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Circadian rhythms and cancer hallmarks: toward advances in immune-based therapeutics, and outcomes

Edited by
Elizabeth Cash, Sandra E. Sephton and Pasquale F. Innominato

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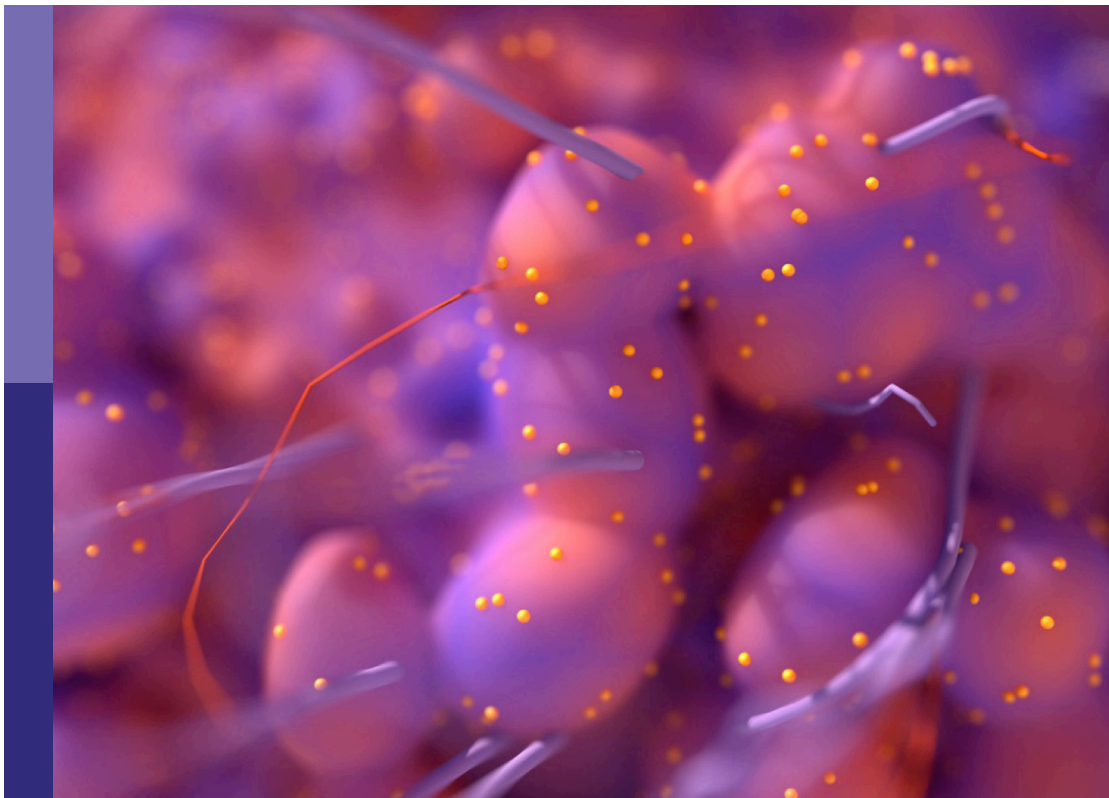

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Circadian rhythms and cancer hallmarks: toward advances in immune-based therapeutics, and outcomes

Topic editors

Elizabeth Cash — University of Louisville, United States

Sandra E. Sephton — Brigham Young University, United States

Pasquale F. Innominato — University of Warwick, United Kingdom

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Peter Brossart,
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*CORRESPONDENCE
Elizabeth Cash
Liz.cash@louisville.edu

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Editorial: Circadian rhythms and cancer hallmarks: toward advances in immune-based therapeutics, and outcomes

Elizabeth Cash[1,2*], Pasquale F. Innominato[3,4,5] and Sandra E. Sephton[6]

[1]Department of Otolaryngology-Head and Neck Surgery and Communicative Disorders, University of Louisville School of Medicine, Louisville, KY, United States, [2]UofL Health-Brown Cancer Center, Louisville, KY, United States, [3]Oncology Department, Ysbyty Gwynedd, Betsi Cadwaladr University Health Board, Bangor, United Kingdom, [4]Cancer Chronotherapy Team, Warwick Medical School, University of Warwick, Coventry, United Kingdom, [5]Research Unit, 'Chronotherapy, Cancers and Transplantation', Faculty of Medicine, Paris-Saclay University, Villejuif, France, [6]Department of Psychology, Brigham Young University, Provo, UT, United States

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Editorial on the Research Topic
Circadian rhythms and cancer hallmarks: toward advances in immune-based therapeutics, and outcomes

Introduction

Circadian rhythms are the daily fluctuations in physiological processes that govern cell cycles and the timing of behaviors (1). Cancer hallmarks are the cellular properties that drive the perennial growth, survival and spread of cancerous cells (2). The disruption of circadian rhythms can contribute to the development of some cancers by affecting the expression of cancer hallmarks (3). Circadian rhythms also temporally regulate cellular immunity, which has important implications for the burgeoning field of immune-based therapeutics (4). Cancer patients exhibiting disrupted circadian rhythms tend to suffer from accelerated tumor growth and metastasis, worse anti-cancer treatment outcomes, and poorer health-related quality of life and overall survival (5). This Research Topic aims to help elucidate the impact of circadian rhythms on processes associated with cancer and its treatments, particularly immunotherapy. We were pleased to accept 11 original manuscripts for this Research Topic.

Molecular processes

Using a variety of various approaches, several groups have explored the molecular underpinnings of the connection between the circadian clock and cancer. Pan et al. extensively reviewed the role of E-box-binding transcription factors in cell physiology and cancer biology, along with their potential as novel therapeutic targets. The broad group

of E-box-binding transcription factors includes two core clock genes, BMAL-1 and CLOCK. This work advances our knowledge of potential therapeutic targets in cancer treatment. Zheng et al. focused on the latest evidence implicating circadian rhythm disruption as a causal factor in endometrial cancer and further explored the potential for mediation of these effects by non-coding RNAs. New research implicating irregular expression of circadian-linked ncRNAs in endometrial cancer cells is described, which may have implications for future targeted therapeutic strategies. Meng et al. deployed a Mendelian randomization analytical approach to large international databases to explore the impact of five genetically independent circadian features on colorectal cancer risk. Strikingly, the authors observed that an individual's chronotype can significantly contribute to the lifetime risk of developing colorectal cancer. Peng et al. offered a multi-gene prognostic model developed using circadian genes that demonstrated predictive performance for gynecologic cancer prognosis. This bioinformatics approach – validated with human data – also provides insights into potential immunotherapy targets by elucidating immune signaling pathways associated with high-risk circadian gene profiles.

Clinical relationships

Several groups have critiqued or provided data suggesting the importance of sleep and circadian influences on cancer risk and outcomes. Gouldthorpe et al., reviewing studies that incorporate objective circadian rhythm measurement, provided a useful compendium of the various indices used to summarize circadian endocrine data, actigraphy data, and sleep-wake cycles, with an urgent call for standardization of measurement. The need for standardization was also highlighted by Jagielo et al., who found that both cortisol dysregulation and abnormal rest-activity rhythms are clearly linked with psychological comorbidities in advanced cancer patients, such as pain, fatigue, nausea, vomiting, and cachexia. Promising data on chronomodulated chemotherapy and circadian-targeting behavioral interventions are discussed. Nettnin et al. highlighted a new and promising approach to treatment that regulates circadian function in gliomas, pointing to the importance of targeting circadian regulation in the tumor microenvironment and the distinct need for research in pediatric cancers. Burch et al. explored the association between formally diagnosed sleep disorders and cancer occurrence in a robust sample of Veterans. They identified an optimal sleep duration for protection against oncogenesis: elevated lifetime cancer risk was noted in those who slept on average less than 6, or more than 8, hours per night. Interestingly, both greater severity and longer duration of sleep disorders showed an impact on cancer incidence. Finally, Cash et al. reported on pilot data that open avenues for further exploration of the links between diurnal cortisol expression, head and neck cancer progression, and the potential role of inflammation. They emphasize the importance of multi-day cortisol sampling. Taken together, these data suggest that by recognizing the timing of treatment in relation to cortisol levels, clinicians could optimize treatment schedules to align with patients' circadian rhythms, potentially enhancing therapeutic outcomes.

Immunotherapy

Through their unique mechanism of action, immune checkpoint inhibitors are influenced by host physiology, including circadian rhythms. Two mini reviews summarized the growing evidence of interactions between host physiology and checkpoint inhibitors. In the first, Hughes et al. critically revised how light exposure, physical exercise and diet, and notably their respective timing over 24 hours, can impact immunotherapy efficacy in patients with cancer. Balachandran et al. reviewed important emerging data suggesting that sleep disturbance is inversely correlated with tumor response to immunotherapy. Nascent links to the microbiome as a mechanism for these effects are considered along with remaining unanswered questions, such as whether these interconnections can be exploited to improve patient response to immunotherapy. These reviews lay the foundations for novel therapeutic avenues with potential circadian-based lifestyle modifications that could be implemented to manipulate cancer immune responsiveness and maximize the benefit of immunotherapy (6).

Conclusion

This Research Topic highlights circadian effects on tumor outcome from two perspectives: that of the host (cancer patient) and that of the tumor and its associated microenvironment. These two perspectives offer unique research questions. To improve immunotherapy outcomes, we must establish the mechanisms of circadian effects on tumor growth and acquisition of cancer hallmarks. This line of inquiry can lead to behavioral and pharmacotherapeutic interventions to improve immunotherapy efficacy.

Research must elucidate the molecular clock function in tumors, and how tumors disrupt circadian rhythmicity within their microenvironment. Host and tumor circadian disruptions are rarely studied within the same organism. Such data will inform on bidirectional effects in host–tumor circadian relationships: e.g., do patients with disrupted rhythms have tumors that suppress or promote their own circadian genes? Research imperatives include establishing the temporal precedence of circadian disruption in the development of cancer, whether bidirectional pathways of circadian regulation exist between host and tumor, and a focus on circadian rhythm disorders in malignant and nonmalignant clinical populations. Standardization of protocols for the assessment of popular measures (e.g., cortisol, melatonin) is needed in addition to the pursuit of lesser-studied measures (e.g., core body temperature, pupillometry, blood pressure dipping). One notable area ripe for inquiry regards the effects of sleep and circadian disruption in cancer treatment efficacy (e.g., immunotherapy). We should leverage biorepositories and epidemiological-level circadian data. Despite the need for more research, clear clinical implications include the need for healthcare providers to assess and treat sleep and circadian

disruption in cancer patients. While new technologies for measuring, for example, clock gene expression, are promising, many protocols remain potentially burdensome for patients. There remains an urgent need for translation of circadian measurement into clinical cancer settings to inform individualized clinical interventions.

Author contributions

EC: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. PI: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. SS: Conceptualization, Investigation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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EDITED BY
Florian A. Siebzehnrubl,
Cardiff University, United Kingdom

REVIEWED BY
Dongyin Guan,
Baylor College of Medicine, United States
Héctor R. Contreras,
University of Chile, Chile

*CORRESPONDENCE
Steve A. Kay
stevekay@usc.edu

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E-box binding transcription factors in cancer

Yuanzhong Pan[1], Pauline J. van der Watt[2,3] and Steve A. Kay[1]*

[1]Department of Neurology, Keck School of Medicine, University of Southern California, Los Angeles, CA, United States, [2]Division of Medical Biochemistry and Structural Biology, Department of Integrative Biomedical Sciences, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa, [3]Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa

E-boxes are important regulatory elements in the eukaryotic genome. Transcription factors can bind to E-boxes through their basic helix-loop-helix or zinc finger domain to regulate gene transcription. E-box-binding transcription factors (EBTFs) are important regulators of development and essential for physiological activities of the cell. The fundamental role of EBTFs in cancer has been highlighted by studies on the canonical oncogene MYC, yet many EBTFs exhibit common features, implying the existence of shared molecular principles of how they are involved in tumorigenesis. A comprehensive analysis of TFs that share the basic function of binding to E-boxes has been lacking. Here, we review the structure of EBTFs, their common features in regulating transcription, their physiological functions, and their mutual regulation. We also discuss their converging functions in cancer biology, their potential to be targeted as a regulatory network, and recent progress in drug development targeting these factors in cancer therapy.

KEYWORDS

E-box, circadian clock, cancer hallmarks, transcription factor, transcription control

Introduction

An E-box is a regulatory motif of DNA, with the consensus sequence 5'-CANNTG-3', that is found abundantly in most eukaryotic genomes. An E-box is a regulatory motif of DNA, with the consensus sequence 5'-CANNTG-3', that is found abundantly in most eukaryotic genomes (see Box 1). Transcription factors (TFs) can bind to E-boxes in the promoter and enhancer region of genes through their basic helix-loop-helix (bHLH) domain or zinc finger domain to regulate their expression. E-box-binding TFs (EBTFs) regulate genes that are diverse in function. During development EBTFs determine the lineage commitment of skeletal muscle, cardiovascular and neuronal tissues, as well as hematopoiesis. In homeostasis they regulate many housekeeping genes and essential physiological processes, such as the cell cycle, circadian rhythm, and metabolism. Therefore, it is not surprising that EBTFs have fundamental functions in maintaining homeostasis and are deeply involved in tumorigenesis.

Several families of EBTFs are widely studied in cancer biology. The MYC family of proteins feature prominently, as around 28% of tumors harbor at least one amplification of a MYC

Box 1 E-box overview.

E-boxes were initially discovered in the promoter region of immunoglobulins to regulate their expression (1, 2), and later found to be widely present in eukaryotic genomes. The most prominent transcriptional regulators that bind E-boxes are the basic Helix-Loop-Helix (bHLH) proteins, which binds to the E-box through their bHLH domain (3). These proteins bind to DNA as hetero- or homodimers, in which the HLH parts interact to form dimers and the basic domain in the longer helix forms an α-helix to insert into the major groove of DNA to form a noncovalent bond. Some zinc finger domains can also bind to E-boxes, but the structural details are not clear. Distinct binding motifs of the proteins may have differential preferences to different variants of E-boxes (3). The binding specificity also depends on other factors such as their binding partner, flanking sequences near the E-box, and chromatin state (4, 5). Once a TF binds to an E-box, it can either activate or repress downstream gene transcription, depending on the cofactor and the cell context.

In homeostasis, E-boxes can be grouped by their binding TFs to regulate specialized physiological functions, such as MYC in the cell cycle, BMAL1 and CLOCK/NPAS2 in the circadian rhythm, HIFs in the hypoxia response, and EMT-TFs in embryonic development. These processes are delicately coordinated by their mutual regulations. One important node that connects their function is that these TF themselves are all regulated by E-boxes in their promoters. In addition, the turnover of cellular EBTFs is regulated by many shared mechanisms including transcriptional feedback control, miRNA control, and protein kinetics mediated by the ubiquitin-proteasomal system. The target genes and binding consequences of EBTFs in a tumorigenic context can be significantly different from those in a normal physiological background. This should be kept in mind when comparing and interpreting different studies on the EBTFs.

Another prominent function of EBTFs is their regulation of the development of specific tissues. Examples include heart and neural crest derivatives expressed (HAND) family TFs in the development of heart and lineage commitment of extraembryonic tissues; myoblast determination protein (MyoD) family factors in the differentiation of skeletal muscle tissues; neurogenic differentiation (NERUOD) family factors in neuronal tissue development; and T cell-acute leukemia protein (TAL1, also known as stem-cell leukemia factor, SCL) family factors in the regulation of hematopoiesis and angiogenesis. Since differentiation and proliferation potentials are in general considered mutually exclusive, these factors are generally not reported to be oncogenic.

Features of the promoters/enhancers in the genes that regulate developmental and essential cell activities have vastly different features, resulting from distinct motif components and binding factors (6). Thus E-boxes can be divided into at least two functional subgroups: those involved in tissue development and those involved in homeostasis maintenance.

paralog, making it one of the most dysregulated oncogenic genes in human cancer (7). Hypoxia-inducible factor (HIF) proteins are also well known EBTFs, and the hypoxic hallmark of solid tumors has attracted much attention to this E-box-binding family of genes, which have also been shown to regulate many other processes in tumor development (8). Further EBTFs include those that regulate the epithelial-mesenchymal transition (EMT). It is generally accepted that these TFs are associated with stem cell features of cancer cells (9, 10). More lately, our lab and others have shown that the master circadian E-box-binding regulators, BMAL1 and CLOCK, also have important functions in several cancer types (11–14). These examples emphasize the importance of EBTFs in cancer biology.

Despite all the progress that has been made, the exact molecular roles of EBTFs in cancer is far from clear. In particular, a basic understanding of how these TFs select and participate in the transcription processes mediated by PolII is still lacking. In recent years, we have witnessed great progress in understanding the PolII transcriptional machinery in detail and the biology of regulatory elements in DNA. Such new knowledge provides unprecedented opportunities to rethink EBTFs in their most native role as DNA-binding proteins. By doing so we might be able to better understand this family of proteins and develop better strategies to target the TFs in cancer.

In this review, we review current understandings of the structure and molecular biology of the EBTF families that have been shown to play important roles in tumorigenesis. We discuss their mutual regulation to gain some insights into how these proteins are coordinated during tumorigenesis and tumor suppression, and we summarize the common processes they convergently regulate. At last, we propose that targeting the whole EBTF network in specific cancer types could be effective in suppressing multiple hallmarks of cancer simultaneously and have potential as a cancer therapeutic strategy.

Important EBTFs in cancer

MYC family proteins

MYC was first discovered as a homolog of the viral oncogene *v-myc* in multiple chick retroviruses; thus the gene was named cellular-MYC (c-MYC) to specify its endogeneity. The MYC family has three members: the most prominent c-MYC, MYCN, which was initially found to be associated with neuroblastoma, and MYCL associated with small cell lung cancer, hence the names (15). The three MYC proteins have relatively limited sequence consensus, but they all share the entirely conserved bHLH-Leucine Zipper (bHLH-LZ) domain that binds to the E-box, and six highly conserved MYC boxes (MYCL lacks MB3a) that are known for interacting with other proteins (16). (Figure 1A, blue block) MYC will be used to refer to c-MYC in this article. Upon heterodimerization with its partner, such as MAX, MYC preferentially bind to the canonical E-box sequence 5'-CACGTG-3' (20, 21).

The role of MYC in human tumorigenesis was exemplified by the translocated MYC coding sequences downstream from the immunoglobulin heavy chain enhancer in Burkitt lymphomas (22–24). Since then, MYC is found to be one of the most dysregulated, usually over-activated, oncogenic gene in human cancers (7). It is classified as a tumors-driving master transcription factor (MTF) in certain cancer types (25). Overexpression of MYC alone is sufficient to trigger a cancerous phenotypic change in cultured cells, and to induce *de novo* tumorigenesis in multiple mouse models (6, 26). The importance of MYC is also underscored by the fact that repression of MYC can result in fast regression of tumors in animal models, making it a promising target for tumor therapy (21). Despite its pivotal role in tumorigenesis and the great attention it attracted, the exact behavior of MYC is still far from clear.

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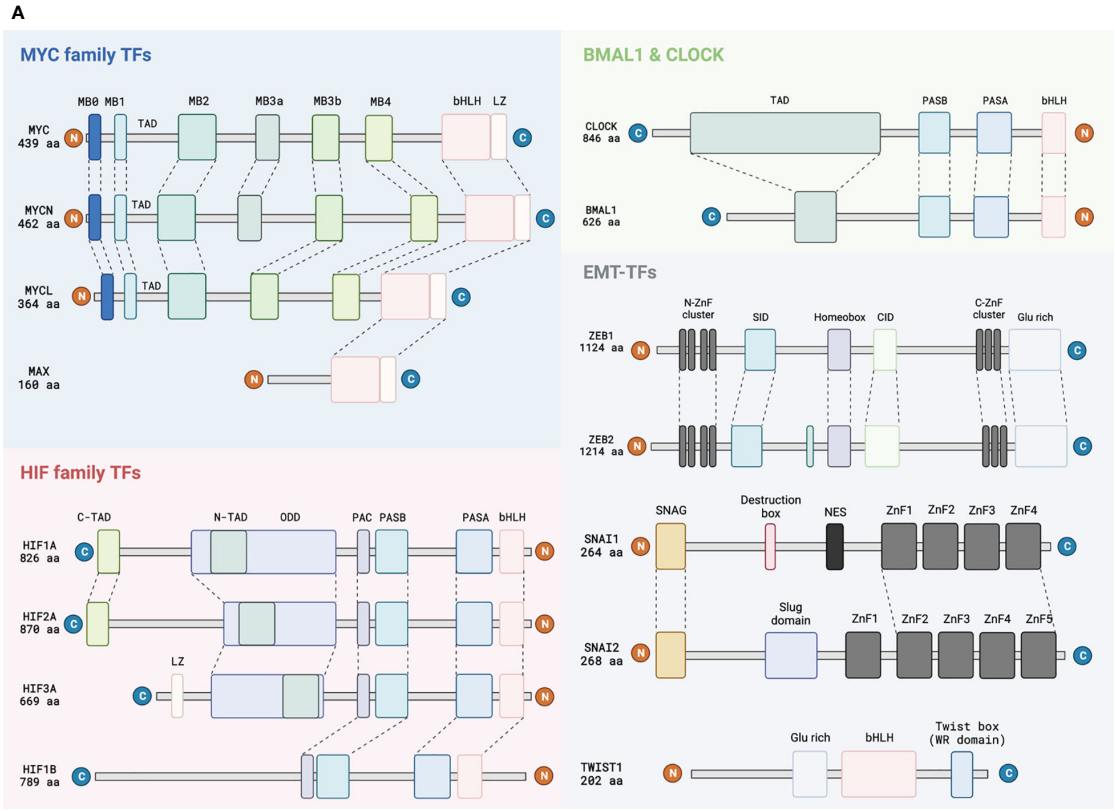


FIGURE 1
Structural overview of EBTFs. **(A)** Functional domains of EBTFs. Proteins can bind to E-boxes through a bHLH domain or a zinc finger domain. MYC family genes contain an extra leucine zipper domain downstream of bHLH. The three MYC family genes share highly conserved MYC-boxes (MB), except for MYCL lacking the MB3a. Other regions of the proteins have low degrees of conservation. MYC also contains a PEST domain (not shown) that might contribute to its fast turnover. MAX is a relatively small protein with a defined bHLH-LZ domain, but functions of the other regions of the protein are not well understood. Apart from MYC, MAX can also dimerize with itself or other bHLH TFs such as MAD and MLX. HIF family TFs feature the bHLH-PAS family TFs, which have a PAS-A and PAS-B domain immediately upstream of the bHLH. The PAS domain provides additional control of protein dimerization and might have a responsive function to environmental cues. A PAS-associated COOH-terminal (PAC) occurs C-terminal to the PAS motifs and is proposed to contribute to the PAS domain fold. The α-subunits of HIF proteins has an oxygen-dependent degradation (ODD) domain that contains two conserved prolyl residue (402 ODD and 564 ODD) that can be hydroxylated and induce proteasome-mediated degradation. They also have two transactivation domains (C-TAD and N-TAD) that facilitate target gene expression. BMAL1 and CLOCK are also bHLH-PAS family TFs, and BMAL1 has a defined TAD in the N-terminus. The ZEB and SNAI family bind E-boxes through their zinc fingers as monomers. ZEB1 and ZEB2 share up to 85% amino acid sequence homology in their zinc finger clusters but have low conservancy elsewhere. They both have a homeobox which seems to not bind DNA. Other defined domains are best known for interacting with other proteins. SNAI family proteins are featured by their conserved SNAG domain which is initially found in SNAI and GFI family proteins and a zinc finger cluster in the C-terminus. TWIST is a small bHLH protein that contains a characteristic TWIST box and binds to DNA as dimers. **(B)** Left: Structure of the bHLH-LZ domain of the MYC : MAX dimer binding to DNA (17). Middle: Structure of the bHLH domain of human BMAL : CLOCK dimer binding to DNA (18). Right: Structure of mouse HIF1A:ARNT bHLH-PAS domain dimer binding to DNA (19).

The fundamental of MYC biology is that MYC function differs when expressed at high levels, as in many tumor cells, versus at relatively low physiological levels (16). MYC expression is ubiquitous but is delicately regulated to be kept at a low level in normal tissue. The turnover of MYC proteins is fast with a half-life of around 30 minutes (27). When in low abundance, MYC mostly binds to E-boxes and their close variants, whereas when overexpressed, it binds to more non-specific binding sites (28). This feature might be a result of the intrinsic disordered properties of the MYC protein, which allows it to dynamically interact with multiple partners in modest affinity. Therefore, in high concentrations, MYC specificity is easily overridden by a mass-action drive, leading to superfluous binding (20). The consequence of MYC binding is complex. There are currently several models describing the mechanism.

Classically, MYC is thought to be a pleiotropic transcription factor that activates, rather weakly, the transcription of genes through binding to the E-boxes in their promoters, as a heterodimer with its canonical cofactor MAX, which is also a bHLH-LZ protein (16). This model implies a group of "target genes" that are regulated by MYC. Attempts to recognize a set of MYC target genes using different large-scale analyses has resulted in sets with surprisingly small overlaps (16). This disparity might be a result of the abundance of E-boxes in the genome and their different open states in different cells, since MYC is generally considered a non-pioneer transcription factor, meaning that it only binds to chromatin regions that are already accessible but cannot open a closed chromatin, as was clearly shown in iPSC studies (29). To some extent, there is consensus over MYC target genes, including the HALLMARK MYC Target gene sets proposed by *Liberzon et al.* in the molecular signature database (MSigDB) (30). Such core common target gene sets have been useful as indicators of MYC activity in hypotheses-generating cases, but they should not be used to preclude potential genes regulated by MYC, especially in cancerous contexts where chromatin accessibility is largely remodeled and mutations in regulatory elements are common, resulting in *de novo* binding sites of TFs.

Apart from being an activator, MYC has also been proposed as a repressor of gene expression (31). The most studied repressive mechanism of MYC is through its interaction with two other proteins, MIZ1 (32) and SP1 (33), to recruit co-repressors. It has been proposed that the repressive function of MYC is of comparable importance to the activating function (34), although genomic-level correlation analysis implies rather weak effects of the repressive function of TFs in general (35). Finally, it is noteworthy that MYC can also regulate RNAPII and RNAPIII-mediated transcription of ribosomal RNA and tRNA (36, 37), but this function is out of the scope of this review.

As a weak activator model is insufficient to explain its broad participation in various physiological processes of the cell and its strong tumorigenic effect, later a "general gene amplifier" model was proposed by two simultaneous papers and introduced a new view of MYC (38, 39). According to this model, MYC can act as an amplifier that increases the overall RNA production of the whole cell. This model can explain some observations in MYC-driven tumorigenesis, but still oversimplifies MYC function since elevated RNA production is neither sufficient nor necessary for tumorigenesis, and it fails to explain the complicated up- and down-regulation of genes after its levels change.

Given the different behaviors of MYC at distinct levels in the cell, a gene-specific affinity model has also been proposed (16, 40). According to this model, promoters of different genes require different levels of MYC protein to activate their transcription. This is possible due to the relatively low affinity of MYC-MAX binding. This model provides an explanation of the paradox of broad DNA-binding and specific gene regulation by MYC, but still lacks the ability to unify MYC function in different tumors and ignores the broad involvement of MYC in multiple processes of RNAPII-mediated transcription.

Recent progress on characterizing the protein interactome of MYC has shed new lights on understanding the function of MYC and further expands the role of MYC function in cancer. The interactome of MYC was revealed by mass-spectrometry analysis of immunoprecipitated MYC or through BioID screening (41, 42). Functional assays combined with selective depletion of certain MYC boxes has also revealed specific functions of different MYC boxes. These studies have helped to define a core group of MYC-associated proteins (16, 43). These MYC interactors mark the broadness of MYC function since they are involved in various fundamental cellular processes, such as chromatin topology and remodeling, the cell cycle, general transcription, and ubiquitination. The interactome also reveals more fundamental functions of MYC in participating in general transcription mechanisms, including the formation of the preinitiation complex, initiation, pausing, elongation, and splicing (43). This aspect of MYC function reinforces the essentiality of MYC in tumorigenesis (16).

These newly revealed mechanisms urge that more basic structural understanding and regulatory element logic are key to dissect the role of MYC in cancer (16).

Hypoxia-inducible Factors

The hypoxia-inducible factor (HIF) family TFs exemplify the bHLH-PAS family of proteins. In general, these proteins are characterized by a PER-ARNT-SIM (PAS) domain, which contains PAS-A and PAS-B located instantly upstream the bHLH domain (44, 45). (Figure 1A, pink block) Similar to MYC and MAX, bHLH-PAS proteins also form heterodimers, with an α-subunit serving as a stimuli-responder or regulator of tissue specificity and the β-subunit expressed more stably and ubiquitously. The PAS domain serves as another layer of dimerization control on top of bHLH for higher specificity, and the PAS-B domain can sometimes serve as a sensing domain that can bind to small molecules in the environment or sensory/regulatory proteins (46). bHLH-PAS TFs have a more defined structure than MYC, which is rather disordered (Box 2). In the case of HIF proteins, there are three α-subunits, HIF1, 2, and 3-α. They can all dimerize with the ubiquitous β-subunit HIF1β (also known as ARNT) (55). Dimerization determines the binding to specific E-box variants. The consensus binding motif of the HIFα-ARNT dimer is 5'-A/G-CGTG-3', which is an E-box variant usually referred to as the hypoxia responsive element (HRE) (55). HIFs mainly serve as activators of gene transcription once bound to DNA. A hypoxia ancillary sequence 5'-

Box 2 Structural insights of bHLH dimerization and DNA-binding.

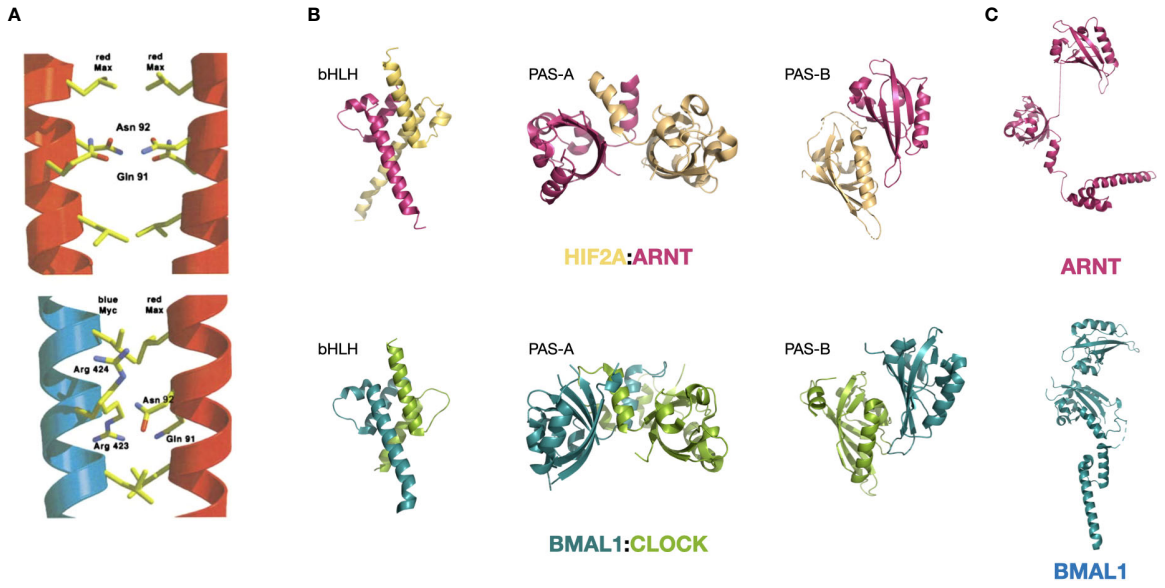
The bHLH domain is a highly abundant DNA-binding domain present in a large number of human proteins. Some bHLH domains co-occur with a leucine-zipper motif, such as that present in MYC and the components in its close regulatory network. These are termed bHLH-LZ proteins. Another important family is the bHLH-PAS TFs in which the tandem PAS-A and PAS-B domains that are located immediately upstream bHLH. All bHLH family proteins bind to DNA as dimers. [figures are adapted from ref. (17) and ref. (19)].

DNA-binding and dimerization features of the bHLH-LZ-containing MYC and MAX: MYC was first found to bind the canonical E-box in a dimer (47). However, MYC alone cannot form a DNA-binding dimer, and this entailed efforts to search for its binding partner, leading to the discovery of MAX (48). The inability of MYC to form homodimer is a result of its LZ region, which exhibits minimal helicity (49). In a putative homodimer of MYC, this region forms a helix that has residues Glu410, Glu417, and Arg424 at the interacting interface of the coil structure that would repel each other through interfacial electrostatic force and destabilizing the putative dimer (50, 51). On the contrary, MAX can form homodimers and bind to DNA (50). MAX dimer does not have the repelling residues that precludes homodimerization, but harbors residues that weaken the affinity (17, 52). These residues become complementary in the MYC : MAX dimer, making the heterodimerization of MYC and MAX the favorable form of existence in the nucleus (17). (see the figure, part A) Upon dimerization, MYC and MAX undergo structural changes and gain a more defined secondary structure, with MYC forming an additional quaternary structure, as shown by circular dichroism (53).

Specificity of the MYC : MAX dimer binding to the canonical E-box sequence 5'-CACGTG-3' is mediated by the conserved His359/Glu363/Arg367 motif in MYC (17). His359 forms a H-bond with the central guanine and lead to the specificity for a purine; Glu363 makes H-bonds with the adenine and the cytosine at position 2 and 3; and Arg 367 forms a H-bond with the guanine in the center and with the phosphate group between the cytosine and adenine at position one and two. Together these residues control the preference of the MYC : MAX dimer towards the 5'-CACGTG-3'version of E-box (17). The heterodimer also has additional contacting sites with the phosphate backbone at Lys371 and Lys389 of MYC and equivalent positions of MAX, which are located in helix 1 and in the loop, respectively (17). Other bHLH-containing proteins with a hydrophobic residue at 367-equivalent position preferentially bind to a Type A E-box 5'-CAGCTG-3' (17).

Structural features of the bHLH-PAS-containing BMAL1:CLOCK and HIF1A:ARNT: bHLH-PAS is a highly conserved motif series that determines protein dimerization, and bHLH-PAS-containing proteins share certain degrees of structural similarity within and across families. The HIF family proteins HIF1A and HIF2A both dimerize with ARNT and the bHLH-PAS domain structure of both dimers are very similar (19). On the other hand, both BMAL1:CLOCK and HIF1A:ARNT dimers feature close interactions in all three domains (bHLH, PAS-A, and PAS-B) with an asymmetric arrangement of the two molecules (19, 54). The interacting modes at the three domains are also similar. The bHLH domain forms a four-helical bundle, which is also observed for other bHLH TFs such as MYC : MAX and USF. The PAS-A domains interact with each other through contacts between the α-helix of one molecule and the β-sheet concave surface of the other molecule. (see the figure, part B) In addition, ARNT and BMAL1 share identical core residues (Arg102, Glu98, and His94) that recognize the 5'-GTG-3' half site of the E-box (18, 19, 54).

Meanwhile, the PAS domains of the two families also exhibit characteristic differences. Prominently, the PAS-A and PAS-B domain of BMAL1 have a connected surface, whereas the two domains are displaced in ARNT (19, 54). (see the figure, part C) This feature of ARNT might be the reason for its ability to dimerize with many other bHLH-PAS members, including HIF1-3A, NPAS1,3-4, AHR and SIM1-2, while BMAL1 has only been shown to dimerize with CLOCK and NPAS2.



CAGGT-3' that is located only several nucleotides downstream from the HRE has been proposed to be necessary for HIF activation of VEGF and EPO – two well-documented HIF-target genes – but this sequence lacks the structural basis for HIF to bind and is likely to be dispensable for other genes (56).

In normoxic conditions, HIF-1α is continuously expressed but undergoes fast hydroxylation mediated by prolyl-4-hydroxylase (PHD) at conserved proline residues. Hydroxylated HIFs will bind to the von Hippel Lindau (VHL) E3 ligase and be polyubiquitinated, then undergo degradation in the proteasome. As oxygen level goes down, HIF α-units are stabilized and dimerize with the β-unit, then bind to DNA and activate the transcription of target genes. Gene activation via HIF1A/2A is associated with two transactivation domains (TADs), with the N-TAD located in the oxygen-dependent degradation domain and the C-TAD at the C-terminus. The C-TAD domain interacts with CBP and p300, recruiting them to the HRE motif of target genes, which modify the local chromatin, and interact with the core transcription machinery to activate gene transcription. Other cofactors of HIF1α/2α include PKM2 (which builds a direct link to the Warburg effect), and a CDK8-mediator which promotes pause release of RNAPII (57, 58). It is

noteworthy that ARNT itself can act as a coactivator of other factors without HIF-α (59). Multiple studies have attempted to identify a set of HIF target genes, using both experimental and computational methods (60, 61). Similar to MYC, and potentially for the same reason, efforts to define target genes of HIFs in different types of cells has resulted in a small intersection set which can serve as a core group of HIF-regulated genes.

The most prominent role of HIF in cancer is its regulation of metabolism in response to the hypoxic tumor microenvironment. But like MYC and other EBTFs, HIFs are also found to be involved in many other aspects of tumorigenesis, including angiogenesis, the immune response, epigenetic regulation, the epithelial-mesenchymal transition (EMT), etc. An example of HIF as a key driver of tumorigenesis is in clear cell renal cell carcinoma (ccRCC), where mutation in VHL is observed in most cases and leads to aberrant accumulation of HIFs (62). HIF1 and HIF2 are both involved in this type of cancer and have complicated interactions, exemplifying how EBTFs from the same family can coordinate to fuel tumorigenesis.

BMAL1 and CLOCK circadian clock proteins

The core mammalian circadian regulators BMAL1 and CLOCK are another example of bHLH-PAS proteins. (Figure 1A, green block) The level of BMAL1 mRNA and protein oscillates with an approximate 24-hour period as a result of a tightly regulated feedback loop, whereas CLOCK levels stay relatively stable. BMAL1 and CLOCK form a heterodimer and bind to the canonical E-box sequence to activate the transcription of target genes, including their own repressors such as the cryptochromes CRY1/2 and the period genes PER1/2/3. PER and CRY proteins can form a complex to repress the transcription mediated by BMAL1-CLOCK, thus forming a negative feedback loop to induce circadian oscillation of gene expression. Two additional layers of feedback control of BMAL1-CLOCK function exist, mediated by the nuclear receptors REV-ERBα, REV-ERBβ and ROR, which make up a tripartite feedback mechanism of circadian gene expression, which is reviewed in detail by *Takahashi* (63).

In mice, the BMAL1-CLOCK dimer activates transcription of target genes in the morning. Then as the PER and CRY protein levels accumulate in the late afternoon, they translocate into the nucleus to interact with BMAL1-CLOCK to repress the transcription mediated by the dimer. PER and CRY are then targeted and degraded by proteasome, leading to reactivated BMAL1 and CLOCK to start a new transcription cycle in the morning (63). BMAL1-CLOCK activation involves chromatin interaction and modification. Like HIFs, BMAL1 and CLOCK also interact with p300 and CBP to acetylate histones for transcription. CLOCK itself has been shown to have histone acetyltransferase activity and can acetylate H3K9 and H3K14 (64).

Unlike MYC and HIF, BMAL1-CLOCK has been proposed to have pioneer properties and can open closed chromatins (65). But in a physiological context, this function seems to have specific requisites for certain cofactors, which can be tissue-specific (66), therefore the actual binding sites of physiological BMAL1-CLOCK still depend on specific contexts, such as in different organs. Again, defining a set of BMAL1-CLOCK target genes by intersecting sets in different contexts results in a small group of genes primarily regulated by BMAL1 and CLOCK (67). This gene set is sometimes referred to as clock-controlled genes (CCGs), but note that CCGs are defined by their 24-hour rhythmic expressions and comprise different genes depending on the context in which they are defined.

A disrupted circadian rhythm at an organismal level has long been marked as a potential risk factor for cancer. However, the molecular function of BMAL1 and CLOCK in tumors has only been studied rather recently. Part of the reason is that mutations in BMAL1 and CLOCK are not commonly observed in cancer, implying that they themselves do not commonly function as mutated drivers of tumor initiation. Nonetheless, BMAL1, CLOCK, and other core clock genes have been shown to be widely dysregulated at the transcriptional level across cancer types (68). This is also true for MYC and HIFs, implying transcriptional mechanism of driving tumorigenesis might exist.

Recent studies have discovered pivotal roles for BMAL1 and CLOCK in multiple types of cancers. For example, in glioblastoma (GBM) stem cells, acute myeloid leukemia (AML), and hepatocellular carcinoma (HCC), BMAL1 is essential for the proliferation of tumor cells (12, 13). Knock-down of BMAL1 can significantly reduce the growth of tumors both *in vitro* and *in vivo*. In GBM, BMAL1 has been shown to gain thousands of new binding sites compared to normal neural stem cells and is rewired to support tumor specific metabolism of both glucose and fatty acids (13). These results echo the case of MYC where EBTF functions vary significantly between tumorous and physiological conditions.

It is noteworthy that, at the cellular level, malignancy does not necessarily disrupt the circadian rhythm of the cell, since cancer cells can either have strong circadian rhythms or be totally arhythmic (13, 69). We recommend that in a tumorigenic context, distinctions should be made between the function of the circadian TFs in tumor cells and the actual circadian rhythm of cells and organisms.

EMT transcription factors

Importantly, the key transcription factors governing the process of epithelial-mesenchymal transition (EMT) are all E-box binding proteins. EMT was first discovered as an essential process during certain stages of embryo development such as gastrulation (70). In cancer cells EMT is featured by upregulation of mesenchymal markers such as vimentin (VIM), and downregulation of epithelial markers such as E-cadherin. EMT in cancer was initially studied in relation to its role in metastasis (71). Although a wide consensus on EMT has not been reached yet (10, 71, 72),now it becomes generally accepted that tumor cells can have intermediate hybrid E/M states spanning a continuous E-M spectrum, and a more hybrid state is associated with more aggressive stem cell properties (72–75).

Multiple families of TFs can induce EMT in cancer, including the SNAIL family, SNAI1 and SNAI2, bHLH-containing proteins TWIST1 and TWIST2, and the zinc-finger E-box binding homeobox family, ZEB1 and ZEB2. They all bind to E-boxes to induce an EMT program in cells, but the specific functions of

different proteins are non-redundant (75). SNAI1 has a zinc finger domain that consists of four zinc finger motifs and can bind to the E-box variant 5'-CAGGTG-3'. The SNAG domain can compete with H3 to prevent lysine-9 from being demethylated, hence activating gene expression (76). The ZEB proteins have two zinc finger clusters that bind to 5'-CAGGTG/A-3' and show higher affinity to promoters that have two E-boxes with variable distances in between, such as in the case of CDH1 (77). TWIST binds to the E-box as a homo- or heterodimer and can act as both a repressor and activator of gene transcription. The different binding preferences of the TFs forms the basis of their distinct functions (77).

EMT-TFs can regulate the expression of a set of common genes and their own specific targets as either repressors or activators. Their most prominent common function is to repress the expression of CDH1 through binding to the E-boxes in the promoter region of the gene. Other common target genes include the interleukins and TGF-β superfamily genes. Currently the most prominent function of the EMT-TFs is their regulation of cancer stem cell-related features such as drug resistance, phenotypic plasticity, immune evasion, etc. (75) Importantly, EMT-TFs can function in non-epithelial types of cancer such as glioblastoma (78). Therefore, researchers have suggested that instead of focusing solely on the EMT program, more attention should be placed in understanding the specific functions of the different EMT-TFs (9).

Other E-box binding TFs reported in cancer

Upstream stimulatory factors (USF) 1 and USF2 are ubiquitously expressed transcription factors that both have a bHLH-LZ domain that binds to the E-box as a heterodimer or homodimer (79). They also contain a USF-specific region (USR) upstream of bHLH that is important for E-box dependent transactivation. USFs are transcription activators that have a small group of defined target genes. USF1 is associated with familial combined hyperlipidemia and was found to bind the promoter of genes that regulate lipid and cholesterol metabolism, which is often dysregulated in cancer cells (80). USF2 can compete with MYC to antagonize the function of MYC (81). USF1 also interacts with p53 and regulates its function (82).

Other bHLH-PAS proteins such as AHR and NPAS in cancer are reviewed in ref (46).

Regulatory features of E-box-containing regulatory elements

Although EBTF families feature distinct functions, certain features of E-box-regulated genes are commonly observed. Some features of regulatory elements in promoters are discussed in Box 3. In an early study that analyzed the promoters of CCGs, it was found that some other motifs are overrepresented in addition to E-boxes, including those of SP1, ZF5, NRF1, and EGR, which represent CG-rich motifs (67). This implies that EBTFs might recruit general TFs under the assistance of SP1. Other overrepresented motifs include NFY and E2F family factors (67). In mechanistic study on the EBTF sterol regulatory element-binding protein (SREBP) family member SREBP1, SP1 and NFY are reported to be partner factors (84). Interestingly, in an analysis that aimed to identify overrepresented motifs in bidirectional promoters (defined as promoters of less than 1kb length and flanked by two protein-coding genes that are transcribed in opposite directions), E-box, E2F, NRF1, NFY, and CG-rich motifs were also reported (85). These reports emphasize the importance of co-motifs in determining the function of E-boxes. Conversely, the TATA-box is usually absent in these bidirectional promoters as well as housekeeping genes (85). Although the TATA-box and E-box are not mutually exclusive in promoters, genes containing both motifs implement highly specialized functions such as regulating certain developmental programs (86, 87) (Figure 2Ai). As any functional promoters or enhancers comprise multiple TF-binding sites, these features imply that E-boxes and other motifs have specific functions in regulating transcription and their distinct combinations encode specific types of transcriptional regulation.

Little is known about the transcriptional regulatory properties of E-boxes. One of their important functions is to convergently coordinate the temporal control of gene expression through oscillators (Figure 2Aii). Oscillator is a general mechanism of dynamic transcriptional control that can be achieved by feedback loops (88, 89). The most prominent example is the 24-hour circadian rhythm implemented by the tripartite feedback loop as described above. The circadian clock is intimately interlocked with the cell cycle, which is another oscillator that is dominated by the dynamics of MYC. The molecular details of the coupling of these two oscillators and their dynamics and system properties are extensively studied with experiments and mathematical modeling (90–94) (Figure 2B). Because EBTFs are usually regulated by E-boxes too, they can form interlocked feedbacks and provides a space for temporal control of various period lengths. The bHLH TF HES1 exemplifies a 2-hour ultradian oscillator through a self-feedback loop via an E-box in its promoter (95). The core inflammatory TF NF-κB is also an example of intrinsic oscillatory gene regulated by EBTFs (96).

Another feature of EBTFs including MYC and BMAL1 is they fit in the Kamikaze model of transcription, where ubiquitin-dependent proteolysis is required for RNAPII elongation, and newly synthesized activators need to be loaded for a new round of transcription burst (97–99). This feature might serve as a mechanism driving the temporal control of cell activities by EBTFs, but the molecular details and the kinetics of this type of transcription is largely unknown (Figure 2Aiii).

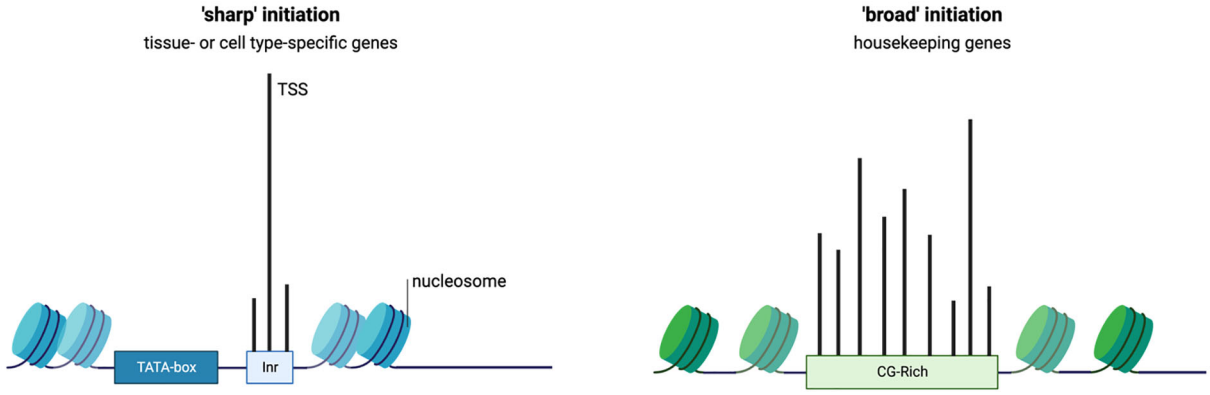
Another key function of E-boxes might be to maintain the robustness of expression of essential genes, since many of the genes containing E-boxes are ubiquitously expressed and regulate basic physiological activities of the cell. Supporting this hypothesis, genes driven by E-box-enriched bidirectional promoters are expressed in a higher frequency than the average of all human genes (85). Furthermore, in *Drosophila* where CpG islands are absent in promoters, E-boxes are often found in promoters of housekeeping genes (100). This intrinsic robustness of E-box-containing genes underlies the importance of E-boxes in cell homeostasis (100) (Figure 2Ai).

Box 3 Functional features of regulatory elements.

Regulatory elements of the genome (enhancers and promoters, etc.) encode the information for spatial and temporal regulation of gene expression. TF-binding motifs are the building blocks of such information and determine the structure of the genome, epigenetic states of the chromatin, and the RNA polymerase-mediated transcription. A promoter region is loosely defined as the region upstream of the TSS that is several hundreds of base pair long. The short region up to 100 bp flanking the TSS is called the core promoter, which is sufficient for the assembly of the pre-initiation complex (PIC). The farther part of the promoter is termed the proximal promoter, which is usually bound by TFs to facilitate transcription and has enhancer activity (83).

Some patterns of promoter components and functions have been recurrently observed. The first type of promoters which is often referred to as 'focused' or 'sharp' promoter features a focused initiation site and is usually found in tissue- or cell-specific genes (6). Focused promoters often contain a eukaryotic core promoter motif such as TATA-box and initiator motifs and lack CG-rich elements. On the contrary, some promoters have multiple closely concatenated TSSs, the uses of which are observed in similar frequency. This type of dispersed promoters is prominently found in housekeeping genes and often have CpG motifs. These promoter types also share specific features of histone modifications, which is discussed in detail in ref (83).

These two types of promoters exemplify the connection between the promoter functions and components. For example, CG-rich motifs are inherently nucleosome-repelling, and their existence might improve the robustness of the promoter to maintain the expression level of essential genes. These properties of regulatory elements form the theoretical basis that TFs binding to the same class of motifs share functional features, and these features are defined by the combination pattern of partner regulatory motifs.



In recent years our understanding of the PolII-mediated gene transcription mechanism has been greatly expanded upon, and its role as a potential therapeutic target in cancer has been explored (101–103). This progress has also revealed new functions of EBTFs in regulating multiple steps of transcription. MYC is a prominent example. Firstly, evidence has shown that MYC can facilitate the formation of the preinitiation complex of PolII by interacting with TATA-box binding protein (TBP) and potentially modulating the energetic landscape of TFIID during preinitiation complex (PIC) formation (104). GTF2F1, which is a component of TFIIF, binds directly to MYC through MB0 and serves another way through which MYC participates in PIC assembly (42). During initial PolII elongation, MYC has been shown to facilitate mRNA capping by recruiting RNGTT and RNMT (105). During productive elongation, MYC can recruit positive transcription elongation factor b (p-TEFb) and enable CDK9 to phosphorylate Ser2 on the CTD of PolII and allow PolII to continue with productive elongation (106, 107). A recent report showed that SPT5, another key regulator of elongation, is recruited by MYC (41). Other EBTFs have also been reported to participate in these steps of RNAPII transcription.

Mutual regulation of different EBTFs in cancer

As EBTFs all bind to E-boxes and share common regulatory features, it is not surprising that they can mutually regulate each other, both within each protein family and across different protein families (Figure 3). This can occur via competitive binding to DNA, direct binding to each other, or regulation of the turnover of each other. These interactions connect EBTFs into a dense regulatory network.

MYC-HIF interactions

MYC and HIF closely interact with each other. HIF1α can inhibit MYC activity through various mechanisms. It can not only bind directly to MAX and interfere with MYC-MAX dimer activity, but also activate expression of other MYC competitors such as MAD and MXI1 (108). HIF1α also competes with MYC for DNA binding, which is exemplified in the promoter region of p21 (109). Additionally, HIF1α has been reported to promote the proteasomal degradation of MYC (110). Paradoxically, MYC has been mostly reported to promote the activity of HIF1α. This is exemplified by decreased HIF1α levels after MYC knock-down in multiple myeloma cells, and stabilization of HIF1α proteins after MYC overexpression (111). Mechanistically, MYC can reduce the binding of HIF1α to the VHL complex and decrease its degradation (112). In addition, MYC increases mitochondrial OXPHOS and ROS production, which inhibits PHD activity in non-hypoxic conditions (113). The paradox also lies in the functional consequence of normal MYC and HIF1α activity. MYC usually promotes the function and biogenesis of mitochondria, whereas HIF1α represses them by activating FOXO3a which consequently represses mitochondrial gene expression and induces BNIP3, triggering mitochondria degradation through autophagy (114).

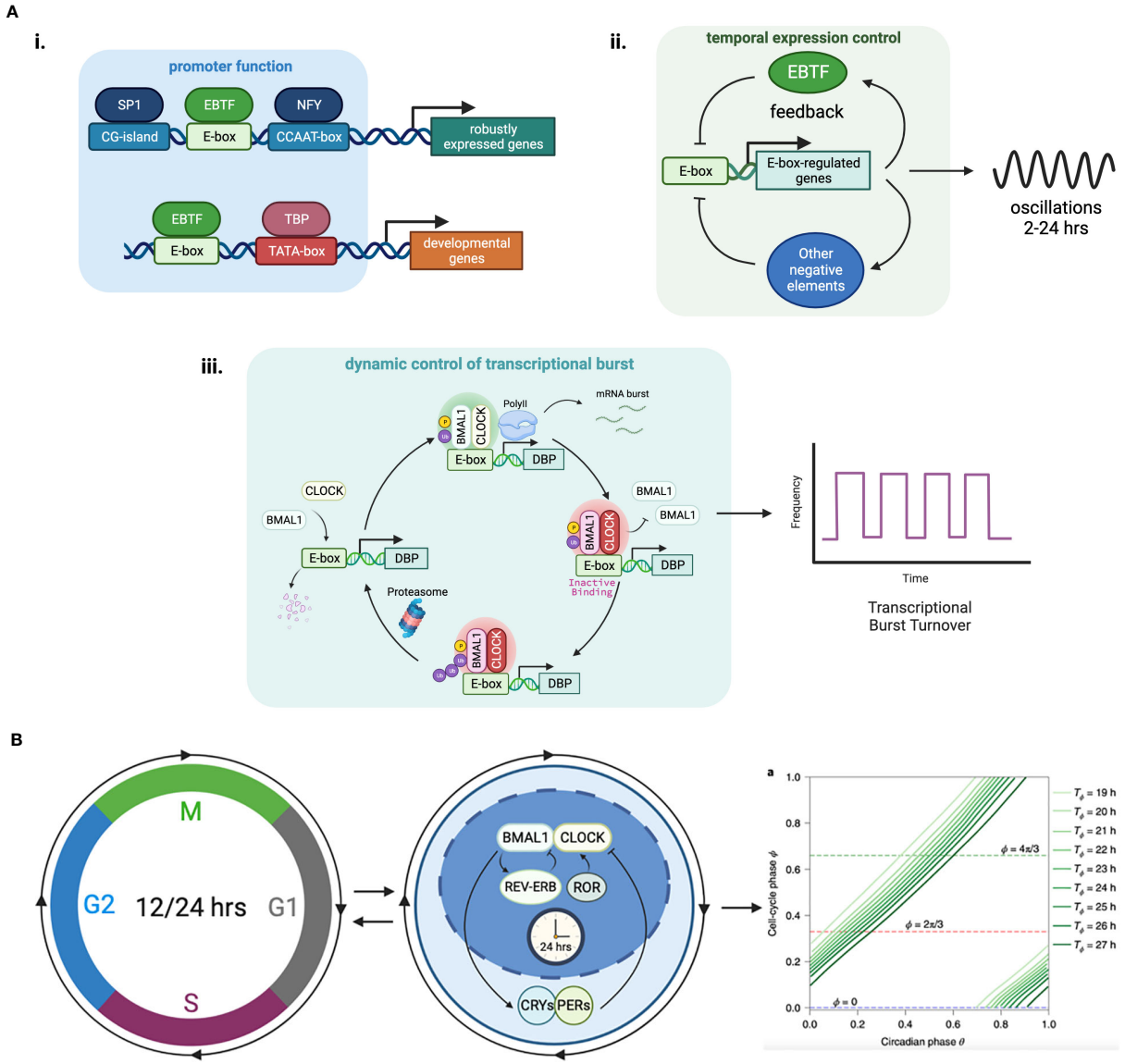


FIGURE 2
Functional features of E-box elements in transcriptional regulation. **(A)** (i) E-boxes are used to generate oscillations of gene expression through negative feedback loops. The negative regulator in the loop can be an EBTF itself, such as in the case of HES1 which features a 2-hour ultradian rhythm, or target genes of EBTFs, such as PER and CRY in the case of BMAL1, which generates a 24-hour rhythm. ii. E-boxes determine different functions of the promoter by working with different partner motifs. For example, in TATA-less promoters that have high CG-content, E-boxes participate in keeping the genes continuously/robustly expressed, whereas in some developmental genes an E-box works together with the TATA-box to initiate downstream developmental programs. iii. EBTFs can serve as a temporal control node in the turnover of transcription burst. The physiological function of such control is unclear. **(B)** Phase lock of circadian clock and cell cycle. Mathematical modeling and experimental validation revealed that the two oscillators of circadian rhythm and cell cycle exhibit multiple phase-locked states that exhibit robustness against molecular fluctuations.

However, in cancer cells MYC and HIF1α are not incompatible since many cancer types have both MYC and HIF1α in high levels. The seeming conflict can be explained by the deregulation of MYC levels. When at high levels, MYC can still maintain its activity stoichiometrically and override the inhibitory effect of HIF1α (115). USP29 has been shown to maintain HIF1α and MYC levels at the same time to promote tumorigenic metabolism (116). In such cases, MYC and HIF1α may cooperatively tailor a gene expression program that takes advantage of the pro-tumorigenic aspect of each protein to fuel tumor growth. For example, both proteins activate genes for glucose import and glycolysis such as LDHA and HKII, which contributes to the Warburg effect in cancer (117). This exemplifies how EBTFs coordinate in a network to fuel cancer progression.

Such cooperation is also observed for HIF2α, although unlike HIF1α, HIF2α is better known to promote MYC activity in cancer. HIF2α enhances MYC function by stabilizing the MYC-MAX dimer in clear cell renal carcinoma cells and colorectal carcinoma cell lines (118). In turn, MYC has been reported to activate HIF2α transcription by directly binding to its promoter in T cell leukemia and maintaining a pool of cancer stem cells (119). By contrast, in physiological endothelial cells, it is reported that HIF2α represses MYC expression (120).

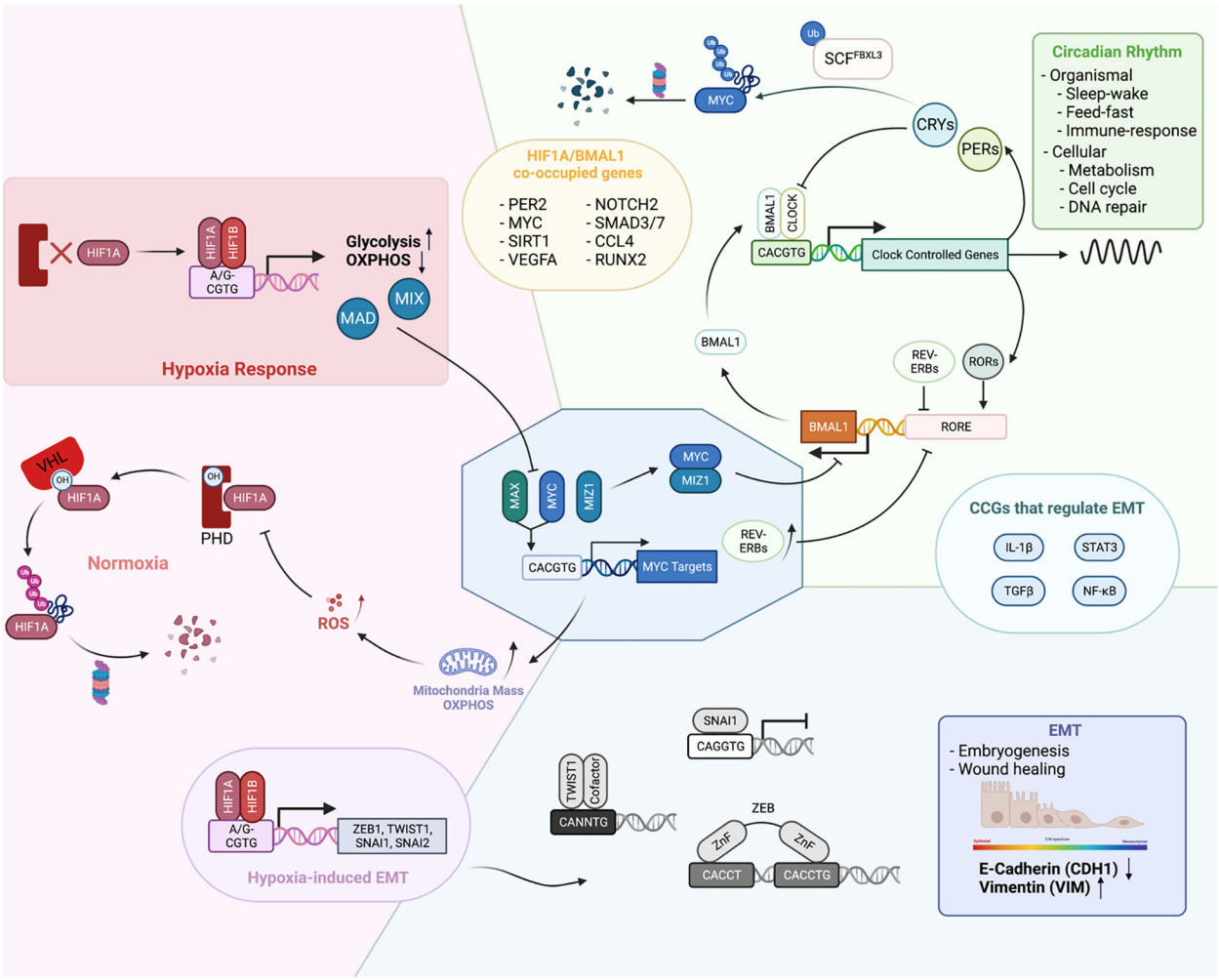


FIGURE 3
Physiological functions of EBTFs and their mutual regulation to form a coordinated network. **(A)** In homeostasis EBTFs each have their prominent functions, including control of the cell cycle by MYC, oxygen sensing by HIF, and control of the circadian rhythm by BMAL1 and CLOCK. The colored blocks highlight the primary physiological function of each EBTF family. MYC tightly regulates the expression of E2F target genes to control the cell cycle. HIF is continuously expressed in cells and rapidly degraded by the proteasome under normal oxygen and ROS levels. When oxygen levels are low and HIF proteins are less hydroxylated, they are stabilized and bind to DNA to activate downstream gene expression. BMAL1 and CLOCK dimerize and bind to DNA to activate clock-controlled genes, which include their own suppressor CRYs and PERs. CRY and PER translocate into the nucleus and form a repressive complex to inhibit BMAL1 and CLOCK transcriptional activity, forming a delayed negative feedback loop. When they are degraded, a new cycle of BMAL1 and CLOCK transcriptional activity is activated. Reported mutual regulations across different EBTFs are exemplified at the interface of each color block corresponding to the two families. As they bind to the same DNA motif, regulate each other, and contain E-boxes in their own promoters, their functions unavoidably converge into a network to coordinate the essential processes of the cell.

MYC-BMAL1/CLOCK interactions

The circadian clock proteins also closely interact with MYC through various mechanisms. Two groups have shown that MYC (both MYC and MYCN) can inhibit BMAL1 and CLOCK function in cancer cells and disrupt the circadian dynamics of the core clock molecules (121, 122). Consequently, periodic glutamine metabolism in cancer cells is altered. MYC disruption of BMAL1 can occur through upregulation of REV-ERBs, which inhibits the transcription of BMAL1, and/or through direct inhibition of BMAL1 transcription by binding to the promoter as an inhibitory MYC-MIZ complex (121, 122). It is noteworthy that these findings were determined in cancer cells with relatively low MYC levels and intact circadian rhythms. But like with HIF1α, high MYC levels and circadian oscillations are not incompatible since high levels of MYC, and intact oscillations have also been observed simultaneously in the same GBM cell line (13).

On the other hand, MYC is a clock-controlled gene itself. CRY2 can cooperatively bind MYC with FXBL3, promoting its ubiquitylation by SCF-FBXL3 and consequent degradation by the proteasome (123). This mechanism is also context-dependent because in mouse spleen, Cry2 knockout had no effect on Myc levels and Cry1/Cry2 double knockout repressed Myc levels (124). Per2 mutation and Bmal1 deletion in lung tumors has also been shown to lead to an increase in Myc levels, although specific detail is not clear (125). Another report using a mouse model suggests an indirect mechanism of Myc control by Bmal1 through catenin or Ctnnb1 as an intermediate effector (124).

HIF-BMAL1 interactions

Another well studied pair of E-box binding proteins that have been shown to regulate each other is the bHLH-PAS TFs HIF1α and BMAL1. Under physiological conditions, two independent groups using different models showed that hypoxic responses mediated by HIF1α are under circadian control, and the hypoxic gene expression pattern is disrupted when BMAL1 is knocked out (126, 127). This is consistent with the presence of the E-box in the HIF1α promoter. In turn, HIF1α can participate in the regulation of circadian rhythm mediated by BMAL1 (127). Pharmacological stabilization of HIF results in a lengthened period and dampened amplitude of Per2 and BMAL1 rhythmicity. HIF1α also has a positive effect on the function of BMAL1 to activate transcription, as is shown in both studies (126, 127). Interestingly, BMAL1 and HIF1α can be co-immunoprecipitated in Co-IP experiments, suggesting that they can at least form a complex together or even dimerize to regulate gene transcription (127). ChIP-seq experiments of both factors in the same osteosarcoma cell line showed that BMAL1 and HIF1α share a large portion of binding sites on chromatin, which constitutes approximately a third of all HIF1α targets and a quarter of BMAL1 targets. These results further underscore their close relationship in regulating gene expression. However, their relationship is not well studied in cancer. Hypoxia-induced HIF activity is proposed to promote the disruption of the circadian rhythm in hepatocellular carcinoma, but a more detailed mechanism still needs to be revealed (128). Correlations between hypoxia/circadian clock and radiation resistance has been noted in glioma, yet mechanistic studies remain to be done (129).

