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A STUDY AROUND THE CLOCK: HUMAN CIRCADIAN RHYTHMS, MECHANISMS, ROLE IN CANCER AND CHRONOTHERAPY

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PRELUDE

We are taught, as medical students, the doctrine of homeostasis. This concept, developed by Claude Bernard in the 19th century is the foundation of the most of our reasoning. So, when reading articles and evaluating the results of clinical studies we easily assume, *a priori*, that the time during the day, month, year or any other time conditions are not of vital importance. We don't use the time factor as an argument to criticize the way trials are designed. We do not even think our teachers are not giving complete information to the patient when they don't indicate precisely the time when the patient should take the prescription. Neither we consider that giving the chemotherapeutic drugs to cancer patients during the daytime at the convenience of the diurnally organization of the hospital is of major consequence. We know that there are biological rhythms, namely hormone fluctuations or menstrual cycle, but we don't learn their implications, their potential in the treatment or they extensively applicability. Hence, we were never taught about chronobiology or chronotherapy.

Some, like the oncologist Bill Hrushesky of the University of South Carolina, consider chronotherapy a foundational idea, which I agree. Its knowledge isn't new, what is new is the discovery of its generality, proprieties and practical relevance in medicine.

In my Master Thesis I will explore this field, applied to the Medical Oncology area, bringing the discussion *de novo* to the Faculty of Medicine, University of Coimbra, Portugal.

ABSTRACT

Objective: The goal of this paper is to discuss biological rhythms, focusing on chronotherapy in cancer. The objectives are to: (1) briefly describe the circadian timing system, its physiology and networks; (2) address causal issues that have prompt progress toward an understanding of mechanisms underlying diseases as circadian-based disorders, specifically cancer; (3) review the concepts and principles of chronotherapy, applied in the medical oncology area; (4) dissect the results obtained by comparative studies between chronotherapy and conventional scheduled cancer treatments; and (5) offer a perspective about the future of chronotherapy and its knowledge in oncology. Methods: Review, synthesis, and interpretation of the literature. Results: Biological rhythms are a ubiquitous feature of life. There is circadian synchronization of endless molecular, physiological, biochemical and behavioral processes. Any deregulation of those rhythms may lead to disease, namely cancer. Likewise, experimental and clinical cancer processes are accelerated under rhythm disruption. On the other hand, anticancer drugs have their pharmacologic effects modified up to several folds accordingly to administration time: improved efficacy is seen when drugs are given near their respective times of best tolerability. Data extrapolated from animal experiments allowed a chronomodulated approach in cancer and randomized trials comparing chronotherapy versus conventional treatments have been performed. Besides the fact that some particular endpoints didn't give always preference to circadian-based therapies, in no case to date has chronotherapy been shown to be less effective than standard approaches. Chronomodulated schedules allow an increase in dose intensity and have a better tolerability profile. Importantly, optimal circadian timing and dosing of anticancer drugs can differ according to gender. Conclusions: Understanding the chronobiology principles has the potential to contribute to improve outcomes, and can open research ground for the development of better prevention and treatment strategies. The fundamental principles of chronotherapy are worthy of further clinical implementation and the future advances towards personalized cancer chronotherapeutics.

KEYWORDS

circadian rhythm

rhythmicity

molecular clock

chronobiology

drug administration schedule

chronotherapy

chronopharmacokinetics

chronopharmacodynamics

drug delivery systems

cancer

clinical trial methodology

personalized medicine

INTRODUCTION

Biological rhythms exist in all living organisms and we can consider that all biological processes and functions are organized at two levels: in space as a physical anatomy, and in time, as a biological time structure [1–3].

A rhythm is a change that is repeated with a similar pattern, probability, and period (Figure 1). If the variable that changes is biological and endogenous, the observed oscillation can be objectively referred to as a biological rhythm. As a rule rhythms are categorized into three major groups: circadian (20–28 h), ultradian (<20 h), and infradian (>28 h) [2].

The circadian rhythm is the most significant influence on physiological processes in the mammals [3,4]. This term, first used by Halberg in 1959, comes from the Latin, *circa* meaning 'approximately' and *dies* meaning 'day' [3]. These about 24-h cycles are expressed as genetic responses to cyclical changes in time in the external environment [5].

One of the earliest references found of rhythmicity in Medicine remounts to the 2nd century AD, when Soranus of Ephesus described the nocturnal occurrence of asthma [1,3]. Although, only in the past few decades we see an increasingly interest in the study of biological rhythms [2,6,7]. Modern technology has facilitated genetic studies to confirm the occurrence of circadian rhythms [8,9] and recent advances identify critical molecular mechanisms that rhythmically control other molecular, physiological, biochemical and behavioral processes, including events such as drug metabolism and detoxification, cell cycle, DNA repair, apoptosis, and angiogenesis [5,10–16].

All these discoveries strengthen chronobiology, the science of biological rhythms, an integrating discipline that has been ranked parallel with the more classical disciplines of development, genetics, and evolution [2]. Chronobiology has given rise to a whole new host of terms that match its applicability as listed in Table 1, whose definitions we should familiarize with to a better understanding of this field.

These acknowledgements, ranging from laboratorial experiments to clinical trials, brought a new insight not only into the understanding of physiology function but also about the pathophysiology of diseases [17,18]. Consequently, it has been opening improved possibilities of treatments and disease management – the chronotherapy. Chronotherapy is the investigative science that elucidates the biological rhythms dependencies of medications [1]. It attempts to time treatment according to biological rhythms in order to achieve the goal of maximizing the desired effects and minimizing the undesired effects [19].

There are many areas in which proper timing of medications or other procedures has been implicated in an improved diagnosis, reduced toxicity, or an increase in efficacy [3,20–23]. Oncology is one of them. Cancer is a systemic disease, defined by an uncontrolled growth of abnormal cells beyond their usual boundaries and which can then invade adjoining parts of the body and spread to other organs [24]. It profoundly affects daily activities as well as cellular metabolism, being associated with a poor quality of life [25]. Powerful anti-cancer agents are accompanied by undesired and even life-threatening toxicities, since these drugs are indiscriminate killers. Therefore, doses of chemotherapy represent an inherent compromise between toxicity in the cancer (the desired target) and the host (the undesired target) while an attempt is made to deliver the maximal dose possible [2].

Cancer chronotherapeutics deal with these problems, appearing as an attractive therapy in which anti-cancer drugs are administered with optimal timing according to the circadian rhythms of anti-cancer action and adverse effects on normal cells [25,26].

The relevance and impact of this emerging and promising research topic is evident in the exponential number of published scientific papers which since the late 1960s are accruing in the thousands per year. Since 1937, when seven scientists met in Sweden to create the first international organization focusing on the study of biological rhythms, the number of

societies studying rhythmic phenomena increased enormously, accompanied by the establishment of scientific journals dedicated to this subject [2].

Herein we propose to discuss the role of chronotherapy in oncology: what is the relevance of chronomodulated drug administration, taking into account rhythm determinants, in cancer?

The initial part of this paper reviews the general principles and organization of human clockwork and introduces the main concepts of chronobiology. Then it concisely explores the bond between circadian rhythms and disease, especially focusing on cancer disorders. The third part of this article aims to assimilate the role of chronotherapy in oncology treatments, ending with some considerations regarding its perspectives.

METHODS

The online search engines PubMed (http://www.ncbi.nlm.nih.gov/PubMed/), Science Direct (sciencedirect.com) and B-on (b-on.pt) were used for this literature search. Published data for this review were identified by searches using ‘circadian rhythms’, ‘cancer’, ‘chronotherapy’, ‘chronopharmaceutics’, ‘chronomodulated therapy’ as keywords, and with English, French, Spanish or Portuguese as language of publication. The selection was restricted to the past ten years, but some relevant citations found on review of the reference lists of retrieved articles were used too and the articles identified were supplemented by papers and books previously identified by the author.

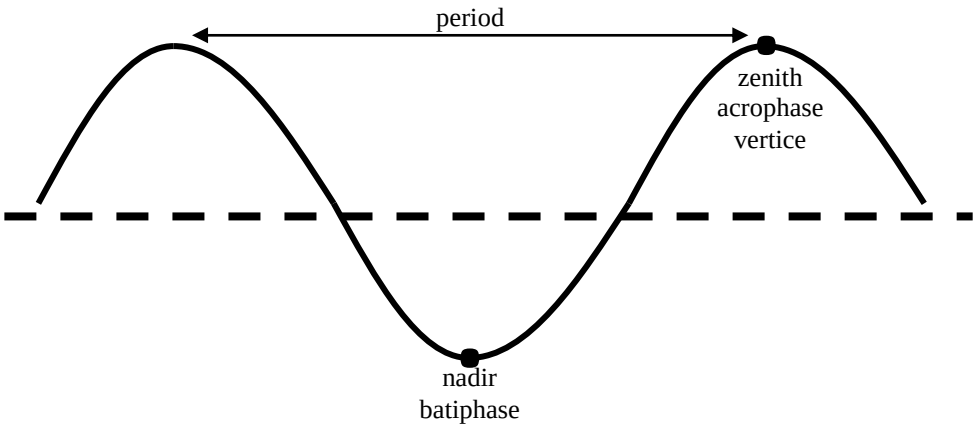

Figure 3. Anatomy of time.* Biological rhythms are characterized by periods (the duration of time to complete a single cycle), levels (the baseline around which the rhythmic variation occurs), amplitudes (a measure of the magnitude of the predictable variability), and phases (peak and trough values relative to the corresponding time scale). Rhythms range from short-period (lasting a second) to long period (lasting a week, month or year), but the major components are in reference of the 24 hour scale: circadian (20–28 h), oscillations of more than one cycle per 24 hours known as ultradian and less than one cycle per 24 hours known as infradian [1]. *Construct influenced by the narrative of Smolensky et al. [1].

Table 3. Definitions of terminology used in the science of biological rhythms (adapted from [3]**)**

Chronobiology	The study of biological rhythms and their mechanisms.
Chronopharmaceutics	The branch of pharmaceutics devoted to the design and evaluation of drug delivery systems that release drug to match the biological requirements of a disease therapy.
Chronopharmacodynamics Chronesthesy	The rhythm-dependent differences in the effects of drugs.
Chronopharmacokinetics	The rhythm-dependent differences in the absorption, distribution, metabolism and elimination of drugs.
Chronopharmacology	A chronobiological approach to pharmacological phenomena. The study of the manner and extent to which the kinetics and dynamics of drugs are affected by biological rhythms and the effect of the drugs on biological rhythms.
Chronotherapy	The investigative science that elucidates the biological rhythms dependencies of medications. The delivery of drugs in synchrony with the rhythm-dependent circadian variation inherent in the human body.
Chronotoxicology	The rhythm-dependent differences in the manifestation and severity of side-effects and intolerance of patients to drugs.
Cancer chronotherapeutics	A field of research that aims at optimizing cancer treatments through the integration of circadian clocks in the design of anticancer drug delivery.

Biological rhythm	Self-sustained and endogenous biological oscillation.
Circadian rhythms	Denote those endogenous, near 24-h physiologic, metabolic, or behavioral rhythms that persist under constant nonentrainment conditions (e.g., constant darkness) and are presumably driven by the molecular clockwork mechanism in the suprachiasmatic nucleus and peripheral tissues.
Circadian timing system	The biological system that generates near 24-h rhythms in cellular and organism physiology and adjusts them to environmental cycles.
Chronotype	Morning type or evening-type tendency of preferred activity time.
Entrainment	The synchronization of a rhythm by a repetitive signal.
Phase advance/delay	Change towards an earlier/later timing relative to the previous timing or to the fixed environmental clock time of a behavioral or physiological process.
Zeitgeber	Literally 'time-giver'. Any signal that acts on a biological clock as a time cue that has the capacity to advance or delay its rhythm and thereby to entrain it to a periodic occurrence of the signal.

I. WHAT IS THE INTERNAL TIME-KEEPING MECHANISM?

THE CLOCKWORK AS A SELECTIVE ADVANTAGE

Earth's rotation and orbit around the sun causes predictable daily and seasonal changes in light and temperature in our environment. Natural selection favored most life forms under this light-dark cycle by equipping them with circadian clocks or biological clocks — endogenous cellular mechanisms for keeping track of time. These clocks impart a survival advantage by enabling an organism to anticipate daily environmental changes and thus tailor its behavior and physiology to the appropriate time of the day, rhythmically, with a period length of about 24-h [9,27–31].

In fact, several studies demonstrated that genotypes with circadian periods that match the light–dark cycle enjoy a fitness gain relative to individuals with faster or slower clocks that cannot 'tune in' so well to the temporal environment [32].

Humans, similarly to all other organisms that inhabit this Earth, have a rhythmic order underlying life. For them, the most obvious circadian rhythm is the cycle of sleep and

wakefulness [32]. When a human being encounters a new day, the body prepares itself for the new tasks ahead and boosts heart rate, blood pressure and temperature. On the other hand, the same parameters decline at the end of the day [2,4,32]. Motor activity is high at daytime and low at night, body temperature reaches a maximum in the early evening, cortisol secretion by the adrenal gland rapidly rises from a nadir near 02h00 to a maximum near 08h00, thus peaking around the waking hours, with lowest values at early night; in contrast, melatonin secretion by the pineal gland usually peaks at early night and it is strongly inhibited by light in humans [13].

CIRCADIAN TIMING STRUCTURE

The circadian timing system has three major components: 1) an input pathway to a self-sustained central circadian pacemaker, 2) the circadian pacemaker itself, and 3) output pathways by which the circadian pacemaker regulates overt rhythms in biochemistry, physiology, and behavior throughout the organism [33] (Figure 2).

The input pathway is made of environmental and social time cues, termed synchronizers or zeitgebers (from German, 'time givers') [33].

