Part II

Disturbed circadian rhythms
Chapter 3

Disturbed peripheral circadian rhythms in hemodialysis patients

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Abstract

The quality of life of hemodialysis (HD) patients is hampered by reduced nocturnal sleep quality and excessive daytime sleepiness. In addition to the sleep/wake cycle, levels of circadian biomarkers (e.g. melatonin) are disturbed in end-stage renal disease (ESRD). This suggests impaired circadian clock performance in HD patients, but the underlying mechanism is unknown. In this observational study, circadian rhythms of sleep, melatonin, cortisol and clock gene expression are compared between HD patients (n=9) and healthy age-matched control subjects (n=9). In addition, the presence of circulating factors that might affect circadian rhythmicity is tested. Reduced sleep quality and increased daytime sleepiness were confirmed in HD patients. Reduced nocturnal melatonin concentrations and affected circadian cortisol rhythms were found, as well as disrupted circadian expression of the clock gene REV-ERBa. HD patient serum had a higher capacity to synchronize cells *in vitro*, suggesting an accumulated level of clock resetting compounds in HD patients. These compounds were not cleared by hemodialysis treatment or related to frequently used medications. In conclusion, the above mentioned results strongly suggest a disturbance in circadian timekeeping in peripheral tissues of HD patients. Accumulation of clock resetting compounds possibly contribute to this. Future studies are needed for a better mechanistic understanding of the interaction between renal failure and perturbation of the circadian clock.
Introduction

The quality of life of hemodialysis (HD) patients is hampered by reduced nocturnal sleep quality and excessive daytime sleepiness.(1,2) Melatonin plays an important role in the sleep-wake rhythm.(3) It is an important biomarker of the body’s circadian clock. Melatonin levels show a circadian rhythm with low levels during the day and high levels at night, thereby providing a ‘nighttime’ signal to the body.(4–6) Levels of melatonin are remarkably lowered in HD patients.(7,8) In addition to the sleep/wake rhythm and melatonin rhythm, other diurnal rhythms, e.g. blood pressure (9) are disturbed in end-stage renal disease (ESRD). Chronic circadian rhythm disruption has been associated with an increased risk to develop morbidities such as obesity, diabetes, cardiovascular events and cancer (10–14), but the causes of circadian rhythm disturbances in HD patients are unknown.

The human circadian system confers temporal organization upon behavior, physiology and metabolism and enables the body to anticipate and adapt to daily recurring changes in the environment.(15,16) It is composed of a central clock in the suprachiasmatic nuclei (SCN) in the hypothalamus in the brain and peripheral clocks in virtually all other cells and tissues.(17) Circadian rhythms are generated by an intracellular molecular oscillator, composed of a set of clock genes and proteins, with an intrinsic period of approximately 24 hours. To keep synchronized with the earth’s day-night cycle, the SCN receives clock-resetting environmental light information from the eye via the retino-hypothalamic tract. (18) In turn, peripheral oscillators are synchronized by the central SCN pacemaker through humoral and neuronal signals.(17,19)

To date, little is known about circadian clock performance in ESRD patients. One preclinical study with nephrectomy-induced CKD in rats associated sleep disruption with changes in the expression of clock genes (i.e. increased rPer1 and rPer2 mRNA levels) in the hypothalamus (20), but the origin of the altered clock gene expression patterns remained unsolved. Likewise, it is not known to what extent changes in serum metabolite levels, inherent to the severely impaired kidney function in HD patients, impacts on circadian clock performance.

The aim of the present study was to compare circadian clock performance in HD patients with that of healthy, age and sex-matched control subjects. To this end, we analyzed sleep quality and melatonin and cortisol rhythms, both important hormonal biomarkers of the clock.(21) Moreover, we assessed circadian clock performance by measuring the expression profiles of four circadian clock genes (Period (PER) 1, PER3, REV-ERBa and BMAL1) in peripheral leukocytes. Finally, we explored whether alterations in serum metabolite composition of HD patients circulating factor may influence peripheral circadian rhythms.
Materials and Methods

Subjects
Participant inclusion and blood sampling was conducted at Meander Medical Centre, Amersfoort, The Netherlands from July 2012 to October 2013. The study protocol was approved by the institutional review board (Netherlands Trial Registry: NTR3631) and written informed consent was obtained from all participants. The study was conducted according to the Declaration of Helsinki. HD patients and community-based healthy volunteers aged 18 to 85 years were eligible for inclusion. HD patients should be on stable, chronic hemodialysis treatment for at least 3 months. Control person should have MDRD-eGFR >60 ml/min/1.73m². Participants were excluded in case of (i) blindness, (ii) fever (>38°C), (iii) C-reactive protein > 20 mg/L, (iv) use of hypnotics during the study period, (v) exposure to anesthesia within 48 hours prior to start study, or (vi) serious co-morbidity that prevented participation in this study according to the investigators.

