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**Managing circadian disruption due to hospitalisation:  
a pilot randomised controlled trial of the CircadianCare inpatient  
management system**

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## ABSTRACT

The objective of the present study was to test the effects of an inpatient management system (CircadianCare) aimed at limiting the negative impact of hospitalisation on sleep by enhancing circadian rhythmicity. Fifty inpatients were randomised to either CircadianCare (n=25; 18 males, 62.4±1.9 yrs) or standard of care (n=25; 14 males, 64.5±2.3). On admission, all underwent a full sleep-wake evaluation; they then completed daily sleep diaries and wore an actigraph for the whole length of hospitalisation. On days 1 (T0), 7 (T1) and 14 (T2, if still hospitalised) salivary melatonin for dim light melatonin onset (DLMO) and 24-hour skin temperature were recorded. In addition, environmental noise, temperature and illuminance were monitored. Patients in the CircadianCare arm followed one of three schedules for light/dark, meal and physical activity timings, based on their diurnal preference/habits. They wore short-wavelength-enriched light-emitting glasses for 45 min after awakening, and short-wavelength light filter shades from 18:00 until sleep onset. While the first, primary registered outcome (reduced sleep onset latency on actigraphy or diary) was not met, based on sleep diaries, there was a trend ( $0.05 < p < 0.1$ ) towards an advance in bedtime for CircadianCare compared to standard of care patients between T0 and T1. Similarly, DLMO time significantly advanced in the small group of patients for whom it could be computed on both occasions, with untreated ones starting from earlier baseline values. Patients sleeping near the window had significantly higher sleep efficiency, regardless of treatment arm. As noise fluctuation increased, so did the number of night awakenings, also regardless of treatment arm. In conclusion, the CircadianCare management system showed positive results in terms of advancing sleep timing and the circadian rhythm of melatonin. Further, our study identified a combination of environmental noise and lighting indices, which could be easily modulated to prevent hospitalisation-related insomnia.



## RIASSUNTO

L'obiettivo del presente studio è stato quello di testare gli effetti di un sistema di gestione di pazienti, chiamato CircadianCare, volto a limitare l'impatto negativo del ricovero sul sonno, migliorando la ritmicità circadiana. Cinquanta pazienti sono stati randomizzati per ricevere il trattamento CircadianCare (n=25; 18 maschi,  $62,4 \pm 1,9$  anni) oppure il trattamento standard (n=25; 14 maschi,  $64,5 \pm 2,3$  anni). Al momento del ricovero veniva eseguita una valutazione completa del ritmo sonno-veglia abituale di tutti i pazienti, comprensivo del cronotipo; per tutta la durata del ricovero i pazienti hanno compilato diari del sonno giornalieri ed indossato un actigrafo. Ai giorni 1 (T0), 7 (T1) e 14 (T2, se ancora ricoverati) venivano misurate la melatonina salivare (dim light melatonin onset, DLMO) e la temperatura cutanea nelle 24 ore. Inoltre, venivano misurati monitorati il rumore, la temperatura e la luce ambientali a cui i pazienti erano esposti. I pazienti nel gruppo CircadianCare hanno seguito uno di tre possibili programmi per la gestione delle ore di luce/buio, degli orari dei pasti e dell'attività fisica, basati sulle loro preferenze/abitudini. Hanno eseguito la terapia della luce, indossando occhiali che infondono luce blu (a lunghezza d'onda corta) per 45 minuti al risveglio, ed occhiali filtranti la luce a lunghezza d'onda corta dalle ore 18:00 fino all'addormentamento. Sebbene il primo endpoint primario (ovvero la riduzione della latenza dell'inizio del sonno registrato con diari del sonno o attigrafia) non sia stato raggiunto, dai diari del sonno è emersa una tendenza ( $0.05 < p < 0.1$ ) verso un anticipo dell'orario di coricamento per i pazienti del gruppo CircadianCare rispetto a quelli del gruppo di controllo tra T0 e T1. Allo stesso modo, l'orario di DLMO è risultato significativamente avanzato nel piccolo gruppo di pazienti per cui è stato possibile calcolarlo a T0 e T1, considerando che i pazienti nel braccio di trattamento standard partivano da valori basali più precoci. L'efficienza del sonno è risultata significativamente maggiore per i pazienti che dormivano vicino alla finestra, indipendentemente dal braccio di trattamento. Con l'aumento delle fluttuazioni del rumore, è aumentato anche il numero di risvegli

notturni, indipendentemente dal braccio di trattamento. In conclusione, il sistema di gestione CircadianCare ha mostrato risultati positivi in termini di anticipo dell'orario del sonno e del ritmo circadiano della melatonina. Inoltre, il nostro studio ha identificato una combinazione di rumore ambientale e indici di illuminazione, che potrebbero essere facilmente modulati per prevenire l'insonnia correlata al ricovero ospedaliero.

# 1. INTRODUCTION

Circadian rhythms are endogenous, self-sustaining biological rhythms with a period of approximately 24 hours, that have an influence on most physiological processes (Douma & Gumz, 2018; Qian & Scheer, 2016; Refinetti, 2020; Weitzman et al., 1971). The name itself comes from the Latin “*circa diem*” (i.e., “approximately one day”). In 2017 Jeffrey C. Hall, Michael Rosbash and Michael W. Young, three American genetists, were awarded with the Nobel Prize in Physiology or Medicine for their discoveries of molecular mechanisms underlying the circadian rhythm regulation on model organisms (the *Drosophila melanogaster*, also known as the fruit fly). Their discoveries had great implications on the understanding of circadian regulation of various plants and animals, including humans. The circadian timing system encompasses a master light-entrainable clock in the suprachiasmatic nuclei (SCN) of the hypothalamus, and peripheral time-keepers in almost every organ of the body (Allada & Bass, 2021). Its rhythmicity depend on both endogenous stimuli (e.g., neural and humoral inputs) and the so called *Zeitgebers* (German for “time givers”), which are external, environmental stimuli that drive circadian entrainment; example of *Zeitgebers* are the light/dark cycle and, to a lesser extent, exercise timing, meal timing and the timing of social interactions (Asher & Schibler, 2011; Reinke & Asher, 2016).

## 1.1 CIRCADIAN AND HOMEOSTATIC SLEEP REGULATION

In humans, the sleep-wake cycle is the most obvious behavioural output of the circadian timing system. Sleep is regulated by the interaction of both homeostatic and circadian processes (Borbély, 1982) (Figure 1). The homeostatic process determines sleep propensity in relation to sleep-wake history: sleep propensity increases during waking, in parallel with the duration of the wake period, and dissipates during sleep; the homeostatic rhythm is mainly marked by the presence and oscillation of the slow wave activity (SWA) on the electroencephalography (EEG)

(Daan et al., 1984; Dijk, 1995; Vassalli A, Dijk DJ., 2009). On the other hand, the circadian process is responsible for the alternation of high or low sleep propensity in relation to dark and light cues and is substantially independent of the preceding sleep-wake history (Borbély, 1982); the circadian rhythmicity of sleep is marked by the 24-hour rhythm of melatonin and core body temperature (Borbély & Achermann, 1999).

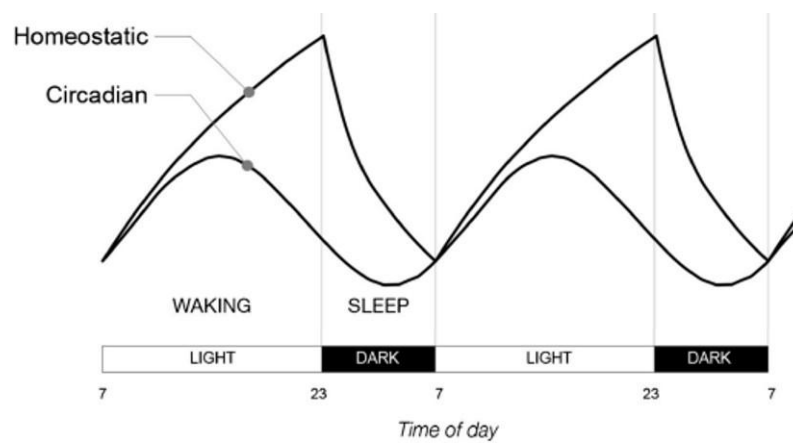


Figure 1. Interaction between Circadian and Homeostatic Processes in sleep regulation (adapted from Borbély AA, 1982)

## 1.2 MASTER CLOCK AND PERIPHERAL CLOCKS

The master pacemaker for the generation of circadian behavioural rhythms in mammals is located in the hypothalamic suprachiasmatic nucleus (SCN), which is composed of approximately 20,000 neurons (Klein et al., 1991). Light reaches SCN neurons by afferent projections from the retina through the retino- hypothalamic tract (Morin LP, Allen CN., 2006). In the retina, direct photic input is received from a non rod-, non cone-photoreceptor cell type known as 'intrinsically photoreceptive retinal ganglion cells' (ipRGCs) (Hattar et al., 2003). These ipRGCs express a photopigment, called melanopsin, which renders them intrinsically photosensitive to short wavelength irradiation (between 460 and 500 nm). Thanks

to their ability to drive non-visual light responses, ipRGCs play a key role in light-mediated regulation of the circadian timing system. In turn, the SCN projects to the pineal gland, modulating the production of melatonin, which can be considered as the neuroendocrine transducer of the light-dark cycle (Cajochen et al., 2003; Cassone, 1990) (Figure 2). Exposure to light robustly suppresses melatonin secretion in an intensity-dependent manner (McIntyre et al., 1989): detection of light activates ipRGCs, that project to the SCN in the hypothalamus, causing the release of inhibitory GABA that suppresses melatonin synthesis in the pineal gland and its release in the cerebrospinal fluid of the third ventricle, thus keeping melatonin concentrations very low during the day. During the darkness, the SCN synaptically triggers noradrenalin release from the superior cervical ganglion (SCG): noradrenalin in turn activates beta-adrenergic receptors in the pineal gland, thus inducing melatonin synthesis and secretion (Bedrosian et al., 2013). Therefore, in healthy individuals, plasma melatonin levels are high at night, and low during the day. Circulating melatonin is hydroxylated/sulphated to 6-sulphatoxymelatonin (aMT6s), primarily in the liver; aMT6s is then excreted in the urine, here its profile mirrors that of plasma melatonin.

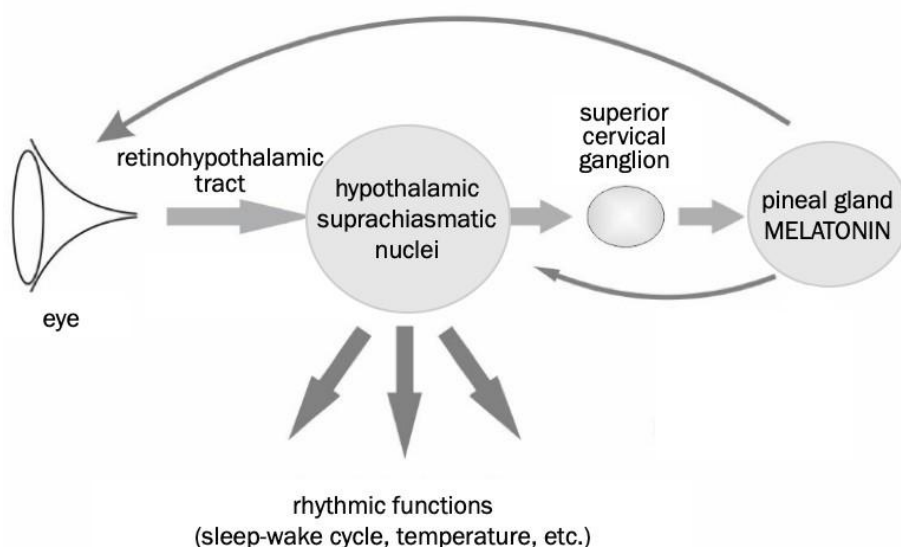


Figure 2. The retinal-SCN-pineal pathway.

The SCN master pacemaker not only entrains to the light/dark cycle by direct effect of light, it also synchronizes other subsidiary cellular oscillators; this is due to the ability of SCN neurons to remain synchronized between each other, even in constant darkness (Aton & Herzog, 2005), and, moreover, to generate a robust circadian rhythm that synchronizes other cells throughout the body, through a variety of pathways (Gachon et al., 2004). In addition to the SCN master clock, other peripheral circadian clocks have been located in many organs/tissues (including liver, muscle, kidney, and heart) (Balsalobre et al., 1998); when isolated in vitro, peripheral clocks maintain a robust circadian rhythmicity for some days, indicating that the SCN is not required for short-term rhythmicity. However, the SCN master plays a hierarchical role, determining synchrony of clock genes expression in peripheral clocks (Figure 3). Synchronization of peripheral clocks occurs via the autonomic nervous system, hormone metabolites, and metabolic pathways. In addition, cues such as feeding patterns, temperature cycles, and physical activity can reset/phase-shift peripheral oscillators (Asher & Schibler, 2011; Reinke & Asher, 2016).

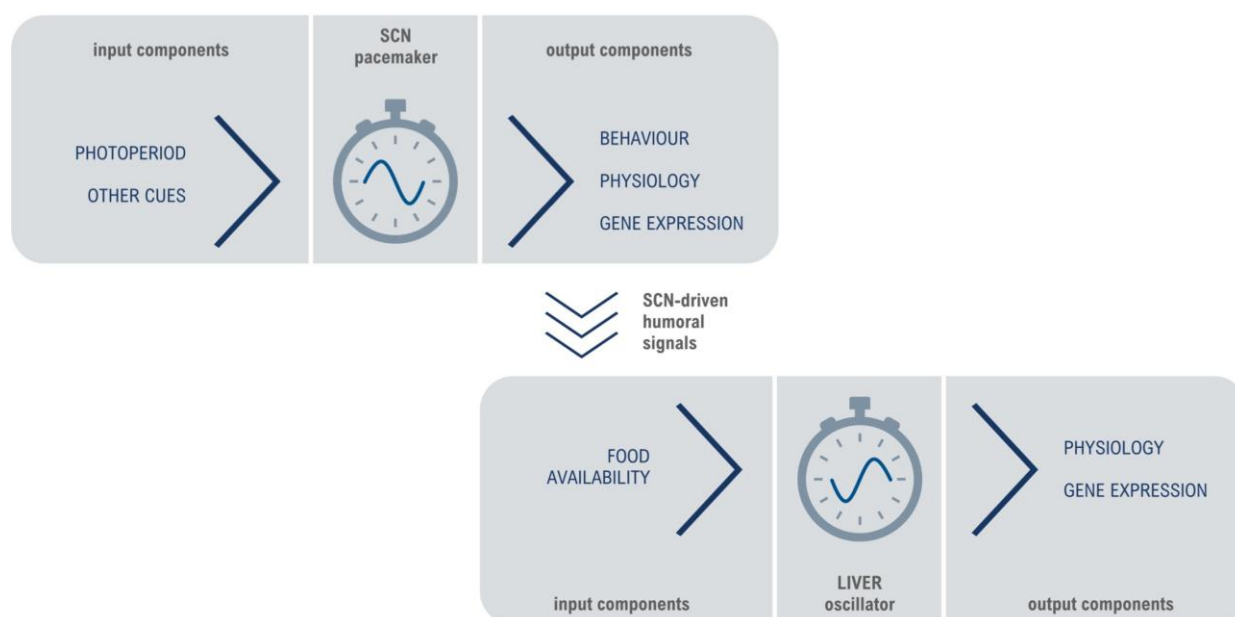


Figure 3. Hypothetical model for entrainment of peripheral clocks by the SCN and local cues (adapted from Balsalobre A, 2002).

### 1.3 CIRCADIAN PHYSIOLOGY AND MISALIGNMENT

Circadian rhythms have an influence on a wide variety of physiological processes: endocrine functions, such as glucocorticoid secretion (Weitzman et al., 1971) gastric acid secretion (Moore & Engler, 1970), blood pressure regulation (Duma & Gumz, 2018), body temperature (Refinetti, 2020); renal functions, including renal plasma flow, glomerular filtration rate, tubular transport activities and diuresis (Firsov & Bonny, 2018); glucose metabolism (Qian & Scheer, 2016); nutrition and regulation of energy balance (Engin, 2017); immune system activity (Kizaki et al., 2015); pulmonary functions (Spengler & Shea, 2000), central nervous system activity and electroencephalographic parameters (Aeschbach et al., 1999). In physiological conditions, the individually controlled biological rhythms are finely synchronised in order to ensure optimal performance (Czeisler & Gooley, 2007). As previously mentioned, circadian rhythms maintain a near 24-hour rhythmicity even in the absence of environmental influences, although the adaptation to the surrounding environment (entrainment) is provided through external time cues (Zeitgebers), such as light (the most powerful environmental time signal), activity/physical exercise, and meal times.

In recent years, evidence has emerged that circadian misalignment, which is mostly due to environmental factors and social constraints, have serious medical consequences. This includes sleep disorders (i.e., sleep curtailment and worsened sleep quality), sleep-related attentional failure and neurocognitive performance failure (Belenky et al., 2003; Van Dongen & Dinges, 2003). Moreover, it has been demonstrated that circadian disruption not only affects sleep, but it has also been related to increased cardiovascular morbidity (Morris et al., 2016; Vetter et al., 2016), increased risk of certain types of cancer (Lin et al., 2015; Masri & Sassone-Corsi, 2018; Walasa et al., 2018), occurrence of neuro-psychiatric disorders (Jagannath et al., 2013) and occurrence of metabolic syndrome (Zimmet et al., 2019). Healthy subjects who are exposed to milder forms of

circadian misalignment, (e.g., evening types or “owls”) are at risk of suffering from relevant sleep loss, weight gain and increased consumption of substances as alcohol/coffee (Roenneberg et al., 2012).

#### 1.4 HOSPITALISATION AND CIRCADIAN RHYTHMICITY

Hospitalisation *per se* weakens circadian rhythmicity and the sleep-wake cycle due to disease itself, an unusual and noisy environment, and modified light (with significant exposure to light at night, especially in hospital rooms with multiple beds), food and activity cues (Aaron et al., 1996; Bano et al., 2014; Giménez et al., 2017; Kamdar et al., 2012). As a result, patients tend to experience delayed, shorter and poorer night sleep, several night awakenings and daytime sleepiness. This disturbed sleep may affect prognosis, also in terms of duration of the inpatient stay (Ryherd et al., 2008).

We have previously documented that, in our medical ward, rolling shutters were rarely moved during the 24 hours, with consequent daylight deprivation and worse sleep (especially for patients whose bed was far from the window) (Bano et al., 2014), highlighting the powerful role of light as a *Zeitgeber* within the arrhythmic hospital environment. Indeed, while we are well accustomed to thinking we need a good night's sleep for a good active day, the opposite is also true, as in order to accumulate sleep propensity, we need an ‘efficient’ wake time, thus a certain amount of light/activity, and timed food intake. Patients’ hospital life is very disruptive in this respect, impinging on the quality of wake as much as that of sleep. This situation is not unlike the jet-lag we experience when we travel across several time zones over a short period of time: similarly, jet-leg symptoms can be alleviated by timed administration of strong light, food/cafeine (Drake et al., 2011) and timed physical activity (Buxton et al., 2003).

Over recent years, we have shown that both morning bright light and caffeine administration can



counteract the excessive daytime sleepiness that accompanies some forms of advanced liver disease (Casula et al., 2015; De Rui et al., 2015; Turco et al., 2018). This is easier to obtain in outpatients (Turco et al., 2018) compared to inpatients (De Rui et al., 2015), most likely in relation to a mixture of disease severity, which tends to be greater in inpatients, and the direct effects of hospitalisations on sleep-wake and circadian rhythmicity.

Further, we subsequently showed that inpatients treated with bright light immediately after awakening and with short-wavelength light filter glasses in the evening had fewer night awakenings, less daytime sleepiness (with potential positive effects also on the subsequent sleep episode) and better mood than their untreated counterparts (Formentin et al., 2020). It is therefore possible that controlling/modulating light and other potentially relevant *Zeitgebers* during an inpatient stay may enhance rhythmicity, improve sleep timing and quality and, ultimately, improve prognosis (Milani et al., 2018).

## 2. AIM

The aim of the present study was to test the effects of an inpatient management system, which we called CircadianCare, aimed at limiting the impact of hospitalisation by enhancing circadian rhythmicity (and, in turn, improving sleep timing and sleep quality) through an assessment of the patient's specific circadian features and needs and an *ad hoc* personalised light-dark, meal and activity or physiotherapy schedule. The main expected outcome was an advance in circadian phase, measured by an advance in sleep onset timing (first registered primary outcome: reduced sleep onset latency based on actigraphy or diary, plus a series of actigraphy/diary based sleep-wake variables) and/or an advance in melatonin onset after 7 days of hospitalisation. This was based on the hypothesis that the personalised CircadianCare schedule would advance phase (mostly due to morning light administration and protection from light in the early evening/night hours, *vide infra*), limit its hospitalisation-related delay and/or rhythmicity weakening and, in turn, improve sleep quality.

### 3. PATIENTS AND METHODS

#### 3.1 PATIENTS

All patients admitted into the Clinica Medica V ward of Padova University Hospital between October 2020 and January 2022 were screened within 24 hours of admission. In principle, all patients screened within 24 hours of admission could be included. Exclusion criteria were: unwillingness or inability to comply with the study procedures, severe cognitive impairment and diagnosed, well-defined sleep-wake disorders. To detect a difference of 10 minutes in sleep onset time between CircadianCare and standard of care after a week of hospitalisation (T1) with an SD of 15 minutes, with a two-sided 0.05 significance and a power of 80%, a sample size of 35 subjects per arm would have been needed. The above was based on what is known on light responses, and not on more articulate, multiple intervention protocols such as ours, thus a less conservative power estimate to detect a difference of 20 minutes in sleep onset time between CircadianCare and standard of care (remaining parameters unchanged) was also made, with 18 subjects per arm being needed. On final review, considering both feasibility in the acute medical setting and the limited, available literature data, 25 subjects per arm were deemed appropriate and study approval sought for based on this power calculation. Despite the difficulties and limitations to screening/recruitment during the pandemic, a total of 142 patients were screened; 48 patients did not meet the inclusion/exclusion criteria and 44 patients were eligible but unwilling to participate

(Figure 4). Thus, the final population included the necessary 50 inpatients (study stop), randomised (1:1 block randomisation) to either the CircadianCare system (n=25) or standard of care (n=25) by use of a computer-generated randomisation list. Information on demographics [sex, age, Body Mass Index (BMI), and level of education], reasons for hospital admission, triage severity code on admission, the Charlson Comorbidity Index (Charlson et al., 1987) and treatment prior to admission were collected for each patient. The study protocol was approved on 20 February 2020 by the Padova University Hospital Ethics Committee (AOP0536) and the study was conducted according to the Declaration of Helsinki (Hong Kong and 2008 Amendments) and Good Clinical Practice (European) guidelines. All participants provided written, informed consent. The study was registered retrospectively (because of administrative/ logistic issues) on ClinicalTrials.gov (NCT05228444). The CONSORT reporting guidelines were used (Schulz et al., 2010). The CONSORT flow diagram is presented in Figure 4.

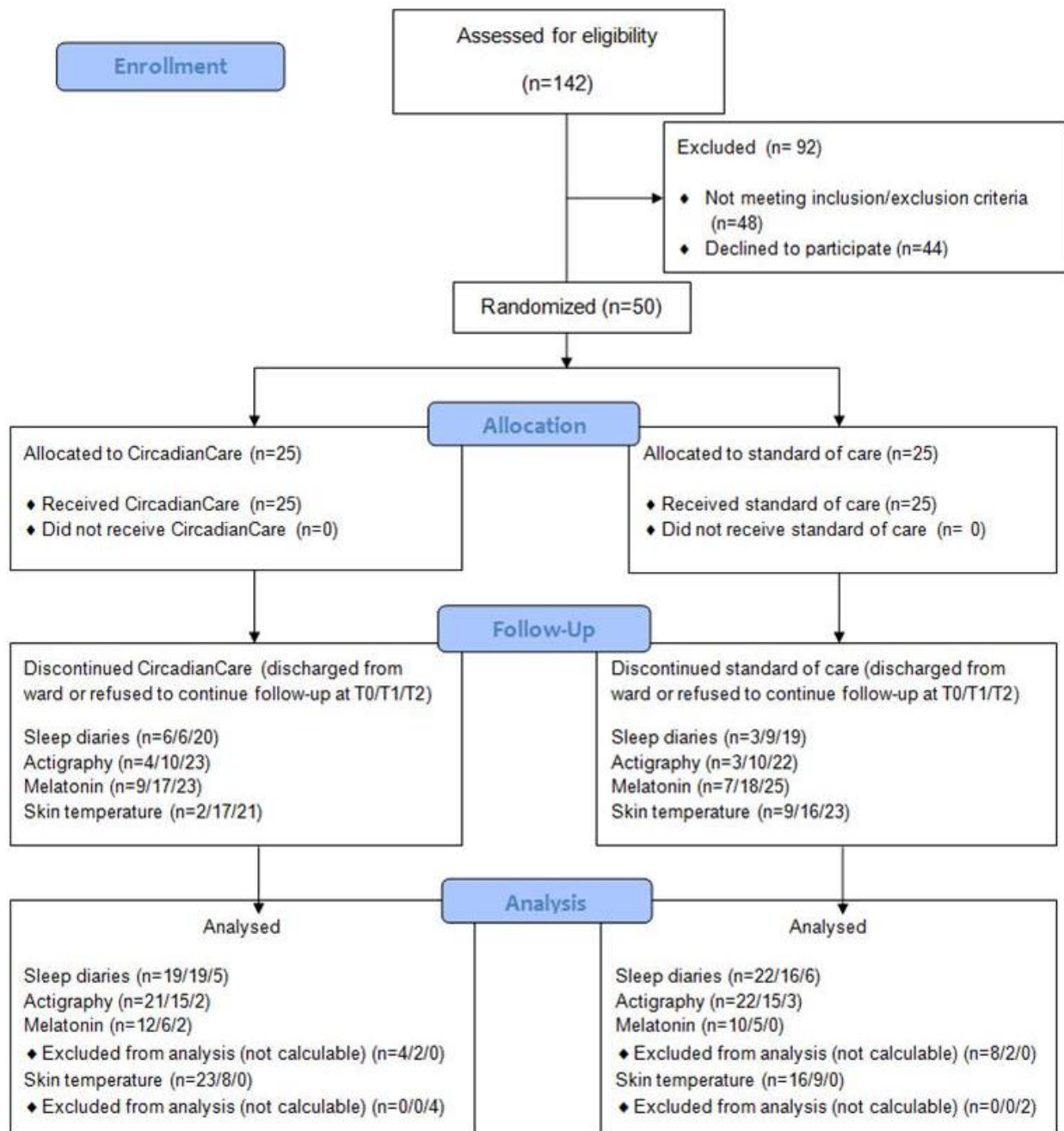


Figure 4. CONSORT flow diagram.

## 3.2 DATA COLLECTION

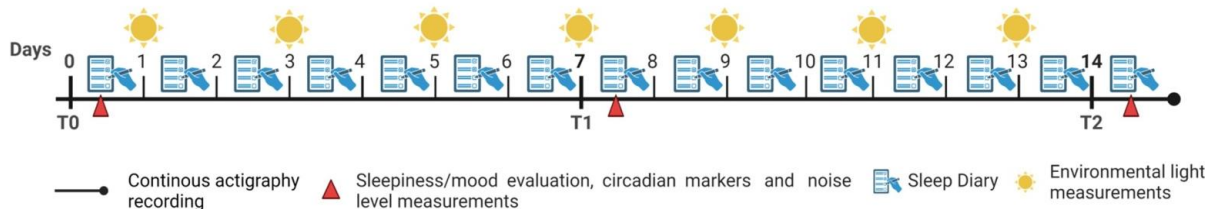
### 5.2.1. Pre-admission sleep-wake profiles

On the day of admission, patients underwent an assessment of their baseline, pre-hospitalisation sleep-wake profiles, based on: 1) the Pittsburgh Sleep Quality Index (PSQI), which evaluates subjective sleep quality over the preceding month and differentiates “good” from “poor” sleepers (Buysse et al., 1989; Curcio et al., 2013); 2) the microMCTQ questionnaire (Ghotbi et al., 2020), which is used to assess chronotype based on habitual sleep timing on work/study vs. free days; 3) the Sleep Timing and Sleep Quality Screening questionnaire (STSQS), to assess habitual sleep-wake timing/quality (Montagnese et al., 2009); and 4) the Horne-Östberg (HÖ) questionnaire (Horne & Ostberg, 1976), which defines diurnal preference and was utilised to split patients into morning (definitely/moderately), intermediate and evening (definitely/moderately) types. For statistical power reasons, “definitely morning” and “moderately morning” types were grouped as “morning”, and “definitely evening” and “moderately evening” as “evening” ones, resulting in three groups. Habitual meal timing at home was also recorded.

On days 1, 7 and 14 (if still admitted) of hospitalisation (Figure 5), or the day before discharge, patients underwent an evaluation of the course of their sleepiness/mood during the waking hours [Karolinska Sleepiness Scale (KSS) and a mood 1-10 Visual-Analogue Scale (VAS)] (Akerstedt & Gillberg, 1990), which they were asked to complete hourly from wake up to sleep onset time.

At the end of hospitalisation, patients’ overall perception of their hospital experience was assessed by a questionnaire for which an Italian, validated version is available (Bai et al., 2018), encompassing satisfaction in relation to information on admission, the medical and nursing staff, general organization and the environment. On admission, standard of care patients were provided

with standard information, which mostly covers visiting hours and doctors' contact hours (for patients and carers). CircadianCare patients were provided with the above plus, because of the protocol, also with information and potential choices for light, meal and activity timing (vide infra).



*Figure 5. Study design, representing a 14-day hospitalisation stay. The icons on the horizontal line indicate the study procedures performed daily, or at the three main study times (T0-baseline, T1-day 7, T2-day 14; red triangles). Continuous actigraphy (black line) and daily sleep diaries (blue paper and pencil) were recorded over the whole inpatient stay. Environmental light was measured every two days (yellow sun); patient skin temperature, salivary melatonin samples, subjective sleepiness/mood, and room noise and temperature were collected at T0, T1 and T2 (red triangles).*

### 5.2.2. Sleep-wake profiles and other circadian markers during the inpatient stay

For the whole length of hospitalisation, patients were asked to complete a daily sleep diary (Figure 5) in the morning, immediately after waking up, in order to limit recall bias. Each diary page also included a VAS for the assessment of sleep quality during the previous night (range: 1=worst to 10=best) (Carney et al., 2012; Lockley et al., 1999). Sleep onset time (as clock time) was then calculated as the sum of the time when the patient stated they tried to sleep plus their sleep latency; time spent in bed as the difference between bedtime and get up time (in hours); sleep duration as the difference between sleep onset and wake up time (in hours). Night awakenings included both spontaneous arousals and sleep interruptions due to environmental factors, which could not be differentiated by the question asked with the diary tool utilised.

In addition, patients underwent continuous actigraphy by MotionWatch 8© (MW8, CamNtech,

Cambridge, UK) (Figure 5), which is a wrist-worn axial accelerometer. The data (number of “counts” per 30-s epochs) were extracted by the MotionWare software (CamNtech, Cambridge, UK). Movements causing the acceleration signal to exceed the threshold of approximately 0.1 g were qualified as activity. Periods in which participants removed the actigraph were recorded by the daily sleep diaries and excluded from analyses. The following indices were obtained: total sleep time (i.e., total duration of all epochs qualified as sleep during time in bed), sleep onset latency (i.e., time to fall asleep), sleep efficiency (i.e., percentage of time spent sleeping out of the total time spent in bed), wake after sleep onset (time spent awake after sleep onset), and number of naps. In addition intra-daily variability (indication of fragmentation of the sleep rhythm, ranging from 0 to 2, with higher scores indicating more fragmentation), and inter-daily stability (indicating the stability of the 24-h activity rhythm across days, ranging from 0 to 1, with higher scores indicating more stable rhythms) (Luik et al., 2013).

On days 1 (T0), 7 (T1) and 14 (T2, if still admitted) of hospitalisation, the following samples/information were also acquired (Figure 5):

- Salivary melatonin samples, collected hourly on five occasions in the evening hours. The first sample was collected 3 hours before the patient’s habitual sleep time (Benloucif et al., 2008; Voultsios et al., 1997). Information was also collected on medications potentially interfering with melatonin levels (Arendt, 2018). Melatonin was then determined using a radioimmunoassay (Melatonin RIA-5503 kit, DRG Instruments GmbH, Germany) according to the manufacturer’s protocol (detection limit: 0.3 pg/mL). The dim light melatonin onset (DLMO) was computed based on a threshold calculated as the mean of baseline plus twice the standard deviations for each individual melatonin profile. Each participant’s DLMO was the point in time when the melatonin concentration exceeded the threshold (Lewy et al., 1980;



Micic et al., 2015). When the threshold was not obviously computable, the time of melatonin rise based on qualitative profile analysis was visually identified (Benloucif et al., 2008; De Rui et al., 2015; Rzepka-Migut & Paprocka, 2020).

- 24-hour-recording of distal and proximal skin temperature, using wireless sensors (iButtons, model no. DS1922L-F5, Maxim Integrated, San Jose, CA, USA) (van Marken Lichtenbelt et al., 2006). Sampling rate was set at 3 minutes (resolution 0.0625°C; approximately 500 temperature values per day). Four sensors were placed on the skin (right mid-thighs on the rectus femoris, right infraclavicular area, abdomen, mid-metatarsal area of the plantar site of the right foot) using medical tape (Longato et al., 2017). Data were extracted using the iButton Viewer software (Dallas Semiconductor, Maxim Integrated Products, Sunnyvale, CA, USA). An artefact rejection procedure was applied to exclude outliers (Rutkove et al., 2007). Temperature measurements were aligned according to try-to-sleep time, as recorded in the sleep diaries, and averaged in 30-minute bins. The data analysis window was set from 4 hours prior to try-to-sleep time until 10 hours after try-to-sleep time. Patients with missing values due to sensor loss or clinical procedures were excluded from the analysis. Proximal skin temperature (PST), distal skin temperature (DST) and the distal-proximal skin temperature gradient (DPG) were calculated as follows (Longato et al., 2017):

- $TPST = 0.359 \times T_{\text{mid-thigh}} + 0.262 \times T_{\text{infraclavicular site}} + 0.379 \times T_{\text{abdomen}}$
- $TDST = 1.017 \times T_{\text{feet}}$
- $DPG = (TDST - TPST)$ .

### 5.2.3. Environmental factors

Type of room (single, double or 4-bed) and bed position (qualified as near/far from the window, where near is less than 1 m and far is more than 3 m), were recorded on admission. On days 1, 7, and 14 (if still admitted) of hospitalisation, or on the day before discharge, the following information was acquired (Figure 5):

- 24-hour recording of the A-weighted sound pressure levels within the ward rooms (one sample per second). In order to carry out this measurement without interfering with the normal activities in the hospital room, a specific acquisition system was set up. A series of sound level meters with accuracy equivalent to class II was arranged by equipping smartphones (Apple iPhone 6 Plus) with external condenser microphones (BSWA Technology Ltd., Type micW i436). The application used for acquiring recordings and saving data was OpenNoise, developed and distributed by the Regional Agency for the Protection of the Environment of Piemonte, Italy (*OpenNoise*, n.d.). Measurement was calibrated in the reverberation room of the acoustic laboratory "LabAcus" of the Department of Industrial Engineering (University of Padua) using two methods: comparison with the measurements obtained from a class I precision sound level meter (NTi XL2) with the method proposed by Kardous and Shawn (Kardous & Shaw, 2016), and with the sound emission of a reference sound source (Brüel & Kjær, Type 4204) by a comparison method for determination of sound power of noise sources according to ISO 3741 standards (*ISO 3741:2010, Acoustics — Determination of Sound Power Levels and Sound Energy Levels of Noise Sources Using Sound Pressure — Precision Methods for Reverberation Test Rooms*, n.d.). From these comparisons, it was possible to obtain the gain value to be applied to the microphone, to align the measurements with those of the precision sound level meter in

reference conditions. Smartphones and microphones were placed on top of the headboard and powered externally to make their presence minimally invasive for patients and to minimize the need for operator interventions. The A-weighted equivalent continuous sound pressure level (LAeq) and the maximum A-weighted sound level (LAm<sub>ax</sub>) were measured during the conventional 8-hour night time period (vide infra). The time history of the A-weighted equivalent continuous sound pressure level was aggregated in 15-minute intervals to fit the resolution of the actigraph used for patient rest/activity monitoring. The equivalent background noise level (LA95eq), evaluated every second by the noise level exceeded 95% of the time during the previous T/6 interval, where T is the measurement time, was estimated according to Alsina-Pagès et al. (Alsina-Pagès et al., 2021). The Intermittency ratio (IR) was computed as in Wunderli et al. (Wunderli et al., 2016). The IR metric expresses the proportion of the acoustic energy contribution in the total energetic dose created by individual noise events above a certain threshold. Thus, it is a descriptor that can effectively integrate the equivalent continuous sound level (LAeq), based on the concept of energetic dose over longer periods, to describe specific changes in environmental noise conditions that can cause awakening (World Health Organization. Regional Office for Europe, 2009). Furthermore, the comparison between the IR and the equivalent background noise level with the same sampling time provides a direct indication of the incidence of sound fluctuations in the variation of the overall sound level better than the maximum sound level at any given moment in time (LAm<sub>ax</sub>). Finally, the LA95eq (dB(A)), IR (%), and the % awake epochs from the actigraphic data (every 15 minutes) were computed during the night. The conventional length of the night period was defined according to Italian law (D.M. 16 March 1998 and D. Lgs. n.66/2003) as the period between 22:00 h and 06:00 h; WHO's Night Noise Guidelines for Europe were used as reference (World Health Organization. Regional Office for Europe, 2009).

- Room illuminance, measured at each patient's eye-level as detailed in Bano et al. (Bano et al., 2014) by a Luxmeter (Konica Minolta T-10A, Marunouchi, Chiyoda, Tokyo, Japan). Measurements were taken four times per day (slot 1: 7.00-10.30 h; slot 2: 12.00-14.00 h, slot 3: 14.30-17.00 h, slot 4: 17.30-21.00 h), with these relatively wide time slots being aimed at minimizing interference with standard hospital activity. At the same times, information was also recorded about headboard lights (on/off), position of the rolling shutters [open (0%),  $\frac{3}{4}$  open (25%), half-way (50%),  $\frac{1}{4}$  open (75%), closed (100%)], and number of people present in the room (patients, personnel and/or visitors).
- 24-hour-recording of room temperature, monitored by an iButton (vide supra) taped on the wall close to the headboard of the patient's bed.

### 3.3 TREATMENT PROTOCOL

Patients in the CircadianCare arm followed one of three different schedules for light/dark, meal and physical activity timing, based on their circadian preference. Such schedules are resumed in Figure 6.



|                   | Morning | Evening | Breakfast | Lunch | Dinner | Morning     | Evening     |
|-------------------|---------|---------|-----------|-------|--------|-------------|-------------|
| Morning type      | 6:45    | 20:00   | 7:00      | 12:30 | 18:30  | 8:00-08:30  | 18:00-18:30 |
| Intermediate type | 7:45    | 21:00   | 8:00      | 13:30 | 19:30  | 9:00-9:30   | 18:30-19:00 |
| Evening type      | 8:45    | 22:00   | 9:00      | 14:30 | 20:30  | 10:00-10:30 | 19:00-19:30 |

Figure 6. Rolling shutter opening/closing, meals and physical activity timing in the CircadianCare arm, categorised by diurnal preference.

#### 5.3.1. Light/dark and noise hygiene

To provide as much natural daylight as possible over the daytime hours, rolling shutters were opened according to the patients' habitual sleep-wake timing (3 morning rounds of rolling shutter opening (06:45, 07:45; 08:45, depending on the patients' habitual sleep-wake timing and immediately prior to the breakfast rounds, Figure 6). Moreover, short-wavelength-enriched morning light was administered via Luminette® light glasses (Lucimed, Villers-le-Bouillet, Belgium, 2000 lux; blue-enriched, Figure 7, Panel A) (Langevin et al., 2014). Patients were asked to wear the light glasses every morning, immediately after awakening, for 45 minutes (e.g. while having breakfast, immediately after their personal

hygiene, etc.). These devices are equipped with 8 Light Emitting Diodes (LEDs) distributed on the upper part of the two lenses (4 on each side), outside the patient's visual field. The LEDs reflect light (2000 lux; blue-enriched 400-750 nm) towards the eye via a diffractive lens, thus focusing it towards the lower part of the retina, regardless of the position of the head (Figure 7, Panel B); this also allows for uniform illumination and for avoidance of glare (Bragard & Coucke, 2013). The aim was to ensure that patients received an adequate amount of intense morning light, favouring the night-day transition of their circadian timing system.

To limit night light exposure, rolling shutters were closed according to the patients' habitual sleep-wake timing and approximately one hour after dinner (3 evening rounds of rolling shutter closing: 20:00, 21:00; 22:00, Figure 6); the aim of the enforced rolling shutter closing was to guarantee darkness at night, especially as the surroundings of the hospital, which the ward windows look onto, are very well-lit by artificial light also overnight. Patients were also asked to wear a pair of short-wavelength light filter shades (MelaMedic, Viborg, Denmark, Figure 8, Panel A) from 18:00 until sleep onset time, plus at any time during the night if the room light was on. These shades filter light in the blue range of the spectrum (up to 530 nm, Figure 8, Panel B), i.e., the one our eye and the circadian timing system are most sensitive to (Brainard et al., 2001; Thapan et al., 2001), thus they limit exposure to wakening light signals to the circadian timing system at times when the natural environment is dark but the artificial one inside the hospital is well lit or the patients use mobile phone, tablets, reading devices which emit relatively strong and blue-enriched light (Gringras et al., 2015). Compliance was verified by regular rounds, together with continuous monitoring by *ad hoc* actigraphs. Finally, each patient received a pair of earplugs, which they were free to wear if and as needed, keeping an *ad hoc* record. Except for sources of environmental disturbance due to

hospital routines, patients were free to go to bed/try to sleep and wake/get up at their chosen times.

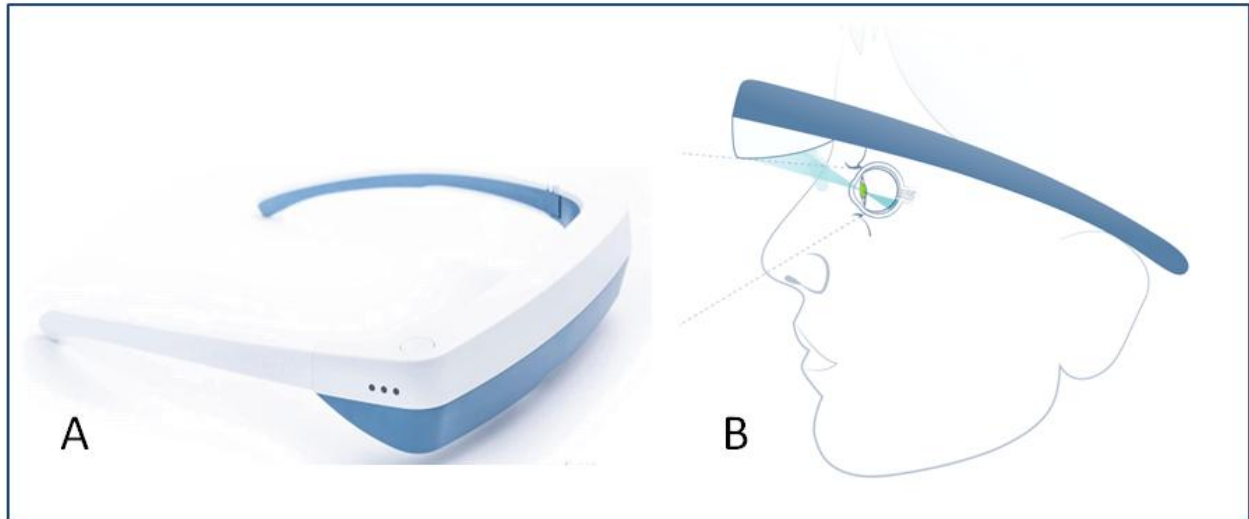


Figure 7. Luminette light glasses (Lucimed, Villers-le-Bouillet, Belgium; panel A). LEDs reflect light (approximately 2000 lux; blue-enriched 400-750 nm) towards the eye via a diffractive lens, thus focusing it towards the lower part of the retina, regardless of the position of the head (Panel B).

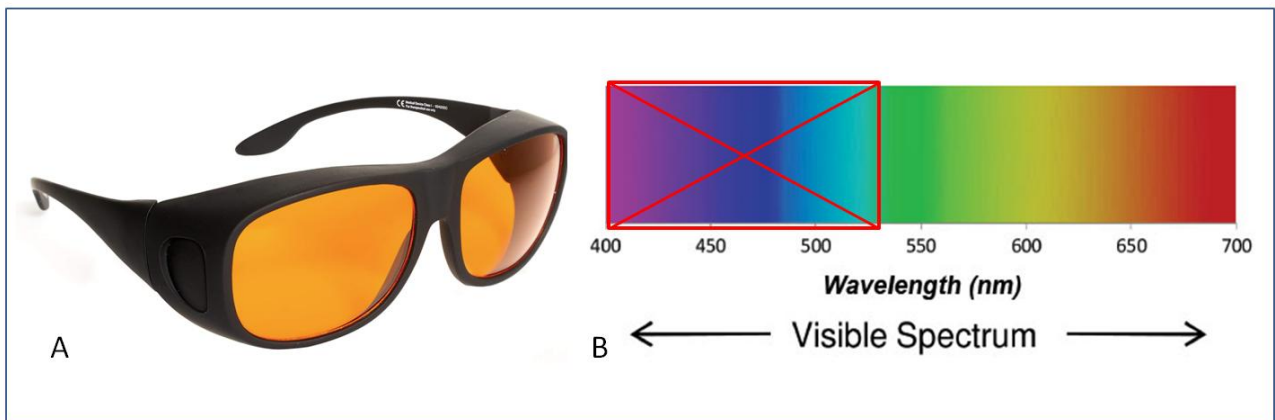


Figure 8. Short-wavelength filter shades (MelaMedic, Viborg, Denmark; Panel A), which filter light in the blue range of the visible spectrum (Panel B.)

### **5.3.2. Meal timing**

Current hospital policy is rigid in relation to meal timing, and generally better suited to patients who are morning types, with early preferred meal timing. Patients in the CircadianCare treatment arm were offered three meal timing options, based on patients' circadian preference (Figure 6):

- 7:00, 12:30, 18:30 h (breakfast, lunch and dinner, respectively)
- 8:00, 13:30, 19:30 h (breakfast, lunch and dinner, respectively)
- 9:00, 14:30, 20:30 h (breakfast, lunch and dinner, respectively).

The patient's diet (i.e. amount of total calories, way they are split between meals, consistency, specific limitations or foods to be avoided) was prescribed individually by the physicians, and not modified for study purposes.

### **5.3.3. Physical activity timing**

Patients were guaranteed the amount of regular, timed physical activity they were capable of, based on their condition. This consisted of: in-bed passive mobilisation for the sickest, in-bed active mobilisation (raise arm across midline, raise leg and extend knees, bend ankle and point toes), bed-to-armchair transition, sitting and standing from chair, aided or unaided short walks inside the room etc. Physical activity timing (Naylor et al., 2000) was chosen between three options: 08:00-08:30 and 18:00-18:30, 09:00-09:30 and 18:30-19:00, 10:00-10:30 and 19:00-19:30, in order to cover most patients' habits and avoid significant overlap with meal distribution and management of the light-dark schedules



(Figure 6).

Comparisons between standard and CircadianCare were performed in terms of:

- patients' overall perception and satisfaction. This was assessed by questionnaires for which Italian, validated versions are available (Bai et al., 2018);
- subjective and semi-quantitative (actigraphy) sleep quality and sleep timing indices during hospitalisation, on a day-by-day basis.

### 3.4 STATISTICAL ANALYSES

Data are reported as mean $\pm$ SEM (or, where indicated, 95% CI) for quantitative variables, as count and percentage for categorical variables. The distribution of variables was tested for normality using the Shapiro Wilk's *W*-test. Differences between groups were evaluated by the Student *t*/Mann-Whitney *U*, ANOVA (*post hoc* Tukey test) or Pearson's  $\chi^2$ , as appropriate. When considering different time points, variables were analysed by repeated measures ANOVA (*post hoc* Tukey test). Correlation analysis was performed by Pearson's *r* or Spearman's rank *R*, as appropriate. Analyses were carried out by the package Statistica, version 13.1 (Dell, Round Rock, Texas, US). Overall information on missing data can be inferred from Figure 4; given the size of the study and the considerable attrition, missing data were treated as such, and imputation was not utilised (complete case analysis at each time point). Finally, also due to attrition and to significant observed variability in the registered outcomes (especially sleep timing and DLMO), these are not necessarily presented in the Results section with the exact, strict order in which they were listed as outcomes on trial registration.

## 4. RESULTS

### 4.1 DEMOGRAPHICS, CLINICAL, AND PRE-ADMISSION SLEEP PROFILES

The treated group (CircadianCare,  $62.4 \pm 1.9$  years) encompassed 18 male patients and 7 female patients, whereas the untreated group (Standard of Care,  $64.5 \pm 2.3$  years) encompassed 14 male and 11 female patients ( $\chi^2=1.390$ ;  $p=0.240$ ). At baseline, CircadianCare and Standard of Care patients were comparable in terms of demographics, diurnal preference, habitual sleep quality (except for number of night awakenings, which were higher in the untreated group:  $1.5 \pm 0.2$  vs.  $2.4 \pm 0.4$ ,  $p=0.047$ ), habitual sleep and meal timing, length of hospitalisation, room type and bed position in relation to the window (Table 1). In particular, the average length of hospitalization was  $11.1 \pm 1.5$  days, with no significant differences between treated and untreated patients ( $11.4 \pm 1.8$  vs  $10.7 \pm 1.1$  days, n.s.; Table 1); the Charlson Comorbidity Index was also comparable in the two groups ( $3.6 \pm 0.6$  in treated vs.  $3.0 \pm 0.4$  in untreated patients, n.s.; Table 1). Further, pre-admission treatment [also in relation to psychoactive, sleep-inducing and medications that may affect melatonin profiles] was comparable in the two groups (Table 1). The two groups were also similar in terms of triage code (white, green, yellow or red), as assigned in the Accident and Emergency Department according to the severity of the underlying disease and patient's conditions ( $\chi^2=4.865$ ;  $p=0.182$ ). The majority of patients were hospitalized for liver/biliary/pancreatic diseases (37%), this being due to the fact that the unit also serves as a tertiary referral hepatology centre. Other diagnoses on admission were infections (including pneumonia, urinary tract infection and erysipelas; 31%), cardiovascular diseases (24%), and gastrointestinal bleeding or acute anemia (8%). Three out of 25 treated patients reported shift work in

**Table 1.** Demographics, pre-admission sleep quality and habitual sleep and meal timing, plus information on the inpatient environment in the CircadianCare and the standard of care arms.

|                                                                                    |                                                                      | CircadianCare<br>(n=25) | Standard of<br>Care (n=25) |
|------------------------------------------------------------------------------------|----------------------------------------------------------------------|-------------------------|----------------------------|
| <b>Demographics</b>                                                                |                                                                      |                         |                            |
| Age (yrs; mean±SE)                                                                 |                                                                      | 62.4±1.9                | 64.5±2.3                   |
| Males [n (%)]                                                                      |                                                                      | 18 (72%)                | 14 (56%)                   |
| Educational attainment (yrs; mean±SE)                                              |                                                                      | 13.8±0.8                | 12.1±0.8                   |
| BMI (kg/m <sup>2</sup> ; mean±SE)                                                  |                                                                      | 26.6±1.0                | 28.0±1.1                   |
| Charlson comorbidity Index (mean±SE)                                               |                                                                      | 3.6±0.6                 | 3.0±0.4                    |
| Triage code (white/green/yellow/red) (n)                                           |                                                                      | 13/3/6/2                | 6/3/10/5                   |
| Reason for admission (infectious/cirrhosis complications/cardiovascular/other) (n) |                                                                      | 9/8/3/5                 | 9/5/5/6                    |
| Currently employed [n(%)]                                                          |                                                                      | 16 (64%)                | 17 (68%)                   |
| Working days per week (n±SE)                                                       |                                                                      | 3.0±0.6                 | 2.2±0.6                    |
| <b>Pre-admission habitual sleep quality and timing questionnaires</b>              |                                                                      |                         |                            |
| HÖ score                                                                           | Total score (mean±SE)                                                | 58.40±1.26              | 60.30±2.33                 |
|                                                                                    | Diurnal preference (morning type/intermediate type/evening type) (n) | 14/11/0                 | 16/5/2                     |
| PSQI total score (mean±SE)                                                         |                                                                      | 7.7±1.1                 | 8.7±0.9                    |
| STSQS questionnaire                                                                | Bed time (hh:mm; mean±SE)                                            | 23:10±00:09             | 22:51±00:17                |
|                                                                                    | Try to sleep time (hh:mm; mean±SE)                                   | 23:23±00:08             | 23:16±00:16                |
|                                                                                    | Sleep latency (min; mean±SE)                                         | 30±9                    | 21±3                       |
|                                                                                    | Sleep onset time (hh:mm; mean±SE)                                    | 23:46±00:10             | 23:37±00:16                |
|                                                                                    | Night awakenings [n; median (interquartile range)]                   | 1.3 (0.5-2.5)           | 2.0 (1.0-3.5)              |
|                                                                                    | Wake up time (hh:mm; mean±SE)                                        | 06:26±00:16             | 06:54±00:19                |
|                                                                                    | Get up time (hh:mm; mean±SE)                                         | 06:47±00:16             | 07:25±00:21                |
|                                                                                    | Sleep quality (1=worst, 10=best; mean±SE)                            | 5.04±0.54               | 4.91±0.54                  |
| MicroMCTQ                                                                          | Sleep time on working days (hh:mm; mean±SE)                          | 22:46±00:30             | 23:13±00:15                |
|                                                                                    | Sleep time on free days (hh:mm; mean±SE)                             | 23:19±00:19             | 23:22±00:17                |
|                                                                                    | Wake up time on working days (hh:mm; mean±SE)                        | 06:04±00:32             | 06:41±00:14                |
|                                                                                    | Wake up time on free days (hh:mm; mean±SE)                           | 07:20±00:16             | 07:10±00:17                |
|                                                                                    | Sleep duration on working days (hours; mean±SE)                      | 7.29±0.34               | 7.47±0.17                  |
|                                                                                    | Sleep duration on free days (hours; mean±SE)                         | 8.01±0.38               | 7.79±0.23                  |
| <b>Habitual meal timing</b>                                                        |                                                                      |                         |                            |
| Habitual breakfast time (hh:mm; mean±SE)                                           |                                                                      | 07:08±00:17             | 07:32±00:21                |
| Habitual lunch time (hh:mm; mean±SE)                                               |                                                                      | 12:26±00:07             | 12:32±00:07                |
| Habitual dinner time (hh:mm; mean±SE)                                              |                                                                      | 19:26±00:13             | 19:40±00:11                |
| <b>Inpatient stay</b>                                                              |                                                                      |                         |                            |
| Length of hospitalization (days; mean±SE)                                          |                                                                      | 11.4±1.8                | 10.7±1.1                   |
| Bed position in relation to the window (near/far)                                  |                                                                      | 11/14                   | 14/11                      |
| Room type (single/double/4-bed) (n)                                                |                                                                      | 1/1/23                  | 1/2/22                     |

BMI: Body Mass Index; HÖ: Horne-Östberg questionnaire; PSQI: Pittsburgh Sleep Quality Index; STSQS: Sleep Timing and Sleep Quality Screening questionnaire; MicroMCTQ: micro Munich ChronoType Questionnaire

the previous 3 months before hospitalization, whereas 1 out of 25 patients in the control group was a shift worker ( $\chi^2=0.835$ ;  $p=0.361$ ). Eight patients in the control group and 9 patients in the treatment group were retired/unemployed, while the others were currently working. The number of working days per week was similar in the two groups ( $2.98\pm0.62$  in treated vs.  $2.24\pm0.61$  in untreated patients, n.s.). No significant differences were observed in diurnal preference and pre-admission sleep quality in treated and untreated patients (HÖ:  $58.40\pm1.26$  vs  $60.30\pm2.33$ , n.s.; PSQI:  $7.76\pm1.08$  vs  $8.75\pm0.99$ , n.s.) (Table 1). Pre-admission sleep timing, as assessed by the Sleep Timing and Sleep Quality Screening questionnaire, was comparable, except for the number of night awakenings, which was significantly higher in the control group ( $1.48\pm0.25$  in treated vs  $2.39\pm0.37$  in untreated,  $p=0.047$ ) (Table 1). In addition, there were no differences when comparing sleep time, wake-up time and sleep duration on working days and free days between treated and untreated patients (Table 1).

Usual breakfast, lunch, and dinner time were comparable in treated and untreated (Table 1).

Patients' diurnal preference was assessed with the HÖ questionnaire (Horne & Ostberg, 1976); 14 patients in the treatment group were classed as "moderately morning" and 11 patients were defined as "intermediate". No patient in the treatment arm was classified as "definitely morning", "moderately evening" or "definitely evening". In the control group, 4 patients were defined as "definitely morning" and 12 as "moderately morning"; 5 patients were classed as "intermediate" and 2 as "moderately evening". No patients in the control group had a "definitely evening" chronotype. For size reasons, "moderately morning" and "definitely morning" patients were grouped

together and will be hereafter defined as “morning” patients. Considering diurnal preference in both groups, since there were only two “evening” patients in the untreated group and none in the treated one, comparisons were performed only between “morning” and “intermediate” patients.

The number of working days per week was higher in “morning” compared to “intermediate” patients [ $3.2 \pm 0.8$ , 95%CI (2.16, 4.20) vs.  $1.8 \pm 0.9$ , 95%CI (-0.60, 2.54),  $F=5.605$ ,  $p=0.023$ ]. Interestingly, the Charlson Comorbidity Index was lower (i.e. meaning less disease burden) in “morning” compared to “intermediate” patients in both groups [ $2.7 \pm 0.4$ , 95%CI (2.09, 3.99) vs.  $4.4 \pm 3.0$ , 95%CI (3.96, 6.79),  $F=4.652$ ,  $p=0.037$ ]. Given the low numbers, this may either be a random series-specific observation, or, as an alternative, may be in line with the notion that morning individuals tend to lead generally healthier lives and to have less difficulties aligning with the timing constraints imposed by the social clock in the industrialised society, and thus carry a generally lower disease risk burden (Allada & Bass, 2021). As expected, patients with a “morning” preference reported a significantly earlier breakfast time compared to “intermediate” patients [ $06:49 \pm 00:17$ , 95%CI (06:15, 07:18) vs.  $07:58 \pm 00:28$ , 95%CI (07:04, 08:27) hh:min;  $F=5.150$ ,  $p=0.029$ ]. In addition, “morning” patients habitually went to bed and tried to sleep earlier than “intermediate” patients [ $22:41 \pm 00:14$ , 95%CI (22:20, 23:02) vs.  $23:24 \pm 00:15$ , 95%CI (22:50, 23:49) hh:min;  $F=4.54$ ,  $p=0.039$  and  $23:02 \pm 00:13$ , 95%CI (22:43, 23:19) vs.  $23:41 \pm 00:13$ , 95%CI (23:13, 00:03) hh:min;  $F=5.73$ ,  $p=0.021$ , respectively]. No other significant differences were observed.

## 4.2 INPATIENT STAY – SLEEP DIARIES AND ACTIGRAPHY

Daily sleep diaries and actigraphic data were regularly obtained for the whole length of hospitalization from all patients. Repeated measures analyses were performed by comparing both sleep diaries and actigraphic data at day 1 (T0), day 7 (T1) and day 14 (T2).

### 6.2.1. Sleep diaries

Sleep diaries were available for 41 patients (19 treated and 22 untreated, respectively) at T0, for 35 patients (19 and 16) at T1 and for 11 patients (5 and 6) at T2. Actigraphic data were available for 43 patients (21 and 22) at T0, for 30 patients (15 and 15) at T1 and for 5 patients (2 and 3) at T2. There was a trend advance in bedtime for treated patients, while untreated patients showed a trend delay in bedtime between T0 and T1 [ $22:38 \pm 00:21$ , 95%CI (21:54, 23:22) at T0,  $22:02 \pm 00:25$ , 95%CI (21:11, 22:54) at T1 in treated vs.  $22:17 \pm 00:20$ , 95%CI (21:35, 22:59) at T0,  $22:49 \pm 00:24$ , 95%CI (22:00, 23:38) at T1 in untreated patients,  $F=3.768$ ,  $p=0.062$ , trend] (Figure 9, Panel A). This was confirmed when also considering T2 (data not shown), despite the marked reduction in patient numbers, and may express the expected, overall positive effect of the CircadianCare system on both wake and sleep (i.e. more active and earlier wake period, and thus earlier bedtime). The number of hours spent in bed significantly increased from T0 to T1 in the treated group [ $8.24 \pm 0.44$ , 95%CI (7.32, 9.14) h at T0 and  $9.08 \pm 0.45$ , 95%CI (8.16, 10.02) h at T1] compared to the untreated group [ $8.65 \pm 0.41$ , 95%CI (7.81, 9.49) h at T0 and  $7.70 \pm 0.42$ , 95%CI (6.83, 8.58) h at T1,  $F=8.503$ ,  $p=0.007$ ] (Figure 9, Panel B).

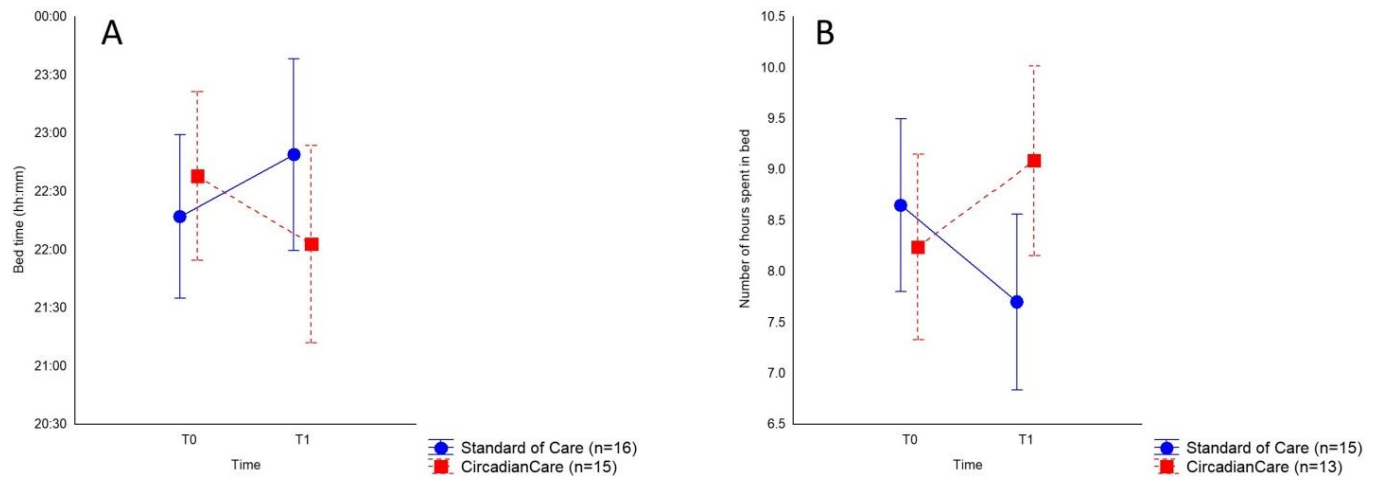


Figure 9. Sleep-diary based bedtime (average and 95% CI) at T0 and T1 (day 7) (panel A;  $p = 0.062$ ) and number of hours spent in bed (average and 95% CI) at T0 and T1 (day 7) (panel B;  $p = 0.007$ ) in the CircadianCare (red squares) and Standard of Care (blue circles) arms.

In addition, treated patients showed an increase in get up latency (i.e. the interval between wake up and get up time, expressed in minutes) compared to untreated patients [ $16 \pm 14$ , 95%CI (-13, 45) min at T0 and  $58 \pm 16$ , 95%CI (24, 91) min at T1 in treated vs.  $47 \pm 14$ , 95%CI (18, 75) min at T0 and  $38 \pm 16$ , 95%CI (5, 70) min at T1 in untreated patients, with a significant interaction,  $F=4.526$ ,  $p=0.043$ ]. This latter finding may relate to an unintended effect of treatment, with some patients in the CircadianCare arm choosing (despite not being instructed to) to remain in bed while wearing the light glasses after waking up in the morning. The number of night awakenings was similar between the two groups at T1. When extending the analysis to T2, the number of night awakenings was greater in the untreated group [ $0.90 \pm 0.46$ , 95%CI (-0.26, 2.25) in treated vs.  $2.43 \pm 0.35$ , 95%CI (1.31, 3.27) in untreated patients,  $F=7.217$ ,  $p=0.036$ ]. Given the baseline differences in this variable between the two groups, changes over time should be interpreted with caution.

The comparison between treated and untreated patients was also performed according to diurnal preference. Considering untreated patients, wake up time was earlier in “morning” compared to “intermediate” patients [ $05:44 \pm 00:14$ , 95%CI (05:19, 07:13) vs.  $07:13 \pm 00:25$ , 95%CI (06:21, 08:04) hh:mm,  $F=7.800$ ,  $p=0.010$ ]; these data were comparable between T0 and T1. Sleep diaries documented a decrease in the number of night awakenings from T0 to T1 for “morning” patients in the treated group [ $3.4 \pm 0.7$ , 95%CI (1.85, 4.97) vs.  $2.2 \pm 0.8$ , 95%CI (0.40, 3.93)] and for “intermediate” patients in the untreated group [ $3.7 \pm 0.9$ , 95%CI (1.84, 5.66) vs.  $1.6 \pm 1.0$ , 95%CI (-0.54, 3.79)], with a significant interaction between time, treatment and diurnal preference ( $F=11.007$ ,  $p=0.004$ ). No significant differences between the two groups were detected in other sleep diary variables.

### **6.2.2. Actigraphic data**

By contrast to sleep diaries, the comparison of actigraphic recordings at T0, T1 and T2 did not show significant differences between treated and untreated patients. This may suggest a decreased reliability of actigraphy within the hospital setting, which is by definition characterised by an overall activity reduction and by bedrest also during the waking hours. Within this context, patient-provided subjective measures may be more reliable than recorded activity, especially when studying/measuring the transitions between sleep and wake states. When limiting the analysis to T0 and T1, the number of daytime naps decreased after one week of inpatient stay in both study arms [ $1.5 \pm 0.3$ , 95%CI (0.89, 2.17) at T0 and  $0.8 \pm 0.3$ , 95%CI (0.27, 1.41) at T1], with a significant effect



of time ( $F=6.211$ ,  $p=0.019$ ). Similarly, the time spent awake after sleep onset also decreased after one week in both study arms [ $1.60 \pm 0.15$ , 95%CI (1.29, 1.91) at T0 and  $1.23 \pm 0.13$ , 95%CI (0.96, 1.51) at T1], with a significant effect of time ( $F=4.982$ ,  $p=0.033$ ). These findings may relate to improved general patients' condition towards the first/second week of hospitalisation. When diurnal preference was considered, the overall number and duration of daytime naps was lower in "morning" compared to "intermediate" patients [number:  $0.7 \pm 0.3$ , 95%CI (0.57, 1.39) vs.  $2.1 \pm 0.4$ , 95%CI (1.18, 2.93),  $F=6.219$ ,  $p=0.020$ ; duration (min):  $16 \pm 8$ , 95%CI (-1.82, 33.01) vs.  $52 \pm 11$ , 95%CI (29.62, 75.38),  $F=6.985$ ,  $p=0.014$ ]. In addition, actigraphic data showed a delay in wake-up time for "intermediate" patients in the untreated group from T0 to T1 [ $05:31 \pm 00:31$ , 95%CI (04:26, 06:36) vs.  $06:59 \pm 00:33$ , 95%CI (05:50, 07:06),  $F=6.569$ ,  $p=0.017$ ] (Figure 10, Panel A). This was not evident in the treated group. Along the same lines, there was a delay in midsleep for "intermediate" patients in the untreated group from T0 to T1 [ $02:20 \pm 00:28$ , 95%CI (01:21, 03:18) vs.  $03:37 \pm 00:29$ , 95%CI (02:37, 04:37)], with a significant interaction between time and diurnal preference ( $F=8.276$ ,  $p=0.008$ ) (Figure 10, Panel C).

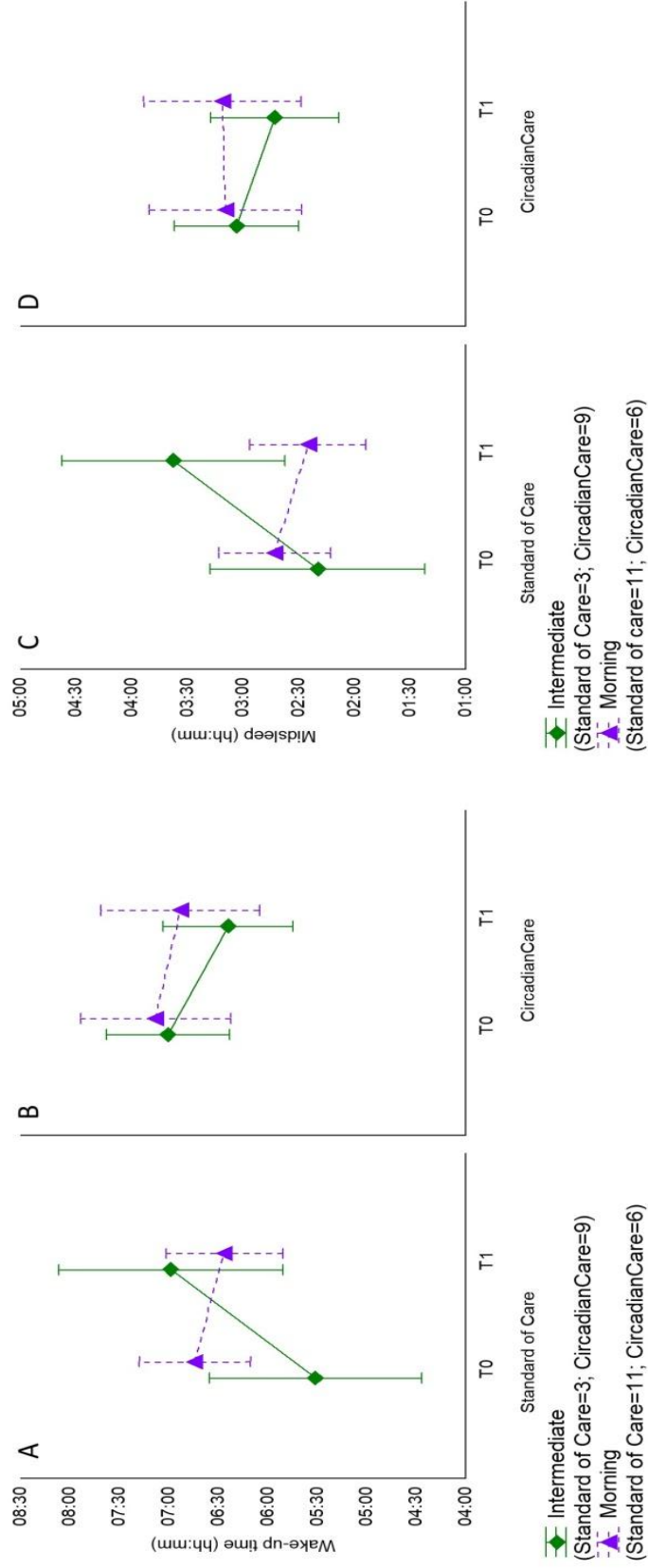


Figure 10. Actigraphy-based wake up time (average and 95% CI; panels A and B;  $p = 0.017$  for intermediate types) and mid-sleep (average and 95% CI; panels C and D;  $p = 0.008$  for intermediate types) in Standard of Care (panels A and C) and CircadianCare (panels B and D) arms in morning (purple triangles) and intermediate types (green diamonds) at T0 and T1 (day 7).

#### 4.3 INPATIENT STAY - SALIVARY MELATONIN SAMPLES

Baseline salivary melatonin was collected in 34 patients at T0, in 15 at T1 and in 2 at T2. The DLMO was calculable in 17 patients (8 treated and 9 untreated) at T0, 9 patients (5 treated and 4 untreated) at T1 and 2 patients (both treated) at T2. While measuring the dim light melatonin offset (DLMOff) rather than DLMO at night is unusual, it was deemed necessary in 5 patients (4 treated and 1 untreated) at T0 and 2 patients (1 treated and 1 untreated) at T1, due to the melatonin profile showing a clear and steady decrease in melatonin levels. This may have been the result of light/dark conditions and/or hospital or disease-dictated sleep-wake habits during the inpatient stay. At T0, the treated group had a later DLMO [ $22:04 \pm 00:22$ , 95%CI (21:10, 22:57)] than the untreated group [ $20:43 \pm 00:27$ , 95%CI (19:39, 21:47)] ( $F=1.71$ ,  $p<0.05$ ). Between T0 and T1 there was an advance in DLMO in treated patients [at T0:  $22:50 \pm 00:18$ , 95%CI (21:42, 23:54) at T1:  $21:48 \pm 00:25$ , 95%CI (21:12, 22:24),  $p<0.05$ ], which may partly relate to the untreated ones having less room for advancement due to the differences at baseline. An example of a patient in the CircadianCare arm whose DLMO was available and calculable at all three time points, with gradual phase advance over the inpatient stay is presented in Figure 11. There were no significant differences in sleep parameters (diaries or actigraphy) between patients grouped by calculable DLMO, calculable DLMOff, and non-calculable DLMO/DLMOff at T0 or T1, suggesting that confounders rather than more specific sleep-wake and/or circadian pathophysiological pathways may contribute to melatonin profile rhythmicity and/or its timing in this population of acute medical inpatients.

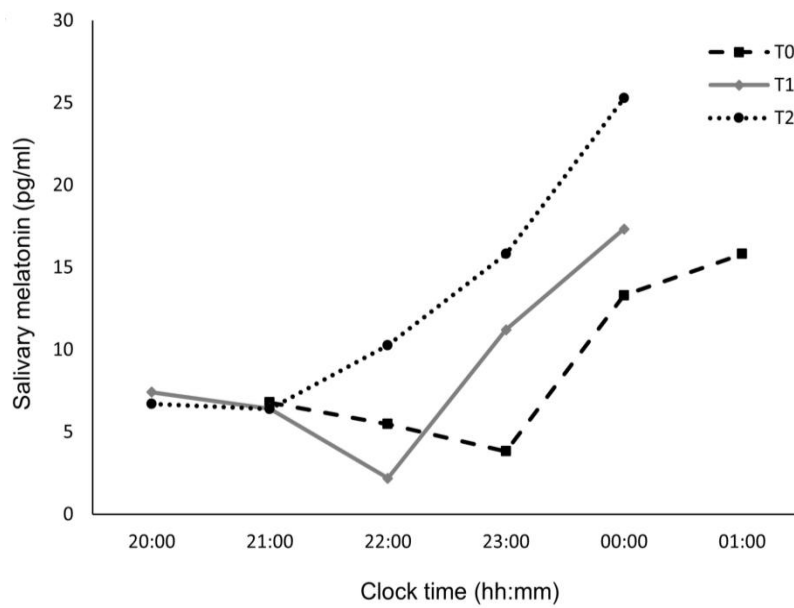


Figure 11. Salivary melatonin profiles of an example patient at baseline (T0, black squares and broken line; calculated DLMO, clock time 23:53), T1 (after one week of hospitalisation, grey diamonds and full line: calculated DLMO, clock time 22:49) and T2 (after two weeks of hospitalisation, black circles and broken line; calculated DLMO, clock time 21:09). The patient was a 71 year old male admitted for abdominal pain due to portal vein thrombosis in known cirrhosis, and was randomised to the CircadianCare arm. While patients with cirrhosis often suffer from insomnia and delayed sleep-wake habits, this was not the case for this patient, whose PSQI score was 3 (normal) on no sleep-inducing medication and whose sleep-wake habits were well within the group average. He was on the beta-blocker carvedilol for portal hypertension, which is a medication known to affect melatonin profiles, which seemed to have a limited or no effect in this case.