Other regulators of the circadian clock, such as PER and CRY, also interact with HIFs but will not be elaborated upon here (130–132).

EMT TFs interactions

The interactions between EMT-TFs and bHLH TFs are relatively less studied directly, although they share multiple phenotypic commonalities. The most understood interaction is with HIFs, which are mainly accounted for by their transcriptional regulation of each other and exemplified by the induction of EMT-TFs by HIF proteins. In multiple cell types, HIF overexpression or hypoxia is sufficient to induce EMT (133). HIF1α can directly bind to the HRE motifs in the promoter region of TWIST1, SNAI1, SNAI2 and ZEB1 to activate their expression. Indirect regulations of EMT-TFs by HIFs are often observed, too. For example, HIF1 can also activate histone modifiers such as HDAC3 to promote SNAI1 activation indirectly (134). Other intermediate genes include WDR5, lncRNA, FoxM1, ILK and PAFAH1B2 (135–137).

It is observed that high MYC levels and EMT often co-occur in cancer, and they can contribute to the same characteristics of later stage tumors. It has been reported that over-expression of MYC can induce EMT in lung cancer and melanoma cells through SNAI1 and ZEB (138, 139). MYC also facilitates TGFβ-induced EMT as a coactivator of the SMAD complex (140). This exemplifies physiological antagonistic factors can cooperate in certain tumors, because in physiological conditions, TGFβ represses MYC expression and inhibits cell proliferation, in turn MYC suppresses the activation of TGFβ-induced genes.

The interaction between circadian regulators and EMT factors has only been noted recently (141). It has been reported lately that the EMT process in cancer is gated by the circadian rhythm in the cell (142). Plus, BMAL1 has been shown to facilitate the EMT in colorectal cancer (143).

Interactions within the same family

In addition to the inter-family mutual regulation, an intra-family interaction layer also exists. Most E-box-binding genes have some extent of redundancy. In physiological conditions, different members of the same family often share a large portion of target genes and implement similar functions in different tissues or at different developing stages. However, in cancer cells they often have independent or even antagonistic functions when they are simultaneously expressed in the same cells.

HIF family members provide prominent examples of such relationships (144). Pioneering work showed that in RCC cells where VHL function is defective, HIF2 fuels tumor growth partly by activating cyclin D1, and has suppressive interactions with HIF1 (145). In mouse xenograft models, HIF2 overexpression significantly enhanced tumor growth *in vivo*, whereas HIF1 overexpression suppressed tumor progression. Consistently, HIF1 overexpression lowered HIF2 protein levels, and vice versa. They also showed that the DNA binding function of HIF2 is responsible for its repressive activity towards HIF1 (145). The antagonistic functions of HIF1 and 2 are also marked in tumor associated stromal cells and have a reversed effect on angiogenesis in the tumor microenvironment (146). Despite all the examples, HIF1 and 2 are not always mutually antagonistic, but can also collaborate to meet different needs of the cancer cells (147).

Along similar lines, ZEB1 has been shown to participate in the initialization and progression of melanoma cells. By contrast, ZEB2 suppresses the onset and metastasis of melanoma in mouse (148, 149). SNAI1 and SNAI2 have also been shown to have opposite effects on the expression of phospholipase D (PLD), which has been proposed as a prognostic marker of breast cancer. PLD also has opposite effects on the expression of SNAI1 and SNAI2. The authors thus proposed a feedback loop model to explain the mutually antagonistic effect of the two factors on each other (150). In a different ovarian tumor model where SNAI1 and SNAI2 were also found to be mutually exclusive, SNAI1 was found to bind to E-boxes in the promoter region of SNAI2 and recruit HDAC to repress SNAI2 expression (151). More examples of EMT-TF mutual regulation can be found in ref (75).

All together, these examples show important features of the intimately connected EBTF network. First, the regulatory relationships among the TFs in normal tissues are largely rewired in cancer, and the new networks depend on tumor type and their evolutionary trajectory, thus are diverse. This explains the controversy that a certain EBTF is oncogenic in some tumor but is tumor-suppressive in others. Another feature is that in the rewired

networks EBTFs are still closely related, because of their intrinsic DNA-binding specificity. On the one hand, targeting strategies on network levels might be needed to inhibit the tumor-fueling EBTF network. On the other hand, such network provides more actionable nodes to targeting certain EBTF or the whole network. Hypothetically, the flexibility of this network might also provide evolutionary spaces for tumor cells to develop plasticity.

Perspectives of EBTFs in cancer

Because of the shared binding specificity and functional interconnectivity of the EBTFs, they also convergently regulate phenotypical hallmarks of tumors.

Tumor initiation

Although all EBTFs discussed above are often dysregulated in cancer, they are rarely mutated (7, 9, 68), and the mechanism of how they "drive" tumorigenesis is still largely unknown. The rarity of their mutations implies the importance of the functional intactness of these proteins in cancer development.

MYC is the earliest and most documented oncogenic TF. Even a small disturbance of MYC homeostasis can induce abnormal phenotypic changes in cells (7). MYC can facilitate the progression of the cell cycle by upregulating genes that promote the passing of checkpoints. Interestingly, pan-cancer analysis showed that MYC amplification is mutually exclusive with many canonical oncogenic drives such as PIK3CA, PTEN, APC, and BRAF (7). This result implies that MYC has its specific mechanisms of driving tumorigenesis, likely through transcriptional regulation of the cell cycle.

Other EBTFs are not recognized as general cancer drivers so far in knock-out or over-expression-based *in vivo* tumor development assays. However, this does not exclude their potential oncogenic role since these models might miss some necessary background or cofactors, such as *de novo* enhancers gained through mutations in non-coding regions or epigenetic changes in the genome.

Metabolism

Cancer cells usually require specific metabolic programs to meet their needs for continuous proliferation. The most prominent consequence of EBTFs in cancer is their ability to rewire the metabolic program of the cells, as a large proportion of metabolic genes contain E-boxes in their promoter region.

MYC, BMAL1-CLOCK and HIFs can all regulate genes that are responsible for glycolysis. Common gene targets include the GLUT family (114, 152), which controls glucose intake into the cell, and most of the enzymes involved in glycolysis. LDHA is also a well-documented gene regulated by MYC, HIF, and BMAL1 (153, 154). These enzymes together may cooperate to fuel the Warburg effect (155).

The TCA cycle turnover is also altered in cancer cells to support cancer progression. Prominently, MYC activates glutamine transporters and feeds more glutamine into the TCA cycle, resulting in the glutamine-addicted metabolic feature of many MYC-driven cancers (156, 157). MYC, HIFs, and BMAL1 can all regulate the source and level of acetyl Co-A entry into the TCA cycle and regulate lipid and cholesterol metabolism (114, 158, 159). Another pivotal TCA metabolite as a common gene target of EBTFs is α-KG, which is of great importance because it is a key node connecting metabolism with histone modification, marking the importance of epigenetic regulation by these factors, and with fatty acid synthesis through ACACA, which is also a shared target gene (159). It is also not surprising that all three factors can directly regulate the synthesis, elimination, and fusion dynamics of mitochondria (110, 160, 161). SREBP1, an essential regulator of cholesterol and fatty acid metabolism, has recently been shown to mediate circadian remodeling and maladaptive response to the over-nutritional environment of non-alcoholic fatty liver disease, which is a major risk of liver cancer (162).

It is noteworthy that BMAL1 and CLOCK are the master regulators of organismal metabolism in response to sleeping and feeding and coordinate metabolism across tissues and organs. This function is reviewed elsewhere (158).

Immune evasion and inflammation

It is critical for tumor cells to evade the surveillance of the immune system, and during tumorigenesis malignant cells evolve multiple mechanisms to suppress the immune reaction against them. All the E-box binding proteins broadly participate in both innate and adaptive immune regulation (8, 163–165).

The recruitment of macrophages and other myeloid cells is the first level of immune regulation. MYC, HIFs, and BMAL1-CLOCK can all regulate cytokines responsible for their recruitment, including CCL family chemokines and interleukins. EBTFs can cooperate in a tumor to promote a conducive microenvironment. For example, MYC and TWIST have been shown to collaboratively support a pro-metastatic phenotype of macrophages through regulating the secretion of CCL2 and IL13 (166).

Immune checkpoint mediated by PD-L1 is another critical mechanism used by cancer cells to evade the immune response. MYC can regulate PD-L1 through either direct binding to its promoter or through post-transcriptional mechanisms in multiple types of cancers (167–169). PD-L1 is also reported to be a direct target of HIF1 (170), whereas BMAL1 regulates PD-L1 expression in an indirect way through lactate metabolism in macrophages (171). Regulation of PD-L1 has also been studied in EMT contexts (172).

NF-κB seems to be a central mediator of EBTF balance in immune regulation. MYC itself is a target of NF-κB (173). BMAL1 can dimerize with RelB and block a subunit of the NF-κB transcription complex (174). CLOCK can acetylate the RelA subunit and GRs to regulate their DNA binding activity (174, 175). Twist 1 can also interact with RELA (176).

Angiogenesis and other tumor microenvironments

EBTFs also remodel other components of the tumor microenvironment, including extracellular matrix (ECM)

components and promoting angiogenesis. Cancer-specific angiogenesis is an important feature of solid tumors and its potential as a therapeutic target has been underscored by the success of recent clinical trials involving anti-angiogenic therapy. VEGF is a central promoting factor of angiogenesis and has been shown to be directly regulated by HIF and BMAL1 (177). VEGF is also reported to be closely regulated by MYC (178, 179). BMAL1 has been shown to be associated with drug resistance of colorectal cancer cells via its regulation of VEGF (180). Other coordinating factors of angiogenesis have also been reported to be under EBTF control.

Recent advances in mechanobiology revealed the important role of ECM components and corresponding signaling pathways in tumorigenesis (181, 182). High ECM stiffness is a driving force of tumorigenesis and itself can result in an abnormal chromatin state (183). Collagen and integrin are the most studied ECM signaling-related molecules in cancer and have been shown to be regulated by EBTFs. The most prominent regulators are the EMT-TFs, which can directly regulate the type and amount of collagen genes produced by cells, and contribute to stem cell features of cancer cells (10, 184). MYC can regulate genes enriched in the ECM, cell adhesion and cell junction gene sets and regulate invasiveness (179). BMAL1-CLOCK has been reported to regulate the secretory pathway of collagens and maintain their homeostasis (185). HIFs also have well-documented functions in regulating ECM components by regulating collagen prolyl and lysyl hydroxylase and integrins (186).

Cancer stem cells

Although the CSC concept still lacks a uniform definition across tumor types, some common features are recurrently observed in certain cancers such as glioblastoma, AML, breast cancer, HCC, etc. CSCs defined in these cancers usually have high heterogeneity, drug- and immune-resistance, and ability to self-renew. Interestingly, all the EBTFs discussed in this review are widely reported to be associated with CSCs (10, 12, 13, 187, 188). These examples imply that transcriptomic features might be able to uniformly define CSCs and guide targeting strategies.

Summarizing all these functions, we propose a network perspective of E-box biology in cancer (Figure 4A) that bridges the fulfillment of phenotypic changes of cancer cells in different levels to meet their progressive needs. We also stratified their functions in line with the ten cancer hallmarks to highlight their specific involvements (Figure 4B).

Targeting EBTFs in cancer

TFs was once thought to be "undruggable" targets because of their intrinsically disordered structure. But recent years new advances in pharmacochemistry provided promising new toolboxes for targeting TFs through various mechanisms such as inducing targeted protein degradation, disrupting protein-protein interaction, and indirect targeting of TF modulators and collaborators (189, 190).

MYC has been the most appealing yet challenging target among EBTFs for its centrality in cancer. The most prominent strategy is to target MYC-MAX dimerization. Early examples include OmoMyc, which is a 90 amino acid MYC mutant that bind to MYC and MAX to disrupt their dimerization (191). Initially OmoMyc was only thought of as a tool because of its size, but recently *in vivo* data showed its potential as a therapeutic (191). Small molecules that disrupt MYC function has also been successfully developed and show favorable pharmacokinetics and tolerability in preclinical studies (192). Multiple other mechanisms have also been explored, such as targeting MYC transcription, translation, and DNA-binding, etc. (193).

HIFs have attracted great interest as a therapeutic target in cancer for many years with multiple tested mechanisms (194). Recently, a small molecule named belzutifan that binds to the PAS-B domain of HIF2α showed exceptional efficacy in VHL-associated RCC in clinical trial (195). The results led to the first-in-kind approval from FDA to treat several cancer types associated with VHL. The success of belzutifan proved the feasibility of faithfully targeting bHLH-PAS TFs with small molecules and the therapeutic potential of these TFs.

Our lab and collaborators have developed several small-molecule-sets to target the circadian network and the activity of BMAL1 and CLOCK, including stabilizers of cryptochrome with precise isoform selectivity (196, 197), REV-ERB agonists (198), and novel inhibitors of casein kinase II (CK2) (199). We also showed the potential of these molecules as cancer therapeutics in pre-clinical models of multiple cancers (12, 13).

Outlook

We progressively reviewed important EBTFs in cancer, their shared binding motif and target gene, close mutual regulation, convergent functions in homeostasis and cancer, and established a network view of the biology of these TFs. Synthesizing these factors in a unified model provide some important implications.

First, because EBTFs lie in a central node that fuels many hallmarks of cancer, targeting this node provides the chance of shutting down multiple hallmarks simultaneously. This is exemplified by the biology of MYC, which established a "coalition model" where MYC interacts with a wide variety of proteins, which cooperate to achieve a collaborative transcriptional program. Thus, it is proposed by the MYC-studying community that instead of targeting individual functions of MYC in different hallmarks, it is much more effective to "chop the MYC tree" to halt cancer cells from progressing (43).

On the other hand, however, this convergent view also imposes major challenges on studying the biology. First the functions of the fundamental connecting node, the E-boxes, remain largely unknown, making it hard to dissect the molecular mechanism of the EBTF network. It is also necessary to understand the determinants of the specificity of different EBTFs to different variants of E-boxes, such as DNA shape at the local motif (5), etc. This will provide a more balanced micro- and macroscopic perspective of EBTF biology in cancer. In addition, studying the properties of a network requires quantitative modeling, but acquiring data for establishing the model necessitates delicate experimental design. Luckily, well-characterized small molecules become available recently and provide handy tools for this purpose.

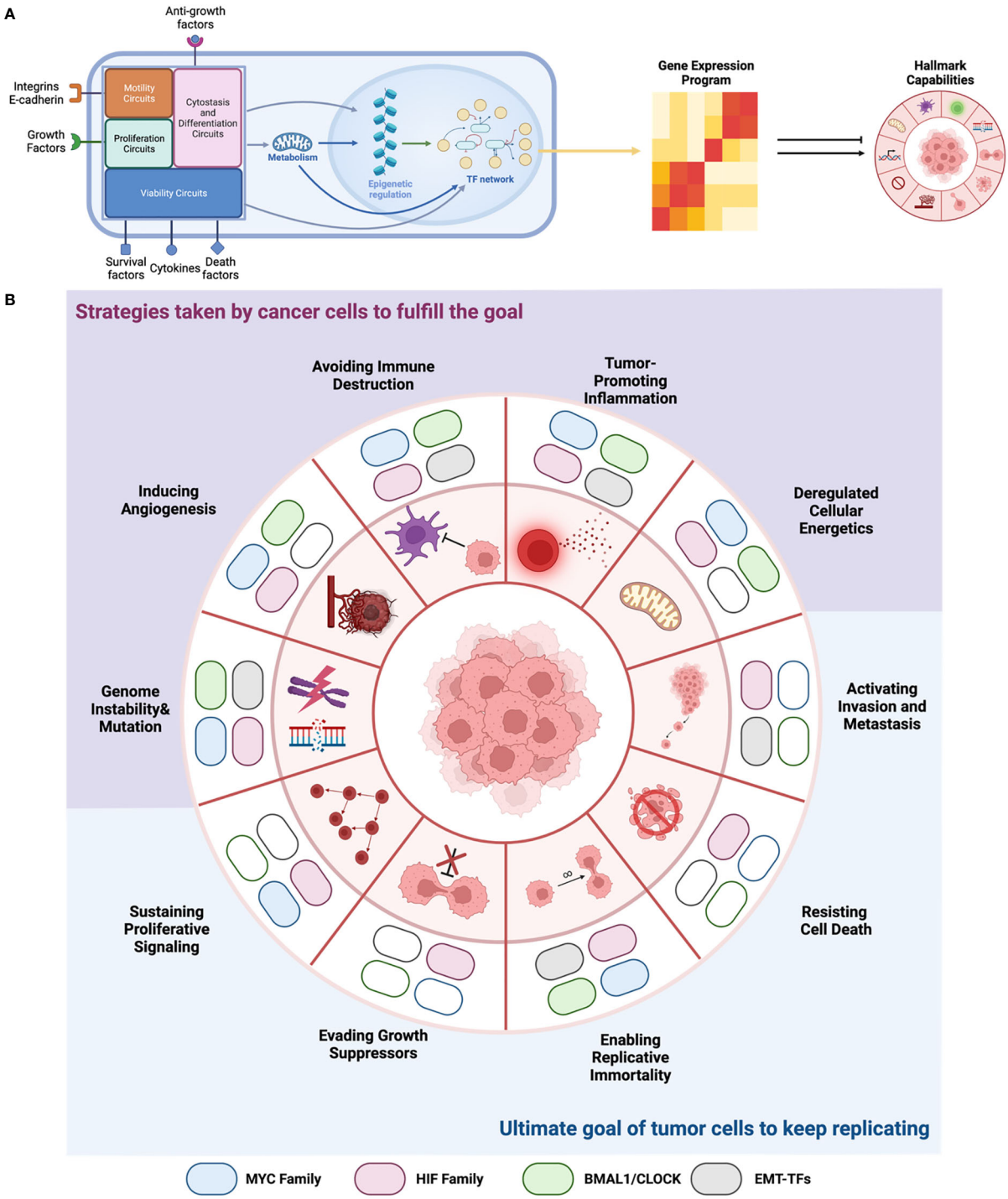


FIGURE 4
EBTFs Contributing to the hallmarks of cancer. **(A)** From a systems point of view, transcription factors are nodes that form the complex transcription regulatory network, which integrate all the information from external and internal signals. Then they decide which genes are transcribed and generate the gene expression program of the cell. The expressed genes then carry on the function to help tumor cells gain the hallmarks to progress. EBTFs form a subset of the whole network and carry out certain cellular functions. **(B)** Summary of EBTFs reported to contribute to the cancer hallmarks. Colored block indicates that the family is reported to contribute to the hallmark. Because the EBTF network regulates essential cellular activities, their function in tumors also contributes more to the pathological changes that cancer cells need to fulfill their ultimate goal of unceasing proliferation. Current results support the strategy of targeting the EBTFs to simultaneously eliminate the functional hallmarks thus halt tumor growth.

Paving the ways for drugs that target EBTFs to clinic will be an important field of study. This will involve biomarker discovery for these targets, recognition of potential benefits, and careful design of pre-clinical and clinical trials, all of which requires better understanding of the biology of EBTFs. A promising first step would be combining EBTF-targeting drugs with current therapeutics. Because of the broad regulation of cancer hallmarks by EBTFs, there's a higher chance that these drugs will synergize with current therapeutics to improve outcome. Successful examples include a MYC small molecule inhibitor, which is shown to synergize with anti-hormone therapy to inhibit prostate cancer and breast cancer cells (200), and several other studies that evaluated the efficacy of HIF inhibitors in combination therapies (Figure 5).

In summary, we depicted a network-based reasoning diagram for proposing and testing new hypotheses and strategies to target EBTFs in cancer. This model will help to account for the many

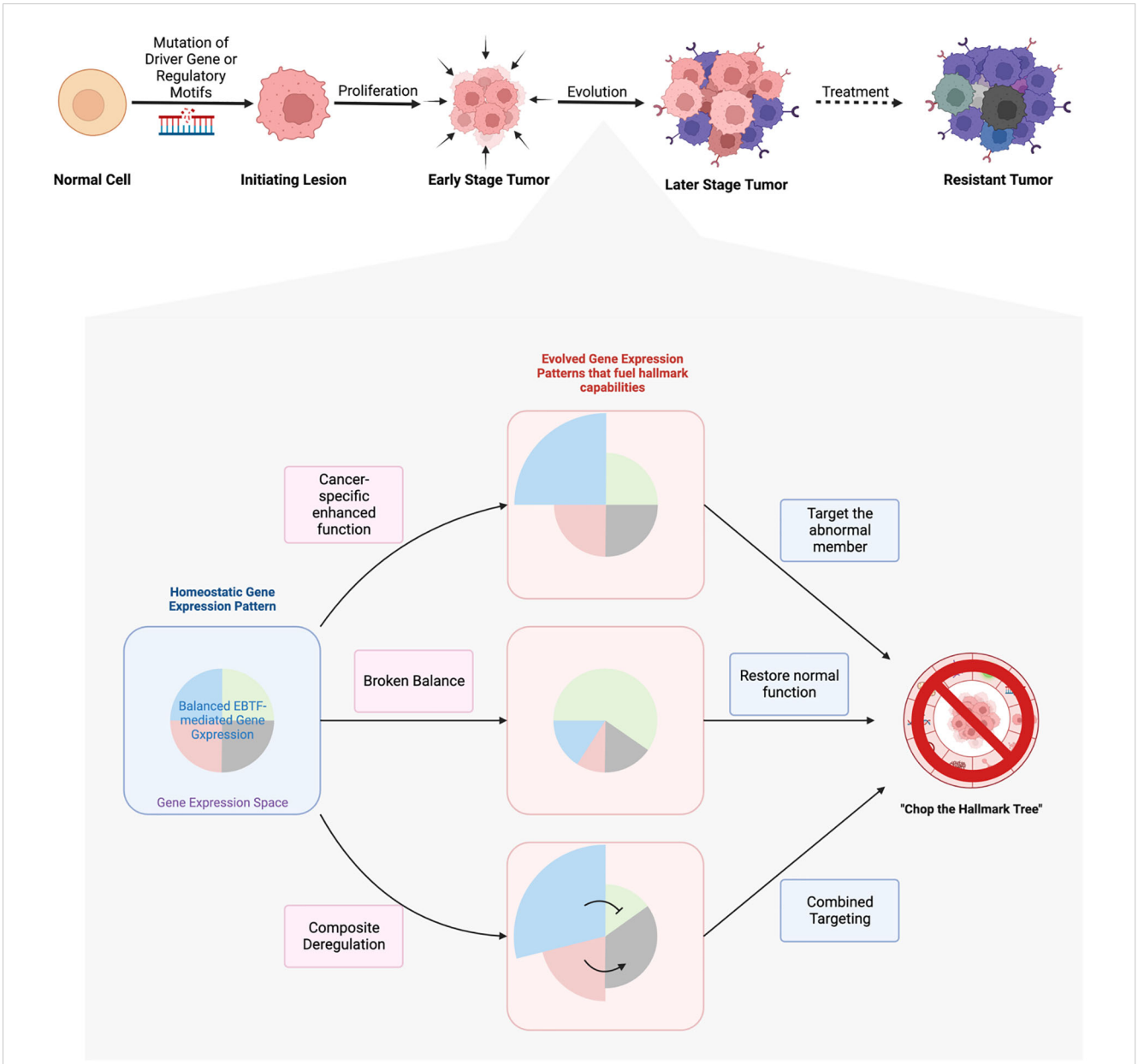


FIGURE 5
Hypotheses of EBTFs dysregulating transcription program during tumorigenesis and targeting strategies. The development of tumor is a long process from initial mutation to the evolved heterogenous tumor mass. Driver mutations in signaling molecules that sustain proliferation signal is the best understood mechanism of tumor initiation. In tumors that lack recurrent driver genes, mutations in the regulatory region of genes might be able to make up a transcriptional program to sustain a proliferation signal in the cell and initiate tumor. After initiation, tumor cells go through thorough evolution to grow into a tumor mass. Because transcription regulation is more flexible than obtaining new mutations, evolution of the transcriptome plays major role in the process of tumor development. In this process the transcriptional balance of TF networks such as EBTFs are broken in to promote the abnormal gene expression and fuel their progression. Such rewiring of the network can be caused by hyperfunction of one or more EBTFs through over-expression, *de novo* enhancers from mutations, and subsequent evolution of the transcriptome. To target these abnormal transcriptional programs, strategies can be taken to precisely eliminate hyperactivity of the master factors, restore function of factors that are suppressed by or competitive against oncogenic factors, or the combination of both.

discrepancies that have been encountered when trying to find a unifying function for one particular factor across different cancers. As chemical tools are becoming more available to regulate activities of EBTFs, we believe targeting EBTFs will be a promising new strategy for cancer therapy.

Author contributions

YP and SK reviewed the literature and wrote the manuscript. PW contributed scientific insights of the topic and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Sandra E. Sephton,
Brigham Young University, United States

REVIEWED BY
Tianyun Qiao,
Fourth Military Medical University, China

*CORRESPONDENCE
Annemarie D. Jagielo
ajagielo@stanford.edu

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Circadian, hormonal, and sleep rhythms: effects on cancer progression implications for treatment

Annemarie D. Jagielo[1,2]*, Catherine Benedict[2] and David Spiegel[2]

[1]PGSP-Stanford Psy.D. Consortium, Palo Alto University, Palo Alto, CA, United States, [2]Department of Psychiatry and Behavioral Sciences, School of Medicine, Stanford University, CA, Stanford, CA, United States

Circadian, hormonal, and sleep rhythm disruptions are commonly experienced concerns among cancer patients throughout the cancer care continuum. This review aims to summarize the existing literature on circadian, hormonal, and sleep rhythms in the oncological population, focusing on circadian disruption and physiological and psychological abnormalities, disease progression, and chronomodulated treatment approaches. The findings demonstrate that subjectively and objectively measured circadian rhythm disruption is associated with adverse mental health and disease outcomes in patients with cancer. Chronomodulated chemotherapy, light therapy, cognitive behavioral therapy for insomnia, and physical activity have shown evidence of effectiveness in improving sleep, and occasionally, disease outcomes.

KEYWORDS

circadian rhythms, cancer, sleep disturbance, cortisol, chronotherapeutics

1 Introduction

Sleep is essential for health and wellbeing. Human beings have an internal 24-hour biological clock fluctuating from periods of sleep to wakefulness (1). During sleep, vital processes occur, including cellular repair, endocrine regulation, and memory consolidation in the brain (1). Disruptions of this sleep pattern can lead to detrimental physical health outcomes, such as the increased risk for diabetes, heart disease, obesity (2), neurodegeneration (3), and overall early mortality (4), as well as mental health challenges including depression (2).

Over half of people with cancer experience sleep disturbances (5), including difficulty in initiating and maintaining sleep, excessive daytime sleepiness, sleep-wake cycle dysregulation, and problems with sleep efficiency and quality (5, 6). Sleep disturbance has been identified as both a consequence of and a potential risk factor for cancer (7). Adjusting to a cancer diagnosis and treatment is stressful and may impact sleep quality (8); sleep disturbance may also be caused by factors such as medications and treatment side

effects (9). Sleep disturbance throughout the cancer continuum from diagnosis to survivorship is associated with increased depression (10), fatigue (10), cognitive challenges (11), diminished quality of life (1, 12), and shortened survival (13).

2 Method

An independent review of the literature was performed by the first author using PubMed and Google Scholar databases. Eligibility criteria included peer-reviewed articles published in English that examined circadian and sleep disruption in the oncological population. Case studies and opinion and commentary papers were excluded. Search dates ranged from the earliest available date to 2023. The search themes were broadly categorized and included cancer-related circadian rhythm observations and associations, disruption and disease progression, and treatment approaches. The search terms included "cancer" and "circadian rhythms," "circadian disruption," "chronotherapy," "CBT-I, "sleep disturbance," "sleep-wake disorders," "cortisol disruption," "circadian disruption," "circadian disruption and psychological outcomes," "wrist actimetry," "diurnal cortisol slope," "light therapy," and "physical activity."

3 Results

3.1 Physiological and psychological abnormalities related to circadian disruption

3.1.1 Sleep disorders in the oncological population

Oncological patients often suffer insomnia, hypersomnolence, sleep-disordered breathing, and sleep movement disorders (7, 14). In a cross-sectional survey examining the prevalence of sleep disturbance in cancer patients, 44% of patients reported fatigue, 41% endorsed restless legs, 31% had insomnia, and 28% reported excessive sleepiness (15). The prevalence of insomnia is two to three times greater among cancer patients compared to the general population (16, 17). Spielman's three-factor model is comprised of predisposing, precipitating, and perpetuating factors of insomnia (18). In the cancer population, predisposing factors include age, family or personal history, comorbid psychiatric symptoms, and female sex (14, 19). Precipitating factors include cancer-related symptoms, emotional distress, hospitalization, and treatment (e.g., chemotherapy, surgery) which may disrupt patients' sleep schedules (14, 19). Perpetuating factors include disease and treatment-related symptoms, poor sleep hygiene, daytime napping, irregular sleep schedules, and sleep myths (14, 19). Head and neck cancers and radiation treatment pose risk factors for obstructive sleep apnea, the most common form of breathing-related sleep disorders (20). Intermittent hypoxia may contribute to tumor growth and metastasis (21, 22).

Sleep disturbance is also closely associated with cancer-related fatigue (CRF), a separate but interrelated condition characterized by a profound sense of exhaustion that can severely disrupt patients' quality of life, mood, and functioning (14, 23). CRF is associated with reduced daytime activity, increased daytime napping, and greater nighttime arousal (14, 24). CRF is a pervasive condition affecting 80-90% of patients undergoing cancer therapy and persists for months or years following treatment completion in roughly 30% of patients (23).

3.1.2 Sleep disruption and psychological outcomes

Cortisol is a catabolic glucocorticoid hormone with a crucial role in the stress response. During periods of physiological or psychological threat, cortisol levels rise, preparing the body for a "fight or flight" response by mobilizing glucose into the blood (25–27). Cortisol levels commonly follow a diurnal cycle by peaking in the morning, declining throughout the day, and reaching a nadir at nighttime (28–30). The increase at night provides adequate blood glucose levels during the prolonged period of fasting at night – 'those who sleep, dine' (31–33). Circadian rhythms reflect the ability of the stress response system to function properly (34). Extended exposure to stress impairs the normal circadian rhythm of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in elevated cortisol secretion throughout the day, when it normally declines.

3.1.3 Disrupted circadian cortisol and psychiatric disorders

Salivary cortisol has been used as an objective biomarker to assess the HPA axis and circadian rhythm disruption as it measures the biologically active, unbound cortisol and is easier to access than blood draws (31, 35). Diurnal cortisol slope variation may be a useful marker of circadian function due to the relationship between circadian rhythms and cortisol (36). Aberrant cortisol rhythms are associated with negative psychological outcomes in the cancer population. For example, metastatic breast cancer patients who reported greater depressive symptoms demonstrated suppressed immunity as measured by lower average induration size in a test of cell-mediated immune response - delayed-type hypersensitivity to intradermal administration of seven common antigens (37). In advanced-stage ovarian cancer, higher cortisol levels (area under the curve) are associated with elevations in depressive symptoms and proinflammatory cytokine interleukin-6 (IL-6) and greater evening cortisol levels (38). Elevation of a single evening cortisol measure is a good marker of flattened diurnal cortisol levels in women with breast cancer (36). Elevated nocturnal cortisol and diminished cortisol variability are associated with enhanced functional disability, vegetative depression, and fatigue among ovarian cancer patients (31). Following primary treatment for epithelial ovarian cancer, diurnal cortisol rhythms normalized and IL-6 decreased (39), suggesting inflammation markers may re-regulate following treatment.

Psychological distress and coping styles can impact circadian rest/activity and cortisol rhythms. Giese-Davis et al. (28) demonstrated that metastatic breast cancer patients who repressed emotion displayed flatter cortisol rhythms compared to self-assured and nonextreme groups. Similarly, distress and

avoidant coping are associated with circadian rest/activity rhythm disruption in presurgical breast cancer patients (40), while avoidance-oriented coping is associated with flattened diurnal cortisol slopes in prostate cancer survivors (41). There is a significant relationship between posttraumatic growth and diurnal cortisol slope in metastatic breast cancer patients, suggesting positive psychological changes may be associated with normalization of cortisol slope and enhanced endocrine functioning (42). These results suggest the psychological sequelae associated with cancer may be related to the dysregulation of the HPA axis.

3.1.4 Subjective sleep measures and psychological outcomes

Sleep disturbance negatively impacts quality of life in patients with mixed cancers (43), breast cancer (44), and ovarian cancer (45). Sleep disturbance is significantly correlated with self-reported depression and fatigue in mixed cancer patients (10), while depression, pain, and life stress predict sleep disturbance in metastatic breast cancer patients (46). Poor sleep quality leads to emotional dysregulation and impaired daily functioning in patients with breast cancer (44). Daytime fatigue and diminished quality of life are qualities associated with shorter cancer survival (47). For patients with lung cancer, frequent nighttime arousals are associated with higher mortality risk (13). In many studies, sleep disturbance occurred early in the illness course (10, 13, 45). Some might expect sleep disturbance to occur solely at the end of life when numerous systems are disrupted. However, these data suggest sleep disturbance may not be the result of having a terminal illness, but rather an earlier factor in the disease progression.

3.2 Circadian disruption and disease progression

3.2.1 Circadian hormonal disruption and tumor growth

Circadian rhythm disruption may impact the ability of the immune system to fight tumor growth. A relationship exists between cancer and circadian clock genes, growth control, and growth effector genes (34). The circadian timing system (CTS) is a molecular clock with at least fifteen genes that regulate the sleep-wake cycle (48, 49). High-quality sleep reflects a robust CTS (47). CTS genes are critical for generating circadian rhythms, while disruptions in this system may promote tumor progression. Indeed, the mPer2 gene plays a crucial role in the circadian clock, and mPer2 gene deficiencies are related to tumor growth in mice (34, 50). Meal timing has been used to reset and reinforce the CTS (48, 51, 52). In mice, the use of meal timing enhances survival (51) and inhibits cancer growth (52). The clinical significance of these findings is supported by a study of 361 patients with colorectal cancer (53). Sleep problems at baseline independently predicted a higher risk of earlier death (HR: 1.36; $p = 0.011$), disease progression (HR: 1.43; $p = 0.002$) and poor treatment response (RR: 0.58; $p = 0.016$). These findings suggest circadian disruption may lead to cancer growth.

3.2.2 Circadian hormonal disruption and cancer progression

The disruption of circadian HPA rhythms is associated with cancer progression (34, 54–59). Patients with advanced cancer often demonstrate flattened diurnal cortisol compared with healthy controls (54, 55, 60, 61). Flattened cortisol slope is a prognostic indicator of early mortality in patients with breast cancer (61), lung cancer (54), and ovarian cancer (62). Abnormal cortisol rhythms characterized by a less rapid decline in cortisol levels late in the day are associated with fatigue in breast cancer survivors as well (63).

3.2.3 Sleep disruption and cancer progression

The actigraphic dichotomy index I<O is a robust metric that reflects individuals' daily patterns by indicating the percentage of in-bed activity counts that are less than median of out-of-bed counts (48, 64–69). A greater I<O index reflects enhanced circadian function and reduced nighttime motor activity (65, 69). I<O is a risk factor for overall survival in metastatic colorectal cancer (48) and breast cancer (70). However, findings regarding the relationship between circadian disruption measured through wrist actimetry and patient-reported subjective sleep data appear to be mixed, although subjective reports of poor sleep are necessarily somewhat unreliable (65, 71–73). Many studies noted a discrepancy between subjective and objective measurements (65, 71, 72) or inconsistent findings (74); yet Grutsch et al. (47) found a correlation between rest/activity rhythms and patient self-reported sleep quality on the Pittsburgh Sleep Quality Index (PSQI) questionnaire. This represents an area for further investigation and highlights the importance of acquiring both objective and subjective patient sleep data.

3.3 Treatment

3.3.1 Chronomodulated chemotherapy

Chronomodulated chemotherapy is the timed administration of chemotherapy based on circadian rhythms to enhance anticancer drug efficacy and/or tolerability and to reduce side effects (68, 75, 76). Modifying the timing of chemotherapy administration based on physiological rhythms can enhance treatment outcomes for patients with cancer (68, 76, 77). In a systematic review of 18 randomized controlled trials conducted by Printezi et al. (75), the use of chronomodulated chemotherapy was associated with reduced toxicity in 61% of studies. Furthermore, 17% of studies demonstrated enhanced efficacy of chronomodulated chemotherapy, as evidenced by overall survival, objective response rate, or time to treatment failure. However, in 11% of studies, chronomodulated chemotherapy reduced some toxic effects but increased others, and one (6%) study reported worse toxicity effects with chronomodulated chemotherapy compared to traditional

chemotherapy. Thus, in most studies, chronomodulated chemotherapy reduced toxicity without necessarily enhancing efficacy (75). A meta-analysis of three phase III trials showed males but not females with metastatic colorectal cancer obtained a survival benefit with chronomodulation vs. conventional chemotherapy (78). Chronomodulated chemotherapy has not yet been widely adopted despite these findings (79).

3.3.2 Light therapy

Light serves as a zeitgeber for the human clock (80). Light therapy can synchronize circadian rhythms and it is used to treat disorders linked to circadian disruption, such as shift-work syndrome, seasonal depression, fatigue, and jet lag (81–86). Light therapy may reduce circadian deterioration during chemotherapy and throughout survivorship. Studies show its benefits in preventing circadian rhythm decline (87, 88) and reducing CRF (81) during chemotherapy. A randomized controlled trial found bright light therapy led to an improvement in cancer survivors' CRF compared to dim red-light exposure (89). A recent randomized controlled trial (83) evaluated the efficacy of bright light therapy compared to dim white light in a sample of 166 (non-)Hodgkin lymphoma survivors. Participants in both conditions reported reductions in CRF and improvements in mood, sleep quality, and quality of life. These results suggest light therapy may serve as a promising intervention to regulate circadian clocks.

3.3.3 Cognitive behavioral therapy for insomnia

Insomnia treatment often includes a pharmacological component. Sedative hypnotics and antidepressants are frequently prescribed (14, 90), but their evidence base is lacking in the oncological population (7, 14). The use of these medications is not a recommended long-term strategy (14). If incorporated into treatment, pharmacotherapy is recommended in combination with Cognitive Behavioral Therapy for Insomnia (CBT-I; 14). CBT-I is the first-line treatment for insomnia, compromising relaxation training, sleep hygiene, cognitive restructuring, stimulus control, and sleep consolidation (91). CBT-I can be delivered in-person, via Telehealth, or self-administered by video, improving access to care (92, 93). CBT-I has demonstrated efficacy in the cancer population. For example, a meta-analysis of 16 trials found CBT-I improved various insomnia outcomes, including insomnia severity, sleep late onset, wake after sleep, sleep time, and sleep efficiency (94). However, these effects were temporary and diminished in short-term follow-up. Similarly, a meta-analysis examining the efficacy of CBT-I for cancer survivors found CBT-I improved patients' insomnia severity, sleep efficiency, sleep latency, and wake after sleep onset, with effects remaining durable for up to 6 months (95). A comprehensive review of 12 studies found CBT-I delivered in various modalities improved cancer patients' insomnia outcomes, mood, quality of life, and CRF (92). CBT-I was more effective than mindfulness-based cancer recovery in changing patients' dysfunctional beliefs regarding sleep (96), although both interventions reduced insomnia severity. Self-administered CBT-I is a cost-effective alternative to professionally based delivery, though slightly less effective (93). Taken together, these results suggest CBT-I has merit in improving cancer patients' sleep outcomes throughout the cancer continuum.

3.3.4 Physical activity

In the general population, physical activity has been shown to improve sleep (97, 98). Exercise may serve as a preferable, low-cost, and readily implementable lifestyle modification for individuals with sleep-related problems. Exercise has been shown to improve the subjective and objective sleep quality of cancer patients and survivors (99–102). Although research is limited in the cancer population, studies have demonstrated yoga (101), tai chi (102), light intensity (100), and wearable technology-based physical activity (99) can reduce sleep disturbance and improve sleep quality in the oncological population.

4 Discussion

4.1 Strengths and limitations

The literature on circadian disruption and psychological outcomes demonstrates a significant relationship between psychosocial factors and sleep disturbance in cancer patients. A strength of the literature is the use of biological indicators such as diurnal cortisol slope to assess circadian disruption (28, 29, 31, 36–42). Additionally, Schrepf et al. (39) and Hoyt et al. (41) utilized longitudinal designs, allowing for observations of changes in diurnal cortisol and outcomes over time. Limitations include the cross-sectional and correlational study designs, limiting causal inferences, and lack of inclusion of healthy control groups in most studies (28, 31, 36–42). Objective biomarkers (i.e., diurnal cortisol slope and wrist actimetry) bolster research on circadian disruption and cancer progression. Wearable biomarkers provide an affordable and accessible means for assessing circadian disruption (66, 71). However, the correlational nature of the literature restricts causal connections, while small sample sizes (54, 56, 59, 63, 70) and homogenous samples (62, 70) limit generalizability.

The chronomodulated chemotherapy literature is strengthened by the use of randomized controlled trials to demonstrate causal effects of toxicity reduction following treatment (75). Limitations include the relative paucity of available research on this subject, heterogeneous study designs, and varied dosage/duration of infusions in the literature that prevented Printezi et al. (75) from examining the independent impact of chronomodulation. The fact that chronomodulation seems to work better among male vs. female patients introduces a source of variability in response that is not yet fully understood (78). Strengths of the light therapy research include the RCT designs (81, 83, 87–89) and the use of both objective and subjective measures to assess sleep/wake patterns (81, 83, 88, 89). Limitations of the studies include relatively small sample sizes (81, 87, 88) and limited adherence to the lightbox intervention (81, 87).

Strengths of the CBT-I literature include the number of meta-analyses included in the systematic review of CBT-I by Gao et al.

(94) and the inclusion of systematic searching and methods sections in the other systematic review articles (92, 95). Limitations include the significant number of patients who do not adhere to the CBT-I protocol (92) and the potential risk of publication bias (92, 94, 95). Finally, strengths of the physical activity literature include the use of actigraphy data to assess objective sleep/physical activity patterns (100, 101), the novelty of the wearable technology-based intervention (99), the use of objective and subjective measures both pre- and post-intervention (99, 101), and random assignment and a partially blinded treatment protocol (102). The limitations include homogenous (102) and relatively small samples (99, 100, 102), lack of a triple-blind study design (101), and cross-sectional study design (100).

4.2 Future directions for research and treatment

Circadian-based chemotherapy administration is an important therapeutic option that may reduce chemotherapy toxicity (75). However, larger randomized controlled trials are needed to inform clinical practice (75). Novel, machine-learning approaches to assess circadian clock disruption in oncology are currently being developed (103, 104) and may have promise as a prognostic biomarker in breast cancer. These circadian-conscious strategies may refine cancer treatment moving forward. Overall, these findings have significant clinical implications and suggest providers should assess for sleep disruption throughout the cancer trajectory and utilize relevant, evidence-based treatments when clinically indicated.

4.3 Conclusion

There is a clear association between circadian disruption and adverse psychological and medical consequences in the oncological population. This review provides evidence that diurnal cortisol variation is associated with many psychological abnormalities, such as depression and fatigue. Further, this review underscored associations between circadian disruption and disease progression. Flattened cortisol slope is a prognostic indicator in a variety of cancer types, while I<O abnormalities constitute a risk factor in metastatic colorectal and breast cancer. Treatments including chronomodulated chemotherapy, light therapy, CBT-I, and physical activity have demonstrated evidence of efficacy in improving sleep, reducing treatment toxicity, and, in some cases, disease outcomes.

Author contributions

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Conflict of interest

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EDITED BY
Sandra E. Sephton,
Brigham Young University, United States

REVIEWED BY
DeeDee Smart,
National Cancer Institute (NIH), United States
Arturo Ortega,
Center for Research and Advanced Studies of the National Polytechnic Institute, Mexico

*CORRESPONDENCE
Craig Gouldthorpe
gouldthc@tcd.ie

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Circadian rhythm disorders in patients with advanced cancer: a scoping review

Craig Gouldthorpe[1,2]*, Jenny Power[2,3] and Andrew Davies[1,2,3]

[1]School of Medicine, Trinity College Dublin, Dublin, Ireland, [2]Academic Department of Palliative Medicine, Our Lady's Hospice and Care Services, Dublin, Ireland, [3]School of Medicine, University College Dublin, Dublin, Ireland

Circadian rhythms can be demonstrated in several biomarkers and behavioural activities, with rhythmical patterns occurring roughly over a 24-h period. Circadian disorders occur in patients with cancer and may be associated with poor clinical outcomes. This scoping review aimed to identify circadian rhythm research and reporting practices, circadian rhythm patterns, circadian rhythm disorders, and relevant associations of circadian rhythm disorders in patients with advanced cancer. Studies involved adult patients with locally advanced or metastatic cancer and used objective measures of circadian rhythmicity. Two independent authors completed initial screening of title and abstracts, full text reviews, data extraction, and data checking. A total of 98 articles were highlighted in the scoping review, which utilised physical activity measures (actigraphy and polysomnography), biomarkers (cortisol and melatonin), or a combination. Several circadian rhythms are commonly disordered amongst patients with advanced cancer and have significant implications for symptom burden, quality of life, and survival. It remains unclear which patients are most at risk of a circadian rhythm disorder. Significant heterogeneity exists in research and reporting practices. Standardising this approach may address discrepancies in the current literature and allow for research to focus on the most relevant parameters and approaches to improving circadian rhythmicity.

KEYWORDS

cancer, circadian rhythms, symptoms, quality of life, survival

Introduction

Circadian rhythms (CRs), repeating patterns approximately every 24 h, can be observed throughout the human body in behavioural activities, such as sleeping and feeding, and biochemical and hormonal changes, such as cortisol and melatonin secretion (1). CRs are coordinated by a central "pacemaker" or "clock" situated in the suprachiasmatic nuclei within the hypothalamus that attempts to synchronise internal body clocks with the 24-h light–dark cycle (1, 2). Additionally, areas within the brain and peripherally, such as endocrine organs, contain self-sustained secondary clocks (2).

In health, two well-established endocrine biomarkers of CRs are melatonin and cortisol, with levels being measurable in several samples, including serum, saliva, and urine (2–4). Serum melatonin begins to rise from around 22:00, peaking at around 04:00, before falling towards a baseline by 10:00, which persists throughout the day. Cortisol levels peak in the early morning, around 08:00, before falling during the day to a baseline at around 00:00 (2).

Physical activity demonstrates a circadian rhythm, with peak physical activity occurring around 14:00 and the most restful period centred around 03:00, although variability does exist between individuals (5). Circadian sleep and physical activity are primarily assessed using polysomnography and actigraphy (6). Although polysomnography and actigraphy are comparable, actigraphy can be applied in various settings and allows for prolonged periods of monitoring (6). Actigraphy utilises a wrist-worn device to detect physical movement during sleep and wake periods, with analysis of data producing several measures of circadian rhythmicity (6). Actigraphy is often accompanied by patient diaries, an approach supported by the American Academy of Sleep Medicine when investigating circadian rhythm sleep disorders (7). Diaries, however, can be burdensome, inaccurately completed, and subject to bias. Adult actigraphy research has focused on sleep–wake activity, particularly sleep onset–offset and the timing of activity phases. Various measures are used within research to describe the robustness of circadian rhythmicity or the timing and relationship of events over 24-h periods (see Table 1).

Circadian rhythmicity can alter during an individual's lifespan and impact on health and disease. With advancing age, activity levels decline, peak activity occurs earlier, sleep becomes shorter and more fragmented, and daytime napping increases (33, 34). Circadian

TABLE 1 Measures of circadian rhythmicity.

Circadian measure	Description
General terms	
Acrophase	The timing of peak level (8)
MESOR	The average level over 24 h (8)
Up-MESOR	The timing of switching between low and high activity (8)
Down-MESOR	The timing of switching between high and low activity (8)
Amplitude	The difference between maximum and minimum level (8)
Double amplitude	The difference between maximum and minimum levels of the cosine function (9)
Cortisol/melatonin specific	
Area under the curve	Total cortisol levels under the curve of all measurements. Larger AUC indicates circadian disruption (10)
F test	To test the zero amplitude hypothesis (11)
Diurnal slope/diurnal decline phase	Rate of decline from cortisol peak. A smaller, more negative value indicates a steeper slope. A larger β-value, closer to 0, indicates a flatter slope, abnormal peaks, or a rising level. Calculated with log-transformed cortisol values undergoing regression analyses (12–15)
Phase angle of entrainment	The timing of the peak of the first waveform relative to awakening (16)
Dim light melatonin onset (DLMO)	Timing when melatonin exceeds a threshold considering mean and standard deviations of melatonin prior to the melatonin rise on 3 days (17)
Diurnal cortisol variability	Difference in cortisol value at earliest collection time and nighttime point (morning – night)/morning (18)
Cortisol variations (VAR)	08:00 cortisol – 20:00 cortisol/08:00 cortisol (19)
Cortisol awakening response	Cortisol slope after awakening (waking and +30 min sample) (15)
CAR_i	Cortisol 30 min after awakening – cortisol at awakening (13)
CAR_{auci}	Area under the curve during first 60 min after awakening (13)
Cortisol variability	Morning cortisol – night cortisol/morning cortisol (18)
Actigraphy specific	
General activity	
Mean activity	Mean of daily activity (20)
Intradaily variability (IV)	A measure of rhythm fragmentation (21)
Interdaily stability (IS)	A measure of rhythm stability between days (21)
VL5	Mean activity value of the 5 least active hours

(Continued)

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TABLE 1 Continued

Circadian measure	Description
L5	Mean timing of the 5 least active hours (21)
VM10	Mean activity value of 10 most active hours (21)
M10	Mean timing of the 10 most active hours (21)
Relative amplitude	(VM10 – VL5)/(VM10 + VL5) (21)
R-squared	Rhythmicity coefficient of the sleep–wake cycle (8)
Bathyphase	Time of lowest activity (22)
Circadian quotient	Amplitude/MESOR (23)
Rhythm quotient	$A_{24HR}/(A_4+A_8+A_{12})$ (23)
Circadian function index (CFI)	A combined measure of IV, IS, and RA (21)
Dichotomy index (I<O)	Activity in bed (I) compared to activity out of bed (O) (8, 24)
Dichotomy index for nighttime restfulness	I<O percentage of activity in bed, which falls below median activity out of bed (15)
Dichotomy index for daytime sedentariness	O<I percentage of activity out of bed, which falls below median activity in bed (15)
Autocorrelation coefficient (r24)	Correlates activity at same time points between different days, considering consistency and regularity. Higher values are more stable (15, 24)
Day-night activity balance	Ratio of activity during the day and night (23)
Night-day sleep balance	Ratio of sleep during the night and day (23)
Night-day sleep duration balance	Not described (23)
Night-day longest sleep balance	Not described (23)
Night-day per cent sleep balance	Not described (23)
Total wake time (day)	Total amount of time spent awake (25)
Movement and fragmentation index	Sum of per cent of mobile minutes and immobile bouts <1 min/no. immobile bouts within a time interval (26)
Inactivity index	Not defined (27)
Rhythm index	A measure of quality and regularity of the inactive state (17)
P1-1	Probability of staying in inactive/rest state (17)
Sleep–wake activity	
Bed time (BT)/time of retiring	Time to bed and lights switched off (27, 28)
Get up time (GUT)/time of waking up	Time woke up in the morning (27, 28)
Total time in bed	Time between BT and GUT (27, 28)
Sleep onset latency	Number of minutes to fall asleep. Time between BT and sleep onset (27–29)
Latency to persistent sleep	Number of minutes to persistent sleep (27)
Wake after sleep onset	Sum of all wake periods whilst in bed OR between sleep onset and offset (26, 27)
Total sleep time	Time between bedtime and wake time (26) OR total time in bed scored as asleep (27)
Total nighttime sleep	Sum of all sleep periods whilst in bed (26)
Sleep midpoint	Midpoint of time in bed (27, 28)
Sleep motor activity (SMA)	Mean number of movements in a given epoch (28)

(Continued)

TABLE 1 Continued

Circadian measure	Description
Diurnal motor activity (DMA)	Mean number of movements in each epoch (28)
Wake minutes	Duration of wake during sleep period (20, 27)
Wake episodes/number of awakenings in the night (NWAK)	Number of wake episodes during sleep period (29)
Mean duration of wake episodes	Mean duration of all wake episodes (27)
Long wake episodes	Number of wake episodes lasting 5 min/+ (27, 30)
Longest wake episode	Duration of the longest wake episode (27)
Sleep fragmentation index (SFI)	Number of awakenings/total sleep time in minutes (27)
Sleep efficiency	Proportion of time asleep whilst in bed (10, 27)
Short burst inactivity index	Zero activity of 1 min/zero activity of any duration (27)
Time napping and sleep minutes	Duration of sleep episodes during wake period (20)
Long sleeps	Frequency of long naps lasting 5 min/+ (30)
Sleep episodes	Number of sleep episodes during wake period (20, 30) OR number of blocks of continuous sleep epochs (27)
Mean duration of sleep episodes	Mean duration of all sleep episodes (27)
Sleep episodes 5 min/+	Number of sleep episodes whose duration lasts 5 min or more (27)
Longest sleep episode	Duration of longest sleep episode (27)
Time awake spent immobile	The percentage of time spent awake and immobile (31).
Early morning awakening	Period of wakening in the morning lasting 30 min or longer (25)
% sleep (up interval)	Per cent of time asleep between two attempted sleep periods (32)
% sleep (down interval)	Percentage of time asleep during attempted sleep time (32)

rhythm disorders (CRDs), where normal rhythmicity is altered, can perpetuate cancer and metabolic, neurodegenerative, psychological, and cardiovascular disease (35). CRDs are common amongst cancer patients, affecting up to 75%, and are associated with increased symptom burden, poorer quality of life, and shorter survival (36, 37). Interestingly, even misalignment between preferred and actual bedtimes is associated with cancer progression (38).

Aims

This review will broadly consider circadian rhythms of cortisol, melatonin, and physical activity in advanced cancer patients, with the aim of:

1. Identifying investigative approaches and reported parameters
2. Identifying circadian rhythm and disordered rhythm patterns
3. Identifying associations with circadian rhythm disorders, focusing on symptoms, quality of life, and survival.

Methodology

Data sources

A literature search was performed using PubMed, Embase, Web of Science, Ebsco host (CINAHL, Psychinfo, and Psycharticles), Scopus, and Cochrane on 20/04/2022. The search was updated on 05/05/2023. Keywords were restricted to title and abstract. No other limitations were placed.

Search terms

An example search strategy within PubMed is as follows: ("circadian"[Title/Abstract] OR "sleep wake"[Title/Abstract] OR "rest activity"[Title/Abstract] OR "chrono*"[Title/Abstract] OR "clock"[Title/Abstract] OR "Chronobiology Disorders"[MeSH Terms]) AND (("advanced"[Title/Abstract] OR "progressive"[Title/Abstract] OR "palliat*"[Title/Abstract] OR "terminal"[Title/Abstract] OR "metast*"[Title/Abstract] OR "end of life"[Title/Abstract]) AND ("cancer*"[Title/Abstract] OR

"malig*"[Title/Abstract] OR "tumo*"[Title/Abstract] OR "neop*"[Title/Abstract] OR "oncol*"[Title/Abstract] OR "Neoplasms"[MeSH Terms])).

Eligibility

Studies were eligible for inclusion if the patients were ≥18 years old with a diagnosis of advanced cancer (locally advanced or metastatic). "Locally advanced" differed between cancer histology and several studies included, rather than focused solely on, patients with advanced cancer. Eligible studies also had to consider objective measures of four markers of circadian rhythm disorders (sleep–wake cycles, rest–activity cycles, cortisol levels, and melatonin levels) and be fully translated into English.

Screening, data extraction, and data synthesis

Two authors (CG and JP) independently screened the title and abstract for potential full-text review. Review papers identified in the initial search were also screened for additional articles. Full-text articles were reviewed independently by two authors (CG and JP). The reference lists of included articles were searched for additional articles. Where full-text copies were not immediately available, the leading author or associated research centre was contacted, and if no full-text made available, the article was excluded. Data were extracted by a single author (CG) and confirmed independently by a second author (JP). The data extraction tool was then coded into main themes including circadian measures, circadian rhythm patterns, and the association of circadian measures with symptoms, quality of life, survival and other relevant factors. The review is presented according to the PRISMA-ScR checklist.

Results

The scoping review highlighted 98 articles, which were mainly observational in nature. The review process can be seen in Figure 1, and the results from individual studies are detailed in Tables 2A–D.

Investigative and reporting practice

Authors utilised actigraphy (n=34), cortisol (n=33), combined assessment methods (n=18), melatonin (n=11), and polysomnography (n=2) in their investigations.

Articles focused on different cancer diagnoses, including breast (n=24), gastrointestinal (n=22), mixed cancer diagnoses (n=22), lung (n=20), gynaecological (n=7), head and neck (n=2), and renal (n=1). All studies included patients with advanced or metastatic cancer; 40 studies focused solely on advanced or metastatic cancer patients.

Heterogeneity was seen in the investigational approach and the reported measures of circadian rhythm. Studies assessing melatonin used between 20 and 190 patients, sampled melatonin at 1–16-h intervals, and used between 2 and 10 different time points. Studies assessing cortisol used between 13 and 210 patients and sampled at 20-min to 12-h intervals. Sampling included fixed times, time slots, and/or were reported in relation to waking and bedtime. Melatonin and cortisol studies lasted between 24 h and 3 days for most studies.

Variation was also seen in the samples utilised. As an example, articles focusing on cortisol measures (n=30) used serum (n=14), saliva (n=16), serum and saliva (n=3), or urine (n=1) samples. Reported measures included descriptive statistics, mean levels (MESOR), variation between peak and trough levels at several time points (amplitude, double amplitude, 12- and 24-h amplitude), timing of peak level (acrophase), the area under the curve, the shape of changing levels between peak and trough levels (diurnal slope, phase angles), cortisol variability, and the change in cortisol levels on waking (cortisol awakening response, CAR).

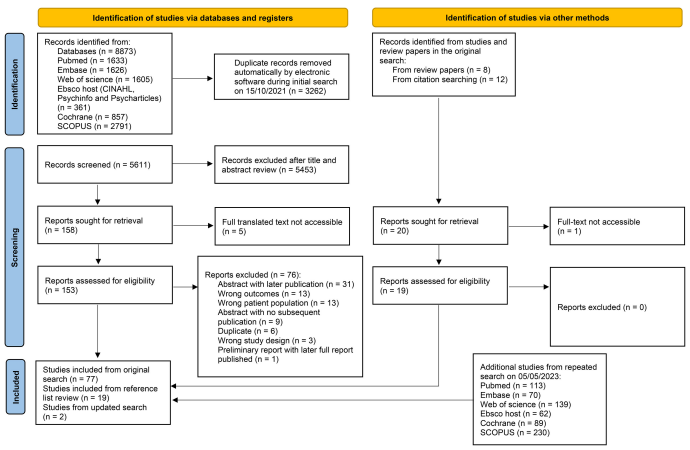

FIGURE 1
A flow chart of article identification, screening, and exclusion. *From:* Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71. For more information, visit: http://www.prisma-statement.org/.

TABLE 2A Melatonin circadian rhythms and their associations in patients with advanced cancer.

Studies with a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Levi et al., 2020 (17)	"IDEAs" study: 25 (21 M) locally advanced or metastatic gastrointestinal cancer patients, median age 66 years "PicaPill" study: 33 (15 M) control subjects, median age 35 years	6-h salivary melatonin levels (19:00, 20:00, 21:00, 22:00, 23:00, 00:00) and the dim-light melatonin onset (DLMO), which represents the time melatonin levels rise above a threshold.	Melatonin levels rose from baseline in all participants between 18:00 and 23:00. Patients with cancer had higher baseline melatonin levels. The rise in melatonin was higher in controls and patients with cancer with relatively less in-bed to out-of-bed physical activity (threefold low I<O group, fivefold high I<O group, and sixfold in control subjects). Patients with cancer and relatively less daytime to night-time activity had earlier DLMOs (1,948 vs. 2,144, p=0.08). Significant inter-individual variation was noted.
Mazzoccoli et al., 2012 (39)	9 (M) stage 2–4 non-small cell lung cancer patients, mean age 51 years 11 (M) control subjects, mean age 44 years	24-h rhythm of serum melatonin: (06:00, 10:00, 14:00, 18:00, 22:00, 02:00)	A 24-h rhythm was found in all subjects with a peak concentration at night, and a trough concentration near waking. Mean values did not differ between the groups at any time points.
Hu et al., 2009 (40)	30 (26 M) "advanced" non-small cell lung cancer patients, mean age 60 years 63 (53 M) control subjects, mean age 67 years	Serum melatonin levels and 24-h rhythm (12:00, 00:00) and urine 6-sulfatoxymelatonin levels (major metabolite of melatonin) (07:00, 16:00)	A 24-h rhythm of melatonin and 6-sulfatoxymelatonin were present in all subjects. Serum melatonin at 00:00 was lower in patients than in control subjects ($p<0.05$). Urine 6-sulfatoxymelatonin at 07:00 and 16:00 was lower in patients than in control subjects ($p<0.05$)
Karasek et al., 2005 (41)	31 (F) stage 0–4 cervical cancer patients, mean age 53 years 14 (F) control subjects, mean age 54 years	Serum melatonin levels and area under the curve (AUC) (08:00, 12:00, 16:00, 20:00, 24:00, 02:00, 04:00, 08:00)	Cancer patients had significantly lower melatonin levels and area under the curve (AUC) than control subjects ($p<0.05$). "Nocturnal" melatonin levels and the AUC were significantly lower in patients with stage 3–4 cancer compared to patients with stage 0–1 cancer ($p<0.05$).
Mazzoccoli et al., 2005 (42)	17 stage 1–2 non-small cell lung cancer patients, mean age 67 years 17 stage 3–4 non-small cell lung cancer patients, mean age 70 years 17 control subjects, mean age 69 years	24-h rhythm of serum melatonin and AUC (06:00, 10:00, 14:00, 18:00, 22:00, 02:00, 06:00)	A 24-h rhythm was present in all three groups. AUC levels were lower in cancer patients ($p<0.05$) and lower in cancer patients with a higher cancer stage (p=ns)
Muc-Wierzgon et al., 2003 (43)	42 (25 M) "advanced" (metastatic) gastrointestinal cancer patients, mean age 61 years 30 (25 M) control subjects, mean age 57 years	Serum melatonin levels, 24-h rhythm (08:00, 14:00, 18:00, 22:00, 02:00, 08:00), amplitude (difference between peak and trough levels) and acrophase (time of peak level)	A 24-h rhythm was noted in all subjects. The maximal peak levels were higher for control subjects, but the minimal trough levels were similar for control subjects and patients. The mean amplitude was higher for control subjects. The acrophase occurred earlier for control subjects (04:35 vs. 08:50).
Ermachenkov et al., 2013 (44)	89 (49 M) gastric cancer patients (8 metastatic) 86 (31 M) colorectal cancer patients (5 metastatic) Mean age 62 years	Diurnal urinary 6-sulfatoxymelatonin levels	Gastric cancer patients with distant metastases had lower diurnal excretion than patients without distant metastases (231±27 ng/h vs. 422±36 ng/h, $p<0.001$). Colorectal cancer patients with metastatic disease had lower diurnal excretion than patients without metastatic disease (176 ±44ng/h vs. 422±36ng/h, $p<0.001$).

(Continued)

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TABLE 2A Continued

Studies with a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Karasek et al., 2000 (45)	23 (F) mixed gynaecological cancer, mean age 50 years, included "invasive ovarian" 16 (F) control subjects, mean age 51 years 7 (F) myomatous uterus patients, mean age 46 years	24-hour rhythms and AUC of serum melatonin (08:00, 12:00, 16:00, 20:00, 22:00, 24:00, 02:00, 04:00, 06:00, 08:00)	There was no significant difference in the 24-h rhythm and AUC between the three groups.
Baranowski et al., 1999 (11)	30 (17 M) stage 4 gastrointestinal cancer patients, mean age 64 years 29 age-matched healthy control subjects (gender not defined)	Serum melatonin: 08:00, 14:00, 18:00, 22:00, 02:00, 08:00	A 24-h rhythm was present for all subjects. Compared to controls, patients with cancer had similar minimum levels (12.1 pg/ml vs. 12pg/ml), lower maximum levels (34.3pg/ml vs. 65pg/ml), and lower average levels (MESOR) (23.1pg/ml vs. 48.2pg/ml)
Tarquini et al., 1999 (46)	39 (17 M) mixed cancer patients (21 metastatic), mean age 72 years 28 (11 M), control subjects, mean age 65 years	24-h serum melatonin levels and amplitude (00:00, 04:00, 08:00, 12:00, 16:00, 20:00, 24:00)	A 24-h rhythm was present for all subjects. The amplitude was smaller in cancer patients than control subjects ($p=0.003$) with higher daytime levels and lower night-time levels. No difference in amplitude was found in relation to cancer stage.
Dogliotti et al., 1990 (47)	Study 1: 132 (90 F), stage 1–4 mixed cancer patients, median age 63 years. 58 (32 M) control subjects, median age 35 years Study 2: 20 stage 1–3 breast cancer patients, median age 60 years Study 3: 18 mixed cancer patients, age and gender not defined, Control subjects age, gender, and number not defined	Study 1: Serum melatonin (08:00, 24:00) Study 2: Serum melatonin (08:00, 24:00) Study 3: 24-h serum melatonin (08:00, 12:00, 16:00, 20:00, 00:00, 04:00, 08:00)	Study 1: Melatonin levels were higher at both time points in patients than controls ($p<0.0001$). Stage 4 breast cancer patients had higher mean melatonin concentration than controls ($p<0.0001$) and higher levels at 24:00 ($p<0.002$) and 08:00 ($p<0.0001$) than stage 1–2 breast cancer patients. Advanced lung cancer patients had higher mean melatonin levels than control at both time-points ($p<0.001$ at 24:00, $p<0.0001$ at 08:00). Highest levels were in patients with SCLC. Advanced GI cancer patients had higher mean melatonin levels than control ($p<0.005$ at 24:00, $p<0.001$ at 08:00). Increased melatonin levels at 08:00 were associated with lower performance status ($r=-37$, $p<0.01$). Study 2: Melatonin levels did not differ in breast cancer patients pre- and post-surgical removal of the primary tumour Study 3: The circadian melatonin rhythm was similar between patients and controls.
Bartsch et al., 1981 (48)	10 (F) stage 1–4 breast cancer patients, mean age 57 years 10 (F) control subjects, mean age 53 years	24-h urinary melatonin (06:00–10:00, 10:00–14:00, 14:00–18:00, 18:00–22:00, 22:00–06:00)	Cancer patients had a lower average melatonin urinary excretion and elevated levels between 06:00 and 10:00 than controls. The differences were not statistically significant. A more synchronised excretion pattern was found in controls
Studies without a control group			
Mormont et al., 2002 (49)	18 (14 M) metastatic colorectal cancer patients, mean age 58 years	24-h rhythms for serum melatonin and serum 6-alphasulfatoxymelatonin on 3–6 h apart for 10–13 times points	15/18 (83%) patients had a 24-h serum melatonin rhythm and 6/18 (33%) had a 24-h 6-alpha-sulfatoxymelatonin rhythm.
Mormont et al., 1998 (50)	18 (14 M) metastatic colorectal cancer patients, age 35–72 years	24-h rhythm of blood melatonin	A group 24-h rhythm was evident for melatonin ($p<0.00001$) and significant circadian melatonin rhythm for 15 patients ($p<0.05$). Wide interindividual variation was noted.