Study design
Participants were allowed to maintain their individual sleep-wake rhythm and food intake prior to and during the study (see figure 1 for study design). Participants entered the hospital at 8:30 AM on day 1 and remained there until 9:30 AM on day 2. They spent the overnight period in an individual room. Blood samples were obtained by venepuncture at 9:00 (day 1), 13:00, 17:00, 21:00, 01:00, 05:00 and 9:00 (day 2) and collected in regular serum collection tubes for serum analysis. In parallel, blood was collected into PAXgeneTM RNA tubes (BD, Erembodegem, Belgium), allowing direct stabilization of intracellular RNA for gene expression studies. In addition, blood samples were taken from HD patients just before and just after HD treatment.

![FIGURE 1. Study design](image)

HD=hemodialysis treatment, ▲ = blood sampling (for RNA isolation and serum collection, both HD patients and controls), ▲ = serum collection (for HD patients only)
Sleep analysis
Sleep parameters were investigated by means of actigraphy, using an Actiwatch 2 device (Respironics Inc., Murrysville, Pennsylvania, USA). Actigraphy is an established sleep monitoring method that records wrist movements and automatically discriminates rest-activity patterns interpreted in terms of sleep and wake periods. In addition, participants were asked to record bedtimes and rise times on a registration form. Respironics Actiware version 5.59 was used to score 1 minute epochs of actigraphic data as sleep or wake. The following parameters were calculated according to standardized methods: ‘sleep onset latency’ (SOL; time period between ‘lights off’ and sleep onset), ‘sleep efficiency’ (SE; actual sleep time divided by time in bed), ‘actual sleep time’ (AST; total duration of recorded sleep periods) and ‘wake after sleep onset’ (WASO; amount of time spent awake after sleep has been initiated and before final awakening). Each episode of actigraphy recordings was carried out during 4 consecutive days and nights, including HD and non-HD days. Daytime sleepiness scores were measured by the Epworth Sleepiness Scale (ESS). This questionnaire measures daytime sleepiness and has been used in HD patients before. Morningness-eveningness of the participants was determined by a chronotype questionnaire, adapted from the Horne and Ostberg Owl and Lark questionnaire.

Hormone assays
Cortisol and melatonin concentrations were measured with online-solid phase extraction in combination with isotope dilution LC-MS/MS. In short, 200 μL of plasma was used for the analysis and deuterated cortisol and melatonin were used as internal standard. Mean intra- and inter-assay coefficients of variation were below 10%. Quantification limits for cortisol and melatonin were 0.2 nM and 8.0 pM respectively.

Gene expression studies
RNA was isolated from leukocytes using the PAXgeneTM Blood miRNA kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions. cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Rochester, NY) and random hexamer primers, as described in the manufacturer’s instructions. The qRT-PCR reaction was performed using the Lightcycler 480 SYBR Green I Kit on a LightCycler 480 II platform (Roche Applied Bioscience, Indianapolis, IN). The protocol was as follows: 10 minutes at 95 °C, 45 cycles of 95 °C, 60 °C and 72 °C (10 seconds each), followed by a melting curve step with continuous acquisition from 65 °C to 97 °C. Relative quantification of gene expression was performed using the second derivative maximum method (Roche Diagnostics), followed by the delta-delta-cycle-
threshold (2-ΔΔCT) as described. Clock gene expression of robust human clock genes \( \text{PER1, PER3, REV-ERB\(\alpha\), BMAL1} \), normalized to the endogenous housekeeping gene \( \beta\)-actin (ACTB) were determined. Primers were acquired from Metabion (Martinsried, Germany), tested for efficiency and specificity of amplification. Primer sequences used: \( \text{PER1: Fwd 5’-GGA CAC TCC TGC GAC CAG GTA CTG-3’, Rev 5’-GGC AGA GAG GCC ACC ACG GAT-3’; PER3: Fwd 5’-GGT CGG GCA TAA GCC AAT G-3’, Rev 5’-GTG TTT AAA TTC TTC CGA GGT CAA A-3’; ACTB: Fwd 5’-TGA CCC AGA TCA TGT TTG AG-3’, Rev 5’-CGT ACA GGG ATA GCA CAG-3’; BMAL1: Fwd 5’-GCC TAC TAT CAG GCC AGG CTC A-3’, Rev 5’-AGC CAT TGC TGC CTC ATC ATT AC-3’; REV-ERB\(\alpha\): Fwd 5’-GCG GCG ATC GCA ACC TCT AG-3’, Rev 5’-GTA GGT GAT GAC GCC ACC TGT GTT-3’}. All gene expression levels at all time points of the participants were calculated relative to the gene expression of the particular gene at the time point 13:00 of the first control person.