Yet, a fundamental feature of all circadian rhythms is their persistence in constant environmental conditions or in the absence of external cues. Though, the period of this endogenous clock is not precisely 24-h, but in most people, it is somewhat longer [9,34]. This explains the need and role of zeitgebers: they do not create rhythms, they adjust (entrain) them. Changes in light, temperature, hormones, meal times and activity play roles in setting our internal clock [8]. In mammals, light is the most potent entraining signal [35], occurring in conjunction with the wake–sleep routine, that sets the inherited pacemaker circadian timekeeping systems to 24-h each day, preventing them from free-running out of phase [9,34,36].

Thus, circadian cells not only respond to environmental change, they anticipate it [37].

The suprachiasmatic nuclei (SCN) of the anterior hypothalamus are the site of the master circadian pacemaker in mammals. The SCN aside the third ventricle and atop the optic chiasm in the anteroventral hypothalamus, each consist of about 8000 cells packed into an approximately 0,5 mm by 1 mm football shape [36], with each half containing about 50,000 neurons in humans [9]. There are two distinct parts: the core and the shell - each of these subregions contains a number of different specialized neuron types with regard to neuroendocrine functions [38]. Its neurons are tightly interconnected and synchronized as an organ by crosstalk coupling mediated by vasoactive intestinal polypeptide and other neurotransmitters [38]. A hallmark feature of the SCN is its circadian pattern of spontaneous action potentials [9].

SCN is the central member of the Photo-Neuroendocrine System: photic information received by the retina is projected to the hypothalamus via the retinohypothalamic tract, which transmits the signals to the SCN and pineal gland. The pineal gland synthesizes melatonin to relay a message of darkness in a feedback loop to the SCN, which encodes genes whose expression is communicated to peripheral body tissues (neural tissues outside of the SCN, as well as tissues throughout the body) [3]. SCN relays phase information to the rest of the brain and body via a combination of neural, humoral, cascades of behavior and systemic signals [9,39].

It's remarkable to note that photic information is received by a non-visual pathway, through a functional retinal photoreceptor identified as a unique subset of melanopsin-containing ganglion cells [38]. Light signals received in the early evening can delay the phase of the circadian clock, while light signals received late in the night serve to advance the phase [40].

In addition to photic signals from the retina, the SCN also receives inputs from a number of other sources conveying functional information about various aspects of the internal and external environments that are integrated to regulate the overall temporal organization [38].

Although referred as the "master clock", the SCN is more correctly described as a "master synchronizer" than a strict pacemaker: the SCN serves to synchronize the individual cells of the body to a uniform internal time more like the conductor of an orchestra rather than the generator of the tempo themselves [9]. Thus, the circadian regulatory network is organized in a hierarchical fashion with signals originating in the SCN orchestrating rhythms in peripheral tissues [39,41,42].

Interestingly, besides being entrained by the SCN, peripheral tissues also contain functional circadian oscillators that are self-sustained at the single cell level, i.e., capable of demonstrating circadian patterns of gene expression when isolated from the SCN [31].

The fundamental mechanism of the generation and maintenance of rhythms is similar in the central and the peripheral clocks [43]. However, peripheral oscillators, also called "slave-oscillators" [44], differ from those of the SCN in their hierarchical position, in the ways they are synchronized and the output pathways elicited can be different and more tissue specific. They are not light sensitive and are independently adjusted by chemical and humoral signals, metabolic factors, and body temperature [9]. Feeding time is the dominant zeitgeber for peripheral mammalian clocks [45,46].

Although somehow independent, their expression depends on a functional SCN pacemaker who sends inputs for proper coordination - otherwise, it's observed a gradual attenuation of the amplitude of clock gene expression in every cell and a loss of synchronization between cells [45]. SCN oscillator overcomes local circadian defects and

signals directly to the molecular clock, what reinforces its important role in coordinating and synchronizing rhythmic behavior throughout the body [47].

Circadian expression in peripheral tissues optimizes cellular physiology by sequestering chemically incompatible reactions to different time windows [48].

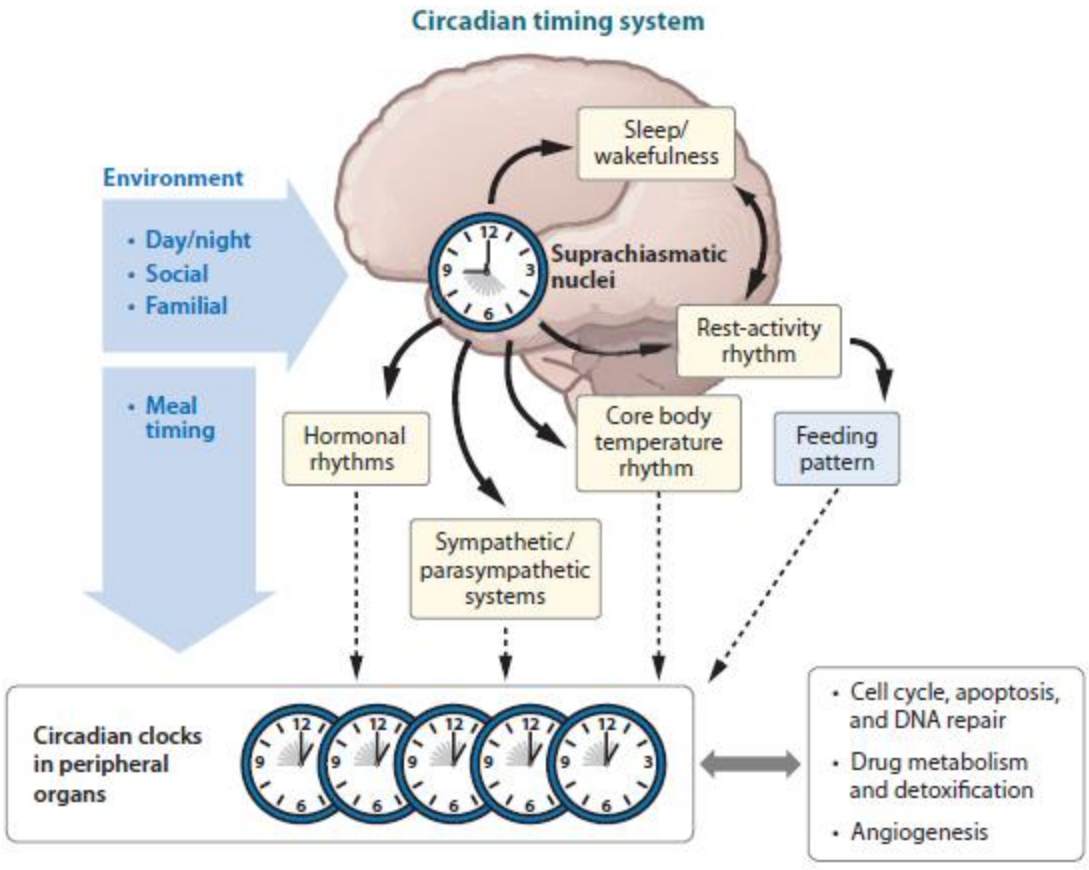

Figure 4. Schematic representation of the circadian timing system. The circadian timing system is composed of a hypothalamic pacemaker, the suprachiasmatic nuclei (SCN), an array of SCN-generated circadian physiology outputs, and molecular clocks in the cells of all peripheral tissues. Molecular clocks rhythmically control xenobiotic metabolism and detoxification, cell cycle, apoptosis, DNA repair, and angiogenesis over a 24-h period. The system is synchronized with time cues provided by light-dark cycles and other environmental factors. Circadian physiology outputs can also serve as circadian biomarkers (see text boxes 1 and 2). Reprinted with permission from Lévi et al. [49].

MOLECULAR CLOCK MACHINERY, BIOLOGICAL PATHWAYS AND NETWORKS

To get an oscillation, the clock requires an activator, a repressor, and some mechanism to delay the repressor's action on the activator [37]. Viewed at a molecular level, this hierarchical multioscillatory system is made of an interconnected network of transcriptional-translational feed-back loops, with a positive and a negative limb [9] (Figure 3).

Core circadian clock genes are defined as genes whose protein products are necessary components for the generation and regulation of circadian rhythms; that is, proteins which form the primary molecular circadian oscillatory mechanism within individual cells throughout the organism [33]. The circadian clocks controlled by the suprachiasmatic nuclei exist within every cell of the body and are comprised of at least twelve genes [50]. Clock-controlled activators and repressors control the rhythmic expression of downstream genes, referred to as clock-controlled genes. Hundreds or even thousands of genes are expressed with a circadian manner in various tissues [9]. Circadian gene expression in each tissue is tissue-specific and optimized to best accommodate that tissue's respective function throughout a circadian cycle [9].

The primary feedback loop comprises at least eight core clock genes: CLOCK (Circadian Locomotor Output Cycles Kaput), Casein kinase Iε (CKIE), Cryptochromes 1 and 2 (CRY1; CRY2), Period genes (PER1; PER2; PER3) and BMAL1 (Brain-muscle Arnt like1: brain and muscle aryl hydrocarbon receptor nuclear translocator). CLOCK and BMAL1 belong to the positive limb, that activate members of a negative limb, such as cryptochrome (CRY) and period (PER) genes by binding to E-box elements within enhancer and promoter sequences of these genes. These 'limbs' are interconnected via two retinoic acid-related orphan nuclear receptors (ROR), Rev-Erbα (RevErbA) and RORα (RORA), that function in the clock to regulate levels of CLOCK and BMAL1. CKIE phosphorylates PER proteins, tags

them for degradation, and can also positively regulate BMAL activity. Stability and degradation rate of the PER and CRY proteins is key to setting the period of the clock. PER and CRY proteins, after reaching a critical concentration, form heterotypic complexes and repress the transcriptional activity of the CLOCK:BMAL complexes, inhibiting the transcription of its own genes. This core system is entrained to the light-dark cycle with CLOCK:BMAL being high during the light period and PER:CRY being high during the dark period [5,9,33,39,47] (for a recent review see [9]).

Lastly, there are also other molecular circadian oscillators which can act independently of the transcription-based clock [9]. Protein-protein interaction, phosphorylation, acetylation, nuclear translocation, and protein degradation, contribute also to create a coordinated molecular cycle [33] – thus non-transcriptional rhythms or post-transcriptional mechanisms [51]. It has been recently proved that CLOCK has intrinsic histone acetyl transferase activity, what links epigenetic control to the circadian clock, through a global regulation of chromatin remodeling [31]. Intercellular processes include those signaling pathways from one circadian cell which impact oscillations in another. To date, neuropeptides and gap junctions have been implicated [37].

The end result of these regulatory pathways is a 24-h rhythmic oscillation of genes of the inner clock. By the use of only a few regulatory elements that precisely time the expression of clock-controlled-genes, the circadian clock orchestrates cellular functions, and ensures they occur at the right time [41].

These pathways influence a (perhaps not) surprisingly extensive range of functions within and outside the clock: metabolic processes (lipid and glucose metabolism, oxidative phosphorylation, adipogenesis), food intake, immune responses, inflammation, DNA synthesis, DNA damage control, cell growth, tumor suppression, xenobiotic responses, (reviewed in [47]); cell cycle [10]; redox state of the cell, and cell signaling [5]; very recent

results demonstrated for the first time that the circadian clock has also a major role in coordinating transcription and translation steps underlying ribosome biogenesis [12].

The opposite is also true: it has been proved that some pathways regulated by the clock can feed back to the clock machinery. For example, many genes that are involved in metabolic pathways such as insulin signaling, folate metabolism and others were found to regulate the core clock components [47]. In this sense, it has been suggested that the feedback transcriptional loop of circadian regulation is closely linked to an enzymatic feedback loop (NAD^+ was identified as an important adjusting signal in peripheral clocks) [31]. Circadian regulation of NAD^+ levels seems to be a crucial regulatory mechanism controlling circadian rhythms, metabolism and cell growth [9,31,43].

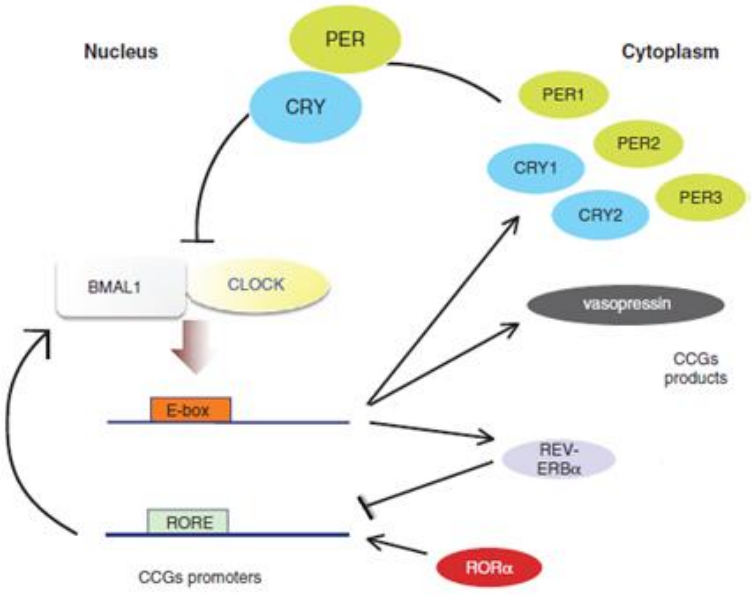

Figure 3. Schematic representation of the transcriptional–translational loops regulating circadian rhythms in mammals. The positive regulators CLOCK–BMAL1 activate genes with E-box elements in their promoters; these are commonly indicated as clock-controlled genes (CCGs). Among the CCGs are also the genes encoding the CRY and PER proteins that act as negative regulators of their own transcription. Most CCGs encode essential regulators of hormonal and metabolic control; here, vasopressin is shown as example. Additional loops of the circadian machinery involve other transcription factors, whose expression is primarily activated by CLOCK–BMAL1, like RevErbA. Adapted from [31].

