Benca *et al.* (2000) examined the effects of amygdala lesions on sleep in rhesus monkey in comparison to normal controls. It was found that sleep was least disrupted in animals

with large bilateral lesions of the amygdala. These animals had more sleep and higher proportion of REM sleep than did animals with smaller lesions and control group. Based on these results, the authors suggested that the amygdala plays a role in sleep regulation in primates and may be important in mediating the effects of emotions and/or stress on sleep.

In addition, recent study of Schneider *et al.,* (2012) on WS mouse models, suggested possible links between the loss of *Gtf2ird1* function and a loss of hypothalamic-pituitary function, that could result in an increased anxiety.

Problems with attention are also very common in WS and they manifest as hyperactivity or ADHD (Udwin & Yule, 1991).

The area of weakness of adaptive behaviour profile in WS includes self-care and daily living skills (Mervis & Klein-Tasman, 2000). Individuals with this syndrome usually achieve approximately 6 year old levels for personal self-care (Plissart *et al.*, 1994).

1.3.4. ***Characteristics of sleep in Williams syndrome***

Parents of children with WS often report sleep-related problems, including difficulty falling asleep and frequent and prolonged awakenings, however, it is still an aspect of WS phenotype that has yet to be adequately characterised (Arens *et al.,* 1998; Annaz *et al.,* 2011; Gombos *et al.,* 2011*;* Mason *et al.,* 2011*;* Ashworth *et al.,* 2013*).* Thus far, very few studies were carried out to look specifically at sleep problems in WS and all of them were based on data collected through questionnaires and PSG (Arens *et al.,* 1998; Annaz *et al.,* 2011; Gombos *et al., 2011; Mason et al., 2011;* Ashworth *et al., 2013).* At the start of the current PhD, two studies reporting sleep

problems in individuals with WS were published (Arens *et al.,* 1998; Goldman *et al.,* 2009), however, several others were published during the course of the work (Annaz *et al.,* 2011; Gombos *et al., 2011; Mason et al., 2011;* Ashworth *et al., 2013*). In one of the studies, parents of 28 children were questioned about the possible sleep problems with their children (Arens *et al.,* 1998). The results of that examination showed that 57% of these children had symptoms compatible with sleep disorders. Of the 16 children screened for possible movement arousal sleep disorder, 7 agreed to have PSG measurements, that revealed that WS children also have a disorder of periodic limb movement in sleep (Arens *et al.,* 1998). Another study examined sleep patterns in adolescents and adults with WS (n = 23) aged 17 to 35 years, using structured interviews, sleep questionnaire and actigraphy. The results showed that 36.4 % had trouble sleeping, 65 % reported two or more awakenings per night and as much as 95.2 % of the participants felt tired during the day (Goldman *et al.,* 2009). In one of the largest questionnaire based studies (n = 64), Annaz and colleagues (2011) reported sleep problems in school-aged children with WS. Based on Children's Sleep Habit Questionnaire (CSHQ) parents reported significantly greater bedtime resistance, sleep anxiety, night walking and daytime sleepiness compared with typically developing controls. One of the most recent studies by Gombos and colleagues (2011) examined sleep architecture, leg movements and the EEG using PSG in adolescents and young adults with WS (n = 9) aged from 14 to 28 years. The data were compared with age- and gender- matched healthy controls (n = 9, 14 to 29 years of age). Obtained data have shown atypical sleep patterns in WS individuals with decreased sleep time, sleep efficiency and REM sleep percentage, and increased wake time after sleep onset, NREM sleep percentage and SWS. The number of leg movements was also higher in WS and the sleep cycles appeared to be irregular

compared to healthy controls. Similar results were obtained by Mason and colleagues (2011) who used a larger number of participants including 35 WS and 35 TD children (age 2-18 years). The study was based on an overnight PSG and questionnaires regarding the participants' sleep and behaviour, which were completed by parents. Obtained results confirmed previous findings that individuals with WS also have decreased sleep efficiency, increased SWS, greater difficulty to fall asleep, higher level of restlessness and arousals from sleep in comparison to the TD control group. The most recent study by Ashworth and colleagues (2013) examined sleep problems and syndromic specificity. The authors compared 6 to 12 years old children in two clinical groups, namely DS (n=22) and WS (n=24). The results showed that children with WS have significant problems initiating sleep, for example bed-wetting and body pains were also reported by parents.

Lastly, based on the hypothesis that sleep problems may affect sleep-dependent learning, Dimitriou and colleagues (2013) investigated the impact of sleep on a motor memory task. The authors examined 12 children with WS and 15 TD children (age range 6 to 12 years, mean = 8.6) on the finger tapping task. Children were trained on the task in the evening and retested the following morning and afternoon. Following sleep period, the control group dramatically improved in performance accuracy but there was no evidence of sleep-related learning in WS. However, the lack of improvement in the WS group cannot be attributed to sleep problems *per se* before more studies can confirm this finding.

To date, there has been no study analysing biological properties of sleep problems in WS. As sleep problems in children with WS can adversely affect daytime activity and the quality of life, as well as social, emotional, health and economic functioning

of the entire family, it is important to address this issue as it will affect the quality of life of the child and family.

Summary

This chapter has discussed sleep characteristics as well as physiological and cognitive phenotypes of individuals with WS and techniques used for sleep monitoring and analysis of endocrine markers of sleep. The effects of sleep problems in WS children are wide-ranging, yet precious little research has focused on the causal mechanism of sleep problems in children with disorders. Sleep problems, behavioural problems, and having a child with a disability all contribute to maternal stress and depression. Chronic sleep disruption may also negatively impact parental perception of the child (Hayes *et al.* 2001). Sleep and its consequences is an area that requires thorough investigation in children with WS as these groups clearly experience significant sleep problems. As has been shown, sleep problems are often amenable to treatment, so alleviating these problems could have a beneficial effect on daytime functioning and a knock-on effect on children's families and maternal well-being. It is hence vital to assess sleep problems in disorder groups in more depth using multidisciplinary approaches.

AIMS OF THE STUDY

As described previously, the evening rise of melatonin secretion and correlated with that fall in body temperature, facilitate sleep. In direct contrast, cortisol whose secretion increases before dawn, prepares us for physical and mental demands of awaking, by increasing heart rate, blood pressure and releasing glucose to our blood stream (Seaward, 2006). If these mechanisms are disturbed, sleep disorders/problems may occur.

The aims of this study are:

1. To define sleep quality and quantity using actigraphy and compare the results to parent-reported sleep data for school-aged TD children and WS.
2. To quantify nocturnal oxyhaemoglobin saturation and indices that can predict OSAS in these groups.
3. To quantify and compare levels of melatonin in children with WS and TD.
4. To quantify and compare levels of cortisol in children with WS and TD.
5. To examine if sleep quality and quantity are related to melatonin and cortisol levels.
6. To assess whether age, gender and environmental factors have an influence on sleep patterns and secretion of melatonin and cortisol.

It is predicted that children with WS have worse sleep quality than age- and gender-matched healthy controls (Gombos *et al.*, 2011; Mason *et al.*, 2011; Ashworth *et al.*, 2013). As problems with settling down by WS individuals have been reported, abnormal levels of cortisol and/or melatonin before night time sleep may be observed as well.

2. METHODOLOGY- ANALYSES OF SLEEP QUALITY AND QUANTITY

In this chapter participants' characteristics and the procedures of objective and subjective sleep analysis are presented. Sleep measurements described in this chapter include actigraphy for monitoring a persons' activity, pulse oximetry for the measurement of pulse rate and oxygen saturation in order to determine whether there are problems related to breathing that may obstruct sleep. This chapter also outlines the standard sleep questionnaire as well as medical questionnaire used to assess whether there are any medical conditions which may have an impact on sleep in study participants.

2.1. Participants

2.1.1. Ethical approval

All studies were approved by the Middlesex University Natural Sciences Ethics sub-Committee (Approval Letter 524) prior to recruitment of participants. In the case of participants with WS, a formal research proposal was submitted and accepted by the WS Professional Advisory Panel. Each parent was sent detailed information about the studies (Appendix 1). Parents then returned a slip indicating whether or not they would like their children to take part in the studies. Each child was also verbally asked if s/he would like to take part in the study.

2.1.2. Participants

2.1.2.1. Children with WS

Primary school-aged children with WS (13 male, 14 female; four to eleven years of age) were identified from the database provided by the Williams Syndrome Foundation, UK. Parents of 35 children were contacted and of these 27 agreed to take part in the studies, yielding an overall response rate of 77%. This number represents approximately 30% of all school-aged children with WS in the United Kingdom. All children with WS were diagnosed clinically and tested positive on the FISH method for detecting hyploinsufficiency for *ELN*.

2.1.2.2. Typically developing control group

The control group consists of 27 typically developing (TD) children (12 male, 15 female) age- and gender- matched with WS group and selected from mainstream schools of Greater London and South-East UK.

2.1.3. Exclusion criteria

Due to the nature of the studies, children were not included in the study if they had co-morbid medical or psychiatric disorders such as ADHD or autism or if they were taking prescribed medication for sleep problems. Upon analysis it was noted that two children with WS were taking melatonin for their sleep problems, thus, the data

obtained for these children were excluded from statistical analysis and analysed separately from the rest of the group. The age range of the participants was 4 to 11 years. This range was chosen in order to avoid puberty.

2.1.4. Challenges with testing young children and children with developmental disorders

Given the rarity of WS, and difficulty in gaining access to young children it is often challenging to recruit large groups, especially if the study is focusing on a relatively narrow age range. However, it is important to employ sufficient participant numbers to disentangle systematic variation between groups from random noise, particularly if the tests involved produce only a small range of scores (e.g. CSHQ having 3-point scale range). Also, travel to collect the samples was critically important and required very careful logistical planning since children were living far away from each other (See Figure 2.1.).

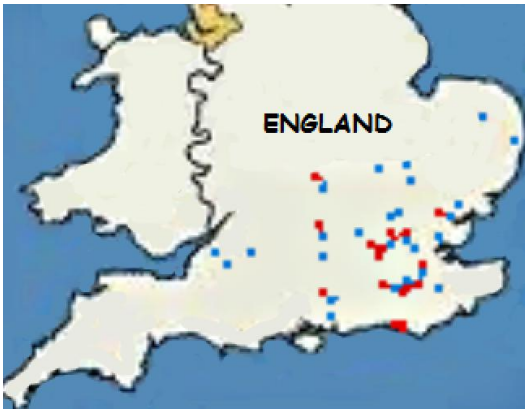


Figure 2.1. Map presenting families visited during the course of this study. Blue dots represent families having a child with Williams syndrome, while red dots represent typically developing children.

2.2. Actigraphy

Each child was given an Actiwatch Mini (CamNTech, Cambridge, UK) (Figure 2.2.) to objectively assess their sleep patterns. This was worn around the non-dominant wrist, as if wearing a watch, and children were asked to wear it continuously for 3 consecutive days and nights. As described in the introduction (Section 1.2.1.2), the actiwatch contains accelerometer measuring activity and movements of an individual. The Actiwatch Activity & Sleep Analysis 7 software (CamNTech, Cambridge, UK) was used to download data from the actiwatch at the default 'medium' sensitivity level. The programme uses an algorithm to score each one-minute epoch as sleep or wake based on movement during that minute, as well as the two preceding and two successive minutes. The activity scores from adjacent minutes influence the total score in the following way: within 1 min of the scored epoch activity levels are reduced by a factor of 5 and this value is added to the scored value; within 2 min of the scored activity epoch, activity values are reduced by a factor of 25 and this value is added to the scored value. A total score of 40 or more determines the epoch as being "Awake". For the determination of "Sleep start" the algorithm looks for a period of at least 10 min of consecutive immobility with no more than 1 epoch of movement within that time, following the "Bed time". The start of this period is classified as "Sleep start". The "Sleep end" is determined by the last epoch from consecutive 6 min period of activity below the threshold. Within this period two epochs above the threshold are allowed. The threshold necessary to equate to "Sleep end" is determined by 6 or less activity counts per minute. During the night, there are at least low activity recordings visible on the actigram, whereas,

in case when actiwatch is removed by a user, no activity recordings (flat line) can be observed (The Actiwatch Mini user guide, Cambridge Neurotechnology Ltd.).

Figure 2.2. Actiwatch Mini (CamNTech, Cambridge, UK).

Several actigraphy variables were computed from the data including:

- **Time in bed:** the total elapsed time between the "Bed Time" and "Get Up" times.
- **Sleep latency:** time from bed time to sleep start.
- **Assumed sleep time:** time from sleep onset to offset.
- **Actual sleep time:** assumed time asleep minus any period(s) awake.
- **Actual sleep (%):** actual sleep time expressed as a percentage of the assumed sleep time.
- **Actual wake time:** the total time spent awake after sleep onset
- **Actual wake (%): a**ctual wake time expressed as a percentage of the assumed sleep time.
- **Night wakings**: number of night wake bouts.

- **Mean sleep bout time:** average duration of periods of sleep.
- **Mean wake bout time:** average duration of periods of intermittent wakefulness.
- **Sleep efficiency:** actual sleep time expressed as a percentage of time in bed.
- **Moving minutes:** the total number of minutes during sleep where the child is moving.
- **Moving time (%):** percentage of time spent moving from sleep onset to offset.
- **Immobile minutes:** the total number of minutes spent immobile from sleep onset to offset.
- **Immobile time (%):** percentage of time spent immobile from sleep onset to offset.
- **Number of immobile phases:** the number of contiguous sections categorised as immobile in the epoch-by-epoch mobile/immobile categorisation.
- **Mean length immobility:** the average length of each of the immobile bouts.
- **One minute immobility phases:** the number of immobile bouts which were less than or equal to one minute in length.
- **One minute immobility (%):** the number of immobile bouts less than or equal to one minute expressed as a percentage of the total number of immobile bouts.
- **Fragmentation index:** The sum of the moving time (%) and the immobile bouts ≤ 1 min (%). This is an indication of the degree of fragmentation of the sleep period and restlessness, and can be used as an indication of sleep quality (or the lack of it). A higher figure denotes increased restlessness.

- **Total activity score:** the total of all the activity counts during the assumed sleep period.
- **Mean activity score:** the total activity score divided by the number of epochs in the assumed sleep period.
- **Average wake movement:** average activity counts per epoch for the wake period (The Actiwatch Mini user guide, Cambridge Neurotechnology Ltd.).

2.3. Pulse oximetry

Pulse oximeters used in the current study were Masimo Rad-8 monitors (Figure 2.3.). Pulse oximetry was used to measure the pulse rate and oxygen saturation over the same period of three consecutive nights as used for the actigraphy measurements, by an infrared sensor attached to a finger or toe. These monitors are increasingly used for the evaluation of sleep disordered breathing (Netzer *et al.*, 2001). Thus, it is expected that data obtained from pulse oximetry will allow us to determine whether there are any problems related to breathing, such as sleep apnoea that may obstruct sleep. Pulse rate was also monitored. The pulse oximetry variables of interest were: mean and median oxygen saturation (SpO_2), mean pulse rate, number of over-4% dips in SpO_2 per hour, delta index 12s (an index of SpO_2 variability over each 12-second epoch where a higher figure indicates increased variability) and the time spent with SpO_2 under 90% calculated in hh:mm:ss as well as the percentage of analysis interval.

Figure 2.3. Masimo Rad-8 monitor (http://masimo.co.uk/).

2.4. Questionnaires

Parents of all participants were asked to complete the CSHQ (Owens *et al.*, 2000a) (Appendix 2), Medical Questionnaire (Appendix 3), sleep diary (Appendix 4) and asses their child puberty stage using Tanner's scale (Appendix 5).

2.4.1. Children's Sleep Habit Questionnaire

Due to the fact that spontaneous parental reports are prone to bias, CSHQ (Owens *et al.*, 2000a) was used in this study. The CSHQ is standard 45-item parent questionnaire that has been used in a number of studies to examine sleep behaviour in school-age children (Liu *et al.*, 2005; Annaz *et al.*, 2011). Parents answer on a three-point scale, which is: "usually" if the sleep behaviour occurred 5-7 times per week; "sometimes" for 2-4 times per week; and "rarely" for 0-1 time per week.

Thirty-three items from the questionnaire (indicated by starts in Appendix 2) are used to calculate total sleep disturbance score, as well as scores in 8 sub-scales: bedtime resistance, sleep onset delay, sleep duration, sleep anxiety, night waking, parasomnias, sleep disordered breathing and daytime sleepiness. The higher the score obtained on this questionnaire, the greater the sleep problems. It has been reported that psychometric properties of CSHQ are satisfactory, showing good internal consistency, validity and test-retest reliability (Owens *et al.*, 2000a). No norms were established for total score and subscale scores for this questionnaire, however, Owens *et al.* (2000a) reported that using 41 as a cut-off score produce the best diagnostic confidence by correctly identifying 80% of the clinical sample.

2.4.2. Medical Questionnaire

Medical Questionnaire, designed for these studies, consists of 45 items divided into four parts. The first section has questions about the demographic data of the family. Sections second and third are the "medical history" and "health habits" sections of the Pediatric Sleep Clinic Questionnaire for children aged 4 to 12 (Owens, http://www.kidzzzsleep.org). Health habits section also includes questions related to child's sport activity. The last section of this questionnaire consists of questions about child's diet, such as amount of caffeinated drinks, main meals and snacks that a child consumes per day. For most of the questions, the care giver must circle one of the options, i.e. "Yes", "No", "Don't know"; some questions were rated on a five-point scale: "None", "Up to 2 hours", "2-4 hours", "4-6 hours", "More than 6 hours", for instance those regarding the time spent on the TV or computer per day. There are also several open-ended questions, such as those on medications or sport activity.

2.4.3. Tanner's scale of puberty

Tanner scale is a scale of physical maturity of children, adolescents and adults based on the development of primary and secondary sexual characteristics, such as the development of pubic and axillary hair, the testicular volume and the size of genitalia and breasts (see Appendix 5 for detailed description of Tanner's stages) (Marshall & Tanner, 1969; Marshall & Tanner, 1970).

2.4.4. Sleep diary

Parents were requested to complete a sleep diary recording their child's bed time and getting up time for the duration of the study. These were used to support analyses of actigraphy data.

Actigraphy, pulse oximetry and CSHQ were all obtained within the same time and during the weekdays to ensure normal routine.

2.5. Statistical analyses

Unless otherwise stated, the data were analysed using SPSS Version 19 (SPSS Inc., Chicago, IL). In other cases the software used was Minitab Version 16 (Minitab Inc., State College, PA). For the group comparison the independent-samples t-test or Mann-Whitney test were used. The application of adequate test was based on the results from the Shapiro-Wilk test of normality. T-test was used for normally distributed data ($p > 0.05$ on Shapiro-Wilk test) and Mann-Whitney for non-normal

distributions ($p < 0.05$ on Shapiro-Wilk test of normality). If the data are normally distributed (t-test) they are appropriately described with mean and standard deviation (SD), while the median and interquartile range (IQR) (the range between 25^{th} and 75^{th} percentiles) are appropriate for non-normal distributions (Lang & Secic, 2006). Analysis of covariance (ANCOVA) were used to control for the age effect while analysing sleep scores and sleep parameters of children with WS and TD group. The outliers of over 3 SD from the mean were excluded.

Summary

This chapter outlined the methodology of sleep analysis including widely used actigraphy for objective sleep measurement as well as standard CSHQ, a more subjective parental report on their children's sleep. Although PSG is a gold standard technique for sleep analysis, it has been reported that actigraphic measurements, such as sleep efficiency and number of awakenings during the night are in agreement with PSG measurements in both adults and children (Corkum *et al.*, 2001; Kushida *et al.*, 2001; Gay *et al.*, 2004). Furthermore, it has been reported that actigraphy is particularly useful in studying infants and children, as well as in special paediatric population as PSG can be difficult to perform (Morgenthaler *et al.*, 2007). The pulse oximetry and medical questionnaire were also presented in this chapter in order to determine whether there are any conditions/factors which may obstruct sleep in these children.

3. RESULTS – ANALYSIS OF QUALITY AND QUANTITY OF SLEEP

The previous chapter focused on methodology of sleep analysis. Based on these methods, this chapter provides a detailed account of sleep characteristics in WS children and the TD controls. The results of qualitative and quantitative sleep analyses including data collected through actigraphy and pulse oximetry as well as questionnaires are reported here.

3.1. Participants' characteristics

The children with WS ranged in age from 4.48 years to 11 years (mean age 7.34 years) and were attending mainstream schools. The healthy control group for comparison of sleep and physiological measures, ranged from 4.04 years to 10.80 years (mean age 7.47 years). Demographic data (Appendix 3) was obtained for each individual. Series of statistical analyses were conducted in order to determine whether groups differed in age, gender, parents' socioeconomic status and puberty stage (Table 3.1.). Age difference between WS children and TD controls as well as BMI was tested using independent samples t-test. No age difference between these two groups was found ($t(50) = -0.240$ $p = 0.81$). In order to assess whether the two groups had any differences on categorical variables such as gender and ethnicity, a Chi-square analyses were conducted. The two groups did not differ on gender (χ^2 (1) = 0.074, p = 0.79), ethnicity (χ^2 (2) = 3.031, p = 0.22) and parent occupation

(professional, clerical, manual and other occupation) (mother's occupation: χ^2 (6) = 6.349, p = 0.39; father's occupation: χ^2 (6) = 5.843, p = 0.44). Eighty eight percent of children with WS and 94 % of controls indicated to be in prepubertal stage using Tanner puberty scale. Using chi-square analyses, it was found that both groups scored similar on this scale (χ^2 (1) = 1.236, p = 0.27).

	WS (n=25)	**TD (n=27)**
Age, years (SD)	7.34 (1.87)	7.47 (2.00)
Age range	4.48 – 11.00	4.04 – 10.80
Gender (M/F %)	48/52	44/56
Ethnicity (% White)	92	78
Ethnicity (% Black)	0	0
Ethnicity (% Other)	8	22
Parents' occupation:		
Professional (%)	33	46
Clerical (%)	20	22
Manual and other (%)	47	32

Table 3.1. Detailed characteristics of groups of study participants. N value for WS group accounts for the differences from N=27, as two children were excluded from the studies due to taking sleep medication.

3.2. Actigraphy

Actigraphs were well tolerated by the children, and data were available for 43 (83 %) of the 52 children involved in the studies. Five children with WS and four TD either refused to wear the actigraphs at all or removed them during the night. These children were not excluded from the rest of the study. The child's bedtime (time the child is in bed with lights off) and get-up time were obtained from sleep diary recorded by parents. Mean night bedtime for children with WS was 20:40 pm (SD = 45 min) and for TD group 21:01 pm (SD = 44 min).

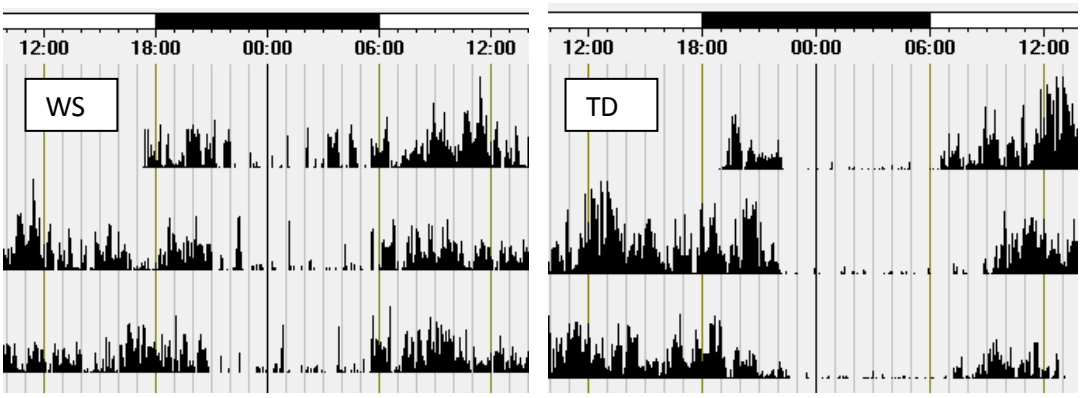


Figure 3.1. Example of actigrams for WS and TD children during three consecutive days and nights. The black bands indicate the level of the child's activity per minute during the 24h cycle. It can be seen that child with WS has a greater level of activity during the night in comparison to TD child, whose nocturnal activity levels are low, as recorded by actigraphy.

In order to investigate if there were any differences between the WS and TD control group on actigraphy scores, analyses of group comparison were performed using t-test (unless otherwise noted). As shown in Table 3.2., children in the WS group were significantly affected by several types of sleep problems.

Category of actigraphic scores	TD (n=22) Mean (SD)/ Median (IQR)	WS (n=21) Mean (SD)/ Median (IQR)	95% CI	p
Bed time (hh:mm)	21:01 (00:44)	20:40 (00:45)	-0.82, 0.10	0.12
Time in bed (hh:mm)	10:24 (00:31)	10:27 (00:50)	-0.34, 0.56	0.62
Sleep latency (hh:mm)	00:34 (00:25)	00:53 (00:29)	1.82, 35.17	**0.03**
Assumed sleep time (hh:mm)	09:37 (00:40)	09:36 (00:51)	-0.49, 0.47	0.98
Actual sleep time (hh:mm)	08:34 (00:38)	08:21 (00:50)	-0.70, 0.24	0.32
Actual sleep (%)	89.63 (3.07)	86.10 (5.70)	-6.16,-0.37	**0.03**
Actual wake time (hh:mm)	01:02 (00:19)	01:16 (00:27)	-0.2, 0.48	0.06
Actual wake (%)	10.65 (3.07)	13.10 (4.37)	0.05, 4.85	**0.04**
Night waking	29.02 (6.51)	28.52 (8.73)	-5.47, 4.47	0.84
Mean sleep bout time (mm:ss)*	17.23 (15.03-20.02)	16.98 (13.81-21.69)	-2.93,3.55	1.00
Mean wake bout time (mm:ss)*	2.07 (2.07-2.28)	2.53 (2.20-3.16)	0.20,0.93	**0.02**
Sleep efficiency (%)	82.55 (5.60)	78.92 (6.40)	-7.47, 0.23	0.06
Moving minutes	78.53 (19.55)	110.88 (59.00)	3.59, 61.11	**0.03**
Moving time (%)	13.59 (2.98)	17.45 (5.71)	0.89, 6.85	**0.01**
Immobile minutes	496.86 (34.73)	465.13 (63.32)	-64.92, 1.45	0.06
Immobile time (%)	86.23 (2.90)	80.92 (9.30)	-9.82,-0.80	**0.02**
Number of immobile phases	49.51 (11.62)	50.46 (11.56)	-6.37, 8.27	0.79
Mean length immobility	10.67 (3.28)	10.03 (3.66)	-2.84,1.56	0.56
One minute immobility phases	8.73 (3.72)	11.27 (5.22)	-0.33,5.41	0.08
One minute immobility (%)	17.32 (3.98)	20.84 (6.07)	0.19, 6.84	**0.04**
Fragmentation index	30.05 (6.71)	39.75 (13.59)	2.74, 16.64	**0.01**
Total activity score*	7269 (5116-9641)	7720 (6221-15966)	-426,5683	0.13
Mean activity score*	12.56 (9.16-15.27)	14.09 (10.92- 25.10)	-0.37,9.37	0.06
Mean score in active periods*	91.05 (72.53-114.83)	90.28 (66.31-115.94)	-17.52,26.47	0.83
Average wake movement	546.18 (156.36)	447.12 (232.29)	-228.9, 30.83	0.13

* group comparison performed on Mann-Whitney

Table 3.2. Comparison of actigraphic scores in WS and TD children using t-test and Mann-Whitney. Table includes mean and standard deviation (SD)/median corresponding to

categories of actigraphic scores on left hand column; 95% confidence interval (95% CI) and p value for determination of significance ($p < 0.05$) are also shown. Statistically significant results are shown in bold.

