Opinion Paper Open Access

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Chronobiology and circadian rhythms establish a connection to diagnosis

Abstract: Circadian rhythms are synchronized by the light/dark (L/D) cycle over the 24-h day. A suprachiasmatic nucleus in the hypothalamus governs time keeping based on melanopsin messages from the retina in the eyes and transduces regulatory signals to tissues through an array of hormonal, metabolic and neural outputs. Currently, vague impressions on circadian regulation in health and disease are replaced by scientific facts: in addition to L/D cyling, oscillation is maintained by genetic (*Clock*, *Bmal1*, *Csnk1*, *CHRONO*, *Cry*, *Per*) programs, autonomous feedback loops, including melatonin activities, aerobic glycolysis intensity and lipid signalling, among others. Such a multifaceted influential system on circadian rhythm is bound to be fragile and genomic clock acitvities can become disrupted by epigenetic modifications or such environmental factors as mistimed sleep and feeding schedules albeit leaving the centrally driven melatonin-dependent pacemakter more or less unaffected.

Keywords: circadian cycle; *clock* genes; day/night rhythm; drug intake time; preanalytics.

DOI 10.1515/dx-2014-0036
Received June 13, 2014; accepted August 26, 2014

Introduction

As we complete this contribution, we experience the solstice of the 2014 year cycle. Based on current knowledge, there is no consistent association of planetary and/or moon cycles unequivocally linked to human physiology and behavior. Nonetheless, cyclic illumination originating from circadian day/night (light/dark; L/D) cycles are increasingly recognized to affect the course of our daily health. The clock-work timing mechanisms coordinate biochemical, physiological and behavioral conduct to maintain synchrony with the environmental cycles of L/D, temperature and nutrients and are now acknowledged to involve cyclic changes in the expression of certain genes, guiding, at least in part, normal clock expression. Such diseases as those associated with chronic inflammation, e.g., inflammatory bowel disease, cancer, neurological disorders or metabolic syndrome, are increasingly studied as relating to circadian organization and microbiota communities [1–4].

The findings are underscored by the recent confirmation of the existence of a photic memory for human cognition involving melanopsin [5], and pursuing a circumscribed path between retinal photoreceptors and hypothalamic tract of the brain [6].

Plants and animals

Revival of interest in light pulsatility began with recent basic science studies on yeast: constant or pulsatile exposure of some fungi to visible wavelengths of light significantly initiates and/or alters respiratory oscillations. The growth of yeast strains that are null for the yeast activator *protein-1* gene that regulates oxidative stress genes is exquisitely sensitive to visible light. This reveals that light can both modulate respiration and induce oxidative stress [7].

In a number of marine species, endogenous rhythms of circalunar periodicity (~29.5 days) and their underlying molecular and genetic basis have been demonstrated. As an example, the palolo worm, *Eunice viridis* of Samoa and Fiji, in its burrows of coral reefs once a year fills the posterior segments with eggs and sperms and fill the coastal waters with millions of discharged gametes: swarming

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occurs regularly on the fist day of the last quarter of the October-November moon. With lobsters, the olfactory attention to detect food might act like tiny clocks by which odor signals reach the crustaceans alertness under time-dependent signal reception [8].

A cyclic 24-h world is encrypted as an internal time-keeping mechanism – the 'circadian' clock – and this has arisen in nearly all organisms allowing them to adapt their physiological and behavioral events.

In mammals, partition clock function is operative with differential tissue time keeping. At the core of all circadian clocks is at least one internal autonomous circadian oscillator using accelerating and retarding elements that form autoregulatory feedback loops – used to generate 24-h timing circuits [4].

The dominant zeitgeber for most species is the L/D cycle, and specialized photoreceptive and phototransductive mechanisms have evolved in all biological clock systems. The circadian watch of mammals is located in the hypothalamus. In addition to L/D as a primary zeitgeber (Figure 1), endogenous rhythms can also be entrained or reset by other cues (sound, body temperature, standing/horizontal position, health/disease) such types of entrainment of biological clocks now being regarded as a topic relevant to medical practice (www.clocksclub.com).

Physiological circadian cycles in healthy human study participants

With the current revival and acknowledgment of the existence of circadian cycling the studies into its exploration have been intensified. Ethical principles, such as those issued by the Society for Research on Biological Rhythms, have been formulated specially for a topic that has long been addressed with skepticism [9] (https://sleep.med.harvard.edu).