#### 4.4 INPATIENT STAY - SKIN TEMPERATURE

At T0, PST was available for 39 patients (23 treated and 16 untreated), whereas DST and DPG were available for 35 patients (22 treated and 13 untreated), with no differences between the two treatment arms (Figure 12, panels A1-A3). As expected, temperature varied significantly over the 24 hours for all three variables (PST:  $F=6.56$ ,  $p<0.001$ ; DST:  $F=7.38$ ,  $p<0.001$ ; DPG:  $F=3.10$ ,  $p<0.001$ ) (Figure 12, panels A1-A3). At T1, PST was available for 17 patients (8 treated and 9 untreated), whereas DST and DPG were available for 15 patients (8 treated and 7 untreated) (Figure 12, panels B1-B3). At T1, no significant difference was observed between the two treatment arms in any of the three temperature indices (Figure 12, panels B1-B3). Qualitative, visual analysis, however, showed that the oscillation in DST amplitude was greater in treated compared to untreated patients (Figure 12, panel B2), again, possibly suggesting rhythm strengthening in the treatment arm over time. Moreover, at T1, temperature varied significantly over the 24 hours in terms of DST and DPG (DST time:  $F=4.82$ ,  $P<0.001$ ; DPG, time:  $F=4.91$ ,  $P<0.001$ ; Figure 12, panels B2 and B3), but not in PST, where the oscillation amplitude diminished at T1 compared to baseline in both treated and untreated patients (Figure 12, panel B1). This may relate to proximal skin temperature being more affected by bedrest or bedcovers, and/or to the contribution to the case series of inpatients with cirrhosis (2 untreated and 1 treated with available data), whom we have previously shown to have increased PST and decreased DPG (Garrido et al., 2017).

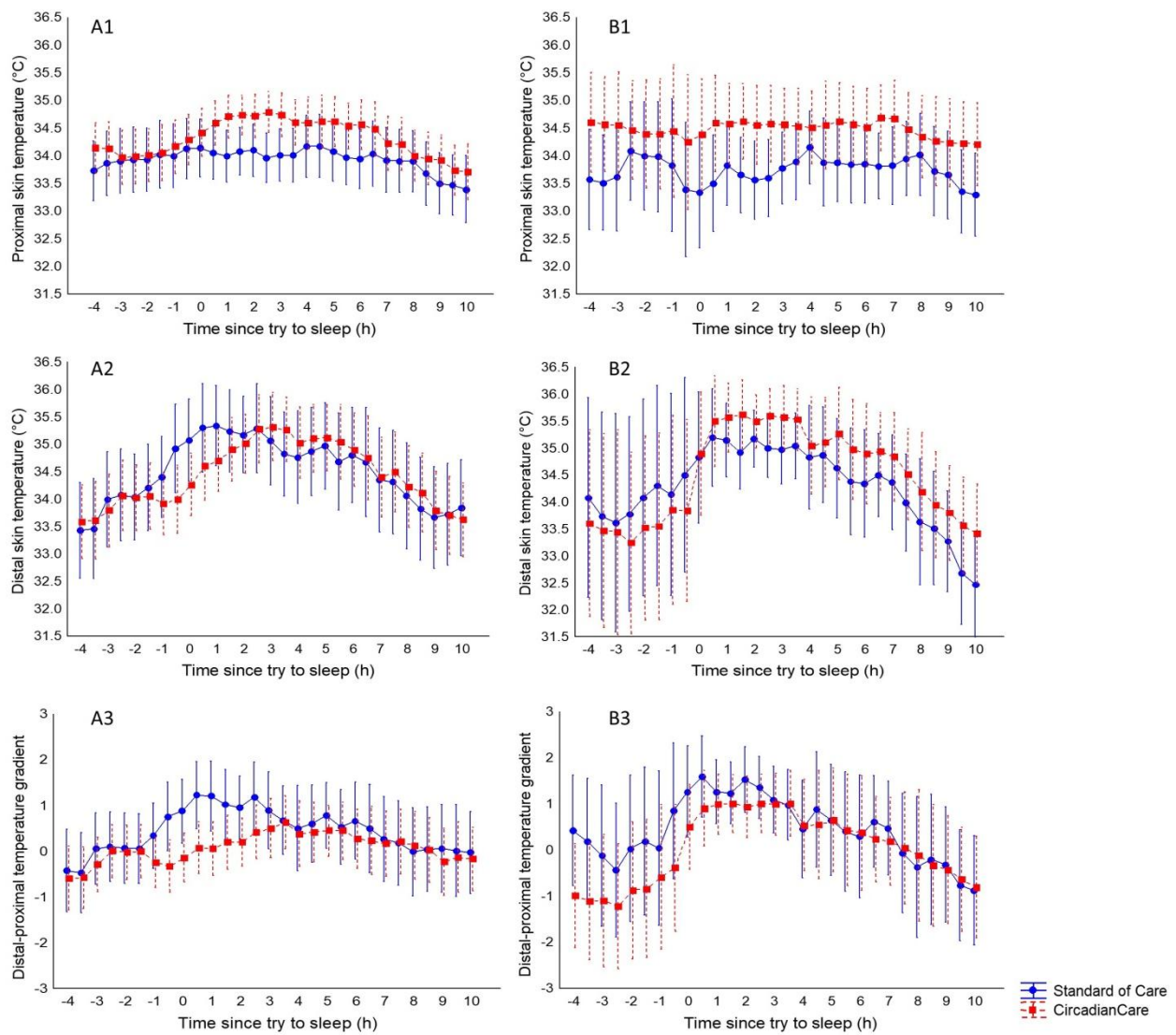


Figure 12. Proximal skin temperature (average and 95% CI; A1 and B1), distal skin temperature (A2 and B2) and distal-proximal gradient (A3 and B3) at T0 (A panels) and T1 (day 7; B panels), aligned according to try-to-sleep time in the CircadianCare (red squares) and Standard of Care (blue circles) arms. No significant differences were observed in relation to treatment. However, on visual analysis, proximal skin temperature seemed to flatten over the inpatient stay in both arms (A1 and B1) and the distal skin temperature oscillation seemed greater in treated compared to untreated patients (A2 and B2).

#### 4.5 INPATIENT STAY - MEDICATION

During the inpatient stay, 11 untreated and 9 treated patients took *pro re nata* sleep-inducing medication; 19 untreated and 16 treated, respectively, took drugs that might have affected melatonin profiles, with no significant difference between the two treatment arms. Moreover, no significant difference between the absolute or relative number of days on *pro re nata* sleep-inducing medication during the inpatient stay was observed between the two treatment arms.

#### 4.6 INPATIENT STAY - SATISFACTION WITH HOSPITALISATION

Patients in the treatment arm were more satisfied than untreated patients in relation to the quality of the information received on admission about the ward organization ( $\chi^2 = 6.74$ ,  $p = 0.034$ ). It is possible that receiving a small amount of additional information (plus having a choice) on light, meal and activity timing in an environment as stressful, disorienting and restrictive as an acute medical ward may contribute to decrease hospitalisation-related discomfort and improve the quality of the inpatient stay.

#### 4.7 INPATIENT STAY - ILLUMINANCE

When considering the entire length of hospitalisation, illuminance levels differed significantly during the day (Table 2). Moreover, illuminance levels differed between patients sleeping near or far from the window in the first two time slots, with a significant interaction between time and bed position (Table 2). When grouping patients by date of admission (i.e. during daylight saving time or standard time), these data were only confirmed in the group admitted during daylight saving time [slot 1:  $623 \pm 74$ , 95%CI (471, 776) vs.  $261 \pm 69$ , 95%CI (117, 403); slot 2:  $679 \pm 96$ , 95%CI (483, 874) vs.  $334 \pm 90$ , 95%CI (151, 518) lux, respectively;  $F = 12.72$ ,  $p = 0.001$ ]. Rolling shutters (average position per time slot) were halfway down for most of the day (Table 2).

**Table 2.** Room illuminance by time of day (divided in four time slots), bed position in relation to the window, and position of the rolling shutters (open, ¾ open, half-way, ¼ open, closed).

| Time of day            | Position of rolling shutter (%; mean±SE) | Illuminance level [Lux; mean±SE (95%CI)] |                   |                                |
|------------------------|------------------------------------------|------------------------------------------|-------------------|--------------------------------|
|                        |                                          | Room                                     | Bed near window   | Bed far from window            |
| 1. From 7:00 to 10:30  | 32±4%                                    | 394±45 (303, 485)                        | 515±58 (399, 634) | 268±59 (147, 387) <sup>•</sup> |
| 2. From 12:00 to 14:00 | 51±3%                                    | 477±54 (367, 587)                        | 636±68 (500, 774) | 311±70 (172, 453) <sup>•</sup> |
| 3. From 14:30 to 17:00 | 58±4%                                    | 251±30 (192, 312) <sup>□,§</sup>         | 309±41 (228, 391) | 192±42 (109, 278)              |
| 4. From 17:30 to 21:00 | 59±3%                                    | 185±26 (134, 237) <sup>#,*</sup>         | 234±35 (164, 308) | 134±36 (63, 208)               |

SE: Standard Error; 0% = window open/100% = window closed; on post-hoc comparison: <sup>•</sup>*p* <0.05, Near vs Far; <sup>•</sup>*p*<0.001, Near vs Far; <sup>□</sup>*p*<0.001, Slot 2 vs Slot 3; <sup>§</sup>*p*=0.059, Slot 1 vs Slot 3; <sup>#</sup>*p*<0.001, Slot 1 vs Slot 4; <sup>\*</sup>*p*<0.001, Slot 2 vs Slot 4.

The position of the rolling shutters correlated with light levels in the afternoon and evening slots (slot 3: *r*=-0.48; *p*<0.001; slot 4: *r*=-0.37; *p*=0.011). This trend was maintained in the patients who slept far from the window (slot 3: *r*=-0.58; *p*<0.01; slot 4: *r*=-0.42; *p*=0.047) but only in the afternoon in the patients who slept near the window (slot 3: *r*=-0.44; *p*=0.033). The total number of people in the room was the highest in slot 4 (range 1-10 people) and the lowest in 2 and 3 (range 1-5 people). Patients sleeping near the window had significantly higher sleep efficiency (defined as the percentage of time spent asleep while in bed, recorded by actigraphy) during their hospitalisation compared to those sleeping far from the window [83±1, 95%CI (80, 86) % vs. 78±2, 95%CI (74, 82) %; *p*=0.042]. No significant relationships were observed between subjective sleep quality, length of hospitalisation or bed position.



#### 4.8 INPATIENT STAY - NIGHT NOISE LEVELS

The noise levels recorded during the night at T0 were constantly higher than the WHO recommended value of 30 dB(A) for 8 hours A-weighted equivalent continuous sound pressure level in care units [average night time ambient noise LAeq: 50.0±0.5dB(A); average background noise level LA95eq: 39.6±0.5dB(A); average LAmax: 80.5±0.5dB(A); average IR: 53.1%]. No significant differences were observed in patients who slept far from or near the window (and thus nearer or further from the corridor, respectively). Noise levels and activity (measured by actigraphy) were compared in 28 (12 treated and 16 untreated) patients at T0 (before the commencement of any treatment) and visual analysis of 9 patients (3 whose bed was close to the window) showed peaks of Intermittency Ratio (IR) that were associated with awakenings of the patient or preceded them (Figure 13, panel A and Figure 14). Although fluctuating ambient noise alone was not the dominant cause for the awakenings, a substantial change in the IR over a relatively short 15-minute interval represented a clear change in room conditions relative to noise. The comparison between the average of the IR (%) and the awake epochs over the conventional 8-hour night led to the definition of four clusters of patients (Figure 13, panel B). Two relatively large groups of patients had a similar number of awakening periods (typically between 5% and 20%) despite two distinct ranges of noise fluctuation (cluster 0 with IR: 40%-60% and cluster 1 with IR: 60%-80%). For these two clusters, as the noise intermittence increased, so did the number of awakenings. Two smaller patient groups had different behaviour: one (cluster 2) had an awakening period of more than 20% despite favourable noise conditions (IR < 30%); the other (cluster 3) exhibited a very high number of awakenings even in average conditions of intermittent noise. Of interest, while the results did not reach statistical significance, patients in clusters 2 and 3 exhibited a tendency towards higher Charlson Comorbidity Index [4.2±0.8, 95%CI (2.3, 6.1) in clusters 2-3 vs. 2.9±2.2, 95%CI (2.0, 3.8) in clusters 0-1] and higher pre-admission PSQI scores

[10.8±5.1, 95%CI (5.8, 15.8) in clusters 2-3 vs. 8.1±5.4, 95%CI (5.6, 10.1) in clusters 0-1], suggesting that their awakenings might have been associated with their clinical condition and/or their chronic sleep disturbance rather than the environmental conditions.

#### 4.9 UNINTENDED EFFECTS

No unintended effects were reported and treatment was well tolerated.

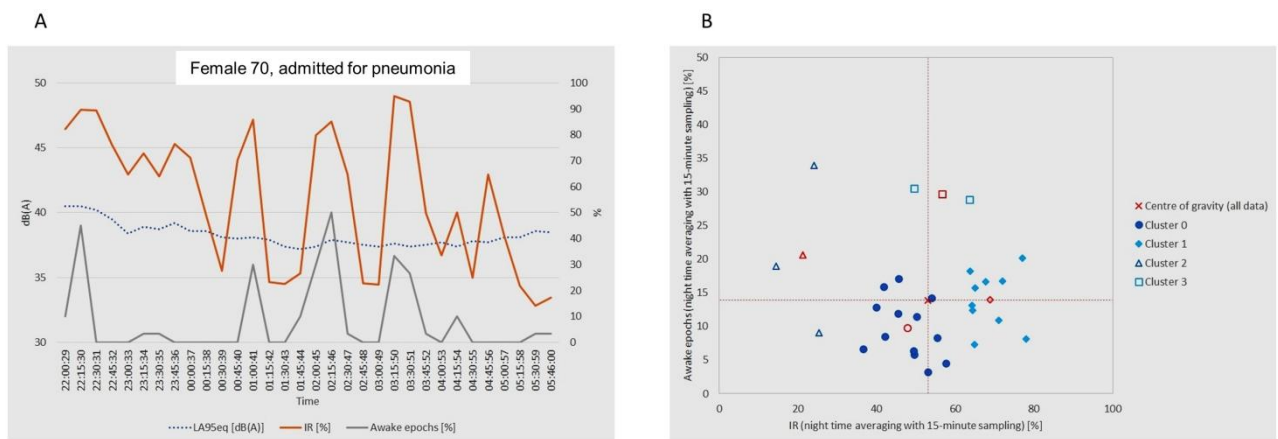


Figure 13. Panel A. Activity and noise levels in one example patient showing a clear relationship between increases in % awake epochs as recorded by actigraphy (grey line) and increased intermittent noise (IR; orange line) during the first night of hospitalisation. The equivalent background noise (LA95eq) is shown as a blue dotted line. Panel B. Cluster analysis of the relationship between % awake epochs and noise intermittency ratio; the centre of each cluster is marked in red. For the two most numerous clusters (0 and 1) a consistent and significant relationship can be observed ( $R=0.866$ ) between ambient noise fluctuation and % awake epochs.



Figure 14. Activity and noise levels in 8 patients (A-H; with sex, age and reason for admission detailed on top of each panel) showing a clear relationship between increases in % awake epochs as recorded by actigraphy (grey line) and increased intermittent noise (IR; orange line) during the first night of hospitalisation. The equivalent background noise (LA95eq) is shown as a blue dotted line. COPD: Chronic Obstructive Pulmonary Disease.

## 5. DISCUSSION

In this pilot study, we tested a novel inpatient management system (CircadianCare) aimed at limiting the negative impact of hospitalisation on sleep by enhancing circadian rhythmicity through personalised light/dark, meal and physical activity timing schedules. While the primary registered outcome (reduced sleep onset latency on actigraphy or diary) was not met, the CircadianCare system resulted in a trend advance in bedtime and, albeit only in the few patients in whom the variable was repeatedly calculable, a significant advance in DLMO time. Patients sleeping near the window showed significantly higher sleep efficiency, regardless of treatment. As noise fluctuation increased, so did the number of night awakenings, also regardless of treatment. Finally, having a “morning” diurnal preference was associated with better sleep quality during hospitalisation, most likely because morning types find it easier to adapt to hospital activity schedules, which tend to start early.

Treated and untreated patients were comparable in terms of demographic characteristics. In our cohort of patients, the Charlson Comorbidity Index was similar in the two groups and its values ( $3.6 \pm 0.6$  in treated vs.  $3.0 \pm 0.4$  in untreated patients) were associated to a 1-year mortality rate of 52%, according to Charlson et al.<sup>72</sup>. Interestingly, our results showed that the Charlson Comorbidity Index was lower in patients with a “morning” chronotype than “intermediate” patients, indicating that the number and severity of comorbidities was lower in the former group. Although derived from a small cohort of inpatients, this observation is in line with the emerging feeling that “evening” chronotypes, who are naturally inclined to be active in the second part of the day and therefore particularly susceptible to societal constraints, may be more exposed to the adverse effects of circadian misalignment in comparison to “morning”

individuals, whose early chronotype may have a certain protective effect within the modern society (Fischer et al., 2017; Roenneberg et al., 2012). Pre-admission sleep quality was similar in treated and untreated patients, with a PSQI score  $>5$  in both study groups, suggesting that sleep quality was generally impaired even before hospitalisation (Buysse et al., 1989). Actigraphic recordings did not document any significant differences between treated and untreated patients. However, the number of daytime naps decreased after one week of inpatient stay in both study arms, and so did the time spent awake after sleep onset. The latter are more likely to relate to the course of disease during hospitalisation rather than to our intervention, or lack thereof. The fact that actigraphy and sleep diaries did not produce overlapping results is most likely due to the fact that these two tools may have a different relationship in hospitalised patients, who are also be bedridden at times, compared to that known for healthy individuals in their home environment (Lehrer et al., 2022; Lockley et al., 1999). It is somewhat intuitive, albeit not well demonstrated, that an activity-based sleep-wake index may be less than ideal within a context which is by definition characterised by an overall reduction in activity.

The lack of significant differences in sleep timing and sleep quality indices (sleep diaries or actigraphy) between patients grouped by DLMO onset, DLMO offset, and non-calculable DLMO, at T0 or T1, may be in relation to the complexity of the condition of the patients, the limited number of DLMO available and the fact that the timing of melatonin production is obviously not the sole determinant of sleep timing/quality (Blume et al., 2022; De Rui et al., 2015; Lavie, 1997), especially in a complex and unusual situation such as hospitalisation. The lack of a relationship between melatonin profiles and subjective or objective sleep-wake variables may also suggest that

confounders such as medication or illness *per se* may play a relevant role on melatonin profile shape and DLMO<sub>n</sub>/Off computability/timing in this and other clinical populations.

Further, after one week of treatment, patients in the CircadianCare arm increased the number of hours spent in bed and the get up latency (time spent in bed after waking up) compared to standard of care patients, possibly because the majority of treated patients chose to stay in bed while wearing the light-glasses after awakening, even though this was not prescribed. While this is not necessarily an unwelcome interpretation of the instructions provided, these may be modified as appropriate in future, similar studies.

Treated patients reported a higher satisfaction regarding the information received on the ward organization, which suggests that provision of such information in a detailed and possibly personalised fashion may be a simple and cheap tool to contain hospitalisation-related discomfort.

The strong effect of diurnal preference on sleep-wake timing/quality was confirmed, even in our hospital setting. For example, patients in the standard of care group with a “morning” diurnal preference woke up earlier than patients with an “intermediate” diurnal preference, and this held true for the entire hospitalisation. The effect of diurnal preference was also evident when examining actigraphic results (delay in wake up time exhibited by “intermediate” patients in the untreated group). It should be highlighted that, in our patient population, the subgroup of patients with an “evening” chronotype was poorly represented. This was probably to be expected, as the mean age was relatively high in both study arms, with a consequent prevalence of early chronotypes (Fischer et al., 2017). This is an important consideration when evaluating

the effectiveness of our intervention, as a significant proportion of patients were already “aligned” to the usual time-schedule of a standard medical ward, where routine activities are generally carried out in the earlier part of the day. Further, this finding could be usefully taken into consideration when designing future, similar trials.

Regarding skin temperature variations, although we did not find significant differences between the two study arms, a 24-hour oscillation in PST and DST was observed, with a tendency to flattening of the PST curve after one week of hospitalisation in all patients. This is in agreement with previously published literature (Cuesta et al., 2017; Garrido et al., 2017). In the distal skin temperature profile, a flattening of the DST curve at T1 was not visible. This suggests that the distal vasodilation occurring at night, which is functionally linked to sleep propensity, is still intact after one week of hospitalisation and it also supports the hypothesis that distal skin temperature is less affected by masking factors (van Marken Lichtenbelt et al., 2006), making it a valuable index also within the context of an acute medical hospitalisation.

Patients sleeping near the window were exposed to more light than those sleeping far from the window, especially in the morning and during daylight saving time. A clear effect of bed position on sleep efficiency was confirmed despite the age and severity of the medical condition (Bano et al., 2014), emphasizing the importance of the amount of natural light an inpatient is exposed to. As we have observed before (Bano et al., 2014), and except for purposes of the CircadianCare protocol, rolling shutters were rarely moved. Given the significant effect that natural light seemed to have on sleep efficiency, small educational campaigns on rolling shutters management aimed at the support hospital staff may be an additional cheap and helpful tool to prevent hospitalisation-related insomnia. By contrast to a previous study (Benedetti et al.,

2001), no significant relationship was observed between bed position and length of hospitalisation, possibly due to the considerably shorter period of hospitalisation in the present study.

With regard to the effects of nocturnal environmental noise in hospital rooms, the baseline A-weighted equivalent continuous sound pressure level and the maximum A-weighted sound level were very similar for all cases analysed, and well above those recommended by the WHO. By contrast, the combination of the IR and the equivalent background noise levels allowed to unveil a relationship between substantial fluctuations in noise levels and night awakenings. It should be noted that the parameters taken into consideration by the WHO to evaluate the effects of noise on sleep focus on the noise determined by vehicle traffic outside buildings. By contrast, the acoustic analysis proposed in this study tried to highlight the effects of noise produced within the wards by the service equipment, as well as by medical operations and activities related to patients' care. The results suggest that better conditions for sleep could be obtained not only by limiting the amount of noise during the night but also by ensuring that noise levels have the smallest possible fluctuations. This could probably be obtained, for example, by educational interventions for nurses on night rounds, and also by changes in the features of medication trolleys (for example operating surfaces materials) and their wheels and other mobile parts, which tend to be noisy.

One of the points of strength of this study is its ecological approach. The CircadianCare management system induced a manifest improvement in some of the sleep diaries indices/actigraphic parameters used to evaluate sleep quality and timing, thus providing an index of feasibility of chronotherapy in the hospital setting. On the other hand, the



study has several limitations. A considerable proportion of patients screened did not provide informed consent and attrition over time was also high, which should be taken into account when planning similar, future studies in the acute medical setting; moreover, this resulted in a relatively small final population, with particular regard to the chronotype-based subgrouping. There were differences in baseline parameters between the two treatment arms (i.e. number of awakenings and DLMO), which might have affected subsequent comparisons. Also, logistics/feasibility issues and the lack of available, published information on the features/variability of certain indices (for example actigraphy and DLMO) in acute medical populations lead to some degree of uncertainty in power estimates and some deviation from the original analyses plan. All the patients enrolled in this protocol had to be fit enough to comply with the required tasks, thus inevitably introducing a selection bias.

Nonetheless, the CircadianCare management system showed some positive results in terms of both sleep and circadian timing. Further, the study documented a significant effect of environmental lighting (bed position in relation to the window) and environmental noise (noise fluctuations during the night) on sleep. These findings may allow to implement minor changes to the service equipment currently used in wards plus in some ward practices, which may be cheap and beneficial, and ultimately to design better wards in future. Finally, our study demonstrates the feasibility of a personalised, multiple intervention chronotherapy protocol in a complex, acute hospitalisation setting.

In conclusion, although our intervention did not significantly reduce the length of hospitalisation, the CircadianCare management system, through an ad hoc and personalized light-dark, meal and activity schedule, showed some positive results in

terms of enhanced circadian rhythmicity and limited circadian misalignment due to hospitalisation, suggesting that the potential of chronotherapy has to be taken into account for hospital planning and design in the future.

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# A Circadian Hygiene Education Initiative Covering the Pre-pandemic and Pandemic Period Resulted in Earlier Get-Up Times in Italian University Students: An Ecological Study

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The aims of the present study were to obtain sleep quality and sleep timing information in a group of university students and to evaluate the effects of a circadian hygiene education initiative. All students of the University of Padova (approximately 64,000) were contacted by e-mail (major campaigns in October 2019 and October 2020) and directed to an *ad hoc* website for collection of demographics and sleep quality/timing information. Participants ( $n = 5,740$ ) received one of two sets of circadian hygiene advice (“A regular life” or “Bright days and dark nights”). Every month, they were then asked how easy it had been to comply and provided with the advice again. At any even month from joining, they completed the sleep quality/timing questionnaires again. Information on academic performance was obtained *post hoc*, together with representative samples of lecture ( $n = 5,972$ ) and examination ( $n = 1,800$ ) timings, plus lecture attendances ( $n = 25,302$ ). Fifty-two percent of students had poor sleep quality, and 82% showed signs of social jetlag. Those who joined in October 2020, after several months of lockdown and distance learning, had better sleep quality, less social jetlag, and later sleep habits. Over approximately a year, the “Bright days and dark nights” advice resulted in significantly earlier get-up times compared with the “A regular life” advice. Similarly, it also resulted in a trend toward earlier midsleep (i.e., the midpoint, expressed as clock time, between sleep onset and sleep offset) and toward a decrease in the latency between wake-up and get-up time, with no impact on

sleep duration. Significant changes in most sleep quality and sleep timing variables (i.e., fewer night awakenings, less social jetlag, and delayed sleep timing during lock-down) were observed in both advice groups over approximately a year, mostly in association with pandemic-related events characterizing 2020. Early chronotype students had better academic performances compared with their later chronotype counterparts. In a multivariate model, sleep quality, chronotype and study subject (science and technology, health and medical, or social and humanities) were independent predictors of academic performance. Taken together, these results underlie the importance of designing circadian-friendly university timetables.

**Keywords:** circadian hygiene, chronotype, sleep, university students, academic performance, pandemic

## INTRODUCTION

Chronotype reflects an individual's natural inclination to place his/her activity/sleep within different intervals of the 24-h day and is partly genetically determined (Ashbrook et al., 2020). Children tend toward earlier, morning chronotypes, whereas adolescents and young adults exhibit a sharp change toward eveningness (which is more pronounced/prolonged in males), but then mature adults/older individuals become progressively more morning-like (Roenneberg et al., 2004). Late chronotype individuals are penalized by the Western “social clock,” which forces them to study/work/function during the early part of the day, counter to their natural biological clock (Gentry et al., 2021). It has been reported that early classes/early school tests in evening-type adolescents adversely affect their academic performance, especially in scientific subjects (Zerbini et al., 2017) and that changes in school start times may help improve sleep (Winnebeck et al., 2020; Meltzer et al., 2021) and, possibly, also grades (Zerbini et al., 2017). Less but similar information is available on the relationship between study timetables, sleep timing/length, and academic performance in university students (Beşoluk et al., 2011; Li et al., 2018; Smarr and Schirmer, 2018). In addition, there are also studies on the effects of sleep deprivation on university students' athletic performance and wellbeing (Hodge et al., 2012; Bolin, 2019). A number of different initiatives/courses or interventions directed at either general or specific university students' populations (Tsai and Li, 2004; Brown et al., 2006; Trockel et al., 2011; Quan et al., 2013; Kloss et al., 2014; Ye and Smith, 2015; Levenson et al., 2016; Hershner and O'Brien, 2018) have shown encouraging results either in terms of increased sleep hygiene literacy (Tsai and Li, 2004; Brown et al., 2006; Quan et al., 2013; Kloss et al., 2014; Ye and Smith, 2015; Hershner and O'Brien, 2018) or actual improvement in sleep habits and/or sleep quality (Brown et al., 2006; Trockel et al., 2011; Levenson et al., 2016; Hershner and O'Brien, 2018).

The aims of this study were to obtain comprehensive sleep quality/timing information and to evaluate the effects of a university welfare-based circadian hygiene initiative—directed at all students—on sleep and academic performance. As the study progressed, it became apparent that novel information could also be obtained on the effects of the pandemic-related lockdown on sleep–wake behavior.

## MATERIALS AND METHODS

### Overview of the Survey and Educational Initiative

An *ad hoc* full-responsive website was created for collection of the relevant information, with either an Italian or English interface to choose from. All active students at the University of Padova (approximately 64,000) were contacted by the university welfare offices on October 28, 2019, with an e-mail message including a brief description of the initiative and its aims, an invitation to participate, and a “call to action” button leading to the website.<sup>1</sup> This could only be accessed by use of the personal Shibboleth University of Padova username/password. Students who joined the initiative went on to receive reminders on the 28th of each subsequent month to access the website again to provide additional information and receive advice. The website was always open for newcomers to join; access after each reminder was possible for 7 days. On joining for the first time, students were asked to provide information on demographics, sleep–wake timing, night sleep quality, and diurnal sleepiness. They then received one of two sets of circadian hygiene advice (“A regular life” or “Bright days and dark nights,” **Figure 1**). Every month after joining, they were asked how easy it had been to comply with the advice and were provided with the advice again. At every even month from joining, they were asked to complete demographic, sleep–wake timing, night sleep quality, and diurnal sleepiness information again. They were also asked how easy it had been to comply, and finally, they were provided with the advice again (**Supplementary Table 1**). An additional information/recruitment campaign was run on October 28, 2020, which excluded any student who had already joined. Information on the participating students' career parameters was obtained from the career office *post hoc*. Representative samples of the timings of electronic room bookings for lectures/examinations and of recorded, anonymous lecture attendances were also obtained *post hoc*.

### Demographics

Date and time of both first accessing and completing all procedures scheduled at time 0 (T0) were obtained. In addition to

<sup>1</sup><https://sleeprhythm-unipd.it>





basic demographics, students also provided information on their height, weight, any problems with eyesight, any disease, and/or any medication they might be on. In relation to eyesight, diseases, and medication, a yes/no answer was a requirement in order to proceed. By contrast, the field to provide additional details on any of these three items could be left empty. Authors SM, CF, and CM, who are all practicing clinicians, independently classified listed eyesight problems, diseases, and drugs as worth considering or negligible; where their classifications differed, an agreement was sought for and reached.

## Sleep–Wake Profile

Carefully developed, user-friendly electronic versions with equal resolution and ease of completion on mobile phones, tablets, and computers of the following questionnaires were then presented on the full-responsive website.

### The Sleep Timing Sleep Quality Screening (STSQS) Questionnaire

This provides a simple, overall assessment of sleep quality rated on a 0–10 visual analog scale (0 = worst, 10 = best sleep ever) and allows collection of information on habitual sleep timing (i.e., bed time, try to sleep time, sleep latency, night awakenings, wake-up and get-up time, Montagnese et al., 2009).

### The Pittsburgh Sleep Quality Index (PSQI)

Responses to 19 questions are used to generate seven components (subjective sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disturbance, use of sleep medication, daytime dysfunction), each of which is scored from 0 (best) to 3 (worst). These component scores are then summated to provide the total PSQI score (range = 0–21); scores of > 5 identify “poor sleepers” (Buysse et al., 1989; Curcio et al., 2013).

### The Epworth Sleepiness Scale (ESS)

Subjects rate their likelihood of “dozing off” in eight different daytime situations, on a scale of 0 (unlikely) to 3 (very likely). The component scores are summated to provide a total score (range = 0–24); a score of  $\geq 11$  is considered abnormal (Johns, 1991; Vignatelli et al., 2003).

### The Self-Morningness/Eveningness Self-ME Question

This is a validated, single-question assessment of chronotype through which participants qualify themselves as definitely morning, morning, evening, or definitely evening types (Turco et al., 2015).

### The Ultrashort Version of the Munich ChronoType Questionnaire $\mu$ MCTQ

This is an adaptation of the MCTQ (Roenneberg et al., 2003) from 17 to 6 essential questions, allowing for a quick assessment of midsleep (i.e., the midpoint, expressed as clock time, between sleep onset and sleep offset), social jetlag (in this instance, the uncorrected difference between midsleep on free and work/study days), and sleep duration (Ghotbi et al., 2020). Social jetlag was both treated as a continuum variable and also qualified as *positive* (difference between midsleep on free and work/study

days > + 20 min), *absent* (−20 min < difference < + 20 min) and *negative* (difference < −20 min) (Korman et al., 2020).

## Circadian Hygiene Advice and Compliance

Two alternative sets of advice (novel in their formulation but grounded in circadian practice; Abbott et al., 2015) were provided (and remained the same on every subsequent “refresh” monthly reminder) with comparable expected benefits (i.e., balancing out because the first type of advice was slightly less prescriptive but probably easier to follow, and the second slightly more prescriptive but probably more difficult to follow) exerted *via* two different forms of habits’ adjustment (Facer-Childs et al., 2019). Both included elements of the recommendations routinely provided to patients with either a formal diagnosis and/or features of delayed sleep phase type (Abbott et al., 2015), which is common in adolescents and young adults.

“**A regular life,**” encouraging participants to regularize habits in relation to sleep–wake timing, meals, and exercise (Figure 1A). This intervention did not include advice on light exposure, as whereas the idea of sleep, meals, and exercise at regular times is extremely intuitive and easily interpreted, that of “light at regular times” seems less so. Therefore, when planning the intervention, we reasoned that advice on “light at regular times” would be prone to misinterpretation.

“**Bright days and dark nights,**” encouraging participants to advance their sleep–wake, meals, and exercise timing and to maximize/minimize light exposure in the first/second part of the day, respectively (Figure 1B).

The user-friendly, short format of the interventions was chosen to maximize the likelihood of them being read and considered by young, healthy individuals on a monthly basis and most likely on the screen of their mobile phone. Along the same lines, we did not modulate advice in relation to photoperiod or daylight saving time (DST), deliberately opting for repeated, identical advice, which could be read, reread (on refresh), and memorized easily, hopefully becoming part of the students’ routine.

This being a university-based welfare initiative, all students were provided with advice; that is, none served as placebo.

Participants subjectively rated their **compliance** with each piece of advice received on a 0 (never) to 10 (all the time) visual analog scale.

## Academic Performance

Information was obtained from the student career offices about each student’s university course, course year, total number of examinations passed, total number of “credits” (i.e., packages of formal lectures/practicals of variable length, depending on study subjects), and average marks (0–30, 18 = pass) at fixed dates of the year, on a 3-monthly interval (October 28, 2019; January 28, 2020; April 28, 2020; July 28, 2020 and October 28, 2020). These data were then matched with the “join the initiative” date and organized as baseline (T0) and subsequent time intervals. Studies were classed as health and medical (M), science and technology (S), and social and humanities (H), based on the Italian Ministry

of University and Research reference tables. Courses have variable duration: most have a 3- plus 2-year structure (in this instance, students who went on to attend their master's degree were qualified as fourth or fifth year), whereas a minority have a 4–6-year duration. Based on the information they provided and on the University of Padova criteria, students were classified as on-site, commuters, or off-site (i.e., non-compulsory lectures and/or registered for examinations only).

Significant differences in sleep–wake and other parameters were observed between students pursuing M, S, and H studies, which were thought to depend, to some extent, on lectures timing/duration and/or examinations timing. Thus, a sample of electronic room bookings for lectures ( $n = 5,972$ ) was obtained *post hoc* on a representative week (November 4–10, 2019, i.e., close to study start; lectures were arbitrarily qualified as *morning/afternoon* if they started prior to/after than 13:00 hh:mm, respectively) and a sample of anonymous students' recorded attendances (electronic swipe in–swipe out,  $n = 25,302$ ; multiple swipe ins–swipe outs,  $n = 7,765$ ) on October 30, 2020. This date was chosen because, due to novel, pandemic-related rules, all students were asked to swipe in–swipe out electronically. Finally, a sample of examination booking times was obtained, on a representative week [September 9–13, 2019 ( $n = 1,800$  bookings)]; again, examinations were arbitrarily qualified as *morning/afternoon* if they started prior to/after than 13:00 hh:mm, respectively.

## Study Approval

The initiative was approved by the University of Padova (July 16, 2019, board meeting). Students were asked to accept/tick a general data protection regulation (GDPR)–compliant informed consent, including the wording “data may be used anonymously, in aggregate fashion for scientific purposes.” The study plan was then approved by the local ethics committee (4948/AO/20–AOP1939).

## Statistical Analysis

Descriptive results are expressed as mean  $\pm$  SD/SE or as count/percentage. Normality was tested for by the Shapiro–Wilk test. Differences between normally distributed variables were examined by Student *t*-test/one-way analysis of variance (ANOVA; *post hoc*: Scheffé test). Differences between non-normally distributed variables were examined by Mann–Whitney *U*-test/Kruskal–Wallis ANOVA (*post hoc*: median test). Bonferroni correction for multiple comparisons was applied as appropriate. The effects of the circadian hygiene initiative and academic performance over time were analyzed by repeated-measures ANOVA (no missing data imputation procedures applied);  $\eta_p^2$  was utilized as an indicator of the effect size. Factorial ANOVA was utilized for multiple grouping factors. A linear regression model was utilized to identify independent predictors of academic performance. The assumption behind the choice of variables for the linear regression was that they were either expected (age) or shown to affect sleep and/or academic performance. These were age, sex, study subject, commuting, sleep quality (continuous total PSQI score), and chronotype. Diagnostics were conducted as follows; normality: inspection of

the histogram and the quantile–quantile plot of the residual; homogeneity of variance (homoscedasticity): inspection of the plot of residuals vs. predicted values; outliers: inspection of residual vs. predicted values and residuals vs. predictors, the leverage and residual plot, evaluation of the Cook Di ( $> 4/n$ ); collinearity: correlation among the predictors and VIF  $> 3$ ; and independence: inspection of the plot of residuals vs. predicted values. Interaction was tested by adding the predictor terms  $X1 \times X2$ ,  $X1 \times X3$ ,  $X2 \times X3$ , and so on, and detected between age and PSQI. Age was treated as a confounder. Analyses were carried out with Statistica, version 13.1 (Dell, Round Rock, TX, United States) and subsequently with version 14.0.0.15 (TIBCO, Palo Alto, CA, United States).

## RESULTS

On December 19, 2020, 5,740 students (38% males; age  $23 \pm 5$  years) had completed the first full set of questions (**Supplementary Table 1**); 55% had responded to the October 2019 call, 29% to the October 2020 call, and 16% had joined at different times between October 2019 and December 2020. Seven hundred forty students (31% males) attended health and medical (M), 2,439 (58% males) science and technology (S), and 2,492 (22% males) social and humanities (H) studies; 69 attended single courses. There were 2,981 students assigned to the “A regular life” and 2,759 to the “Bright days and dark nights” advice group; 1,144 students had one/more diseases, and 1,359 were on medication, with significant overlap ( $\chi^2 = 869$ ,  $p < 0.0001$ ); 1,295 reported minor eyesight issues.

## Sleep–Wake Profiles

There were 2,972 students (52%) who had an abnormal PSQI, with the most heavily affected components being subjective sleep quality, sleep latency, sleep disturbance, and daytime dysfunction; 714 (12%) had an abnormal ESS. Based on the Self-ME, 570 students qualified themselves as definitely morning, 1,896 as morning, 2,233 as evening, and 1,041 as definitely evening. As expected, the Self-ME classification was reflected in the more specific sleep timing variables obtained from the STSQS questionnaire. In addition, definitely evening/evening types had significantly worse PSQI scores compared with their more morning counterparts [ $6.9 \pm 3.2/6.4 \pm 3.0$  vs.  $5.8 \pm 2.9/5.8 \pm 3.0$ ;  $F_{(3, 5736)} = 34$ ,  $p < 0.0001$ ; all *post hoc* significant for definitely evening/evening vs. definitely morning and morning types]. Based on the  $\mu$ MCTQ, 240 students had negative, 755 no, and 4,703 positive social jetlag; 42 did not provide sufficient information. Average midsleep on study and free days was  $03:54 \pm 01:06$  and  $05:06 \pm 01:12$  (hh:mm), respectively. Average sleep duration on study and free days was  $7.5 \pm 1.2$  and  $8.4 \pm 1.2$  h, respectively.

Female students had earlier sleep–wake timing habits, more night awakenings, worse sleep quality, and more daytime sleepiness compared with male students (**Table 1**). In addition, they had earlier midsleep on both study and free days, and they more commonly classified themselves as morning chronotypes. Finally, their sleep duration was longer on free days (**Table 1**).

**TABLE 1** | Sleep–wake indices (mean  $\pm$  SD) on joining the initiative at T0, by sex.

| Questionnaire | Variable                                                  | Females<br>( <i>n</i> = 3,566) | Males<br>( <i>n</i> = 2,174) |
|---------------|-----------------------------------------------------------|--------------------------------|------------------------------|
| STSQS         | Bed time (hh:mm)                                          | 23:18 $\pm$ 00:54              | 23:42 $\pm$ 01:06*           |
|               | Try to sleep time (hh:mm)                                 | 23:51 $\pm$ 00:57              | 24:06 $\pm$ 01:04*           |
|               | Sleep latency (min)                                       | 24 $\pm$ 24                    | 20 $\pm$ 18*                 |
|               | Calculated sleep onset time (hh:mm)                       | 24:15 $\pm$ 01:06              | 24:26 $\pm$ 01:09*           |
|               | Night awakenings ( <i>n</i> )                             | 1.0 $\pm$ 1.2                  | 0.8 $\pm$ 1.0*               |
|               | Wake-up time (hh:mm)                                      | 07:34 $\pm$ 01:09              | 07:39 $\pm$ 01:15            |
|               | Get-up time (hh:mm)                                       | 07:56 $\pm$ 01:16              | 08:00 $\pm$ 01:21            |
|               | Latency between wake-up and get-up time (min)             | 21 $\pm$ 28                    | 20 $\pm$ 31                  |
| PSQI          | Total score (0–21)                                        | 6.5 $\pm$ 3.2                  | 5.8 $\pm$ 2*                 |
| ESS           | Total score (0–24)                                        | 6.7 $\pm$ 3.6                  | 5.7 $\pm$ 3.3*               |
| Self-ME       | Definitely morning/morning/evening/definitely evening (%) | 11/34/38/16                    | 8/31/40/21*                  |
| $\mu$ MCTQ    | Sleep onset study days (hh:mm)                            | 24:03 $\pm$ 01:14              | 24:15 $\pm$ 01:15*           |
|               | Sleep offset study days (hh:mm)                           | 07:34 $\pm$ 01:17              | 07:42 $\pm$ 01:21*           |
|               | Sleep duration study days (hours)                         | 7.5 $\pm$ 1.2                  | 7.4 $\pm$ 1.1                |
|               | Midsleep study days (hh:mm)                               | 03:49 $\pm$ 01:05              | 04:00 $\pm$ 01:09*           |
|               | Sleep onset free days (hh:mm)                             | 24:27 $\pm$ 01:15              | 01:06 $\pm$ 01:20*           |
|               | Sleep offset free days (hh:mm)                            | 09:14 $\pm$ 01:21              | 09:24 $\pm$ 01:29*           |
|               | Sleep duration free days (hours)                          | 8.4 $\pm$ 1.2                  | 8.3 $\pm$ 1.1*               |
|               | Midsleep free days (hh:mm)                                | 05:00 $\pm$ 01:09              | 05:15 $\pm$ 01:16*           |
|               | Social jetlag (hours)                                     | 1.2 $\pm$ 1.3                  | 1.3 $\pm$ 1.4                |

\* $p < 0.001$  (Bonferroni-corrected threshold:  $0.05/20 = 0.0025$ ).

STSQS, Sleep Timing Sleep Quality Screening questionnaire; PSQI, Pittsburgh Sleep Quality Index; ESS, Epworth Sleepiness Scale; Self-ME, self-morningness/eveningness questionnaire;  $\mu$ MCTQ, ultrashort Munich ChronoType Questionnaire.

Commuters had earlier sleep–wake timing habits [i.e., bed time: 23:24  $\pm$  01:00 vs. 23:30  $\pm$  01:00 hh:mm;  $t_{(4,144)} = 4.8$ ;  $p < 0.001$ ] compared with on-site students. Students attending H were older than those attending M, who, in turn, were older than those attending S studies (Table 2). Students attending M had earlier sleep–wake timing compared with students attending S/H studies. Finally, students attending H had worse sleep quality than those attending M/S studies (Table 2).

Students with diseases had more night awakenings [ $1.1 \pm 1.2$  vs.  $0.9 \pm 1.1$ ;  $t_{(5,737)} = -5.0$ ,  $p < 0.0001$ ] and worse sleep quality [PSQI:  $7.0 \pm 3.5$  vs.  $6.1 \pm 2.9$ ;  $t_{(5,737)} = -8.6$ ,  $p < 0.0001$ ]; the same applied to students on medication.

Students who joined in October 2020 (during the pandemic;  $n = 1,697$ ) had later sleep–wake timing [i.e., wake-up time: 07:48  $\pm$  01:12 vs. 07:24  $\pm$  01:06 hh:mm;  $t_{(4,825)} = -9.9$ ,  $p < 0.001$ ], less social jetlag [ $1.2 \pm 1.7$  vs.  $1.3 \pm 1.1$  h;  $t_{(4,799)} = 3.9$ ,  $p < 0.001$ ], longer study days sleep duration [ $7.6 \pm 1.2$  vs.  $7.4 \pm 1.1$  h;  $t_{(4,808)} = -6.3$ ,  $p < 0.001$ ], better sleep quality

(PSQI abnormal 49% vs. 52%;  $\chi^2 = 4.6$ ,  $p < 0.05$ ), and less daytime sleepiness (ESS abnormal 10% vs. 14%;  $\chi^2 = 14.9$ ,  $p < 0.001$ ) compared with the pre-pandemic October 2019 cohort ( $n = 3,137$ ). Of note, the two cohorts were comparable in age and female-to-male ratio, and any students who joined in between or later were excluded for purposes of this specific comparison.

## Circadian Hygiene Intervention and Self-Reported Compliance

Questionnaire completion rates decreased over time (Supplementary Table 1). While several hundred students completed the questionnaires at any given T (Supplementary Table 1), a total of 30 completed all even study times up to T12 in the “A regular life” group, and 28 in the “Bright days and dark nights” group. The two groups were comparable for sleep quality/timing, and female-to-male, study subject, and chronotype ratios. Significant differences between the two intervention groups were observed in get-up time and in the latency between wake-up and get-up time until T10 (earlier get-up time and shorter latency in the “Bright days and dark nights” group). Statistical significance was lost when T12, which coincided with the transition from DST to standard time (ST), was included (Figures 2A,B). At this moment, it was the behavior of the “A regular life” group that changed the most (Figures 2A,B), with the return to ST facilitating them more than their counterparts who had already been advised to go to bed and get up earlier. Along the same lines, a trend difference [ $F_{(5, 355)} = 0.8$ ,  $0.05 < p < 0.1$ ] between the two intervention groups was observed in midsleep on study days until T10 (earlier midsleep in the “Bright days and dark nights” group). All other sleep quality/timing variables were comparable between the two groups (Supplementary Table 2).

Significant changes in most sleep quality (i.e., fewer night awakenings) and sleep timing (i.e., less social jetlag) variables were observed in both groups over time, most likely in relation to the combination of the shift to/from DST and the course of pandemic-related events that characterized 2020 (Figures 2A–C). This is why time points were not cumulated to increase statistical power. When students were classified into four groups based on the Self-ME, their actual sleep–wake timing reflected their Self-ME at T0. Over time, the four Self-ME groups responded in a similar fashion but to a different extent to the 2020 events (Figure 2C). When sleep–wake timing was compared between commuters and on-site students at T0 (October 2019) and T6 (full lockdown in April 2020), commuters’ sleep–wake habits became more similar to those of on-site students, and their social jetlag decreased (Supplementary Figure 1).

Compliance completion rates decreased over time (Supplementary Table 1); 40 students completed all odd study times in the “A regular life” group, and 45 in the “Bright days and dark nights” group. Differences between the two groups were observed in compliance to sleep-related advice, which was greater in the “A regular life” group (Figure 3A). Compliance with all types of advice increased over time in both groups (Figures 3A–D). Consistent differences in compliance to different types of advice were observed in



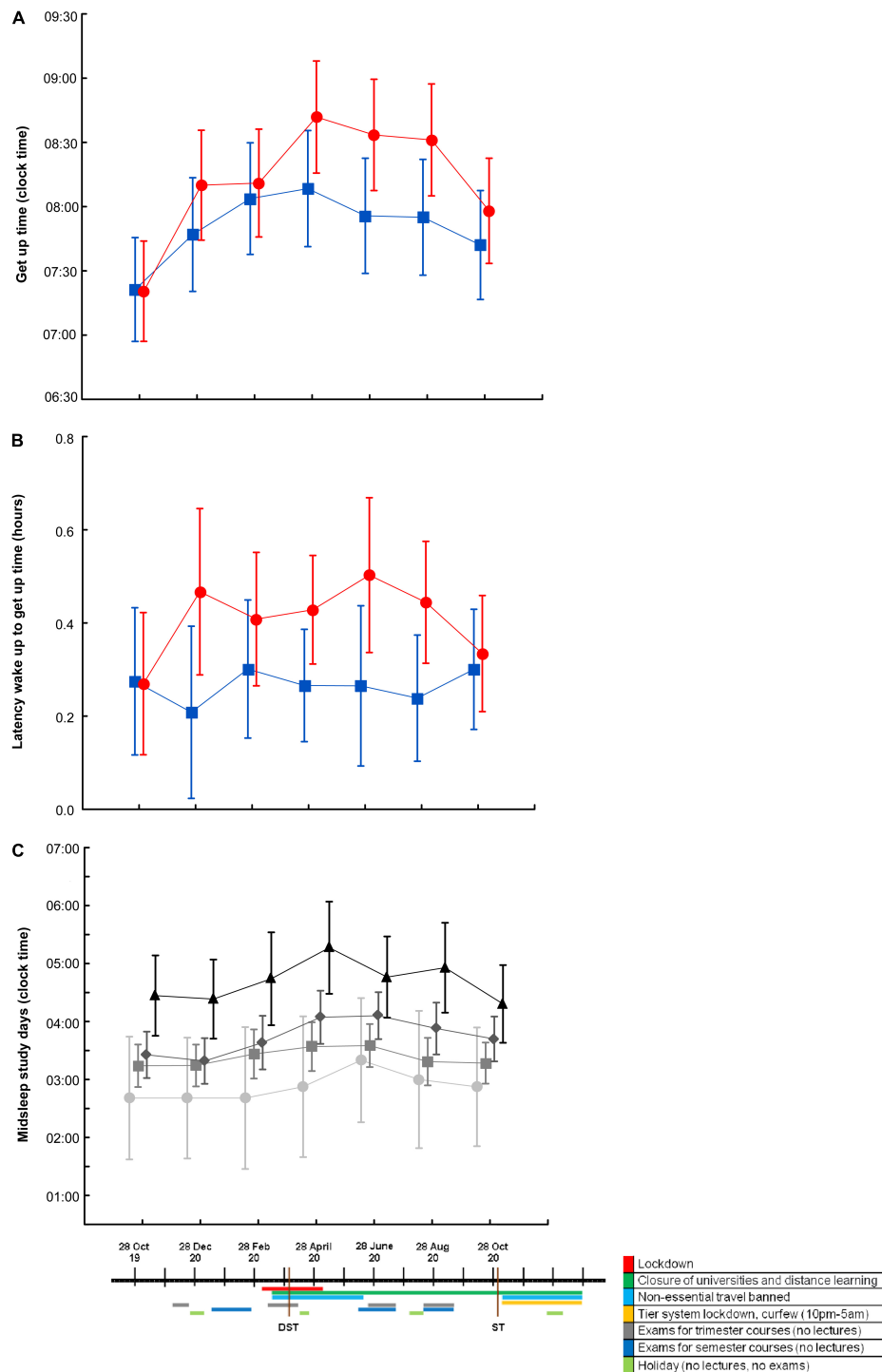
**TABLE 2 |** Sleep–wake indices [mean  $\pm$  SE (95% CI)] on joining the initiative at T0, by study subject.

| Questionnaire Variable |                                                           | Social and humanities<br>(H; $n = 2,492$ ) | Health and medical<br>(M; $n = 740$ )  | Science and technology<br>(S; $n = 2,439$ ) |
|------------------------|-----------------------------------------------------------|--------------------------------------------|----------------------------------------|---------------------------------------------|
| Age (years)            |                                                           | 24.0 $\pm$ 0.1<br>(23.8–24.2)              | 23.2 $\pm$ 0.2***<br>(22.8–23.5)       | 22.4 $\pm$ 0.1 ###,ooo<br>(22.2–22.6)       |
| STSQS                  | Bed time (hh:mm)                                          | 23:26 $\pm$ 00:01<br>(23:24–23:29)         | 23:19 $\pm$ 00:02 ***<br>(23:15–23:24) | 23:27 $\pm$ 00:01°<br>(23:25–23:30)         |
|                        | Try to sleep time (hh:mm)                                 | 24:00 $\pm$ 00:01<br>(23:58–24:03)         | 23:46 $\pm$ 00:02 ***<br>(23:41–23:50) | 23:56 $\pm$ 00:01 #,ooo<br>(23:53–23:58)    |
|                        | Sleep latency (min)                                       | 24 $\pm$ 0<br>(24–25)                      | 21 $\pm$ 1 *<br>(20–23)                | 21 $\pm$ 0 ###<br>(20–22)                   |
|                        | Calculated sleep onset time (hh:mm)                       | 24:25 $\pm$ 00:01<br>(24:22–24:27)         | 24:07 $\pm$ 00:02 ***<br>(24:02–24:12) | 24:17 $\pm$ 00:01 ###,ooo<br>(24:14–24:19)  |
|                        | Night awakenings (n)                                      | 1.06 $\pm$ 0.02<br>(1.01–1.10)             | 0.82 $\pm$ 0.04 ***<br>(0.73–0.90)     | 0.83 $\pm$ 0.02 ###<br>(0.78–0.87)          |
|                        | Wake-up time (hh:mm)                                      | 07:45 $\pm$ 00:01<br>(07:42–07:48)         | 07:22 $\pm$ 00:02 ***<br>(07:17–07:27) | 07:31 $\pm$ 00:01 ###,°<br>(07:28–07:34)    |
|                        | Get-up time (hh:mm)                                       | 08:08 $\pm$ 00:01<br>(08:05–08:11)         | 07:41 $\pm$ 00:02 ***<br>(07:35–07:46) | 07:51 $\pm$ 00:01 ###,oo<br>(07:48–07:54)   |
|                        | Latency between wake-up and get-up time (min)             | 23 $\pm$ 0<br>(22–24)                      | 18 $\pm$ 1 ***<br>(16–20)              | 20 $\pm$ 0 ###<br>(18–21)                   |
|                        |                                                           |                                            |                                        |                                             |
| PSQI                   | Total score (0–21)                                        | 6.6 $\pm$ 0.1<br>(6.5–6.7)                 | 5.9 $\pm$ 0.1 ***<br>(5.7–6.2)         | 6.0 $\pm$ 0.1 ###<br>(5.9–6.1)              |
| ESS                    | Total score (0–24)                                        | 6.3 $\pm$ 0.1<br>(6.2–6.5)                 | 6.5 $\pm$ 0.1<br>(6.2–6.7)             | 6.2 $\pm$ 0.1<br>(6.0–6.3)                  |
| Self-ME                | Definitely morning/morning/evening/definitely evening (%) | 10/33/38/19                                | 11/33/41/15                            | 9/33/39/19                                  |
| $\mu$ MCTQ             | Sleep onset study days (hh:mm)                            | 24:12 $\pm$ 00:01<br>(24:09–24:15)         | 23:55 $\pm$ 00:02 ***<br>(23:50–24:00) | 24:07 $\pm$ 00:01°oo<br>(24:04–24:10)       |
|                        | Sleep offset study days (hh:mm)                           | 07:45 $\pm$ 00:01<br>(07:42–07:48)         | 07:19 $\pm$ 00:02 ***<br>(07:13–07:24) | 07:35 $\pm$ 00:01 ###,ooo<br>(07:32–07:38)  |
|                        | Sleep duration study days (hours)                         | 7.56 $\pm$ 0.02<br>(7.51–7.60)             | 7.40 $\pm$ 0.04 **<br>(7.32–7.49)      | 7.45 $\pm$ 0.02 ##<br>(7.41–7.50)           |
|                        | Midsleep study days (hh:mm)                               | 03:58 $\pm$ 00:01<br>(03:56–04:01)         | 03:37 $\pm$ 00:02 ***<br>(03:32–03:41) | 03:51 $\pm$ 00:01 ###,ooo<br>(03:48–03:53)  |
|                        | Sleep onset free days (hh:mm)                             | 24:55 $\pm$ 00:01<br>(24:52–24:58)         | 24:47 $\pm$ 00:02<br>(24:42–24:53)     | 24:55 $\pm$ 00:01<br>(24:52–24:58)          |
|                        | Sleep offset free days (hh:mm)                            | 09:18 $\pm$ 00:01<br>(09:15–09:21)         | 09:11 $\pm$ 00:03<br>(09:05–09:17)     | 09:20 $\pm$ 00:01<br>(09:16–09:23)          |
|                        | Sleep duration free days (hours)                          | 8.38 $\pm$ 0.02<br>(8.34–8.43)             | 8.40 $\pm$ 0.04<br>(8.31–8.49)         | 8.40 $\pm$ 0.02<br>(8.36–8.45)              |
|                        | Midsleep free days (hh:mm)                                | 05:06 $\pm$ 00:01<br>(05:04–05:09)         | 04:59 $\pm$ 00:02<br>(04:54–05:04)     | 05:07 $\pm$ 00:01°<br>(05:05–05:10)         |
|                        | Social jetlag (hours)                                     | 1.16 $\pm$ 0.03<br>(1.11–1.21)             | 1.42 $\pm$ 0.05 *<br>(1.32–1.51)       | 1.29 $\pm$ 0.03°<br>(1.24–1.34)             |

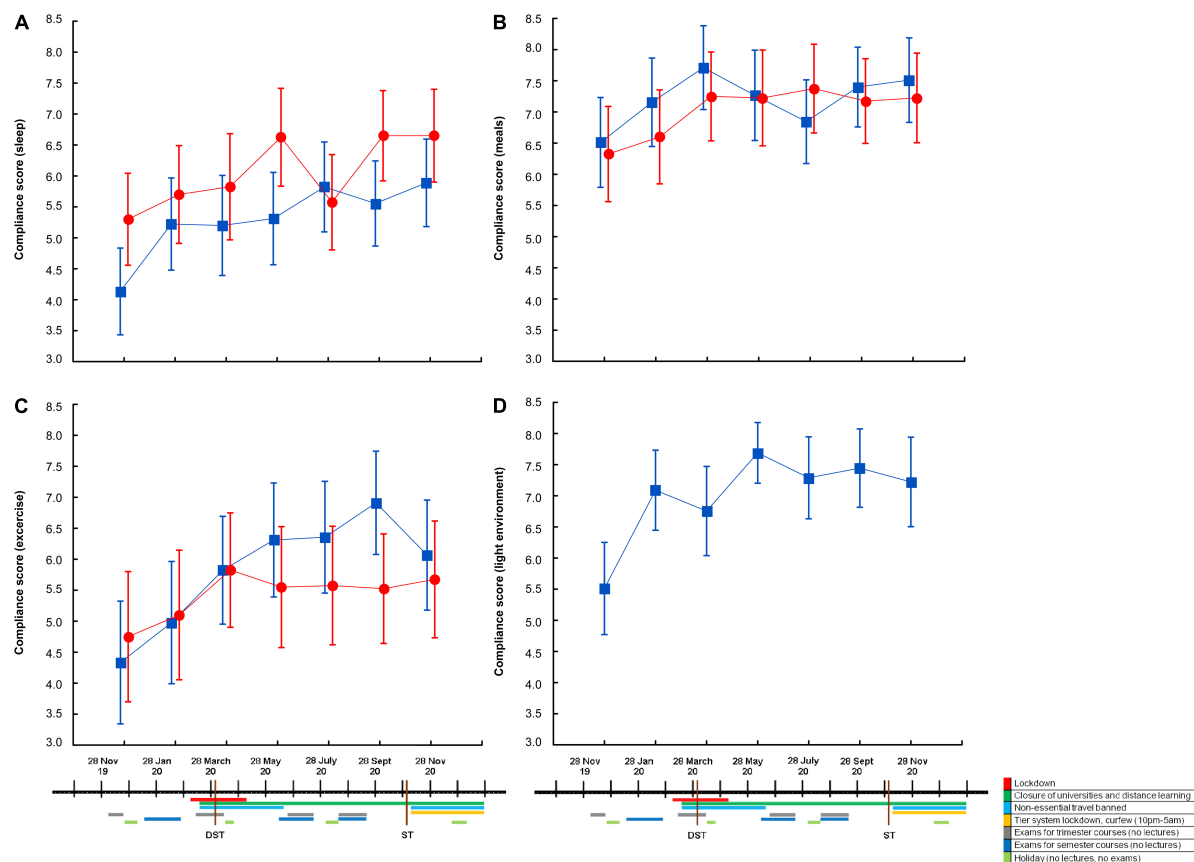
\*\*\* $p < 0.001$ . \*\* $p < 0.01$  H. \* $p < 0.05$ . vs. M. ### $p < 0.001$ . ## $p < 0.01$ . # $p < 0.05$  H vs. S. °oo $p < 0.001$ . °o $p < 0.01$ . ° $p < 0.05$  M vs. S. STSQS, Sleep Timing Sleep Quality Screening questionnaire; PSQI, Pittsburgh Sleep Quality Index; ESS, Epworth Sleepiness Scale; Self-ME, self-morningness/eveningness questionnaire;  $\mu$ MCTQ, ultrashort Munich ChronoType Questionnaire.

both groups [meals > sleep > exercise in the “A regular life” group;  $F_{(2, 7,168)} = 511$ ,  $p < 0.001$ , all *post hoc* significant; meals and light environment > exercise > sleep in the “Bright days and dark nights” group;  $F_{(3, 9,843)} = 324$ ,  $p < 0.001$ , all *post hoc* significant except for meals vs. light environment]. These relationships held true when analyzed at T1 (highest single completion rate with 920 students in the “A regular life” and 841 in the “Bright days and dark nights” groups), at complete odd time points cumulated (280 vs. 315) and at all time points cumulated (3,585 vs. 3,282) (Supplementary Table 3).

More compliant students exhibited better sleep quality on both total PSQI and on the STSQS 1–10 self-reported sleep quality score [both ANOVA and correlation analyses; i.e., PSQI vs. compliance to sleep advice:  $n = 2,188$ ;  $r^2 = -0.099$ ,  $p < 0.0001$ ], irrespective of advice group. Later chronotypes exhibited lower compliance compared with morning chronotypes [both ANOVA and correlation analyses], in most instances irrespective of advice group. In the case of compliance to sleep advice, this was also higher in the “A regular life” group, irrespective of chronotype [advice group:  $F_{(1, 1,753)} = 81$ ,  $p < 0.0001$ ; chronotype:  $F_{(3, 1,753)} = 27$ ,



**FIGURE 2 |** Sleep timing indices by circadian hygiene education group and by chronotype, over time and in relation to relevant 2020 events. Get-up time **(A)** and latency between wake-up and get-up time **(B)** in the “A regular life” (red circles;  $n = 30$  at each time point) and the “Bright days and dark nights” (blue squares;  $n = 28$  at each time point) groups. Differences were significant up to August 2020, that is T10 [get-up time:  $F_{(5,365)} = 17.0$ ,  $p$  time  $< 0.0001$  ( $\eta_p^2 = 0.189$ ),  $F_{(1, 73)} = 4.2$ ,  $p$  intervention  $= 0.042$  ( $\eta_p^2 = 0.055$ ),  $F_{(5,365)} = 1.2$ ,  $p$  interaction n.s. ( $\eta_p^2 = \text{n.s.}$ ); latency:  $F_{(5,365)} = 2.1$ ,  $p$  time  $= 0.06$  ( $\eta_p^2 = \text{n.s.}$ ),  $F_{(1, 73)} = 5.9$ ,  $p$  intervention  $= 0.02$  ( $\eta_p^2 = 0.075$ ),  $F_{(5,365)} = 1.7$ ,  $p$  interaction n.s. ( $\eta_p^2 = \text{n.s.}$ )] and were abolished/reduced when October 2020 (T12), which coincided with the week after the transition from daylight saving time (DST) to standard time (ST), was included [get-up time:  $F_{(6,336)} = 11.9$ ,  $p$  time  $< 0.0001$ ,  $F_{(1, 56)} = 2.4$ ,  $p$  intervention n.s.;  $F_{(6,336)} = 1.5$ ,  $p$  interaction n.s.; latency:  $F_{(6,336)} = 0.9$ ,  $p$  time n.s.,  $F_{(1, 56)} = 3.1$ ,  $p$  intervention  $= 0.08$ ;  $F_{(6,336)} = 2.0$ ,  $p$  interaction n.s.]. Midsleep on study days **(C)** in definitely morning (light gray circles), morning (gray squares), evening (dark gray diamonds), and definitely evening (black triangles) chronotypes [ $F_{(3,345)} = 5.3$ ,  $p$  time  $< 0.001$ ,  $F_{(3, 69)} = 4.0$ ,  $p$  chronotype  $< 0.005$ ,  $F_{(15, 345)} = 1.2$ ,  $p$  interaction n.s., at T10;  $F_{(6,312)} = 4.7$ ,  $p$  time  $< 0.001$ ,  $F_{(3, 52)} = 5.6$ ,  $p$  chronotype  $< 0.005$ ,  $F_{(18, 312)} = 0.9$ ,  $p$  interaction n.s., at T12]. Values are expressed as means  $\pm$  95% confidence intervals. n.s., not statistically significant.



**FIGURE 3 |** Compliance to different types of advice by circadian hygiene education group, over time, and in relation to relevant 2020 events. Self-reported compliance to advice relating to sleep (A), meals (B), exercise (C), and light environment (D) in the “A regular life” (red circles;  $n = 40$  at each time point) and the “Bright days and dark nights” (blue squares;  $n = 45$  at each time point) groups. Significant differences between the two groups were observed in compliance to sleep advice (A), which was greater in the “A regular life” group [ $F_{(6, 498)} = 6.0$ ,  $p$  time  $< 0.0001$ ,  $F_{(1, 83)} = 4.1$ ,  $p$  group  $= 0.045$ ,  $F_{(6, 498)} = 1.6$ ,  $p$  interaction n.s.]. Compliance to all types of advice increased over time in both groups ( $p$  time always significant, with no further differences between groups and no interactions). Values are expressed as means  $\pm$  95% confidence intervals. n.s., not statistically significant.

$p < 0.0001$ ; advice group  $\times$  chronotype:  $F_{(3, 1,753)} = 11$ ,  $p$  not statistically significant (n.s.).

## Academic Performance

Females had better academic performance than males at all time points [i.e.,  $26.3 \pm 2.4$  (out of 30, 18 = pass) vs.  $25.8 \pm 2.6$  on study entrance T0,  $t_{(3,604)} = 6.6$ ,  $p < 0.0001$ ]. Morning had better academic performance than evening chronotypes, within each study subject (Figure 4A). Students with good sleep quality (PSQI  $< 5$ ) had better marks at all time points [i.e.,  $26.3 \pm 2.4$  vs.  $25.9 \pm 2.5$  on study entrance T0,  $t_{(3,604)} = 3.8$ ,  $p < 0.001$ ].

As expected, the number of examinations/credits increased over time. Average marks also increased over time (Figure 4B). Students' academic indices were comparable between the two circadian hygiene advice groups at T0 and remained so over time.

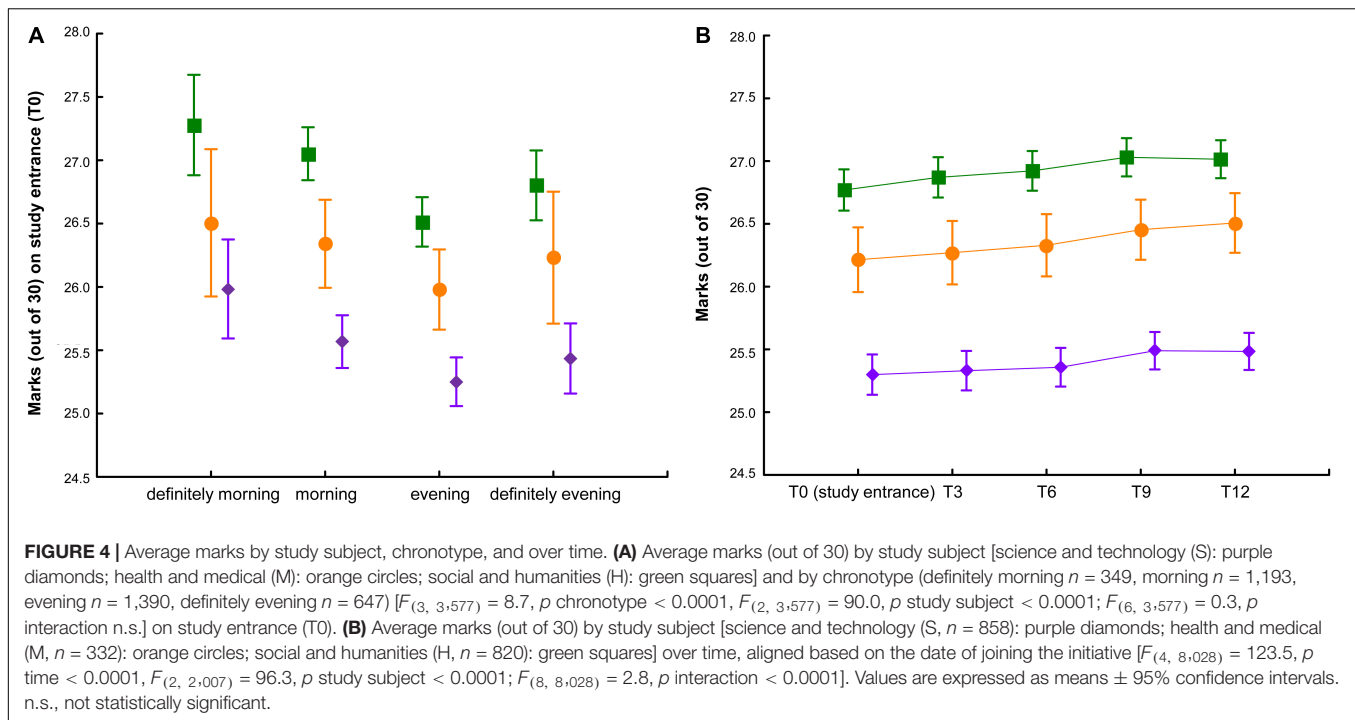
There were institutional differences in the timing of classes for M, S, and H students. Based on lectures room bookings and attendance data, M students started earlier than S students, who started earlier than H students. Further, the total lecture

time was longer for M compared with S/H students. Morning examinations were scheduled earlier for M compared with S/H students, and afternoon examinations were scheduled later for H compared with M/S students (Table 3).

When variables shown to affect sleep and/or academic performance [sex, study subject, commuting, sleep quality (continuous) and chronotype, plus age (continuous) as an adjustment factor] were included in a linear regression model, study subject ( $M > H > S$ ), age (positive correlation), sleep quality (positive correlation), and sex ( $F > M$ ) were associated with a higher number of examinations passed/credits acquired, whereas study subject ( $H > M > S$ ), chronotype ( $M > E$ ), and sleep quality (positive correlation) were associated with better marks (Supplementary Table 4).

## DISCUSSION

Poor sleep quality was common, with more than half of the students reporting abnormal, albeit mild, sleep disruption.



**TABLE 3 |** Lecture and examination booking times, plus electronic attendance records, by study subject.

|                      |                         | Social and humanities (H) |             | Health and medical (M) |             | Science and technology (S)          |             |
|----------------------|-------------------------|---------------------------|-------------|------------------------|-------------|-------------------------------------|-------------|
| Lecture bookings     | Morning start (hh:mm)   | 10 : 32 $\pm$ 01 : 33     | $n = 1,141$ | 09 : 43 $\pm$ 01 : 21* | $n = 621$   | 10 : 19 $\pm$ 01 : 29 <sup>§#</sup> | $n = 2,070$ |
|                      | Morning end (hh:mm)     | 12 : 34 $\pm$ 01 : 34     |             | 12 : 30 $\pm$ 01 : 23  |             | 12 : 23 $\pm$ 01 : 34 <sup>§</sup>  |             |
|                      | Afternoon start (hh:mm) | 15 : 14 $\pm$ 01 : 03     | $n = 667$   | 14 : 30 $\pm$ 01 : 01* | $n = 387$   | 14 : 52 $\pm$ 00 : 56 <sup>§#</sup> | $n = 1,036$ |
|                      | Afternoon end (hh:mm)   | 17 : 15 $\pm$ 01 : 05     |             | 17 : 25 $\pm$ 01 : 09  |             | 17 : 03 $\pm$ 01 : 03 <sup>§#</sup> |             |
|                      | Length (min)            | 122 $\pm$ 28              | $n = 1,808$ | 171 $\pm$ 64*          | $n = 1,008$ | 127 $\pm$ 47 <sup>§#</sup>          | $n = 3,106$ |
| Examination bookings | Morning start (hh:mm)   | 09 : 50 $\pm$ 01 : 09     | $n = 508$   | 09 : 28 $\pm$ 01 : 04* | $n = 451$   | 09 : 42 $\pm$ 00 : 58 <sup>#</sup>  | $n = 841$   |
|                      | Afternoon start (hh:mm) | 14 : 53 $\pm$ 00 : 48     |             | 14 : 32 $\pm$ 00 : 49* |             | 14 : 34 $\pm$ 00 : 39 <sup>§</sup>  |             |
| Single swipe         | Start (hh:mm)           | 10 : 49 $\pm$ 02 : 17     | $n = 4,648$ | 09 : 52 $\pm$ 02 : 02* | $n = 3,783$ | 10 : 33 $\pm$ 02 : 20 <sup>§#</sup> | $n = 7,062$ |
|                      | Length (min)            | 127 $\pm$ 27              |             | 184 $\pm$ 57*          |             | 131 $\pm$ 57 <sup>§#</sup>          |             |
| Multiple swipes      | First swipe in (hh:mm)  | 09 : 48 $\pm$ 01 : 35     | $n = 1,890$ | 09 : 11 $\pm$ 01 : 17* | $n = 1,969$ | 09 : 52 $\pm$ 02 : 03 <sup>#</sup>  | $n = 3,806$ |
|                      | Last swipe out (hh:mm)  | 15 : 34 $\pm$ 01 : 56     |             | 15 : 40 $\pm$ 02 : 11  |             | 14 : 53 $\pm$ 02 : 21 <sup>§#</sup> |             |

\* $p < 0.001$  H vs. M. <sup>§</sup> $p < 0.001$  H vs. S. <sup>#</sup> $p < 0.001$  M vs. S on post hoc comparisons (Bonferroni-corrected threshold:  $0.05/11 = 0.0045$ ).