This means that the circadian timing system is more pervasive than one would expect and that there are a rich network of pathways in both senses, at a global level – i.e. the capacity for mutual regulation of gene expression of all components by all components [37] - giving to circadian control a major role: circadian regulation may be a fundamental housekeeping function of the cell [5].

TEXT BOX 1| CIRCADIAN BIOMARKERS | The integrity of the circadian system can be accessed through its outward manifestations that have a robust circadian rhythmicity and are consequently considered circadian biomarkers. In clinical experiments researchers may use wrist actimetry monitoring (for a review see [124]), that is an actigraph, which is similar to a watch worn by the patients on the nondominant wrist. It detects wrist movements and records noninvasively the number of accelerations per minute to evaluate rest-activity cycle, and then the obtained data is associated with adequate mathematical parameters [62]. Hormonal markers may be evaluated through blood tests or salivary samples. Cortisol and melatonin are the most commonly used (for proposed functions on the melatonin profile and methodology of evaluation see [125]). Core body temperature is also an indicator. It is both a biomarker of the circadian timing system whose pattern is generated by the suprachiasmatic nuclei and an effector of the circadian coordination of peripheral clocks, through the involvement of heat shock and cold-induced proteins [9]. A non-invasive approach is given by the monitoring of skin surface temperature rhythms. Wireless skin surface temperature patches obtain relevant information on phase and amplitude of the dynamics of the circadian timing system and ultradian rhythms [119]. There are also cell-based and molecular approaches to assess circadian rhythms in humans, by skin biopsy or blood sample. One possibility is using ex vivo fibroblasts from skin biopsies following transformation with a lentiviral-luciferase circadian reporter [5,49]. At last, appropriate questionnaires may also be done, and are especially relevant to access diurnal preference or chronotype [34]. An ambulatory method combining several information to allow accurately detecting the circadian phase while subjects maintain their normal life style was recently validated [126].

Once elucidated the basic mechanisms of human circadian clock, in the next sections we will make the bond to clinical medicine: it will be highlighted the influence of the circadian clock in health conditions, mainly focusing on cancer, and then it will be discussed how these acknowledgments can be used in favor of better treatments.

TEXT BOX 2 | EXPERIMENTAL MODELS IN THE STUDY OF CIRCADIAN RHYTHMS | In animal models, precise period and phase estimates of circadian rhythms can be made by real-time measurements using circadian reporter genes in cultured cells [5]. It's important to note that rest-activity rhythm is a reliable marker of the circadian system function in both rodents and humans and the results are usually extrapolated from experimental models to human subjects in this field [26]. On the other hand, the rest-activity pattern is different between mice and humans, since mice are active during night. Thus, it is the "stage of rhythm" and not "time of day" which would be expected to ultimately result in consistent data and further a successful chronotherapy when extrapolating from preclinical findings to humans [2].

The role of circadian clock in the context of chemotherapy, growing tumor and their interactions has been evaluated using experimental models, including mutant mice with alteration of constituents of the circadian clock signaling process, longer, shorter or ablated circadian period, light-induced functional rhythm alterations and lesions of the suprachiasmatic nuclei as compared with 'wild type' animals [26,50], and also computer simulations [15,80].

II. IS THE DISRUPTION OF CIRCADIAN RHYTHMS LINKED TO CANCER?

CIRCADIAN RHYTHMS IN SYMPTOMS AND DISEASE

When circadian cycles are disrupted, either by genetic or environmental insults, disorders of diverse physiological processes can occur [5], what is not a surprise given the pervasive influence of the circadian clock on bodily functions, as seen above.

A number of human diseases display a circadian component, and in some cases, human disorders and diseases have been shown to occur as a consequence of faulty circadian clocks [9]. Klerman distinguishes two types of interaction between the circadian system and clinical pathophysiology: *primary* circadian, in which abnormalities in the circadian system itself cause the pathophysiology, and *secondary* circadian, in which the underlying pathophysiology is not circadian based, but the expression of this pathology is altered by circadian or diurnal events [17]. Besides the relevance of this differentiation, since we explored the complexity of clock networks with reciprocal relationships and with an apparently astonishing omnipresence, this distinction won't be strictly taken into account in the organization of the reasoning that follows.

The rapport between several diseases and circadian variations is well documented, even though for some of them the extensive underlying physiology is not yet accurately known. There are many examples in the literature regarding diseases diurnal rhythmicity in pathophysiology and symptoms: cardiovascular-related disorders, whose events are more common in the morning hours; respiratory function and disease symptoms, in which it's observed a nocturnal worsening of asthma and an exacerbation of allergic rhinitis in the morning; glucose tolerance and insulin secretory rates, and a number of metabolic complications that may result from miscommunication with the circadian clock and metabolic pathways; rheumatoid arthritis symptoms in the early morning correlated to the rhythm of

human cytokine production peaking in the morning, when cortisol (anti-inflammatory) is lowest and melatonin (pro-inflammatory) is still high; psychiatric and neurological disease (epilepsy, Alzheimer, Parkinson); sleep disorders; infectious diseases; pain; duodenal ulcer; coagulation disorder and thrombosis; cancer (reviewed in [3,17,20]).

THE CLOCK-CANCER LINK: FROM JET LAG AND SHIFTWORK EXAMPLES TO EPIDEMIOLOGICAL STUDIES AND EXPERIMENTAL DATA

The clock-cancer association has mainly been evaluated in two situations where the interactions circadian rhythms *versus* well-being are extremely evident: jet lag and shift-work, including night-work. They reflect the adaptive features of the system to its environment. Jet lag consists in a cluster of symptoms of poor sleep, fatigue, poor appetite and gastrointestinal disturbances for some time following rapid changes in time zones. This desynchronization of external and internal time also causes deregulation of physiological processes within the body [40]. Jet lag is a byproduct of robust circadian timekeeping, in essence ignoring temporal displacement. Jet lag diminishes, however, due to the system's sensitivity toward light cues: it's both the ability to maintain robust circadian rhythms in the face of perturbations and uncertainty and the competence of being reset and better adjusted [37]. But, while the rhythms of the SCN adjust rapidly to the new time zone, rhythms in peripheral tissues remain out of synchrony with the new time zone, and each other, for several cycles [40]. The significance of this delayed re-entrainment is felt by airline pilots, cabin crew and regular travelers. Flight attendants who frequently fly across different time zones show cognitive defects associated with the reduction in the temporal lobe volume. Several studies suggest that the incidence of cancer, psychological disorders, metabolic syndrome, diabetes, and cardio-vascular diseases is higher in these individuals (reviewed in [47]).

In the case of shiftworkers, there is a big incidence of accidents, family and social stress, errors, and poor workplace performance. Working at night not only requires the presence of light, which suppresses natural melatonin production and causes sleep disturbances [35], but also shifts the activity–rest rhythm from a diurnal habit to a nocturnal one, and can lead to altered meal times. It results in a loss of coordination between oscillators as they are reset by the different zeitgeber signals [17,52].

A sufficient number of high-quality epidemiological studies correlate lifestyle with disturbed circadian rhythms and an increased incidence of cancer [35]. People in repeatedly altered environmental cycles had a statistically significantly increased risk of breast or colorectal cancer compared with that of people who are exposed to regular our cycles [53–55]. Besides, circadian rhythm disruption increased the risk of prostate, lung, ovarian, endometrial and hepatocellular carcinoma (reviewed in [4]). The circadian disruption hypothesis was particularly corroborated by a large Nurses' Health Study that concluded that the risk of breast cancer was statistically significantly elevated in female nurses who worked on rotating night shifts, being the incidence higher among individuals who spend more hours per week and years working at night [55].

In our modern '24/7' society, the social and economic pressures that oppose internal temporal order are a growing source of circadian stress, implicated in the etiologies of many chronic illnesses and cancer [32,56], making this a pertinent question in the public health domain [4,17,55]. An expert working group for the World Health Organization International Agency for Research on Cancer recently concluded that shift work that causes circadian rhythm disruption is 'probably carcinogenic to humans' (estimated risk level 2A, that is, close to full evidence) [57]. Thus, circadian rhythm disruption is at the same level as some carcinogens such as acrylamide, biomass fuel, chloramphenicol, nitrogen mustard, lead compounds, diesel engine exhaust fumes and human papillomavirus type 68 [58].

In cancer patients, severe alterations of the rest-activity circadian rhythm influenced survival, suggesting that disturbed rhythms predict poor survival outcome. In a group of patients with metastatic colorectal cancer those with severe circadian rhythmicity alterations showed a fivefold increase in the risk of death when compared to individuals with a better rest-activity pattern, who had superior tumor response and longer survival [59]. Besides, disruption of circadian rhythms has been found associated with breast tumor progression as well and those patients with metastatic breast cancer who had impaired circadian rhythms had earlier mortality than patients with an intact rhythm [60]. Most importantly, the prognostic value of rhythm alterations was independent from all conventional known clinical factors [59–62].

Individual rest-activity cycle also showed to be a quantitative indicator for quality of life [59], what is in agreement with the idea that the disruption of circadian rhythmicity is an important pathophysiologic pathway in the etiology of fatigue (one component of quality of life and a highly prevalent symptom in cancer patients) in cancer [63]. Fatigue and appetite loss were also more evident among individuals with altered circadian rhythmicity [64]. Other quality of life indicators that may be affected are insomnia, depression, and nutritional deficiency [65]. This is further elucidated by an interplay between cytokine signaling pathways, the hypothalamic-pituitary-adrenal axis, the autonomic nervous system, and efferent pathways of the suprachiasmatic nucleus that control circadian physiology: some cancer patient's symptoms are related to tumor or host generated cytokines and could reflect cytokine effects on the circadian timing system [66].

Investigations in experimental models are in synchrony with those clinical evidences that severe perturbations of circadian rhythms increase the risk of cancer, determine its progression and account for overall survival. Filipski conducted several studies in mice to understand these problematic [67–71]. Mice subjected experimentally to chronic jet-lag or

with disrupted 24-h rest-activity cycle through SCN destruction showed drastically accelerated malignant growth in two transplantable tumor models, when compared to controls. It was observed a functional disturbance of molecular clock (absence of mRNA expression of clock genes PER2 and RevErbA) resulting in down regulation of the tumor suppressor p53 and overexpression of the proto-oncogene c-Myc, two effects which may favor cancer growth [67,69]. Moreover, PER2 mRNA expression in control tumor-bearing mice, showed significant synchronized circadian variations both in host tissue and in tumor, indicating that a temporal control of gene expression in the tumor is sensitive to host circadian signals [71]. When the team tried to entrain circadian clocks of chronic jet-lagged mice with feeding, the experiments revealed that meal timing moderately reversed the chronic jet lag-induced alterations in clock genes expression and slowed tumor growth [68]. These last results put emphasis in the idea that potentially restoring circadian rhythms should improve prognosis. From those experiments in general results, then, that both an anatomical structure such as the SCN and lifestyle-related factors such as the light-dark exposure cycle and feeding schedules can impact on tumor growth rate. This effect can be mediated through host circadian physiology, central circadian coordination, and/or molecular clocks in healthy and/or malignant tissues [10].

THE CLOCK-CANCER LINK: MOLECULAR MACHINERY UNDERLIES PHYSIOLOGY

In view of the fact that cancer is the uncontrolled growth and spread of cells [24], the underlying mechanisms that explain cancer as a circadian-related disorder involve mainly the cross-talks between the circadian clock and the cell cycle and DNA repair processes [10].

Circadian clock shares several conceptual and molecular similarities with the cell cycle. Both consist of interlocked autoregulatory loops, and they both rely on sequential phases of transcription, translation, post-translational modification and degradation [43]. To divide, cells undergo a sequence of molecular and biochemical events that gate and monitor the traverse of the cell division cycle through four distinct sequential phases called Gap 1 (G1), S (for DNA synthesis), G2, and M (for mitosis). The first three are termed interphase, and in G1 cell enlarges, during S phase DNA synthesis and duplication occurs and in G2 the cell checks that DNA-replication is complete and prepares for cell division. The final phase termed M involves the separation of chromosomes (mitosis), followed by cell division. Two key classes of regulatory molecules, cyclins and cyclin-dependent kinases, determine a cells' progress through the cell cycle [10,47].

For some cells, the cell cycle itself seems to be synchronized with circadian time, that leads directly to the entrainment of the cell cycle or indirectly through circadian variations in the level of a growth factor inducing cell entry into the G1 phase, which explains why cell division often operates on a 24-h time scale [16,33]. Time dependent cellular contexts such as hormone levels can affect not just the cell cycle but also immune and humoral function [72].

Interconnected with the cell cycle are also the DNA repair, apoptosis, and necrosis systems that limit genomic instability and prevent genetic mutations to accumulate and eventually result in malignant transformation [10].

It has been demonstrated that many of the cyclins, tumor suppressor genes, and oncogenes that are involved in regulating processes like DNA synthesis, mitotic indices, and DNA repair show circadian rhythmicity in their expression and are under the control of core clock genes [47,73]. The coupling seems to be made through clock-controlled genes that may either have E-box or ROR elements in their promoters. Cell cycle genes affected by the molecular circadian clock include cell cycle checkpoint-related genes such as cyclins D, B, E

and A, the proto-oncogene c-Myc, the antimitotic kinase Wee, the tumor suppressor gene p53 (that regulates apoptosis and arrests the cell cycle in G1 phase through activating p21 transcription, playing a critical role in the G1-S checkpoint), p21 (prevents cell cycle progression from S to G2 phase), and caspases, among others [73]. Some of them, such as the Wee1 kinase and c-Myc transcription factor, have been identified as direct transcriptional targets of the circadian CLOCK–BMAL1 transactivation complex [73]. Apart from controlling the expression of cell-cycle genes and tumor-suppressor genes at the transcriptional and post-transcriptional levels, the core circadian genes are also involved directly in modulating the intracellular signaling pathways of cell proliferation [74].