A synopsis is presented on Table 1. Life expectancy-extension connects circadian cycling to geriatrics [14]; as yet, prevailing work so far devoted to medical rhythm research looks closer at circadian variations of physiopathological translational topics in younger participants because the research protocols with healthy volunteers are stressful.

Background studies

Molecular biology, next-generation DNA sequencing (NGS), bioinformatics and biotechnology have confirmed original work on genetic backgrounds of circadian clockworks [15]. The way the endogenous molecular machine

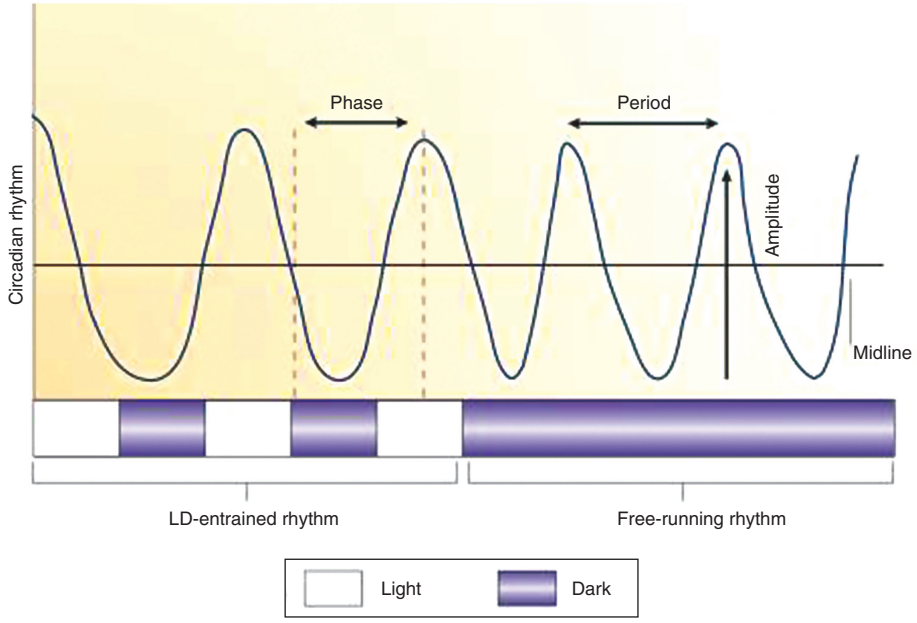

Figure 1 Schematic representation of cyclic and/or circadian activity for any given analyte.
The dominant oscillator for most species is the light/dark (LD) cycle for photoreceptive and olfactory pathways. Some oscillators, known as circadian osciallators, follow approximately 24 h and form the circadian biological clock. From Bell-Pedersen et al. [4], with permission from the author.

Table 1 Possible clinical inference from circadian variations of selected medial laboratory analyses.

Analyte	Results	Clinical significance	Setting/location	References
PAI-1	Morning trough values	Frequency of myocardial infarction in the morning	USA	[10]
Thromb-elastogram	Individual *viz* circadian about equal	Further studies warranted	Hemostasis ward Switzerland	[11]
TSH	Nadir in the late afternoon, evening elevation	Acutely ill patients: elevated TSH	Lower levels in Blacks	[12]
Prolactin	Nadir midmorning	Good clinical practice to respect circadian variation	Across the human race	[13]

monitors the time cycles is currently being explored and recognized to work under control of the transcription factors genes *CLOCK* and *Bmal1* and their corepressor genes *Per* and *Cry* by impinging on nuclear receptors all of which are transcriptionally activated (Table 2) [21]. As with many other biological achievements, it all comes down to phosphorylation reactions, in the present case of serine/threonine protein kinases and casein kinases whose members govern cytoplasmic and nuclear reactions, including DNA replication and repair [22].

They implicate phosphorylation of a variety of proteins, including the enzymes partaking in the reactions in an on/off manner.

Some of those proteins have been shown to phosphorylate proteins of the period family of circadian rhythm proteins on their own. Ancestry-related genetic data are emerging: thus, changes in the *Per* gene can cause advanced sleep phase syndrome (FASPS), a disorder characterized by very early sleep onset and offset. Individuals are 'morning larks' with a 4 h advance of the sleep (http://ghr.nlm.nih.gov/gene/Per2).