Although direct comparisons are not necessarily easy, as published studies on poor sleep in university students are heterogeneous for country, student populations, questionnaire/assessment tools, and/or questionnaire thresholds utilized, our results seem slightly more encouraging than those of Reis et al. (2021), who recently reported a prevalence of insomnia of 67.7% in college students in Portugal, and those of Driller et al. (2021), who reported 67% between bad and moderate sleepers among first-year health degree undergraduates in New Zealand. Despite reasonable sleep duration, social jetlag (Wittmann et al., 2006; Korman et al., 2020) was also common. All such abnormalities were less pronounced in the October 2020 compared with the October 2019 cohort, suggesting that long periods of full/partial lockdown and distance learning

(thus less pressure from the “social clock”) made it easier for students to follow their inclination toward later sleep timing and to sleep better. This is supported also by the large decrease in social jetlag observed in commuters between October 2019 and April 2020 and is in line with similar, albeit mostly retrospective and unpaired observations in varying populations (Blume et al., 2020; Gao and Scullin, 2020; Korman et al., 2020; Leone et al., 2020; Cellini et al., 2021; Hallman et al., 2021; Partinen et al., 2021) and also in university students (Wright et al., 2020; Marelli et al., 2021). Most of the above studies on the effects of COVID-19-related lockdown measures and circadian/sleep variables in students and other populations were initiated during the pandemic, asking participants to provide pandemic and pre-pandemic information (the latter by recall)



at a single moment in time. This methodology can be, of course, accepted but is far from ideal, because recollection of sleep–wake information (as recollection of other subjective variables) has been associated with both recall bias (Sukul et al., 2020) and summary distortion (Montagnese et al., 2009). By contrast, we were able to acquire repeat circadian/sleep information prior to and during the pandemic in a clean, longitudinal fashion, and with high time resolution. The “Bright days and dark nights” advice resulted in earlier get-up time/earlier midsleep and decreased latency between wake-up and get-up time, with no impact on sleep duration. These are desirable, relevant effects in young adults (Bjorvatn and Pallesen, 2009), and were observed within the context of a “low-intensity” intervention to promote healthy circadian behaviors in university students. One must remember that expectations on the real-life effects of educational interventions in healthy individuals are low as unsolicited advice is rarely complied with, there is an inherent resistance to changing habits (dietary advice is the most common reference in this respect; Kapur et al., 2008; Martis et al., 2018), and this is even more pronounced in healthy individuals, who have limited incentive to do so. Also, although we opted for a comprehensive baseline sleep–wake assessment, the intervention was circadian in its essence and meant to affect sleep timing rather than sleep quality variables, which is what happened until T10, which corresponded to the last time of data collection before the transition from DST to ST. The effect on sleep timing was observed despite students reporting difficulties in complying with sleep advice and may be due, in part, to them finding it easier to follow light environment advice. This type of light advice, in a population that makes large use of portable devices in the evening/early night hours for study and recreational purposes, may indeed be crucial (Gringras et al., 2015), also explaining part of the differences in the effects of the “Bright days and dark nights” and the “A regular life” advice. Being a university-based educational initiative, the study obviously lacks a placebo arm and some of the methodological rigor associated with pre-ordained design. On the positive side, the ecological nature of the presented results provides a phenomenal amount of real-life information, which may be useful to plan similar initiatives or more formal studies in future (i.e., amount of material to fill in, amount of advice provided and its format, timing between questionnaire administration, compliance rates to different types of advice, etc.). The initial response to the initiative, as measured by standard e-mail marketing indices, compares favorably with average responses to Italian work/education-related initiatives in which candidate participants were contacted by e-mail.<sup>2</sup> However, completion of questionnaires/compliance decreased considerably over time, suggesting feasibility issues with the initiative recall/refresh frequency, which may need modulating. It should be highlighted, however, that our intervention, lasting 1 year and based on monthly monitoring, was much longer and included considerably more time points than the few published similar interventions (Hershner and O’Brien, 2018; Illingworth et al., 2020; van Rijn et al., 2020; Semsarian

et al., 2021). For example, Semsarian et al. (2021) report 35% participation on second contact; ours was similar, with 32% of students responding at T1 (**Supplementary Table 1**). In addition, lower completion rates after T0 do not necessarily imply lack of compliance with the advice received on joining the initiative. In addition, should more or less compliant students adhere to some or all the received “Bright days and dark nights” advice over long periods (i.e., it is very easy to imagine them making a habit of using the night-shift function on their portable devices), its positive effects may cumulate over time. Selection bias in relation to varying compliance levels is possible but unlikely, as students who completed all procedures at all times did not have distinctive demographic/sleep features. Further, students with later chronotypes and worse sleep quality also had slightly but significantly lower average compliance levels. Finally, while it is possible to hypothesize that the “Bright days and dark nights” advice provided to the small number of students with a definitely morning chronotype and/or a negative social jetlag may have been counterproductive, it should be highlighted that even definitely morning students had a social jetlag, which was close to 1 h, thus most likely benefiting from the suggestions received.

The published, original, and also review/meta-analytic literature on education (mostly sleep but also circadian) aimed at adolescents (Chung et al., 2017; Illingworth et al., 2020; van Rijn et al., 2020) or university students (Dietrich et al., 2016; Hershner and O’Brien, 2018; Semsarian et al., 2021) is not conclusive; it is based on shorter programs, yielding generally short-lived effects. In addition, it suggests that more consistent interventions compared with ours in terms of provision of information on the science/medical evidence behind the advice delivered tend to result in increased knowledge but not necessarily in significant changes in sleep/circadian habits (Illingworth et al., 2020; van Rijn et al., 2020). Along the same lines, and despite being appreciative of the importance of entrainment dynamics [synchronization between the endogenous and the solar clock (Roenneberg and Merrow, 2007)], our intervention was aimed at reducing the time gap between the students’ endogenous clock and the social one, because for the time being, lesson/examination times, like most social constraints, cannot be changed in the real world. So, while the advice provided might not have necessarily favored/paralleled entrainment in natural conditions, it will have hopefully helped the students functioning in their real world, with all its imposed time constraints, including lesson/examination times and DST.

The time course of sleep timing/quality between October 2019 and October 2020 suggests that the mixture of full/partial lockdown, distance learning, and the transition to/from DST all had profound and intertwining effects, with any form of relaxation of the “social clock” resulting in delayed sleep timing, longer sleep duration, and better sleep quality. These changes were always more obvious in evening types. Evening types also had worse academic performance compared with their more morning counterparts, within any study subject area. This is only partially in line with previous observations in high school students, whose performance

<sup>2</sup><https://www.mailup.com/>

was affected by chronotype mostly for scientific subjects (Zerbini et al., 2017).

Average marks increased over time, albeit very slightly. This might be explained by the fact that there is a selection process throughout university, with specific subjects—which may be more interesting for the students—being usually concentrated toward the end of their careers. However, it should be highlighted that the Italian system does not allow for a significant amount of choice/changes of the core curriculum, and these choices are probably of limited impact over the course of a single year, which was our analysis period. Alternative possibilities include some form of progressive familiarization with the examination system, especially for younger students, or even distance learning during 2020 affecting both students' preparation and professors' attitudes.

In the current study, both sleep quality and chronotype independently predicted academic performance, together with the study subject. Based on these data and the observed, institutional differences in lecture timing/duration and examinations timing between study subjects, two approaches could be envisaged, at least in Italian universities: (i) delaying science and technology timetables by 30–60 min (overall study day duration would allow to accommodate this as the last swipe out for the science and technology students was between 30 and 45 min earlier compared with the other two study subjects; **Table 3**); (ii) acquiring chronotype information from students (by the one-question Self-ME, which is fast and convenient), and taking these into account when designing timetables, with a view to limit pressure on evening chronotypes. This not only seems feasible and worth investigating but may also become necessary in the post-pandemic era, which will presumably be still characterized by the need to avoid large groups of students attending lectures at any given time. Thus, should lectures/examinations timetable shifts remain necessary in large universities such as ours, they should probably be better designed to maximize academic performance. Similar approaches (i.e., acquisition and use of chronotype information to design work shifts) have already proven effective in terms of sleep length in workers from major industries (Vetter et al., 2015). In addition, the advice shown to be easier to follow (i.e., advice on meal timing and light environment from “Bright days and dark nights”) could readily be provided to students when they register at the university as part of their induction package.

The main limitations of this study include: (i) the lack of a placebo arm, (ii) a decrease in adherence to completion of questionnaires/compliance over time, and (iii) the possibility that the “Bright days and dark nights” advice provided to the small number of students with a definitely morning chronotype may

have been unnecessary. Despite these limitations, this large and comprehensive set of baseline data, the response and compliance to the educational initiative, the time course of sleep timing in the year of the pandemic, and the observed differences in academic performance by chronotype all underlie the importance of designing circadian-friendly university timetables.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Azienda Ospedale – Università Padova. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

SM, LZ, CF, PF, DM, ST, DS, and RC designed the study and developed the study methods. SM, LZ, GG, CM, CI, PF, ST, DS, and RC analyzed the data. AP, DM, RR, and RV contributed to the interpretation of the data. SM and RC drafted the manuscript. All authors critically reviewed the manuscript and approved the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnins.2022.848602/full#supplementary-material>

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Liver, Pancreas and Biliary Tract

# Evolution of hepatic encephalopathy over time: ecological data from a tertiary referral centre for hepatology

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## ABSTRACT

**Objectives:** Few data on hepatic encephalopathy (HE) over time are available, thus our aim was to study its evolution in patients with varying degree of HE on first assessment.

**Methods:** Eighty-six patients with cirrhosis (age =  $58 \pm 11$  years; males = 72) were evaluated 2–10 times for liver transplantation selection purposes, differential diagnosis or treatment optimization. The presence/severity of HE was assessed by clinical and neuropsychiatric indices [ Psychometric Hepatic Encephalopathy Score (PHES) and electroencephalography (EEG)] and the severity of liver disease by the Model for End-Stage Liver Disease (MELD) score. Treatment was instituted/modified after each evaluation.

**Results:** Amongst 23 unimpaired patients, 56/6% remained unimpaired, 35/3% developed covert HE, 9/0% developed overt HE on second/third evaluation. Amongst 32 patients with covert HE, 25/10% became unimpaired, 44/19% remained covert, 31/13% developed overt HE. Finally, amongst 32 patients with overt HE, 19/16% became unimpaired, 25/13 % became covert and 56/25% remained overt. PHES results improved in patients with overt HE and EEG worsened over time (despite remaining normal) in unimpaired patients. In patients with multiple evaluations, HE evolution was manifold and difficult to predict.

**Conclusions:** HE evolution over time is variable and largely dependent on HE history/management. These data support the concept that HE is an essentially reversible condition.

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## 1. Introduction

Hepatic encephalopathy (HE) is a continuum of neuropsychiatric impairment, ranging from mild neurophysiological or neuropsychological abnormalities to coma, caused by liver cirrhosis and/or portal-systemic shunting [1–6]. The development of a first episode of overt HE marks significant progression in the natural history of cirrhosis, as it is associated with poorer prognosis [7,8] and worsened quality of life [9–12].

Patients with overt HE are exposed to a 40% cumulative risk of recurrent overt HE at 1 year [5,13] and minimal or covert HE are associated with a significant risk of developing overt HE [14–17]. Over recent years, the reversibility of HE has been extensively debated, with cognitive abnormalities after repeated episodes overt

HE (and despite adequate treatment) and/or after liver transplantation being interpreted by some authors [18–20] as proof of irreversibility and by others as suggesting pre-existing confounders/predisposing factors affecting both cognitive performance *per se* and also the likelihood of developing overt HE [21–23].

Few data [24] are available regarding HE evolution over time in single patients, especially based on comprehensive neuropsychiatric assessment.

Our aim was to re-evaluate this issue by retrospective analysis of a large set of repeated, comprehensive neuropsychiatric evaluations (performed for clinical reasons on the request of the treating clinicians) of patients with cirrhosis and varying degree of HE on first evaluation.

## 2. Material and methods

Eighty-six patients with cirrhosis (age  $58 \pm 11$  years; 72 males) referred to the HE outpatient clinic of the Regional Centre for Liver Diseases at Padova University Hospital between June 2009 and January 2019 were included. They had been evaluated from 2 up to 10 times (Fig. 1), for purposes of liver transplantation work up (55%), HE treatment optimization (28%) or neuropsychiatric differential diagnosis (17%). Relevant demographic and clinical data are

**Abbreviations:** HE, hepatic encephalopathy; MELD, Model for End-Stage Liver Disease; PHES, Psychometric Hepatic Encephalopathy Score; MPZS, mean PHES Z score; MDF, mean dominant frequency.

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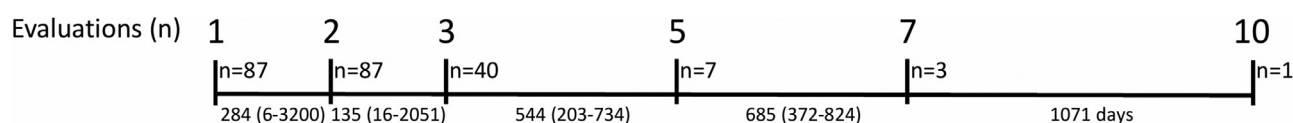


Fig. 1. Number of patients evaluated from 2 to 10 times and median (range) of the time (in days) between evaluations.

presented in Table 1. A set of clinical information were acquired on first and, where relevant, subsequent evaluations: aetiology of cirrhosis, ascites, presence of spontaneous/surgical/transjugular portal-systemic shunt, asterixis, venous ammonia (obtained on the day as an urgent measurement within the local laboratory for patients followed up in Padova and/or brought in from their respective hospitals/outpatient blood clinics for patients referred to from outside the Padova area) and Model for End-Stage Liver Disease (MELD) score (Table 1).

The presence/degree of HE (i.e., HE status) on the day of study was assessed by clinical, neuropsychological (PHES) and neurophysiological (EEG) indices on each evaluation.

## 2.1. Clinical evaluation

The assessment included the acquisition of neuropsychiatric history and a neurological examination.

## 2.2. Neuropsychological evaluation

The PHES battery is composed by five paper and pencil tests (the Trail Making Test A and B, the Digit Symbol test, the Line Tracing test and the Serial Dotting test) [25,26]. Both the mean z score (MPZS) and rounded sum of each test z score (PHES), adjusted for age and education in relation to Italian norms were obtained. A PHES score  $\leq -4$  was considered abnormal [26]. The Animal Naming Test [27] was also available for a subset of patients.

## 2.3. Neurophysiological evaluation

EEG was recorded by 21-channels, with the ground placed on Fpz and the reference on Oz (Brainquick 3200; Micromed, Mogliano Veneto, Italy). Impedance was kept below 5 k $\Omega$  and the signal was filtered in the range of 0.33–60 Hz. Sampling frequency was 256 Hz and conversion resolution 0.19 mV/digit. After selection of an artifact-free EEG section, spectral analysis was performed on the bi-parietal derivation P3-P4; the mean dominant frequency (MDF) and the relative amount of slow EEG activity within the  $\theta$  and  $\delta$  frequency bands were calculated [28,29]. The EEG was considered abnormal if MDF was  $\leq 7.3$  Hz or if relative  $\theta/\delta$  power was  $\geq 35\%/ \geq 44\%$  [29].

## 2.4. HE status on the day of assessment

Patients were classed as: *unimpaired* if they had no signs of overt HE, normal PHES and EEG, regardless of HE history and/or treatment; having *covert HE* if they showed PHES and/or EEG abnormalities or mild clinical signs of brain dysfunction (slight slowing, irritability etc, i.e. grade I HE according to the West Haven criteria) without disorientation for time or asterixis; and having *overt HE* if they met grade II or higher West Haven criteria [5]. Patients who had been evaluated before 2014 (the year when the joint EASL-AASLD guidelines were published) and diagnosed as having grade I HE were then reclassified, together with patients with minimal HE, as having covert HE [5].

Treatment was instituted/modified according to both guidelines and local experience after each evaluation [30].

As most of the patients (71%) had a positive HE history, often they were already on secondary prophylaxis with non-absorbable

disaccharides and/or non-absorbable antibiotics on first assessment (75%), according to international guidelines (i.e. lactulose or lactitol alone or in combination with rifaximin in case of recurrent episodes of overt HE within six months; rifaximin alone can be used for lactulose/lactitol intolerance) [30]. Even though treatment for covert HE is not routinely recommended, it is normally instituted in our center to treat the symptoms of mild HE and facilitate differential diagnosis; finally, dietary change, supplementation with fermentable fibers, probiotics, branched-chain amino acids and in-between-meals snacks are also often recommended [30].

Permission for retrospective data analyses was obtained from the local Ethics Committee.

## 2.5. Statistical analyses

Normality was tested for by the Shapiro-Wilk test. Results are expressed as mean  $\pm$  SD or median (range) for quantitative variables, as count and percentage for categorical variables. Patients with different grades of HE were compared by ANOVA for quantitative variables (post hoc Tuckey test) and by chi-square or Fisher's exact test, as appropriate, for categorical variables. Factorial ANOVA was utilised for multiple grouping factors. Multivariate analyses were performed both to identify significant independent predictors on first evaluation and to test for significant changes in neuropsychiatric indices between first and second evaluation. Analyses were carried out with the packages Statistica, version 13.1 (Dell, Round Rock, Texas, US) and SAS 9.4 (SAS Institute Inc, Cary, NC, USA).

## 3. Results

On first assessment, 23 (26%) patients were classed as unimpaired, 32 (37%) as having covert and 32 (37%) as having overt HE. Unimpaired patients were significantly younger compared with patients with any degree of HE (Table 1). As expected, the MELD score in patients with overt HE was significantly higher compared to that of unimpaired patients. Similarly, the percentage of patients on HE treatment and with a history of overt HE was significantly higher amongst patients who had overt HE on first evaluation (Table 1). With reference to neuropsychiatric indices, EEGs were significantly slower in patients with overt HE, followed by patients with covert HE; MPSZ/PHES performance was better in both unimpaired and patients with covert HE compared to patients with overt HE (Table 1). Unexpectedly, no differences in ammonia levels were observed between the three groups (Table 1). However, when both HE status on the day of study and HE history were taken into account, there was a trend ( $0.05 < p < 0.1$ ) for patients with a positive history to have higher ammonia levels, regardless of their HE status on the day of assessment. Amongst unimpaired patients, 56% remained unimpaired on the second evaluation and 6% on the third; 35% had covert HE on the second evaluation and 3% on the third; finally, 9% had overt HE on the second evaluation and none on the third (Fig. 2). Amongst patients with covert HE, 25% were unimpaired on the second and 10% on the third evaluation, 44% remained covert on the second evaluation and 19% on the third evaluation; finally, 31% had overt HE on the second and 13% on the third evaluation (Fig. 2). Lastly, amongst patients with overt HE, 19% became unimpaired on the second and 16% on the third evaluation, 25% had covert HE on the second and 13% on the third evaluation; finally, 56% still had overt HE on the second

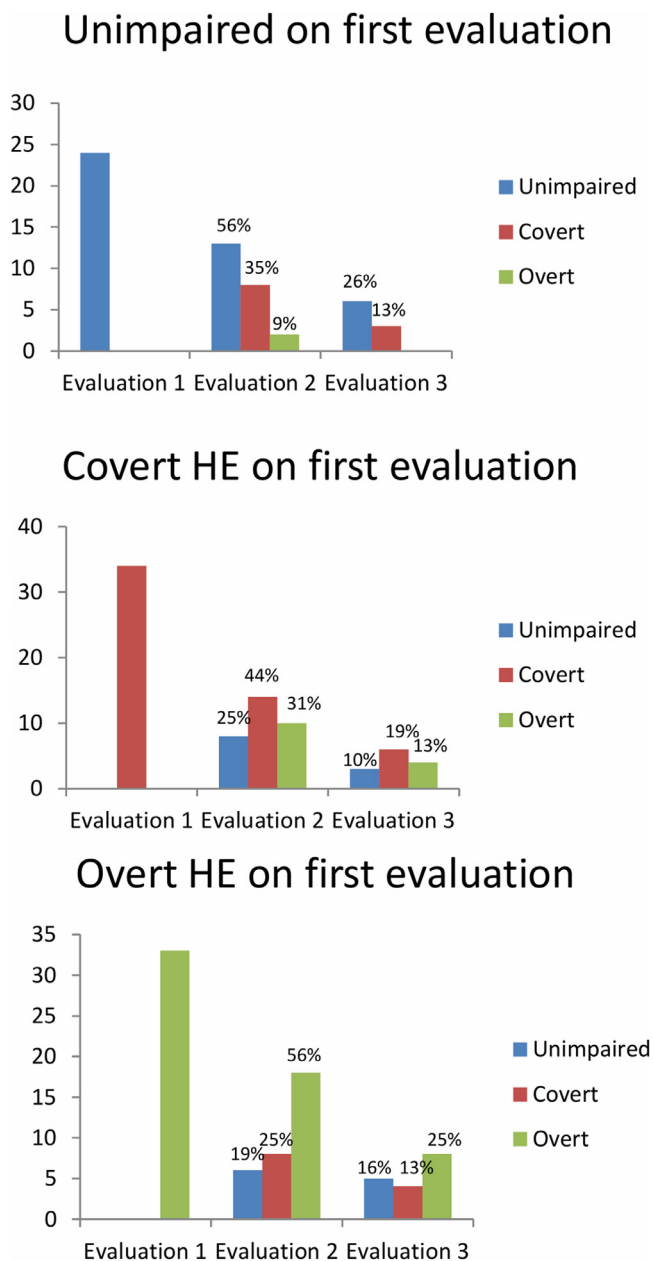
**Table 1**

Demographic, clinical, laboratory, neuropsychological and neurophysiological indices in patients, as a whole and by presence/degree of hepatic encephalopathy on first, second and third assessment.

|                                                       | First evaluation         |                                    |                                        |                                       | Second evaluation                  |                                        |                                       | Third evaluation                   |                                        |                                       |
|-------------------------------------------------------|--------------------------|------------------------------------|----------------------------------------|---------------------------------------|------------------------------------|----------------------------------------|---------------------------------------|------------------------------------|----------------------------------------|---------------------------------------|
|                                                       | All patients<br>(n = 86) | Unimpaired<br>patients<br>(n = 23) | Patients with<br>covert HE<br>(n = 31) | Patients with<br>overt HE<br>(n = 32) | Unimpaired<br>patients<br>(n = 27) | Patients with<br>covert HE<br>(n = 29) | Patients with<br>overt HE<br>(n = 30) | Unimpaired<br>patients<br>(n = 14) | Patients with<br>covert HE<br>(n = 13) | Patients with<br>overt HE<br>(n = 11) |
| Age (years; mean ± SD)                                | 58.7 ± 10.3              | 53.07 ± 8.05                       | 59.60 ± 9.08*                          | 61.86 ± 11.41**                       | 54.88 ± 8.48                       | 60 ± 9.26                              | 63.33 ± 11.50                         | 56.41 ± 10.07                      | 58.01 ± 10.93                          | 66.48 ± 7.07                          |
| Education (years in school; mean ± SD)                | 10.94 ± 4.56             | 11.73 ± 4.61                       | 11.97 ± 4.44                           | 9.35 ± 4.34                           | 12.09 ± 3.71                       | 11.74 ± 4.06                           | 9.1 ± 4.13                            | 11 ± 2.83                          | 11.27 ± 4.36                           | 8.11 ± 3.34                           |
| Sex (% males)                                         | 80                       | 83                                 | 90                                     | 69 <sup>‡</sup>                       | 81                                 | 76                                     | 80                                    | 93                                 | 61*                                    | 64                                    |
| Aetiology (% alcohol/viral/other)                     | 50/40/10                 | 48/48/4                            | 52/35/13                               | 50/37/13                              | 48/45/7                            | 55/28/17                               | 47/47/6                               | 71/29/0                            | 54/23/23                               | 55/36/9                               |
| MELD (mean ± SD)                                      | 12.14 ± 4.31             | 10.32 ± 2.69                       | 11.56 ± 3.62                           | 14.04 ± 5.19**                        | 13.53 ± 6.69                       | 12.68 ± 4.37                           | 13.87 ± 4.68                          | 11.60 ± 5.50                       | 11.18 ± 2.40                           | 14.80 ± 3.27                          |
| Spectral EEG indices                                  |                          |                                    |                                        |                                       |                                    |                                        |                                       |                                    |                                        |                                       |
| MDF (Hz; mean ± SD)                                   | 9.2 ± 2.14               | 10.99 ± 0.97                       | 9.19 ± 1.40**                          | 7.95 ± 2.46***,§§                     | 10.01 ± 1.60                       | 8.84 ± 1.90**                          | 8.42 ± 2.25***,§§§                    | 9.69 ± 1.25                        | 8.82 ± 1.85***                         | 8.72 ± 2.16***                        |
| δ (%; mean ± SD)                                      | 11 ± 13                  | 6 ± 3                              | 8 ± 8                                  | 18 ± 18***,§§§                        | 7 ± 4                              | 10 ± 11                                | 15 ± 13***,§§§                        | 8 ± 5                              | 10 ± 8                                 | 11 ± 7                                |
| Θ (%; mean ± SD)                                      | 35 ± 19                  | 16 ± 6                             | 40 ± 16***                             | 44 ± 20***                            | 28 ± 18                            | 41 ± 19***                             | 42 ± 20***                            | 28 ± 19                            | 43 ± 20***                             | 44 ± 21***                            |
| α (%; mean ± SD)                                      | 37 ± 19                  | 53 ± 13                            | 37 ± 15**                              | 24 ± 17***,§§                         | 45 ± 17                            | 34 ± 16***                             | 28 ± 16***,§§§                        | 48 ± 20                            | 32 ± 18***                             | 28 ± 16***                            |
| β (%; mean ± SD)                                      | 17 ± 11                  | 25 ± 11                            | 15 ± 8**                               | 14 ± 11***                            | 20 ± 13                            | 15 ± 9**                               | 15 ± 11***,‡                          | 16 ± 7                             | 15 ± 9**                               | 17 ± 12**                             |
| MPZS (mean ± SD)                                      | -1.11 ± 1.24             | 0.07 ± 0.55                        | -0.74 ± 0.61                           | -2.24 ± 1.21***,§§§                   | -0.42 ± 1.26                       | -0.71 ± 1.11                           | -1.51 ± 1.17***,§§                    | -0.15 ± 0.53                       | -0.72 ± 1.04                           | -1.48 ± 1.13***,‡                     |
| PHES (mean ± SD)                                      | -3.74 ± 4.2              | -0.30 ± 1.25                       | -1.84 ± 2.35                           | -8.11 ± 3.41***,§§§                   | -1.28 ± 4.50                       | -2.21 ± 3.72                           | -5.76 ± 4.22***,§§                    | -0.50 ± 1.52                       | -2.64 ± 3.26                           | -5.09 ± 3.99***,‡                     |
| ANT (mean ± SD)                                       | 13.15 ± 5.22             | 17.4 ± 4.5                         | 13.8 ± 4.2                             | 9 ± 3.8***,§§                         | 13.6 ± 4.6                         | 15.3 ± 4.6                             | 11.4 ± 6.3                            | 13.0 ± 6.1                         | 10.3 ± 1.4                             | 5                                     |
| Ammonia (mmol/L; mean ± SD)                           | 81.11 ± 43.52            | 80.30 ± 51.54                      | 82.12 ± 47.30                          | 80.88 ± 38.15                         | 66.31 ± 32.32                      | 87.91 ± 43.28                          | 74.83 ± 34.09                         | 81.50 ± 22.59                      | 95.33 ± 52.94                          | 73.00 ± 26.83                         |
| HE history (% positive)                               | 71                       | 45                                 | 70                                     | 96***,‡                               | 64                                 | 85                                     | 83                                    | 75                                 | 67                                     | 100 <sup>‡</sup>                      |
| Portal-systemic shunt (%ab-sent/spontaneous/surgical) | 35/60/5                  | 44/56/0                            | 44/52/4                                | 21/71/8                               | 41/35/24                           | 39/50/11                               | 27/68/5                               | 33/45/22                           | 25/62/13                               | 0/87/13                               |
| HE treatment (% positive)                             | 75                       | 54                                 | 73                                     | 93***,‡                               | 88                                 | 96                                     | 100                                   | 100                                | 100                                    | 100                                   |

Abbreviations: ANT, Animal Naming Test; EEG, Electroencephalogram; HE, Hepatic Encephalopathy; MDF, Mean Dominant Frequency; MELD, Model for End-Stage Liver Disease; MPZS, Mean PHES Z Score; PHES, Psychometric Hepatic Encephalopathy Score.

<sup>‡</sup> Information available in 10/14/3 unimpaired patients, 17/10/6 patients with covert and 13/12/1 patients with overt HE on first/second/third evaluation, respectively.\*  $p < .050$ ; \*\*  $p < .010$ ; \*\*\*  $p < .001$  on post-hoc in the comparison between unimpaired patients and patients with covert HE.\*\*  $p < .010$ ; \*\*\*  $p < .001$  on post-hoc in the comparison between unimpaired patients and patients with overt HE.‡  $p < .050$ ; §§  $p < .010$ ; §§§  $p < .001$  on post-hoc in the comparison between covert HE and overt HE patients.



**Fig. 2.** Hepatic encephalopathy (HE) status over time in patients classed as unimpaired (blue - upper panel), covert (red - middle panel) and overt (green - lower panel) on first assessment.

and 25% on the third evaluation (Fig. 2). No significant differences were observed between unimpaired patients with/without a history of HE or on/off treatment in terms of baseline performance and/or evolution over time. MPZS tended to improve [ $F(2) = 3.17$ ,  $p = 0.055$ ] in patients with overt HE. Slow EEG activity (theta) increased, thus the EEG worsened, without becoming abnormal, [ $F(2) = 4.89$ ,  $p = 0.010$ ] from the first to the second evaluation in unimpaired patients (Fig. 3). These trends were not confirmed after adjustment for HE history, HE treatment and MELD.

Amongst the parameters HE status, MELD, age and HE history on first evaluation, HE status [ $F(2)=7.63$ ,  $p = 0.001$ ] was the only independent predictor of HE status on second evaluation. When ammonia levels on first evaluation were included, previous HE history [ $F(1) = 8.29$ ;  $p = 0.006$ ], HE status [ $F(2) = 5.39$ ;  $p = 0.009$ ] and ammonia [ $F(1) = 6.89$ ;  $p = 0.012$ ] were significant predictors of HE status on second evaluation.

Finally, in patients ( $n = 7$ ) with multiple evaluations (from 5 to 10), HE evolution was manifold and difficult to predict (Fig. 4). Three main patterns were observed: two patients (subjects 1 and 6) remained substantially stable over time; three patients (subjects 4, 5 and 7) improved their HE status; two patients (subjects 2 and 3) worsened after initial improvement.

#### 4. Discussion

In this study HE evolution over time was investigated by retrospective analysis of a large set of repeated, comprehensive neuropsychiatric evaluations of patients with cirrhosis and varying degree of HE on first evaluation. The novelty of our study was that patients were tested comprehensively several times, even if timing was not necessarily planned in advance because all tests were performed for clinical and not for study purposes. Two previous studies of repeated evaluations over time are not directly comparable with ours, because the first one considered a much shorter period between evaluations (3 days) [19], while the second one used only two sub-tests of the PHES battery and was limited to covert HE patients [24]. Of note, in the latter no differences were observed between first and second evaluation on either trial-making test A or the digit symbol test [24].

In the present cohort, the majority of patients had already been diagnosed with some degree of HE, had spontaneous shunt and were already on ammonia-lowering treatment. These are expected features as our outpatients clinic is part of a tertiary referral center for liver disease, and could explain why the levels of ammonia were generally high but still predictive.

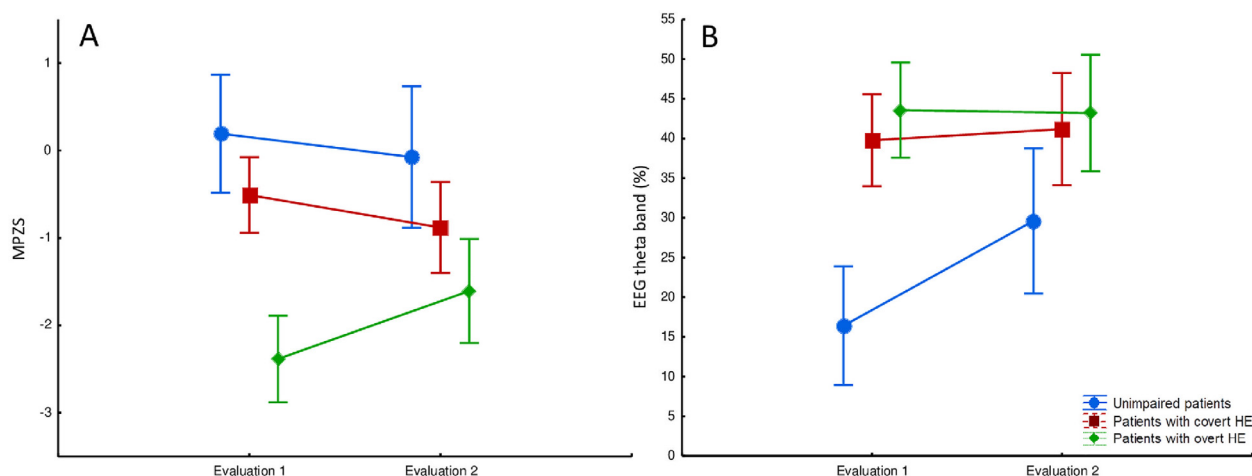
When divided by presence/degree of HE on first evaluation, patients with any degree of HE were older compared with their unimpaired counterparts, and MELD was higher in patients with overt HE compared with unimpaired patients. These features seem to be in line with the findings of Cordoba and colleagues (2014), who observed that patients with a long stable disease: i) are usually older, ii) have a history of HE, and iii) have no acute but mild long-lasting liver failure [31].

In line with our previous findings [32], after a course of ammonia-lowering treatment 44% of patients with overt HE on first evaluation were classed as covert or unimpaired on second evaluation, and there was a significant improvement in MPZS. This improvement after treatment supports the contention that HE is a substantially reversible condition [21–23]. The persistence of abnormal cognitive performance in some of our patients despite proper ammonia-lowering treatment, might be explained by the combination of worsening liver disease and some degree of cognitive decline, as already highlighted in one previous study from our group [22]. Unimpaired patients without a history of HE (and on no treatment [6]) who were re-evaluated because of worsening liver function or for purposes of orthotopic liver transplantation, exhibited an increase in the percentage of slow activity on the EEG (even if within the normal range), most likely in relation to progression of the underlying liver disease.

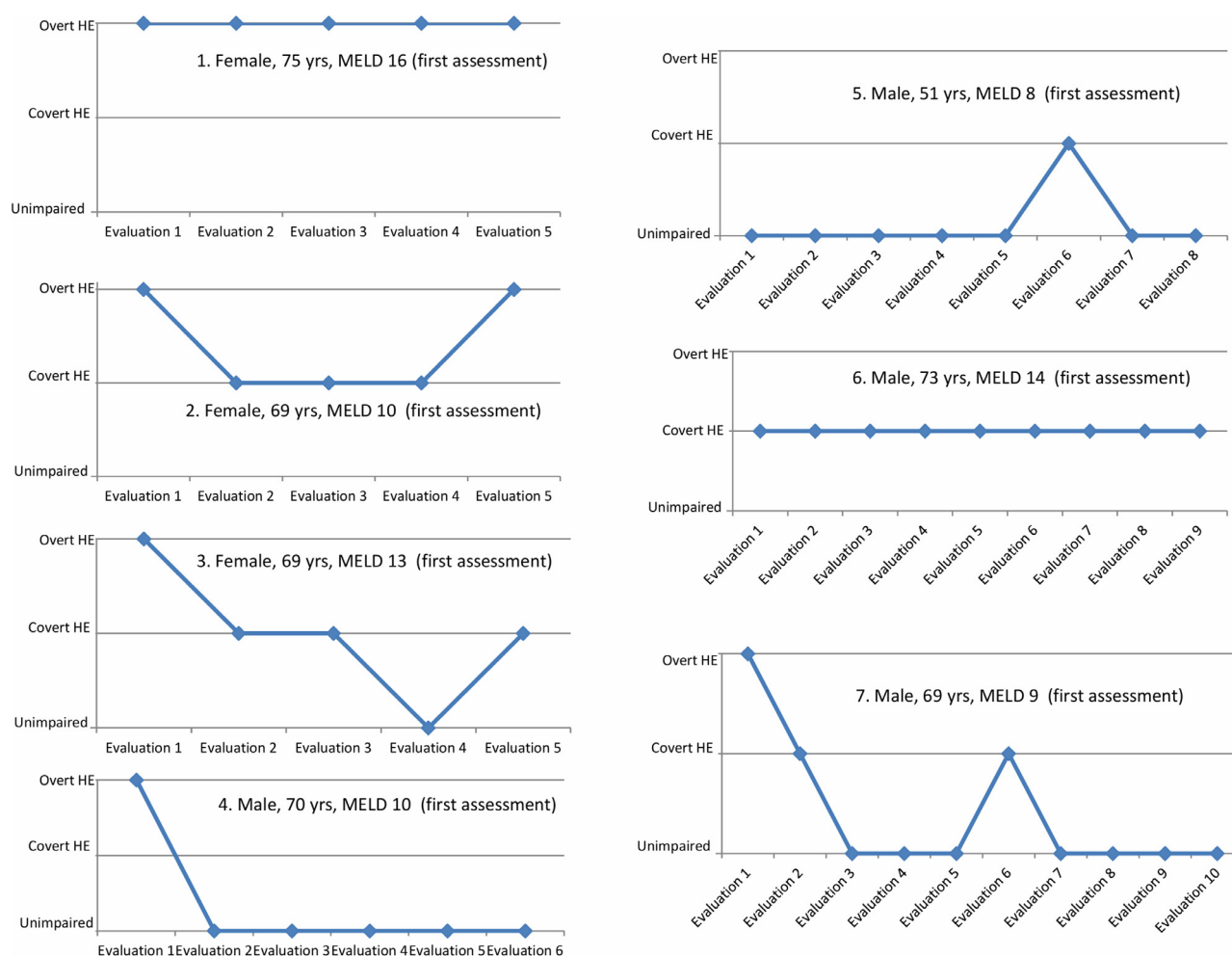
The multivariate analysis showed that HE status on first evaluation, HE history and ammonia levels were significant predictors of HE status on second evaluation, with limited role for age and MELD. This is in line with one previous study from our group [32] and may be due to the features of the patient population, who was referred largely because of HE/difficulties with managing HE and is therefore not representative of the general features of patients with cirrhosis.

The study has a number of limitations: since it is retrospective in nature, data were collected over different time intervals, based on clinical needs; in a future study, a similar cohort of patients should be tested prospectively at fixed time intervals. Moreover, selection bias could have occurred, as patients with several evalu-





**Fig. 3.** **Panel A:** MPZS score of unimpaired patients (blue circles), patients with covert hepatic encephalopathy (HE) (red squares) and patients with overt HE (green diamonds) on the first (left) and second (right) evaluation. **Panel B:** EEG relative theta power (%) of unimpaired patients (blue circles), patients with covert HE (red squares) and patients with overt HE (green diamonds) on the first (left) and second (right) evaluation.



**Fig. 4.** Evolution of hepatic encephalopathy (HE) (y axis) over time (x axis) in 7 patients evaluated 5–10 times. Three patterns may be recognized: two patients (subjects 1 and 6) remained substantially stable over time in spite of HE therapy optimization, possibly in relation to cerebral-vascular comorbidity; three patients (subjects 4, 5 and 7) improved their HE status and then remained substantially stable over time; two patients (subjects 2 and 3) worsened after initial improvement, possibly due to worsening liver function. [MELD score (Model for End Stage in Liver Disease)].

ations are more likely to have had severe or difficult to manage HE, and, in the case of those tested for transplant work-up, worsening or changing clinical conditions.

In conclusion, while there is obvious HE risk associated with the presence of any degree of HE on first assessment, our data suggest that the evolution of HE over time is extremely variable and largely dependent on HE history and clinical management. These data also support the concept that HE is an essentially reversible condition.

### Declaration of Competing Interest

All authors declare that they have no conflicts of interest.

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## MINI REVIEW

# Circadian rhythms and the liver

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## Abstract

This narrative review briefly describes the mammalian circadian timing system, the specific features of the liver clock, also by comparison with other peripheral clocks, the role of the liver clock in the preparation of food intake, and its relationship with energy metabolism. It then goes on to provide a chronobiological perspective of the pathophysiology and management of several types of liver disease, with a particular focus on metabolic-associated fatty liver disease (MAFLD), decompensated cirrhosis and liver transplantation. Finally, it provides some insight into the potential contribution of circadian principles and circadian hygiene practices in preventing MAFLD, improving the prognosis of advanced liver disease and modulating liver transplantation outcomes.

## KEYWORDS

decompensated cirrhosis, liver clock, liver transplantation, MAFLD, mammalian circadian timing system, metabolism

## 1 | INTRODUCTION

Translational and clinical chronobiological studies are flourishing thanks to the enormous amount of knowledge that has accumulated over the past decades in molecular chronobiology and its physiological correlates. This has been such that in 2017 the Nobel prize for Physiology or Medicine was awarded to Jeff Hall, Michael Rosbash and Michael Young “for their discoveries of molecular mechanisms controlling the circadian rhythm”.<sup>1</sup> The circadian timing system encompasses a master clock in the suprachiasmatic nuclei (SCN) of the hypothalamus and peripheral timekeepers in every organ of the body, and most tissues and cells.<sup>2</sup> In addition, clocks are also present

in areas of the brain other than the SCN.<sup>3</sup> Circadian clocks control our physiology over the 24 h affecting, for example, the sleep–wake cycle.<sup>4</sup> Several studies have linked persistently disturbed circadian rhythmicity, for example due to shift work, to an increased incidence of cardiovascular accidents,<sup>5</sup> higher rates of substance misuse<sup>6</sup> and certain types of cancer.<sup>7</sup> Remarkable, isolated pieces of clinical research have linked circadian rhythmicity to other major health outcomes, for example, documenting an increase in the incidence of myocardial infarction after the switch to daylight saving time,<sup>8</sup> better outcomes of aortic valve replacement in the afternoon compared to morning surgery<sup>9</sup> and increased intravasation/metastatic proficiency of circulating tumour cells during the rest phase

**Abbreviations:** ETS, Erythroblast Transformation Specific; HCC, Hepatocellular Carcinoma; HE, Hepatic Encephalopathy; MAFLD, Metabolic-Associated Fatty Liver Disease; NAD<sup>+</sup>, Nicotinamide Adenine Dinucleotide; NAMPT, Nicotinamide Phosphoribosyltransferase; OSA, Obstructive Sleep Apnea; PPARs, Peroxisome Proliferator-Activated Receptors; PPAR $\alpha$ , Peroxisome Proliferator Activated-Receptor alpha; PPAR $\gamma$ , Peroxisome Proliferator Activated-Receptor gamma; SCN, Suprachiasmatic Nuclei; SIRT1, Sirtuin 1 Deacetylase; TTFL, Transcription-Translation-Feedback Loop.

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in both patients and mouse models of breast cancer.<sup>10</sup> This narrative review describes the mammalian circadian timing system and the peculiarities of the liver clock and examines liver disease and its management—with a particular focus on cirrhosis—from a chronobiological perspective.

## 2 | THE MAMMALIAN CIRCADIAN TIMING SYSTEM

The molecular model of the mammalian circadian timing system encompasses a set of so-called clock genes, which encode proteins involved in the mechanism generating the circadian oscillation. This is self-generated in each of the approximately 20000 neurons clusterings within the SCN. The system consists of interlocking transcription-translation-feedback-loops (TTFLs), the main one being organized in “positive elements” (Figure 1)—the transcription factors CLOCK and BMAL1—which form heterodimers and trigger the transcription of a number of genes containing, in their promoters, the so-called E/E' box motifs.<sup>11</sup> Amongst these genes, some encode the “negative elements” (Figure 1), the clock proteins PER1, PER2, PER3, CRY1 and CRY2. These also form heterodimers (PER/CRY) which, after a delayed accumulation process in the cytoplasm, are transported into the nucleus, where they inhibit the activity of CLOCK and BMAL1, and, therefore, temporarily block the transcription of their own genes.<sup>2</sup> This auto-repression is the core of the negative feedback loop. An overlapping loop in which CLOCK/BMAL1 heterodimers trigger transcription of the genes *Rev-erbs* and *Rors*, the products of which modulate the expression of BMAL1 in antiphase to the first loop, makes the circadian oscillation more robust (Figure 1).<sup>2</sup> It is now known that tens of additional proteins are also involved in the finer regulation of the TTFL, modulating chromatin structure,<sup>12,13</sup> and miRNAs also play a role in TTFL regulation.<sup>14</sup> Further, an additional core component of the TTFL has been identified and named *Chrono*, which seems to function as a core clock repressor (by inhibiting the activity of CLOCK/BMAL1) and is also under epigenetic control.<sup>15</sup>

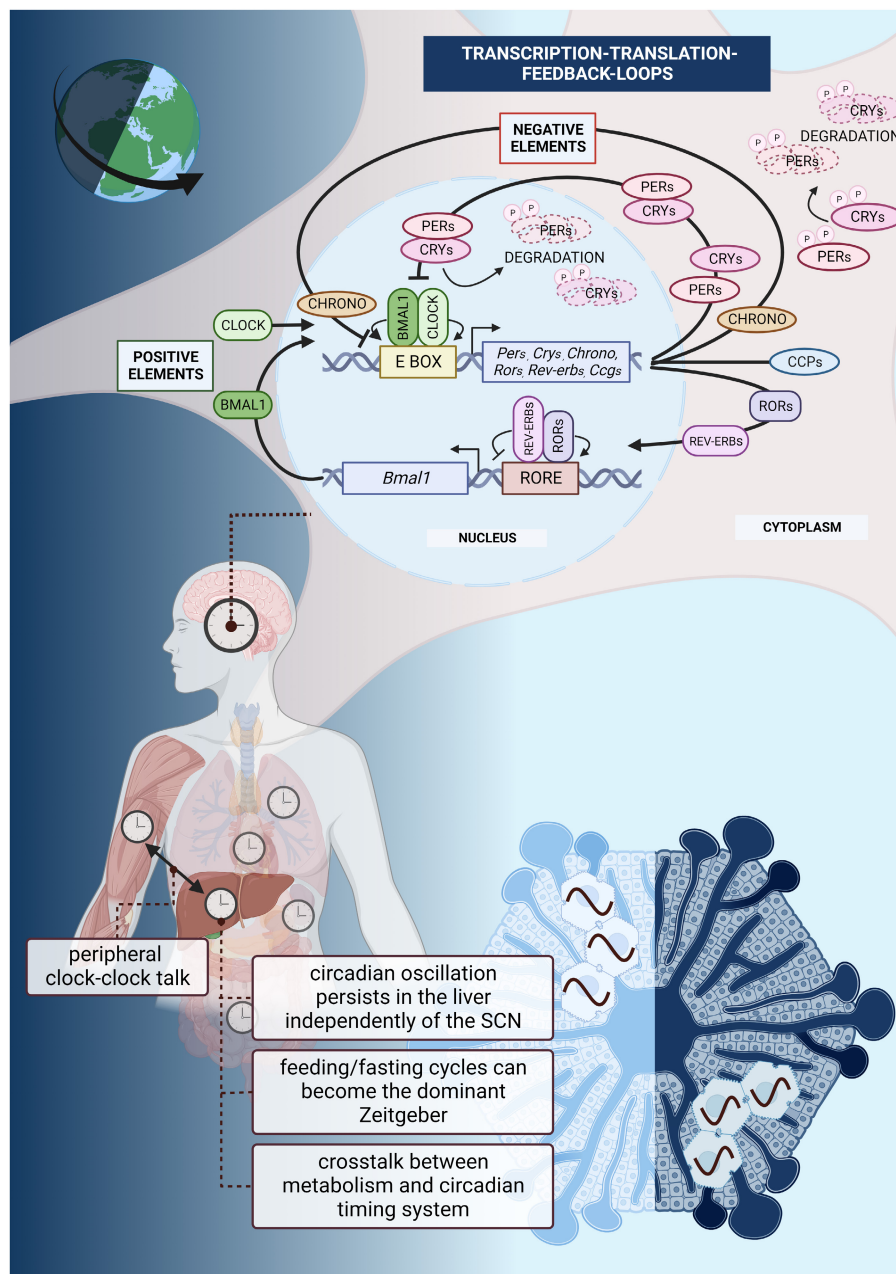
The circadian clock oscillation is qualified as *endogenous* as it is self-sustained even in the absence of any external stimuli, signals or cues. The term *phase* (or internal time) refers to the time when a feature of the circadian rhythm occurs (i.e. the peak or the nadir of the expression of a clock gene or protein), in relation to an external or otherwise fixed time reference such as solar time, civil time etc. In humans, reliable markers of the phase of the SCN clock are the hormones melatonin and cortisol and core body temperature.<sup>16</sup> Environmental signals (known as time givers or *Zeitgebers*), the most important being natural light, are capable of synchronizing the master clock with the day-night cycle generated by the rotation of the Earth around its axis (Figure 1). This process, which also encompasses cues such as fasting/feeding and physical or social activity patterns, is called *entrainment*.

### Key points

- The circadian timing system encompasses a master circadian clock in the hypothalamus and peripheral time-keepers in most organs.
- The liver clock is a robust oscillator and is at the centre of the cross-talk between the circadian timing system and metabolism. Abnormalities in such cross-talk play a role in the pathogenesis of fatty liver and the vicious circle underpinning its progression.
- Cirrhosis is associated with functional abnormalities of the liver and master clocks, and the master-peripheral clocks communication system.
- A better understanding of such abnormalities may help improve the management of decompensated cirrhosis and the outcomes of liver transplantation.

Major recent discoveries have further added to the understanding of the complexity and plasticity of the master SCN clock. First, it is now accepted that SCN astrocytes also play an important role in generating mammalian circadian rhythmicity. Ablation of the clock gene *Bmal1* in mice SCN astrocytes lengthens the period of the SCN clock and that of rest-activity behaviour.<sup>17</sup> Further, SCN astrocytes express clock genes in a rhythmic fashion, exhibit circadian  $\text{Ca}^{2+}$  oscillations with a broad night peak, in *quasi* antiphase with the sharper daytime peak observed in SCN neurons, and show circadian oscillations in glutamate release in phase with the  $\text{Ca}^{2+}$  peak.<sup>18</sup> Thus, SCN astrocytes function as a *bona fide* circadian clock, and the SCN overall output oscillation is the result of the interaction between the *quasi*-antiphasic oscillations of clock neurons and astrocytes. Secondly, neighbouring clock neurons have been shown to have synchronous but not necessarily simultaneous electrical and transcriptional/translational activity within different SCN areas (e.g. ventral and dorsal). There is also data to suggest that the phase difference between such activity may reflect the length of the *photoperiod*, that is, the light hours within the 24-h day.<sup>19</sup> This anatomical and functional SCN organization may provide organisms with information relevant to their adaptation to seasonal changes, suggesting that the master circadian clock may also have a calendar-type function.

Peripheral clocks in addition to the SCN have been located in virtually every organ and tissue of the body (Figure 1), including other regions of the brain.<sup>3</sup> When isolated, these maintain circadian rhythms of gene expression for days, indicating that the master SCN clock is not indispensable for short-term rhythmicity.<sup>20</sup> However, the SCN master clock is the only one to be directly regulated by the light-dark cycle and plays a hierarchical role, affecting the phase and synchrony of clock gene expression in peripheral clocks. This process occurs via the autonomic nervous system and humoral



**FIGURE 1** Schematic representation of the circadian timing system, including its hierarchy (bottom left: master and peripheral clocks), a hint at its being an adaptation to the Earth's rotation and therefore the light-dark cycle (top left), the core of the molecular mechanism generating the oscillation in the mammalian clock neurons (top right) and the specific features of the liver clock (bottom). With reference to the top right panel, the system consists of interlocking transcription-translation-feedback-loops. In the main one, the transcription factors CLOCK and BMAL1 form heterodimers and trigger the transcription of a number of genes containing, in their promoters, the so-called E box. Amongst these genes, some encode the clock proteins PER1, PER2, PER3, CRY1 and CRY2, which also form heterodimers (PER/CRY). After delayed accumulation in the cytoplasm, these enter the nucleus, where they inhibit the activity of CLOCK and BMAL1 and therefore temporarily block the transcription of their own genes. A synergic contribution to the inhibition of the CLOCK/BMAL1 transcription factors is provided by CHRONO. An overlapping loop in which CLOCK/BMAL1 heterodimers modulate the expression of the genes *Rev-erbs* and *Rors*, the products of which regulate the expression of BMAL1, acts in antiphase to the first loop.

signals.<sup>11</sup> Nonetheless, cues such as fasting/feeding, temperature and activity patterns can reset/phase-shift peripheral oscillators<sup>21</sup> (Figure 1). Finally, and despite an obvious hierarchical relationship, signals from peripheral clocks have been shown to generate SCN feedback in specific circumstances.<sup>22,23</sup>

### 3 | THE LIVER CLOCK

A large number of gastrointestinal processes, especially in the liver,<sup>24,25</sup> are under circadian control. These include, for example, nutrient uptake and processing, which align gastrointestinal function



to the cycling of food availability and supply.<sup>26</sup> This way the circadian timing system allows the organism to anticipate and prepare for food intake,<sup>27</sup> a still poorly characterized yet crucial function in gastrointestinal physiology.

An endogenous hepatic circadian oscillation was first identified in 1998,<sup>28</sup> when the concept of a peripheral clock was extremely innovative. The liver clock has since been shown to generate a very robust oscillation and, by comparison with the clock of other organs, to function for a relatively long time once isolated from the master SCN clock.<sup>20</sup> The molecular machinery that generates the circadian oscillation in peripheral clocks, and, therefore, also the liver clock, is almost identical to that of the SCN. However, the liver clock has a set of interesting features (Figure 1).

- Feeding cycles can become the dominant *Zeitgeber* for the liver clock, so much so that extreme changes in meal frequency (i.e., meals that are either very distant or very close to one another in time) can result in the acquisition of a remarkable degree of independence, if not complete uncoupling from the SCN control.<sup>29</sup> It has been speculated that this may have an adaptive value.
- The relevance of feeding cycles to the liver clock and their effect on its phase<sup>30</sup> underpin a crosstalk between metabolism and the circadian timing system.<sup>31</sup> This is essential because feeding/fasting patterns can modify some of the molecular characteristics of clock proteins, synchronizing the local clock to their periods. For example, the synthesis of Nicotinamide Adenine Dinucleotide (NAD<sup>+</sup>) involves the gene encoding phosphoribosyltransferase (NAMPT), which is regulated by the CLOCK/BMAL1 heterodimer.<sup>32</sup> Thus, the synthesis of NAD<sup>+</sup> is rhythmic, which results in the modulation of the activity of several proteins in the temporal domain.<sup>33</sup> Amongst these, sirtuin 1 deacetylase (SIRT1) affects, in turn, the transcriptional efficiency of the CLOCK/BMAL1 heterodimer and the stability of the PER2 clock protein, which both feedback to the circadian clock and contribute to the molecular oscillation affecting, for example, its phase and its amplitude.<sup>33</sup> Thus, the control of metabolite sensors like NAD<sup>+</sup> allows the circadian clock to be informed of the energy status of cells, and to reset oscillations based on metabolic signals.<sup>21</sup>
- Sinturel et al. have recently shown that circadian oscillations persist in the liver of freely moving mice without a functioning SCN or functioning oscillators in cells other than hepatocytes.<sup>34</sup> They have also shown that when these SCN-lesioned animals are maintained in constant conditions, the amplitude of their overall body rhythmicity is considerably reduced, suggesting that the SCN is still important to maintain synchrony amongst non-SCN clocks. The authors propose<sup>34</sup> that signals—as yet unidentified—from extra-SCN clocks are required for phase coherence of peripheral clock rhythms. This hypothesis is supported also by recent evidence that rhythmic liver gene expression depends also on intercellular communication between different liver cell types.<sup>35</sup> In line with what discussed above, this last study also demonstrates that hepatocyte clock-driven changes in the rhythmicity of the transcriptome and metabolome of other liver cells are modulated

by food availability.<sup>35</sup> Disease-related alterations in liver structure would be expected to compromise intrahepatic intercellular communication and synchrony, and also the response of the liver to signalling from the SCN and/or other oscillators.

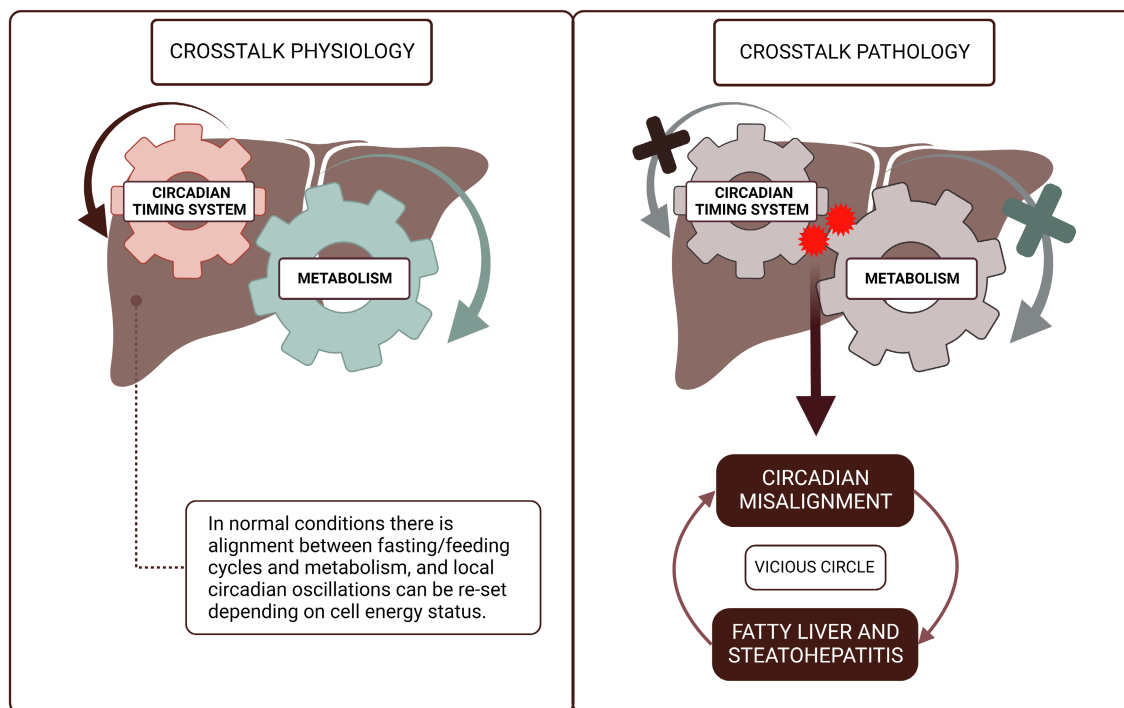
- Currently, the interaction between peripheral clocks is a hot topic in chronobiology. Whilst data are sparse, the most convincing ones come, yet again, from studying the liver clock. Greco et al.<sup>36</sup> have recently demonstrated that the liver clock and feeding patterns are sufficient for temporal carbohydrate homeostasis. However, liver rhythms tied to redox and lipid metabolism require the integrity of the skeletal muscle clock, suggesting peripheral clock-clock talk (Figure 1). This observation has implications for the relationship between cirrhosis and sarcopenia (*vide infra*). Along similar lines, it is expected that improved methods to study circadian rhythmicity in the human gut microbiome<sup>37</sup> will allow to perform studies on the interaction between the liver and gut/microbiota clocks, possibly offering more complete explanations for the therapeutic effects of microbiota manipulation in liver diseases and their complications.<sup>38</sup>
- Strikingly and based on a single set of in vivo experiments in mice, it has even been hypothesized that a small portion of the autonomous hepatic oscillation (i.e., in the absence of the SCN) may directly depend on the light–dark cycle.<sup>23</sup> Ray et al.<sup>39</sup> have also reported that in animals lacking the core clock component BMAL1, liver slices (previously synchronized with dexamethasone) retained a 24-h rhythm of gene expression, protein abundance and protein phosphorylation for 2–3 days in the absence of any light or temperature cues. The authors suggest that such oscillations might result from the interplay between Erythroblast Transformation Specific (ETS) family transcription factors and redox oscillations for transcriptional and non-transcriptional regulation, respectively. Both the above observations need to be confirmed but remain intriguing.
- Information on hepatic temporal regulation has recently been coupled with the much better-known information on hepatic spatial organization, or zonation (i.e., the complex microarchitecture that allows both compartmentalisation and interplay between anabolic and catabolic activities within the liver), which has traditionally been analysed as a static phenomenon. By use of single-cell RNA sequencing, it has been demonstrated that the expression of many genes in the liver is both zoned and circadian, in most instances with multiplicative effects of the space–time interplay.<sup>40</sup> Such genes are implicated in lipid, carbohydrate and amino acid metabolism, and also in processes involving protein chaperones.<sup>40</sup> The effects of diseases affecting liver histology/anatomy to varying degrees on such a complex spatio-temporal organization are difficult to predict but obviously important.

Finally, whilst *ultradian rhythmicity*, i.e. intrinsic biological rhythmicity with a period shorter than 24 h, is considerably less explored than circadian rhythmicity and also beyond the scope of this review, it should be highlighted that evidence is emerging on the role of both 8- and 12-h rhythms—which cycle at the second and third harmonics of circadian frequency—in controlling liver function (for a review, please refer to Ref. [41]).

#### 4 | MISALIGNMENT AND FATTY LIVER—A TOXIC RELATIONSHIP

The term *misalignment* refers to situations in which entrainment (i.e., the process through which the endogenous circadian timing system synchronizes with the environment, *vide supra*) is compromised.<sup>42</sup> This is generally because of weak or inefficient *Zeitgebers*, often in relation to a set of time constraints imposed by the society we live in, which are collectively known as the *social clock*.<sup>43</sup> These include the times around which schools, firms, offices, commercial activities and transport are organized, but also the presence of time zones and, for part of the year, daylight saving time.<sup>43</sup> The availability of artificial light over the 24 h, together with the widespread use of devices such as televisions, computers and smartphones in the evening and night hours has also profoundly modified the timing of exposure to the natural light–dark cycle, resulting in poor light–dark hygiene.<sup>44,45</sup> Misalignment of varying degrees and different origins results in shorter and worse sleep, and it has also been associated with an increased risk of systemic hypertension,<sup>46</sup> overweight/obesity,<sup>47</sup> cardiovascular events,<sup>5</sup> reduced fertility, the likelihood of misusing cigarettes and alcohol,<sup>6</sup> and certain types of cancer.<sup>7</sup> These risks are likely to be mediated, at least in part, by shortened/disturbed sleep itself and have been more consistently associated with the most severe and prolonged forms of misalignment, such as night shift work.<sup>5,7</sup> However, other and generally milder forms of misalignment (i.e., those related to extreme positions within a time zone, daylight saving time, a society functioning mostly in the morning and thus penalizing individuals who are genetically inclined to delayed sleep–wake habits, i.e., late *chronotypes*) are very prevalent in the Western world, and it has been hypothesized that they may contribute to the current metabolic syndrome epidemic<sup>6</sup> and, in turn, that of metabolic (dysfunction) associated fatty liver disease (MAFLD). It should be highlighted how isolating any contribution of misalignment to an increased risk of MAFLD or alcohol-related fatty liver/steatohepatitis is extremely difficult phenotypically because of the confounding effects of sleep–wake abnormalities of other origins, the most obvious example being obstructive sleep apnea (OSA). OSA is associated with the development and evolution of MAFLD, independently of obesity or other common risk factors.<sup>48</sup> The OSA–MAFLD association is thought to relate to the degree of nocturnal hypoxemia, which leads to tissue hypoxia.<sup>49</sup> In turn, tissue hypoxia has been associated with insulin resistance, atherosclerosis, dysfunction in hepatic lipid metabolism and hepatic steatosis.<sup>48</sup> Despite significant confounding, a number of human studies have started to highlight some circadian-type abnormalities within the sleep–wake profile of MAFLD patients<sup>50</sup> and an increased prevalence of MAFLD in misaligned (albeit widely qualified as exhibiting “mistimed sleep, late sleep or irregular chronotype”) compared to non-misaligned individuals has also been reported.<sup>51</sup> Some of the pathophysiological mechanisms potentially underpinning these epidemiological observations are also starting to emerge. Several human studies using lipidomics have documented that hundreds of plasma lipids are regulated in the circadian domain, including fatty acids, triglycerides, glycerophospholipids, sterol lipids and sphingolipids.<sup>52</sup> A high-fat diet leads to significant remodelling of

the liver clock output, disrupting normal circadian cycles and, somewhat unexpectedly, also producing *de novo* oscillating transcripts.<sup>53</sup> The mechanisms underlying this remodelling involve impairment in CLOCK/BMAL1 chromatin recruitment and the rhythmic activation of alternative pathways through the transcriptional regulator peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ , also known as the glitazone reverse insulin resistance receptor).<sup>53</sup> The experiment also demonstrated how a short, three-day exposure to the high-fat diet was sufficient to start molecular reprogramming and how switching to a normal diet after a longer period of high-fat diet lead to the resolution of the diet-induced perturbations.<sup>53,54</sup> This may have implications, for example, for the relationships between binge eating and MAFLD.<sup>55</sup> Another clinically relevant hypothesis is the possibility that glitazones, which are pharmacological PPAR $\gamma$  ligands used in the management of diabetes, might be able to mimic the PPAR $\gamma$  effects described above.<sup>54</sup> Another study documented how diet-induced obesity caused remodelling of circadian enhancer activity in the mouse liver, triggering detectable oscillations in fatty acid synthesis and oxidation.<sup>56</sup> The same diet caused a high amplitude circadian rhythm in PPAR $\alpha$ , which was also required for fatty acid oxidation.<sup>56</sup> Of note, peroxisome proliferator-activated receptors (PPARs) are under study as drug targets for steatohepatitis.<sup>57</sup> One human study has shown that a significant proportion of circulating metabolites implicated in food metabolism uncouple from the phase of the SCN after simulated shift work schedules, losing rhythmicity or acquiring rhythmicity that appears to be driven by the behavioural schedule (i.e., that of shift work, sleep/wake and feeding/fasting patterns).<sup>58</sup> Further, by use of unbiased high-throughput proteomics, acetylomics and circadian transcriptomics, Gaucher and co-workers<sup>59</sup> have shown how acute and chronic alcohol consumption differentially reprogram circadian gene expression in the mouse liver. In turn, genetic disruption of the liver clock worsens both abnormalities in lipid metabolism and steatosis in mice fed with alcohol.<sup>60</sup> Randomized clinical trials on the effects of modulating meal timing (*chrononutrition*) on weight, adiposity, energy expenditure and hormones/metabolites circadian profiles are sparse but promising,<sup>61</sup> and suggest that circadian hygiene, especially in relation to meal timing, may come to play an important role in preventing MAFLD, and in breaking the vicious circle through which abnormalities in the cross-talk between metabolism and the circadian timing system sustain the onset and progression of fatty liver and steatohepatitis (Figure 2). Finally, and as for the association between MAFLD and hepatocellular carcinoma (HCC) [also in the absence of significant liver fibrosis/cirrhosis<sup>62</sup>], a single but large and apparently solid US study has documented that an increase in East to West longitude within a given time zone is associated with a statistically significant increase in HCC risk in individuals under 65 years of age,<sup>63</sup> adding HCC to the list of neoplasms for which misalignment seems to represent a risk factor.<sup>64</sup> Of note, the impact on hepatocarcinogenesis depended on misalignment from residing to the West of a time zone, in agreement with the concept that jet-lag East, and thus the attempt to move endogenous timing forward, is generally more difficult and more unpleasant (more pronounced and more prolonged jet-lag symptoms) than the opposite process.<sup>65</sup>



**FIGURE 2** Physiology (left) and pathology (right) of the crosstalk between the circadian timing system and metabolism, which occurs in the liver and may contribute to the pathophysiology of fatty liver and steatohepatitis.

## 5 | CIRCADIAN DISRUPTION IN CIRRHOSIS

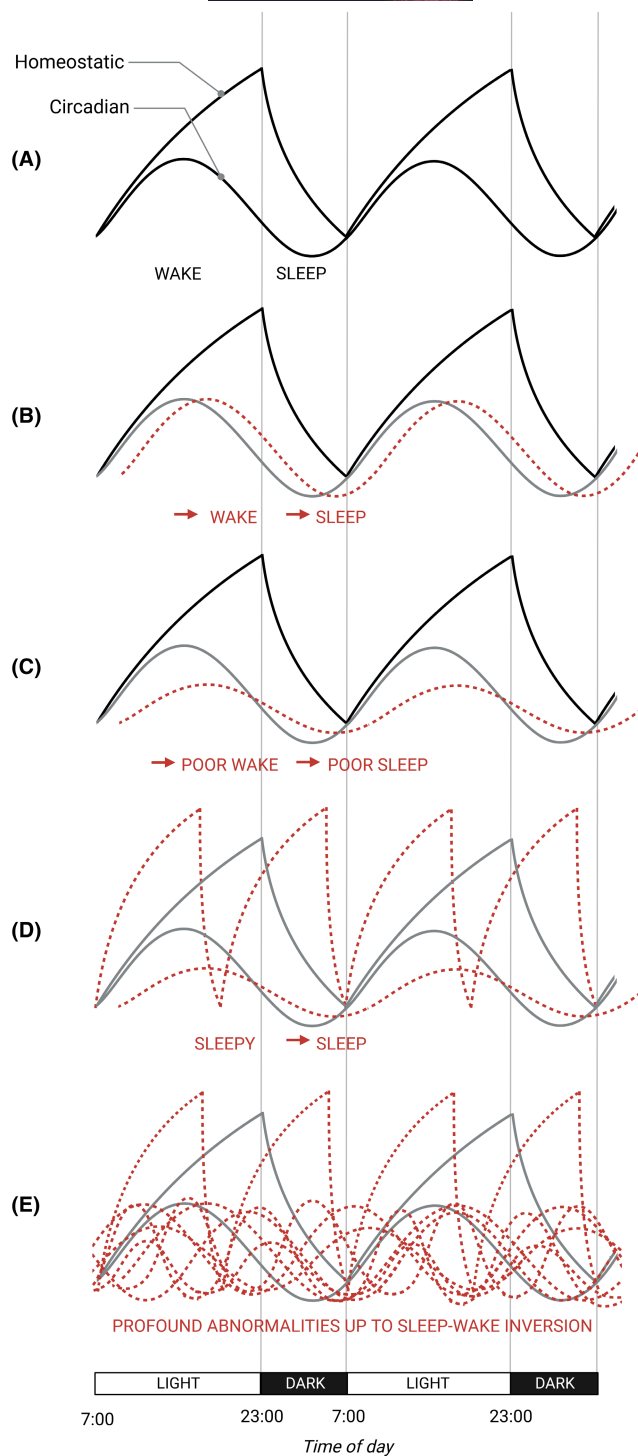
Patients with cirrhosis exhibit several circadian abnormalities. These were originally characterized with a view to identify and hopefully correct any circadian impairment that may contribute to the sleep–wake disturbances these patients commonly report, i.e. difficulties falling asleep, frequent night awakenings, delayed sleep–wake habits and excessive daytime sleepiness up to sleep–wake inversion.<sup>66–68</sup> Such sleep–wake disturbances are difficult to manage with standard hypnotics both because of delays in hepatic metabolism and enhanced sensitivity of patients with cirrhosis to psychoactive medication.<sup>69,70</sup> Further, whilst all sleep–wake abnormalities exhibited by patients with cirrhosis were traditionally considered to be part of the hepatic encephalopathy (HE) syndrome, in more recent years it has become clear that only excessive daytime sleepiness and the striking phenomenon of sleep–wake inversion is associated with HE of varying severity.<sup>71,72</sup> By contrast, insomnia seems to be more of a feature of cirrhosis itself, also by comparison with other chronic diseases of similar severity<sup>73,74</sup> and even in the absence of any cognitive impairment. It is however obvious that as sleep–wake disturbance impinges on cognitive performance, the relationship between sleep–wake disturbance and HE will always be difficult to study.<sup>75</sup> The circadian phenotype of patients with cirrhosis can be summarized as follows:

- Delayed sleep–wake habits (60–90 min), which tend to associate with worse sleep quality.<sup>73,76</sup> Delayed sleep–wake habits seem to be independent of the presence/degree of HE<sup>73</sup> and most likely also of the

aetiology of cirrhosis<sup>76,77</sup> but solid data on the latter issue are lacking.

- Reduced SCN sensitivity to dark/light cues plus minor impairment in hepatic clearance of melatonin, mostly at night, when the levels of the hormone are high.<sup>78–80</sup> The combination of the above determines an overall delay in the melatonin peak and abnormally high daytime melatonin levels. Similar abnormalities are observed in the 24-h rhythm of cortisol,<sup>81</sup> and they parallel the delays in the sleep–wake patterns.<sup>76</sup> The beneficial effect of the administration of strong morning light (*chronotherapy*) on sleep timing, sleep quality and urinary melatonin rhythmicity<sup>82–84</sup> also supports the concept that central circadian disruption may underpin part of the sleep–wake abnormalities exhibited by patients with cirrhosis. Since it is now accepted that the SCN overall output oscillation is the result of the *quasi* antiphasic oscillation of clock neurons and astrocytes<sup>18</sup> and it is also well established that hyperammonaemia causes both functional and morphological astrocyte abnormalities,<sup>85</sup> we have recently hypothesized and demonstrated that hyperammonaemia acts directly on the SCN to disrupt its output oscillation and, in turn, daily rhythms in physiology and behaviour.<sup>86</sup> Critically, these effects were reversed by ammonia removal or by glutamate receptor antagonists.<sup>86</sup>
- Abnormal skin temperature, with high average values and limited variation over the 24 h,<sup>87</sup> most likely in relation to the peripheral vasodilation/abnormalities in blood distribution which characterize the disease. This translates into an inability to prepare for sleep with the physiological mechanisms of vasodilation and warming of the extremities and has been shown to be associated with increased sleep latency.<sup>87</sup> Whilst skin temperature is not a circadian parameter and cannot be used as an equivalent of core body temperature, when measured at multiple sites it provides a





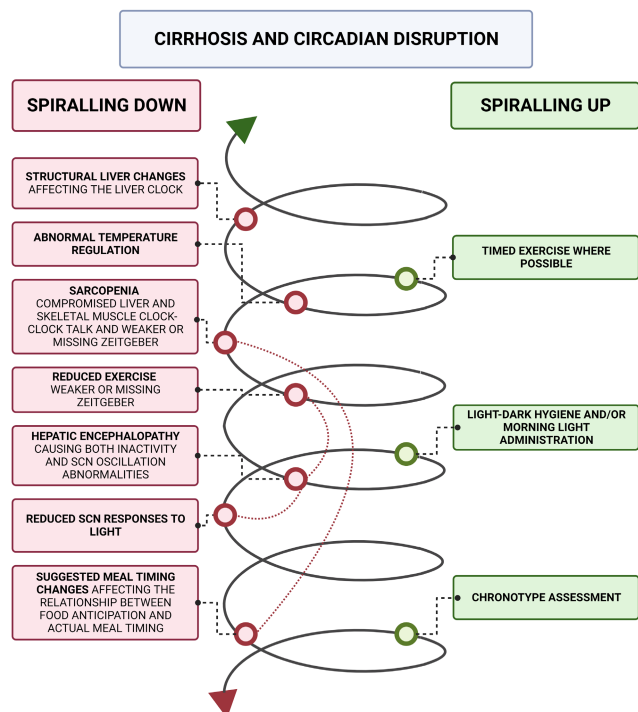
reliable estimate of heat loss, which plays an important part in the circadian variation of core body temperature. We were able to explore and confirm the relationship between skin and core body temperature changes in bile duct-ligated rats.<sup>88</sup>

As our understanding of the circadian timing system and its relationship with *homeostatic sleep regulation* (i.e., the phenomenon mediated by the neurotransmitter adenosine whereby our propensity to sleep increases over the waking hours, regardless of the time of day<sup>89,90</sup>) evolves, it becomes more and more difficult to attribute

**FIGURE 3** (A) Normal interaction between the circadian oscillation in sleep propensity over the 24 h and the increase in homeostatic sleep pressure (i.e., the increase in sleep propensity that accumulates during the waking hours) in a healthy person: the greater the distance between the two curves (23:00), the higher the sleep propensity/likelihood of falling asleep (adapted from Ref. [89]). (B) Abnormal interaction between the homeostatic regulation (black line) and the delayed circadian rhythm (red broken line) in a patient with cirrhosis with or without HE; grey line: reference circadian oscillation in the healthy population. The lack of synchrony between the two processes leads to a delayed sleep phase-type sleep disorder, which could contribute to the observed difficulties in commencing and maintaining sleep (adapted from Ref. [95]). (C) Abnormal interaction between the homeostatic regulation (black line) and the delayed circadian rhythm (red broken line) in a patient with cirrhosis with HE, in whom daytime somnolence, inactivity, reduced muscle mass etc. reduce the amplitude of the delayed circadian oscillation; grey line: reference circadian oscillation in the healthy population. (D) Abnormal interaction between homeostatic fluctuations (red broken line) and delayed plus blunted circadian rhythmicity (red broken line) in a patient with cirrhosis and HE; grey lines: reference circadian oscillation and homeostatic build-up in the healthy population. Hyperammonaemia/HE results in magnified and short-lived adenosine responses to the build-up of sleep pressure during the waking hours. This may translate into an inability to generate slow-wave, restorative sleep and a less efficient recovery from sleep deprivation (adapted from Ref. [93]). (E) Abnormal interaction between homeostatic fluctuations (red broken line) and profoundly desynchronised circadian rhythm (multiple red broken lines) in a patient with cirrhosis and a bout of severe HE; grey lines: reference circadian oscillation and homeostatic build-up in the healthy population. Hyperammonaemia/HE results in magnified and short-lived adenosine responses and completely desynchronises the output oscillation from the master clock. This results in profound sleep-wake abnormalities, up to sleep-wake inversion.

any specific circadian/sleep-wake feature observed in a patient with cirrhosis to cirrhosis *per se*, HE or their combination (Figure 3). It would seem reasonable to attribute mostly to HE excessive daytime sleepiness,<sup>72,91</sup> which also results in inactivity and thus in weakened *Zeitgebers* (Figures 3 and 4), impaired light perception and consequent melatonin/cortisol rhythm abnormalities,<sup>80,81</sup> desynchronisation of the master circadian clock output,<sup>86</sup> abnormalities in the homeostatic response to sleep deprivation<sup>92</sup> and in the ability to produce deep, restorative sleep<sup>72</sup> (Figure 3). It should also be highlighted that patients with cirrhosis - and those with HE in particular - are extremely sensitive to psychoactive medication,<sup>69</sup> including that used in attempts to treat their sleep-wake abnormalities, which often result in prolonged sedation and paradoxical reactions<sup>93</sup> or bouts of HE,<sup>70</sup> sustaining sleep-wake and possibly also circadian abnormalities in a vicious circle. Finally, alcohol itself and drugs that are commonly used in patients with cirrhosis, for example beta-blockers, are known to affect melatonin synthesis,<sup>94</sup> potentially worsening any HE-related abnormalities in the rhythm of this crucial circadian hormone.