(Continued)

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TABLE 2A Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Vivani et al., 1992 (51)	7 (6 M) small cell lung cancer patients, median age 51 years Patients administered IL-2	Baseline and weekly serum melatonin (08:00, 16:00, and 24:00)	Abnormal melatonin 24-h rhythms were found in all patients at baseline which was absent in 5 patients and had an earlier acrophase in 2 patients. The mean values were not significantly higher than those at 08:00 or 16:00.

M, male; F, female; AUC, area under curve; ns, not significant.

TABLE 2B Cortisol circadian rhythms and their associations in patients with advanced cancer.

Studies with a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Levi et al., 2020 (17)	IDEAs study: 25 (21 M) locally advanced or metastatic gastrointestinal cancer patients, median age 66 PicaPill study: 33 (15 M), control subjects, median age 35	2-day salivary cortisol (3-hourly) and 7-day wrist actigraphy and chest accelerometry and 1-day melatonin samples hourly at 19:00	A consistent diurnal change in cortisol levels was seen in most controls and patients, irrespective of their dichotomy index (I<O), which represents the relative difference between in-bed and out-of-bed physical activity. Those with a high I<O (i.e., relatively less in-bed to out-of-bed activity) had a larger circadian cortisol amplitude (difference between peak and trough concentrations). No significant difference was found in other cortisol parameters between I<O groups.
Zeitzer et al., 2016 (52)	97 (F) recurrent or metastatic breast cancer patients, age 57.6 24, health age-matched controls, age 57.1 (gender not identified)	Combination: 28-h plasma cortisol (20–60-min intervals) and polysomnography and 2-week actigraphy (Actiwatch 2) and sleep diary	There were no differences in the cortisol amplitude, MESOR (mean value) or absolute/relative timing between groups ($p>0.09$). There were no differences in the diurnal cortisol rhythm between groups ($p>0.11$). Abnormal cortisol peaks, midway through the sleep episode, were seen in a subset of patients and were associated with increased wake episodes ($p=0.004$), metastases to bone or organs rather than local recurrence ($r=-0.37$, $p=0.002$), use of steroids ($r=0.26$, $p=0.03$), ER negative status ($r=-0.25$, $p=0.04$) and higher a stage of initial diagnosis ($r=0.31$, $p=0.009$). In a multivariate analysis, metastases to bone ($p=0.02$) and ER negative status ($p=0.048$) continued to be associated with the abnormal cortisol peaks. Abnormal cortisol peaks were not related to psychological traits ($p>0.018$). Larger abnormal peaks were associated with a shorter disease-free interval ($r=-0.30$, $p=0.004$). The disease-free interval (DFI) and the diurnal cortisol rhythm were not associated ($p>0.10$).
Du et al., 2013 (53)	25 (15 M) stage 2–4 lung cancer patient with depression, mean age 55.1 39 (23 M) stage 2–4 lung cancer patients without depression, mean age 57.0 21 (8 M) patients with depression, mean age 53.8 41 (21 M) control subjects, mean age 55.9	24-h salivary cortisol (08:00, 16:00, 00:00, 06:00)	Lung cancer patients with depression had a flattened circadian cortisol pattern (less diurnal variation) compared to other groups. Lung cancer patients also had higher salivary cortisol at 00:00 compared to lung cancer patients without depression ($p<0.001$). The salivary cortisol area under the curve (AUC) was significantly higher in patients with depression only than the other groups ($p = 0.021$). Salivary cortisol diurnal variation (VAR) was significantly lower in lung cancer patients with depression than other groups ($p<0.001$).

(Continued)

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TABLE 2B Continued

Studies with a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Kim et al., 2012 (13)	52 (37 M) stage 3a–4 lung cancer patients, mean age 60.8 56 (32 M), control subjects, mean age 60.7	2-day salivary cortisol (waking, +30 min, +60 min, 21:00)	The cortisol awakening rise (CAR) represents the rapid rise in cortisol on awakening, and levels at 0, + 30 min and +60 min were significantly higher in controls than in patients ($p<0.05$). CARauci and CARi were higher in controls than in patients ($p<0.05$). Cortisol levels at 21:00 were similar between patients and controls. Flatter diurnal cortisol slopes were seen in patients compared to controls ($p<0.001$). Decreased cortisol levels and abnormal secretory patterns were seen in patients with an ECOG PS of 3 and 4 compared to controls ($p<0.001$). A positive correlation was seen between the diurnal cortisol slope and clinical disease stage ($p<0.01$). CARi and CARauci were not associated with clinical disease stage. For patients, gender, number of metastatic sites, chemotherapy status, body mass index, and smoking status were independent of the cortisol profile ($p>0.05$).
Mazzoccoli et al., 2012 (39)	9 (M) stage 2–4 non-small cell lung cancer patients, mean age 51.0 11 (M) control subjects, mean age 43.6	Combination: serum cortisol and melatonin (06:00, 10:00, 14:00, 18:00, 22:00, 02:00)	Prominent 24-h cortisol rhythms were seen in all subjects with peaks at night for melatonin and near waking for cortisol. Mean cortisol values did not differ between groups at any time points. The overall 24-h mean for cortisol was higher in cancer patients than controls ($p=0.001$). An increased cortisol slope was associated with increasing disease severity ($p<0.001$).
Weinrib et al., 2010 (54)	100 (F) stage 1–4 ovarian cancer patients, mean age 58.19 77 (F) benign disease, mean age 51.04 33 (F) control subjects, mean age 52.79	Salivary cortisol (waking, 16:00–18:30, bedtime)	Mean afternoon cortisol for ovarian cancer patients was 55% higher than for healthy women ($p<0.0001$) and similar to patients with benign disease ($p=0.07$). Nocturnal cortisol levels for ovarian cancer were 41.5% higher than benign disease ($p=0.02$) and 103% higher than healthy women ($p=0.0001$). Cortisol variability of ovarian cancer patients was lower than for benign disease ($p=0.023$) and healthy women ($p<0.0001$). Adjusted for age and disease stage in the ovarian group, a higher nocturnal cortisol, and lower cortisol variability was associated with greater fatigue ($p=0.005$ and $p = 0.01$). Lower cortisol variability also associated with poorer physical well-being ($p=0.007$). Depression scores were associated with a higher nocturnal cortisol ($p=0.059$) and lower cortisol variability ($p=0.028$). A more advanced cancer stage was associated with a higher morning ($r=0.23$, $p=0.02$) and afternoon ($r=0.32$, $p=0.002$) cortisol, but not nocturnal cortisol ($r=0.13$, $p=33$). Adjusted for age and disease stage in ovarian cancer group: higher nocturnal cortisol associated with poorer physician-related PS (rated on a 0–4 scale) ($p=0.043$) and patient-related PS ($p=0.035$). Lower cortisol variability was also associated with poorer physician-rated PS ($p=0.01$) and poorer patient-rated PS ($p=0.004$).
Wu et al., 2008 (55)	13 (9 M) stage 2–4 nasopharyngeal cancer, median age 41 14 (8 M) healthy control subjects, mean age 24.5	24-h plasma cortisol (4 hourly sampling)	Patients had a lower cortisol MESOR compared to control (200.31 ± 14.38 nmol/L vs. 243.77 ± 14.96 nmol/L, $p=0.30$) The acrophase was later for patients than controls (09:14 vs. 08:41) The amplitude was higher for patients than controls (64.01 vs. 61.94) A clear cortisol circadian rhythm with peaks in the morning was seen in both groups
Mazzoccoli et al., 2005 (42)	17 healthy subjects, mean age 68.8 17 stage 1–2 non-small cell lung cancer patients, mean age 67.2 17 stage 3–4 non-small cell lung cancer patients, mean age 69.5	Combination: 24-h serum cortisol and melatonin (4 hourly)	No significant different in cortisol levels was seen amongst groups. Cancer patients did not show a clear rhythm of cortisol secretion.

(Continued)

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TABLE 2B Continued

Studies with a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Abercrombie et al., 2004 (12)	17 (gender not defined) metastatic breast cancer patients, mean age 57.6 31 (F) control subjects, mean age 56.0	3-day salivary cortisol at waking (mean collection time, 07:17), 12:00, 17:00, and 21:00	Patients had significantly flatter diurnal cortisol slopes than controls ($p<0.05$). Disease severity was associated with flatter diurnal cortisol slopes ($p=0.07$) and higher mean cortisol levels ($p<0.05$). Mean cortisol levels between groups were not significantly different at different time points. Diurnal cortisol slopes and mean cortisol levels did not correlate with psychological measures ($p>0.32$).
Baranowski et al., 1999 (56)	30 (17 M), "advanced" gastric, pancreatic, or colorectal cancer patients, mean age 63.5 20 control subjects (gender not defined), mean age 59.5	Serum cortisol (08:00, 14:00, 18:00, 20:00, 02:00, 08:00)	A "well-defined circadian rhythm" was seen in control subjects, with a MESOR (average) of 116.8pg/ml, amplitude (difference between peak and trough) of 85.5pg/ml and acrophase (timing of peak concentration) of 07:20. In patients, a later acrophase of 08:08 at 520±11.8ng/ml was noted, and the trough occurred at 18:00 at 279.7±3.7 ng/ml.
Mormont et al., 1998 (9)	3 study cohorts 1. 19 (7 M) control subjects, 7 young (mean age, 24), 6 elderly women (mean age, 74.7), 6 elderly men (mean age, 71.7) 2. 19 (F) advanced ovarian cancer patients, mean age 59 3. 18 (14 M) advanced metastatic colorectal cancer patients, mean age 58	Serum cortisol Retrospective studies—4 circadian time series at 3-monthly intervals Prospective study—5–6 samples in 1st and 4th day of chemotherapy	The mean cortisol peak occurred at 08:00. The cortisol trough occurred earlier in controls (20:00) than in ovarian and colorectal cancer patients (00:00 or 01:00). Mean serum cortisol amplitude was 30% lower in cancer patients compared to controls (ovarian $p=0.01$, colorectal cancer $p=0.002$). There was no significant influence of age, gender, performance status, percentage of liver replacement, or number of metastatic sites on the mean estimate of circadian amplitude in the colorectal cancer patients. Ovarian cancer patients with a WHO PS 3–4 had a significantly lower MAX-MIN and lower mean H8-16 cortisol than those with a performance status of 2 or less.
Singh et al., 1998 (57)	25 (F), early (TNM B-1) and advanced (TNM B-2) breast cancer patients, aged 25–60 years 15(F), control subjects, aged 25–40 years	24-hour urine 17-ketogenic steroid (17-KGS) and 17-ketosteroid (17-KS) at 6 hourly collections 17-KGS and 17-KS are metabolites that may be derived from adrenal steroids and androgens from the gonads	A significant circadian rhythm of urinary 17-KGS in controls and early-stage breast cancer (all $p<0.001$) with an acrophase of 18:14 in controls and 18:55 in early-stage breast cancer. In advanced-stage breast cancer the acrophase occurred at 16:26 with elevated values at almost all time points compared to controls and early-stage breast cancer patients. A significant circadian rhythm of urinary 17-KS was noted in controls. The acrophase was 21:18. A circadian rhythm of urinary 17-KS was also found in early-stage breast cancer with an acrophase around 20:59. An irregular circadian rhythm of urinary 17-KS was noted in patients with advanced breast cancer with an acrophase of 20:16, when compared to controls and early-stage breast cancer
Payer et al., 1997 (58)	11 (8 M), bowel cancer patients (4 metastatic) median age 65 17 (13 M), ulcerative colitis patients, median age 32.5, 28 (15 M) patients with large bowel polyps, median age 32.5 13 (10 M) health controls, median age 21	Serum cortisol (08:00, 12:00, 16:00, 20:00, 04:00, 08:00)	Cancer patients had a lower cortisol amplitude ($p<0.05$) and shorter 12-hour acrophase ($p<0.05$) than other groups.
Singh et al., 1995 (59)	25 (F) early and advanced breast cancer patients, aged 25–60 15 (F) control subjects, aged 25–40	Serum 17-hydroxycorticosteroid (OHCS) (06:00, 12:00, 18:00, 00:00)	Control subjects had a mean 17-OHCS of 19.21µg/dl at 06:00, which reduced throughout the day to minimum concentrations at 00:00. A significant difference at time points was found ($p<0.001$). The MESOR was 13.2±0.55µg/dl, and amplitude was 5.43µg/dl. The amplitude was significantly different from zero. The acrophase occurred at 08:56. Patients with advanced breast cancer had higher 17-OHCS at 06:00 than controls (34.56µg/dl), and an earlier acrophase of

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TABLE 2B Continued

Studies with a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
			04:38. Breast cancer patients have a higher MESOR than controls (p<0.001).
Singh et al., 1987 (60)	10 (F) advanced breast cancer patients, aged 35–60 Patient group split into two groups according to their circadian pattern (not further defined) 10 (F) control subjects, aged 25–40	24-h serum cortisol at 8-hourly intervals from 08:00	Control subjects had a mean 17-OHCS at 08:00 of 18.03μg/dl with a minimum level at 00:00. The amplitude was significant from baseline (p=0.001) suggesting a marked circadian rhythm. Group 2 (6 patients) had deranged circadian rhythms with no significant difference in mean values between time points or in the change in cortisol level from baseline. Group 3 (4 patients) had a mean 17-OHCS at 08:00 of 25.20μg/dl with a minimum at 00:00. A normal circadian rhythm was seen with a significantly amplitude from baseline (p=0.05).
DeMeester et al., 1979 (61)	76 stage 1–3 non-oat-cell bronchogenic carcinoma 15 control subjects with suspected carcinoma but found to have benign disease Age and gender not defined	Serum cortisol (08:00, 16:00, 00:00)	Stage 1 patients' cortisol levels at all time points did not significantly differ from controls. Stage 2–3 patients had higher 08:00 and 16:00 levels than control subjects and stage 1 (p<0.05). Stage 3 metastatic patients had similar 00:00 levels to 08:00 peak of controls. 66 patients maintained a normal diurnal rhythm with significantly higher 08:00 levels to 00:00. Two patients lost their diurnal variation and in 8 patients the rhythm was reversed with 00:00 levels higher than 08:00. Elevated cortisol at 00:00 was associated with progressive disease but not length of survival.
Bishop et al., 1970 (62)	80 inoperable lung cancer patients admitted for radiotherapy/chemotherapy, 45 inoperable or metastatic cancer patients (not lung) 35 control subjects admitted for minor surgery Age and gender not defined	Serum cortisol (basal 08:30–09:30, midnight 23:00–24:00, 08:30–09:30 the morning after 2mg dexamethasone)	Cancer patients had significantly higher 8 a.m. cortisol than controls. A reduction in the diurnal cortisol variation was seen in cancer patients. All cancer patients showed less cortisol suppression following dexamethasone than control. No significant correlation was found between TNF-alpha and cortisol. Significance levels not reported
Studies without a control group			
Cheung et al., 2021 (10)	30 (16 M) stage 3b-4 non-small cell lung cancer patients Group 1—Aerobic exercise, mean age 61.00 Group 2—Tai-Chi, mean age 61.11 Group 3—Self-management group, mean age 58.36	Combination: Salivary cortisol rhythms (0.5, 4, 8, and 12 h after waking) and 3-day actigraphy (AMI)	The diurnal cortisol slope (representing the decline in cortisol levels during the day following the morning peak) and the cortisol area under the curve values at baseline were identified but no correlations reported. No control group was included for comparison.
Allende et al., 2020 (63)	99 (F) metastatic or recurrent breast cancer patients, median age 54	3-day salivary cortisol at waking, +30, 12:00, 17:00, and 21:00	The diurnal cortisol slope data was split at the median point to distinguish flat and steep slopes. Flat and steep diurnal cortisol slopes had significantly different salivary cortisol levels at 12:00 (p=0.0086), 17:00 (p<0.0001), and 21:00 (p<0.0001), but not at waking (p=0.4795) or waking +30 min (p=0.1364). This suggests that the differences between flat and steep cortisol slopes occur at 12:00 or later. Flatter diurnal cortisol slopes were associated with an escape from dexamethasone suppression (p=0.0042).

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TABLE 2B Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Oh et al., 2019 (64)	46 (39 M), stage 2-4 small cell lung cancer and non-small cell lung cancer patients (15 patients had metastatic disease), 23 ≤65 years old, 23 >65 years old	24-h salivary cortisol (waking, +30 min, +60 min, 21:00–22:00)	Cortisol concentrations differed between patient and controls (p<0.001). The cortisol awakening response (CAR) represents changes in cortisol levels within given time periods. CARi (the cortisol increase in the first 30 min after waking) and CARauc (the cortisol increase in the first 60 min from waking) were both significantly smaller in the patients compared to controls (p<0.01). A flatter diurnal cortisol slope was seen in the patients compared to controls (p<0.001). Patients with Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) of 3–4 had a small CAR (p<0.001) and flatter diurnal cortisol slope (p<0.001) compared to than patients with an ECOG PS of 1–2. Patients with metastatic disease had a small CARauc than those without metastatic disease (p=0.003). Total MD Anderson Symptom Inventory scores, fatigue, and interference with general activity, work, and walking were all significant associated with a reduced CAR and a flatter diurnal cortisol slope (p<0.05).
Rebholz et al., 2018 (65)	57 (F) stage 0–5 breast cancer patients, mean age 52.32	Combination: 3-day salivary cortisol (waking, +30 min, 16:00, bedtime) and 3-day actigraphy (AMI)	The diurnal cortisol slope was not significantly correlated with QoL.
Cash et al., 2015 (15)	43 (F), stage 0–4 breast cancer patients, mean age 52.49	Combination: 3-day salivary cortisol (waking, +30 min, 16:00, "prior to going to bed") and 3-day actigraphy (Micro Mini-Motionlogger) and sleep diary	A higher CAR was associated with elevated levels of VEGF, TGF-beta, and MMP-9 (markers associated with angiogenesis, immunosuppression, epithelial–mesenchymal transition, tumour invasion and metastasis).
Hsiao et al., 2015 (66)	62 (F) stage 0–3 breast cancer patients, 25.8% had metastatic disease, mean age 35.3	24-h salivary cortisol (waking, +30 min, +45 min, 12:00, 17:00, 21:00)	A flatter diurnal cortisol slope was associated with greater tumour size (p=0.01) an increase of body mass index over 8 months (p=<0.001) and a persistently later waking time over 8 months (p=0.006). Factors that were not significantly associated with the cortisol slope included metastatic status, physical activity levels, time of going to bed, sleep problem index, and depressive symptoms.
Schrepf et al., 2015 (18)	113 (F) stage 1-4 ovarian, primary peritoneal or fallopian tube cancer patients, mean age 57.99	Salivary cortisol (waking, 17:00, and bedtime)	Increasing age was associated with a higher evening cortisol (p=0.004) but not cortisol variability or slope. "High grade" disease, and poorer physical well-being were associated with a higher night cortisol, a flattened diurnal cortisol slope, and reduced cortisol variability (all p<0.05). "Late" stage disease was also associated with higher evening cortisol (p=0.05). Shorter survival was seen with elevated night cortisol prior to surgery (HR, 1.802, p<0.001), and the diurnal cortisol slope (HR, 1.633, p=0.001). Longer survival was seen with cortisol variability (HR, 0.644, p<0.001). Estimated median survival for low evening cortisol was 7.3 years compared to 3.3 years in those with a high evening cortisol. Elevated night cortisol, a flattened diurnal cortisol slope, and reduced cortisol variability were associated with higher levels of inflammation indicated by ascitic and plasma IL-6 (all <0.05). The diurnal cortisol slope was associated to cortisol variability (r = 0.88, p<0.001). Night cortisol was correlated with cortisol variability (r=−0.727, p<0.001) and the diurnal cortisol slope (r=0.758, p<0.001).
Zeitzer et al., 2015 (16)	97 (F) recurrent or metastatic breast cancer patient, "age" 57.4	Combination: 27-h inpatient serum cortisol (20–60-minintervals from 3 h prior to bedtime to 1 h after wake time), salivary cortisol 09:00 on day 1, awakening and 30 min on day 2 and 2-week outpatient actigraphy (actiwatch 2) and sleep diary logs	The cortisol diurnal slope varied depending on the analytical method used. 10/91 (11%) had a "positive" slope from 06:00. A flatter diurnal cortisol slope was associated with a lower morning peak and elevated evening trough. Plasma and salivary cortisol concentrations were correlated (p<0.001).

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TABLE 2B Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Diaz et al., 2014 (67)	99 (F) metastatic breast cancer patients, median age 54	2-day salivary cortisol (waking, +30 min, 12:00, 17:00, and 21:00)	Post-traumatic growth (positive psychological change following cancer diagnosis or treatment) was associated with a steeper cortisol slope (p=0.039). 'Relating to others' subscale was also associated with a steeper cortisol slope (p=0.039). Income, education, marital status, age, time since recurrence, PR status, and metastatic sites were unrelated to cortisol slope (p>0.05).
Palesh et al., 2014 (68)	97 (F) metastatic or locally advanced breast cancer patients, mean age 54.6	Combination: 2-day salivary cortisol (waking, +30 min, 12:00, 17:00, and 21:00) 3-day actigraphy (micro-mini-motion logger) and sleep diary	Salivary diurnal cortisol was associated with survival (HR, 1.03; p=0.85) (character of the slope was not noted).
Sephton et al., 2013 (69)	62 (34 F) stage 1–4 non-small cell lung cancer and small cell lung cancer patients, mean age 64	2-day salivary cortisol (waking, +45 min, 16:00, 21:00) Single serum cortisol (time not defined)	A lack of normal diurnal variation was associated with shortened survival (HR, 68,052.8; p=0.009). The cortisol AUC and CAR were not associated with survival time. Flattened cortisol rhythms were associated with more advanced lung cancer (r=0.35, p=0.003), poor performance status (r=−0.29, p=0.012), being male (p=0.028), low total lymphocytes (r=−0.39, p=0.002), and low cytotoxic T lymphocyte count (r=−0.30, p=0.017). The diurnal cortisol slope was not significantly associated with CAR, age at diagnosis, cancer type, time since radio- or chemotherapy, socioeconomic status, prior martial disruption, depressive symptoms, fatigue, or sleep difficulties. The diurnal salivary cortisol slope was not associated with serum cortisol levels.
Cohen et al., 2012 (70)	202 (156 M), metastatic renal cell cancer patients, mean age 59	3-day salivary cortisol at waking +45 min, 8 h, 12 h, and bedtime)	The cortisol slope was significantly associated with survival (HR, 1.88; p=0.002). The cortisol slope was not associated with psychosocial variables or CES-D scores.
Dedert et al., 2012 (71)	57 (F) stage 1–4 breast cancer patients, mean age 52	Combination: 3-day salivary cortisol (3 days—waking, 30 min after, 16:00 and bedtime) and actigraphy and sleep diary	Intrusion and avoidant coping were not related to cortisol measures. A higher autocorrelation coefficient (a measure of physical activity circadian consistency between days) was associated with a steeper diurnal cortisol slope (r=−0.41, p=0.003).
Brivio et al., 2010 (72)	14 (10 M) metastatic non-small cell lung cancer, pancreatic cancer, prostate cancer, and malignant melanoma patients, median age 67	Serum cortisol (08:00 and 16:00) at baseline and following melatonin treatment	Patients had abnormal cortisol circadian rhythms (defined as a lack of decline of 30% in the cortisol level from the morning to the afternoon). No significant difference was reported in morning or afternoon mean cortisol levels between stable and progressive disease (significance levels not reported).
Sephton et al., 2009 (73)	72 (F), stage 4 metastatic breast cancer, mean age 54.5	3-day salivary cortisol (08:00, 12:00, 17:00, 21:00)	Greater depression symptoms were associated with higher morning cortisol (p<0.02) and accentuated diurnal cortisol rhythms (p≤0.05). Depression scores were uncorrelated with mean cortisol levels.
Lutgendorf et al., 2008 (74)	25 (F) low malignant potential patients, mean age 51.24 26 (F) early-stage ovarian cancer patients, mean age 55.6 86 (F) advanced ovarian cancer patients, mean age 60.22	Salivary cortisol (waking, +30 min, 15:00–18:00, 20:00–24:00)	Salivary cortisol was increased for all patients with advanced cancer patients having approximately 3 times the healthy population normal values. Cortisol AUC was significantly higher in advanced-stage patients compared to the low malignant potential patients (p=0.047). Diurnal cortisol levels did not significantly differ between groups or over the day (p>0.06 and p>0.73). Higher evening cortisol levels were associated with higher total depression (p=0.026) and vegetative depression (p=0.005).

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TABLE 2B Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Palesh et al., 2008 (75)	99 (F), metastatic breast cancer patients, mean age 54.65	Combination: 2-day salivary cortisol (waking, +30 min, 12:00, 17:00, 21:00) and 3-day actigraphy (micro-mini-motionlogger)	A flatter cortisol slope was associated with longer average wake episodes at night (r=0.21, p=0.04). No significant relationship was found between mean waking cortisol or cortisol rise and other sleep measures.
Giese-Davis et al., 2006 (14)	29 (F) stage 4 breast cancer patients, mean age 53.21 All received supportive-expressive group therapy (SET)	3-day salivary cortisol (08:00, 12:00, 17:00, 21:00)	Steeper diurnal cortisol slopes were associated with shorter duration of negative affect (p=0.02). 08:00 and mean cortisol levels were not associated.
Jehn et al., 2006 (19)	114 (38 M), mixed stage 4 cancer patients, (71 had "progressive" disease, 43 had "stable" disease) Mean age of patients with depression 62.7 Mean age of patients without depression 59.4	Serum cortisol (08:00 and 20:00)	Cortisol concentrations were significantly higher in patient with depression at 08:00 (p=0.003) and 20:00 (p<0.001). Cortisol diurnal variation (VAR) was significantly decreased in cancer patients with depression compared to those without depression (11.7% vs. 60.6%, p = 0.001).
Mussi et al., 2006 (76)	40 (22 F), colorectal cancer patients (13 patient had nodal disease, 10 had liver metastases), median age 66	Serum cortisol (23:00 and 08:00)	Patients with liver involvement had a higher evening cortisol (p<0.0005). Nodal involvement did not impact on cortisol levels. 28% had an altered circadian rhythm defined as 23:00 level >50% of 08:00 level. This was more frequent if there was nodal involvement and metastatic spread (p<0.005). Cortisol levels and circadian rhythm were unrelated to CD4+ lymphocyte count (a prognostic marker).
Spiegel et al., 2006 (77)	99 (F) metastatic breast cancer patients, median age 54	2-day salivary cortisol (waking, +30, 12:00, 17:00, 21:00)	The cortisol slope was correlated with cortisol rise within 30 min of waking (r=0.29, p=0.004), but not with the waking level (p=0.19). A flatter slope was associated with a higher 21:00 level (r=0.85, p<0.0001) and escape from cortisol suppression (r=0.30, p=0.005). No significant association was reported between the cortisol slope and CRF administration or social stress. Antidepressant use was associated with higher waking cortisol (r=0.21, p=0.04) and lower cortisol rise (r=−0.32, p=0.001). Lower income status was associated with flatter cortisol slope (r=−0.28, p=0.008). Patients with progesterone receptor positive breast cancer had a lower waking cortisol rise (r=0.22, p=0.04). The cortisol slope was unrelated to demographics, disease-free interval, or treatment. Patients with progesterone receptor positivity had lower waking cortisol rise (p=0.04).
Rich et al., 2005 (78)	80 (52 M) metastatic colorectal cancer patients Group 1—40 patients with a "good" rhythm (r24 >0.47 <0.77), median age 59.6 Group 2 - 40 patients with a "dampened" rhythm (r24 >0.03, <0.35), median age 60	Combination: 2-day serum cortisol (08:00 and 16:00) and 3-day actigraphy (Actigraph)	Patients with a "good" activity rhythm had higher cortisol ratios (between 08:00 and 16:00) compared to those with a "dampened" activity rhythm. Mean cortisol levels did not differ significantly between groups. Mean cortisol was prognostic (p<0.0001). IL-6 and TGF-alpha were positively correlated with mean serum cortisol (p=0.0001). IL-6 was negatively correlated with circadian cortisol ratio (p = 0.042).

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TABLE 2B Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Giese-Davis et al., 2004 (79)	91 (F) metastatic breast cancer patients, mean age at diagnosis of metastatic disease 49.08-55.93	3-day salivary cortisol (mean times 08:06, 12:26, 17:25, 20:26)	40% patients displayed the usual morning cortisol peak with a decline across the day. 56% had peaks later in the day and 4% had flattened rhythms. Patient's demonstrating psychological repression had flatter slopes than the self-assured (p=0.01) and non-extreme groups (p=0.02). Mean cortisol levels were not significantly different between groups.
Mormont et al., 2002 (80)	Round the clock study (RTCS): 18 (14 M) metastatic colorectal cancer patients, mean age 58 Two-time point study (TTPS): 192 (128 M) metastatic colorectal cancer patients, mean age 58	RTCS: Cortisol (13 blood and salivary samples 1st and 4th day of 4-day chemotherapy) TTPS: Cortisol (2 day—blood and saliva 08:00 and 16:00, + saliva 23:00 all before chemotherapy)	A significant circadian rhythm was seen in the serum of 8/18 (44.4%) patients and in the saliva of 6/16 patients (37.5%). Patients with marked circadian rhythms had lower 08:00 and 16:00 levels. Marked cortisol rhythms were not associated with longer survival than those with altered rhythms and cortisol did not predict the clinical outcome. A higher performance status and per cent of liver replacement was associated with higher cortisol concentrations at 08:00 and 16:00. Salivary and serum cortisol were correlated, particularly for those with stronger circadian rhythms.
Mormont et al., 2002 (49)	18 (14 M) metastatic colorectal cancer patients, mean age 58	Combination: salivary cortisol and serum cortisol, melatonin and 6-alphasulfatoxymelatonin (3–6 hourly over 10–13 time points on day 1 and 4) and 3-day actigraphy (Actigraph)	A circadian cortisol rhythm was seen in serum cortisol for 8 patients, and 6 patients had a significant salivary cortisol rhythm. Interindividual variation in markers of circadian rhythm.
Mormont et al., 2000 (37)	192 (128 M) metastatic colorectal cancer patients, mean age 58	Combination: 2-day blood cortisol (08:00 and 16:00) and 3-day actigraphy (actigraphy) and sleep diary	Mean cortisol was higher in patients with a low r24 (a measure of physical activity circadian consistency) (r=−0.17, p=0.04), low I<O (a measure of activity in and out of bed) (r=−0.24, p=0.07), poor performance status (p=0.0005) or severe liver involvement (p=0.0001). The estimate of cortisol circadian rhythm (difference in cortisol values) was correlated with r24 (r=0.16, p=0.04) but not I<O or mean activity. Cortisol was not prognostic, and the circadian rhythm did not estimate treatment response.
Sephton et al., 2000 (81)	104 (F), metastatic breast cancer patients, mean age 53.2	3-day salivary cortisol (08:00, 12:00, 17:00, 21:00)	Flatter diurnal cortisol slopes were associated with shorter survival (HR, 464.9; p = 0.0036). The change in survival between slopes was seen up to 7 years later. The diurnal cortisol slopes were split at the median value, 77% with "flat" rhythms averaged 3.2 years survival, whereas 60% with "steep" rhythms averaged 4.5 years survival. The diurnal cortisol slope remained prognostic after adjusting for age at initial diagnosis, disease-free interval, and oestrogen receptor status. Flatter diurnal cortisol slopes were associated with taking megestrol (p=0.000), more nocturnal awakenings (p=0.003), marital disruption (p=0.040), fewer circulating NK cells (p=0.007), and suppressed NK cell activity (p=0.05). Steeper slopes were associated with metastases to the chest wall or adjacent lymph nodes (p=0.023).
Mormont et al., 1998 (50)	18 (14 M) metastatic colorectal cancer patients, age 35–72	Combination: 6 hourly blood for cortisol and melatonin (9–11 time points) on day 1 and 4 and 3-day actigraphy	Study 1: 7 of 18 (39%) patients had significant cortisol circadian rhythms (p<0.05). Group circadian rhythms for cortisol were validated (p<0.00001). Wide interindividual variation was noted between cancer patients.

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TABLE 2B Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Touitou et al., 1996 (36)	13 metastatic breast cancer patients, mean age 52 (gender not defined) 20 stage 2a–4 ovarian cancer patients, mean age 57	24-h serum cortisol (4 hourly intervals)	Serum 08:00 cortisol was low in 9 patients. 8 breast cancer patients had flattened cortisol patterns, a shift in the peak or trough time, or a plateau with high morning values. 15 ovarian cancer patients had high cortisol levels throughout a 24-h period and/or erratic peak and trough locations and/or flattened profiles.
Touitou et al., 1995 (82)	13 (F) stage 1–4 metastatic breast cancer, median age 52	48-h serum cortisol (4 hourly intervals)	The cortisol acrophase was near 0930 ± 110. A significant circadian rhythm was seen in patients with WHO PS1 or no liver metastases. Circadian cortisol rhythm was lost for patients with liver metastases.
Touitou et al., 1990 (83)	13 (F) metastatic breast cancer patients, mean age 52	36–48-h serum cortisol (4 hourly intervals)	6 patients had normal rhythmicity with a peak at 08:00 and trough 00:00. 7 patients had altered peak and/or high concentration between 04:00–12:00 and/or flattened rhythms.

TABLE 2C Actigraphy-related circadian rhythms and their associations in patients with advanced cancer.

Studies with a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Levi et al., 2020 (17)	Combined data IDEAs study: 25 (21 M) locally advanced or metastatic gastrointestinal cancer patients, median age 66 PicaPill study: 33 (15 M), control subjects, median age 35	7-day actigraphy and chest accelerometry and 2-day salivary cortisol (3-hourly) and 1-day melatonin samples (hourly from 19:00)	13 patients had a dichotomy index (I<O, ratio of activity in and out of bed) of ≤97.5% on chest accelerometry. Patients had worse I<O (p=0.008), levels of activity (p<0.0001), and rest probability P1-1 (probability of remaining in a rest state) than controls (p=0.005). The activity amplitude (between peak and trough activity levels), r24 (autocorrelation coefficient, a measure of physical activity consistency between days), RI (rhythm index, measure of quality, regularity, and consistency of rest state), average centre-of-rest time, and rest duration were not significantly different. Hospital Anxiety and Depression Scale (HADS) scores, performance status, and Pittsburgh Sleep Quality Index (PSQI) scores did not differ significantly between I<O groups. Large inter-subject variability was noted. 91% of patients with a I<O >97.5% were physical active 30 min/+ a day compared to 15% of patients with a I<O of ≤97.5% (p=0.001). Controls aged 40/+ had a reduced I<O (p=0.01), P1-1 (p=0.0009) and phase-advanced activity acrophase (earlier peak activity time) and centre-of-rest time (p=0.01). Patients aged 40/+ had similar I<O values to older controls. For patients, the I<O was associated with day-to-day variability in sleep duration (r=−0.53, p=0.009), self-reported exercise (r=0.48, p=0.02), rest–activity amplitude (r=0.73, p<0.0001), median activity out-of-bed (r=0.68, p=0.0003), level of activity (r=0.56, p=0.005), day-to-day variability in self-reported retiring time (r=0.49, p=0.02), physiological chest temp (p=0.03), and chronotype (a measure of sleep timing preference) score (r=−0.43, p=0.04). The circadian amplitude in rest–activity and sleep duration variability were the best predictors of a patient's I<O.
Zeitzer et al., 2016 (52)	97(F) recurrent or metastatic breast cancer patients, age 57.6	2-week actigraphy (Actiwatch 2) and sleep diary &	No reported associated with actigraphy derived data.

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TABLE 2C Continued

Studies with a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
	24 health age-matched controls, age 57.1 (gender not defined)	28-h plasma cortisol (20–60-min intervals) and polysomnography	
Natale et al., 2015 (28)	226 (149 M) metastatic colorectal cancer patients, mean age 58.4 182 (103 F) control subjects, mean age 38.7	At least 4-day actigraphy (Basic Mini-Motionlogger)	Patients had a lower median I<O than controls (97.8% vs. 99.6%) The lowest patient I<O was 75.7% compared to 97.2% in controls. Patients spent a longer time in bed (67 min longer on average) than controls but slept a similar amount of time. Patients had longer sleep onset latency, mean sleep motor activity, wake after sleep onset, number of awakenings more than 5 and lower sleep efficiency compared than controls. I<O was significantly correlated with sleep motor activity (movements during sleep within a given time frame), wake after sleep onset, number of awakenings more than 5 and sleep efficiency (all p=0.0001). Younger patients went to bed, and woke up, later than older patients, and had a delay in the midpoint of sleep. The actigraphic parameter to best discriminate cancer patients was I<O.
Grutsch et al., 2011 (30)	84 (65 M), stage 2b–4 non-small cell lung cancer patients, mean age 62 35 control subjects from the Ambulatory Monitoring Inc. database (not further defined)	2-7-day actigraphy (Mini Motionlogger Basic Model 4)	Actigraphy parameters were abnormal for all cancer patients compared to controls. Same figures for actigraphy parameters as Du-Quiton et al., 2010). Higher daytime activity was associated with lower PSQI daytime dysfunction (r=−0.61, p=0.006), higher PSQI sleep quality (r=−0.48, p=0.014), and less use of self-reported sleep medication (r=−0.58, p<0.003). Higher daytime inactivity was associated with more daytime dysfunction (r= 0.54, p=0.017), lower PSQI global sleep quality (r=0.41, p=0.014), and higher self-reported use of sleep medication (r=0.39, p=0.05). A higher 24-h rhythm quotient was associated with less daytime dysfunction (r=−0.58,2p <0.01). Patients who slept well during the night and less in the day slept for longer regardless (p<0.03) Higher levels of night-day sleep balance were associated with less nighttime sleep disturbance (r=−0.44, p=0.067), less daytime dysfunction (r=−0.43, p=0.065) and better global PSQI sleep (r=−0.36, p=0.071)
Grutsch et al., 2011 (20)	84 (65 M) stage 2b-4 non-small cell lung cancer patients 42 inpatients, mean age 57 42 outpatients, mean age 66 35 control subjects from reference database, age 20-50 (gender not defined)	2–7-day actigraphy (Ambulatory Monitoring Inc.)	All actigraphy parameters were abnormal in patients. A flatter activity circadian rhythm was observed for patients. A higher r24 was associated with less insomnia (r=−0.48, p=0.003) Loss of appetite was associated with decreased peak activity (r=−0.41, p=0.005) and the circadian quotient (the amplitude/MESOR) (r=0.4, p=0.015) A higher r24 was associated with higher quality-of-life index Health/Functioning domain scores (r=0.34, p=0.05). Higher satisfaction with health was associated with more stable circadian structures. Fatigue was associated with a diminished circadian quotient (r=−0.40, p=0.04), rhythm quotient (4=−0.41, p=0.03) and night-day sleep balance (r=−0.52, p<0.01). A more robust rhythm was associated with less fatigue. A higher rhythm quotient was associated with less pain (r=−0.39, p=0.04). Loss of appetite was negatively associated with night-day sleep balance (r=−0.47, p<0.01). Peak activity significantly correlated with all the Power and Ferrans QoL index domains (all p≤0.02). Robustness of circadian measures reflects all quality-of-life measured aspects. Higher r24 was associated with higher health/functioning domain scores (r=0.45, p=0.02) and global health scores (r=0.53, p<0.01). Night-day sleep balance was correlated with the health/functioning domain (r=0.39, p=0.04), social/economic domain (r=0.40, p=0.04) and psychological/spiritual domain (r=0.45, p=0.02). A larger difference in nocturnal and daytime activity correlated with the score in each QLI measure. Night-day balance was correlated with the EORTC QLQ C30 domains of role (r=0.56, p<0.01) and cognitive function (r=0.45, p=0.02).

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TABLE 2C Continued

Studies with a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
			A more robust circadian rhythm was associated with greater patient satisfaction with health/functioning, and better overall quality of life.
Du-Quiton et al., 2010 (23)	84 (65 M) stage 2b–4 non-small cell lung cancer patients 42 inpatients, mean age 57 42 outpatients, mean age 66 Control subjects from the Ambulatory Monitoring Inc. database	3–7-day actigraphy (Ambulatory Monitoring Inc. action W-2)	All actigraphy parameters were abnormal for cancer patients compared to controls ($p<0.05$). Patients were 20%–50% less active than controls. Patients had at least 3 times longer daytime inactivity, 4 times longer sleep/inactivity periods in the day (20.9% vs. 4.7%) than controls. Patients had more fragmented sleep, longer waking episodes and less nighttime sleep than controls. The longest patient night sleep episode was less than half of the controls. Patients had lower sleep efficiency (79.8% vs. 95.9%), shorter longest nighttime sleep duration (91.7 min vs. 255.6 min), less activity (126.9 accelerations/min vs. 182.6), shorter daytime wake time (797.5 min vs. 947.1 min), shorter sleep time at night (284.0 min vs. 417.8 min), and shorter % of time asleep at night (72.5% vs. 93.0%) than controls (all $p<0.05$). Patients took longer to fall asleep at night (20.8 min vs. 12.1 min), were awake more in the night (95.0 min vs. 31.1 min), slept for longer in the day (208.8 min vs. 47.1 min), and had longer longest daytime sleep periods (43.0 min vs. 23.6 min) than controls ($p<0.05$). Overall daytime and nighttime sleep were not associated with anxiety or depression. There were no statistically significant associations between sleep-activity circadian rhythm and anxiety or depression amongst inpatients.
Fouladiun et al., 2007 (84)	39 (28 M) mixed cancer patients (32 metastatic), mean age 71 31 control subjects, two cohorts mean ages 49 and 74 (genders not defined)	≥3-day actigraphy (Actigraph)	Patients were less active in the day on weekdays and weekends than controls ($p<0.01$). Physical-rest activity was not different to age-matched and recently hospitalised non-cancer patients. Subjectively scored physical function and pain were predicted by objectively measured physical activity ($p<0.0001$).
Le Guen et al., 2007 (85)	29 (25 M) stage 1–4 limited and extensive small cell lung cancer patients, mean age 59 14 (12 M) obstructive sleep apnoea patients treated with nasal continuous positive airway pressure, mean age 55	5-day actigraphy (Actiwatch) and sleep log	Actigraphy parameters differed between the two groups. During the night, cancer patients had longer sleep times (7.4h vs. 6.5h, $p=0.03$), lower sleep efficiency (78% vs. 88.1%, $p=0.002$), higher mean activity scores (31.5 vs. 9.6, $p<0.001$), a higher fragmentation index (not described) (51.7 vs. 28.4, $p=0.002$), and lower immobile time (75.2% vs. 87.4%, $p=0.004$). Sleep latency was not significantly different between groups. Patients had lower daytime mean activity scores than controls (186.5 vs. 274.8, $p=0.001$) The PSQI and ESS scores were not significantly correlated with any actigraphic parameters.
Pati et al., 2007 (86)	31 (19 M) stage 2–4 mixed cancer patients, median age 43 35 (22 M) control subjects, median age 35	4-day actigraphy (Actiwatch) and sleep diary	Activity levels were higher, activity patterns more regular, and day–night activity more distinct in controls compared to patients. Cancer patients demonstrated more frequent episodes of activity. Activity rhythms occurred in a 24-h pattern for most participants except 4 patients and 1 control who had activity rhythms lasting 6 or 12 h. MESOR, amplitude and I<O were lower in patients than controls (all $p<0.001$). The activity acrophase occurred early (~1 h) in patients than controls ($p<0.001$). The mean r24 of the patient group was lower, but not significantly different, than the control group. Patients spent more time in bed ($p<0.02$), had more wake episodes ($p<0.001$), a higher nap frequency ($p<0.001$), more total naps ($p<0.001$), and longer naps ($p<0.001$) than controls.
Fernandes et al., 2006 (32)	25 (F) mixed cancer diagnoses patients (72% metastatic) median	3-day actigraphy (Actimeters) and sleep diary	All actigraphy parameters were significantly different between the two groups. Patients had lower median r24 values (0.28 vs. 0.46, $p<0.001$), longer wake after sleep onset (68.34 min vs. 25.67 min,

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TABLE 2C Continued

Studies with a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
	age 67 25 (F) control subjects, median age 63		p=0.004), and lower sleep efficiency (85.75% vs. 94.63%, p=0.001). Sleep latency was not significantly different between groups. Actigraphy parameters were not significantly correlated with EORTC QLQ-C30 fatigue score in patients or controls.
Levin et al., 2005 (87)	33 stage 3–4 non-small cell lung cancer patients, 2 cohorts MRMC site: 21 (12 F), mean age 56.71 VAMC site: 12 (M) mean age 71.75 35 control subjects	4–7-day actigraphy (Ambulatory Monitoring Inc.)	Actigraphy parameters were significantly different between patients and controls. The I<O for the patient cohorts was 90.0±2.2 and 78.9 ± 2.4. During wakeful periods patients had less daily activity (mean 92.8 min vs. 127 min), more sleep time (mean 195 min vs. 46.5 min and % asleep 21.8 vs. 4.7), and more sleep episodes (17.8 vs. 5.4) than controls. During nighttime periods, cancer patients spent more time awake (134.1 min vs. 31.1 min), had more sleep interruptions (14.6 vs. 6.9), and spent less time sleeping (71.2% vs. 93.0%). The r24 varied depending on the ECOG PS (PS0 0.31, PS1 0.17, PS2 0.21) The duration of long sleep episodes was longer in PS0 patients than PS2 (129 min vs. 96.5 min, p<0.05). No significant differences in I<O, peak activity and the circadian quotient were seen between groups. The ultradian (4 hourly rhythms) were significantly different (p=0.046). Circadian quotient was lower with a higher PS (PS0 0.55±0.08, PS1 0.53±0.04, and PS2 0.47±0.06). Rhythm quotient was lower with a higher PS (PS0 1.05±0.16, PS1 1.02±0.09, and PS2 0.94±0.16).
Chevalier et al., 2003 (88)	10 (5 M) metastatic colorectal cancer patients, median age 61 15 (M) control subjects (age not defined)	3-day actigraphy (Actigraph)	The median r24 for controls was 0.57 and 1 control had an r24 <0.40. Mean activity counts ranged from 118 to 163 with a median of 145. The peak activity time (acrophase) ranged between 14:20 and 20:10 with a median acrophase of 17:00. The median r24 for patients was 0.57 and 2 patients had an r24 <0.40. Mean activity counts ranged from 76 to 275 counts with a median 148. The acrophase ranged from 13:18 to 17:54 with a median acrophase of 15:24.
Studies without a control group			
Patel et al., 2023 (89)	44 (17 F) mixed locally advanced or metastatic cancer diagnoses, median age 66	72-h actigraphy (Actiwatch)	Mean I<O 88.9% (70.9%–98.1%) I<O correlated with r24, mean activity, sleep efficiency, WASO I<O not associated with survival (based on median or quartile groups) I<O negatively correlated with ECOG-PS (p<0.0005) Autocorrelation coefficient 0.16 (0.04–0.37), unrelated to survival No actigraphy-derived sleep parameters associated with survival Sleep efficiency and later get up time associated with survival
Block et al., 2022 (90)	30 (22 F), stage 2–4 mixed cancer diagnosis, median age 55	7-day actigraphy (Ambulatory Monitoring Inc) Day 6 overnight and morning urine 6-sulfatoxymelatonin	Baseline actigraphy from 22 patients r24 median 0.48, mean 0.47 (0.08–0.63) Intradaily stability median 0.73, mean 0.73 (0.43–0.86) Intradaily variability median 0.48, mean 0.53 (0.37–0.88) Relative amplitude median 0.87, mean 0.86 (0.76–0.95)
Padron et al., 2022 (91)	Cognitive behavioural therapy for insomnia and pain (CBTi.p).: 18 (F) stage 1–3 mixed gynaecological cancer patients, mean age 58.9 Psychoeducation (PE): 17 (F) stage 1–4 mixed gynaecological cancer patients, mean age 59.9	14-day actigraphy (Actiwatch-L) and sleep diary a post-surgical polysomnography	Actigraphy and sleep diary data were not correlated. Baseline mean values (polysomnography vs. actigraphy) CBTi.p.: sleep efficiency was 75.5% vs. 80.8%, sleep onset latency (time between being in bed and falling asleep) was 24.7 min vs. 29.3 min, and wake after sleep onset was 91.1 min vs. 50.5 min PE: sleep efficiency was 71.0% vs. 79.4%, sleep onset latency was 36.2 min vs. 33.6 min, wake after sleep onset was 94.8 min vs. 53.4 min Baseline objective data (CBT.i.p and PE): 29% and 24% of patients had a sleep efficiency >85% 76% and 50% of patients had sleep onset latency <30 min 6% and 13% of patients had wake after sleep onset <30 min

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TABLE 2C Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Cheung et al., 2021 (10)	30 (16 M) stage 3b–4 non-small cell lung cancer patients Group 1—Aerobic exercise, mean age 61.00 Group 2—Tai-Chi, mean age 61.11 Group 3—Self-management group, mean age 58.36	3-day actigraphy (Ambulatory Monitoring Inc.) and salivary cortisol rhythms (0.5, 4, 8, and 12 h after waking)	Patients had a total sleep time of (1) 283.03 min, (2) 240.46 min, and (3) 295.48 min. Sleep efficiency (total sleep time to time in bed ratio) was (1) 91.13%, (2) 89.69%, and (3) 90.70%
Bernatchez et al., 2020 (92)	57 (30 M) mixed cancers patients (45 stage 3–4, 53 metastatic), mean age 65.8	7-day actigraphy (Actiwatch-64) and sleep diary	No objective measures were significantly correlated with time to death or quality of life (QoL). Higher sleep efficiency was associated with less pain and depressive symptoms (all r=−0.23, p≤0.05). Longer napping time was associated with increased pain (r=0.38, p≤0.01), fatigue (r=0.37, p≤0.01), daytime sleepiness (r=0.38, p≤0.01), and depressive symptoms (r=0.23, p≤0.05). Gastrointestinal symptoms were associated with increased sleep onset latency (r=0.33, p≤0.01), nighttime awakenings (r=0.30, p≤0.05), and time in bed (r=0.34, p≤0.01). Maladaptive sleep behaviours were associated with longer sleep onset latency (r=0.27, p≤0.05), increase nighttime awakening (r=0.24, p≤0.05), early morning awakening (r=0.24, p≤0.05), and poorer sleep efficiency (r=−0.35, p≤0.01). None of the objective measures were significantly correlated to erroneous sleep beliefs or 24-h light exposure.
Jakobsen et al., 2020 (29)	40 (24 M), metastatic mixed cancer patients, median age 70	Overnight actigraphy (Actiwatch 2) and polysomnography	Actigraphy: mean total sleep time was 418 min (SD±138), mean sleep onset latency was 35 min (SD±61), mean number of awakenings was 24 (SD±15), mean total time awake was 40 min (SD±21), and mean per cent of time in bed asleep was 78% (SD±23). Actigraphy measured total sleep time (r_s=0.61, p<0.005) and sleep efficiency (r_s=0.48, p<0.005) were associated with patient reported outcomes measures (PROMs). Sleep onset latency, number of awakenings, and wake after sleep onset were not associated with PROMs. Longer wake after sleep onset (actigraphy) was "significantly" associated with worsening of total subjective sleep quality (r_s = 0.45, significance level not reported).
Fujisawa et al., 2019 (31)	51 (20 M), stage 4 non-small cell lung cancer patients, mean age 66.8	3-day actigraphy (Actiwatch 2) and activity log	The hazard ratio was 1.48 for each 10% increment of time awake spent immobile (p<0.05). The odds ratio for death within 6 months was 2.99 for each 10% increment of time awake spent immobile (p<0.05). Eastern Cooperative Oncology Group (ECOG) performance status (PS) was non-discriminatory for survival in patients with a PS 0–1. For those who died at 6 months, discriminating factors for survival were 10% increment of time awake spent immobile (OR 2.99, p<0.05), ECOG PS >1 (OR 9.23, p<0.05), and the percentage of time awake spent immobile (OR 5.09, p<0.05). Time awake spent immobile correlated with Functional Assessment of Cancer Therapy—Trial Outcome Index (FACT-TOI) (p<0.01) and ECOG PS (p<0.01).
Komarzynski et al., 2019 (27)	31 (17 M), mixed cancer patients (24 metastatic), median age 61	30-day actigraphy (Micro Motionlogger)	Median sleep efficiency was 92.0% (20.2%–100%), the sleep midpoint was 03:29 (00:38–10:19), wake time was 08:00 (03:49–15:39), bedtime was 23:07 (19:29–05:00), sleep onset latency was 5 min, wake after sleep onset was 42 min, number of wake episodes was 11, and total sleep time was 7hr50 (6h50–8h44). Subjective sleep disturbance was associated with objective parameters (p≤0.05). Worse MD Anderson Symptom Inventory (MDASI) sleep scores were found in those with lower sleep efficiency (r=−0.13, p=0.002), larger number of wake episodes (r=0.12, p=0.005), longer wake after sleep onset (r=0.14, p<001), and worse sleep fragmentation index (r not defined) (p=0.01).

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TABLE 2C Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
			The number of wake episodes was associated with fatigue (p=0.02), drowsiness (p=0.03) and interference with activity (p=0.03). Sleep efficiency was associated with daytime drowsiness (p = 0.01). MDASI sleep correlated with sleep efficiency, wake minutes, wake episodes, sleep episodes, and longest sleep episodes (all p<0.01). MDASI sleep correlated with wake after sleep onset (p<0.001). MDASI fatigue correlated with total sleep time, total time in bed and sleep episodes ≥5 min (all <0.01). MDASI drowsiness correlated with wake after sleep onset and total time in bed (all p<0.01). MDASI interference with activity correlated with sleep episodes ≥5 min and longest sleep episode (all p<0.01).
Bernatchez et al., 2018 (8)	55 (29 M) mixed cancer patients (44 stage 3–4, 52 distant metastases), mean age 65.9 Actigraphy data available for 55 patients, outliers removed and 51 analysed	7-day actigraphy (Actiwatch-64) and sleep diary	The R-squared (rhythmicity coefficient of the sleep–wake cycle) was 0.27 (0.09–0.51), acrophase was 13:35 (10:34–20:10), MESOR (average activity level) was 45.4 (3.6–167.8), amplitude was 47.0 (5.4–178.8), up-MESOR (mean time from low to high activity) was 8.18 (2.00–14.00), and down-MESOR (mean time from high to low activity) was 19.23 (16.20–22.00). Less rhythmic sleep–wake cycle, as characterised by lower amplitude (r=0.32), MESOR (r=0.27), and R-squared (r=0.31), was associated with shorter survival (all p≤0.05). No circadian activity rhythm parameter was correlated with pain, fatigue, depression, or maladaptive sleep behaviours. A lower amplitude (r=0.24, p≤0.01), lower MESOR (r=0.27, p≤0.05), and later acrophase (r=−0.23, p≤0.01) was associated with poorer global QoL. A higher down-MESOR was associated with poor global (r=−0.31, p≤0.05) and functioning QoL (r=−0.30 p≤0.05). A more robust rest-activity rhythm (higher amplitude (r=0.33, p≤0.05), MESOR (r=0.42, p≤0.01), and r-squared (r=0.24, p≤0.05) was associated with greater 24-h exposure to light intensity >1,000 lux. There were no significant differences between any rest–activity rhythm variable between ECOG 2 vs. 3.
Bernatchez et al., 2018 (25)	57 (26 M) mixed cancer patients (40 stage 3–4, 48 distant mets) 51 analysed, mean age 66.4	7-day actigraphy (Actiwatch-64) and sleep diary	The wake after sleep onset ranged between 48.2 and 70.9 min. The longest daytime napping was 100.3 min. Patients with no perceived sleeping difficulty had a mean sleep onset latency of 10.2 min, wake after sleep onset of 48.2 min, total wake time of 68.5 min, total sleep time of 479.9 min, time in bed of 548.4 min and sleep efficiency of 87.3%. Subjective assessments differed from objective assessment, significance not reported. No significant differences for sleep-wake parameters were noted between men and women.
Cash et al., 2018 (93)	55 (33 M) stage 0–4 head and neck cancer patients, mean age at diagnosis 58.5	6-day actigraphy (Mini-Motion logger) and sleep diary	The mean rest–activity rhythm (RAR) correlation coefficient (r24) was 0.132 and mean nighttime restfulness was 92.82%. The activity acrophase occurred at 14:46. 2-year survival was impacted by RAR disruption (HR, 0.073; p = 0.012), lower nighttime restfulness (HR, 0.910; p=0.009), and the acrophase (HR, 1.196; p=0.288). Depression was associated with RAR disruption (r=−0.338, p=0.041). Overall and somatic depressive symptoms were associated with activity phase shifts (morning to evening) (r=0.370, p=0.024).
Innominato et al., 2018 (94)	11 (5 M) advanced or metastatic colorectal or pancreatic cancer patients, median age 60	30-day actigraphy (Micro-Motionlogger)	Activity was prominent at daytime and restful at nighttime. Mean I<O values ranged from 96.3% to 98.5%. The average sleep efficiency was 81.9%–90.8% and <70% in approximately 1/10 of the nights. The average total sleep time was 8.6–9.7 h with an average midpoint of sleep of 01:02–05:30. I<O was independent predictive factor for all selected MDASI PROMs.
Innominato et al., 2018 (95)	Cohort 1: 237 metastatic colorectal cancer patients, not receiving anticancer Cohort 2: 31 advanced or	Cohort 1: 3-day actigraphy (Mini-Motionlogger) Cohort 2: 30-day actigraphy (Micro-Motionlogger)	The I<O was ≤97.5% in 54.9% of cohort 1 and 44.4% in cohort 2. A reduced I<O was significantly associated with increasing fatigue (p<0.0001), anorexia (p<0.0001), pain (p<0.0001), and sleep trouble (p<0.003). An increased I<O was significantly associated with greater values of global quality of life (p<0.0001), physical (p<0.0001), and social (p<0.0001) functioning but not role (p=0.02) functioning.

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TABLE 2C Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
	metastatic gastrointestinal cancer patients		Patients with a reduced I<O values had more interference with enjoyment of life, activity, relations with others and work (all p< 0.0001).
Chang and Lin, 2017 (96)	82 (59 M) stage 1–4 lung cancer patients, mean age 66.54	3-day actigraphy (Ambulatory Monitoring Inc.) and sleep diary	The I<O was 88.50, and higher I<O was associated with improved QoL (p<0.001).
Palesh et al., 2017 (22)	237 (148 M) metastatic colorectal cancer patients, median age 60.4	3-day actigraphy (Mini-Motionlogger)	Median values were a total sleep time of 7hr22, sleep efficiency of 90.56%, sleep latency of 8m40s, wake after sleep onset of 46m15s, I<O of 96.9%, bathyphase (lowest activity time) of 02:33, and average activity of 99 accelerations/minute. Patients with subjective sleep complaints had more circadian disruption (lower I<O) compared to those without sleep complaints (p=0.005). 40.1% of patients had subjective and circadian disruption (I<O <97.5%), 25.3% had subjective disruption alone, 14.8% had circadian disruption alone, and 19.8% had neither. Lowest health-related quality of life scores (including global, physical, social, and role functioning), and highest symptom scores (including fatigue and appetite loss), were seen in those with subjective and circadian disruption. Subjective difficulty sleeping was not associated with actigraphy sleep parameters.
Chen et al., 2016 (24)	Intervention group: 56 (24 M) stage 1–4 lung cancer patients, mean age 64.64 Standard care group: 55 (25 M) stage 1-4 lung cancer patients, mean age 62.51	3-day actigraphy (Micro-Mini actigraph) and sleep diary	Baseline (mean values): The r24 values were 0.42 (I) and 0.36 (C). A poor r24 was seen in 11/56 in the intervention group and 18/55 in the standard care group (26% overall). The I<O values were 94.68 (I) and 92.65 (C). A poor I<O was seen in 11/56 in the intervention group and 23/55 in the standard care group (30% overall). The total sleep time was 380.32 (I) and 395.06 (C). The sleep efficiency was 88.94 (I) and 88.36 (C). The sleep onset latency was 27.14 (I) and 31.85 (C). The wake after sleep onset was 45.86 (I) and 50.56 (C).
Ortiz-Tudela et al., 2016 (21)	24 (11 M), gastrointestinal cancer patients (21 metastatic), median age 63	4 and 8 days of wrist temperature activity and body position (accelerometery)	The L5 timing (centre of least 5 active hours) was 01.13 +/- 42 min. I<O before chemotherapy was 83.88%. Females had stronger and less fragmented rhythms than males. Significant gender differences were seen for interdaily stability (IS, a measure of rhythm stability) (p = 0.002), intradaily variability (IV, a measure of rhythm fragmentation) (p = 0.001), relative amplitude (RA, the difference between the mean of ten consecutive hours with the highest values and the mean of five consecutive hours with highest values divided by the combined value of both) (p = 0.001), circadian function index (CFI, combined IV/IS/RA) (p < 0.001) and I < O (p = 0.008).
Rebholz et al., 2018 (65)	57 (F) stage 0–5 breast cancer patients, mean age 52.32	3-day actigraphy (Ambulatory Monitoring Inc.) and 3-day salivary cortisol (waking, +30 min, 16:00, bedtime)	The r24 was 0.28 and wake after sleep onset was 0.15. African American females had more wake after sleep onset (p=0.017) and lower r24 (p=0.037) which persisted after adjusting for age and cancer stage. Wake after sleep onset and r24 were not significantly correlated with QoL.
Cash et al., 2015 (15)	43 (F) stage 0-4 breast cancer patients, mean age 52.49	3-day actigraphy (Micro Mini-motionlogger) and sleep diary and salivary cortisol (3 days—on waking, +30 min, 16:00, pre-bed)	The mean r24 was 0.27, nighttime restfulness (proportion of activity in bed that falls below the median out of bed activity) was 97.21%, daytime sedentariness (proportion of activity out of bed that is below the median in bed activity) was 6.59%, mean sleep time was 386.94 min, sleep efficiency was 89%, and wake after sleep onset was 13 min. Uncoordinated rest-activity rhythm, such as poor inter-daily stability, was associated with elevated markers VEGF, TGF-beta, and MMP-9 (associated with angiogenesis, immunosuppression, epithelial-mesenchymal transition, tumour invasion, and metastasis).

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TABLE 2C Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Dean et al., 2015 (97)	29 (18 M) stage 2a–4 non-small cell lung cancer patients, mean age 66.6	7-day actigraphy (Motionlogger) and sleep diary	Baseline actigraphy demonstrated poor sleep with 45% of patients sleeping <6 h The mean sleep efficiency was 77%, sleep latency was 51 min, and sleep duration was 5.9 h. Baseline sleep efficiency and wake after sleep onset were not associated with FACT-TOI, FACT-L, FACT-G, or the subscales. There were, however, associated after receiving chemotherapy.
Zeitzer et al., 2015 (16)	97 (F) recurrent or metastatic breast cancer patients, age 57.4	2-week actigraphy (Actiwatch 2) and sleep diary logs and Serum cortisol (20–60-min intervals from 3 h prior to bedtime to 1 h after wake time) Salivary cortisol 09:00, awakening and 30 min	No relevant findings from actigraphy
Chang and Lin, 2014 (98)	68 (34 M) stage 2–4 mixed cancer patients, median age 54.0	3-day actigraphy (Ambulatory Monitoring Inc.) and sleep diary	The median I<O was 89.5%. 34 patients were classified having a "disrupted" circadian rhythm (I<O ≤89.4) and 34 patients were classified as having a "regular" circadian rhythm (I<O ≥89.5) Survival was longer for patients with a "regular" circadian rhythm than a "disrupted" rhythm (3.9 months vs. 1.8 months, p<0.001, HR 2.19, p=0.006) Controlling for age, gender, cancer diagnosis, cancer stage, PSQI, Brief Pain Inventory-Chinese version (BPI-C), and Karnofsky Status (KPS) scale, a "dampened" circadian rhythm impacted survival (HR 4.59 p=0.001). Significant difference in cumulative patient survival rates were reported between "disrupted" and "regular" rhythm (p=0.005).
Levi et al., 2014 (99)	436 (273 M) colorectal cancer patients (427 metastatic), median age 59	2-7-day actigraphy (Mini-Motion logger)	The median I<O was 97.5% and a lower I<O was associated with poor WHO performance status (PS0 98.2%, PS1 96.5% PS1 and PS2/+ 91.5%, p<0.001). Women had a higher I<O than men (98.0% vs. 97.1%, p=0.04). Overall survival was longer in patients with an I<O >97.5% than those with an I<O ≤97.5% (21.6 months vs. 11.9 months, p<0.001). A 1% increase of I<O had a HR 0.954 (p=0.003). Progression free survival was also longer in patients with an I<O >97.5% than those with an I<O ≤ 97.5% (9.3 months vs. 5.8 months, p<0.001). Median I<O varied between patients without metastases (99.4%), with one metastatic site (97.4%), and with two or more metastatic sites (97.1%) (p=0.03).
Ma et al., 2014 (100)	68 (34 M) mixed advanced cancer patients, mean age 52.40	3-day actigraphy (Actigraph) and sleep diary	The median and mean r24 were 0.19 (−0.03–0.64). The median was I<O 89% and the mean I<O 85.29% (51%–100%). Patients spent a mean total time in bed of 461.19 min, mean total sleep time was 314.92 min, mean sleep efficiency was 75.95%, mean wake after sleep onset was 99.48 min, mean sleep onset latency was 40.65 min, and mean waking episodes was 12.66. Higher r24 was associated with longer total sleep time (r=0.26), improved sleep efficiency (r=0.29), shorter sleep onset latency (r=−0.24), and less wake after sleep onset (r=−0.28) (all p<0.05). Higher I<O was associated with longer total sleep time (r=0.38, <p<0.001) and shorter sleep onset latency (r=−0.49, p<0.01), improved sleep efficiency (r=0.30, p<0.05) and less wake after sleep onset (r=−0.25, p<0.05). r24 and I<O were negatively correlated with worst pain, pain intensity, and global PSQI score (all p<0.01). r24 predicted sleep quality and pain intensity (p<0.001).
Ortiz-Tudela et al., 2014 (101)	49 (25 M) mixed cancer patients (47 metastatic), median age 61.6	3–10-day actigraphy (Mini-Motionlogger)	A mean rhythmic 24-h pattern was observed for the whole group of patients. Circadian disruption during or after chemotherapy was significantly associated with developing clinically relevant fatigue (OR, 5.1; p=0.028), or body weight loss (OR, 6.1; p=0.05).