This contribution to *Diagnosis* appears timely, for several reasons

1. A recent prize-winning study from the Centre for Chronobiology at the University of Basel, Switzerland (www.chronobiology.ch) retrospectively analyzed sleep structure.

   Electro-encephalo-graphic (EEG) activities during non-rapid-eye-movement (NREM, an indicator of deep sleep) sleep of the partakers were recorded and secretion of the hormones melatonin and cortisol was analyzed.

   Under stringently controlled laboratory conditions the study finds that around a full moon, EEG delta activity during NREM sleep decreases by 30%, and time to fall asleep increases by 5 min. EEG-documented total sleep duration became reduced by 20 min; melatonin levels decreased. The authors surmize their work as first reliable evidence that a lunar rhythm can modulate sleep structure in humans when measured under desynchronized conditions of a circadian laboratory study protocol without time cues [23].
2. The neuronal histaminergic system makes us undergo a sleep-wake cycle, incidentally disturbed by air travel across time zones or shift working that induce circadian rhythm misalignments occasionally followed by inadequate and poor-quality sleep, and sleep disorders, e.g., apnoea and insomnia.

   Wakefulness-promoters such as modafinil and caffeine might get those affected back on track.

Table 2 Selection of genetic loci exerting influence on circadian cycling.

Gene locus	Chromosome	Function	References
CLOCK	2q37.3	Positive transcription factor	[16] https://www.youtube.com/watch?v=XzcdZ-MAyus
Bmal1: *ARNTL*	1 (rats) hypertensibility locus	Transactivates *CLOCK* and *CLOCK*-controlled genes	[17]
Csnk1 (casein-kinase 1)	15q22.1-q22.31 (mouse)	Regulates rapid eye movement sleep in mice	[18]
Chrono (Gm129)		Glucocorticoid receptor function	[19, 20]
Per (period circadian clock)	17 (human) zinc involvement	maintains circadian rhythms in cells	[17]
Cry	5	Core clock component	http://ghr.nlm.nih.gov

Shifting the circadian pacemaker using appropriately timed melatonin and/or bright light can be achieved. The cyclic variations of menses tuning by estrogen/progesteron go far beyond menstrual cycle considerations and extend to thyroidal hormones or insulin. A circadian locomotor output cycles kaput gene (*CLOCK*) polymorphism, recently adressed in a journal suggestively named "Chronobiology International" is now known to affect persistence of circadian rhythm.

The circadian system also regulates daily ups and downs in lipid metabolism and adipose tissue function. Using lipidomics-based approaches to profile the time course of lipid concentrations in blood plasma in healthy individuals [24] it was seen that they are subject to circadian variation; the rhythmicity spans storage, transport, and signaling equally. Opposing reactions of lipogenesis and fatty acid oxidation are the consequence [25]; the key transcription factor protein Clock in control of circadian regulation enhances atherosclerosis by increasing intestinal cholesterol absorption [26].

The implications for lipid metabolism disorders (http://www.eas-society.org/) linked to circadian clock disruption is clinically relevant indeed when relating to the variations to eating habits. Lipid metabolism shows a strong dependence of the circadian rhythm, which controls lipolysis rate, release of free fatty acids, triglyceride and hormone-sensitive lipase activity [26–31] and intestinal lipid absorption [32, 33].

3. Clinical importance of circadian rhythm on metabolic diseases.

Circadian rhythms do regulate metabolism and energy homeostasis to a certain extent. Circadian disruption and shortness of sleep have an important impact to the development of the metabolic syndrome [30]. A cluster of related risk factors (e.g., dyslipidemia, hypertension, hyperglycemia) make this syndrome risky for cardiovascular disorders and resistance to insulin eventually leading to type 2 diabetes mellitus (T2DM) [31]. Disturbance of the clock affects both the intrinsic and the peripheral metabolic systems, e.g., of liver, intestine, heart, retina and/or adipous tissue. In this regard suprachiasmatic nuclei of the hypothalamus controls thermoregulation, hunger and satiety, food intake, adrenal corticosterone and pituitary hormone release, sympathetic activation, as well as energy metabolism (e.g., lipolysis, gluconeogenesis, insulin sensitivity, basal metabolic rate) [32]. The neuropeptides involved in the regulation of hunger and satiety (energy metabolism) are the neuropeptide Y, the Aguti-related proteins and other satiation peptides like melanocortine. These peptides are regulated and stimulated through chronobiological impulses from the hypothalamus and underlie circadian dependency [33]. The level of leptin during the night-time acts as a satiety hormone, supporting the fasting state and nocturnal rest. In this context obesity correlates not only with high level of leptin but also with a decrease in the amplitude of the rhythm and reduction of circadian rhythmicity [34]. The same can be observed in the other adipocytokines like adiponectine and resistin too, which are linked to accentuated obesity, T2DM and metabolic syndrome [35]. The neuroendocrine system in the hypothalamus senses nutrients (glucose and lipids) and through a complex, in part feedback-type, pathway detects and then regulates circulating metabolic hormones such as leptin, insulin, thyroid hormones, adiponectin and ghrelin, which exhibit circadian oscillation [30, 33, 36, 37]. As a result, diurnal variation in glucose, glucose tolerance [38] insulin, triglycerides and adipose-derived hormones becomes apparent [39].