There are further, mostly unexplored mechanisms through which cirrhosis and especially decompensated cirrhosis might affect the circadian system (Figure 4). For example, abnormal liver architecture/



**FIGURE 4** Schematic representation of the circadian abnormalities (proven or hypothetical) which characterize and then self-sustain in patients with cirrhosis (red, left and spiralling down) and a set of simple tests or hygienic measures that may help manage them (green, right and spiralling up). The dashed red connections between red circles highlight proven or hypothetical pathophysiological relationships.

vascularization may lead to liver clock dysfunction. Compromised glycogen storage and protein synthesis may affect the physiological, rhythmic oscillations in glucose/protein metabolism which anticipate and follow meals. Sympathetic/parasympathetic imbalance may compromise the autonomic transmission of signals from the SCN to the liver clock. In addition, malnutrition and sarcopenia lead to a reduced ability for physical activity and exercise,<sup>75</sup> which in healthy individuals act as accessory *Zeitgebers* for the circadian timing system. Similarly, HE reduces daytime alertness, weakening the wake-sleep cycle. The combination of all such mechanisms<sup>95</sup> may lead to an abnormal SCN-liver clock interaction, and possibly also to the uncoupling of the liver clock from the SCN. There is also evidence that stressors such as sleep fragmentation and sleep deprivation, which are extremely common in patients with cirrhosis, impinge on the 24-h rhythm of the healthy human metabolome<sup>58</sup> and that the liver clock also responds to signals from the microbiome,<sup>96</sup> which is heavily modified both by cirrhosis itself<sup>97</sup> and by the treatment of its complications.<sup>98</sup> Hepatic xenobiotic detoxification is also under temporal control, which provides some bases for the dosing time-dependence of drug efficacy and toxicity in otherwise healthy individuals.<sup>99</sup> This is likely to be relevant to the management of patients with cirrhosis and acute liver failure, who are on multiple drugs with stand-alone and combined pharmacokinetics/pharmacodynamics which become very difficult to predict.<sup>100</sup>

Finally, the relationship between malnutrition/sarcopenia which is frequently associated with decompensated cirrhosis and circadian disruption deserves special attention (Figure 4). The onset of malnutrition/sarcopenia marks a defining point in the evolution of cirrhosis, with all its major complications (HE, uncontrolled ascites, spontaneous bacterial peritonitis and hepatorenal syndrome) being more common in malnourished patients.<sup>101</sup> Malnutrition is also associated with the severity of cirrhosis, so much so that the original version<sup>102</sup> of the Child-Pugh score<sup>103</sup> included a nutritional status index, which was subsequently dropped because its assessment was perceived to be too operator-dependent. Ultimately, malnutrition/sarcopenia seems to be more of a feature than a complication of cirrhosis, and its aetiology has been generally attributed to combined abnormalities in oral intake, absorption and metabolism of nutrients.<sup>104</sup> The possibility that it may also relate to liver clock dysfunction and central-peripheral clock desynchrony has remained substantially unexplored. However, given the features of the liver clock we have already discussed, and the structural abnormalities that characterize a cirrhotic liver, it seems a plausible hypothesis. Whichever its exact origin, a premature switch from satiety to fasting state metabolism, and thus from carbohydrate to amino acid utilization for energy production, ultimately sustains the hypercatabolic state which characterizes cirrhosis. Accordingly, commonly utilized treatment strategies have been focused on increasing caloric intake and/or shortening the interval between meals.<sup>105</sup> The main emphasis has been on late-evening snacks, which are meant to cover the longest inter-meal interval (evening meal to breakfast), and thus avoid the overnight utilization of amino acids as energy substrates.<sup>106,107</sup> Accordingly, malnourished patients with cirrhosis are encouraged to avoid long periods of fasting and to split their food intake into small, frequent meals. This is difficult operatively, as these patients struggle to cope with three main meals and three snacks (frequent hyporexia, dysgeusia, early satiety and prescription of unpalatable diets<sup>104</sup>), and has not been consistently shown to be beneficial, with the exception of iatrogenic malnutrition deriving from inappropriate or excessively strict dietary prescriptions.<sup>108</sup> A circadian perspective of fasting/feeding patterns may help to explain the difficulties in managing malnutrition in advanced liver disease. Firstly, and as already discussed, the circadian timing system allows to anticipate food intake and the timing of such anticipatory/preparatory behaviour is not easily modified. Further, it has been demonstrated that in healthy individuals a 5-h delay in meal timing resulted in a comparable delay in the phase of the rhythm of glucose in laboratory conditions. Such abnormal glucose rhythms were not accompanied by changes in markers of the phase of the master clock, or in the rhythms of insulin and triglycerides.<sup>109</sup> Therefore meal timing seems to affect glucose more than lipid metabolism, and to dissociate their rhythmicity. The study also concluded that the absence of changes in SCN phase markers in relation to changes in meal timing suggests that feeding patterns can synchronize peripheral clocks in humans.<sup>109</sup> In another human experiment studying the temporal response of lipids and hepatic proteins to combined light and food cues, greater shifts were observed for lipids and hepatic

proteins compared to melatonin, with albumin and triglyceride exhibiting peak times close to that of melatonin, and cholesterol and high-density lipoprotein being offset by approximately 12 h.<sup>110</sup> It is therefore reasonable to hypothesise that in patients with cirrhosis changes in meal timing, and especially the introduction of the evening snack, may have rather unpredictable and possibly even damaging effects on an already very deranged carbohydrate and protein metabolism. Finally, the recent observation<sup>36</sup> that liver rhythms tied to redox and lipid metabolism require the integrity of the skeletal muscle clock offers a novel, potential explanation for the vicious circle between the onset of sarcopenia and the worsening in liver function that is so commonly observed in decompensated cirrhosis.

## 6 | LIVER TRANSPLANTATION WITHIN A CIRCADIAN PERSPECTIVE

To date, limited attention has been devoted to liver transplantation from a circadian perspective. However, the liver clock is an extremely robust autonomous oscillator,<sup>39</sup> thus the timing of transplantation may modulate transplant outcomes. Further, the phase of a transplanted liver may be more or less close to that of the recipient's, most likely impinging on the time it will take for the transplanted liver to be "captured" by the recipient's SCN. This process may be slowed or impaired also by the fact that the transplanted liver is denervated,

and thus receives SCN signals only via humoral pathways, and not via both humoral and neural pathways as in healthy individuals. In addition, this process generally takes place in an intensive care environment, where *Zeitgebers* are profoundly abnormal (i.e., continuous light, continuous feeding etc.). It is more than likely that all the above will impinge, at least to some extent, on immediate and mid-term transplant prognosis. Also, it is well known to chronobiologists that dexamethasone synchronizes the phase of circadian liver gene expression across the 24 h,<sup>111</sup> thus the use of steroids prior to, during or immediately after transplantation may have effects other than the anti-inflammatory and immunosuppressant expected ones, and could probably be timed in a constructive fashion. All these issues are difficult but not impossible to study in the clinical setting, and even retrospective, collaborative studies might be useful.

## 7 | CONCLUSIONS AND FUTURE WORK

In conclusion, it would appear that both a circadian perspective in analysing the pathophysiology of liver disease and the application of chronobiological principles to its treatment may contribute to the understanding and management of MAFLD,<sup>112</sup> the complications of cirrhosis and those of liver transplantation (Table 1). Another interesting application, which has not been covered in this review, may be the treatment of pruritus and fatigue in primary biliary cholangitis,

TABLE 1 Future research agenda

| Review section                                 | Topics for future studies                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| The liver clock                                | How disease-related structural changes in liver architecture affect intercellular communication and synchrony within the liver<br>How disease-related structural changes in liver architecture affect interactions with the master clock and other peripheral clocks<br>Liver and gut/microbiota clock interaction, also in relation to gut/microbiota clock modulation as part of liver disease management                                                                                                                                                                                        |
| Misalignment and fatty liver                   | Baseline circadian profile of individuals who go on to develop MAFLD/NASH over time<br>Long-term effects on MAFLD/its evolution of circadian hygiene measures, particularly in relation to food and exercise timing<br>Time-course of the metabolomic, lipidomic and acetyloomic profiles of MAFLD patients<br>Role of misalignment in the development of HCC in MAFLD/NASH                                                                                                                                                                                                                        |
| Circadian disruption in cirrhosis              | Relationship between delayed sleep phase syndrome features and the aetiology of cirrhosis<br>Effects of timed melatonin administration (with or without timed light administration) on delayed sleep phase syndrome<br>Effects of proximal temperature modulation on delayed sleep onset<br>Effects of timed food administration on nutritional status and sarcopenia<br>Circadian and 24-h metabolomic effects of the prescribed evening snack in malnourished patients with cirrhosis                                                                                                            |
| Circadian disruption in hepatic encephalopathy | Exact relationship between circadian and cognitive impairment by interventional, controlled studies<br>Effects of timed melatonin administration (with or without timed light administration) on sleepiness and cognitive performance<br>Relative role of the neuronal and astrocytic components of the master clock in the response to hyperammonaemia<br>Relative role of circadian and homeostatic sleep regulation in the pathogenesis of sleep-wake inversion<br>Role of the impairment in liver and muscle clock-clock talk in the relationship between sarcopenia and cognitive performance |
| Circadian perspective of liver transplantation | Transplantation outcomes in relation to: <ul style="list-style-type: none"> <li>• timing of transplantation</li> <li>• chronotype of donor and recipient</li> <li>• circadian-friendly intensive care/general wards versus standard of care</li> <li>• timed steroid utilization prior to and after transplantation</li> </ul>                                                                                                                                                                                                                                                                     |

which has been shown to exhibit diurnal variation<sup>113</sup> and also to benefit from light therapy.<sup>84</sup> Ultimately, the specific features of the liver clock and those of the sleep–wake profile of patients with cirrhosis have already made hepatology a well-established *niche* model for the study of disease-related circadian abnormalities. As knowledge continues to grow on the interactions between peripheral clocks, tools start to become available for an easier assessment of human internal circadian time<sup>114</sup> and chrononutrition becomes a discipline with a clearer pathophysiological basis<sup>115</sup> and better-established protocols, hepatology may also become an ideal field to test the value of circadian hygiene advice and that of chrononutrition in both disease prevention and disease management.<sup>116</sup> Like all translational processes, this will require interaction between different disciplines and different professions, and a considerable amount of patience, goodwill and open-mindedness from all parts involved. Hepatologists will need to learn to factor time into their pathophysiological and clinical reasoning more and more, and chronobiologists will need to accept that clinical studies in any healthcare setting will always be less controlled and “noisier” than the ones they are used to.<sup>117</sup> Still, this process has already started, and it only needs to be made smoother and faster, for the benefit of both liver patients and society at large.

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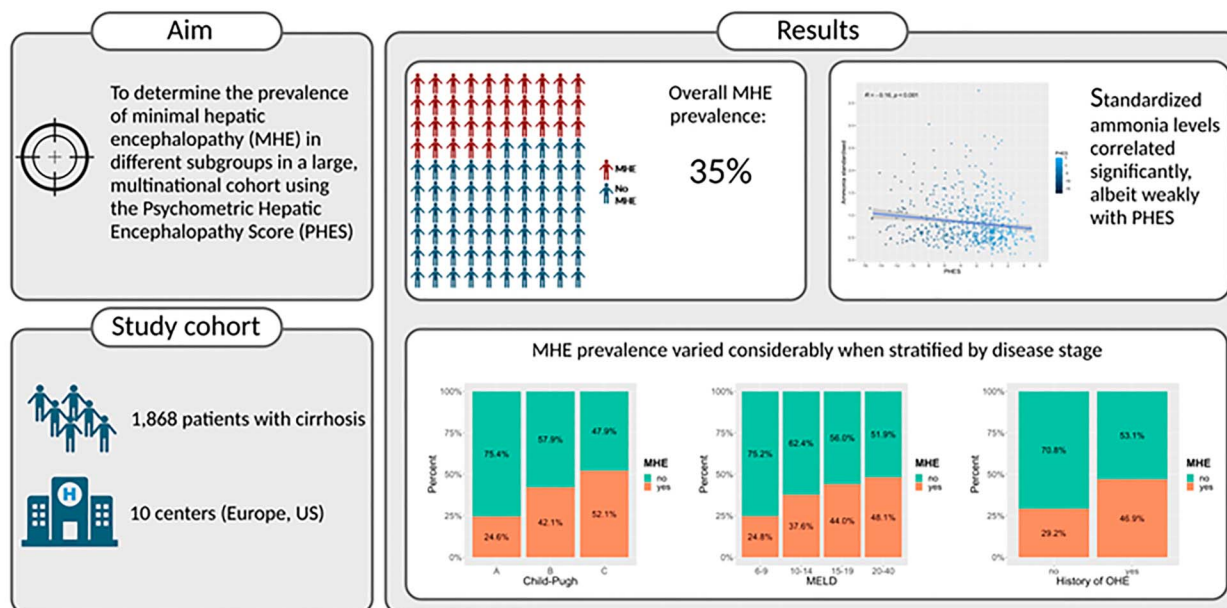
# Prevalence of Minimal Hepatic Encephalopathy in Patients With Liver Cirrhosis: A Multicenter Study

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**INTRODUCTION:** The prevalence of minimal hepatic encephalopathy (MHE), in particular in different subgroups, remains unknown. This study aimed to analyze the prevalence of MHE in different subgroups to identify patients at high risk and to pave the way for personalized screening approaches.

**METHODS:** In this study, data of patients recruited at 10 centers across Europe and the United States were analyzed. Only patients without clinical signs of hepatic encephalopathy were included. MHE was detected using the Psychometric Hepatic Encephalopathy Score (PHES, cut-off  $< \text{or} \leq -4$  depending on local norms). Clinical and demographic characteristics of the patients were assessed and analyzed.

## Prevalence of MHE in patients with cirrhosis



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**RESULTS:** In total, 1,868 patients with cirrhosis with a median model for end-stage liver disease (MELD) of 11 were analyzed (Child-Pugh [CP] stages: A 46%, B 42%, and C 12%). In the total cohort, MHE was detected by PHES in 650 patients (35%). After excluding patients with a history of overt hepatic encephalopathy, the prevalence of MHE was 29%. In subgroup analyses, the prevalence of MHE in patients with CP A was low (25%), whereas it was high in CP B or C (42% and 52%). In patients with a MELD score <10, the prevalence of MHE was only 25%, but it was 48% in patients with a MELD score ≥20. Standardized ammonia levels (ammonia level/upper limit of normal of each center) correlated significantly, albeit weakly with PHES (Spearman  $\rho = -0.16$ ,  $P < 0.001$ ).

**DISCUSSION:** The prevalence of MHE in patients with cirrhosis was high but varied substantially between diseases stages. These data may pave the way for more individualized MHE screening approaches.

**KEYWORDS:** MHE; frequency; point prevalence; PSE syndrome test; PHES; Psychometric Hepatic Encephalopathy Score

**SUPPLEMENTARY MATERIAL** accompanies this paper at <http://links.lww.com/AJG/C914>

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## INTRODUCTION

Hepatic encephalopathy (HE) is one of the most frequent and serious complications in patients with cirrhosis. HE manifests with a wide spectrum of neuropsychiatric abnormalities caused by liver insufficiency and/or portosystemic shunting (1) and can be roughly divided into 2 groups according to current guidelines (2). Overt HE (OHE) is the more severe form and defined by clinically detectable symptoms. By contrast, covert HE (CHE) is an umbrella term combining the 2 lowest grades, named minimal HE (MHE) and HE grade 1. Per definition, MHE/CHE can only be diagnosed using specialized tests. The current gold standard for the detection of MHE is the psychometric hepatic encephalopathy score (PHES). PHES is a paper-and-pencil testing battery containing 5 subtests and has been validated in several countries (3). Although MHE is associated with impaired quality of life, falls, increased risk of road traffic accidents, and a poorer prognosis, studies demonstrate that physicians frequently do not test for MHE in routine clinical practice (4–9). This may be explained by the time-consuming nature of several testing strategies and, in particular, a lack of information on whom to screen. Thus, reliable data on the prevalence of MHE in different subgroups are needed.

The true prevalence of MHE in patients with cirrhosis is currently unknown and frequently estimated to range from 30% to 70% (1,10). This large variance in prevalence data is explained by the analysis of predominantly small and heterogeneous cohorts, plus by the use of different tests and different thresholds. Currently, there is only 1 large study including 1,114 patients from 20 Indian centers, where the pooled prevalence of MHE was 59.7%. However, this study is limited by only including patients from 1 country and a heterogeneous cohort with various disease stages (11). Therefore, it was the aim of this study to provide an overview on the prevalence of MHE in a large multinational cohort of patients with cirrhosis and to report more specific estimates of the MHE prevalence across different disease stages and etiologies of cirrhosis. These data could be used to implement further psychometric testing for patients at high risk of MHE in a precision medicine setting.

## METHODS

This retrospective study analyzed data of 1,868 patients, who underwent MHE testing with PHES at the following centers with expertise in diagnosing MHE: Mainz, Siegen, Lübeck, Hannover,

Halle (all Germany), Paris (France), Padua, Rome (both Italy), Esbjerg (Denmark), and Ann Arbor (Michigan, USA). At each center, the primary etiology of underlying liver disease was determined according to clinical, serological, and histological findings. Diagnosis of cirrhosis was established by histology, conclusive appearance on ultrasound, elastography or radiological imaging, endoscopic features of portal hypertension, or medical history. The severity of liver disease was determined by calculating the model for end-stage liver disease (MELD), Child-Pugh (CP) scores, and the albumin-bilirubin (ALBI) scores (12–14). MELD scores were calculated centrally using the original MELD formula of Kamath et al (12). The 3 ALBI grades were defined by cut-offs of  $\leq -2.60$  (grade 1),  $> -2.60$  to  $\leq -1.39$  (grade 2), and  $> -1.39$  (grade 3) (14).

Patients were not included in this study, if they had a history of any other disease leading to mental alterations (e.g., dementia or a history of stroke). At some centers, patients with self-reported ongoing alcohol consumption were included for this study provided that they were not under the effects of alcohol when tested with PHES. Other exclusion criteria varied between centers and are detailed for each center in Supplementary Table S1 (see Supplementary Digital Content 1, <http://links.lww.com/AJG/C914>).

Some data have been published in previous studies (6,15).

## Diagnosis of MHE

All patients were examined at the respective hospitals by an experienced hepatologist to rule out clinical signs of HE. Patients with clinically obvious concurrent diseases affecting cognitive testing, such as dementia, were not included in this study. In each patient, PHES was performed to diagnose MHE. PHES is a paper-and-pencil testing battery including 5 subtests (number connection test A and B, serial dotting test, digit symbol test, and line tracing test). PHES was scored at each center using the validated country-specific norms (Germany (3), United States (16), Italy (17), and France (18)). The Danish center used the German norms (3). A score  $< -4$  was considered pathological for centers from Germany and Denmark (3). For centers from Italy, the United States, and France, a score  $\leq -4$  was considered pathological.

## Determination of ammonia

Venous ammonia levels were measured in a subset of patients according to a standard operating procedure of the respective





**Figure 1.** Flow chart of patients eligible for study inclusion. DE, Germany; DK, Denmark; FR, France; IT, Italy; MHE, minimal hepatic encephalopathy; US, United States.

center that involved rapid sample transport on ice to the central laboratory within a few minutes. Because upper limits of normal vary between laboratories, we decided to calculate standardized ammonia levels for each center to make ammonia levels comparable: actual ammonia level/ammonia upper limits of normal of the respective center.

### Ethics

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki (seventh revision, 2013). For this study, we used anonymized electronic medical records without directly identifiable data. According to German regulations and the recommendations of the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, no ethical approval is required for this type of study. Anonymized data were analyzed as aggregates with no protected health information available. Regarding a subset of patient data that were recorded in a prospective setting, the respective study protocols were approved by the ethics committees of the respective centers.

### Statistical analysis

IBM SPSS Statistic version 27 and 29 (IBM, Armonk, NY) as well as R 4.1.3 (R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2022. URL <https://www.R-project.org/>) were used for data analysis and graphic design. Quantitative data are expressed as medians with interquartile ranges (IQR: Q1–Q3). Missing data were excluded from the respective analysis (complete-case analysis). Pairwise comparisons for continuous

variables were performed using the Mann-Whitney *U* test or an independent samples *t* test after testing for normal distribution using the Shapiro-Wilk method. Categorical variables are displayed as frequencies and percentages. For comparison of 2 or more patient groups, a  $\chi^2$  test was applied. Correlation analyses between standardized ammonia levels and PHES were conducted using the Spearman rank correlation, given their non-normal data distribution (Shapiro-Wilk test). To identify factors associated with the prevalence of MHE, we conducted multivariable logistic regression analyses including all significant variables on univariable analysis. For all tests, we used a 0.05 level to define statistically significant deviations from the respective null hypothesis.

### RESULTS

In total, data of 1,868 patients with cirrhosis were available for analyses (Figure 1). The cohort was predominantly male ( $n = 1,241$ , 66.4%), and the median age was 60 years (IQR 53–67). CP stages at the day of PHES testing were as follows: CP A 46% ( $n = 813$ ), CP B 42% ( $n = 731$ ), and CP C 12% ( $n = 215$ ). Median MELD was 11 (IQR 9–15). In total, 22%/58%/20% ( $n = 342/905/316$ ) patients were graded as ALBI grade 1/2/3. A history of OHE was noted in 30% ( $n = 518$ ) of the cohort, and 55% ( $n = 898$ ) had a history of ascites. The most common etiology of cirrhosis was harmful alcohol use ( $n = 795$ , 43%), followed by viral hepatitis ( $n = 431$ , 23%). Demographics and clinical characteristics are displayed in Table 1. In addition, demographics and clinical characteristics of patients without a history of OHE are shown in Supplementary Table S2 (see Supplementary Digital Content 1, <http://links.lww.com/AJG/C914>). The patient characteristics differed significantly between centers. The detailed characteristics of the cohorts of each center are displayed in Supplementary Table S3a–c (see Supplementary Digital Content 1, <http://links.lww.com/AJG/C914>).

### Comparison of patients with and without MHE

Patients with MHE were older, more likely to have alcohol-related cirrhosis, and had higher MELD, ALBI, and CP scores compared with patients without MHE. Moreover, standardized ammonia levels differed significantly between patients with and without MHE ( $P = 0.015$ , Table 1). However, there was only a weak correlation between results in PHES and standardized ammonia levels (Spearman  $\rho = -0.16$ ,  $P < 0.001$ , Figure 2). Detailed comparisons between patients with and without MHE are displayed in Table 1.

To identify independent variables associated with the presence of MHE, we conducted a multivariable logistic regression analysis including all univariable significant variables from Table 1 ( $n = 1,113$ ). Here, age, sodium, creatinine, albumin, history of ascites, and history of OHE were associated with the presence of MHE (Table 2). Given that ammonia levels were only available in a subset of the cohort, we repeated the multivariable regression model in this subcohort and included ammonia into the model. Here, standardized ammonia was not associated with the presence of MHE (odds ratio 1.157, 95% confidence interval 0.667–2.006,  $P = 0.603$ , data not shown). In addition, a logistic regression model was fitted after excluding patients with a history of OHE (Table 2). Here, age, sodium, creatinine, albumin, hemoglobin, and history of ascites were associated with the presence of MHE.

**Table 1.** Demographics and clinical characteristics of the study cohort

| Variable                       | Total cohort<br>(n = 1,868)      | Missing (n), total<br>cohort | MHE<br>(n = 650)                 | Missing (n),<br>MHE | No MHE<br>(n = 1,218)            | Missing<br>(n),<br>no MHE | P      |
|--------------------------------|----------------------------------|------------------------------|----------------------------------|---------------------|----------------------------------|---------------------------|--------|
| Age, yr                        | 60 (53; 67)                      | 1                            | 62 (54; 68)                      | 0                   | 60 (52; 66)                      | 1                         | <0.001 |
| Male gender                    | 1,241 (66.4)                     | 0                            | 442 (68.0)                       | 0                   | 799 (65.6)                       | 0                         | 0.295  |
| Etiology                       |                                  |                              |                                  |                     |                                  |                           |        |
| Alcohol                        | 795 (43.2)                       | 29                           | 310 (48.3)                       | 8                   | 485 (40.5)                       | 21                        | 0.006  |
| Viral hepatitis                | 431 (23.4)                       |                              | 138 (21.5)                       |                     | 293 (24.5)                       |                           |        |
| Others/mixed                   | 613 (33.3)                       |                              | 194 (30.2)                       |                     | 419 (35.0)                       |                           |        |
| Prognostic scores              |                                  |                              |                                  |                     |                                  |                           |        |
| MELD score                     | 11 (9; 15)                       | 97                           | 12 (10; 16)                      | 28                  | 11 (8; 14)                       | 69                        | <0.001 |
| ALBI score                     | -2.02 (-2.53; -1.49)             | 305                          | -1.77 (-2.30; -1.32)             | 91                  | -2.13 (-2.63; -1.64)             | 214                       | <0.001 |
| Child-Pugh A/B/C               | 813/731/215 (46.2/<br>41.6/12.2) | 109                          | 200/308/112 (32.3/<br>49.7/18.1) | 30                  | 613/423/103 (53.8/<br>37.1/9.0)  | 79                        | <0.001 |
| ALBI 1/2/3                     | 342/905/316 (21.9/<br>57.9/20.2) | 305                          | 75/327/157 (13.4/<br>58.5/28.1)  | 91                  | 267/578/159 (26.6/<br>57.6/15.8) | 214                       | <0.001 |
| Clinical variables             |                                  |                              |                                  |                     |                                  |                           |        |
| MHE                            | 650 (34.8)                       | 0                            | 650 (100.0)                      | 0                   | 0 (0.0)                          | 0                         | n.a.   |
| PHES                           | -2 (-6; 0)                       | 0                            | -7 (-10; -5)                     | 0                   | -1 (-2; 1)                       | 0                         | <0.001 |
| History of ascites             | 898 (55.0)                       | 235                          | 376 (66.9)                       | 88                  | 522 (48.7)                       | 147                       | <0.001 |
| Ascites at PHES<br>examination | 644 (39.7)                       | 246                          | 309 (54.3)                       | 81                  | 335 (31.8)                       | 165                       | <0.001 |
| History of SBP                 | 71 (8.6)                         | 1,039                        | 34 (11.6)                        | 358                 | 37 (6.9)                         | 681                       | 0.019  |
| History of OHE                 | 518 (29.6)                       | 120                          | 243 (40.4)                       | 48                  | 275 (24.0)                       | 72                        | <0.001 |
| Varices                        | 766 (59.8)                       | 588                          | 252 (60.7)                       | 235                 | 514 (59.4)                       | 353                       | 0.657  |
| Laboratory parameters          |                                  |                              |                                  |                     |                                  |                           |        |
| Sodium, mmol/L                 | 138 (136; 140)                   | 265                          | 137 (134; 140)                   | 71                  | 138 (136; 141)                   | 194                       | <0.001 |
| Creatinine, mg/dL              | 0.85 (0.70; 1.09)                | 173                          | 0.91 (0.72; 1.20)                | 39                  | 0.83 (0.70; 1.00)                | 134                       | <0.001 |
| Bilirubin, mg/dL               | 1.3 (0.8; 2.3)                   | 122                          | 1.5 (0.9; 2.6)                   | 26                  | 1.2 (0.8; 2.2)                   | 96                        | <0.001 |
| Albumin, g/L                   | 35 (30; 39)                      | 293                          | 32 (28; 37)                      | 89                  | 36 (31; 40)                      | 204                       | <0.001 |
| INR                            | 1.2 (1.1; 1.4)                   | 161                          | 1.3 (1.2; 1.5)                   | 36                  | 1.2 (1.1; 1.4)                   | 125                       | <0.001 |
| WBC, /nL                       | 5.7 (4.2; 7.5)                   | 997                          | 5.4 (4.3; 7.5)                   | 362                 | 5.7 (4.2; 7.5)                   | 635                       | 0.801  |
| Haemoglobin, g/dL              | 12.2 (10.4; 13.8)                | 449                          | 11.4 (9.7; 13.1)                 | 139                 | 12.6 (10.7; 14.1)                | 310                       | <0.001 |
| Platelets, /nL                 | 114 (77; 164)                    | 660                          | 107 (76; 161)                    | 193                 | 119 (78; 168)                    | 467                       | 0.249  |
| Ammonia<br>standardised        | 0.71 (0.50; 1.04)                | 1,248                        | 0.74 (0.53; 1.16)                | 449                 | 0.68 (0.50; 0.97)                | 799                       | 0.015  |

*P* < 0.05 was set as statistically significant (bold).

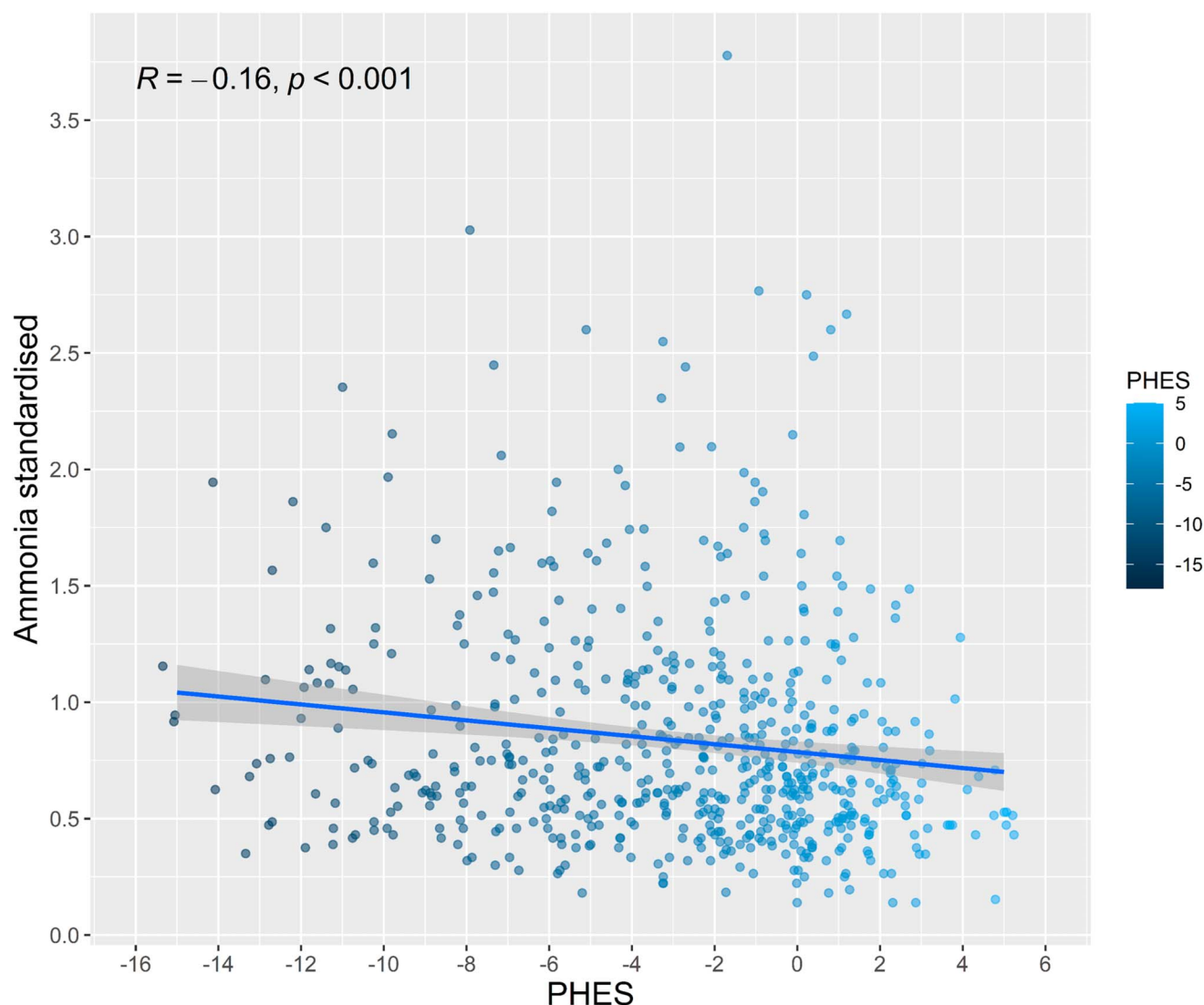
Data are expressed as medians (IQR) or as frequencies and percentages. Percentages refer to the patients with available data. Continuous variables were tested for their distribution in both groups (MHE + vs MHE-) using the Shapiro-Wilk test.

ALBI, albumin-bilirubin; INR, international normalized ratio; IQR, interquartile range; MELD, model for end-stage liver disease; MHE, minimal hepatic encephalopathy; n.a., not applicable; OHE, overt hepatic encephalopathy; PHES, Psychometric Hepatic Encephalopathy Score; SBP, spontaneous bacterial peritonitis; WBC, white blood cell.

### Prevalence of MHE

The overall prevalence of MHE was 34.8% (n = 650) in the total cohort (Figure 3). The prevalence of MHE was considerably higher in patients with a history of OHE compared with patients without a history of OHE (46.9% vs 29.2%, *P* < 0.001, Figure 3). In patients with CP A cirrhosis, the prevalence of MHE was low (24.6%), higher in patients with CP B (42.1%), and was highest in patients with CP C (52.1%) (Figure 3).

Similarly, higher prevalence of MHE was detected with increasing MELD (MELD <10: 24.8%, MELD 10–14: 37.6%, MELD 15–19: 44.0%, and MELD >20: 48.1%) or ALBI strata (ALBI 1: 21.9%, ALBI 2: 36.1%, and ALBI 3: 49.7%) (Figure 3). Additional prevalence data of MHE stratified by other demographic and clinical characteristics are displayed in Supplementary Table S4 (see Supplementary Digital Content 1, <http://links.lww.com/AJG/C914>).



**Figure 2.** Correlation between ammonia standardized and PHES. Spearman  $\rho = -0.16$ ,  $P < 0.001$ . PHES, Psychometric Hepatic Encephalopathy Score.

Given that a history of OHE has a relevant impact on the prevalence of OHE, we repeated the aforementioned analyses in the subcohort of patients without a history of OHE ( $n = 1,230$ ). The MHE prevalence data of these analyses stratified by various disease stages are shown in Figure 4.

To further visualize the prevalence of MHE according to disease characteristics, we developed a decision tree for the total cohort stratified by the history of OHE, MELD, and CP strata (Table 3). Here, the MHE prevalence was 18.9% in the lowest risk group of patients without a history of OHE, a MELD  $<10$ , and CP A. By contrast, in patients with CP C or with CP B and a history of OHE, the prevalence was close to 50% or even exceeded 50%.

To further analyze the prevalence of MHE in patients with different underlying etiologies of liver cirrhosis, we conducted separate subgroup analyses. In the total cohort, the prevalence of MHE was higher in patients with alcohol-related liver disease (39.0%) compared with patients with viral hepatitis (32.0%,  $P = 0.015$ ) or the group of other/mixed etiologies (31.6%,  $P = 0.004$ ). However, in most subgroups analyses, stratified by CP stages, MELD, or ALBI strata, the prevalence of MHE was not

significantly higher in patients with alcohol-related liver disease compared with viral hepatitis or the group of other/mixed etiologies (see Supplementary Table S5, Supplementary Digital Content 1, <http://links.lww.com/AJG/C914>).

## DISCUSSION

In this study, we provide detailed data on the prevalence of MHE in patients with cirrhosis in a large multinational cohort. We were able to demonstrate that the prevalence of MHE defined by PHES increases according to the severity of liver cirrhosis either expressed by a higher MELD or CP stage or the occurrence of decompensation events. Here, the lowest prevalence was found in patients with early cirrhosis (no history of OHE, MELD  $<10$  and CP A) with 18.9% and the highest in patients with severe decompensated cirrhosis (history of OHE, MELD  $>15$ , and CP C) with 54.5%.

Several studies and even the current EASL clinical practice guidelines state that the prevalence of MHE/CHE is 30%–70% (1,2,10). This wide range is explained by the fact that prior studies on the topic of MHE included a maximum of 2 or 3 centers, used different tests and thresholds, and several cohorts were restricted

**Table 2.** Multivariable logistic regression models to analyze the association of variables with minimal hepatic encephalopathy in the total cohort and in the cohort of patients without a history of OHE

| Variables                                                                                                                                                     | OR (95% CI)         | P                |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|------------------|
| Total cohort (n = 1,113)                                                                                                                                      |                     |                  |
| Age (yr)                                                                                                                                                      | 1.028 (1.014–1.041) | <b>&lt;0.001</b> |
| Alcohol-related cirrhosis (ref: other etiology)                                                                                                               | 1.059 (0.802–1.400) | 0.684            |
| Sodium (mmol/L)                                                                                                                                               | 0.932 (0.900–0.965) | <b>&lt;0.001</b> |
| Creatinine (mg/dL)                                                                                                                                            | 1.262 (1.008–1.580) | <b>0.042</b>     |
| Bilirubin (mg/dL)                                                                                                                                             | 0.999 (0.960–1.039) | 0.956            |
| Albumin (g/L)                                                                                                                                                 | 0.955 (0.931–0.979) | <b>&lt;0.001</b> |
| INR                                                                                                                                                           | 1.030 (0.646–1.643) | 0.900            |
| Hemoglobin (g/dL)                                                                                                                                             | 0.943 (0.882–1.007) | 0.079            |
| History of OHE (ref: no)                                                                                                                                      | 1.821 (1.373–2.415) | <b>&lt;0.001</b> |
| History of ascites (ref: no)                                                                                                                                  | 1.592 (1.181–2.147) | <b>0.002</b>     |
| Patients without a history of OHE (n = 774)                                                                                                                   |                     |                  |
| Age (yr)                                                                                                                                                      | 1.025 (1.009–1.041) | <b>0.003</b>     |
| Alcohol-related cirrhosis (ref: other etiology)                                                                                                               | 1.031 (0.724–1.468) | 0.865            |
| Sodium (mmol/L)                                                                                                                                               | 0.928 (0.888–0.969) | <b>&lt;0.001</b> |
| Creatinine (mg/dL)                                                                                                                                            | 1.634 (1.126–2.371) | <b>0.010</b>     |
| Bilirubin (mg/dL)                                                                                                                                             | 1.003 (0.959–1.049) | 0.898            |
| Albumin (g/L)                                                                                                                                                 | 0.970 (0.942–1.000) | <b>0.048</b>     |
| INR                                                                                                                                                           | 1.145 (0.647–2.026) | 0.643            |
| Hemoglobin (g/dL)                                                                                                                                             | 0.883 (0.812–0.960) | <b>0.004</b>     |
| History of ascites (ref: no)                                                                                                                                  | 1.521 (1.053–2.196) | <b>0.025</b>     |
| P < 0.05 was set as statistically significant (bold).<br>CI, confidence interval; OHE, overt hepatic encephalopathy; OR, odds ratio; ref, reference category. |                     |                  |

to highly selected subgroups of cirrhosis (10,19). One study evaluated the prevalence of MHE in 1,114 patients from 20 centers in India and reported a prevalence rate of 59.7% (11). However, this study was limited by only including centers from 1 country and lacked a fine granular analysis of subgroups. Importantly, detailed analyses of the subgroup of patients without a history of OHE are lacking. To the best of our knowledge, our current study comprises the largest multicenter cohort ever published in the field of MHE and provides a robust overview on prevalence rates in different stages of cirrhosis. The overall prevalence of MHE in our cohort was lower compared with the study by Rathi et al (11). This is most likely explained by differences in patient characteristics (mean MELD score of 15 [Rathi et al (11)] vs median MELD 11 in this study). In this context, our study clearly demonstrates that an increasing severity of liver disease, either expressed by a higher MELD or CP stage or the occurrence of decompensation events, are the most important factors associated with an increasing prevalence of MHE. However, racial or ethnic differences cannot be fully excluded.

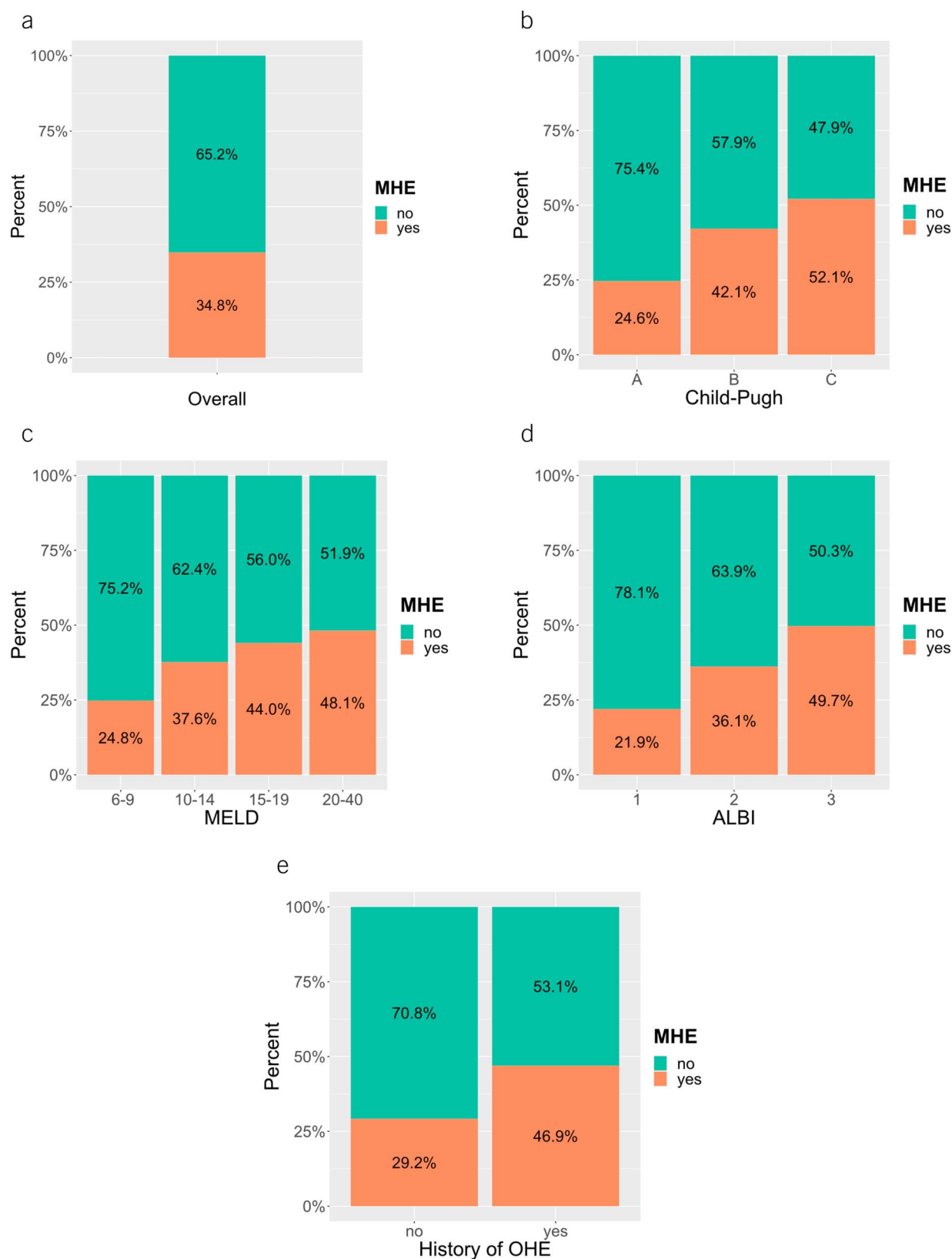
It remains a matter of debate which patients should undergo MHE testing, and currently testing with specialized tests is

frequently not performed in routine practice (8,9). As testing for MHE is time-consuming, especially when using PHES, it is of utmost importance to gain knowledge on groups in whom testing may not be necessary due to very low or high risk, respectively. Owing to the large sample size of our cohort, we were able to build a robust decision tree based on MELD and CP stage strata estimating the prevalence of MHE. Here, we could demonstrate that the prevalence of MHE is only 1 in 5 patients (18.9%) without a history of OHE, a MELD score <10, and CP A. By contrast, the prevalence of MHE in patients with CP C, a history of OHE, and a MELD score  $\geq 15$  was 54.5%. These data could be the backbone for future randomized controlled trials evaluating the value of treatment of MHE and primary prophylaxis for OHE in these high-risk patients for having MHE. In this context, it has to be mentioned that the efficiency of multiple treatment strategies, such as lactulose, is proven for the reversal of MHE (20,21), whereas well-designed and placebo-controlled studies for the prevention of OHE are currently lacking (22,23).

Historically, HE was considered to be a completely reversible complication of cirrhosis. However, during the past decade, studies emerged indicating a residual cognitive deficit after a bout of OHE with special focus on working memory, response inhibition, and learning impairment (24,25). Moreover, there is still an ongoing debate on residual cognitive deficits in those patients after liver transplantation (26–28). Our study adds to this body of evidence by demonstrating a remarkably higher prevalence of cognitive deficits (MHE) defined by PHES in patients with a history of OHE compared with patients without a history of OHE with similar cirrhosis disease stages (as shown in Table 3). Although, it has to be acknowledged that in these patients, chronic forms of HE may not be completely excluded.

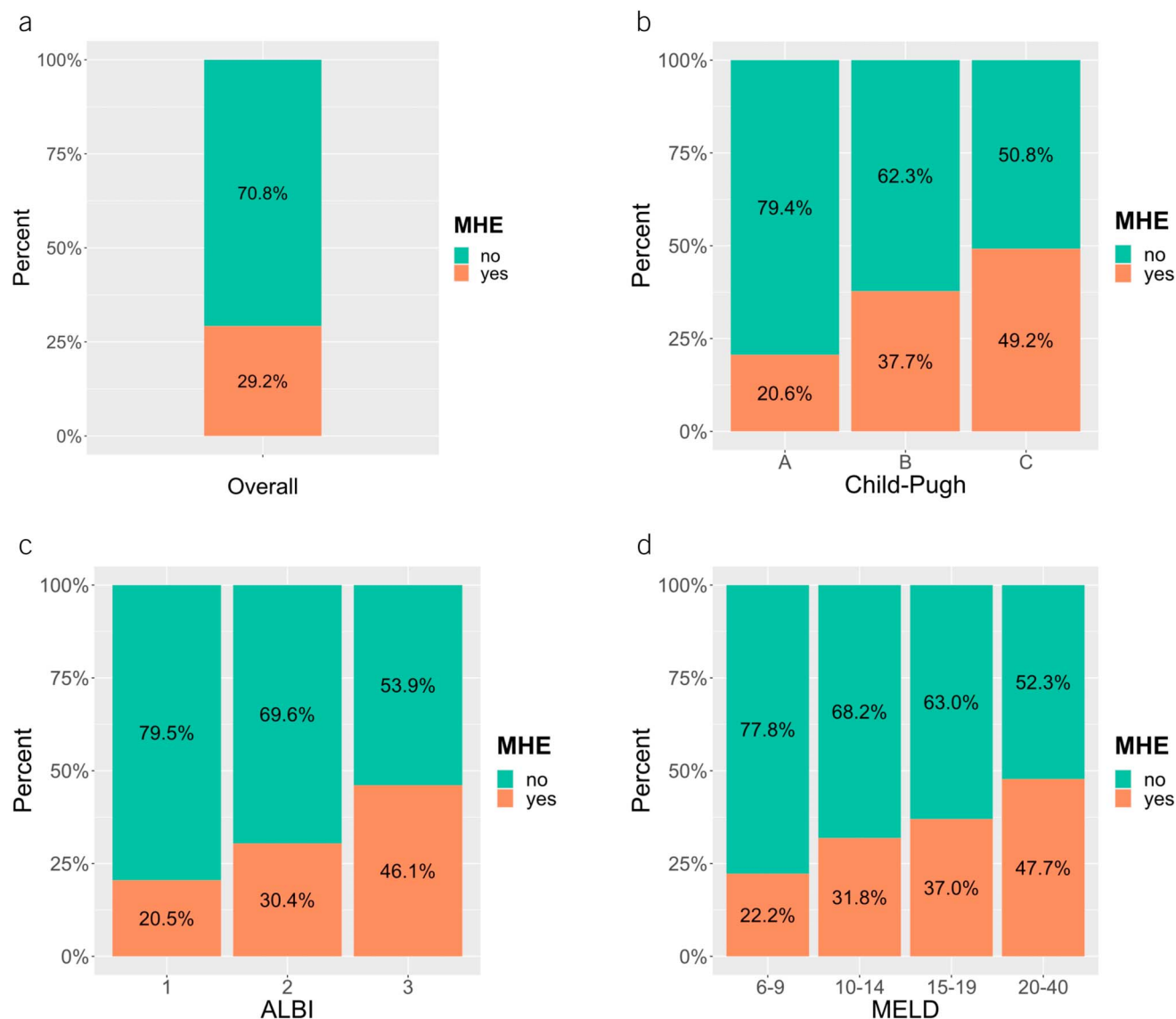
Alcohol leads directly or indirectly to brain damage resulting in altered brain structure and function. In addition, in heavy drinkers, factors such as poor diet including vitamin deficiency are associated with poorer brain functioning (29). In the current study, we analyzed and stratified patients with different etiologies of cirrhosis by disease severity. Here, we did not find a clinically relevant difference in MHE prevalence between different etiologies. In line, alcohol-related cirrhosis was not associated with MHE in multivariable analysis. Although these analyses may indicate that a history of harmful alcohol use may not significantly limited testing for MHE using PHES, these results have to be interpreted with caution. Owing to the retrospective design, we are unable to provide detailed information on ongoing drinking status and are, therefore, unable to perform appropriate sensitivity analyses. Therefore, we cannot exclude a potential bias of ongoing alcohol use on PHES testing.

Ammonia is a proven cornerstone in the complex pathophysiology of HE (1). In addition, the prognostic value of ammonia measurement for predicting hospitalizations and mortality in outpatients with cirrhosis was recently demonstrated by a large multicenter study and subsequent validation (30,31). However, the correlation between serum ammonia levels and the presence of MHE is controversial, and studies are conflicting and limited by small sample sizes (32–34). Our current study expands the existing literature by demonstrating a significant, albeit weak correlation between standardized serum ammonia levels and results in PHES in a very large cohort of patients with cirrhosis. Our data indicate that ammonia may have no relevant value in MHE diagnosis. By contrast, markers of systemic inflammation seem to have a better correlation with the presence of MHE and



**Figure 3.** MHE prevalence in different subgroups. Overall MHE prevalence (a) and stratified by the Child-Pugh stage (b), MELD (c), ALBI (d), and history of OHE (e). ALBI, albumin-bilirubin; MELD, model for end-stage liver disease; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy.





**Figure 4.** MHE prevalence in different subgroups including only patients without a history of OHE. Overall MHE prevalence in patients without a history of OHE (a) and stratified by Child-Pugh stage (b), ALBI (c), and MELD (d). ALBI, albumin-bilirubin; MELD, model for end-stage liver disease; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy.

may be of greater diagnostic utility. However, this should be confirmed in future prospective studies, and it has to be acknowledged that our study was not designed to give insights into the pathophysiology of MHE. Therefore, our current findings have to be interpreted cautiously.

One of the most important strengths of our study is the large patient cohort, recruited at various centers with interest in HE, allowing robust subgroup analyses of the prevalence of MHE. However, there are also limitations that have to be acknowledged. First, this was a retrospective analysis and patients were not specifically recruited for this study. Moreover, patients were only included if testing with PHES was available. This may introduce selection bias. Moreover, most participating hospitals are tertiary care centers with special interest in HE. Therefore, our findings may not be generalizable to every patient with cirrhosis. Second, we defined MHE by PHES. This seems reasonable given that PHES is the accepted gold standard for the detection of MHE. However,

International Society for Hepatic Encephalopathy and Nitrogen Metabolism guidelines recommended that at least 2 testing strategies should be used for the detection of MHE in multicenter studies (35); although, the prognostic value of this approach has been questioned in a recent US study (10). We decided to use PHES due to the fact that the commonly used secondary tests vary between centers, and therefore, results would not be comparable in such a large study. Third, due to the retrospective design of our study, we were unable to apply any fidelity measures regarding PHES and ammonia standardization between centers. Fourth, the fact that both the use of psychoactive medications and mental illness was not excluded in a structured manner in all centers before testing with PHES may have introduced a potential bias. Last, we do not have detailed information on the number, duration, or precipitating factors of OHE episodes in the subgroup of patients with a history of OHE and are, therefore, unable to perform robust subgroup analyses under consideration of these factors.

**Table 3.** MHE prevalence stratified by CP stage, MELD and history of OHE

|          | History of OHE, n (%) |                      |
|----------|-----------------------|----------------------|
|          | No                    | Yes                  |
| CP A     |                       |                      |
| MELD <10 | n = 403<br>76 (18.9)  | n = 67<br>22 (32.8)  |
| MELD ≥10 | n = 216<br>51 (23.6)  | n = 81<br>35 (43.2)  |
| CP B     |                       |                      |
| MELD <15 | n = 297<br>116 (39.1) | n = 177<br>91 (51.4) |
| MELD ≥15 | n = 138<br>50 (36.2)  | n = 60<br>30 (50.0)  |
| CP C     |                       |                      |
| MELD <15 | n = 19<br>9 (47.4)    | n = 9<br>4 (44.4)    |
| MELD ≥15 | n = 99<br>49 (49.5)   | n = 66<br>36 (54.5)  |

CP, Child-Pugh; MELD, model for end-stage liver disease; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy.

In conclusion, we provide fine granular prevalence data on MHE in cirrhosis in a large multinational cohort. We found that the prevalence of MHE varied considerably between different disease stages of cirrhosis, ranging from 18.9% in patients with compensated cirrhosis to more than 50% in patients with decompensated cirrhosis. In addition, we developed a decision tree to identify low and high-risk populations for having MHE in whom testing in routine practice may be omitted. These data may not only guide physicians regarding the MHE risk in their patients but also in the context of sample size estimation for future studies. Data from this study may pave the way for a more personalized approach for MHE screening.

## CONFLICTS OF INTEREST

**Guarantor of the article:** Christian Labenz, MD.

**Specific author contributions:** Performed research: S.J.G., C.M., L.Z., S.G., E.J.N., S.D., M.G., A.F.E., P.B., C.R., P.S., P.R.G., J.L., D.T., A.Z., A.S.L., K.W., J.U.M., M.M.L., S.N., S.M., and C.L. Contributed to acquisition of data: S.J.G., C.M., L.Z., S.G., E.J.N., S.D., M.G., A.F.E., P.B., C.R., P.S., P.R.G., J.L., D.T., A.Z., A.S.L., K.W., J.U.M., M.M.L., S.N., S.M., and C.L. Designed the experiments and analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, and statistical analysis: S.J.G. and C.L. All authors approved the final version of the manuscript and the authorship list.

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**Potential competing interests:** S.J.G.: Travel expenses: Ipsen and Gilead. J.L.: Consulting: Alphasigma, Norgine. Lecture fees: Norgine. P.R.G.: Lecture fees and consulting: Merz Pharmaceuticals. P.P.B.: Research grant from Vedanta Biosciences. Consultant for Nexilco. C.R.: Lecture fees: Falk, Gore, Grifols, CSL Behring. Research grants: DFG, Schwiete Stiftung. D.T.: Lecture fees: Alfasigma, Abbvie, Gilead. C.L.: Travel expenses and consulting: Norgine, Merz

Pharmaceuticals. Lecture fees: Norgine, Merz Pharmaceuticals. Research grants: Norgine, Merz Pharmaceuticals. All other authors disclose no potential financial or non-financial conflict of interests regarding this study.

**Ethics:** The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki (seventh revision, 2013). For this study, we used anonymized electronic medical records without directly identifiable data. According to German regulations and the recommendations of the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, no ethical approval is required for this type of study. Anonymized data were analyzed as aggregates with no protected health information available. Regarding a subset of patient data that were recorded in a prospective setting, the respective study protocols were approved by the ethics committees of the respective centers.

## Study Highlights

### WHAT IS KNOWN

- ✓ Minimal hepatic encephalopathy (MHE) is associated with poor quality of life, falls, and impaired prognosis.
- ✓ Little is known about the exact prevalence of MHE, in particular, in different subgroups of patients with cirrhosis.

### WHAT IS NEW HERE

- ✓ This study enrolled 1,868 patients with cirrhosis from 10 centers in Europe and the United States who were tested for MHE by the Psychometric Hepatic Encephalopathy Score.
- ✓ Overall MHE prevalence was found to be 35%, but the prevalence varied considerably when stratified by the disease stage.
- ✓ After excluding patients with a history of overt hepatic encephalopathy, the prevalence of MHE was 29%.
- ✓ The prevalence of MHE in patients with Child-Pugh A was low (25%), whereas it was high in Child-Pugh B or C (42% and 52%).
- ✓ Standardized ammonia levels (ammonia level/upper limit of normal of each center) correlated significantly, albeit weakly with the Psychometric Hepatic Encephalopathy Score (Spearman  $\rho = -0.16$ ).

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## An isolated insular stroke mimics a bout of overt hepatic encephalopathy in a patient with cirrhosis

To the Editor:

The manifestations of hepatic encephalopathy (HE) vary from mild neuropsychiatric abnormalities to coma.<sup>1,2</sup> Diagnosis and differential diagnosis can be challenging, especially in the hospital setting, where patients may suffer from other metabolic encephalopathies or neurological/psychiatric disorders.<sup>1–3</sup> Moreover, HE might also occur on top of pre-existing disease, such as dementia.<sup>1</sup> Any potential HE precipitating factor should be identified and treated promptly.<sup>1</sup> In patients with delirium and cirrhosis, even those with a working diagnosis of HE, a set of laboratory tests including a full blood count, glucose, electrolytes, blood gases, C-reactive protein, urinalysis, alcohol/other drugs, thyroid-stimulating hormone and ammonia should be performed. A normal blood ammonia level has a high negative predictive value.<sup>1,4</sup>

Herein, we present the case of a 52-year-old male with a history of mixed aetiology cirrhosis (HBV and alcohol) diagnosed in 2021 after a first episode of ascites. At the time, alcohol consumption was active and F1 oesophageal varices plus a gastric ulcer were detected on oesophagogastroduodenoscopy. No portal-systemic shunt was documented on abdominal imaging. He was discharged on treatment with a proton-pump inhibitor, carvedilol, tenofovir, and diazepam as needed. In December 2022, the patient was admitted to A&E because of confusion and slurred speech, and his wife also reported he was “capable of saying just a few words”. In A&E, he was normal on physical and neurological examination, except for spatial and temporal disorientation, slurred speech and lethargy. He was admitted to our ward with a working diagnosis of HE, despite normal ammonia levels (20 mmol/L) on no ammonia-lowering drugs. He had high potassium (5.8 mmol/L) and slightly low sodium (134 mmol/L); C-reactive protein, haemoglobin, creatinine, prothrombin time, bilirubin, blood alcohol levels, venous pH and thyroid-stimulating hormone were all normal; MELD (model for end-stage liver disease) and MELD-Na were 7.<sup>5</sup> A brain CT scan was negative for acute events but documented some degree of vasculopathy.

In the ward, the patient was initially treated with nil by mouth, intravenous fluids and antibiotics; HE precipitating factors were not identified and ammonia remained normal on repeated testing. Symptoms started to improve at 36 hours. The patient was less confused and less sleepy and reported he had been taken to A&E because he “wanted to speak, but the words would not come out”. This was considered to suggest non-fluent aphasia, the CT scan was repeated and documented thickening of the left insular cortical ribbon (Fig. 1), which is compatible with an insular stroke. Treatment with aspirin (100 mg QD)<sup>6</sup> and intravenous proton-pump inhibitors was instituted, taking into consideration the low hemorrhagic risk (F1 varices) and the high risk of stroke; non-selective beta-blockers were re-started to minimize the risk of gastro-

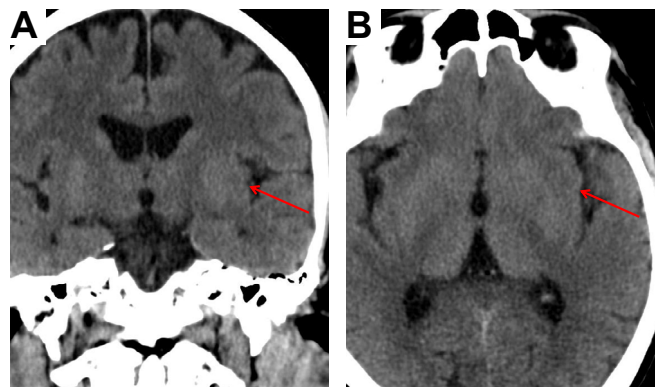


Fig. 1. Coronal and axial view of the patient's brain CT at 36 hours after admission. The red arrows indicate swelling of the cortex and junctional white matter of the left insula.

intestinal bleeding prior to repeating an oesophagogastroduodenoscopy, which was unchanged. An electroencephalogram performed after three days was near-normal, except for some slow activity over the temporal regions. For logistical reasons, it was not possible to perform a cerebral MRI; this was performed over a month after the event and was substantially negative.

The insula is a small but anatomically and functionally complex part of the human cortex, located within the Sylvian fissure<sup>7</sup> and implicated in motor, somato-sensory, vestibular, autonomic, speech and cognitive functions.<sup>8</sup> The insula is supplied by a proximal branch of the middle cerebral artery, thus vascular occlusions often involve adjacent structures supplied by the same artery.<sup>9</sup> Such occlusions have poor functional outcomes when the insula is involved.<sup>9</sup> By contrast, isolated insular strokes (IIS) are rare, clinically heterogeneous and difficult to diagnose, but tend to have a good prognosis.<sup>8,9</sup> Typical IIS symptoms are motor deficits, speech disorders and sensory impairment, whereas atypical ones include sleepiness, confusion, agitation, anxiety and spatial-temporal disorientation,<sup>9</sup> besides autonomic, auditory and vestibular disturbance.<sup>9</sup> Our patient presented with one typical (aphasia) and three atypical (spatial-temporal disorientation, mental confusion and sleepiness) IIS symptoms. The latter are, of course, also part of the HE syndrome.<sup>2</sup>

In conclusion, the diagnosis of HE is challenging, even when the *a priori* probability is high. This case was further complicated by the significant overlap between IIS and HE symptoms.

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## Authors' contributions

CM: patient care and manuscript drafting; RG: patient care and manuscript drafting; FC: imaging interpretation and patient care; PA: revision of the manuscript for important intellectual content; SM: patient care, drafting and revision of the manuscript.

## Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2023.03.004>.

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# Holding opposing ideas and the half-life of truth

To the Editor:

In a stimulating editorial in this issue of *Journal of Hepatology*,<sup>1</sup> Vincent Di Martino contrasts our current study<sup>2</sup> with previous ones on methotrexate hepatotoxicity. It is worthwhile revisiting the key observations from our previous studies and rehearsing interpretations that were made in these articles. The first of our cohort studies questioning 'are serial liver biopsies justified?' involved 121 liver biopsies from 66 individuals and found 'advanced hepatic fibrosis with low-dose methotrexate therapy to be much less frequent than previously reported'.<sup>3</sup> None in this study developed cirrhosis or discontinued treatment on the basis of liver biopsy findings. The putative hypothesis that was put forward regarding the mechanism consistently stressed the interaction of the drug with the host and environmental factors.<sup>4,5</sup> Another study based on data from United States Organ Procurement and Transplantation Network involving 158,904 adults who had been listed for, and/or received liver transplantation

concluded that the 'burden of end-stage methotrexate-related liver disease to be exceedingly small, suggesting the need for reappraisal of current hepatotoxicity surveillance guidelines'.<sup>6</sup> The discussion section in this article started with the quote 'Methotrexate hepatotoxicity and the premature reporting of Mark Twain's death: both greatly exaggerated'.<sup>6</sup> In another article, we pointed out that the 'evidence base published to date overwhelmingly supports the notion that features of the metabolic syndrome such as diabetes and obesity accentuate the risk of methotrexate related liver disease'.<sup>7</sup>

It was Francis Scott Fitzgerald, one of the great American writers of the 20<sup>th</sup> century who wrote about 'holding two opposing ideas in mind at the same time and still retain the ability to function'. The 'null hypothesis' proposes that no relationship and significance exists in a set of observed variables and measured phenomena. A scientist's craft is to question, and loyalty rests with the observations; it is essential to recognise that truth is contextual rather than absolute.



# Working Memory in Patients with Varying Degree of Hepatic Encephalopathy (HE): A Pilot EEG-fNIRS Study

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## Abstract

It is known that patients with covert hepatic encephalopathy (CHE) exhibit working memory abnormalities, but to date there is no study comparing patients with cirrhosis with/without CHE and controls with both electrophysiological and hemodynamic data collected at the same time.

Here we collected behavioral [accuracy and reaction times (RTs), electrophysiological (evoked potentials) and hemodynamic (oxygenated and deoxygenated haemoglobin) correlates of an n-back task [formed by a control (0-back) condition, a low (1-back) and a high (2-back) working memory load conditions] in patients with cirrhosis with/without CHE: (1) at baseline (n=21, males=15, 58±8 yrs), and by comparison with controls (n=21, males=15, 57±11 yrs) and (2) after a 3-month course of rifaximin (n=18, males=12, 61±11 yrs), and by comparison to baseline.

All patients showed slower RTs ( $p < 0.0001$ ) and lower P2 amplitude compared with controls ( $p = 0.018$ ); moreover, patients with CHE showed reduced accuracy ( $p < 0.0001$ ) compared with controls, and patients without CHE showed higher oxygenated haemoglobin in the central dorsolateral prefrontal cortex in the 2-back compared with patients with CHE. Post-rifaximin, oxygenated haemoglobin increased in the central frontopolar cortex. In addition, in patients without CHE the RTs of the 2-back became comparable to those of the 0-back and P3 showed higher amplitude.

In conclusion, the presence of cirrhosis seemed to have more effects than CHE on working memory at baseline. A course of treatment with rifaximin was more beneficial to patients without CHE, who probably had more room for improvement in this complex task.

**Keywords** Hepatic encephalopathy · Working memory · Rifaximin · EEG · fNIRS

## Introduction

Hepatic encephalopathy (HE) is defined as a continuum of neuropsychiatric impairment caused by liver insufficiency and/or portal-systemic shunting, ranging from abnormalities detected on neurophysiological or neuropsychological testing up to coma [1–6]. HE is usually classified as overt (OHE) or covert (CHE) [7, 8]; when a patient has OHE the neurological and psychiatric alterations are clinically

detectable, while CHE is characterized by minor (usually detected by family, caregivers or doctors to whom the patient is well known) or even no signs or symptoms but the presence of abnormalities on neuropsychological and/or neurophysiological testing [8]. In further detail, the most commonly observed neurophysiological alteration is a slowing of the electroencephalogram (EEG) [9], while neuropsychological abnormalities pertain to domains such as those of attention, motor speed, visuo-spatial abilities [10, 11] and working memory [12–14]. More specifically, CHE can be detected by wake EEG and different neuropsychological tests [15, 16]. The most widely used test is the Psychometric Hepatic Encephalopathy Score (PHES), a battery including the Digit Symbol Test, Trail Making Test A, Trail Making Test B, the Serial Dotting Test and the Line Tracing Test [10, 11]. Other tools include the Stroop test, Scan test, the Continuous Reaction Time Test, the Cognitive Drug

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Research test battery, the Repeatable Battery for the Assessment of Neuropsychological Status and the Animal Naming Test [15, 16].

Working memory allows to store and manipulate mental representations of goals, sequence of actions and task-relevant information for a limited period of time [17], for instance remembering a phone number for the time needed to dial it after having heard or read it. One widely used task to investigate working memory is the so-called n-back, which also allows to directly manipulate working memory load. The task consists in remembering and reporting the identity/position of a stimulus over time (i.e., in the 2-back it is required to report if the stimuli is the same or different to the one that had appeared two computer screens backwards) [18, 19]. During an n-back task it is possible to record at the same time: (1) behavioral data such as reaction times and accuracy, (2) EEG correlates such as frequency bands and evoked potentials, and (3) neuroanatomical correlates as marked by brain activation. Since EEG correlates have good time but poor spatial resolution, and neuroanatomical correlates have good spatial but poor time resolution, the two techniques combined complement each other in a productive fashion. Neuroanatomical correlates can be recorded by functional magnetic resonance imaging (fMRI) or by functional near infrared spectroscopy (fNIRS). fMRI can map the whole brain while the fNIRS study is limited to cortical activation. However, fNIRS has higher time resolution and can be used in ecological environments, such as outpatient departments, the bedside and other relevant clinical settings [20, 21].

Patients with CHE have been shown to exhibit worse working memory behavioral performance (longer reaction times and higher number of errors) compared to controls [11]. In addition, when performing an n-back task, these patients exhibited less brain activation compared to controls in brain regions implicated in working memory management (i.e., bilateral middle/medial frontal gyrus and superior/inferior parietal lobule and right inferior frontal gyrus) [22]. Finally, patients with cirrhosis, with or without cognitive impairment, exhibit a reduction in the amplitude of the P2, the evoked potential of the EEG that underlies attention, compared to controls [23].

Ahluwalia and colleagues (2014) [24] have shown how patients with CHE exhibited a greater number of correct responses and higher cortical (left parietal operculum, temporal occipital fusiform, right temporal pole and bilateral insular) and subcortical activation (caudate, thalamus and hippocampus) after 8 weeks of treatment with rifaximin, suggesting an improvement in cognitive performance. Our aim was to compare patients with cirrhosis with/without HE and controls by electrophysiological and hemodynamic data collected simultaneously. Thus we present behavioral,

electrophysiological (evoked potentials - EEG) and hemodynamic (oxygenated and deoxygenated haemoglobin - fNIRS) correlates of an n-back task in patients with cirrhosis with and without CHE: (1) at baseline, and by comparison with controls matched by age and sex, and (2) after a 3-month course of rifaximin, and by comparison to baseline.

## Methods

### Participants

Healthy controls were recruited from local associations to perform the task once. Patients were recruited from the liver out-patients clinic at Padua University Hospital to perform the task twice, at baseline and after a 3-month treatment course with rifaximin; none had a history of overt HE or were on ammonia-lowering treatment.

At baseline, 21 patients (15 males, age =  $58 \pm 8$  yrs and educational attainment =  $11 \pm 4$  yrs) and 21 controls (15 males, age =  $57 \pm 11$  yrs and educational attainment =  $14 \pm 5$  yrs) were recruited; among the patients, 8 had an abnormal PHES score (PHES score  $\leq 4$  was considered abnormal [10]) and thus were classified as having CHE. Demographic and clinical data are reported in Table 1. At baseline and after three months of treatment with rifaximin 18 patients (12 males, age =  $61 \pm 11$  yrs and educational attainment =  $9 \pm 3$  yrs) were recruited; among them 8 had an abnormal PHES score and thus were classified as having CHE. Demographical and clinical data are reported in Table 2. All participants signed an informed consent. The study was approved by the Ethics Committee of the University of Padova Hospital (study number 4033/AO/16).

### Protocol, Task and Recordings

All participants underwent: the Mini Mental State Examination (MMSE) [25] to investigate general cognitive functioning; the Cognitive reserve index questionnaire (CRIq) [26] to assess cognitive reserve; the Beck depression inventory II (BDI-II) to test for the presence of depressive symptoms [27]; the Animal Naming Test (ANT) to record verbal fluency [28] and Psychometric Hepatic Encephalopathy Score (PHES) to highlight the presence of CHE [9]. Laboratory (bilirubin and albumin) and standard outpatient clinical indices were also recorded.

All participants performed the n-back task once [29, 30] and patients who underwent treatment with rifaximin performed it twice.