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TABLE 2C Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Palesh et al., 2014 (68)	97 (F) metastatic or locally advanced breast cancer patients, mean age 54.6	3-day actigraphy (Micro Mini-Motionlogger) and sleep diary and 2-day salivary cortisol (waking, +30 min, 12:00, 17:00, and 21:00)	Patients had mean total sleep time of 390 min, wake after sleep onset of 71.2 min, 14.5 wake episodes, and 4.8 min duration of wake episodes. The mean sleep efficiency was 84.6%, total time in bed of 478.3 min, and a sleep latency of 11.5 min. A higher sleep efficiency was associated with lower mortality (HR, 0.96; p<0.001). A 10% increase in sleep efficiency reduced subsequent mortality by 32%. The mean survival with a sleep efficiency of ≥85% was 68.9 ± 4.0 months compared with 33.2 ± 4.3 for a lower sleep efficiency (p<0.001). The sleep efficiency effect on survival remained after adjusting for age, oestrogen receptor status, treatments received, metastases, depression, and cortisol (HR, 0.94; p<0.001). There was no association between sleep duration and survival.
Dean et al., 2013 (102)	35 (34 M) stage 1-4 lung cancer patients, mean age 63.5	7-day actigraphy (Motionlogger) and sleep diary	77% patients had a sleep latency of more than 30 min and 61% slept <5 h per night. 88% of patients had a sleep efficiency of <85% and 91% were awake more than 30 min after sleep onset. Considering "good" and "poor" sleepers, actigraphy measures of sleep and measures of mood were not significantly different. Subjective and objective measures of sleep efficiency, sleep latency, sleep hours and wake after sleep onset were significantly different (all <0.05). There were no significant associations between the sleep diary and actigraphy variables of interest.
Dedert et al., 2012 (71)	57 (F) stage 1–4 breast cancer patients, mean age 52	3-day actigraphy and sleep diary and 3-day salivary cortisol (waking, 30 min after, 16:00, and bedtime)	The mean r24 was 0.27. Nighttime inactivity was 97.3%, and daytime inactivity was 6.1%. Intrusive thoughts were associated with a lower r24 and with daytime sedentariness. There was no association between intrusive thoughts and nighttime inactivity. More avoidant coping was associated with a lower r24 (p<0.05) and daytime inactivity (p<0.001). A higher autocorrelation co-efficient was significantly correlated with a steeper diurnal cortisol slope (p = 0.003).
Dhruva et al., 2012 (103)	73 (F) breast cancer patients (43.8% locally advanced), mean age 55.1	2-day actigraphy (Ambulatory Monitoring Inc.) and sleep diary	87% of patients had an "excessive" number of awakenings, 46% had abnormal wake after sleep onset, and 58% had total sleep times below healthy adult values. 25.6% had abnormal sleep onset latency and the mean value was 14.7. 46.2% had abnormal wake after sleep onset and the mean value was 11%. 87.2% had an abnormal number of awakenings with the mean number being 15.1. 57.7% had abnormal total sleep time with the mean of 419.8 min. 26.9% had abnormal sleep efficiency and the mean was 85.5%.
Innominato et al., 2012 (104)	77 (65 M) metastatic colorectal cancer patients, median age 62.3	At least 3-day actigraphy (Mini-Motionlogger)	The median and mean I<O were 97.5% and 95.1%. 39 patients (50.6%) had altered I<O of <97.5%. There was no significant association between I<O and progression free survival. Overall survival was associated with a more robust circadian rhythm (22.3 months vs. 14.7 months, p=0.013). This was independent of gender, treatment schedule, number of metastatic sites, rank of chemotherapy course of interest, and performance status on day 1 (p = 0.004). Patients with an altered rhythm during chemotherapy had a higher risk of earlier death (HR, 2.12; p=0.004). There were no significant differences between I<O and response rate or overall grade 3–4 toxicity rate. No clinical or biological parameters predicted the occurrence of a rhythm disturbance on treatment. Baseline disruption did not predict subsequent disruption, and there was no significant correlation between baseline and on-treatment I<O). There was no significant difference in toxicity in relation to circadian parameters.

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TABLE 2C Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Gibbins et al., 2009 (26)	60 (27 M) mixed advanced and incurable cancer patients, median age 67	7-day actigraphy (Actiwatch W-4) and sleep diary	Sleep efficiency was >90% for most patients with 12% of patients having a sleep efficiency <86%. Patients had 14–17 naps per day, lasting approximately 9 min each and 42%–48% of the day was spent immobile. Cancer diagnosis was not significantly related subjective or objective data. Subjective sleep quality was not correlated with objective sleep efficiency, sleep fragmentation, or daytime activity.
Innominato et al., 2009 (105)	130 (74 M) metastatic colorectal cancer patients, median age 60	At least 3-day actigraphy (Mini-Motionlogger)	The mean r24 was 0.38, and the median r24 was 0.37. The mean I<O was 94.3, and the median I<O was 97.0. The I<O and r24 were associated (r = 0.74, p<0.001) I<O and r24 were also associated with meanAct (mean activity levels) (p<0.001). There were no significant associations between progression-free survival and any CircAct parameter (I<O, r24, meanAct). Patients in the lowest I<O quartile (<92.4%) had the poorest survival (12.0 months), and patients in the highest I<O quartile (≥99.2%) had better survival (23.5 months). I<O and r24 were related to survival (HR, 0.95; p<0.0001 and HR, 0.20; p=0.004). Higher I<O, r24 and meanAct were associated with improved QoL and role functioning and less fatigue and appetite loss (all p≤0.01). Higher I<O and r24 were associated with improved social functioning, and less pain and dyspnoea (p≤0.01). Higher I<O and meanAct were associated with improved physical functioning (p≤0.01). Higher I<O was associated with less insomnia (p≤0.01). Actigraphy parameters were not correlated with emotional or cognitive functioning, nausea/vomiting, constipation, or diarrhoea. Patients with a good WHO PS had a more robust CircAct (I<O (p=0.01), r24 (p=0.0014), meanAct (p=0.047) that those with a poor PS. Lower I<O and r24 was associated with poorer WHO PS (PS0 I<O 98.2, r24 0.44, PS1 I<O 95.7, r24 0.36, PS2 I<O 95.3, r24 0.24). Use of analgesia was associated with a disturbed circadian rhythm (p<0.05 for all parameters). CircAct parameters did not correlated with age, gender, body mass index, number of metastases, site of primary tumour, blood tests, previous chemotherapy, or per cent of liver involvement, p>0.10). CircAct parameters did not predict for tumour response or toxicity.
Palesh et al., 2008 (75)	99 (F) metastatic breast cancer patients, mean age 54.65	3-day actigraphy (Micro Mini-Motionlogger) and 2-day salivary cortisol (waking, +30 min, 12:00, 17:00, 21:00)	Cancer patients had a time in bed of 478.5 min, sleep onset latency of 11.50 min, wake after sleep onset of 71.44 min, sleep efficiency of 84.5%, and 15 wake episodes. Longer time in bed was associated with lower pain intensity (r=−0.23, p=0.03), depression (r=0.21, p=0.05), younger age (r=−0.24, p=0.02), and chemotherapy (r=−0.29, p=0.006). Longer mean sleep latency was associated with lower perceived stress scale scores (r=−0.22, p=0.04) and the use of radiation (r=0.21, p=0.05). Mean sleep efficiency was associated with the dominant metastatic site being the chest alone vs. bone or viscera (r=0.27, p=0.01). Longer wake after sleep onset was negatively related to metastases to the chest only (r=−0.31, p=0.003) and positively correlated to hormone therapy (r=0.24, p=0.03). Higher mean number of wake episodes were associated with younger age (r=−0.23, p=0.03), metastases to the chest only (r=−0.23, p=0.03), sleep medication (r=0.26, p=0.012), and hormone therapy (r=0.23, p=0.03).
Rich et al., 2005 (78)	80 (52 M) metastatic colorectal cancer patients Group 1 (40) – "Good" circadian rhythm (r24 >0.47, <0.77),	3-day actigraphy (Actigraph) and 2-day serum cortisol (08:00 and 16:00)	"Good" circadian rhythm patients had a higher median r24 (0.58 vs. 0.22), I<O (98.45% vs. 91.36%), O>I (96.57% vs. 83.89%), and mean activity levels (107.5 vs. 84.0) than patients with "dampened" circadian rhythm (all p≤0.0001) "Good" circadian rhythm patients survived longer than "dampened" circadian rhythm patients (13.8 months vs. 11.1 months, p=0.0176). r24 and I<O were prognostic (p=0.001 and p<0.0001 in a univariate analysis).

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TABLE 2C Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
	median age 59.6 Group 2 (40) – "Dampened" rhythm (r24 >0.03, <0.35), median age 60		Patients with "Dampened" circadian rhythms had higher fatigue (60% vs. 40%, p=0.003), nausea/vomiting (33% vs. 10%, p=0.007), and appetite loss (50% vs. 28%, p=0.004) than patients with "good" circadian rhythms. "Good" circadian rhythm patients had higher QoL emotional and social functioning scores (p<0.0001). PS was worse in patients with "dampened" circadian rhythms (p<0.0008).
Mormont et al., 2002 (106)	192 (128 M) metastatic colorectal cancer patients, mean age 58	3–5-day actigraphy (Actigraph)	r24 was associated with I<O (r=0.67, p<0.001) and both r24 and I<O were associated with mean activity (r=0.38 and 0.21, p<0.01). I<O and r24 were highest in patients with a performance status 0 and lowest in those with a performance status 2. Higher I<O, r24 and mean activity was associated with less fatigue, appetite loss, and nausea/vomiting (all p≤0.05) Higher I<O was associated with less pain, constipation, and dyspnoea (all p≤0.002). Higher I<O and r24 were associated with less depression (all p≤0.01). Higher I<O and r24 were associated with improved global quality of life, physical functioning, social functioning, and emotional functioning (all p≤0.04).
Mormont et al., 2002 (49)	18 14(M) metastatic colorectal cancer patients, mean age 58	3-day actigraphy (Actigraph) and serum melatonin, 6-alphasulfatoxymelatonin and salivary cortisol Blood and saliva collected 3–6 h apart over 10–13 time points on day 1 and day 4	Actigraphy demonstrated a mean r24 of 0.37, median r24 of 0.41, mean I<O of 92.8 and median I<O of 94.2. 13 patients had an r24 >0.28, 10 patients had I<O >25% quartile Interindividual variation was noted in circadian rhythms of activity, hormonal, and haematological markers of circadian system function.
Mormont et al., 2000 (37)	192 (128 M) metastatic colorectal cancer patients, mean age 58	3-day actigraphy (Actigraph) and sleep diary % 2 days of blood cortisol (08:00 and 16:00)	The r24 ranged from -0.06 to 0.77 with a median 0.42. The dichotomy index (I<O) ranged from 49%-100% with a median of 97%. Patients with a higher r24 or I<O had longer survival (p<0.0001). Patients in the upper quartiles had a longer 2-year survival than those in the lower quartiles (38% vs. 8%). Higher I<O and r24 were associated with improved global quality of life and physical functioning, and lower depression scores (p≤0.01). Higher I<O, r24, and mean activity were associated with less fatigue and less appetite loss (p<0.001). Higher I<O was also associated with less pain (p=0.002). Higher mean cortisol was seen in patients with a low r24 (r=−0.17, p=0.04), or low I<O (r=−0.24, p=0.007). Cortisol rhythmicity was correlated with the r24 (r=0.16, p = 0.04). Self-rated sleep disturbances were not correlated to the rest–activity rhythm or mean activity levels. A poor performance status was associated with lower r24 and I<O (p<0.0001), and lower mean activity (p = 0.04). The probability of an objective response was significantly influenced by r24 (p=0.02) and I<O (p<0.0001).
Mormont et al., 1998 (50)	Study 1: 18 (14 M) metastatic colorectal cancer patients, age 35–72 Study 2: 109 (72 M) metastatic colorectal cancer patients, median age 59	Study 1: 3-day actigraphy and melatonin and cortisol (blood 9–11 time points, 6 hourly) Study 2: At least 3-day actigraphy activity	9/18 patients had a marked circadian activity rhythm. Wide interindividual variation was noted. High r24 (p=0.0005), dichotomy indices (p=0.0005) and activity levels (p=0.0002) were related to survival. Increases in r24 were correlated with less fatigue (r=−0.33, p=0.03), less appetite loss (r=−0.34, p=0.01), less depression (r=−0.24, p=0.04), improved global quality of life (r=0.29, p=0.009), improved social functioning (r=0.28, p=0.01), and performance status (p=0.008).

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TABLE 2D Polysomnography-related circadian rhythms and their associations in patients with advanced cancer.

Studies with a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
Zeitzer et al., 2016 (52)	97 (F) recurrent or metastatic breast cancer, age 57.6 24 age-matched control subjects, age 57.1 (gender not identified)	28-h polysomnography and plasma cortisol (20–60-minute intervals) and 2-week actigraphy (Actiwatch 2) and sleep diary	Abnormal cortisol peaks were associated with polysomnography sleep disruption (p=0.004).
Silberfarb et al., 1993 (107)	Cancer patients: 17 (10 M) limited stage small-cell lung cancer or stage 2–4 non-small cell lung cancer, mean age 60.1 15 (F) stage 1–3 breast cancer, mean age 57.5 32 sex- and age-matched control subjects 32 patients with insomnia	3-day polysomnography and 2-week sleep diary	Cancer patients had longer total time in bed than any other group (vs. normal p<0.001, vs. patients with insomnia p<0.003). There was no difference in total time asleep between cancer groups (p=0.05) Sleep efficiency was lower in cancer patients (lung cancer vs. control subjects (p=0.007) and breast cancer vs. control subjects, (p=0.10)). Sleep latency was longer for lung cancer patients than control (p=0.03). Lung cancer patients had more difficulty remaining asleep than breast cancer patients (p=0.01) and control subjects (p=0.001).
Studies without a control group			
Padron et al., 2022 (91)	Cognitive behavioural therapy for insomnia and pain (CBT.i.p) group: 18 (F) stage 1–3 mixed gynaecological cancers, mean age 58.9 Psychoeducation (PE) group: 17 (F) stage 1–4 mixed gynaecological cancers, mean age 59.9	14-day polysomnography and actigraphy (Actiwatch-L) and sleep diary	Actigraphy and sleep diary data were not correlated. Baseline mean values (polysomnography vs. actigraphy) CBTi.p.: sleep efficiency was 75.5% vs. 80.8%, sleep onset latency was 24.7 min vs. 29.3 min, and wake after sleep onset was 91.1 min vs. 50.5 min PE: sleep efficiency was 71.0% vs 79.4%, sleep onset latency was 36.2 min vs. 33.6 min, and wake after sleep onset was 94.8 min vs. 53.4 min Objective (CBT.i.p and PE): 29% and 24% of patients had a SE >85% at baseline 76% and 50% of patients had SOL <30 min 6% and 13% of patients had WASO <30 min
Jakobsen et al., 2020 (29)	40 (24 M) mixed metastatic cancer diagnoses, median age 70	Overnight polysomnography and actigraphy (Actiwatch 2)	Results detail actigraphy findings and associations. Polysomnography findings not reported.
Parker et al., 2008 (108)	114 (58 M) stage 3–4 mixed cancer diagnoses, mean age 55.2	24-h polysomnography	Patients slept approximately 382 min/night (normal, 340–466 min) and 17.5% had <5 h sleep/night. Nocturnal sleep latency was <30 min (normal defined as <30 min), and sleep efficiency was 77.2% (normal defined as 86%–100%). >60 brief arousals occurred per hour and approximately 6 of the awakenings lasted at least 60 s/h. Patients slept in the daytime for an average of 89.3min. Total daytime sleep was associated with less nocturnal total sleep time (r_s=−0.21, p=0.025) and less sleep efficiency (r_s=−0.25, p=0.008), and more wake episodes (nocturnal index of awakenings) lasting at least 60 s (r_s=0.27, p=0.003). Females had higher sleep efficiency than males (80.0% vs. 74.9%, p=0.042). White patients had more nocturnal total sleep time (409.8 min vs. 364.4 min, p=0.017) and higher sleep efficiency than non-white patients (80.2% vs. 75.3%, p=0.021). Patients living with another had greater nocturnal total sleep time (392.5 min vs. 336.9 min, p=0.035) and shorter sleep latency (23.5 min vs. 57.2 min, p=0.018) than patients living alone. A higher level of education was associated with a higher sleep efficiency (79.2% vs. 74.6%, p=0.013).

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TABLE 2D Continued

Studies without a control group			
Article	Participants	Circadian measures	Outcomes relevant to review
			Lung cancer patients had higher index of awakenings lasting at least 60 seconds than breast cancer patients (3.9 vs. 2.4, p=0.002). Total sleep time was increased in patients that use SSRIs (430.4 min vs. 372.7 min, p=0.009) and reduced in patients taking beta blockers (345.7 min vs. 388.5 min, p<0.047) Antineoplastics agents were associated with an increased sleep efficiency (83.4% vs. 76.1%, p=0.000). Beta blockers significantly decreased sleep efficiency (72.3% vs. 78.1%, p=0.047)

M, male; F, female.

Actigraphy studies included between 11 and 436 patients, with a duration of monitoring varying from an overnight study to 30 days. The number of reported actigraphy parameters were much larger, with overlapping characteristics. Across sleep and wake periods, the timing and duration of activities, the relative proportions between different activities, and the variability both within and between 24-h periods were considered. At least 50 different actigraphy parameters were reported. When normal actigraphy values were stated, they differed between studies. The dichotomy index (I<O), for example, which measures proportional in-bed activity to out of bed activity, was reported in 20 studies. Unfavourable, or disordered, I<O values ranged from <89.5% to ≤97.5% (17, 24, 28, 94, 95, 98, 99, 101, 104). Favourable, or regular, I<O values varied between ≥89.5% and ≥99% (8, 17, 22, 24, 28, 49, 94, 95, 98–101, 104).

Finally, polysomnography studies included between 35 and 121 patients, with the duration of monitoring varying from an overnight study to a 14-day study.

Many articles did not describe normal circadian patterns or values.

Melatonin

Melatonin patterns in advanced cancer

Significant interindividual variation in melatonin circadian rhythms was noted (9, 17). 24-h melatonin rhythms, with a peak-to-trough pattern, were noted in non-small cell lung, gastrointestinal, mixed gynaecological, and mixed cancer cohorts (9, 11, 17, 39, 40, 42, 43, 45, 46). Abnormal 24-h rhythms were noted in two studies and affected 17% of the patients with metastatic colorectal cancer and 100% of patients with small cell lung cancer (49, 51). Detailed abnormalities included a smaller evening melatonin rise and earlier dim-light melatonin onset (DLMO) for patients with a gastrointestinal cancer who demonstrate more in-bed to out-of-bed physical activity (lower I<O) (17). Smaller evening melatonin rises were also noted in patients with non-small cell lung, gastrointestinal, and cervical cancers, particularly in advanced stages (40, 41, 43, 46). Advanced breast and lung cancer patients had higher mean melatonin levels compared to early-stage disease or controls (47).

Symptoms, quality of life, and survival

No statistically significant associations were reported between the melatonin circadian rhythm parameters and symptoms, quality of life, or survival in any of the included studies.

Cortisol

Cortisol patterns in advanced cancer

Normal and abnormal rhythms were noted across cancer cohorts to varying degrees. Ten studies reported abnormal

cortisol circadian rhythms in cancer patients (9, 36, 49, 60, 61, 72, 76, 79, 82, 106). This included mixed cancer cohorts (100%), ovarian cancer (75%), breast cancer (60%), and colorectal cancer (28%–56%) (36, 49, 50, 72, 76, 79, 83). Four studies noted normal cortisol circadian rhythms, including in patients with gastrointestinal ("most"), non-small cell lung (100%), nasopharyngeal cancer, and breast cancer patients (17, 39, 52, 55). Where present, abnormalities included a flattened cortisol slope, or reduced diurnal variation, coinciding with a lower morning cortisol rise and/or an increased evening cortisol (9, 12, 13, 16, 42, 54, 55, 58, 61–64, 72, 76, 77). These changes, along with the area under the curve, were more pronounced with later stage, higher grade or metastatic disease, and poorer performance status (9, 12, 13, 18, 54, 61, 64, 66, 69, 74, 76, 82). Cortisol levels at separate time points were not significantly different between cancer stages, or when compared with controls, but the overall mean 24-h cortisol was higher in cancer patients (39, 42, 52, 74). The timing of peak cortisol level (acrophase) ranged from 04:38 to 09:30 (55, 59, 82).

The change from peak to trough (cortisol slope) was unrelated to education level, marital status, age, time since recurrence, PR status, and metastatic sites in a breast cancer cohort (67). Patients with progesterone-receptor-positive breast cancer did, however, have a smaller cortisol awakening response (77). A flatter cortisol slope was associated with being male (69).

Salivary and serum cortisol were positively correlated, particularly when a strong circadian rhythm was present (16, 80).

Survival

Eight studies reported on survival, of which six report prognostic relevance of cortisol circadian rhythms. Patients with gynaecological cancer and either elevated night-time cortisol (HR, 1.802; $p<0.001$) or a flatter cortisol slope (HR, 1.633; $p=0.001$) had shorter survival (18). Conversely, patients with gynaecological cancer and more cortisol variability across the day had longer survival (HR, 0.644; $p<0.001$) (18). A lack of 24-h rhythmicity was prognostic in patients with lung cancer (HR, 68,052.8; $p=0.009$) and non-significantly in breast cancer (HR, 1.03; $p=0.85$) (68, 69). A flatter cortisol slope was prognostic in breast (HR, 464.9; $p=0.0036$) and renal cell cancer (HR, 1.88; $p=0.002$) (70, 81). Mean cortisol levels were prognostic in colorectal cancer (78). The early morning cortisol rise (CAR), area under the curve, and cortisol level at 00:00 were not prognostic in patients with lung cancer (61, 69). Altered cortisol rhythms in a study of patients with colorectal cancer were unrelated to survival (80).

Furthermore, a study of patients with breast cancer highlighted abnormal cortisol peaks, rather than the diurnal rhythm, to be associated with a shorter disease-free interval ($r= -0.30$, $p=0.004$) (52).

Physical and psychological symptoms

A smaller cortisol awakening response, flatter cortisol slope, and less diurnal variability were associated with increased total symptom scores, individual scores for fatigue, and interference with general activity, work, and walking (54, 64).

Within lung, ovarian, and mixed cancer cohorts, reduced diurnal cortisol variation, elevated evening cortisol, elevated morning cortisol, and higher area under the curve were associated with depression (19, 53, 54, 73, 74). Patients with steeper cortisol slopes, and therefore healthier rhythms, expressed less negative affect during psychological therapy and demonstrated more post-traumatic psychological growth following diagnosis, and those with flatter cortisol slopes were found to repress emotions (14, 67, 79). Abnormalities, including higher waking cortisol and lower cortisol awakening response, were associated with antidepressant use in patients with breast cancer (77). Some studies reported no significant correlations between cortisol levels, or cortisol slope, and psychological measures (12, 70, 73).

Quality of life

Flattened cortisol slopes, less cortisol variability, and elevated evening cortisol were associated with reduced physical well-being in patients with ovarian cancer (18, 54). Conversely, cortisol rhythms were also not correlated with quality-of-life measures for patients with breast cancer (65).

Other

Abnormal cortisol peaks during sleep, coinciding with waking episodes, were reported in a subset of metastatic breast cancer patients (52). More frequent and longer lasting wake episodes and a progressive later waking time were also seen with flatter cortisol slopes (66, 75, 81).

The cortisol slope was correlated with the CAR, rather than waking level, and flatter slopes were associated with higher evening cortisol levels and an escape from cortisol suppression (63, 77).

Actigraphy

Actigraphy patterns in advanced cancer

Of the studies comparing patients with cancers to controls, 90% found abnormal actigraphy activity parameters in patients with cancer (17, 20, 23, 28, 30, 32, 85–88). Due to several reported parameters, the dichotomy index (I<O), 24-h autocorrelation coefficient (r24), and sleep efficiency (SE) are noted as examples.

Between 30%-95% of patients had a disrupted dichotomy index (I<O), suggesting proportionally more in-bed to out-of-bed activity, with average group values of 79%–98% (individual values of 49%–100%) (17, 21, 22, 24, 28, 37, 78, 87, 89, 94–96, 98–100, 104–106). Lower values suggest proportionally higher in-bed to out-of-bed activity. I<O values were lower in men, those with metastatic disease, and a poorer performance status (89, 99, 106). The I<O was reported to be the most discriminative parameter for cancer patients, although a large inter-subject variability was noted (17, 28).

Approximately 26%–28% of patients had a disordered 24-h autocorrelation coefficient (r24), representing the dissimilarity of rest–activity rhythms (RARs) between days, with average group values of 0.19–0.57 (individual values of −0.06–0.77) (15, 24, 32, 37, 49, 65, 71, 78, 88, 100, 105, 106). A r24 approaching 1 represents a prominent RAR (105). r24 values were lower in those with poorer performance status and in African-American women (37, 65, 105, 106).

Approximately 12%–88% of patients had disordered sleep efficiency, which measures sleep during time in bed, with average group values of 71%–92% (individual values, 20.2%–100%) (10, 15, 20, 22–27, 29, 30, 32, 68, 75, 85, 87, 91, 94, 97, 100, 102, 103). Higher values suggest that more time in bed has been spent sleeping, and lower values suggest that more time in bed has been spent awake.

Some studies reported circadian actigraphy parameters were unrelated to performance status, whilst others reported that time awake spent immobile (TASI), I<O, r24, and mean activity were significantly correlated with performance status (8, 17, 31, 37, 50, 78, 89, 99, 105, 106).

Survival

A total of 12 studies commented on survival and all linked circadian disruption to survival. Stronger RARs evidence by improved dichotomy index (I<O), 24-h autocorrelation coefficient (r24), physical activity amplitude and MESOR, nighttime restfulness, sleep efficiency, and time awake spent immobile were associated with longer survival in patients with colorectal, breast, head and neck, non-small cell lung, and mixed cancer diagnoses (8, 9, 31, 37, 68, 78, 89, 93, 98, 99, 104, 105). In a mixed cancer cohort, disordered I<O was not prognostic; however, r24 and sleep efficiency were prognostic (89).

Examples of prognostic relevance include colorectal and mixed cancer cohort patients with an I<O <97.5%, or below median I<O, having a reduced overall survival (OS) of between 2.1 and 9.7 months, and reduced progression-free survival (PFS) of 4.2 months (98, 99, 104). Similarly, patients with colorectal cancer and an I<O ≥99.2% had 11.5 months longer survival than those with an I<O <92.4% (105). The I<O was an independent prognostic factor when accounting for factors including age, gender, performance status, cancer diagnosis and stage, previous chemotherapy, and surgery (98, 99, 104). Similarly, sleep efficiency was an independent risk factor for patients with breast cancer whereby those with a sleep efficiency >85% had over a double survival compared to those with poor SE (68). However, I<O, r24, mean activity, and sleep activity parameters were reported also reported to not be significantly correlated with overall survival or progression-free survival (68, 89, 92, 104, 105).

Physical and psychological symptomatology

Abnormal circadian activity rhythms were associated with pain, fatigue, drowsiness, nausea, vomiting, anorexia, and weight loss (20, 27, 37, 50, 78, 101, 106). Higher I<O values were specifically associated with less pain, fatigue, anorexia, sleep disturbance, constipation, and dyspnoea, and improved sleep quality (95, 100, 105, 106). Higher r24 values were specifically associated with less insomnia, daytime dysfunction, fatigue, anorexia, pain, and dyspnoea (20, 30, 100, 105). Higher sleep efficiency was associated with less pain (92). Greater time to sleep once in bed (sleep onset latency, SOL), wake after sleep onset (WASO), and time in bed (TIB) were associated with gastrointestinal symptoms in a mixed cancer cohort (92). Increase time spent napping was associated with increased pain, fatigue, and daytime sleepiness (92). No association between circadian activity parameters and pain or fatigue was found in a mixed cancer cohort (8).

Lower sleep efficiency, I<O, r24, and mean activity along with increased time spent napping or in bed were all associated with increased depression (37, 50, 75, 92, 93, 106). A lower r24 and more daytime inactivity were associated with intrusive thoughts and avoidant coping in patients with breast cancer (71). Studies also reported that anxiety and depression were not associated with sleep–activity rhythms, including the I<O (8, 17, 23).

Quality of life

Circadian disruption was associated with interference with activity, work, relations, and enjoyment of life for patients with colorectal cancer (95). Improved r24, I<O, and meanAct were associated with improved global QoL, along with health, physical, social, and functioning subscores (20, 50, 78, 95, 96, 105, 106). A lower amplitude and MESOR and a later acrophase were associated with worse global QoL in a mixed cancer cohort (8). The strongest correlation between an actigraphy parameter and quality of life measure in a mixed cancer cohort was the 24-h correlation coefficient (32). Studies also noted that circadian parameters were not associated with the fatigue, emotional, or cognitive subscales of quality-of-life measures (105, 106). One study of patients with breast cancer noted that WASO and r24 were unrelated to global QoL (65).

Other

There were mixed reports regarding chemotherapy response and circadian rhythmicity in patients with colorectal cancer. One study noted that disordered rhythmicity during chemotherapy was associated with earlier death but not to objective response or toxicity, while another study noted objective response to be influenced by r24 and I<O (37, 104). Patients receiving chemotherapy who also had evidence of circadian disruption were more likely to experience weight loss and fatigue (101).

I<O appeared to correlate with circadian temperature rhythms, self-reported physical activity, and chronotype (17). More robust circadian rhythms were associated with greater light exposure (8).

Subjective and objective measures differed for physical activity but were closely correlated for sleep (27, 102). Subjective sleep disruption and circadian disruption can occur together or independently (22). Although total sleep time (TST), SE, and

WASO were associated with subjective sleep quality, physical activity measures were also not significantly different between those who report their sleep as good or poor (26, 29, 37, 85, 102).

Subjective scores of pain and physical function correlated with objective physical activity, and those using analgesia had more abnormal circadian activity rhythms (84, 105). Daytime sleep, or inactivity, was related to sleep medication use, night-time sleep disturbance, daytime dysfunction, night-time sleep, and sleep quality (30, 38, 108). Sleep efficiency was reported to be correlated with chest metastases, hormone use, and radiotherapy (75). Patients with a higher r24 had less daytime dysfunction and less insomnia (20, 30). Circadian disruption was associated with tumour progression markers (15).

Polysomnography

Patients with cancer spent more time in bed were noted to have multiple nocturnal awakenings and had an average sleep efficiency of up to 77.2% (91, 107, 108). Increased daytime sleep was associated with less night-time sleep and more nocturnal awakenings in a mixed cancer cohort (108). Medications were also found to impact on sleep. Anticancer therapies were associated with increased sleep efficiency, whereas beta blocker use was associated with reduced sleep efficiency (108). Sleep efficiency was higher in women, white patients, and those with a higher education level (108).

Correlations between measures of circadian rhythm

Increased diurnal physical activity variability was associated with increased diurnal melatonin and cortisol variability along with an earlier DLMO (17). Salivary cortisol levels appeared unrelated to I<O, cortisol rhythmicity positively was correlated with r24, and more robust actigraphy rhythms were associated with a steeper cortisol slope (17, 37, 71).

The dichotomy index was correlated with r24, mean activity, sleep motor activity, sleep efficiency, and WASO (28, 89). r24, I<O, and mean activity were also correlated (106).

Higher I<O and r24 were associated with improved sleep efficiency (100).

Polysomnography-derived values for sleep efficiency were lower, and wake after sleep onset higher, than actigraphy-derived values (91).

Discussion

This review supports, expands upon, and updates several previous reviews of circadian rhythmicity in patients with advanced cancer. It highlights that, for several patients with advanced cancer, disordered cortisol, melatonin, and physical activity circadian rhythms are associated with increased symptom burden, poorer quality of life, and shortened survival. Other important associations with CRDs include poorer performance status and raised biomarkers of tumour progression.

A review of rest–activity rhythms in advanced cancer patients found that CRDs are particularly evident amongst men, those undergoing chemotherapy, and those who were symptomatic (109). Additionally, circadian disruption may be seen across the cancer trajectory, with worse biopsychosocial outcomes reported in cancer survivors who have disordered cortisol rhythms (110). This review highlights that circadian rhythms may be maintained in some patients with cancer and that wide inter-individual variation exists. Future research aimed at identifying patients that are at risk of circadian rhythm disorders, and impacted by their associations, is important, particularly when considering interventional studies to improve circadian rhythms and patient outcomes.

Articles were predominantly observational in nature, and many studies lacked a control group. Causality is difficult to establish, particularly due to the bi-directional relationship between cancers and circadian rhythm disorders, and the influence from external factors. CRDs impact on several neuroendocrine-immune functions, including inflammatory responses and hormonal secretion, and predispose individuals to developing cancer (111). Cancer in turn generates a pro-inflammatory state, and increased circulating cytokines levels can disrupt circadian rhythms (111). Rest–activity patterns are influenced by age, sex, race, education, and voluntary behaviour (6, 112). Cortisol values are influenced by sex, age, body mass index, menstrual cycle, sleep disturbances, renal disease, and acute illness, for example (4). The review highlights studies of patients prior to, during, and after anticancer therapies, and within the inpatient and community setting. Limited information on previous and current therapeutic regimes, and location of metastatic disease, limits the ability to synthesise findings. Potential modifying factors of circadian rhythmicity should be reported and taken into account when reviewing findings (113).

CRDs and their impact are not solely seen in cancer patients. Circadian disruption has been reported in patients with neurodegenerative conditions including Alzheimer's disease, Parkinson's disease, and Huntington's disease (114). Despite conflicting findings, evidence highlights altered rest–activity, body temperature, melatonin, and cortisol rhythms within this population and associations with physical and psychological well-being, and quality of life (114). At present, the similarities in circadian disruption between clinical conditions are not clear.

Furthermore, the review highlights heterogeneity in the investigation and reporting of circadian rhythms in cancer patients and a lack of threshold values to identify circadian parameter abnormalities. Several reporting measures, overlapping definitions, and an absence of clear definitions were found in the investigation of circadian rhythms, particularly when using actigraphy. Heterogeneity in actigraphy research is not limited to cancer populations. A review of 126 actigraphy studies of children highlighted a lack of standardisation in actigraphy practice, including the reporting of epoch length, artefact detection, and definition of variables (115). Additionally, within cohorts of bipolar disorder patients, over 30 possible actigraphy parametric and non-parametric measures were reported (116). In this review, only four actigraphy parameters (I<O, r24, mean activity, and SE) were

associated with at least three of the areas of interest (physical symptoms, psychological symptoms, quality of life measures, and survival). Although a wealth of information can be obtained using actigraphy, the reporting parameters should be aligned with the overall study objectives to allow a clear message in the literature. Analysis of actigraphy data takes many forms and lacks standardisation (6, 112). Similarly, variable sampling protocols, analysis, and reporting practices has been seen in cortisol and melatonin studies (3, 110). When faced with such heterogeneity in approaches, it is challenging to make firm conclusions, and standardisation may improve research practice. The development of recommendations to identify, and subsequently report, optimal sampling processes, particularly the frequency and timing of samples, and the calculation of circadian parameters are required.

Actigraphy data can report the timing of events, duration of events, or relationship between events. Although studies may focus on "sleep–wake" or "rest–activity" periods, there is significant overlap. Diagnostic criteria have been formulated for circadian rhythm sleep–wake disorders by American Academy of Sleep Medicine (117). The diagnosis considers the timing of sleep onset and offset, and the presence of jet lag or shift work, to categorise patients into seven different diagnoses. Many studies of advanced cancer patients reported actigraphy measures across the 24-h period rather than focusing on this timing of sleep onset–offset. The circadian activity rhythm disorders in cancer patients are likely separate to intrinsic circadian sleep–wake rhythm disorders. Recent international consensus recommendations have been developed for the assessment and diagnosis of circadian rest–activity rhythm disorders (CARDs) (118). The recommendations outline key modifiers of circadian rhythmicity, areas to consider within a clinical history, patient sleep and activity diary, and accelerometery during assessment, and criteria to diagnose a CARD. Diagnostic criteria of other forms of CRDs do not currently exist.

The scoping review was strengthened by using independent authors at multiple stages of the review process. Additional evidence was actively sought through hand searching review papers and reference lists. The scoping review is inclusive of available evidence and placed minimal limitations in the search strategy. It made no attempt to critically analyse the quality of evidence. Although the review aimed to focus on advanced cancer patients, several studies included non-advanced cancer patients. This approach may dampen associations, but it was felt to be more inclusive and to provide a broader insight of the topic. Furthermore, the review did not exclude several confounding factors in selected articles, such as medications and chemotherapy. This information was not available in several studies, and through exclusion, it would have limited the generalisability of the findings. Studies reporting on circadian rhythmicity in patients with cancer would benefit from detailed information on recent and existing modifiers of circadian rhythmicity, and the presence and location of metastatic disease.

Conclusion

Cancer patients, particularly those with advanced disease, are at risk of circadian rhythm disorders and significant associated complications. It remains unclear which subset of patients are most susceptible. Conflicting results within the review highlight the need for further studies to identify patient populations that are most impacted by circadian rhythm disorders. Current investigative approaches require a multiple sampling approach (blood, urine, and saliva) or a prolonged period of activity monitoring. In the clinical setting, and advanced cancer population, this may require an alternative approach. Current gaps in the literature are highlighted in Box 1. There needs to be an attempt to standardise research approaches and reporting practice within circadian rhythm research and to develop criteria to identify circadian rhythm disorders. Research standardisation and targeted approaches may help in future research aimed at developing management approaches to circadian rhythm disorders.

Box 1:

Gaps in the current literature.

- What are the risk factors for a patient with cancer to develop a circadian rhythm disorder?
- How do circadian rhythm disorders differ between malignant and non-malignant clinical conditions?
- How do circadian rhythm disorders different between malignant subgroups?
- What are the optimal measurement and analytical approaches when assessing cortisol, melatonin, and rest–activity circadian rhythms?
- What are the abnormal threshold values for cortisol, melatonin, and rest–activity parameters when diagnosing a circadian rhythm disorder?
- Do current investigative approaches translate into the clinical setting, considering the ease and acceptability for patients and clinicians?

Author contributions

CG was responsible for the conceptualisation of the review and development of the search strategy. CG and JP conducted the scoping review, data extraction, and data checking. CG wrote the initial draft of the manuscript with editorial input from JP and AD. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Elizabeth Cash,
University of Louisville, United States

REVIEWED BY
Mario Cioce,
Campus Bio-Medico University, Italy
Paolo Martini,
University of Brescia, Italy

*CORRESPONDENCE
Qi-yang Shi
wsqy214@163.com
Shu Lin
shulin1956@126.com

†These authors have contributed equally to this work

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Latest advances in the study of non-coding RNA-mediated circadian rhythm disorders causing endometrial cancer

Ling-tao Zheng[1†], Shao-rong Chen[1†], Liang-yu Zhou[1], Qiao-yi Huang[1], Jia-ming Chen[1], Wei-hong Chen[1], Shu Lin[2,3*] and Qi-yang Shi[1*]

[1]Department of Gynaecology and Obstetrics, The Second Affiliated Hospital of Fujian Medical University, Quanzhou, Fujian, China, [2]Centre of Neurological and Metabolic Research, The Second Affiliated Hospital of Fujian Medical University, Quanzhou, Fujian, China, [3]Group of Neuroendocrinology, Garvan Institute of Medical Research, Sydney, NSW, Australia

Endometrial cancer (EC) is one of the most common gynecological cancers, and its risk factors include obesity and metabolic, genetic, and other factors. Recently, the circadian rhythm has also been shown to be associated with EC, as the severity of EC was found to be related to night work and rhythm disorders. Therefore, circadian rhythm disorders (CRDs) may be one of the metabolic diseases underlying EC. Changes in the circadian rhythm are regulated by clock genes (CGs), which in turn are regulated by non-coding RNAs (ncRNAs). More importantly, the mechanism of EC caused by ncRNA-mediated CRDs is gradually being unraveled. Here, we review existing studies and reports and explore the relationship between EC, CRDs, and ncRNAs.

KEYWORDS

endometrial cancer, circadian rhythm disorders, non-coding RNAs, clock genes, miRNA, lncRNA

1 Introduction

Endometrial cancer (EC) is a malignancy of the endometrial epithelium. The annual incidence of EC is very high. Worldwide, 417,367 cases of EC were reported in 2020, making it the sixth most common cancer among women (1). Continuous exposure to exogenous or endogenous estrogen without progesterone antagonism is a risk factor for endometrial cancer. Other risk factors, such as obesity, tamoxifen use, insulin resistance, type 2 diabetes, and polycystic ovary syndrome, can increase the risk of EC (2). The main symptom of EC is abnormal uterine bleeding, which can be accompanied by vaginal secretions and uterine infection (3). When a patient presents with any of these symptoms, abdominal and pelvic examinations should be considered (4). The primary clinical treatment for EC is surgery, including total hysterectomy, bilateral salpingo-

oophorectomy, and adjuvant therapy. However, the 5-year survival rates of patients with stage IVA and IVB EC are only 17% and 15%, respectively, although 67% of these patients display early signs of the disease (5). Therefore, finding a new treatment strategy is urgent.

The circadian rhythm is a stable regulatory system in the human body and is regulated by several hormones, particularly melatonin (MLT). Some studies have shown that MLT is involved in the regulation of epithelial-mesenchymal transformation and subsequent tumor invasion (6–10) as well as in inhibiting osteosarcoma (6) and ovarian cancer stem cells (7). At the molecular level, circadian rhythms are regulated by clock genes (CGs). Many diseases are caused by the abnormal expression of these genes, including cancer, endocrine, cardiovascular, and psychological diseases (11, 12). As a genetic disease, cancer is caused by uncontrolled growth and one reason for this is changes in circadian pathway genes (13). Furthermore, non-coding RNAs (ncRNAs), including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs), are involved in the regulation of CGs, such as miR-576-5p (14), miR-126-5p (15).

In this paper, we reviewed the latest progress in EC caused by CRDs and mediated by ncRNAs. Additionally, we attempted to summarize the relationship between ncRNAs, circadian rhythms, and EC.

2 Endometrial cancer

Historically, proliferative lesions that occur in glands without cytological atypia are called "hyperplasia" and have a 2% cancer risk, while those with cytological atypia are called "atypical hyperplasia" and have a 23% cancer risk (16). Endometrial intraepithelial neoplasia (EIN) is now recognized to precede atypical endometrial hyperplasia and is considered a precursor lesion of endometrial cancer (17).

The etiology of EC is not completely clear; however, it includes a variety of risk factors, such as BMI, as analyzed by Aune et al. (18). Using data from 22,320 cases, high BMI at 18–25 years of age, waist and hip circumferences, waist-to-hip ratio, height, and weight gain (over 10kg) were associated with an increased risk of EC. In other words, there is a positive correlation between body fat, weight gain, height, and the risk of EC. Renehan et al. (19) also reported that every 5 kg/m^2 increase in BMI raises the risk of developing EC. Fisher et al. (20) reported that although tamoxifen reduced the incidence of breast cancer, it increased the risk of EC. Crosbie et al. (2) reported that insulin resistance, hyperinsulinemia, type 2 diabetes, and polycystic ovary syndrome (PCOS) could promote endometrial hyperplasia, which might be associated with EC.

Estrogen promotes endometrial hyperplasia; periodic menstruation and estrogen-antagonistic progesterone work together to maintain endometrial health. In obese women, the adipose tissue converts adrenal androgen to estrogen, forming a hyperestrogenic state (2). This state may interfere with the normal proliferation of the endometrium and increase the risk of EC. Additionally, Modugno et al. (21) showed that obesity is a chronic pro-inflammatory state that promotes the development of an inflammatory microenvironment and is accompanied by high levels of circulating c-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α). These inflammatory markers may mediate the changes in the endometrial immune microenvironment.

Tamoxifen can stimulate endometrial hyperplasia. The stimulating effect of the long-term use of tamoxifen may be the mechanism that increases the risk of EC (20). Similarly, insulin resistance, hyperinsulinemia, type 2 diabetes, and PCOS can reduce the circulation levels of estrogen-binding proteins, insulin-like growth factor (IGF)-1, sex hormone-binding globulin, and IGF-binding protein, and improve their efficiency to stimulate endometrial growth (2) (Figure 1).

Total hysterectomy and bilateral salpingo-oophorectomy are cornerstones of EC treatment and can be performed using open or minimally invasive techniques. Minimally invasive surgery is the first treatment choice for early-stage EC when the uterus is completely resectable. Minimally invasive surgery has the advantages of a short hospital stay, less blood loss, less pain, and low perioperative incidence (22–25).

3 Circadian rhythm disorders

Sleep and wakefulness have distinct circadian rhythms and are two bodily functional states. Sleep restores vitality, strengthens the immune system, maintains brain and heart health, consolidates memory (26). The circadian rhythm regulates the sleep cycle, and an appropriate circadian rhythm results in normal cell growth and survival. Various factors regulate circadian rhythms, one of the most important of which is MLT. Light-generated neural signals regulate MLT metabolism via the retinohypothalamic tract (RHT)-suprachiasmatic nucleus (SCN)-paraventricular nucleus (PVN)-brainstem-spinal cord (levels T1–T3)-superior cervical ganglion (SCG)-pineal gland pathway (27). Since MLT promotes sleep and reduces daily activity performance, inappropriate light stimulation can produce circadian rhythm disorders (CRDs) and disrupt MLT metabolism in the pineal gland (27).

Sun et al. demonstrated an increase in the number of macrophages in tissues and organs during circadian rhythm disturbances. Several studies have suggested a potential link between circadian rhythms and the cell division cycle (28–30) as well as malignancy (31). This occurs when the timing of daily activities is out of sync with an individual's innate chronotype (32). CRDs may cause defects in the regulation of cell proliferation (33). For example, disturbances in circadian rhythms caused by night work may increase the risk of breast and prostate cancer (34–36) as well as EC (32, 37).

3.1 Clock genes

The first clock gene discovered in *Drosophila* is called the "period" gene (38, 39). There are at least 12 known CGs in

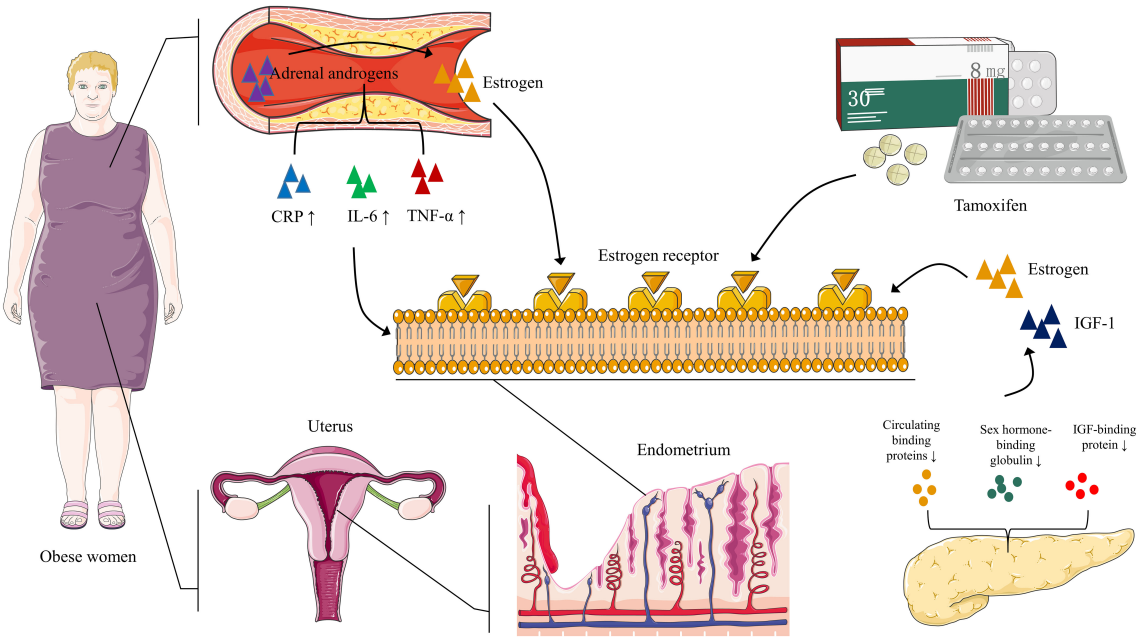

FIGURE 1
EC: pathways to carcinogenesis In obese women, adipose tissue aromatizes adrenal androgens into estrogen, leading to a hyperestrogenic state. Obesity also promotes high circulating CRP, IL-6, and TNF-α levels, which may be associated with changes in the endometrial immune microenvironment. Prolonged tamoxifen use promotes estrogen binding to its receptor, further enhancing the hyperestrogenic state. Insulin resistance, hyperinsulinemia, type 2 diabetes, and PCOS inhibit circulating binding proteins of estrogen and insulin-like growth factor (IGF)-1, sex hormone-binding globulin, and IGF-binding protein. As a result, estrogen and IGF-1 bioavailability increases, stimulating the endometrium.

mammals: Period1 (*PER1*), Period2 (*PER2*), Period3 (*PER3*), Cryptochrome 1 (*CRY1*), Cryptochrome 2 (*CRY2*), Circadian Locomotor Output Cycles Kaput (*CLOCK*), the transcription factor Aryl Hydrocarbon Receptor Nuclear Translocator-Like (*ARNTL*), Timeless (*TIM*) (13), Retinoic acid-related Orphan Nuclear Receptor (*ROR*) (40–42), Neuronal PAS domain protein-2 (*NPAS2*) (42, 43), Nuclear Receptor Subfamily 1 Group D members 1 and 2 (*NR1D1* and *NR1D2*, respectively, also known as REV-ERB alpha and beta, respectively) (42, 44), and Casein Kinase I Epsilon (*CSNK1E*) (42, 45). Among these, *PER1*, *PER2*, and *NPAS2* are associated with EC (46, 47). AT-rich interaction domain 1A (*ARID1A*) may be involved in the progression of EC by regulating the CGs *BHLHE41* and *ARNTL* (48). The following sections discuss the relationship between CGs and EC.

3.1.1 Period1 and period2

PER1 and *PER2* may be involved in mechanisms underlying EC onset and progression. Wang et al. (46) found that a high expression of *PER1* and *PER2* was associated with a better prognosis in EC. In their study, western blotting showed that the expression of PER1 and PER2 decreased in the rhythm group, whereas the expression of breast carcinoma amplified sequence 4 (BCAS4), tubulin beta-2B chain (TUBB2B), and Roof Plate-Specific Spondin-4 (RSPO4) increased in the breast cancer group. The high expression of PER1 indicates that the survival time of patients with EC is longer, while the high expression of TUBB2B indicates a lower survival rate. TUBB2B is related to diffuse and symmetrical aberrations in cerebral cortex development, and its importance in the central nervous system reveals its potential role in regulating circadian rhythms. In addition, they transfected Ishikawa cells with overexpressed PER1 plasmid and found that the apoptosis rate was significantly increased after 24h, and cell invasion was disturbed after 24h and 48h (46). A loss of *PER* expression suppresses the diurnal oscillation of decidualized human endometrial stromal cells (49). It is reported that *BCAS4*, *TUBB2B*, and *RSPO4* regulate cancer development by interacting with other proteins (50–52). All in all, the severity of EC is associated with CRDs, and factors such as the CGs PER1 and PER2 may regulate the mechanisms of EC onset and development.

3.1.2 AT-rich interaction domain 1A

The switch/sucrose non-fermentable (SWI/SNF) complex is a nucleosome-remodeling factor found in both eukaryotes and prokaryotes (53). Through transcriptional control, it participates in the regulation of gene expression and is essential for cancer growth (54). SWI/SNF is a multi-subunit complex that includes AT-rich interaction domain 1A (*ARID1A*). *ARID1A* is one of the most commonly mutated genes in human cancers, such as colorectal cancer (55, 56), gastric cancer (57, 58), pancreatic cancer (59), esophageal adenocarcinoma (60), liver cancer (61), ovarian clear cell carcinoma (62), and endometrioid carcinoma (63–65). As reported by Hanyang Hu et al. (48), *ARID1A* regulates the binding of ER to clock gene enhancers in EC. *ARID1A* depletion affects chromatin accessibility and ER binding in enhancers, leading

for the downregulation of CGs *ARNTL* and *BHLHE41*, eventually favoring attenuation of endometrial cancer cell proliferation and metastasis. In addition, a decreased *ARID1A* expression was linked to shorter progression-free survival in patients with endometrial-associated cancer. In summary, *ARID1A* and circadian rhythm genes can be regarded as novel diagnostic markers and potential targets for the treatment of EC (53).

3.1.3 Neuronal PAS domain protein-2

NPAS2, the longest CG in humans, is a mammalian transcription factor with a length of 176.68 kb (47). The PAS domain of *NPAS2* binds to heme as a prosthetic group, making heme-based signal transduction possible, thereby playing a key role in generating circadian rhythms (66). *NPAS2* participates in the cell cycle and the DNA damage response (67). A recent study showed that high *NPAS2* expression is associated with poor survival in patients with EC (47). The data from that study show that *NPAS2* is positively correlated with poor prognosis in EC. In addition, overexpression of *NPAS2* significantly induces the proliferation of Ishikawa cells, while silenced *NPAS2* inhibits the growth of AN3CA cells, and these situations are likely due to the influence of *NPAS2* expression on the G1 and S phases of the cell cycle. This suggests that *NPAS2* can be used as an indicator for the diagnosis and treatment of EC. Moreover, the researchers analyzed and predicted the expression correlation between miRNAs and *NPAS2* in UCEC using the starBase database and found that *NPAS2* was negatively correlated with *miR-17-5p* (R=-0.119, p=2.09E-02) and *miR-93-5p* (R=-0.091, p=7.96E-02), and positively correlated with *miR-106a-5p* (R=0.111, p=3.21E-02) and *miR-381-3p* (R=0.198, p=1.11E-04) (47).

To sum up, *PER1* and *PER2* may regulate EC pathogenesis and progression, *ARID1A* affects EC cell growth and metastasis, and *NPAS2* affects EC cell proliferation and apoptosis. Focusing on these CGs and exploring corresponding targeted therapy may lead to a potential tool for improving the effectiveness of EC therapy (Table 1).

4 Endometrial cancer and circadian rhythm disorders

A study showed that age, education, smoking, type of work, marital status, fertility, menopause, gynecological history, hypertension, and shift time were all related to the severity of EC (46). When uncontrollable factors (such as age and menopause) were excluded, the correlation between rhythm-related factors and EC was the strongest (R ≈ 0.1). In order to control the diurnal functioning of the whole body, the circadian rhythm makes the behavior pattern consistent with ambient light and darkness, supporting body function by predicting and coordinating the necessary metabolic procedures (68). Disorders of the circadian rhythm disrupt the metabolic balance in the body. Endometrial proliferation, secretion, and shedding occur periodically, and the disruption of this cycle elevates the risk of disease. Working at night is one such case. Viswanathan et al. (69) reported that night shift work might increase the risk of EC. Besides, Von Behren et al. (32) explored the relationship between EC and chronotypes and found that post-menopausal women with evening chronotypes were more likely to develop EC, especially those with a body mass index (BMI) of 30 or higher. According to a study of sleep/night shift characteristics of patients with EC conducted by Wang et al. (46), the severity of EC is associated with night shift and rhythm disorders. In addition, people who work at night are exposed to inappropriately timed light, causing cortisol, body temperature, and MLT rhythms to be out of sync (70). As discussed above, the effect of CRDs on EC is ultimately mediated by CGs.

TABLE 1 CGs, their expression in EC, and the corresponding prognosis.

Clock genes	Expression level	Prognosis	References
PER1 and PER2	Low	Poor	(46)
ARID1A	High		(48)
NPAS2	High		(47)

In EC cells, CGs *PER1*, and *PER2* were at low expression levels, while *ARID1A* and *NPAS2* were at high expression levels. The expression levels of all these genes indicate the poor prognosis of EC.

5 Non-coding RNAs

NcRNAs affect circadian rhythms through the gene-effector protein-circadian rhythm axis. NcRNAs are mainly composed of miRNAs, lncRNAs, and circRNAs (71, 72), and they play an important role in tumor development (73, 74). Here, we discuss the influence of miRNAs and lncRNAs on CRDs. MiRNAs are small ncRNAs, 19–24 nucleotides long, whereas lncRNAs are longer than 200 nucleotides. LncRNAs control gene expression by altering the function of transcription, splicing, translation, or miRNAs (75). Ray et al. (13) found that a subset of ncRNAs changes in cancer tissue, with target sites on certain CG mRNAs that can directly influence the abundance of these clock genes; another subset of ncRNAs targets specific oncogenes or tumor suppressor genes and is directly regulated by CGs. The potential use of ncRNAs in disease diagnosis has become widespread. Gharib et al. (76) examined the levels of *miR-31* in 100 patients with breast cancer and their adjacent normal breast tissues using RT-PCR and concluded that *miR-31* is expressed at low levels in breast cancer. Similarly, Zhao et al. (77) concluded that serum *miR-205-5p* is a valuable biomarker for lung cancer diagnosis because it promotes the proliferation and metastasis of lung cancer cells by regulating TP53INP1. Herein, we summarize the information that has become available in recent years.

By selectively targeting the ZBT4/Sp1 axis, *miR-576-5p* may affect the ability of EC cells to proliferate, migrate, and invade (14). *Circ_0002577* downregulates *miR-126-5p* in concert with MACC1 to promote EC invasion and metastasis (15). *MiR-1271-5p* overexpression prevents EC cell proliferation, migration, and invasion by targeting its downstream target, CTNND1, and induces cell death (78). *MiR-202-5p* (79), *miR-197* (80), *miR-298* (81), and *miR-105* (82) have similar effects to *miR-1271-5p* and can

target downstream genes to prevent the proliferation, migration, and invasion of EC cells. CLOCK-controlled *miR-455-5p* regulates circadian rhythms by accelerating the degradation of clock mRNA (83). In short, the above-mentioned miRNAs related to CGs are usually stable in normal tissues but are irregularly expressed in abnormal tissues, especially in tumors (84). Nonetheless, we did not observe many EC-related miRNAs in the data we collected. Future studies should explore additional miRNAs related to EC and CRDs.

LncRNAs also play a important role in EC, as lncRNA binding to miRNAs promotes or inhibits the proliferation of EC cells. For example, the lncRNA *OIP5-AS1* inhibits the proliferation and invasion of EC cells by suppressing *miR-200C-3p*, which in turn regulates PTEN/AKT (85). Thus, lncRNAs, like miRNAs, play an indispensable role in the development of EC. Again, from the information collected, we did not observe many lncRNAs associated with EC. Further exploration of the interaction of lncRNAs and miRNAs with EC will help discover new therapeutic options for treating EC. To summarize the information we collected, we have compiled Figure 2.

6 Discussion

The biological clock is a 24-h self-service oscillator controlled by CGs. Every cell in the human body has a day-night oscillator controlled by a master clock. This oscillator provides rhythmicity to specific cells and organs through rate-limiting metabolic program stages (68). The breakdown of the circadian rhythm disrupts the rhythmic nature of every cell and organ, resulting in a wide range of diseases. The circadian rhythm is regulated by CGs. The disorder of circadian rhythm is usually accompanied by abnormal expression of CGs, which also involves ncRNAs. This review summarizes recent perspectives on EC and CRDs, and collects relevant CGs and ncRNAs, including *PER1*, *PER2*, *NAPS2*, and *ARID1A*. However, based on our review, the only ncRNAs associated with CGs and EC are *miR-17-5p, miR-93-5p,* and *lncRNA SNHG14*. *MiR-17-5p* expression is negatively correlated with *NAPS2* expression (47). Gao et al. (86) showed that miR-17-5p inhibited *CLOCK* translation, downregulated *NAPS2* levels, and increased *CRY1* expression. Furthermore, *miR-17-5p* directly targeted p21 to affect the migration and invasion of EC cells (87). Zhang et al. (88) showed that the lncRNA *SNHG14* inhibited EC migration and invasion via the *miR-93-5p*/ZBTB7A axis. In other words, *SNHG14* expression negatively correlated with *miR-93-5p*, and high *SNHG14* expression inhibited EC development (Figure 3).

From existing references, it can be seen that ncRNAs are upstream molecules of CGs, which means that CGs are regulated by ncRNAs. In addition, lncRNAs regulate miRNAs. Notably, this result is usually achieved by lncRNAs acting as molecular sponges. When CRDs occur, the human body activates ncRNAs in a uncertain way, thereby regulating the expression level of CGs. Abnormal levels of CGs expression ultimately lead to the occurrence of EC. From a macro perspective, long-term CRDs can cause EC. It is noteworthy that *NAPS2* may be regulated by *mi-93-5p* and is negatively correlated, while lncRNA *SNHG14* is also negatively correlated with *mi-93-5p*. This implies that *NAPS2* is positively correlated with lncRNA *SNHG14*. Then, according to Zhang et al. (88), lncRNA *SNHG14* is lowly expressed in EC patients, which means that *NPAS2* should also be at low expression levels. However, the study by Zheng et al. (47). demonstrated that elevated levels of *NAPS2* in EC patients. This

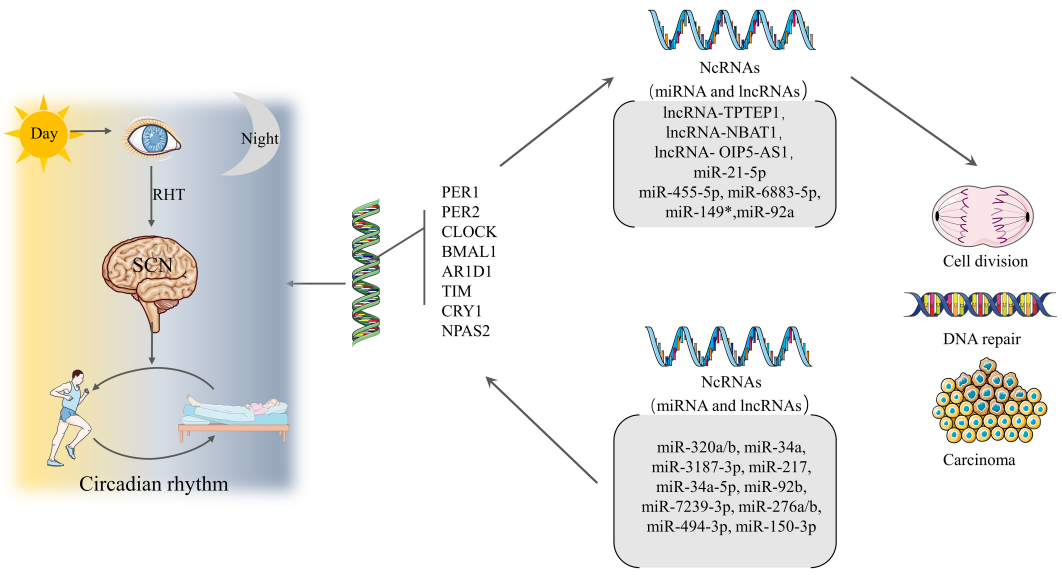

FIGURE 2
Formation of circadian rhythms and associated regulation Light travels through the retina and through the RHT to the SCN, forming the circadian rhythm that guides the mode of operation of daytime activity and nighttime rest. Circadian rhythms are regulated by CGs. A properly functioning CGs can direct ncRNAs to regulate cell division and DNA repair, while dysregulation can lead to cancer. Conversely, ncRNAs can affect the expression of CGs leading to CRDs and a range of diseases.