Cell culture and real time imaging of clock performance

HEPA1-6 cells, stably expressing a lentiviral \( \text{Per2::luciferase} \) clock reporter construct were cultured in DMEM medium, supplemented with ultra-glutamine and 10% fetal calf serum (Lonza, Basel, Switzerland). For \textit{in vitro} testing of the clock synchronizing capacity of patients and control serum, HEPA1-6 cells were seeded in 30 mm dishes in culture medium supplemented with HEPES (25 mM final concentration; Lonza, Basel, Switzerland) and luciferin (10 mM final concentration) and allowed to attach and stabilize for one day. Next, dishes were placed in a LumiCycle bioluminescence imager to monitor clock performance (see below). When the intracellular clocks in the cell population were desynchronized (72 hour after seeding), serum from HD patients or control subjects was added to the medium and cultures were monitored for the reappearance of bioluminescence rhythms. As a positive control, cells were synchronized with dexamethasone (100 nM final concentration). Likewise, the clock synchronizing capacity of frequently used drugs was tested. To this end pharmacological concentrations of acenocoumarol, acetaminophen, acetylsalicylic acid, amlodipine, furosemide, insulin, metoprolol, nadroparin and oxazepam were added to pooled human serum from the blood bank Erasmus MC. In all experiments, the amplitude (peak/through) of the oscillation was used as a readout for synchronization potential. Real time bioluminescence recordings of luciferase activity (60 seconds measurements at 10 minute intervals) were performed using a LumiCycle 32-channel automated luminometer (Actimetrics), placed in a dry incubator at 37 °C. Prior to placing dishes in the LumiCycle, the lid was replaced by a glass coverslip and sealed with Parafilm. Data were analyzed using Actimetrics software.
Data analysis

Mean values and standard deviations of actigraphy, ESS and morningness-eveningness scores, melatonin concentrations per time point, and in vitro amplitudes of bioluminescence were calculated and/or plotted. Comparisons between control and patient results were made with Student’s T test.

Individual mRNA expression profiles and cortisol curves were subjected to analysis by the CircWave V1.4 programme (http://www.euclock.org/results/item/circ-wave.html). This program determines the significance of circadian oscillation of an individual curve. Because of its square wave pattern (25), this circadian analysis was not appropriate for the melatonin curves.

Results

Nine HD-patients and 9 age- and sex-matched control subjects completed the study protocol and were included in the analysis. The clinical characteristics of patients and controls are summarized in table 1. As expected, hemoglobin levels were lower and creatinin levels were higher in HD patients compared with controls. In line with the notion that diabetes mellitus is frequently related to the development of ESRD (26), the HD group contained more diabetic patients.

Sleep is affected in HD patients

We confirmed previous results showing that sleep is disturbed in HD patients.(27) HD-patients suffered from a longer sleep onset latency (mean 25.1 ± 13.7 minutes versus 8.9 ± 8.5 minutes, p=0.01), a lower sleep efficiency (mean 70.2 ± 8.1% versus 82.9 ± 10.9%, p=0.02) and a higher number of awake minutes during the night after initial sleep onset (mean 104.8 ± 27.9 minutes versus 54.6 ± 41.6 minutes, p=0.01), when compared with healthy controls. Furthermore, HD patients reported elevated daytime sleepiness. Mean Epworth Sleepiness Scale scores (± SD; a score >9 indicating elevated daytime sleepiness) were 10.0 ± 4.8 and 3.9 ± 2.0 respectively (p<0.005). The morningness-eveningness scores, as assessed by the chronotype questionnaire, did not differ significantly between patients and controls.
TABLE 1. Clinical characteristics