The N-back was administered using a computer screen (17-inches display with a resolution of  $1024 \times 768$  pixels) placed at approximately 50 cm from the participant

**Table 1** Demographic, clinical, laboratory and neuropsychological indices in controls and patients, as a whole and by the presence of CHE

|                                                | Controls<br>(n=21) | Patients<br>(n=21) | No CHE<br>(n=13) | CHE<br>(n=8)              |
|------------------------------------------------|--------------------|--------------------|------------------|---------------------------|
| Age (yrs, mean $\pm$ SD)                       | 58 $\pm$ 9         | 57 $\pm$ 8         | 58 $\pm$ 9       | 59 $\pm$ 7                |
| Sex (male, %)                                  | 71                 | 71                 | 85               | 67                        |
| Educational level (yrs, mean $\pm$ SD)         | 14 $\pm$ 6         | 11 $\pm$ 4**       | 11 $\pm$ 3       | 11 $\pm$ 5                |
| Aetiology<br>(% alcohol/metabolic/viral/other) |                    | 28/39/22/11        | 19/36/36/9       | 43/43/0/14                |
| MMSE (mean $\pm$ SD)                           | 28 $\pm$ 1         | 26 $\pm$ 3**       | 27 $\pm$ 3       | 26 $\pm$ 3                |
| BDI-II (mean $\pm$ SD)                         | 4 $\pm$ 3          | 11 $\pm$ 8**       | 10 $\pm$ 11      | 10 $\pm$ 6                |
| ANT (mean $\pm$ SD)                            | 23 $\pm$ 6         | 17 $\pm$ 4         | 17 $\pm$ 4       | 16 $\pm$ 4                |
| CRI-q total score (mean $\pm$ SD)              | 118 $\pm$ 20       | 105 $\pm$ 16*      | 104 $\pm$ 15     | 107 $\pm$ 19              |
| PHES (mean $\pm$ SD)                           |                    | -4 $\pm$ 4         | -1 $\pm$ 2       | -9 $\pm$ 3 <sup>ooo</sup> |
| Ammonia ( $\mu$ mol/L; mean $\pm$ SD)          |                    | 67 $\pm$ 63        | 73 $\pm$ 78      | 55 $\pm$ 44               |
| Portal-systemic shunt (absent/present) (%)     |                    | 47/53              | 50/50            | 43/57                     |
| Pugh (mean $\pm$ SD)                           |                    | 6.3 $\pm$ 1.3      | 6.4 $\pm$ 1.2    | 6.1 $\pm$ 1.5             |
| Child (% A/B/C)                                |                    | 69/31/0            | 67/33/0          | 71/29/0                   |

\*  $p < 0.05$  \*\* $p < 0.01$  in patients vs. controls<sup>o</sup>  $p < 0.05$  <sup>oo</sup> $p < 0.001$  in patients with normal vs. patients with abnormal PHES

MMSE = Mini Mental State Examination; DBI = Beck depression inventory II; ANT = Animal Naming Test; CRI-q = Cognitive reserve index questionnaire; PHES = Psychometric Hepatic Encephalopathy Score

**Table 2** Demographic, clinical, laboratory and neuropsychological indices in patients before and after rifaximin, as a whole and by the presence of CHE

|                                                | pre-rifaximin  |                  |               | post-rifaximin |               |               |
|------------------------------------------------|----------------|------------------|---------------|----------------|---------------|---------------|
|                                                | All patients   | No CHE<br>(n=10) | CHE<br>(n=8)  | All patients   | No CHE        | CHE           |
| Age (yrs, mean $\pm$ SD)                       | 61 $\pm$ 11    | 59 $\pm$ 11      | 64 $\pm$ 10   |                |               |               |
| Sex (male, %)                                  | 67             | 80               | 50            |                |               |               |
| Educational level (yrs, mean $\pm$ SD)         | 9 $\pm$ 3      | 10 $\pm$ 3       | 8 $\pm$ 3     |                |               |               |
| Aetiology<br>(% alcohol/metabolic/viral/other) | 33/44//17/6    | 10/50/20/20      | 50/37/13/0    |                |               |               |
| MMSE (mean $\pm$ SD)                           | 27 $\pm$ 3     | 27 $\pm$ 3       | 26 $\pm$ 3    |                |               |               |
| BDI-II (mean $\pm$ SD)                         | 11 $\pm$ 6     | 11 $\pm$ 7       | 11 $\pm$ 6    | 8 $\pm$ 5*     | 10 $\pm$ 4    | 8 $\pm$ 5***  |
| ANT (mean $\pm$ SD)                            | 16 $\pm$ 4     | 16 $\pm$ 5       | 16 $\pm$ 4    | 16 $\pm$ 7     | 16 $\pm$ 6    | 16 $\pm$ 7    |
| CRI-q total score (mean $\pm$ SD)              | 102 $\pm$ 12   | 102 $\pm$ 6      | 102 $\pm$ 26  |                |               |               |
| PHES (mean $\pm$ SD)                           | -4 $\pm$ 4     | -1 $\pm$ 2       | -4 $\pm$ 4    | -3 $\pm$ 4     | -1 $\pm$ 4    | -3 $\pm$ 4    |
| Ammonia ( $\mu$ mol/L; mean $\pm$ SD)          | 65 $\pm$ 63    | 74 $\pm$ 78      | 39 $\pm$ 9    | 47 $\pm$ 23    | 43 $\pm$ 21   | 41 $\pm$ 13   |
| Portal-systemic shunt (absent/present) (%)     | 29/61          | 38/62            | 17/83         | 27/73          | 29/71         | 29/71         |
| Pugh (mean $\pm$ SD)                           | 6.1 $\pm$ 1.11 | 6.2 $\pm$ 1.3    | 5.8 $\pm$ 0.8 | 6.1 $\pm$ 1.4  | 6.4 $\pm$ 1.3 | 5.8 $\pm$ 0.8 |
| Child (% A/B/C)                                | 69/31/0        | 62/38/0          | 80/20/0       | 62/38/0        | 50/50/0       | 80/20/0       |

\*  $p < 0.05$  \*\*\* $p < 0.001$  in patients pre vs. post Rifaximin course

MMSE = Mini Mental State Examination; DBI = Beck depression inventory II; ANT = Animal Naming Test; CRI-q = Cognitive reserve index questionnaire; PHES = Psychometric Hepatic Encephalopathy Score

in a quiet, dimly lit room by E-prime software (Psychology Software Tools, Pittsburgh, PA). The stimulus was the letter O (66  $\times$  156 pixels), which moved within a 3  $\times$  3 grid (340  $\times$  255 pixels) on the blank screen. The stimulus lasted 1500 ms and the inter-stimulus interval, in which only the grid was visible, was kept constant at 500 ms; the response button was the letter “h” (i.e. the central key of

the keyboard) which was marked with white tape on the keyboard. The task encompassed three different conditions. In the control condition (0-back), which does not involve working memory, participants were asked to respond if the stimulus appeared in the center of the grid. In the low working memory load condition (1-back) participants were asked to respond if the stimulus appeared in the same position as



in the previous computer screen and in the high working memory load condition (2-back) participants were asked to respond if the stimulus appeared in the same position as two computer screens before (Fig. 1). The experimental conditions had six blocks each and they were alternated with the control condition of 12 blocks; the order of the experimental conditions was randomized and counterbalanced amongst participants. The experiment was self-paced: participants could decide at the beginning of each block when to start the block by pressing the spacebar. The total number of stimuli was 552, split in blocks of 33 stimuli each.

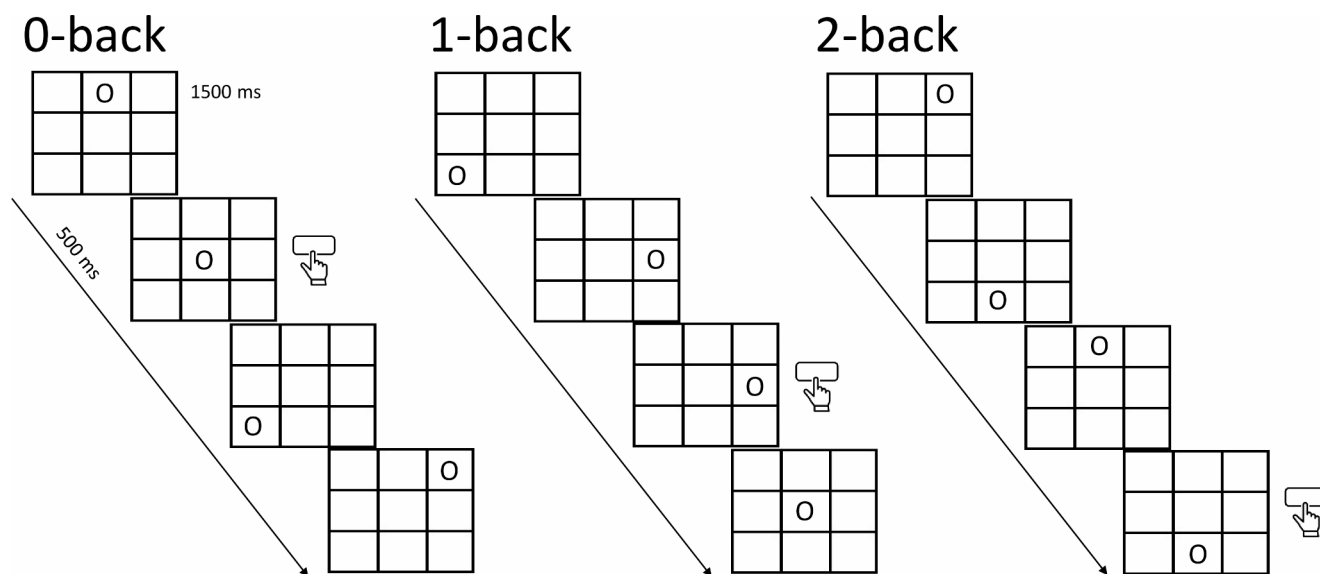
Behavioral, EEG and fNIRS data were simultaneously recorded during the task. Behavioral data [reaction times (RTs) and accuracy] were recorded using E-prime (Psychology Software Tools, Pittsburgh, PA). EEGs (evoked potentials) were recorded using the Brain Quick system 98 (Brainquick 3200; Micromed, Mogliano Veneto, Italy). Based on the 10–5 international system [31], electrodes were placed on: AFp1, AFp2, F7, AFF5h, FCz, AFF6h, F8, T3, C3, Cz, C4, T4, T5, P3, Pz, P4, T6, O1, Oz, O2, FC5, FC1, FC2, FC6, CP5, POz, CP1, CP2, CP6, PO3 and PO4 with the online reference on CPz and the ground on FCz (Fig. 2, panel A). Eight additional channels measured the electrooculogram (two immediately under the orbital fossa and two on the outer canthus of each eye), the electrocardiogram (one on each wrist) and offline reference (one on each mastoid). The impedance was kept under 5K $\Omega$  and the sampling rate was 256. fNIRS (oxygenated and deoxygenated haemoglobin) was recorded using a continuous wave NIRScout system (NIRx Medical Technologies LLC). Based

on the 10–5 international system and by use of 15 optodes placed on the forehead, twenty channels were obtained [31]; the 8 sources emitting light at 760 and 850 nm were placed on FPz, AF7, AF8, AF3, AF4, Fz, F3, F4, while the 7 detectors on Fp1, Fp2, AFz, F1, F2, F5 and F6 (Fig. 2, panel B). The sampling rate was set at approximately 7.8 Hz.

## Data Processing and Statistical Analyses

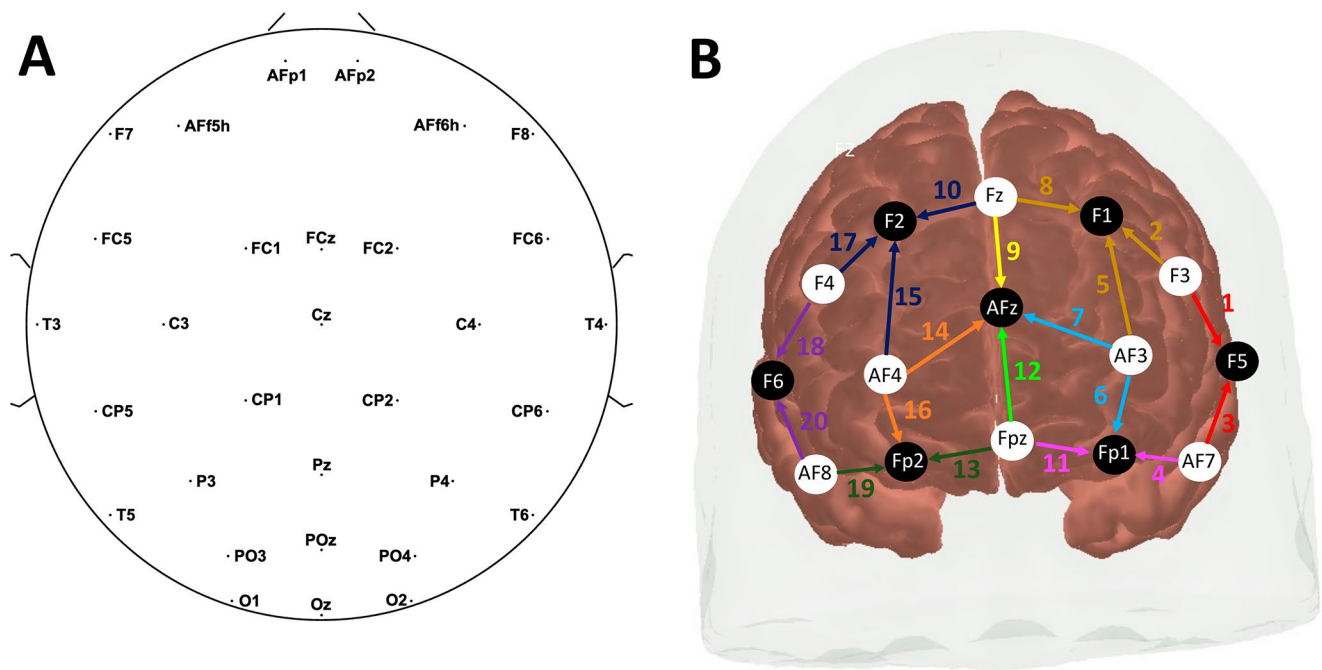
Reaction times (RTs) and accuracy were extracted via the E-prime software and tested for normality (to find they were not normally distributed). RTs and accuracy of patients and controls were compared by two Wald tests with working memory load (0, 1 and 2 back) as the within factor and group (patients and controls) as the between factor. RTs and accuracy of patients with/without CHE and controls were compared by two Wald tests with working memory load (0-, 1- and 2-back) as the within factor and group (patients with and without CHE) as the between factor. RTs and accuracy of patients before and after treatment with rifaximin were compared by two Wald tests with working memory load (0-, 1- and 2-back) and time (before and after rifaximin) as within factors. RTs and accuracy of patients and with/without CHE before and after treatment with rifaximin were compared by two Wald tests with working memory load (0-, 1- and 2-back) and time (before and after rifaximin) as within factors and group (patients with and without CHE) as between factor.

EEG data were processed using EEGLAB version 13.3.4 b [32]. The EEG signal was filtered 30 Hz low pass, 0.1 Hz



**Fig. 1** Experimental design for the visuo-spatial n-back task; the stimuli are represented by the letter O that lasts 1500 ms and moves within the 3×3 grid on the screen; the inter-stimulus interval is 150 ms. Participants are asked to respond, as shown by the hand that is pressing the keyboard, when the stimulus is in the same position as *n* stimuli

before. The experiment encompassed three conditions: control condition (0-back) on the left, low working memory load condition (1-back) in the center, and high working memory load condition (2-back) on the right



**Fig. 2** EEG electrodes (Panel **A**) and fNIRS optodes (Panel **B**) placement. Electrodes are placed and labelled according to the 10–20 International System [29]. Regarding fNIRS, sources (i.e. the optodes that emitted the light) are represented by white dots, detectors (i.e. the

optodes that detect the light) by black dots and the channels (i.e. the source/detector pairs) by lines, labelled with numbers. The channels represented by lines with the same colour belong to the same cortical area

high pass and 50 Hz as notch filter to remove noise and artifacts; in addition, ocular, muscular and other artefacts were removed by Independent Component Analysis. Epochs were extracted from –200 to 1500 ms pre/post stimulus and any incorrect responses were removed; finally, data were re-referenced to T3/T4 and epoch rejection was performed with a cut-off of 100  $\mu$ V, to remove any further noise or artifacts. After visual inspection, the time points selected for the analysis were the amplitude and the latency of the two evoked potentials found between 200 and 400 ms on FC1, FC2 and FCz and between 300 and 800 ms on P3, P4, Pz, PO3, PO4, POz; the data were not normally distributed. Amplitude and latency of the two evoked potentials of patients and controls were compared by two Wald tests with working memory load (0-, 1- and 2-back) as the within factor and group (patients and controls) as the between factor. Amplitude and latency of the two evoked potentials of patients with/without CHE and controls were compared by two Wald tests with working memory load (0-, 1- and 2-back) as the within factor and group (patients with and without CHE and controls) as the between factor. Amplitude and latency of the two evoked potentials in patients before and after treatment with rifaximin were compared by two Wald tests with working memory load (0-, 1- and 2-back) and time (before and after rifaximin) as the within factors. Amplitude and latency of the two evoked potentials of patients with/without CHE before and after treatment with

rifaximin were compared by two Wald tests with working memory load (0-, 1- and 2-back) and time (before and after rifaximin) as the within factors and group (patients with and without CHE) as the between factor.

fNIRS data were processed using Homer2 [33]. Artifacts related to movement during the task were removed by applying the wavelet motion correction technique ( $iqr=0.5$ ) and other types of artifacts were removed by principal component analysis by low filtering the data (cut off was 0.5 Hz and the explained variance was set to 85%). Oxygenated ( $\Delta$ HbO) and deoxygenated ( $\Delta$ HbR) haemoglobin were computed using the modified Beer–Lambert law with a subject-specific DPF [34] and extracted from –5 to 60 s pre/post block onset for each working memory load and for each channel. Based on channel sensitivity calculated by the AtlasViewer toolbox [35] and MRICro [36], channels were divided in 10 cortical areas: right (channels 18 and 20) and left (channels 1 and 3) frontal cortex, right (channels 13 and 19) and left (channels 4 and 11) orbitofrontal cortex, right (channels 14 and 16), left (channels 6 and 7) and central (channel 12) frontopolar cortex and right (channels 10, 15 and 17), left (channels 2, 5 and 8) and central (channel 9) dorsolateral prefrontal cortex. After visual inspection,  $\Delta$ HbO and  $\Delta$ HbR concentration changes were extracted in the 15–30 s time window and averaged, as metric for statistical analysis; both  $\Delta$ HbO and  $\Delta$ HbR were not normally distributed.

$\Delta\text{HbO}$  and  $\Delta\text{HbR}$  of patients and controls were compared by two Wald tests with working memory load (0-, 1- and 2-back) and cortical areas (right and left frontal cortex, right and left orbitofrontal cortex, right, left and central frontopolar cortex and right, left and central dorsolateral prefrontal cortex) as the within factors, and group (patients and controls) as the between factor.  $\Delta\text{HbO}$  and  $\Delta\text{HbR}$  of patients with/without CHE and controls were compared by two Wald tests with working memory load (0-, 1- and 2-back) and cortical areas (right and left frontal cortex, right and left orbitofrontal cortex, right, left and central frontopolar cortex and right, left and central dorsolateral prefrontal cortex) as the within factors, and group (patients with and without CHE and controls) as the between factor.  $\Delta\text{HbO}$  and  $\Delta\text{HbR}$  of patients before and after treatment with rifaximin were compared by two Wald tests with working memory load (0-, 1- and 2-back), cortical areas (right and left frontal cortex, right and left orbitofrontal cortex, right, left and central frontopolar cortex and right, left and central dorsolateral prefrontal cortex) and time (before and after rifaximin) as the within factors.  $\Delta\text{HbO}$  and  $\Delta\text{HbR}$  of patients with/without CHE before and after treatment with rifaximin were compared by two Wald tests with working memory load (0-, 1- and 2-back), cortical areas (right and left frontal cortex, right and left orbitofrontal cortex, right, left and central frontopolar cortex and right, left and central dorsolateral prefrontal cortex) and time (before and after rifaximin) as the within factors, and group (patients with and without CHE) as the between factor.

Comparisons with one/two within factors we were carried out by the nparLD package (*post hoc*: Wilcoxon test adjusted by Bonferroni) [37] and comparisons with more than two within factors by the Manova.RM package (*post hoc*: Tuckey test adjusted by Bonferroni) [38] using R, version 1.2.5 (R core team, 2013). Correlations between laboratory indices and behavioural, neuroanatomical and neurophysiological indices were carried out by the Pearson r correlation coefficient using R, version 1.2.5 (R core team, 2013).

## Results

Patients showed lower educational attainment [ $11 \pm 3$  vs.  $14 \pm 6$  yrs,  $t(105)=3.07$ ,  $p<0.01$ ], MMSE scores [ $26 \pm 3$  vs.  $28 \pm 1$ ,  $t(90)=4.96$ ,  $p<0.01$ ], higher BDI-II score [ $11 \pm 8$  vs.  $4 \pm 3$ ,  $t(67)=-5.87$ ,  $p<0.01$ ] and lower CRI-q scores [ $105 \pm 16$  vs.  $118 \pm 201$ ,  $t(111)=3.69$ ,  $p<0.05$ ] compared to controls. By contrast, there were no differences in the above indices in patients with/without CHE. Post-rifaximin, amongst patients with CHE at baseline, 5 (63%) still had CHE, 2 (25%) became unimpaired and 1 (12%) did not

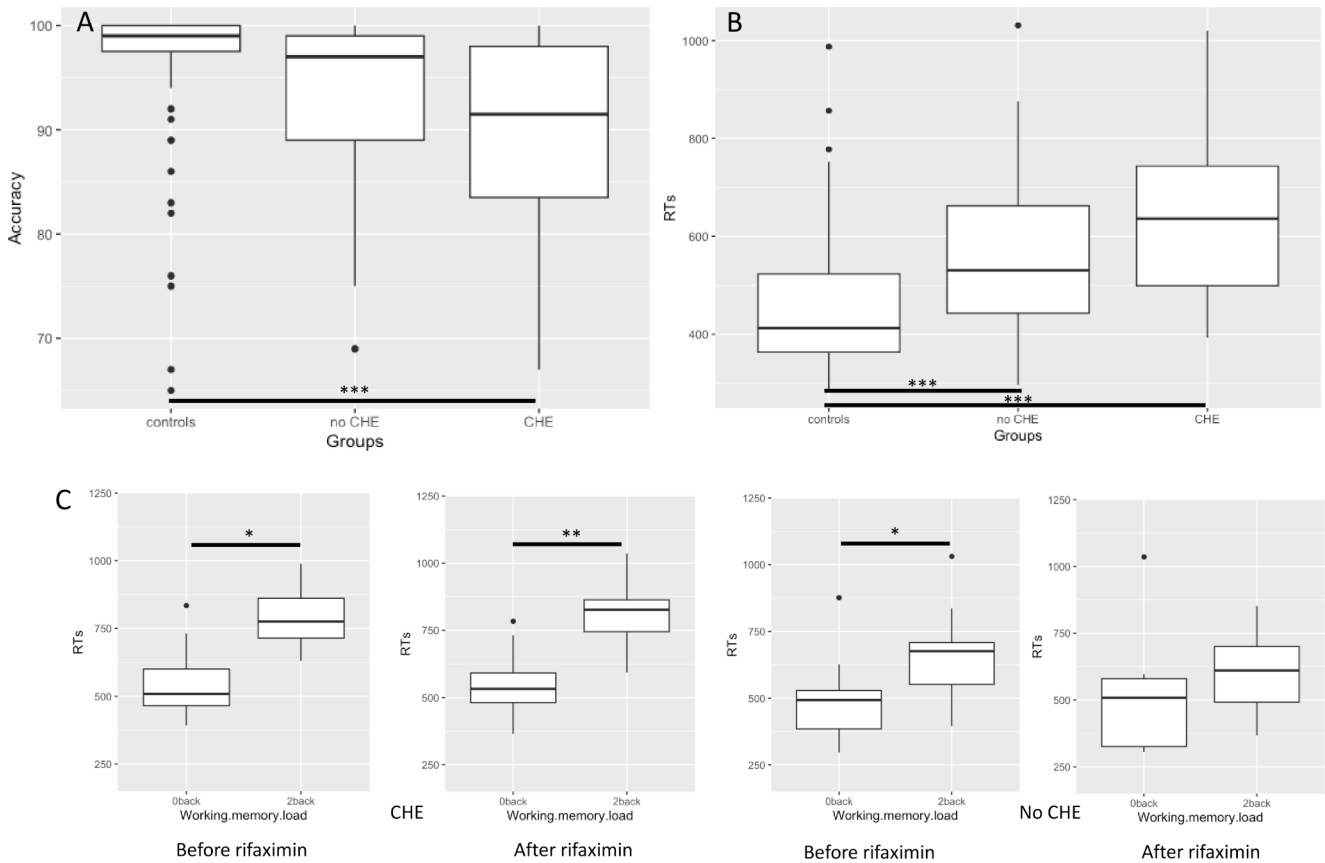
complete the tests because of logistic reasons. Amongst patients with no CHE at baseline, 9 (90%) remained unimpaired while 1 (10%) developed CHE after three months of rifaximin.

## Behaviour

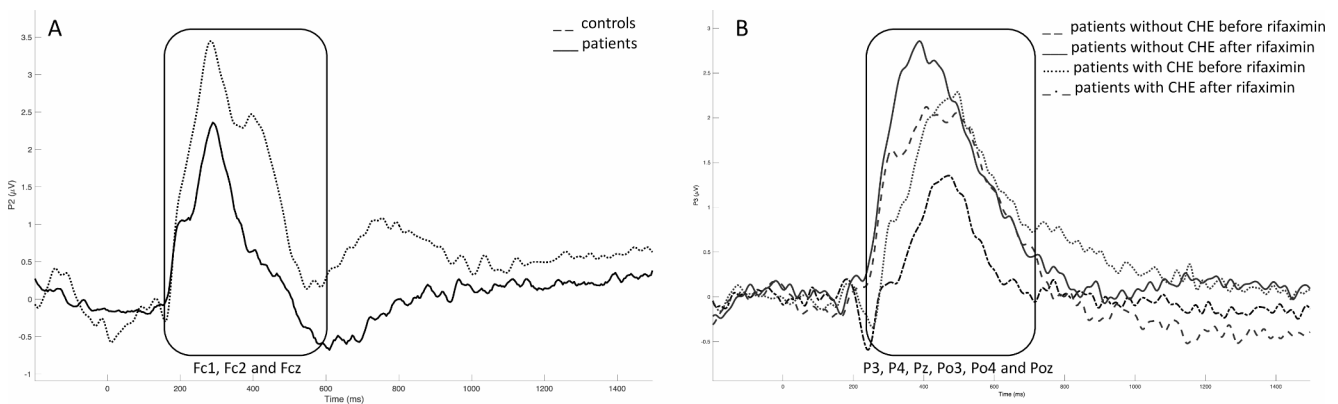
Patients showed less accuracy [median (range)=96 (67/100) %] and slower reaction times [median (range)=544 (296/1030) ms] compared to controls [accuracy: median (range)=99 (65/100) %] [ $\chi^2$  (1,  $N=42$ )=16.3,  $p<0.0001$ ] [RTs: median (range)=412 (67/100) ms] [ $\chi^2$  (1,  $N=42$ )=16.9,  $p<0.0001$ ]. The increase in working memory load was associated with worse performance [accuracy:  $\chi^2$  (2,  $N=42$ )=88.2,  $p<0.000$ ] [RTs:  $\chi^2$  (2,  $N=42$ )=135.8,  $p<0.000$ ], but no interaction between group and load was observed. Patients with CHE showed worse accuracy [median (range)=91 (67/100) %] compared with controls [median (range)=99 (67/100) %] [main effect:  $\chi^2$  (2,  $N=42$ )=47,  $p<0.0001$ ; *post hoc*:  $p<0.0001$ ] (Fig. 3, Panel A), while both patients with [median (range)=636 (396/1019) ms] and without [median (range)=530 (296/1030) ms] CHE showed slower RTs compared to controls [median (range)=412 (67/100) ms] [main effect:  $\chi^2$  (2,  $N=42$ )=20.5,  $p<0.0001$ ; *post hoc*:  $p<0.0001$ ] (Fig. 3, Panel B). The increase in working memory load was associated with worse performance [accuracy:  $\chi^2$  (2,  $N=42$ )=89.1,  $p<0.0001$ ] [RT:  $\chi^2$  (2,  $N=42$ )=1048,  $p<0.0001$ ], but no interaction between group and load was observed.

Accuracy increased after treatment with rifaximin [before: median (range)=97 (65/100) %] [after: median (range)=98 (70/100) %] [ $\chi^2$  (1,  $N=18$ )=11,  $p<0.0001$ ] in both patients with and without CHE. By contrast, RTs showed a three-way interaction between time (before/after treatment with rifaximin), working memory load and presence/absence of CHE [ $\chi^2$  (2,  $N=18$ )=7.5,  $p=0.022$ ]. In further detail, in patients without CHE the difference in RTs between 2- [median (range)=676 (395/1030) ms] and 0-back [median (range)=493 (296/775) ms] observed at baseline disappeared after the treatment with rifaximin [2-back: median (range)=610 (368/852) ms, 0-back: median (range)=508 (305/1036) ms]; *post hoc*:  $p=0.370$ ] (Fig. 3, Panel C). By contrast, in patients with CHE the RTs of the 2-back remained slower compared those of 0-back post-rifaximin [before rifaximin – 2-back: median (range)=775 (630/989) ms; 0-back: median (range)=508 (393/835) ms] [after rifaximin – 2back: median (range)=826 (592/1036) ms; 0-back: median (range)=531 (365/1783) ms, *post hoc*:  $p=0.006$ ] (Fig. 1, Panel C). The increase in working memory load was associated with worse performance [accuracy:  $\chi^2$  (2,  $N=18$ )=102.4,  $p<0.0001$ ] [RT:  $\chi^2$  (2,  $N=18$ )=69,





**Fig. 3** Boxplot of base-10 logarithm of accuracy (Panel A) and reaction times (Panel B and Panel C) in the controls and patients with/without CHE (Panel A and B) and in the patients before and after rifaximin (Panel C)



**Fig. 4** Grand average of the P2 (Panel A) in controls (solid line) and patients (dotted line) and grand average of the P3 (Panel B) in patients without CHE before (dashed line) and after (solid line) rifaximin and in patients with CHE before (dotted line) and after (dashed and dotted line) rifaximin

$p < 0.0001$ ], but no interaction between group and load was observed.

## EEG

Controls showed higher amplitude on FC1, FC2 and FCz at 200–400 ms [median (range)=4.99 (-0.29/11.22)  $\mu$ V] compared to patients [median (range)=3.07 (-0.10/7.57)

$\mu$ V] [ $\chi^2$  (1, N=39)=5.5,  $p=0.018$ ] (Fig. 4, Panel A). Working memory load and the presence/absence of CHE did not produce any EEG effect. When comparing patients before/after treatment with rifaximin, a two-way interaction between presence/absence of CHE and time (before/after treatment with rifaximin) was observed in amplitude on P3, P4, Pz, PO3, PO4 and POz at 300–800 ms [main effect:  $\chi^2$  (1, N=15)=8.7,  $p=0.030$ ]; in further detail,

while patients without CHE showed an increase in amplitude after rifaximin [pre: median (range)=0.90 (0.12/1.89)  $\mu\text{V}$ ; post: median (range)=1.02 (0.03/2.50)  $\mu\text{V}$ ], patients with CHE showed a decrease [pre: median (range)=0.56 (0.11/2.71)  $\mu\text{V}$ ; post: median (range)=0.38 (0.03/2.25)  $\mu\text{V}$ ] (Fig. 4, Panel b).

## fNIRS

On both  $\Delta\text{HbO}$  [ $\chi^2$  (18,  $N=34$ )=75.7,  $p<0.0001$ ] and  $\Delta\text{HbR}$  [ $\chi^2$  (18,  $N=34$ )=34.3,  $p=0.001$ ] a three-way interaction between group (controls/patients), cortical areas and working memory load was observed. In further detail, controls [median (range)=7.67 (-19.75/40.40)  $\mu\text{mol}$ ] showed higher activation in the  $\Delta\text{HbO}$  in the central dorsolateral prefrontal cortex in the 0-back compared to patients [median (range)=0.77 (-13.75/14.18)  $\mu\text{mol}$ ]. Similarly, controls showed higher activation compared to patients in the  $\Delta\text{HbR}$  both in the central frontopolar cortex in the 0-back [controls: median (range)=-0.84 (-15.62/4.92)  $\mu\text{mol}$ ; patients: median (range)=-0.20 (-5.18/1.82)] and in the right dorsolateral prefrontal cortex in the 2-back [controls: median (range)=-1.407 (-5.02/1.66)  $\mu\text{mol}$ ; patients: median (range)=-0.54 (-1.10/2.09)  $\mu\text{mol}$ ]. When considering the presence/absence of CHE, a three-way interaction group (patients with/without CHE), cortical areas and working memory load was observed for both  $\Delta\text{HbO}$  [ $\chi^2$  (31,  $N=34$ )=268.7,  $p<0.0001$ ] and  $\Delta\text{HbR}$  [ $\chi^2$  (31,  $N=34$ )=539.8,  $p<0.0001$ ]. In further detail, patients without CHE [median (range)=10.45 (-2.18/4.28)  $\mu\text{mol}$ ] showed higher  $\Delta\text{HbO}$  in the central dorsolateral prefrontal cortex in the 2-back compared with patients with CHE [median (range)=0.03 (-1.22/21.8)  $\mu\text{mol}$ ] (Fig. 5).

In the comparison before/after treatment with rifaximin both the two-way interaction between time (before/after) and cortical areas [ $\chi^2$  (9,  $N=17$ )=49.3,  $p<0.0001$ ] and the three-way interaction between time (before/after), cortical areas and working memory load [ $\chi^2$  (18,  $N=18$ )=400.5,

$p<0.0001$ ] were significant in  $\Delta\text{HbO}$ . In further detail, after treatment with rifaximin  $\Delta\text{HbO}$  increased in the central frontopolar cortex [before: median (range)=2.66 (-16.77/30.17)  $\mu\text{mol}$ ; after: median (range)=8.19 (-10.46/34)  $\mu\text{mol}$ ] (Fig. 6).

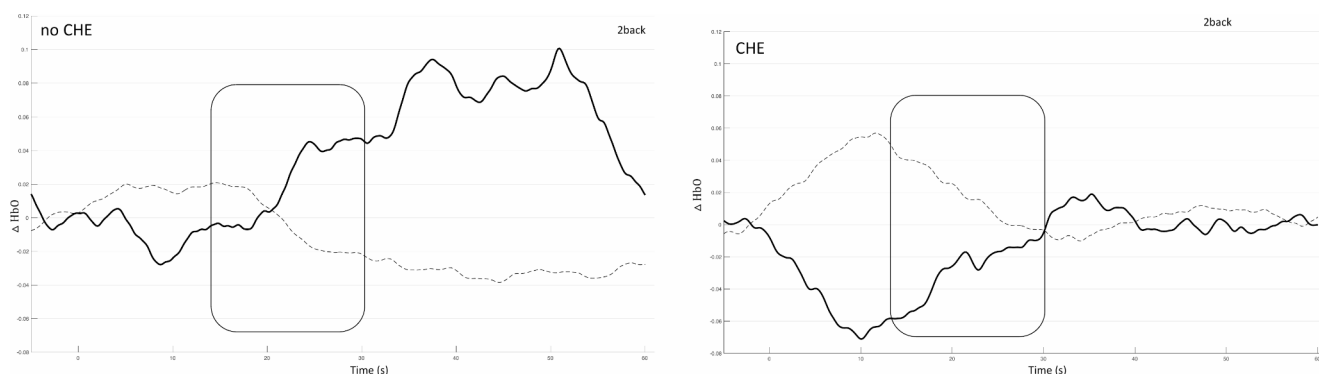
## Correlations Between Laboratory and Behavioural, EEG and fNIRS Indices

Ammonia levels were positively correlated with RTs [ $r(14)=2.374$ ,  $p=0.032$ ]. No other statistically significant correlations were observed, most likely in relation to the fact that, as a group, patients were fairly homogeneous in terms of liver function (all Child A or B) and clinically stable at the time of study.

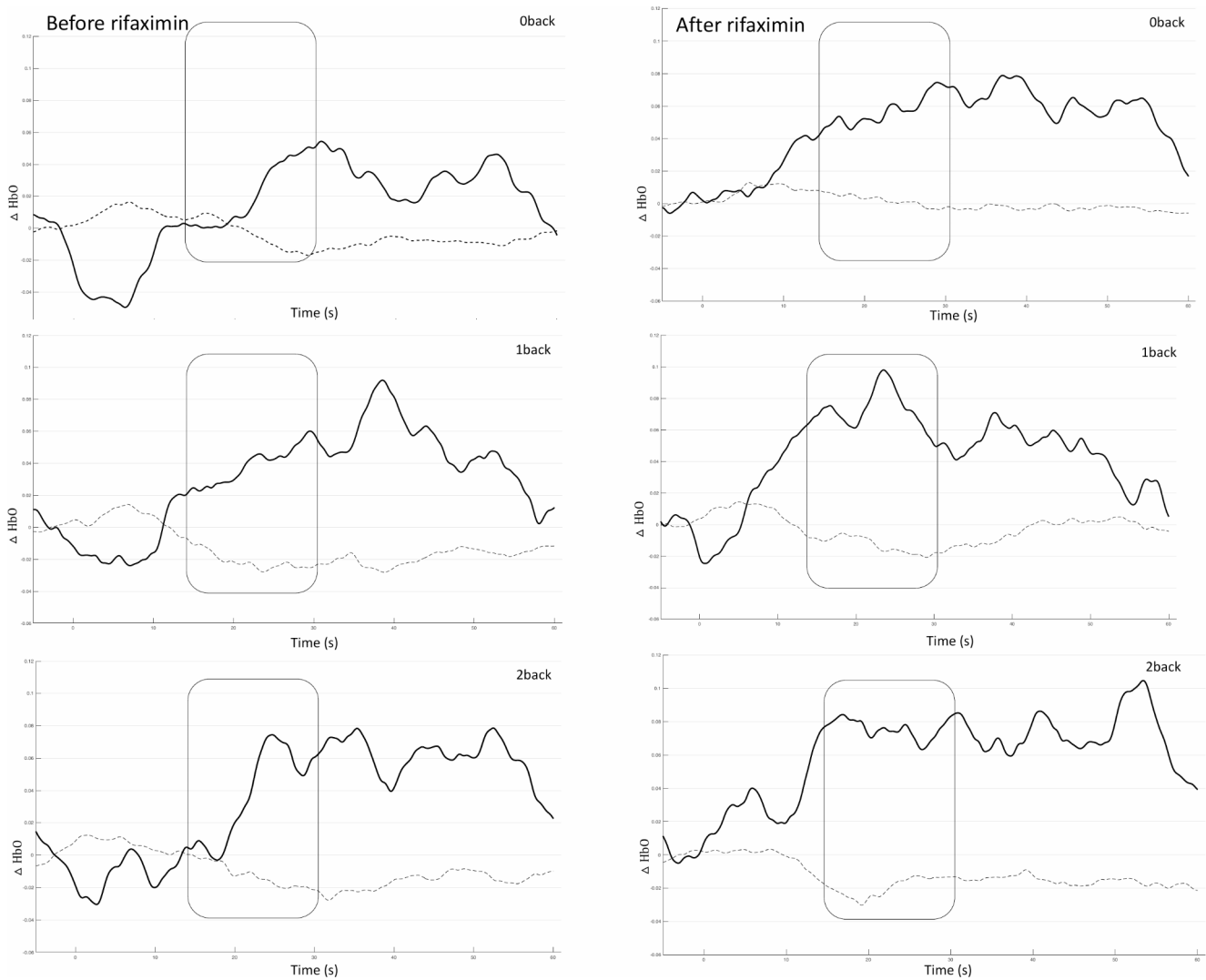
## Discussion

In this study the behavioral, psychophysiological, and hemodynamic correlates of a visuo-spatial n-back task with varying working memory load were compared between controls and patients with cirrhosis and with/without CHE. In addition, behavioral, psychophysiological, and hemodynamic correlates of a visuo-spatial n-back task with varying working memory load were compared before and after a 3-month treatment with rifaximin in patients with cirrhosis as a whole, and split based on the presence/absence of CHE.

Patients with cirrhosis, regardless of the presence of CHE, showed slower reaction times compared to controls; this is in line with one previous study [22] but in contrast with another one that did not find any differences [23]. Regarding the EEG, P2 showed lower amplitude on both task conditions in patients with cirrhosis compared to controls, in line with the findings of Ciecko-Michlaska and colleagues (2012). The alteration of P2 amplitude may relate to an impaired orientation of the attention to the stimulus, and in particular to discrimination between n-back matching and



**Fig. 5** Grand average of the oxygenated (solid line) and deoxygenated (dashed line) haemoglobin in the 2-back condition in patients without (right panel) and with (left panel) CHE



**Fig. 6** Grand average of the oxygenated (solid line) and deoxygenated (dashed line) haemoglobin in the 0- (upper figures), 1- (middle figures) and 2-back (lower figure) conditions in patients without (right panel) and in patients with (left panel) CHE

non-matching stimuli, that therefore either required or did not require a response, respectively [23, 39]. In addition, compared to controls, patients with cirrhosis showed less deoxygenated haemoglobin in the 2-back in the right dorsolateral prefrontal cortex, i.e. the portion of the cortex which is expected to be involved during a visuo-spatial n-back task [40].

Patients with CHE were less accurate than controls, which is in line with previous studies [22, 23]. Further, they showed less oxygenated haemoglobin in the 2-back in the central dorsolateral prefrontal cortex compared with patients without CHE. This seems to be coherent with a diminished response relating to HE rather than cirrhosis

per se and confirms the relation between HE and impaired working memory in haemodynamic terms, which is a novel finding.

After treatment with rifaximin, all patients showed higher accuracy and higher oxygenated haemoglobin in the central frontopolar cortex. Since the central frontopolar cortex is involved when tasks require two or more separate cognitive operations to be behaviorally performed [39], the effect seems to be more related to a general improvement in cognition rather than a specific improvement in working memory. This is also supported by the improvement in both accuracy and oxygenated haemoglobin, that did not differ

when considering the condition and/or the two working memory loads.

Patients without CHE showed faster reaction times in the 2-back compared to the 0-back task, and higher P3 amplitude after treatment with rifaximin compared to patients with CHE, while patients with CHE showed slower RTs in the 2-back compared with the 0-back, and lower P3 amplitude after rifaximin. Since P3 amplitude is thought to be a correlate of the attentional resources allocated to a task [41] and the 2-back was the most demanding task in terms of working memory, it seems that only patients without CHE were able to recruit further attentional resources to improve their performance after treatment. The lack of an effect on P3 latency (which would have been expected) may depend on the high variability of the evoked potential amongst patients with CHE, which was previously documented by our group [42].

This study has some limitations, to include the low number of participants and the difference in educational attainment between patients and controls. In addition, cognitive dysfunction in domains other than working memory is also common in mild HE and this was not specifically tested (other than with PHES). Also, the MMSE might not be adequate to detect mild cognitive impairment, especially in highly educated people. Moreover, when considering the results, it should be noted that patients with cirrhosis showed worse general cognitive functioning (MMSE), worse depressive symptoms (BDI-II) and less cognitive reserve compared to controls, even if those indices were within the normal range, and these differences may have influenced the results, at least to some extent.

In summary, this study confirms that EEG and fNIRS can be successfully used together in patients with cirrhosis, with/without CHE. In addition, we showed that while cirrhosis per se was associated with slower speed and altered attention, CHE was associated with lower accuracy. However, both conditions (i.e., cirrhosis and CHE) were associated with lower cognitive activation during the most difficult working memory task. Moreover, a 3-month rifaximin treatment course was associated with an improvement in accuracy and cognitive activation in patients with cirrhosis and with faster speed in the most difficult task and higher amplitude in the evoked potentials related to working memory task in patients without CHE, while patients with CHE showed a worsening of those indices.

In conclusion, the presence of cirrhosis seemed to have more of an effect than that of CHE on working memory at baseline. A course of treatment with rifaximin was more beneficial to patients without than those with CHE, who probably had more room for improvement in this complex task.

**Author Contributions** LZ, PAm and SM conceived and designed the study. CM, SF and PAn recruited the patients. LZ acquired the data. LZ, CM, DE, PAm, and SM analysed/interpreted the data. LZ and SM drafted the manuscript. SF and PAn reviewed the manuscript for important intellectual content. All authors have read and approved the final version of the manuscript.

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**Data Availability** The datasets generated during the current study are available from the corresponding author on reasonable request.

## Declarations

**Competing Interests** The authors declare no competing interests.

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# Minimal hepatic encephalopathy is associated with a higher risk of overt hepatic encephalopathy and poorer survival

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**Background and aims.** Minimal hepatic encephalopathy (MHE) is a frequent complication in patients with liver cirrhosis. Its impact on predicting the development of overt hepatic encephalopathy (OHE) and survival has not been studied in large multicenter studies.

**Methods.** Data from patients recruited at eight centers across Europe and the United States were analyzed. MHE was detected using the psychometric hepatic encephalopathy score (PHES). A subset was also tested with the simplified animal naming test (S-ANT1). Patients were followed for OHE development and death/liver transplantation (LTx).

**Results.** A total of 1462 patients with a median model of end-stage liver disease of 11 were included (Child-Pugh (CP) stages: A 47%/B 41%/C 12%). Median follow-up time was 19 months, during which 336 (23%) patients developed an OHE episode and 464 (32%) reached the composite end point of death/LTx (369 deaths, 95 LTx). In multivariable analyses, MHE (defined by PHES) was associated with the development of OHE (subdistribution hazard ratio 1.74,  $p < 0.001$ ) and poorer LTx-free survival (hazard ratio 1.53,  $p < 0.001$ )

in the total cohort as well as in the subgroup of patients without a history of OHE. In subgroup analyses, MHE (defined by PHES) was associated with OHE development in patients with CP B, whereas there was no association in patients with CP A or C. In the subgroup of patients with available S-ANT1, MHE (defined by S-ANT1) was independently associated with OHE development. Combined testing (PHES+S-ANT1) was superior to single testing for predicting OHE and poorer LTx-free survival.

**Conclusions.** This large multicenter study demonstrates that screening for MHE is a useful tool for predicting OHE and poorer survival.

**Keywords:** animal naming test, covert hepatic encephalopathy, decompensated cirrhosis, psychometric hepatic encephalopathy score

**Abbreviations:** ALBI, albumin–bilirubin; ANT, animal naming test; CHE, covert hepatic encephalopathy; CI, confidence interval; CP, Child-Pugh; HE, hepatic encephalopathy; HR, hazard ratio; INR, international normalized ratio; IQR, interquartile range; ISHEN, International Society of hepatic encephalopathy and nitrogen metabolism; LTx, liver transplantation; MELD, model of end-stage liver disease; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy; PHES, psychometric hepatic encephalopathy score; S-ANT1, simplified animal naming test; SD, standard deviation; sHR, subdistribution hazard ratio

## Introduction

Liver cirrhosis is widely prevalent worldwide and the main cause of about 1.32 million deaths yearly [1]. The prognosis of cirrhosis is mainly determined by the occurrence of decompensation events. Hepatic encephalopathy (HE)—a neurophysiological complication with detrimental impact on patients' quality of life and huge burden for caregivers [2, 3]—is a common complication in patients with cirrhosis. According to the current guidelines, HE can be divided into covert (CHE) and overt HE (OHE) [4]. CHE is an umbrella term combining the two lowest grades—minimal HE (MHE) and HE grade 1 according to the West-Haven-Criteria, whereas OHE covers grades 2–4. The prevalence of MHE is heavily dependent on the liver disease stage and ranges from <25% in patients with Child-Pugh (CP) A cirrhosis to >50% in patients with CP C [5]. The incidence rates of OHE are less well studied and have been reported to be approximately 10% and 25% per year in patients with CP A and B cirrhosis, respectively [6]. One of the most important predictors for OHE development is the presence of MHE. However, the accuracy of prediction is variable and MHE is not a prerequisite for and may not be detected prior to diagnosis of OHE given the lack of systematic screening for MHE among patients with cirrhosis [6–11]. Most currently available studies on the influence of MHE on OHE development are limited by either small to mediocre sample sizes with heterogeneous cohorts precluding subgroup analyses and different testing strategies used for the detection of MHE. Additionally, it is still a matter of debate on which test should be used to define MHE. Thus, definitive conclusions on the predictive ability of MHE, for example, defined by the psychometric hepatic encephalopathy score (PHES) or the simplified animal naming test (S-ANT1), for OHE development cannot be drawn. The aims of this study were to investigate the association between MHE defined by PHES or the S-ANT1 and OHE development as well as mortality in a large multinational cohort of patients with cirrhosis.

## Patient and methods

This retrospective study analyzed data of 1462 patients, who underwent MHE testing with PHES at the following centers with expertise in diagnosing MHE: Mainz, Siegen, Lübeck (all Germany), Paris (France), Padua, Rome (both Italy), Esbjerg (Denmark), and Ann Arbor (Michigan, US). The details of this study are also described elsewhere

[5]. At each center, the primary etiology of the underlying liver disease was determined according to clinical, serological, and histological findings. Diagnosis of cirrhosis was established by histology, conclusive appearance on ultrasound, elastography or radiological imaging, endoscopic features of portal hypertension, or medical history. The severity of liver disease was determined by calculating the model for end-stage liver disease (MELD), CP scores, and the albumin–bilirubin (ALBI) scores [12–14]. MELD scores were calculated centrally using the original MELD formula of Kamath et al. The three ALBI grades were defined by cut-offs of  $\leq -2.60$  (grade 1),  $> -2.60$  to  $\leq -1.39$  (grade 2), and  $> -1.39$  (grade 3).

Patients were not included in this study if they had a history of any other disease leading to mental alterations (e.g., dementia or a history of stroke). At some centers, patients with self-reported ongoing (mild) alcohol consumption were included provided that they were not under the effects of alcohol during testing with PHES or animal naming test (ANT). Other exclusion criteria varied between centers and are detailed for each center in Table S1.

### *Diagnosis of minimal hepatic encephalopathy*

All patients were examined at the respective hospitals by an experienced hepatologist to rule out clinical signs of HE. For each patient, PHES was performed to diagnose MHE. PHES is a paper-and-pencil testing battery including five subtests (number connection test A and B, serial dotting test, digit symbol test, and line tracing test). PHES was scored at each center using the validated country-specific norms (Germany [15], US [16], Italy [17], and France [18]). The Danish center used the German norms. A score  $< -4$  was considered diagnostic of MHE for centers from Germany, France, and Denmark, whereas the centers from Italy and the US used a score  $\leq -4$ .

In addition, a subgroup of 651 patients (from Germany and Italy) was tested with the ANT. The detailed procedure of ANT is described elsewhere [19]. In brief, patients were asked to name as many animals as possible in 1 min and the number of named animals was defined as the ANT score. Repeats and errors were excluded from the calculations. To adjust for the influence of age and education, we calculated the S-ANT1 which has been proposed by Campagna et al. [19]. For centers from Italy, a score  $< 15$  animals was considered



diagnostic of MHE [19], whereas a score <20 animals was used in centers from Germany and Denmark [20].

#### *Follow-up evaluation*

Patients were followed during regular clinic visits or via electronic chart review at the respective centers for the occurrence of OHE. The presence of OHE was diagnosed after detailed neurological examination according to the West-Haven-Criteria by an experienced hepatologist. Moreover, patients were followed for the occurrence of either death or liver transplantation (LTx). Patients who did not reach the end point of death/LTx were censored at the date of last contact.

#### *Ethics*

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki (7th revision, 2013). This study used anonymized electronic medical records without directly identifiable data. According to German regulations and the recommendations of the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, no ethical approval is required for this type of study. Anonymized data were analyzed as aggregates with no protected health information available. For the subset of patients with data recorded in a prospective setting (Mainz, Siegen, Lübeck), the respective study protocols were approved by the Ethics Committees of the respective centers, and all patients provided informed consent for participation.

#### *Statistical analysis*

R 4.1.3 (R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>) and RStudio version 2022.12.0.353 (Posit Team (2022). RStudio: Integrated Development Environment for R. Posit Software, PBC, Boston, MA. URL <http://www.posit.co/>) were used for data analysis and graphic design. The {table1} R package (v1.4.3; Benjamin Rich 2023) was used for calculating demographics and clinical characteristics. Quantitative data are expressed as median with range. Pairwise comparisons for continuous variables were performed using the Mann–Whitney *U* Test. Categorical variables are displayed as frequencies and percentages. For the comparison

of two or more patient groups, a chi-squared test was applied.

Missing data were excluded from the respective analysis (complete-case analysis). Median follow-up time was calculated using the reverse Kaplan–Meier method and the {prodlm} R package (v2019.11.13, Thomas A. Gerds 2019).

Cumulative incidence functions for competing risk analyses and Fine and Gray competing risk regression analyses were conducted using the {tidycmprsk} R package (v0.2.0, Daniel D. Sjöberg and Teng Fei 2022).

To identify the impact of MHE on OHE development, multistate models with three states were used to calculate OHE incidences and to fit Fine and Gray regression models (coding: 0: alive without LTx and no OHE event at the end of follow-up, 1: OHE event during follow-up, 2: death or LTx during follow-up). Gray's test was used to assess differences between groups in cumulative incidence function. Follow-up time was defined as time from study inclusion to censoring, OHE, death, or transplantation, respectively.

To analyze the impact of MHE on overall survival, analyses were conducted using cumulative incidence functions with a three-state model (0: alive and not transplanted at the end of follow-up; 1: death, 2: LTx). Gray's test was used to evaluate differences between groups. To identify independent variables associated with poorer overall survival, we conducted multivariable regression analyses using the method of Fine and Gray.

To analyze the impact of MHE on transplantation-free survival, Kaplan–Meier curves and Cox proportional hazards regression models were fitted using the {survminer} R package (v0.4.9; Alboukadel Kassambara, Marcin Kosinski, and Przemyslaw Biecek 2021) and the {survival} R package (v3.5-3, Therneau T 2023). Differences between groups were assessed with the log-rank test. For these survival analyses, a two-state model with death and LTx as a composite end point was applied (0: alive, not transplanted; 1: death or LTx).

Regarding multivariable Fine and Gray as well as Cox regression analyses, models were adjusted for age, bilirubin, international normalized ratio, creatinine, albumin, and history of OHE. These variables were selected because they have been found

to be relevant risk factors for developing OHE in previous studies. Continuous variables were kept continuous without categorization, dichotomization, or transformation and were modeled as linear.

For all tests, a 0.05 level was used to define statistically significant deviations from the respective null hypothesis.

## Results

In total, data from 1462 patients with cirrhosis were available for analyses (Fig. S1). The cohort was predominantly male (66.4%) with a median age of 61 years (range 23–87). A history of OHE was noted in 419 patients (29.1%) and MHE according to PHES was present in 503 patients (34.4%). Additional demographics and clinical characteristics are displayed in Table 1. In addition, demographics and clinical characteristics of patients without a history of OHE and the subgroup of patients with available testing with S-ANT1 are shown in Tables S2 and S3, respectively. The patient characteristics differed between centers. The detailed characteristics of the cohorts of each center are displayed in Table S4.

### *Association between MHE and development of OHE during follow-up*

All included patients were followed for the development of the first OHE episode after enrollment or death/LTx. Median follow-up time was 18.8 months (interquartile range 10.3; 29.9). In total, 336 (23%) patients experienced at least one OHE episode. Additionally, 274 (19%) patients died or received a LTx before an episode of OHE.

In the total cohort, the cumulative OHE-incidences differed significantly between patients with MHE defined by PHES compared to patients without MHE ( $p < 0.001$ , Fig. 1). The cumulative OHE-incidences for patients with MHE at 6, 12, and 24 months for patients with MHE were 19%, 28%, and 38%, respectively, whereas they were 7%, 12%, and 20% for patients without MHE (Table 2). Sensitivity, specificity, positive predictive value, and negative predictive value of MHE defined by PHES for the prediction of an OHE event during one year is displayed in Table S5. To further analyze the association between MHE and OHE development, we stratified the cohort according to CP stages. Here, cumulative OHE-incidences differed significantly between patients with and without MHE

in the subgroup of patients with CP B cirrhosis ( $p < 0.001$ ), whereas there was no significant difference in patients with CP A ( $p = 0.09$ ) or CP C ( $p = 0.7$ ) (Fig. 1, Table 2). The same was also done for different MELD groups (Fig. S5). Next, we analyzed the association between MHE defined by PHES and OHE development in the subgroup of patients with alcoholic, viral, or other/mixed cirrhosis. Here, patients with MHE had a significantly higher risk of OHE development in all subgroups (1-year cumulative incidence (MHE– vs. MHE+) for alcohol: 10% vs. 26% ( $p < 0.001$ ), viral: 15% vs. 41% ( $p < 0.001$ ), and others/mixed: 12% vs. 21% ( $p = 0.029$ )). To identify variables independently associated with the development of OHE, we fitted multivariable regression models using the method of Fine and Gray under consideration of the competing events of death or LTx (Table 3). MHE defined by PHES was independently associated with the development of OHE (subdistribution hazard ratio [sHR] 1.74, 95% CI 1.34–2.27,  $p < 0.001$ ). In subgroup analyses, the association between MHE and OHE development only remained significant in patients with CP B (sHR 2.23, 95% CI 1.57–3.18,  $p < 0.001$ ), whereas there was no significant association in patients with CP A (sHR 1.40, 95% CI 0.75–2.61,  $p = 0.3$ ) or CP C (sHR 1.10, 95% CI 0.68–1.78,  $p = 0.7$ ) (Table 3).

Given that patients with a history of OHE are at higher risk for recurrent OHE, we repeated our analyses in the subgroup of patients without a history of OHE ( $n = 1021$ ). Again, the cumulative OHE-incidences differed significantly between patients with and without MHE ( $p < 0.001$ , Fig. 2). This was also true in a subgroup analysis after excluding patients with a prescription of rifaximin and/or lactulose or in those with a prescription of lactulose and/or rifaximin (Fig. S2). The cumulative OHE-incidences for patients with MHE at 6, 12, and 24 months were 16%, 25%, and 37%, respectively, whereas they were 5%, 9%, and 16% for patients without MHE (Table 2). In subgroup analyses stratified by CP stage, cumulative OHE-incidences differed significantly between patients with and without MHE in the subgroup of patients with CP A ( $p = 0.01$ ) or CP B cirrhosis ( $p < 0.001$ ), whereas there was no significant difference in patients with CP C ( $p = 0.5$ ) (Fig. 2). In multivariable analysis, MHE remained significantly associated with the development of OHE (sHR 2.23, 95% CI 1.57–3.17,  $p < 0.001$ , Table S6). In subgroup multivariable analyses, the association between MHE and OHE development remained

Table 1. Demographics and clinical characteristics of the study cohort.

|                           | Total cohort<br>(n = 1462) | MHE–<br>(n = 959)    | MHE+<br>(n = 503)    | p-Value |
|---------------------------|----------------------------|----------------------|----------------------|---------|
| <b>Age (years)</b>        |                            |                      |                      |         |
| Median [min, max]         | 61.0 [23.0, 87.0]          | 60.0 [23.0, 87.0]    | 62.0 [27.0, 87.0]    | 0.03    |
| Missing                   | 1 (0.1%)                   | 1 (0.1%)             | 0 (0%)               |         |
| <b>Gender</b>             |                            |                      |                      |         |
| Male                      | 971 (66.4%)                | 618 (64.4%)          | 353 (70.2%)          | 0.03    |
| Female                    | 491 (33.6%)                | 341 (35.6%)          | 150 (29.8%)          |         |
| <b>Etiology</b>           |                            |                      |                      |         |
| Alcohol                   | 637 (43.8%)                | 388 (40.8%)          | 249 (49.6%)          | 0.005   |
| Viral                     | 356 (24.5%)                | 242 (25.4%)          | 114 (22.7%)          |         |
| Others/Mixed              | 460 (31.7%)                | 321 (33.8%)          | 139 (27.7%)          |         |
| Missing                   | 9 (0.6%)                   | 8 (0.8%)             | 1 (0.2%)             |         |
| <b>MELD score</b>         |                            |                      |                      |         |
| Median [min, max]         | 11.0 [6.00, 34.0]          | 10.0 [6.00, 29.0]    | 12.0 [6.00, 34.0]    | <0.001  |
| Missing                   | 67 (4.6%)                  | 50 (5.2%)            | 17 (3.4%)            |         |
| <b>ALBI score</b>         |                            |                      |                      |         |
| Median [min, max]         | –2.06 [–3.75, 0.272]       | –2.19 [–3.75, 0.272] | –1.79 [–3.40, 0.143] | <0.001  |
| Missing                   | 328 (22.4%)                | 224 (23.4%)          | 104 (20.7%)          |         |
| <b>Child Pugh</b>         |                            |                      |                      |         |
| A                         | 648 (46.6%)                | 497 (54.7%)          | 151 (31.3%)          | <0.001  |
| B                         | 574 (41.2%)                | 333 (36.6%)          | 241 (49.9%)          |         |
| C                         | 170 (12.2%)                | 79 (8.7%)            | 91 (18.8%)           |         |
| Missing                   | 70 (4.8%)                  | 50 (5.2%)            | 20 (4.0%)            |         |
| <b>ALBI grade</b>         |                            |                      |                      |         |
| 1                         | 260 (22.9%)                | 205 (27.9%)          | 55 (13.8%)           | <0.001  |
| 2                         | 659 (58.1%)                | 425 (57.8%)          | 234 (58.6%)          |         |
| 3                         | 215 (19.0%)                | 105 (14.3%)          | 110 (27.6%)          |         |
| Missing                   | 328 (22.4%)                | 224 (23.4%)          | 104 (20.7%)          |         |
| <b>MHE (S-ANT1)</b>       |                            |                      |                      |         |
| No                        | 336 (51.6%)                | 283 (66.1%)          | 53 (23.8%)           | <0.001  |
| Yes                       | 315 (48.4%)                | 145 (33.9%)          | 170 (76.2%)          |         |
| Missing                   | 811 (55.5%)                | 531 (55.4%)          | 280 (55.7%)          |         |
| <b>History of ascites</b> |                            |                      |                      |         |
| No                        | 660 (45.7%)                | 494 (52.1%)          | 166 (33.5%)          | <0.001  |
| Yes                       | 784 (54.3%)                | 454 (47.9%)          | 330 (66.5%)          |         |
| Missing                   | 18 (1.2%)                  | 11 (1.1%)            | 7 (1.4%)             |         |
| <b>History of OHE</b>     |                            |                      |                      |         |
| No                        | 1021 (70.9%)               | 730 (77.0%)          | 291 (59.1%)          | <0.001  |
| Yes                       | 419 (29.1%)                | 218 (23.0%)          | 201 (40.9%)          |         |
| Missing                   | 22 (1.5%)                  | 11 (1.1%)            | 11 (2.2%)            |         |
| <b>Sodium (mmol/L)</b>    |                            |                      |                      |         |
| Median [min, max]         | 138 [112, 149]             | 139 [118, 149]       | 137 [112, 147]       | <0.001  |
| Missing                   | 215 (14.7%)                | 156 (16.3%)          | 59 (11.7%)           |         |
| <b>Creatinine (mg/dL)</b> |                            |                      |                      |         |
| Median [min, max]         | 0.840 [0.100, 7.51]        | 0.814 [0.100, 7.51]  | 0.900 [0.300, 6.73]  | <0.001  |
| Missing                   | 85 (5.8%)                  | 67 (7.0%)            | 18 (3.6%)            |         |

(Continued)

Table 1. (Continued)

|                              | Total cohort<br>(n = 1462) | MHE–<br>(n = 959)  | MHE+<br>(n = 503)  | p-Value |
|------------------------------|----------------------------|--------------------|--------------------|---------|
| <b>Bilirubin (mg/dL)</b>     |                            |                    |                    |         |
| Median [min, max]            | 1.28 [0.100, 46.5]         | 1.17 [0.175, 33.3] | 1.50 [0.100, 46.5] | <0.001  |
| Missing                      | 87 (6.0%)                  | 70 (7.3%)          | 17 (3.4%)          |         |
| <b>Albumin (g/L)</b>         |                            |                    |                    |         |
| Median [min, max]            | 35.0 [14.0, 54.0]          | 36.0 [15.0, 54.0]  | 32.0 [14.0, 51.1]  | <0.001  |
| Missing                      | 238 (16.3%)                | 167 (17.4%)        | 71 (14.1%)         |         |
| <b>INR</b>                   |                            |                    |                    |         |
| Median [min, max]            | 1.24 [0.800, 3.30]         | 1.21 [0.800, 3.30] | 1.30 [0.900, 3.23] | <0.001  |
| Missing                      | 122 (8.3%)                 | 96 (10.0%)         | 26 (5.2%)          |         |
| <b>Platelets (per nL)</b>    |                            |                    |                    |         |
| Median [min, max]            | 115 [12.0, 1150]           | 120 [19.0, 1150]   | 107 [12.0, 644]    | 0.1     |
| Missing                      | 475 (32.5%)                | 344 (35.9%)        | 131 (26.0%)        |         |
| <b>Lactulose<sup>a</sup></b> |                            |                    |                    |         |
| No                           | 962 (65.9%)                | 699 (73.0%)        | 263 (52.5%)        | <0.001  |
| Yes                          | 497 (34.1%)                | 259 (27.0%)        | 238 (47.5%)        |         |
| Missing                      | 3 (0.2%)                   | 1 (0.1%)           | 2 (0.4%)           |         |
| <b>Rifaximin<sup>a</sup></b> |                            |                    |                    |         |
| No                           | 877 (74.8%)                | 634 (80.6%)        | 243 (63.1%)        | <0.001  |
| Yes                          | 295 (25.2%)                | 153 (19.4%)        | 142 (36.9%)        |         |
| Missing                      | 290 (19.8%)                | 172 (17.9%)        | 118 (23.5%)        |         |

Abbreviations: ALBI, albumin–bilirubin; INR, international normalized ratio; MELD, model for end-stage liver disease; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy; S-ANT1, simplified animal naming test; SD, standard deviation.

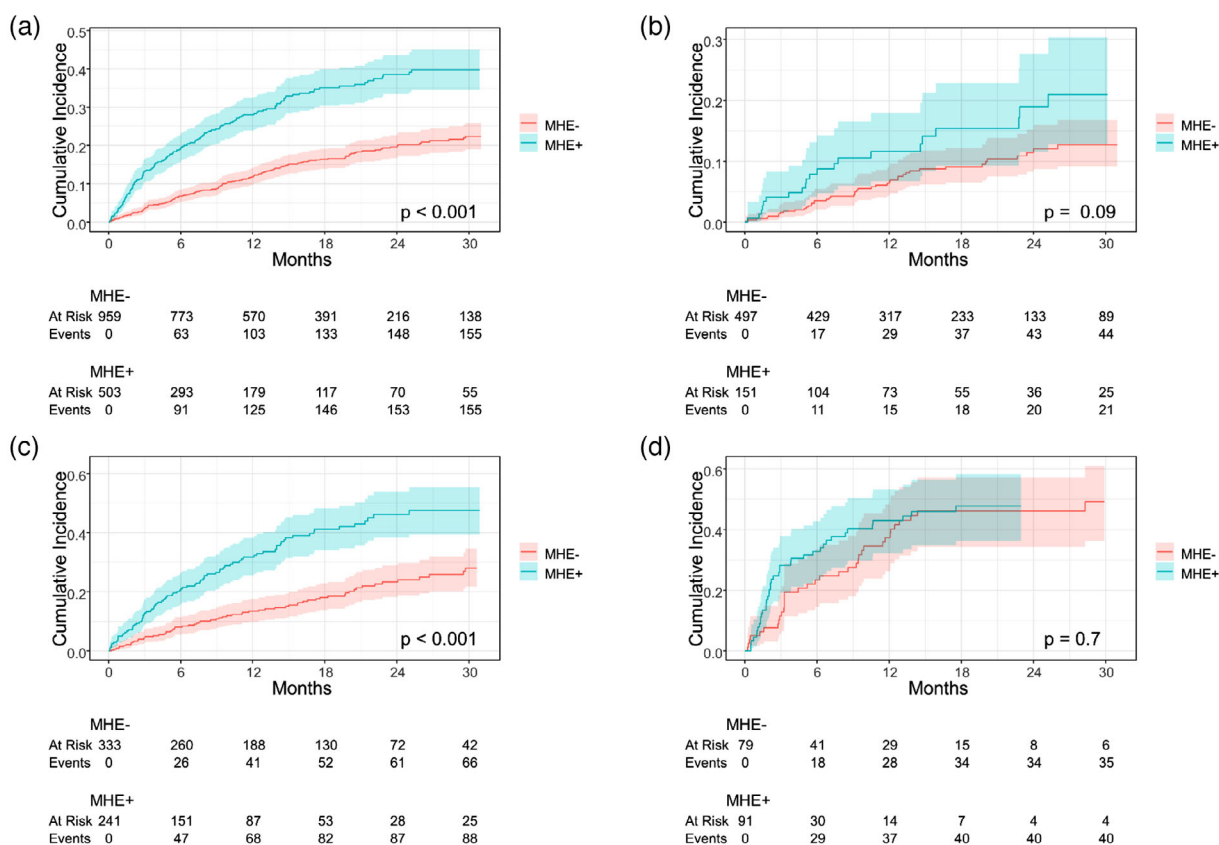
<sup>a</sup>Intake at study inclusion. Percentages refer to the patients with available data.

significant in patients with CP A cirrhosis (sHR 2.14, 95% CI 1.02–4.49,  $p = 0.043$ ) as well as CP B (sHR 2.92, 95% CI 1.76–4.82,  $p < 0.001$ ), whereas there was no significant association in patients with CP C (sHR 1.16, 95% CI 0.60–2.21,  $p = 0.7$ ) (Table S6).

We also investigated cumulative OHE-incidences in patients with a history of OHE. No significant difference was detected in patients with or without MHE (Fig. S6).

A subgroup of 651 patients was also tested with S-ANT1 at baseline and MHE according to S-ANT1 was present in  $n = 315$  (48%). In this subgroup, 20.7% developed an episode of OHE during follow-up, whereas 25.8% of the cohort without available S-ANT1 developed an OHE episode (Table S7). In the total cohort as well as in the subgroup of patients without a history of OHE, the cumulative OHE-incidences differed significantly

between patients with MHE defined by S-ANT1 compared to patients without MHE ( $p = 0.002$  and  $p = 0.006$ , Fig. 3). In multivariable analysis, MHE according to S-ANT1 remained significantly associated with OHE development (sHR 1.56, 95% CI 1.11–2.21,  $p = 0.011$ ) (Table S8). We also compared the cumulative OHE incidences of patients with abnormal results in both PHES and S-ANT1 ( $n = 170$ ) to patients with abnormal results in only one test ( $n = 198$ ) and patients with no abnormal results ( $n = 283$ ). The cumulative OHE incidences differed significantly between the groups (Fig. 3,  $p < 0.001$ ). This finding also remained true after excluding patients with a history of OHE (Fig. 3,  $p < 0.001$ ). In multivariable analysis, abnormal results in both PHES and S-ANT1 had a stronger association with the development of OHE (sHR 2.44, 95% CI 1.59–3.74,  $p < 0.001$ ) compared to abnormal results in only one test (sHR 1.47, 95% CI 0.95–2.27,  $p = 0.082$ ) (Table S8).



**Fig. 1** Cumulative incidence of overt HE (OHE). A three-state model was fitted with death and liver transplantation as competing events. Differences between groups were calculated with Gray's test. Cumulative OHE incidence in patients with versus without MHE defined by PHES in (a) all patients, (b) patients with Child-Pugh A, (c) patients with Child-Pugh B, and (d) patients with Child-Pugh C. MHE, minimal hepatic encephalopathy (defined by PHES); PHES, psychometric hepatic encephalopathy score.

#### Association between MHE and death/liver transplantation during follow-up

In total, 464 (32%) patients died ( $n = 369$ ) or underwent LTx ( $n = 95$ ) during follow-up. Patients with MHE according to PHES had significantly poorer overall survival and transplantation-free survival compared to patients without MHE ( $p < 0.001$  each, Fig. 4). When stratifying the cohort according to CP stage, MHE according to PHES remained significantly associated with poorer transplantation-free survival across all CP stages ( $p < 0.01$  each, Fig. S3). In multivariable Cox regression analysis, MHE was independently associated with poorer transplantation-free survival (hazard ratio [HR] 1.53, 95% CI 1.22–1.92,  $p < 0.001$ , Table 4). In competing risk regression analysis using the method of Fine and Gray, MHE remained independently associated with poorer overall

survival even when LTx was defined as a competing event (sHR 1.66, 95% CI 1.27–2.18,  $p < 0.001$ , Table S9).

In the subgroup of patients with available S-ANT1 results, MHE defined by S-ANT1 was not significantly associated with poorer transplantation-free survival (HR 1.27, 95% CI 0.94–1.72,  $p = 0.12$ , Table S10) in multivariable Cox regression analysis. Additionally, in multivariable competing risk regression analysis, MHE defined by S-ANT1 was not significantly associated with overall survival when treating LTx as a competing risk (sHR 1.30, 95% CI 0.91–1.84,  $p = 0.2$ ) (Table S9).

We also investigated the association between combined testing with PHES and S-ANT1 and transplantation-free as well as overall survival. In Cox regression analysis, pathologic results in both



**Table 2.** Cumulative OHE incidences at 6, 12, and 24 months in different subgroups stratified by MHE status.

|                                                        | MHE (PHES)                           | No MHE (PHES)                        |                              |
|--------------------------------------------------------|--------------------------------------|--------------------------------------|------------------------------|
| Time                                                   | Cumulative OHE<br>incidence (95% CI) | Cumulative OHE<br>incidence (95% CI) | <i>p</i> -Value <sup>a</sup> |
| <b>Total cohort (n = 1462)</b>                         |                                      |                                      |                              |
| 6 months                                               | 19% (16%, 23%)                       | 6.8% (5.3%, 8.5%)                    | <0.001                       |
| 12 months                                              | 28% (24%, 32%)                       | 12% (9.8%, 14%)                      |                              |
| 24 months                                              | 38% (33%, 44%)                       | 20% (17%, 23%)                       |                              |
| <b>Child-Pugh A (n = 648)</b>                          |                                      |                                      |                              |
| 6 months                                               | 7.9% (4.2%, 13%)                     | 3.6% (2.1%, 5.5%)                    | 0.09                         |
| 12 months                                              | 12% (6.7%, 18%)                      | 6.6% (4.5%, 9.3%)                    |                              |
| 24 months                                              | 19% (12%, 28%)                       | 12% (8.7%, 16%)                      |                              |
| <b>Child-Pugh B (n = 574)</b>                          |                                      |                                      |                              |
| 6 months                                               | 21% (16%, 26%)                       | 8.1% (5.4%, 11%)                     | <0.001                       |
| 12 months                                              | 32% (26%, 38%)                       | 13% (9.9%, 18%)                      |                              |
| 24 months                                              | 46% (38%, 54%)                       | 23% (18%, 29%)                       |                              |
| <b>Child-Pugh C (n = 170)</b>                          |                                      |                                      |                              |
| 6 months                                               | 33% (23%, 43%)                       | 23% (15%, 33%)                       | 0.7                          |
| 12 months                                              | 43% (32%, 53%)                       | 37% (26%, 48%)                       |                              |
| <b>Patients without a history of OHE (n = 1021)</b>    |                                      |                                      |                              |
| 6 months                                               | 16% (12%, 21%)                       | 5.4% (3.9%, 7.2%)                    | <0.001                       |
| 12 months                                              | 25% (20%, 31%)                       | 8.8% (6.8%, 11%)                     |                              |
| 24 months                                              | 37% (30%, 44%)                       | 16% (13%, 19%)                       |                              |
| <b>Child-Pugh A without a history of OHE (n = 530)</b> |                                      |                                      |                              |
| 6 months                                               | 5.3% (1.9%, 11%)                     | 2.7% (1.4%, 4.6%)                    | 0.01                         |
| 12 months                                              | 10% (5.0%, 18%)                      | 4.9% (3.0%, 7.5%)                    |                              |
| 24 months                                              | 20% (11%, 31%)                       | 8.9% (6.0%, 12%)                     |                              |
| <b>Child-Pugh B without a history of OHE (n = 357)</b> |                                      |                                      |                              |
| 6 months                                               | 19% (13%, 27%)                       | 5.9% (3.3%, 9.6%)                    | <0.001                       |
| 12 months                                              | 31% (22%, 40%)                       | 9.7% (6.1%, 14%)                     |                              |
| 24 months                                              | 48% (36%, 59%)                       | 18% (13%, 25%)                       |                              |
| <b>Child-Pugh C without a history of OHE (n = 105)</b> |                                      |                                      |                              |
| 6 months                                               | 32% (20%, 46%)                       | 25% (14%, 38%)                       | 0.5                          |
| 12 months                                              | 45% (30%, 59%)                       | 33% (21%, 47%)                       |                              |

Abbreviations: CI, confidence interval; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy; PHES, psychometric hepatic encephalopathy score.

<sup>a</sup>Gray's test.

PHES and S-ANT1 had a stronger association with poorer transplantation-free survival (HR 1.97, 95% CI 1.36–2.85,  $p < 0.001$ ) compared to pathologic results in only one test (HR 1.51, 95% CI 1.04–2.20,  $p = 0.032$ ) (Table S10). In multivariable competing risk regression analysis, pathologic results in both PHES and S-ANT1 were associated with poorer overall survival when LTx was defined as a competing event (sHR 2.03, 95% CI 1.33–3.09,  $p < 0.001$ ), whereas pathologic results in only one

test were not (sHR 1.26, 95% CI 0.81–1.95,  $p = 0.3$ ) (Table S9).