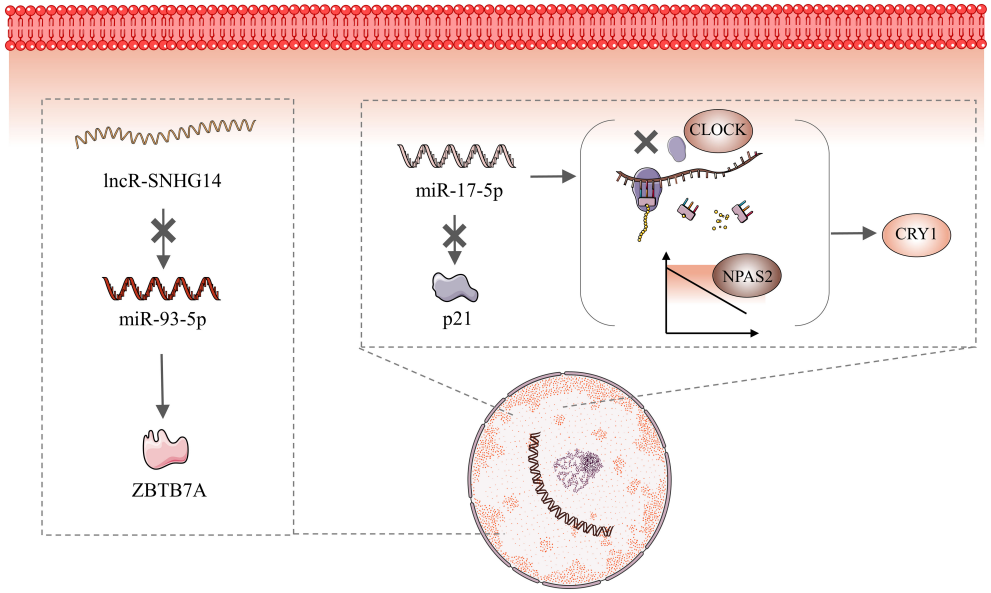

FIGURE 3
Molecular Mechanisms of *MiR-17-5P* and *miR-93-5 PMiR-17-5P* maintains circadian stability via inhibiting *CLOCK* translation, downregulating *NAPS2* levels, and increasing *CRY1* expression. In addition, *miR-17-5p* directly targets p21 to affect the migration and invasion of endometrial cancer cells. Elevated SNHG14 expression inhibits miR-93-5P, which then directly targets ZBTB7A.

conclusion is contradictory to their relationship. So far, we cannot explain this result, and we speculate that it may be the result of the action of multiple molecular pathways.

Interestingly, data reported by Costas et al. (89) did not support the carcinogenic role of CRDs in EC. This result is contrary to the findings of our previously mentioned study by Viswanathan et al. (69): the Nurses' Health Study I found a significant elevated risk (RR=1.47) of endometrial cancer among long-term rotating night workers (>20 years). We observed that the study by Costas et al. (89). included only 180 cases while the study by Viswanathan et al. (69). included 53,487 women. The number of samples may have had an impact on the results of the study, and we believe that the results of a study with a large sample may be more convincing. What's more, night work was defined as a working schedule that involved partly or entirely working between 00:00 and 06:00, while the latter defined night work as working at least three nights per month, in addition to daytime or evening shifts in that month. It can be seen that the target populations of these two studies are fundamentally different. It is possible that this is one of the reasons for the inconsistency of these two results. Although it is unclear why similar studies have reached inconsistent conclusions, the mechanism underlying the occurrence and development of EC needs to be further explored to resolve the conflicting evidence.

7 Conclusion and prospects

Circadian rhythms enable organisms to move regularly and maintain the balance between action and recovery, as disrupting this balance may lead to disease progression. We attempted to synthesize existing information on the role of CGs, miRNAs, and lncRNAs in developing EC to enhance our understanding of their participation in disease biology. This review summarizes the carcinogenic pathways associated with circadian gene ncRNAs. Deepening our understanding of these pathways is crucial for future EC research and can be extended to studies of other tumors. In addition, the carcinogenic pathway of CGs and ncRNAs provides a new direction for exploring new therapeutic targets. We believe that this area should be explored in more detail in the future.

Author contributions

LZ: Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. SC: Supervision, Writing – review & editing. LZ: Writing – review & editing. QH: Supervision, Writing – review & editing. JC: Supervision, Writing – review & editing. WC: Supervision, Writing – review & editing. SL: Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing. QS: Funding acquisition, Methodology, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Elizabeth Cash,
University of Louisville, United States

REVIEWED BY
Patrick Schwartz,
University of Wisconsin-Madison, United States

*CORRESPONDENCE
Diwakar D. Balachandran
DBalachandran@mdanderson.org

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The Impact of Immunotherapy on Sleep and Circadian Rhythms in Patients with Cancer

Diwakar D. Balachandran[1*], Lara Bashoura[1], Ajay Sheshadri[1], Ellen Manzullo[2] and Saadia A. Faiz[1]

[1]Department of Pulmonary Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, [2]Department of Internal Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, United States

Immunotherapy has revolutionized treatments for both early and advanced cancers, and as their role evolves, their impact on sleep and circadian rhythms continues to unfold. The recognition, evaluation, and treatment of sleep and circadian rhythm disturbance leads to improved symptom management, quality of life and treatment outcomes. An intricate complex relationship exists in the microenvironment with immunity, sleep and the tumor, and these may further vary based on the cancer, addition of standard chemotherapy, and pre-existing patient factors. Sleep and circadian rhythms may offer tools to better utilize immunotherapy in the care of cancer patients, leading to better treatment outcome, reduced symptom burden, and increased quality of life.

KEYWORDS

immunotherapy, sleep, circadian rhythm, immune checkpoint inhibitors, immunity

Introduction

Immunotherapy has revolutionized treatments for both early and advanced cancers, and as their role evolves, their impact on sleep and circadian rhythms continues to unfold (1, 2). Sleep disturbance may occur at any time during the spectrum of the cancer care continuum including prior to diagnosis, during treatment and years into survivorship. Sleep disruption during cancer treatment can perpetuate and exacerbate the symptom burden for cancer patients (3–6). The recognition, evaluation, and treatment of sleep and circadian rhythm disturbance leads to improved symptom management, quality of life and treatment outcomes (7). Immune checkpoint inhibitors (ICIs) modulate the immune system to target cancer cells, and the interaction between immunity, sleep and circadian rhythm has been well described. Thus, ICIs likely contribute another dimension to the

Abbreviations: ICI, immune checkpoint inhibitor; irEA, immune mediated adverse event; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand-1; CTLA-4, cytotoxic T lymphocyte antigen-4; BMAL, brain and muscle ARNT–like protein 1; PER2, period 2; REV-Erbα, reverse erythroblastsosis virus alpha; RORα retinoic acid receptor–related orphan receptor alpha; Th1, T helper 1 cell; TNFα, tumor necrosis factor alpha; IL-1, interleukin-1; IFNγ, interferon gamma.

complex interactions between cancer and sleep (3, 4, 7–10). Our objectives are to review the importance of sleep and circadian rhythms in cancer care, to discuss the interplay between sleep, circadian rhythms and immunity and to highlight the interplay of ICIs with sleep, circadian rhythms, immunity and symptom burden (Figure 1).

Immune checkpoint inhibitors

ICI's main function is to disable T-cell regulation to amplify the impact of the T-cell mediated killing of cancer cells. Immune checkpoints are immune cell functions governed by receptor-ligand interactions which control the activation or inhibition of immune responses. Activation of the immune system is a desired outcome to achieve tumor control but can also lead to autoimmunity and toxicity (11). The discovery of monoclonal antibodies against the inhibitor immune checkpoint CTLA–4 (cytotoxic T lymphocyte antigen 4), PD-1 and PD-L11 (programmed cell death 1 and programmed cell death ligand-1, respectively) have resulted in dramatic antitumor responses by the upregulation of immune activation at various stages of the immune cycle (12).

ICI therapy has been transformational in the care of cancer patients, and they have become a pillar for cancer care including neoadjuvant, adjuvant, primary therapy and the treatment of metastatic disease of numerous cancer types (12–14). Now more than a decade after the Federal Drug Administration (FDA) approval of the first ICI, there is nascent recognition of their impact that these medications may have on sleep and circadian rhythms (15, 16). Since the approval of ipilimumab to treat metastatic melanoma in 2011, several ICIs have been approved to treat a growing list of cancers, and many regimens even include dual immunotherapy to maximize impact. The efficacy and safety have been established in a myriad of clinical trials, but immune-mediated adverse events (irAEs) resulting in organ dysfunction resembling autoimmune diseases can occur (17). The most common irAEs include rash, diarrhea and fatigue, but endocrinopathies, myocarditis, hepatitis, pneumonitis, nephritis and nervous system issues may also occur (18). Thus, ICIs, their irAEs and the underlying cancer may all impact sleep.

Fundamentals of sleep and circadian rhythms

Sleep is a fundamental and basic need for life. Sleep is formally studied by polysomnography and is divided into both non-rapid eye movement (NREM) and rapid eye movement (REM) sleep (19). NREM sleep is further divided into 3 stages including: Stage 1, light sleep or drowsiness; Stage 2 which comprises the largest portion of non-REM sleep; Stage 3, deep or slow-wave sleep. The sensitivity of the cortical response to auditory, tactile, and visual stimuli are correspondingly more depressed during stage 3 sleep compared with stage 1 sleep. REM sleep or paradoxical sleep is a metabolically active period of sleep with saccadic rapid eye movements as the hallmark of this important stage of sleep. Both NREM and REM sleep have critical restorative functions and are essential for cognition, learning, and memory (20). Sleep impacts immune function in a fundamental manner affecting both innate and

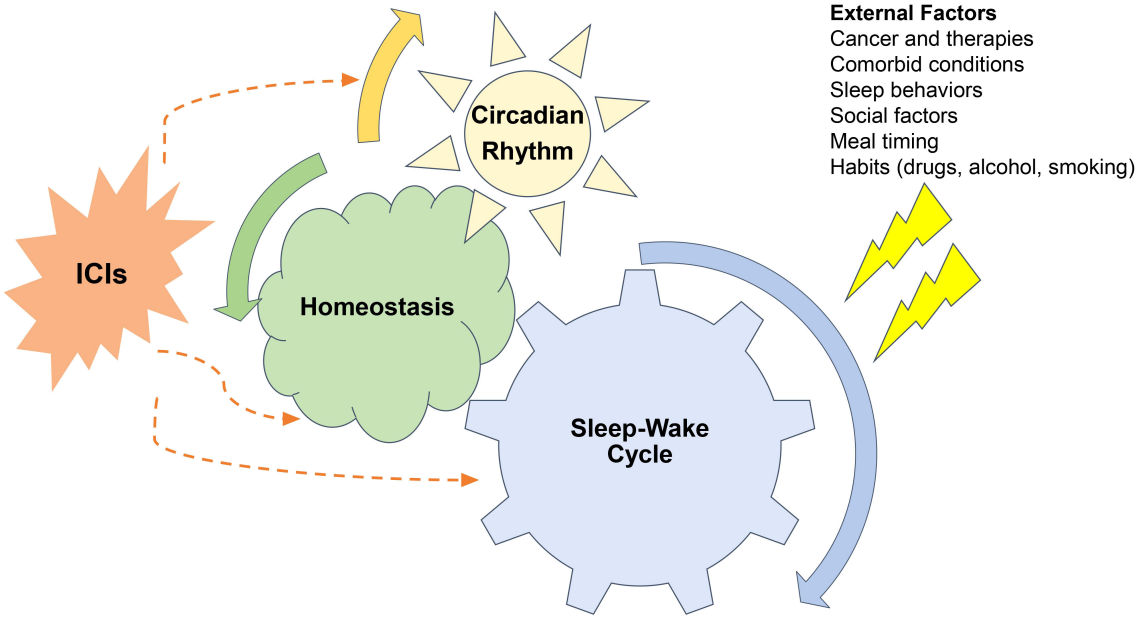

FIGURE 1
Relationships between sleep-wake cycles, circadian rhythms, cancer and immune checkpoint inhibitors (ICI). Sleep wake cycles are controlled by the brains neural networks and are governed by circadian rhythms. External factors related to cancer, comorbid conditions, sleep behaviors, social factors, meal timings, and other habits all can perturb both sleep-wake cycles and circadian rhythms which disturbing homeostasis. These disturbances impact the function of immune checkpoint inhibitors both on their on-target therapeutic and off- target auto-immune effects.

adaptive immunity (21). Immune function is enhanced by both better sleep quality and optimal sleep duration (20). Perturbations of sleep quality and duration fundamentally impact health and disease and have been shown to impact cancer incidence, cancer-related symptoms, and cancer outcomes (10). Identification of these of these symptoms and disorders is enhanced by the utilization of validated subjective and objective tools to assess sleep and circadian rhythms (Table 1).

The control of sleep is determined by a defined set of hypothalamic and brainstem nuclei primarily with pathways to the thalamus and cortex and is best explained by the 2 process models of sleep regulation with both homeostatic and circadian control. Homeostatic control refers to the concept of "sleep debt" which accrues during wakefulness increases the impetus to sleep (29). Circadian, meaning about a day, rhythms are an approximately 24-hour cyclic rhythm which govern both sleep and wakefulness and are a critical input into sleep regulation and helps maintain and consolidate nocturnal sleep as the homeostatic process abates during the sleep period, as sleep debt is paid off (30). Circadian rhythms originate in the suprachiasmatic nucleus in the brain and fundamentally control biology on a behavioral, physiologic, cellular, and molecular level. Circadian rhythms also drive the visible behaviors of sleep and wakefulness (30). Disrupted circadian rhythms have also been shown to impact health and disease, including cancer incidence, cancer symptoms, and cancer outcomes (31). Furthermore, immune regulation and many aspects especially cellular immunity are also under circadian control (32).

TABLE 1 Tools to evaluate sleep and circadian rhythms.

Tool	Significance
Patient-reported outcomes	**Pittsburgh Sleep Quality Index Questionnaire.** A survey that measures sleep quality over the last 1 month. With a maximum score of 24, a score ≥ 5 represents disturbed sleep (22)
	Epworth Sleepiness Scale. An 8-question survey which measures daytime sleepiness. With a maximum score of 24, a score ≥ 10 represents increased daytime sleepiness (23).
	Insomnia Severity Index. A 7-question survey with maximum score of 28 with a score ≥15 indicative of moderate clinical insomnia (24).
	STOP-BANG Questionnaire. An 8-question tool incorporating symptoms (snoring, fatigue), medical history (hypertension), and anthropometric data (age, gender, BMI, neck circumference). With a maximum score of 8, total signifies the following: ▪ a score of <3 low risk for OSA ▪ ≥3 and <5 intermediate risk for OSA ▪ ;≥5 high risk for OSA (25).
Diagnostic testing	Polysomnography: a multi-parameter sleep test recoding EEG, EOG, and EMG, respiratory and cardiac data to evaluate sleep and sleep disorders. Usually, facility based (26).
	Ambulatory sleep testing: multi-parameter sleep testing done at home, primarily to detect sleep apnea (27).
	Actigraphy: wrist-worn accelerometer which measure activity count and can be used to evaluate sleep parameters such as total sleep or rest time, sleep latency, sleep efficiency and circadian rhythms (28).

BMI, body mass index; OSA, obstructive sleep apnea.

These relationships provide a basis on which to better understand the intersection between the circadian clock, cancer and immunotherapy (Figure 2).

Sleep, circadian rhythms, and cancer

Sleep and circadian disruption are considered risk factors for cancer (33). Sleep disruption is at least 3 times as prevalent in cancer population as in the general population (34). Sleep and circadian disruption have also been shown increase with the severity of disease and the degree of disruption has prognostic value (31). Furthermore, sleep disruption clusters with other symptoms of cancer including pain, mood disturbance, and fatigue (9). Improved sleep quality in cancer patients is associated with a better prognosis and treatment response. Innominate and colleagues demonstrated an 8-month increase in overall survival in patients receiving chemotherapy for metastatic colon cancer who were shown to be objectively at rest by actigraphy during their time in bed (35). Sleep disorders such as obstructive sleep apnea has been shown to increase both cancer incidence and mortality (36, 37). Gozal posits that sleep fragmentation is oncogenic and hypothesizes several mechanisms for this relationship through sleep disruption's impact on inflammation and immunity, the autonomic nervous system, the hypothalamic- pituitary axis, oxidative stress and hormonal pathways (33, 38).

The circadian clock is a major regulatory pathway for the cell cycle. Clock genes regulate the cell cycle and therefore modulate gene replication, gene expression, and cellular proliferation (39). In a chronic jet lag model, spontaneous hepatocellular carcinoma occurred in mice following a mechanism very similar to that observed in obese humans (40). Genetic abnormalities in clock genes have been shown to be both oncogenic as well as onco-suppressive (41–49). In studies with circadian clock gene mutant animal models, clock gene BMAL1 deficient knockout mice were shown to have greater progression of hematologic malignant disease and breast cancer cell metastasis (50, 51). Furthermore, core circadian genes Per2 and Bmal1 were shown to have cell-autonomous tumor-suppressive roles in transformation and lung tumor progression (52). Disrupted circadian rhythms in humans have consequences for health and disease and highlight the importance of maintaining robust circadian rhythms of sleep and wakefulness (20). Shift work has been classified as a carcinogen due the higher rate of cancer (53, 54). Circadian rhythm has also been shown play a role in the overall symptom burden of cancer and modify levels of fatigue, depression, and sleep disturbance. Circadian rhythm disturbance has been demonstrated as common regulating factor in the manifestation symptom clusters (35, 55–59). Cancer cells carry mutations which disassociate cells from normal circadian control of the cell cycle compared with heathy tissue, and this difference is used to amplify the therapeutic window in cancer chronotherapy (60, 61). Cancer chronotherapy has been shown to improve on-target effects while minimizing off-target adverse effects (31, 62, 63). Circadian rhythm disruption has prognostic value as cancer patients with attenuated circadian rhythms often also have

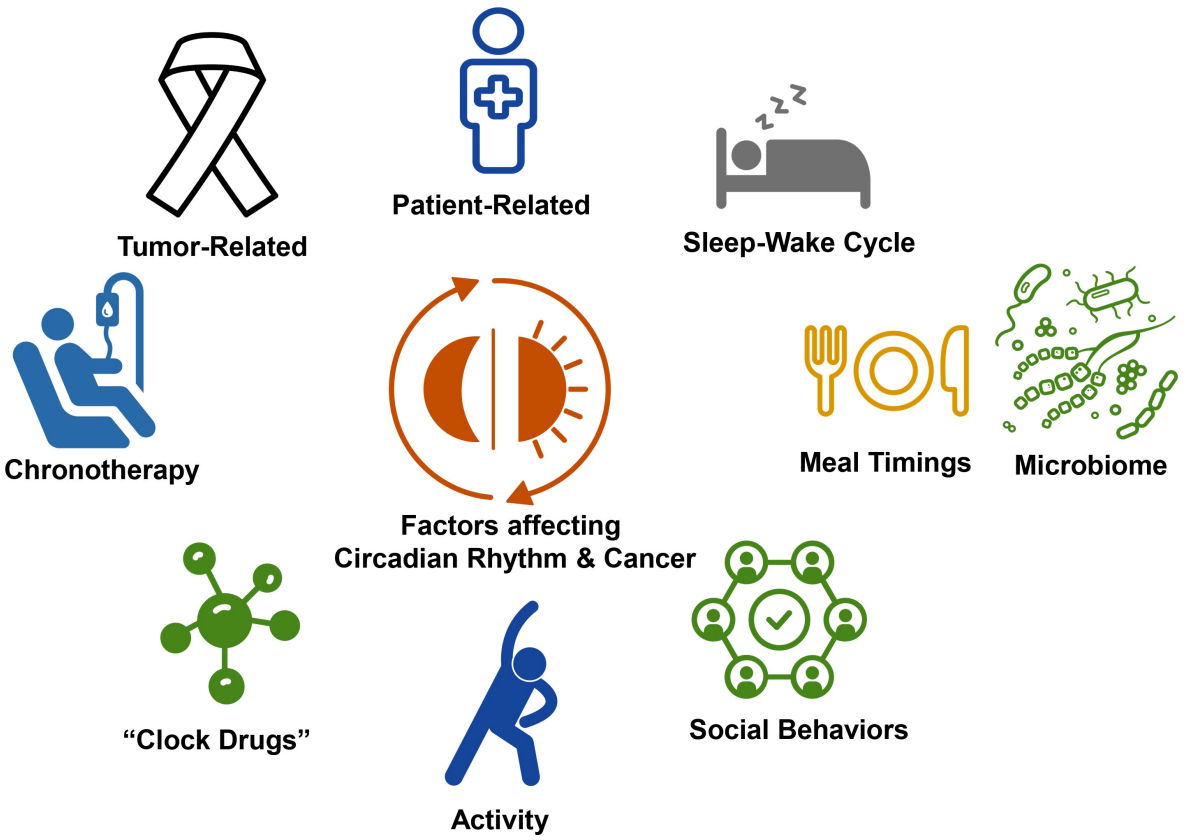

FIGURE 2
Factors impacting circadian rhythms and cancer. The intersection between circadian, rhythms, and cancer is governed by multiple factors, including patient related factors, such as comorbidities and habits and tumor, related factors. Activity, social behaviors, and meal timings entrain circadian rhythms and sleep wake cycles and may be targets for chronoregulation. There is increasing recognition that the microbiome is bi-directionally impacted by circadian rhythms and sleep-wake cycles. Further opportunity for intervention using circadian rhythm related strategies include chronotherapy and the development of clock drugs, the targeting clock gene expression and activity.

higher mortality (64). These factors show that circadian rhythms are important not only for behavioral control of sleep but for the regulation and treatment of cancer (39).

The impact of sleep and circadian rhythms in cancer patients is primarily twofold. First, quality of life is often dramatically impaired due to insomnia, excessive daytime sleepiness, and fatigue. These often debilitating symptoms decrease opportunities for beneficial lifestyle habits such as exercise, social interaction, and healthy diet (65). Second, sleep and circadian rhythms are coupled to improved symptom control, better treatment outcomes, and extended survival in cancer patients. About 30 to 55% of cancer patients have impaired circadian rest-activity rhythms with attenuated rhythms correlating to more advanced and aggressive cancer. Interventions to improve both sleep disorders and circadian rhythm disorders have been shown to ameliorate cancer symptoms and may impact cancer outcomes (66–68).

Circadian rhythms regulate both immunity as well as the cycle of hormones such as cortisol which impacts immune cell function, and through this control of cortisol secretion, the circadian clock function as a gate that controls many aspects of immune function in cancer including cancer cell antigen release and presentation, activation of effector immune cells, trafficking, tumor infiltration and elimination of cancer cells. These regulatory rules in both tumor surveillance and prevention of oncogenesis highlight the circadian clock's relevance to cancer immunotherapy (69).

Sleep, circadian rhythms, and immunity

Sleep and circadian rhythms help regulate the immune system impacting health and disease and in the context of cancer, play a role in tumor surveillance and regulation. Sleep and immunity are now thought to bi-directionally linked (20, 70). The impact of sleep and circadian rhythm on inflammatory response, leukocytes and hormones further exemplifies their potential influence with immunotherapy.

Numerous studies have shown that stimulation of the immune system by microbial challenges triggers an inflammatory response promoting sleepiness and in turn that sleep modifies the inflammatory response. Infections can increase NREM sleep through the production of cytokines such as IL-1 and TNFα (21, 71, 72). Just as sleep impacts cognitive learning and memory, it is thought that sleep also impacts the immune system's ability to

recognize learn and recall infectious stimuli modulating the adaptive immune response (20). Sleep enhancement during infection can promote host defense and increase the response to vaccination (73–76). Sleep also affects immunological memory as summarized in vaccination studies. For example, with influenza virus specific antibody titers measured 10 days after vaccination more than doubled in participants who kept their usual bedtime of 7.5 to 8 hours compared to those restricted to 4 hours of sleep. This study also showed that sleep enhances the production of Th1 effector cytokine interferonγ (77). Circadian rhythms regulate the production and function of inflammatory cytokines. For example, TNFα secretion varies in circadian fashion based on the time of an endotoxin challenge (78). Circadian rhythm and clock genes impact gene expression of key cytokines. Disruption of clock gene expression seems to be a common outcome of acute infection and it is suggested by some that the circadian clock itself is an innate immune system sensor that is disabled by infection (79).

Sleep along with circadian factors exerts a significant influence on circulating leukocyte number in the bloodstream. Studies have reported that sleep reduces the numbers of various leukocyte subsets of blood. This is thought to be due to redistribution of cells from the circulation into tissues rather than an effect on proliferation (76). Studies using sleep deprivation show an accumulation of lymphocytes in both tissue and blood. Chronic sleep deprivation which leads to the development of habitual short sleep times may cause the development of low-grade inflammation. This chronic inflammatory state is associated with an increased risk of several diseases including cancer and a decreased ability to mount an adaptive immune response (20). Clock genes and immune processes also regulate the differential maturation of leukocyte subsets as clock genes are required for the differentiation of type II lymphoid cells. Leukocyte trafficking which represents the movement of cells from the bone marrow to the bloodstream and into target organs is also under circadian control. In Bmal1 knockout mice macrophages are unable to sustain mitochondrial function, enhancing succinate dehydrogenase (SDH)-mediated mitochondrial production of reactive oxygen species as well as Hif-1α-dependent metabolic reprogramming and inflammatory damage precipitating an inflammatory and tumor- promoting cellular milieu (80). Clock genes also control neutrophil maturation (81). Furthermore specific clock genes Rev-Erbα and RORα deficient mice have negative effects on the development of and activation of dendritic cell and other antigen presenting cells (APC) critical to pathways involved with immune mediated cancer cell targeting (82). Therefore, it is increasingly clear that circadian gating is part of the core program of the immune system, and thus alteration of this regimen is likely to have widespread ramifications for disease pathogenesis (32). Furthermore, it is in slow wave sleep where high levels of growth hormone, prolactin and aldosterone and nadir levels of cortisol are present. These hormonal factors also may alter T-cell interactions. Low levels of cortisol during slow-wave sleep may allow efficient antigen-presenting cell–T-cell interactions which are important for immunomodulation and targeting cancer cells which contributes to the efficacy of cancer immune therapy (83).

Sleep, circadian rhythms, and cancer immunotherapy

Sleep and immunotherapy

There is a paucity of data on the relationship between sleep disturbance and immunotherapy. In an early study in lung cancer patients undergoing treatment with ICIs, Zarogoulis and associates recorded sleep disturbance using phone questionnaires and polysomnography. Interestingly for immunotherapy patients with a PD-L1 expression greater than 50%, disease response was rapid and associated sleep disturbances decreased rapidly. In contrast, in patients on standard chemotherapy, those with both partial response and stable disease continued to have sleep disturbances. They concluded that although ICIs did not induce sleep disturbance, treatment response may improve sleep disturbances (84). Another study by the same group showed that upon diagnosis, lung cancer patients had sleep disturbances including early morning awakenings, late sleep onset, prolonged nocturnal waking periods, daytime sleepiness, and unrefreshed sleep. These symptoms improved quickly in patients with a PDL-1 expression greater than 80% during the first 4 months of treatment due to the rapid response of the tumor to immunotherapy. There was no difference in symptom control seen between patients who received nivolumab or pembrolizumab (85).

Recent studies have demonstrated that ICIs can impact symptoms which cluster with sleep disruption such as cancer-related fatigue (CRF). These symptoms may present coincidentally as cancer-related symptoms clusters sharing common inflammatory, hormonal, autonomic nervous system, and hypothalamic-pituitary axis abnormalities. Hajjar and colleagues found that in 88 patients with advanced metastatic cancers undergoing immunotherapy, fatigue was identified in 66%. High level fatigue was found in 34%. This study is among the first to describe the microbiome in patients on immunotherapy which also has an important immune mediated impact, potentially affecting ICI effectiveness and irAEs. The study was able to show that there was a correlation of *Eubacterium hallii* that was negatively associated with fatigue severity scores whereas those patients with *Cosenzaea* sp. had higher fatigue scores (86). In a meta-analysis in subjects on ICI therapy, CTLA-4 inhibitors are associated with a higher risk of all and high-grade fatigue compared with control regimens, whereas PD-1 inhibitors are associated with a lower risk of all- and high-grade fatigue compared with control regimens. Although ICIs have revolutionized the treatment of certain cancers, many patients do not respond to ICIs and treatment outcomes vary disproportionately between cancer types (87). Therefore there is a significant interest how lifestyles, diet, and psychosocial factors including sleep quality determine the outcomes and ICI management (88). The impact of ICI therapy on sleep and related symptoms and the association with microbiome composition present opportunities to potentially augment the efficacy and tolerance of immunotherapy.

Circadian rhythm and immunotherapy

The human immune system is equipped to keep unnatural cell growth in check and aide in cancer suppression (69). Given the circadian clock's influence on immune recognition and elimination of cancer cells, it is unsurprising that there is evolving evidence for the connection between circadian rhythms and cancer immunotherapy. In a metastatic melanoma murine model, BMAL1 is responsible for T-cell activation and the expression of CTLA-4, PD-1 and PD-L1 (89). Enrichment of clock gene pathways in animal studies increase PD-L1 expression and enhance T cell receptor signaling (90). In normal lung tissue, clock genes Per1 and Cry2 have been linked to the expression of CD4+ T cells and PD-1 expression follows a circadian rhythm (91). In clock gene RORγ knockout mice, the presence of PD-1 Type 17 cells and levels of PD-1 is decreased (92).

The circadian clock also affects the functional response of CD8 T-cells to antigen presentation by dendritic cells, the core aspect of the immune response against pathogens and cancer cells. This early T-cell receptor response was shown to impact T-cell receptor signaling such as activation, proliferation factor functions. The influence of circadian rhythms on the functional aspect of T-cell development, response to antigens, and trafficking is well recognized (69). Although there is no direct evidence that eloquently describes interplay between circadian rhythms and immunotherapy response, it is an exciting area of study and has the potential to transform immunotherapy (88).

Circadian rhythms are being considered for the prevention and management diseases including cancer (60). There are 3 broad approaches using our knowledge about circadian rhythms to impact cancer outcomes. First, lifestyle management, including sleep hygiene, diet composition and timing, and exercise timing, may be used to entrain a robust circadian rhythm. Consistent daily behavior patterns and sleep and eating may significantly reduce the risk of cancer (88). Studies in mice, utilizing rhythmic food intake have shown that these signals driven contribute to driving rhythms in liver gene expression and metabolic functions outweighing the influence of even the cell-autonomous hepatic clock (93). In addition, microbiome modification by lifestyle changes is an area of active study in patients on immunotherapy, as recently reviewed by Wargo and associates (94). Secondly, chronotherapy, or the timing of medication administration, can be used to target tumors while preventing adverse off-target effects. Nelson and Levi have shown that the time of day of infusion of both PD-1 and PD-L1 inhibitors showed clinically significant association with increased survival in patients which a variety of cancer types (95, 96). Finally, recent advancements on how to enhance our circadian clock through pharmacological targeting of circadian clock components that are already providing new preventive and therapeutic strategies for several diseases, including metabolic syndrome and cancer (97). A new class of drugs targeting circadian clock genes ("clock drugs") act through targeting components of the clock circadian clock and have shown early promise in modulating immunotherapy (98). Clock drugs directly target the circadian clock components including, RORγ, REV–ERB's (99). Furthermore, specific clock genes REV-ERBα and RORα deficient mice have negative effects on the development of and activation of dendritic cell and other antigen presenting cells critical to pathways involved with immune-mediated cancer cell targeting (82). In a series of experiments done by Hu and colleagues, RORγ agonists can act as monotherapy *in vivo* and display anti-tumor properties, including boosting the activity of TH17 cells. When treated with RORγ agonists, T-cells are more resistant to PD-L1 inhibition which is critical in suppressing anti-tumor activity. Supplementation of a RORγ agonist during ex-vivo expansion during chimeric antigen receptor (CAR)-T cell engineering, increased the antitumor activity of TH17 cells. The function of the CAR Type 17 cells is elevated when re-exposed to tumor with increase cytokine production including IL-17A and IFNγ. Moreover, mice with RORγt-primed T cells are protected after cancer cell inoculation (92, 100).

Circadian rhythms may also indicate treatment prognosis in immunotherapy with a recent study finding that in patients undergoing immunotherapy for lung adenocarcinoma, a circadian rhythm gene related signal could serve as an independent indicator for prognosis. This circadian rhythm genetic marker was found to be upregulated in cancer samples (101). These data taken together, suggest that circadian rhythms are likely to be harnessed in the future to augment the impact of immunotherapy in the treatment of cancer.

Future directions

As the role of ICIs expand, the impact of sleep, circadian rhythms, immunity, and immunotherapy requires further study. There are several unanswered and significant questions.

- Are sleep and circadian rhythms a biomarker for prognosis in patients on immunotherapy as they have been shown in other therapies?
- What are the interactions between sleep and circadian and other modifiable lifestyle factors such as diet?
- Do these factors impact the microbiome and thereby affect treatment related outcomes with immunotherapy?
- How will chronotherapy best be utilized to boost on target effects of immunotherapy?
- Can we exploit the interconnection between the circadian clock genes and the immune system to drive more effective and safer immunotherapy approaches, minimizing auto-immunity?

It is the answers to these and other intriguing questions that lie the heart reaping of the promise of immunotherapy, that further exploration of these complex relationship hold.

Conclusion

Immunotherapy has transformed cancer treatment, and given its impact on the immune system, the role of ICI as it relates to sleep and circadian rhythm continue to unfold. Clearly an intricate complex relationship exists in the microenvironment with immunity, sleep and the tumor, and these may further vary based on the cancer, addition of standard chemotherapy, pre-existing patient factors and irAEs. Sleep and circadian rhythms may offer tools to better utilize immunotherapy in the care of cancer patients, leading to better treatment outcome, reduced symptom burden, and increased quality of life.

Author contributions

DB: Conceptualization, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing. LB: Conceptualization, Writing – review and editing. AS: Conceptualization, Writing – review and editing. EM: Conceptualization, Writing – review and editing. SF: Conceptualization, Visualization, Writing – review and editing.

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Conflict of interest

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Laura Senovilla,
Spanish National Research Council (CSIC), Spain

REVIEWED BY
Marina Maria Bellet,
University of Perugia, Italy

*CORRESPONDENCE
Pasquale F. Innominato
pasquale.innominato@wales.nhs.uk

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Circadian lifestyle determinants of immune checkpoint inhibitor efficacy

Bethan R. Hughes[1,2], Sadiq Shanaz[1], Seline Ismail-Sutton[1], Nicholas I. Wreglesworth[1,2], Christian P. Subbe[2,3] and Pasquale F. Innominato[1,4,5]*

[1]Oncology Department, Ysbyty Gwynedd, Betsi Cadwaladr University Health Board, Bangor, United Kingdom, [2]School of Medical Sciences, Bangor University, Bangor, United Kingdom, [3]Department of Acute Medicine, Ysbyty Gwynedd, Bangor, United Kingdom, [4]Cancer Chronotherapy Team, Warwick Medical School, University of Warwick, Coventry, United Kingdom, [5]Research Unit 'Chronotherapy, Cancers and Transplantation', Faculty of Medicine, Paris-Saclay University, Villejuif, France

Immune Checkpoint Inhibitors (ICI) have revolutionised cancer care in recent years. Despite a global improvement in the efficacy and tolerability of systemic anticancer treatments, a sizeable proportion of patients still do not benefit maximally from ICI. Extensive research has been undertaken to reveal the immune- and cancer-related mechanisms underlying resistance and response to ICI, yet more limited investigations have explored potentially modifiable lifestyle host factors and their impact on ICI efficacy and tolerability. Moreover, multiple trials have reported a marked and coherent effect of time-of-day ICI administration and patients' outcomes. The biological circadian clock indeed temporally controls multiple aspects of the immune system, both directly and through mediation of timing of lifestyle actions, including food intake, physical exercise, exposure to bright light and sleep. These factors potentially modulate the immune response also through the microbiome, emerging as an important mediator of a patient's immune system. Thus, this review will look at critically amalgamating the existing clinical and experimental evidence to postulate how modifiable lifestyle factors could be used to improve the outcomes of cancer patients on immunotherapy through appropriate and individualised entrainment of the circadian timing system and temporal orchestration of the immune system functions.

KEYWORDS

cancer, immunotherapy, circadian, diet, exercise, light, lifestyle

Introduction

Discoveries in immunotherapy have revolutionised cancer treatment. In 2018, James Allison and Tasuku Honjo won the Nobel Prize in Medicine for their work investigating the proteins CTLA-4 and PD-1 (1). Found on T cells, these proteins acted as checkpoint

molecules, moderating T cell activation and preventing over-activation. As T cells are also involved in immunosurveillance of cancer cells (2), tumours can exploit CTLA-4 and PD-1 expression to evade host immune response. Inhibiting these checkpoint molecules can therefore enhance the antitumour immune response (3).

The antibodies subsequently developed to target checkpoint molecules and block their function are referred to as immune checkpoint inhibitors (ICI) and are the most widely used form of immunotherapy in cancer clinics. Currently, ICI are licensed to treat a wide array of cancers, including melanoma, lung, head and neck, renal, mesothelioma, breast, oesophageal, gastric, colorectal, biliary tract and urothelial carcinomas (4).

However, primary or acquired resistance remains a problem even with ICI (5). In addition, over activation of T cells can endanger self-tolerance, with the unavoidable risk of developing potentially life-threatening autoimmune adverse effects even years following initial treatment (6, 7).

In patients unlikely to respond to ICI therapy, accurate prediction of efficacy and tolerability would allow clinicians to minimise adverse effects and delays to these inherently time-pressured treatment plans. For many reasons, including difficulties developing appropriate *in-vitro* assays, the determinants of ICI efficacy, tolerability, and deleterious interactions are not fully understood, but are generally appreciated to be multifactorial and likely involve both modifiable and non-modifiable factors.

Many biomarkers both from the original tumour and circulating cells have emerged as areas of research interest into the impact of ICI efficacy and/or tolerability (8, 9). Alongside these factors, recent reports have highlighted the relevance of the circadian timing system (10). In turn, the CTS function is influenced by a host of lifestyle factors (11, 12). Lifestyle factors are of particular interest to clinicians, as they allow outcomes to not only be predicted, but to be potentially manipulated as well. This aspect of non-pharmacological interventions in oncology is indeed rising growing interest recently (13, 14). In this review, we critically summarise existing evidence on key lifestyle factors of interest – diet, physical exercise, and bright light exposure – with regards to ICI efficacy, through CTS manipulation, and impact on the immune system and the microbiome. We then discuss how these factors all interact to form a complex web which, with further understanding, may be manipulated by the empowered patient in conjunction with clinicians and various specialised healthcare professionals to optimise response to cancer immunotherapy (15, 16).

The circadian timing system

The human body has an inherent timekeeping ability. Its internal 'clock' is thought to have evolved thanks to the survival advantage conferred by the ability to predict bodily requirements and adapt accordingly (17, 18). Circadian (i.e., with a period of about 24 hours) rhythms reflect the nature of the world humans have evolved in – being that environmental properties change with time in a predictable pattern based on the Earth's rotation. In humans, the CTS hierarchically involves a central pacemaker, the suprachiasmatic nucleus in the ventral hypothalamus, and peripheral oscillators, temporally coordinated by hormonal, neural and physiological cues (19). Timing within cells themselves involve transcription-translation feedback loops and post-translation modifications involving a set of core clock genes, which encode proteins with limited half-lives (17). The rhythmic oscillations in core clock genes coordinate, in a tissue specific function and directly and indirectly, circadian transcription of selected genes, which ultimately engender variation in cellular functions over the 24-hour period, including cancer- and immunity-related hallmarks (20–22).

Although the CTS does not require external input, it can be entrained using external stimuli, such as light exposure. Other stimuli which can entrain the CTS include feeding times, exercise, and social schedules (11, 12, 23–26). Consequentially, manipulation of exposure to rhythmic entraining cues can be used to enhance or shift the CTS function (27), with potential benefit for patients' wellbeing (28).

Timekeeping behaviour is also important to the immune system. Intrinsic clocks have been demonstrated to be present in a number of innate immune cells, causing rhythmic gating of function as well as regulating temporal spatial abundance (29). Natural killer cell cytolytic activity was found to be suppressed in correlation with altered clock gene expression in rats experiencing a simulation of chronic shift-lag, which was also associated with increased lung tumour growth (30). Additionally, experimental disruption of host circadian rhythms has shown to create an immunosuppressive remodelling in the tumour microenvironment, promoting cancer-cell proliferation and metastatic spread (31, 32). Evidence of circadian rhythmicity has also been found in the adaptive immune system in regulating $CD8^+$ T-cell and dendritic cell differentiation and trafficking, with implications in cancer immunotherapy (29, 33). Studies in night shift work in humans corroborate experimental evidence on a negative effect of circadian disruption on immune system physiology (34).

The circadian rhythmicity of the innate and adaptive immune system ensures proportionate responses to infections, whereas dysregulation presents acutely in an inflammatory cytokine syndrome or manifests long-term as chronic inflammatory conditions, with relevant therapeutic implications in oncology as well as in many other medical conditions and procedures (35, 36).

The taxonomic composition of the microbial ecosystem, principally but not solely in the gut, has been associated with the incidence and clinical course of many different diseases, as well as with response to specific treatments (37). The mechanisms involved, which can display circadian oscillations (38, 39), include direct vagal stimulation, inflammation processes, and production of cytokines and metabolites (38, 40). Of paramount interest here is the growing evidence of the impact of the gut microbiota on ICI efficacy (41). Alongside this, the CTS and microbiome have been demonstrated to have intertwined relationships illustrated best by research showing how in combination they can synchronize bi-directionally the body's metabolic response to diet (42) as well as light (43), exercise (44) and socialisation (45). Thus, the gut

microbiome, itself potentially modifiable through iatrogenic interventions (46) takes a pivotal role in the rhythmic interplay among malignant processes, metabolism and immunity (13, 38, 47–49). Indeed, as a developing theme, the gut microbiome has been demonstrated as having effects on innate immunity, adoptive immunity and intriguingly direct within the tumour microenvironment (50).

The link between the immune system and the CTS has been used to investigate potential ways of optimising response to various cancer treatments, including ICI. In particular, the time of day of ICI administration has been shown to be an independent prognostic factor for overall survival in several cancer types, with consistent findings disfavouring late afternoon administration (51). As the CTS can be entrained through modifications to lifestyle determinants, it therefore stands to reason that via the CTS certain lifestyle modifications could ultimately positively impact overall survival in cancer patients receiving ICI (Figure 1).

Light exposure

Photic signals from the retina to the suprachiastmatic nucleus (SCN) encodes time of day information regarding the environmental surroundings (52). Photic signalling integrated in the SCN modify cellular and molecular activity of astrocytes, neurones and synchronises peripheral clock activity of organs (52, 53). The SCN interacts with peripheral clocks via inputs from the endocrine and adrenergic nervous system (54) resulting in activation of immune cells (55).

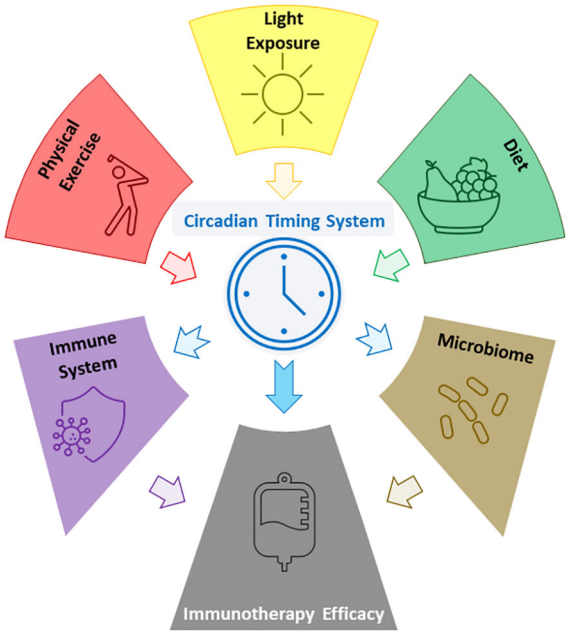

FIGURE 1
Figure showing some of the reported relations between lifestyle factors, the CTS, the microbiome, and ICI efficacy.

However, photic signalling information can be altered in cancer patients because of insufficient physiological bright light exposure during the day and experience of artificial light at night (ALAN). For example, ALAN affects both innate and adaptive immune function in invertebrates, birds, and rodents with robust pineal melatonin rhythm (43). Abnormal photic exposure not consistently encoding time-of-day information can affect innate and adaptive immune activity (44, 45, 56, 57), potentially impacting the efficacy of ICI. Although the impact of circadian photic schedule on ICI remains putative to date, in other conditions such as in psychiatry, light-based chronotherapy has shown positive therapeutic interactions with pharmacological and other behavioural interventions (58–60). Although prone to intrinsic constraints in the anatomical region of measured light exposure, contemporary digital tools allow for circadian evaluation of light exposure schedule and intensity, with potential cancer chronoimmunotherapeutic overtones (61, 62).

It is shown trafficking of immune cells are affected in a time of day dependent fashion (63, 64) Photic exposure entrains immune cell trafficking via the adrenergic nervous system (63, 64). No direct evidence exists to our knowledge on the impact of photic scheduling and entraining effects on outcomes of ICI, chemotherapy or other targeted therapies used in cancer. There are experimental and clinical data to support the impact of daytime bright light exposure and avoidance of artificial light at night, on both the immune and microbiome activity (43, 65–68). For instance, in the absence of light, the sympathetic nervous system triggers the pineal gland to produce melatonin, which synchronises SCN activity to peripheral clocks of immune cells (57, 69). Melatonin regulates innate, cellular and humoral responses of the immune system through modulating production of cytokines and oxidative stress (57). Additionally, melatonin acts as an immunostimulant under basal or immunosuppressed conditions, providing more effective early immune response against external stressors, such as viruses or parasites (70). However, in transient or chronically exacerbated immune response states, melatonin exerts negative regulation and can be regarded as an anti-inflammatory molecule (71).

The activity of the immune system is also influenced by Vitamin D, which is produced because of sunlight's effect on keratinocytes (72, 73). Furthermore, Vitamin D modulates the gut microbiome and its metabolic activity, which is shown to be influenced by ALAN (69, 74). Thus, Vitamin D deficiency is linked with inflammatory bowel disease (75), obesity (76), diabetes (77), pro-inflammatory cytokine production (78), intestinal barrier disturbance (79), gut dysbiosis (80) and immune-mediated disease (81).

Physical exercise

Physical exercise has been shown to strongly entrain the human circadian timing system, particularly through its effects on skeletal muscle and the cardiovascular system (82, 83). Furthermore, peripheral clocks within cardiovascular cells are key for modulating endothelial function, vasodilation, resistance, blood pressure, heart rate and several other key functions (84). Aerobic exercise induces neuroendocrine changes including increased production and release of melatonin and lower cortisol levels at

night. This allows resynchronisation of the circadian clock, resulting in better sleep quality, and lower blood pressure and heart rate (82).

Entrainment of the circadian rhythm via exercise occurs even through low-intensity exercise and may be partly driven by changes in body temperature during physical activity (85). The degree to which physical exercise increases the body temperature is also dependent on the circadian phase, being larger in the rest phase than in the active phase (84). Moreover, combining photic cues, and non-photic exercise cues, has been shown to result in entrainment of the human clock at a faster rate than those with limited exercise (82).

Altogether, appropriate physical exercise in terms of timing, intensity, duration and type, adapted to the individual constraints of cancer patients, can be exploited to entrain the CTS and increase the robustness of circadian rhythms. Mobile health devices can lend useful tools to implement tailored circadian-based exercise schedules, even in cancer patients on ICI (16, 86, 87).

Although no direct evidence on the impact of physical exercise on outcomes on ICI is available to date, its impact on both the immune system and the microbiome supports its individualised manipulation to try to increase circadian-based ICI efficacy. Indeed, physical exercise has several immunomodulatory effects, including immune cell mobilisation in the blood, particularly PD-1+ CD8+ T cells redirected to peripheral tissues, which are crucial for host defence against tumours (88, 89).

An additional study analysing mice with pancreatic cancer demonstrated an improved responsiveness to immunotherapy in mice that exercised regularly, compared to those that did not. Mice who had regular exercise also had a greater antitumor response and an increased volume and influx to tumours of NK and CD8+ T cells (90).

Moreover, regular exercise influences the gut/brain axis, leading to an anti-inflammatory, immunoregulatory state and enriched gut microflora diversity (91). Indeed, multiple factors have been associated with intestinal dysbiosis in cancer patients (92), and physical exercise, alongside diet, could be a potentially modifiable element to ameliorate the gut microbiome in order to maximise benefit from ICI.

Diet

Our eating habits generally follow a broad pattern that repeats every 24 hours. This pattern will vary from person to person and culture to culture, however commonly it may include three meals of various composition at a similar time each day. Interactions between the CTS and diet can therefore be divided into those relating to meal timing and those related to meal composition. Evidence suggests both these factors interact with the CTS (25, 93).

Food is one of the main synchronisers of the peripheral clocks (94). Both meal timing and meal composition can disrupt and re-programme the CTS by altering clock gene expression, causing reorganisation of liver metabolic pathways and altered pancreatic insulin secretion (95, 96). Thus, with regards to circadian entrainment, both timing, including fasting duration, and composition of the meal are relevant and could be potentially manipulated for therapeutic purposes. Modern digital tools can provide monitoring capability of feeding and fasting habits over the 24-h period and a way to behavioural dietary interventions (16, 86, 97–99).

Furthermore, diet can influence markers of immune function, with an association between diet and incidence of several immune-mediated diseases including allergy, diabetes, and cancer reported (100). Moreover, fasting can influence immune responses in tumour-laden mice, with twice-monthly fasting resulting in higher white blood cell count and reduction in neoplasms despite no change in calorie intake (101). The influence of circadian dietary pattern on the immune system has also been explored, with studies showing associations between circadian feeding cycle, fasting period and alterations in both adaptive and innate immune response, with potential therapeutic implications (56, 102, 103).

Modifying diet also affects the gut microbiome, with different diets associated with noticeably different abundances and diversity of gut microbiota (104–106).

Interactions between outcomes on ICI and diet are thought to often occur via the microbiome, with studies reporting correlations between diversity and relative abundance of specific species, such as *Akkermansia* and *Ruminococcaceae* (107–109).

Contrarily to light exposure and physical exercise, there is clinical evidence on the impact of diet type on ICI efficacy. Although there is some discordance between studies, and heterogeneity with regards to cut-offs, adherence and duration of particular diets, disease types and clinical outcomes, there is an overall trend towards better outcomes associated with what is regarded as an healthy diet in humans in general by the WHO (110). For instance, high amount of fruit and vegetable, and low amount of dairy portions, were significantly associated with clinical benefit from ICI therapy (111). Specifically, increased fibre intake (threshold of 20g per day or more), higher adherence to a Mediterranean diet (rich in whole grains, fish, nuts, fruit, and vegetables, and low in red and processed meat), and a periodic fasting-mimicking diet (consisting of a nutritional composition that mimics fasting) displayed beneficial impact in patients receiving ICI in various studies (112–114).

Moreover, normal (> 30 ng/dL) vitamin D3 levels, whether naturally-occurring or through oral supplementation, were associated with significantly better outcomes (115). Furthermore, experimental evidence suggests an impact of ketogenic (low carbohydrate, low protein, and high fat) diet, of dietary amino-acid restriction and of polyphenols administration on ICI efficacy (116–118).

Interestingly, defecation frequency was also relevant, with emptying bowels less than daily associated with poor response to ICI (111).

Discussion

Immune checkpoint inhibitors have provided enormous benefit in the management of an ever-expanding array of cancer types, with dramatic increases in overall survival in those who respond. Their

current main weaknesses lie in a variable response rate and risk of toxicity. Recent studies have consistently reported increased efficacy of ICI therapy when infusions were administered in the morning, and that timing of immunotherapy is an independent prognostic factor for overall survival (10, 51, 119–126). This suggests a link between ICI efficacy and the CTS, which is responsible for circadian variations in many physiological features. In reporting a correlation between time of administration and efficacy, the findings suggest the CTS could be harnessed by clinicians to improve ICI efficacy. In order to do this, it is important a patient's CTS is entrained, as any benefit could be impaired by CTS disruption. Indeed, circadian disruption (evaluated with continuous wrist-actigraphy or with diurnal salivary cortisol slope) has been associated with shorter overall survival in various cancers, but not yet in those treated with ICI (127, 128).

Conveniently, the CTS can be entrained by numerous lifestyle factors which have also been shown in studies to have independent effects on the immune system, the microbiome and sometimes on ICI efficacy, as shown above and as brilliantly discussed by others very recently (13). Both ICI therapy and circadian systems are complex, and further research will be needed to better understand the science of their interactions in order to harness this insight for therapeutic purposes.

Although extensive research is aiming at identifying tumour-associated or host-related factors predicting for ICI efficacy or tolerability, most of them are immutable and intrinsic to the patient and the disease, thus potentially impossible to be manipulated (e.g., PD-L1 expression levels) or very hard to be meaningfully modified in a relatively short timeframe (e.g., body mass index) (129). Similarly, the use of some drugs (e.g., antibiotics, proton-pump inhibitors and obviously steroids) has been shown to impair ICI efficacy in retrospective studies (130). Yet, most likely these drugs have been prescribed for a therapeutic reason and arguably it would not be easy to avoid them altogether in clinical practice.

Conversely, lifestyle interventions, including light exposure, physical exercise and diet, could be allegedly manipulated more easily to obtain the maximal therapeutic benefit from ICI (Table 1). Thus, a circadian-based optimisation of entraining cues and timing of administration could safely improve the outcomes of cancer patients treated with ICI.

However, this would require dedicated observational and interventional studies, with a robust translational component, in order to precisely and dynamically personalise lifestyle modifications. Indeed, the intertwining between these factors are multiple and complex, and involve hormonal messaging (e.g., melatonin, Vitamin D), unavoidable interactions (e.g., between outdoors physical activity and exposure to bright sunlight), and indirect microbiome-mediated mechanisms.

Further, they are all intrinsically bound to occur at a certain time of the day, thus impacting on the CTS and its temporal control of the immune system and of pharmacological determinants (136, 137).

Thus, although with this brief overview we have critically discussed photic stimuli, physical exercise and dietary factors, encompassing clinical and experimental findings, we believe that additional research will be of great interest and should be warranted in furthering our understanding of the effects of lifestyle factors on ICI efficacy as a whole, through modulation of the CTS, and the temporal organisation of the immune system and the microbiota.

However, difficulties with this approach should be acknowledged, including the intrinsic heterogeneity in populations, studies and

TABLE 1 Table of potential lifestyle interventions, their effects, and clinical considerations for studies/deployment.

Intervention	Effects	Aspects to be considered/ optimised
Physical Exercise	* Entrainment of the CTS, increased production of melatonin and lower cortisol release at night (82) * Anti-inflammatory and immunoregulatory effects (91) * Immune cell mobilisation to peripheral bloodstream, including PD-1 CD8+ T cells, which are vital for host tumour defence (88, 89) * Enrichment of gut microbiome (91)	* Regularity and frequency of exercise * Time of day exercise is conducted * Intensity and type of exercise * Duration of exercise * Circadian phase whilst exercising and change in body temperature
Light Exposure	* Causes suprachiasmatic nucleus neurons (master clock) to alter clock gene expression (53, 131, 132) * Clock genes expressed synchronise peripheral clocks to the daily light dark cycle (120, 133) * Affects both innate and adaptive immunity (43, 45, 57, 134) * Alters gut microbiome and its metabolic activity (69, 135)	* Timings of bright (outdoors) light exposure * Duration of bright (outdoors) light exposure * Intensity of artificial bright light exposure * Timing of avoidance of artificial light at night exposure * Feasible and realistic intensity (and spectrum) of acceptable artificial light at night
Diet	* Entrainment of the CTS (94) * High levels of fruit and veg consumption associated with improved ICI efficacy (111) * Low dairy consumption also associated with improved ICI efficacy (111) * Increased fibre intake associated with improved progression-free survival (112) * Normal vitamin D levels (with or without supplementation) associated with improved response rate (115) * Increased adherence to Mediterranean diet associated with increased chance of response to ICI (114) * Fasting-mimicking diet associated with increased ICI efficacy (113) * Opening bowels daily associated with improved ICI efficacy (111)	* Fibre intake * Vitamin D levels * Mediterranean diet adherence * Fruit and vegetable consumption * Dairy consumption * Frequency of defecation * Spacing of meals throughout day * Duration of fasting period * Consistency of meal timings

outcomes, and, for instance, microbiome composition across cohorts (138), as well as the tolerance to interventions to factor into a patient's cancer treatment plan.

This tolerance is indeed equally relevant when using lifestyle modifications as treatments. Consideration should be given as to the likeliness of patients with cancer and undergoing cancer treatment being able to enact and maintain lifestyle changes without unduly impacting their quality of life. For the patient, the impact of certain lifestyle modifications may not outweigh the possible benefit of increased ICI efficacy in a trade-off which will be personal to the patient.

It is difficult to discuss the potential for lifestyle modifications to optimise cancer treatment further without taking the time to emphasise the importance of the individual patient. Not only will optimisation of cancer treatment have to consider cancer subtype and patient chronotype, but also the patient's symptoms, comorbidities, habits, health beliefs, socio-economic status, social support, self-determination, and values in helping them make informed decisions on how best to utilise lifestyle modifications to optimise their cancer management. Practical implementation of such approaches could also be challenging, without appropriate support. Tellingly, surveys carried out exploring how often patients implement lifestyle changes after cancer diagnosis found that 41 to 65% of patients made dietary changes post-cancer diagnosis and 14 to 27% increased their level of exercise (139, 140). Future research could also make use of digital technologies to monitor circadian biorhythm to further refine our understanding of the correlation between the CTS and outcomes on ICI (127).

In summary, building on evidence showing the CTS plays a role in increasing ICI efficacy and circadian disruption have deleterious effect on cancer patients survival, we argue CTS precise and personalised entrainment by lifestyle factors such as photic stimuli, diet composition and timing, and physical exercise could be harnessed to potentially increase ICI efficacy. Conveniently, existing evidence suggests these behavioural interventions shown to improve outcomes on ICI – either directly or via the gut microbiota – regularly are associated with healthier lifestyle habits, with intrinsic health benefits. Combining these findings, the CTS could feasibly be entrained by a patient-tailored combination of lifestyle determinants of ICI efficacy to maximise response, with future research offering patients and clinicians an expanding evidence base on which to draw from.

Author contributions

BH: Data curation, Formal Analysis, Investigation, Methodology, Resources, Visualization, Writing – original draft, Writing – review & editing. SS: Data curation, Formal Analysis, Investigation, Resources, Writing – original draft, Writing – review & editing. SI: Data curation, Formal Analysis, Investigation, Resources, Writing – original draft, Writing – review & editing. NW: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. CS: Formal Analysis, Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing. PI: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Sandra E. Sephton,
Brigham Young University, United States

REVIEWED BY
William H. Walker II,
West Virginia University, United States
Xiangqi Meng,
Harbin Medical University, China

*CORRESPONDENCE
Laura M. Prolo
lmprolo@stanford.edu

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Review: therapeutic approaches for circadian modulation of the glioma microenvironment

Ella A. Nettnin[1], Thien Nguyen[2], Sophia Arana[1], Maria Isabel Barros Guinle[1], Cesar A. Garcia[1], Erin M. Gibson[3] and Laura M. Prolo[1,4]*

[1]Department of Neurosurgery, Stanford University School of Medicine, Stanford, CA, United States, [2]Division of Pediatric Hematology/Oncology, Lucile Packard Children's Hospital, Palo Alto, CA, United States, [3]Department of Psychiatry and Behavioral Sciences, Stanford University, Stanford, CA, United States, [4]Division of Pediatric Neurosurgery, Lucile Packard Children's Hospital, Palo Alto, CA, United States

High-grade gliomas are malignant brain tumors that are characteristically hard to treat because of their nature; they grow quickly and invasively through the brain tissue and develop chemoradiation resistance in adults. There is also a distinct lack of targeted treatment options in the pediatric population for this tumor type to date. Several approaches to overcome therapeutic resistance have been explored, including targeted therapy to growth pathways (ie. EGFR and VEGF inhibitors), epigenetic modulators, and immunotherapies such as Chimeric Antigen Receptor T-cell and vaccine therapies. One new promising approach relies on the timing of chemotherapy administration based on intrinsic circadian rhythms. Recent work in glioblastoma has demonstrated temporal variations in chemosensitivity and, thus, improved survival based on treatment time of day. This may be due to intrinsic rhythms of the glioma cells, permeability of the blood brain barrier to chemotherapy agents, the tumor immune microenvironment, or another unknown mechanism. We review the literature to discuss chronotherapeutic approaches to high-grade glioma treatment, circadian regulation of the immune system and tumor microenvironment in gliomas. We further discuss how these two areas may be combined to temporally regulate and/or improve the effectiveness of immunotherapies.

KEYWORDS

circadian, tumor microenvironment (TME), chronotherapy, glioma, glioblastoma, pediatric high-grade glioma

1 Introduction

Circadian rhythms are endogenous behavioral, physiological, and molecular rhythms that follow an approximately 24-hour cycle (1). This cycle involves transcriptional-translational negative feedback loops that are regulated by the core clock components; these include brain and muscle ARN-t like protein 1 (*BMAL1*), circadian locomotor output

cycles kaput (*CLOCK)*, period proteins *(PER1, PER2, PER3)*, and cryptochrome proteins (*CRY1, CRY2)* (2). These clock components form a positive limb, which activates the expression of downstream circadian genes, and a negative limb, which dampens expression of downstream circadian genes (3). Together, BMAL1 and CLOCK form a heterodimer that comprises the positive limb of the circadian clock (4). This BMAL1-CLOCK heterodimer drives transcription of downstream genes including the PER and CRY genes, which comprise the negative limb of the clock and feed back into the nucleus to suppress their own transcription (4). Other components of the clock include REV-ERBα, which inhibits BMAL1 transcription and thus regulates the positive limb of the circadian clock (5).

These circadian genes regulate an array of physiological processes throughout the body and can influence immune system functioning, cell cycle, metabolism, apoptosis, DNA repair, and epithelial-mesenchymal transition (EMT) (6–8). Notably, several of the pathways regulated by the circadian system overlap with oncologic mechanisms of survival; therefore, alteration of the circadian clock may be instrumental in modulating survival and progression of these various cancers. The circadian clock's regulation of such widespread processes has led to the emerging strategy of chronotherapy (9). This method involves timing the administration of treatments to a patient's circadian rhythm to maximize benefit and minimize adverse effects (9). Understanding circadian regulation of oncologic mechanisms of survival, and how to use this knowledge to enhance treatment efficacy, will be a crucial step in advancing the treatment of cancer.

Gliomas are the most common primary brain tumor, with high-grade gliomas or glioblastoma being the most common primary malignant brain tumor in adults (10). Prognosis for glioblastoma remains poor, with a median survival of 15 months, and most patients do not survive beyond two years (10, 11). There is evidence that high-grade gliomas express circadian genes that follow diurnal rhythms (12) and appear to have deregulated expression levels compared to low-grade gliomas and surrounding non-glioma brain tissue (13–17). Expression of PER1 and PER2—genes involved in the negative limb of the circadian clock—is found to be lower in glioma cells compared to the surrounding non-cancer tissue (15). Similarly, studies have shown decreased expression of CRY1 and CRY2 in glioma cells (16). Expression of CLOCK, on the other hand, is found to be upregulated in high-grade glioma compared to low-grade glioma and non-cancerous cells (14, 18). Thus, research in gliomas has shown downregulation of circadian genes in the negative limb and an upregulation of genes in the positive limb. Experimental downregulation of CLOCK and BMAL1 in glioma stem cells (GSCs) results in cell cycle arrest and apoptosis, suggesting that this circadian deregulation is crucial to the growth of GSCs (12). In this review, we highlight the role of the circadian clock in gliomagenesis and explore how this relationship can be exploited therapeutically. We reviewed the literature for chronotherapeutic approaches to treatment, and we focus on the interplay of glioma regulation and the tumor microenvironment (TME).

2 Chronotherapeutic approach to high grade glioma treatment

Outcomes for patients with high-grade gliomas (grade III and grade IV glioblastoma) remain poor despite decades of clinical trials, highlighting the need for enhanced treatment efficacy (19). Recent studies showing circadian regulation of glioma pathogenesis suggest that administering treatment based on a patient's circadian rhythms, or chronotherapy, may be a promising treatment strategy (9, 20–28). Studies exploring chronotherapeutic approaches to glioma treatment are summarized in Table 1.

2.1 Conventional treatments

The standard treatment for high-grade gliomas follows the 2005 Stupp protocol which is resection followed by concurrent chemotherapy with temozolomide and radiation followed by maintenance chemotherapy (11, 29, 30). Treatment for low-grade gliomas consists of resection when amenable followed by surveillance; for unresectable tumors, biopsy followed by targeted therapy (ie. BRAF or MEK inhibitors) for progressive tumors (30–32). In this section, we review the literature on chronotherapy in these conventional glioma treatments, with a specific focus on high-grade gliomas. The efficacy and side effects profile of these treatments may be regulated by circadian rhythms in gene expression, highlighting the importance of exploring chronotherapeutic approaches to augment standard glioma treatment strategies.

2.1.1 Temozolomide

Standard treatment for glioblastoma involves the DNA-alkylating agent TMZ, which acts by methylating DNA at the O6-guanine residue site (33). By methylating the DNA, TMZ induces DNA cross-linking and eventually cell apoptosis (33). Methylguanine methyltransferase (MGMT), a DNA repair enzyme, can remove the O6-methylguanine thus conferring resistance to TMZ. A subset of glioblastoma, however, express methylated MGMT which makes the repair enzyme inactive and leads to tumor cell TMZ sensitivity and prolongs patient survival (33). Recent work has suggested that adult gliomas demonstrate differential responses to TMZ depending on time of administration, suggesting a beneficial role for a chronotherapeutic approach to TMZ administration (20, 22, 23). One retrospective study on adult glioblastoma found that patients who received TMZ in the mornings had a 3.6 month longer overall survival than patients who received TMZ in the evenings (20). On further risk stratification, this survival benefit was extended to a 6 month increased overall survival in patients with O6-methylguanine-DNA methyltransferase (MGMT)-methylated glioblastoma, who received TMZ in the morning compared to MGMT-methylated patients treated in the evening (20). Given these findings, a follow-

TABLE 1 Summary of treatments and clinical outcomes for circadian regulation of gliomas.