These are highly regulated processes. Any disruption can lead to cancer [47]. So, proliferation and genomic instability are favored, apoptosis is down-regulated, and malignant progression is accelerated [4]. Importantly, the effect of circadian clock disruption on cellular response to DNA damage and cancer predisposition may depend on the mechanism by which the clock is disrupted [75].

Therefore, no doubt remains that the rhythm of the circadian clock and cancer are interlinked. Experimental examples from animal models were already given by the studies conducted by Filipski et al. above cited. Other molecular reports lend additional support to the data and further established convincing links between some clock genes and tumorigenesis.

Briefly, the main associations are given. The downregulation of clock gene PER2 is associated with increased cell proliferation, while its overexpression promots apoptosis [74,76]. Disruption of the PER1 and PER2 genes also causes dysfunction of cell cycle checkpoints and susceptibility to DNA damage-induced malignancies [50,76]. Besides laboratory studies, these findings (RNA or protein downregulation of PER1 or PER2) were also found in human cancers [11]. The translated protein product of the CLOCK gene has an important role in encouraging cell cycle progression [50]. In an evaluation of microsatellite

instability in colorectal cancers CLOCK was also identified as one of the possible microsatellite instability genes. It may also not affect colorectal cancer cell growth directly but they do affect cellular responses to DNA damage, having a possible 'caretaker' role rather than being a classic tumor suppressor gene in this context [50]. BMAL1 is a putative regulator of the p53 pathway. Depletion of BMAL1 caused cell cycle disruption, increased apoptosis and was associated with downregulation of Wee1, cyclin B and p21 as well as upregulated expression of cyclin E. Knockdown of BMAL1 induced mitotic catastrophe in cells that highly expressed BMAL1 [50]. ROR elements regulate the proto-oncogene N-myc transcription [50].

Mutations of core circadian genes or disturbed expression rates, affecting specific molecular mechanisms already clarified by recent researches were observed in various forms of cancer such as hematologic malignancies, non–Hodgkin lymphoma, acute lymphocytic leukemia, chronic myeloid leukemia, mesothelioma, neuroblastoma, glioma, and pancreatic, liver, prostate, breast, epithelial ovarian, endometrial, and colorectal cancers (reviewed in [11,28,76]). Importantly, significant associations between clock genes polymorphisms and increased or decreased cancer risk are reported [11].

Regarding DNA repair, it's important to notice that since the repair of DNA lesions contributes to the resistance of chemotherapy with DNA damaging agents such as cisplatin, understanding the fundamental molecular mechanism regulating DNA repair pathways is important for cancer therapy [14]. DNA damage or genotoxic stress can also modulate circadian rhythms [5,25].

Of particular interest are the functions of free radical scavenging and enhancement of immune functions mediated by melatonin, which is effective in protecting against oxidative damage and, thus, it can protect macromolecules like DNA from oxidative damage and prevent carcinogenesis [40].

A direct molecular coupling exists between the circadian clock, energy metabolism and cell survival [31,43,77]. Circadian regulation of NAD^+ levels seems to be the crucial regulatory mechanism intersecting those pathways. Thus, defects in a clock-mediated metabolic pathway leading to cancer is proposed by the increase of NAD^+ levels in cancer cells in which NAD^+ is used at a higher rate for glycolysis. In hepatic cancer, for example, the metabolic gene phosphoenolpyruvate carboxykinase 2 that encodes a gluconeogenic enzyme is decreased [43].

It has also been suggested a circadian regulation of malignant tumors that can involve the circadian timing system control of vascular endothelial growth factor-mediated neo-angiogenesis [47,49].

At the tissue level, clock gene related rhythm alterations may be explicated by the desynchronization of the individual cancer cells that form into a solid tumor, hypothesis that is supported by the observation of a decreased expression of PER genes at a single time point in comparison with reference tissues [20]. This means that the difference in re-entrainment rates of central and peripheral clocks are a compounding factor in tumor development: while tissues are out of phase with their normal controlling systems, abnormal growth and cell proliferation may occur. This fact was demonstrated for the particular case of breast cancer, where breast cancer epithelial cells showed disruption of the inner clock. Circadian deregulation of gene expression has emerged to be as important as deregulation of estrogen signaling in breast tumorigenesis [29].

As an aside, it is of interest that the role of the histone deacetylase sirtuin 1 (SIRT1), a crucial modulator of the circadian clock machinery, was implicated in the pathways that intersect the regulation of circadian rhythms, ageing and cancer. Ageing has been described to be associated with the modification of circadian rhythmicity and the incidence of cancer increases with advanced age [43].

III. TIMING TREATMENT

CONVENTIONAL CANCER TREATMENTS: CONSTANT DOSING AND LIMITATIONS UNDER THE DEFINITIONS OF TOXICITY, EFFICACY AND OUTCOME

Conventional chemotherapy protocols only consider drug doses, duration, and frequency of infusions. As a result, treatment times vary among and within patients, being scheduled according to hospital routine and staff working hours [49].

The drugs used are the result of the development of anticancer treatments whose efforts and strategies have focused mainly on the eradication of cancer cells without paying much attention to the host [25]. The main therapeutic objectives have involved attempts to prevent or impair cell division and/or angiogenesis and/or to induce apoptosis in cancer cells [22]. Agents used in clinical practice are mainly: Antimetabolites (5-fluorouracil, Gemcitabine, L-alanosine), Topoisomerase inhibitors (Irinotecan, Mitoxantrone, Etoposide), Intercalating agents (Theprubicin), Alkylating agents (Cisplatin, Carboplatin, Oxaliplatin), Mitosis inhibitors (Vincristine, Vinblastine, Vinorelbine, Docetaxel), Cell cycle inhibitors (Seliciclib), Cytokines (Interferon-α, Interleukin-2), vascular endothelial growth factor inhibitors and Radiation (reviewed in [49]). They are usually very toxic. Indeed, only 5% of the anticancer agents selected for clinical development successfully complete all clinical phases and become registered as medications, being the reason precisely the inexistence of a full warranty of being safety [49].

Such problem implies the determination of the maximum tolerated dose – a delicate balance to maximizing efficacy on cancer cells under the constraint of a maximal allowed toxicity on healthy tissues. Thus, besides all the developments and research, outcomes of patients receiving anticancer treatments remain complicated both by unpredictable severe

toxicities and also by poor antitumor efficacy [49]. These endpoints need to be clearly defined to a precise evaluation of the therapeutic success.

Toxicity can be defined as the drug activity on healthy cells [47]. It can be objectively measured according to established criteria, such as the Common Toxicity Criteria grading score (www.eortc.be/services/doc/ctc). The most common toxicities are associated to bone marrow, gut, skin or oral mucosa, since they are fast-growing tissues, whose proliferation is gated throughout each day by the circadian clock [76] - proliferative cells usually display increased susceptibility to anticancer agents, whether these cells are malignant or healthy [48]. One can consider acute and long-term toxicity.

Efficacy stands for the drug activity on cancer cells, its therapeutic value [47]. The endpoints to evaluate therapeutic success are usually objective response rate and survival increase in life span. It is also considered the time to treatment failure, and freedom from distant metastasis. Methodology to measure changes in solid tumor size in response to treatment was standardized by the World Health Organization and by the Responsive Evaluation Criteria in Solid Tumors Group. Tumor evolution can be complete (disappearance of all symptoms and signs of disease for a minimum of 4 weeks), having a partial (disease shrinkage by 50%) response, no change or progressive disease. The tools used in that evaluation are given by radiology (mainly computed tomography scan, and also ultrasonography, X-Ray, and magnetic resonance imaging), cytology and histology techniques. Blood analysis of tumor markers also help to assess response [78].

Overall survival is a traditional gold standard end point, with the virtues of being unambiguously defined and clearly critically important to the patient, but it requires extended follow-up and may be confounded by subsequent therapies or procedures [79]. Intermediate end points are useful in isolating the clinical benefit associated with a specific regimen or strategy and for providing the information in a shorter time frame relative to overall survival.

Progression free survival is an end point generally considered to have been reached when a patient experiences disease progression or death [79].

Outcomes appear as the sum of the equilibrium between those considered factors, which also determine clinically the quality of life.

CHRONOTHERAPY: PRINCIPLES AND METHODOLOGY

In the case of chronotherapy, drugs administered are those that are already being used to treat patients, thus with the same intrinsic limitations to efficiently optimize treatment. Therefore, it's to be noted that the exploration of chronomodulated schedules does not constitute an alternative to the use of new drugs against cancer. In fact, chronotherapy critically contributed to the demonstration of oxaliplatin tolerability and activity against colorectal cancer and to its availability today. This drug was considered as too toxic to pursue its development by the pharmaceutical industry following conventional Phase I clinical testing. However, experimental chronotherapeutic studies revealed threefold change in tolerability according to dosing time in mice. Oxaliplatin was the first anticancer drug that has undergone chronotherapeutic development long before its approval. Translation of these findings revealed that chronotherapy displayed the best safety profile of oxaliplatin as compared with the patients receiving constant-rate infusion [25] (See text boxes 3 and 4 for experimental methodology).

Contrasting with conventional chemotherapy, chronotherapy consists in the administration of each drug according to a delivery pattern with precise circadian times. Circadian chronomodulated schedules stipulate the time courses and parameters of the delivery profile for each anticancer medication over the 24-h period to achieve the best therapeutic index. This includes times of onset and offset of infusion and variation of flow

rate, ranging from constant to sinusoidal or gradually increasing or decreasing, as well as drug sequence [49]. The underlying principle is that if there are diurnal changes in the physiology, if there are oscillations that determine variations in drug interactions with their molecular targets, if there are diurnal patterns of activities that may affect the disease, or if there may be different times of vulnerability to treatment and side effects of medications, then there may be optimal times to administer drugs or other therapies. For example, if cardiovascular risk is highest in the early morning and at approximately wake time, then medications affecting blood pressure, platelet aggregability, and other factors affecting cardiovascular risk should be administered so that they are at their peak effectiveness at that time. Regarding cancer therapy the goal is to administer medications at times when the cancer cells might be most vulnerable but the healthy cells are less vulnerable, improving the therapeutic index [26].

TEXT BOX 3 | EXPERIMENTAL METHODOLOGY IN CHRONOTHERAPY | The optimal circadian timings are staggered along the 24-h period and cannot be predicted thus far by the knowledge of pharmacologic class or that of main target organs for toxicity neither by the current understanding of how circadian rhythms impact on cancer drug metabolism and activity [11,49]. The methodology used to demonstrate the circadian influence in anticancer drug tolerability involves the synchronization of nocturnally active mice or rats with an alternation of 12 h of Light and 12 h of Darkness (LD12:12). The same drug dose is administered to different groups of rodents, with each group corresponding to a different circadian stage, also called Zeitgeber Time (ZT). Usually, six circadian stages, occurring 4h apart, are tested. Time usually is expressed in ZT-hours or in hours after light onset [49]. The conditions that humans live in are far more variable. However, humans and rodents have comparable rhythms [27], and data of expected times of least toxicity in human patients has been extrapolated from those experimentally demonstrated in these laboratory animals and tested in clinical trials in human cancer patients [84]. There are more than one hundred phase I (to determine dose-limiting toxicities) and II (to achieve the best chronomodulated schedule) clinical trials of cancer chronotherapeutics that have involved patients with advanced or metastatic cancer of almost all origins [49]. Phase III trials compare the “best” chronomodulated schedule to the “best” conventional delivery scheme in order to contribute in defining new standards of care.

Chronotherapy rejects the postulate that intends that with a certain plasmatic concentration constant in time the drug will have the same effect around the 24-h. On the other hand, the pharmacotherapy of medical conditions has traditionally respected the concept of homeostasis, with drug-delivery systems designed to achieve constancy in the concentration of medications over time as an assumed means of achieving constancy of therapeutic effect, regardless the patient physiological condition [1]. But striving to maintain relatively constant concentrations of one or more medications throughout the 24-h may be detrimental due to the elicitation of severe adverse effects when medication is delivered in too great a dose at the wrong biological time [1,80].

TEXT BOX 4 | DRUG DELIVERY SYSTEMS | The development of nonimplantable programmable in time drug delivery systems represents an important advance in the clinical progress of cancer chronotherapeutics. This dedicated technology enabled intravenous chronomodulated delivery of up to four anticancer drugs without hospitalization of the patient [22].

Currently there are many chronopharmaceutical technologies available that allow chronotherapy in daily clinical practice. They are pulsatile drug delivery systems: drug is released suddenly after well-defined lag time or time gap according to circadian rhythm of disease states, thus drug is released when necessity comes [127]. Oral chemotherapy is also amenable to chronotherapeutic optimization and oral multiple-unit preparations are also available regarding chronoprogramed release formulations for cancer [25]. There are many systems (based on capsule, osmosis, membranes' characteristics; sensible to different stimuli such as pH, temperature, magnetic field, ultrasound, electric field, chemical stimuli, light or mechanical force; externally regulated) from infusion pumps to controlled-release microchips (for more details on scientific and technological aspects of the design and manufacture of controlled-released formulations see [3,20]).

Multichannel, programmable in-time pumps are those that have allowed testing of the clinical relevance of the chronotherapy principle in fully ambulatory patients. Thus, the delivery of anticancer drugs can be performed routinely in the patient's home or during their usual activities, with minimal or no medical or nursing intervention [25].