<table>
<thead>
<tr>
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<th>Hemodialysis patients</th>
<th>Control persons</th>
<th>p-value</th>
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<tr>
<td>Male / female</td>
<td>9 / 0</td>
<td>9 / 0</td>
<td>0.31</td>
</tr>
<tr>
<td>Age (years)</td>
<td>65.4 ± 12.1</td>
<td>59.4 ± 12.3</td>
<td>0.31</td>
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<tr>
<td>Body Mass Index (kg/m²)</td>
<td>28.3 ± 5.3</td>
<td>25.9 ± 3.1</td>
<td>0.27</td>
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<td>Hemoglobin (mmol/L)</td>
<td>7.0 ± 0.5</td>
<td>9.4 ± 0.6</td>
<td>&lt;0.001</td>
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<tr>
<td>Creatinin (μmol/L)</td>
<td>1021 ± 229</td>
<td>88.4 ± 6.9</td>
<td>&lt;0.001</td>
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<tr>
<td>eGFR* (ml/min/1.73m²)</td>
<td>hemodialysis</td>
<td>82.1 ± 10.1</td>
<td></td>
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<tr>
<td>Dialysis (hrs/week)</td>
<td>10.8 ± 2.0</td>
<td>n.a.</td>
<td>0.58*</td>
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<tr>
<td>Smoking</td>
<td>3</td>
<td>1</td>
<td></td>
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<tr>
<td>Diabetes mellitus</td>
<td>6</td>
<td>0</td>
<td>0.009*</td>
</tr>
<tr>
<td>ESS</td>
<td>10.0 ± 4.8</td>
<td>3.9 ± 2.0</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Chronotype questionnaire score</td>
<td>19.0 ± 3.1</td>
<td>21.7 ± 2.9</td>
<td>0.08</td>
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<tr>
<td>Sleep total (min.)</td>
<td>364 ± 92</td>
<td>381 ± 60</td>
<td>0.67</td>
</tr>
<tr>
<td>Sleep onset latency (min.)</td>
<td>25.1 ± 13.7</td>
<td>8.9 ± 8.5</td>
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<tr>
<td>Sleep efficiency (%)</td>
<td>70.2 ± 8.1</td>
<td>82.9 ± 10.9</td>
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<td>WASO (min.)</td>
<td>104.8 ± 27.9</td>
<td>54.6 ± 41.6</td>
<td>0.01</td>
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</tbody>
</table>

Data are expressed as mean ± SD or number of patients where appropriate.
* Calculated by the CKD-epi formula
eGFR = estimated Glomerular Filtration Rate, ESS = Epworth Sleepiness Scale, WASO = wake after sleep onset (in minutes)

Melatonin and cortisol oscillations are affected in HD patients

Being important outputs of the circadian clock, we have measured cortisol and melatonin rhythms in the serum of HD patients and control subjects (figures 2A and B). As expected, mean melatonin peak levels were higher in control subjects than in HD patients. At 1 AM, the mean melatonin concentration of the control persons was significantly higher than that of the HD-patients (91.9 ± 61.8 pmol/L versus 29.7 ± 41.2 pmol/L, p=0.025) and at 5 AM, their mean melatonin concentration tended to be higher (111.9 ± 86.3 pmol/L versus 39.6 ± 53.3 pmol/L, p=0.051). Likewise, cortisol rhythms differed between patients and controls. Significant circadian oscillation of cortisol was found in 2 HD-patients and in 6 control subjects. At 5 AM, the mean cortisol concentration of the control persons was significantly higher than that of the HD-patients (251 ± 83 nmol/L versus 169 ± 54 nmol/L, p=0.026). These findings are in line with previously reported circadian melatonin and cortisol measurements in ESRD patients, demonstrating reduced nocturnal melatonin concentrations (7,8,28) and elevated cortisol nadir in the evening (29), as compared to
healthy subjects. Moreover, our data suggest that the cortisol and melatonin rhythms are less robust in HD patients.