			Sample	Patient Demographics: N (% Caucasian) Mean age Female (%)	Circadian Effect on Treatment Efficacy	Circadian Effect on Treatment Side Effects
Conventional Treatments	**TMZ**	Damato et al., 2021 (20)	Patients with glioblastoma	N = 166 (96.4% Caucasian) Mean age = 60.1 years Female (%) = 36.75	Morning TMZ increased OS compared to evening (1.43, 1.12–1.92 vs 1.13, 0.84–1.58 years). Effect increased to 6 months longer survival for MGMT-methylated patients treated in the morning.	N/A
		Damato et al., 2022 (21)	Patients with WHO grade III and IV or high-risk WHO grade II gliomas	N = 35 (91% Caucasian) Mean age = 56.31 years Female (%) = 43	No difference in survival was observed between AM- and PM-treated patients.	No difference in adverse events between AM- and PM-treated patients
		Slat et al., 2017 (22)	Mesenchymal glioblastoma astrocytes in mice	N/A	Maximum TMZ sensitivity occurred near the daily peak in BMAL1 expression.	N/A
		Chai et al., 2022 (23)	Patients with glioma (TCGA and CGGA databases)	N = 1,589 Female (%) = 42	Optimal TMZ administration should be at peak BMAL1 expression, which occurs at night.	N/A
	Radiotherapy	Zhanfeng et al., 2015 (24)	Glioma tissue in rats	N/A	Radiation delivered at peak PER2 expression led to increased apoptosis and decreased proliferation compared to trough PER2 expression.	N/A
		Zhu, Wang, Hu, & Wang, 2019 (25)	Human glioma cells (U343 glioma cell line)	N/A	High expression of PER1 was associated with increased sensitivity to radiation.	N/A
		Sapienza et al., 2021 (26)	Patients with high-grade glioma	N = 109 Mean age = 62.6 years Female (%) = 43	Treatment time (morning vs afternoon) did not affect survival outcomes.	Treatment time (morning vs afternoon) did not affect adverse events.
Novel Treatments	**P38 MAPK inhibitors**	Goldsmith et al., 2018 (27)	Rat glioma cells (IM3)	N/A	Glioma invasiveness is inhibited when p38 MAPK inhibitor is applied at the nadir of phosphorylated p38 MAPK expression. Invasiveness is not affected when treatment is applied at peak phosphorylated MAPK expression.	N/A
	1A-116	Trebucq et al., 2021 (28)	Human glioma cells (LN229)	N/A	Survival was increased when mice were treated with 1A-116 at ZT12 compared to ZT3 and vehicle conditions.	N/A

up phase II clinical trial was performed and demonstrated that chronotherapy with TMZ is feasible (21). Although this study found no difference in overall survival or adverse effects between patients treated with TMZ in the morning versus evening, the authors note that their small sample size and heterogenous patient population limit what can be concluded in terms of survival benefit (21). In summary, increased TMZ sensitivity in the AM may be driven by diurnal and differential expression of MGMT (34, 35) and may be a suitable target for larger studies in the future.

Another proposed mechanism of differential efficacy of TMZ in gliomas may relate to direct interaction of TMZ to circadian gene expression. One hypothesis is that TMZ sensitivity is specifically tied to the cyclic expression of BMAL1, the binding partner of CLOCK that helps drive transcription of downstream circadian genes (PERs and CRYs) and an essential part of the circadian clock (36, 37). Investigators used both primary human glioblastoma cells and primary mesenchymal murine glioblastoma astrocytes to show that cells are most sensitive to TMZ when it is administered near the daily peak in BMAL1 expression (22). This temporal effect disappeared after a CRISPR-mediated loss of BMAL1, suggesting that the chronotherapeutic sensitivity of TMZ is dependent on BMAL1 (22). Another study took a bioinformatics approach utilizing a database with information on drug sensitivity and gene expression profiles, demonstrating that higher expression of BMAL1 is significantly correlated to higher TMZ sensitivity (23). These findings link the TMZ chronotherapeutic outcomes to the molecular components of the circadian clock. Our understanding of chronotherapy for TMZ remains limited by the lack of large, prospective randomized control trials and future studies should explore how to best time TMZ administration to maximize benefit (38). One promising approach could involve the use of high-throughput sequencing modalities to delineate transient changes

in circadian gene expression, which would allow for more accurate dosing of TMZ therapy. RNA sequencing analysis has previously been used to identify molecular pathways involved in TMZ resistance in glioblastoma and could similarly be used to explore circadian regulation of TMZ sensitivity (39, 40). By employing high-frequency output, clinicians may be able to appropriately time treatment to the tumor circadian rhythms in their patients.

2.1.2 Radiotherapy

Unlike TMZ, chronotherapeutic strategies for radiation therapy treatment of gliomas remain controversial. Broadly, the expression of circadian genes *Per1* and *Per2* are thought to modulate the efficacy of radiotherapy in the treatment of gliomas (24, 25). Per1 expression levels are found to modulate the transcription of a variety of genes, including *p53* target genes and checkpoint components for DNA repair (25). Decreasing PER1 expression also results in decreased expression of the *Chk2-P53* signaling pathway and C-Myc, which are integral components of DNA-damage repair and apoptosis, respectively (25). As a result, PER1 is seen to play an important role in regulating the DNA damage response and subsequent apoptosis caused by radiation (25). Zhu et al. demonstrated that downregulating PER1 in human glioblastoma cells *in vitro* using an shRNA lentivirus resulted in minor DNA damage and reduced apoptosis in response to radiation compared to controls (25). Similarly, Zhanfeng et al. found this same positive correlation *in vivo*, showing that high expression of PER2 in glioma murine tissue was associated with an increased sensitivity to radiation. Specifically, there were higher levels of apoptosis and lower levels of proliferation when radiation was delivered at peak PER2 levels versus trough PER2 levels (24). This suggests there is a benefit of optimizing timing of radiotherapy based on circadian cycling (24). However, the survival benefits seen *in vitro* in U343 glioma cells and *in vivo* in glioma-bearing rats were not found in humans. In a retrospective study of 109 patients with high-grade gliomas, Sapienza et al. found no significant difference in progression-free survival or overall survival between patients treated with radiotherapy in the morning versus the afternoon (26). This may be because: i) other mechanisms overpower a modest survival benefit of radiation timing in humans, ii) that the time of radiation effect on tumor cells is much longer than what would occur within a circadian cycle, iii) there are other confounders such as concordant steroid administration that may reset the circadian clock, or iv) that there is not a measurable effect in humans. Despite this finding, the outcomes and toxicity-related benefits of chronoradiotherapy have been shown in other cancers (41–43). For example, a retrospective review on patients with rectal cancer found that patients who received radiotherapy after 12:00 pm had improved response rates compared to patients who were primarily treated before 12:00 pm (43). Similar benefits to chronoradiotherapy were shown for prostate cancer (42) and for bone metastases (41). These conflicting results suggest the need for further exploration into the potential benefits of chronotherapeutic approaches to radiation therapy in malignant brain tumors. As mentioned above, modern sequencing technologies may prove useful in elucidating the role for chronotherapeutic approaches to radiotherapy. Past studies have utilized total RNA sequencing to understand mRNA expression changes underlying radiotherapy resistance in glioblastoma (44), thus a similar analysis of circadian gene expression may allow for effective chronotherapeutic radiation therapy.

2.2 Novel treatments

Given the poor outcomes with the standard high-grade glioma treatment of surgical resection, chemotherapy, and radiotherapy, there is an urgent need for new therapeutic strategies. To further optimize novel therapies, we discuss the potential of chronotherapeutic approaches to augment new promising treatments in high-grade gliomas.

2.2.1 Immunotherapy

The development of immunotherapies, such as chimeric antigen receptor (CAR) T-cells, was a promising advancement in treatment for high-grade gliomas, yet survival outcomes remain poor with immunotherapy. Barriers to immunotherapies such as CAR T-cell therapy in gliomas include off-target effects, poor infiltration into the tumor, and an immunosuppressive tumor microenvironment (45). CAR T-cell response can be enhanced by the addition of a synthetic agonist for the circadian gene Retinoid-related orphan receptor γ (*RORγ*) (46, 47). Hu et al. demonstrate that adding RORγ, a circadian regulator of BMAL1 (48), to melanoma tumor-specific T cells *in vitro* and transferring them to tumor-bearing mice results in reduced tumor growth and improved T cell survival (47). Similarly, in a separate study, they show that adding RORγ during *ex vivo* expansion of a patient's immune cells increases the antitumor activity of T-helper 17 (Th17) cells that are engineered with a CAR (46). This antitumor activity is seen to persist long-term, with elevated levels of cytokines detected months after infusion (46). The antitumor activity of RORγ involves increasing production of cytokines such as Interleukin-17 and Granulocyte macrophage colony-stimulating factor, as well as co-stimulatory receptors tumor necrosis factor receptor superfamily member 9 (CD137) and Cluster of Differentiation (CD) 226 (47). RORγ also decreases immunosuppression by attenuating the activity of regulatory T cells (Tregs) and reducing expression of CD39, CD73, Programmed Cell Death Protein 1 (PD-1), and T cell immunoreceptor with Ig and ITIM domains (TIGIT) (47). These results offer promising evidence that manipulating the circadian clock may enhance the efficacy of CAR T-cell treatment. Future research should examine whether these findings extend to high-grade gliomas and whether time of administration affects the efficacy and side effect profile of CAR T-cell therapy.

2.2.2 Small molecule inhibitors

Several small molecule inhibitors have limited efficacy in the treatment of gliomas, despite targeting key growth pathways. Given their molecular potential, their response may be enhanced through further regulation of the tumor's circadian rhythm. These small molecular inhibitors target the epidermal growth factor (EGF), and

mitogen-activated protein kinase (MAPK) pathways and may have maximal therapeutic effect by understanding the chronoregulation of gliomas.

2.2.2.1 Epidermal growth factor receptor inhibitors

EGFR is one of the most common mutation sites in glioblastoma, making it an important therapeutic target (49). Mutations in the glioblastoma EGFR are primarily gene amplifications, suggesting the use of EGFR inhibitors as a potential treatment (49). Despite this promise, EGFR inhibitors have limited efficacy in treating glioblastoma (50). Recent work in mice has demonstrated circadian control of EGFR signaling, with EGFR signals being low during the active phase and high during the resting phase (51, 52). Lauriola et al. used xenograft athymic nude mouse models injected subcutaneously with N87 human gastric cancer cells to further show that administration of an EGFR inhibitor drug during the resting phase, when EGFR levels were elevated, resulted in a lower tumor volume compared to administration during the active phase (51). Advances in sequencing technologies have elucidated correlations between circadian genes and clinically actionable genes such as EGFR (53), further paving the way for more personalized chronotherapeutic approaches to treatment. Together, these results suggest that chronotherapy may be key in successfully applying EGFR inhibitors as a treatment for high-grade gliomas.

2.2.2.2 p38 MAPK inhibitors

Inhibitors of p38 MAPK have garnered attention as potential therapeutic agents given the correlation between high activity of p38 MAPK and poor prognosis in cancers, such as glioblastoma (3, 54). The p38 MAPK pathway is a signaling pathway known to play an important role in various cell processes including apoptosis, proliferation, and differentiation (27, 54). Increased p38 MAPK activity is associated with decreased apoptosis and less sensitivity to TMZ, suggesting that p38 MAPK inhibitors may sensitize tumors to chemotherapy (54). The low efficacy and high rate of off-target effects, however, limit the current potential of these inhibitors (55). Recently, there has been evidence of circadian regulation of the p38 MAPK activity, therefore highlighting a connection between the p38 MAPK pathway and the circadian clock (56–58). One study demonstrated this circadian regulation of p38 MAPK activity *in vitro* using murine glioma cells (27). The authors showed that p38 MAPK levels are cyclic in human astroglia, but remain elevated and arrhythmic in murine glioma cells (27). By inhibiting p38 MAPK in glioma cells at a time of day when levels are normally low in the human astroglia, there is a significant reduction in glioma invasiveness (27). These findings suggest that the therapeutic use of p38 MAPK inhibitors for high-grade glioma could be improved by timing administration of the drugs to complement the circadian rhythms in p38 MAPK activity.

2.2.2.3 1A-116

The novel drug 1A-116 was recently proposed as a potential treatment for tumors such as glioblastoma (59–61). This drug is a small molecule that reduces Rac1 activation levels by inhibiting interactions between Rac1 and guanine nucleotide exchange factors (GEFs) (60). These Rac1-GEF interactions are crucial for fundamental cellular processes such as proliferation, migration, cytoskeletal reorganization, and apoptosis (60). As a result of inhibiting Rac1 activation, 1A-116 is seen to effectively reduce tumor progression in a variety of cancers including gliomas (59, 61). One study demonstrated circadian regulation of 1A-116 efficacy, showing increased survival time for mice treated with the drug at the end of the light period compared to those treated at the beginning of the light period (28). The study also found that administration of 1A-116 at the time when PER1 levels are high and BMAL1 levels are low resulted in the strongest effects on cell proliferation, apoptosis, and migration (28). These temporal responses disappeared upon knocking down BMAL1, suggesting a dependence of 1A-116 sensitivity on circadian regulation (28). Mechanisms driving these time-dependent effects may include cyclic expression of Rac1, circadian regulation of downstream components in the affected pathways, and circadian regulation of 1A-116 entry into tumor cells (28). Together, these results offer promising evidence for novel glioma treatments, which can have an enhanced benefit when administration is timed to circadian rhythms.

3 Circadian modification of the tumor microenvironment

The heterogenous and ever-changing nature of high-grade gliomas are due, in part, to their ability to modify the tumor microenvironment (TME) to overcome selection pressures from the outside environment (62). The TME refers to the non-cancerous cells in the tumor including fibroblasts, endothelial cells, neurons, and immune cells, as well as non-cellular components such as the extracellular matrix, cytokines, and growth factors (63). There is a reciprocal relationship between cancer cells and the TME, and the dynamic nature of the glioma TME contributes to the low efficacy of various treatments, including immunotherapies, by creating an immunosuppressive environment (62, 63). Circadian clock components regulate the TME through effects on angiogenesis, inflammation, and immune suppression, thus influencing the TME's role in tumor progression (62, 63). Targeting circadian regulation of the TME therefore offers a promising therapeutic strategy by interfering with glioma pathogenesis.

3.1 Extracellular microenvironment

In this section, we summarize the literature on circadian regulation of the glioma TME, focusing on the adaptive measures and changes in the tumor's surroundings (Figure 1). The non-cancerous cells and environmental components that comprise the TME include endothelial cells, immune cells, cytokines, and the extracellular matrix (63). We discuss circadian regulation of angiogenesis, EMT, and immune targets, all critical to glioma proliferation and invasion.

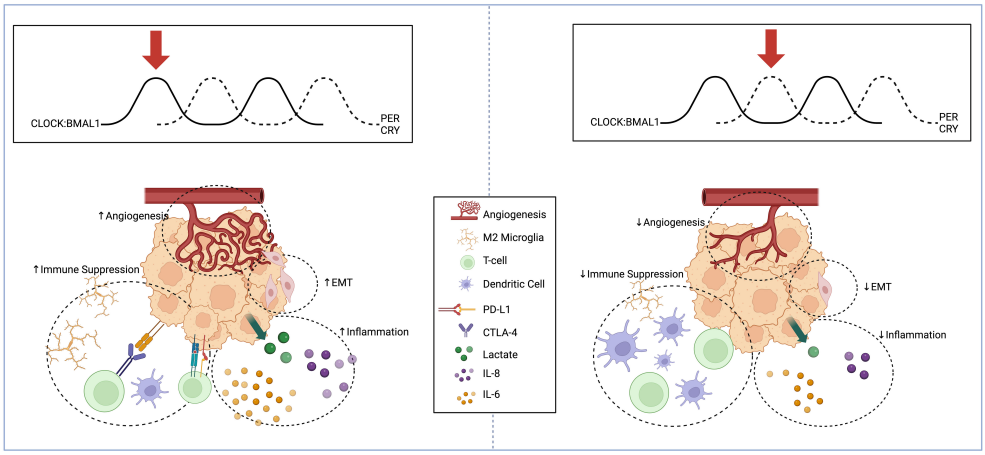

FIGURE 1
Schema displaying circadian regulation of high-grade glioma extracellular microenvironment at two different points in the circadian cycle. High-grade glioma microenvironment represented at two different points in the circadian cycle. When BMAL1 and CLOCK levels are high and PER and CRY levels are low (Left), there is an increase in pro-angiogenic factors, mesenchymal differentiation, inflammatory cytokine release, and immunosuppressive microglia. When BMAL1 and CLOCK levels are low and PER and CRY levels are high (Right), there is reduced angiogenesis, suppression of EMT, reduced inflammatory markers, and reduced recruitment of immunosuppressive microglia and expression of PD-L1. Created with BioRender.com.

3.1.1 Angiogenesis

Gliomas can modify their TME through angiogenesis, mediated through the vascular endothelial growth factor (VEGF) pathway and resulting in the generation of new blood vessels to deliver necessary nutrients for further tumor growth and expansion (64, 65). This process is thought to involve circadian regulation, according to results of a study by Pang et al. (66). In this study, authors utilize gene set enrichment analysis on patient glioblastoma samples from publicly available databases to show that angiogenesis is one of the most enriched pathways in glioblastoma tumors with high BMAL1 expression compared to those with low BMAL1 expression (66). Additionally, they use glioblastoma patient-derived cells to show that angiogenesis is greatly reduced in glioblastoma cells with depletion of either CLOCK or BMAL1 (66). Furthermore, the administration of SR9009, a selective androgen receptor modulator that activates the circadian gene *Rev-Erbα*, decreases angiogenesis in glioblastoma *in vitro* and reduces intratumoral blood vessels *in vivo* in murine glioma models (66). Together, these findings highlight the effect of circadian regulation on angiogenesis.

More specifically, circadian genes regulate the expression of several angiogenic factors. For example, the Pang et al. study suggests that CLOCK and BMAL1 drive expression of the pro-angiogenic factor periostin (POSTN) in endothelial cells (66). They propose a mechanism through which the CLOCK-BMAL1 complex regulates expression of the olfactomedin like 3-hypoxia-inducible factor 1-alpha (HIF-1α) axis, which upregulates POSTN and activates TANK-binding kinase 1 signaling in endothelial cells (66). Similarly, a study by Wang et al. found that expression of BMAL1 positively correlates with microvascular density, as well as angiogenic factors HIF-1α, Angiopoietin 2 (ANG2), and VEGF in gliomas (67). Correspondingly, inhibition of BMAL1 *in vitro* using primary glioma cells results in decreased expression of HIF-1α and VEGF (67). It is likely that BMAL1 promotes angiogenesis through its modulation of these angiogenic factors. Similar results are observed in other cancer types. For example, overexpression of CLOCK in human colorectal carcinoma cell lines correlates with increased expression of angiogenesis-related genes such as *HIF-1α*, *ARNT* and *VEGF*, with CLOCK knockdown showing the opposite results (68). A study in sarcoma and melanoma showed circadian rhythms in VEGF expression are regulated by *Period* and *Cryptochrome1*, and *in vivo* chronotherapeutic administration of anti-angiogenic agents during early light phase decrease tumor growth to a greater degree than administration during early dark phase (69). Additionally, a study in zebrafish embryos showed that BMAL1 positively regulates VEGF expression while PER2 negatively regulates expression (70). Altogether, these findings elucidate the mechanism underlying circadian regulation of angiogenesis through angiogenic factors such as HIF-1α and VEGF, and they highlight the therapeutic potential of targeting this pathway (Figure 2A).

3.1.2 Epithelial-mesenchymal transition

The epithelial-mesenchymal transition (EMT) allows glioma cells to migrate and invade into surrounding tissue (64) and circadian regulation of EMT may provide a novel target for manipulation of the glioma microenvironment to inhibit the invasive nature of high-grade gliomas. In glioblastoma tissue and in cell culture, CLOCK was found to positively regulate glioblastoma migration by upregulating activity of transcription factor nuclear factor-κB (NF-κB), thus promoting mesenchymal differentiation (18, 71). NF-κB is a ubiquitous transcription factor, and one of its roles in glioblastoma involves inducing expression of mesenchymal transcription factors and mesenchymal proteins such as CD44, vimentin, and N-cadherin (71). Additionally, a study by Yu et al. in glioblastoma cells found that knocking down the nuclear

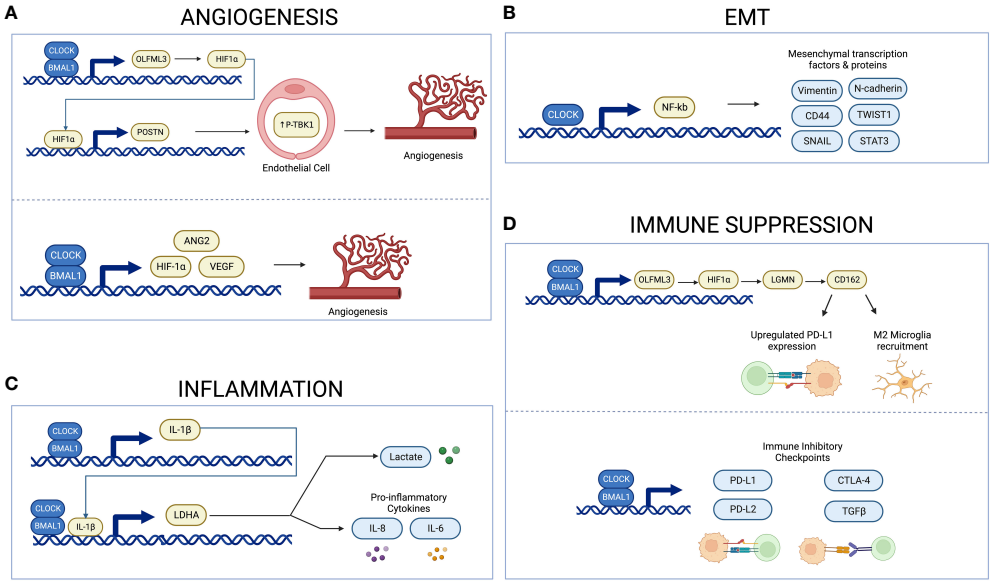

FIGURE 2
Schema illustrating circadian regulation of angiogenesis, inflammation, EMT, and immune suppression. The CLOCK-BMAL1 complex regulates expression of proangiogenic factors and microvascular density **(A)**. CLOCK upregulates NF-kB activity, thus inducing expression of mesenchymal proteins and transcription factors **(B)**. The CLOCK-BMAL1-IL-1B-LDHA axis regulates expression of lactate and proinflammatory cytokines **(C)**. The CLOCK-BMAL1 complex regulates recruitment of immunosuppressive microglia and expression of immune inhibitory checkpoints **(D)**. Created with BioRender.com.

receptor REV-ERBß, a repressor of circadian genes, appears to suppress EMT and metastasis of glioblastoma cells (72). Similarly, circadian regulation of EMT has been shown in other cancers such as colorectal cancer. Specifically, increased expression of CLOCK or BMAL1 in colorectal cancers correlates with increased mesenchymal markers and decreased epithelial markers (73). Conversely, silencing CLOCK or BMAL1 has the expected opposite effect, downregulating mesenchymal markers and increasing epithelial ones (68, 73). In breast cancer cells, reduced expression of the circadian gene PER2 was found to correlate with increased expression of the pro-EMT genes Snail Family Transcriptional Repressor 2 (*SLUG*), Snail Family Transcriptional Repressor 1 (*SNAI1*), and Twist-related protein 1 (*TWIST1*) (74). These results suggest a link between the circadian system and increased EMT in various cancer models (Figure 2B). Future studies should determine whether this mechanism is important in glioblastoma as well.

3.1.3 Inflammation

The circadian clock plays a critical role in regulating the immune system through modulation of cytokine expression such as proinflammatory cytokine interleukin-1ß (IL-1ß) (75). Inflammation in the context of cancer is often tumor-promoting by supplying growth factors, proangiogenic factors, and enzymes that modify the extracellular matrix (64). Tumor-derived lactate plays several roles in tumor development, including serving as a proinflammatory mediator that increases cytokines such as IL-1ß, and lactate dehydrogenase A (LDHA) is integral in producing lactate (75, 76). Circadian genes CLOCK and BMAL1 are found to regulate expression of IL-1ß and LDHA in gliomas, with suppression of CLOCK and BMAL1 leading to a reduction in LDHA and IL-1ß levels (75). Similarly, knocking down BMAL1 or CLOCK diminishes the IL-1ß-induced increase in lactate and proinflammatory cytokines such as IL-8 and IL-6 (75). Increased expression of each part of this CLOCK-BMAL1- IL-1ß-LDHA feedback loop is correlated with poor prognosis and shorter survival (75). These findings therefore demonstrate an autocrine signaling loop that could be targeted to disrupt the dysregulated inflammation and metabolism that benefits glioma cells (Figure 2C).

Glucocorticoids, such as dexamethasone, are often used to alleviate cerebral edema and inflammation from intracranial tumors (77). Recently, there have been more studies linking chronopharmacology of glucocorticoid administration and the expression of circadian genes in the immune cells (78). For example, a study by Fonken et al. showed that glucocorticoids entrain circadian clock gene expression in microglia by inducing the expression of the *Per1* gene (78). This study also showed that microglia demonstrate temporal fluctuations in inflammatory cytokines such as tumor necrosis factor α (TNFα), IL-1β and IL6 (78). These findings show that microglia demonstrate circadian rhythms in inflammatory responses, and these rhythms can be influenced by administration of glucocorticoids (78). Glucocorticoids are widely used among patients with all brain tumors, and these results suggest an important link between steroid administration and circadian-regulated changes in immune cells in the glioma TME. Future studies should examine whether there are differential effects in treatment response, based on what time of day the glucocorticoids are administered.

3.1.4 Immune suppression

Recently, circadian markers such as CLOCK and BMAL1 have been tied to immune suppression in glioblastomas by increasing microglia infiltration. High CLOCK expression is positively correlated with an increase in microglia and a decrease in CD8 activated T-cells and dendritic cells (79). In addition, both CLOCK and BMAL1 expression correlate with expression of microglial markers (79) and the infiltrating microglia are biased towards the immunosuppressive (M2) phenotype (79, 80). Overall, by increasing the expression of immunosuppressive microglia in the TME, CLOCK and BMAL1 genes can effectively minimize the response of glioma to immunotherapies.

Because of their interference with circadian recruitment of microglia, these circadian genes can decrease immune suppression in glioma. For example, administration of SR9009, the REV-ERBα agonist that inhibits BMAL1 expression, decreases intratumoral immune-suppressive microglia in GSCs (80). Similarly, depletion of CLOCK or BMAL1 in murine models results in reduced infiltration of microglia and improved overall survival (79). These studies highlight circadian regulation of immune suppression in the glioma TME and demonstrate how this regulation can be targeted therapeutically.

A specific signaling pathway between GSCs and microglia, the CLOCK-olfactomedin-like 3 (OLFML3)-HIF1α-legumain (LMGN)-cluster of differentiation 162 (CD162) axis, has been identified and serves as a potential therapeutic target for glioblastoma (Figure 2D) (80). GSCs utilize CLOCK and BMAL1 to transcriptionally regulate LMGN and OLFML3, two chemokines that promote recruitment of microglia (79, 80). Expression of the circadian gene CLOCK specifically demonstrates a positive correlation with LMGN and OLFML3 in glioblastoma (79, 80). Inhibiting any part of this CLOCK–OLFML3–HIF1α–LGMN axis results in reduced immunosuppressive microglial recruitment and prolonged survival in glioblastoma murine models (80). Similarly, treatment with either SR9009 or anti-CD162 enhances survival in this glioblastoma mouse model in response to anti-programmed cell death protein 1 (PD1) therapy (80). This finding is significant because gliomas are known to upregulate immune checkpoint molecule programmed death-ligand 1 (PD-L1) in order to suppress the immune response and evade immunotherapies (81). Targeting the CLOCK–OLFML3–HIF1α–LGMN–CD162 axis reduces PD-L1 expression and augments anti-PD1 therapy in glioblastoma mice, overall highlighting the therapeutic potential of this pathway (80).

The circadian clock is associated with immune evasion in tumors (82). In particular, a study by Wu et al. found that the circadian clock is associated with an immune evasion phenotype (82). Using RNA-sequencing data to infer level of immune cell infiltration, they demonstrated a positive correlation of core circadian genes with infiltration of Tregs and Mast cells, but a negative correlation with infiltration of Th2 cells, Th1 cells, natural killer T cells, CD8 T cells, CD8 naïve T cells, and CD4 T cells (82). This finding shows the broad impact of the circadian clock on the immune cells that infiltrate the TME. Using bioinformatics approaches, the authors demonstrate that the disrupted circadian clock in cancer contributes to T-cell exhaustion through persistent elevation of inhibitory checkpoints (82). Specifically, there was a positive correlation between all clock genes and immune inhibitory checkpoints PD-L1, PD-L2, cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and transforming growth factor beta (TGFB) (Figure 2D) (82). Treatments aimed at targeting the circadian system in gliomas may therefore diminish the ability of these tumors to evade the immune system.

3.2 Intracellular mechanisms

In the previous section we reviewed the role of the extracellular tumor microenvironment on circadian rhythms. Here we will discuss potential intracellular pathways that may be targeted for chronotherapy.

3.2.1 MGMT-methylation

As previously mentioned, MGMT plays a critical role in promoting TMZ-resistance in gliomas by repairing double-stranded DNA breaks (33). Studies by Damato et al. demonstrate a significant 6-month difference in survival between patients treated in the AM and PM, highlighting the role of the circadian system on TMZ sensitivity in MGMT-methylated gliomas (20). Further studies by Marchenay et al. and Martineau-Pivoteau et al. demonstrate circadian rhythms in MGMT protein activity in both human and mouse cells, respectively (34, 35). Marchenay et al. use serial blood samples across a 24-hour period from healthy volunteers to demonstrate circadian rhythms in MGMT activity in circulating mononuclear cells (34). Similarly, Martineau-Pivoteau et al. use liver samples obtained from mice at eight different circadian times to demonstrate circadian rhythms in MGMT activity, with the highest activity occurring during the active period (35). These findings demonstrate circadian regulation of MGMT-methylation, and understanding this cycling may allow for more precise timing of TMZ administration to maximize overall survival.

3.2.2 IDH 1/2 mutations

Mutations in isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2) are associated with improved survival rates among patients with glioblastoma and are positive prognostic predictors of overall survival (83, 84). However, there are few studies examining the role of IDH mutations in tumorigenesis and in modulating the circadian cancer pathway. Although most studies exploring circadian regulation of gliomas do not stratify based on IDH mutation status, De La Cruz Minyety et al. found that PER gene expression predicts survival in high-grade glioma patients independently of IDH mutational status (84). Interestingly, another study of *in vitro* glioma cells demonstrated that IDH1 mutations are associated with lower expression of BMAL1, CLOCK, PER genes, and CRY genes compared to their wildtype counterparts (85). Together these findings suggest that IDH mutations may regulate circadian gene expression, but do not show clear evidence for circadian regulation of IDH mutational status. Given the paucity

of research into this important prognostic factor for glioblastoma, further studies should elucidate whether IDH mutational status could be used as a marker for assessing the effectiveness of chronotherapy.

3.2.3 Growth factor axis

High-grade gliomas are known to express growth factors and growth factor receptors (86), and several malignant behaviors such as proliferation, invasion, angiogenesis, and decreased apoptosis involve growth factor signaling (87). For example, Insulin Growth Factor 1 (IGF-1) receptor signaling is one mechanism through which GSCs become resistant to radiotherapy (88). Specifically, acute radiation increases expression of IGF1R and secretion of IGF-1, which activates the phosphatidylinositol 3-kinase (PI3K)-Protein kinase B (Akt) pathway to prevent apoptosis and promote survival (88, 89). Recent work by Alonso-Gomez et al, Mazzoccoli et al, and Chaurdhari et al. revealed IGF-1 levels in the liver and serum exhibit circadian rhythms (90–92). Additionally, circadian regulation of IGF-1 has been shown to regulate cancer progression in non-small cell lung cancer cells (90–93). IGF-1 entrains the circadian clock in the liver, highlighting the two-way relationship between circadian-regulated targets and the circadian clock itself (94–96). Similarly, nerve growth factor (NGF) was found to entrain the circadian clock in hamsters, and epidermal growth factor (EGF) is seen to induce clock gene expression in neural stem cells (97–99). Likewise, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) induce robust PER1 expression in murine fibroblasts (100). Together, these data show that regulation of growth factors represents another bi-directional link between the circadian clock and cancer biology. This link should be further researched and targeted to potentially decrease radiotherapy resistance in glioblastoma.

4 Pediatric high grade gliomas

Cancer is a leading cause of death amongst children, with brain tumors, specifically, being the top cause of cancer-related death in children (101). However, there is a lack of effective treatment options for childhood brain tumors and most treatments were developed for adult cancers and applied to children despite the tumors being quite different in nature (102). We discuss the available data on circadian regulation of pediatric high-grade gliomas and how the clock may be targeted for treatment.

4.1 Circadian regulation of the tumor microenvironment in pediatric gliomas

Immune cells play a crucial role in the TME of both high- and low-grade pediatric gliomas (103, 104). Pediatric high-grade gliomas are enriched in genes related to microglia and macrophages, and tumor-associated macrophages are specifically found to promote the growth of low-grade pediatric gliomas (103–105). Daginakatte et al. use Neurofibromatosis-1 (NF1), a glioma predisposition syndrome, to evaluate the role of microglia in glioma growth (106). They show that NF1-heterozygous microglia demonstrate increased proliferation, motility, and genes associated with microglial activation (106). When they inhibit the microglia activity *in vivo* using optic pathway glioma mouse models, there is reduced low-grade glioma proliferation (106). The mechanism underlying microglia stimulation of pediatric glioma growth is not yet fully known; one hypothesis is that microglia release chemokines and paracrine factors, such as hyaluronidase, to increase glioma proliferation (105). These findings suggest a similar role for immune cells in the survival of pediatric high-grade gliomas, but there remains a gap in our understanding the role of these cell types play in tumor progression. Future research should explore whether there is a survival benefit in immune cell targeted treatments at different times of the circadian cycle in pediatric high-grade gliomas.

4.2 Chronotherapy in pediatric tumors

Although there are no studies to date on the application of chronotherapy to pediatric high-grade gliomas, studies have shown a benefit of chronotherapy in pediatric patients with acute lymphoblastic leukemia (107, 108). These studies have found increased progression free survival in pediatric patients with acute lymphoblastic leukemia when chemotherapy is administered in the evening versus the morning, with a higher risk of relapse for those being treated in the morning (107, 108). Specifically, Schmiegelow et al. found a higher probability of event free survival for patients administered oral methotrexate (MTX) and 6-mercaptopurine (6MP) in the evening versus the morning, with a median follow-up of 78 months (108). Similarly, Rivard et al. found that for patients surviving disease-free for more than 78 months, there was a greater risk of relapse for patients taking MTX and 6MP in the morning versus the evening (107). A potential benefit of timing of chemotherapy for pediatric high-grade gliomas should be explored in future studies. Future studies should also explore the use of chronotherapy for other pediatric high-grade glioma treatments, such as CAR T-cell therapy. Pediatric high-grade gliomas such as diffuse intrinsic pontine gliomas (DIPGs) are fatal, but the recent clinical success of CAR T-cell therapy in DIPGs (109) highlights a potential area for chronotherapeutic augmentation. Overall, there is a dearth of research into chronotherapy in pediatric tumors, and more research is needed in this area in order to expand treatment options and efficacies among these patients.

While the cyclic expression of circadian genes may affect the efficacy of administered drugs, the pharmacological agents also affect the circadian system. Steroids such as dexamethasone, for example, which are often administered to children with brain tumors, may affect the circadian clock. Studies on children with acute lymphocytic leukemia have shown that dexamethasone dampens circadian activity rhythms in these patients, which leads to decreasing daily trends of peak activity during dexamethasone treatment (110). This study also shows that increased fatigue is associated with the dampened circadian activity rhythms, but notes that the relationship between dexamethasone and fatigue is complex and requires further investigation (110). Similarly, a

study on children with central nervous system cancers demonstrated dysregulated circadian activity rhythms as a result of chemotherapy, and circadian dysregulation is known to affect health-related outcomes including quality of life, responsiveness to chemotherapy, relapse, and fatigue (111). Further research is needed to explore the health outcomes related to circadian dysregulation and interventions that restore rhythmicity. Overall, these results demonstrate the potential use of chronotherapeutic treatment of pediatric high-grade glioma to both increase the efficacy of cancer treatment as well as decrease negative side effects of the treatments.

5 Conclusion

In summary, deregulation of circadian genes is thought to play a role in the pathogenesis of high-grade gliomas. Circadian regulation of the TME and the immune system may be a route through which gliomas manipulate their environment to enhance their survival. Because of the link between the circadian clock and glioma pathology, chronotherapy offers a promising adjunct to the low efficacy and high side effect profile of existing glioma treatments. Future work should explore the potential of targeting circadian regulation of the TME and timing immunotherapies to maximize their benefit and minimize side effects. Rapidly advancing technologies such as high-throughput sequencing offer the unique opportunity to dissect the heterogenous and dynamic TME and can be used to further elucidate circadian regulation of the TME. For example, these technologies can be used to understand circadian-regulated changes in TME composition and to identify predictive biomarkers related to these circadian-driven changes. Additionally, most of the present research exploring circadian regulation of gliomagenesis focuses on adult tumors. Future work should focus on circadian regulation of pediatric gliomas because of the distinct lack of research and therapies directed towards this patient population. Effectively timing treatments based on circadian regulation is a novel approach that may improve survival in pediatric high-grade gliomas. Ongoing research into the circadian regulation of TME and immune targets may result in the improved therapeutic outcomes that are urgently needed for high-grade gliomas.

Author contributions

EN: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. TN: Conceptualization, Writing – review & editing. SA: Visualization, Writing – review & editing. MB: Visualization, Writing – review & editing. CG: Writing – review & editing. EG: Writing – review & editing. LP: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Zhengming Chen,
Cornell University, United States

REVIEWED BY
Mario Cioce,
Campus Bio-Medico University, Italy
Masoud Sadeghi,
Islamic Azad University, Iran
Antonino Maniaci,
Kore University of Enna, Italy
Anna Brzecka,
Wroclaw Medical University, Poland

*CORRESPONDENCE
James B. Burch
james.b.burch@vcuhealth.org

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Sleep disorders and cancer incidence: examining duration and severity of diagnosis among veterans

James B. Burch[1]*, Alexandria F. Delage[2,3], Hongmei Zhang[4], Alexander C. McLain[2], Meredith A. Ray[4], Austin Miller[5], Swann A. Adams[2,6] and James R. Hébert[2,7]

[1]Department of Epidemiology, School of Population Health, Virginia Commonwealth University, Richmond, VA, United States, [2]Department of Epidemiology and Biostatistics, Arnold School of Public Health, University of South Carolina, Columbia, SC, United States, [3]Palmetto GBA, Columbia, SC, United States, [4]Division of Epidemiology, Biostatistics, and Environmental Health, School of Public Health, University of Memphis, Memphis, TN, United States, [5]Alabama College of Osteopathic Medicine, Dothan, AL, United States, [6]Department of Biobehavioral Health and Nursing Science, College of Nursing, University of South Carolina, Columbia, SC, United States, [7]South Carolina Statewide Cancer Prevention & Control Program, University of South Carolina, Columbia, SC, United States

Introduction: Sleep disruption affects biological processes that facilitate carcinogenesis. This retrospective cohort study used de-identified data from the Veterans Administration (VA) electronic medical record system to test the hypothesis that patients with diagnosed sleep disorders had an increased risk of prostate, breast, colorectal, or other cancers (1999-2010, N=663,869). This study builds upon existing evidence by examining whether patients with more severe or longer-duration diagnoses were at a greater risk of these cancers relative to those with a less severe or shorter duration sleep disorder.

Methods: Incident cancer cases were identified in the VA Tumor Registry and sleep disorders were defined by International Classification of Sleep Disorder codes. Analyses were performed using extended Cox regression with sleep disorder diagnosis as a time-varying covariate.

Results: Sleep disorders were present among 56,055 eligible patients (8% of the study population); sleep apnea (46%) and insomnia (40%) were the most common diagnoses. There were 18,181 cancer diagnoses (41% prostate, 12% colorectal, 1% female breast, 46% other). The hazard ratio (HR) for a cancer diagnosis was 1.45 (95% confidence interval [CI]: 1.37, 1.54) among those with any sleep disorder, after adjustment for age, sex, state of residence, and marital status. Risks increased with increasing sleep disorder duration (short [<1-2 years] HR: 1.04 [CI: 1.03-1.06], medium [>2-5 years] 1.23 [1.16-1.32]; long [>5-12 years] 1.52 [1.34-1.73]). Risks also increased with increasing sleep disorder severity using cumulative sleep disorder treatments as a surrogate exposure; African Americans with more severe disorders had greater risks relative to those with fewer treatments and other race groups. Results among patients with only sleep apnea, insomnia, or another sleep disorder were similar to those for all sleep disorders combined.

Discussion: The findings are consistent with other studies indicating that sleep disruption is a cancer risk factor. Optimal sleep and appropriate sleep disorder management are modifiable risk factors that may facilitate cancer prevention.

KEYWORDS

apnea, cancer, insomnia, risk, sleep, veteran

Introduction

Sleep disorders arise due to complex circumstances that can include genetic, environmental, behavioral, and psychosocial risk factors (1, 2). Veterans comprise a vulnerable population for sleep disorder occurrence and their rates are elevated relative to the general population (3, 4). Furthermore, secular trends in sleep disorder diagnoses have been increasing, both among Veterans and within the general population (3–5). For example, a national study of sleep disorder diagnoses among Veterans from 2000 to 2010 found a ~6-fold increase in sleep disorder prevalence during that period, with the largest increases among those with combat experience or post-traumatic stress disorder (3). Because of these increasing trends, an association between sleep disorders and cancer incidence would have important public health implications.

Several pathophysiological processes may mediate the association between chronic sleep disruption and carcinogenesis, including pathological changes in immune (via inflammation- and oxidative stress-related mechanisms), endocrine, neurological, metabolic and circadian systems (6–9). For example, the intermittent hypoxia and subsequent oxidative stress that occurs with sleep apnea has been cited as a biologically plausible, experimentally supported mechanism (7, 9). Results from longitudinal studies of sleep disorders and cancer incidence tend to show increased risks although results have been inconsistent. For example, several investigators reported no association between insomnia symptoms and prostate cancer (PrCA) incidence (10–12), whereas others report higher risks for PrCA among insomniacs after ~10 or more years of follow-up (13). Insomnia has also been associated with increased risks for breast (BrCA), colorectal (CRC) or other cancer types, particularly after a decade or more of follow-up (14–16). Some studies among sleep apnics reported an elevated incidence of BrCA, PrCA, CRC (17–21) whereas other studies of patients with obstructive sleep apnea (OSA) reported lower risks (22–24). A national matched retrospective cohort among Veterans found that OSA was associated with an increased incidence of BrCA, PrCA, CRC and several other cancers after a median follow-up of 7.4 years (25). It has been suggested that cancer risk increases with OSA severity (17, 26, 27). However, few studies, if any, have examined the role of cumulative sleep-related treatments or duration of diagnosis as proxies for dose-response.

Several studies that assessed sleep either subjectively (sleep quality) or quantitatively (nightly sleep duration) reported no association with cancer incidence (28–31). However, studies among women in Singapore (32) and Ohsaki, Japan (33) both found a modest association between postmenopausal BrCA risk and long sleep duration within a 24-hour period, consistent with a USA study where long sleep (≥9 hours per night) was associated with a modest increase in BrCA risk, although short nightly sleep duration had no association (34). Among men in the Ohsaki cohort, those who reported trouble falling asleep or staying asleep had increased PrCA risks (35) whereas men who slept ≥9 hours per night had lower PrCA risks relative to those with normal sleep (33). These examples indicate that, despite decades of research among millions of patients, there are still inconsistencies and uncertainties among studies examining relationships between sleep disturbances and cancer risk. Recent systematic reviews and meta-analyses addressing this issue highlight heterogeneous sleep assessment methods as one possible explanation, and indicate a need for more studies using clinically diagnosed sleep disorders (8, 16, 17, 31, 36–38).

Racial disparities in sleep disturbances have also been described. African Americans (AAs) have shorter sleep duration, lower sleep efficiency, and unfavorable sleep stages including less slow wave sleep and worse sleep continuity relative to European Americans (EAs) (39, 40). AAs also have an elevated incidence of certain cancers relative to EAs, both in the general (colorectal, gastric, lung, renal, hepatic, prostate) and Veteran (gastric, hepatic, prostate) population (41, 42). However, few studies have examined the extent to which sleep disparities may contribute to racial cancer disparities (40). The VA's electronic medical record (EMR) system includes data for millions of Veterans and serves as a valuable resource for examining relationships between sleep disorders and cancer incidence.

Methods

This retrospective cohort study used data from the VA EMR to test the hypothesis that Veterans in the southeastern Veterans Integrated Service Network 7 (VISN-7, includes AL, GA, SC) with a diagnosed sleep disorder had increased cancer risk relative to those without a sleep disorder. Analyses were performed to evaluate whether the duration or severity of the sleep disorder diagnosis enhanced risk, and whether cancer risk was modified by race.

Following regulatory approvals, electronic medical records for patients seeking care at least once at a VISN-7 facility between January, 1999 and July, 2010 were retrieved from MedSAS Dataset and Department of Veterans Affairs (VA) Corporate Data Warehouse files. During the study period, the VISN-7 included 9 tertiary care medical centers, 14 community-based outpatient clinics, and 18 primary care clinics serving ~1.3 million Veteran patients, and it was sixth largest among VISNs nationally for percentage of patients in chronic care. Patients younger than 18 years old and those without age information were excluded. Data elements were linked via social security number by a VA data manager, and then scrambled and replaced with a unique patient identification number so that data used by the study investigators were de-identified.

Sleep disorder cases were defined as patients with at least two occurrences of a diagnosis ≥30 days apart based on American Academy of Sleep Medicine International Classification of Sleep Disorder (ICSD) categories (43). The first occurrence of the in- or out-patient diagnosis was used to define date of diagnosis for: sleep disturbances (ICD-9 780.50-59), nonorganic sleep disorders (ICD-9 307.40-49), organic insomnia (ICD-9 327.00-09), organic hypersomnia (ICD-9 327.10-19), organic sleep apnea (ICD-9 327.20-29), and circadian rhythm sleep disorders (ICD-9 327.30-39). Time since diagnosis was calculated by summing the number of months since initial sleep disorder diagnosis and was used in the statistical analyses either as a continuous variable or categorized into three groups based on tertiles defining short, medium and long duration of diagnosis (<1-2.2, 2.3-5.2, 5.3-12.4 years, respectively). Sleep disorder severity was approximated as the cumulative number of treatments by summing sleep-related prescriptions, clinical procedures and surgeries into a single continuous variable. Participants were then grouped into four categories: those without any treatment, and those with few (1 treatment/prescription), moderate (cumulative count: 2-18) or frequent (19 - 1,150) treatments.

Data from the VA Tumor Registry were accessed to identify patients with an incident, primary tumor of the: prostate (ICD-9 185), female breast (ICD-9 174), colorectum (ICD-9 153-4), lung (ICD-9 162), pancreas (ICD-9 157) kidney (ICD-9 189), brain (ICD-9 191), bladder (ICD-9 188), liver (ICD-9 155), ovary (ICD-9 183), esophagus (ICD-9 150), or stomach (ICD-9 151). Patients with a cancer diagnosis prior to the beginning of the study, and those with *in situ* (ICD-9 230-234) and benign (ICD-9 210-229) tumors were excluded. Patients with a rare cancer or an etiology unrelated to the study hypothesis (e.g., infectious) were excluded, which included tumors of the: small intestine (ICD-9 152), skin (ICD-9 172-173), uterus (ICD-9 179 and 182), cervix (ICD-9 180), thyroid (ICD-9 193), lips, oral cavity, pharynx (ICD-9 140-149), or a lymphoma (ICD-9 201). Demographic variables used in the analyses as covariates included: age at cohort entry (18-34 yrs, 35-44 yrs, 45-54 yrs, 55-64 yrs, ≥65 yrs), sex, marital status, and state of residence (AL, GA, SC). Prior to the creating the final analytic data set, Veteran Medicare data were merged by a VA data manager using the social security number to assign race (EA, AA, and Other/Unknown) for each patient based on VA Information Resource Center (VIReC) guidelines (44, 45).

Statistical analyses examining the relationship between sleep disorders and subsequent cancer diagnoses were conducted using extended Cox regression models (SAS 9.3, SAS Institute, Inc, Cary, NC). This method included sleep disorder diagnosis as a time-varying covariate and is applied when the proportional hazards assumption is not met (46, 47). Survival time within the cohort was defined as months from entry until cancer diagnosis or censoring. Patients with only one visit at a VISN-7 facility were censored at the time of entry into the cohort. Hazard ratios with corresponding 95% confidence intervals for each combination of the sleep disorder variable and cancer outcome were computed for both crude and adjusted (age, sex, marital status, state of residence) models with statistical significance set at alpha = 0.05. Initial analyses evaluated all sleep disorders combined and subsequent analyses were performed for the following subgroups: insomnia, sleep apnea, and all other sleep disorders. Analyses of sleep disorder duration were used to assess the latency of tumor development following a sleep disorder diagnosis, and also to address the potential for reverse causality. A time-varying model was implemented where exposure (i.e., sleep disorder diagnosis) was assessed at each failure point in the model, with the amount of time since diagnosis included as a continuous variable in the Cox extended hazard analysis (47). A sleep disorder diagnosis is not always required for a given sleep disorder treatment (i.e., some prescriptions or clinical procedures can be used to treat conditions other than a sleep disorder, or can be used for patients without a sleep disorder diagnosis). Therefore, to prevent the introduction of bias, cumulative sleep-related treatments were computed for all participants regardless of their sleep disorder status, and a Cox extended regression model was used in which the cumulative treatment was multiplied by the total time in the study to account for the lack of proportionality. Additional analyses focused on potential effect modification by race, which was assessed by including an interaction term between race and each sleep disorder variable used in the model, and results were presented as hazard ratios and 95% confidence intervals within each race stratum. In some instances, the stage of disease was missing from the tumor registry. To evaluate the potential introduction of bias, sensitivity analyses included an indicator variable ('1' for those without cancer stage and '0' for others) with the Cox extended regression models. Those analyses produced the same conclusions as the results presented below.

Results

The final study population included 663,869 eligible patients after applying inclusion and exclusion criteria (Figure 1). At baseline, the study population was 88% male, 45% EA, 26% AA, 29% Other/Unknown, and 67% were ≥45 years old (Table 1). There were 56,055 patients with sleep disorders (8.4% of the study population); sleep apnea and insomnia were most common (46% and 40% of all sleep disorders, respectively). Sleep disorder patients were more likely to be African American (30% vs. 26%), male (92% vs. 88%) and divorced (19% vs. 8%) compared to those without a sleep disorder (Table 1). The average (± SD) duration of a sleep

disorder diagnosis was 7.2 ± 3.1 years. Patients with a sleep disorder had an average of 66 ± 98 sleep-related treatments, whereas those without a sleep disorder had an average of 21 ± 56 treatments. The average follow-up time among all patients in the cohort was 10.8 ± 2.5 years. There were 18,181 cancer cases diagnosed during the study period (2.7% of the study population), and the average age at diagnosis was 64 ± 10 years.

Crude and adjusted HRs for cancer incidence in relation to sleep disorder diagnoses are presented in Table 2. Patients with any sleep disorder diagnosis, sleep apnea, insomnia, or other types of sleep disorders had increased risks for PrCA, all hypothesized cancers, and other selected cancers (lung, pancreatic, kidney, brain, bladder, liver, ovarian, esophageal, gastric, melanoma). Adjusted HRs for CRC were elevated among those with any sleep disorder or with sleep apnea, whereas there were no statistically significant HRs for female BrCA for any of the sleep disorders (Table 2).

When the duration of time since diagnosis was evaluated, patients with any sleep disorder had statistically significant increased HRs for all, PrCA, CRC, and for other cancers, with adjusted HRs increasing as the time after initial diagnosis increased (Table 3). The results for female BrCA did not indicate a statistically significant increase in cancer risk related to the duration of any sleep disorder (Table 3). The results were similar when the data were grouped by those with only insomnia, sleep apnea, or other sleep disorders (results not shown). When cumulative sleep-related treatments were evaluated, statistically significant increases in cancer risk were observed for each type of cancer that was assessed (including BrCA), and HRs increased with an increasing number of prescriptions and clinical procedures (Table 4).

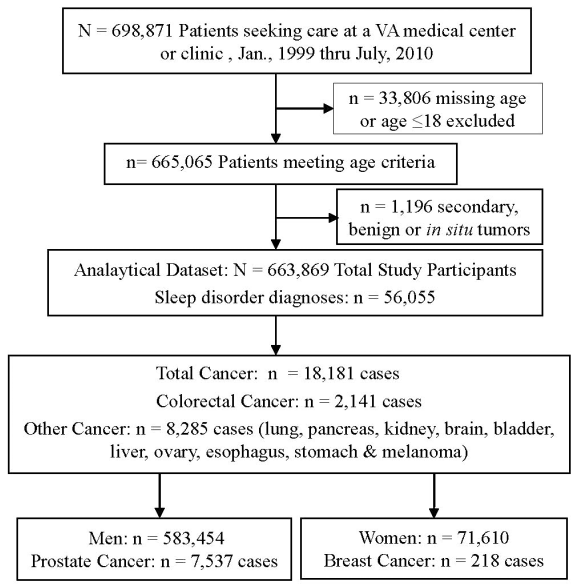

FIGURE 1
Flow diagram of study population after applying inclusion and exclusion criteria. Patients in the Department of Veterans Affairs (VA) Southeast United States Service Network (VISN-7). Sleep disorder and cancer diagnoses were obtained through the VA's Corporate Data Warehouse and Tumor Registry, respectively.

TABLE 1 Population characteristics among veterans in the southeast USA (1999-2010, VISN-7).

Characteristic	Total Population (N = 663,869) % (n)	Sleep Disorder (n = 56,055) % (n)	No Sleep Disorder (n = 607,814) % (n)
Age (years)			
18-34	15.4 (102,501)	13.4 (7,524)	15.6 (94,977)
35-44	17.9 (118,952)	19.4 (10,893)	17.8 (108,059)
45-54	26.4 (174,996)	33.4 (18,714)	25.7 (156,282)
55-64	15.7 (104,475)	16.4 (9,164)	15.7 (95,311)
≥65	24.5 (162,945)	17.4 (9,760)	25.2 (153,185)
Race			
African American	26.2 (173,942)	30.1 (16,884)	25.8 (157,058)
European American	44.9 (298,339)	53.8 (30,141)	44.1 (268,198)
Other[1]	28.9 (191,588)	16.1 (9,030)	30.0 (182,558)
Sex			
Male	87.9 (583,454)	92.4 (51,803)	87.5 (531,651)
Female	10.8 (71,610)	6.8 (3,800)	11.2 (67,810)
Unknown	1.3 (8,805)	0.8 (452)	1.4 (8,353)
Marital Status			
Married	51.5 (342,144)	59.2 (33,159)	50.8 (308,955)
Never Married	7.8 (51,846)	6.2 (3,491)	7.9 (48,355)
Divorced	17.8 (118,148)	19.3 (10,827)	7.7 (107,321)
Widowed	6.9 (45,455)	6.1 (3,394)	6.9 (42,061)
Unknown	16.0 (106,306)	9.3 (5,184)	16.6 (101,122)
Cumulative Sleep-Related Treatments[1]			
None (0)	41.2 (273,450)	9.7 (5,456)	44.1 (267,994)
Few (frequency = 1)	7.8 (51,858)	4.6 (2,574)	8.1 (49,284)
Moderate (2 - 18)	25.9 (171,793)	29.3 (16,428)	25.6 (155,365)

(Continued)

TABLE 1 Continued

Characteristic	Total Population (N = 663,869) % (n)	Sleep Disorder (n = 56,055) % (n)	No Sleep Disorder (n = 607,814) % (n)
Cumulative Sleep-Related Treatments[1]			
Frequent (19 - 1,150)	25.1 (166,768)	56.4 (31,597)	22.2 (135,171)
Cancer Diagnosis			
None	97.3 (645,688)	97.7 (54,784)	97.2 (590,904)
All Cancer	2.7 (18,181)	2.3 (1,271)	2.8 (16,910)
Prostate	1.1 (7,537)	0.92 (515)	1.2 (7,022)
Colorectal	0.32 (2,141)	0.39 (216)	0.32 (1,925)
Female Breast	0.03 (218)	0.03 (15)	0.03 (203)
Other[2]	1.3 (8,285)	0.94 (525)	1.3 (7,760)

[1] Hispanic, Asian, American Indian, Pacific Islanders, or unknown. [2] Includes: lung, pancreas, kidney, brain, bladder, liver, ovary, esophagus, stomach, skin (melanoma). VISN-7, Veterans Integrated Service Network 7 (AL, GA, SC).

When effect modification by race was examined, few differences were noted among race groups for the main analyses that evaluated the risk of a sleep disorder diagnosis (Supplementary Table S1A-D), or for analyses that assessed the duration of sleep disorder diagnoses (Supplementary Table S2). However, when cumulative sleep-related treatments were stratified by race, cancer risk among AAs was elevated relative to EAs, particularly for all cancer, PrCA, CRC, and Other cancers, and among patients in the 'Frequent' cumulative treatment category (Supplementary Table S3). For female BrCA, the trends were similar but there were no statistically significant HRs. Results among those in the Other/Unknown race category tended to be intermediate between the AA and EA groups (Supplementary Table S3).

Discussion

In this retrospective cohort study among 663,869 Veterans in the southeastern USA, patients with a sleep disorder diagnosis had increased cancer risks over an average of 11 ± 2.5 years of follow-up. For those with any sleep disorder, the HR was greatest, though imprecise, for BrCA 1.69 (0.88, 3.24), and was lowest for CRC 1.34 (1.16, 1.54). The results are generally consistent with other studies of cancer incidence in populations with sleep disorders, including studies among patients with moderate to severe sleep apnea or insomnia (8, 16, 17, 25, 31, 36, 37). For example, a national study

TABLE 2 Cancer incidence among veterans with sleep disorders (1999-2010, N=663,869, VISN-7).

Cancer Site	Crude		Adjusted[2]	
	Hazard Ratio	95% CI	Hazard Ratio	95% CI
All Cancer	**Any Sleep Disorder**			
	1.75	(1.66, 1.86)	1.45	(1.37, 1.54)
Prostate	1.73	(1.58, 1.90)	1.50	(1.37, 1.64)
Colorectal	1.52	(1.32, 1.75)	1.34	(1.16, 1.54)
Female Breast	2.87	(1.50, 5.49)	1.69	(0.88, 3.24)
Other[1]	1.75	(1.60, 1.91)	1.45	(1.33, 1.59)
	Insomnia			
All Cancer	1.73	(1.58, 1.89)	1.39	(1.27, 1.52)
Prostate	1.64	(1.43, 1.90)	1.39	(1.20, 1.60)
Colorectal	1.46	(1.17, 1.82)	1.22	(0.98, 1.51)
Female Breast	2.98	(1.21, 7.30)	1.78	(0.73, 4.38)
Other[1]	1.84	(1.61, 2.10)	1.46	(1.28, 1.67)
	Sleep Apnea			
All Cancer	1.70	(1.55, 1.84)	1.44	(1.32, 1.57)
Prostate	1.72	(1.51, 1.96)	1.52	(1.38, 1.73)
Colorectal	1.48	(1.21, 1.81)	1.38	(1.12, 1.69)
Female Breast	3.31	(1.22, 8.99)	1.90	(0.69, 5.17)
Other[1]	1.59	(1.39, 1.82)	1.37	(1.20, 1.57)

(Continued)

TABLE 2 Continued

Cancer Site	Crude		Adjusted[2]	
	Hazard Ratio	95% CI	Hazard Ratio	95% CI
	Other Sleep Disorders[3]			
All Cancer	1.68	(1.46, 1.94)	1.47	(1.28, 1.70)
Prostate	1.69	(1.35, 2.11)	1.54	(1.23, 1.92)
Colorectal	1.53	(1.08, 2.16)	1.41	(0.99, 1.99)
Female Breast[4]	–	–	–	–
Other[1]	1.68	(1.35, 2.09)	1.47	(1.18, 1.83)

[1] Includes: lung, pancreas, kidney, brain, bladder, liver, ovary, esophagus, stomach, skin (melanoma). [2] Adjusted for: age, sex (except for gender specific cancers), marital status, state or residence. CI: confidence interval. [3] Includes: hypersomnias, parasomnias, circadian rhythm sleep disorders, movement disorders, arousal disorders. VISN-7, Veterans Integrated Service Network 7 (AL, GA, SC). [4] Data too sparse for evaluation.

among Veterans with sleep apnea reported an approximate doubling of cancer risk after a median follow-up of 7.4 years (25). To the authors' knowledge, no other study has assessed cancer risk in relation to the duration of a sleep disorder diagnosis over time, and there were incremental increases in cancer HRs with increasing sleep disorder duration for each type of cancer that was evaluated, suggesting a form of dose-response. In other studies, increased cancer risks were observed in relation to subjective or short-term quantitative sleep disruption measures, but not necessarily in those with a sleep disorder diagnosis. For example, multiple studies have evaluated nightly sleep duration in relation to cancer risk. Short duration sleep (usually defined as ≤6 hours per night) has been associated with PrCA (33), BrCA (48), CRC (49, 50), and total cancer (51), and long nightly sleep has been linked with increased risk of BrCA (34), CRC (50, 52) or other cancers relative to those with normal nightly sleep (usually 7-8 hours per night) (8, 16, 24, 36). The authors also are unaware of other research that evaluated cancer incidence in relation to cumulative sleep-related treatments as a surrogate for disease severity. In a retrospective cohort study (N=33,711), OSA severity and nocturnal hypoxemia were each associated with an increased incidence of cancer of any type (15% and 30% increased risk, respectively) over a median of seven years follow-up (26). In a recent meta-analysis, a monotonic increase in cancer incidence of any type was observed with progressive increases in OSA severity based on apnea-hypopnea indices, with pooled risk estimates ranging from 1.14 (mild), to 1.36 (moderate)

TABLE 3 Sleep disorder duration and cancer incidence among veterans (N=663,869, 1999-2010, VISN-7).

Cancer Site	Duration of Sleep Disorder[1]	Crude		Adjusted[2]	
		Hazard Ratio	95% CI	Hazard Ratio	95% CI
All Cancer	Short	1.08	(1.07, 1.10)	1.04	(1.03, 1.06)
	Medium	1.48	(1.39, 1.58)	1.23	(1.16, 1.32)
	Long	2.20	(1.94, 2.50)	1.52	(1.34, 1.73)
Prostate	Short	1.07	(1.05, 1.10)	1.04	(1.02, 1.07)
	Medium	1.43	(1.28, 1.59)	1.24	(1.11, 1.38)
	Long	2.04	(1.65, 2.53)	1.53	(1.24, 1.90)
Colorectal	Short	1.08	(1.06, 1.11)	1.05	(1.03, 1.08)
	Medium	1.48	(1.31, 1.67)	1.29	(1.14, 1.46)
	Long	2.18	(1.70, 2.79)	1.65	(1.29, 2.12)
Female Breast	Short	1.13	(0.96, 1.33)	1.03	(0.87, 1.23)
	Medium	1.86	(0.82, 4.23)	1.17	(0.49, 2.80)
	Long	3.46	(0.67, 17.85)	1.38	(0.24, 7.83)
Other Cancer[3]	Short	1.08	(1.05, 1.10)	1.04	(1.01, 1.06)
	Medium	1.45	(1.30, 1.61)	1.20	(1.07, 1.33)
	Long	2.10	(1.70, 2.59)	1.43	(1.15, 1.77)

[1] Any sleep disorder of short, medium or long duration since diagnosis (<1-2.2, 2.3-5.2, 5.3-12.4 years, respectively). [2] Adjusted for: age, sex (except gender specific cancers), marital status, state of residence. [3] Includes lung, pancreas, kidney, brain, bladder, liver, ovary, esophagus, stomach, skin (melanoma). CI, Confidence Interval; VISN-7, Veterans Integrated Service Network 7 (AL, GA, SC).

TABLE 4 Cumulative sleep-related treatments and cancer incidence among veterans (N=663,869, 1999-2010, VISN-7).

Cancer Site	Variable	Cumulative Treatments[1]	Crude		Adjusted[2]	
			Hazard Ratio	95% CI	Hazard Ratio	95% CI
All	Treatment	–	0.96	(0.96, 0.97)	0.96	(0.95, 0.96)
	Treatment*Time	None	1.01	(1.01, 1.01)	1.01	(1.01, 1.01)
		Few	1.22	(1.20, 1.24)	1.26	(1.24, 1.28)
		Moderate	1.49	(1.45, 1.53)	1.60	(1.54, 1.65)
		Frequent	2.22	(2.09, 2.35)	2.55	(2.38, 2.72)
Prostate	Treatment	–	0.95	(0.95, 0.96)	0.94	(0.94, 0.95)
	Treatment*Time	None	1.01	(1.01, 1.01)	1.01	(1.01, 1.01)
		Few	1.31	(1.27, 1.35)	1.35	(1.30, 1.39)
		Moderate	1.71	(1.61, 1.81)	1.81	(1.70, 1.93)
		Frequent	2.91	(2.58, 3.29)	3.28	(2.88, 3.74)
Colorectal	Treatment	–	0.99	(0.98, 0.99)	0.98	(0.97, 0.99)
	Treatment*Time	None	1.00	(1.00, 1.01)	1.00	(1.00, 1.01)
		Few	1.10	(1.06, 1.14)	1.10	(1.06, 1.15)
		Moderate	1.21	(1.11, 1.30)	1.22	(1.12, 1.33)
		Frequent	1.45	(1.24, 1.70)	1.49	(1.25, 1.77)
Female Breast	Treatment	–	0.99	(0.97, 1.00)	0.97	(0.95, 0.99)
	Treatment*Time	None	1.00	(1.00, 1.01)	1.01	(1.00, 1.01)
		Few	1.10	(0.99, 1.23)	1.17	(1.02, 1.34)
		Moderate	1.22	(0.98, 1.51)	1.36	(1.04, 1.78)
		Frequent	1.49	(0.97, 2.29)	1.85	(1.08, 2.18)
Other[3]	Treatment	–	0.97	(0.96, 0.97)	0.96	(0.96, 0.97)
	Treatment*Time	None	1.01	(1.01, 1.01)	1.01	(1.01, 1.01)
		Few	1.20	(1.18, 1.23)	1.24	(1.22, 1.27)
		Moderate	1.45	(1.39, 1.51)	1.54	(1.48, 1.61)
		Frequent	2.09	(1.93, 2.27)	2.38	(2.18, 2.60)

[1] Cumulative sum of each participant's sleep-related prescriptions, clinical procedures, and surgeries during the study period; Few (1 treatment or prescription), Moderate (2 - 18), Frequent (19 - 1,150). [2] Adjusted for: age, sex (except gender specific cancers), marital status, state of residence. [3] Includes: lung, pancreas, kidney, brain, bladder, liver, ovary, esophagus, stomach, skin (melanoma). CI, confidence interval; VISN-7, Veterans Integrated Service Network 7 (AL, GA, SC).

and 1.59 (severe) (27). Results in the current study are similar to these observations. Incremental increases in HRs were observed for each of the cancers evaluated as cumulative sleep-related treatments increased relative to those without such treatment. This may also indicate dose-response although other interpretations are possible. A greater number of treatments may suggest an iatrogenic effect of increased cancer risk among those with extended sedative or hypnotic prescription medication use (53), or it may reflect different behaviors, such as heightened health care vigilance or an increased probability of tumor detection by a clinician.