The constant-rate delivery of medications need not result in constancy in desired effects [1] and can cause also problems such as resistance and tolerability, besides side effects [21]. Indeed, continuous intravenous infusion of 5-fluorouracil (5-FU) or doxorubicin resulted in circadian changes in plasma drug levels, despite a flat infusion rate [81]. The same was observed for other drugs [49]. In the particular case of a constant rate infusion of 5-FU, it was observed a peak concentration in the early rest span in both mice and in cancer patients [13]. So, treatment effects vary according to dosing time and circadian timing can modify from two up to several folds the tolerability of anticancer medications in experimental models and in cancer patients [48]. Twenty-four hour changes have been demonstrated for each of the four processes that determine the disposition of more than 300 drugs in rodents and in humans, i.e., absorption, distribution, metabolism, and elimination [48] (Figure 4).

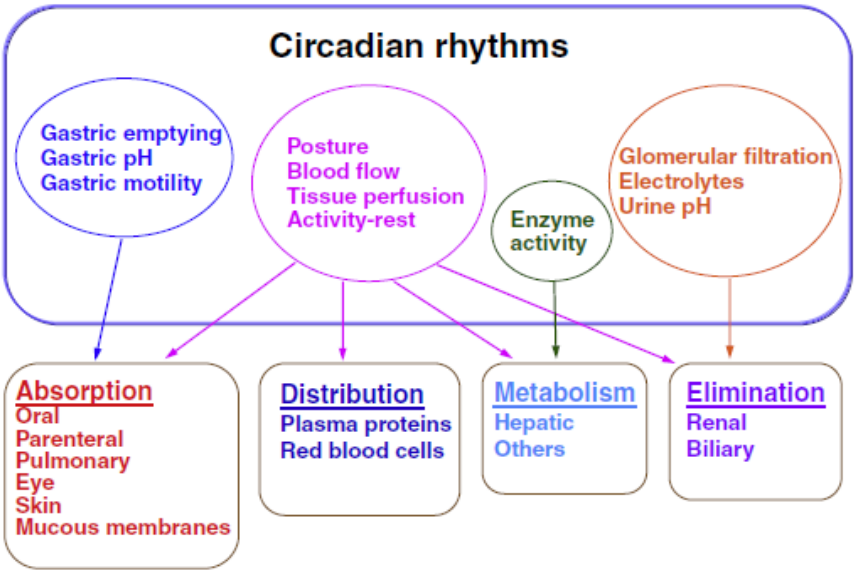

Figure 4. Effects of circadian rhythms on absorption, distribution, metabolism, and elimination of drugs. Reproduced from [21].

More than 40 anticancer agents were timely tested in mice or rats [22]. Large toxicity differences occur irrespective of delivery route — oral, intravenous, intraperitoneal, or intra-arterial— or the number of daily or weekly administrations [49], determining a circadian-dependence of toxicity rhythms. For instance, a treatment with the anticancer drug

cyclophosphamide that causes 20% mortality when administered at dusk (ZT10-ZT14) results in 100% mortality when administered at dawn (ZT22-ZT02) [5], revealing the circadian sensitivity to chemotherapeutic agents.

Quite strikingly, the administration of a drug at a circadian time when it is best tolerated has usually achieved best antitumor activity [49]. Even when combined, chemotherapeutic agents display the least toxicity near their respective times of best tolerability as single agents [49]. This overlap between chronotolerance and chronoefficacy result from an array of cellular rhythms involving drug detoxification and/or bioactivation enzymes as well as drug transporters. Thus, the xenobiotic detoxification system plays an important role in the timing of drug metabolism. It manifests itself in circadian pharmacokinetics and pharmacodynamics, consequently producing circadian changes in drug effectiveness and toxicity [48]. Reproducible coincidence between times of highest efficacy and least toxicity for the majority of anticancer agents suggest that common mechanisms may be involved. In general, the main reasons for efficacy and tolerability having the same time window can be simply resumed as an inherently poor circadian entrainment of tumors and a persistent circadian entrainment of healthy tissues [26].

So, the temporal dissociation between healthy and cancer tissues provides the main rationale of cancer chronotherapy [49] (Figure 5).

These mechanisms can be dissected trough analyze of a vulgar antimetabolite agent. Fluoropyrimidine 5-fluorouracil (5-FU) is a chemotherapeutic agent commonly used in gastrointestinal cancers. Like many anticancer drugs, it exerts its cytotoxicity at specific phases of the cell division cycle. Cells that are undergoing DNA synthesis (so called S-phase cells) are more susceptible to this drug. 5-FU is a prodrug that after intracellular activation disrupts DNA synthesis and repair, induces lethal DNA damage, and alters RNA processing and function, thus affects cell metabolism and viability. The target enzyme for this drug is

thymidilate synthase; the dehydropyrimidine dehydrogenase is the rate-limiting enzyme for 5-FU catabolism [82]. Both of them are under circadian rhythmicity regulation. That's what explains that experimental data revealed that 5-FU is best tolerated at the early rest span, precisely when the rate-limiting enzyme activity is high and the target enzyme activity is low, i.e., intracellular 5-FU catabolism is accelerated at night, resulting in a lower exposure of the intracellular targets to the drug active metabolites [15]. Extrapolating these results to human, it was verified that administration of 5-FU with a peak flow at 04h00 resulted in a better therapeutic index. This temporal window coincides with the minimum proportion of S-phase cells in oral mucosa as well as in bone marrow, the healthy cells one won't to be affected, and that are toxicity targets for 5-FU. Therefore, at night when the clearance of 5-FU is increased, the proportion of healthy cells potentially damaged by 5-FU is decreased [83].

Circadian based schedules (Figure 6) should achieve low toxicity to optimize efficacy. This paradigm seems to be specific for chronotherapy, contrasting with the current concept of conventional chemotherapy, in which the maximum tolerated dose (what corresponds to the maximum tolerated toxic effects) of an anticancer drug represents the optimal therapeutic dose [64]. Conversely, host clocks are disrupted whenever anticancer drugs are administered at their most toxic time [49].

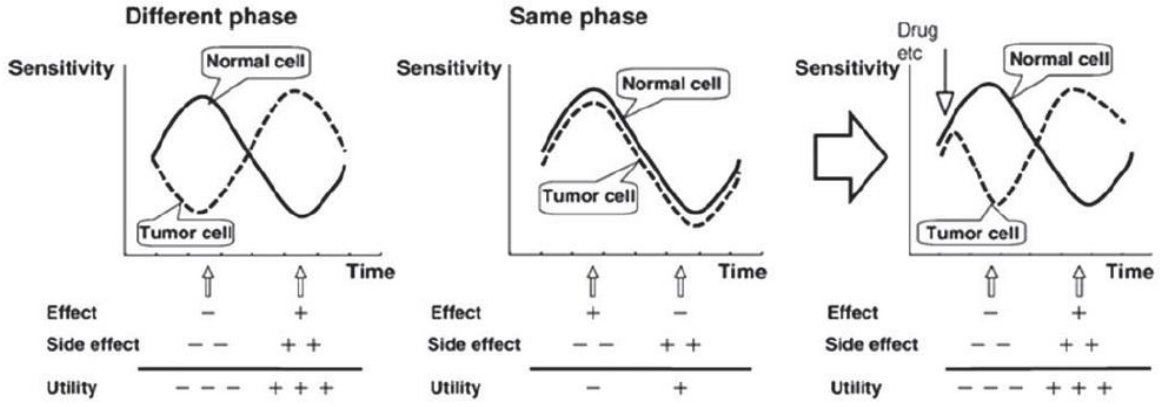

Figure 5. Schematic showing a typical chronomodulated chemotherapy strategy to synchronization drug treatment with the cell cycle for enhanced drug sensitivity and tolerance. Reprinted from [3].

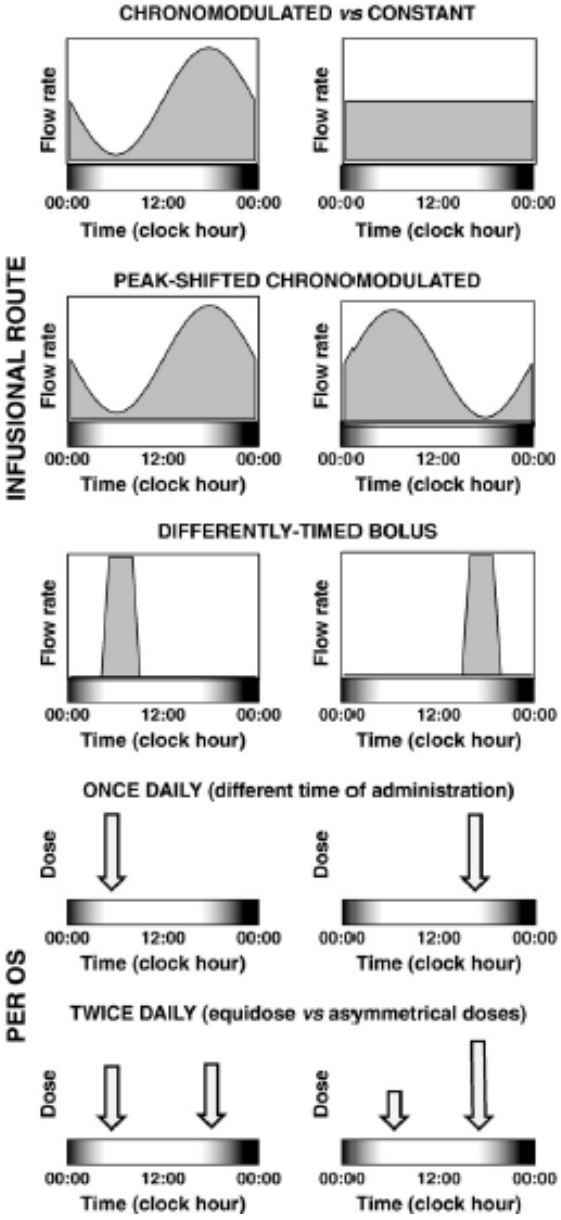

Figure 6. Examples of chemotherapy administration profiles used in comparative trials that stipulate drug-delivery timing along the 24-h day. The upper three panels show drug-delivery profiles involving intravenous administration route, with comparison between a chronomodulated and a continuous delivery, between chronomodulated profile with different peak-flow times, and between bolus perfusions administered at different circadian times. The bottom two panels show two approaches of oral administration route of chemotherapy, for drugs given once daily, for which two different hours of administration are compared, or for drugs given twice a day, for which the comparison involves 50% of the daily dose given at either time points with unequal doses, with a greater fraction of the daily dose given at one time point and a lower fraction at the other time point. Reproduced from [11].

Given the experimental data and reasoning inferred, theoretical advantages of circadian-based anti-cancer therapy can be elicited.

First of all, the dosing time-related reduction of drugs toxicity may result in an improvement in quality of life, although high doses of chemotherapy are being delivered. Thus, patients experience fewer symptoms and display less healthy tissue damage. Secondly, the administration of a higher maximum tolerated dose at the least toxic circadian time, as compared to other dosing times, may result in an improvement in efficacy outcomes, what can be observed by shrinkage of the tumor burden [84]. Third, chemotherapy can be administered without hospitalization, at home or during the usual activities of the patient. Altogether, this can translate into a favorable impact on overall survival with greater quality of life during and after the treatment [84].

APPLIED CHRONOTHERAPY

The first chronotherapy to be widely applied in clinical practice was introduced in the 1960s [1].

In the oncology field, case reports from four decades ago and recently published in the literature are very enthusiastic. A remarkable case exemplify timing someone's therapy less based on the best time extrapolated from concomitant studies on laboratory rodents and mostly on changing the time of chemotherapy based on mood and other self-rating parameters and objective comparisons of variables related to hematotoxicity. Franz Halberg and his colleagues published a case report [85] from a patient who had in 1973 a very rare and highly malignant ovarian tumor who underwent early autorhythmometry-guided timed treatment. That young lady presented with a malignant ovarian endodermal sinus tumor with spillage into the peritoneal cavity and had a poor prognosis. The first four courses of chemotherapy

were given at different circadian stages and autorhythmometry (mood, vigor, nausea, temperature) and complete blood counts were followed to determine the patient's time of highest tolerance. Remaining treatment courses were given at the best tolerance time found to the patient. She had 10% 2-year survival rate at admission, but after chronochemotherapy she was alive and well more than 30 years after. Another interesting case is also reported in the same article: a man who had read about timing treatment performed marker rhythmometry on himself and changed his living schedule (sleep-wakefulness, meals) to shift his circadian rhythms so he could receive chemotherapy at a time convenient to the doctor, but corresponding to the presumed optimal time determined by his body temperature rhythm [85].

More than isolated case reports, some clinical trials had suggested significant clinical benefits from specific circadian timing of chemotherapy or radiotherapy**.**

A Canadian study done in 1985 showed that children who were being treated for acute lymphoblastic leukemia did have improved long term survival when treated in the evening instead of the morning. The survival rate of those 118 children differed markedly depending on the time of maintenance chemotherapy: 80% of the patients dosed with 6-mercaptopurine and methotrexate in the evening were alive and disease-free 5 years after disease onset, as compared with 40% of the children receiving the same drugs in the morning ($p < 0.001$) [86]. These findings suggested that residual malignant lymphoblasts might be more susceptible to antimetabolites in the evening than in the morning [26]. Although these studies were not randomized, the results were robust enough and the magnitude of the time-related difference were so impressive that circadian-timed maintenance therapy is currently a consensus treatment for childhood acute lymphoblastic leukemia [13].

In the same year, researchers at South Carolina's Veteran's hospital found that ovarian cancer patients also had improved survival rates (four-fold greater 5-year rate of survival)

with fewer toxicity events when their medications were given at the optimal time of day. Patients treated with the morning doxorubicin and evening cisplatin schedule had fewer infections and fewer bleeding episodes; required fewer red blood cells transfusions; had less nausea, fewer vomiting episodes, and less emesis; had less severe renal damage; and experienced less frequent and less severe peripheral neuropathy (reviewed in [7,87]).