Chronic desynchronization of circadian rhythm contributes to increased risk of cardiovascular disease, higher prevalence of insulin resistance, obesity, metabolic syndrome and diabetes in shift workers [40–42]. Timing of food intake at night-time is associated with an increase of postprandial blood triglycerides, compared to meals taken during the daytime [39]. Male C57BL/6 mice can be accustomed to a 12 h light:12 h dark dycle since birth then fed a regular diet at differential time intervals. Thus, research on nocturnal mice, when anti-cyclically fed during daytime, revealed a higher fat percentage of their body weight than dark-fed animals. It was concluded, that circadian anticycling of food intake contributes to weight gain [43]. Similar to the murine model, we observe a macronutrient preference in men, i.e., carbohydrates at breakfast and fatty meals later in the day. The tolerance to glucose compounds and gastrointestinal transit slows down closer to bed time [44]. The effect of timing in nutrient intake has important effects on metabolism. Thus, if glucose is ingested during the evening compared to matutinal absorption, oral glucose tolerance will be worse as a result of a reduction in insulin sensitivity towards the end of the day [45].

In shift workers, delay in going to sleep, eating late in the evening/night and irregular meal times is linked to obesity, hypertriglyceridemia, low high-density lipoprotein, abdominal fat, diabetes and cardiovascular diseases [46]. When a night-eating syndrome was induced in voluntary medical students

by skipping their breakfast but consuming much (>50% of their daily food intake) in the evening and at night with the sleep from 0130 h to 0830 h the next morning, plasma concentrations of melatonin, leptin, glucose and insulin were measured at timed intervals. In a comparative diurnal lifestyle group they showed peaks at 0300 h, whereas the night peaks decreased in the nocturnal lifestyle group. Changes in the patterns of melatonin and leptin were highly consistent with that of night-eating syndrome (NES). Plasma glucose concentration maintained a high level in the NES group between midnight and early morning while insulin secretion decreased markedly during this period. What impresses us most from this study, is the damage done by the strong association between glucose and insulin in the diurnal lifestyle group after meals appearing in the NES group. The conclusion is evident that nocturnal life leads to the impairment of insulin response to glucose [47].

Metabolism and energy homeostasis are regulated by the expression and activity of specific metabolic enzymes, transcription activators and transport systems, which influence the core clock mechanism [48].

Signals from the anterior hypothalamus clock or from the local endogenous clock may regulate rhythmic gene expression in the peripheral tissue [49].

Some years ago, Naylor et al. demonstrated that mice homozygous for a *Clock* mutation exhibited significant increases in daily wake time (+2 h) and a reduction of REM sleep followed by sleep deprivation. These mice developed alterations in energy metabolism, including greatly attenuated diurnal feeding and activity rhythms, hyperphagia, and obesity with attendant symptoms of the metabolic syndrome like hyperleptinemia, hyperlipidemia, hepatic steatosis and hyperglycemia with an increased risk of T2DM [50, 51]. A circadian rhythm in blood glucose concentration has been observed in humans and mice with a significant increase of the serum level just before the beginning of the main activity period, demonstrating fluctuations in hepatic glucose export, glucose uptake and insulin sensitivity [52]. The glycogen metabolism of the liver is also linked to circadian oscillations with changes in glycogen synthase and storage, glycogen phosphorylase and glucose-6-phosphatase [53]. When 34 bedridden patients fed through nasoenteric tube had calorimetric energy expenditure measurements, it appeared that the values obtained were higher with the intermittend *viz* continuous infusion [54].