3.3. Pulse oximetry

Ten children with WS and 9 TD children refused to wear a sensor on their finger or toe. For the additional 3 WS children and 2 TD insufficient data was collected. Thus, the pulse oximetry data were available for 28 children out of 52 involved in the study (54%). Pulseoximetric measures included mean and median saturation of oxygen (SpO_2), mean pulse rate, number of dips of SpO_2 per hour over 4%, delta 12 s index (index of SpO_2 variability over each 12-second epoch, where a higher figure indicates increased variability), time spent under 90% of SpO_2 calculated in mm:ss, as well as in a percentage of analysis interval.

In order to investigate if there were any differences between the WS and TD control group on pulse oximetry scores, Mann-Whitney/t-test for group comparison was performed. The Mann-Whitney test was used for the data that did not demonstrate normal distribution ($p < 0.05$ on Shapiro-Wilk test of normality), while t-test for normally distributed data ($p > 0.05$ on Shapiro-Wilk test). As shown in Table 3.3. children with WS had significantly higher levels of pulse rate compared to TD controls. No difference was observed between both groups in mean and median SpO_2, as well as on the rest of the pulseoximetric scores.

Pulse oximetry parameters	TD (n=16) Median (IQR)/ Mean (SD)	WS (n=12) Median (IQR)/ Mean (SD)	95% CI	p value
Mean $Sp0_2$*	96.37 (2.37)	95.22 (2.73)	-2.38,0.43	0.13
Median $Sp0_2$*	97.06 (1.73)	96.08 (2.35)	-2.00,0.00	0.12
Mean pulse rate**	74.33 (6.92)	87.49 (7.49)	6.03,16.99	**<0.001**
Dips/hr > 4%**	4.26 (3.55)	5.22 (3.81)	-1.80,3.71	0.48
Delta index 12 s**	0.51 (0.16)	0.59 (0.19)	-0.05,0.21	0.22
Time spent <90% SpO_2 (mm:ss)*	14.38 (31.17)	22.38 (32.14)	-16.2, 66.0	0.60
Time spent <90% SpO_2 (% of analysis interval)*	3.36 (6.84)	3.38 (3.94)	-7.80, 4.07	0.50

* group comparison performed using Mann-Whitney
** group comparison performed using t-test

Table 3.3. Comparison of pulse oximetry scores in WS and TD children using Mann-Whitney and t-test. Table includes median and IQR/ mean and SD corresponding to categories of actigraphic scores on left hand column; 95% confidence interval (95% CI) values and p value for determination of significance are also shown.

3.4. Children's Sleep Habit Questionnaire

3.4.1. Detailed characteristics of sleep disturbances in children with WS

In the answer for question "Does your child have a sleep problem or have they had a sleep problem in the past?" 15% of the children with WS were reported as having a sleep problem in the past and 65% were reported as having a current problem from which 23% demonstrate phases of sleep problems. The sleep problems described by parents were: difficulty to relax and settle down during bed time, thus taking a long time to fall asleep, and difficulty to go back to sleep after night awakening. As shown in Table 3.4., 41% of parents reported that their children never or rarely fall asleep within 20 min, 30% do it sometimes and as little as 33% usually sleeps the right amount each night. Restlessness during sleep was reported in 48% of WS children and up to 59% have trouble falling asleep when they are away from home (33%- to demonstrate this problem sometimes; 26%- often). Other frequently reported sleep problems included enuresis in 48% of the WS sample. A relatively large number of parents (53%) reported their children to be tired during the day (42%- sometimes; 11% often). However, despite appearing tired, none of children were reported to nap during the day and only 4% fall asleep spontaneously during daytime activities. Bruxism was a rare problem occurring in less than 5% of WS group (3.8%). Parents reported bruxism, sleeptalking, restlessness, sleepwalking and breathing problems by passively observing their children. The mean amount of reported sleep for WS children was 9.5 h (SD = 1.3), similar to TD group with the average amount of sleep 10.2 h (SD = 0.5) per night.

Sleep problems reported in CSHQ	WS children			TD children		
	% U	% S	% R/N	% U	% S	% R/N
BEDTIME RESISTANCE AND ANXIETY:						
Going to bed at same times	85.2	11.1	3.7	100.0	0	0
Falling asleep within 20 min	29.6	29.6	40.7	56.5	21.7	21.7
Falling asleep in other's bed	14.8	14.8	70.4	0	13.6	86.4
Needing parent in the room to sleep	7.4	0	92.6	4.3	0	95.7
Resisting to go to bed	3.7	37.0	59.3	17.4	39.1	43.5
Struggling at bed time	7.4	40.7	51.9	0	0	100.0
Trouble sleeping away	25.9	33.3	40.7	4.3	8.7	87.0
Afraid to sleep alone	11.1	14.8	74.1	4.3	4.3	91.3
Afraid to sleep in the dark	22.2	18.5	59.3	17.4	4.3	78.3
NIGHT WAKINGS AND SLEEP DURATION:						
Moving to other's bed	14.8	18.5	66.7	0	21.7	78.3
Awaking once during the night	37.0	25.9	37.0	9.1	40.9	50.0
Awaking more than once	7.7	30.8	61.5	0	0	100.0
Sleeping too little	33.3	37.0	29.6	0	26.1	73.9
Sufficient sleep	33.3	40.7	25.9	95.7	4.3	0
Consistent sleep	63.0	33.3	3.7	91.3	8.7	0
SLEEP DISORDERED BREATHING:						
Snoring loudly	0	29.6	70.4	4.3	8.7	87.0
Holding breath during sleep	0	7.7	92.3	0	0	100.0
Snorting and gasping	0	11.1	88.9	0	4.3	95.7
PARASOMNIAS:						
Wetting the bed	48.1	14.8	37.0	4.3	4.3	91.3
Sleeptalking	3.7	18.5	77.8	0	34.8	65.2
Restlessness	48.1	22.2	29.6	4.5	27.3	72.7
Sleepwalking	0	0	100.0	0	0	100.0
Grinding teeth during sleep	0	3.8	96.2	0	8.7	91.3
Awaken screaming, sweating	0	11.1	88.9	0	0	100.0
Awaken by frightening dream	0	25.9	74.1	0	18.2	81.8
DAYTIME SLEEPINESS						
Awakening by others in the morning	22.2	18.5	59.3	26.1	56.6	17.4
Waking up in a bad mood	7.4	22.2	70.4	0	22.7	77.3
Hard time getting out of bed	11.1	14.8	74.1	9.1	27.3	63.6
Taking a long time to be alert	0	18.5	81.5	0	18.2	81.8
Seeming tired during the day	11.5	42.3	46.2	0	17.4	78.3
Falling asleep while watching TV	7.7	7.7	84.6	0	0	100.0
Falling asleep in a car	11.5	7.7	80.8	0	0	100.0
Falling asleep during daytime	0	3.8	96.2	0	0	100.0
Napping during the day	0	0	100.0	0	0	100.0

Table 3.4. The percentage of specific sleep problems in WS and TD children reported by parents in CSHQ. U indicates usually (5-7 nights/week), S- sometimes (2-4 nights/week) and R/N- rarely/never (0-1 nights/week).

3.4.2. Comparison of WS and TD groups

In order to investigate whether there were any differences between the WS and TD control groups on the total scores and subscales of CSHQ scores, one-way between-groups Mann-Whitney test was applied. As shown in Figure 3.2 children with WS compared to TD controls had significantly elevated scores on bedtime resistance ($p < 0.01$), sleep onset delay ($p = 0.03$), sleep duration (<0.001), sleep anxiety ($p < 0.01$), excessive night waking ($p = 0.04$) and parasomnias ($p < 0.01$), as well as on total CSHQ score (<0.001).

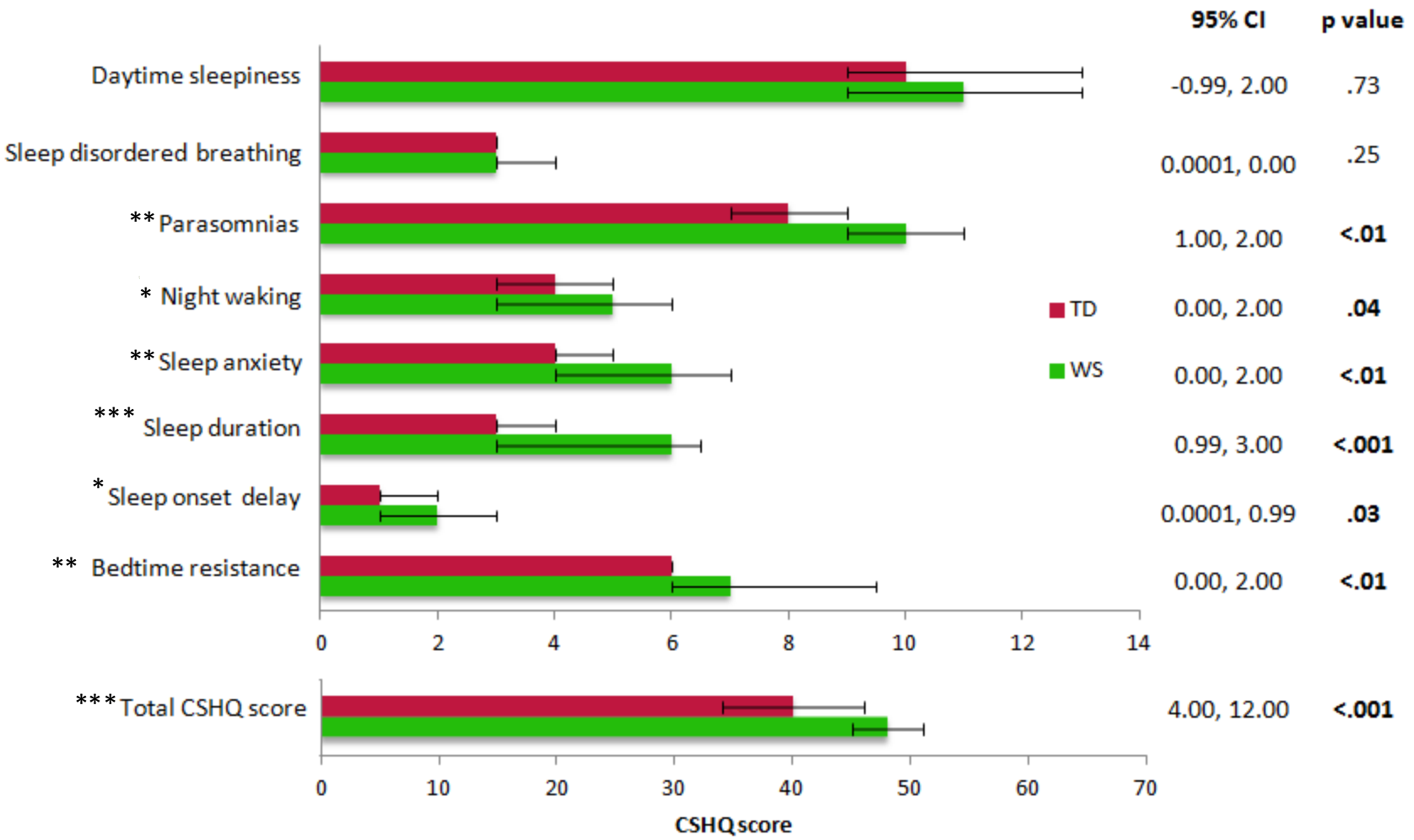


Figure 3.2. Comparison of CSHQ scores in WS and TD children using Mann-Whitney. Figure represents median and IQR of all scores. 95% confidence interval (95% CI) and p values for determination of significance are also shown.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Lastly, sleep scores and parameters of children with WS and TD group were also analysed using ANCOVA with age as the covariate to control for the age effects. There was an expected general trend for a decrease in sleep time with increasing chronological age. This trend was significant for time in bed in WS group ($F(1,22) = 11.36$, $p < 0.01$) but not TD group ($F(1,20) = 0.29$, $p = 0.60$). Similar situation was observed for assumed sleep time (WS: $F(1,21) = 14.78$, $p = 0.001$; TD: $F(1,20) = 0.60$, $p = 0.45$), and the actual sleep time (WS: $F(1,21) = 7.85$, $p = 0.01$; TD: $F(1,20) = 0.41$, $p = 0.53$).

3.5. Medical questionnaire

Parents of one child from the TD group failed to return completed medical questionnaire, thus the data were available for 53 children out of 54 involved in the study (98 %). See Tables 3.5. and 3.6. for percentage of children who were reported with specific problems.

Specific health problems (%)	TD (n=26)	WS (n=25)	χ^2	p
Allergies	11	4	1.00	0.32
Asthma	11	0	3.06	0.08
Frequent sinus infections	0	4	0.98	0.32
Frequent colds/respiratory infections	22	20	0.02	0.88
Frequent strep throats/tonsillitis	8	4	0.31	0.58
Pain due to chronic medical condition	0	4	1.06	0.30
Difficulty breathing by nose	4	8	0.31	0.58
Frequent complain of heartburn	0	4	1.06	0.30
Gastroesophageal reflux	0	14	2.19	0.14
Abnormal glucose levels	0	4	1.06	0.30
Thyroid problems	0	12	3.31	0.07

Table 3.5. Percentages of specific health problems which may affect sleep and/or levels of melatonin and cortisol in WS and TD children. The outcome of the Chi-square analyses are also shown in the table. No significant difference was found between WS and TD children in any of these parameters.

Medical questionnaire variables (%)	TD (n=26)	WS (n=25)	χ^2	p
Extracurricular sport activities	72	52	2.01	0.16
TV in bedroom	12	29	2.42	0.12
PC/game console in bedroom	23	12	1.08	0.30
Shared bedroom	50	33	1.42	0.23
Bedroom on the main road	8	16	0.85	0.36

Table 3.6. Percentages of specific medical questionnaire parameters which may affect sleep patterns and/or levels of melatonin and cortisol. The outcome of the Chi-square analyses are also shown in the table. No significant difference was found between WS and TD children in any of these parameters.

Further questions related to the amount of time spent watching TV/playing on the computer each school day and during the weekend. Parents answered on the six-point scale: "none", "up to 2 hrs", "2-4 hrs per day", "4-6 hrs per day", "more than 6 hrs per day" and "don't know". In the WS group, 72% of children spend up to 2 hrs and 28% between 2 and 4 hrs watching TV on school days, while for TD group these numbers are 65% and 31% respectively. One TD child did not watch TV at all on school days. Regarding playing computer/game console during the school days, in WS group 40% of children do not use it at all, while 60% spent up to 2 hrs playing games. In TD control group, 31% of children did not spent any time on the computer on school days, 65% did it for up to 2 hrs daily and one child spent between 2-4 hrs playing games. During the weekend, 24% of children with WS spent up to 2 hrs watching TV, 48% 2-4 hrs, 24% 4-6 hrs and 1 child over 6 hrs. For the TD group

these numbers are respectively: 15%, 73%, 8% and 1 child watching TV for over 6 hrs. Twenty-four % of children with WS did not use a computer on the weekend days at all, 56% did it up to 2 hrs, 16% between 2-4 hrs and 1 child over 6 hrs. In TD group, 23% of children do not spent any time on computer/game consoles during the weekend, 54% spent up to 2 hrs per day and a further 23% between 2-4 hrs. Next question related to watching TV and/or videos in 30 min before falling sleep. Parents answered on a five-point scale: "every night", "5-6 nights per week", "3-4 nights per week", "1-2 nights per week" and "not at all". It was noted that 42% of WS children did not watch TV in 30 min before sleep at all, while 17% did it every night. For the TD children, these numbers are 38% and 23% respectively. Due to the fact that data were not normally distributed ($p < 0.05$ on Shapiro-Wilk test of normality) Mann-Whitney analyses were conducted to determine whether there are any differences between both groups in any of these variables (Table 3.7.).

Medical questionnaire variables	95% CI	P value
TV on school days	0.00, 0.0001	1.00
PC/game console on school days	0.00, 0.0001	0.40
TV on weekend days	-0.0001, 0.0002	0.69
PC/game console on weekend days	0.00, 0.0002	0.88
TV 30 min before sleep	-0.99, 1.00	0.79

Table 3.7. Comparison of specific variables from medical questionnaire between WS and TD children using Mann-Whitney test. Table presents 95% confidence interval values (95% CI) and p value for the determination of significance. It is shown that there are no significant differences in these parameters between both study groups ($p > 0.05$).

Analyses of the dietary habits of the WS and TD groups showed that 88% of WS children and 89% of TD controls did not drink any caffeinated drinks at all (($\chi^2(2) = 1.20$, $p = 0.55$) and there was no difference in the number of main meals consumed ($\chi^2(2) = 1.29$, $p = 0.52$). However, the WS children had significantly more snacks daily compared to TD controls ($\chi^2(2) = 6.00$, $p = 0.05$). The consumption of food prior bedtime (time from last meal to bedtime) has not been analysed.

This data from Medical questionnaire was used for the analysis of covariance to estimate its effect on sleep and endocrine markers of sleep in both study groups (see Section 5.9.).

3.6. Comparison of actigraphy and CSHQ measures

Pearson's product-moment correlations were used to investigate the similarity between parent report and actigraphy, where analogous variables existed. This was conducted for the variables: sleep onset delay, bedtime resistance, sleep duration and night wakings from CSHQ, and sleep latency, assumed and actual sleep times, time in bed, number and duration of night wakings as well as sleep fragmentation from actigraphy (see Table 3.8.).

Sleep latency was likely to correlate with parentally reported sleep onset delay. The significant correlation was observed for the WS ($r(21) = 0.439$, $p = 0.05$) (Figure 3.3.), but not TD group ($r(22) = 0.328$, $p = 0.13$). Significant positive correlation was also observed between parentally reported night wakings and the mean wake bout time computed from actigraphs, however, only in TD children ($r(22) = 0.441$, $p = 0.04$) and not in the WS group ($r(21) = 0.141$, $p = 0.55$).

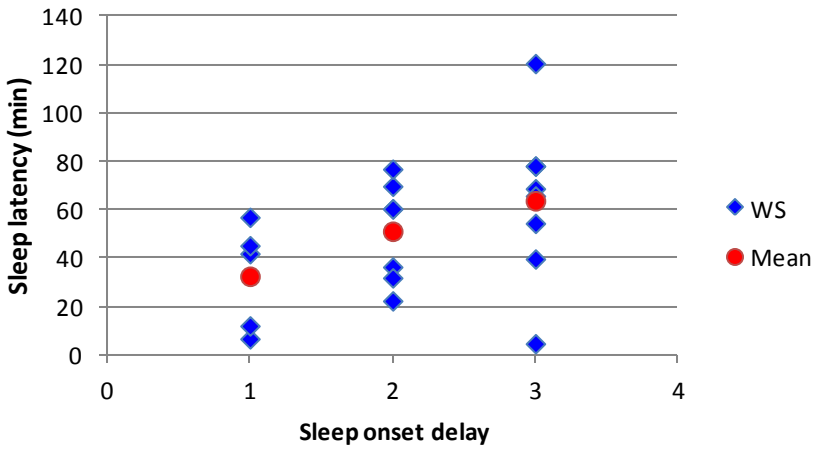


Figure 3.3. Comparison of sleep onset delay scores of Children's sleep habit questionnaire (CSHQ) and sleep latency (min) measured by actigraphy in children with WS. The higher sleep latency score demonstrates more pronounced problems with sleep onset in children. Positive relationship can be observed between the scores on time taken to fall asleep measured by both methods, CSHQ and actigraphy.

No significant relationships were found between night wakings and sleep fragmentation (WS: r(22) = -0.006, p = 0.98; TD: r(19) = 0.133, p = 0.59), bedtime resistance and sleep latency (WS: r(21) = 0.314, p = 0.18; TD: r(22) = -0.147, p = 0.51), sleep duration and assumed sleep time (WS: r(21) = -0.017, p = 0.94; TD: r(20) = -0.430, p = 0.06), sleep duration and actual sleep time (hr) (WS: r(21) = 0.120, p = 0.61; TD: r(20) = -0.359, p = 0.12), sleep duration and the percentage of actual sleep time in the night (WS: r(22) = 0.106, p = 0.65; TD: r(20) = 0.095, p = 0.69), sleep duration and time in bed (WS: r(22) = 0.034, p = 0.88; TD: r(20) = -0.415, p = 0.07) and for the number of night wakings reported by parents in CSHQ as well as computed by actigraphy (WS: r(21) = -0.239, p = 0.31; TD: r(19) = -0.228, p = 0.35). Many of the correlations were negative, especially in TD group (nearly 50 % of negative correlations), indicating that parents' knowledge of their children's sleep is limited. Due to the nature of parental questionnaire as well as the subjectivity and inaccuracy of CSHQ for reporting sleep characteristics, it should not be used as a substitute for objective measures, such as actigraphy and PSG. However, many of the CSHQ parameters, such as child waking up in negative mood, pain reports or appetite cannot be measured by actigraphy, and in addition, sleep diary is necessary for analysis of actigraphy data, thus, parent report should still be valued.

Parental report (CSHQ)	Actigraphy parameters	Group	r value	P value
Sleep onset delay	Sleep latency	TD(22)	0.33	0.13
		WS(21)	0.44	**0.05**
Night wakings	Mean wake bout time	TD(22)	0.44	**0.04**
		WS(21)	0.14	0.55
	Sleep fragmentation	TD(19)	0.13	0.59
		WS(22)	-0.01	0.98
Bedtime resistance	Sleep latency	TD(22)	-0.15	0.51
		WS(21)	0.31	0.18
Sleep duration	Assumed sleep time	TD(20)	-0.43	0.06
		WS(21)	-0.02	0.94
	Actual sleep time	TD(20)	-0.36	0.12
		WS(21)	0.12	0.61
	The percentage of actual sleep time in the night	TD(20)	0.10	0.69
		WS(22)	0.11	0.65
	Time in bed	TD(20)	0.415	0.07
		WS(22)	0.03	0.88
Night waking	Number of wakings	TD(19)	-0.23	0.35
		WS(21)	-0.24	0.31

Table 3.8. Pearson's product moment correlation analysis between parental report (Children's Sleep Habit Questionnaire- CSHQ) and actigraphy. Only two significant correlations can be observed and are indicated in bold. Many of the correlations were negative, especially in TD group (nearly 50 % of negative correlations), indicating that parents' knowledge of their children's sleep is limited and parental reports are not reliable methods of sleep analysis in children.

Summary

This chapter outlines the results obtained from the measurements undertaken at participant's home environment including questionnaires, actigraphy and pulse oximetry. It has been shown, that children with WS demonstrate significant sleep disturbances including increased sleep latency, moving time during the night and fragmentation index, which is the indication of restlessness as well as bedtime resistance and sleep anxiety. Furthermore, no breathing-related problems, which could affect sleep, were found in any of the study groups.

4. METHODOLOGY- LABORATORY ANALYSES OF BIOMEDICAL MARKERS OF CIRCADIAN RHYTHM AND SLEEP

Previous chapters focused on the analysis of sleep patterns in WS and TD children. However, as stated in the aims, the current work focused on the analysis of endocrine markers of circadian rhythm, namely melatonin and cortisol. Thus, the following section outlines a detailed description of sample collection from participants as well as the enzyme based immunoassay techniques used for analysis of salivary melatonin and cortisol as well as urinary MT6s. Lastly, the development and application of a ultra-high performance liquid chromatography- tandem mass spectrometry (UHPLC-MS/MS) method for simultaneous measurement of MT6s, cortisol and cortisone in urine is described.

4.1. Sample collection

Given compliance and ethical concerns regarding repeated sample collection from children, salivary levels of cortisol and melatonin as well as urinary levels of cortisol, cortisone and MT6s were measured. The environmental conditions, such as stress, cause a physiologic increase in cortisol levels, thus, parents of all the children were instructed to ensure normal night routine for each child and were further asked whether anything abnormal happened in a day/night of collection. Saliva and urine samples were all obtained within the same time as actigraphy, pulse oximetry and CSHQ measurements and during the weekdays to ensure normal routine. Samples

were collected over the period of one year through all the seasons. The reports regarding seasonal changes of melatonin and cortisol are inconclusive. The delay of the time of maximum excretion was found for both melatonin and cortisol during Winter in comparison to samples collected in Summer (Kennaway & Royles, 1986). On the other hand, it has also been reported that the excretion of 6-sulfatoxymelatonin and amplitude of cortisol excretion is remarkably stable between summer and winter solstices (Kennaway & Royles, 1986) and in addition, no influence of season of the year on levels of melatonin was found (Sack *et al.*, 1986).

4.1.1. Saliva

Due to circadian rhythmicity of melatonin and cortisol, saliva samples were obtained at three time points: afternoon between 4-6 pm, evening before bedtime, and morning immediately after awakening. Sample collection at these three time points provided a means of determining whether sleep hormones in WS follow similar or different pattern compared to TD control group. In order to ensure that the time between first collection and bedtime sample did not vary strongly across study participants, parents were instructed, that the time difference between these collection points should fall within 3-4 h. Several studies have reported high stability and intra-day reproducibility of melatonin (Benloucif *et al.*, 2008; Mirick & Davis, 2008) and cortisol (Kudielka & Kirschbaum, 2003; Stone *et al.*, 2001) in the same individual. Thus, in the current work, samples were collected over the period of one day. It has also been established that salivary levels of melatonin and cortisol closely parallel those found in corresponding blood samples (Voultsios et al. 1997; Dorn *et al.* 2007).