Circadian expression of REV-ERBα is disturbed in HD patients
Given the reduced amplitude of melatonin and cortisol rhythms in HD patients, we next questioned whether circadian rhythmicity in peripheral clock gene expression is affected in HD patients. To this end, we measured PER1, PER3, REV-ERBα and BMAL1 mRNA rhythms in white blood cells by quantitative RT-PCR. Analysis of individual gene expression profiles of the control subjects (supplementary figure S1) revealed that REV-ERBα is the most reliable circadian marker. Significant REV-ERBα mRNA rhythms were observed in 6 out of 9 control subjects, whereas significant PER1, PER3 and BMAL1 mRNA rhythms were detected in only 2, 3 and 2 controls, respectively (figure 2C). Strikingly, none of the HD-patients showed a circadian REV-ERBα mRNA rhythm (p=0.009). Moreover, REV-ERBα mRNA, but also PER3 and BMAL1 mRNA levels were consistently lower in HD patients (average values shown in figure 2D-G). Taken together, these data suggest that circadian gene expression is markedly affected in peripheral blood cells of HD patients.

Serum from HD patients has a higher capacity to synchronize cells in vitro
Peripheral clocks are daily synchronized by the central SCN clock though cyclic release of humoral factors.(19) Given the severely impaired kidney function in CKD patients, it is tempting to speculate that abnormal levels of serum metabolites (including humoral factors involved in peripheral clock synchronization) underlie altered levels in the blood of CKD patients. We therefore explored the hypothesis that the dampened amplitude of cortisol, melatonin and peripheral clock gene expression patterns may (in part) be caused by impaired clearance of clock synchronizing factors by the kidney resulting in increased, saturating levels of clock synchronizing factors. Under this condition, peripheral cells constitutively, rather than cyclically are triggered to reset their clocks, causing desynchronization of these peripheral clocks. To test this hypothesis, we have made use of the phenomenon that asynchronous circadian clocks of cultured peripheral cells (e.g. fibroblasts, hepatocytes) can be temporarily synchronized by addition of serum to the medium (known as serum shock). The higher the serum concentration, the higher will be the amplitude of synchronized oscillation.
FIGURE 2. Melatonin, cortisol and clock gene rhythms
Panel A: mean oscillation (±SD) of melatonin. The horizontal axis reflects the time of day in hours and the vertical axis reflects melatonin concentration in serum (pM). The detection limit of melatonin was 8 pM. Individual results below the detection limit, were set at 0 pM. Panel B: mean oscillation (±SD) of cortisol. The horizontal axis reflects the time of day in hours and the vertical axis reflects cortisol concentration in serum (nM). Panel C: Characterization of circadian rhythmicity. The vertical axis reflects the percentage of individuals in whom a significant circadian rhythm was calculated with CircWave. The horizontal axis reflects each circadian marker. The actual number of individuals is shown above each bar. Panels D to G: Mean (±SD) oscillations in expression of clock genes PER1, PER3, REV-ERBα and BMAL1. The horizontal axes reflect the time of day in hours. The vertical axes reflect clock gene mRNA expression (relative copy number/β-actin, all arbitrarily normalized to the relative expression at 13:00 of the first control person). The black lines represent patients, the dashed lines represent controls. n=9 for both control and patient groups. * p<0.05

We first tested the effect of increasing concentrations of pooled human control serum on the amplitude of bioluminescence rhythms in Hepa1-6 cells, expressing a Per2::luciferase clock reporter gene. As shown in supplementary figure S2, human serum can dose dependently synchronize the circadian clock. Importantly, when using serum from HD patients, the amplitude of oscillation was higher at all serum concentrations tested, with the most significant difference observed at 6% serum (supplementary figure S2). These data suggest that HD patient serum has a higher clock synchronizing potential.

We next compared the clock synchronizing capacity of serum taken from control subjects and HD patients from our study groups at a fixed concentration of 6% serum. In line with the previous experiment, the serum of HD patients has a higher capacity to synchronize the circadian clocks of cultured HEPA1-6 cells than serum of healthy controls (figure 3 upper panels), as reflected by the larger amplitude of bioluminescence (2.61 ± 0.61 and 1.89 ± 0.32 for patient and control sera respectively, p=0.008).