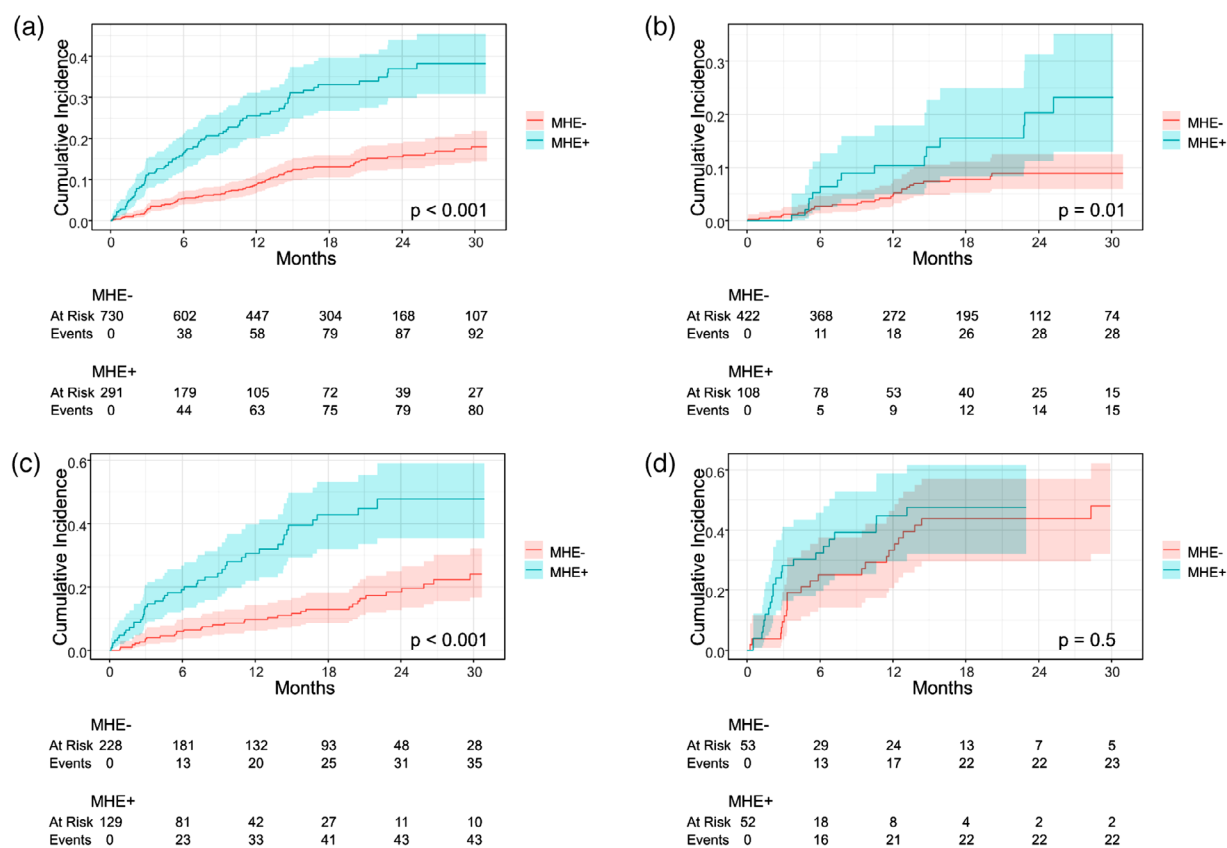
To adjust for within-center dependence, we fitted a Fine and Gray regression model including seven centers as dummy variables with Mainz as the largest center in the reference group, respectively (Table S11). Here, MHE remained independently associated with OHE development with a sHR of 1.61 (95% CI 1.23–2.09,  $p < 0.001$ ).

**Table 3.** Multivariable competing risk regression analysis for the development of overt HE in the total cohort and stratified by Child-Pugh (CP) stage.

| Variables                 | Total cohort |      |            |                  |  | CP A |      |            |                  |  | CP B |      |            |                  |  | CP C |      |            |      |  |
|---------------------------|--------------|------|------------|------------------|--|------|------|------------|------------------|--|------|------|------------|------------------|--|------|------|------------|------|--|
|                           | N            | sHR  | 95% CI     | p                |  | N    | sHR  | 95% CI     | p                |  | N    | sHR  | 95% CI     | p                |  | N    | sHR  | 95% CI     | p    |  |
| <b>MHE (PHES)</b>         |              |      |            |                  |  |      |      |            |                  |  |      |      |            |                  |  |      |      |            |      |  |
| No                        | 746          |      |            |                  |  | 404  |      |            |                  |  | 275  |      |            |                  |  | 67   |      |            |      |  |
| Yes                       | 417          | 1.74 | 1.34, 2.27 | <b>&lt;0.001</b> |  | 125  | 1.40 | 0.75, 2.61 | 0.3              |  | 212  | 2.23 | 1.57, 3.18 | <b>&lt;0.001</b> |  | 79   | 1.10 | 0.68, 1.78 | 0.7  |  |
| <b>Albumin (g/L)</b>      | 1,163        | 0.94 | 0.92, 0.96 | <b>&lt;0.001</b> |  | 529  | 0.91 | 0.85, 0.98 | <b>0.009</b>     |  | 487  | 0.98 | 0.95, 1.01 | 0.2              |  | 146  | 0.95 | 0.89, 1.01 | 0.11 |  |
| <b>Creatinine (mg/dL)</b> | 1,163        | 1.22 | 1.03, 1.46 | <b>0.023</b>     |  | 529  | 1.17 | 0.52, 2.64 | 0.7              |  | 487  | 1.28 | 1.07, 1.54 | <b>0.009</b>     |  | 146  | 0.82 | 0.49, 1.37 | 0.5  |  |
| <b>History of OHE</b>     |              |      |            |                  |  |      |      |            |                  |  |      |      |            |                  |  |      |      |            |      |  |
| No                        | 845          |      |            |                  |  | 445  |      |            |                  |  | 308  |      |            |                  |  | 92   |      |            |      |  |
| Yes                       | 318          | 1.72 | 1.32, 2.23 | <b>&lt;0.001</b> |  | 84   | 2.95 | 1.71, 5.09 | <b>&lt;0.001</b> |  | 179  | 1.34 | 0.95, 1.91 | 0.10             |  | 54   | 1.35 | 0.81, 2.23 | 0.3  |  |
| <b>Bilirubin (mg/dL)</b>  | 1,163        | 1.01 | 0.98, 1.05 | 0.4              |  | 529  | 0.77 | 0.29, 2.08 | 0.6              |  | 487  | 1.04 | 1.01, 1.07 | <b>0.011</b>     |  | 146  | 0.96 | 0.89, 1.03 | 0.3  |  |
| <b>INR</b>                | 1,163        | 1.57 | 1.07, 2.32 | <b>0.022</b>     |  | 529  | 2.78 | 0.55, 14.0 | 0.2              |  | 487  | 0.77 | 0.44, 1.34 | 0.4              |  | 146  | 1.50 | 0.71, 3.18 | 0.3  |  |
| <b>Age (years)</b>        | 1,163        | 1.01 | 1.00, 1.03 | <b>0.025</b>     |  | 529  | 1.03 | 0.99, 1.06 | 0.11             |  | 487  | 1.02 | 1.00, 1.03 | 0.063            |  | 146  | 1.01 | 0.98, 1.05 | 0.4  |  |

Note: Patients with missing data were excluded from analysis (complete-case analysis). *p*-Values in bold show significant values.

Abbreviations: CI, confidence interval; CP, Child-Pugh; INR, international normalized ratio; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy; PHES, psychometric hepatic encephalopathy score; sHR, subdistribution hazard ratio.



**Fig. 2** Cumulative incidence of overt HE (OHE) in patients without a history of OHE. A three-state model was fitted with death and liver transplantation as competing events. Differences between groups were calculated with Gray's test. Cumulative OHE incidence in patients with versus without MHE defined by PHES in (a) all patients without a history of OHE, (b) patients with Child-Pugh A without a history of OHE, (c) patients with Child-Pugh B without a history of OHE, and (d) patients with Child-Pugh C without a history of OHE. MHE, minimal hepatic encephalopathy (defined by PHES); PHES, psychometric hepatic encephalopathy score.

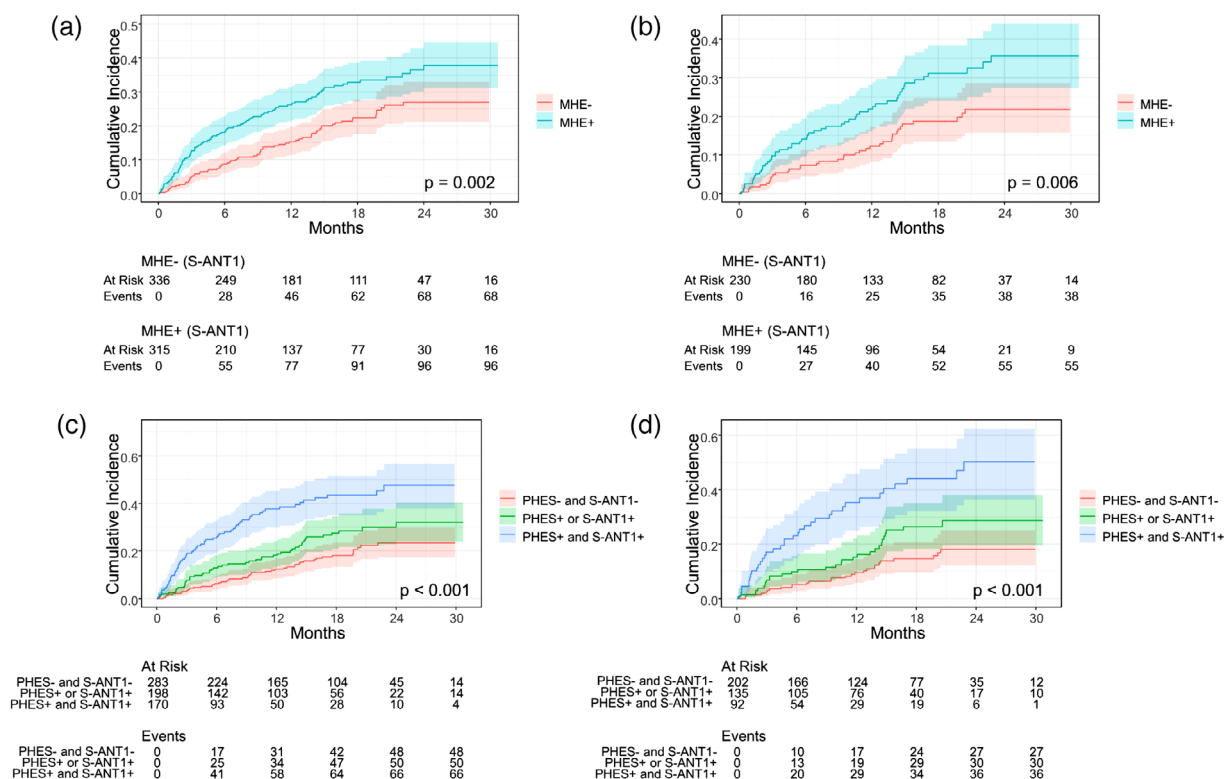
## Discussion

In this study, we provide detailed data on the predictive value of MHE regarding hard clinical end points in patients with cirrhosis in a large multinational cohort. We were able to demonstrate that MHE according to PHES is associated with a higher risk for OHE development; however, this association was restricted to patients with CP B cirrhosis in the total cohort. Moreover, we found a robust association between MHE defined by PHES and poorer (transplantation-free) survival. The findings regarding OHE prediction were also consistent in a subgroup of patients with available S-ANT1. Additionally, we were able to demonstrate that when using combined testing, abnormal results in both PHES and S-ANT1 are superior to abnormal results

in only one test in identifying a group at greater risk of OHE in the subgroup with both tests available.

MHE<sup>></sup> is a common complication in patients with cirrhosis and its prevalence strongly depends on the grade of decompensation of cirrhosis and the respective test used to define the disease [5]. Consequently, testing strategies for MHE are highly debated and studies demonstrated only poor correlation between different tests, such as PHES, critical flicker frequency, or Stroop EncephalApp [21, 22]. This is most likely explained by the fact that in patients with MHE not every sign or symptom may be present at any given time. That's why the international guidelines and the International Society of Hepatic Encephalopathy and Nitrogen Metabolism recommended the use of two





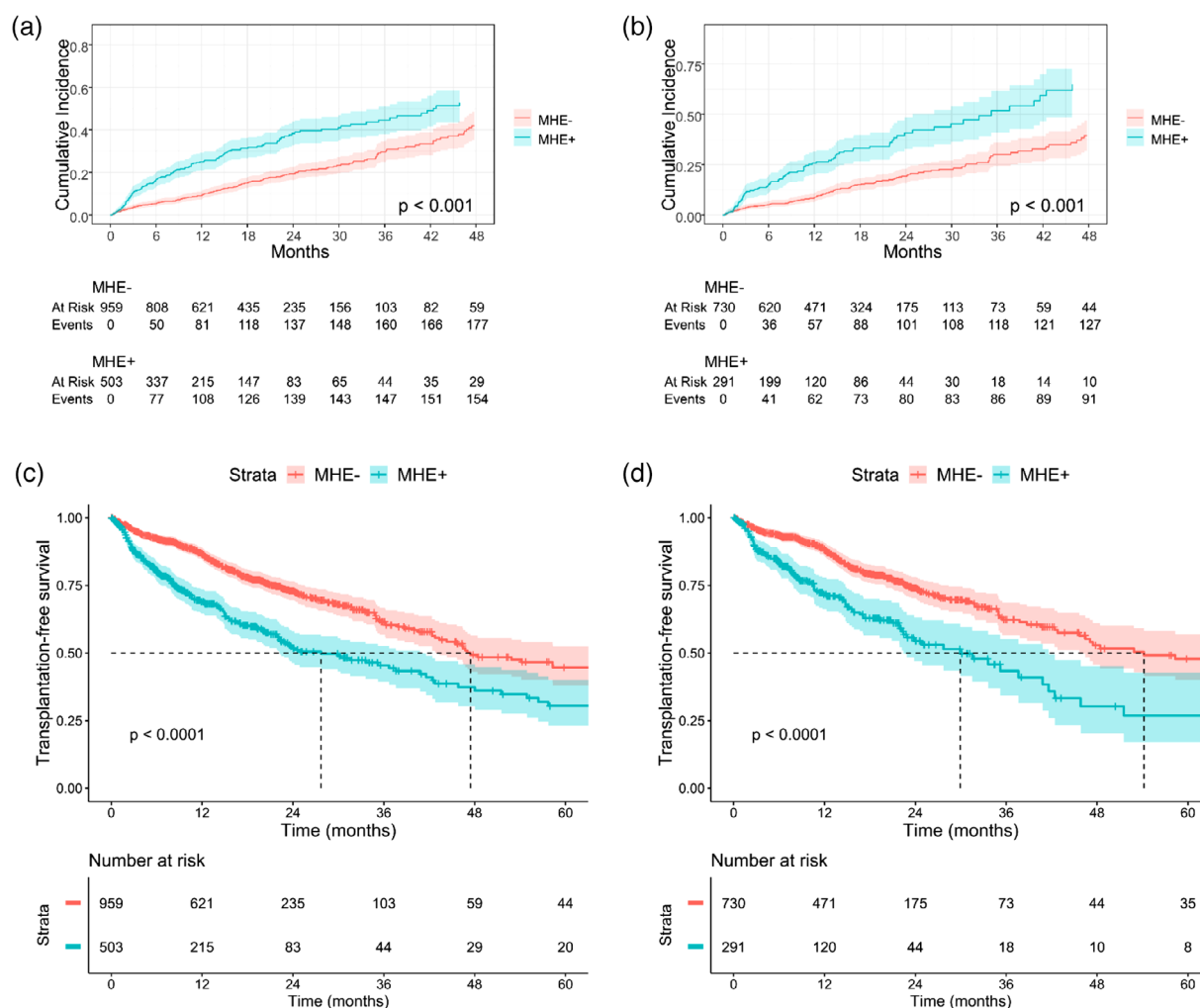
**Fig. 3** Cumulative incidence of overt HE (OHE). Cumulative OHE incidence stratified by MHE status defined by S-ANT1 in (a) total cohort and (b) subgroup of patients without a history of OHE. Cumulative OHE incidence stratified according to their results in PHEs and S-ANT1 (group 1: no MHE according to PHEs and S-ANT1; group 2: MHE according to PHEs or S-ANT1; group 3: MHE according to both PHEs and S-ANT1) in (c) total cohort and (d) subgroup of patients without a history of overt HE. Differences between groups were calculated with Gray's test. MHE-, MHE negative defined by S-ANT1; MHE+, MHE positive defined by S-ANT1; PHEs, Psychometric Hepatic Encephalopathy Score; S-ANT1, simplified animal naming test.

**Table 4.** Multivariable Cox regression analysis for transplantation-free survival.

| Variables                 | N    | HR   | 95% CI     | p      |
|---------------------------|------|------|------------|--------|
| <b>MHE (PHEs)</b>         |      |      |            |        |
| No                        | 746  |      |            |        |
| Yes                       | 417  | 1.53 | 1.22, 1.92 | <0.001 |
| <b>Albumin (g/L)</b>      | 1163 | 0.94 | 0.92, 0.96 | <0.001 |
| <b>Creatinine (mg/dL)</b> | 1163 | 1.46 | 1.29, 1.67 | <0.001 |
| <b>History of OHE</b>     |      |      |            |        |
| No                        | 845  |      |            |        |
| Yes                       | 318  | 1.16 | 0.92, 1.47 | 0.2    |
| <b>Bilirubin (mg/dL)</b>  | 1163 | 1.07 | 1.05, 1.09 | <0.001 |
| <b>INR</b>                | 1163 | 2.17 | 1.59, 2.97 | <0.001 |
| <b>Age (years)</b>        | 1163 | 1.02 | 1.01, 1.04 | <0.001 |

Abbreviations: CI, confidence interval; HR, hazard ratio; INR, International Normalized Ratio; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy; PHEs, psychometric hepatic encephalopathy score.

independent testing strategies for the detection of MHE in multicenter studies in the past [23]. However, this approach has not been extensively validated and a recent study by Duarte-Rojo et al. challenged this concept regarding outcome prediction [11]. When focusing on PHEs, the existing literature is quite heterogeneous indicating a potential association between MHE defined by PHEs and development of OHE, whereas studies regarding a potential association with overall survival are conflicting [7–10]. This may most likely be explained by only mediocre sample sizes with a lack of power or single/bicentric study settings. Our current study solidifies the association between PHEs and hard clinical outcome events, such as OHE and death/LTx, in a large multinational setting. Due to the large sample size, we were also able to perform robust subgroup analyses in different CP stages for the first time. The by far strongest association between MHE and OHE development was found



**Fig. 4** Survival of patients stratified by MHE status. Cumulative incidence of death in patients with versus without MHE defined by PHES, with liver transplantation as a competing event in (a) total cohort and (b) subgroup of patients without a history of overt HE. Differences between groups were calculated with Gray's test. (c) Kaplan-Meier curves showing transplantation-free survival stratified according to MHE status defined by PHES in (c) total cohort and (d) subgroup of patients without a history of overt HE. Differences between groups were calculated with the log-rank test. MHE-, MHE negative defined by PHES; MHE+, MHE positive defined by PHES; PHES, psychometric hepatic encephalopathy score.

in patients with CP B cirrhosis. This finding is of great clinical importance given that most clinicians are aware of MHE. However testing is frequently neglected given the effort needed for cognitive testing in the busy daily routine [24]. In this setting, our study robustly indicates that testing for MHE with PHES for predicting OHE development is especially useful in patients with CP B cirrhosis and should be implemented into routine care for risk stratification. Evidence already suggests that prevention of a first OHE episode may be achiev-

able, and our data may also serve as a blueprint for designing future trials on primary prophylaxis [25–27]. In this context, it seems mandatory that future studies on primary prophylaxis of OHE in patients with CP B cirrhosis need stratification for the presence of MHE, because OHE frequency differs markedly between patients with and without MHE. Another important finding of our study is that primary prophylaxis does not appear to be justified in patients with CP A cirrhosis without a history of OHE irrespective of MHE status given the

low 1-year cumulative incidence of OHE of <10% even in patients with MHE. This is also in line with a single center study published by Tapper et al. (10% 1-year OHE incidence in patients with CP A and portal hypertension) [6]. On the other side, our data suggest that primary prophylaxis trials in patients with CP C cirrhosis independent of MHE status should be strongly encouraged given the high probability for OHE during the subsequent year (>33%).

Despite the availability of various tools for detecting MHE, screening is often neglected in clinical practice [24, 28]. An important explanation is the often busy daily schedules and limited time for patient contact. In this context, the implementation of testing with S-ANT1 may enhance cognitive testing in patients with cirrhosis. Our study indicates in a multicenter setting that MHE defined by S-ANT1 is robustly associated with OHE development. Nevertheless, it has to be kept in mind that S-ANT1 has its weaknesses and the results must be interpreted in the context of a wide variety of influencing factors [29].

There is an ongoing debate on whether testing for MHE should be conducted using one or more testing strategies in routine care as well as study settings [23, 30]. The rationale behind this recommendation is that although the requirement of abnormal results in a combination of tests decreases the prevalence of MHE, pathologic results in two tests could identify patients at the highest risk of OHE. However, a US-based multicenter study by Duarte-Rojo et al. found that a combination of PHES, inhibitory control test, or Stroop EncephalApp was not superior for predicting OHE compared to PHES or Stroop EncephalApp alone [11]. By contrast, our study showed that patients with pathologic results in both PHES and S-ANT1 had a significantly higher risk of OHE and lower probability of transplant-free survival compared to abnormal results in only one test. The study by Duarte-Rojo et al. and ours are not easily comparable given that except for PHES different testing strategies were used to define MHE. Moreover, our current study cannot answer whether the predictive ability of a combination of PHES and S-ANT1 is better for predicting OHE episodes than a combination of PHES and, for example, an oral glutamine challenge or even genetic risk scores [31, 32]. Additionally, we acknowledge that results in S-ANT1 were only available for a subset of the cohort; therefore, our findings need validation in

future prospective studies. In this context, our results lead to the hypothesis that the assessment of different cognitive domains may improve the prediction of OHE. Here, the combination of PHES and S-ANT1 could be potentially beneficial as they investigate partly different cognitive domains.

One of the most important strengths of our study is the large multicenter patient cohort and sufficient follow-up time allowing robust subgroup analyses. However, there are also limitations. First, this was a retrospective analysis. Therefore, patients were not specifically recruited for this study following a dedicated protocol and no monitoring of the quality of testing was possible. Moreover, information on neurotoxic drugs, narcotic analgesia, precipitating events of the OHE episodes or ammonia levels at diagnosis of OHE are not available in this study. Second, patients were only included if testing with PHES was available. This may introduce relevant selection bias as in some parts of the cohort testing with PHES might have only been performed in case of suspicion of MHE. This may lay a focus on sicker patients and therefore our findings might not be generalizable to every patient with cirrhosis. However, we are unable to speculate on the direction of this selection bias. Third, we are unable to provide ideal cut-offs for PHES or S-ANT1 for predicting clinical outcomes in this study. This is explained by the fact that we used country-specific norms for scoring PHES and S-ANT1; therefore, raw results are not completely comparable between centers. Fourth, there is missing data on some laboratory characteristics in some patients. However, these values were missing at random and are not expected to have a major impact on our results. Fifth, data on S-ANT1 were only available in a subgroup of patients; therefore, these analyses lack statistical power. Therefore, these results have to be interpreted with more caution. Sixth, detailed information on the underlying etiology of cirrhosis in the “mixed/other” group was unavailable and consequently precluded additional analyses. Last, we performed a lot of statistical tests in this study and did not adjust for multiple testing. This might cause type I error and *p*-values should be interpreted with caution.

In conclusion, we provide detailed data on the predictive utility of MHE regarding hard clinical end points in patients with cirrhosis in a large multinational cohort. We were able to demonstrate that MHE according to PHES is associated with a higher risk for OHE development, with the strongest asso-

ciation in patients with CP B cirrhosis. Additionally, we were able to demonstrate the superiority of combined testing with PHES and S-ANT1 compared to single testing for OHE prediction. These data need to be validated in prospective, large cohorts to be adopted into routine clinical practice.

### Author contributions

*Performed research:* Simon Johannes Gairing, Chiara Mangini, Lisa Zarantonello, Stefania Gioia, Elise Jonasson Nielsen, Sven Danneberg, Patricia P. Bloom, Philippe Sultanik, Peter Robert Galle, Joachim Labenz, Dominique Thabut, Anna S. Lok, Jens Uwe Marquardt, Mette Munk Lauridsen, Silvia Nardelli, Sara Montagnese, and Christian Labenz. *Contributed to acquisition of data:* Simon Johannes Gairing, Chiara Mangini, Lisa Zarantonello, Stefania Gioia, Elise Jonasson Nielsen, Sven Danneberg, Patricia P. Bloom, Philippe Sultanik, Peter Robert Galle, Joachim Labenz, Dominique Thabut, Anna S. Lok, Jens Uwe Marquardt, Mette Munk Lauridsen, Silvia Nardelli, Sara Montagnese, and Christian Labenz. *Designed the experiments and analyzed the data:* Simon Johannes Gairing and Christian Labenz. *Contributed reagents/materials/analysis tools:* Simon Johannes Gairing and Christian Labenz. *Wrote the paper:* Simon Johannes Gairing and Christian Labenz. *Statistical analysis:* Simon Johannes Gairing and Christian Labenz.

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### Conflict of interest statement

Simon Johannes Gairing: Travel expenses: Ipsen and Gilead. Joachim Labenz: Consulting: Alphasigma, Norgine. Lecture fees: Norgine. Peter R. Galle: Lecture fees and consulting: Merz Pharmaceuticals Patricia P. Bloom: Research grant from Vedanta Biosciences. Consultant for Nexlixo. Dominique Thabut: Lecture fees: Alfasigma, Abbvie, Gilead. Christian Labenz: Travel expenses and consulting: Norgine, Merz Pharmaceuticals. Lecture fees: Norgine, Merz Pharmaceuticals. Research grants: Norgine, Merz Pharmaceuticals. All other authors disclose no potential financial

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### Ethics statement

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki (7th revision, 2013). This study used anonymized electronic medical records without directly identifiable data. According to German regulations and the recommendations of the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, no ethical approval is required for this type of study. Anonymized data were analyzed as aggregates with no protected health information available. Regarding a subset of patient data that were recorded in a prospective setting, the respective study protocols were approved by the Ethics Committees of the respective centers and patients provided written informed consent.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supporting Information ■



# The Alarm Clock Against the Sun: Trends in Google Trends Search Activity Across the Transitions to and from Daylight Saving Time

SHORT PAPER

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ubiquity press

## ABSTRACT

The human circadian timing system depends on the light/dark cycle as its main cue to synchronize with the environment, and thus with *solar time*. However, human activities depend also on *social time*, i.e. the set of time conventions and restrictions dictated by society, including Daylight Saving Time (DST), which adds an hour to any degree of desynchrony between *social* and *solar time*. Here, we used Google Trends as a data source to analyze diurnal variation, if any, and the daily peak in the relative search volume of 26 Google search queries in relation to the transitions to/from DST in Italy from 2015 to 2020. Our search queries of interest fell into three categories: sleep/health-related, medication and random non sleep/health-related. After initial rhythm and phase analysis, 11 words were selected to compare the average phase of the 15 days before and after the transition to/from DST. We observed an average phase advance after the transition to DST, and a phase delay after the transition to civil time, ranging from 25 to 60 minutes. Advances or delays shorter than 60 minutes, which were primarily observed in the sleep/health-related category, may suggest that search timing for these queries is at least partially driven by the endogenous circadian rhythm. Finally, a significant trend in phase anticipation over the years was observed for virtually all words. This is most likely related to an increase in age, and thus in earlier chronotypes, amongst Google users.

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Circadian Clock; Social Clock

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## INTRODUCTION

The Sun has been our most important reference point to tell the time for thousands of years, with the Sun's highest position marking noon in *solar time*. The human circadian clock, an autonomous oscillator regulating most physiological functions [1], uses the light/dark cycle as its main cue to synchronize with the environment and runs according to *solar time* [2–4]. *Solar time* is place-specific, depending on longitudinal position on Earth, with a span of 15° in longitude corresponding to one hour. However, presently the timing of our activities (e.g. work and school schedules) is mainly based on *social time*, i.e. a set of time conventions and restrictions directed by society, which negatively impinge on the entrainment of the endogenous circadian clock with the natural environment [2]. The resulting desynchrony is increased by one hour for part of the year, when we transition to Daylight Saving Time (DST). The use of DST is currently under debate in Europe and has been associated with negative health effects, including sleep curtailment/disturbance [5], and also an increase in cardiovascular events [6]. Further, the DST period has been associated with an increase in attendances to the emergency room [7] and in the likelihood of occurrence of road traffic accidents, observed both in studies based on recorded accidents [8] and experiments with driving simulators [9].

Google Trends [10] is a freely accessible tool providing the relative search volume (RSV) for any Google search query of interest, within a given time frame and location. Important insights can be obtained from these data. For example, the number of searches for “I cannot smell” matched almost perfectly with the incidence rate of COVID-19, hinting at anosmia as a symptom of COVID-19 before it was officially recognized as such [11, 12]. Further, Google Trends has been often used to examine the spread of disease (e.g. influenza) analyzing seasonal and diurnal RSV patterns [13, 14]. The aim of this study was to use Google Trends for the analysis of diurnal variation, if any, in RSV in relation to the transitions to/from DST in Italy for a number of search queries falling into one of three categories: sleep/health-related, medication and random non sleep/health-related.

## METHODS

### GOOGLE TRENDS

The RSV can be extracted from Google Trends specifying: timeframe, geographical location and category to search within [e.g. “health”, “finance” or “news”]. The RSV is calculated from an anonymized representative random sample of the total search frequency. It is scaled on a range

from 0–100, with the value 100 being given to the highest search frequency within the selected time frame and location for the search query [15]. Absolute values are not provided. Besides the RSV of a search query, Google Trends also contains a “compare” function which shows the RSV of two or more search queries. This allows for comparison between related search queries with a view, for example, to choose the most/least commonly searched term amongst a series of synonyms.

### WORD SELECTION

Our search queries of interest fell into three distinct areas: sleep/health-related [e.g. “insomnia” (insomnia)], medication area [e.g. “Xanax”, which is the Italian commercial name for a commonly utilized sleep-inducer, i.e. lorazepam], and random non sleep/health-related [e.g. “taxi”]. The Google Trends “compare” function was used to select the most representative word out of two or more synonyms, and the synonym with the highest RSV was chosen. This resulted in a final list of 26 words (Supplementary Table 1).

### GOOGLE TRENDS DATA EXTRACTION

Hourly search query data in Italy was extracted for all 26 words using the available pytrends script for Python 3 [18]. For each word, the RSVs were extracted separately in 1-week segments for a period of 15 days prior to and 15 days after the transition to/from DST in the years 2015 – 2020. In Italy, the transition to/from DST occurs on the last Sunday in March and the last Sunday in October at 01:00 UTC, respectively. The geographical location was set as “Italy”. The files were downloaded in Coordinated Universal Time (UTC) using the following filters: “Italy”, “Time zone offset 0” (i.e. the difference in hours and minutes from UTC), and “All categories” (i.e. category to search within). Since the time was set to UTC, there was no one-hour time shift during the switch to/from DST in the extracted data. The Italian standard time, CET, is UTC+1 and the Italian DST, CEST, is UTC+2.

### NORMALIZATION

Hourly data from Google Trends were extracted in 1-week segments, with two days overlap between consecutive weeks. These two days – with the first week being used as reference – were utilized to normalize the data (which are available as percentages of RSV ranges within each single week) and thus make percentages comparable across the explored period. The ratio of RSVs of the overlapping days was used to calculate a normalization coefficient and normalization then performed by word and time period (transition to and from DST), separately.

## DAILY RHYTHM AND PHASE DETERMINATION

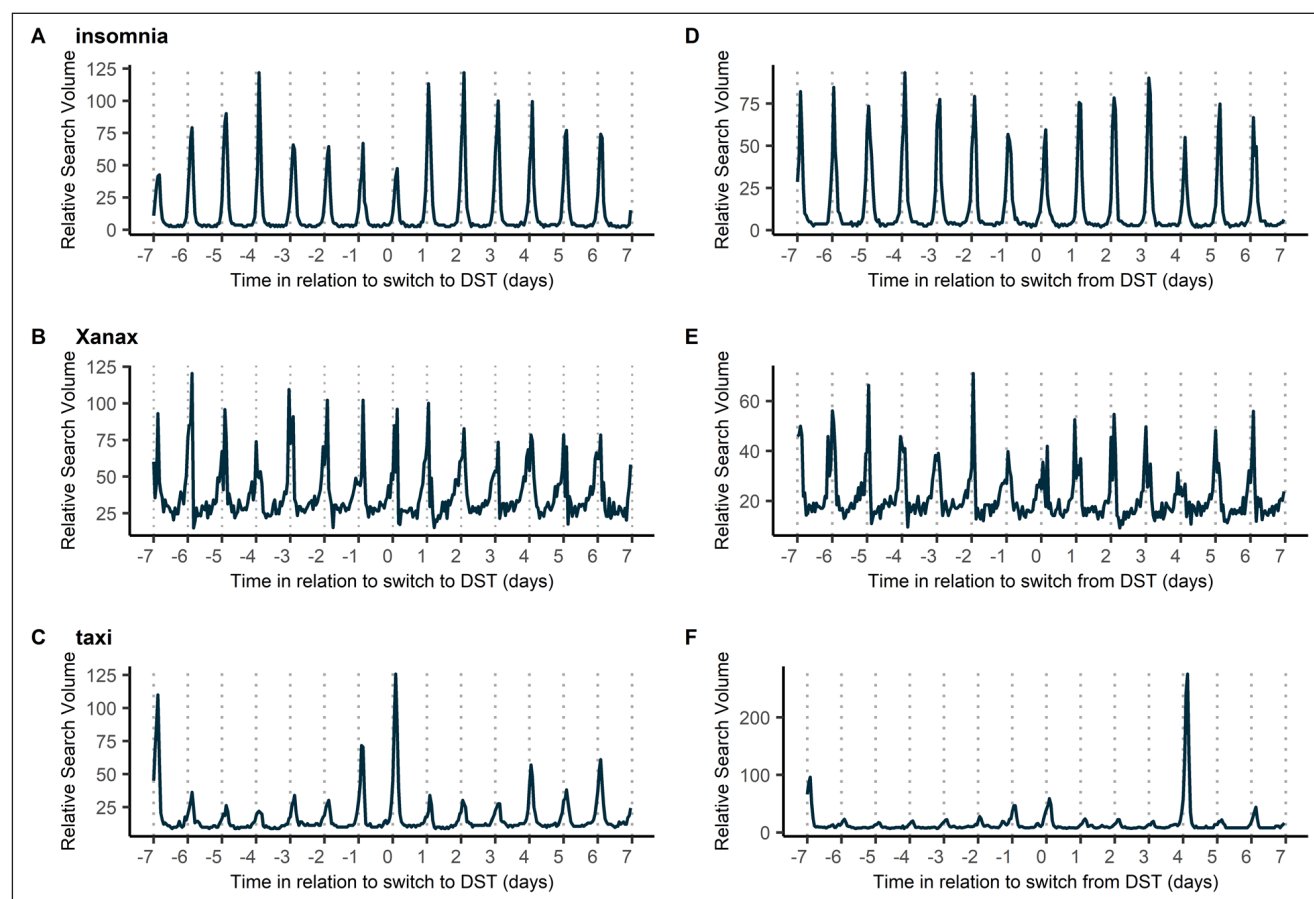
The hourly RSVs were plotted to obtain a qualitative impression of the presence/lack of any rhythmicity over the 24 hours for the selected search queries. The primary peak phase was estimated using the software *ChronoSapiens*© version 12, which has been specifically developed for human activity analysis [17]. This software also provided the significance of the fit, the Pearson relation coefficient between fit and raw data and the Rhythmicity Index (RI, i.e. a rhythm quality measure calculated by multiplying the regression coefficient with the range of oscillation). The peak phase was reported if the significance of the fit was  $<0.05$ . If the fit of a given day had a p-value of  $>0.05$ , that day was removed from the dataset. The average phase of the 15 days prior to and the 15 days after the switch to/from DST were compared both by year (2015–2020) and with all years combined for a sub-selection of the words, based on the RI and significance of the fit [16, 17]. The phase difference was also computed.

## STATISTICAL ANALYSES

The phase prior to and after the switch to/from DST for each year were plotted and outliers identified using the boxplot method (with outliers defined as data points located outside the boxplot whiskers, i.e.  $1.5 \times$  interquartile range) [19] and removed. Mean phase averages were compared by two-tailed unpaired student t-test. These analyses were performed with R-studio version 4.2.1, using the “ggplot2” package [20] to create the boxplots. To detect any trends over time, a repeated measures ANOVA was carried out with “average phase pre or post the switch to/from DST” as fixed factor and “time” as random factor with Statistica™, version 13.1 (Dell, Round Rock, Texas, USA).

## RESULTS

The hourly RSVs were plotted to obtain a qualitative impression of the presence (or lack thereof) of any rhythmicity over the 24 hours. The plots of the three words (one from each



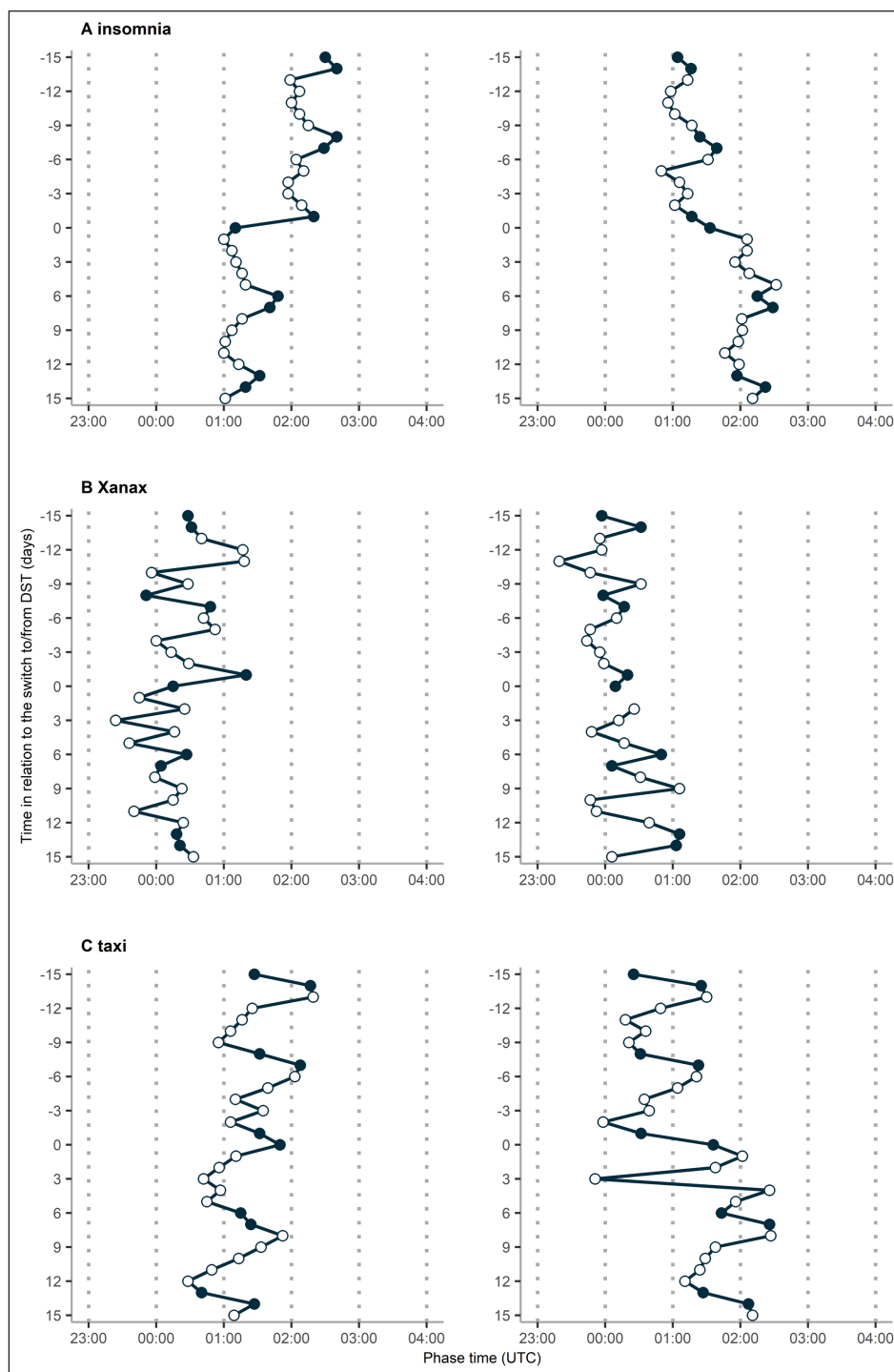
**Figure 1** Hourly Relative Search Volumes (RSV) for the words “insomnia”, “Xanax” and “taxi” in 2018, chosen as an example year. The RSVs are plotted over a period of 7 days prior to and 7 days after the transition to (A, B, C) and from (D, E, F) DST, which is marked as “0” on the x axis. The area between two dotted lines indicates 24 hours, with the dotted line representing midnight in UTC. Daily rhythms are visible for all three words, being most obvious for insomnia (A, D) with distinct peaks just after midnight. For the word taxi there is a visible increase in RSV during weekends (i.e. days -7, -1, 0 and 6 in both E and F). Additionally, there is a substantial increase in RSV for the word taxi on day four after the switch from DST, which is celebrated as Halloween in Italy (of note, the Halloween peak in taxi searches was present in all years analyzed).



category) “insomnia”, “Xanax” and “taxi” around the transition to/from DST in 2018 are presented in [Figure 1](#) as an example, with diurnal variation being visible in all three plots on qualitative analysis. Of these three words, the phases of the 30 days around the transition to/from DST in 2018 are presented in [Figure 2](#), documenting a phase

advance after the transition to DST and a phase delay after the transition from DST. This was consistent also for the remaining words ([Table 1](#)).

Based on the presence of a 24-hour rhythm, the RI and significance of the fit (Supplementary Table 2), 11 words were selected for further analysis, including chamomile, insomnia,



**Figure 2** Phase of three relevant words for the 15 days prior to and after the switch to (left) and from (right) DST. The phase values for the words “insomnia”(A), “Xanax”(B) and “taxi”(C) in 2018, chosen as an example year, are shown. Time is expressed as UTC. Weekend days are marked as full and weekdays as open circles. The day of the switch to/from DST is marked as “0” on the y axis. A phase advance can be observed over the Spring transition, while in Autumn there is a phase delay, both being most obvious for the word insomnia.

| WORD                                   | SEASON | PHASE PRE (HH:MM) | PHASE POST (HH:MM) | Δ (MIN) | p VALUE |
|----------------------------------------|--------|-------------------|--------------------|---------|---------|
| <b>SLEEP/HEALTH</b>                    |        |                   |                    |         |         |
| chamomile                              | Spring | 23:39             | 23:04              | -35     | <0.0001 |
| chamomile                              | Autumn | 23:00             | 23:30              | 30      | <0.0001 |
| emergency room                         | Spring | 02:16             | 01:40              | -36     | <0.0001 |
| emergency room                         | Autumn | 01:10             | 02:07              | 57      | <0.0001 |
| insomnia                               | Spring | 02:18             | 01:19              | -59     | <0.0001 |
| insomnia                               | Autumn | 01:14             | 02:11              | 57      | <0.0001 |
| melatonin                              | Spring | 00:49             | 00:13              | -36     | <0.0001 |
| melatonin                              | Autumn | 00:14             | 00:46              | 32      | <0.0001 |
| sleep                                  | Spring | 01:30             | 00:52              | -38     | <0.0001 |
| sleep                                  | Autumn | 00:43             | 01:23              | 40      | <0.0001 |
| stress                                 | Spring | 00:45             | 00:20              | -25     | <0.0001 |
| stress                                 | Autumn | 00:13             | 00:45              | 32      | <0.0001 |
| <b>MEDICATION</b>                      |        |                   |                    |         |         |
| painkiller                             | Spring | 02:46             | 02:02              | -44     | <0.001  |
| painkiller                             | Autumn | 01:14             | 02:06              | 52      | <0.0001 |
| Xanax                                  | Spring | 00:41             | 00:11              | -30     | <0.0001 |
| Xanax                                  | Autumn | 23:55             | 00:22              | 27      | <0.0001 |
| <b>RANDOM NON SLEEP/HEALTH-RELATED</b> |        |                   |                    |         |         |
| spa                                    | Spring | 10:34             | 09:37              | -57     | <0.001  |
| spa                                    | Autumn | 09:40             | 10:39              | 59      | 0.004   |
| taxi                                   | Spring | 01:39             | 00:55              | -44     | <0.0001 |
| taxi                                   | Autumn | 00:51             | 01:42              | 51      | <0.0001 |
| weather forecast                       | Spring | 05:16             | 04:24              | -52     | <0.0001 |
| weather forecast                       | Autumn | 04:08             | 05:01              | 53      | <0.0001 |

**Table 1** Average phase of the RSV (in UTC) and phase difference (in minutes) prior to and after the transition to (Spring) and from (Autumn) DST, by word, word category and season.

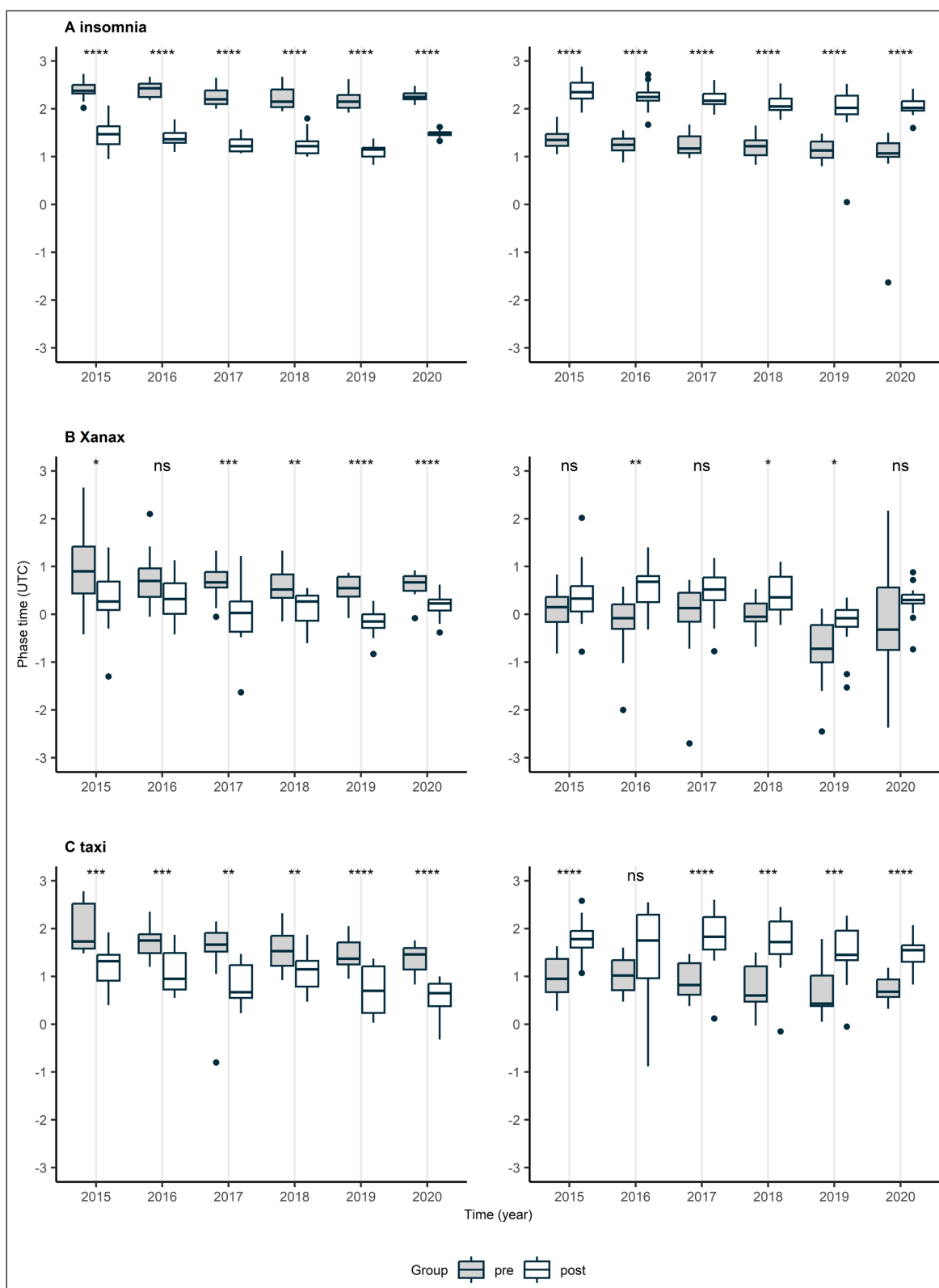
melatonin, emergency room, sleep and stress for the sleep/health-related area; painkiller and Xanax for the medication area; weather forecast, spa and taxi for the random non sleep/health-related area. When averaging the phase 15 days prior to and after the switch to/from DST per year and season, a phase advance was observed after the transition to DST and a phase delay after the transition from DST. The boxplots of the words “insomnia”, “Xanax” and “taxi” are presented in Figure 3 (for all remaining words, please refer to Table 1). A significant trend in phase anticipation of the search timings was observed for virtually all words over the years, both in Spring and in Autumn (Figure 3).

The significant phase advance and delay after the transition to/from DST, respectively, were also observed when combining all study years as an average (Table 1).

The time difference in phase varied between words and was consistently close to one hour only for “insomnia”, “weather forecast” and “spa”, for all other words the time difference was less than one hour (Table 1). The size of the phase difference was comparable for the transition to DST (in Spring) and from DST (in Autumn), except for the word “emergency room”, for which the phase difference was smaller in Spring when compared to Autumn (36 and 57 minutes, respectively, Table 1).

## DISCUSSION

When looking for information, a widespread approach for many people is to “Google it”. Using the data collected



**Figure 3** Average phase prior to and after the switch to (left) and from (right) DST, by year. The average phase of the 15 days prior to [median, lower/upper quartile, min/max within the 1.5\*interquartile range and outliers (black circles); in grey] and the 15 days after (in white) the switch to/from DST for the words insomnia (A), Xanax (B) and taxi (C) is presented. Time is expressed in years. Phase is expressed in relation to midnight in UTC. Significant results of a two-tailed unpaired student t-test between pre and post the switch are indicated with an asterisk, with \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ . On repeated measures ANOVA, a significant, progressive phase anticipation was observed over the years, both in Spring (factor time: insomnia  $p < 0.0001$ ; Xanax  $p < 0.01$ ; taxi  $p < 0.0001$ ) and in Autumn (factor time: insomnia  $p < 0.0001$ ; Xanax  $p < 0.001$ ; taxi  $p = 0.023$ ).

on these Google searches, we found diurnal variation in the RSV for various sleep/health, medication-related and random non sleep/health related search terms. We observed a phase advance after the transition to DST and a phase delay after the transition to civil time by up to 60 minutes in all analyzed search terms. Advances or delays shorter than 60 minutes (for example for the words “chamomile”, “melatonin” and “stress”) may suggest that the timing of the search query is at least partially driven by the circadian clock rather than by social timing.

Diurnal variation in RSV has been previously observed [21–23]. For instance, a study by Zitting et al. [22] found diurnal variation for the word “insomnia” with a peak in searches around 3 am, corresponding to the insomnia peak in the current study. However, to the best of our knowledge, no studies have used Google Trends data to explore the impact of the transition to/from DST on the diurnal pattern of searches yet. During DST, *social time* is moved one hour forward, while *solar time* remains unchanged, increasing the desynchrony between the two. If the search timing for a given query is driven by *social time*, it is expected that the phase will shift by one hour after the transition to/from DST. This was the case for the words “insomnia” (i.e. one probably expects to become sleepy depending on the time they read on their watch), “spa” and “weather forecast”. However, the timing of a Google search can also be driven by physiology/the endogenous circadian clock, considering that one might search on Google based on their physical state (e.g. searching for “stress” when feeling stressed). We found that the remaining analyzed words (i.e. “chamomile”, “melatonin”, “emergency room”, “sleep”, “stress”, “painkiller”, “Xanax” and “taxi”) had an average phase shift of less than 60 minutes, suggesting that their search may be partially driven by the circadian clock. Interestingly, most of the sleep/health-related words, which may be more closely related to physiology rather than to the social clock, shifted by 40 minutes or less (9 out of 12, when subdivided by season) while the phase of most of the random words shifted by more than 50 minutes, i.e. very close to the shift in social time (5 out of 6, when subdivided by season).

Another interesting finding was the progressive phase anticipation over the years – present in all analyzed terms – suggesting that, on average, people searched earlier. This might be explained by an increase in age, and thus in earlier chronotypes, amongst Google users over the years. People tend to become “more morning” with age [24] and indeed, the percentage of internet users in Italy between 2015 to 2020 increased mostly in people aged 55–74 [25]. As Google Trends does not provide information on users’ characteristics, this hypothesis cannot be confirmed. Excluding the lack of demographic information, the reason to search for a particular term

is not known either. This limits our data interpretation. However, for this study, the timing of the search was of greater importance than the intent. Despite these limitations, using Google Trends as a data source has numerous benefits. For instance, Google Trends data provides direct measurements of search activity with a resolution as low as one hour, free from errors due to completion/interpretation of surveys or questionnaires. Furthermore, the study population is large and data from both the present and past years are readily accessible without having to actively recruit participants.

In conclusion, we found a phase shift ranging from 25 to 60 minutes in the 15 days after the transition to/from DST in RSV. This suggests that the timing of Google searches might be driven by a mixture of the *social clock* switch and the endogenous circadian rhythm.

## ADDITIONAL FILES

The additional files for this article can be found as follows:

- **Table S1.** List of all search queries. DOI: <https://doi.org/10.5334/jcr.230.s1>
- **Table S2.** Indices of model fitting used to select words that were amenable to phase analysis/peak calculation. DOI: <https://doi.org/10.5334/jcr.230.s2>

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## COMPETING INTERESTS

The authors have no competing interests to declare.

## AUTHOR CONTRIBUTIONS

Conceptualization, SM, RC and AB; Methodology, SM, RC, AB, PC and LZ; Investigation, EDD, LZ, CM, CF, GG and DR; Writing – Original Draft. EDD and SM; Writing – Review & Editing, AB, RC and SM; Supervision, RC and SM; Funding, SM.

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# Managing Circadian Disruption due to Hospitalization: A Pilot Randomized Controlled Trial of the CircadianCare Inpatient Management System

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**Abstract** The objective of the present study was to test the effects of an inpatient management system (CircadianCare) aimed at limiting the negative impact of hospitalization on sleep by enhancing circadian rhythmicity. Fifty inpatients were randomized to either CircadianCare ( $n=25$ ; 18 males,  $62.4 \pm 1.9$  years) or standard of care ( $n=25$ ; 14 males,  $64.5 \pm 2.3$  years). On admission, all underwent a full sleep-wake evaluation; they then completed daily sleep diaries and wore an actigraph for the whole length of hospitalization. On days 1 (T0), 7 (T1), and 14 (T2, if still hospitalized), salivary melatonin for dim light melatonin onset (DLMO) and 24-h skin temperature were recorded. In addition, environmental noise, temperature, and illuminance were monitored. Patients in the CircadianCare arm followed 1 of 3 schedules for light/dark, meal, and physical activity timings, based on their diurnal preference/habits. They wore short-wavelength-enriched light-emitting glasses for 45 min after awakening and short-wavelength light filter shades from 18:00 h until sleep onset. While the first, primary registered outcome (reduced sleep-onset latency on actigraphy or diary) was not met, based on sleep diaries, there was a trend ( $0.05 < p < 0.1$ ) toward an advance in bedtime for CircadianCare compared to standard of care patients between T0 and T1. Similarly, DLMO time significantly advanced in the small group of patients for whom it could be computed on both occasions, with untreated ones starting from earlier baseline values. Patients sleeping near the window had significantly higher sleep efficiency, regardless of treatment arm. As noise fluctuation increased, so did the number of night awakenings, regardless of treatment arm. In conclusion, the CircadianCare management system showed positive results in terms of

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advancing sleep timing and the circadian rhythm of melatonin. Furthermore, our study identified a combination of environmental noise and lighting indices, which could be easily modulated to prevent hospitalization-related insomnia.

**Keywords** hospitalization, misalignment, chronotherapy, chronobiotic, light, noise

Circadian rhythms are self-sustaining rhythms with a period of approximately 24 h that have an influence on most physiological processes (Weitzman et al., 1971; Qian and Scheer, 2016; Douma and Gumz, 2018; Refinetti, 2020). The circadian timing system encompasses a master light-entrainable clock in the suprachiasmatic nuclei of the hypothalamus, and peripheral time-keepers in almost every organ of the body (Allada and Bass, 2021). Its regulation depends on both internal (neural and humoral) and environmental stimuli, called *Zeitgebers*, such as the light/dark cycle and, to a lesser extent, exercise timing, meal timing, and the timing of social interactions (Asher and Schibler, 2011; Reinke and Asher, 2016). In humans, the sleep-wake cycle is the most obvious behavioral output of the circadian timing system and it also indirectly determines light/dark exposure.

In recent years, evidence has emerged that circadian disruption and misalignment have serious medical consequences, including sleep curtailment and worsened sleep quality, increased cardiovascular morbidity (Morris et al., 2016; Vetter et al., 2016), and increased risk of certain types of cancer (Lin et al., 2015; Masri and Sassone-Corsi, 2018; Walasa et al., 2018). Hospitalization *per se* weakens circadian rhythmicity and the sleep-wake cycle due to disease itself, an unusual and noisy environment, and modified light (with significant exposure to light at night, especially in hospital rooms with multiple beds), food, and activity cues (Aaron et al., 1996; Kamdar et al., 2012; Bano et al., 2014; Giménez et al., 2017). As a result, patients tend to experience delayed, shorter, and poorer night sleep, several night awakenings, and daytime sleepiness. This disturbed sleep may affect prognosis, also in terms of duration of the inpatient stay (Ryherd et al., 2008).

We have previously documented that, in our medical ward, rolling shutters were rarely moved during the 24 h, with consequent daylight deprivation and worse sleep (especially for patients whose bed was far from the window; Bano et al., 2014), highlighting the powerful role of light as a *Zeitgeber* within the hospital environment. Furthermore, we subsequently showed that inpatients treated with bright light immediately after awakening and with short-wave-length light filter glasses in the evening had fewer night awakenings, less daytime sleepiness (with potential positive effects also on the subsequent sleep episode), and better mood than their untreated

counterparts (Formentin et al., 2020). It is therefore possible that controlling/modulating light and other potentially relevant *Zeitgebers* during an inpatient stay may enhance rhythmicity, improve sleep timing and quality, and, ultimately, improve prognosis (Milani et al., 2018).

Thus, the aim of the present study was to test the effects of an inpatient management system, which we called CircadianCare, aimed at limiting the impact of hospitalization by enhancing circadian rhythmicity (and, in turn, improving sleep timing/quality) through an assessment of the patient's specific circadian features/needs and an *ad hoc* personalized light/dark, meal, and activity or physiotherapy schedule. The main expected outcome was an advance in circadian phase, measured by an advance in sleep onset timing (first registered primary outcome: reduced sleep onset latency based on actigraphy or diary, plus a series of actigraphy-/diary-based sleep-wake variables) and/or an advance in melatonin onset after 7 days of hospitalization. This was based on the hypothesis that the personalized CircadianCare schedule would advance phase (mostly due to morning light administration and protection from light in the early evening/night hours, *vide infra*), limit its hospitalization-related delay and/or rhythmicity weakening, and, in turn, improve sleep quality.

## PATIENTS AND METHODS

### Patients

All patients admitted into the Clinica Medica V ward (a general medical 54-bed ward with 10 hepatology beds) of Padova University Hospital between October 2020 and January 2022 were screened within 24 h of admission. In principle, all patients screened within 24 h of admission could be included. Exclusion criteria were: unwillingness or inability to comply with the study procedures, severe cognitive impairment, and diagnosed, well-defined sleep-wake disorders. To detect a difference of 10 min in sleep onset time between CircadianCare and standard of care after a week of hospitalization (T1) with a standard deviation (SD) of 15 min, with a two-sided 0.05 significance and a power of 80%, a sample size of 35 subjects per arm would have been needed. The above was based on what is known on light responses, and

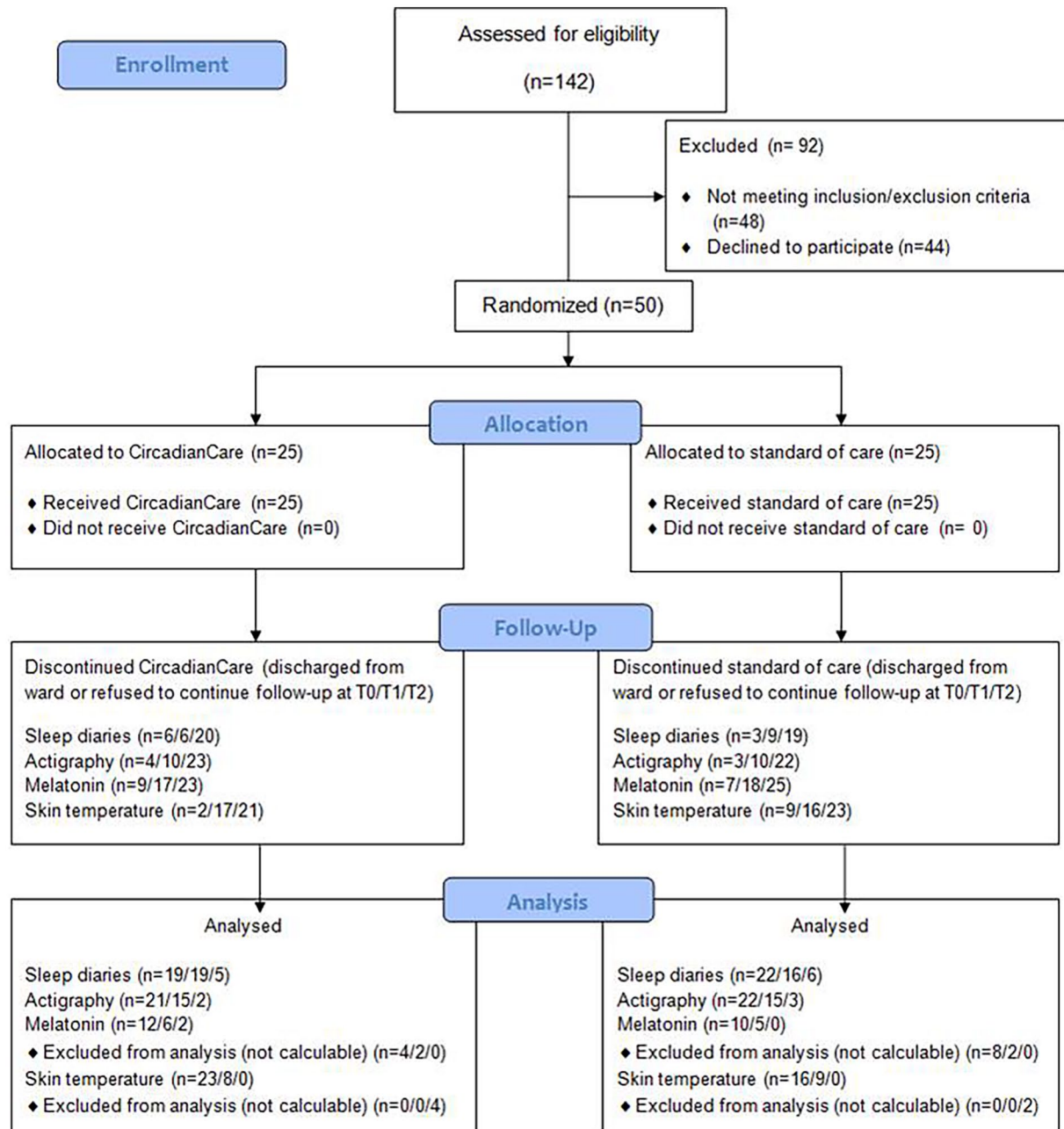
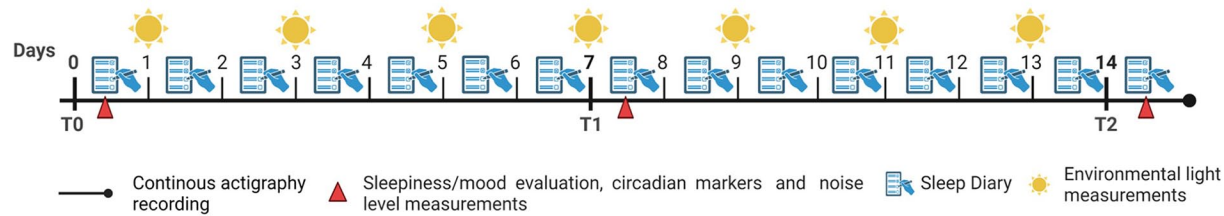


Figure 1. Consolidated Standards of Reporting Trials (CONSORT) flow diagram.

not on more articulate, multiple intervention protocols such as ours, thus a less conservative power estimate to detect a difference of 20 min in sleep onset time between CircadianCare and standard of care (remaining parameters unchanged) was also made, with 18 subjects per arm being needed. On final review, considering both feasibility in the acute medical setting and the limited, available literature data, 25 subjects per arm were deemed appropriate and study approval sought for based on this power calculation. Despite the difficulties and limitations to

screening/recruitment during the pandemic, a total of 142 patients were screened; 48 patients did not meet the inclusion/exclusion criteria, and 44 patients were eligible but unwilling to participate (Figure 1). Thus, the final population included the necessary 50 inpatients (study stop), randomized (1:1 block randomization) to either the CircadianCare system (n=25) or standard of care (n=25) by use of a computer-generated randomization list. Sequentially numbered, sealed envelopes were then utilized and singly provided by author LZ to clinician author



**Figure 2.** Study design, representing a hypothetical 14-day hospitalization stay. The icons on the horizontal line indicate the study procedures performed daily, or at the 3 main study times (T0: baseline, T1: day 7, T2: day 14; red triangles). Continuous actigraphy (black line) and daily sleep diaries (blue paper and pencil) were recorded over the whole inpatient stay. Environmental light was measured every 2 days (yellow sun); patient skin temperature, salivary melatonin samples, subjective sleepiness/mood, and room noise and temperature were collected at T0, T1, and T2 (red triangles). Color version of the figure is available online.

CM, who wrote the screened patient code on the outside of the envelope prior to opening it. Authors LZ, CF, GG, EDD, DR, RC, DJS, DB, LB, ADB, PA and SM remained blinded after assignment of the interventions. Information on demographics (sex, age, body mass index, and level of education), reasons for hospital admission, triage severity code on admission, the Charlson Comorbidity Index (Charlson et al., 1987), and treatment prior to admission were collected for each patient. The study protocol was approved on 20 February 2020 by the Padova University Hospital Ethics Committee (AOP0536) and the study was conducted according to the Declaration of Helsinki (Hong Kong and 2008 Amendments) and Good Clinical Practice (European) guidelines. All participants provided written, informed consent. The study was registered retrospectively (because of administrative/logistic issues) on ClinicalTrials.gov (NCT05228444). The Consolidated Standards of Reporting Trials (CONSORT) reporting guidelines were used (Schulz et al., 2010). The CONSORT flow diagram is presented in Figure 1 and the CONSORT checklist is included in the Supplementary Material.

## Data Collection

**Pre-admission Sleep-Wake Profiles.** On the day of admission, patients underwent an assessment of their baseline, pre-hospitalization sleep-wake profiles, based on: (1) the Pittsburgh Sleep Quality Index (PSQI), which evaluates subjective sleep quality over the preceding month and differentiates “good” from “poor” sleepers (Buysse et al., 1989; Curcio et al., 2013); (2) the ultrashort version of the Munich ChronoType Questionnaire (MicroMCTQ) (Ghotbi et al., 2020), which is used to assess chronotype based on habitual sleep timing on work/study versus free days; (3) the Sleep Timing and Sleep Quality Screening (STSQS) questionnaire, to assess habitual

sleep-wake timing/quality (Montagnese et al., 2009); and (4) the Horne-Östberg (HÖ) questionnaire (Horne and Ostberg, 1976), which defines diurnal preference and was utilized to split patients into morning (definitely/moderately), intermediate, and evening (definitely/moderately) types. For statistical power reasons, “definitely morning” and “moderately morning” types were grouped as “morning,” and “definitely evening” and “moderately evening” as “evening,” resulting in 3 groups. Habitual meal timing at home was also recorded.

On days 1, 7, and 14 (if still admitted) of hospitalization (Figure 2), or the day before discharge, patients underwent an evaluation of the course of their sleepiness/mood during the waking hours (Karolinska Sleepiness Scale and a mood 1-10 Visual Analogue Scale [VAS]; Akerstedt and Gillberg, 1990), which they were asked to complete hourly from wake up to sleep onset time.

At the end of hospitalization, patients’ overall perception of their hospital experience was assessed by a questionnaire for which an Italian, validated version is available (Bai et al., 2018), encompassing satisfaction in relation to information on admission, the medical and nursing staff, general organization, and the environment. On admission, standard of care patients were provided with standard information, which mostly covers visiting hours and doctors’ contact hours (for patients and carers). CircadianCare patients were provided with the above plus, because of the protocol, also with information and potential choices for light, meal, and activity timing (*vide infra*).

**Sleep-Wake Profiles and Other Circadian Markers During the Inpatient Stay.** For the whole length of hospitalization, patients were asked to complete a daily sleep diary (Figure 2) in the morning, immediately after waking up, to limit recall bias. Each diary page also included a VAS for the assessment of sleep quality during the previous night (range: 1=worst to 10=best; Lockley et al., 1999; Carney et al., 2012).



Sleep onset time (as clock time) was then calculated as the sum of the time when the patient stated they tried to sleep plus their sleep latency; time spent in bed as the difference between bedtime and get up time (in hours); sleep duration as the difference between sleep onset and wake-up time (in hours). Night awakenings included both spontaneous arousals and sleep interruptions due to environmental factors, which could not be differentiated by the question asked with the diary tool utilized.

In addition, patients underwent continuous actigraphy by MotionWatch 8© (MW8, CamNtech, Cambridge; Figure 2), which is a wrist-worn axial accelerometer. The data (number of "counts" per 30-sec epochs) were extracted by the MotionWare software (CamNtech, Cambridge). Movements causing the acceleration signal to exceed the threshold of approximately 0.1 g were qualified as activity. Periods in which participants removed the actigraph were recorded by the daily sleep diaries and excluded from analyses. The following indices were obtained: total sleep time (i.e., total duration of all epochs qualified as sleep during time in bed), sleep onset latency (i.e., time to fall asleep), sleep efficiency (i.e., percentage of time spent sleeping out of the total time spent in bed), wake after sleep onset (time spent awake after sleep onset), and number of naps. In addition, intra-daily variability (indication of fragmentation of the sleep rhythm, ranging from 0 to 2, with higher scores indicating more fragmentation) and inter-daily stability (indicating the stability of the 24-h activity rhythm across days, ranging from 0 to 1, with higher scores indicating more stable rhythms; Luik et al., 2013) were obtained.

On days 1 (T0), 7 (T1), and 14 (T2, if still admitted) of hospitalization, the following samples/information were also acquired (Figure 2):

- Salivary melatonin samples collected hourly on 5 occasions in the evening hours. The first sample was collected 3 h before the patient's habitual sleep time (Voultsios et al., 1997; Benloucif et al., 2008). Information was also collected on medications potentially interfering with melatonin levels (Arendt, 2018). Melatonin was then determined using a radioimmunoassay (Melatonin RIA-5503 kit, DRG Instruments GmbH, Marburg) according to the manufacturer's protocol (detection limit: 0.3 pg/mL). The dim light melatonin onset (DLMO) was computed based on a threshold calculated as the mean of baseline plus twice the SDs for each individual melatonin profile. Each participant's DLMO was the point in time when the melatonin concentration exceeded the threshold (Lewy et al., 1980; Micic et al., 2015). When the threshold was not obviously

computable, author DJS (blinded to any information relating to the patient and/or their treatment) visually identified the time of melatonin rise based on qualitative profile analysis (Benloucif et al., 2008; De Rui et al., 2015; Rzepka-Migut and Paprocka, 2020).

- The 24-h recording of distal skin temperature (DST) and proximal skin temperature (PST) was performed using wireless sensors (iButtons, model no. DS1922L-F5, Maxim Integrated, San Jose, California; van Marken Lichtenbelt et al., 2006). Sampling rate was set at 3 min (resolution 0.0625 °C; approximately 500 temperature values per day). Four sensors were placed on the skin (right mid-thighs on the rectus femoris, right infraclavicular area, abdomen, and mid-metatarsal area of the plantar site of the right foot) using medical tape (Longato et al., 2017). Data were extracted using the iButtonViewersoftware (Dallas Semiconductor, Maxim Integrated Products, Sunnyvale, California). An artifact rejection procedure was applied to exclude outliers (Rutkove et al., 2007). Temperature measurements were aligned according to try-to-sleep time, as recorded in the sleep diaries, and averaged in 30-min bins. The data analysis window was set from 4 h prior to try-to-sleep time until 10 h after try-to-sleep time. Patients with missing values due to sensor loss or clinical procedures were excluded from the analysis. PST, DST, and the distal-proximal skin temperature gradient (DPG) were calculated as follows (Longato et al., 2017):

$$\begin{aligned}
 - T_{PST} &= 0.359 \times T_{\text{mid-thigh}} + 0.262 \times T_{\text{infraclavicular site}} \\
 &\quad + 0.379 \times T_{\text{abdomen}} \\
 - T_{DST} &= 1.017 \times T_{\text{feet}} \\
 - DPG &= (T_{DST} - T_{PST}).
 \end{aligned}$$

*Environmental Factors.* Type of room (single, double, or 4-bed) and bed position (qualified as near/far from the window, where near was less than 1 m and far was more than 3 m) were recorded on admission. On days 1, 7, and 14 (if still admitted) of hospitalization, or on the day before discharge, the following information was acquired (Figure 2):

- The 24-h recording of the A-weighted sound pressure levels within the ward rooms (1 sample/sec). To carry out this measurement without interfering with the normal activities in the hospital room, a specific acquisition system was set up. A series of sound level meters with accuracy equivalent to Class II was arranged by equipping smartphones (Apple iPhone 6 Plus) with external condenser microphones (BSWA

Technology Ltd., Type micW i436). The application used for acquiring recordings and saving data was OpenNoise, developed and distributed by the Regional Agency for the Protection of the Environment of Piemonte, Italy (Arpa Piemonte, n.d.). Measurement was calibrated in the reverberation room of the acoustic laboratory “LabAcus” of the Department of Industrial Engineering (University of Padua) using two methods: comparison with the measurements obtained from a Class I precision sound level meter (NTi XL2) with the method proposed by Kardous and Shaw (2016), and with the sound emission of a reference sound source (Brüel & Kjær, Type 4204) by a comparison method for determination of sound power of noise sources according to ISO 3741 standards (International Organization for Standardization, 2010). From these comparisons, it was possible to obtain the gain value to be applied to the microphone, to align the measurements with those of the precision sound level meter in reference conditions. Smartphones and microphones were placed on top of the headboard and powered externally to make their presence minimally invasive for patients and to minimize the need for operator interventions. The A-weighted equivalent continuous sound pressure level (LAeq) and the maximum A-weighted sound level (L<sub>Amax</sub>) were measured during the conventional 8-h night time period (*vide infra*). The time history of the LAeq was aggregated in 15-min intervals to fit the resolution of the actigraph used for patient rest/activity monitoring. The equivalent background noise level (LA95eq), evaluated every second by the noise level exceeded 95% of the time during the previous  $T/6$  interval, where  $T$  is the measurement time, was estimated according to Alsina-Pagès et al. (2021). The intermittency ratio (IR) was computed as in Wunderli et al. (2016). The IR metric expresses the proportion of the acoustic energy contribution in the total energetic dose created by individual noise events above a certain threshold. Thus, it is a descriptor that can effectively integrate the LAeq, based on the concept of energetic dose over longer periods, to describe specific changes in environmental noise conditions that can cause awakening (World Health Organization. Regional Office for Europe [Bonn, Germany], 2009 – this should be marked as a reference, as in a few lines here below). Furthermore, the comparison between the IR and the LA95eq with the same sampling time provides a direct indication of the incidence of

sound fluctuations in the variation of the overall sound level better than the maximum sound level at any given moment in time (L<sub>Amax</sub>). Finally, the LA95eq (dB(A)), IR (%), and the percent of awake epochs from the actigraphic data (every 15 min) were computed during the night. The conventional length of the night period was defined according to Italian law (D.M. 16 March 1998 and D. Lgs. n.66/2003) as the period between 2200h and 0600h; World Health Organization (WHO) Night Noise Guidelines for Europe were used as reference (World Health Organization. Regional Office for Europe, 2009).