Parents of all the participants were trained in how to collect saliva samples and the collection protocol was given to each parent (Appendix 6 and 7). Parents were asked to collect samples in a dimly lit room as high intensity of light is known to suppress level of melatonin. Saliva was collected using saliva collection devices including inert polymer swab (Salimetrics Europe, UK). On the day of collection every child was asked to avoid caffeinated drinks, chocolate and bananas, as these can affect levels of hormonal markers of sleep. Every child was asked to, avoid any food or drink for at least 15 min and to rinse their mouth with water before sampling. A swab was placed in the mouth for 3 to 5 min until it was thoroughly wet, and then placed into a storage tube. If this method of sampling caused any discomfort to the child, they could instead spit directly into the tube. Every sample was labelled with the time and date of collection and then stored in a household fridge for maximum of 24 h before they were transported in ice to a laboratory freezer. In cases when it was not feasible to transport the samples within 24 h, parents were asked to freeze the specimens. In these cases when the samples collected from parents were already frozen, they have been placed directly to the laboratory freezer, then thawed and centrifuged on the day of analysis. Samples were transported to the laboratory in ice box and centrifuged for 10 min at 1500 g in order to extract saliva. Volume capacity of thoroughly saturated swab was 1 ml. Saliva collected at the bottom of the centrifuge tubes was subsequently divided into 2-3 aliquots and stored at -20°C until assayed. It has been reported that salivary melatonin is stable for at least 3 days even when kept at room temperature, and for months (probably years) when kept frozen (at -20°C or lower) (Weber *et al.*, 1999). It has also been reported that storage of uncentrifuged swabs for 24 h at 4°C or for 2 months in a freezer does not affect the recovery of melatonin from the swab. Thus, there is no need to centrifuge samples

within short period of time after collection (Bagci *et al.*, 2009). Cortisol levels also demonstrate good stability, salivary cortisol is stable for at least a year at -20°C (Garde & Hansen, 2005).

4.1.2. Urine

First void urine samples were collected into 200 ml storage pots which were labelled with the date and time of collection. No food or drink was given prior to collection. As in the case of saliva samples, urine specimens were stored by parents of participating children in the household fridge for maximum of 24 h before they were transported to a laboratory freezer. In cases where it was not possible to transport the samples within 24 h of collection then they were stored in a freezer. Following transfer to the laboratory, uncentrifuged urine samples were divided into 4-5 aliquots and stored at -20°C. In cases where the samples collected were already frozen, they were transported in ice to a laboratory freezer. They were thawed and centrifuged on the day of analysis. Bojkowski *et al.* (1987) stored urine samples without preservative at room temperature for 5 days, and at -20°C for 2 years and found that urinary metabolite of melatonin (MT6s) is stable under both conditions. Cortisol levels also demonstrate good stability in the urine, the samples can be stored in a freezer for at least 9 months (Barrett *et al.*, 2005).

4.2. Analyses of salivary melatonin

Salivary melatonin was analysed in duplicates using a competitive ELISA kit from IBL International (Germany). The assay is based on the competition between biotinylated melatonin and melatonin in the sample for a fixed number of antibody biding sites (Figure 4.1.).

Prior to analyses, each sample was thawed, mixed and centrifuged for 10 min at 3500 rpm. An aliquot (100 µl) of each sample, control and standards were added to wells coated with anti-rabbit IgG polyclonal antibodies. In the next step 50 µl of antiserum was added to each well, except blank wells. Plates were then incubated overnight for 16-20 h at 4 °C. After each incubation process, plates were washed five times with 'wash buffer' and excess solution was removed by inverting the plate onto a paper towel and tapping it. In the next step biotin solution (100 µL) was added to each well and the plate was incubated for 2 h at room temperature. After this incubation step and plate washing, 100 µl of enzyme conjugate (streptavidin conjugated to horseradish peroxide) was added to each well and the plate was incubated for additional 1 h at room temperature. Following the last wash step, 100 µl of tetramethylbenzidine (TMB) substrate was added to each well and the plate was incubated for 15 min at room temperature. TMB chromophore (blue) was formed in each well. Directly after this incubation step 100 µl of TMB stop solution (1M sulphuric acid) was added to each well, which produced a yellow colour. In the last step, the plate was shaken briefly and the absorbance was measured using a FLUOstar OPTIMA plate reader (BMG LABTECH) set at wavelength of 450 nm within 15 min after pipetting stop solution. Final results were calculated from 4 Parameter Logistic fit, using OPTIMA MARS data analysing software (BMG

LABTECH). The concentration of analyte in the sample was determined using a standard curve. The amount of biotinylated antigen bound to the antibody, as measured by the intensity of colour, is inversely proportional to the analyte concentration of the sample. Samples with melatonin levels exceeding highest level of standard curve were diluted with standard A, reassayed and the results were multiplied by the corresponding dilution factor. The sensitivity of the assay was 0.3 pg/ml. The assay was carried out according to manufacturer's instructions with modification in washing steps (five washing steps instead of four) (Melatonin direct saliva ELISA instruction of use, http://www.ibl-international.com).

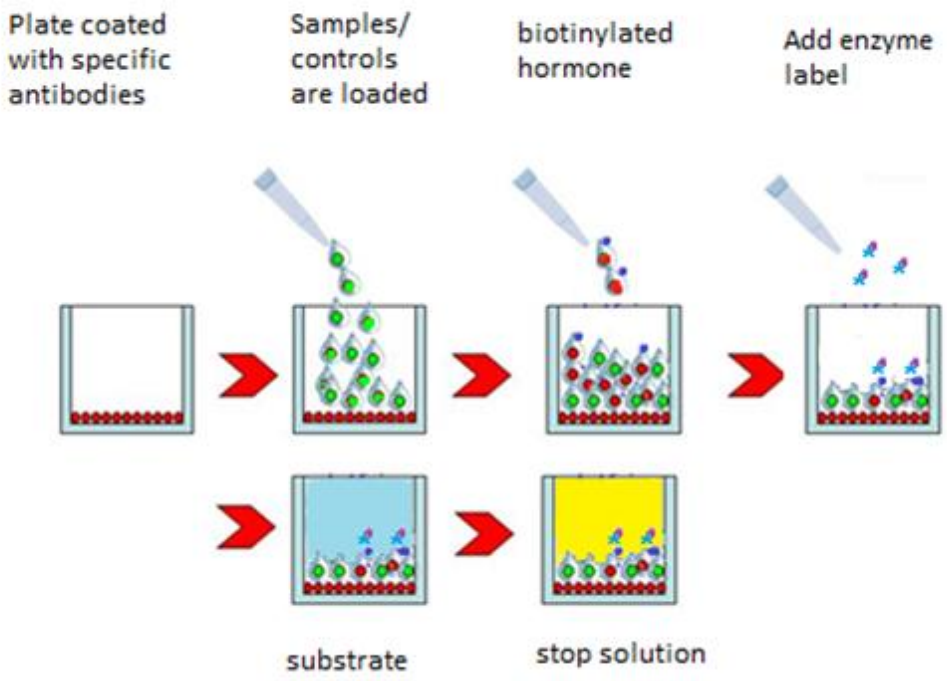


Figure 4.1. Principle of competitive ELISA method.

4.3. Analyses of salivary cortisol

Salivary cortisol was assayed in duplicates using competitive enzyme immunoassay kit from Salimetrics Europe (Suffolk, UK). On the day of analysis, saliva samples were thawed, vortexed and centrifuged at 3000 rpm for 15 min. Multiple freeze-thaw cycles were avoided. Samples were allowed to reach room temperature before they were transferred to an assay plate. This assay was based on the competition between standards and unknowns with cortisol linked to horseradish peroxide for the antibody biding sites. In the first step an aliquot of each sample, standard or control (25 µL) was added to a microtiter plate coated with monoclonal anti-cortisol antibodies. Next, 200 µl of cortisol conjugated to horseradish peroxide was added to each well and the plate was incubated for 55 min at room temperature. The plate was then washed five times with washing buffer. An aliquot (200 µL) of the substrate TMB was added to each well. The plate was then placed on a plate rotator for 5 min at 500 rpm and incubated for additional 25 min at room temperature. Bound cortisol peroxidase was measured by the reaction of peroxidase enzyme on the TMB substrate. This reaction produced a blue colour. A yellow colour was formed after stopping the reaction with 50 µl of stop solution (3 M sulphuric acid). Following 3 min of mixing on the plate rotator at 500 rpm, the optical density was determined using a standard FLUOstar OPTIMA plate reader (BMG LABTECH) set at a wavelength of 450 nm. Final results were calculated from 4 Parameter Logistic fit using OPTIMA MARS data analysing software (BMG LABTECH). The concentration of analyte in the sample was determined using a standard curve. The amount of cortisol peroxidase detected, as measured by the intensity of colour, was inversely proportional to the amount of cortisol present. Samples with cortisol levels

exceeding highest level used to generate a standard curve were diluted with assay diluent and reassayed. The sensitivity of the assay was 30 pg/ml. The assay was carried out according to manufacturer's instructions with modification in washing steps (five washing steps instead of four) (High sensitivity salivary cortisol enzyme immunoassay kit protocol, http://www.salimetrics.com).

4.4. Analyses of MT6s using ELISA

Urinary Mt6s was analysed using competitive ELISA kit from IBL International (Germany). The assay is based on the competition between unknown amount of antigen present in the sample with a fixed amount of enzyme labelled antigen for the biding sites of the antibodies coated onto the wells.

Prior to analyses, each sample was thawed, mixed and centrifuged for 10 min at 3500 rpm. A small volume (10 µl) of each standard, control and urine sample was transferred to an eppendorf tube, mixed with 500 µL of assay buffer and vortexed. Further, 50 µl of each diluted standard, control and samples were transferred into the respective walls of the microtiter plate. Next, 50 µl of enzyme conjugate was added to each well, followed by 50 µl of melatonin sulphate antiserum. The plate was further incubated for 2 h at room temperature on orbital shaker (500 rpm). The plate was then washed five times with washing buffer and 100 µl of the TMB substrate solution was added to each well. The plate was then incubated for 30 min at room on a plate rotator at 500 rpm. Chromophore was formed in inverse proportion to the amount of MT6s. This reaction produced a blue colour. Directly after this incubation step 100 µl of TMB stop solution (1M sulphuric acid) was added to each well, which

produced a yellow colour. In the last step, the plate was shaken briefly on a shaker and the absorbance was measured using a FLUOstar OPTIMA plate reader (BMG LABTECH) set at a wavelength of 450 nm. Final results were calculated from 4 Parameter Logistic fit, using OPTIMA MARS data analysing software (BMG LABTECH). The absorbance reading was used to determine level of analyte present in a sample. The concentration of analyte in the sample was determined using a standard curve. The intensity of colour was inversely proportional to the analyte concentration in the urine sample. The sensitivity of the assay was 1 ng/ml. The assay was carried out according to manufacturer's instructions with modification in washing steps (five washing steps instead of four) (Melatonin sulfate ELISA instruction of use, http://www.ibl-international.com).

For the analysis of salivary melatonin and cortisol as well as urinary MT6s using immunoassays, each sample was run in duplicate and average value was calculated and used for further data analyses. In these cases when the results of duplicate samples did not agree within 10 % (van Emmon, 2006), samples were re-run and the concentrations that were within 10 % were taken for further analysis.

4.5. Analyses of urinary levels of MT6s, cortisol and cortisone using UHPLC-MS/MS

UHPLC-MS/MS is an analytical technique that combines separation of compounds using chromatography with detection using a mass spectrometer. The liquid chromatography component is used to physically separate compounds and the mass

spectrometer is used to detect the compounds on the basis of their *m/z* ratio. In the current work a UHPLC-MS/MS method (described in details in Section 1.2.2.) was developed for the measurement of cortisol, cortisone and MT6s in urine of children with WS and a TD control group. In order to account for the dilution of urine, creatinine was analysed separately using UHPLC-MS/MS method as well and the levels of cortisol, cortisone and MT6s were divided by creatinine levels.

4.5.1. Chemicals

Standards of cortisol and cortisone were purchased from Sigma-Aldrich (Dorset, UK). Due to the lack of commercially available standards for MT6s, for the development of LC-MS/MS method and calibration curve, standards for melatonin sulphate from an ELISA kit (IBL International, Germany) were used. Creatinine standard was purchased from Acros Organics (Geel, Belgium). 6-α-methylprednisolone used as internal standard for cortisol and cortisone, indole-3-acetamide used as IS for MT6s as well as 1-(3-aminopropyl)imidazole used as IS for creatinine were also obtained from Sigma-Aldrich (Dorset, UK). Acetonitrile of LiChrosolv LCMS-grade was purchased from Merck Millipore (Feltham, UK).

4.5.2. Preparation of stock and standard solutions

Cortisol (1 mg) and cortisone (1 mg) were dissolved in 10 ml of methanol to give 0.1 mg/ml stock solutions. These solutions were used to prepare standard solutions in methanol at concentrations of 2.78, 5.56, 16.67, 50 and 100 ng/ml. Standard

solutions from MT6s ELISA kit were used at concentrations of 3.11, 6.22, 18.67, 56 and 112 ng/ml. Creatinine (5 mg) was dissolved in 5 ml of deionised water to give a 1 mg/ml stock solution. Standard in the concentration range 0.125-0.75 mg/ml for calibration studies were prepared using this stock solution. All the standard solutions for the calibration curves were spiked with the internal standards.

Normal levels of urinary cortisol range between 1.5-37 ng/ml and cortisone between 2.8-73 ng/ml in 3 to 8 years old children (Mayo Clinic, http://www.mayomedicallaboratories.com). Due to the fact that these represents mean daily levels of both analytes, morning levels were expected to be closer towards higher values. In addition, taking into account the prevalence of anxiety in WS, it was expected that WS children may have higher levels of cortisol and cortisone. Thus, the standard range for calibration curve was selected in concentrations between 2.8 and 100 ng/ml. Subsequently, normal levels of creatinine in children range between 0.25 mg/ml to 0.75mg/ml (Vasudevan & Sreekumari, 2007; MacGregor & Methven, 2011), thus, concentrations chosen for standard curve ranged between 0.125-0.75 mg/ml.

4.5.3. Sample preparation

Frozen urine samples were thawed and centrifuged for 5 min at 1500 g, and then diluted in water (1:1 v/v). For the analysis of cortisol, cortisone and MT6s, 500 ml of diluted samples were spiked with a small volume (2.5 µl) of the internal standards working standards (10 µg/ml) and vortexed for 30 s. For the analysis of creatinine,

600 ml of diluted samples were spiked with 30 µl of the internal standards working standards (10 µg/ml) and vortexed.

4.5.4. LC-MS/MS

A LC-MS/MS system comprising of a *Shimadzu* UHPLC *Nexera* system interfaced to a triple quadrupole mass spectrometer *LCMS-8030* (Shimadzu Corporation, Japan) equipped with an ESI source. The chromatographic separations were performed using a Kinetex 2.1 x 50 mm, 2.6 µm, C18 (Phenomenex, Maccelsfield, UK). The UHPLC system was composed of two LC-30AD pumps, a column oven (CTO-20AC) maintained at 40 ºC and a DGU-20A3 solvent degasser. A binary gradient elution profile composed of eluent 'A' 0.1 % formic acid in water and 'B' 0.1 % formic acid in acetonitrile was used. The eluent was delivered at a flow rate of 0.4 ml/min. For the separation of cortisol, cortisone, MT6s and their internal standards a linear gradient from 2% to 90% 'B' over 4 min followed by a hold at 90 % 'B' for 0.5 min then a drop back to 2% 'B' with a final reequilibration at 2 % 'B' for 1.5 min was used. For the separation of creatinine and its IS a linear gradient from 2% to 90% % 'B' over 2 min followed by a hold at 90 % 'B' for 0.5 min then a drop back to 2% 'B' with a final reequilibration at 2 % 'B' for 1.5 min was used. The desolvation line temperature was maintained at 300 °C while the heatblock temperature was set at 400 °C. An interface voltage of 4.5 kV was applied to the electrospray probe. Nitrogen was used as the nebulising gas at a flow rate of 2.8 l/min, additionally, the flow rate of drying gas was 16 l/min. Detection and quantification of all the compounds apart from MT6s was accomplished using multiple reaction monitoring (MRM). MT6s was analysed using the mass

spectrometer in the single ion monitoring (SIM) mode. An injection volume of 5 µl was used for the main method (analysis of cortisol, cortisone and MT6s) and 0.1 µl was injected for the analysis of creatinine. The ESI was operated in a positive ion mode using the MRM transitions or *m/z* values detailed in Table 4.1. includes mass ion transitions for the compounds. The raw data were processed using Lab Solution Software.

Compound	Molecular ion (m/z)	Product ion (m/z)
Cortisol	363.25	121.05
Cortisone	361.05	163.1
6-sulfatoxymelatonin*	328.8	-
6-α-methylprednisolone	375.25	161
Indole-3-acetamide	174.9	130
Creatinine	113.9	86
1-(3-aminopropyl-imidazole)	126.1	69.1

Table 4.1. Mass ion transitions for analytes measured in urine. Note 6-sulfatoxymelatonin was analysed using the *m/z* for the molecular ion.

4.5.5. Method validation

Quality control (QC) samples were a mixture of three control urine samples spiked with known amount of all internal standards (see sample preparation Section 4.5.3.). These samples were run nine times for analysis of cortisol, cortisone and MT6s, and five times for analysis of creatinine.

The intra-assay precision was determined by analysing 15 replicates of cortisone and cortisol at concentrations of 2.78, 5.56, 16.67, 50 and 100 ng/mL, 10 replicates of MT6s at concentrations of 3.11, 6.22, 18.67, 56 and 112 ng/mL and 18 repeat injections of creatinine at concentrations of 0.125, 0.25, 0.5 and 0.75 mg/mL. The results were corrected to peak areas of internal standards prior to calculation of repeatability.

The inter-assay variability was measured by 12 replicate injections of cortisol and cortisone at concentrations of 2.78, 5.56, 16.67 and 50 ng/mL, 10 repeat injections of, MT6s at concentrations of 3.11, 6.22, 18.67 and 56 ng/mL and 20 replicate injections of creatinine at concentrations of 0.125, 0.25, 0.5 and 0.75 mg/mL. These samples were analysed over two consecutive days and the HPLC peak areas were corrected to peak areas of internal standards.

4.6. Statistical analyses

The statistical analyses for group comparisons were performed according to the description in Section 2.5. ANCOVA was used for the analysis of relationship between sleep parameters and levels of endocrine markers of sleep and circadian

rhythm, namely melatonin, cortisol and their metabolites, as well as to control for the effect of health/dietary and sleep habits on sleep in children with WS and TD group.

Summary

The current chapter included description of sample collection as well as the enzyme based immunoassay techniques used for the analysis of salivary melatonin and cortisol as well as urinary MT6s. In the last part of this chapter, the novel UHPLC-MS/MS method for the analysis of 6-sulfatoxymelatonin, cortisol and cortisone in urine was described. The salivary analyses of these hormones were carried out to observe changes occurring prior to bedtime. In order to determine nocturnal levels of melatonin and cortisol and to answer the question whether indeed we can observe differences in levels of these hormones between WS and TD children, the novel UHPLC-MS/MS method was developed for urine.

5. RESULTS- LABORATORY ANALYSES OF BIOMEDICAL MARKERS OF CIRCADIAN RHYTHM AND SLEEP

The following chapter includes detailed description of the data obtained by analysis of melatonin, cortisol and their metabolites in saliva and urine of children with WS and TD control group. The comparison of ELISA and UHPLC-MS/MS methods for analysis of MT6s is also included. Furthermore, the association between sleep parameters and levels of endocrine markers of sleep is described as well.

5.1. Salivary melatonin

Twenty- two children out of 27 in the WS group and all TD children collected at least one out of three saliva samples. Of 22 WS children who collected saliva samples, 2 were excluded because it was noted that they were taking melatonin as medication by the time the assessments were due to be made. Levels of melatonin in saliva were determined using ELISA (see Section 4.2.). The standard curve for melatonin analyses was in range 0.5 to 50 pg/ml (Figure 5.1.). Each sample was run in duplicate and average value was calculated and used for further data analyses. The average recovery quoted by the manufacturer of this assay is 96.8%. High individual variability in the amount of melatonin secreted was observed between participants in both groups. In the WS group melatonin levels ranged from 0.19-17.40 pg/ml in the afternoon, 0.06-26.47 pg/ml in the evening and 0.12-14.71 pg/ml in the morning. For the TD control group these values were 0.06-9.13 pg/ml, 0.15-26.38 pg/ml and 0.06-38.10 pg/ml respectively (see Figure 5.2.).

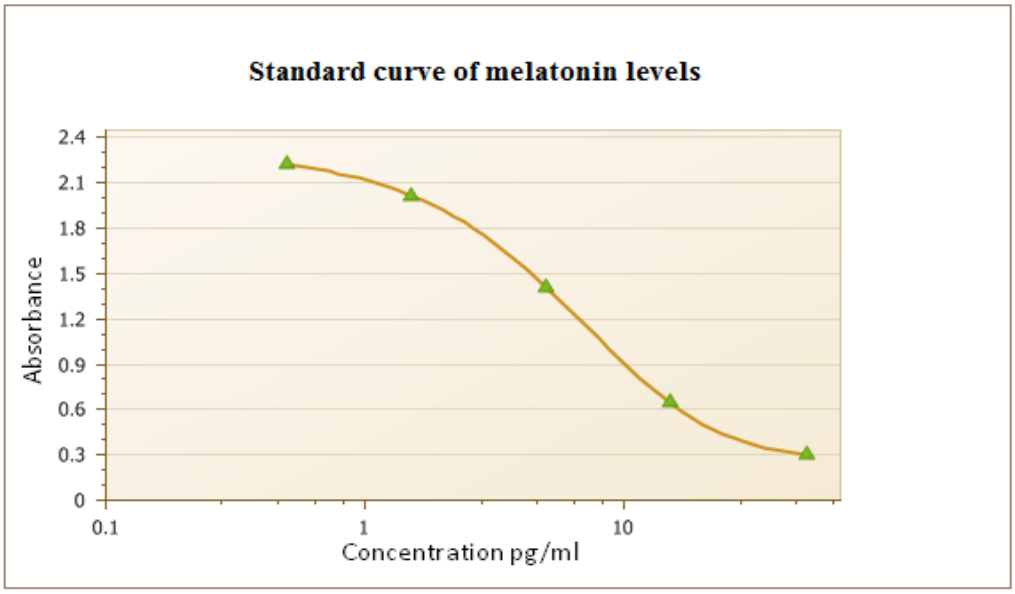


Figure 5.1. Standard curve of melatonin, $r^2 = 0.999$**.** The x axis represents concentration of the standards and y axis corresponding absorbance value.

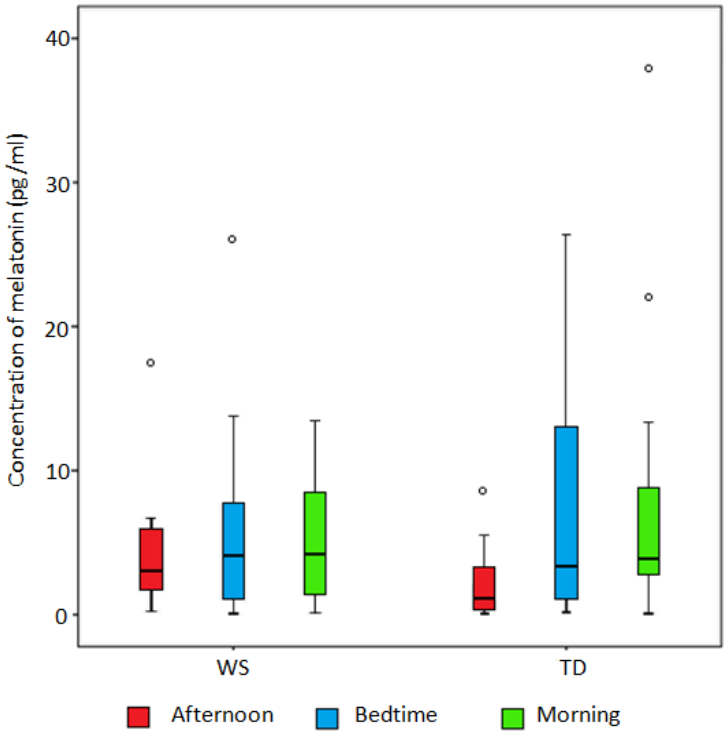


Figure 5.2. Raw levels of salivary melatonin in the afternoon, bedtime and morning samples from children with WS and TD control group. Data are shown as a median and quartiles. Circles represents outliers. High individual variability in the amount of melatonin secreted was observed between participants in both groups.

In order to compensate for individual variation and to observe the changes of melatonin before night time sleep, the morning level of this hormone was set to 100% and the afternoon and evening levels were normalised as a percentage of the morning value (Nowak *et al.*, 1987; Cinaz *et al.*, 2013).

Mann-Whitney analyses were used for group comparison. There was no significant difference in the levels of normalised melatonin in the afternoon and evening between WS and TD groups (Table 5.1., Figure 5.3.).

	Groups	Median	95% CI	p value
Normalised melatonin afternoon (%)	TD (n=25)	40.37	-9.43, 55.70	0.13
	WS (n=17)	72.22		
Normalised melatonin bedtime (%)	TD (n=21)	80.48	-72.7, 35.0	0.78
	WS (n=19)	61.03		

Table 5.1. Comparison of normalised levels of melatonin in WS and TD children using Mann-Whitney test. Table includes median for afternoon and evening levels of melatonin calculated as a percentage of the morning value. 95% confidence interval values (95% CI) and p value are also shown for the determination of significance. N value accounts for the differences from N = 27, as some children failed to provide all three saliva samples and two were excluded because they were taking melatonin as sleep medication.

As described in Section 1.1.5, the evening rise of melatonin secretion facilitates sleep. Thus, the increase in level of this hormone between afternoon to evening should be observed. In order to investigate changes in level of melatonin before bedtime, the ratio between raw, not-normalised levels of melatonin in evening and afternoon samples was calculated for each participant. There was a median fold

increase of 1.83 in TD control group, whereas there was no such increase observed for the WS children (Table 5.2., Figure 5.3.).

	Groups	Minimum	Maximum	Median	95% CI	p value
Increase of melatonin levels from afternoon to bedtime	TD (n=20)	0.36	10.73	1.83	-1.53, -0.04	**0.04**
	WS (n=17)	0.19	2.72	0.96		

Table 5.2. Increase of melatonin levels between afternoon and evening saliva samples collected from children with WS and TD controls. Table includes minimum and maximum value, as well as median of the ratio of melatonin between afternoon and evening samples. 95% confidence interval values (95% CI) and p value are also shown for the determination of significance. N value accounts for the differences from N=27, as some children failed to provide all three saliva samples and two were excluded because they were taking melatonin as sleep medication.