Muscle-specific ablated mice (*Bmal1* knockout) suffer from impaired insulin-stimulated glucose uptake with reduced protein level of the glucose transporter type 4 gene, *GLUT4*. This mechanism shows the importance of the now well-acknowledged muscle clock for the shift of glucose (predominant fuel for skeletal muscle) from the rest phase to the active phase [55].

One of the first therapeutic behavioral interventions against obesity and metabolic disorders is the regularization of the sleep-wake pattern with sleep-hygiene practices, which improves day-time alertness, nocturnal sleep efficiency, weight loss, to optimize metabolic processes, i.e., the cardiometabolic profile [35, 56, 57]. Van Someren et al. demonstrated that total daily energy intake ingested as one meal at dinner-time causes a rise in body weight unless the participants eat the same meal in the morning [58]. Meal times and physical activity are also relevant aspects in the chronobiological time keeping for prevention and treatment of overweight and obesity [45, 59]. Of course, interindividual differences in blood glucose and insulin levels are substantial [60].

4. The immune and microbial defense systems' functional performance has been described recently to follow circadian rhythm waves.

Clonal expansion/functional bursts of particular lymphocyte subsets bear the potential for selective immune defense increments/autoimmune attack under circadian influence.

The proinflammatory interleukin-17-producing CD4(+) T helper [T(H)17] cells which protect against bacterial and fungal infections at mucosal surfaces are regulated by a nuclear time keeping receptor: a special class of transcription factors, i.e., proteins which are central to the differentiation and long-term survival of lymphocytes, act on T(H)17 cell development underscored by the circadian clock: lighting up immunity→ differentiation of the T (H) 17 cells in the intestine suggest that not only nutrition but also light are important environmental functions that directly regulate cytokine homoeostasis and immune response [61, 62].

Mice can be colonized to higher levels with *Salmonella enterica serovar Typhimurium* during the early rest period compared with other times of the day – this was associated with induction of maximum performance of proinflammatory genes in the "mouse evening". Before such murine clock-regulated mechanism of the immune response against an enteric pathogen can be translationally funneled into chronopharmacologic

antiinfectious strategies, obviously, human studies need to confirm the existence of a circadian clock in the immune response to acute infections [63].

Human pathology and chronobiology

1. Healthy young volunteers were recently recruited under the auspices of the Kantonale Ethikkommission Lucerne, Switzerland, to twice a day thromboelastometry measurements (8:00 a.m., 4:00 p.m.) indicating that hemostasis oriented clinicans are beginning to scrutinize influence of circadian rhythms in their patient care [11]. Whereas the Lucerne study could not direct us towards a clear cut difference, a recently published study involving plasminogen activator inhibitor (PAI) found peak values at 6:30 a.m. [10]. Twelve healthy volunteers ~26 years old, followed a protocol designed to desynchronize daily behavioral rhythms from the internal zeitgeber and across 24 h hourly blood samples were PAI-1 tested.

   One hour prior to habitual wake time the prothrombogenic PAI-1 levels peak. The endogenous circadian system takes part in daily changes of PAI-1 seen during normal sleep/wake cycle in humans.
2. Circadian timing of anticancer medications has improved treatment tolerability and efficacy several fold, but as it shows intersubject variability, it is a topic currently under focus by clinical oncologists [64–66].
3. Circadian rhythm dysbalance due to environmental light pollution and reduction of sleep duration have been accused of reducing well-being and longevity [58, 67, 68]. Circadian timing in the elderly loses zeitgeber performance; this is aggravated in patients suffering from Alzheimer's disease and now, at least in part, has been recognized by the presence of *CLOCK* gene 3111T/C C>T [14]. Human cohort studies like these compare clinically well-defined patients to apparently healthy cohorts with the latter definitely needing to be confirmed as normal controls [69]. Reduction of insulin-sensitivity in the elderly [70] as recently put forward by our own work with senior citizens >60 years of age [71], may be due to reduction in suprachiasmatic nucleus pacemaker impairment [72]. Shifts in muscle fatty acid metabolism from oxidation to lipogenesis may be the cause of evening reduction of insulin sensitivity (see "Clinical importance of circadian rhythm on metabolic diseases"), as recently inferred from a study done on metabolically normal, yet overweigh women [60].