Large scale multicentre international chronotherapy trials were firstly initiated by the Chronotherapy Group of the European Organization for Research and Treatment of Cancer, with the intention of investigating the relevance of chronomodulated or timed administration of cancer therapy based on biological rhythms [88].

APPLIED CHRONOTHERAPY TO THE COLORECTAL CANCER

5-fluorouracil: 5FU
folinic acid: FOL
leucovorin: LV
oxaliplatin : L-OHP
irinotecan: CPT11

The chronotherapy principle was tested in a large population of patients with metastatic colorectal carcinoma. Colorectal cancer is the third most common cancer in men and the second in women worldwide, being its incidence generally higher in men. The risk of the disease increases with age. In 2012 there were 1361 thousands cases and 694 thousands of deaths caused by this cancer in both genders. In western countries is the second most common fatal cancer after lung cancer. In Europe, it represents 12,9% of all newly diagnosed cancers and is responsible for 12,2% of all cancer deaths. Portugal is the fourth country of the European Union with more new cases, with an observed number of deaths increased by 3% per year between 2000 and 2005 [89,90]. Metastases are present in 25% to 30% of patients at the time of diagnosis and will develop in another 25% [91]. Nine to ten Portuguese die everyday with this type of cancer [92]. Chemotherapy drugs used in colorectal cancer aim inhibiting cellular proliferation or having cytotoxic properties. Besides

drug developments in the past decades (5-fluorouracil and leucovorin as single agents or in combination, modulated or not by oxaliplatin, irinotecan, and more recently with bevacizumab and cetuximab) (reviewed in [93]), the treatment effect is still not very satisfactory [91]. In addition, in many cases, toxicity exceeds tolerable limits before the effectiveness of a regimen is fully exploited [79]. The golden standard for chemotherapy of advanced colorectal cancer implies to combine to a 5-fluorouracil (5FU) – folinic acid (FOL) base, a platinum derivative (LOHP-oxaliplatin) or irinotecan (CPT11) [94]. These combination as first-line treatment has been the standard of care in most patients for almost a decade producing response rates of roughly 50%, progression-free survival of 6–8 months and overall survival of 14–16 months (reviewed in [95]). The median survival for patients with advanced disease treated with FU-LV without effective salvage therapy has been consistently reported as 11 to 13 months [96].

From October 1998 to February 2002, 564 patients with metastatic colorectal cancer from 36 institutions in 10 countries were enrolled in the largest study conducted by the Chronotherapy Group of the European Organization for Research and Treatment of Cancer (Protocol EORTC 05963). Patients were randomized to receive chronomodulated or conventional infusion of 5-fluorouracil, leucovorin, and oxaliplatin as first-line treatment for metastatic colorectal cancer (conventional 2-day administration schedule without any timing stipulation (FOLFOX2) versus 4-day chronomodulated schedule (chronoFLO4)). The trial intended to treat each patient at the near maximum tolerated dose. Both regimens achieved similar median survival times more than 18 months with an acceptable toxicity [97].

Previous studies ([98,99]; and others also reviewed in [13,48]) had already established the activity and safety of a chronomodulated delivery of this three-drug regimen. Lévi et al. compared in two consecutive European randomized trials chronomodulated vs. fixed infusion delivery (chronoFLO5 versus constant-rate infusion over 5 days every 3 weeks in a total of

278 patients) and concluded that response rate was increased and toxicity was less severe in the patients receiving chronomodulated delivery compared with constant-rate infusion, being the median survival near 17 months. Objective response rate (percentage of patients whose metastases regressed by ≥50%) was significantly greater (51% versus 29%; $p < 0.001$). It reduced fivefold the incidence of severe mucositis and halved that of peripheral sensory neuropathy; patients, with previously unresectable metastases, underwent surgical resection of them [98]. Fewer toxicity allowed the delivery of higher daily doses [100] and both endpoints objective response rate and duration of patient survival ranked among the highest ones ever attained for the treatment of metastatic colorectal cancer in a multicenter setting (66% and 18,5 months, respectively). These chronotherapeutic studies played a major role in the development of a new medico-surgical strategy with curative intent for patients with initially unresectable liver metastases from colorectal cancer [13]. Results were confirmed with a minimum ten-year patient follow-up duration [101].

This more recent international randomized two-arm phase III trial was the first with an adequate sample size to address survival. The chronomodulated schedule produced a survival advantage over FOLFOX2 in men: women did significantly worse, whereas men did significantly better when receiving chronoFLO4 compared with FOLFOX2. In women, the risk of an earlier death with chronoFLO4 was increased by 38% compared with FOLFOX2 (median survival times of 16,3 months in chronoFLO4 and 19,1 months in FOLFOX2). In men, the risk of death was decreased by 25% with chronoFLO4 compared with FOLFOX2 (median survival times of 21,4 months in chronoFLO4 and 18,3 months in FOLFOX2), with 2-year survival rates of 43,6% and 34,3%, respectively. Gender was the single most important survival predictor. It was found a different toxicity profile between regimens: the main dose-limiting toxicities were diarrhea for chronoFLO4 and neutropenia for FOLFOX2 [97].

An update [102] to the individual data of the patients included in three international randomized phase III trials (with a total of 842 patients, 345 females and 497 males), confirmed that male and female patients with metastatic colorectal cancer responded differently to the circadian timing of 5-FU-LV and L-OHP-based therapy. For men, the time from the date of randomization to the date of death from any cause was significantly prolonged on the chronomodulated schedule (median values of 20,8 months (18,7 to 22,9) versus 17,5 months (16,1 to 18,8)). In contrast, females on chronotherapy displayed a poorer overall survival when compared with conventional therapy (16,6 months versus 18,4 months). 5-year survival rate was 14,4% for men on chronomodulated arms and 7,9% for those on conventional regimens. On the other hand, 15,6% of women on conventional therapy survived after 5 years, when compared with 5,3% of those on circadian-based therapy. Besides, response rate was also higher in males on chronomodulated schedule, as the opposite was observed in female patients.

Some studies did link chronotherapy to improved survival rates in colorectal cancer. It's the case of a rigorous meta-analysis that evaluated five [93,97–99,103] randomized controlled trials (with a total of 958 participants) comparing chronomodulated chemotherapy versus conventional chemotherapy for advanced colorectal cancer. It concluded that chronomodulated chemotherapy had a significantly superior overall survival (hazard ratio = 0,82; 95% confidence interval); the objective response rate was not significantly different between two arms. Regarding toxicity, it was observed a higher incidence of mucositis and asthenia in the chronomodulated schema, which demonstrated lower incidence of neutropenia, being the remaining parameters evaluated (diarrhea, vomiting and nausea and peripheral sensory neuropathy) similar in both arms, what led the researchers to affirm that chronochemotherapy produces a 'significant survival benefit without additional severe toxicities' [91]. Gender differences were not examined separately as a possible source of

interaction between the treatments under comparison and this evaluation included 387 women and 571 men. Regarding overall survival, this endpoint was defined as the time from assignment to death from any cause, with all cured patients (not died at the date last known alive) being omitted from the comparison of survival times.

APPLIED CHRONOTHERAPY TO THE COLORECTAL CANCER: DRUGS ASSOCIATION

Associations of other drugs to the classical 5-FU based-chemotherapy for colorectal cancer have also been evaluated, comparing chronomodulated with conventional schedules. For instance, Price and colleagues [104] conducted a large randomized phase III study of chronotherapy with 5-FU in 320 advanced colorectal cancer patients. They were randomized to receive a prolonged continuous fixed rate infusion of 5-FU at 300 mg/m^2/d continuously or a chronomodulated infusion of 450–600 mg/m^2/d of 5-FU infused at a constant rate from 22h15 to 09h45 every 6 weeks. Both treatment arms also included mitomycin-C at 7 mg/m^2. Quality of life assessments were comparable on both arms. The overall median dose intensity was 28,8% higher for the chronomodulated infusion arm ($p < 0.001$), what led them to conclude that the chronomodulation of 5-FU did appear to improve the tolerability of the combination. On the other hand, there was no significant response or survival benefit observed in the chronomodulated group [104]. In fifty-six patients with previous failure of several treatment regimens, the addition of cetuximab to chronotherapy allowed safe and effective therapeutic control of metastases, including their complete resection; the results were twice as high as those reported for first-line combination of cetuximab with conventional chemotherapy or for third line chronotherapy [105]. Accordingly, the POCHER trial reported an overall response rate of 79,1% and achieved 60% complete resectability of colorectal liver metastases [106].

APPLIED CHRONOTHERAPY TO THE COLORECTAL CANCER: REGIONAL INFUSIONS

Regional chronomodulated infusions were also evaluated in this disease. Chronomodulated hepatic arterial infusion for patients with heavily pretreated metastatic colorectal cancer, being most patients refractory to several current standard therapeutic options, revealed to be well tolerated, and an important rescue approach for selected patients. Such strategy could overcome drug resistance and even permitted further resection of metastasis and prolonged patient survival [107].

APPLIED CHRONOTHERAPY TO THE COLORECTAL CANCER: ORAL THERAPY

Oral chemotherapy associated to intravenous infusions has also been studied in chronomodulated regimens for colorectal cancer. Oral chemotherapy optimization is suggested in clinical chronopharmacology studies for oral fluoropyrimidines. 141 patients were enrolled in a randomized trial comparing a chronomodulated schema with a control arm of short-time infusion of oxaliplatin in combination with capecitabine (taken via oral) as first-line therapy in patients with advanced colorectal cancer. Capecitabine is converted to 5-FU by enzymathic steps, but its chronopharmacokinetics is not precisely known and drug absorption process after oral administration exhibits circadian variation due to daily variations in gastrointestinal dynamics. Besides, the maximum concentration of 5-FU expected in plasma is seen 2-h after ingestion of capecitabine and doesn’t correspond to the peak dose timing recommended by ‘original’ chronomodulated 5-FU infusion regimen. It couldn’t be excluded that a stricter adherence to the original chronomodulated administration schedule could be beneficial [95].

APPLIED CHRONOTHERAPY BEYOND COLORECTAL CANCER

Investigations extended beyond colorectal cancer. Encouraging results that support the clinical benefices of chronomodulated schedules of cancer treatment came from several studies of different tumor types: non-small cell lung cancer, advanced ovarian cancers (reviewed in [25]); renal carcinoma treated with floxuridine or interferon, breast carcinoma with mitoxantrone, 5-FU, and leucovorin, lung carcinoma with 5-FU, LV, and cisplatin or carboplatin (reviewed in [26]); biliary tract, pancreas, stomach, pleura [108]; œsophageal cancer [109]. In head-and-neck cancer, chronotherapy with paclitaxel, carboplatin, and 5-FU had a significantly more effective response (71,43% versus 42,86%), with higher and longer survival and lower incidence of adverse events [110]. Chronochemotherapy of cisplatin and 5–FU followed by traditional radiotherapy in nasopharyngeal carcinoma significantly reduced stomatitis [111].

APPLIED CHRONOTHERAPY IN RADIOTHERAPY

The chronotherapy principles were also applied to radiotherapy treatments and to therapeutic approaches combining chemoradiotherapy. Bjarnason et al. confirmed that morning radiotherapy led to significant improvements in weight loss and an apparent reduction in oral mucositis in subsets of patients than afternoon radiotherapy in head-and-neck cancer. The results are explained by the circadian rhythm in the oral mucosa cell cycle. Gender differences in radiotherapy toxicity were also observed [112]. In patients with advanced low-rectal adenocarcinoma it was observed a high response rate with minimal toxicities when administered chronomodulated 5-FU before irradiation, suggesting an advantage not only as a chronotherapy but also as a radiation sensitizer [113]. Other clinical studies reinforce the role of chronomodulated radiosensitizing chemotherapy schedules. Chronomodulated capecitabine for unresectable hepatocellular carcinoma combined to

intensity-modulated radiation therapy resulted in relatively low acute and late toxicity with promising local control [114]. When applied a schema of 5-FU chronomodulated chemoradiotherapy for pancreatic adenocarcinoma median survival rate was greater than that after most chemoradiotherapy programs, with relatively low acute toxicity [115]. The underlying mechanisms of chronomodulated radiosensitization might be related to circadian rhythm of tumor hypoxia, cell cycle redistribution, DNA damage, and expression of relevant molecules, as suggested by studies of human nasopharyngeal carcinoma (actually an epidemic health problem in southern China and Southeast Asia) using mouse models [116].

APPLIED CHRONOTHERAPY: DISCUSSING CLINICAL TRIALS

In the Table 2, there's a list of results obtained from clinical trials comparing chronotherapy with standard chemotherapy treatments applied to some types of solid tumors. Only a few large randomized controlled trials were performed to date. Besides phase III trials, the table includes the results from a meta-analysis and a phase II trial that does a comparative study. For a list including also retrospective, phase I and II trials and smaller phase III trials, (between 1985 and 2010) see the reference [11].

Table 4. List of clinical trials comparing chronotherapy with conventional methodology.