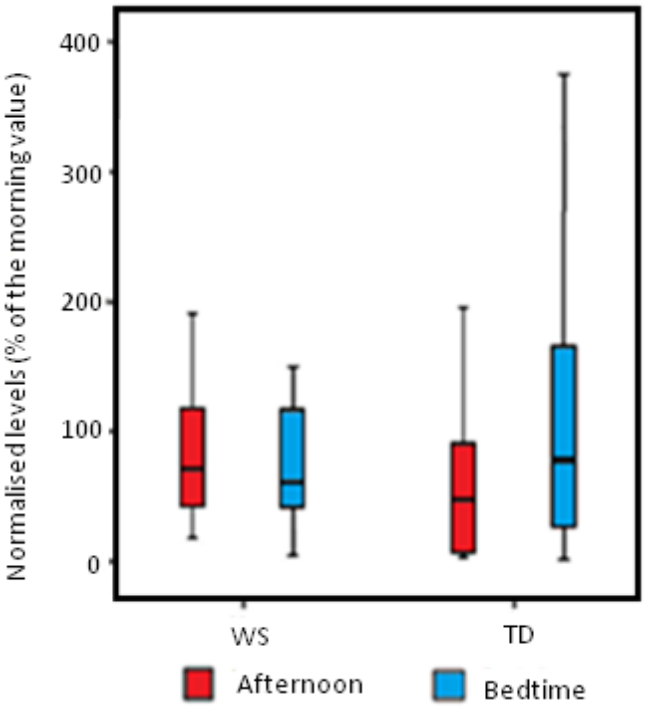


Figure 5.3. Normalised levels of salivary melatonin in the afternoon and bedtime in children with WS and TD control group. Data are shown as a median and quartiles. There was a significant difference in the ratio between afternoon and bedtime levels of salivary

melatonin between WS and TD children ($p = 0.04$) with less pronounced increase in melatonin levels in WS prior to bedtime.

5.2. Salivary cortisol

Twenty- two children out of 27 in the WS group and all TD children collected at least one out of three saliva samples. Cortisol levels rise rapidly during the first 30 min after awakening (Clow *et al.*, 2010), hence it seemed necessary to collect the samples as soon as the child was awake. No difference was observed between both groups in the time from waking up to collection of the samples using independent samples t-test ($t(35) = 0.861$, $p = 0.40$).

Levels of cortisol were determined using an enzyme immunoassay. The standard curve for this assay was in range from 0.012 to 3 µg/dl (Figure 5.4.). Each sample was run in duplicates and average value was calculated for further analyses. The average recovery quoted by the manufacturer of this assay is 100.82%. The level of cortisol was found to be at a maximum in the morning and lowest at bedtime, indicating circadian rhythmicity of salivary cortisol levels. Cortisol levels in WS group ranged from 0.01 to 0.21 µg/dl in the afternoon, 0.01 to 0.14 µg/dl in the evening and 0.04 to 0.75 µg/dl in the morning. For TD control group these values are respectively 0.02 to 0.16 µg/dl, 0.01 to 0.12 µg/dl and 0.09 to 0.89 µg/dl (see Figure 5.5.). As described in the Section 1.1.6. cortisol levels are highest in the morning and decline through the day. Obtained values reflect this pattern, the highest levels are observed in the morning, lower in the afternoon and finally the lowest at bedtime.

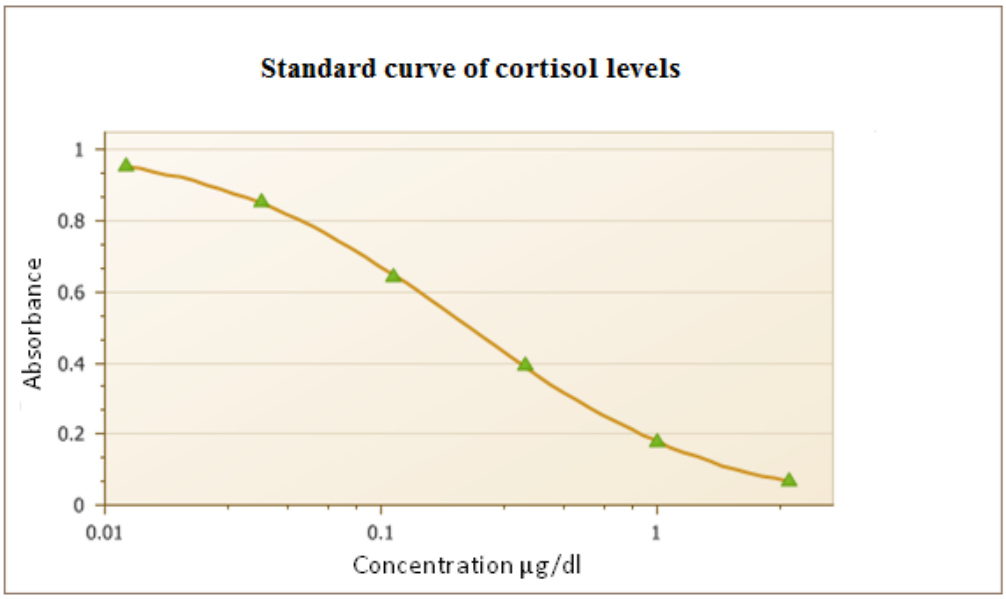


Figure 5.4. Standard curve of cortisol, r^2 = 0.999. The x axis represents concentration of standard solutions, while y axis corresponding absorbance value.

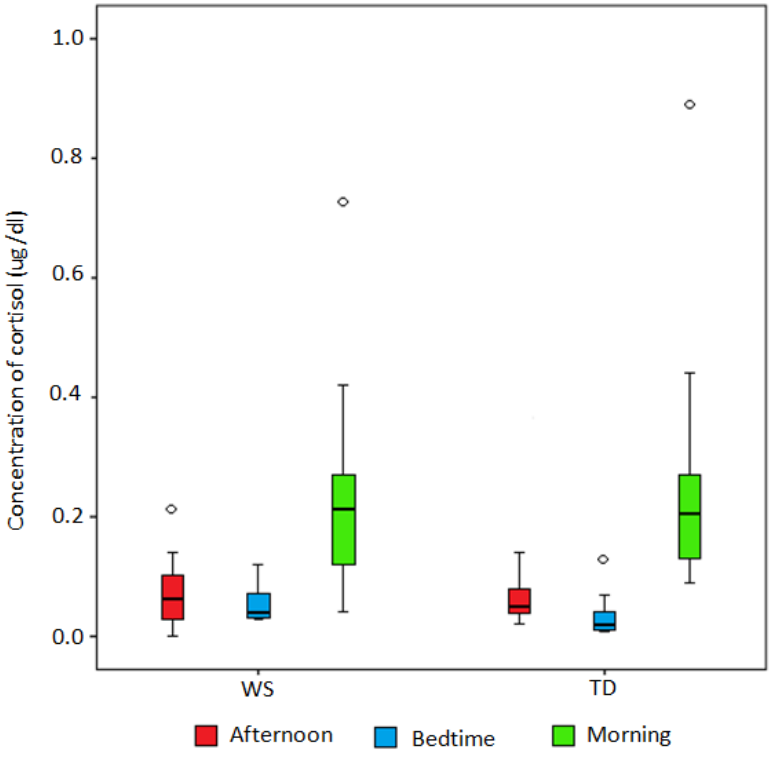


Figure 5.5. Raw levels of salivary cortisol in the afternoon, bedtime and morning samples from children with WS and TD control group. Data are shown as a median and quartiles. Circles represents outliers. High individual variability in the amount of melatonin secreted was observed between participants in both groups.

In order to compensate for individual variation and to observe the changes of cortisol before night time sleep, the morning level of this marker was set to 100% and the afternoon and evening levels were normalised as a percentage of the morning value (Cinaz *et al.*, 2013).

For further group comparison Mann-Whitney test was applied. There was no significant difference in the levels of normalised cortisol in the afternoon between WS and TD groups but a significantly higher value was observed in the evening before bedtime in WS participants (Table 5.3., Figure 5.6.).

Two TD data points and 1 data point from WS group were excluded as outliers (scored over 3 values of SD from the mean).

	Groups	Median	95% CI	p value
Normalised cortisol afternoon (%)	TD (n=25)	22.22	-9.02, 14.11	0.69
	WS (n=20)	27.17		
Normalised cortisol evening (%)	TD (n=20)	15.29	-0.56, 29.99	**0.03**
	WS (n=20)	33.45		

Table 5.3. Comparison of normalised levels of cortisol in WS and TD children using Mann-Whitney test. Table includes median for afternoon and evening levels of melatonin calculated as a percentage of morning value. 95% confidence interval values (95% CI) and p value are shown for the determination of significance. N value accounts for the differences from N=27, as some children failed to provide all three saliva samples and two were excluded due to taking melatonin as a sleep medication.

A more detailed investigation of the cortisol levels was carried out calculating the ratio of raw, not-normalised cortisol levels between afternoon and evening samples. There was a median fold decrease of 1.37 in level of cortisol in individuals with WS,

whereas for the TD control group a decrease of 2.25 was observed ($p < 0.01$) (Table 5.4. and Figure 5.6.).

	Groups	Minimum	Maximum	Median	CI	p value
Decrease of cortisol levels from afternoon to bedtime	TD (n=22)	0.21	10.00	2.25	-2.61, -0.40	**<0.01**
	WS (n=19)	0.09	3.50	1.37		

Table 5.4. Decrease of cortisol levels between afternoon and evening saliva samples collected from children with WS and TD controls. Table includes minimum and maximum value, as well as median of the ratio of cortisol between afternoon and evening samples. 95% confidence interval values (95% CI) and p value are also shown for determination of significance. N value accounts for the differences from N=27, as some children failed to provide all three saliva samples and two were excluded due to taking melatonin as a sleep medication.

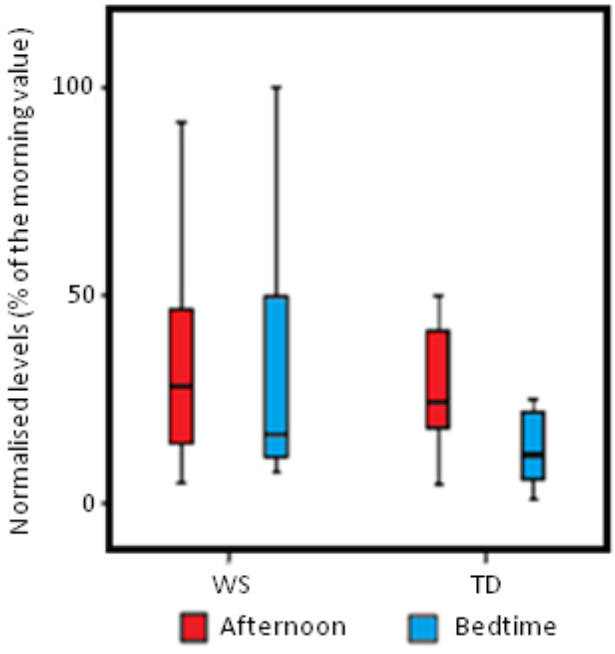


Figure 5.6. Normalised levels of salivary cortisol in the afternoon and bedtime in children with WS and TD control group. Data are shown as a median and quartiles. There was a significant difference in the ratio between afternoon and bedtime levels of salivary cortisol ($p < 0.01$) between WS and TD children with shallower drop of cortisol levels in WS prior to bedtime. Furthermore, children with WS demonstrated significantly higher levels of bed-time salivary cortisol ($p = 0.03$).

5.3. Analysis of MT6s using ELISA

First void morning urine samples (21 from WS and 24 from TD children) were analysed using ELISA. The standard curve for MT6s analyses was in range from 1.7 to 420 ng/ml (Figure 5.7.). Each sample was run in duplicate and average value was calculated and used for further analyses. The average recovery quoted by the manufacturer of this assay is 105.8%.

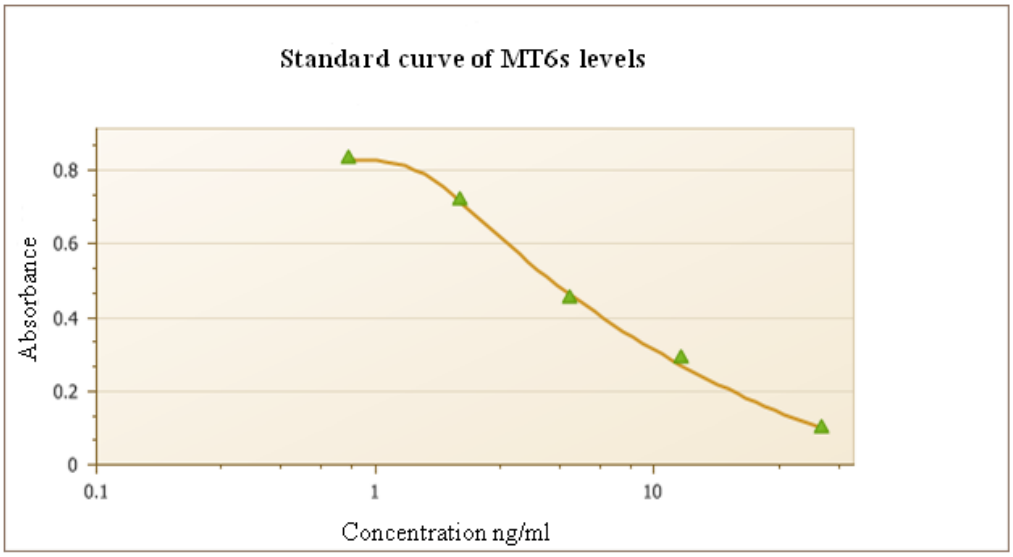


Figure 5.7. Standard curve of MT6s levels, r^2 = 0.998. The x axis represents concentration of the standard solutions, while y axis represents corresponding absorbance value.

The MT6s levels were corrected to levels of creatinine to account for the dilution of urine. MT6s concentration in samples from WS children ranged between 8.91-398.70 ng/mg creatinine and in TD group ranged from 54.61 to 488.51 ng/mg of creatinine. Mann-Whitney test were used for group comparison. As shown in Table 5.5. children with WS scored significantly lower levels of MT6s compared to healthy

controls. One TD sample was removed as an outlier over 3 values of SD from the mean.

	Groups	Median	95% CI	p value
MT6s levels in urine (ng/mg creatinine)	TD (n=23)	171.60	-151.5, -20.4	**<0.01**
	WS (n=21)	83.40		

Table 5.5. Comparison of levels of 6-sulfatoxymelatonin (MT6s) in WS and TD children using Mann-Whitney. Table includes median of the compound concentration; 95% confidence interval values (95% CI) and p value for determination of significance are also shown. N value accounts for the differences from N=27, as some children failed to provide urine sample and two were excluded due to taking melatonin as a sleep medication.

ANCOVA tests were used to investigate whether MT6s levels were associated with chronological age of groups. No statistical effect of age on MT6s levels measured using ELISA was observed in the WS and TD groups (WS: $F(1,21) = 0.811$, $p = 0.38$; TD: $F(1,23) = 1.071$, $p = 0.31$). The effect of bed-wetting on levels of MT6s in WS was not significant ($F(1,21) = 1.68$, $p = 0.21$). Using independent samples t-test, gender difference was analysed in both study groups. It was found that urinary levels of MT6s do not differ between boys and girls in WS ($t(18) = -0.523$, $p = 0.61$) and TD groups ($t(21) = -0.976$, $p = 0.34$).

5.4. Effect of chronological age, gender and the time of sample collection on levels of salivary melatonin and cortisol

Levels of melatonin and cortisol were analysed using ANCOVA with chronological age covariate to control for age effects (Table 5.6.). There was no effect of age on the levels of salivary melatonin in samples collected in the afternoon (WS: $F(1,17) = 0.002$, $p = 0.96$; TD: $F(1,25) = 0.001$, $p = 0.98$) and bedtime samples (WS: $F(1,19) = 0.006$, $p = 0.94$; TD: $F(1,21) = 0.382$, $p = 0.54$). Similarly there was no significant effect of age on salivary cortisol in the samples taken in the afternoon (WS: $F(1,20) = 1.361$, $p = 0.26$; TD: $F(1,25) = 0.655$, $p = 0.43$) and bedtime (WS: $F(1,21) = 0.034$, $p = 0.855$; TD: $F(1,22) = 0.713$, $p = 0.408$), for both groups. No gender difference in levels of afternoon and bedtime melatonin as well as bedtime cortisol was found in either study groups using independent samples t-test (afternoon melatonin: WS: $t(15) = -0.266$, $p = 0.79$, TD: $t(23) = -1.653$, $p = 0.11$; bedtime melatonin: WS: $t(17) = 0.079$, $p = 0.94$, TD: $t(19) = -0.933$, $p = 0.36$; bedtime cortisol: WS: $t(19) = -1.100$, $p = 0.29$, TD: $t(20) = -0.013$, $p = 0.99$). For the afternoon cortisol levels it was noted that girls have significantly higher levels than boys in WS group ($t(18) = -2.414$, $p = 0.03$). Similar findings were observed for TD control group with marginal significance ($t(23) = -1.792$, $p = 0.09$). Furthermore, no difference was observed between both groups in the time taken to collect saliva samples from the moment the child woke up ($t(36) = 0.850$, $p = 0.401$), as well as time taken from saliva collection to sleep start ($t(30) = 0.746$, $p = 0.461$) using independent-samples t test. In addition, as the time of collection may influence the results of the current study, raw/non-normalised levels of melatonin and cortisol were analysed using ANCOVA with collection times as covariates (Table 5.6.). No effect of the time taken to collect

saliva samples from the moment the child woke up was found on morning melatonin levels (WS: $F(1,16) = 0.034$, $p = 0.857$; TD: $F(1,20) = 0.878$, $p = 0.361$), as well as cortisol levels in morning sample (WS: $F(1,18) = 0.041$, $p = 0.842$; TD: $F(1,20) = 0.220$, $p = 0.645$). Similarly, no effect of the time taken from saliva collection to sleep start was found on evening melatonin levels (WS: $F(1,15) = 0.713$, $p = 0.414$; TD: $F(1,14) = 0.001$, $p = 0.973$), as well as evening cortisol levels (WS: $F(1,14) = 0.613$, $p = 0.449$; TD: $F(1,14) = 0.860$, $p = 0.372$).

Parameter for which effect on levels of melatonin and cortisol was analysed	Time point/hormone	Group	P value
Age	Afternoon melatonin	TD(25)	0.98
		WS(17)	0.96
	Bedtime melatonin	TD(21)	0.54
		WS(19)	0.94
	Afternoon cortisol	TD(25)	0.43
		WS(20)	0.26
	Bedtime cortisol	TD(22)	0.41
		WS(21)	0.86
Time taken to collect saliva from the moment the child woke up	Morning melatonin	TD(20)	0.36
		WS(16)	0.86
	Morning cortisol	TD(20)	0.65
		WS(18)	0.84
Time taken from bedtime saliva collection to sleep start	Bedtime melatonin	TD(14)	0.97
		WS(15)	0.41
	Bedtime cortisol	TD(14)	0.37
		WS(14)	0.45

Table 5.6. Effect of chronological age and time of sample collections on levels of salivary melatonin and cortisol in children with WS and TD control group using analysis of covariance (ANCOVA). Neither of these parameters affected the levels of analysed hormones at different time points.

5.5. Ultra-high performance liquid chromatography- tandem mass spectrometry analyses

5.5.1. UHPLC-MS/MS analysis

In this method, chromatographic separation of the analytes was achieved using a C18 reverse phase column. The mass spectrometer was operated in a positive ion mode to detect the compounds. The total run time for the analyses of cortisol, cortisone and MT6s was 6 min including reequilibration, although peaks of interest eluted within 3 min. For the analysis of creatinine, the total run time was 4 min, however, the peaks of interest eluted within 0.5 min. The MRM chromatograms for cortisol, cortisone, and the internal standards indole-3-acetamide and 6-α-methylprednisolone together with single ion chromatogram of 6-sulphatoxymelatonin are shown in the Figures 5.8. and 5.9.

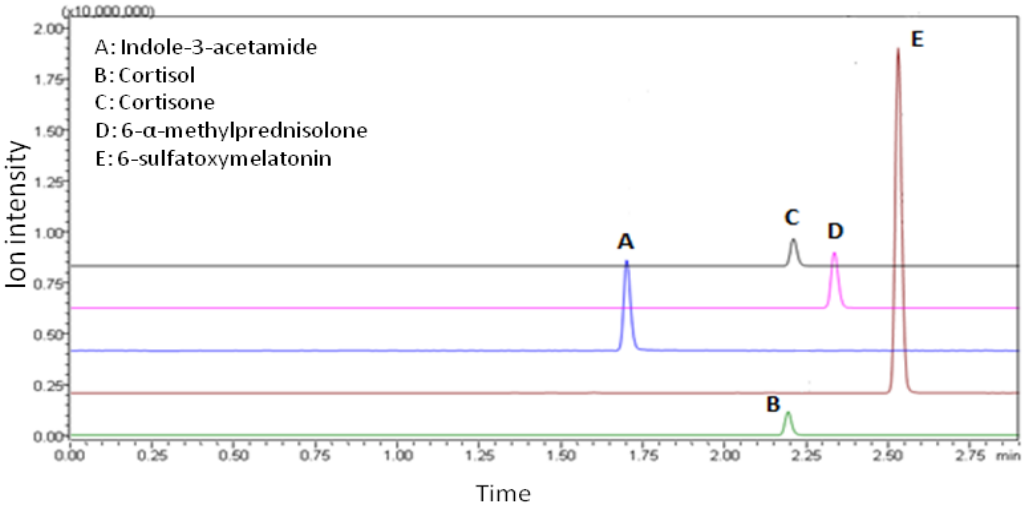


Figure 5.8. Multiple reaction monitoring (MRM) chromatograms of indole-3-acetamide (A), cortisol (B), cortisone (C), 6-α-methylprednisolone (D) and single ion

chromatogram of MT6s (E) in urine. Conditions used to obtain these are described in Section 4.5.

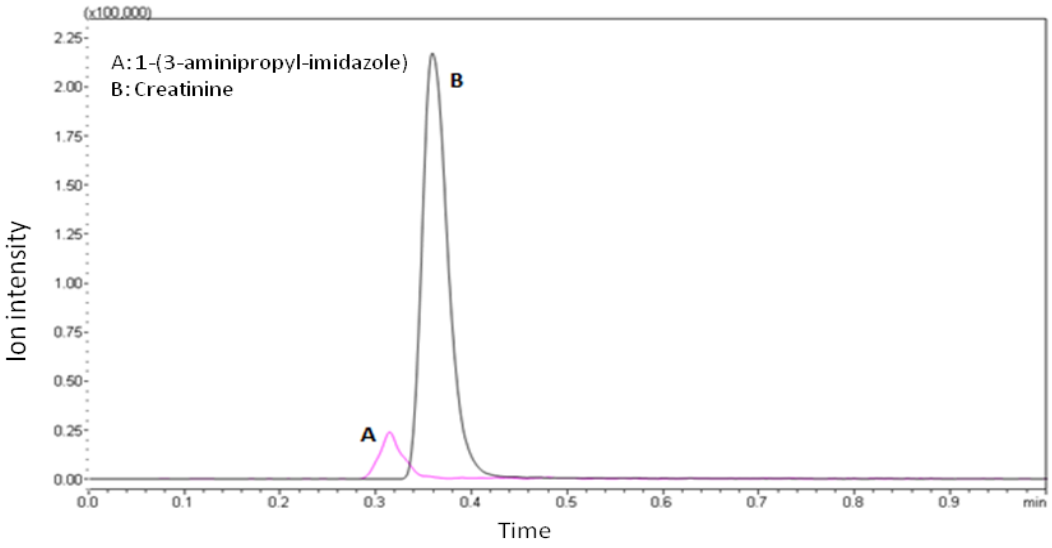


Figure 5.9. Multiple reaction monitoring (MRM) chromatograms of 1-(3-aminopropyl-imidazole) (A) and creatinine (B) at concentration of 500 ng/ml and 0.25 mg/ml respectively.

Narrow and symmetrical peaks were obtained. The resolution between compounds was calculated to be: between cortisol and cortisone was 0.4; between 6-α-methylprednisolone and cortisol and as well as cortisone was 2.55 and 2.18 respectively; between MT6s and its IS was calculated to be 12.62 and between creatinine and its IS was 0.83.

Retention time (RT) for each compound, as well as coefficient of variance (CV%) values for internal standards and QC results are presented in the Table 5.7.

Compound	RT	CV% for RT	r^2	CV% for IS standard solutions	CV% for IS samples	QC CV%
Cortisol	2.19	0.43	0.999	17.91	12.73	12.42
Cortisone	2.21	0.18	0.999	17.91	12.73	7.91
6-Sulfatoxymelatonin	2.51	1.99	0.998	14.83	19.19	11.03
Creatinine	0.36	1.39	0.999	8.04	16.84	11.85
6-α-methylprednisolone*	2.33	0.17	*these three compounds are used as IS thus no values for r^2, CV% for IS and QC CV% can be given			
Indole-3-acetamide*	1.69	0.39				
1-(3-aminipropyl-imidazole)*	0.31	1.33				

Table 5.7. Retention times (RT), r^2 values and coefficient of variance (CV%) for RT, internal standards (IS) and quality control samples (QC) for cortisol, cortisone, 6-sulfatoxymelatonin, creatinine as well as RT and CV% for RT for internal standards.

The percentage CVs for retention time for all the analytes ranged between 0.17 to 1.99%. The intra-assay CVs ranged from 0.02 to 20.83% at concentrations of 2.78-100 ng/ml for cortisol and cortisone, 3.11-112 ng/ml for MT6s and 0.125-0.75 mg/ml for creatinine. The higher values of CV were observed for the lowest concentrations of analysed compounds. It has been reported that CVs around 20 % are acceptable if the concentrations used for CV analysis were at the lower limit of quantification or near (Bioanalytical Method Validation, 2001). Inter-assay CVs ranged from 0.18 to 22.50% for the glucocorticoids (cortisol and cortisone), MT6s and creatinine at concentration ranging from 2.78 to 50 ng/ml, 3.11 to 56 ng/ml and 0.125 to 0.75 mg/ml respectively.

Linearity was estimated by dividing the observed value by the expected value for each calibration sample. Linear calibrations were obtained for glucocorticoids and MT6s in the range of 2.78 to 50 ng/ml and 3.11 to 56 ng/ml respectively. The percentages of the expected results for cortisol ranged between 92 % - 105 % for cortisone from 92 % to 107 % and for MT6s between 93 % - 120 %.

Calibration curves

Logarithmic axis scales were chosen in order to avoid compressing small values down into bottom of the graph. The R^2 values for the curves ranged between 0.998 and 0.999 (Figures 5.10. to 5.13.).