Circadian cycles in patients undergoing clinical laboratory workup

A few diseases are known to have occurrences at different daytimes or at night. For example, heart attacks occur most frequently in the morning a few hours after waking up, recently suggested to be due, at least in part, to morning rises of plasminogen activator inhibitor, PAI-1 [10]. Temporal lobe epileptic seizures usually occur in the late afternoon or early evening, and asthma is generally worst at night [73, 74].

Encouraged by the progress in basic research on circadian rhythm in human physiology, laboratory medicine specialists are beginning to include daytime hour of diagnostic venipunctures in their preanalytical considerations. A recent study from Marseille found that inorganic phosphorus, osteocalcin and parathormone assessed in 20 healthy volunteers displayed the lowest level around noon when tested between 09:00 a.m. and 05:00 p.m. [75].

A challenge similar to the one encountered with the establishment of reference intervals (RI) it seems to be the notoriously difficult access to healthy voluntary probands who are ready to undergo several venipunctures over 24 h.

Thus, cortisol peaks between 7:00 and 9:00 a.m. (~45–225 μg/L) and reaches nadir between 3:00 p.m. and 5:00 p.m. (~30–165 μg/L). The saliva cortisol concentrations oscillates from 8:00 a.m. to 10:00 p.m. from 0.15–1.00 μg/dL to 0.07 and 0.22 μg/dL Some hold that glucocorticoid circadian rhythm is regulated by gating mechanisms deploying their zeitgeber in the adrenal cortical clock [76]. Both the hypothalamic gonadotropin-releasing hormone (GnRH), and the pituitary luteinizing (LH) and follicle-stimulating hormones (FSH) have pulsatile secretion profiles [77] and that these hormones affect the secretion of estrogens within the human hypothalamic-pituitary-gonadal axis.

Salivary estradiol cycles from 4 pg/mL to 14 pg/mL in four to five waves during a 24 h cycle [78]. Because of night-time increase of water reabsorption, i.e., reduced nocturnal diuresis, a circadian rhythm of plasma arginine vasopressin independent of sex and puberty stage is observable [79]. Prospective studies of the circadian variation of anti Müllerian hormone, gonadotropins, sex steroids and androgens was recently conducted in healthy menstruating and ovulating women with blood sampling

every other hour during 24 h [80]. Significant nadir late-night values were seen. A circadian profile was performed in each study and control subject during a 24-h period by blood sampling every second hour, starting at 8:00 a.m. and continuing until 8:00 a.m. the following day.

A significant circadian rhythm of TSH is observed in healthy people with lowest serum concentrations 7:00 p.m.–8:00 p.m. (about 4 mIU/L) the next morning levels reaching 50% increments, which makes the time of blood sampling for laboratory medicine purpose crucial; such substantial circadian variance must be considered in discussions about the reference range settings of TSH. The mechanism of circadian TSH secretion is becoming increasingly clear, with the nuclear corepressor, NCOR, now known to impinge on thyroid hormone receptors bound to TSH subumit genes [12]. The 24-h mean concentrations of prolactin in plasma were the same in all groups, whereas those of LH and FSH were twice as high in the elderly as in the young men and eight and 23 times higher, respectively, in the elderly women. The 24-h mean plasma levels of melatonin in the elderly were half those in the young, but were not influenced by the sex or mental condition of the subjects.

It is International Federation of Clinical Chemistry's recommendation that number of laboratory exams have their conditions defined using a checklist which declares specimen-drawing in the morning vs. in the afternoon [81].

Outlook

With this eclectic text, we focus on a connection between time-dependent, e. g. circadian functional performance of the organism with real-time fitness and disease outbreak and medical laboratory validation.

Whereas we acknowledge that laboratory medicine for diagnostic and patient-follow up purpose is now mindful of these 24 h cycles, drug-development industry, regenerative medicine as well as organ transplantation may take such observations more consciously into account. At present it is out the authors' authority to assign chronobiology an importance in practicing medicine. At least the topic receives continuing attention by researchers, and is funded extensively none the least in quest of extending a healthful life. Physicians would be at a loss to ignore potential influence of chronobiology on their patients' wellbeing.

Acknowledgments: The continued endeavor of Mrs. Simone Inderbitzin in maintaining the education program of the Labormedizinisches Zentrum Dr. Risch is kindly acknowledged.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.
Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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