To eliminate the possibility that the observed higher synchronizing capacity was caused by the medication of HD patients, commercially available human pooled serum was spiked with pharmacological concentrations of frequently prescribed drugs (acenocoumarol, acetaminophen, acetylsalicylic acid, amlodipine, furosemide, insulin, metoprolol, nadroparin and oxazepam) and tested for modulation of clock synchronizing capacity. None of the drugs tested was capable of synchronizing HEPA1-6 cells at pharmacological concentrations (supplementary figure S3).

To test whether hemodialysis influences the synchronizing capacity of the patients’ serum, e.g. by removing clock resetting factors from the bloodstream, patient serum was collected just before and right after hemodialysis. No significant difference in synchronization capacities was observed (figure 3 lower panels), reflected in mean amplitudes of 2.50 ± 0.52 versus 2.63 ± 0.65 before and after hemodialysis treatment, respectively. Hemodialysis does not reduce the clock synchronizing capacity of serum.
FIGURE 3. Cell synchronization
Upper panels (A): in vitro synchronization capacity of HD patients’ serum (n=9) versus controls’ serum (n=9) on HEPA1-6 cells carrying a Per2::luciferase reporter construct. Upper left panel: graphical representation of the amplitude of oscillation. A higher amplitude indicates a higher degree of synchronization. Upper right panel: mean baseline corrected bioluminescence recordings of controls (dashed line) and patients (solid line). Lower panels (B): in vitro synchronization capacity of HD patients’ serum (n=9) on HEPA1-6 cells carrying a Per2::luciferase reporter construct before and after hemodialysis treatment. Lower left panel: graphical representation of the amplitude of oscillation. A higher amplitude indicates a higher degree of synchronization. Lower right panel: mean baseline corrected bioluminescence recordings of serum before (solid line) and after (dotted line) dialysis. ** p<0.01
Discussion

The present study assessed peripheral diurnal oscillations in human HD patients at a behavioral, hormonal and molecular level. Patients and control subjects were allowed to follow their own daytime routine inside and around the hospital in order to stay close to “real life” conditions and preserve the naturally occurring variation. The disturbance of the sleep wake cycle in HD patients was confirmed by lower nighttime sleep quality and higher daytime sleepiness in addition to a reduced amplitude of melatonin and cortisol rhythmicity. In order to identify alterations in peripheral circadian oscillators, we compared the expression levels of four clock genes in leukocytes of HD-patients with those of healthy age-matched control subjects. We observed a marked difference in the circadian expression profile of patients compared to controls. Overall, PER3, BMAL1 and REV-ERBα mRNA levels were lower in HD patient leukocytes, suggesting that intracellular peripheral clocks are dampened. Moreover, when analyzing the presence of circadian oscillations, we observed a statistically significant difference between HD patients and control subjects for REV-ERBα rhythms, which did not show significant oscillation in any of the patients. The other genes tested appeared less informative, as significant rhythms could only be detected in a limited number of control subjects. Nonetheless, even for BMAL1 and PER1, we could not detect significant mRNA rhythms in HD patients, further supporting the dampening of the circadian clock in HD patients. Taken together, even in the absence of a constant routine protocol (e.g. standardized light and meal times), our behavioral, hormonal and gene expression data point to impaired circadian clock performance in HD patients.

Apart from a reduction in clock gene expression in individual cells, at the population level of cells, dampening of circadian amplitude can also originate from a desynchronization of individual cellular clocks. We hypothesized that circadian dysregulation in HD patients may (in part) be caused by elevated levels of circadian clock resetting compounds, which accumulate in the blood from HD patients due to impaired renal clearance. In this scenario, the central clock still functions normally, in HD patients, but clock synchronizing humoral signals from the SCN are not sufficiently cleared and accumulate in the bloodstream at concentrations that exceed threshold levels and accordingly continuously, rather than rhythmically, trigger peripheral clocks to reset. This ultimately causes peripheral oscillators to desynchronize, resulting in a lower amplitude, or even absence of peripheral circadian rhythms, as found for melatonin, cortisol and REV-ERBα expression. The concept of the presence of certain serum factors being responsible for circadian changes has already suggested before in clinical research on increasing age.(30)
In support of the above hypothesis, using an \textit{in vitro} clock synchronization assay with Per2::luciferase Hepa1-6 cells, we have shown that HD patient serum has a higher clock resetting potential than control serum. As commonly used HD related drugs did not have any influence on cell synchronization, the synchronizing factor(s) is (are) of endogenous origin. Hemodialysis treatment did not reduce the synchronizing potential of patient serum. An explanation for this observation could be that hemodialysis does not remove these compounds to substantially lower concentrations (i.e. below threshold values). Which serum components are responsible for the effect observed remains to be determined. Since melatonin and cortisol synthesis and secretion as well as sleep/activity all result from circadian regulation within peripheral (i.e. non-SCN) tissues, their changes in HD patients might all share the same origin.