- Room illuminance was measured at each patient’s eye level as detailed in Bano et al. (2014) by a luxmeter (Konica Minolta T-10A, Marunouchi, Chiyoda, Tokyo, Japan). Measurements were taken 4 times per day (Slot 1: 07:00-10:30h, Slot 2: 12:00-14:00h, Slot 3: 14:30-17:00h, and Slot 4: 17:30-21:00h), with these relatively wide time slots being aimed at minimizing interference with standard hospital activity. At the same time slots, information was also recorded about headboard lights (on/off), position of the rolling shutters (open [0%], three-fourth open [25%], halfway [50%], one-fourth open [75%], and closed [100%]), and number of people in the room (patients, personnel, and/or visitors).
- The 24-h recording of room temperature was monitored by an iButton (*vide supra*) taped on the wall close to the headboard of the patient’s bed.

### Treatment Protocol

Patients in the CircadianCare arm followed 1 of 3 different schedules for light/dark, meal, and physical activity timing, based on their circadian preference (Figure 3).

*Light/Dark and Noise Hygiene.* To provide as much natural daylight as possible over the daytime hours, rolling shutters were opened according to the patients’ habitual sleep-wake timing (Figure 3). Moreover, short-wavelength-enriched morning light was administered via Luminette light glasses (Lucimed, Villers-le-Bouillet, Belgium, 2000 lux; blue-enriched; Langevin et al., 2014). Patients were asked to wear the light glasses every morning, immediately after awakening, for 45 min (e.g., while having breakfast). These devices are equipped with 8 light-emitting diodes (LEDs) distributed on the upper part of the 2 lenses (4 on each side), outside



|                   | Morning | Evening | Breakfast | Lunch | Dinner | Morning     | Evening     |
|-------------------|---------|---------|-----------|-------|--------|-------------|-------------|
| Morning type      | 6:45    | 20:00   | 7:00      | 12:30 | 18:30  | 8:00-08:30  | 18:00-18:30 |
| Intermediate type | 7:45    | 21:00   | 8:00      | 13:30 | 19:30  | 9:00-9:30   | 18:30-19:00 |
| Evening type      | 8:45    | 22:00   | 9:00      | 14:30 | 20:30  | 10:00-10:30 | 19:00-19:30 |

Figure 3. Rolling shutter opening/closing, meals, and physical activity timing (clock time or clock time range) in the CircadianCare arm, categorized by diurnal preference.

the patient's visual field. The LEDs reflect light toward the eye via a diffractive lens, thus focusing it toward the lower part of the retina, regardless of the position of the head; this also allows for uniform illumination and for avoidance of glare (Bragard and Coucke, 2013).

To limit night light exposure, rolling shutters were closed according to the patients' habitual sleep-wake timing (Figure 3). Patients were also asked to wear a pair of short-wavelength light filter shades (MelaMedic, Viborg, Denmark) from 18:00 h until sleep onset time, plus at any time during the night if the room light was on. These shades filter light in the blue range of the spectrum (up to 530nm), that is the one our eye and the circadian timing system are most sensitive to (Brainard et al., 2001; Thapan et al., 2001). Finally, each patient received a pair of earplugs, which they were free to wear if and as needed.

**Meal Timing.** Meal timing was chosen based on patients' circadian preference (Figure 3); patients' diet was prescribed individually by the physicians, and not modified for study purposes.

**Physical Activity Timing.** Patients were guaranteed the amount of regular, timed physical activity they were capable of, based on their condition. This consisted of: in-bed passive mobilization for the sickest, in-bed active mobilization (raise arm across midline, raise leg and extend knees, bend ankle and point toes), bed-to-armchair transition, sitting and standing from chair, aided or un-aided short walks inside the room, and so on. Physical activity timing was chosen between 3 options, again depending on pre-admission habits and preferences (Figure 3).

## Statistical Analyses

Data are reported as mean  $\pm$  standard error of the mean (or, where indicated, 95% confidence interval [CI]) for quantitative variables, and as count and percentage for categorical variables. The distribution of variables was tested for normality using the Shapiro-Wilk's  $W$  test. Differences between groups were evaluated by the Student  $t$ /Mann-Whitney  $U$ , analysis of variance (ANOVA; *post hoc* Tukey test), or Pearson's  $\chi^2$ , as appropriate. When considering different time points, variables were analyzed by repeated measures ANOVA (*post hoc* Tukey test). Correlation analysis was performed by Pearson's  $r$  or Spearman's rank  $R$ , as appropriate. Analyses were carried out by the package Statistica, version 13.1 (Dell, Round Rock, Texas). Overall information on missing data can be inferred from Figure 1; given the size of the study and the considerable attrition, missing data were treated as such, and imputation was not utilized (complete case analysis at each time point). Finally, also due to attrition and to significant observed variability in the registered outcomes (especially sleep timing and DLMO), these are not necessarily presented in the Results section with the exact, strict order in which they were listed as outcomes on trial registration.

## RESULTS

### Demographics, Clinical, and Pre-admission Sleep-Wake Profiles

At baseline, treated (CircadianCare) and untreated (standard of care) patients were comparable in terms

**Table 1. Demographics, pre-admission sleep quality and habitual sleep, and meal timing, plus information on the inpatient environment in the CircadianCare and the standard of care arms.**

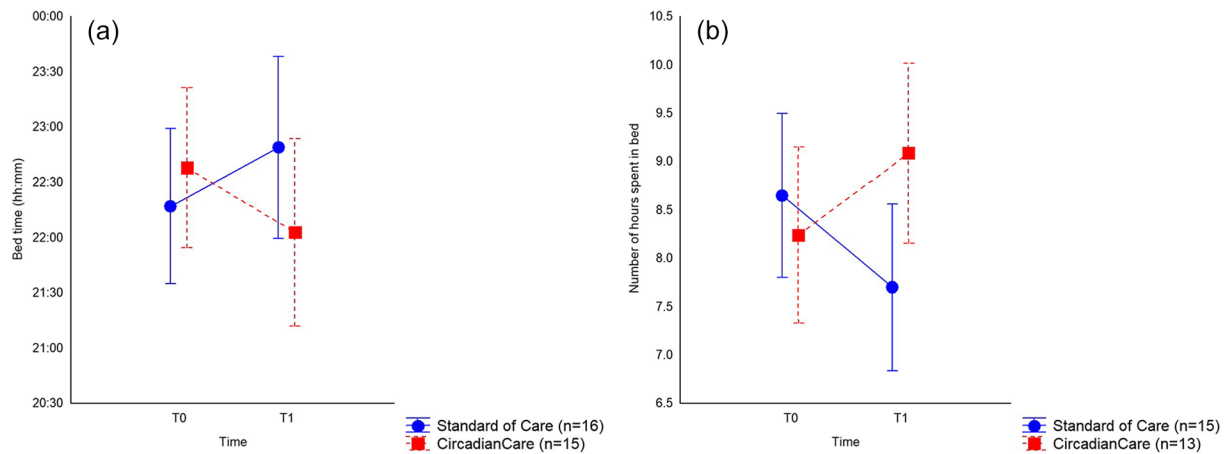
| Variable                                                                                  | CircadianCare ( <i>n</i> = 25) | Standard of Care ( <i>n</i> = 25) |
|-------------------------------------------------------------------------------------------|--------------------------------|-----------------------------------|
| <b>Demographics</b>                                                                       |                                |                                   |
| Age (years; mean $\pm$ SE)                                                                | 62.4 $\pm$ 1.9                 | 64.5 $\pm$ 2.3                    |
| Males, <i>n</i> (%)                                                                       | 18 (72%)                       | 14 (56%)                          |
| Educational attainment (years; mean $\pm$ SE)                                             | 13.8 $\pm$ 0.8                 | 12.1 $\pm$ 0.8                    |
| BMI (kg/m <sup>2</sup> ; mean $\pm$ SE)                                                   | 26.6 $\pm$ 1.0                 | 28.0 $\pm$ 1.1                    |
| Charlson Comorbidity Index (mean $\pm$ SE)                                                | 3.6 $\pm$ 0.6                  | 3.0 $\pm$ 0.4                     |
| Triage code (white/green/yellow/red; <i>n</i> )                                           | 13/3/6/2                       | 6/3/10/5                          |
| Reason for admission (infectious/cirrhosis complications/cardiovascular/other; <i>n</i> ) | 9/8/3/5                        | 9/5/5/6                           |
| Currently employed ( <i>n</i> [%])                                                        | 16 (64%)                       | 17 (68%)                          |
| Working days per week ( <i>n</i> $\pm$ SE)                                                | 3.0 $\pm$ 0.6                  | 2.2 $\pm$ 0.6                     |
| <b>Pre-admission habitual sleep quality and timing questionnaires</b>                     |                                |                                   |
| <b>HÖ score</b>                                                                           |                                |                                   |
| Total score (mean $\pm$ SE)                                                               | 58.40 $\pm$ 1.26               | 60.30 $\pm$ 2.33                  |
| Diurnal preference (morning type/intermediate type/evening type; <i>n</i> )               | 14/11/0                        | 16/5/2                            |
| PSQI total score (mean $\pm$ SE)                                                          | 7.7 $\pm$ 1.1                  | 8.7 $\pm$ 0.9                     |
| <b>STSQS questionnaire</b>                                                                |                                |                                   |
| Bed time (h; mean $\pm$ SE)                                                               | 23:10 $\pm$ 00:09              | 22:51 $\pm$ 00:17                 |
| Try to sleep time (h; mean $\pm$ SE)                                                      | 23:23 $\pm$ 00:08              | 23:16 $\pm$ 00:16                 |
| Sleep latency (min; mean $\pm$ SE)                                                        | 30 $\pm$ 9                     | 21 $\pm$ 3                        |
| Sleep onset time (h; mean $\pm$ SE)                                                       | 23:46 $\pm$ 00:10              | 23:37 $\pm$ 00:16                 |
| Night awakenings ( <i>n</i> ; median [interquartile range])                               | 1.3 (0.5-2.5)                  | 2.0 (1.0-3.5)                     |
| Wake-up time (h; mean $\pm$ SE)                                                           | 06:26 $\pm$ 00:16              | 06:54 $\pm$ 00:19                 |
| Get-up time (h; mean $\pm$ SE)                                                            | 06:47 $\pm$ 00:16              | 07:25 $\pm$ 00:21                 |
| Sleep quality (1 = worst, 10 = best; mean $\pm$ SE)                                       | 5.04 $\pm$ 0.54                | 4.91 $\pm$ 0.54                   |
| <b>MicroMCTQ</b>                                                                          |                                |                                   |
| Sleep time on working days (h; mean $\pm$ SE)                                             | 22:46 $\pm$ 00:30              | 23:13 $\pm$ 00:15                 |
| Sleep time on free days (h; mean $\pm$ SE)                                                | 23:19 $\pm$ 00:19              | 23:22 $\pm$ 00:17                 |
| Wake-up time on working days (h; mean $\pm$ SE)                                           | 06:04 $\pm$ 00:32              | 06:41 $\pm$ 00:14                 |
| Wake-up time on free days (h; mean $\pm$ SE)                                              | 07:20 $\pm$ 00:16              | 07:10 $\pm$ 00:17                 |
| Sleep duration on working days (hours; mean $\pm$ SE)                                     | 7.29 $\pm$ 0.34                | 7.47 $\pm$ 0.17                   |
| Sleep duration on free days (hours; mean $\pm$ SE)                                        | 8.01 $\pm$ 0.38                | 7.79 $\pm$ 0.23                   |
| <b>Habitual meal timing</b>                                                               |                                |                                   |
| Habitual breakfast time (h; mean $\pm$ SE)                                                | 07:08 $\pm$ 00:17              | 07:32 $\pm$ 00:21                 |
| Habitual lunch time (h; mean $\pm$ SE)                                                    | 12:26 $\pm$ 00:07              | 12:32 $\pm$ 00:07                 |
| Habitual dinner time (h; mean $\pm$ SE)                                                   | 19:26 $\pm$ 00:13              | 19:40 $\pm$ 00:11                 |
| <b>Inpatient stay</b>                                                                     |                                |                                   |
| Length of hospitalization (days; mean $\pm$ SE)                                           | 11.4 $\pm$ 1.8                 | 10.7 $\pm$ 1.1                    |
| Bed position in relation to the window (near/far; <i>n</i> )                              | 11/14                          | 14/11                             |
| Room type (single/double/4-bed; <i>n</i> )                                                | 1/1/23                         | 1/2/22                            |

Abbreviations: BMI = body mass index; HÖ = Horne-Östberg; PSQI = Pittsburgh Sleep Quality Index; STSQS = Sleep Timing and Sleep Quality Screening; MicroMCTQ = micro Munich ChronoType Questionnaire; SE = standard error.

of demographics, diurnal preference, habitual sleep quality (except for number of night awakenings, which were higher in the untreated group:  $1.5 \pm 0.2$  vs  $2.4 \pm 0.4$ ,  $p = 0.047$ ), habitual sleep and meal timing, length of hospitalization, room type, and bed position in relation to the window (Table 1). Furthermore, pre-admission treatment (also in relation to psychoactive, sleep-inducing, and medications that may affect melatonin profiles) was comparable in the two groups (Table 1). Considering diurnal preference in both groups, since there were only 2 “evening” patients in the untreated group and none in the treated one, comparisons were performed only

between “morning” and “intermediate” patients. The number of working days per week was higher in “morning” compared to “intermediate” patients ( $3.2 \pm 0.8$ , 95% CI [2.16, 4.20] vs  $1.8 \pm 0.9$ , 95% CI [−0.60, 2.54],  $F = 5.605$ ,  $p = 0.023$ ). Interestingly, the Charlson Comorbidity Index was lower (i.e., meaning less disease burden) in “morning” compared to “intermediate” patients in both groups ( $2.7 \pm 0.4$ , 95% CI [2.09, 3.99] vs  $4.4 \pm 3.0$ , 95% CI [3.96, 6.79],  $F = 4.652$ ,  $p = 0.037$ ). Given the low numbers, this may either be a random series-specific observation or, as an alternative, may be in line with the notion that morning individuals tend to lead generally healthier lives and to





**Figure 4.** Sleep diary-based bedtime (mean and 95% CI) at T0 and T1 (day 7; Panel a;  $p=0.062$ ) and number of hours spent in bed (mean and 95% CI) at T0 and T1 (day 7; Panel b;  $p=0.007$ ) in the CircadianCare (red squares) and standard of care (blue circles) arms. Abbreviation: CI = confidence interval. Color version of the figure is available online.

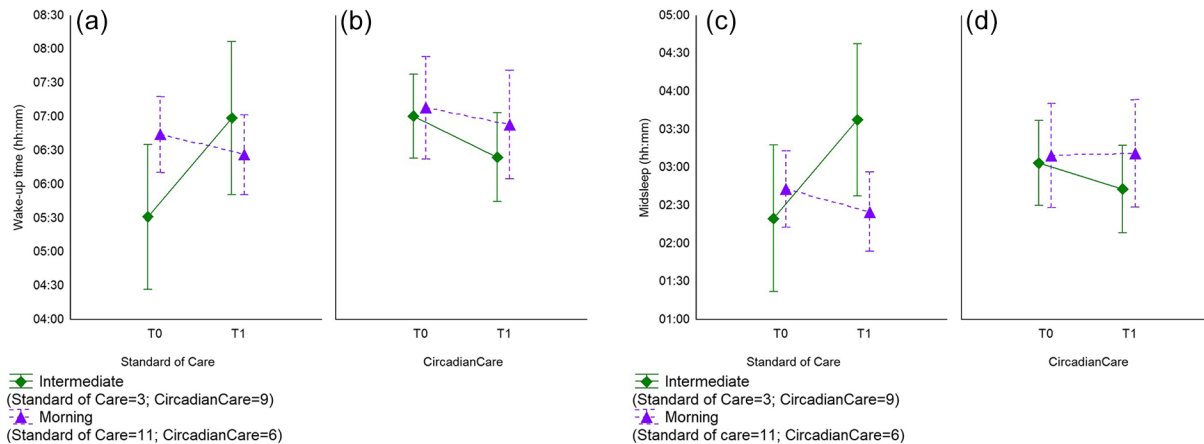
have less difficulties aligning with the timing constraints imposed by the social clock in the industrialized society, and thus carry a generally lower disease risk burden (Allada and Bass, 2021). As expected, patients with a “morning” preference reported a significantly earlier breakfast time than “intermediate” patients ( $06:49 \pm 00:17$ h, 95% CI [06:15, 07:18] vs  $07:58 \pm 00:28$ h, 95% CI [07:04, 08:27],  $F=5.150$ ,  $p=0.029$ ). In addition, “morning” patients habitually went to bed and tried to sleep earlier than “intermediate” patients ( $22:41 \pm 00:14$ h, 95% CI [22:20, 23:02] vs  $23:24 \pm 00:15$ h, 95% CI [22:50, 23:49],  $F=4.54$ ,  $p=0.039$  and  $23:02 \pm 00:13$ h, 95% CI [22:43, 23:19] vs  $23:41 \pm 00:13$ h, 95% CI [23:13, 00:03],  $F=5.73$ ,  $p=0.021$ , respectively). No other significant differences were observed.

### Inpatient Stay—Sleep Diaries and Actigraphy

Sleep diaries were available for 41 patients (19 treated and 22 untreated, respectively) at T0, for 35 patients (19 and 16) at T1, and for 11 patients (5 and 6) at T2. Actigraphic data were available for 43 patients (21 and 22) at T0, for 30 patients (15 and 15) at T1, and for 5 patients (2 and 3) at T2. There was a trend advance in bedtime for treated patients, whereas untreated patients showed a trend delay in bedtime between T0 and T1 ( $22:38 \pm 00:21$ h, 95% CI [21:54, 23:22] at T0,  $22:02 \pm 00:25$ h, 95% CI [21:11, 22:54] at T1 in treated vs  $22:17 \pm 00:20$ h, 95% CI [21:35, 22:59] at T0,  $22:49 \pm 00:24$ h, 95% CI [22:00, 23:38] at T1 in untreated patients,  $F=3.768$ ,  $p=0.062$ , trend; Figure 4, Panel a). This was confirmed when also considering T2 (data not shown), despite the marked reduction in patient numbers, and may express the expected,

overall positive effect of the CircadianCare system on both wake and sleep (i.e., more active and earlier wake period, and thus earlier bedtime). The number of hours spent in bed significantly increased from T0 to T1 in the treated group ( $8.24 \pm 0.44$ h, 95% CI [7.32, 9.14] at T0 and  $9.08 \pm 0.45$ h, 95% CI [8.16, 10.02] at T1) compared to the untreated group ( $8.65 \pm 0.41$ h, 95% CI [7.81, 9.49] at T0 and  $7.70 \pm 0.42$ h, 95% CI [6.83, 8.58] at T1,  $F=8.503$ ,  $p=0.007$ ; Figure 4, Panel b). In addition, treated patients showed an increase in get up latency (i.e., the interval between wake-up and get-up time) compared to untreated patients ( $16 \pm 14$ min, 95% CI [−13, 45] at T0 and  $58 \pm 16$ min, 95% CI [24, 91] at T1 in treated vs  $47 \pm 14$ min, 95% CI [18, 75] at T0 and  $38 \pm 16$ min, 95% CI [5, 70] at T1 in untreated patients,  $F=4.526$ ,  $p=0.043$ ). This latter finding may relate to an unintended effect of treatment, with some patients in the CircadianCare arm choosing (despite not being instructed to) to remain in bed while wearing the light glasses after waking up in the morning. The number of night awakenings was similar between the two groups at T1. When extending the analysis to T2, the number of night awakenings was greater in the untreated group ( $0.90 \pm 0.46$ , 95% CI [−0.26, 2.25] in treated vs  $2.43 \pm 0.35$ , 95% CI [1.31, 3.27] in untreated patients,  $F=7.217$ ,  $p=0.036$ ). Given the baseline differences in this variable between the two groups, changes over time should be interpreted with caution.

The comparison between treated and untreated patients was also performed according to diurnal preference. Considering untreated patients, wake-up time was earlier in “morning” compared to “intermediate” patients ( $05:44 \pm 00:14$ h, 95% CI [05:19, 07:13] vs  $07:13 \pm 00:25$ h, 95% CI [06:21, 08:04],  $F=7.800$ ,  $p=0.010$ ); these data were comparable between T0



**Figure 5.** Actigraphy-based wake-up time (mean and 95% CI; Panels a and b;  $p=0.017$  for intermediate types) and mid-sleep (mean and 95% CI; Panels c and d;  $p=0.008$  for intermediate types) in standard of care (Panels a and c) and CircadianCare (Panels b and d) arms in morning (purple triangles) and intermediate types (green diamonds) at T0 and T1 (day 7). Abbreviation: CI = confidence interval. Color version of the figure is available online.

and T1. Sleep diaries documented a decrease in the number of night awakenings from T0 to T1 for “morning” patients in the treated group ( $3.4 \pm 0.7$ , 95% CI [1.85, 4.97] vs  $2.2 \pm 0.8$ , 95% CI [0.40, 3.93]) and for “intermediate” patients in the untreated group ( $3.7 \pm 0.9$ , 95% CI [1.84, 5.66] vs  $1.6 \pm 1.0$ , 95% CI [−0.54, 3.79]), with a significant interaction between time, treatment, and diurnal preference ( $F=11.007$ ,  $p=0.004$ ). No significant differences between the two groups were detected in other sleep diary variables.

By contrast to sleep diaries, the comparison of actigraphic recordings at T0, T1, and T2 did not show significant differences between treated and untreated patients. This may suggest a decreased reliability of actigraphy within the hospital setting, which is by definition characterized by an overall activity reduction and by bed rest also during the waking hours. Within this context, patient-provided subjective measures may be more reliable than recorded activity, especially when studying/measuring the transitions between sleep and wake states. When limiting the analysis to T0 and T1, the number of daytime naps decreased after 1 week of inpatient stay in both study arms ( $1.5 \pm 0.3$ , 95% CI [0.89, 2.17] at T0 and  $0.8 \pm 0.3$ , 95% CI [0.27, 1.41] at T1), with a significant effect of time ( $F=6.211$ ,  $p=0.019$ ). Similarly, the time spent awake after sleep onset also decreased after 1 week in both study arms ( $1.60 \pm 0.15$ , 95% CI [1.29, 1.91] at T0 and  $1.23 \pm 0.13$ , 95% CI [0.96, 1.51] at T1), with a significant effect of time ( $F=4.982$ ,  $p=0.033$ ). These findings may relate to improved general patients’ condition toward the first/second week of hospitalization. When diurnal preference was considered, the overall number and duration of daytime naps were lower in “morning” compared to “intermediate” patients (number:  $0.7 \pm 0.3$ , 95% CI [0.57, 1.39] vs  $2.1 \pm 0.4$ , 95% CI [1.18, 2.93],  $F=6.219$ ,  $p=0.020$ ;

duration:  $16 \pm 8$  min, 95% CI [−1.82, 33.01] vs  $52 \pm 11$  min, 95% CI [29.62, 75.38],  $F=6.985$ ,  $p=0.014$ ). In addition, actigraphic data showed a delay in wake-up time for “intermediate” patients in the untreated group from T0 to T1 ( $05:31 \pm 00:31$  h, 95% CI [04:26, 06:36] vs  $06:59 \pm 00:33$  h, 95% CI [05:50, 07:06],  $F=6.569$ ,  $p=0.017$ ; Figure 5, Panel a). This was not evident in the treated group. Along the same lines, there was a delay in mid-sleep for “intermediate” patients in the untreated group from T0 to T1 ( $02:20 \pm 00:28$  h, 95% CI [01:21, 03:18] vs  $03:37 \pm 00:29$  h, 95% CI [02:37, 04:37]), with a significant interaction between time and diurnal preference ( $F=8.276$ ,  $p=0.008$ ; Figure 5, Panel c).

### Inpatient Stay—Salivary Melatonin Samples

Baseline salivary melatonin was collected in 34 patients at T0, in 15 patients at T1, and in 2 patients at T2. The DLMO was calculable in 17 patients (8 treated and 9 untreated) at T0, 9 patients (5 treated and 4 untreated) at T1, and 2 patients (both treated) at T2. While measuring the dim light melatonin offset (DLMOff) rather than DLMO at night is unusual, it was deemed necessary in 5 patients (4 treated and 1 untreated) at T0 and 2 patients (1 treated and 1 untreated) at T1, due to the melatonin profile showing a clear and steady decrease in melatonin levels. This may have been the result of light/dark conditions and/or hospital or disease-dictated sleep-wake habits during the inpatient stay. At T0, the treated group had a later DLMO ( $22:04 \pm 00:22$ , 95% CI [21:10, 22:57]) than the untreated group ( $20:43 \pm 00:27$ , 95% CI [19:39, 21:47],  $F=1.71$ ,  $p<0.05$ ). Between T0 and T1, there was an advance in DLMO in treated patients (at T0  $22:50 \pm 00:18$  h,

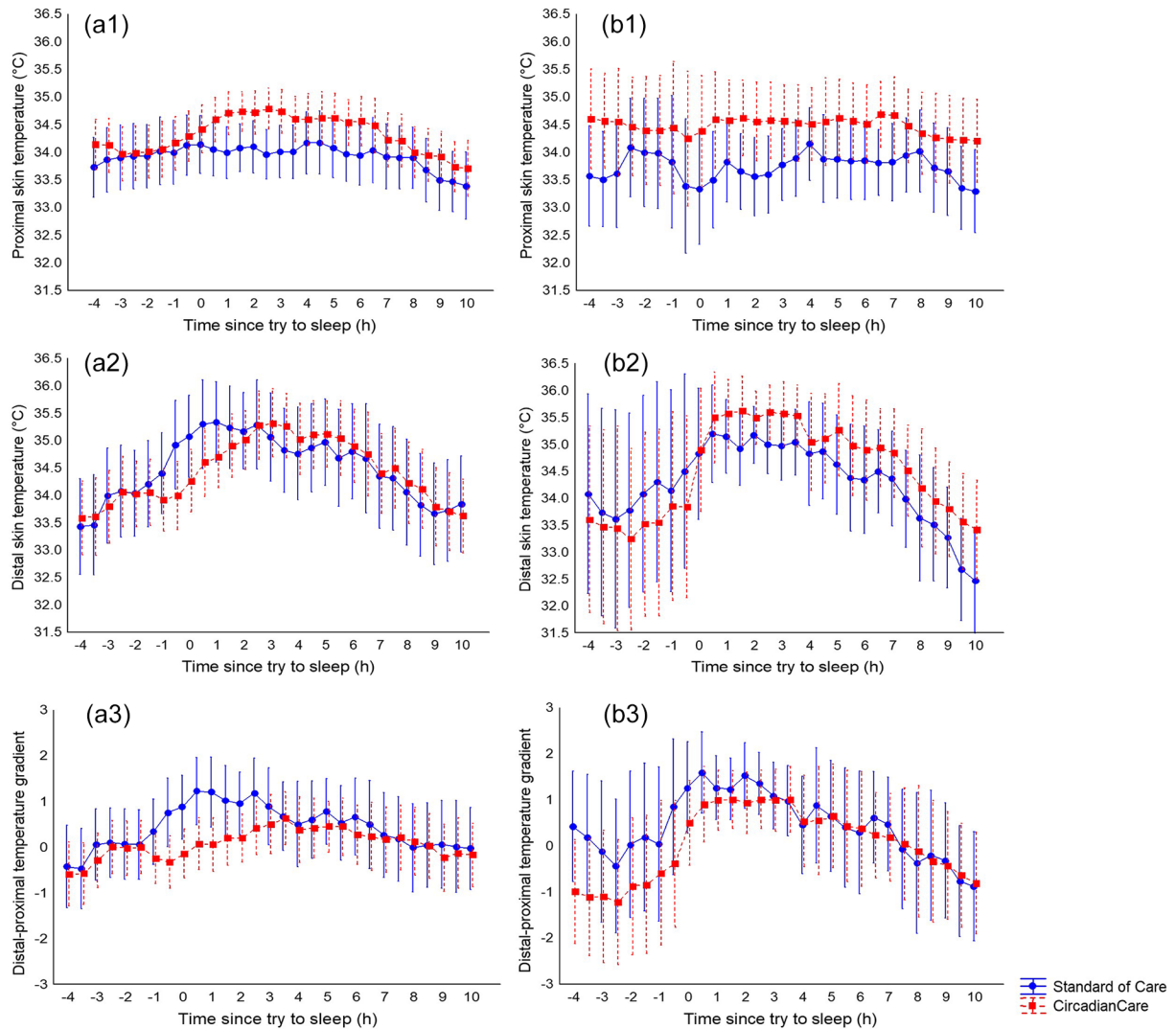


Figure 6. Proximal skin temperature (mean and 95% CI; Panels a1 and b1), distal skin temperature (Panels a2 and b2), and distal-proximal skin temperature gradient (Panels a3 and b3) at T0 (Panels a1-a3) and T1 (day 7; Panels b1-b3), aligned according to try-to-sleep time in the CircadianCare (red squares) and standard of care (blue circles) arms. No significant differences were observed in relation to treatment. However, on visual analysis, proximal skin temperature seemed to flatten over the inpatient stay in both arms (a1 and b1) and the distal skin temperature oscillation seemed greater in treated compared to untreated patients (a2 and b2). Abbreviation: CI = confidence interval.

95% CI [21:42, 23:54]; at T1: 21:48  $\pm$  00:25h, 95% CI [21:12, 22:24],  $p < 0.05$ ), which may partly relate to the untreated ones having less room for advancement due to the differences at baseline. An example of a patient in the CircadianCare arm whose DLMO was available and calculable at all 3 time points, with gradual phase advance over the inpatient stay, is presented in Suppl. Fig. S1. There were no significant differences in sleep parameters (diaries or actigraphy) between patients grouped by calculable DLMO, calculable DLMOff, and non-calculable DLMO/DLMOff at T0 or T1, suggesting that confounders rather than more specific sleep-wake and/or circadian pathophysiological pathways may

contribute to melatonin profile rhythmicity and/or its timing in this population of acute medical inpatients.

### Inpatient Stay—Skin Temperature

At T0, PST was available for 39 patients (23 treated and 16 untreated), whereas DST and DPG were available for 35 patients (22 treated and 13 untreated), with no differences between the two treatment arms (Figure 6, Panels a1-a3). As expected, temperature varied significantly over the 24 h for all 3 variables (PST:  $F = 6.56$ ,  $p < 0.001$ ; DST:  $F = 7.38$ ,  $p < 0.001$ ; DPG:  $F = 3.10$ ,  $p < 0.001$ ; Figure 6, Panels a1-a3). At T1, PST was available for 17 patients (8 treated and 9 untreated),

**Table 2.** Room illuminance by time of day (divided into 4 time slots), bed position in relation to the window, and position of the rolling shutters.

| Time of Day (h)        | Position of Rolling Shutter<br>(%; Mean $\pm$ SE) | Illuminance Level (Lux; Mean $\pm$ SE [95% Confidence Interval]) |                         |                                      |
|------------------------|---------------------------------------------------|------------------------------------------------------------------|-------------------------|--------------------------------------|
|                        |                                                   | Room                                                             | Bed Near Window         | Bed Far From Window                  |
| 1. From 07:00 to 10:30 | 32 $\pm$ 4%                                       | 394 $\pm$ 45 (303, 485)                                          | 515 $\pm$ 58 (399, 634) | 268 $\pm$ 59 (147, 387) <sup>a</sup> |
| 2. From 12:00 to 14:00 | 51 $\pm$ 3%                                       | 477 $\pm$ 54 (367, 587)                                          | 636 $\pm$ 68 (500, 774) | 311 $\pm$ 70 (172, 453) <sup>b</sup> |
| 3. From 14:30 to 17:00 | 58 $\pm$ 4%                                       | 251 $\pm$ 30 (192, 312) <sup>c,d</sup>                           | 309 $\pm$ 41 (228, 391) | 192 $\pm$ 42 (109, 278)              |
| 4. From 17:30 to 21:00 | 59 $\pm$ 3%                                       | 185 $\pm$ 26 (134, 237) <sup>e,f</sup>                           | 234 $\pm$ 35 (164, 308) | 134 $\pm$ 36 (63, 208)               |

0% = window open/100% = window closed; on *post hoc* comparison.

<sup>a</sup> $p < 0.05$ , near versus far; <sup>b</sup> $p < 0.001$ , near versus far.

<sup>c</sup> $p < 0.001$ , Slot 2 versus Slot 3; <sup>d</sup> $p = 0.059$ , Slot 1 versus Slot 3; <sup>e</sup> $p < 0.001$ , Slot 1 versus Slot 4; <sup>f</sup> $p < 0.001$ , Slot 2 versus Slot 4.

whereas DST and DPG were available for 15 patients (8 treated and 7 untreated; Figure 6, Panels b1-b3). At T1, no significant difference was observed between the two treatment arms in any of the 3 temperature indices (Figure 6, Panels b1-b3). Qualitative, visual analysis, however, showed that the oscillation in DST amplitude was greater in treated compared to untreated patients (Figure 6, Panel b2), again, possibly suggesting rhythm strengthening in the treatment arm over time. Moreover, at T1, temperature varied significantly over the 24 h in terms of DST and DPG (DST time:  $F = 4.82$ ,  $p < 0.001$ ; DPG, time:  $F = 4.91$ ,  $p < 0.001$ ; Figure 6, Panels b2 and b3), but not in PST, where the oscillation amplitude diminished at T1 compared to baseline in both treated and untreated patients (Figure 6, Panel b1). This may relate to PST being more affected by bed rest or bedcovers, and/or to the contribution to the case series of inpatients with cirrhosis (2 untreated and 1 treated with available data), whom we have previously shown to have increased PST and decreased DPG (Garrido et al., 2017).

### Inpatient Stay—Medication

During the inpatient stay, 11 untreated and 9 treated patients took *pro re nata* sleep-inducing medication; 19 untreated and 16 treated, respectively, took drugs that might have affected melatonin profiles, with no significant difference between the two treatment arms. Moreover, no significant difference between the absolute or relative number of days on *pro re nata* sleep-inducing medication during the inpatient stay was observed between the two treatment arms.

### Inpatient Stay—Satisfaction With Hospitalization

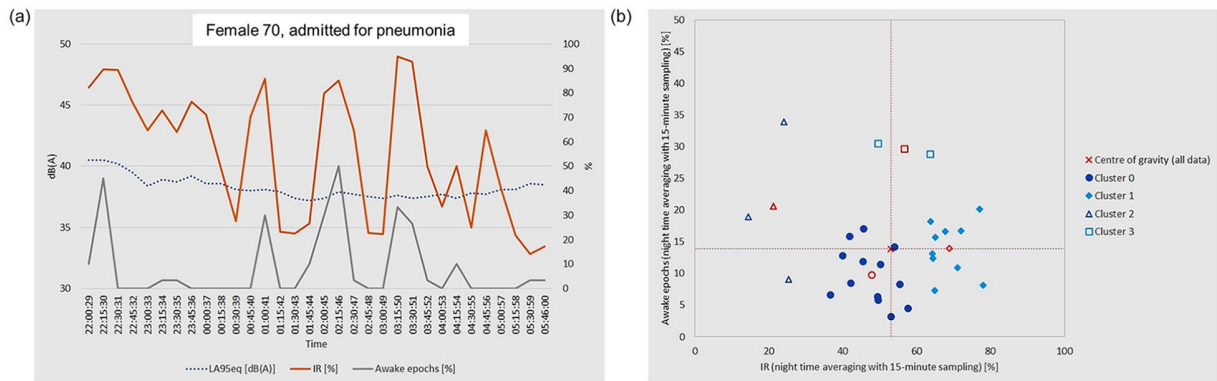
Patients in the treatment arm were more satisfied than untreated patients in relation to the quality of

the information received on admission about the ward organization ( $\chi^2 = 6.74$ ,  $p = 0.034$ ). It is possible that receiving a small amount of additional information (plus having a choice) on light, meal, and activity timing in an environment as stressful, disorienting, and restrictive as an acute medical ward may contribute to decrease hospitalization-related discomfort and improve the quality of the inpatient stay.

### Inpatient Stay—Illuminance

Illuminance levels differed significantly during the day (Table 2). Moreover, illuminance levels differed between patients sleeping near or far from the window in the first 2 time slots, with a significant interaction between time and bed position (Table 2). When grouping patients by date of admission (i.e., during daylight saving time or standard time), these data were only confirmed in the group admitted during Daylight Saving Time (Slot 1:  $623 \pm 74$  lux, 95% CI [471, 776] vs  $261 \pm 69$  lux, 95% CI [117, 403]; Slot 2:  $679 \pm 96$  lux, 95% CI [483, 874] vs  $334 \pm 90$  lux, 95% CI [151, 518], respectively,  $F = 12.72$ ,  $p = 0.001$ ). Rolling shutters (average position per time slot) were half-way down for most of the day (Table 2). Moreover, the position of the rolling shutters correlated with light levels in the afternoon and evening slots (Slot 3:  $r = -0.48$ ,  $p < 0.001$ ; Slot 4:  $r = -0.37$ ,  $p = 0.011$ ). This trend was maintained in the patients who slept far from the window (Slot 3:  $r = -0.58$ ,  $p < 0.01$ ; Slot 4:  $r = -0.42$ ,  $p = 0.047$ ) but only in the afternoon in the patients who slept near the window (Slot 3:  $r = -0.44$ ,  $p = 0.033$ ). The total number of people in the room was the highest in Slot 4 (range: 1-10 people) and the lowest in Slots 2 and 3 (range: 1-5 people). Patients sleeping near the window had significantly higher sleep efficiency (defined as the percentage of time spent asleep while in bed, recorded by actigraphy) during their hospitalization compared to those sleeping far from the window ( $83 \pm 1$ , 95% CI [80, 86] vs  $78 \pm 2$ ,





**Figure 7.** (a) Activity and noise levels in one example patient showing a clear relationship between increases in percent of awake epochs as recorded by actigraphy (gray line) and increased intermittent noise (intermittency ratio; orange line) during the first night of hospitalization. The LA95eq is shown as a blue dotted line. (b) Cluster analysis of the relationship between percent of awake epochs and noise intermittency ratio; the center of each cluster is marked in red. For the two most numerous clusters (0 and 1), a consistent and significant relationship can be observed ( $R=0.866$ ) between ambient noise fluctuation and percent of awake epochs.

95% CI [74, 82],  $p=0.042$ ). No significant relationships were observed between subjective sleep quality, length of hospitalization, or bed position.

### Inpatient Stay—Night Noise Levels

The noise levels recorded during the night at T0 were constantly higher than the WHO recommended value of 30 dB(A) for 8 h LAeq in care units (average night time ambient noise LAeq:  $50.0 \pm 0.5$  dB(A), average LA95eq:  $39.6 \pm 0.5$  dB(A), average LAmx:  $80.5 \pm 0.5$  dB(A), and average IR: 53.1%). No significant differences were observed in patients who slept far from or near the window (and thus nearer or further from the corridor, respectively). Noise levels and activity (measured by actigraphy) were compared in 28 (12 treated and 16 untreated) patients at T0 (before the commencement of any treatment) and visual analysis of 9 patients (3 whose bed was close to the window) showed peaks of IR that were associated with awakenings of the patient or preceded them (Figure 7, Panel a and Suppl. Fig. S2). Although fluctuating ambient noise alone was not the dominant cause for the awakenings, a substantial change in the IR over a relatively short 15-min interval represented a clear change in room conditions relative to noise.

The comparison between the average of the IR (%) and the awake epochs over the conventional 8-h night led to the definition of 4 clusters of patients (Figure 7, Panel b). Two relatively large groups of patients had a similar number of awakening periods (typically between 5% and 20%) despite two distinct ranges of noise fluctuation (Cluster 0 with IR: 40%-60% and Cluster 1 with IR: 60%-80%). For these two clusters, as the noise intermittence increased, so did the number of awakenings. Two smaller patient groups had different behavior: one (Cluster 2) had an

awakening period of more than 20% despite favorable noise conditions (IR <30%); the other (Cluster 3) exhibited a very high number of awakenings even in average conditions of intermittent noise. Of interest, while the results did not reach statistical significance, patients in Clusters 2 and 3 exhibited a tendency toward higher Charlson Comorbidity Index ( $4.2 \pm 0.8$ , 95% CI [2.3, 6.1] in Clusters 2 and 3 vs  $2.9 \pm 2.2$ , 95% CI [2.0, 3.8] in Clusters 0 and 1) and higher pre-admission PSQI scores ( $10.8 \pm 5.1$ , 95% CI [5.8, 15.8] in Clusters 2 and 3 vs  $8.1 \pm 5.4$ , 95% CI [5.6, 10.1] in Clusters 0 and 1), suggesting that their awakenings might have been associated with their clinical condition and/or their chronic sleep disturbance rather than environmental conditions.

### Unintended Effects

No unintended effects were reported and treatment was well tolerated.

## DISCUSSION

In this pilot study, we tested a novel inpatient management system (CircadianCare) aimed at limiting the negative impact of hospitalization on sleep by enhancing circadian rhythmicity through personalized light/dark, meal, and physical activity timing schedules. While the primary registered outcome (reduced sleep onset latency on actigraphy or diary) was not met, the CircadianCare system resulted in a trend advance in bedtime and, albeit only in the few patients in whom the variable was repeatedly calculable, a significant advance in DLMO time. Patients sleeping near the window showed significantly

higher sleep efficiency, regardless of treatment. As noise fluctuation increased, so did the number of night awakenings, also regardless of treatment. Finally, having a “morning” diurnal preference was associated with better sleep quality during hospitalization, most likely because morning types find it easier to adapt to hospital activity schedules, which tend to start early.

Actigraphic recordings did not document any significant differences between treated and untreated patients. However, the number of daytime naps decreased after 1 week of inpatient stay in both study arms, and so did the time spent awake after sleep onset. The latter are more likely to relate to the course of disease during hospitalization rather than to our intervention, or lack thereof. The fact that actigraphy and sleep diaries did not produce overlapping results is most likely due to the fact that these two tools may have a different relationship in hospitalized patients, who are also bedridden at times, compared to that known for healthy individuals in their home environment (Lockley et al., 1999; Lehrer et al., 2022). It is somewhat intuitive, albeit not well demonstrated, that an activity-based sleep-wake index may be less than ideal within a context which is by definition characterized by an overall reduction in activity.

The lack of significant differences in sleep timing and sleep quality indices (sleep diaries or actigraphy) between patients grouped by DLMO onset, DLMO offset, and non-calculable DLMO, at T0 or T1, may be in relation to the complexity of the condition of the patients, the limited number of DLMO available, and the fact that the timing of melatonin production is obviously not the sole determinant of sleep timing/quality (Lavie, 1997; De Rui et al., 2015; Blume et al., 2022), especially in a complex and unusual situation such as hospitalization. The lack of a relationship between melatonin profiles and subjective or objective sleep-wake variables may also suggest that confounders such as medication or illness *per se* may play a relevant role on melatonin profile shape and DLMO/DLMOOff computability/timing in clinical populations.

Furthermore, after 1 week of treatment, patients in the CircadianCare arm increased the number of hours spent in bed and the get up latency (time spent in bed after waking up) compared to standard of care patients, possibly because the majority of treated patients chose to stay in bed while wearing the light glasses after awakening, even though this was not prescribed. While this is not necessarily an unwelcome interpretation of the instructions provided, these may be modified as appropriate in future, similar studies.

Treated patients reported a higher satisfaction regarding the information received on the ward organization, which suggests that provision of such

information in a detailed and possibly personalized fashion may be a simple and cheap tool to contain hospitalization-related discomfort.

The strong effect of diurnal preference on sleep-wake timing/quality was confirmed, even in our hospital setting. For example, patients in the standard of care group with a “morning” diurnal preference woke up earlier than patients with an “intermediate” diurnal preference, and this held true for the entire hospitalization. The effect of diurnal preference was also evident when examining actigraphic results (delay in wake-up time exhibited by “intermediate” patients in the untreated group). It should be highlighted that, in our patient population, the subgroup of patients with an “evening” chronotype was poorly represented. This was probably to be expected, as the mean age was relatively high in both study arms, with a consequent prevalence of early chronotypes (Fischer et al., 2017). This is an important consideration when evaluating the effectiveness of our intervention, as a significant proportion of patients was already “aligned” to the usual time schedule of a standard medical ward, where routine activities are generally carried out in the earlier part of the day. Furthermore, this finding could be usefully taken into consideration when designing future, similar trials.

Regarding skin temperature variations, although we did not find significant differences between the two study arms, a 24-h oscillation in PST and DST was observed, with a tendency to flattening of the PST curve after 1 week of hospitalization in all patients. This is in agreement with previously published literature (Cuesta et al., 2017; Garrido et al., 2017). In the DST profile, a flattening of the DST curve at T1 was not visible. This suggests that the distal vasodilation occurring at night, which is functionally linked to sleep propensity, is still intact after 1 week of hospitalization and it also supports the hypothesis that DST is less affected by masking factors (van Marken Lichtenbelt et al., 2006), making it a valuable index also within the context of an acute medical hospitalization.

Patients sleeping near the window were exposed to more light than those sleeping far from the window, especially in the morning and during Daylight Saving Time. A clear effect of bed position on sleep efficiency was confirmed despite the age and severity of the medical condition (Bano et al., 2014), emphasizing the importance of the amount of natural light an inpatient is exposed to. As we have observed before (Bano et al., 2014), and except for purposes of the CircadianCare protocol, rolling shutters were rarely moved. Given the significant effect that natural light seemed to have on sleep efficiency, small educational campaigns on rolling shutters management aimed at hospital staff may be an additional, cheap and helpful tool to prevent hospitalization-related

insomnia. By contrast to a previous study (Benedetti et al., 2001), no significant relationship was observed between bed position and length of hospitalization, possibly due to the considerably shorter period of hospitalization in the present study.

With regard to the effects of nocturnal environmental noise in hospital rooms, the baseline LAeq and the LAmx were very similar for all cases analyzed, and well above those recommended by the WHO. By contrast, the combination of the IR and the LA95eq allowed to unveil a relationship between substantial fluctuations in noise levels and night awakenings. It should be noted that the parameters taken into consideration by the WHO to evaluate the effects of noise on sleep focus on the noise determined by vehicle traffic outside buildings. By contrast, the acoustic analysis proposed in this study tried to highlight the effects of noise produced within the wards by the service equipment, as well as by medical operations and activities related to patients' care. The results suggest that better conditions for sleep could be obtained not only by limiting the amount of noise during the night but also by ensuring that noise levels have the smallest possible fluctuations. This could probably be obtained, for example, by educational interventions for nurses on night rounds, and also by changes in the features of medication trolleys (e.g., operating surfaces materials) and their wheels and other mobile parts, which tend to be noisy.

The study has several limitations. A considerable proportion of patients screened did not provide informed consent and attrition over time was also high, which should be taken into account when planning similar, future studies in the acute medical setting. There were differences in baseline parameters between the two treatment arms (i.e., number of awakenings and DLMO), which might have affected subsequent comparisons. Also, logistics/feasibility issues and the lack of available, published information on the features/variability of certain indices (e.g., actigraphy and DLMO) in acute medical populations lead to some degree of uncertainty in power estimates and some deviation from the original analyses plan. Nonetheless, the CircadianCare management system showed some positive results in terms of both sleep and circadian timing. Furthermore, the study documented a significant effect of environmental lighting (bed position in relation to the window) and environmental noise (noise fluctuations during the night) on sleep. These findings may allow to implement minor changes to the service equipment currently used in wards plus in some ward practices, which may be cheap and beneficial, and ultimately to design better wards in future. Finally, our study demonstrates the feasibility of a personalized, multiple

intervention chronotherapy protocol in a complex, acute hospitalization setting.

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## CONFLICT OF INTEREST STATEMENT

The authors have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## NOTE

Supplementary material is available for this article online.

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## SHORT REPORT



# Personalising circadian hygiene educational initiatives aimed at university students—“He who has ears to hear, let him hear”

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## Summary

The aim of the present study was to characterise “early drop-outs” ( $n = 3185$ ) out of a group of university students ( $n = 7766$ ) engaged in an ongoing circadian education initiative, to evaluate its efficacy and direct its developments. The initiative is aimed at improving sleep timing/quality through one of two sets of circadian hygiene advice covering the timing of sleep, meals, exercise and light exposure, and it has already been shown to have a positive effect on sleep timing. This second, interim analysis confirmed the high prevalence of disturbed night sleep and social jetlag amongst students at Padova University. Three-thousand, one-hundred and eighty-five (41.0%) students were early drop-outs. These were more commonly males (46.4 versus 37.6%;  $\chi^2 = 58$ ,  $p < 0.0001$ ), had later sleep–wake habits, more daytime sleepiness and worse night sleep quality. Chronotype distribution was also different, with a slight but significantly higher proportion of extremely evening/evening types amongst early drop-outs ( $\chi^2 = 10$ ,  $p < 0.05$ ). These results suggest that the more evening the student, the lower their likelihood of choosing/being able to follow circadian advice.

## KEYWORDS

academic performance, chronotype, circadian education, sleep, university students

## 1 | INTRODUCTION

Health-related surveys and educational initiatives, especially when advertised and/or provided online, suffer from multiple types of selection bias, to include a personal interest in the object of the initiative, computer/social media literacy, and other factors. Unless the features of the population to which the initiative is aimed at are known in detail beforehand, it is difficult to evaluate participation and attrition in a meaningful fashion post hoc. Here we make use of a group of “early drop-outs”, and of initial, self-reported compliance with the circadian education initiative SleepRhythm Unipd (fully described in our

first, published paper on the initiative: Montagnese et al., 2022) to evaluate some aspects of its efficacy and direct its developments. In brief, the initiative—which started in October 2019, is ongoing and described in detail in Montagnese et al. (2022)—is aimed at improving sleep timing and sleep quality through one of two sets of circadian hygiene advice, covering the timing of sleep, meals, physical exercise and light exposure.

## 2 | METHODS

All methods used in this study are described in full detail in Montagnese et al. (2022). All active students at the University of Padova were

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**TABLE 1** Chronotype distribution, by sex.

|                | Extremely morning | Morning      | Evening      | Extremely evening | Total (n) |
|----------------|-------------------|--------------|--------------|-------------------|-----------|
| Males (n, %)   | 250 (8.4%)        | 934 (31.5%)  | 1181 (39.8%) | 604 (20.3%)       | 2969      |
| Females (n, %) | 557 (11.6%)       | 1602 (33.4%) | 1833 (38.2%) | 805 (16.8%)       | 4797      |
| Total (n)      | 807               | 2536         | 3014         | 1409              | 7766      |

Note:  $\chi^2 = 34$ ,  $p < 0.0001$ .

**TABLE 2** Age, mood and sleep–wake features of students who did/did not engage in follow-up.

|                                                | Follow-up<br>n = 4581 | No follow-up<br>n = 3185 |
|------------------------------------------------|-----------------------|--------------------------|
| Age (years)                                    | 23.2 ± 5.4            | 23.3 ± 5.1               |
| Mood (1–10)                                    | 5.9 ± 1.9             | 5.8 ± 1.8                |
| ESS (total score, 0–24)                        | 6.2 ± 3.5             | 6.4 ± 3.6**              |
| PSQI (total score, 0–21)                       | 6.7 ± 3.2             | 6.8 ± 3.1*               |
| Sleep-onset time (work/study days; clock time) | 00:06 ± 01:12         | 00:12 ± 01:18***         |
| Wake up time (work/study days; clock time)     | 07:30 ± 01:18         | 07:42 ± 01:36***         |
| Midsleep (work/study days; clock time)         | 03:48 ± 01:12         | 03:54 ± 01:18***         |
| Sleep duration (work/study days; hr)           | 7.4 ± 1.3             | 7.4 ± 1.4                |
| Sleep-onset time (free days; clock time)       | 00:54 ± 01:18         | 01:00 ± 01:30***         |
| Wake up time (free days; clock time)           | 09:12 ± 01:30         | 09:18 ± 01:18*           |
| Midsleep time (free days; clock time)          | 05:00 ± 01:12         | 05:12 ± 01:24***         |
| Sleep duration (free days; hr)                 | 8.4 ± 1.3             | 8.3 ± 1.4                |
| Social jet lag (hr)                            | 1.2 ± 1.1             | 1.2 ± 1.3                |

Note: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.0001$  (Bonferroni-corrected for sleep–wake timing variables).

Abbreviations: ESS, Epworth Sleepiness Scale; PSQI, Pittsburgh Sleep Quality Index.

offered the possibility to join the SleepRhythm Unipd initiative. On joining it for the first time, they were asked to provide information on demographics, health-related indices, sleep–wake timing, night sleep quality and diurnal sleepiness. They then received one of two sets of circadian hygiene advice: “A regular life” or “Bright days and dark nights” (figure 1 in Montagnese et al., 2022). Both included elements of the recommendations routinely provided to individuals with either a formal diagnosis and/or features of delayed sleep phase type (Abbott et al., 2015), which are common in adolescents and young adults. “A regular life” encouraged participants to regularise their habits. “Bright days and dark nights” encouraged participants to advance their sleep–wake, meals and exercise timing, and to maximise/minimise light exposure in the first/second part of the day, respectively (figure 1 in Montagnese et al., 2022). Every month after joining, participants were asked how easy it had been to comply

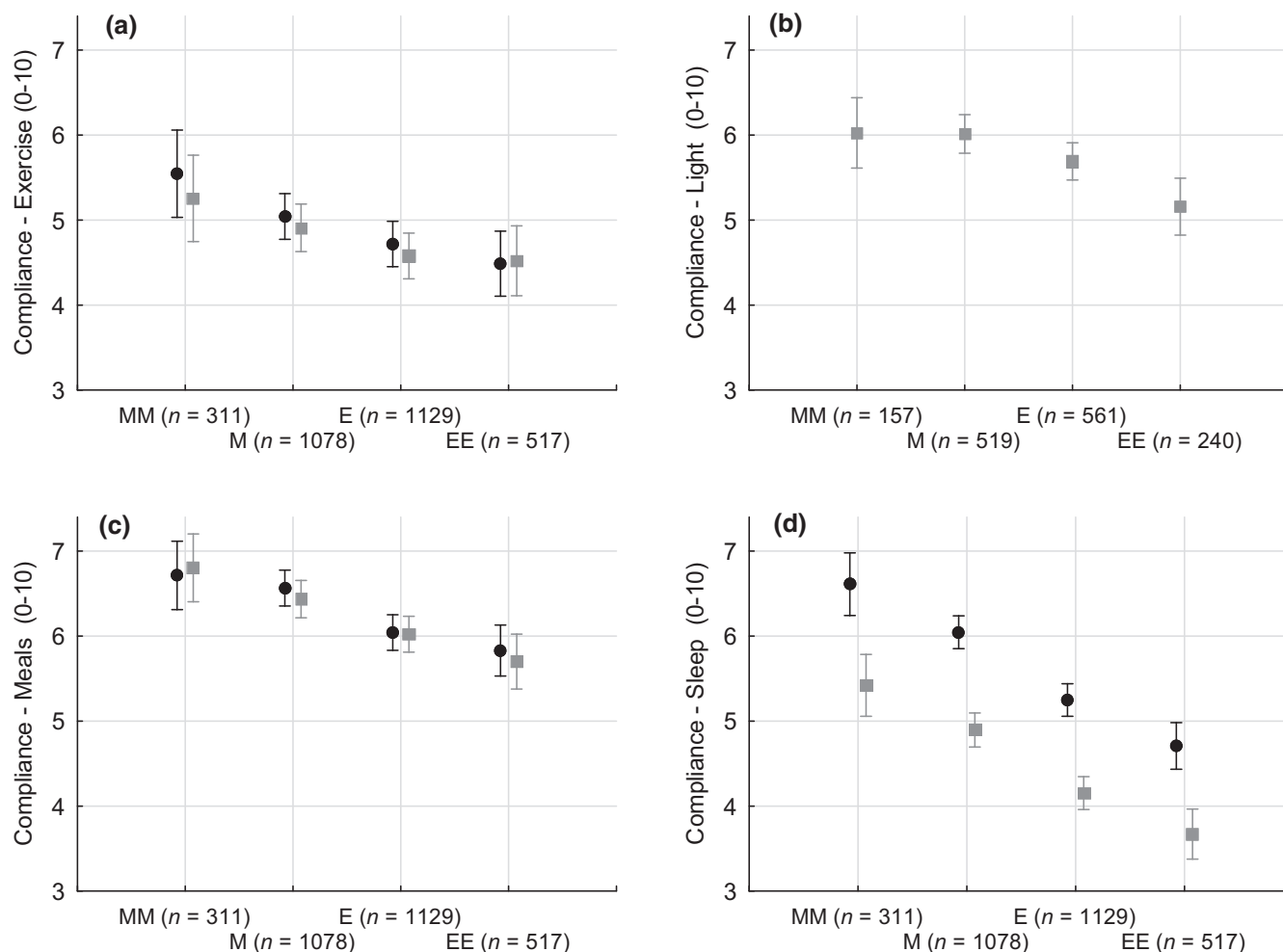
(compliance was subjectively rated on a 0–10 visual-analogue scale) with each piece of advice, and were provided with the same advice again. At every even month from joining, they were asked to complete demographic and sleep–wake information again. They were also asked how easy it had been to comply, and provided with the advice again. Date and time of completion of all procedures scheduled at time 0 (T0) were recorded. The month of entry into the study was recorded and corrected, where appropriate, for the presence of daylight saving time (DST) for use as a surrogate marker of light exposure. Study entry was also qualified as prior to, during (March 2020–September 2021) or after distance learning was instated/interrupted.

Electronic versions of the following questionnaires were administered, and scored according to pertinent Italian norms: the Sleep Timing Sleep Quality Screening (STSQS) questionnaire (Montagnese et al., 2009); the Pittsburgh Sleep Quality Index (PSQI; Buysse et al., 1989); the Epworth Sleepiness Scale (ESS; Johns, 1991); the Self-Morningness/Eveningness (Self-ME) questionnaire (Turco et al., 2015); the ultrashort version of the Munich ChronoType Questionnaire ( $\mu$ MCTQ; Ghotbi et al., 2020).

Descriptive results are expressed as mean ± SD or as count/percentage. Normality was tested for by the Shapiro–Wilk test. Differences between normally/non-normally distributed variables were examined by the Student's *t*-test/one-way analysis of variance (ANOVA; post hoc: Scheffé test) and by the Mann–Whitney *U*-test/Kruskal–Wallis ANOVA, respectively. Bonferroni correction was applied as appropriate. Factorial ANOVA was utilised for multiple grouping factors. A linear regression model was utilised to identify independent predictors of sleep–wake indices (amongst age, sex, chronotype, corrected month of entry and distance learning). Analyses were carried out with Statistica, version 14.0.0.15 (TIBCO, Palo Alto, CA, USA).

### 3 | RESULTS

Between 28 October 2019 and 15 May 2023, 7766 students (4797 [61.8%] females, age 23 ± 5 years) joined the initiative; 3868 (49.8%) were assigned to the “Bright days and dark nights” and 3898 (50.2%) to the “A regular life” advice groups, and there were no significant differences between the two groups on any demographic, health-related or sleep–wake variable. One-thousand, four-hundred and seven (18.2%) students reported having one or more diseases; amongst those who chose to qualify such diseases (free writing), 12 (0.15%) reported suffering from “insomnia”. One-thousand, eight-hundred



**FIGURE 1** Self-reported compliance (1–10 visual-analogue scale, study time T1, that is, 1 month after joining; mean  $\pm$  95% confidence interval) to different types of advice (advice on exercise [a], light [b], meals [c], and sleep [d]) in the “Bright days and dark nights” (grey squares) and “A regular life” (black circles) groups, by chronotype (on the x-axis). Chronotype for factorial ANOVA was significant in all instances ( $p < 0.0001$ ; a–d). In the case of compliance to sleep advice (d), the advice group (“Bright days and dark nights” versus “A regular life”) was also significant (chronotype:  $F = 61$ ,  $p < 0.0001$ ; advice group:  $F = 132$ ,  $p < 0.0001$ ; interaction n.s.). Advice on light was provided only in the “Bright days and dark nights” group (b). E, evening; EE, extremely evening; M, morning; MM, extremely morning.

and forty-three (24%) students reported taking medication. As expected, there was significant overlap between students reporting diseases and those reporting being on medication ( $\chi^2 = 1319$ ,  $p < 0.0001$ ). Significantly more female students reported having one/more diseases (20.3% versus 15.1%;  $\chi^2 = 32$ ,  $p < 0.0001$ ). While reports of “insomnia” were rare, they were more common in males (Table S1).

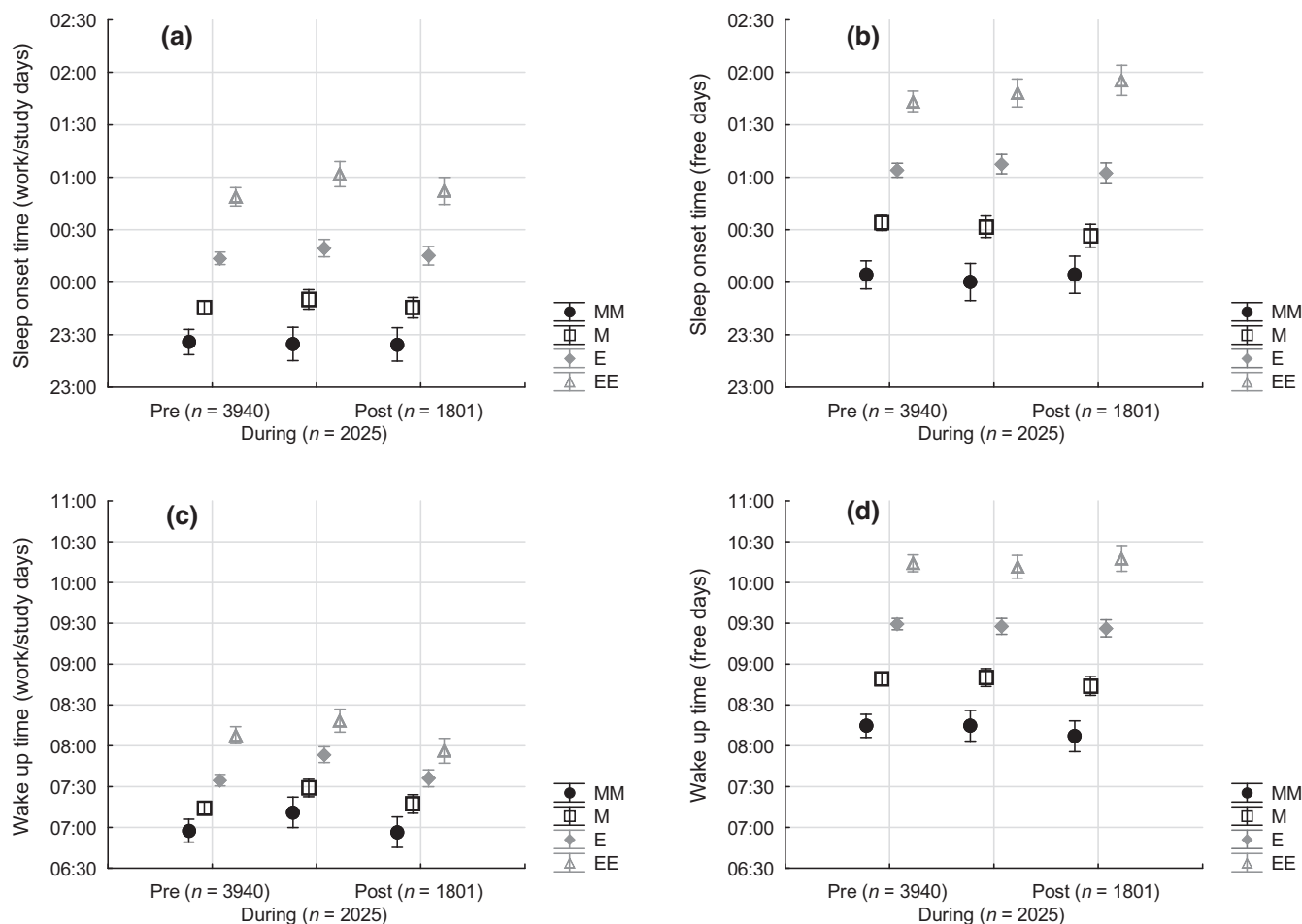
Four-thousand, six-hundred and forty students (59.7%) had abnormal night sleep quality (PSQI score  $> 5$ ), and 967 (12.4%) had excessive daytime sleepiness (ESS  $\geq 11$ ). In both instances, abnormal scores were more common in females (62.9 versus 54.5%,  $\chi^2 = 54$ ,  $p < 0.0001$  and 14.6 versus 8.9%,  $\chi^2 = 54$ ,  $p < 0.0001$ , respectively). Midsleep on work/study and free days was  $03:48 \pm 01:12$  and  $05:06 \pm 01:18$ , respectively, and in both instances it was significantly later in males ( $03:54 \pm 01:18$  versus  $03:42 \pm 01:12$ ,  $p < 0.0001$ ;  $05:12 \pm 01:24$  versus  $05:00 \pm 01:18$ ,  $p < 0.0001$ ); social jet lag was  $1.2 \pm 1.2$  hr, and it was significantly larger in males ( $1.3$

$\pm 1.3$  hr versus  $1.2 \pm 1.3$  hr,  $p < 0.0001$ ). Chronotype distribution, by sex, is presented in Table 1.

As expected, extremely evening/evening students had significantly delayed sleep–wake habits compared with their extremely morning/morning counterparts, and they also had longer sleep latency, worse mood, worse night sleep quality, more daytime sleepiness, shorter sleep duration on work/study days, longer sleep duration on free days and more social jet lag (Table S2).

Three-thousand, one-hundred and eighty-five (41.0%) students never filled in a follow-up compliance or questionnaire. Depending on study entrance date, they had a period of time to do so that ranged between 2 and 43 months. The likelihood of never providing follow-up data was higher in males (46.4% versus 37.6%;  $\chi^2 = 58$ ,  $p < 0.0001$ ). Age and reported disease/insomnia were comparable in the two groups. The percentage of students allocated to the two types of advice was also comparable in the two groups. Students who never provided follow-up information had later sleep–wake habits,





**FIGURE 2** Sleep-onset (mean  $\pm$  95% confidence interval; top panels, a, b) and wake up times (mean  $\pm$  95% confidence interval; bottom panels, c, d) on work/study days (left panels, a, c) and free days (right panels, b, d), by chronotype (extremely morning: black circles; morning: empty squares; evening: grey diamonds; extremely evening: empty triangles) and distance learning (on the x-axis). On factorial ANOVA, chronotype was significant in all instances while distance learning was significant only on work/study days. The effect of distance learning was more obvious on wake up times on work/study days (c); here, the differences between chronotypes were less obvious than in all other instances, most likely as a result of the use of an alarm clock (c). (a) Chronotype:  $F = 344$ ,  $p < 0.0001$ ; distance learning:  $F = 3$ ,  $p < 0.05$ ; interaction n.s. (b) Chronotype:  $F = 385$ ,  $p < 0.0001$ ; distance learning n.s.; interaction n.s. (c) Chronotype:  $F = 132$ ,  $p < 0.0001$ ; distance learning:  $F = 19$ ,  $p < 0.0001$ ; interaction n.s. (d) Chronotype:  $F = 447$ ,  $p < 0.0001$ ; distance learning n.s.; interaction n.s. E, evening; EE, extremely evening; M, morning; MM, extremely morning.

more daytime sleepiness and worse night sleep quality compared with their counterparts who provided follow-up information (Table 2). Chronotype distribution was also different, with a slight but significantly higher proportion of extremely evening/evening types amongst the early drop-outs ( $\chi^2 = 10$ ,  $p < 0.05$ ; Table S3). All the above analyses were repeated in the subgroup of students who had had a minimum of 12 months to provide follow-up data (7016 in total, of whom 2804 [40%] were early drop-outs), and the features of the early drop-outs in this sample overlapped those of the larger one.

Three-thousand and thirty-five (39%) students completed the first compliance questionnaire, of which 1963 (64.7%) were females and 1072 (35.3%) males, with a significantly lower proportion of males compared with the initial T0 sample ( $\chi^2 = 8$ ,  $p < 0.01$ ). Extremely evening/evening types were also significantly less prevalent compared with the T0 sample ( $\chi^2 = 19$ ,  $p < 0.001$ ). Sleep quality was comparable

in the two groups. Compliance was comparable in the “Bright days and dark nights” and “A regular life” groups for the advice on meal and exercise timing. Compliance for the advice on sleep timing was significantly lower in the “Bright days and dark nights” group ( $4.4 \pm 2.4$  versus  $5.6 \pm 2.4$ ,  $p < 0.001$ ). Compliance was significantly lower in extremely evening/evening chronotypes compared with their extremely morning/morning counterparts for all types of advice provided, with no effect of “Bright days and dark nights” versus “A regular life”, except for compliance with the advice on sleep timing. This was significantly lower in the “Bright days and dark nights” group, which advised sleep-wake timing anticipation rather than simply regular sleep-wake timing (chronotype:  $F = 61$ ,  $p < 0.0001$ ; advice group:  $F = 132$ ,  $p < 0.0001$ ; interaction n.s.; Figure 1).

Distance learning was associated with significant, large and temporary delays in sleep-wake timing, especially during work/study

days. When the sleep timing effects of the combination of distance learning and chronotype were assessed, both were significant for work/study days, while only chronotype was significant for free days (Figure 2).

Models including age, sex, chronotype, month of entry into the study corrected for DST and distance learning as predictors of sleep–wake timing/quality indices showed that chronotype was a significant predictor for all sleep–wake timing/quality indices. All other predictors were also significant for most indices, with sleep quality and sleep offset on free days being generally—and not unexpectedly—insensitive to month of entry into the study and distance learning (Table S4).

## 4 | DISCUSSION

This second, interim analysis of the circadian education initiative SleepRhythm Unipd confirmed the high prevalence of disturbed night sleep and social jetlag amongst students of Padova University, especially extremely evening/evening ones. Extremely evening/evening students also reported lower levels of compliance and were more likely to leave the initiative early. So were males compared with females. These observations are in line with the notion that an individual's ability to modify their natural inclination towards placing the main sleep episode in a certain period of the 24 hr, especially during adolescence and early adulthood, is limited (Hagenauer & Lee, 2012). It also suggests that circadian education for university students may be more effective for evening rather than extremely evening types, and extremely evening types may benefit, as an alternative, from delays in lecture timings and/or light therapy. Delayed or flexible lecture timings have been shown to be beneficial for sleep duration (Biller et al., 2022) and, in some instances, also for grades in high school (Dunster et al., 2018). Recently, daily schedules have been shown to influence sleep timing/duration also in undergraduate college students (Lu et al., 2023). Of interest, we were previously able to document how a short course of morning light administration/evening shades resulted in a significant decrease in sleep-onset latency in a small group of university students (Montagnese et al., 2021). Adequately lit, dedicated communal areas may pleasantly serve the same purpose. Also, while the first interim analysis of the SleepRhythm Unipd initiative documented worse academic performance in evening/extremely evening students compared with their morning/extremely morning counterparts in all study subjects, it also showed a tendency of extremely evening students to perform better than evening ones (Montagnese et al., 2022). In view of the present data on early drop-outs and compliance, it is possible to hypothesise that as their evening propensity increases, students may make less of an effort to comply with social timing constraints, study at their most productive time and ultimately perform better. Further, while in high school absenteeism is more likely to result in worse academic performance (Zerbini et al., 2017), this may not be the case in university. Finally, it is worth highlighting that in our study chronotype was defined on a previously validated single question (Turco et al., 2015), thus making it possible to imagine rapid and logistically

easy screening of all students—for example on a voluntary basis on enrolment—in order to personalise strategies to combat the negative effects of misalignment on sleep duration and academic performance (Creswell et al., 2023). Within a productive society, sleep loss amongst young adults and impaired academic performance are costly in the medium- and long-run (Hafner et al., 2017). By contrast, educational campaigns, light therapy and changes to university timetables may be more or less complex logistically but are cheap, especially once instated. It is therefore reasonable to imagine that any such intervention and especially their combination will be cost-effective.

## AUTHOR CONTRIBUTIONS

**Gianluca Giusti:** Writing – original draft; formal analysis; data curation; visualization; investigation. **Esther D. Domenie:** Investigation; writing – original draft; visualization; formal analysis; data curation. **Lisa Zarantonello:** Methodology; formal analysis; project administration; data curation. **Chiara Mangini:** Visualization; writing – review and editing; data curation. **Paolo Ferrari:** Conceptualization; methodology; software; project administration; data curation; formal analysis. **Rodolfo Costa:** Conceptualization; investigation; funding acquisition; writing – review and editing; formal analysis; project administration; methodology; supervision. **Sara Montagnese:** Conceptualization; investigation; funding acquisition; writing – original draft; methodology; formal analysis; project administration; data curation; supervision; resources.

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## CONFLICT OF INTEREST STATEMENT

PF is the Chief Executive Officer and owner of reMedia Srl., which received compensation for the development of the initiative full-responsive website.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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# Overt Hepatic Encephalopathy: More Than Meets the Eye

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**INTRODUCTION:** We aimed to assess the reliability of a qualitative approach to overt hepatic encephalopathy (OHE) diagnosis compared with the semiquantitative, and recommended one.

**METHODS:** The above 2 methods were compared in 411 outpatients (71% males,  $60 \pm 10$  years, model for end-stage liver disease  $13.5 \pm 5.0$ ).

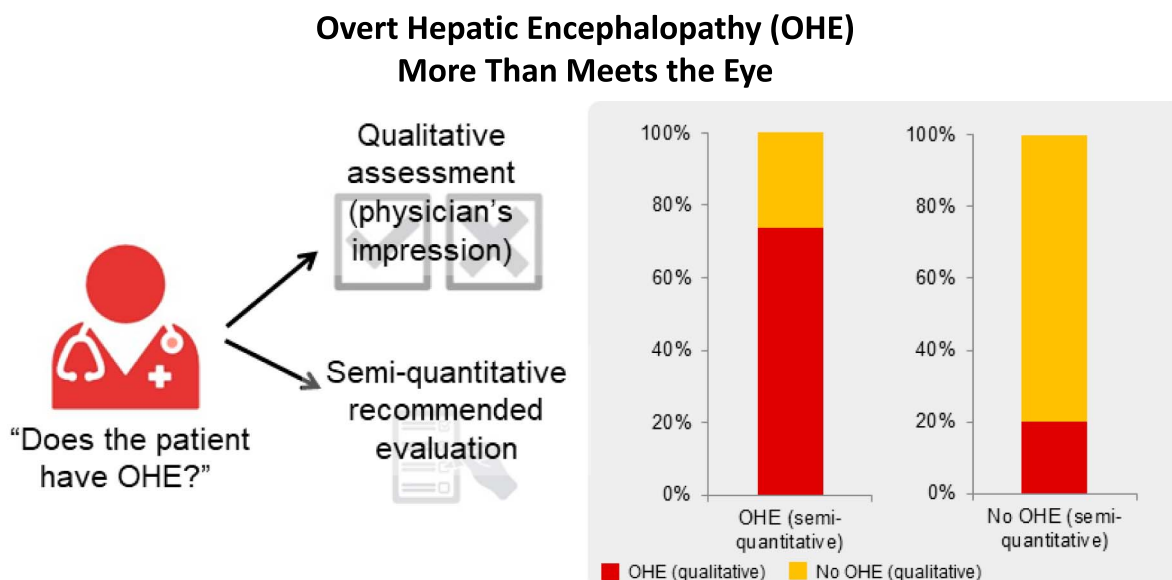
**RESULTS:** Of the 73 patients with OHE on quantitative assessment, 19 (26%) were missed on qualitative assessment, with no difference in the likelihood of the physician missing grades II or III. Sixty-eight (20%) of the 270 patients with no OHE on quantitative assessment were wrongly qualified as having OHE.