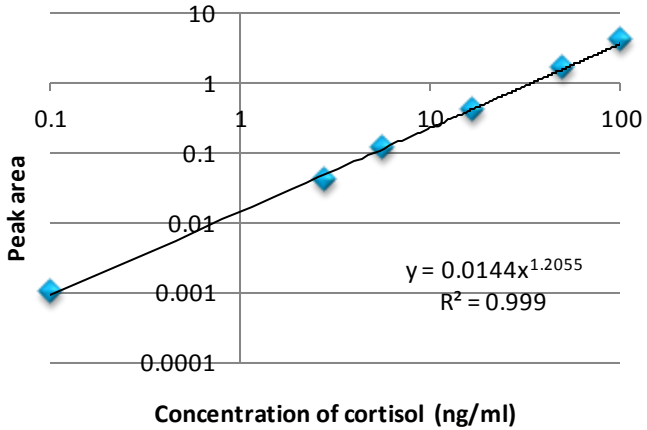


Figure 5.10. Standard curve of cortisol

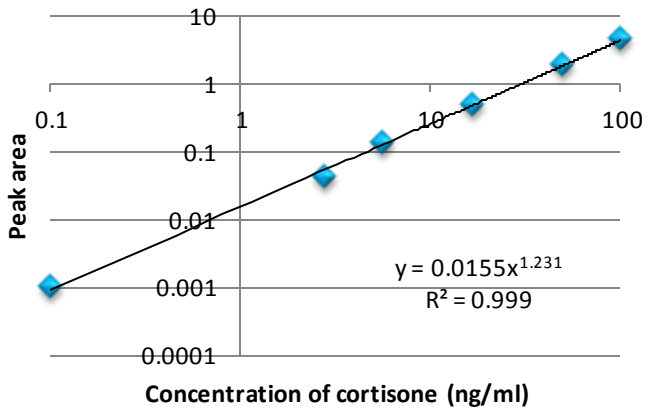


Figure 5.11. Standard curve of cortisone

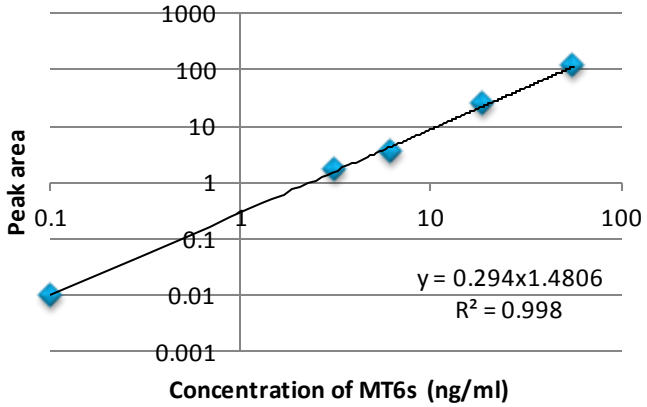


Figure 5.12. Standard curve of MT6s

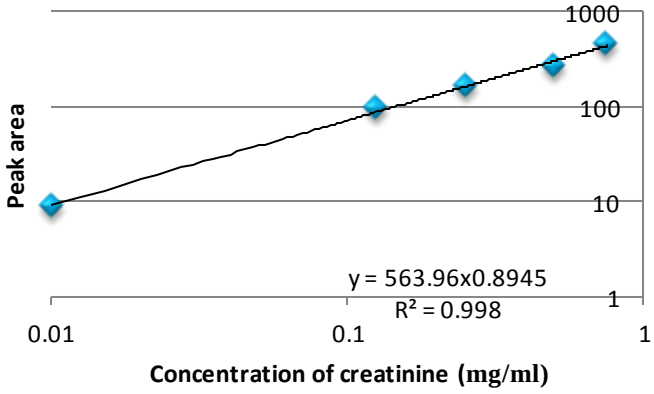


Figure 5.13. Standard curve of creatinine

5.5.2. Urine samples analysis

Obtained values were multiplied by the dilution factor. Levels of urinary cortisol, cortisone and MT6s uncorrected for creatinine in children with WS and TD control group are presented in a Table 5.8. Levels similar to those were reported previously (Lin *et al.*, 1997; Mayo Clinic, http://www.mayomedicallaboratories.com).

Compound (ng/ml)	Groups	Minimum	Maximum	Median	SD
Cortisol	TD (n=24)	4.85	32.68	18.08	7.76
	WS (n=22)	7.32	52.46	18.99	11.05
Cortisone	TD (n=24)	8.90	49.20	21.60	9.66
	WS (n=22)	11.42	61.81	26.89	12.00
MT6s	TD (n=24)	0.36	3.66	1.62	0.98
	WS (n=20)	0.23	2.08	0.86	0.47

Table 5.8. Levels of urinary cortisol, cortisone and 6-sulfatoxymelatonin (MT6s) uncorrected for creatinine in children with WS and TD control group. Table includes minimum, maximum and median levels of all the compounds, as well as standard deviation (SD).

Levels of cortisol, cortisone and MT6s in urine samples were further corrected with respect to level of creatinine prior to statistical analysis. The corrected values obtained in this study were: cortisol in WS group 18.84-139.61 ng/mg creatinine and in the TD children 14.61-93.52 ng/mg creatinine, levels of cortisone were 26.50-265.99 ng/mg creatinine and 17.96-110.15 ng/mg creatinine for WS and TD

respectively. High individual variability in the amount of MT6s secreted was also observed in both groups. Level of MT6s in urine samples in children with WS ranged from 0.60 to 6.96 ng/mg creatinine and in the TD from 1.27 to 13.52 ng/mg creatinine.

Further, Mann-Whitney test for group comparisons was used. Table 5.9. shows that level of cortisone is significantly higher in WS children then TD controls. Also, levels of MT6s excreted in urine were found to be significantly lower in WS group.

Creatinine levels were within the normal range (Sugita *et al.*, 1992; Trombetta & Foote, 2009) and ranged between 0.10-0.40 mg/ml in WS group and 0.20-0.53 mg/ml for TD children. No statistically significant difference was observed for levels of creatinine between both study groups ($U(46) = 246.0$, $p = 0.69$).

Compound (ng/mg creatinine)	**Group**	**Median**	**95% CI**	**p value**
Cortisol	TD (N=24)	48.13	-13.52, 18.98	0.90
	WS (N=22)	47.32		
Cortisone	TD (N=22)	48.82	-0.47, 39.63	**0.05**
	WS (N=21)	64.20		
MT6s	TD (N=24)	4.39	-3.44, -0.48	**<0.01**
	WS (N=20)	3.22		

Table 5.9. Comparison of urinary levels of cortisol, cortisone and 6-sulfatoxymelatonin (MT6s) in WS and TD children using Mann-Whitney test. Table includes median concentration of compounds. The 95% confidence interval values (95% CI) and p value for determination of significance are also shown. Significant results are shown in bold.

5.5.3. Cortisol/cortisone ratio

Using Pearson's moment correlations it was found that urinary levels of cortisol and cortisone significantly correlate in WS and TD children (WS: $r(22) = 0.562$, $p < 0.01$; TD: $r(24) = 0.638$, $p = 0.001$) (Figure 5.14.). Furthermore, cortisol/cortisone ratio was also analysed as an indication of HSD type II activity. The median cortisol/cortisone ratio in WS was lower in comparison with TD group (0.59 and 0.79 for WS and TD groups respectively), however, the level of significance was not achieved ($U(46) = 184.0$, $p = 0.08$).

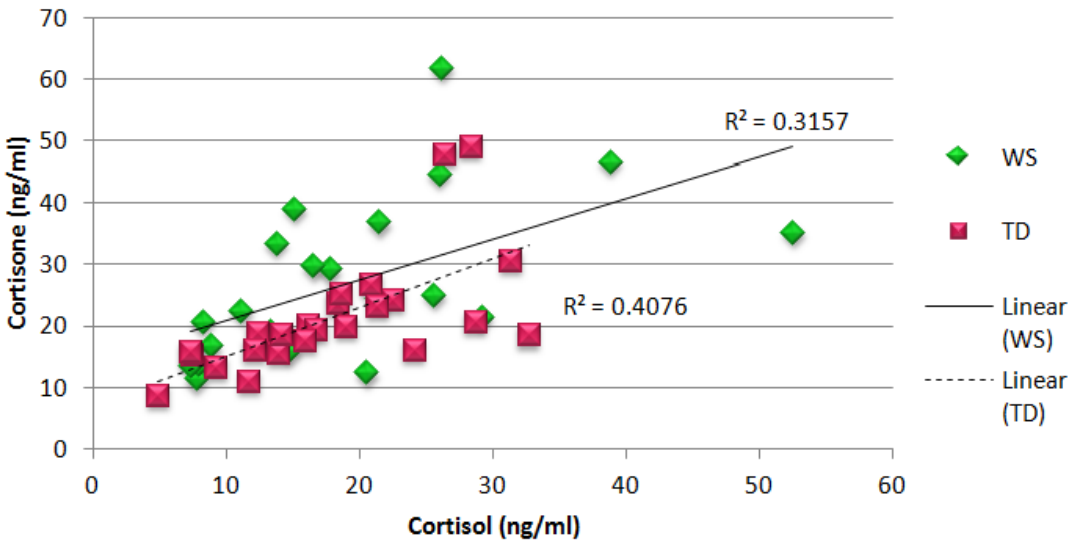


Figure 5.14. The comparison of levels of cortisol and cortisone measured by UHPLC-MS/MS method. Using Pearson's moment correlations it was found that urinary levels of cortisol and cortisone significantly correlate in WS and TD children ($p < 0.01$).

5.5.4. Effect of chronological age, gender, bed-wetting and time of samples collection on levels of cortisol, cortisone and MT6s

Levels of urinary cortisol, cortisone and MT6s were compared using ANCOVA with age as a covariate to control for the age effects (see Table 5.10). No age effect was observed in both study groups for the urinary levels of cortisol (WS: $F(1,22) = 0.431$, $p = 0.52$; TD: $F(1,24) = 0.879$, $p = 0.36$), cortisone (WS: $F(1,22) = 0.629$, $p = 0.44$; TD: $F(1,23) = 1.202$, $p = 0.29$) and MT6s (WS: $F(1,20) = 0.487$, $p = 0.49$; TD: $F(1,24) = 0.340$, $p = 0.57$).

In addition, in both study groups no gender difference was found in levels of cortisol (WS: $t(20) = 0.207$, $p = 0.84$; TD: $t(22) = -1.641$, $p = 0.12$), cortisone (WS: $t(20) = -1.125$, $p = 0.27$; TD: $t(21) = -1.327$, $p = 0.20$) and MT6s (WS: $t(18) = -1.346$, $p = 0.20$; TD: $t(22) = -1.063$, $p = 0.30$) using independent samples t-test.

Four children with WS were reported to wet the bed on the night of sample collection (See Table 5.10.). The bed-wetting parameter was used as a covariate to assess whether the results were affected. No effect of bed-wetting was observed in the data obtained from WS children for urinary analysis of cortisol ($F(1,21) = 0.35$, $p = 0.56$), cortisone ($F(1,21) = 2.10$, $p = 0.16$) and MT6s ($F(1,19) = 1.13$, $p = 0.30$).

Furthermore, the time (min) from get up to sample collection was measured and analysed using independent samples t-test. No significance was found for this parameter ($t(33) = 1.108$, $p = 0.24$). The time from get up to urine collection was also used as a covariate in ANCOVA analysis (See Table 5.10.). No significant effect of this parameter was found for levels of urinary cortisol (WS: $F(1,19) = 0.971$, $p = 0.34$; TD: $F(1,20) = 0.677$, $p = 0.42$), cortisone (WS: $F(1,19) = 0.008$, $p =$

0.93; TD: F(1,20) = 0.439, p = 0.52) and MT6s (WS: F(1,17) = 0.106, p = 0.75; TD: F(1,19) = 0.001, p = 0.98).

Parameter for which effect on levels of melatonin and cortisol was analysed	Time point/hormone	Group	P value
Age	Cortisol	TD(24)	0.36
		WS(22)	0.52
	Cortisone	TD(23)	0.29
		WS(22)	0.44
	MT6s	TD(24)	0.57
		WS(20)	0.49
Bed-wetting	Cortisol	WS(21)	0.56
	Cortisone	WS(21)	0.16
	MT6s	WS(19)	0.30
Time from get up to sample collection	Cortisol	TD(20)	0.42
		WS(19)	0.34
	Cortisone	TD(20)	0.52
		WS(19)	0.93
	MT6s	TD(19)	0.98
		WS(17)	0.75

Table 5.10. Effect of chronological age, bed-wetting and sample collection time on urinary levels of cortisol, cortisone and 6-sulfatoxymelatonin (MT6s) in children with WS and TD control group using analysis of covariance (ANCOVA). Neither of these parameters affected levels of analysed hormones in urine. No effect of bed-wetting in TD group is given, as none wet the bed.

5.6. Sleep characteristic in children on melatonin as a sleep medication

Two of the WS children were taking melatonin as a medication for their sleep problems and were excluded from the main analysis of the results. However, sleep parameters and evening levels of melatonin were analysed in these children separately from the rest of WS group. Figure 5.15. shows the evening levels of melatonin in WS group, TD controls and WS children taking melatonin as a sleep medication. The level of melatonin in these two children was over 8 fold higher than in WS population without any sleep medicine and over 6 fold higher than TD control group.

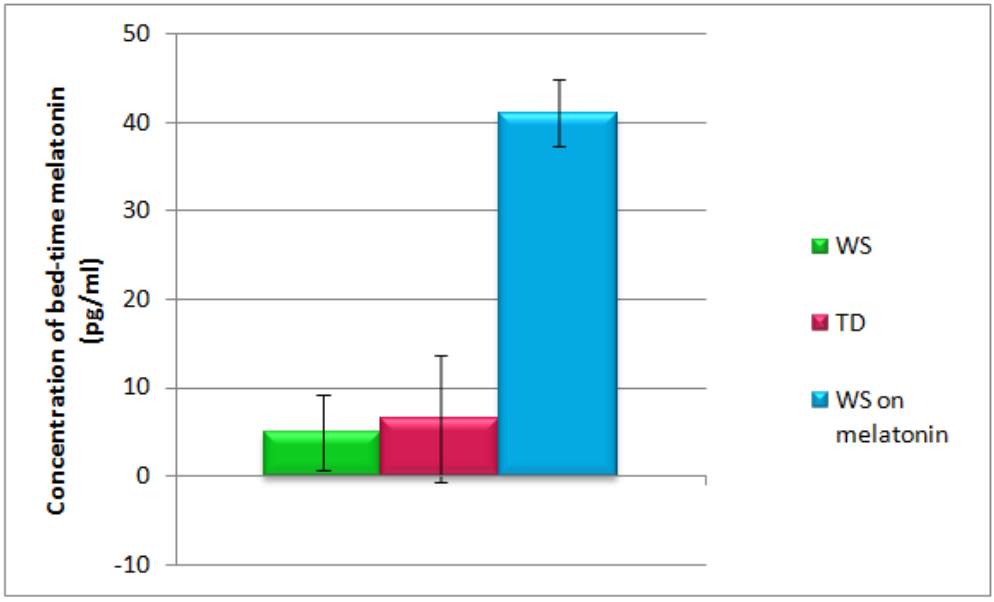


Figure 5.15. Concentration of salivary melatonin (pg/ml) in WS and TD children compared to two WS children taking melatonin as a sleep medicine. The level of melatonin in these two children was over 8 fold higher than in WS population without any sleep medicine and over 6 fold higher than TD control group.

Such high levels however, did not improve sleep efficiency (67.97 compared to 79.56 ± 6.90 in WS group and 83.56 ± 4.87 in TD controls), sleep latency (86.67 min

compared to 52.83 ± 28.73 in WS and 28.73 ± 18.80 in TD children) or mean activity scores during the night measured by actigraphy (58.82 in children on melatonin, 17.27 ± 8.22 in WS population, 12.43 ± 4.22 in control group) (Figure 5.16.). No standard deviation for WS group taking melatonin as sleep medication is given, as one of these children failed to keep an actiwatch on, thus the actigraphic data was collected for only one child.

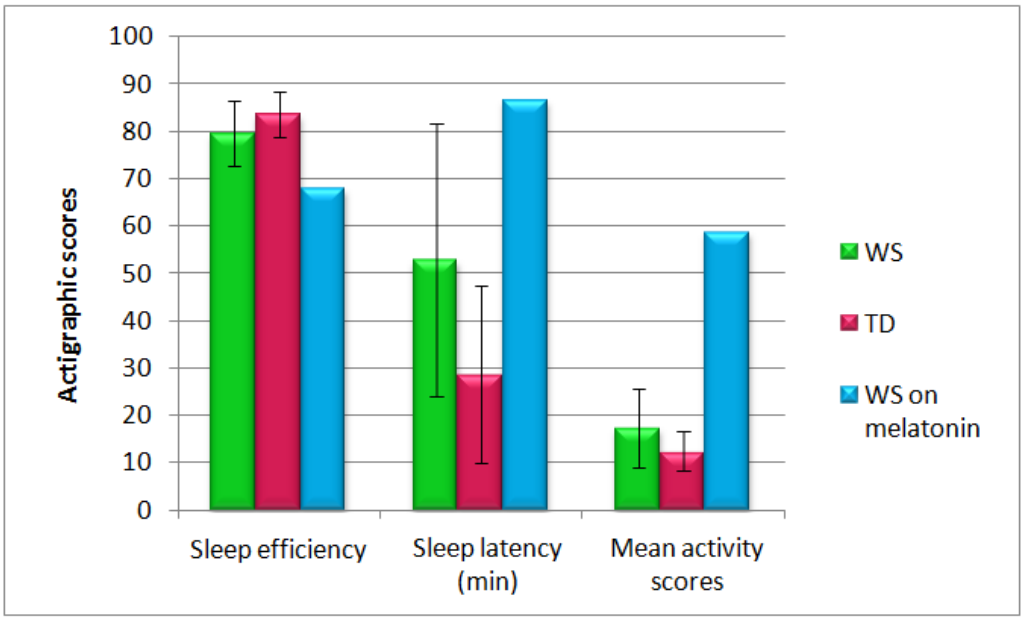


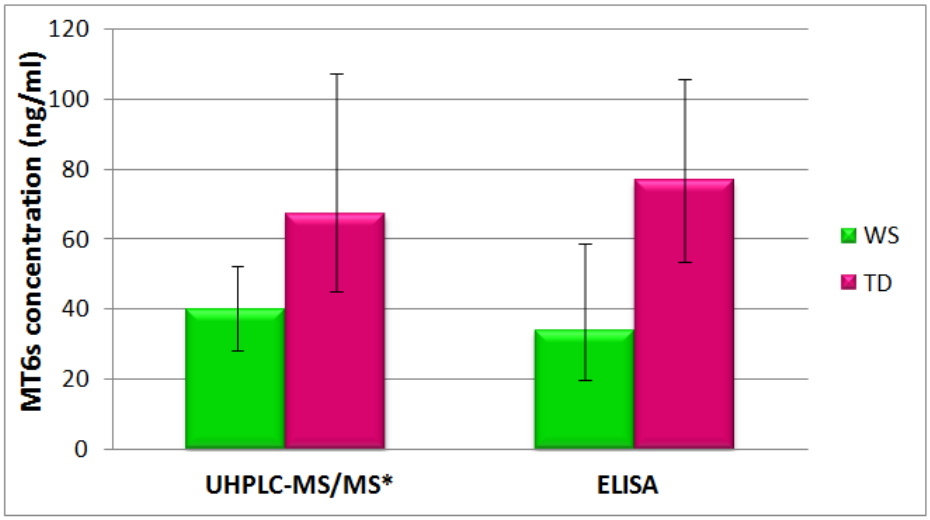
Figure 5.16. Comparison of mean and standard deviation of sleep efficiency, sleep latency (min) and mean activity score measured by actigraphy during three consecutive nights in children with WS, TD children and a child taking melatonin as a sleep medication.

The results of urinary MT6s in two children taking melatonin as sleep medication were not conclusive. The levels of MT6s using ELISA were found to be 48.79 and 310.93 ng/mg creatinine, while the mean value for WS group is 119.69 ± 105.41 ng/mg creatinine. A similar situation is observed for MT6s values obtained by UHPLC-MS/MS method. The two children scored 4.17ng/mg creatinine and 12.17ng/mg creatinine, while the mean and SD for the rest of WS group are 2.99 ±

1.43ng/mg creatinine. As there were only two children taking melatonin as a sleep medication, and only one of them kept the actiwatch on over the course of the study, these data are not significant, but indicate that effect of melatonin on sleep in children with neurodevelopmental disorders should be investigated further.

5.7. Comparison of UHPLC-MS/MS and ELISA method for analysis of MT6s

As shown in Sections 5.3. and 5.5. significant difference in levels of MT6s in urine samples between WS and TD children has been found using both- UHPLC-MS/MS and ELISA methods (Figure 5.17.). However, the values obtained using ELISA were much higher (69.29 ± 47.57 ng/ml) in comparison with those measured by UHPLC-MS/MS (1.27 ± 0.87 ng/ml). Pearson's product moment correlations were used to investigate the similarity between MT6s levels analysed using UHPLC-MS/MS and ELISA. The significant positive relationship was observed between both methods ($r(41) = 0.39$, $p = 0.01$) (Figure 5.18.).



* MT6s levels multiplied by 50

Figure 5.17. Comparison of 6-sulfatoxymelatonin (MT6s) levels obtained using ultra-high performance liquid chromatography- tandem mass spectrometry (UHPLC-MS/MS) and enzyme-linked immunosorbent assay (ELISA) in children with WS and TD control group.

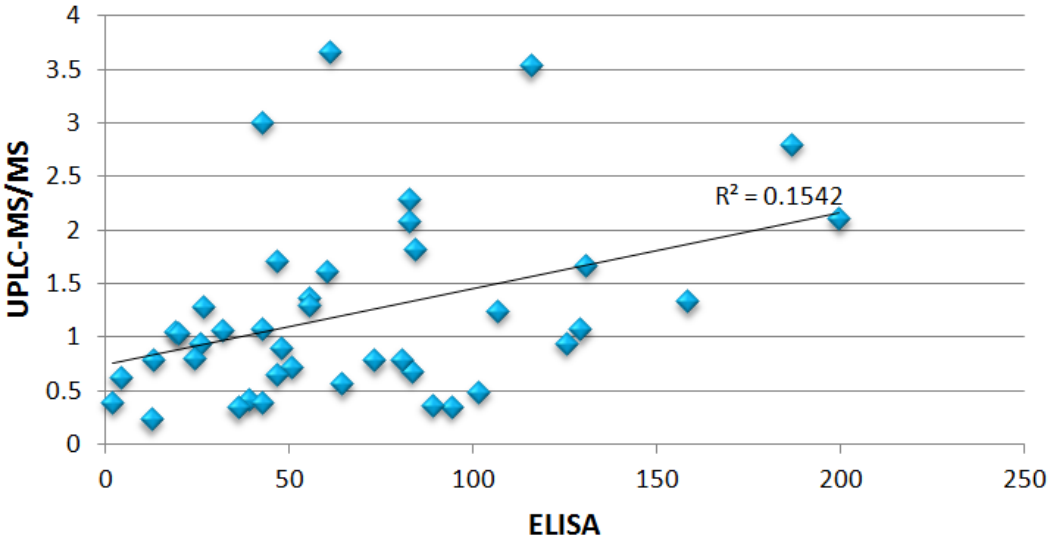


Figure 5.18. Comparison of 6-sulfatoxymelatonin (MT6s) levels obtained using enzyme-linked immunosorbent assay (ELISA)and ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) methods from all study participants (WS and TD children).

5.8. Correlation between sleep measures and levels of melatonin and cortisol

Pearson's product moment correlations were used to determine whether the sleep problems observed in WS children can be explained by abnormal secretion of melatonin and cortisol. The analyses were performed using SPSS for Windows Version 19 (SPSS, Inc., Chicago, IL) as well as on the Minitab Version 16 (Coventry, UK). As the current work focuses on investigation of sleep problems, it is particularly interesting to observe changes of hormonal markers of sleep at and prior to bedtime. Thus, bedtime samples as well as ratios between afternoon and bedtime levels are most relevant to relate with the sleep parameters data. Several significant correlations have been, however, found for afternoon levels as well (please see sections below).

5.8.1. Correlations between CSHQ data and salivary melatonin and cortisol

One of the CSHQ parameters, which scored significantly higher in WS group are parasomnias. It was shown that afternoon levels of salivary melatonin significantly correlate with parasomnias subscale of CSHQ in WS ($r(17) = 0.631$, $p < 0.01$) but not TD controls ($r(25) = -0.268$, $p = 0.20$) (Table 5.11.). As shown on Figure 5.19., the relationship between these parameters follow a different pattern in both groups. As the melatonin is a hormone, which facilitates sleep, it is expected that sleep disturbances, in this case parasomnias, will decrease with increased concentration of

melatonin, and this pattern is observed in the control group. However, in the WS group, the relationship between parasomnias and afternoon levels of melatonin is directly proportional. Furthermore, using 2 sample t-test of the gradients of the two regression fits, it was found that there is a significant difference in relationship between salivary melatonin and parasomnias between WS and TD groups ($t(31) = 3.33$, $p < 0.01$).

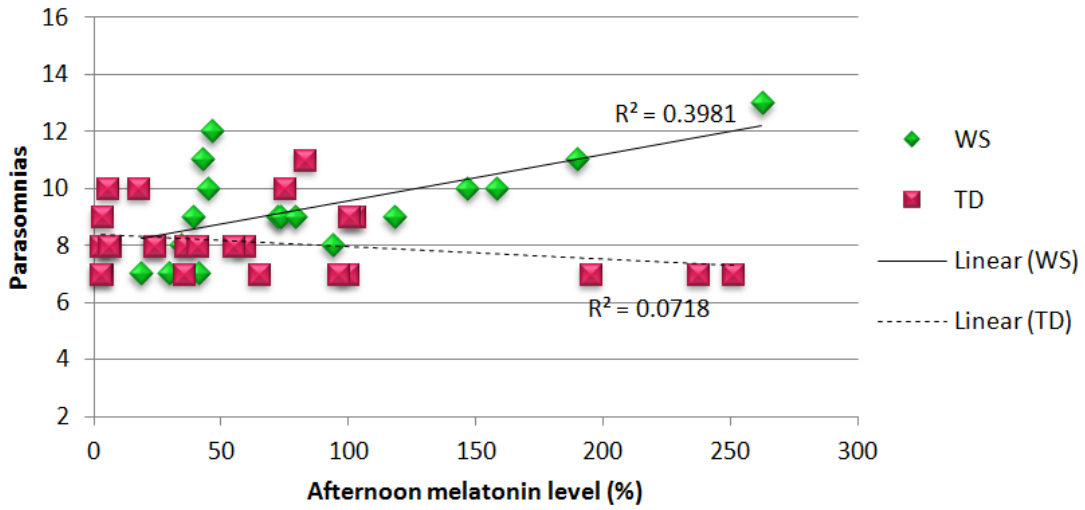


Figure 5.19. The correlation between frequency of parasomnias reported by parents in Children's Sleep Habit Questionnaire (CSHQ) and normalised levels of afternoon melatonin (%) in saliva samples collected from WS and TD children. The significant positive correlation was observed between afternoon levels of salivary melatonin and parasomnias subscale of CSHQ in WS group ($r(17) = 0.631$, $p < 0.01$) but not in TD controls ($r(25) = -0.268$, $p = 0.20$). It can be seen that the relationship between these parameters follow a different pattern in both study groups. Parasomnias decrease with increased concentration of melatonin in the control group. However, in the WS group, the relationship between parasomnias and afternoon levels of melatonin is directly proportional. Using 2 sample t-test of the gradients of the two regression fits, significant difference in relationship between salivary melatonin and parasomnias was found between WS and TD groups ($t(31) = 3.33$, $p < 0.01$).