Regardless of the interpretation, cancer risks among those with more sleep-related treatments were greatest among AAs. These results should be interpreted cautiously because evidence for effect modification by race was seen only in analyses of sleep disorder severity, and because all Veterans should have equal access to care. Nonetheless, cancer surveillance data indicate that Veteran AAs have elevated rates of gastric, hepatic, and PrCA incidence relative to EAs (41, 42), which merits further investigation. Racial cancer disparities arise from complex biological, social, and behavioral factors that can include discrimination and stress (39, 40, 54–58). AAs may differ in their endogenous circadian timing relative to EAs (59, 60), and they are more likely than EAs to have poor sleep (39, 40), to participate in shiftwork (61, 62), and to have excessive stress (allostatic overload or 'weathering') (54, 63). The autonomic nervous system (ANS) is coupled to sleep and other circadian processes (64–66) as well as the stress response (57, 63, 65, 67, 68). Sleep disturbances elicit stress and chronically can disrupt the ability of the ANS to modulate sympathetic nervous system activity (69, 70). A recent study reported an increased odds of metabolic syndrome, a cancer risk factor (71), among those with both poor

sleep and low heart rate variability (HRV, an indicator of dysfunctional autonomic activity), relative to those with normal sleep and HRV (72). This and other evidence indicates that chronic stress leads to a pro-inflammatory state of sympathetic overactivation and ANS dysregulation (73, 74) that can promote tumorigenesis (73–77). These pathophysiological processes may underlie the etiology of racial cancer disparities (57, 58, 63, 64, 68, 74).

This study had some noteworthy strengths and limitations. Strengths include a robust sample size and the use of electronic medical records to ascertain both sleep disorders and cancer diagnoses through the VA's Corporate Data Warehouse and Tumor Registry, which enhanced validity and provided clinically meaningful exposure and outcome data. The population was restricted to patients in the VA health care system in the southeastern US region (VISN-7), which limits generalizability. The exclusion of tumors with an infectious etiology also limits the generalizability of the results among those with 'Other Cancers' since immune dysfunction associated with sleep disturbances and adaptive stress may impact sensitivity to virally mediated cancer progression. Veteran patients are predominantly male (89% in this study population), whereas the distribution of sex in the general population is closer to 50%. For this reason, the statistical power for analyses of BrCA risk may have been insufficient. Veterans who did not utilize a VA medical center, migrated from the study area, or transitioned out of the VA health care system were lost to follow-up, which could have introduced a selection bias. Sleep disorders can occur in heterogeneous populations and prescription use can have multiple indications. The extent to which those factors may influence cancer risk is uncertain. Also, the presence of undiagnosed sleep disorders or other sleep disturbances in the study population may have resulted in exposure misclassification that likely resulted in a bias towards the null. A major study limitation was the inability to control for several important cancer risk factors, such as body mass index, smoking, socioeconomic status, comorbid disease, and family cancer history. Additionally, there was no information on either physical activity or diet, both of which influence carcinogenesis processes including inflammation, and are associated with cancer risk (78, 79). However, a recent study among Veterans reported only modest differences between the crude and adjusted HRs for a cancer diagnosis among those with sleep apnea after adjusting for age, sex, year of cohort entry, smoking, alcohol use, obesity, and comorbid disease (crude HR: 2.16, CI: 2.13-2.20 vs. adjusted HR: 1.97, CI: 1.94-2.00) (25). Among its strengths, this study included innovative analyses of the duration of sleep disorder diagnosis and cumulative sleep-related treatments as potential indicators of dose-response. The increasing HRs with increasing duration or severity of sleep disorder reduced the possibility of confounding but does not rule it out entirely. Similarly, the potential for reverse causality was minimized as the duration of sleep disorder diagnosis or the number of sleep-related treatments increased. The results from this study were consistent when the analyses were performed only among those with sleep apnea, insomnia, or other sleep disorder diagnoses. This suggests that sleep disturbances in general may share a common mechanism of carcinogenesis.

Sleep problems tend to be chronic and persistent (80, 81), and trends in sleep disorders have been increasing both in Veterans and in the general population (3, 4). Night work is an extreme and relevant example of how chronic sleep loss can elicit pathological effects that increase cancer risk (82, 83). The weight of evidence documenting linkages between sleep disruption and cancer incidence has continued to expand (8, 16, 17, 25, 31, 36, 37), although some inconsistencies and uncertainties remain that may be due to differences among study designs, populations studied, or other methodological considerations, particularly sleep assessment validity and representativeness, and uncertainties concerning the nature of the dose-response relationship between sleep disturbances and cancer risk. Results from the current analysis support other evidence suggesting that cancer risks associated with sleep disruption may take up to a decade or more to manifest. This has clinical implications and introduces a window of opportunity for intervention. Concerns over long term sleep medication use (53, 84) suggest a need for alternatives that are safe, effective, low-cost and sustainable. One possibility is supplemental use of melatonin, a hormone derived from the essential amino acid, tryptophan, which facilitates sleep timing and other circadian processes. Multiple studies support its use for insomnia and other sleep problems, particularly in older individuals (85, 86). Melatonin also has established antiproliferative, antioxidant, immune enhancing, and anti-inflammatory properties, and evidence for its effectiveness in treating BrCA, PrCA, CRC and other cancers is promising (87, 88). However, its role in cancer prevention has not been thoroughly studied. Another promising class of compounds are used for insomnia treatment by acting upon the orexin (hypocretin) receptor system that modulates sleep/wakefulness (89). These agents selectively induce apoptosis in several tumor types and thus have promising therapeutic potential (8). Other alternative sleep disorder treatments include interventions that act upon ANS activity, such as HRV biofeedback, which is effective at improving sleep and reducing inflammation using a nonpharmacological, mechanism-driven technique (resonance frequency breathing) that can reduce sympathetic overactivation and promote ANS homeostasis (73, 74, 90, 91). Additional research is needed to further optimize the timing, frequency and intensity of these and other innovative sleep promoting, cancer prevention strategies in order to fully characterize their effectiveness and long term benefits.

In summary, the results of this study are consistent with other data indicating a link between sleep perturbations and cancer risk. Sleep disorder diagnoses and clinical treatments were examined rather than short-term measures of nightly sleep or self-reported sleep quality. Patients with a longer duration or greater severity of sleep disorder diagnosis had the greatest risks. The findings suggest that optimal sleep and appropriate sleep disorder management may represent modifiable risk factors for facilitating cancer prevention. Carefully controlled clinical trials are needed to more rigorously evaluate these possibilities.

Data availability statement

The data analyzed in this study was obtained from USA Department of Veterans Affairs (VA) Corporate Data Warehouse

and MedSAS data files. Requests to access the datasets should be directed to the VA Informatics and Computing Infrastructure (VINCI) office (VINCI@VA.GOV).

Ethics statement

The study was conducted in accordance with applicable laws and regulations governing use of secondary data in human research. Data were coded to protect the identity of study subjects. This study was reviewed and approved by the Institutional Review Boards of the Columbia Veterans Affairs Health Care System (formerly WJB Dorn Department of Veterans Affairs Medical Center) and the University of South Carolina, Columbia, SC.

Author contributions

JBB: Conceptualization, Methodology, Funding acquisition, Project administration, Investigation, Data curation, Software, Formal Analysis, Validation, Writing – original draft, Writing – review & editing. AFD: Conceptualization, Methodology, Project administration, Investigation, Data curation, Software, Formal Analysis, Validation, Writing – original draft, Writing – review & editing. HZ: Conceptualization, Methodology, Investigation, Data curation, Software, Formal Analysis, Validation, Writing – review & editing. ACM: Conceptualization, Methodology, Investigation, Data curation, Software, Formal Analysis, Validation, Writing – review & editing. MAR: Investigation, Data curation, Software, Formal Analysis, Validation, Writing – review & editing. AM: Writing – original draft, Writing – review & editing. SAA: Conceptualization, Methodology, Writing – review & editing. JRH: Conceptualization, Methodology, Writing – review & editing.

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Conflict of interest

Author AD is currently employed by Palmetto GBA, LLC. When the study was conducted, she was employed by the University of South Carolina.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1336487/full#supplementary-material

SUPPLEMENTARY TABLE 1A
All Sleep Disorder Diagnoses and Cancer Incidence Stratified by Race, Veterans in the Southeast USA (1999-2010, VISN-7).

SUPPLEMENTARY TABLE 1B
Insomnia Diagnoses and Cancer Incidence Stratified by Race, Veterans in the Southeast USA (1999-2010, VISN-7).

SUPPLEMENTARY TABLE 1C
Sleep Apnea Diagnosis and Cancer Incidence stratified by Race, Veterans in the Southeast USA (1999-2010, VISN-7).

SUPPLEMENTARY TABLE 1D
Other Sleep Disorder Diagnoses and Cancer Incidence stratified by Race, Veterans in the Southeast USA (1999-2010, VISN-7).

SUPPLEMENTARY TABLE 2
Duration of Sleep Disorder Diagnosis and Cancer Incidence Stratified by Race, Veterans in the SE USA (VISN-7, 1999-2010).

SUPPLEMENTARY TABLE 3
Cumulative Sleep-Related Treatments and Cancer Incidence Among Veterans, Stratified by Race (VISN-7, 1999-2010).

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EDITED BY
Signe Friesland,
Karolinska University Hospital, Sweden

REVIEWED BY
Pavol Dubinsky,
East Slovakia Institute of Oncology, Slovakia
Olgun Elicin,
University Hospital Bern, Switzerland

*CORRESPONDENCE
Elizabeth Cash
liz.cash@louisville.edu

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Evening cortisol levels are prognostic for progression-free survival in a prospective pilot study of head and neck cancer patients

Elizabeth Cash[1,2]*, Isak Beck[1], Brooks Harbison[3], Christy Albert[1] and Sandra E. Sephton[4,5]

[1]Department of Otolaryngology–Head and Neck Surgery and Communicative Disorders, University of Louisville School of Medicine, Louisville, KY, United States, [2]University of Louisville Healthcare–Brown Cancer Center, Louisville, KY, United States, [3]Psychiatry and Behavioral Medicine, Medical College of Wisconsin, Madison, WI, United States, [4]Department of Psychology, Brigham Young University, Provo, UT, United States, [5]Department of Psychological and Brain Sciences, University of Louisville, Louisville, KY, United States

Introduction: Cortisol rhythm disruptions predict early mortality in renal, colorectal, lung, and metastatic breast cancer. In head and neck cancer (HNC), various cortisol indices are known to correlate with adverse psychological and biological (e.g., inflammatory) outcomes, but links to mortality have yet to be demonstrated. We hypothesize that the prognostic value of diurnal cortisol aberrations will hold in HNC. Prior work leads us to predict that flattened or elevated diurnal cortisol profiles will be associated with elevations of serum inflammatory and tumor-promoting cytokines in this population, and that these immune markers would themselves predict poor progression-free survival.

Method: We prospectively recruited a pilot sample of HNC patients (N=40) at a multidisciplinary HNC clinic. Most patients presented with late-stage oral/oropharyngeal cancer, were older than 50, male, and subsequently received combined-modality (surgery and/or radiotherapy with or without chemotherapy) treatment with curative intent. Saliva was collected twice daily for six days to assess diurnal slope, mean, waking, and evening cortisol levels. Serum was assayed for an exploratory panel of inflammatory and tumor-promoting cytokines. Two years post study-entry, disease progression and survivorship status were abstracted from medical records. Bivariate correlations, linear regressions, and Cox Proportional Hazards models tested hypotheses.

Results: Elevations of evening cortisol and diurnal mean levels were each associated with shorter progression-free survival (evening: Hazard Ratio [HR] =1.848, 95% Confidence Interval [CI]=1.057-3.230, p=.031; diurnal mean: HR=2.662, 95% CI=1.115-6.355, p=.027). Bivariate correlations revealed that higher levels of the serum inflammatory marker interferon (IFN)-γ were linked with elevated evening (r=.405, p=.014) and mean (r=.459, p=.004) cortisol. Higher expression of IFN-γ also predicted poorer progression-free survival (HR=4.671, 95% CI=1.409-15.484, p=.012).

Discussion: Elevated evening and diurnal mean cortisol were both prognostic; suggesting cortisol secretion is both dysregulated and elevated among patients who subsequently experienced accelerated disease progression. These exploratory data from 40 HNC patients mirror relationships between cortisol and survival identified among patients with numerous other tumor types. This pilot study highlights the need for research on effects of cortisol rhythm disruption among HNC patients. Future research in larger samples should also examine the role of inflammatory and tumor-promoting factors–both systemically and within the tumor microenvironment–as potential mediators of cortisol rhythm disruption.

KEYWORDS

head and neck cancer, cortisol, progression-free survival, interferon-gamma, circadian rhythm disruption

1 Introduction

Among cancer patients, disrupted circadian rhythms are associated with poor quality of life and accelerated disease progression. Clinical and preclinical studies have linked circadian disruption to cancer initiation and progression (1–3). Paralleling this, poor central circadian control is linked with lower treatment efficacy in patients with cancer (2). Consequently, circadian rhythms are being increasingly considered in cancer research, with recent lines of inquiry examining circadian rhythms as a therapeutic target for new cancer treatments (4).

The hypothalamic-pituitary-adrenal (HPA) rhythm is an endocrine axis serving as the body's principal circadian regulator through modulating the expression of the stress hormone cortisol. Typical HPA rhythm in healthy individuals is characterized by the expression of cortisol at high levels in the morning and nadir levels during sleep (5). Between 30-70% of advanced cancer patients show idiosyncratic changes in cortisol rhythm: unsynchronized peaks and troughs, erratic fluctuations, or consistently high or low levels (6). Viewed in aggregate, diurnal cortisol profiles of advanced cancer patients appear "flattened" (7, 8). Flattened cortisol rhythms are strongly linked with accelerated tumor growth (9–11) as well as early mortality from renal, lung, colorectal, and metastatic breast cancer (7, 8, 12, 13). Notably, elevation of evening cortisol levels - which contribute to flattened diurnal cortisol slopes - are prognostic for shorter survival among patients with ovarian cancer (14).

At the cellular level, cortisol coordinates the circadian rhythms of peripheral cells, including patterns of activity, growth and metabolism, mitosis, DNA repair, apoptosis, senescence, and autophagy. When circadian rhythms are disrupted, changes in these processes can lead to the acquisition of cancer hallmarks by tumor cells and result in rapid proliferation and metastasis, escape from apoptosis, angiogenesis, drug resistance, and cause an immunosuppressive shift in the tumor microenvironment that favors tumor cell proliferation (1, 15–18). Additionally, tumors with the ability to upregulate their own internal circadian clocks grow faster, leading to shorter host survival (19).

Scholars have recently begun to examine relationships between cortisol and oncologic processes among head and neck cancer (HNC) patients. Single time point salivary measures have demonstrated feasibility, including among patients with cancers in the oral cavity and those suffering from xerostomia (20). Studies have revealed that HNC-related elevations in basal cortisol can persist over time (21), can become increasingly pronounced with advancing disease (22, 23), and may be associated with increased incidence of regional head and neck metastases (24). Among newly diagnosed HNC patients, elevated diurnal mean and evening salivary cortisol, measured on a single day, have also been associated with poorer HNC-specific health-related quality of life (25). However, no studies to our knowledge have rigorously assessed diurnal cortisol aberrations among patients with HNC, which requires multiple samples over successive days of data collection (26).

Among HNC patients, aberrations in cortisol indices are known to correlate with adverse psychological and biological outcomes. This may be due in part to the direct effects of cortisol on immune function: Cortisol is an important driver of immune cell trafficking and cytotoxic activity (27–29). In an epidemiological study of HNC patients, diurnal salivary cortisol aberrations–as well as anxiety, depression, poor sleep quality, fatigue, and reduced quality of life–were linked to elevations in the serum inflammatory markers C-reactive protein (CRP) and IL-6 (30). In a case-control study examining a broad array of cytokines, only four analytes differed significantly between HNC patients and controls: patients had lower serum interferon (IFN)-γ, interleukin (IL)-13, macrophage inflammatory protein-1β (MIP-1β); and elevated levels of IFN-γ-inducible protein-10 (IP-10; 31). In contrast, another study that compared data from HNC patients with controls (32) found a different pattern of results, with significantly elevated IFN-γ. IL-13 was not measured, but similar to the study by Kaskas et al. (31), IP-

10 was elevated, as were additional factors including IL-1ra, IL-2, IL-5, IL-6, IL-8, and IL-17. Links between diurnal cortisol, cytokines, and HNC mortality have yet to be demonstrated.

In this prospective pilot sample of newly diagnosed HNC patients, we aimed to rigorously assess diurnal cortisol rhythms, using methods appropriate for reliable calculation of diurnal slope, waking, evening, and diurnal mean cortisol values (26, 33). We hypothesized that disruptions in diurnal cortisol rhythm collected during the diagnostic and treatment planning phase would be associated with poorer subsequent two-year progression-free survival. In secondary, exploratory analyses, we tested associations of diurnal cortisol variables with serum inflammatory and tumor-promoting cytokines; and where appropriate, we tested the prognostic value of these cytokines.

2 Methods

2.1 Participants

Participants in this study were prospectively recruited as part of a pilot study on circadian rhythm disruption among patients with primary HNC. After IRB approval (study #14.0607), patients who were recently diagnosed with new primary HNC were invited to participate during their initial treatment planning appointment at a Multidisciplinary Head and Neck Cancer Clinic. Those who were not fluent in English, did not have pathologically-proven malignancy, would be treated at another facility, had comorbid alcohol abuse, had a history of severe psychiatric illness, lived farther than 120 miles from the treatment center, or were severely immunocompromised (e.g., HIV+; concurrent use of immunosuppressants for organ transplant) were excluded.

2.2 Procedures

Data collection occurred between September 2014 and June 2016. A total of 197 patients were screened; of those, 132 met inclusion criteria. During the initial treatment-planning appointment, eligible participants were approached by study personnel and invited to participate. Reasons for declining included: acute pain or distress (n=6); limited time to complete data collection prior to beginning treatment (n=16); and lack of interest in participation (n=55).

A total of 55 participants enrolled. A blood draw was collected at the time of enrollment for assessment of serum inflammatory and tumor-promoting cytokines. Participants were instructed on how to collect and store saliva samples at waking and bedtime for the subsequent six days. They were also instructed on the use of actigraphy watches, from which data were used to confirm participant adherence to the saliva sample timing instructions. Upon return of data, participants were compensated with a $50 prepaid gift card. Fifteen of 55 patients did not provide complete data; thus, the final sample included 40 participants.

2.3 Measures

2.3.1 Sociocultural and demographic variables

Participants self-reported on demographic characteristics, including age, sex, race, marital status, educational achievement, ethnicity, and annual income.

2.3.2 Clinical variables

Information about participant disease status and treatment was abstracted from medical records, including pack-years of smoking history, cancer stage at diagnosis, cancer site, human papillomavirus (HPV) status, current medications, and subsequent cancer treatment received. Two years post study-entry, disease progression and survivorship status were abstracted from medical records, yielding two variables for use in Cox regressions: tracking time (number of days from study entry to the date of recorded progression of disease or death; or for those with no disease progression or death, tracking stopped at two years/730 days), and status (a dichotomous variable indicating whether or not subjects experienced disease progression or death). The two variables, tracking time and status, were used in survival analyses to indicate progression-free survival.

2.3.3 Cortisol

2.3.3.1 Data cleaning and scoring procedures for diurnal salivary cortisol

Cortisol was collected via salivary sampling at waking and bedtime on six consecutive days. Cotton Salivettes (Sarstedt, Inc.; Newton, NC) were used, and dates and times were recorded by participants at each sample collection. Participants were asked to refrain from eating, drinking, smoking, brushing their teeth, using mouthwash, and chewing gum for half an hour before each sample collection. They were instructed to hold the cotton in their mouths for around two minutes or until saturated to ensure sufficient saliva was collected. Participant adherence to the saliva collection schedule was monitored via actigraphy data (see Salivary Data Collection Procedure Adherence, below). Participants stored completed saliva samples in their refrigerator, typically for one to two days, before returning them to the research team. Upon receipt at the lab, a trained research assistant centrifuged, aliquoted, and stored saliva samples at -80°C.

Assays were conducted using an enzyme-linked immunoassay (ELISA) developed specifically for saliva (Salimetrics, Inc., State College, PA). The lower limit of assay sensitivity was .003 μg/dL. The cortisol inter-assay CV was 5.9% for low controls and 5.9% for high controls; intra-assay CVs were 5.8% for low controls and 2.9% for high controls.

2.3.3.2 Salivary data collection procedure adherence

As participant compliance with salivary data collection procedures/schedule is critical (34), actigraphy data were used to verify salivary data collection procedural compliance. Participants wore an actigraphy watch (Mini-Motionlogger; Ambulatory Monitoring Systems, Inc., Ardsley, N.Y.) during the six days of salivary data collection. Watches were removed in situations where

a watch was not allowed to be worn (e.g., the watch would interfere with medical equipment). There were a few instances wherein the watches were briefly removed (e.g., during a clinical procedure), and minutes of data from those instances were deleted. These instances were few and short in span (e.g., <30 minutes) and did not overlap with saliva collection times.

2.3.3.3 Data cleaning and transformation for cortisol variables

Of the entire array of saliva samples (N=552), n=79 samples were modified: For 6.3% (n=35) of bedtime samples, collection time was modified based on accelerometry data (e.g., if actigraphy showed the patient was sleeping at the minute of self-reported saliva collection; typically the difference was <5 minutes). When self-reported waking saliva collection times differed from the accelerometry-based estimate of first awakening by <15 minutes, accelerometry wake times were favored due to their objectivity; this occurred for 7.9% (n=44) of the wake samples. Finally, 6.7% (n=37) were omitted from analyses based on *a priori* exclusion criteria: values greater than 4 SDs from the mean (n=4) or waking values with large irreconcilable discrepancies between self-reported collection time and accelerometry data (e.g., >15 minutes; n=33). These steps yielded 515 samples for analysis. The average wake sample collection time was 07:57 (SD=2:11), and average bedtime collection time was 23:40 (SD=2:51).

Because cortisol data are typically skewed due to natural circadian-linked elevations in the morning (35), raw salivary cortisol values were log-transformed to meet parametric analytic assumptions. Calculated cortisol variables included diurnal cortisol slope, log mean waking value, log mean bedtime value, and diurnal log mean cortisol. The diurnal cortisol slope was calculated as the unstandardized β of log-transformed cortisol regressed on collection time over all 12 samples. Mean wake values were calculated using all 6 log-transformed wake samples; mean evening values were calculated using all 6 log-transformed bedtime samples. Diurnal cortisol mean was calculated using all 12 log-transformed waking and bedtime samples (26).

2.3.4 Serum inflammatory and tumor-promoting cytokines

A trained phlebotomist drew single blood samples using Vacutainer (Beckton, Dickinson and Company; Franklin, NJ) tubes. Serum was collected as close to study entry as possible, and always prior to definitive chemoradiation. Since biomarkers of interest are known to exhibit circadian patterns of release in systemic circulation (36), the timing of blood draw was restricted as much as possible. Mean blood collection time was 11:38 a.m. (SD=30 min; median=11:41 a.m.); none were postprandial. Serum aliquots were frozen at -80°C within 1 hour of blood draw. A MILLIPLEX MAP Human High Sensitivity T Cell Magnetic Bead Panel immunoassay (MilliporeSigma; Billerica, MA) was used to quantify granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-1β, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17α, macrophage inflammatory protein (MIP)-1α, MIP-1β, and tumor necrosis factor (TNF)-α. Assay plates were imaged on a Luminex 100/200, and data were analyzed using Luminex xPONENT 3.1 software (ThermoFisher Scientific; Waltham, MA). Respectively, the detection limits, intra-assay, and inter-assay CVs were: GM-CSF (1.22, 9.38%, 5.63%), IFN-γ (0.61, 13.82%, 5.18%), IL-1β (0.49, 6.93%, 1.52%), IL-5 (0.49, 3.95%, 5.58%), IL-6 (0.18, 6.41%, 1.82%), IL-7 (0.37, 1.36%, 4.25%), IL-10 (0.73, 2.37%, 2.58%), IL12 (0.49, 2.10%, 3.96%), IL-13 (0.24, 12.71%, 4.48%), IL-17α (0.73, 7.61%, 4.57%), MIP-1α (0.31, 1.73%, 2.72%), MIP-1β (0.92, 4.91%, 5.46%), and TNF-α (0.43, 10.56%, 6.7%).

2.4 Analyses

2.4.1 Primary analysis

Cox proportional hazards models were used to test the hypothesis that disrupted diurnal cortisol rhythms would be associated with poorer two-year progression-free survival.

2.4.2 Secondary analysis

Our *a priori* hypothesis guiding secondary, exploratory analyses was that serum inflammatory and tumor-promoting cytokines would be associated both with cortisol variables and survival outcomes. Bivariate correlations tested associations between cortisol variables, survival variables, and serum inflammatory and tumor-promoting cytokines. Cytokines that were significantly correlated both with any cortisol variable and either survival variable were further explored in secondary analyses using linear regressions, in which cortisol variables were predictors and cytokines were outcome variables. For cytokines that demonstrated a significant association with cortisol in regression analyses, Cox models incorporating both tracking time and status were employed to test the prognostic value of the cytokine for two-year progression-free survival. All statistical tests were 2-sided with alpha set at .05 (SPSS 29; IBM, Armonk, New York). Because these were exploratory analyses, we did not correct for multiple comparisons (37).

Spearman rank correlations were used to assess the contributions of traditional prognostic indicators, including cancer stage, site of disease, age at diagnosis, sex, race, marital status, and tobacco history in pack-years. Those that correlated with both the predictor for each model (e.g., cortisol or IFN-γ) and survival, were considered possible confounds.

3 Results

Demographic and clinical characteristics of the pilot sample are provided in Table 1. The sample was predominantly married White males, who had attended some high school through some college (e.g., vocational degree), and earned less than $70,000 per year. The average number of days between initial cancer diagnosis and enrollment was 7.7 (SD=17.22, range 0-87). Because of changes in practice patterns at our clinic during the time of study recruitment, 12 patients had undergone an extirpative surgical procedure prior to collection of salivary and serum data. However, statistical comparison of summary statistics for each study variable (salivary cortisol indices and serum inflammatory/tumor-promoting

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TABLE 1 Sample characteristics (N=40).

		N	%	Median	M	SD
Age at Diagnosis (range 24 - 73)		40		57.0	56.7	10.9
Pack-Years (range 0 - 80)		40		2.0	16.8	22.5
Male Sex		24	60.0%			
Marital Status	Never married	5	12.5%			
	Married	23	57.5%			
	Divorced	7	17.5%			
	Widowed	3	7.5%			
	Separated	1	2.5%			
Race	White	33	82.5%			
	Non-White	6	15.0%			
Site of Disease	Oral	11	27.5%			
	Oropharyngeal	11	27.5%			
	HPV-positive	9	81.8%			
	HPV-negative	2	18.2%			
	Laryngeal	6	15.0%			
	Other	10	25.0%			
Stage	Early	12	30.8%			
	Late	29	69.2%			
Treatment Received	Surgery Only	6	15.0%			
	Surgery + Radiation	8	20.0%			
	Surgery + Radiation + Chemotherapy	12	30.0%			
	Radiation +/- Chemotherapy	14	35.0%			
Diurnal Cortisol	Diurnal Slope (log(µg/dL/hr))	39		-.092	-.085	.057
	Waking Cortisol (µg/dL)	40		.391	.431	.243
	Evening Cortisol (µg/dL)	39		.092	.178	.265
	Diurnal Mean (µg/dL)	40		.241	.303	.225
Events at Two-Year Follow-Up	Locoregional Recurrence	4	10.0%			
	Distant Metastases	5	12.5%			
	Deaths	2	5.0%			
Two-Year Progression Free Survival, Months (range 0 - 24)		40		6.7	12.2	8.3

*HPV, human papillomavirus.

cytokines) revealed no significant differences between preoperative and postoperative patients (all p's >.099); data from all pre- and post-operative patients were included in analysis.

3.1 Tests of hypotheses

3.1.1 Primary analyses

The proportional hazards model was constructed with diurnal cortisol slope entered as a continuous variable; however, in this small sample, the slope values did not meet assumption of linearity, so the Cox model was reconstructed entering diurnal slope stratified at the median value: below median/steeper slopes N=21, M=-.128, SD=.028; above median/flattened slopes, N=19, M=-.039, SD=.042. Diurnal slope was not significantly associated with progression-free survival (HR=3.495, 95% CI=0.901-13.556, p=.070). Elevated evening cortisol and diurnal cortisol mean were both significantly associated with poorer progression-free survival (evening: HR=1.848, 95% CI=1.057-3.230, p=.031; diurnal mean: HR=2.662, 95% CI=1.115-6.355, p=.027; Figure 1).

3.1.2 Secondary exploratory analyses

Because recent data from our laboratory have highlighted inflammation as a mediator of survival effects in HNC (38), we explored the role of inflammatory and tumor-promoting immune markers in the cortisol–survival analyses. Immune markers included in the analyses are listed in Table 2.

Bivariate Spearman correlations revealed no significant association of any cytokine with diurnal cortisol slope, waking cortisol, or progression-free survival tracking time. However, one of the 13 cytokines studied, IFN-γ, was associated both with cortisol (evening and mean levels) and progression-free survival (status; Table 2), suggesting it may meet criteria for statistical mediation in a larger sample of patients. On this pilot basis, linear regressions revealed higher IFN-γ levels were significantly associated with evening cortisol (r=.405, p=.014) and diurnal mean cortisol (r=.459, p=.004), but not diurnal cortisol slope. Thus, an exploratory Cox regression was performed with IFN-γ entered as a continuous variable. Higher IFN-γ predicted poorer progression-free survival (HR=4.671, 95% CI=1.409-15.484, p=.012).

In assessing for possible confounds, we observed that no clinical or demographic variables were associated with both the predictor and progression-free survival. Thus, no adjustments were made to tests of hypotheses.

4 Discussion

4.1 Diurnal cortisol predicts progression-free survival

Our study provides the first exploratory evidence suggesting that elevated evening and diurnal mean cortisol are prognostically relevant in HNC. These data suggest future studies should test hypotheses that cortisol secretion is both dysregulated and elevated among HNC patients who subsequently experience accelerated disease progression. The current finding is consistent with prior work demonstrating strong links between flattened cortisol rhythms and early mortality among renal, lung, colorectal, and metastatic breast cancer patients (7, 8, 12, 13). Further, results underscore the notion that evening cortisol may represent a key physiological marker of cancer-relevant circadian disruption among patients with HNC, and that evening levels may be an adequate standalone measure of HPA rhythm disruption relevant to survival.

Diurnal HPA rhythms are typically marked by high cortisol levels in the morning hours (peaking 30-45 minutes after first waking) with steady declines in expression level throughout the day until a nadir is reached during sleep. Evening samples may, on their own, be sufficient to discriminate among patients with healthy 'steep' versus unhealthy 'flattened' cortisol rhythms (39). Elevated evening cortisol levels have been shown to predict early cancer mortality among ovarian cancer patients (14). Consistent with this, our pilot data demonstrate that evening cortisol predicts poorer progression-free survival. Figure 2 suggests that at an aggregate level among this small sample of HNC patients, evening cortisol may distinguish steep from flattened cortisol slopes more effectively than waking values. As often noted, variability appears lower in evening cortisol levels in comparison with waking, represented in our figure by the restricted confidence interval for evening cortisol. Had our sample been larger, we may have also observed prognostic effects of diurnal cortisol slope. Further, as different clinical conditions are associated with markedly different profiles of circadian cortisol secretion aberrations, we suggest that future research not exclude waking cortisol levels and slopes. Future, larger studies should also explore the prognostic value and psychophysiological relevance of intraindividual variability in cortisol rhythms, including for example day-to-day slope stability (40).

Our time-consistent six-day schema for cortisol sampling represents a major strength of this study. Robust measurement of chronic physiological stress via diurnal cortisol sampling at consistent points across multiple days (26) grants us heightened rigor and more consistent results. Most studies examining cortisol among HNC patients employ simpler data collection strategies, such as single-day or single time-point cortisol measures (21, 22, 24, 30). Variability in results across studies is compounded by differing time points at which samples are collected and variance in calculation methodologies for cortisol summary variables, making summative conclusions difficult to draw. Moreover, we address a shortcoming of prior cancer research by examining these relationships over the course of multiple days in the short timeframe between cancer diagnosis and treatment planning, a period relatively free of the potential confounds of medically-induced treatment effects.

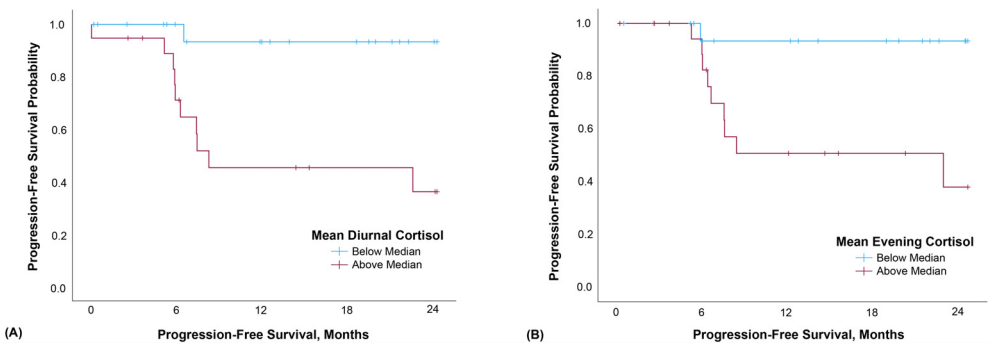

FIGURE 1
Mean diurnal **(A)** and evening **(B)** cortisol were both significantly associated with progression-free survival. Data were split at the median and Kaplan-Meier curves generated for illustrative, not analytic, purposes.

TABLE 2 Raw mean and SD values for an exploratory panel of serum inflammatory and tumor-promoting immune markers and their associations with cortisol and survival parameters.

Analyte (pg/mL)	Mean	SD	Evening Cortisol	Mean Diurnal Cortisol	Progression-Free Survival Status
GM-CSF	54.028	45.404	.189	.254	.193
IFN-γ	11.801	7.192	.335*	.435**	.427**
IL-1β	1.696	1.021	.407*	.373*	.211
IL-5	3.929	5.644	.236	.230	.433**
IL-6	5.015	3.629	.187	.224	.410*
IL-7	12.456	5.296	.322	.323*	.307
IL-10	9.180	7.345	.315	.376*	.254
IL-12	3.414	3.456	.110	.082	-.066
IL-13	36.364	52.989	.098	.122	.088
IL-17α	15.050	13.315	.393*	.408*	.277
MIP-1α	33.152	14.466	.278	.233	.394*
MIP-1β	31.812	11.820	.199	.159	.262
TNF-α	7.629	3.405	.093	.176	.275

Bivariate Spearman correlations between log transformed immune markers, diurnal cortisol measures, and progression-free survival were performed. IFN-γ was the only marker significantly correlated with both diurnal cortisol and progression-free survival and therefore the only marker further explored in secondary analyses.
*p <.05.
**p <.01.

Among cancer patients, multiple factors may contribute to disrupted HPA rhythms. Diagnosis and treatment may exact psychological stress that is frequent and repeated, conveying a high risk for depression and possibly leading to HPA axis dysregulation (41, 42). Among presurgical oral cavity cancer patients, those presenting with greater symptoms of depression exhibit higher circulating levels of cortisol—measured at a single time point—compared to those with benign disease (23). In turn, elevated depressive symptoms predict disease progression and poorer treatment outcomes, including greater neck node metastases (43), poorer tumor response to medical treatment, and poorer overall survival in HNC (38, 44, 45). Depression and concomitant inflammation can exacerbate HPA dysregulation (46). Thus depression and cortisol dysregulation may feed on one another, creating a cycle that negatively influences cancer treatment and survivorship outcomes (46, 47).

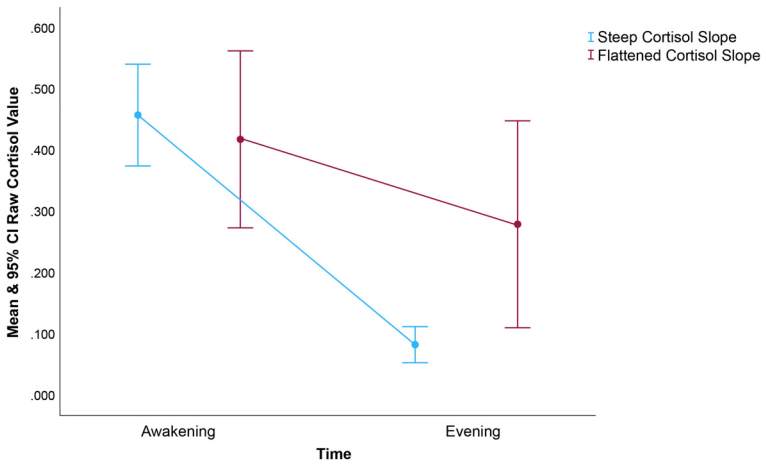

FIGURE 2
Raw mean and 95% CI cortisol based on a median split of the diurnal cortisol slope within our sample. As noted, prior studies found diurnal cortisol slope prognostic for cancer mortality, a finding not replicated in this small (n=40) sample of HNC patients. Rather, we found that only the evening and overall mean cortisol levels predicted survival. To inform future research, we felt it important to describe interrelationships between cortisol summary variables. It's worth noting that evening cortisol appears to distinguish steep from flattened slopes more distinctly than do waking values. The restricted CI for evening cortisol among those with steep, healthier cortisol is also interesting. This figure demonstrates the magnitude of the difference in evening cortisol level associated with differences in cortisol slope.

Sleep disruption is another likely component in the nexus of cancer-related depression, cortisol rhythm disruption, and inflammation (48, 49). Animal studies show that both acute and chronic stress profoundly affect sleep architecture and circadian rhythms (50). Sleep loss delays the recovery of the HPA axis from stimulation, possibly involving an alteration in negative glucocorticoid feedback regulation (51). Finally, tumors are known to disrupt multiple physiological processes including neural, endocrine, metabolic, and immune functions (9). Each of these may impact sleep – comprising multiple potential indirect pathways of cancer effects on sleep and circadian regulation (52, 53).

Preclinical *in vivo* models of cancer have similarly demonstrated that psychosocial factors affect tumor cells, modulate anti-cancer immunity, and impact the tumor microenvironment; providing insights for potential interventions aimed at slowing cancer progression and improving treatment response (54). Among HNC patients, burgeoning evidence points to psychoneuroimmune relationships across various immune markers and tumor-growth factors. In an epidemiological study of HNC patients, IL-6 was observed to be a key marker linked with diurnal salivary cortisol aberrations and anxiety, depression, poor sleep quality, fatigue, and reduced quality of life (30). Among patients with non-HPV associated HNCs, higher reported levels of perceived stress, anxiety and depressive symptoms have been linked to circulating levels of the tumor angiogenesis marker vascular endothelial growth factor (VEGF; 55). Similar patterns have been shown in studies assessing the effects of cortisol exposure, showing that it regulates circadian rhythms of activity, growth and metabolism in peripheral cells; and drives immune cell trafficking and cytotoxic activity (27–29). Given the similarities observed in psychoneuro-endocrine and -immune relationships across multiple cancer types, these pathways warrant further study among HNC patients.

4.2 IFN-γ predicts progression-free survival

These exploratory analyses revealed IFN-γ was associated with progression-free survival in this small sample of newly-diagnosed HNC patients. We view these interesting results with caution for a number of reasons. First, IFN-γ displays both tumor-promoting and tumor-suppressive roles in oncologic processes. This cytokine—produced predominantly by natural killer and cytotoxic T cells—promotes tumor defenses by activating type 1 (cellular) immunity (56) and suppressing type 2 (global allergy and inflammatory) responses (57). As such, IFN-γ has long been considered central in antitumor immunity (58). It is known to enhance immune responses against tumors by upregulating tumor expression of MHC class 1 molecules, making cancer cells easier for immune cells to recognize and destroy (59). IFN-γ also enhances the cytotoxic activity of NK cells and cytotoxic T lymphocytes (60). Indeed, IFN-γ has been used as an adjuvant treatment based on its cytostatic, pro-apoptotic, antiproliferative, and anti-angiogenic functions (58).

Paradoxically, IFN-γ also has a role in tumor promotion: cancer cells exposed to IFN-γ demonstrate increased capability for immune evasion (60). Within the tumor microenvironment (TME), T cell production of IFN-γ creates a widespread cytokine field shared by most tumor cells as well as infiltrating immune cells. Thus, effects of IFN-γ are likely active within a broader tumor-associated cytokine field, rather than by very discrete cytokine hotspots (61). However, concentrations of IFN-γ in the TME determine whether the function of this cytokine is pro- or anti-tumorigenic (58). IFN-γ contributes to tumor promotion or eradication both directly and indirectly by cooperating with other TME mediators, so effects of this cytokine can likely not be understood without considering other aspects of the intracellular milieu (58). With regard to effects on tumor growth, levels of IFN-γ within the TME are probably more informative than are the serum levels measured here. However, serum measurement may still point to valid processes, as changes in serum cytokines may mirror those in the TME in some instances (62, 63). Research designs contrasting systemic biomarker expression against potential cytokine hotspots in the TME will be highly informative.

Among HNC patients, recent research also highlights contrasting findings when assessing IFN-γ: In one study, downregulated serum IFN-γ was noted in patients compared with controls (31). In another, elevated serum levels were observed among node-negative patients who demonstrated better disease-specific survival (32). In light of our observation that elevated IFN-γ associates with worse HNC outcomes, these findings underscore the need for greater clarity into the roles of IFN-γ in HNC.

4.3 Implications

Our results point to additional targets for future examination in larger studies, including circadian rhythm disruption that may extend to rest-activity rhythms and sleep parameters. A recent examination highlighted measurement strategies for estimating circadian disruption, and found that both rest-activity and HPA rhythms were consistently prognostic for cancer mortality (64). In support, our group has previously shown rest-activity rhythms to be prognostic in HNC (44). Disruptions at the cellular level should also be given consideration. For example, telomerase is regulated by the circadian time-keeping machinery, coordinated in peripheral cells by cortisol rhythms (65). In turn, telomerase is a crucial regulator of cancer progression that induces replicative immortality and inhibits apoptosis. As such, it should be a target in future studies of circadian effects on tumor progression (66). To our knowledge, none of these associations have been studied in HNC patients.

Clinically, immunotherapy is becoming increasingly utilized for HNC. Circadian disruption may have deleterious effects on immunotherapy outcomes: HNC patients who receive infusion treatments later in the day (versus early morning when the adaptive immune response may be under better circadian regulation) may suffer suboptimal survival outcomes (67). Potentially compounding the effects of treatment timing misalignment, cortisol dysregulation has the potential to influence immunotherapy efficacy. Rises in circulating glucocorticoids induced by chronic stress lead to a reduced ability to mount effective anti-programmed death (PD)-1-induced anti-tumor

immune responses in mice, and similar effects were observed after corticosteroid administration (68). Patterns parallel to this have been seen among corollary patient samples: higher distress has been associated with elevated circulating glucocorticoids which, in turn, have been associated with suboptimal anti-PD-1 therapeutic response (68), shorter progression-free and overall survival times (69), and altered IFN-γ signaling pathways (70). As these stress-related immune marker and cancer effects have been implicated in immunotherapy outcomes (71), these intriguing findings warrant further, prospective study where enhanced predictive capacity could potentially be furnished through assessment of diurnal fluctuations of endogenous glucocorticoid (i.e., salivary cortisol) expression. Studies of this nature would also allow for better elucidation of the potential for mediation of these effects by IFN-γ.

Our results point to clinically-relevant targets with the potential to improve cancer patient well-being. For example, development of a clinically-available, robust diurnal salivary collection paradigm could provide valuable information to oncologists, as these data might be useful for flagging cases at elevated risk for accelerated disease progression. Similarly, depression stands out as a highly feasible target for renewed clinical attention (38). Behavioral interventions, including cognitive-behavioral therapy (CBT) and Mindfulness-Based Stress Reduction, have shown promise in reducing depression and managing cortisol dysregulation among patients with other cancer types (72–74). Similarly, screening for poor sleep quality among HNC patients using validated sleep evaluation tools - such as actigraphy - can help identify those at risk of persistent poor sleep quality and allow for early, effective interventions (e.g., CBT) targeting sleep disturbances and related symptoms (75, 76), which may in turn offer benefit for managing cortisol dysregulation (74).

4.4 Limitations

This study is limited by the small sample size, small number of subjects who experienced cancer progression and death, and large number of serum inflammatory and tumor-promoting cytokines analyzed. Our methodology did not allow us to comment on whether variations in serum cytokine measurements were a result of host versus tumor effects, nor can we comment on aspects of the tumor microenvironment that may have played a role in tumor response and treatment outcomes. These limitations make it difficult to draw firm conclusions from our data. Instead, our pilot study suggests that rigorous, multi-day diurnal salivary data collection methods are feasible even among HNC patients where potentially high levels of pain and interference from the tumor location have often been considered impediments to data collection.

4.5 Conclusions

This pilot study suggests for the first time that diurnal cortisol expression has prognostic relevance in HNC. We observed that elevated evening and diurnal mean cortisol were both prognostic, suggesting cortisol secretion is both dysregulated and elevated among patients who subsequently experienced accelerated disease progression. Our data represent a valid approach to robustly measuring diurnal cortisol expression among patients with HNC, and add to a growing body of literature suggesting diurnal cortisol aberrations portend poorer cancer outcomes. Future research in larger samples could add more definitive characterization to these findings, elucidate the role of psychosocial factors at this nexus, and further examine the role of inflammatory and immune markers (both systemically and within the tumor microenvironment) as potential mediators of the tumor-promoting effects of cortisol rhythm disruption.

Data availability statement

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

Ethics statement

The studies involving humans were approved by University of Louisville Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

EC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. IB: Investigation, Writing – original draft, Writing – review & editing. BH: Investigation, Writing – original draft, Writing – review & editing. CA: Data curation, Formal analysis, Project administration, Supervision, Writing – original draft, Writing – review & editing. SS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Elizabeth Cash,
University of Louisville, United States

REVIEWED BY
Venkaiah Betapudi,
United States Department of Health and Human Services, United States
Shitong Lin,
Huazhong University of Science and Technology Wuhan, China

*CORRESPONDENCE
Lan Li
kmlanmm@sina.com
Lan Zhang
zhanglan@kmmu.edu.cn

†These authors have contributed equally to this work

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Transcriptome profiling revealed multiple circadian rhythm-related genes associated with common gynecological cancers

Lan Peng†, Meiping Jiang†, Kangming Li†, Shuhui Yu, Chunfang Zhao, Lan Zhang* and Lan Li*

Department of Radiation Oncology, The Third Affiliated Hospital of Kunming Medical University (Yunnan Cancer Hospital, Yunnan Cancer Center), Kunming, China

Background: Studies have shown that more than half of the human genome expression is affected by circadian rhythms, which includes genes involved in cell cycle control, DNA repair and apoptosis that are critical in cancer biology. However, the roles of circadian rhythm-related genes (CRRGs) in cervical cancer (CC) and other common gynecologic cancers remain unclear.

Methods: The transcriptome data and clinical information related to CC and other common gynecologic cancers were extracted from the UCSC Xena and Gene Expression Omnibus (GEO) databases. In this study, the differentially expressed CRRGs of CC (target genes) were obtained, and the functional enrichment analysis of these target genes was performed by "clusterProfiler". Then, the biomarkers of CC were screened out to construct the survival risk model (risk score). Moreover, function and tumor micro-environment (TME) analyses in different risk groups were performed for further study of the potential mechanism of CC. Furthermore, the prognostic value and function analyses of biomarkers in three common gynecologic cancers were performed to reveal the potential agreement or heterogeneity regulations.

Results: A total of 19 target genes were associated with pyrimidine metabolism. The survival risk model was constructed with six biomarkers, including APOBEC3B, CDA, HELLS, RHOB, SLC15A3, and UPP1. Among these, APOBEC3B, HELLS, and SLC15A3 were identified as positive factors, while CDA, RHOB, and UPP1 were identified as negative factors in CC. It is notable that multiple immune-related signaling pathways were associated with the clinical risk of CC, and the immunotherapy sensitivity was worse in the high-risk group. In addition, we found that most of biomarkers had the prognostic values in other common gynecologic cancers. It was notable that the mechanisms by which these biomarkers influence gynecologic cancers were associated with extracellular matrix (ECM) receptor interaction, focal adhesion, etc.

Conclusion: This study identified six circadian rhythm-related biomarkers, including APOBEC3B, CDA, HELLS, RHOB, SLC15A3, and UPP1, which were

associated with the prognosis of CC. The mechanisms by which these biomarkers influence gynecologic cancers were associated with ECM receptor interaction, focal adhesion, and other functions. These findings might help to deepen the understanding of the agreement or heterogeneity of CRRGs in the pathological processes of common gynecologic cancers.

KEYWORDS

gynecological cancers, circadian rhythms, prognosis, biomarkers, function, regulatory mechanism

1 Introduction

Cervical cancer (CC), endometrial cancer, and ovarian cancer are three common gynecologic cancers that pose a significant threat to the health of women worldwide, resulting in substantial economic burdens (1). CC is ranked as the fourth most frequently diagnosed cancer and the fourth leading cause of cancer death in women, with 604,127 new cases of cervical cancer and 341,831 cancer deaths worldwide in 2020. Endometrial cancer and ovarian cancer are the sixth and eighth most common cancers in women globally, respectively, which is also in the forefront of the incidence in the global female population (2). The accelerated process of population aging has resulted in an overall increase in the incidence rate of gynecologic cancers in China in recent years (3, 4). Despite notable advancements in conventional therapeutic modalities, including surgery, radiotherapy, and chemotherapy, the dismal prognosis of recurrent and advanced gynecologic cancer patients persists as a significant challenge in clinical practice (5, 6). Targeted therapy, biological therapy, and immunotherapy have made breakthrough advances in the treatment of this portion of refractory cervical cancer (7, 8). Therefore, it is necessary to identify accurate prognostic biomarkers and molecular targets to achieve accurate and personalized treatment of gynecologic cancer patients.

The term "circadian rhythms" is used to describe the periodic changes in biochemical, physiological, and behavioral functions that occur in almost all eukaryotic organisms in order to adapt to the 24-hour rotation period of the Earth (9). Recent studies have demonstrated that alterations in the expression of circadian rhythm-related genes (CRRGs) are associated with an increased risk, progression and poor prognosis of various diseases, including cancer (10–12). The core circadian rhythm genes Per2 and Bmal1 have been demonstrated to act in a synergistic manner to promote lung tumorigenesis in conjunction with Kras and p53. A deficiency in these genes results in elevated expression of the oncogenic transcription factor c-Myc, which in turn drives glycolysis and glutaminolysis, thereby promoting the proliferation of tumor cells (13). The rhythmic gene NFIL3 has been demonstrated to promote the proliferation and metastasis of tumor cells through the inhibition of NFKBIA transcription and the subsequent enhancement of NF-κB signaling activity (14). A number of circadian rhythm genes have been linked to the prognosis of various cancers, including head and neck squamous cell carcinoma, breast cancer, and liver cancer, have been identified (15–18). In recent years, it has been found that the expression of multiple CRRGs, with PER2 being the most notable, is reduced in CC cells (19). However, there is currently no research examining the prognostic value of CRRGs in CC and other common gynecological cancers.

In this study, we pioneered an innovative approach integrating comprehensive bioinformatics analysis and public data to identify a set of CRRGs that exhibit unique expression patterns in these cancers compared to healthy tissues. Furthermore, a new prognostic model of CC has been constructed, and the relationship between CRRGs and immunotherapy and the tumour microenvironment has been deeply explored. The objective was to gain further insight into the functional mechanisms of CRRGs and to investigate their functional roles in other common gynecologic cancers. This was done in order to address the persistent gaps in understanding the complex interactions between CRRGs and gynecologic cancers, with the aim of developing a deeper understanding of the disease and of therapeutic strategies.

2 Materials and methods

2.1 Data extraction and pre-processing

In this study, 1,280 CRRGs were obtained from the Circadian Gene Database (CGDB) (Supplementary Table 1). The RNA sequencing data, mutation data, survival and clinical information of CC, ovarian serous cystadenocarcinoma (OV) and uterine corpus endometrial carcinoma (UCEC) were downloaded from the Gene Expression Omnibus (GEO), Figshare and UCSC Xena databases. Among them, the GSE7803 dataset contains 21 CC and 10 healthy

control (HC) cervical epithelium samples, and the GSE9750 dataset contains 33 CC and 24 HC cervical epithelium samples. These two datasets were combined and removed batch effect by "sva" R package (version 3.44.0), and the combined dataset was utilized for screening the differentially expressed genes (DEGs). The TCGA-CC dataset contains 291 CC samples with survival information, which was used as the training dataset to screen the biomarkers and build the survival risk model. The CGCI-HTMCP-CC dataset contains 117 CC samples with survival information, which was used as the validation dataset to verify the availability of survival risk model. Furthermore, the GSE168652 dataset contains 1 CC and 1 HC tissue samples, which was the single-cell sequencing dataset and was used to study the cellular localization of the biomarkers. Besides, the TCGA-OV dataset contains 373 OV samples, and the TCGA-UCEC dataset contains 543 UCEC samples with survival information, which were further used to evaluate the prognostic value and function of biomarkers in other common gynecologic cancers.

2.2 Functional enrichment analysis of target genes

In this study, we employed the R package "limma" to identify differentially expressed genes (DEGs) with thresholds set at ($|\log_2 FC| > 1.5$, adj.*p*.value < 0.05). We subsequently compared these DEGs between the 54 cancerous cell (CC) and 34 healthy cell (HC) samples within the merged dataset (20). Then, the differentially expressed CRRGs (target genes) were obtained by intersecting the DEGs and CRRGs using "venn". Besides, the functional enrichment analysis of these target genes was conducted by "clusterProfiler" R package (version 4.2.2) (adj.*p*.value < 0.05) (21).

2.3 Screening of the biomarkers and construction of the survival risk model of CC

In this study, the biomarkers of CC that obtained by univariate cox and least absolute shrinkage and selection operator (LASSO) analyses were screened for constructing the survival risk model (risk score). We utilized univariate Cox regression, also known as univariate Cox proportional hazards modeling, which was a widely adopted statistical technique for examining the impact of a single independent variable on the hazard of an event within survival data. We assessed the validity of the proportional hazards assumption through the application of the Schoenfeld residual test. Additionally, we implemented LASSO regression, known as Least Absolute Shrinkage and Selection Operator, a prevalent regression method designed to manage high-dimensional datasets. By incorporating a regularization term into the regression analysis, LASSO enabled us to both select relevant variables and mitigate issues related to coefficient shrinkage and multicollinearity. The risk score was calculated by the algorithm: $\text{Riskscore} = \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_n X_n$. The Kaplan-Meier (K-M) survival curve, risk curve and receiver operating characteristic (ROC) curve were used to predict the accuracy of survival risk model. And the survival of different groups was compared by Log-Rank test. Moreover, the validation dataset (CGCI-HTMCP-CC dataset) was used to verify the applicability of this survival risk model. Besides, the correlations between risk score and different clinical characteristics (age, stage, grade, pathologic T, M and N) were compared by "wilcoxon".

2.4 Function and tumor micro-environment analyses

In this research, we employed GSVA (Gene Set Variation Analysis), an R package designed to assess the enrichment of gene sets across various samples. The toolkit encompassed a range of methodologies, among which ssGSEA (Single-sample Gene Set Enrichment Analysis) stood out as a non-parametric and unsupervised approach. This method allowed us to compute enrichment scores for gene sets independently within each individual sample. By utilizing these analytical tools, we were able to gain insights into the differential expression patterns of gene sets and their potential implications for biological processes and disease states. The gene-set variation analysis (GSVA) was utilized for studying the Gene Ontology (GO) functions and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the genes in different risk groups by "GSVA" R package (version 1.42.0) (22). On the other hand, the proportions of 28 immune cells and 17 immune reactions between different risk groups were calculated by "single sample gene set enrichment analysis (ssGSEA)" algorithm and compared by "wilcoxon", respectively. The Wilcoxon test is a nonparametric statistical test used to compare the difference in medians between two independent groups of samples. It is applied to the assumption that the data do not satisfy a normal distribution. Next, the correlations between biomarkers and differential immune cells/reactions, and the correlations between risk score and differential immune cells/reactions were calculated by "spearman", respectively. Then, the differences of 48 immune checkpoints between different risk groups were compared for assessing the immune reaction. Moreover, the immunophenotype (IPS) score and tumor immune dysfunction and exclusion (TIDE) score were calculated for assessing the immunotherapy sensitivity. Furthermore, the stromal score and immune score were calculated for assessing the tumor purity.

2.5 Patient cohorts and immunohistochemistry

The study was approved by the Ethics Committee of Yunnan Cancer Hospital (KYCS2024-276). We enrolled 20 recurrent or metastatic cervical cancer patients who were treated with immune checkpoint inhibitors including Cadonilimab, Zimberelimab and

Toripalimab plus chemotherapy or chemoradiotherapy from January 2022 to April 2024. All patients had had formalin-fixed and paraffin-embedded (FFPE) tissues for immunohistochemistry and imaging data for monitoring tumor response to immunotherapy. 16 patients were classified as have clinical complete response (cCR) and 4 patients were classified as progression disease (PD). Patients with partial respond (PR) and stable disease (SD) were not included.

After antigen retrieval, 4 um thick FFPE sections were incubated with primary antibodies against SLC15A3 (1:100, PA5-48477, ThermoFish, America) or CD8 (ready-to-use reagent, ZA-0508, ZSGB-BIO, China) at 4℃ overnight. The next day, slides were incubated with horseradish peroxidase conjugated secondary antibody for 30 min at 37℃, followed by detecting using 3,3′-diaminobenzidine (DAB). SLC15A3 staining was measured using the H-score method. The H-score were calculated as follows: (3 × percentage of strongly stained tumor cells) + (2 × percentage of moderately stained tumor cells) + (percentage of weakly stained tumor cells), ranging from 0 to 300. The density of $CD8^+$ lymphocytes in the tumor were measured by quantifying positively stained cells in five random square areas (1 mm^2 each).

2.6 Drug sensitivity analysis

In addition, the maximum inhibitory concentration (IC50) of 138 chemotherapy drugs in different risk groups was compared to study the drug sensitivity of CC by "pRRophetic" R package (version 0.5) (23).

2.7 Cellular localization analysis of biomarkers

In this study, quality control of the single-cell dataset (GSE168652) was studied by the "Seurat" R package (version 4.1.0) (genes in cell > 200, cell coverage number > 3, 200 < nFeature_RNA < 5,000, nCount_RNA < 12,000, and percent.mt < 15%) (24). The top 2,000 highly variable genes were screened using "FindVariableFeatures" function. The principal component were clustered by "UMAP", and the major cell types were annotated by "SingleR" R package (version 1.8.0) (25). Based on these analyses, the expressions of biomarkers in major cell types were calculated for explaining the cellular localization.

2.8 Prognostic value analyses of the biomarkers and clinical correlation analyses in three common gynecologic cancers

On the one hand, the K-M survival curves of all biomarkers in OV and UCEC were plotted to determine the prognostic value of biomarkers in common gynecologic cancers by "survminer" R package (version 0.4.9). On the other hand, the expressions of all biomarkers in different clinical characteristics groups of CC, OV, and UCEC were compared to study the clinical correlation, respectively. Among them, the clinical characteristics of CC contains age, stage, grade, pathologic T, M and N, and the clinical characteristics of OV and UCEC contain age, stage, grade.

2.9 Function enrichment and mutation analyses of biomarkers in three common gynecologic cancers

The median expression value of each biomarker was used to divide the samples into high and low expression groups, and gene set enrichment analysis (GSEA) was performed to study the KEGG pathways of each biomarker by "clusterProfiler" R package in TCGA-CC dataset, TCGA-OV dataset, and TCGA-UCEC dataset, respectively ($|NES| > 1$, NOM $P < 0.05$, and $q < 0.25$) (20). Besides, the mutation situation of all these biomarkers in the three cancers were analyzed by "TCGAmutations" R package (version 0.3.0), respectively.

2.10 Statistical analysis

All analyses were conducted using R language. Differences between two groups were compared by "Wilcoxon" test. If not specified above, $p < 0.05$ was regarded as statistically significant.

3 Results

3.1 19 Target genes were associated with pyrimidine metabolism

There were 310 DEGs (98 up-regulated and 212 down-regulated) between 54 CC and 34 HC samples in combined dataset (Figure 1A). Then, totals of 19 target genes, including APOBEC3B, APOL1, CDA, DUSP1, EMP1, ESR1, FCGBP, GATM, KANK1, HELLS, IL1R2, MAL, RHCG, RHOB, SLC15A3, SNX10, UPP1, ZNF135, ZSCAN18 were obtained by intersecting 310 DEGs and 1,280 CRRGs (Figure 1B). As the perspective of function, these 19 target genes were significantly enriched to pyrimidine ribonucleoside catabolic process, glycosyl compound catabolic process, and etc. 279 GO functions. Besides, these target genes were associated with pyrimidine metabolism, glycine, serine and threonine metabolism, and etc. six KEGG pathways (Figures 1C, D, Supplementary Tables 2, 3).

3.2 Six biomarkers were used to construct the survival risk model of CC

In this study, six biomarkers, including APOBEC3B, CDA, HELLS, RHOB, SLC15A3, and UPP1 were identified, among them, APOBEC3B, HELLS, and SLC15A3 were positive factors (Hazard

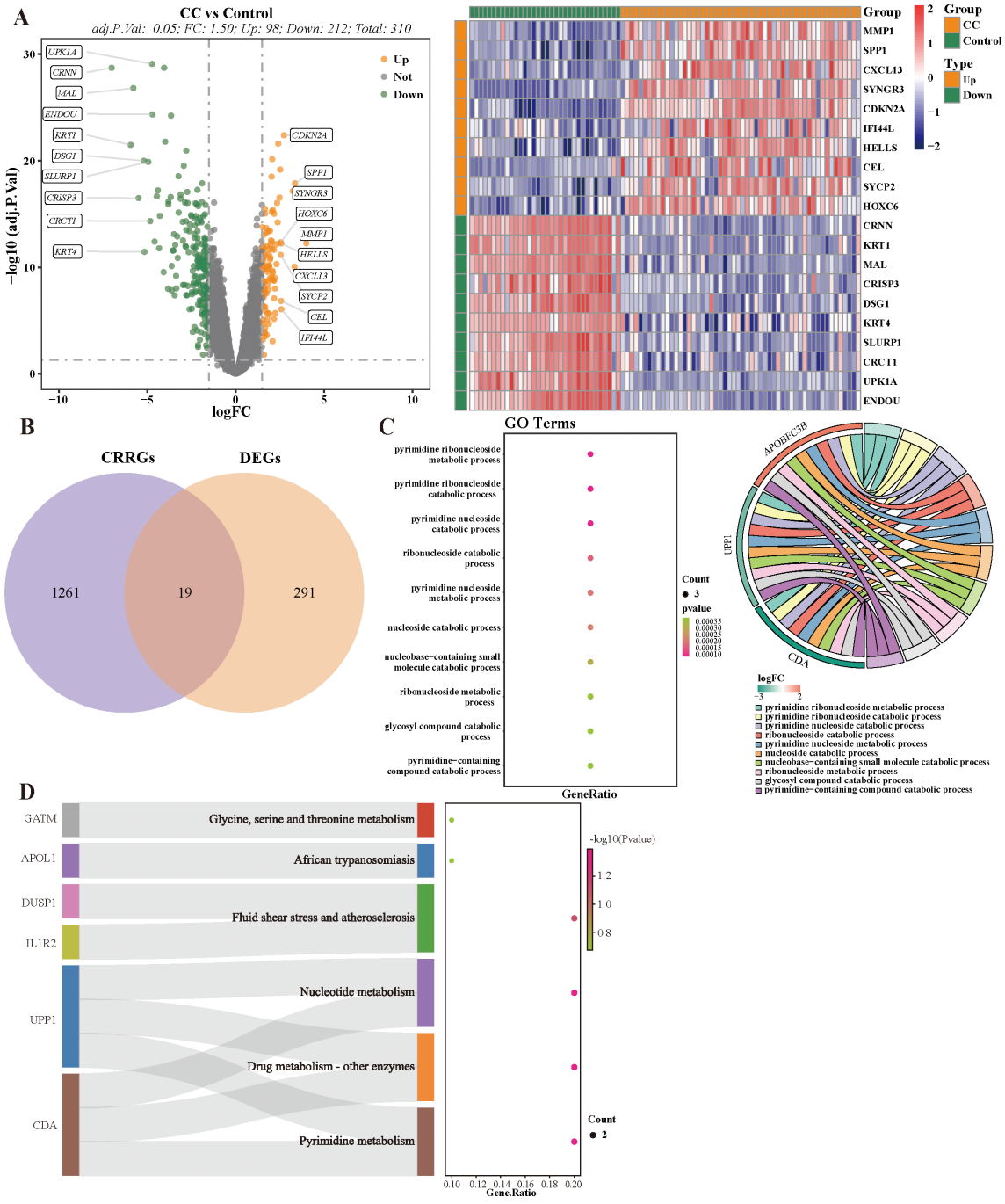

FIGURE 1
Identification and Functional enrichment analysis of candidate CRRGs. **(A)** Volcano plot and heat map of differentially expressed 310 DEGs. **(B)** Venn diagram of CRRGs and DEGs between cervical cancer and healthy control. **(C, D)** Results of GO and KEGG nrichment pathways.