Reference	Gallion, 2003 [117]	Giacchetti, 2006 [97]	Price, 2004 [104]	Lévi, 1997 [98]	Lin, 2013 [111]	Liao, 2010 [91]	Garufi, 2006 [103]
Trial type	Phase III	Phase III	Phase III	Phase III	Phase III	Meta analysis	Phase II
Cancer Disease	endometrial	metastatic colorectal	advanced colorectal	metastatic colorectal	advanced nasopharyngeal carcinoma	advanced colorectal	metastatic colorectal
N	342	564	320	186	125	958	68
Drugs	doxorubicin DDP	5-FU LV L-OHP	5-FU mitomycin-C	5-FU FA L-OHP	DDP 5-FU RT	5-FU FA L-OHP CPT-11	CPT-11 5-FU FA

Chrono. arm (*C*)	Doxorubicin at 6h00 DDP at 18h00 both: 60 mg/m^2 IV during 30 min	chronoFLO4 .FU-LV: from 22h15 to 09h45, peak at 04h00 .L-OHP: from 10h15 to 21h45, peak at 16h00	5-FU: 450–600mg/m^2/d, constant rate from 22h15 to 09h45, every 6 weeks	.FU-FA: from 22h00 to 10h00, peak at 04h00 .L-OHP: from 10h00 to 22h00, peak at 16h00	sinusoidal chronomodulated infusion 5-FU: peak at 04h00 DDP: peak at 16h00	*	CPT-11 : 6-h sinusoidal infusion, from 02h00 to 08h00, peak at 5h00 every 2 weeks
Standard arm (*S*)	at any convenient time, doxorubicin followed immediately by DDP (same doses)	FOLFOX2 .L-OHP and LV: 2-hour infusion on day 1 and LV only on day 2, starting between 09h00 and 16h00 .FU: constant rate for 22 hours on days 1 and 2	5-FU: prolonged continuous fixed rate infusion of 300 mg/m^2/d	Flat infusion	Flat intermittent constant rate infusion	*In each study, both arms using same drugs and doses, differing only by the timing of drug admin.	CPT-11: 180 mg/m^2 on day 1, 1-h infusion every 2 weeks 5-FU and FA, from 22h00 to 10h00, peak flow at 04h00, from day 2 to 5 *(in both arms)*.
Significant Difference in survival	No Median survival time was *C:*13,2 months *S:* 11,2 months	No *C*: 19,6 months *S*: 18,7 months Gender-difference observed, men did better with *C*.	No C: 16,3 months S: 15,8 months	No *C*: 14.9 months *S*: 16,9 months	No	Yes Significant overall survival benefit in favor of *C* (HR: 0,82)	Yes Better with *C* (median survival 28 months versus 18 months).
Significant Difference in toxicity	Yes Leukopenia *C:* 63% *S:* 73%	Yes *C*: more diarrhea *S:* more neutropenia	Yes *C*: more diarrhea	Yes Grade 3 stomatitis higher in *S* 76% (versus 14% in *C*)	Yes Incidence of stomatitis during RT was significantly lower in *C*	Yes *C:* more asthenia, mucositis; less neutropenia	No Major toxicity was diarrhoea: 10 patients in *S* and 13 in *C*.
Difference in objective response	No *C*: 49% *S*: 46%	No	No *C*: 30,3% *S*: 38%	Yes *C*: 51% *S*: 29%	No	No	Yes Benefit with *C* (25.7% response rate for 7 months versus 18,2%).
Comments	Median total doses higher in *C*.	Gender was the single most important predictor factor 50 patients of each arm underwent surgical ressection of residual metastases .	No differences in global quality of life. Dose intensity 28,8% higher in *C*.	22% crossed over to *C*. Median 5-FU dose intensity higher in *C*.		Meta-analysis of five randomized controlled trials.	*C* safe, active and can be integrated with oxaliplatin (EORTC 05011) for the treatment of advanced CRC.

N: number of patients; C: chronomodulated arm; S: standard arm; 5-FU: 5-fluorouracil; LV: leucovorin; L-OHP: oxaliplatin; FA: folinic acid; DDP: cisplatin ; RT: radiotherapy; admin: administration; CRC: colorectal cancer; HR: hazard ratio.

Interpretation is complicated by the multiple differences between the chronotherapy and control arms that extend beyond the timing of drug administration. These include differences in sequence, dose, and duration that may affect clinical outcomes independent of chronobiology [118].

Besides, the precise administration time was not systematically registered in most control arms, what can lead to mixture and similarity of circadian timings in both the control and timed arms. This further makes sensible circadian analysis problematic. Some trials have compared chronotherapeutic delivery to a control administration protocol without any time specification. The control administration is determined by patient or hospital convenience. The problem is that this conventional approach cannot be used as a reliable control arm for testing the impact of circadian-based therapies, especially if the expected best time of administration takes place during the day. Conversely, constant-rate infusion over at least 24-h eliminates any circadian timing hypothesis for drug administration. Experimental and clinical data show that a constant-rate infusion schedule lasting an integral multiple of 24-h constitutes an adequate control for proof of principle demonstration of chronotherapeutics delivery schedules, if the pharmacologic properties of the drug permit it, which is also supported by several mathematical models that include circadian clocks [11,49].

The design of some studies can be criticized and, thus, the assertions made in consequence of their results may be questioned. For instance, the trial conducted by the Gynecologic Oncology Group Study on the evaluation of chronochemotherapy in endometrial cancer [117] was accused to have some important limitations: cisplatin timing was not completely random in the control arm, and was actually quite similar in both groups, control arm doesn't specify drug timing, and it was overlooked the fact that most of the chronobiology effects in this schema would be mainly related to the time span between the

two drugs administration. In those sense, the conclusion of "no significant circadian therapy benefit" is questioned and said that the assertion is not supportable by the data [87].

Along studies, patient's characteristics also differ, being chemotherapy-naïve in some or pre-treated in another. Since drugs used in cancer treatments can disrupt the rest-activity rhythms in cancer patients, and this disruption is associated with systemic toxicities [25], those patients that were already submitted to standard chemotherapy treatments before chronomodulated schedules may have more a disrupted circadian timing system that can compromise the effectiveness of circadian-based treatments. A recent study evaluating a triple combination of chronomodulated 5-fluorouracil/folinic acid, carboplatin and irinotecan in advanced colorectal cancer reported an excellent tumoral activity with over 70% response rate and 15,6 months overall survivals, but the more prolonged survivals were observed in chemotherapy naive patients (> 50% of them are alive at more than 2 years) [94].

The evaluation of a therapeutic success is made through the endpoints defined. Here, we witness that, after all, the two largest randomized chronotherapy studies in colorectal cancer [97,104] show no benefit in antitumour efficacy as assessed by objective response, overall survival, and progression or failure-free survival. Also, the first randomized, multicenter phase II trial designed to evaluate a chronomodulated schedule of 5-FU with oxaliplatin in the United States conducted by Ramanathan et al. concluded that in that study did not seem to be an advantage to using chronomodulated schedules in terms of toxicity and efficacy. The trial evaluated and compared the efficacy of 4 different oxaliplatin/5-FU regimens in 129 patients: (1) continuous 5-FU infusion plus oxaliplatin (n = 23); (2) weekly 5-FU bolus with LV plus oxaliplatin (n = 40); (3) oxaliplatin with 2-day infusion 5-FU/LV (FOLFOX4, n = 41); and (4) chronomodulated 5-FU plus oxaliplatin (n = 25). They further added that chronomodulated regimens are unlikely to be used in community practice in the

United States [93]. One can't help noticing that these assertions are made after a relatively small sample size of patients in each treatment arm.

Almost all the trials referred do, in fact, show a difference in tolerability or dose intensity that favors chronomodulation.

It's important to emphasize that in the study conducted by Price chronomodulated infusion was administered as a flat rate, without peak delivery and not as a sinusoidal infusion, as would be expected from previous results extrapolated from mice [13]. Moreover, it was suggested that overall survival may not be the most appropriate endpoint to assess the efficacy of a first-line treatment in metastatic colorectal cancers, given that subsequent chemotherapy lines and metastases surgery also influence outcome. Also, partial response could not be confirmed, mostly because patients underwent metastases surgery immediately after documentation of the best response [97]. Consistent with this, other parameters, such as progression-free survival, time to treatment failure, and 60-day mortality, were suggested to be used in addition to overall survival to evaluate efficacy and clinical usefulness of first-line protocols [79,96].

Taking together, on the one hand only some particular results are not completely favoring chronomodulated schemas, but on the other hand several of them are giving an important preference to chronotherapy. Considering that, and all the possible limitations implicated, one may surely state that can learn from the results obtained in several clinical trials that the method of delivery of anti-cancer therapies may be just as important as the agent itself [115].

HURDLES

The extent of individual benefit from circadian-timed therapy can differ as a function of environmental, genetic, and epigenetic factors [48].

Gender of a patient was the single significant predictor of the relative advantage of chronomodulated chemotherapy as the most effective delivery schedule of 5-FU-LV- L-OHP in advanced colorectal cancer, independently of all known baseline patient characteristics [97,102]. Exploring the reasons behind these differences, one should look first to preclinical studies: male mice were used for most of the preclinical chronotherapeutic studies of 5-FU and oxaliplatin that were the basis for the chronomodulated schedule used in subsequent clinical trials and men predominated in human translational studies that supported the concept of these drugs' chronomodulation [102]. Secondly, there are differences in drug metabolism and detoxification between male and female patients: clearance of 5-FU is down-regulated in women, when compared to men [48]. Besides, dihydropyrimidine dehydrogenase (the enzyme responsible for this drug clearance) gene polymorphisms were only robust and highly statistically significant to predict toxicity in men [102]. Gender-specific toxicity mechanisms are also supported by the fact that men had lesser side effects than females not limited to chronomodulated delivery [13]. The occurrence of excessive toxicity in women may cause circadian disruption, and thus the impairment of chronotherapeutic mechanisms [13,119]. Third, a different circadian genotypic profile between males and females with colorectal cancer is possible. Male patients may have more robust circadian rhythms and the rhythmicity of some circadian biomarkers differ between male and female subjects, having only a few hundred rhythmic transcripts in common and with similar rhythmicity patterns from the two-thousand analyzed [11]. In sum, gender is a critical determinant of the optimal schedule of the reference three-drug chemotherapy against colorectal cancer which offered best survival. This is particularly exemplified by the data referred to the patients alive at 9 years, who may be considered cured from a disease usually deemed as incurable: this was achieved in 9,2% of the male patients treated with the chronomodulated schema and in 9,6% of the female patients receiving a conventional infusion of the same three drugs [102]. The optimal

chronomodulated schedule could also differ between male and female patients [102]. Further research is required to explore optimal chemotherapy timing in females. For now it's known that in men, the same chronoFLO treatment significantly increased tumor response and survival compared with conventional delivery, independent of other prognostic factors. It would be interesting to search for gender differences in trials that involved other tumor types.

Besides gender dissimilarities, humans can be grouped into different chronotypes, ranging from larks to owls [2,34]. Patients can also display distinct patterns in circadian physiology, possibly related to the impact of the disease on their circadian timing system components. Hence, the physiological significance of daytime differs between human individuals. In the simplest cases, the different circadian phases can be explained simply by period length [48]. Diurnal sleep patterns also change across the life cycle [17].

The impact in circadian timing system it's also a challenge since drugs and disease itself can disturb the robust nature of biological clocks [49,80]. Robustness is the ability to sustain daily oscillations with an accurate period length day after day. Biological clocks can be influenced by many stimuli while ignoring most others: they are susceptible to specific environmental oscillation and can be buffered against inappropriate signals. Thus, they are remarkably robust to perturbation [37]. However, a disruption may impair the coordination of drug metabolism and pharmacodynamics over the 24-h cycle [49]. Malignant tumors and cancer-bearing hosts may exhibit nearly normal or markedly altered circadian rhythms. Such alterations seem to depend upon tumor type, growth rate and level of differentiation. These alterations consist of amplitude damping, phase shifts, and/or appearance of ultradian rhythms; they usually worsen in the course of cancer progression [84].

Optimally timed treatments assume gross circadian synchrony of the population to be treated, with intent to diminish toxicity and enhance therapeutic efficacy [87]. But a standardized chronomodulated delivery schedule may not be appropriate for an entire patient

population [13]. Since intra and inter-individual differences may not be negligible, chronotherapy may be further improved by selecting treatment timing according to individual circadian marker rhythms, further diminishing between individual differences, and further enhancing the therapeutic index of cancer treatment [18]. Mathematical and systems biology approaches currently develop and integrate theoretical, experimental, and technological tools in order to further optimize and personalize the circadian administration of cancer treatments [49].

The design of trials to evaluate the relevance of chronotherapy may also raise some important questions, as already seen. To conduct a trustful trial, circadian-based questions need to be asked in a way that can elicit clear answers; otherwise no useful information can result [87]. Applying chronobiology concepts in oncology is by itself already a challenge, due to the characteristics of each tumor with entirely different profiles, that differ according to type, evolution and differentiation, due to the patients intra and inter-individual variability and due to the particularities and diversity of anti-cancer drugs. So, in this area it may not be as easy as a switching to morning–evening administration or vice versa, that is simple and inexpensive, as done for some relevant classes of drugs commonly used by internists [23]. Conversely, the gains obtained in this particular disease could have significant impact in the care of patients.

Economics aspects of cancer chronotherapy are also questions to be addressed to evaluate the feasibility and economy of the chronotherapeutic procedures. If one consider, for example, the need of schedule a large quantity of patients to be submitted to radiotherapy in a same limited period of time a logistic problem may arise, since the equipment available may be insufficient to respond to that demand. Chronomodulated infusion seems to be more expensive, requiring dedicated electronic pumps and several disposable materials. However, Tampellini et al. [120] compared the direct costs of two regimens (chronochemotherapy and

FOLFOX regimen in the treatment of patients with metastatic colorectal cancer) and concluded that there is a pharmaco-economic benefit resulting from the decrease in toxicity in chronochemotherapy. In fact, direct costs for a single chronotherapy cycle appeared to be comparable to a single course of the standard regimen. The major material cost of chronochemotherapy devices was balanced by a better tolerability profile. They further stated that the improvement in quality of life with chronochemotherapy affecting indirect costs, such as reduction of work, and intangible costs is worthy of further pharmacoeconomic attention [120]. Even though, a large heterogeneity exists with regard to the economic implications of cancer chronotherapeutics across countries [19], as a further prospective study taking into account the cost-effectiveness ratio of the treatments to provide a more comprehensive comparison of the regimens is still lacking.