It was also expected that sleep improves with increasing levels of melatonin. Thus, due to the fact that the higher CSHQ score, the greater sleep problems, it is expected that the total CSHQ score decreases with the rise of bedtime levels of melatonin. The significant negative correlation was observed between bedtime melatonin levels in saliva and the total score of CSHQ in TD ($r(21) = -0.500$, $p = 0.02$), indicating that the higher melatonin concentration, the lower sleep disturbance level in children (Figure 5.20.). This relationship was not observed for individuals with WS ($r(19) = 0.079$, $p = 0.75$). However, using residual diagnostic it was found that the difference in relationship between salivary melatonin levels at bedtime and total score of CSHQ was not significant between WS and TD groups ($t(38) = 0.89$, $p = 0.38$).

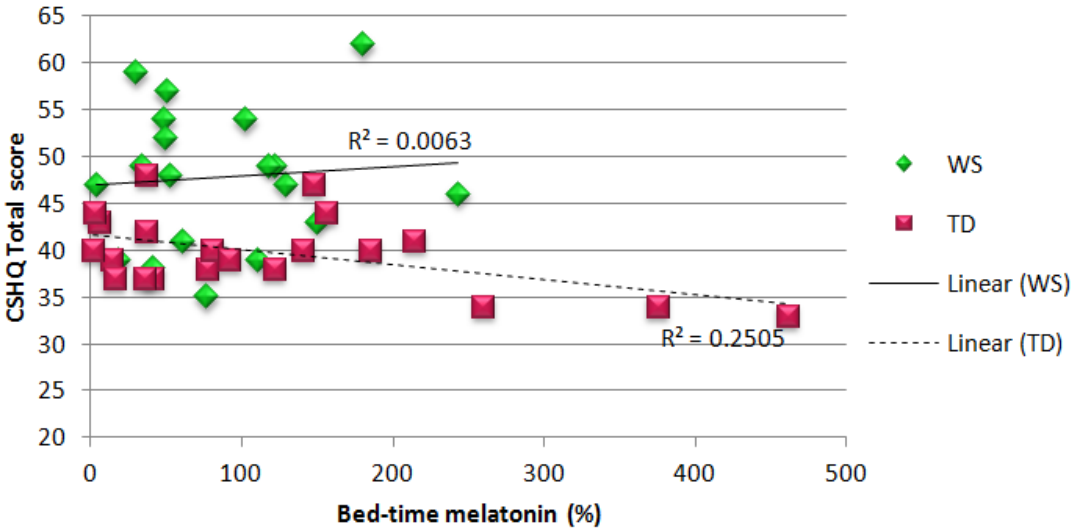


Figure 5.20. The correlation between Total Children's Sleep Habit Questionnaire (CSHQ) score and bedtime melatonin levels (%) in saliva samples collected from WS and TD children. A significant negative correlation was observed between bedtime melatonin levels in saliva and the Total score of CSHQ in TD ($r(21) = -0.500$, $p = 0.02$), indicating that the higher melatonin concentration, the lower sleep disturbance level. This was not observed in WS ($r(19) = 0.079$, $p = 0.75$). Using residual diagnostic it was found that the difference in relationship between salivary melatonin levels at bedtime and total score of CSHQ was not significant between WS and TD groups ($t(38) = 0.89$, $p = 0.38$).

Also, some evidence of correlation between afternoon levels of cortisol and sleep onset delay in WS has been found ($r(19) = 0.399$, $p = 0.09$), but this relationship is not observed in the TD group ($r(25) = -0.045$, $p = 0.83$). Using 2-samples t-test it was shown, that there is no significant difference in relationship between afternoon cortisol levels in saliva and sleep onset delay score of CSHQ between WS and TD groups ($t(42) = 1.46$, $p = 0.15$).

Endocrine marker of sleep	Sleep parameter	Group	r value	p value
Saliva	**CSHQ**			
Afternoon melatonin	Parasomnias	TD(25)	-0.27	0.20
		WS(17)	0.63	**<0.01**
Bedtime melatonin	Total CSHQ score	TD(21)	-0.50	**0.02**
		WS(19)	0.08	0.75
Afternoon cortisol	Sleep onset delay	TD(25)	-0.05	0.83
		WS(19)	0.40	0.09
Saliva	**Actigraphy**			
Afternoon melatonin	Sleep latency	TD(25)	-0.54	**0.01**
		WS(17)	0.25	0.37
Bedtime melatonin		TD(21)	-0.49	**0.04**
		WS(19)	0.06	0.83
Afternoon cortisol		TD(20)	0.45	**0.04**
		WS(17)	0.51	**0.04**
Afternoon cortisol	Sleep efficiency	TD(19)	-0.58	**0.01**
		WS(16)	-0.09	0.75

	Wake after sleep onset	TD(19)	0.44	0.06
		WS(16)	0.24	0.37
	Time spent asleep after sleep onset (%)	TD(19)	-0.44	0.06
		WS(17)	-0.58	**0.01**
Drop of cortisol from before bedtime	Number of immobile phases	TD(16)	0.52	**0.04**
		WS(17)	-0.10	0.72
Urine	**CSHQ**			
Cortisone	Sleep onset delay	TD(24)	0.43	**0.03**
		WS(20)	0.15	0.52
Cortisol		TD(24)	0.38	0.07
		WS(20)	-0.05	0.84
	Actigraphy			
Cortisol	Total actigraphy score	TD(19)	0.44	0.06
		WS(19)	0.08	0.75
Cortisone	Sleep fragmentation	TD(16)	0.67	**<0.01**
		WS(19)	0.09	0.72
	Moving minutes during the night	TD(16)	0.45	0.08
		WS(19)	0.02	0.94

Table 5.11. Pearson's product moment correlation analysis between endocrine markers of sleep (melatonin and cortisol) and parental report (Children's Sleep Habit Questionnaire- CSHQ) as well as actigraphy. Table presents r value and p value. Significant correlations are presented in bold.

5.8.2. Correlations between actigraphy data and salivary melatonin and cortisol

Several correlations were found between levels of melatonin and cortisol and sleep latency (Table 5.11.). As expected, TD children that have higher levels of salivary melatonin before sleep took shorter time to fall asleep. This was not observed for WS group suggesting that melatonin may not function properly as a sleep marker in this disorder. From the actigraphic data the sleep latency adversely correlated with afternoon ($r = -0.540$, $p = 0.01$) and bedtime ($r = -0.494$, $p = 0.04$) levels of salivary melatonin in TD group, however, no such correlation was observed for the WS children ($r = 0.249$, $p = 0.37$ and $r = 0.058$, $p = 0.83$ for afternoon and bedtime samples respectively) (Figure 5.21.). In addition, the difference in relationship between afternoon levels of melatonin and sleep latency proved to be significant between WS and TD children ($t(33) = 2.15$, $p = 0.04$). This was not observed for bedtime melatonin levels and sleep latency ($t(31) = 1.00$, $p = 0.33$).

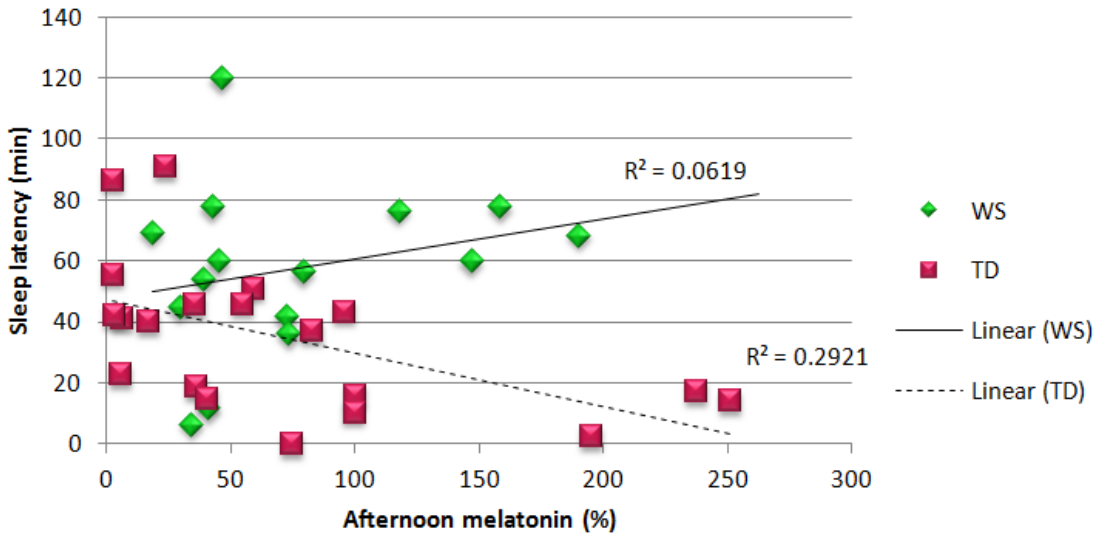


Figure 5.21. The correlation between sleep latency (min), and normalised levels of afternoon melatonin (%) in saliva samples collected from WS and TD children. The significant negative correlation was found between afternoon melatonin levels and sleep latency in TD children ($r(20) = -0.540$, $p = 0.01$), indicating that the higher melatonin levels, the shorter time they took to fall asleep. No such correlation was observed for the WS children ($r(15) = 0.249$, $p = 0.37$). In addition, the difference in relationship between afternoon levels of melatonin and sleep latency proved to be significant between WS and TD children ($t(33) = 2.15$, $p = 0.04$).

The afternoon levels of salivary cortisol correlated positively with sleep latency ($r(20) = 0.450$, $p = 0.04$) and negatively with sleep efficiency ($r(19) = -0.577$, $p = 0.01$) in TD controls. Thus, the higher the level of cortisol the longer time taken by the child to fall asleep and thus, the lower sleep efficiency score. This typical relationship was observed in WS individuals for sleep latency ($r(17) = 0.514$, $p = 0.04$), but not for sleep efficiency ($r(16) = -0.087$, $p = 0.75$). However, using 2-sample t-test, no statistically significant difference in relationship between afternoon cortisol levels and sleep latency ($t(34) = -0.41$, $p = 0.69$) as well as afternoon cortisol level and sleep efficiency ($t(33) = 1.30$, $p = 0.20$) was observed between both study groups.

In TD children it was also found that the higher levels of cortisol in the afternoon, the longer time spent awake after sleep onset and accordingly, the shorter time spent asleep during the night with marginal significance of $r(19) = 0.442$, $p = 0.06$ for actual wake % and $r(19) = -0.436$, $p = 0.06$ for actual sleep %. These typical relationship was found in WS children for actual sleep % ($r(17) = -0.580$, $p = 0.01$), but not actual wake % ($r(16) = 0.242$, $p = 0.37$). No statistically significant difference in relationship between afternoon levels of cortisol and actual wake % in the night ($t(33) = -1.31$, $p = 0.20$) as well as actual sleep % ($t(34) = 0.88$, $p = 0.38$) was found for both study groups.

The significant correlation was also found between the drop of cortisol levels from afternoon to bedtime and the number of immobile phases in TD controls. The higher drop of cortisol observed, the more immobile phases recorded by actigraphs in TD children ($r(16) = 0.519$, $p = 0.04$), but not in WS individuals ($r(17) = -0.095$, $p = 0.72$). However, the difference in this relationship between the two groups was not significant ($t(31) = -1.13$, $p = 0.26$).

No significant correlations were found between raw levels of melatonin and cortisol in morning, afternoon and bedtime samples and sleep parameters from CSHQ as well as actigraphy.

5.8.3. Correlations between CSHQ data and urinary cortisol and cortisone

The significant correlation was found between urinary levels of cortisone and sleep onset delay in TD children ($r(24) = 0.432$, $p = 0.03$), but not in the WS group ($r(20) = 0.152$, $p = 0.52$). However, the difference in this relationship between both study groups did not prove to be significant ($t(42) = -0.61$, $p = 0.55$) (Table 5.11.).

The marginal significance was also observed for urinary cortisol and sleep onset delay in TD ($r(24) = 0.375$, $p = 0.07$), but again not WS children ($r(20) = -0.048$, $p = 0.84$). However, this time as well, the significant difference in this relationship between both groups was not observed ($t(42) = -1.34$, $p = 0.19$).

No correlations between CSHQ data and levels of MT6s were found.

5.8.4. Correlations between actigraphy data and urinary MT6s, cortisol and cortisone

The marginal significance was found for the relationship between urinary levels of cortisol and total activity score from actigraphic measurements in TD group ($r(19) = 0.436$, $p = 0.06$) indicating that the activity score increased in children with higher cortisol levels (Table 5.11.). This relationship was not observed in WS children ($r(19) = 0.079$, $p = 0.75$) and using 2-sample t-test it was shown that there is no significant difference in relationship between urinary cortisol and total activity scores between both study groups ($t(36) = -0.97$, $p = 0.34$). Furthermore, it was also observed that the higher levels of cortisone in first void morning urine, the higher

sleep fragmentation scores in TD children ($r(16) = 0.673$, $p < 0.01$). Again, this typical relationship was not observed in WS group ($r(19) = 0.088$, $p = 0.72$). No significant difference in relationship between urinary cortisone levels and sleep fragmentation index ($t(33) = -1.23$, $p = 0.23$) was found between WS and TD children.

A marginal significance was also found in TD group for the Pearson's product moment correlations between urinary cortisone levels in the first void morning urine and number of immobile phases ($r(17) = 0.448$, $p = 0.07$) as well as moving minutes during the night ($r(16) = 0.446$, $p = 0.08$). No significant correlations for these parameters were observed in WS children (number of immobile phases: $r(19) = 0.081$, $p = 0.74$; moving minutes during the night: ($r(19) = 0.017$, $p = 0.94$). Marginal significance was found for the difference in relationship between cortisone levels and number of immobile phases between both study groups ($t(34) = -1.83$, $p = 0.07$). The difference in relationship between cortisone levels and moving minutes during the night did not prove to be significant between WS and TD children ($t(33) = -1.27$, $p = 0.21$). No correlations between CSHQ data and levels of MT6s were found.

5.9. The effect of health problems and sleep habits on sleep parameters and levels of melatonin and cortisol

Several variables from Medical questionnaire (Section 3.5.), including frequent colds, thyroid problems, watching TV before sleep and having shared bedroom were analysed as covariates using ANCOVA test. It was shown that the frequent colds

significantly predicts the occurrence of parasomnias ($F(1,48) = 5.445$, $p = 0.02$). However, there was still significant difference on the parasomnias score between WS and TD children after controlling for the effect of frequent colds ($F(1,45) = 9.512$, $p < 0.01$). The significant effect of watching TV within 30 min before bedtime on bedtime resistance has been found ($F(1,50) = 19.706$, $p < 0.001$). Similar findings were observed for the effect of having TV/video in bedroom on bedtime resistance ($F(1,50) = 4.183$, $p = 0.04$). However, after controlling for the effect of these two parameters, the significant difference on bedtime resistance between WS and TD groups was still observed (watching TV before bedtime: $F(1,47) = 11.535$, $p = 0.001$; having TV/video in bedroom: $F(1,47) = 5.099$, $p = 0.03$).

No effect of having TV in bedroom, the time spent using computer/watching TV, watching TV within 30 min before bedtime, having shared bedroom and bedroom on the main road when traffic can be heard at night was found for sleep efficiency and sleep latency. Interestingly, the time spent using a computer have significant effects on bed-time melatonin levels ($F(1,36) = 8.962$, $p < 0.01$). Furthermore, when controlling for the effect of this parameter, the significant difference in levels of bed-time melatonin were found between WS and TD children ($F(1,36) = 3.856$, $p = 0.05$). Using Pearson's moment correlations it was noted that the more time spent on the computer during the schooldays, the lower levels of melatonin observed in TD children ($r(20) = -0.518$, $p = 0.02$). The trend towards this relationship was observed in WS children, however, it did not prove to be significant ($r(19) = -0.385$, $p = 0.10$).

No effect of problems with thyroid gland and glucose levels as well as extracurricular sport activities on levels of melatonin and cortisol was found.

No significant effects were found using raw data, what could be due to the high individual variation of both endocrine markers.

Summary

The effects of sleep problems in WS children are wide-ranging, yet precious little research has focused on the causal mechanism of sleep problems in children with disorders. The current chapter includes a description of the data obtained from laboratory analyses of melatonin, cortisol and their metabolites using enzyme based immunoassays and UHPLC-MS/MS method developed for this study. Abnormalities in secretion of both, melatonin and cortisol, as well as their metabolites have been found in children with WS. These findings may indicate contributing or an underlying factor of sleep problems observed among individuals with this disorder. Furthermore, different patterns of relationship between sleep parameters and levels of endocrine markers of sleep were observed between children with WS and TD control group. It was also noted that time spent using a computer can significantly affect levels of melatonin at bedtime. Sleep and its consequences is an area that requires thorough investigation in children with WS as this group clearly experience significant sleep problems. As sleep problems in children with WS may adversely affect daytime activity and the quality of life, as well as social, emotional and health functioning of the entire family, it is particularly important to assess sleep problems in disorder groups in sufficient detail.

6. DISCUSSION, CLINICAL IMPLICATIONS AND FUTURE WORK

The motivation for this research has its roots in a lack of expertise regarding the causality of sleep problems affecting individuals with WS. Since sleep disturbances can have a disruptive impact not only on developing brain of a child, but also functioning of a family, it is necessary to examine sleep using multi-system analyses. Furthermore, it was beneficial for clinical applications to analyse both melatonin and cortisol simultaneously in order to assess interrelationships of these substances, such as their effect on diurnal rhythm and sleep in both TD and WS children.

This chapter contains a discussion of the research findings and how they relate to the existing research. It covers the limitations and implications of the present study and suggestions are made for future research.

6.1. Discussion

The aim of this thesis was to get a deeper understanding of mechanisms contributing to sleep problems observed in children with WS. This study is the first to present a quantitative approach to sleep problems in WS in relation to parental reports, actigraphy and measures of endocrine markers of sleep.

6.1.1. CSHQ, actigraphy and pulse oximetry

Analysis of sleep using CSHQ, actigraphy and pulse oximetry allowed for comparisons with previously published studies and provided a platform for further in depth examination of sleep in comparison with endocrine markers of circadian rhythm. The summary of sleep studies done to date including results of current investigation are presented in Table 6.1. The CSHQ data from the current study demonstrated that individuals with WS are significantly affected by several types of sleep disturbances, such as sleep onset delay, bedtime resistance, sleep anxiety, parasomnias and night wakings (see Section 3.4.) These findings are in line with the previous studies that have been reported (Bódizs *et al.*, 2009; Goldman *et al.*, 2009; Mason *et al.*, 2009; Annaz et *al.*, 2011; Ashworth *et al.*, 2013). Furthermore, in the current study, parents of 41% of children with WS reported that their children never or rarely fell asleep within 20min and that only 33% have sufficient sleep each night. This was comparable to the study of Goldman and colleagues (2009), which has shown that over 36% of individuals with WS have trouble sleeping. The CSHQ data reported by Annaz and colleagues (2011) are also very similar having shown significant bedtime resistance, sleep anxiety and night waking in a WS group compared to healthy controls of similar age.

Actigraphic measurements recorded over three consecutive days and nights provided further, more objective evidence of sleep disturbance. Several studies confirmed the reliability of actigraphy data obtained over this period of time (Glod *et al.*, 1997; Hering *et al.*, 1999; Gertner, 2002). It was found that children with WS had significantly increased sleep latency and fragmentation index, which is the indication of restlessness (see Section 3.2.). The decrease in sleep efficiency was also observed albeit only marginally significant ($p = 0.06$) requiring larger group samples. In

addition, number of wakings and moving time during the night was higher in WS population in comparison with TD control group. These findings are similar to those previously reported (Gombos *et al.*, 2011; Mason *et al.*, 2011). A recent study by Gombos *et al.* (2011) has shown atypical sleep patterns in children with WS; with decreased sleep efficiency and increased wake time after sleep onset. Similar results were reported by Mason *et al.* (2011) demonstrating decreased sleep efficiency with higher level of restlessness and arousals from sleep in WS population. Both studies have been based on PSG analysis.

Furthermore, investigating similarities between parental reports and actigraphy, it was shown that only sleep latency and parentally reported sleep onset delay were related in WS group (see Section 3.6.). It has been previously reported that actigraphy and parental reports in TD children provide differing, but complimentary information about a child's sleep habits (Holley *et al.*, 2010). The positive association between actigraphy and parental report of sleep concerns was shown in studies of Allik *et al.* (2006) and Malow *et al.* (2006) but not in the other studies (Hering *et al.*, 1999; Wiggs & Stores, 2004; Wiggs *et al.*, 2005). The inaccuracy of parent report underlines the importance of the objective measures for sleep assessment especially in children at risk. Furthermore, it is recommended that parent reports of sleep characteristics, such as CSHQ should not be used as a substitute of objective measures. However, despite its inaccuracy, parent report may be more useful for uncovering specific information regarding sleep, for example parasomnias, such as bed-wetting. Another finding from the current investigation was an expected general trend in a decrease in sleep time with increasing chronological age, which has been demonstrated previously (Ashworth *et al.*, 2013).

Actigraphy has some important limitations. The ability to precisely measure sleep latency depends on parents accurately reporting bedtime with lights out in the sleep diary. Furthermore, although it is assumed that Actiwatch Mini is comparable with other validated devices, as far as I am aware of, there are no published validation studies. Also, the sensitivity and specificity for use with children in clinical groups is unknown. It has also been shown that actigraphs can overestimate sleep efficiency due to scoring epochs of wakeful inactivity as sleep (Pollak *et al.*, 2001). The study of Pollak *et al.* (2001), however, has not used Actiwatch Mini applied in the current work. Despite these limitations, the use of a TD control group allowed for a useful comparison and valuable data could be obtained. Actigraphy is a useful tool for monitoring movement and studying sleep-wake cycles. It proved to be particularly valuable for the present study as its simplicity made it ideal for use with children with intellectual disability.

It has also been shown that intrinsic sleep disorders, such as sleep apnoea, have the potential to disrupt sleep and promote night waking (Gozal, 2008). Thus, oxyheamoglobin saturation was also analysed using pulse oximetry monitors, (Section 3.3.), which are easy to use in a home environment and provide accurate information on oxygen levels (Urschitz, 2003). In line with previous studies (Arens *et al.*, 1998; Mason *et al.*, 2009; Gombos *et al.*, 2011), no indication of sleep apnoea was found in individuals with WS. However, the use pulse oximetry has its limitations. The occurrence of OSAS is likely to be under-diagnosed using this method, since hypoxia is not always present. It detects around two thirds of OSAS cases (Williams *et al.*, 1991; Golpe *et al.*, 1999; Brouillette *et al.*, 2000; Magalang *et al.*, 2003). This method is also sensitive to the effects of body movements and poor blood flow, which is another limitation of using pulse oximetry. However, new

models are better at correcting for this (Netzer *et al.*, 2001). Furthermore, pulse oximetry data could not be obtained for all of the children as over 30% refused to wear a sensor or removed it during the night, that is a common occurrence when working with children with disabilities (Ashworth, 2013). Also, although parents were shown how to use the device correctly, some inadequate recordings might be due to incorrect placement of the sensor. Thus, in some cases the data could not be used due to insufficient artefact-free recording time. Despite these limitations, pulse oximetry provides a non-invasive, straightforward technique that is considerably less time-consuming and expensive than PSG. However, as studies examining sleep in WS using PSG have not found any indication of sleep apnoea (Arens *et al.*, 1998; Mason *et al.*, 2009; Gombos *et al.*, 2011), further analysis using less reliable pulse oximetry method might not provide any additional useful data in individuals with this disorder. Thus, this technique did not prove to be valuable for analysis of sleep in WS.

Study reference	Sample size	Age (years)	Method	Parameter measured	Findings in WS group
Current study	n (WS) = 27 n (TD) = 27	4 – 11	CSQH Actigraphy Pulse oximetry Immunoassays UHPLC-MS/MS	CSHQ sleep parameters: bedtime resistance, sleep onset delay, sleep anxiety, night waking, parasomnias, sleep disordered breathing, daytime sleepiness, sleep duration as well as total score; Actigraphy parameters including: bedtime, time in bed, actual sleep time, sleep latency, sleep efficiency, moving time, fragmentation index, night wakings; Cortisol, melatonin and their metabolites	CSHQ findings: significantly greater level of parasomnias, sleep anxiety, night waking, as well as bedtime resistance, sleep duration and sleep onset delay; Actigraphy findings: increased sleep latency and moving time after sleep onset and fragmentation index, lower actual sleep time; Pulse oximetry findings: no breathing related problems that might affect sleep have been found; Endocrine markers of sleep: significantly higher levels of salivary cortisol prior to bed time, as well as lack of increase of salivary melatonin and decrease of cortisol before bed time. Also, significantly higher levels of cortisone and lower levels of MT6s in first void morning urine
Arens *et al.*, 1998	n (WS) = 28 n (TD) = 10	1.5 – 10	Telesurvey PSG	Movement arousal sleep disorder	Telesurvey findings: Movement arousal in over 50 % of WS individuals PSG findings: Significantly higher prevalence of periodic limb movement syndrome in comparison to control group
Goldman *et al.*, 2009	n (WS) = 23	17 – 35	Sleep habit questionnaires: Epworth Sleepiness Scale, questionnaire regarding daytime sleepiness in adults Actigraphy	Sleep parameters: sleep efficiency, night wakings, daytime sleepiness, night movement	Findings from questionnaires: Tiredness during the day in 95 % of participants, excessive daytime sleepiness in 34 %, frequent awakenings during the night . Findings from actigraphy: mean sleep efficiency 74.4 %, mean sleep latency of 37 min and 56 min awaken after sleep onset

Annaz *et al.*, 2011	n (WS) = 64 n (TD) = 92	6 – 12	CSHQ	Sleep parameters included: bedtime resistance, sleep onset delay, sleep anxiety, night waking, parasomnias, sleep disordered breathing, daytime sleepiness, sleep duration as well as total score	Greater bedtime resistance, sleep anxiety, night waking and daytime sleepiness in WS children compared to healthy controls
Gombos *et al.*, 2011	n (WS) = 9 n (TD) = 9	14 – 28	PSG	Sleep architecture, leg movements, EEG spectra	Atypical sleep pattern in WS individuals including decreased sleep time and sleep efficiency, increased wake time after sleep onset, increased percentage of NREM sleep and SWS, decreased REM sleep percentage, greater number of leg movements compared to healthy controls
Mason *et al.*, 2011	n (WS) = 35 n (TD) = 35	2 – 18	PSG Sleep questionnaire	Sleep architecture, respiratory-related sleep arousals, limb movements, night awakenings	PSG findings: decreased sleep efficiency, increased respiratory-related arousals, increased SWS Sleep questionnaire findings: more difficulty falling asleep, greater restlessness, more arousals from sleep than controls
Bódizs *et al.*, 2012	Study 1: n (WS) = 9 n (TD) = 9 Study 2: n (WS) = 20 n (TD) = 20	Study 1: 14 – 29 Study 2: 6 – 29	EEG	Sleep EEG alternations	Alternations in EEG recordings including redistribution of NREM sleep EEG power towards higher frequency
Ashworth *et al.*, 2013	n (WS) = 24 n (TD) = 52	6 – 12	CSHQ Actigraphy	CSHQ sleep parameters: bedtime resistance, sleep onset delay, sleep anxiety, night	CSHQ findings: problems initiating sleep, bed-wetting, body pain, sleep onset delay, night wakings

				waking, parasomnias, sleep disordered breathing, daytime sleepiness, sleep duration as well as total score Actigraphy parameters: bedtime, time in bed, actual sleep time, sleep latency, sleep efficiency, moving time, fragmentation index, night wakings	Actigraphy findings: greater sleep latency, reduced sleep efficiency, increased percentage of moving time after sleep onset

Table 6.1. Summary of studies on sleep in WS reported to date and the summary of the current work. Table presents study cohort, age range of participants, methods used and parameter measured as well as findings of sleep studies in WS. Underlined author's names represents studies reported prior to start of current Ph. D. Abbreviations: CSHQ – Children's Sleep Habit Questionnaire, EEG- electroencephalography, MT6s- 6-sulfatoxymelatonin, NREM- non-rapid eye movement, PSG- polysomnography, REM- rapid eye movement, SWS- slow wave sleep, UHPLC-MS/MS- ultra-high performance liquid chromatography-tandem mass spectrometry.