At first sight, our data seem to contradict the preclinical study of Hsu and coworkers (20), in which sleep disruption in nephrectomized rats was shown to be associated with a significant upregulation of \textit{rPer1} and \textit{rPer2} expression in the hypothalamus. Yet, closer examination of the data suggest that, despite higher expression levels, the amplitude of \textit{rPer1} and \textit{rPer2} rhythms is reduced, which would be in line with our study (figure 2). On the other hand, differences may originate from the fact that Hsu and coworkers investigated clock gene expression in brain tissue (i.e. hypothalamus), which apart from humoral signaling, is also subject to neuronal signaling from the SCN.

Our results point to perturbation of circadian control in HD-patients, but it cannot be ascertained that the observed differences are solely renal function related. Although sleep deprivation itself influences clock gene expression (31), it is not likely that disturbed sleep is fully responsible for our observations. Diabetes could as well be a confounding factor, as most HD patients and none of the controls have diabetes. This reflects the well-known clinical situation in which end-stage renal disease occurs as a consequence of long-term poorly regulated glucose levels. However, both at gene expression level and synchronizing potential the patients without diabetes do not cluster closer to the controls, suggesting that it is not diabetes that is causing the rhythm alterations. This is further validated by the following observations: i) serum with higher glucose concentrations does not induce a higher amplitude of oscillation (supplementary figure S4) and ii) insulin does not induce a significant circadian oscillation (supplementary figure S3).

In conclusion, we have identified disturbances in circadian rhythmicity of sleep, melatonin, cortisol and clock gene expression in HD patients. Notably, clock gene expression (including circadian amplitude) in peripheral cells was shown to be reduced in HD patients. Furthermore, our data suggest that impaired peripheral clock performance may originate from compromised humoral clock resetting from the SCN. Serum of
HD patients has a higher clock synchronizing capacity than serum of matched control subjects. This strongly suggests a disturbance in circadian timekeeping in HD patients, induced by reduced renal clearance. Further studies are needed in order to understand the mechanism of the interaction between renal failure and perturbation of the circadian clock. Importantly, efforts should be made to determine the nature of accumulated clock resetting compounds. Understanding the origin of circadian rhythm disturbances in HD patients is important in order to further optimize current treatment strategies and to identify novel therapeutic targets.

Declaration of interest

The authors of this manuscript have no conflicts of interest to disclose.

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Chapter 3

References

Disturbed peripheral circadian rhythms in hemodialysis


FIGURE S1. Individual oscillations in expression of clock genes PER1, PER3, REV-ERBα and BMAL1
The horizontal axes reflect the time of day in hours. The vertical axes reflect clock gene mRNA
expression (relative copy number/β-actin, all arbitrarily normalized to the relative expression at 13:00
of the first control person). The black lines represent patients, the dashed lines represent controls,
n=9 for both groups.
FIGURE S2. Determination of optimal serum concentration to detect differences in in vitro synchronization capacity between hemodialysis patient serum (n=1, black dots) and pooled human control serum (grey diamonds).
A larger amplitude indicates a higher degree of synchronization. * p < 0.05, ** p < 0.01
FIGURE S3. Mean bioluminescence recordings of HEPA1-6 cells carrying a Per2::luciferase reporter construct
Cells are synchronized with pooled human serum (panel A, mock treatment), serum containing dexamethasone 1 μM (panel B, positive control) and serum spiked with pharmaceutical drug concentrations (Panels C to J) (n=2). Panels K and L: mock treatment and insulin treatment respectively.
FIGURE S4. Mean amplitude results of bioluminescence recordings of HEPA1-6 cells carrying a Per2::luciferase reporter construct. Cells are synchronized with hemodialysis patient serum spiked with increasing glucose concentration. A higher amplitude indicates a higher degree of synchronization. There was no significant correlation between glucose concentration and amplitude.