**DISCUSSION:** Qualitative clinical evaluation of OHE is not reliable, and recommendations should be followed.

**KEYWORDS:** cirrhosis; hepatic encephalopathy; neuropsychology; electroencephalography; ammonia

**SUPPLEMENTARY MATERIAL** accompanies this paper at <http://links.lww.com/AJG/D241>.

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**Qualitative OHE assessment results in a significant proportion of both false negatives (26%) and false positives (20%)**

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## INTRODUCTION

Although a semiquantitative assessment of overt hepatic encephalopathy (OHE) has been repeatedly and formally recommended (1,2), the impact of its replacement by unstructured interviews and/or physicians' impressions, which are still common practice, has never been formally assessed. The concept behind the recommendation is that verbal abilities tend to be preserved even in severe OHE (3,4), which can therefore be easily missed in conversation, unless questions ascertaining temporal and spatial orientation are actually asked.

The aim of this study was to assess the reliability and the impact, if any, of a qualitative approach to OHE diagnosis compared with the semiquantitative one recommended in the most recent set of Italian guidelines (2) (see Supplementary Figure 1, Supplementary Digital Content 1, <http://links.lww.com/AJG/D241>).

## METHODS

A total of 411 patients (71% males,  $60 \pm 10$  years of age) evaluated in our dedicated HE clinic between April 2009 and June 2023 were included.

Before any formal assessment, patients were classified as having/not having OHE based on a qualitative impression of the physician—generally, a senior resident (i.e., a fourth- to fifth-year hepatology specialist trainee) or a consultant hepatologist—who clerked them into clinic, took their history, and went on to examine them. The doctor ticked *yes* or *no* in response to the question: *Does the patient have overt hepatic encephalopathy?* on the outpatient clinic form, before any formal neuropsychiatric assessment.

Patients were then formally evaluated by neuropsychological tests and electroencephalography (EEG), as fully described in Mangini et al (5), and qualified as unimpaired, when they were

clinically normal and both the psychometric hepatic encephalopathy score (also summarized as the so-called mean PHES Z-score (6,7)) and the EEG were normal, having covert HE (CHE) when they were clinically normal but the Psychometric Hepatic Encephalopathy Score (PHES) and/or the EEG were abnormal, or having OHE based on a recommended semiquantitative modification of the West Haven criteria (2). This includes orientation to time (5 questions) and space (4 questions) plus the Glasgow Coma Scale (GCS) (8). OHE was graded as II (oriented to space, disoriented to time, or presence of asterixis), III (disoriented to time and space with a GCS  $\geq 8$ ), and IV (disoriented to time and space with a GCS  $< 8$ ; coma) (2). From 2016 onward, the animal naming test (ANT) was also administered (9).

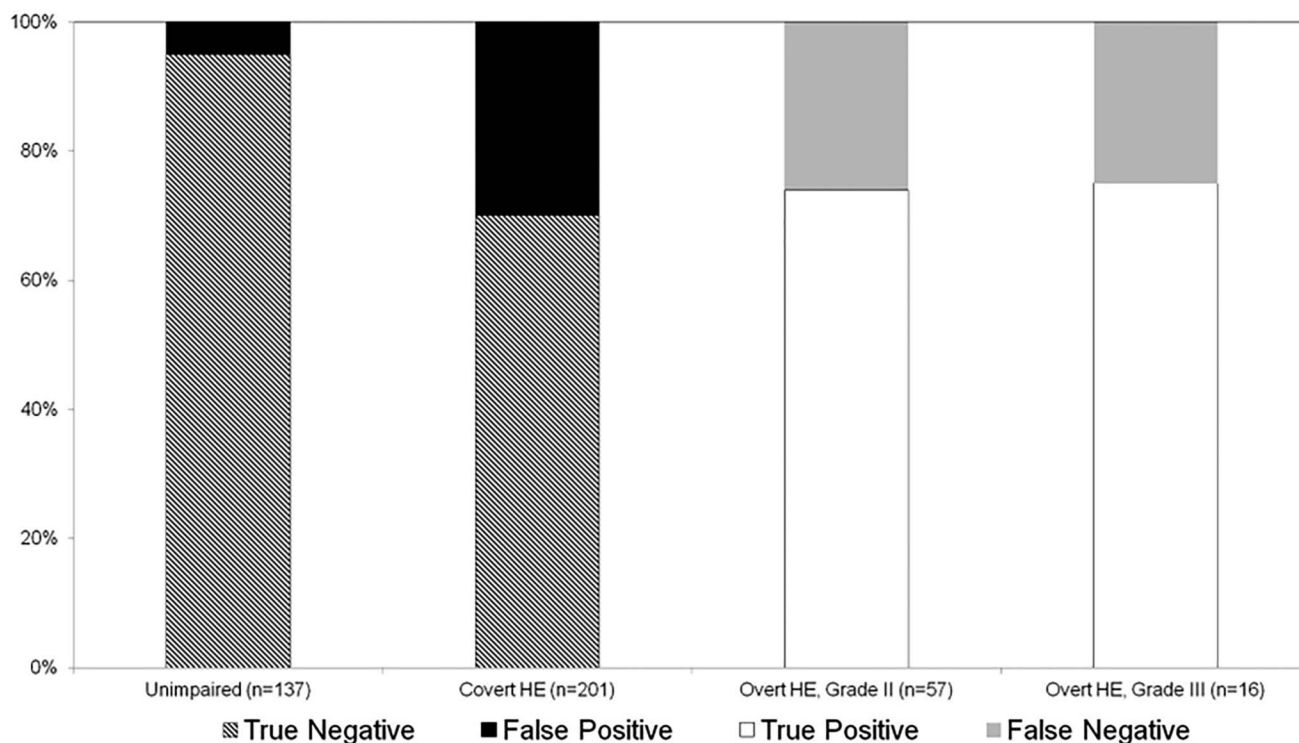
The semiquantitative and qualitative OHE diagnoses were compared, and patients qualified as true positives (TPs) when the impression of the physician was consistent with the formal diagnosis of OHE; false negatives (FNs) when the physician did not recognize OHE; false positives (FPs) when patients were qualified as having OHE by the physician, but they did not meet the formal diagnostic criteria; and true negatives (TNs) when neither the physician nor the formal criteria confirmed an OHE diagnosis.

Permission for retrospective data analyses was obtained from the local Ethics Committee.

Results are expressed as mean  $\pm$  SD or as count and percentage, as appropriate. Comparisons were performed by ANOVA (*post hoc* Tukey test) or by  $\chi^2$ , as appropriate. Analyses were performed with the package Statistica, version 13.1 (Dell, Round Rock, TX).

## RESULTS

One hundred twenty-two (30%) patients were qualified as having OHE on qualitative assessment.



**Figure 1.** Percentage true-positive, false-negative, false-positive, and true-negative results, by degree of hepatic encephalopathy (HE).

**Table 1.** Demographic, hepatic failure, and neuropsychiatric indices, by agreement between quantitative and qualitative assessment of hepatic encephalopathy

|                                                                    | Available data (n) | All patients (n = 411) | TP (n = 54, 13%) | FN (n = 19, 5%) | FP (n = 68, 16%)                   | TN (n = 270, 66%)                 |
|--------------------------------------------------------------------|--------------------|------------------------|------------------|-----------------|------------------------------------|-----------------------------------|
| Sex (% males)                                                      | 411                | 71                     | 76               | 47              | 76                                 | 70                                |
| Age (yr), mean $\pm$ SD                                            | 411                | 60 $\pm$ 10            | 62 $\pm$ 1       | 64 $\pm$ 2      | 61 $\pm$ 1                         | 59 $\pm$ 1                        |
| Etiology (% alcohol/viral/metabolic/other)                         | 401                | 34/30/6/30             | 24/40/6/30       | 47/16/5/32      | 43/25/7/25                         | 34/31/5/30                        |
| MELD, mean $\pm$ SD                                                | 318                | 13.3 $\pm$ 5.0         | 14.3 $\pm$ 0.7   | 14.3 $\pm$ 1    | 14.5 $\pm$ 0.9                     | 12.7 $\pm$ 0.3                    |
| Ascites (% absent/mild/severe or refractory)                       | 370                | 57/29/14               | 52/33/15         | 29/53/18        | 46/39/15                           | 62/24/14                          |
| Asterixis (% absent/rare/frequent or continuous) <sup>EEE</sup>    | 340                | 79/12/9                | 19/41/40         | 41/35/24        | 80/13/7                            | 93/6/2                            |
| Portal-systemic shunt (% absent/spontaneous/surgical) <sup>E</sup> | 281                | 45/49/6                | 30/56/14         | 23/77/0         | 48/50/2                            | 49/45/6                           |
| Ammonia-lowering treatment (% yes/no) <sup>EEE</sup>               | 385                | 73/27                  | 90/10            | 88/12           | 83/17                              | 66/34                             |
| Ammonia ( $\mu$ mol/L), mean $\pm$ SD                              | 258                | 67 $\pm$ 44            | 96 $\pm$ 10      | 67 $\pm$ 7      | 73 $\pm$ 6                         | 59 $\pm$ 3 <sup>+</sup>           |
| ANT, mean $\pm$ SD                                                 | 300                | 13 $\pm$ 5             | 10 $\pm$ 1       | 9 $\pm$ 1       | 12 $\pm$ 1 <sup>^ \$</sup>         | 15 $\pm$ 1 <sup>+ ° ##</sup>      |
| MPZS, mean $\pm$ SD                                                | 384                | -0.9 $\pm$ 1.5         | -2.1 $\pm$ 0.2   | -2.6 $\pm$ 0.3  | -1.1 $\pm$ 0.1 <sup>^ \$\$\$</sup> | -0.5 $\pm$ 0.1 <sup>+ ° ##</sup>  |
| Spectral EEG indices, mean $\pm$ SD                                |                    |                        |                  |                 |                                    |                                   |
| MDF (Hz)                                                           | 383                | 9.3 $\pm$ 2.1          | 7.5 $\pm$ 0.3    | 7.2 $\pm$ 0.5   | 8.7 $\pm$ 0.2 <sup>^ \$</sup>      | 10.0 $\pm$ 0.1 <sup>+ ° ###</sup> |
| $\delta$ (%)                                                       |                    | 11 $\pm$ 12            | 20 $\pm$ 2       | 24 $\pm$ 5      | 12 $\pm$ 1 <sup>^ \$</sup>         | 9 $\pm$ 0 <sup>+ °</sup>          |
| $\theta$ (%)                                                       |                    | 35 $\pm$ 19            | 49 $\pm$ 2       | 47 $\pm$ 4      | 42 $\pm$ 2                         | 29 $\pm$ 1 <sup>+ ° ###</sup>     |
| $\alpha$ (%)                                                       |                    | 36 $\pm$ 18            | 21 $\pm$ 2       | 20 $\pm$ 3      | 31 $\pm$ 2 <sup>\$</sup>           | 41 $\pm$ 1 <sup>+ ° ##</sup>      |
| $\beta$ (%)                                                        |                    | 23 $\pm$ 103           | 11 $\pm$ 1       | 9 $\pm$ 2       | 15 $\pm$ 1                         | 21 $\pm$ 1 <sup>+ ° #</sup>       |

ANT, animal naming test; EEG, electroencephalogram; FN, false negative; FP, false positive; MDF, mean dominant frequency; MELD, model for end-stage liver disease; MPZS, mean PHES Z-score; PHES, Psychometric Hepatic Encephalopathy Score; TN, true negative TP, true positive.

<sup>^</sup> $P < 0.05$ , <sup>^^</sup> $P < 0.01$ , <sup>^^^</sup> $P < 0.001$ , significance of the difference between TP and FP; <sup>\$</sup> $P < 0.05$ , <sup>\$\$</sup> $P < 0.01$ , <sup>\$\$\$</sup> $P < 0.001$ , significance of the difference between FN and FP; <sup>+</sup> $P < 0.001$ , significance of the difference between TP and TN; <sup>°</sup> $P < 0.05$ , <sup>°°</sup> $P < 0.01$ , significance of the difference between FN and TN; <sup>#</sup> $P < 0.05$ , <sup>##</sup> $P < 0.01$ , <sup>###</sup> $P < 0.001$ , significance of the difference between FP and TN.

<sup>EEE</sup>Asterixis:  $\chi^2 = 145$ ,  $P < 0.001$ ; <sup>E</sup>portal-systemic shunt:  $\chi^2 = 13$ ,  $P < 0.05$ ; <sup>EEE</sup>ammonia-lowering treatment:  $\chi^2 = 18$ ,  $P < 0.001$ .

On quantitative evaluation, 137 (33%) patients were qualified as unimpaired, 201 (49%) as having CHE, 57 (14%) as having grade II OHE, and 16 (4%) as having grade III OHE. Of the 73 patients with OHE, 19 (26%) were missed on qualitative assessment (FNs), with no difference in the likelihood of the physician missing grades II and III (Figure 1). Sixty-eight (20%) unimpaired/CHE patients were qualified as having OHE on qualitative assessment (FPs), of whom 61 (90%) had CHE and 7 (10%) were unimpaired (Figure 1).

Demographic, hepatic failure, and neuropsychiatric features of the patients, by agreement between quantitative and qualitative HE assessment, are presented in Table 1 and Figure 2. The latter (Figure 2, panels b–d) highlights how FNs had virtually identical neuropsychiatric profiles as true positives. By contrast, FPs had slightly worse neuropsychiatric performances than TNs, suggesting that the physician was probably capable of detecting CHE. Finally, although overall model for end-stage liver disease scores were low (Table 1), they were even lower in TNs (Figure 2, panel a).

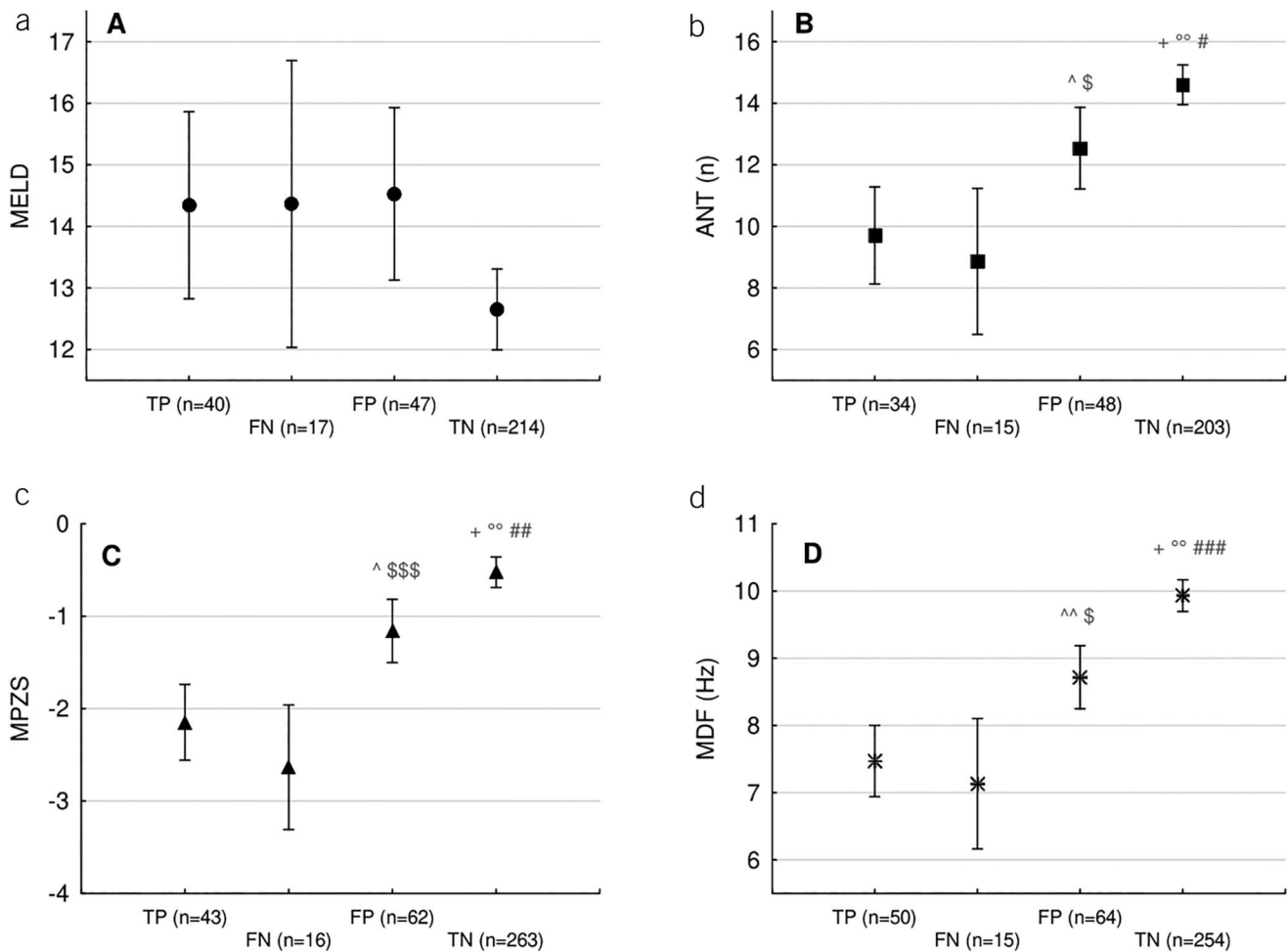
## DISCUSSION

Qualitative clinical evaluation of OHE is not reliable, with approximately a quarter of patients with grades II and III OHE being missed. This confirms the appropriateness of the recommended

evaluation tools and most likely also the theory behind them. The time taken to perform the semistructured interview, which is less than 2 minutes, seems a very reasonable investment by comparison with the missed diagnoses of grades II/III OHE patients, some of whom may deserve immediate management or hospitalization (10–12). By contrast, the large majority (90%) of FPs were CHE and not unimpaired, suggesting that the physician was actually capable of detecting a mild degree of neuropsychiatric impairment, which did not result in temporal disorientation or asterixis (2). Although the data on FPs may be confounded by the fact that the study was conducted in a tertiary referral center for hepatology with a research interest in HE, the data on FNs are even more worrying for the same reason. Neurologists and geriatricians need and use semiquantitative and quantitative tools to assess their patients' performance (13,14), and one cannot see why hepatogastroenterologists should not (15). In conclusion, although our data need to be replicated in multicenter studies, they support existing guidelines to routinely perform semiquantitative OHE assessment, which is clinically actionable and might reduce both underdiagnosis and overdiagnosis and treatment of OHE.

## CONFLICTS OF INTEREST

**Guarantor of the article:** Sara Montagnese, MD, PhD.



**Figure 2.** Mean  $\pm$  95% confidence interval of the variables: model for end-stage liver disease (MELD); (a) animal naming test (ANT, the higher the number of animals listed in 60 seconds, the better the performance); (b) mean Psychometric Hepatic Encephalopathy Score Z-score (MPZS, the higher the Z-score, the better the performance); (c) and the spectral EEG parameter mean dominant frequency (MDF, the higher the frequency, the better the EEG); (d) by classes of agreement between quantitative and qualitative assessment of hepatic encephalopathy. FN, false negatives; FP, false positives; TN, true negatives; TP, true positives. ^  $P < 0.05$ , ^^  $P < 0.01$ , significance of the difference between TP and FP; \$\$\$  $P < 0.001$ , significance of the difference between FN and FP; +  $P < 0.05$ , ++  $P < 0.01$ , +++  $P < 0.001$ , significance of the difference between TP and TN; °  $P < 0.05$ , °°  $P < 0.01$ , °°°  $P < 0.001$ , significance of the difference between FN and TN; #  $P < 0.05$ , ##  $P < 0.01$ , ###  $P < 0.001$ , significance of the difference between FP and TN on *post hoc* comparisons (Tukey test).

**Specific author contributions:** S.M.: study concept and design. D.E., C.M., L.Z., and S.M.: acquisition of data. D.E., C.M., L.Z., and S.M.: analysis and interpretation of data. D.E., C.M., and S.M.: drafting of the manuscript. P.A.: critical revision of the manuscript for important intellectual content. D.E., C.M., L.Z., and S.M.: statistical analysis. All authors have approved the final draft submitted.

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**Potential competing interests:** None to report.

**Data sharing statement:** The data that support the findings of this study are available on reasonable request from the corresponding author.

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## RESEARCH LETTER

OPEN

# PHES scores have limited impact on the risk of overt HE in patients with minimal HE

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## Abstract

**Background:** Minimal hepatic encephalopathy, defined by the portosystemic hepatic encephalopathy score (PHES), is associated with a higher risk of subsequent OHE. It remains unclear if there is a stepwise increase in OHE risk with worse PHES results.

**Methods:** In this multicenter study, patients with minimal hepatic encephalopathy, as defined by abnormal PHES, were followed for OHE development.

**Results:** In all, 207 patients were included. There was no stepwise increase in OHE risk with worse PHES results.

**Conclusions:** Abnormal PHES is associated with a higher OHE risk, but we found no stepwise increase in OHE risk with worse PHES results below the established cutoff.

**Abbreviations:** MHE, minimal hepatic encephalopathy; PHES, psychometric hepatic encephalopathy score; OHE, overt hepatic encephalopathy.

Supplemental Digital Content is available for this article. Direct URL citations are provided in the HTML and PDF versions of this article on the journal's website, [www.hepcommjournal.com](http://www.hepcommjournal.com).

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## INTRODUCTION

HE is a severe complication of cirrhosis and is associated with poor prognosis.<sup>[1]</sup> Therefore, prevention of overt hepatic encephalopathy (OHE), and especially severe OHE requiring hospitalization, is of pivotal importance to improve quality of life and prognosis. It is well-known that patients with minimal hepatic encephalopathy (MHE) defined by the psychometric hepatic encephalopathy score (PHES-MHE) are at higher risk for developing OHE compared to patients without MHE.<sup>[2]</sup> However, not every patient with MHE develops an episode of OHE in the near future, and accordingly, primary prophylaxis is recommended only on a case-by-case basis.<sup>[3]</sup> Therefore, identifying high-risk patients in the subgroup of patients with MHE could be beneficial to establishing strategies to prevent OHE in routine clinical practice and for purposes of future interventional trials.<sup>[4]</sup> This study aimed to investigate whether there is a stepwise increase in OHE risk with worse PHES results in patients with PHES-MHE.

## METHODS

This retrospective study analyzed data of 1462 patients, who underwent testing with the PHES and had available follow-up data. Patients were studied in 8 centers with expertise in diagnosing MHE: Mainz, Siegen, Lübeck (all Germany), Paris (France), Padua and Rome (Italy), Esbjerg (Denmark), and Ann Arbor (Michigan, USA). For this study, we only included patients with PHES-MHE, no history of OHE, and no prescription for lactulose or rifaximin (excluded: 959 without PHES-MHE, 212 with PHES-MHE but with a history of OHE, 84 with a prescription for lactulose or rifaximin). Additional details of this study regarding the diagnosis of cirrhosis and all exclusion criteria for each center can be found in previous publications based on the same cohort (more details in the Supplemental Methods, <http://links.lww.com/HC9/A879>).<sup>[2,5]</sup>

All patients were examined at the respective hospitals to rule out signs of OHE, and MHE was subsequently diagnosed by PHES (more details are provided in the Supplemental Methods, <http://links.lww.com/HC9/A879>). PHES was scored at each center using the validated country-specific norms (Germany and Denmark,<sup>[6]</sup> United States,<sup>[7]</sup> Italy,<sup>[8]</sup> France<sup>[9]</sup>). A score  $< -4$  was considered diagnostic of PHES-MHE for centers from Germany, France, and Denmark, while the centers from Italy and the United States used a score  $\leq -4$ . For subsequent analyses and to make results between centers more comparable, we calculated a scoring system adjusted PHES scores (adjusted PHES) by dividing the PHES score by the number of subtests. As an example for German patients: if a patient had a PHES score of -12, this score was divided by the number of subscores of PHES (in Germany 6 subscores), resulting in an adjusted PHES of -2. All

patients were followed up for the occurrence of OHE and liver transplantation-free survival.

The study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. For this study, we used anonymized electronic medical records without directly identifiable data. According to German regulations and the recommendations of the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, no ethical approval is required. Regarding a subset of patient data that were recorded in a prospective setting, the respective study protocols were approved by the Ethics Committees of the respective centers, and informed consent was obtained from each participant.

Statistical analyses and graphic design were performed with R 4.1.3 (R Core Team, 2022). We examined the correlation between OHE risk and the adjusted PHES as a continuous variable and as a categorical variable divided into quartiles. Death/liver transplantation was classified as a competing event in the multi-state model analyses.

Detailed information on the statistical analyses is provided in the Supplemental Methods, <http://links.lww.com/HC9/A879>.

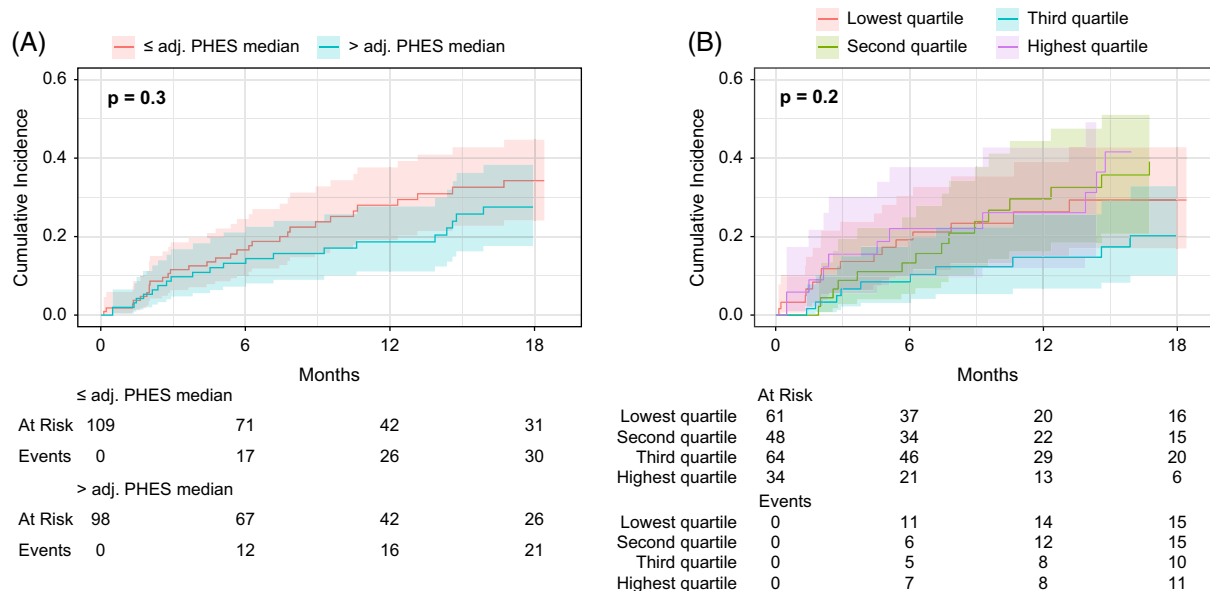
## RESULTS

Baseline characteristics of the patient cohort ( $n = 207$ ) are displayed in Supplemental Table S1, <http://links.lww.com/HC9/A879>. The median adjusted PHES was  $-1.20$  (range  $-2.67$ ,  $-0.80$ ). The median follow-up time was 18.5 months (95% CI: 13.9–20.6). In total, 59 (29%) patients developed an OHE episode during follow-up. Additionally, 41 (20%) patients reached the end point of death/liver transplantation during follow-up before an episode of OHE.

To analyze the association between worse results in the adjusted PHES and the risk of OHE development, the cohort was split for 2 separate analyses: (i) into 2 groups according to the median and (ii) into 4 groups according to the quartiles of the results in adjusted PHES. In both analyses, the cumulative OHE incidences did not differ significantly between patients with poorer or better results in PHES (Figure 1A–B). In multivariable competing risk regression analyses, adjusted PHES as a continuous variable (sHR 1.05, 95% CI: 0.55–2.01,  $p = 0.9$ ) or categorized into quartiles (each  $p \geq 0.2$ ) were not associated with the development of OHE (Supplemental Table S2, <http://links.lww.com/HC9/A879>). No association was observed in multivariable models only including patients with Child-Pugh A or B/C (Supplemental Table S2, <http://links.lww.com/HC9/A879>) either.

## DISCUSSION

MHE, defined by an abnormal PHES, is associated with a higher risk of developing OHE.<sup>[2]</sup> Whether PHES



**FIGURE 1** Cumulative overt HE (OHE) incidences. Cumulative OHE incidences in the total cohort stratified by (A) adjusted PHES median and (B) adjusted PHES quartiles. Abbreviations: Inf, infinity; OHE, overt hepatic encephalopathy; PHES, Psychometric Hepatic Encephalopathy Score.

results below these cutoffs are associated with a further increase in the risk of future OHE episodes, as well as the rationale for this study, was unknown. In this multicenter study, we found no stepwise increase in OHE risk with worse PHES results below the cutoff defining MHE.

Identification of high-risk patients for OHE development is important for the management of patients with cirrhosis<sup>[4]</sup> and MHE is a well-known risk factor for OHE; however, our study demonstrates that within the group of patients with MHE, worse results in PHES cannot be taken to reflect a higher OHE risk. This may relate to the fact that cognitive function varies from patient to patient, and PHES results are not only affected by MHE. This means that one may not know a patient's "baseline" cognitive performance in PHES by testing the patient only once. Therefore, an important research question might be whether serial testing, particularly in patients with results below the cutoff defining MHE, and detection of subsequent changes in PHES results might be more informative for predicting OHE risk.<sup>[10]</sup> Future studies should focus on serial testing and determine potential thresholds for a predictive delta of changes in PHES. In the context of 1-time testing with PHES, our study highlights that additional instruments are needed for more granular risk stratification. Here, biomarkers could be a promising adjunct, as recently demonstrated for ammonia.<sup>[11]</sup> The combination of a biomarker and a phenotypic index may also be a reasonable and promising route. Importantly, our findings should not deter physicians from considering the effects of varying degrees of PHES abnormalities on everyday functioning and quality of life, even within the MHE category, and especially from treating patients with MHE.

A limitation of this study that has to be acknowledged is that we cannot account for the potential initiation of HE treatment during follow-up, as this piece of information was not available in this data set. Additionally, this database lacks information on, for example, non-liver-related comorbidities and we are therefore unable to adjust for these factors. Another limitation is that potentially not all OHE episodes might be captured if they were not registered in the respecting hospitals participating in this study.

In conclusion, our study provides evidence that in patients with PHES-MHE, there is no stepwise increase in OHE risk with worse PHES results.

## AUTHOR CONTRIBUTIONS

Performed research: Simon Johannes Gairing, Chiara Mangini, Lisa Zarantonello, Stefania Gioia, Elise Jonasson, Sven Danneberg, Patricia P. Bloom, Philippe Sultanik, Peter Robert Galle, Joachim Labenz, Dominique Thabut, Anna S. Lok, Jens Uwe Marquardt, Mette Munk Lauridsen, Silvia Nardelli, Sara Montagnese, and Christian Labenz. Contributed to acquisition of data: Simon Johannes Gairing, Chiara Mangini, Lisa Zarantonello, Stefania Gioia, Elise Jonasson, Sven Danneberg, Patricia P. Bloom, Philippe Sultanik, Peter Robert Galle, Joachim Labenz, Dominique Thabut, Anna S. Lok, Jens Uwe Marquardt, Mette Munk Lauridsen, Silvia Nardelli, Sara Montagnese, and Christian Labenz; Designed the experiments and analyzed the data; contributed reagents/materials/analysis tools; wrote the paper; and statistical analysis: Simon Johannes Gairing and Christian Labenz. Critical revision: All authors.

## ETHICS

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki (7<sup>th</sup> revision, 2013). For this study, we used anonymized electronic medical records without directly identifiable data. According to German regulations and the recommendations of the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, no ethical approval is required for this type of study. Anonymized data were analyzed as aggregates with no protected health information available. Regarding a subset of patient data that were recorded in a prospective setting, the respective study protocols were approved by the Ethics Committees of the respective centers.

## GUARANTOR OF THE ARTICLE

Christian Labenz.

All authors approved the final version of the manuscript and the authorship list.

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## CONFLICTS OF INTEREST

Simon Johannes Gairing: Travel expenses: Ipsen and Gilead. Research grant: Schwiete Stiftung. Joachim Labenz: Consulting: Alphasigma, Norgine. Lecture fees: Norgine. Peter R. Galle: Lecture fees and consulting: Merz Pharmaceuticals. Patricia P. Bloom: Research grant from Vedanta Biosciences. Consultant for Nexilico. Cristina Ripoll: Lecture fees: Falk, Gore, Grifols, CSL Behring. Research grants: DFG, Schwiete Stiftung. Dominique Thabut: Lecture fees: Alfasigma, Abbvie, Gilead. Christian Labenz: Travel expenses and consulting: Norgine, Merz Pharmaceuticals. Lecture fees: Norgine, Merz Pharmaceuticals. Research grants: Norgine, Merz Pharmaceuticals, Schwiete Stiftung. The remaining authors have no conflicts to report.

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# Proton pump inhibitor use and risk of hepatic encephalopathy: A multicentre study

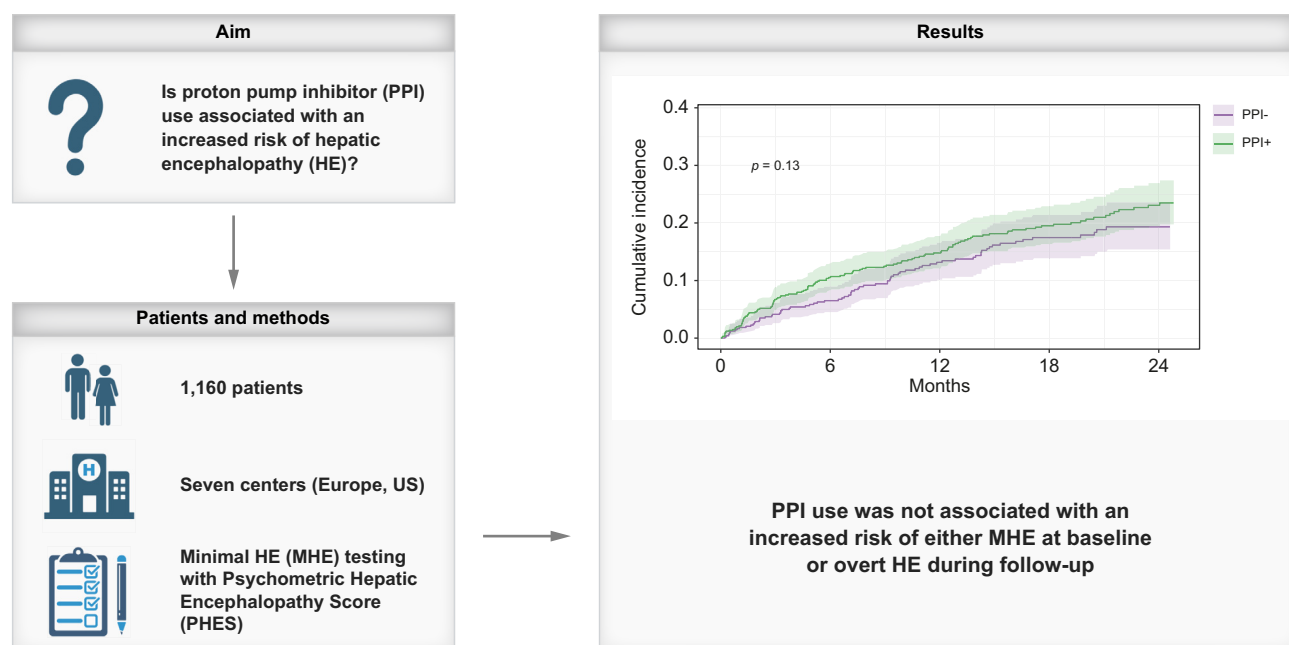
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## Graphical abstract



## Highlights:

- 58% of patients with cirrhosis had a PPI prescription, of whom only 26.5% had a generally accepted indication.
- PPI use was not associated with an increased risk of minimal hepatic encephalopathy at study inclusion.
- PPI use was not associated with an increased risk of overt hepatic encephalopathy during follow-up.

## Impact and implications

Data on the association between proton pump inhibitor (PPI) use and hepatic encephalopathy (HE) are conflicting. In this study, PPI use was not associated with a higher risk of minimal HE at baseline or overt HE during follow-up in patients with cirrhosis. Based on these findings, prescription of a PPI for a generally accepted indication should not be prohibited in patients with cirrhosis.

# Proton pump inhibitor use and risk of hepatic encephalopathy: A multicentre study

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**Background & Aims:** Data on the association between proton pump inhibitor (PPI) use and hepatic encephalopathy (HE) are conflicting, and data from multicentre studies are scarce. The aim of this study was to dissect the potential association between PPI use and minimal (MHE) and overt HE (OHE).

**Methods:** Data from patients with cirrhosis recruited at seven centres across Europe and the US were analysed. MHE was defined by the psychometric hepatic encephalopathy score (PHES). PPI use was recorded on the day of testing with PHES. Patients were followed for OHE development and death/liver transplantation.

**Results:** A total of 1,160 patients with a median MELD of 11 were included (Child-Pugh stages: A 49%/B 39%/C 11%). PPI use was noted in 58% of patients. Median follow-up time was 18.1 months, during which 230 (20%) developed an OHE episode, and 224 (19%) reached the composite endpoint of death/liver transplantation. In multivariable analyses, PPI use was neither associated with the presence of MHE at baseline nor OHE development during follow-up. These findings were consistent in subgroup analyses of patients with Child-Pugh A or B cirrhosis and after excluding patients with a history of OHE. PPI use was also not associated with a higher risk of OHE, neither in patients with an indication for treatment nor in patients without an indication.

**Conclusions:** PPI use is not associated with a higher risk of HE in patients with cirrhosis. Based on these findings, at present, a prescription should not be prohibited in case of a generally accepted indication.

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## Introduction

Cirrhosis is prevalent worldwide and is the main cause of about 1.32 million deaths per year.<sup>1</sup> One of the most severe complications of cirrhosis is hepatic encephalopathy (HE) – a neurophysiological complication that negatively impacts patients' quality of life and poses a huge burden for caregivers.<sup>2,3</sup> According to the current guidelines, HE can be divided into covert HE and overt HE (OHE).<sup>4</sup> Covert HE is an umbrella term combining the two lowest HE grades – minimal HE (MHE) and HE grade 1 according to the West Haven Criteria, while OHE covers grades 2–4. The prevalence of MHE correlates with the stage of liver disease and ranges from <25% in patients with Child-Pugh (CP) A cirrhosis to >50% in patients with CP C.<sup>5</sup> The incidence rates of OHE are less well studied and have been reported to be approximately 10% and 25% per year in patients with CP A and B cirrhosis, respectively.<sup>6</sup> Given that MHE is associated with a poorer quality of life and a higher risk of OHE and that OHE is associated with a poorer prognosis and a huge burden for caregivers,<sup>2,3,7,8</sup> it is of the utmost importance to identify potentially modifiable factors associated with a higher risk of HE.

In recent years, several studies have investigated the association between PPI use and HE, and most found a significant association between PPI intake and the risk of HE. PPI might modify the intestinal microbiome, which may increase the production of ammonia and other toxic compounds involved in the pathogenesis of HE.<sup>9,10</sup> Thus, the hypothesis arose that there might be an association between PPI use and HE. However, most studies on this topic had limitations in terms of their design (cross-sectional and relying on ICD-codes,<sup>11</sup> a lack of MHE testing at baseline,<sup>12</sup> or only including a single-centre cohort<sup>13</sup>). Currently, only two studies investigating the association between PPI use and HE considered MHE testing at baseline.<sup>13,14</sup> Surprisingly, the results were conflicting: the study by Nardelli *et al.* found a robust association between PPI use and MHE as well as OHE, while the study by Acharya *et al.* found no association.<sup>13,14</sup> Both studies were based on cohorts from one (Nardelli *et al.*) or two (Acharya *et al.*) centres and were not adequately powered for subgroup analyses. Therefore, it was the aim of this study to investigate a potential association between PPI use and M/OHE in a large multinational cohort of patients with cirrhosis and to conduct subgroup analyses according to the severity of cirrhosis.

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## Patients and methods

This retrospective study analysed data on 1,160 patients who underwent MHE testing with PHES at the following centres with expertise in diagnosing MHE: Mainz, Siegen, Lübeck (all Germany), Paris (France), Padua (Italy), Esbjerg (Denmark), and Ann Arbor (Michigan, US). The details of this study are also described elsewhere.<sup>5,15</sup> At each centre, the primary aetiology of the underlying liver disease was determined according to clinical, serological, and histological findings. Diagnosis of cirrhosis was established by histology, conclusive appearance on ultrasound, elastography or radiological imaging, endoscopic features of portal hypertension, or medical history. The severity of liver disease was determined by calculating the model for end-stage liver disease (MELD), CP, and the albumin-bilirubin (ALBI) scores.<sup>16–18</sup> MELD scores were calculated centrally using the original MELD formula of Kamath *et al.* The three ALBI grades were defined by cut-offs of  $\leq -2.60$  (grade 1),  $> -2.60$  to  $\leq -1.39$  (grade 2), and  $> -1.39$  (grade 3).

Patients were not included in this study if they had a history of any other disease leading to mental alterations (e.g., dementia or a history of stroke). At some centres, patients with self-reported ongoing (mild) alcohol consumption were included, provided that they were not under the effects of alcohol during testing with PHES. Other exclusion criteria varied between centres and are detailed for each centre elsewhere.<sup>5</sup>

### PPI use

In all patients, PPI use at study inclusion was recorded. Additionally, we recorded type of PPI (pantoprazole, omeprazole, esomeprazole, rabeprazole, lansoprazole) and the underlying indication for treatment, if there was one. Established indications were as follows: gastroesophageal reflux disease or symptoms of reflux, gastric or duodenal ulcer disease, and indication for ulcer prophylaxis.

### Diagnosis of MHE

All patients were examined at the respective hospitals by an experienced hepatologist to rule out clinical signs of OHE. For each patient, PHES was performed to diagnose MHE. PHES is a paper-and-pencil testing battery including five subtests (number connection test A and B, serial dotting test, digit symbol test and line tracing test). PHES was scored at each centre using the validated country-specific norms (Germany,<sup>19</sup> US,<sup>20</sup> Italy,<sup>21</sup> France<sup>22</sup>). The Danish centre used the German norms. A score  $\leq -4$  was considered diagnostic of MHE for centres from Germany, France and Denmark, while the centres from Italy and the US used a score  $\leq -4$ .

### Follow-up evaluation

Patients were followed during regular clinic visits or via electronic chart review at the respective centres for the occurrence of OHE. The presence of OHE was diagnosed after a detailed neurological examination according to the West Haven criteria by an experienced hepatologist. Moreover, patients were followed for the occurrence of death or liver transplantation. Patients who did not reach the endpoint of death/liver transplantation were censored at the date of last contact.

## Ethics

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki (7<sup>th</sup> revision, 2013). For this study, we used anonymised electronic medical records without directly identifiable data. According to German regulations and the recommendations of the Ethics Committee of the Landesärztekammer Rheinland-Pfalz, no ethical approval is required for this type of study. Anonymised data were analysed as aggregates with no protected health information available. For the subset of patients with data recorded in a prospective setting, the respective study protocols were approved by the Ethics Committees of the respective centres, and all patients provided informed consent for participation.

## Statistical analysis

Statistical analysis was performed with R 4.1.3 (R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>), RStudio version 2023.9.1.494 (Posit team (2023). RStudio: Integrated Development Environment for R. Posit Software, PBC, Boston, MA. URL <http://www.posit.co/>) and IBM SPSS Statistic Version 29 (Armonk, NY: IBM Corp.). Baseline characteristics were calculated with the {table 1} R package (v1.4.3; Benjamin Rich 2023). Continuous data are given as median with range, and pairwise comparisons were performed with the Mann-Whitney *U* test. Categorical variables are shown as frequencies and percentages, and a chi-squared test was calculated for comparison of two or more groups. Median follow-up time was calculated using the reverse Kaplan-Meier method ({prodlm} R package (v2019.11.13, Thomas A. Gerds 2019)).

To identify variables associated with the prevalence of MHE, we conducted multivariable logistic regression analyses, including established risk factors for MHE and PPI use.

The {tidycmprsk} R package (v0.2.0, Daniel D. Sjöberg and Teng Fei 2022) was used for cumulative incidence functions for competing risk analyses and Fine and Gray competing risk regression analysis. For this purpose, a multi-state model with three states was used (0: alive without liver transplantation and no OHE event at the end of follow-up, 1: alive with an OHE event during follow-up and without liver transplantation prior to the event, 2: death or liver transplantation during follow-up without OHE event prior to death or liver transplantation). Differences between groups in cumulative incidence functions were calculated with Gray's test. Follow-up time was defined from study inclusion to censoring, OHE, death, or transplantation. Multivariable models were adjusted for biologically plausible and established risk factors for OHE. In addition, univariable competing risk regression was performed, and variables with a univariable *p*  $< 0.05$  were included in a multivariable model (plus PPI). Missing data were excluded from the analyses (complete case analysis).

## Results

In total, data from 1,160 patients with cirrhosis were available for analysis (Fig. S1). The cohort was predominantly male (65.3%) with a median age of 60 years (range 23–87). A history of OHE was noted in 335 patients (29.4%), and MHE, according

Table 1. Demographics and clinical characteristics of the study cohort.

|                           | Total cohort (N = 1,160) | PPI- (n = 491)      | PPI+ (n = 669)      |
|---------------------------|--------------------------|---------------------|---------------------|
| Age (years)               |                          |                     |                     |
| Median [Min, Max]         | 60.0 [23.0, 87.0]        | 59.0 [23.0, 87.0]   | 60.0 [26.0, 86.0]   |
| Missing                   | 1 (0.1%)                 | 1 (0.2%)            | 0 (0%)              |
| Gender                    |                          |                     |                     |
| Male                      | 758 (65.3%)              | 326 (66.4%)         | 432 (64.6%)         |
| Female                    | 402 (34.7%)              | 165 (33.6%)         | 237 (35.4%)         |
| Etiology                  |                          |                     |                     |
| Alcohol                   | 553 (48.0%)              | 236 (48.3%)         | 317 (47.8%)         |
| Viral                     | 174 (15.1%)              | 83 (17.0%)          | 91 (13.7%)          |
| Others/mixed              | 425 (36.9%)              | 170 (34.8%)         | 255 (38.5%)         |
| Missing                   | 8 (0.7%)                 | 2 (0.4%)            | 6 (0.9%)            |
| MELD score                |                          |                     |                     |
| Median [Min, Max]         | 11.0 [6.00, 34.0]        | 10.3 [6.00, 33.0]   | 11.2 [6.00, 34.0]   |
| Missing                   | 66 (5.7%)                | 27 (5.5%)           | 39 (5.8%)           |
| Child-Pugh                |                          |                     |                     |
| A                         | 539 (49.4%)              | 262 (56.7%)         | 277 (44.1%)         |
| B                         | 429 (39.4%)              | 156 (33.8%)         | 273 (43.5%)         |
| C                         | 122 (11.2%)              | 44 (9.5%)           | 78 (12.4%)          |
| Missing                   | 70 (6.0%)                | 29 (5.9%)           | 41 (6.1%)           |
| ALBI grade                |                          |                     |                     |
| 1                         | 210 (24.8%)              | 113 (32.2%)         | 97 (19.6%)          |
| 2                         | 478 (56.4%)              | 179 (51.0%)         | 299 (60.3%)         |
| 3                         | 159 (18.8%)              | 59 (16.8%)          | 100 (20.2%)         |
| Missing                   | 313 (27.0%)              | 140 (28.5%)         | 173 (25.9%)         |
| MHE (PHES)                |                          |                     |                     |
| MHE-                      | 775 (66.8%)              | 348 (70.9%)         | 427 (63.8%)         |
| MHE+                      | 385 (33.2%)              | 143 (29.1%)         | 242 (36.2%)         |
| History of ascites        |                          |                     |                     |
| No                        | 506 (44.3%)              | 235 (48.6%)         | 271 (41.2%)         |
| Yes                       | 636 (55.7%)              | 249 (51.4%)         | 387 (58.8%)         |
| Missing                   | 18 (1.6%)                | 7 (1.4%)            | 11 (1.6%)           |
| History of OHE            |                          |                     |                     |
| No                        | 803 (70.6%)              | 355 (74.0%)         | 448 (68.1%)         |
| Yes                       | 335 (29.4%)              | 125 (26.0%)         | 210 (31.9%)         |
| Missing                   | 22 (1.9%)                | 11 (2.2%)           | 11 (1.6%)           |
| Sodium (mmol/L)           |                          |                     |                     |
| Median [Min, Max]         | 138 [116, 146]           | 139 [118, 145]      | 138 [116, 146]      |
| Missing                   | 202 (17.4%)              | 89 (18.1%)          | 113 (16.9%)         |
| Creatinine (mg/dl)        |                          |                     |                     |
| Median [Min, Max]         | 0.848 [0.320, 7.51]      | 0.826 [0.385, 4.15] | 0.870 [0.320, 7.51] |
| Missing                   | 76 (6.6%)                | 34 (6.9%)           | 42 (6.3%)           |
| Bilirubin (mg/dl)         |                          |                     |                     |
| Median [Min, Max]         | 1.24 [0.100, 33.3]       | 1.20 [0.175, 29.1]  | 1.30 [0.100, 33.3]  |
| Missing                   | 78 (6.7%)                | 33 (6.7%)           | 45 (6.7%)           |
| Albumin (g/L)             |                          |                     |                     |
| Median [Min, Max]         | 35.0 [14.0, 52.0]        | 36.0 [14.0, 52.0]   | 34.9 [15.0, 51.2]   |
| Missing                   | 223 (19.2%)              | 97 (19.8%)          | 126 (18.8%)         |
| INR                       |                          |                     |                     |
| Median [Min, Max]         | 1.22 [0.800, 3.30]       | 1.20 [0.900, 3.30]  | 1.26 [0.800, 3.23]  |
| Missing                   | 113 (9.7%)               | 51 (10.4%)          | 62 (9.3%)           |
| Lactulose                 |                          |                     |                     |
| No                        | 723 (62.4%)              | 342 (69.7%)         | 381 (57.0%)         |
| Yes                       | 436 (37.6%)              | 149 (30.3%)         | 287 (43.0%)         |
| Missing                   | 1 (0.1%)                 | 0 (0%)              | 1 (0.1%)            |
| Rifaximin                 |                          |                     |                     |
| No                        | 864 (74.5%)              | 384 (78.2%)         | 480 (71.9%)         |
| Yes                       | 295 (25.5%)              | 107 (21.8%)         | 188 (28.1%)         |
| Missing                   | 1 (0.1%)                 | 0 (0%)              | 1 (0.1%)            |
| Reason for PPI treatment* |                          |                     |                     |
| Reflux/GERD               |                          |                     | 71 (10.6%)          |
| Ulcer disease             |                          |                     | 38 (5.7%)           |
| Dyspepsia                 |                          |                     | 30 (4.5%)           |
| Ulcer prophylaxis         |                          |                     | 24 (3.6%)           |
| Mixed/others              |                          |                     | 14 (2.1%)           |
| PPI indication            |                          |                     |                     |
| No                        |                          |                     | 492 (73.5%)         |
| Yes                       |                          |                     | 177 (26.5%)         |

(continued on next page)



Table 1. (continued)

|                        | Total cohort (N = 1,160) | PPI- (n = 491) | PPI+ (n = 669) |
|------------------------|--------------------------|----------------|----------------|
| PPI type               |                          |                |                |
| Pantoprazole           |                          |                | 382 (59.7%)    |
| Esomeprazole           |                          |                | 29 (4.5%)      |
| Lansoprazole           |                          |                | 115 (18.0%)    |
| Omeprazole             |                          |                | 100 (15.6%)    |
| Rabeprazole            |                          |                | 13 (2.0%)      |
| No information on type |                          |                | 1 (0.2%)       |

\*73.5% had no PPI indication. ALBI, albumin-bilirubin; GERD, gastroesophageal reflux disease; MELD, model for end-stage liver disease; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy; PPI, proton pump inhibitor.

to PHES, was present in 385 patients (33.2%). Additional demographics and clinical characteristics are displayed in Table 1. In addition, demographics and clinical characteristics of patients without a history of overt HE, as well as characteristics of the patients from the respective centres, are shown in Tables S1 and S2, respectively.

### Comparison of PPI users and non-users

PPI use was noted in 58% of the patients at study inclusion. The most frequently prescribed PPI was pantoprazole (59.7%) followed by lansoprazole (18.0%) and omeprazole (15.6%). Only 26.5% of the patients with a PPI prescription had a hard indication for treatment. Details on the distribution of PPI and the indications are displayed in Table 1, Tables S1, and S2. Compared to PPI non-users, PPI users had a higher MELD score and a higher rate of cirrhosis-related complications.

### PPI use and the presence of MHE

Comparisons of the characteristics of patients with and without MHE are provided in Table S3. Patients with MHE were in a more severe stage of cirrhosis as described by a higher MELD score or CP stage. Notably, PPI use was more frequent in patients with than without MHE (63% vs. 55%,  $p = 0.024$ ). To assess the independent association between PPI use and the presence of MHE, a multivariable logistic regression model based on established and biologically plausible risk factors for MHE (albumin, MELD, history of OHE, gender, age) and PPI use was constructed. There was no significant association between PPI use and MHE in the total cohort nor in the sub-cohort of patients without a history of OHE (Table 2).

### PPI use and the development of OHE

All patients were followed for the development of the first OHE episode after enrolment or death/liver transplantation. Median follow-up time was 18.12 months (95% CI 17.82–18.44). In total, 230 (19.8%) patients experienced at least one OHE

episode. Additionally, 224 (19.3%) patients died or received a liver transplantation before an episode of OHE.

In the total cohort, the cumulative OHE incidences did not differ significantly between PPI users and non-users ( $p = 0.13$ , Fig. 1A). To identify variables independently associated with the development of OHE, we fitted multivariable regression models using the method of Fine and Gray under consideration of the competing events death or liver transplantation. Again, established and biologically plausible risk factors for OHE, as well as PPI use, were included in the models. In the total cohort, PPI use was not associated with a higher risk of OHE in multivariable regression analysis (subdistribution hazard ratio [sHR] 1.13, 95% CI 0.81–1.59,  $p = 0.5$ ; Table 3). This finding remained robust in a separate model including lactulose or rifaximin use at study inclusion (PPI use: sHR 1.08, 95% CI 0.77–1.51,  $p = 0.7$ ) (Table S4). In addition, we fitted a multivariable competing risk regression model including only variables with a  $p$  value  $<0.05$  in univariable analysis (Table S5 and Table S6). PPI use was not significantly associated with the development of OHE in univariable analysis (sHR 1.23, 95% CI 0.94–1.60,  $p = 0.13$ ), but was nonetheless included in the multivariable model. Here, PPI use showed no significant association (sHR 1.16, 95% CI 0.82–1.64,  $p = 0.4$ ).

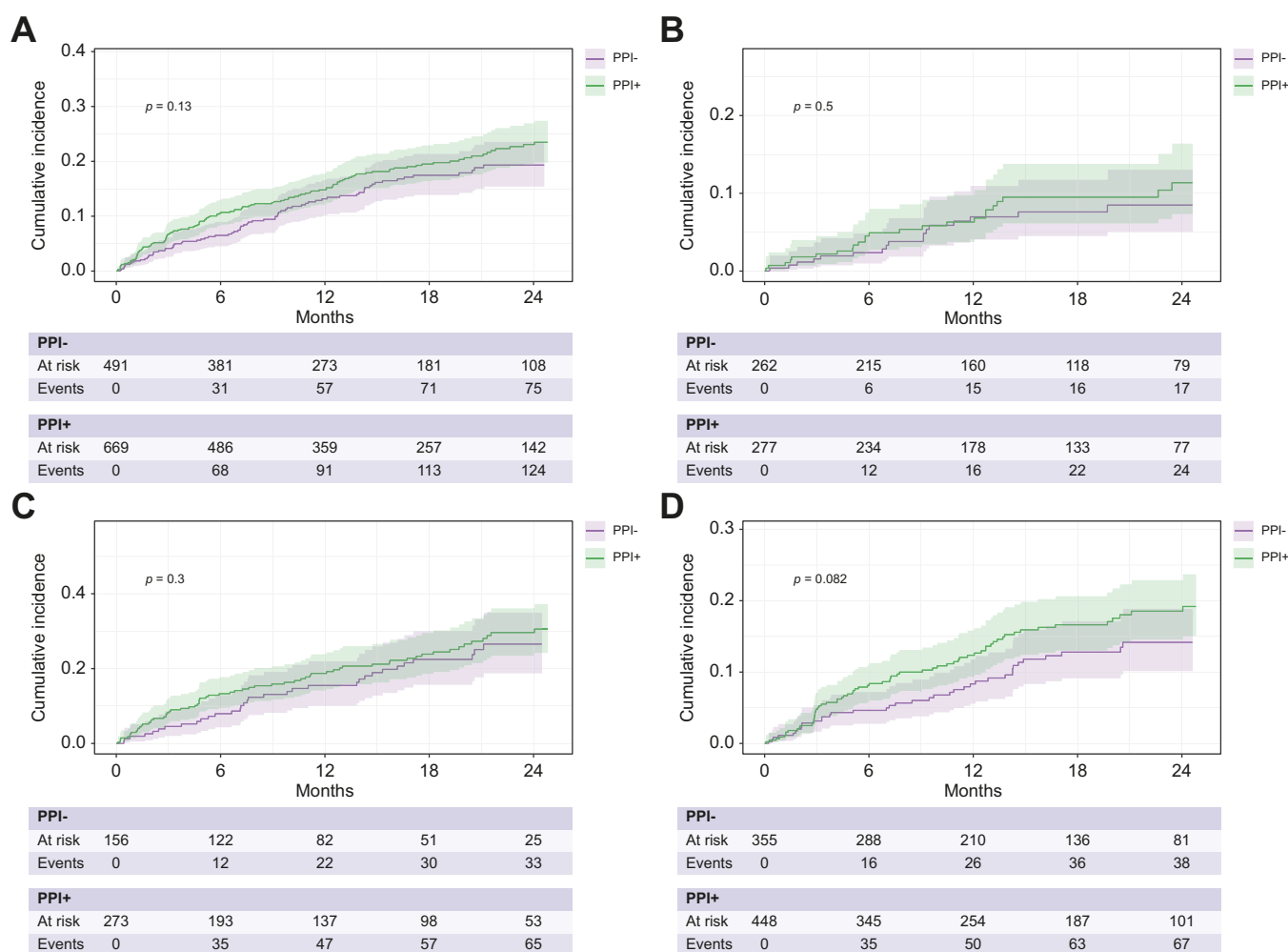
Given that OHE incidence differs markedly between patients with CP A and B cirrhosis, we conducted subgroup analyses in these two groups. We did not conduct a subgroup analysis in patients with CP C due to the low case and event numbers. PPI use was not associated with a higher risk of OHE in patients with CP A or B cirrhosis, neither in univariable nor in multivariable analyses (Fig. 1B,C, Table 3 and Table S4).

Given that patients with a history of OHE are at higher risk for recurrent OHE, we repeated our analyses in the subgroup of patients without a history of OHE ( $n = 803$ ) and in the subgroup of patients without a history of OHE and no prescription of either lactulose or rifaximin ( $n = 468$ ). Again, there was no difference between PPI users and non-users in the cumulative OHE incidences or in multivariable regression analysis (Fig. 1D and Fig. S2, Table 4, Tables S7, and S8).

Table 2. Multivariable logistic regression analysis for the association between variables and MHE prevalence in the total cohort and in patients without a history of OHE.

| Variables      | Total cohort |           |                  | Patients without a history of OHE |           |                  |
|----------------|--------------|-----------|------------------|-----------------------------------|-----------|------------------|
|                | OR           | 95% CI    | $p$ value        | OR                                | 95% CI    | $p$ value        |
| PPI use        | 1.07         | 0.80–1.43 | 0.7              | 1.02                              | 0.72–1.46 | 0.99             |
| Albumin (g/L)  | 0.94         | 0.92–0.97 | <b>&lt;0.001</b> | 0.95                              | 0.93–0.98 | <b>&lt;0.001</b> |
| MELD           | 1.02         | 0.99–1.05 | 0.3              | 1.05                              | 1.01–1.09 | <b>0.018</b>     |
| History of OHE | 1.80         | 1.29–2.38 | <b>&lt;0.001</b> | —                                 | —         | —                |
| Gender         | 0.70         | 0.51–0.94 | <b>0.018</b>     | 0.60                              | 0.42–0.86 | <b>0.006</b>     |
| Age (years)    | 1.02         | 1.01–1.03 | <b>0.029</b>     | 1.02                              | 0.99–1.03 | 0.09             |

Patients with missing data were excluded from analysis (complete case analysis).  $P$  values in bold show significant values. MELD, model for end-stage liver disease; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy; OR, odds ratio; PPI, proton pump inhibitor.



**Fig. 1. Cumulative incidence of overt HE (OHE).** A three-state model was built with death and LTX as competing events. Differences between groups were calculated with Gray's test. Cumulative OHE incidence in patients with vs without PPI use in (A) all patients ( $p = 0.13$ ), (B) patients with Child-Pugh A ( $p = 0.5$ ), (C) patients with Child-Pugh B ( $p = 0.3$ ), and (D) patients without a history of OHE ( $p = 0.082$ ). Abbr.: PPI, proton pump inhibitor.

We also analysed cumulative OHE incidences in patients with and without an indication for PPI treatment. There was no significant difference between the cumulative OHE incidences either between patients with or without an indication for PPI use, nor between patients without an indication for PPI use and those without PPI use or those with an indication for PPI use and those without PPI use (Fig. 2).

## Discussion

In this study, we provide evidence against an association between PPI use and the presence of MHE or the development of OHE in patients with cirrhosis. This finding held true regardless of CP stage, and PPI indication. Factors associated with a higher OHE risk were related to worse liver function – MELD,

**Table 3. Multivariable Fine and Gray regression analysis for OHE development.**

| Variable       | Total cohort |      |           |                  | Child-Pugh A |      |           |                  | Child-Pugh B |      |           |              |
|----------------|--------------|------|-----------|------------------|--------------|------|-----------|------------------|--------------|------|-----------|--------------|
|                | N            | sHR  | 95% CI    | p value          | N            | sHR  | 95% CI    | p value          | N            | sHR  | 95% CI    | p value      |
| PPI use        |              |      |           |                  |              |      |           |                  |              |      |           |              |
| No             | 381          | —    | —         |                  | 214          | —    | —         |                  | 133          | —    | —         |              |
| Yes            | 527          | 1.13 | 0.81–1.59 | 0.5              | 220          | 1.63 | 0.84–3.16 | 0.2              | 237          | 1.12 | 0.71–1.76 | 0.6          |
| MHE            |              |      |           |                  |              |      |           |                  |              |      |           |              |
| No             | 599          | —    | —         |                  | 331          | —    | —         |                  | 211          | —    | —         |              |
| Yes            | 309          | 1.33 | 0.94–1.87 | 0.11             | 103          | 1.02 | 0.44–2.33 | >0.9             | 159          | 1.90 | 1.21–2.99 | <b>0.005</b> |
| MELD           | 908          | 1.06 | 1.03–1.10 | <b>&lt;0.001</b> | 434          | 1.18 | 1.03–1.34 | <b>0.016</b>     | 370          | 1.05 | 0.99–1.11 | 0.10         |
| Albumin (g/L)  | 908          | 0.93 | 0.91–0.96 | <b>&lt;0.001</b> | 434          | 0.89 | 0.81–0.97 | <b>0.011</b>     | 370          | 0.96 | 0.93–1.00 | 0.062        |
| History of OHE |              |      |           |                  |              |      |           |                  |              |      |           |              |
| No             | 661          | —    | —         |                  | 362          | —    | —         |                  | 229          | —    | —         |              |
| Yes            | 247          | 2.00 | 1.43–2.79 | <b>&lt;0.001</b> | 72           | 4.26 | 2.16–8.37 | <b>&lt;0.001</b> | 141          | 1.70 | 1.09–2.66 | <b>0.020</b> |
| Age (years)    | 908          | 1.02 | 1.00–1.03 | 0.067            | 434          | 1.00 | 0.97–1.03 | >0.9             | 370          | 1.02 | 1.00–1.04 | <b>0.045</b> |

Patients with missing data were excluded from analysis (complete case analysis). P values in bold show significant values. MELD, model for end-stage liver disease; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy; PPI, proton pump inhibitor; sHR, subdistribution hazard ratio.

**Table 4. Multivariable Fine and Gray regression analysis for OHE development in patients without a history of OHE.**

| Variable      | N   | sHR  | 95% CI    | p value          |
|---------------|-----|------|-----------|------------------|
| PPI use       |     |      |           |                  |
| no            | 288 | —    | —         |                  |
| yes           | 373 | 1.33 | 0.80–2.19 | 0.3              |
| MHE           |     |      |           |                  |
| no            | 467 | —    | —         |                  |
| yes           | 194 | 1.93 | 1.21–3.07 | <b>0.006</b>     |
| MELD          | 661 | 1.07 | 1.03–1.12 | <b>0.002</b>     |
| Albumin (g/L) | 661 | 0.92 | 0.88–0.95 | <b>&lt;0.001</b> |
| Age (years)   | 661 | 1.02 | 1.00–1.04 | 0.067            |

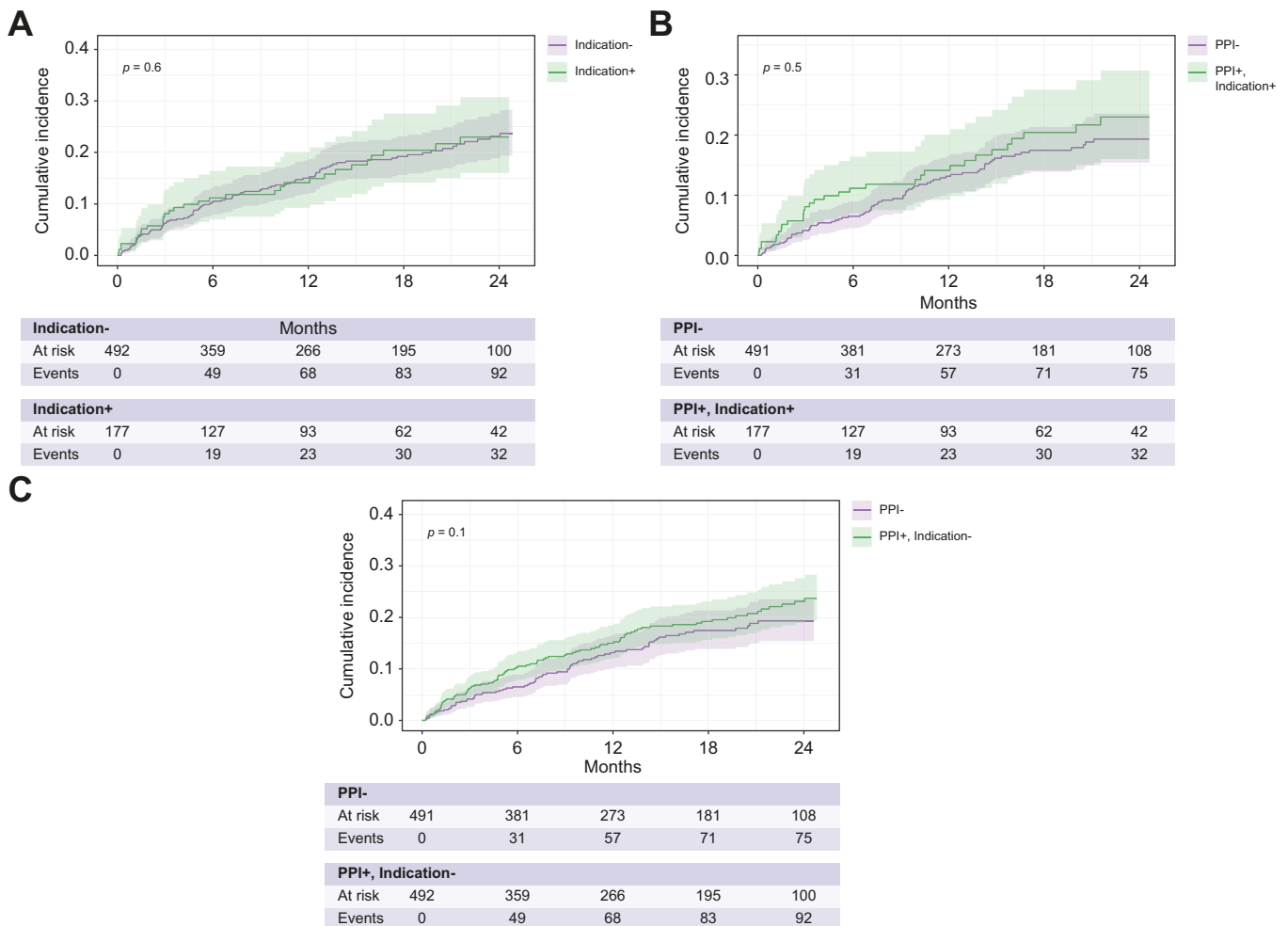
Patients with missing data were excluded from analysis (complete case analysis). P-values in bold show significant values. MELD, model for end-stage liver disease; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy; PPI, proton pump inhibitor; sHR, subdistribution hazard ratio.

albumin, and a history of OHE – as previously shown in this cohort.<sup>15</sup> Our findings fuel the debate on whether or not PPI use is associated with complications of cirrhosis.<sup>23,24</sup>

PPIs are among the most frequently prescribed medications in cirrhosis. In our cohort, nearly 60% used PPI, of whom three out of four had no valid and documented indication. A similar observation was made in another study.<sup>25</sup> Overall, the data

illustrates an overuse, which is inexpedient irrespective of its effects on the prognosis of patients with cirrhosis. Two prior studies found an association between PPI use and adverse events in patients with cirrhosis,<sup>26,27</sup> but high-quality data on the association between PPI use and HE are scarce. Important studies arguing for an association between PPI use and HE were published by Dam *et al.* and Nardelli *et al.*<sup>12,13</sup> Acharya *et al.* analysed data from a large cohort of patients with MHE testing at baseline using PHES. In contrast to Dam *et al.* and Nardelli *et al.* and similarly to our findings, they found no association between PPI use and MHE or OHE during follow-up.<sup>14</sup> However, the follow-up analysis in Acharya's study only included patients with prior OHE, and the cohort mainly consisted of males. Our study expands the existing literature by providing data from a large multicentre cohort in which 70% of patients were OHE-naïve, all with available PHES testing at baseline and with sufficient power to detect associations between PPI use and HE. Our data argue against an association between PPI use and HE and conflict with most published studies on this topic.

This discrepancy might be partly explained by publication bias affecting the publication of negative studies. Additionally, some studies might be biased by confounding by indication for



**Fig. 2. Cumulative incidence of overt HE (OHE).** Cumulative OHE incidence in (A) patients with vs without an indication for PPI use ( $p = 0.6$ ), (B) patients with an indication for PPI use and those without PPI use ( $p = 0.5$ ), (C) patients with PPI use but no indication and those without PPI use ( $p = 0.1$ ). Abbr.: PPI, proton pump inhibitor.

a PPI prescription, as recently described in a secondary analysis of the ATTIRE trial.<sup>28</sup> Here, China *et al.* found that patients with PPI use had a significantly different clinical course (e.g. significantly higher number of suspected variceal bleeds in the PPI group) compared to those not prescribed PPIs. This fact may explain the significant association between PPI use and HE grade III/IV during hospitalisation in their study. Supporting this assumption, the authors found no association between PPI use and potential pathophysiological links, such as the degree of bacterial translocation or systemic inflammation.<sup>28</sup>

An important strength of our study, separating it from the one with a similar design published by Nardelli *et al.*,<sup>13</sup> is our large sample size, allowing for good power across sub-analyses that all consistently showed no association between PPI use and HE. Our conclusions are further strengthened by a low number of randomly missing data points and the inclusion of only patients with full datasets in multivariable analyses.

The limitations of our study were that although most patients were prospectively recruited in the respective centres, the collection of PPI data and the subsequent analyses were retrospective with related weaknesses. The most important limitation is the lack of information on the start and duration of PPI use. Our data include information on PPI use at the time of psychometric testing and the indications were reviewed in the patient charts. It could be possible that PPI use is associated with the occurrence of adverse events in a time-dependent manner in patients with cirrhosis, which cannot be sufficiently

analysed in this current study. Additionally, it could be possible that some patients might be resistant to the potentially harmful effects of PPI, if such effects exist. This could only be sufficiently addressed by implementing a “new user design” only including patients with a PPI prescription at the day of cognitive testing and subsequent testing during follow-up. However, these data are not available in the current study. Given the aforementioned limitations, we are unable to apply causal inference methods in this observational dataset. Therefore, it has to be acknowledged that our study can only detect associations and is unable to prove causality. Last, although the patients have a prescription for PPI, patients may not be taking them. Here, epidemiological studies, including prescription time data from prescription databases and actual drug purchase databases, might be more accurate.

Nevertheless, our data support that PPIs in a primarily OHE-naïve cohort might not be associated with an increased risk of OHE. However, the issue of causality can only be clarified by a randomized and prospective study, which is currently recruiting as part of the STOPPIT trial.<sup>29</sup>

In conclusion, we provide evidence against an association between PPI use and the presence of MHE and OHE development. This observation applied regardless of liver disease severity and PPI indication. Although PPI should only be prescribed when indicated, these data suggest that PPI use should not be restricted in patients with cirrhosis with a solid indication.

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## Abbreviations

ALBI, albumin-bilirubin; CP, Child-Pugh; HE, hepatic encephalopathy; HR, hazard ratio; MELD, model for end-stage liver disease; MHE, minimal hepatic encephalopathy; OHE, overt hepatic encephalopathy; PHES, psychometric hepatic encephalopathy score; PPI, proton pump inhibitor; sHR, subdistribution hazard ratio.

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## Conflicts of interest

Simon Johannes Gairing: Travel expenses: Ipsen and Gilead. Joachim Labenz: Consulting: Alfaasigma, Norgine. Lecture fees: Norgine. Peter R. Galle: Lecture fees and consulting: Merz Pharmaceuticals. Patricia P. Bloom: Research grant from Vedanta Biosciences. Consultant for Nexilico. Dominique Thabut: Lecture fees: Alfaasigma, Abbvie, Gilead. Christian Labenz: Travel expenses and consulting: Norgine, Merz Pharmaceuticals. Lecture fees: Norgine, Merz Pharmaceuticals. Research grants: Norgine, Merz Pharmaceuticals. All other authors disclose no potential financial or non-financial conflict of interests regarding this study.

Please refer to the accompanying ICMJE disclosure forms for further details.

## Authors' contributions

Performed research: S.J.G., C.M., L.Z., E.J.N., H.D., P.B., P.S., P.R.G., J.L., D.T., J.U.M., M.M.L., S.M., C.L. Contributed to acquisition of data: S.J.G., C.M., L.Z., E.J.N., H.D., P.B., P.S., P.R.G., J.L., D.T., J.U.M., M.M.L., S.M., C.L. Designed the experiments and analysed the data: S.J.G., C.L. Contributed reagents/materials/analysis tools: S.J.G., C.L. Statistical analysis: S.J.G., C.L. Wrote the paper: S.J.G., C.L. Critical revision of the manuscript: all authors.

## Data availability statement

Data are available from the corresponding author (C.L.) on reasonable request.

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## Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhepr.2024.101104>.

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*Author names in bold designate shared co-first authorship*

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