6.1.2. *Endocrine markers of circadian rhythm*

The data collected through CSHQ and actigraphy indicated that children with WS indeed suffer from sleep problems, thus, levels of endocrine markers of circadian rhythm, namely melatonin, cortisol and their metabolites were investigated. Both of these markers are known to affect and regulate sleep/wake patterns.

6.1.2.1. Salivary melatonin

To date, very few studies have been carried out to analyse endocrine sleep markers in children with developmental disorders and none of them included individuals with WS (Potocki *et al.*, 2000; Tordjman *et al.*, 2005; Corbett *et al.*, 2006). Alterations in secretion of both endocrine markers (melatonin and cortisol) in WS have been found in the current work. As shown in the Section 5.1. high individual variability in the amount of melatonin secreted was observed in children in WS and TD groups. High variation of melatonin levels has been reported previously (Wetterberg, 1978; Waldhauser & Dietzel, 1985; Burgess & Fogg, 2008; Minami *et al.*, 2009). Burgess and Fogg (2008) determined that the peak value of salivary melatonin ranges between 2-84 pg/ml, while Minami and colleagues (2009) reported the morning levels of MT6s in adult healthy volunteers (n=16) as 26.9 ± 44.0 (representing mean and SD respectively). It has been also reported, that the ability to synthesize melatonin differs among individuals. There are low and high secretors of this hormone (Wetterberg, 1978; Waldhauser & Dietzel, 1985). Bergiannaki *et al.* (1995) suggested that this distinction most likely reflects genetically determined variable levels of the noradrenergic secretory drive and/or variable N-

acetylotransferase/hydroxyindole-O-methyltransferase enzymatic activity during the night. In the current study, it was found that the median increase in the level of salivary melatonin in TD control group was 1.83 fold between afternoon and bedtime samples, whereas for the individuals with WS no such increase was observed (median change of 0.96 fold). This finding may possibly explain the causality of problems with settling down and falling asleep affecting children with this syndrome, as the nocturnal secretion of melatonin is known to facilitate sleep (Arendt, 2005). Similarly the study reported by Potocki *et al.* (2000) has shown abnormalities in the circadian rhythm of melatonin in patients with Smith-Magenis syndrome (n = 19). Tordjman and colleagues (2005) also reported significantly reduced nocturnal production of MT6s in children and adolescents with autism spectrum disorder (n = 49).

6.1.2.2. Children taking melatonin as a sleep medication

Melatonin is taken on average by 14% of children with WS (Annaz *et al.*, 2011) and more surprising most toddlers with WS are taking melatonin (personal communication with the Williams Syndrome Foundation UK). However, there is no published evidence of any clinical efficacy for such hormonal sleep therapy in this disorder. Upon analysis it was noted that two children were taking melatonin as sleep medication and had to be excluded from analysis post study. It was shown that very high levels of salivary melatonin (8 fold higher than in the rest of WS group) observed in these children did not improve sleep quality (see Section 5.6.), suggesting that this hormone may not be effective for treating sleep problems in WS. However, as this was observed for only one child (the second child failed to keep an

actiwatch on), so this indication should be investigated further. Furthermore, results of urinary MT6s analysed by both, ELISA and UHPLC-MS/MS in these two children were not conclusive. One child had approximately 3-6 fold higher levels of MT6s than the other. This situation may be explained by the short biological half-life of melatonin, which according to different sources ranges between 30 min (Brown *et al.*, 1997) and 1 h (Claustrat *et al.*, 1986; Mallo *et al.*, 1990). Melatonin supplementation is usually taken at least an hour prior to habitual sleep time. Thus, when the child passed urine in the time between taking melatonin and going to bed, the higher levels of this hormone may not be observed in the first void morning urine. Nocturnal excretion of urine has not been measured in the current study and there are no studies reporting urine excretion in WS. Gringras *et al.* (2012) reported significant decrease in sleep onset latency in children with various neurodevelopmental disorders (n = 146) while given oral melatonin. However, there are also studies showing no evidence that melatonin is effective in treating sleep problems, such as secondary sleep disorders (n = 97) (Buscemi *et al.*, 2006). Furthermore, despite limited evidence of the effectiveness of exogenous melatonin and some concerns regarding safety, melatonin is being prescribed as a medication for sleep disorders to children with neurodevelopmental delay by an increasing number of physicians (Czeisler, 1997; Stone *et al.*, 2000; Owens *et al.*, 2003), and there is no standardisation as a wide variations of dose are being used (Phillips & Appleton, 2004; Waldron, *et al.*, 2005). This indicates a major question as to any clinical benefit from melatonin administration and whether these children should be given melatonin to help with their sleeping problems. The results of a current pilot study suggest that more research is needed in order to establish standard dosage and investigate efficacy of melatonin treatment in children with WS and possibly other

neurodevelopmental disorders. Furthermore, prior to introducing melatonin treatment, levels of this hormone should be examined at the individual level.

6.1.2.3. Salivary cortisol

Unlike melatonin, cortisol has not been studied as a sleep marker in children with developmental disabilities caused by various genetic disorders, such as WS or DS. Physiological levels of cortisol are highest in the morning and decline through the day (McVicar *et al.*, 2007, Clow *et al.*, 2010). Values obtained in the current study reflect this pattern (Section 5.2.). The highest level was observed in the morning and the lowest at bedtime in both TD and WS study groups. It was also found that there was a lack of decrease of salivary cortisol levels from afternoon to bedtime in many of the WS children (median fold decrease of cortisol levels in WS was 1.37 as opposed to 2.25 in TD group). In addition, significantly higher bedtime levels of this hormone in children with WS were observed in comparison to TD control group. Since cortisol is often described as a stress hormone and its actions include increasing blood pressure, heart rate and releasing glucose into the circulation (Seaward, 2006), high levels of cortisol before bedtime could potentially cause/contribute to sleep problems such as difficulty with relaxing and falling asleep (Talbott, 2007). Thus, findings of the current study may be another explanation of sleep problems such as delayed sleep onset affecting children with WS.

6.1.2.3.1. Cortisol and anxiety

WS individuals display tendency towards anxiety as well as specific phobias and worrying (Dykens, 2003; Morris, 2006; Leyfer *et al.*, 2009), that could result in higher levels of cortisol or *vice versa* and as a result may experience disordered sleep. Furthermore, as a prevalence of sleep problems is high in WS, it could be hypothesised that the bedtime struggles may also affect cortisol levels and/or cause sleep anxiety. However, no correlation was found between sleep anxiety parameter of CSHQ and cortisol levels in saliva and urine. Similar to the current study, Lense *et al.* (2013) analysed diurnal cortisol profile in adults with WS. However, the objective of that study was to examine cortisol as a biomarker of stress in both, novel (during a residential summer camp) and familiar settings (at home), so any association with sleep disturbance was not explored. Nevertheless, participants with WS demonstrated elevated cortisol levels late in the day in the novel setting when social demands were higher. The current study used natural home environment, what was the familiar setting for children, as opposed to sleep laboratory or hospital settings, in order to minimise effect of study procedure on participating children, such as stress effect on levels of cortisol. In addition, parents were trained how to collect samples, thus, children did not have to deal with an unfamiliar person. However, if collection procedure would still cause additional stress in children with WS and result in the rise of levels of cortisol, significantly greater concentration of this hormone would be observed between WS and TD children in all three saliva samples (morning, afternoon and bedtime). This difference was found only for the bedtime sample suggesting that the increase of anxiety and cortisol levels in WS children are unlikely to be caused by the sample collection procedure.

It is proposed that deletion of *GTF2IRD1* contributes to increased anxiety and in turn higher cortisol levels in individuals with WS (Schneider *et al.,* 2012). Schneider *et al.* (2012) and Howard *et al.,* (2012) have shown elevated ACTH release in *Gtf2ird1* homozygous mice, what could be due to altered regulation of stress in the hypothalamus. As described in Section 1.1.6, ACTH acts on the adrenal cortex causing the synthesis of cortisol, thus, increased levels of ACTH cause elevated cortisol secretion. In addition, expression studies have shown that *GTF2IRD1* is densely expressed in the hypothalamus and pituitary throughout murine embryonic development and this expression continuous to be high in the pituitary through adulthood (Palmer *et al.,* 2007). This indicate the importance of the *GTF2IRD1* in regulating anxiety response in adulthood and could be possible explanation of anxiety affecting not only children, but also adults with WS. Furthermore, the elevated endocrinological response to stress as well as decreased circadian activity was previously reported in *Gtf2ird1* knock-out mice (Schneider *et al.*, 2012).

Thus, taking into account the abnormalities in secretion of endocrine markers of sleep found in the current work and the previously reported genotype/phenotype correlations of the genes deleted in WSCR, the model linking genetics with neurophysiological and biochemical parameters of sleep in WS was created (Figure 6.1.). To date, there is no published model representing these interactions in WS.

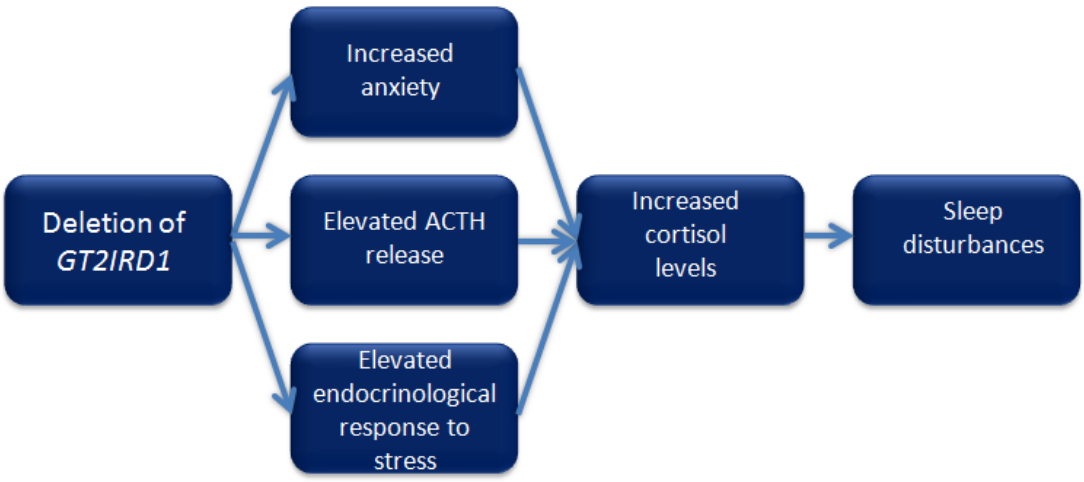


Figure 6.1. The model of interaction between genetics and biochemical as well as neurophysiological sleep parameters in WS. It has been reported that *GT2IRD1* knock-out mice demonstrate elevated adrenocorticotropic hormone (ACTH) release as well as elevated endocrinological response to stress (Howard *et al.*, 2012; Schneider *et al.*, 2012). It is proposed that deletion of *GTF2IRD1* contributes to increased anxiety and in turn higher cortisol levels in individuals with WS (Schneider *et al.*, 2012). Higher levels of cortisol may consequently cause/contribute to sleep problems observed in WS, such as sleep onset delay and sleep anxiety.

Corbett and colleagues (2006) also analysed salivary cortisol in children with autism spectrum disorder (n=12) compared to healthy controls (n=10). The expected circadian variation of this hormone was seen in all study groups, but the objective was to evaluate the response to environmental stressors. The authors reported that children with autism demonstrated significant elevations of cortisol levels following exposure to novel, non-social stimulus, however, no significant difference in circadian variation (morning/afternoon/evening samples) as well as in baseline levels of cortisol was found between both study groups. The tendency towards cortisol hypersecretion in this disorder was also reported by Richdale and Prior (1992). The elevation of cortisol levels was observed during school integration in those children who were integrated into the normal school system. The authors suggested that this

tendency might indicate environmental stress response in children with ASD. Furthermore, Kiess *et al.* (1995) reported that analysis of cortisol levels in saliva provides a reliable tool for the determination of physiology of cortisol and characteristics of its metabolism.

Analyses of salivary cortisol and melatonin from this study have indicated possible contributing/underlying factors for sleep problems in WS. However, it did not provide the data regarding the nocturnal levels of sleep hormones. There is a possibility that melatonin levels increase later in the night in children with WS than in TD control group. Thus, analysing nocturnal levels of this hormone would answer whether melatonin levels are/are not similar to those observed in healthy control group. Furthermore, although saliva is a diagnostic medium that can be easily collected at repeated intervals, the collection from children has its disadvantages. Children with WS often experience dryness of mouth, which I have observed during the course of my work. Thus, in some cases it was difficult to wet the swab thoroughly as it is recommended (Salimetrics, 2013) and collect enough saliva for immunoassay analysis. These difficulties would not be experienced while collecting urine. In addition, it has been shown that the concentration of melatonin and cortisol in the first void urine samples reflect their nocturnal levels (Smith & French, 1997; Benloucif *et al.*, 2008).

The current study used natural home environment as opposed sleep laboratory or hospital settings. This is an important factor when examining children with developmental disorders or young typically developing children, in order to eliminate the possibility that response differences are due to WS sensitivity to novel, non-social stimuli as demonstrated in those with autism (Richdale & Prior, 1992; Corbett *et al.*, 2006) as well as adults with WS (Lense *et al.*, 2013). In addition,

although the bedtime varies among children, melatonin and cortisol patterns are dependent on individual routine, melatonin levels rises approximately 2 h before habitual night-time sleep (Wulff, 2012), thus, different timing should not cause inconsistency, and in turn alter the results. Also, cortisol levels rise rapidly during the first 30 min after awakening (Wilhelm *et al.*, 2007; Clow *et al.*, 2010), hence it seemed necessary to collect the samples as soon as the child was awake. In this study no effect of collection times on levels of melatonin and cortisol was found.

6.1.2.4. Novel analysis of simultaneous analysis of cortisol, cortisone and MT6s

The new method is presented that enables simultaneous determination of most robust circadian markers, namely cortisol, cortisone and MT6s in urine. The structure of internal standards was similar to the structure of analysed compounds. Both, 6-α-methylprednisolone and indole-3-acetamide were previously used as internal standards for analysis of cortisol (Turpeinen *et al.*, 1997) and MT6s (Minami *et al.*, 2009). As shown in Section 5.5., the short run time with all peaks of interest eluting within 3 min was obtained. This demonstrates the feasibility of the method to carry out large multi-scale studies when the determination of the circadian rhythm of melatonin and cortisol require rapid sample processing. Another advantage of this method is direct sample injection, that eliminates the necessity for lengthy preparative steps, resulting in considerable saving of both time and chemicals. Simultaneous analysis of melatonin and cortisol, as well as testosterone was already reported (Jensen *et al.*, 2011), however, the medium used were saliva samples collected from four healthy volunteers. In field studies it is often a problem to collect

sufficient amounts of saliva for analysis (Hansen *et al.*, 2008). The amount of saliva samples necessary for the analysis of melatonin and cortisol using LC-MS based methods in the previously reported studies range between 0.1 ml to 1 ml (Eriksson *et al.*, 2003; Motoyama *et al.*, 2004; Turpeinen *et al.*, 2009). In the current work the injection volume of diluted urine samples was 5 µl for analysis of cortisol, cortisone and MT6s. In contrast, the 0.5 ml of urine was necessary for analyses of cortisol in previously reported LC-MS/MS methods (Nassar *et al.*, 2001; Taylor & Singh, 2002; Kushnir et *al.*, 2003). In addition, Jensen e*t al.* (2011) in their method for simultaneous analysis of melatonin, cortisol and testosterone used liquid-liquid extraction prior to injection to the LC-MS/MS system. Following liquid-liquid extraction samples were evaporated to dryness and redissolved in over 5-fold lower volume of solution (methanol), which allowed for increase in concentration of hormones. Thus, their method obtained very good sensitivity with the limits of detection of approximately 1 pg/ml for melatonin and 10 pg/ml for cortisol with the run time of 6.5 min, while the lowest values detected by the UHPLC-MS/MS method involving direct sample injection, developed in the current study are 106 pg/ml for MT6s, 2.23 ng/ml for cortisol and 2.78 ng/ml for cortisone. However, analysis of samples from WS and TD children showed that the method is sensitive enough to provide clinical data that will allow diagnosis of endocrine-related sleep problems. It has been reported that the advantages of analysis of cortisol and cortisone using LC-MS/MS methods include higher sensitivity and specificity as well as elimination of drug interference and a high throughput and short chromatographic run time (Taylor *et al.*, 2002). The UHPLC-MS/MS method developed in this study could enable easier diagnosis of different types of sleep disorders, where there are abnormalities in the secretion of melatonin and/or cortisol.

One of the limitations of the current study was inability to obtain a good standard of MT6s due to two factors, namely, it is commercially unavailable and the synthesis was not possible in the current laboratory. In order to overcome this problem it was decided to use the MT6s standard from an ELISA kit for MT6s. As these standards were prepared in a buffer for ELISA analysis (containing sodium ions) (Melatonin sulfate ELISA instruction of use, http://www.ibl-international.com), ion suppression or enhancement could be observed during UHPLC-MS/MS measurements of MT6s. Ion suppression/enhancement results from the presence of less volatile compounds that can change the efficiency of droplet formation or droplet evaporation, that can consequently affect the amount of charged ion in the gas phase that ultimately reaches the detector. Compounds often causing ion suppression includes salts, ion-pairing agents, metabolites, drugs or proteins (Annesley, 2003). Sodium can either form a salt or act as an ion-pairing agent. This may possibly explain why MT6s levels obtained with UHPLC-MS/MS were much lower (approximately 55 fold) than those measured by ELISA (Sections 5.3. and 5.5.). Regardless this difference, the significant positive correlation was found between MT6s levels analysed by these two methods.

6.1.2.5. Urinary levels of melatonin and cortisol

In line with previous findings regarding analyses of salivary melatonin and cortisol, children with WS demonstrated significantly lower levels of MT6s in the first void morning urine ($p = 0.03$) in comparison with healthy controls (see Section 5.5.). Thus, it can be concluded that apart from the lack of increase of melatonin levels

before bedtime, overall nocturnal levels of this hormone are decreased in individuals with WS as well. This may explain the problems with maintaining good quality sleep at night observed in this disorder. In addition, it was shown, that children with WS demonstrate significantly higher levels of cortisone in first void morning urine in comparison with healthy controls, this may be another explanation of sleep problems affecting individuals with this disorder and confirmed the results obtained for salivary bedtime cortisol levels. The summary including data obtained for cortisol, melatonin and their metabolite using immunoassays and UHPLC-MS/MS is shown in Table 6.2. WS individuals also display tendency towards anxiety (Morris, 2006), that could result in higher levels of cortisol or *vice versa* and as a result may experience disordered sleep. Moreover, although four children with WS were reported to wet bed during the night prior sample collection, no effect of bed-wetting was observed in WS children for urinary analysis of cortisol, cortisone and MT6s using ANCOVA (all $p > 0.05$).

Method	Matrix	Compound	Time point	Median	P value
Immunoassays	Saliva % of the morning value	Melatonin	Afternoon	TD = 40.37 WS = 72.22	0.13
			Bedtime	TD = 80.48 WS = 61.03	0.78
			Ratio between bedtime/afternoon level (increase of melatonin level)	TD = 1.83 WS = 0.96	**0.04**
		Cortisol	Afternoon	TD = 22.22 WS = 27.17	0.69
			Bedtime	TD = 15.29 WS = 33.45	**0.03**
			Ratio between afternoon/bedtime level (decrease of cortisol level)	TD = 2.25 WS = 1.37	**<0.01**
	Urine ng/ mg creatinine	MT6s	Morning, first void	TD = 171.60 WS = 83.40	**<0.01**
UHPLC-MS/MS	Urine ng/ mg creatinine	MT6s	Morning, first void	TD = 4.39 WS = 3.22	**<0.01**
		Cortisol		TD = 48.13 WS = 47.32	0.90
		Cortisone		TD = 48.82 WS = 64.20	**0.05**

Table 6.2. Summary table presenting median levels of normalised melatonin and cortisol in saliva as well as 6-sulfatoxymelatonin (MT6s), cortisol and cortisone in first void morning urine analysed in samples collected from children with WS and TD age- and gender-matched control group using immunoassays and ultra-high performance liquid chromatography- tandem mass spectrometry (UHPLC-MS/MS). It can be seen that urinary analyses reflect data obtained for the saliva indicating: increased salivary cortisol at bedtime and greater urinary cortisone in the first void morning urine in WS group; lack of increase of melatonin prior bedtime and lower MT6s levels in morning urine indicating decreased nocturnal secretion in children with WS. Significant results obtained at 95 % confidence interval are presented in bold.

Since melatonin and cortisol secretion is affected by the environment, as many environmental variables as possible should be controlled and regulated. In order to completely manage environmental factors, it would be necessary to conduct the

study at a sleep laboratory, however, this could cause stress to children and result in the rise of cortisol levels.

6.1.2.6. Comparison of immunoassay based method and UHPLC-MS/MS for analysis of endocrine markers of sleep

Both, salivary melatonin and cortisol were analysed using standard enzyme immunoassays. General clinical analysis of both melatonin and cortisol are mostly based on the use of immunoassay based methods, mainly because of the convenience and limited number of clinical laboratories with liquid chromatography tandem mass spectrometry (LC-MS/MS) equipment (Inder *et al.*, 2012). The novel UHPLC-MS/MS method developed in this study could significantly contribute to the clinical laboratory field. There is no regular clinical method used for analysis of melatonin and cortisol as well as their metabolites, with some laboratories using immunoassay based methods, mainly RIA (ARUP Laboratories, http://ltd.aruplab.com; Cambridge Specialist Laboratory Services Information Manual, 2011), while other LC-MS/MS methods (Cardiff and Vale University Heath Board, 2013/2014; NMS Labs, http://www.nmslabs.com). Using immunoassay based techniques, hand pipetting necessary for these methods can create errors. On the other hand, these errors would be avoided while using automated HPLC system. Furthermore, using immunoassay based methods only one analyte and limited number of samples can be measured at one time. These assays require several hours to complete. For instance, 6-sulfatoxymelatonin ELISA requires 2.5 hrs incubation time, thus, running whole assay takes several hours and only 40 samples can be analysed in duplicate. The UHPLC-MS/MS method developed in the current work allow for analysis of over

one hundred samples during the course of the day and multiple compounds are analysed at the same time. Another disadvantage of using immunoassays is cross-reactivity with structurally similar compounds. As an example, ELISA kit for analysis of cortisol in urine reported over 30 % cross-reactivity with other analytes (cortisone, 11-α-deoxycortisol and corticosterone) (IBL International Cortisol (urine) ELISA instruction of use http://www.affinitydiagnostics.ca/; DiaMetra Urinary "free" cortisol ELISA instruction of use http://www.alpco.com/). LC-MS/MS methods could overcome these limitations and minimise the variability. In addition, no evidence was found for simultaneous analysis of melatonin and cortisol in clinical laboratories, while there is an increasing evidence suggesting that simultaneous measurements of more than one biomarker in the same sample provides greater confidence to the acceptance or rejection of specific diagnoses (Lin *et al.*, 1997; Taylor *et al.*, 2002; Remer & Maser-Gluth, 2007). Thus, in light of these problems, the specific UHPLC-MS/MS method for simultaneous analysis of MT6s, cortisol and cortisone was developed and could enable easier diagnosis of different types of sleep disorders, where there are abnormalities in the secretion of melatonin and/or cortisol. The LC-MS based methods have however some disadvantages. They require elaborate and expensive instrumentation. Nevertheless, in these laboratories that already work on these machines, measurements of endocrine markers of sleep using method developed in the current work is very cost effective. The cost of all chemicals and standards to run couple of hundred samples is up to £300 for cortisol, cortisone and MT6s to be analysed simultaneously. Whereas, cost of one plate for analysis of MT6s in only 40 samples is over £500. In addition, there is no standard immunoassay kit for analysis of cortisone. In summary, once the instrumentation is in place, the use of UHPLC-MS/MS method for analysis of cortisol, cortisone and

MT6s is cost effective, no cross-reactivity with similar compounds is observed, the method enables simultaneous analysis of many compounds at the same time and has a much shorter run time in comparison to enzyme immunoassays. All these factors demonstrate the feasibility of utilising the method for large multi-scale studies, where high throughput is required for studying the circadian rhythm of melatonin and cortisol secretion.

6.1.3. Correlations between sleep parameters and endocrine markers of sleep

When analysing associations between levels of endocrine markers of sleep with sleep parameters, several typical correlations were observed for the TD control group, but not WS children (Section 5.8.). As expected, it was noted that the higher levels of salivary melatonin at bedtime, the lower sleep disturbance score (Total score of CSHQ) and the shorter time taken to fall asleep by TD children. These typical relationships were not observed for WS group. In addition, it was found that TD children having higher bedtime and afternoon levels of salivary cortisol, as well as higher levels of cortisol and cortisone in the first void morning urine experienced greater accounts of sleep problems including increased sleep latency and activity scores during the night, reduced sleep efficiency as well as longer time spent awake after sleep onset. The increased afternoon levels of salivary cortisol in WS also affected time taken to fall asleep in children with this disorder. In addition, no gender difference as well as no effect of age or sample collection times (time between the collection of evening saliva sample and child's bedtime as well as time taken to

collect saliva samples from the moment the child woke up) was observed on levels of melatonin, cortisol and their metabolites.