Besides the challenges chronotherapy has to face, and all the hurdles it has to handle to, in no case to date it has been shown to be less effective than standard scheduling, except for those gender differences reported, with circadian-based studies being published in peer reviewed, highly respected medical journals.

All these observed, the question is: why chronotherapy is not a widely approach in cancer treatments? Several reasons are found and have almost nothing to do with the science, including money, attitude problems, logistics, and dogma, as reviewed in another thesis [7].

One of the particular issues raised is the general ignorance regarding chronobiology. Most oncologists still have never heard of chronotherapy. Likewise, patients and health insurance companies lack of any awareness of the potentials of chronotherapy [7]. Also, a large quantity of articles is not in English. Besides, the massive volume of information that confronts the working clinician makes easy that some findings become overlooked: in 1997, when one of the first large chronotherapy studies in colorectal cancer conducted by Lévi was

published in the Lancet, 3414 other articles were published relating to colorectal cancer alone [7].

Another explanation resides in the fact that chronotherapy is mainly a foundational idea: it challenges medical dogmas [7]. It ceases to be legitimate to talk about constancy [2]. Accepting the concept, it may be proved that giving medications at any random time of day may, in fact, be harmful to the patient [1,7,25,64]. In this sense, chronotherapy may be a huge liability: who could be liable for having delivered drugs at the wrong times? The idea behind chronotherapy directly threatens one of the central pillars of physicians' self image: the notion that the doctors themselves may be actively causing harm by ignoring facts that there are in the general medical literature for 30 years is hardly accepted [7] - a situation that Hrushesky compared to Semmelweis, who in the 19th century first suggested that medical students might be transmitting diseases to their patients by not washing their hands.

Regarding money justifications, pharmaceutical companies are the major financial sponsors of clinical cancer research based on the need to be able to sell a product and turn a profit. Chronotherapy offers nothing for a drug company to sell. At best, it improves the drugs that are already on the market, those upon which oncologists already depend. They have no obvious financial incentive to improve a drug's efficacy by specifying a time of day [7]. Moreover, it’s difficult to get the pharmaceutical industry to invest in novel ideas when a potential liability issue emerges.

Some of the hurdles that exist for further research are merely bureaucratic [85].

Unlike researchers, clinicians tend to be more precautious about new ideas, especially in the cancer field. They require a great deal of scientific evidence before they change their practices [7]. Is there enough evidence to support chronotherapy?

Indeed, chronopharmacology has had limited influence on current standard treatment recommendations for human cancers. Some studies resulted, in fact, in a new consensus of

treatment, as seen for childhood acute lymphoblastic leukemia [86]. Others contributed to the development of a new medico-surgical strategy with curative intent for patients with initially unresectable liver metastases from colorectal cancer [13]. The majority of randomized experiments do show a difference in tolerability or dose intensity that generally favors chronomodulation [118]. The largest study in cancer chronotherapy did provide evidence supporting the use of its principles in male patients [97]. The clinical development of standardized chronotherapeutics was the first to reveal the safety and antitumor activity of oxaliplatin in patients with metastatic colorectal cancer [49]. There are also multiple small studies in almost all cancer origins that recapitulate the same or very similar chronotherapy values, with statistically significant results, but that need larger prospective assessments to draw definite solid conclusions [118]. Yet, chronotherapy has not entered the mainstream of clinical oncology. The simplest and only true means to solve this dilemma is to conduct well designed and definitive clinical trials [118], knowing that large trials are hard to organize and require substantial resources [7]. Another limit chronotherapy has to face is the rapid pace of progress in developmental therapeutics and regimens, and the need it has to be incorporated as an exploitable advantage for cancer chemotherapy [22].

Basically, chronotherapy is a new therapeutic concept that must contend with the interests of drug companies, insurance providers and medical systems resistant to change, along with unscientific objections [7]. Its principles are universally applicable, but to become an established therapeutic methodology it needs to prevail over the current limitations. To be more than a concept, an idea, or an argument, it is going to need continued confirmation as well as powerful advocacy [7]. These are maybe the major hurdles for chronotherapy.

FUTURE PRESPECTIVES

Circadian-based cancer treatments deserve further investigation and several approaches promise to be easily applied in clinical daily routine in the near future.

First of all, the dosing time for existing drugs may be explored and tested in cancer treatments [47]. Also, chronotolerance and chronopharmacology should be taken into account in all new drugs formulation and protocols [49].

Secondly, research may focus in drugs to target the molecular clock. Therapeutic delivery to the dynamics of the cross-talk among the circadian clock, the cell division cycle, and pharmacology pathways represents a new multidisciplinary challenge to concurrently improve quality of life and survival through personalized cancer chronotherapeutics [10]. For example, BMAL1 that is constitutively expressed in pleural mesothelioma is a potential therapeutic target in treatment of this specific disease [50]. Also, since small intestinal and colonic polyps were increased under altered expression of PER2 gene product, which seems to suppresses tumorigenesis, this another circadian core clock gene may represent a novel target for colorectal cancer prevention and control [76]. Compounds that affect circadian rhythm exist with attendant future therapeutic possibilities. These include casein kinase I inhibitors and a candidate small molecule KL001 that affects the degradation of cryptochrome. A synthetic ROR agonist, SR1078 stabilized p53 and induced apoptosis in preclinical studies, demonstrating that ROR agonists may have anti-cancer therapeutic potential [50]. Knowledge of the molecular clock with it is points of coupling with the cell cycle may prove a new strategy to intelligently target cancer cells. Besides, it was suggested that telomere length and telomerase function that are a measure of the days, weeks and years in the life of a mammalian cell, and they too can be corrupted, contributing to malignancy, can be interesting therapeutic targets [50].

Third, the early detection of circadian disruption before and during treatment through monitoring of circadian biomarkers allow the adjustment and modification of drug doses in order to significantly improve outcomes [49]. Moreover, rhythm monitoring prior to chronochemotherapy allows the determination of the circadian phase of the individual, permitting the delivery of drugs both at their optimal internal timing and proper dose levels [119]. More than a therapeutic individualization, rhythm monitoring in large scale patients leads to the identification of responders to chronomodulated chemotherapy and the definition of sub-groups of patients [49].

Fourth, the prevention of circadian disruption, and/or the restoration or even the manipulation of functional clocks, ensuring an adequate synchronization are of vital importance [121]. This way, the risk of wrongly administering the treatment at the best timing extrapolated from experimental data in a patient out of synchrony, and, thus at a stage of rhythm that is not the best for that individual, will be minimized.

Besides therapeutic adjustment, rhythm biomarkers monitoring allows the properly reinforcement of the circadian timing system. This can be achieved through circadian lifestyle interventions (meal timing, adequate physical exercise, morning light exposure) [65] and small molecules, such as seliciclib [122] - both revealed to be capable of inhibit cancer growth. The administration of chronobiotic agents, like synthetic melatonin as an adjuvant therapy is also an option [35].

It is to be recalled that there is also the possibility of pharmacotherapy based on the intra and inter-individual variability of clock genes [18].

Lastly, correlative studies opened new avenues regarding achievable lifestyle recommendations to prevent circadian disruption as it may be seemed as a public health concern [40]. The knowledge acquired can further be used to develop lighting regimens or sources that mitigate the adverse consequences of altered light exposure and reduce circadian

misalignment in shift workers [35]. For example, bright light exposure at the beginning of the night, along with attenuation of light (sun-glasses) in the morning, was found to aid night workers in maintaining alertness at night while sleeping during the day [65].

SUMMARY AND CONCLUSION

Circadian rhythms are a feature of life [2]. They have an evolutionary origin and are of vital physiological importance. The organization of the internal time-keeping mechanism is operated hierarchically by the feedback loops of the clock genes in the mammalian central pacemaker, and in peripheral tissues [5,27,32,42]. The circadian timing system regulates endless functions in the human body, including molecular, physiological, biochemical and behavioral processes [32,50].

The growing understanding of the importance of biological rhythms and chronobiology in human disease led to the recognition that timing is not a negligible factor in Medicine, especially in the oncology area where patients usually have an unfavorable prognostic and a poor quality of life.

Disruption of circadian rhythms has been linked to mammalian tumorigenesis and tumor progression [25,67–71], and has been used as an independent prognostic factor of survival time for patients with certain metastatic cancers [59–62]. The impact of these discoveries is corroborated by epidemiological studies and circadian-related disorders are currently a public health concern [17,28,58].

Scientific research clarified that the balance between health and disease may be highly dependent on the proper synchrony within and between oscillating systems. Genetic or functional disruption of the molecular circadian clock has been found in various cancers. Also, significant associations between clock genes polymorphisms and increased or decreased

cancer risk are reported. There is a clear our circadian rhythm for cell growth and proliferation, DNA synthesis, apoptosis, responses to genotoxic stress, and activities of drug catabolic enzymes in humans, which lastly also determines the pharmacologic effects of anticancer agents [25].

Hence, in clinical practice, time of drug administration matters, affecting by several folds the efficacy and side effects of many treatments. Circadian sensibility to anti-cancer agents manifested itself by an overlap between chronotolerance and chronoefficacy [49]. Based on the asynchrony that exists in cell proliferation and drug metabolism between normal and malignant tissues, experimental models (including laboratorial studies with mice, cell-based assays and mathematical model systems) allowed the translation of those results into cancer clinical trials. Thus, the theoretical concepts of chronobiology and chronotherapy principles were challenged by practical results.

Indeed, chronotherapy has been demonstrated to be a valuable treatment approach in several types of cancer, using classical drugs, drugs combinations and radiotherapy. To date, only a very few large randomized controlled clinical trials were done. The extrapolation of the least toxic times of chemotherapy from mice to human beings was particularly validated in male patients with metastatic colorectal cancer in clinical Phase III trials, with significant improvements in tolerability and efficacy [97,98].

Besides the fact that some particular endpoints didn't give always preference to circadian-based therapies, in no case to date has chronotherapy been shown to be less effective than standard approaches. Indeed, chronomodulated schedules allow an increase in dose intensity and have a better tolerability profile improving quality of patients' life, which favors the circadian-based treatments. The use of programmable in-time pumps accounts for chronotherapy convenience and feasibility in ambulatory regimen, and represented an

important advance in cancer chronotherapeutics. Since cancer is associated with high mortality rates, the quality of the time patients do have is extremely important.

So far, however, only a very small number of studies resulted, in fact, in a new consensus of treatment. The clinical development of standardized chronotherapeutics was the first to reveal the safety and antitumor activity of oxaliplatin in patients with metastatic colorectal cancer, to trigger a new medical and surgical strategy with curative intent for this disease, and to specify the need for chronomodulated infusion protocols for nonhospitalized patients [49]. Robustness of circadian-related results in the treatment of acute lymphoblastic leukemia also influenced the treatment strategy [86,123]. Yet, chronopharmacology affected modestly the current conventional therapeutic recommendations for human cancers, facing hurdles that include money, attitude problems, logistics, and dogma [7]. To become an established therapeutic methodology chronotherapy has to defeat the current limitations.

Since chronotherapy is not the searching for the ideal anti-cancer agent, but it attempts the optimization of treatments according to time, its great exploitable advantage is the possibility to apply this concept to all treatment approaches in cancer, what makes chronobiology principles virtually universally pertinent.

The future progress of chronotherapeutics requires its integration into the development of anticancer drugs, ranging from screening to clinical phases. Appropriate well designed large clinical trials capable of support definitive conclusions are warranted. Besides, the molecular clock has to be looked as a promising therapeutic target, along with the monitoring of circadian rhythmicity, prevention of its disruption and restoration of its functionality. In addition, gender and other intra and inter individual differences in the optimal circadian timing and dosing of anticancer drugs need to be further investigated.

Answering the question raised in the introduction of this paper, it is the moment to affirm that it was already proved that chronotherapy has a relevant role in oncology, which

demands to be further explored and applied in clinical practice. Besides long time known, chronobiology is only now taking the first steps asserting itself through chronotherapy. It is submitted that the challenge is precisely to make the bond between the fields of research to routine clinical practice.

To conclude, it is to be recalled that understanding the chronobiology principles has the potential to contribute to improve outcomes, and can open research ground for the development of new prevention and treatment strategies, defining better standards of care. The fundamental principles of chronotherapy are worthy of further clinical implementation and the future is advancing towards personalized cancer chronotherapeutics.

LIST OF ABREVIATIONS USED IN THIS PAPER

24-h: 24 hours;

5-FU: 5-fluorouracil;

BMAL1: Brain-muscle Arnt like 1 - brain and muscle aryl hydrocarbon receptor nuclear translocator;

ChronoFLO: 5-fluorouracil, leucovorin, and oxaliplatin delivered on a circadian-based, chronomodulated schedule;

CKIE: Casein kinase Iε;

CLOCK: Circadian Locomotor Output Cycles Kaput;

CPT11: irinotecan;

CRY1; CRY2: Cryptochromes 1 and 2;

DNA: deoxyribonucleic acid;

FOL: folinic acid;

FOLFOX: conventional delivery schedule associating 5-FU, leucovorin, and oxaliplatin;

L-OHP: oxaliplatin;

LV: leucovorin;

mRNA: messenger ribonucleic acid;

NAD^+: nicotinamide adenine dinucleotide;

PER1; PER2; PER3: Period (gene or protein);

RevErbA; RORA; ROR elements: retinoic acid-related orphan nuclear receptors;

RNA: ribonucleic acid;

SCN: suprachiasmatic nuclei;

ZT: Zeitgeber Time.

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