Reviewing sections 3.6. and 5.8. regarding correlations between parental reports and actigraphy, as well as correlation between sleep measures and levels of melatonin and cortisol, it could be observed that only sleep latency from actigraphy was correlated to both, endocrine markers of sleep and parental reports. As seen in Section 3.6. sleep latency and parentally reported sleep onset delay were related in WS group. Furthermore, it was also observed that the higher levels of afternoon cortisol in saliva in WS, the longer time these children took to fall asleep. Some additional possible effects could be seen if the sample size was larger. However, due to the rarity of WS, most studies have small sample sizes. The studies of Bodizs *et al.* (2009), as well as Gombos *et al.* (2011) investigating sleep in WS using PSG included 9 participants with this disorder. The largest WS group in sleep research study included 64 children, however, it was based on parent-report questionnaire rather than empirical research (Annaz *et al.*, 2011). Although, the sample size in this thesis accounts for ~30% of school-age children with WS (from Williams Syndrome Foundation database), the greatest number of participants could add power to analyses where significant effects were expected but not found. Due to time constraints as well as the unavailability of participants and equipment, this was not possible.

6.2. **Clinical implications**

Managing sleep in developmental disorders is paramount not only for the health of a child but also for cognitive functioning and family life as whole. It has been reported that sleep deprivation negatively affects cognition (Killgore, 2010), while abnormally high secretion of cortisol during sleep is associated with poorer performance of declarative memory and executive function (Li *et al.*, 2006; Scher *et al.*, 2010; Evans *et al.*, 2011). This study indicates that sleep problems should be treated at individual levels. A number of studies showed that levels of both melatonin and cortisol could be managed through behavioural management or pharmacologically. For instance, cortisol levels could be reduced with a specific diet containing low-glycemic-index foods and foods rich in vitamin C as well as omega 3 fats (e.g. salmon, walnuts, almonds) (Moss, 2009). In addition, opiates as well as some benzodiazepines (alprazolam, temazepam) can decrease levels of cortisol (Carlson, 2011). Melatonin is also widely used medication for sleep problems (Owens *et al.*, 2003). Furthermore, the new method for simultaneous analysis of MT6s, cortisol and cortisone in urine could be used clinically for measuring circadian disruption.

Results from the current work regarding each child as well as summarised data were presented to the parents of a particular child, either personally or in a form of a letter. According to the research ethic's committee, it is not permitted to give clinical advice based on research study.

6.3. Future work

Both cortisol and melatonin play significant role in circadian rhythm and sleep/wake cycle, therefore it is necessary to look closely at these endocrine markers in individuals suffering from sleep disorders/problems. Although secretion of melatonin and cortisol proved to be stable across days and weeks amongst a wide range of individuals, and typical bedtime routine was ensured for the children, it would be beneficial to conduct a detailed study with sampling on more than one day. Also, obtaining repeated saliva samples would be useful in order to assess dim light melatonin onset as well as cortisol awakening response.

Future work should also be focused on extending age groups, since sleep problems are shown in toddlers with WS (Axelsson *et al.*, in press), and also on extending developmental trajectory to adolescence as well as cross-syndrome approach to examine if these findings are specific to WS or syndrome general (Annaz & Ashworth, 2011). Clinical studies are necessary to examine the modulation of melatonin and cortisol in individuals suffering from abnormalities in the levels of these hormones. It would be valuable to extend analysis of melatonin and cortisol on other neurodevelopmental disorders and individual suffering from sleep problems. In addition, despite very high levels of melatonin, sleep quality was shown to be poor in a child taking melatonin as a sleep medication. Thus, the efficacy of such treatment should be further investigated in children with WS and possibly other neurodevelopmental disorders.

Future work could also include analysis of clock genes. Clock genes are components of the circadian clock critical to the generation of circadian rhythm (Dunlap, 1999; Albrecht & Ripperger, 2009). It would be beneficial to ascertain whether sleep as

well as melatonin and cortisol production anomalies observed in individuals with WS are due to an alternation of the molecular mechanisms of the circadian clock. Several studies suggest that human clock gene variants contribute to phenotypic differences observed in various behavioural and physiological processes and can have an impact on circadian rhythm (Albrecht, 2002; Viola *et al.*, 2007; Lee *et al.*, 2010; Franken, 2013; Zhang *et al.*, 2013). Furthermore, it has been shown that in the *Per2*/*Cry1* mutant mouse, the HPA axis regulating cortisol secretion is defective (Oster *et al.*, 2006), which may be another rationale for analysis of clock genes in individuals demonstrating abnormalities in levels of endocrine markers of circadian rhythm. In contrast, it has also been reported that expression of clock genes (such as *Per1*, *Cry1*, *Npas4*) can be influenced by secretion of both, melatonin and cortisol (Yamamoto *et al.*, 2005; Imbesi *et al.*, 2009; Mavroudis *et al.*, 2012; Zeman & Herichová, 2013).

It would also be beneficial to investigate the expression of melatonin receptors. It has been shown that the genetic inactivation of melatonin receptors MT1 and MT2 result in increased wakefulness and decreased NREM sleep time (Ochoa-Sanchez *et al.*, 2011; Comai *et al.*, 2013). Studies by Imbesi *et al.* (2009) indicated also that melatonin receptor 1 is involved in the regulatory action of melatonin on neuronal clock gene expression. Thus, melatonin receptor MT1 may control clock gene expression and in turn generation of circadian rhythm.

As described in the Sections 1.1.7. and 1.3.2. as well as in the current section, mouse models have been widely used to determine the function of clock genes, melatonin receptors as well as the genes from the WSCR (Yamamoto *et al.*, 2005; Oster *et al.*, 2006; Li *et al.*, 2009; Schneider *et al.*, 2012; Comai *et al.*, 2013). The mouse models of various genetic disorders, including WS, play an important role in defining

genotype/phenotype correlations. Moreover, mice are ideal candidates to model human genetic disorder, due to the fact that 99 % of a mouse genome sequence has direct counterparts in humans, thus, genomic alternations should affect the same biological pathways throughout development and result in similar phenotypes (Gunter & Dhand, 2002). There are several other studies using mouse models for the analysis of various sleep disorders, such as narcolepsy and sleep-disordered breathing (Tagaito *et al.*, 2001; Zhang *et al.*, 2007; Scammell *et al.*, 2009). Thus, it would be beneficial to analyse sleep disturbances in mouse model of WS. Future work should also include the examination of correlations between genes deleted in the WSCR and levels of endocrine markers of sleep. This association would be particularly interesting between cortisol levels and the *GT2IRD1* gene, as decreased circadian activity and elevated endocrinological response to stress, was previously reported in *GT2IRD1* knock-out mice (Schneider *et al.*, 2012). In addition, Schneider *et al.* (2012) and Howard *et al.*, (2012) have shown elevated ACTH release in *GTF2IRD1* homozygous mice, what is another rationale why the effect of *GT2IRD1* deletion on cortisol secretion should be investigated further.

In light of the findings of the current thesis, and accounting for its limitations, it is clear that sleep problems should be treated at individual levels. Increased bedtime cortisol and less pronounced rise in melatonin levels before sleep may play a role in the occurrence of sleep disturbances, such as delayed sleep onset, observed in children with WS. Since, both markers play a significant role in our circadian rhythm and sleep/wake cycle it is necessary to look closely at these endocrine markers in individuals suffering from sleep disorders. Sleep problems in children with WS may adversely affect daytime activity and the quality of life, as well as social, emotional,

health and economic functioning of the entire family. Hence, finding their cause is of great importance for affected children and their families.

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APPENDIX 1

INFORMATION SHEET FOR PARENTS

Middlesex University

Sleep patterns and hormonal markers of sleep, such as melatonin and cortisol levels in children with Williams syndrome

We would like to invite you and your child to participate in this research project. You and your child should only participate if you want to; choosing not to take part will not disadvantage you or your child in any way. Before you decide whether you want to take part, it is important for you and your child to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

What is the study about?

The study is to investigate the secretion of salivary melatonin and cortisol as well as urinary metabolites such as metabolite of melatonin and cortisol, with the heart rate and respiratory rate variability and its contribution to the sleeping pattern in the children with Williams Syndrome.

Why is the study being done?

Melatonin and cortisol play an important role in sleep. Sleep disturbances have been shown to have an adversely effect on daytime activities of children and of the entire family. The aim of this study is to investigate causes of sleep problems in children with Williams syndrome.

What are the benefits of taking part?

Your family participation in this project will increase our understanding of sleep problems in developmental disorders such as Williams syndrome.

What does taking part mean?

You will be asked to fill in the questionnaires and to take saliva and urine samples 3 times in one day of your child. All collection devices will be provided. We will also measure pulse rate and respiratory rate of your child over the three nights using sensor attached to your child's finger or toe. Your child's activity and sleep/wake cycle will be also monitored with actiwatch. It is priority for us not to cause any inconvenience to your child. All participants will be asked to take part voluntarily and can withdraw at any stage of the project.

Who will have access to the research records?

Only the researchers will have access to the data collected during this study. Your child's identity will be confidential (a code will be used for all documents and data collected, not your child's name) and is safeguarded by the Data Protection Act 1998 (DPA). The DPA places an obligation on those who record or use personal information, but also gives the rights to people whom information is held. This project is conducted in a manner that is consistent with the World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. Middlesex University has Licenses for the storage of the tissue for research issued by The Human Tissue Authority Human Tissue Act 2004).

Do I have to take part in the study?

If you or your child decides, now or at a later stage, that you wish not to participate in this research project that is entirely your right and will not in any way prejudice your involvement in future studies. You or your child does not have to give a reason for withdrawal.

Will I get notified of the outcome of the study?

We are more than happy to provide you with information of our findings. We will send this to you as soon as the project is completed.

What will happen to the results of the study?

Results will be published as a part of doctoral dissertation as well as scientific publications and Williams Syndrome Foundation newsletter. The copy of publication may be obtained from researcher. Participants will not be identified in any report or publication.

Who has reviewed the study?

Study was reviewed by the Middlesex University, School of Health & Social Sciences, Natural Sciences Ethics sub-Committee.

Whom do I speak to if I have a question?

If you have any questions or comments discuss them with Dr. Dagmara Annaz via email: d.annaz@mdx.ac.uk or call on 020 8411 4695 or Anna Sniecinska, MSc, email: a.sniecinska@mdx.ac.uk, 020 8411 6223, 077 9533 8880

APPENDIX 2

Child's Sleep Habits
(Preschool and School-Aged)

Coding _____

The following statements are about your child's sleep habits and possible difficulties with sleep. Think about the past week in your child's life when answering the questions. If last week was unusual for a specific reason (such as your child had an ear infection and did not sleep well or the TV set was broken), choose the most recent typical week. Answer USUALLY if something occurs **5 or more times** in a week; answer SOMETIMES if it occurs **2-4 times** in a week; answer RARELY if something occurs **never or 1 time** during a week. Also, please indicate whether or not the sleep habit is a problem by circling "Yes," "No," or "Not applicable (N/A).

Bedtime

Write in child's bedtime: _____

	3 Usually (5-7)	2 Sometimes (2-4)	1 Rarely (0-1)	Problem?		
Child goes to bed at the same time at night	☐	☐	☐	Yes	No	N/A
☆ Child goes to bed at the same time at night	☐	☐	☐	Yes	No	N/A
☆ Child falls asleep within 20 minutes after going to bed	☐	☐	☐	Yes	No	N/A
☆ Child falls asleep alone in own bed	☐	☐	☐	Yes	No	N/A
☆ Child falls asleep in parent's or sibling's bed	☐	☐	☐	Yes	No	N/A
Child falls asleep with rocking or rhythmic movements	☐	☐	☐	Yes	No	N/A
Child needs special object to fall asleep (doll, special blanket, etc.)	☐	☐	☐	Yes	No	N/A
☆ Child needs parent in the room to fall asleep	☐	☐	☐	Yes	No	N/A
Child is ready to go to bed at bedtime	☐	☐	☐	Yes	No	N/A
Child resists going to bed at bedtime	☐	☐	☐	Yes	No	N/A
☆ Child struggles at bedtime (cries, refuses to stay in bed, etc.)	☐	☐	☐	Yes	No	N/A
☆ Child is afraid of sleeping in the dark	☐	☐	☐	Yes	No	N/A
☆ Child is afraid of sleep alone	☐	☐	☐	Yes	No	N/A

Sleep Behavior

Child's usual amount of sleep each day: _____ hours and _____ minutes
(combining nighttime sleep and naps)

	3 Usually (5-7)	2 Sometimes (2-4)	1 Rarely (0-1)	Problem?		
☆ Child sleeps too little	☐	☐	☐	Yes	No	N/A
☆ Child sleeps too much	☐	☐	☐	Yes	No	N/A
Child sleeps the right amount	☐	☐	☐	Yes	No	N/A
☆ Child sleeps about the same amount each day	☐	☐	☐	Yes	No	N/A
☆ Child wets the bed at night	☐	☐	☐	Yes	No	N/A
☆ Child talks during sleep	☐	☐	☐	Yes	No	N/A
☆ Child is restless and moves a lot during sleep	☐	☐	☐	Yes	No	N/A
☆ Child sleepwalks during the night	☐	☐	☐	Yes	No	N/A
☆ Child moves to someone else's bed during the night (parent, brother, sister, etc.)	☐	☐	☐	Yes	No	N/A

CSHQ

Coding

Sleep Behavior (continued)

	3 Usually (5-7)	2 Sometimes (2-4)	1 Rarely (0-1)	Problem?
Child reports body pains during sleep. If so, where? _____	☐	☐	☐	Yes No N/A
☆ Child grinds teeth during sleep (your dentist may have told you this)	☐	☐	☐	Yes No N/A
☆ Child snores loudly	☐	☐	☐	Yes No N/A
☆ Child seems to stop breathing during sleep	☐	☐	☐	Yes No N/A
☆ Child snorts and/or gasps during sleep	☐	☐	☐	Yes No N/A
☆ Child has trouble sleeping away from home (visiting relatives, vacation)	☐	☐	☐	Yes No N/A
Child complains about problems sleeping	☐	☐	☐	Yes No N/A
☆ Child awakens during night screaming, sweating, and inconsolable	☐	☐	☐	Yes No N/A
☆ Child awakens alarmed by a frightening dream	☐	☐	☐	Yes No N/A

Waking During the Night

	3 Usually (5-7)	2 Sometimes (2-4)	1 Rarely (0-1)	Problem?
☆ Child awakes once during the night	☐	☐	☐	Yes No N/A
☆ Child awakes more than once during the night	☐	☐	☐	Yes No N/A
Child returns to sleep without help after waking	☐	☐	☐	Yes No N/A

Write the number of minutes a night waking usually lasts: _____

Morning Waking

Write in the time of day child usually wakes in the morning: _____

	3 Usually (5-7)	2 Sometimes (2-4)	1 Rarely (0-1)	Problem?
☆ Child wakes up by him/herself	☐	☐	☐	Yes No N/A
Child wakes up with alarm clock	☐	☐	☐	Yes No N/A
☆ Child wakes up in negative mood	☐	☐	☐	Yes No N/A
☆ Adults or siblings wake up child	☐	☐	☐	Yes No N/A
☆ Child has difficulty getting out of bed in the morning	☐	☐	☐	Yes No N/A
☆ Child takes a long time to become alert in the morning	☐	☐	☐	Yes No N/A
Child wakes up very early in the morning	☐	☐	☐	Yes No N/A
Child has a good appetite in the morning	☐	☐	☐	Yes No N/A

Coding

Daytime Sleepiness

	3 Usually (5-7)	2 Sometimes (2-4)	1 Rarely (0-1)	Problem?		
Child naps during the day	☐	☐	☐	Yes	No	N/A
Child suddenly falls asleep in the middle of active behavior	☐	☐	☐	Yes	No	N/A
☆ Child seems tired	☐	☐	☐	Yes	No	N/A

During the past week, your child has appeared very sleepy or fallen asleep during the following (check all that apply):

	1 Not Sleepy	2 Very Sleepy	3 Falls Asleep
Play alone	☐	☐	☐
☆ Watching TV	☐	☐	☐
☆ Riding in car	☐	☐	☐
Eating meals	☐	☐	☐

APPENDIX 3

MEDICAL HISTORY QUESTIONNAIRE

1. Your name: ……
2. Your child's full name: …… ...Gender: M ☐ F ☐
3. Your child's date of birth: ……
4. Child's ethnicity……
5. What's the parents' occupation?

Mother

☐ High managerial, administrative or professional (director/chairman/general manager)

☐ Intermediate managerial, administrative or professional (public service managers/HE/FE teachers /headmasters)

☐ Supervisory, clerical and junior managerial, administrative or professional (nurses /junior doctors/primary/secondary teachers/secretarial)

☐ Skilled manual workers (emergency services/electrician/carpenter etc)

☐ Semi and unskilled manual workers (retail /shop assistants etc)

☐ Housewife/ househusband

☐ Other……

Father

☐ High managerial, administrative or professional (director/chairman/general manager)

☐ Intermediate managerial, administrative or professional (public service managers/ Education (HE / FE teachers / Headmasters)

☐ Supervisory, clerical and junior managerial, administrative or professional (nurses /junior doctors/primary/secondary teachers/secretarial)

☐ Skilled manual workers (emergency services/electrician etc)

☐ Semi and unskilled manual workers (retail / shop assistants etc)

☐ Housewife/ househusband

☐ Other……

6. Where there any problems with this pregnancy or delivery (prematurity, high blood pressure, etc.)?

……

……

7. What was the birth weight? ……

8. What is your child's weight and height? Weight……/height……

9. Has your child had a genetic blood (FISH) test? Yes ☐ No ☐
   If yes, what's the result: positive ☐ negative ☐

10. Is your child: right handed ☐ left handed ☐ don't know ☐

11. Does your child wear glasses or contact lenses? Yes ☐ No ☐

12. Has your child been officially diagnosed with a developmental disorder such as ADHD/ ADD/Autism/ Asperger's/ other? Yes ☐ No ☐
    If yes, please specify: ……

13. Is your child in any pain due to chronic medical conditions? Yes ☐ No ☐
    If yes, please
    describe:……
    ……

14. Has your child ever had any operations (other than tonsils/adenoids removal)?
    Yes ☐ No ☐
    If yes, type of operation? …… Age ……
    …… Age ……
    …… Age ……

15. Have your child's tonsils or adenoids been removed?
    a. Tonsils: Yes ☐ At what age? ……
       For what reason: ……
    b. Adenoids: Yes ☐ At what age?……
       For what reason: ……
    c. Describe briefly any changes you noticed in your child's sleep or waking behaviour after removal of tonsils or adenoids: ……
       ……

16. If NO, do you think the tonsils or adenoids are a problem? Yes ☐ No ☐ Don't know ☐

17. Has your child ever broken his/her nose or other facial bones? Yes ☐ No ☐

18. Does your child have difficulty breathing through his/her nose? Yes ☐ No ☐

19. In the past year, has your child had strep throats/tonsillitis? Yes ☐ No ☐
    Frequent colds/ respiratory infections? Yes ☐ No ☐
    Frequent sinus infections? Yes ☐ No ☐

20. Does your child have allergies? Yes ☐ No ☐ Possibly ☐
    If yes, to what? ……

21. Does your child have asthma? Yes ☐ No ☐
    If yes, how many asthma attacks has your child had in the last year?…… None ☐

22. Does your child frequently complain of heartburn? Yes ☐ No ☐ Don't know ☐
    Has he/she ever been diagnosed with gastroesophageal (stomach) reflux?
    Yes ☐ No ☐ Only when younger ☐

23. Has your child ever been tested for glucose level in blood? Yes ☐ No ☐ Don't know ☐
    If yes, is your child's glucose level in blood abnormal/over the range? Yes ☐ No ☐

24. Does your child suffer from hypercalcaemia? Yes ☐ No ☐ Don't know ☐

25. Has your child ever been tested for level of thyroid hormones (THS, T3, T4)?
    Yes ☐ No ☐ Don't know ☐
    If yes, which hormone and what was the result? ……
    ……

26. Has your child had any injuries requiring medical evaluation and/or treatment or loss of consciousness? If yes, please describe: ……
    ……

27. List any prescription or over- the counter medications your child has taken in the last month:

Type:……..Reason for medication……

Type:……..Reason for medication……

Type:……..Reason for medication……

28. Does your child take part in any extracurricular sport activities? Yes ☐ No ☐

If yes, how many hours a week and what kind of sport?

Type: ……..Hours/week……

Type: ……..Hours/week……

Type: ……..Hours/week……

29. Do you have any additional comments about your child's medical history?

……

……

……

HEALTH HABITS

30. How much television and/or videos does your child watch on school days?

☐ none ☐ 2-4 hours per day ☐ More than 6 hours per day

☐ up to 2 hours ☐ 4-6 hours per day ☐ Don't know

31. How much time does your child spend on the computer on school days?

☐ none ☐ 2-4 hours per day ☐ More than 6 hours per day

☐ up to 2 hours ☐ 4-6 hours per day ☐ Don't know

32. How much television and/or videos does your child watch on weekend days?

☐ none ☐ 2-4 hours per day ☐ More than 6 hours per day

☐ up to 2 hours ☐ 4-6 hours per day ☐ Don't know

33. How much time does your child spend on the computer on weekend days?

☐ none ☐ 2-4 hours per day ☐ More than 6 hours per day

☐ up to 2 hours ☐ 4-6 hours per day ☐ Don't know

34. Does your child watch TV and/or videos in the 30 minutes before falling asleep?

☐ Every night
☐ 5-6 nights per week
☐ 3-4 nights per week
☐ 1-2 nights per week
☐ Not at all

35. Does your child have a television set in his/her bedroom? Yes ☐ No ☐

36. Does your child have a computer or game console (including handheld such as Nintendo DS) in his/her bedroom? Yes ☐ No ☐

37. Does your child share a bedroom? Yes ☐ No ☐

38. Is child's bedroom on a main road where traffic can be heard at night? Yes ☐ No ☐

39. Please list any other factors that you think may affect your child's sleep.

.....
.....
.....
.....

DIET

40. Is your child vegetarian? Yes ☐ No ☐
41. How much caffeinated drinks does your child drink?

☐ More than 3 glasses per day
☐ Between 1 and 3 glasses per day
☐ Less than one glass per day
☐ None
☐ Don't know

42. How many main meals does your child eat per day?

☐ Less than 3
☐ 3
☐ 4
☐ 5 or more
☐ Not eating regularly

43. How many snacks does your child eat per day?

☐ None
☐ 1 or 2
☐ 3
☐ 4 or more

43. Does your child have gluten-free diet? Yes ☐ No ☐

44. Do you have any additional comments about your child's diet?

.....

.....

.....

.....

Many thanks for filling out this questionnaire. Your answers will be treated in the strictest confidence and will be used only for this research project.

APPENDIX 4

Child's name……

D.O.B……

Please fill in the table below as accurately as possible for each night that your child is wearing the Actiwatch.

Date	Getting up time	Bedtime (in bed with light out)	Time and duration of any daytime naps	Time and duration of any night time awakenings

Any Comments:

APPENDIX 5

Tanner Stage of Puberty

Please circle the right stage for your child

Boys:

Stage 1: Prepubertal

Stage 2: Enlargement of scrotum and testes; scrotum skin reddens and changes in texture

Sparse growth of long, slightly pigmented hair, straight or curled, at base of penis

Stage 3: Enlargement of penis (length at first); further growth of testes

Darker, coarser and more curled hair

Stage 4: Increased size of penis with growth in breadth and development of glans; testes and scrotum larger, scrotum skin darker

Hair adult in type, but covering smaller area than in adult; no spread to medial surface of thighs

Stage 5: Adult genitalia

Girls:

Stage 1: Prepubertal

Stage 2: Breast bud stage with elevation of breast; enlargement of areola

Sparse growth of long, slightly pigmented hair, straight or curled along the labia

Stage 3: Further enlargement of breast and areola; no separation of their contour

Darker, coarser and more curled hair

Stage 4: Nipple form a secondary mound above level of breast

Hair adult in type, but covering smaller area than in adult; no spread to medial surface of thighs

Stage 5: Mature stage, adult genitalia

APPENDIX 6

Collection mode

The room should be dimly lit (table lamp)

1.15 minutes before each saliva sample, rinse child's mouth thoroughly with water

2. Put on the gloves and remove the swab from the tube

3. Wearing gloves put the swab into the child's mouth between the teeth and cheek

4. Ask your child keep it for 3-5 minutes, until the swab is thoroughly soaked with saliva.

5. If this doesn't work, ask your child to spit into the big tube provided in the pack.

6. Put the swab back into the tube and put the lid on securely.

7. Record time on the tube

8. Put the tube/s in the fridge as soon as saliva is collected.

APPENDIX 7

SAMPLE COLLECTION REMINDER:

☐ Drinking: Drinks containing artificial colorants, caffeine (coffee, black or green tea, iced tea, cola) are not allowed on the evening of collection

☐ Eating: Nothing should be eaten during the collection time. The last meal must be taken at least 15 minutes before starting the collection. Bananas and chocolate should not be eaten during entire day before the collection.

☐ Teeth should not be washed at least 30 minutes prior to saliva collection

☐ Please collect saliva and urine samples at following times: first one in the afternoon (4-6pm), next one in the evening before going to bed, and the last one in the morning, just after awaking. Please note the time of collection on the tubes and pots.

☐ Please collect saliva samples under the muted light: light from reading lamp or from the television is preferable

☐ If by mistake, you forget to collect the first urine sample in the morning, please do this next day instead. It is extremely important that we get the very first urine sample

☐ Keep all saliva urine samples in fridge

☐ Give actiwatch to your child

☐ Please note down the time when your child go to bed and get up in the morning

☐ Please set up the masimo pulsoximetry for three nights

☐ Please fill in the questionnaires

Publications

Dimitriou D, **Sniecinska AM**, Iles RK (2013) Abnormal endocrine and behavioural sleep markers in a child with Williams syndrome and siblings. *Journal of Sleep Disorders and Therapy 2:1*.

Sniecinska AM, Iles RK, Butler SA, Jones H, Bayford R, Dimitriou D. (submitted) Sleep disturbances, elevated cortisol and low melatonin in children with Williams syndrome.

Sniecinska AM, Shah AJ, Titman C, Dimitriou D, Butler SA, Bayford R. (in preparation) Determination of urinary cortisol, cortisone and 6-sulfatoxy-melatonin by Ultra Performance Liquid Chromatography- Tandem Mass Spectrometry and its application to the assessment of the sleep disorders in Williams syndrome.