Ratio < 1), and CDA, RHOB, and UPP1 were negative factors (Hazard Ratio > 1) of CC (Figures 2A, B).Then, the survival risk model was constructed by the algorithm: risk score = -0.1629 × APOBEC3B + 0.0142 × CDA - 0.1827 × HELLS + 0.1390 × RHOB - 0.4112 × SLC15A3 + 0.2217 × UPP1. The risk curve and K-M curve showed that there were significant survival differences between these two risk groups (p = 0.000003) (Figures 2C, D). Besides, the area under ROC curves (AUC values) was higher than 0.7 (Figure 2E). Moreover, the CGCI-HTMCP-CC dataset was used to verify the applicability of this survival risk model. The results of risk curve and K-M curve were consistent with the training dataset (Figures 2F, G). These results indicated that this survival risk model was applicable for CC. Besides, the risk score was significantly different between pathologic T1 and pathologic T3 (p < 0.05) (Figure 2H).

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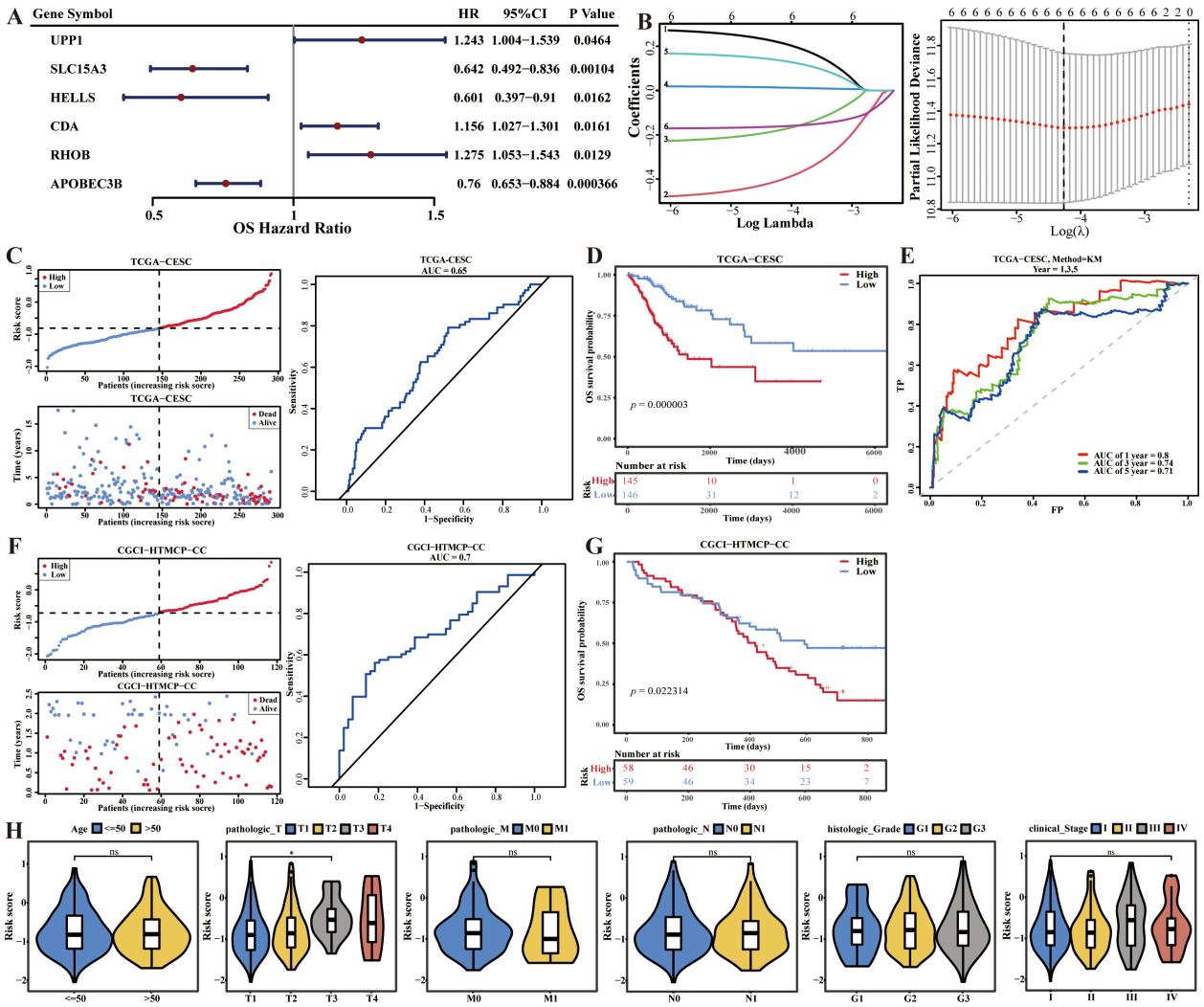

FIGURE 2
Survival risk model of CC construction, validation and evaluation. **(A)** Forest plot for univariate Cox regression analysis. **(B)** Cross-validation curve and lasso coefficient curves of LASSO regression. **(C)** Risk curve, survival status and ROC curve analysis of risk model in the training set. **(D)** K-M curve compares the survival between two risk groups in the training set. **(E)** Time-dependent ROC curves analysis in the training set. **(F)** Risk curve, survival status and ROC curve analysis of risk model in the validation set. **(G)** K-M curve compares the survival between two risk groups in the validation set. **(H)** Correlations analysis of risk scores and clinical traits.

3.3 Multiple immune-related signaling pathways were associated with clinical risk of CC

As the perspective of GO functions, the positive regulation of mast cell chemotaxis, positive regulation of cell adhesion molecule production, interleukin 21 (IL-21) production, BMP signaling pathway, and etc. functions were significantly up-regulated, and the negative regulation of interferon alpha (INF-α) production, microglial cell migration, IL-27 mediated signaling pathway, and etc. functions were significantly down-regulated in high risk group (Figures 3A–C, Supplementary Table 4). As the perspective of (KEGG) pathways, the extracellular matrix (ECM) receptor interaction, focal adhesion, TGF beta signaling pathway and etc. 46 pathways were up-regulated, and B cell receptor signaling pathway, T cell receptor signaling pathway, and etc. 30 pathways were down-regulated in high risk group (Figure 3D, Supplementary Table 5).

3.4 The level of immune escape was higher and the immunotherapy sensitivity was worse in high-risk group

In this study, 28 immune infiltrating cell enrichment scores for CESC samples in the training set were calculated based on the ssGSEA algorithm using the R package “GSVA”. The 28 immune infiltrating cell enrichment scores were plotted as box plots based on comparisons between high and low risk samples. There were seven significantly decreased immune cells (activated B cell, activated CD4 T cell, activated CD8 T cell, effector memory CD8 T cell, immature dendritic cell, immature B cell, and MDSC) and only one increased immune cell (neutrophil) in high risk group ($p < 0.05$) (Figure 4A). Among them, SLC15A3 was significantly positively correlated with the majority of differential immune cells, which perhaps implied that the key mechanism of SLC15A3 in CC was associated with immune-related functions. In addition, there was the strongest significantly

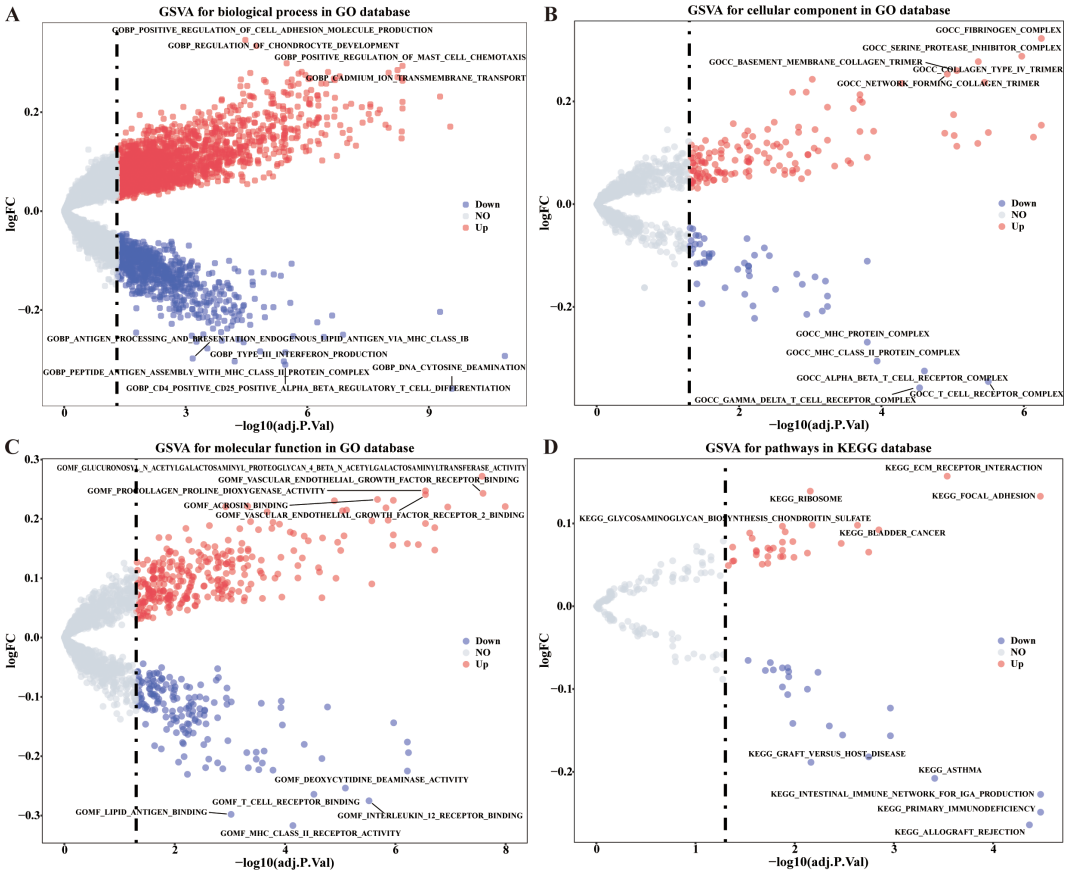

FIGURE 3
GSVA analysis of differentially expressed CRRGs in different risk groups. **(A–C)** Enriched items in GO analysis. **(D)** Enriched items in KEGG pathway analysis.

positive correlation between SLC15A3 and effector memory CD8 T cell (R = 0.64, p < 2.2e-16), and the strongest significantly negative correlation between HELLS and neutrophil (R = -0.3, p = 1.7e-07) (Figure 4B). Spearman correlation analysis was performed to analyze the correlation between 8 differential immune infiltrating cells and risk scores. The results showed that the risk score was significantly positively correlated with the proportions of neutrophil, and was significantly negatively correlated with other seven immune cells decreased in high risk group, which was consistent with their expression results (Figure 4C).

Considering the strong positive correlation between SLC15A3 and CD8+ T cell, we hypothesized that CC patients with high SLC15A3 expression may benefit from immunotherapy. Immunohistochemistry was used to delineate the SCL15A3 protein expression and CD8+ T cell immune infiltrates in a CC immunotherapy cohort in our department. We found higher H-scores of SLC15A3 in the clinical complete response (cCR) CC patients compared with the progression disease (PD) CC patents (p = 0.0225) (Figures 4D, E). The Spearman correlation again demonstrated a positive correlation between SLC15A3 expression and CD8+ T cell densities in CC patients (p = 0.007, r = 0.584) (Figure 4F). Future investigations should be tested to explore whether SLC15A3 could serve as a predictive biomarker for immunotherapy in CC patients.

In addition, 17 immune response pathway enrichment scores were plotted as box plots based on comparisons between high and low risk samples. The expression of three immune responses (cytokines, TGFb family members and TGFb family member receptors) was found to be significantly higher in the high-risk group than in the low-risk group, whereas antigen processing and expression, the BCR signaling pathway, the TCR signaling pathway, and the TNF family member receptor were significantly higher in the low-risk group than in the high-risk group (p<0.05) (Figure 4G). Seven differential immune response pathways and six risk model genes were subjected to Spearman correlation analysis. Among them, Antigen_Processing_and_Presentation had the strongest positive correlation with SLC15A3, with a correlation coefficient of 0.53. Cytokines had the strongest negative correlation with HELLS, with a correlation coefficient of -0.43 (Figure 4H). Similarly, the correlation results between risk score and differential immune reactions were consistent with their expression results (Figure 4I).

In this study, 48 immune checkpoint molecules extracted from the literature. Compare the differences in expression of the 48 immune checkpoint molecules in the training set between high and low risk groups. Thirty-eight immune checkpoints were found to be significantly different between risk groups, with most of the immune checkpoints in the high-risk group being lower than

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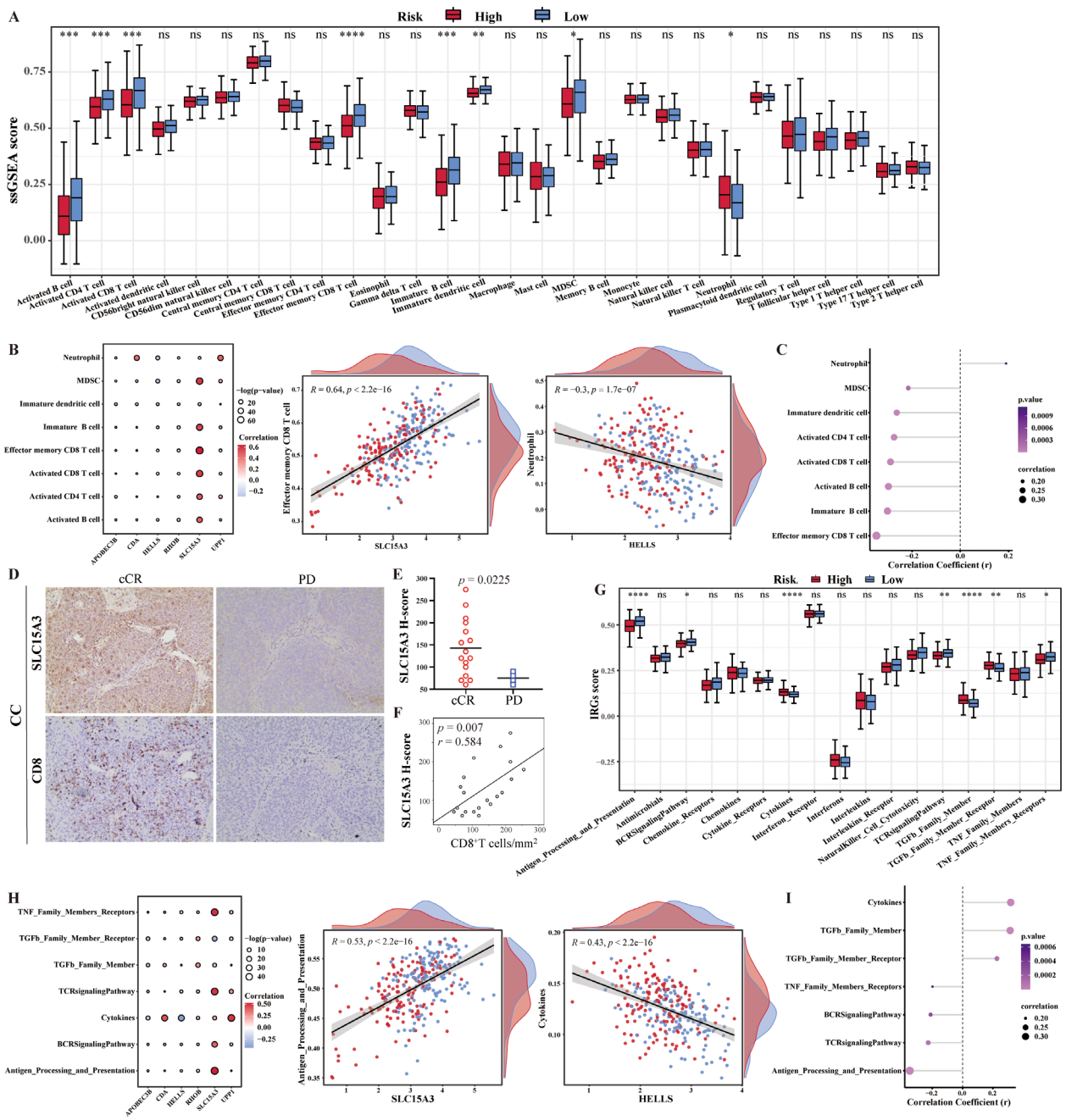

FIGURE 4
Characteristics of tumor immune cell infiltration and immune reactions in different risk groups. **(A)** The box plot showed the difference in immune cells infiltration between the two risk groups. **(B)** Spearman correlation analysis showed the relationship between differential immune cells and 6-CRRGs. **(C)** Analysis of the correlation between risk score and differential immune cells. **(D)** Representative immunohistochemical images of SLC15A3 and CD8 from tumors of progression disease (PD) and clinical complete response (cCR) cervical cancer patients. **(E)** SLC15A3 H-scores in cCR and PD cervical cancer patients. **(F)** Spearman correlation between CD8+ cell densities and SLC15A3 H-scores in cervical cancer patients. **(G)** The box plot showed the difference in immune reactions between the two risk groups. **(H)** Spearman correlation analysis showed the relationship between immune reactions and 6-CRRGs. **(I)** Analysis of the correlation between risk score and differential immune reactions. *p < 0.05, **p < 0.01, ***p < 0.001, ****p <0.0001, ns: No statistical significance.

those in the low-risk group (Figure 5A). Notably, the IPS score was significantly lower and the TIDE score was significantly higher in high risk group ($p < 0.05$) (Figures 5B, C). In addition, the immunization scores were significantly higher in the low-risk group than in the high-risk ($p < 0.05$) (Figure 5D). All these results revealed that the level of immune escape was higher in high risk group and the immunotherapy sensitivity/response was better in low risk group.

In addition, the samples in high risk group were more sensitive to *Bryostatin.1*, *Bicalutamide*, and etc., and the samples in low risk group were more sensitive to *Metformin*, *Mitomycin.C* and etc. ($p < 0.001$) (Figure 5E).

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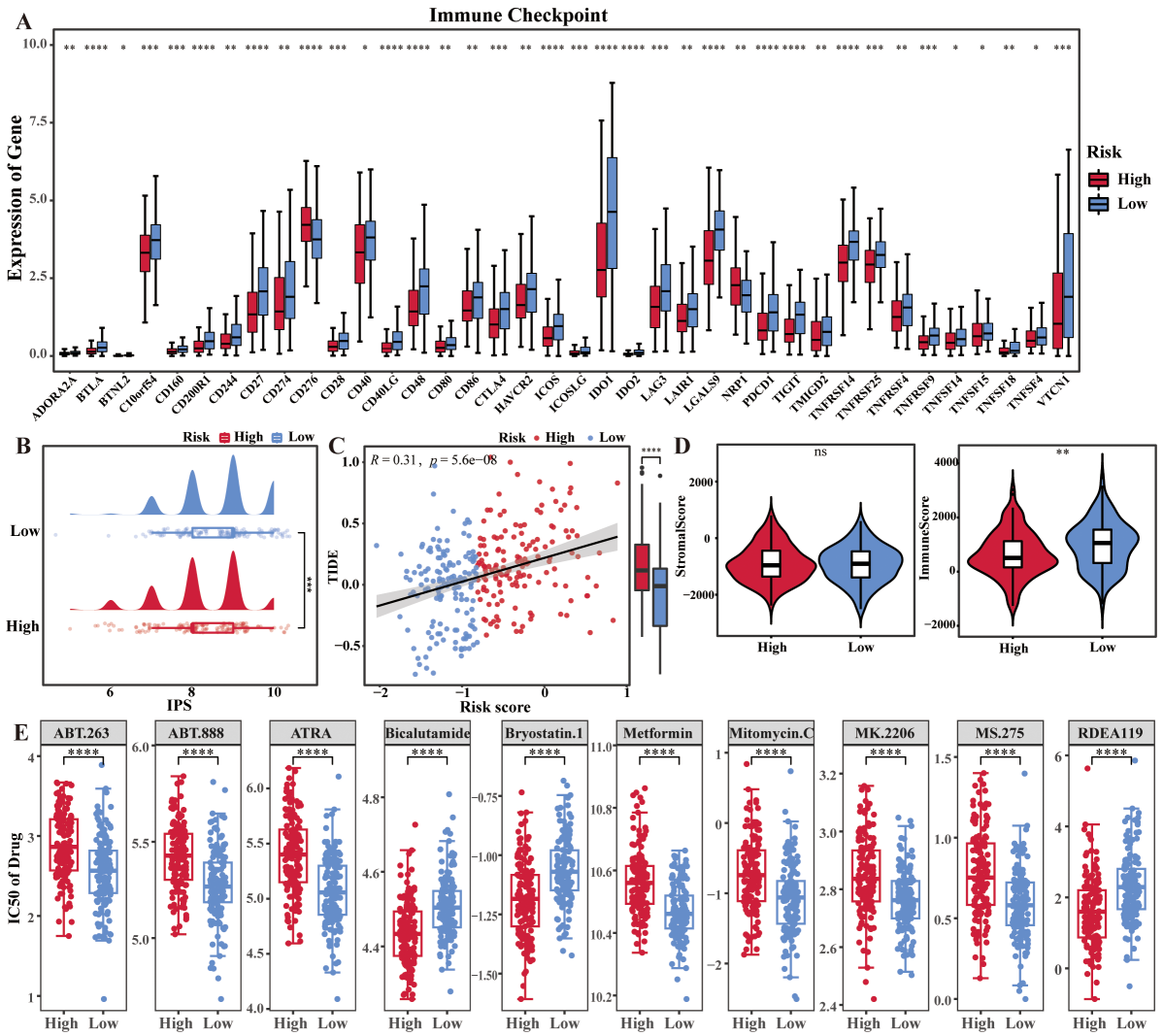

FIGURE 5
Immune escape levels and immunotherapy sensitivity in two risk groups of cervical cancer patients. **(A)** Box plots showed the differences of immune checkpoints in the two risk groups. **(B–D)** IPS score, TIDE score, stromal score and immune score in the two risk groups. **(E)** Drug sensitivity analysis in the two risk groups. *p < 0.05, **p < 0.01, ***p < 0.001, ****p <0.0001, ns: No statistical significance.

3.5 Cellular localization analysis of biomarkers

In this study, cells were classified into 13 clusters and identified as five major cell types, including dendritic cell (DC), endothelial cells, epithelial cells, smooth muscle cells, and T cells (Figures 6A, B). Among them, the proportion of epithelial cells was highest in CC sample, and the proportion of smooth muscle cells was highest in HC sample (Figure 6C). Notably, UPP1 was predominantly localized in epithelial cells, while RHOB was predominantly localized in smooth muscle cells. Compared with healthy controls (HC), RHOB was significantly less expressed in smooth muscle cells, but significantly more expressed in dendritic cells (DC) and T cells in ovarian cancer (OV). Similarly, SLC15A3 was significantly more expressed in DC in OV ($p < 0.05$) (Figures 6D, E).

3.6 The biomarkers also have the prognostic values in other common gynecologic cancers

We used the R package "survminer" to plot the KM curves of model genes based on high and low expression, and to investigate the correlation between model genes and prognosis. The K-M survival analyses results showed that CDA and HELLS were the positive factors ($p = 0.0199$, $p = 0.0324$), and APOBEC3B and SLC15A3 were also the negative factors ($p = 0.0001$, $p = 0.0064$) of OV (Figure 7A), APOBEC3B and UPP1 were the positive factors ($p = 0.0114$, $p = 0.0023$) of UCEC (Figure 7B). Model gene expression in TCGA-CESC based on differences in different clinical indicators (Age, clinical_Stage, pathologic_T, pathologic_M, pathologic_N, histologic_Grade). On the other hand, the expression of CDA was

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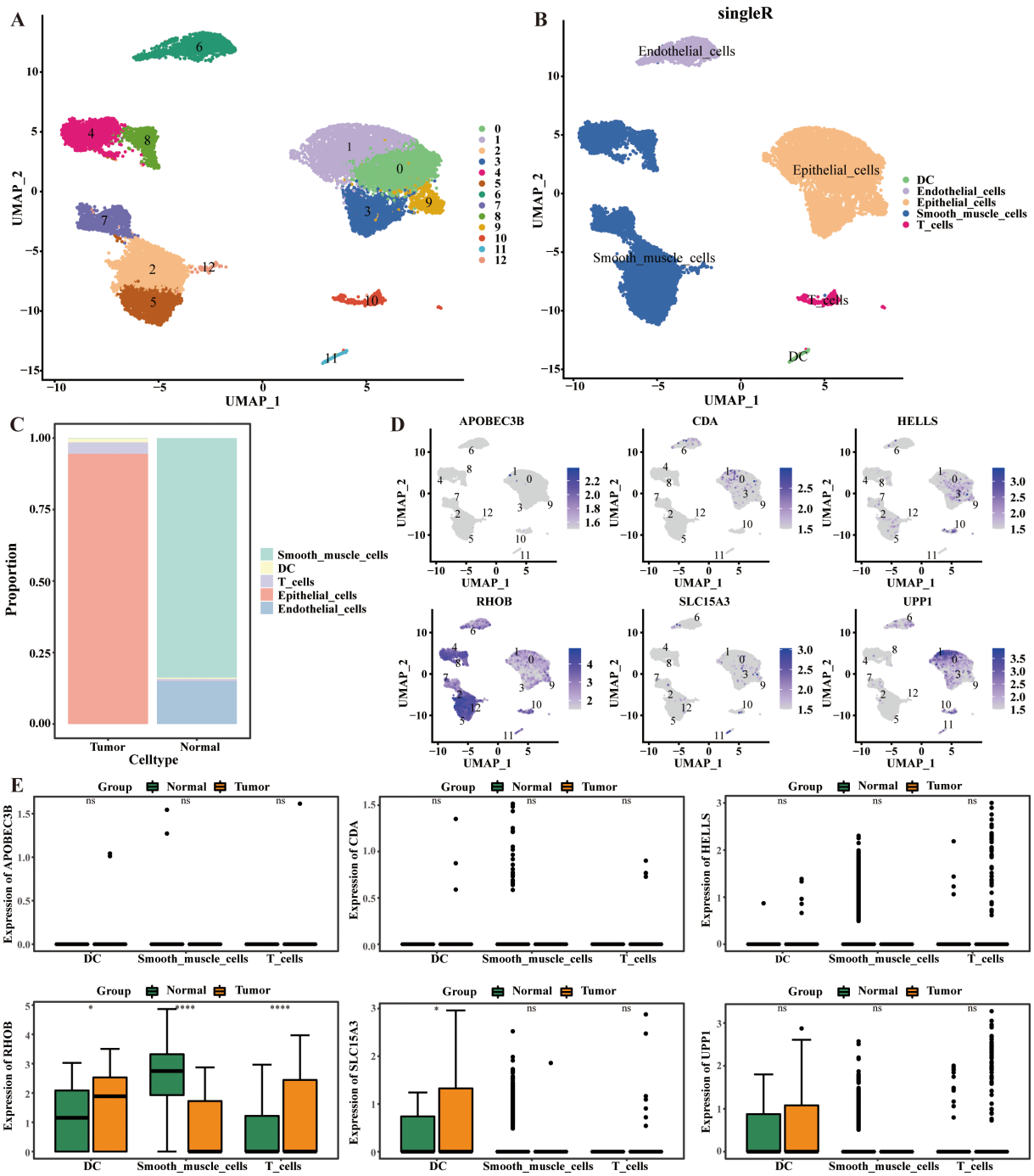

FIGURE 6
Cellular localization analysis of 6-CRRGs. **(A, B)** UMAP visualization in all the cells displayed with different colors for clusters and cell lineages. **(C)** The proportions of various cells in CC and normal cervical tissue. **(D, E)** Expression of 6-CRRGs in major cell types.

significantly higher in age ≤ 50 group than age > 50 group, the expression of UPP1 was significantly higher I n pathologic M0 group than pathologic M1 group in CC ($p < 0.05$) (Figure 7C). The expression of SLC15A3 was significantly higher in grade 3 group than grade 2 group in OV ($p < 0.01$) (Figure 7D). The expressions of APOBEC3B and SLC15A3 were significantly higher and the expression of RHOB was significantly lower in grade 3 group than grade 2 group, and the expression of APOBEC3B was significantly higher in stage IV than stage I in UCEC ($p < 0.05$) (Figure 7E).

3.7 Function enrichment analyses of biomarkers in three common gynecologic cancers

In CC, the ECM receptor interaction was significantly highly enriched in the high CDA, RHOB, and UPP1 expression groups and low APOBEC3B, HELLS, and SLC15A3 expression groups. Besides, all these biomarkers, except for RHOB, were associated with the complement and coagulation cascades, and all these biomarkers,

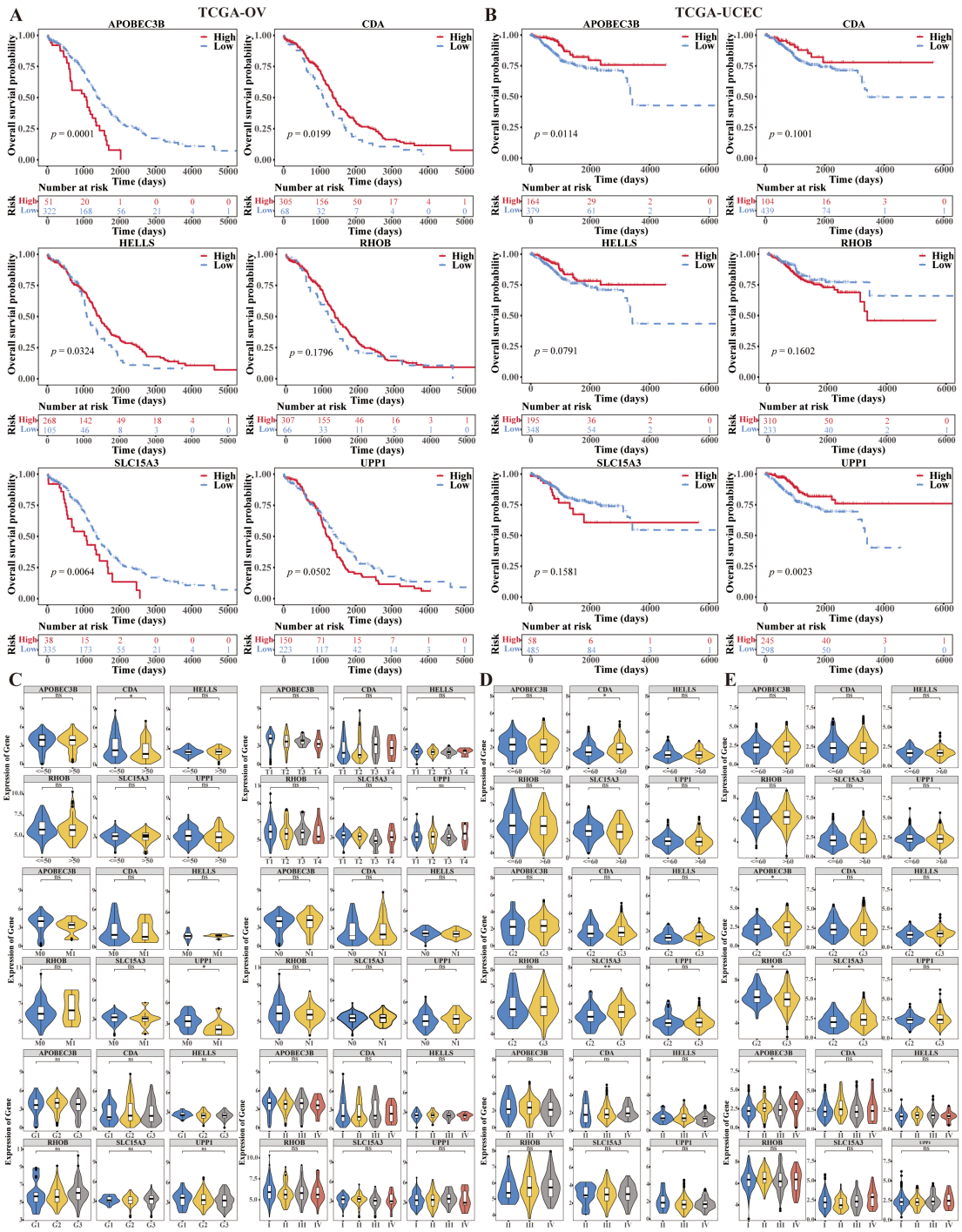

FIGURE 7
Prognostic value of 6-CRRGs in ovarian cancer patients and uterine corpus endometrial carcinoma patients. **(A)** Survival analysis of 6-CRRGs in OV patients between two risk groups. **(B)** Survival analysis of 6-CRRGs in UCEC patients between two risk groups. **(C–E)** Correlation between the expression of 6-CRRGs and clinical characteristics in CC, OV, UCEC patients.

except for SLC15A3, were associated with the focal adhesion (Figure 8A, Supplementary Table 6). In OV, all these biomarkers were associated with the antigen processing and presentation, cell adhesion, chemokine signaling pathway, and etc. KEGG pathways. Besides, all these biomarkers, except for APOBEC3B, were associated with the oxidative phosphorylation, leukocyte transendothelial migration, and JAK STAT signaling pathway, and all these biomarkers, except for HELLS, were associated with the focal adhesion (Figure 8B, Supplementary Table 7). In UCEC, the complement and coagulation cascades were significantly highly enriched in the high CDA, RHOB, and UPP1 expression groups and low APOBEC3B, HELLS, and SLC15A3 expression groups (Figure 8C, Supplementary Table 8).

On the other hand, we found that APOBEC3B was associated with the pathway of cell cycle in all these three common gynecologic

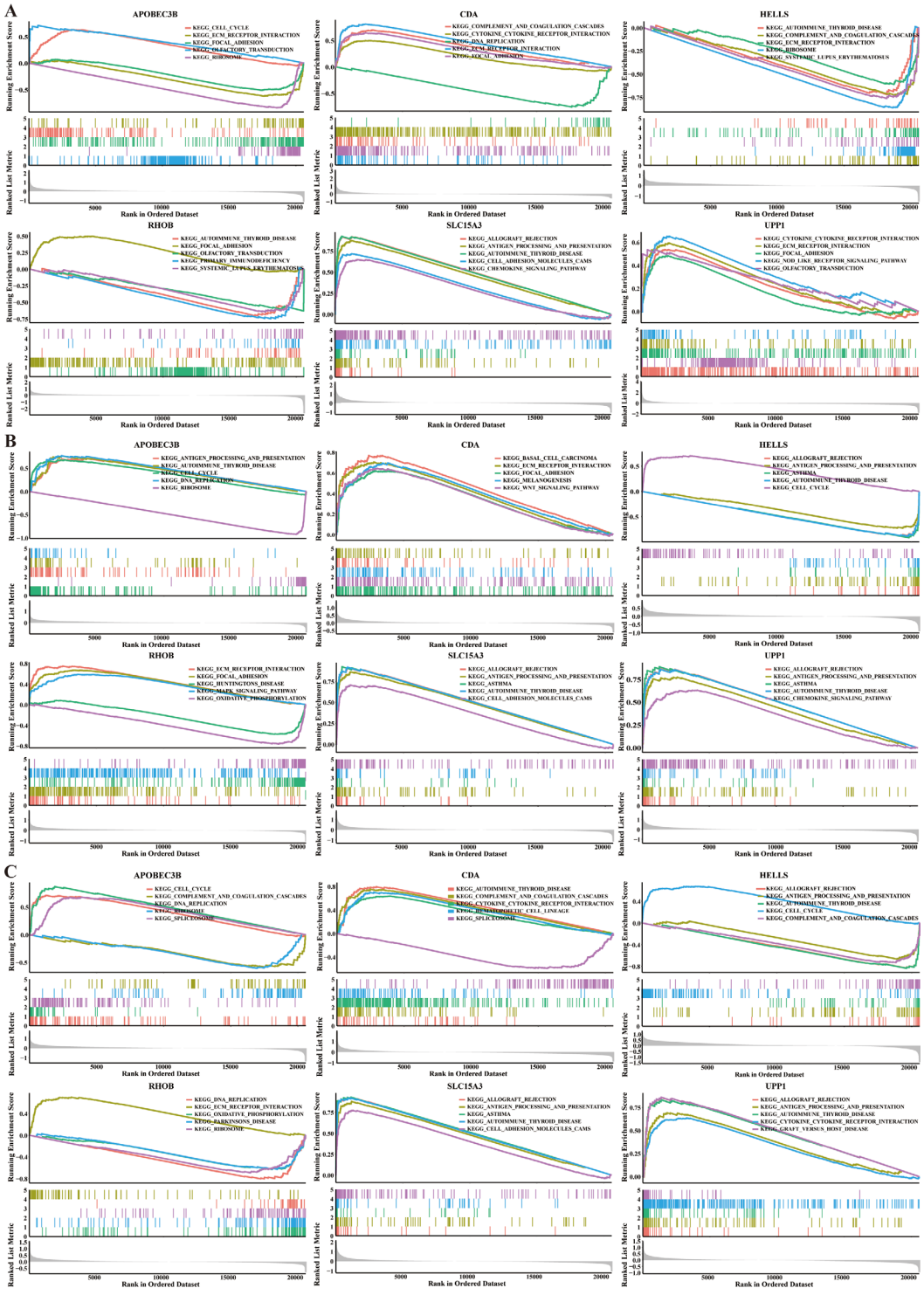

FIGURE 8
The GSEA of biomarkers in three common gynecologic cancers. **(A)** CC. **(B)** OV. **(C)** UCEC.

cancers. CDA was associated with the pathways of ECM receptor interaction, focal adhesion, complement and coagulation cascades, cytokine receptor interaction, etc., in all these three common gynecologic cancers. HELLS was associated with the pathways of complement and coagulation cascades, cell cycle, cell adhesion, etc., in all these three common gynecologic cancers. RHOB was associated with the pathways of ECM receptor interaction, focal adhesion and etc. in all these three common gynecologic cancers. SLC15A3 was associated with the Toll like receptor, Nod like receptor, T cell receptor, B cell receptor, JAK STAT signaling pathways, FC gamma γ mediated phagocytosis and etc. in all these three common gynecologic cancers. UPP1 was associated

with the pathways of Toll like receptor, Nod like receptor, JAK STAT, chemokine signaling pathways, ECM receptor interaction, focal adhesion, etc., in all these three common gynecologic cancers.

3.8 Mutation analyses of biomarkers in three common gynecologic cancers

In the end, the mutation situation of all these biomarkers in the three cancers was analyzed, and the results showed that missense mutation was the major mutation type in CC, OV, and UCEC. Besides, the mutation type of RHOB in CC was frame shift ins, the mutation type of CDA in OV was frameshift insertions (Figures 9A-C).

4 Discussion

Gynecological cancers have imposed a significant burden on women worldwide in terms of public health and the economy. Hermyt et al. (26) have reported that changes in miRNA activity may regulate the elevated expression of circadian rhythm genes NPAS2 and CSNK1D in endometrial cancer tissues. Additionally, they have observed that Clock and PER3 exhibit reduced expression in endometrial cancer tissues. Han et al. demonstrated that the overexpression of CRY1 and NANOG in cervical cancer is significantly associated with poor prognosis and resistance to chemoradiotherapy, indicating their potential as therapeutic targets for CC (27). Another CRRG, PER2, has been confirmed to be indirectly regulated by methylation and exhibits low expression in cervical cancer. Its overexpression inhibits the proliferation of drug-resistant cervical cancer cells through the PI3K/AKT pathway and promotes apoptosis, thereby improving the efficacy of cisplatin chemotherapy for CC (28). Yeh et al. discovered that ARNTL as a highly methylated target in ovarian cancer cells and demonstrated that its overexpression enhances the chemotherapy sensitivity of cisplatin (29). Therefore, it is imperative to explore the CRRGs associated with common gynecological cancers in order to identify potential therapeutic targets.

Bioinformatics is a field of study that integrates techniques from statistics, computer science, and biology. This interdisciplinary approach provides a comprehensive understanding of genetic data, potentially revealing new biological insights that may have been overlooked by single-discipline analyses. Bioinformatics has become an indispensable tool for modern biological research. Pathway analysis in bioinformatics enables the prediction of the effects of genetic variants on biological pathways, thereby providing greater contextual information about disease mechanisms (30). It can predict disease risk, identify drug targets and optimise drug

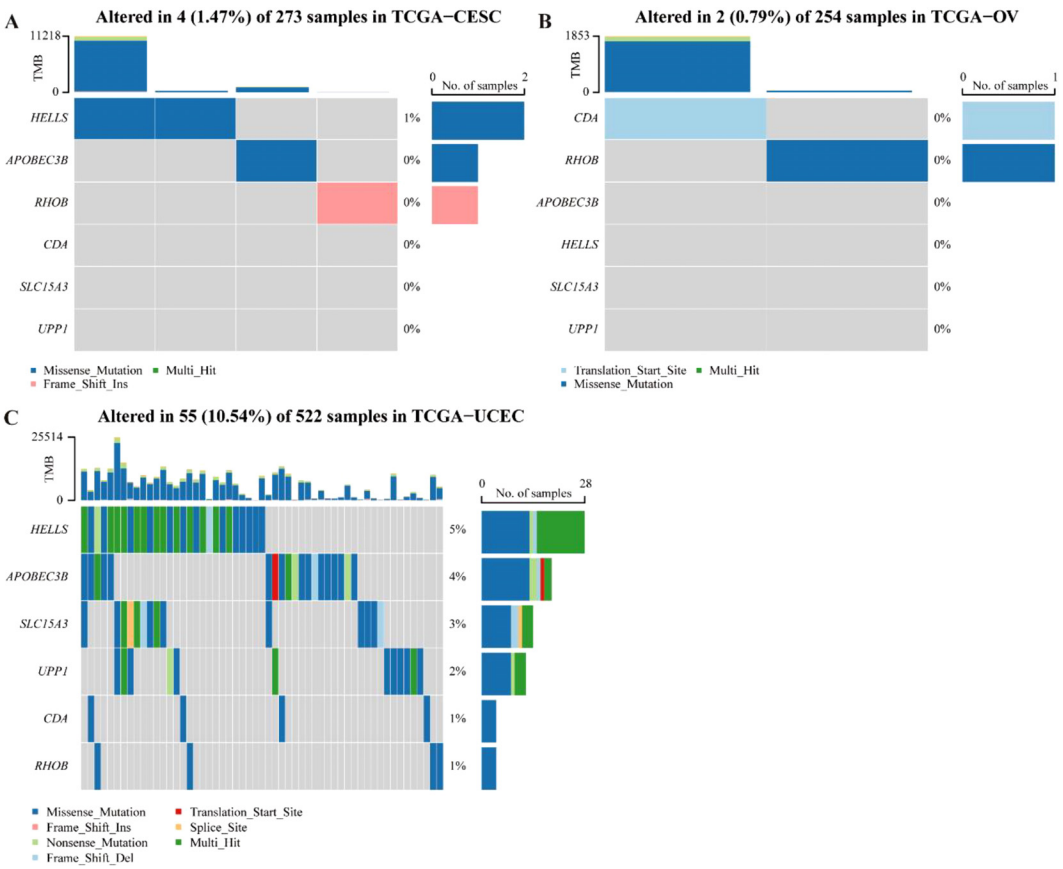

FIGURE 9
Mutation occurrence analyses of 6-CRRGs in three common gynecologic cancers. **(A)** CC. **(B)** OV. **(C)** UCEC.

dosing based on genetic information (31). Some studies have demonstrated that the use of bioinformatics analysis has the potential to identify abnormal *FAM64A* mRNA expression as a biomarker for oncogenesis, progression, invasiveness, and prognosis of gynecological malignancies (32). Similarly, abnormal BAG3 expression has the potential to serve as a marker for tumorigenesis, invasiveness, and prognosis, providing a new avenue for the treatment of cancers (33). Additionally, REG4 mRNA expression has been identified as a potential biomarker for gynecological cancers or a therapeutic target (34). Furthermore, it has been demonstrated that LASSO, in conjunction with bioinformatics analysis techniques, is a valuable approach for the identification of prognostic genes in gynecological cancers (35). This indicates that bioinformatics analysis methods are an effective means of predicting disease risk and identifying drug targets.

In this study, a multi-gene prognostic model based on the HCGA-CC cohort was constructed with good predictive performance, utilizing 6-CRRGs, including APOBEC3B, HELLS, SLC15A3, CDA, RHOB and UPP1. Furthermore, these biomarkers have been demonstrated to possess prognostic value in OV and UCEC. Previous studies have documented the overexpression of APOBEC3B in gynecological cancer (36–38). The overexpression of APOBEC3B has been demonstrated to induce TP53 gene mutation, which has been shown to significantly promote tumor cell proliferation, migration, chemotherapy resistance and recurrence through the p53 pathway (39, 40). HELLS was found to be highly expressed in a variety of cancer cells and serves as a poor prognostic biomarker. HELLS was overexpressed in cervical cancer and promoted the proliferation of cervical cancer by regulating the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) (41). It has been demonstrated that HELLS plays a role in the promotion of homologous recombination of DNA double-strand breaks and contributes to the repair of heterochromatin regions during the G2 phase. The downregulation of HELLS has been demonstrated to inhibit tumor cell proliferation, colony formation, and induce G2/M cell cycle arrest, thereby representing a potential effective treatment for cervical cancer (42–44). UPP1 has been identified as a predictor of poor survival in cervical cancer, with a strong correlation to common carcinogenesis pathways and inflammation-related pathways. It plays a role in promoting tumor aggressiveness and predicting the effectiveness of immune checkpoint inhibitor therapy by modulating the immune microenvironment or mediating immune responses (45). To date, no relevant studies have been identified on other genes in cervical cancer. However, RHOB has been shown to play a role in the development of other cancers. RHOB maintains cell-cell adhesion in epithelial-derived cancer cells by regulating the levels and localization of E-cadherin. Downregulation of RHOB expression has been observed to enhance migration following a decrease in intercellular adhesion, thereby promoting tumor progression (46).

GSVA analysis of differentially expressed CRRGs revealed that the high-risk group was associated with the BMP signaling pathway, focal adhesion, and ECM receptor interaction pathways, among others. It has been demonstrated that the BMP signaling pathway is responsible for the occurrence, migration, and resistance to chemotherapy of ovarian cancer and endometrial cancer. This is achieved by regulating the stemness and epithelial-mesenchymal transition (EMT) of tumor cells. Conversely, the BMP signalling pathway has been shown to have a tumor-suppressing effect on CC (47). Gruber et al. demonstrated that the expression of β3-integrin was present in the majority of cervical cancer patients who underwent radiotherapy, and that the prognosis for patients with positive β3 integrin expression was significantly worse (48). The activated leukocyte cell adhesion molecule (ALCAM) is primarily involved in cell adhesion and signal transduction processes. Abnormal expression of ALCAM has been described in various tumors and is associated with cancer progression (49–51). Ihnen et al. have demonstrated that overexpression of ALCAM in cervical cancer tissue is associated with increased sensitivity to chemotherapy and radiotherapy (52). Zhang et al. found that Twist2 plays a role in the proliferation and invasion of renal cancer cells by regulating the expression of ITGA6 and CD44 in the ECM-receptor interaction pathway. In addition, abnormal activation of the ECM-receptor interaction signaling pathway may contribute to the development of breast cancer (53). Zhang et al. have shown that Twist2 is involved in the progression of renal cancer by regulating ITGA6 and CD44 in the ECM-receptor interaction pathway (54).

It has been demonstrated that demographic information has a significant impact on the risk and prognosis of gynecological cancers. Alimena et al. have assessed differences in cervical cancer survival by age and race, and have shown that young Black women are likely to have a higher stage of disease and lower overall survival (55). Recent studies have indicated the existence of racial/ethnic disparities in HPV infection, which may also be a contributing factor to the observed differences in cervical cancer (56). Furthermore, marital status is indirectly associated with the risk of gynecological cancer, exerting an influence on lifestyle, sexuality and mental health. It has been demonstrated that a woman's marital status is associated with mortality from invasive cervical cancer infection (57). In our study, we combined clinical data and found that the CDA gene was highly expressed in age ≤ 50 group, which may be associated with early tumour development or progression. These findings enhance our comprehension of the biological mechanisms underlying gynecological cancers and furnish crucial insights for investigating the role of CDA in disease prognosis and treatment. They also provide robust scientific evidence in support of personalized and precise prevention, screening and treatment strategies for gynecological cancers.

The role of immune cells in the tumor microenvironment is a key area of research, with a particular focus on their potential influence on tumor occurrence, progression, prognosis, and tolerance to anticancer treatment (58). It has been demonstrated that circadian rhythm disorders are associated with the inhibition of immune cell infiltration (59). In our study, ssGSEA analysis revealed a notable decline in the abundance of seven distinct immune cell types, including activated B cells, activated CD4 T cells, activated CD8 T cells, effector memory CD8 T cells, immature dendritic cells, immature B cells, and MDSCs, in the high-risk group. Conversely, there was a notable increase in the neutrophil population. Matsumoto et al. observed a significant correlation between intratumoral neutrophil density and shorter progression-

free survival (PFS) in patients with cervical cancer (CC) who underwent definitive radiotherapy (60). Subsequent analysis revealed that the high-risk group exhibited a reduced number of immune checkpoints, heightened immune escape, and diminished sensitivity to immunotherapy in comparison to the low-risk group. In contrast with our findings, Wang et al. observed that circadian rhythm genes were dysregulated in glioma, and anti-tumour immunocytes and immunosuppressive cells were significantly enriched in the high-risk group, which were more sensitive to immunotherapy (61). As reported by Chi et al., the majority of immune checkpoints were significantly upregulated in the low-risk group, indicating that patients in this group may benefit from immune checkpoint blockade (ICB) therapy (18). This finding aligns with our own observations. The rhythmic genes identified in our study offer promising avenues for immune checkpoint therapy in patients with gynecological malignancies and may lead to improved prognoses for patients.

The objective of our study is to elucidate the functional role of biomarkers associated with circadian rhythms in common gynecological cancers through a bioinformatics approach. Prior research has indicated a correlation between the severity of endometrial cancer (EC) and nocturnal work and rhythm disturbances (62). This is attributed to the involvement of clock genes (CG), microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) in the etiology of EC (63). The risk of EC was significantly elevated among women who work long rotating night shifts, particularly in the presence of obesity, which doubles the risk (64, 65). Overexpression of the circadian rhythm gene NPAS2 was linked to unfavorable prognosis and clinicopathological characteristics of UCEC (66). In conclusion, the existing literature indicates that common gynecological cancers are associated with CRRGs. Furthermore, a recent study integrated the clinicopathological characteristics of CESC patients with risk scores to construct a Nomogram. The model validated the hypothesis that the 6-ANRG profile and its associated Nomogram may serve as a key factor in the management of patients with CESC (67). Another study constructed a risk score model containing 11 necroptosis-related lncRNAs, which showed potential in predicting the prognosis and immune response of CESC patients (68). The AUC values of the aforementioned models exceeded 0.6, which corroborates the findings of the present study. Collectively, these results substantiate the reliability and validity of these models in clinical applications. In light of the above, the risk model of circadian rhythm-related biomarkers in common gynecological cancers constructed in the present study is therefore deemed to be reliable.

The findings of our study contribute to the understanding of the mechanisms underlying the role of CRRGs in the development of common gynecological cancers. It should be noted, however, that the present study is not without limitations. Firstly, our study examined the expression of circadian rhythm-related genes at the transcriptome level, which may limit the generalizability of the findings in exploring interactions at the global transcript level. Secondly, this study employed a bioinformatics approach to investigate the functional role of biomarkers associated with circadian rhythms in common gynecological cancers. While the effectiveness of this analysis method has been demonstrated in numerous studies, the lack of validation experiments represents a limitation. As a result, the study will be repeated with a larger sample size, more effective methods will be employed, and the results will be further validated through additional experiments, including gene function experiments, immunoprecipitation experiments, protein expression analyses, and studies of transcriptional regulatory mechanisms. This will serve to enhance the depth and significance of the study. Furthermore, a significant number of the datasets employed in this study lack comprehensive clinical and demographic information, which precludes the assessment of the influence of socioeconomic factors on disease incidence and treatment outcomes. In the future, we will consider the impact of genetic variation across different racial and ethnic backgrounds, as well as the direct links between marital status and biological mechanisms, in order to assess the expression patterns of CRRGs in different groups. This could help to identify potential biological differences.

5 Conclusion

In conclusion, our findings indicate that the expression of 6-CRRGs, including APOBEC3B, HELLS, SLC15A3, CDA, RHOB and UPP1, is associated with the prognosis of patients with common gynecological cancers. This is due to their influence on ECM receptor interaction and focal adhesion pathways, among others. Our results may provide new insights into the prognostic mechanisms of common gynecological cancers and potential targets for immunotherapy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author/s.

Ethics statement

This study was reviewed and approved by the Research Ethics Committee of the Third Afliated Hospital of Kunming Medical University (Approval no. KYCS2024-276). Written informed consent was obtained from all participants included in the study.

Author contributions

LP: Writing – original draft, Writing – review & editing. MJ: Writing – review & editing, Data curation, Methodology, Visualization. KL: Writing – review & editing, Data curation, Methodology, Visualization. SY: Data curation, Methodology, Visualization, Writing – review & editing. CZ: Data curation, Methodology, Visualization, Writing – review & editing. LZ: Project administration, Writing – review & editing. LL: Project administration, Supervision, Writing – review & editing

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2025.1422122/full#supplementary-material

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EDITED BY
Pasquale F. Innominato,
University of Warwick, United Kingdom

REVIEWED BY
Tianyun Qiao,
Fourth Military Medical University, China
Qingchao Tang,
The Second Affiliated Hospital of Harbin Medical University, China

*CORRESPONDENCE
Shixin Liu
liushixindoctor@163.com

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Associations between sleep traits and colorectal cancer: a mendelian randomization analysis

Xiangyue Meng[1], Enshuo Fan[1], Dan Lv[1], Yongjing Yang[2] and Shixin Liu[2*]

[1]Department of Traditional Chinese Medicine, Changchun University of Chinese Medicine, Changchun, China, [2]Department of Radiotherapy, Jilin Cancer Hospital, Changchun, China

Background: Although many researches have shown a relationship between sleeping habits and the risk of developing colorectal cancer (CRC), there is a lack of data from randomized controlled trials (RCTs) to support this point. Hence, this study used Mendelian randomization (MR) to robustly assess whether five primary sleep characteristics are directly linked with the risk of CRC occurrence.

Methods: In the performed study, the main Mendelian randomization analysis was conducted using approaches such as Inverse Variance Weighting (IVW), MR Egger, and weighted median method. To this end, five genetically independent variants associated with the sleep-related characteristics (chronotype, sleep duration, insomnia, daytime napping, and daytime fatigue) were identified and used as instrumental variables. Publicly accessible GWAS (Genome-Wide Association Study) data were used to identify these variants to investigate the putative causal relationships between sleep traits and CRC. Additionally, we conducted sensitivity analyses to minimize possible biases and verify the consistency of our results.

Results: Mendelian randomization analyses showed that an morning chronotype reduces the risk of CRC with the IVW method, hence, odds ratio (OR) of 1.21 and 95% confidence interval (CI) of 0.67-0.93, which is statistically significant at P = 5.74E-03. Conversely, no significant evidence was found to suggest that sleep duration, insomnia, daytime napping, or daytime sleepiness have a direct causal impact on CRC risk according to the IVW analysis.

Conclusions: Findings from our Mendelian randomization analyses suggest that an individual's chronotype may contribute to an increased risk of CRC. It is advisable for individuals to adjust their sleep patterns as a preventative measure against CRC.

KEYWORDS

colorectal cancer, chronotype, sleep duration, insomnia, daytime sleepiness, daytime napping, Mendelian randomization

Introduction

CRC occupies the third position in terms of prevalence among all cancers and is the second most common cause of death related to cancer (1). Data from 2023 show that CRC was diagnosed in approximately 153,020 individuals, resulting in 52,550 deaths. The evolution of surgical interventions and advancements in systemic treatments have enhanced the five-year survival rate for CRC, which has risen from 50% to 65% in various European regions (2). Despite an overarching decrease in CRC's incidence and mortality rates, there's an alarming uptrend in its occurrence among those under 50 years of age (3). Epidemiological studies have consistently identified several lifestyle and dietary elements as potential risk factors for CRC, such as extended periods of sitting, smoking, high alcohol consumption, and diets predominated by red or processed meats (4).

The investigation into the relationship between various lifestyle habits and the risk of cancer has intensified among researchers. Beyond the traditionally acknowledged risk factors such as physical activity levels, dietary habits, tobacco usage, alcohol consumption, and body weight, recent studies have identified sleep patterns—including the amount of sleep and the body's natural sleep-wake cycle—as contributing factors to cancer risk, particularly with breast cancer highlighted in the literature (5, 6). Yet, the limited exploration of how sleep duration and insomnia related to colorectal cancer has remained unclear, with only a handful of observational studies addressing this connection (7–9). Moreover, research delving into the genetic underpinnings of the impact of sleep traits on colorectal cancer is exceedingly rare.

MR represents a statistical methodology that employs genetic variants as instrumental variables for probing the potential causal relationships between exposures and outcomes, utilizing data from observational studies (10). This approach emulates the conditions of a randomized clinical trial through the principle that genetic variants are randomly allocated at the moment of conception (11). This methodology boasts significant advantages. First, it utilizes genetic variation as a form of 'natural experimentation', where the random distribution of alleles at conception inherently disconnects these genetic factors from the environmental and lifestyle variables that frequently obscure the true relationships in observational research. Second, because the sequence and progression of a disease do not alter an individual's germline genetic composition, MR effectively navigates around the pitfalls of reverse causation and confounding variables (12). Thus, MR is recognized as a potent and reliable strategy for elucidating causal links.

In an effort to expand upon previous research, this investigation employed Mendelian randomization, analyzing data from a comprehensive genetic study examining sleep patterns and GWAS concerning colorectal cancer. By exploring the potential causal relationships between sleep behaviors and the risk of colorectal cancer, this research aims to contribute towards the development of more precise prevention and treatment strategies for this disease.

Materials and methods

Data sources

Data pertaining to sleep traits and colorectal cancer have been compiled and made accessible online (See Table 1). This study did not necessitate ethical approval or informed consent, as it exclusively utilized data from previously published sources.

Exposure

Daytime napping

A daytime nap refers to a brief period of sleep occurring during daylight hours. Summary data on the habit of napping were derived from a GWAS that included 452,633 adults of European descent registered in the UK Biobank (13). Participants were surveyed about their napping habits through a questionnaire that inquired, *"Do you engage in daytime napping?"* with the options for responses being *"yes"* or *"no"*.

Chronotype

The term chronotype refers to an individual's inclination to either go to bed and wake up early or stay up late and rise later, with variations between these two extremes, which are also known as circadian preferences. Genetic associations related to chronotype were derived from published GWAS involving 403,195 individuals of European ancestry who were part of the UK Biobank (14). Participants provided information regarding their chronotype by answering the question "Do you consider yourself to be?" with

TABLE 1 Summary of genome-wide association studies (GWAS) datasets in our study.

Phenotype	Author, published year	Consortium	Sample size	PMID	Population
Chronotype	Jones SE et al, 2019 (14)	UKB	403,195	30696823	European
Sleep duration	Dashti HS et al, 2019 (15)	UKB	446,118	30846698	European
Insomnia	Jansen PR et al, 2019 (17)	UKB and 23andMe	1,331,010	30804565	European
Daytime sleepiness	Wang H et al, 2019 (18)	UKB	452,071	31409809	European
Daytime napping	Dashti HS et al,2021 (13)	UKB	452,663	33568662	European
Colorectal cancer	2023	FinnGen	314,193	NA	European

several possible responses, including "Definitely a 'morning' person," "More of a 'morning' than 'evening' person," "More of an 'evening' than 'morning' person," "Definitely an 'evening' person," "Do not know," or "Prefer not to answer".

Sleep duration

The assessment of sleep duration within the UK Biobank involved 446,118 participants of European descent. This parameter was evaluated by querying participants on their total sleep hours over a 24-hour period, requiring responses in whole numbers. On average, participants reported 7.2 hours of sleep daily. For analytical purposes, sleep duration was treated as a continuous variable, which facilitated the categorization into two distinct groups: those with short sleep duration (less than 7 hours) and those experiencing long sleep duration (more than 9 hours). Furthermore, an interval defining normal sleep duration was established for those with 7 to less than 9 hours of sleep (15).

Insomnia

Insomnia, a prevalent sleep disturbance, manifests through challenges in initiating sleep or premature awakenings with subsequent inability to return to sleep, significantly deteriorating individuals' quality of life (16). Analysis of GWAS summary statistics from a combined cohort of 1,331,010 participants, encompassing individuals from the UK Biobank and the 23andMe database, has elucidated a genetic predisposition to insomnia (17). The evaluation of insomnia-related symptoms necessitates that participants respond to the query, "Do you experience difficulty in initiating sleep at night, or find yourself waking up during the night?"

Daytime sleepiness

Insights into the link between daytime sleepiness and genetic factors were derived through analysis of GWAS data, which encompassed a cohort of 452,071 individuals of European ancestry registered with the UK Biobank (18). To evaluate daytime sleepiness, subjects provided responses to queries concerning unintentional sleep episodes, their alertness during the day, and the effort required to stave off sleep while engaged in work or educational activities.

Outcome

The collection of genetic data related to CRC was facilitated through the FinnGen consortium, recognized as one of the preeminent genetic databases across Europe (https://www.finngen.fi/en). This comprehensive study, undertaken within the FinnGen project's scope, involved the participation of 6,509 individuals diagnosed with CRC, juxtaposed against a substantial cohort of 287,137 controls. For researchers and interested parties, the dataset was made accessible through a specific link, designated in Table 1, enabling detailed examination and further analysis.

Study design

In the context of MR studies, it is imperative to adhere to three critical presuppositions (19): Firstly, there should be a robust association between the genetic markers and the exposures (namely, sleep traits). Secondly, these genetic markers must not be influenced by any potential confounding variables. Thirdly, the relationship between the genetic markers and the outcome (in this case, colorectal cancer or CRC) should be mediated exclusively through the exposures (again, sleep traits). This methodology was also recently employed to explore the impact of sleep characteristics on the likelihood of developing various conditions, such as liver cancer and Systemic Lupus Erythematosus (20).

Selection of genetic instruments

In our study to pinpoint optimal IVs for investigating the influence of sleep traits, we meticulously followed a structured protocol. Initially, we sifted through GWAS data to identify significant SNPs, adhering to stringent criteria (P-value less than 5×10^{-8} and r^2 less than 0.1), ensuring only the most relevant genetic markers were considered. To control for the influence of weak IVs and potential distortion of results, the F-statistic formula, $F = R^2(n - k - 1)/k(1 - R^2)$, was used, where 'n' is the total number of individuals in the exposure group, 'k' is the number of IVs used, and R^2 is the proportion of variance in the exposure An F-value less than 10 was considered as a poor relationship between the IVs and the exposure which might result in the bias in the analysis. To maintain the independence of the chosen IVs, we checked LD among the SNPs, with the r^2 threshold set to below 0.001 and the clumping distance of 1Mb (21), hence preventing common genetic signals. Finally, we meticulously chose SNPs for sleep traits, which coincided with allele frequencies at CRC outcomes, leaving out any palindromic SNPs to prevent uncertainty. When situations arose where direct SNPs linked with the exposure were absent in the outcome dataset, we performed substitute SNPs that had a high linkage disequilibrium (R^2 greater than 0.8) with pertinent traits, improving the strength and perinatal of our instrumental variable selection.

MR analysis

In the main MR analysis, we used the IVW approach. This approach involves a regression analysis in which the effect of SNPs on the outcome is plotted against their effect on the exposure, ignoring the intercept and using the inverse of the variance of the outcome as weights. Within the IVW framework, it is important to eliminate SNPs that display pleiotropic behavior. This exclusion is critical since the existence of horizontal pleiotropy violates one of the MR basic premises—no horizontal pleiotropic effects. These effects distort the causal inference derived from the MR analysis hence resulting in the identification of wrong causative links (22).

The MR-Egger approach is unique in the sense that it incorporates an intercept in the weighted linear regression analysis, using this intercept to measure the degree of horizontal pleiotropic effects (23). The IVW method, unlike the MR-Egger approach, is useful when genetic variants present with directional pleiotropy. It requires that pleiotropic effects remain uncorrelated with variant to exposure association and also assumes the absence of measurement error. However, it should be pointed out that these method is limited concerning the power of the other methods.

Method of weighted median (WM) requires cause to be linked to at least more than half variables considered effective. This approach, in combination with MR-Egger, helps to improve the estimates given by the IVW procedure.

Sensitivity analysis

A comprehensive sensitivity analysis was performing in order to guarantee the validity and consistency of the results of our research. This method was developed to reveal any hidden biases, with special emphasis on gene pleiotropy and data consistency differences. Our analysis employed two sophisticated methods: and the MR-PRESSO technique and MR-Egger regression. The two approaches are equally good at managing issues that arise from horizontal pleiotropy. Particularly, MR-PRESSO method has a feature that allows the detection of outlier SNPs that can be removed from the analysis. That is why the method is suitable for the detailed evaluation of the influence of isolative SNPs on the results of the study. This stage also provides an opportunity for a critical comparison of the initial results and those which have been corrected for the presence of outlying values (22). Cochran's Q test was used to further investigate the consistency of SNP estimates, and hence any estimate variances. Another important element of our methodology is the 'leave-one-out' sensitivity analysis which, systematically dropped all IVs to test its individual contribution to the aggregated MR estimates. Should the omission of any IV have a significant impact on the MR estimates as compared to the pooled data, the overall results are said to be sensitive to that IV and accordingly, the findings should be interpreted with caution.

We carefully identified secondary phenotypes connected to each SNP specified as an IV using the PhenoScanner to minimize the effect of possible horizontal pleiotropy from the confounding variables. This step entailed elimination of SNPs that could introduce bias in the association of sleep traits and CRC risk factors, subsequently improving the specificity of the analysis (24).

Statistics analysis

In the present research, we applied the approach of Two-Sample Mendelian Randomization (TSMR) to examine the potential causative relationship between sleep characteristics and CRC. This analytical process was facilitated through the application of R software, version 4.4.0, making use of specialized packages: Two Sample MR, version 0.5.6 and MR-PRESSO, version 2.1 (25). The statistical significance threshold for our study was P value less than 0.05 which implied a relationship existed between the variables of interest and the health outcome that was being investigated.

Result

The detailed descriptions of the IVs are provided across Supplementary Tables S1-S5. Supplementary Table S6 elaborates on the specifics of the instrument variables, including their Beta coefficients, standard errors (SE), and P values. The layout of the research methodology is depicted in Figure 1. The MR findings are comprehensively presented in Table 2 and visualized in Figure 2.

Chronotype and CRC

In the study spearheaded by Jones et al. (14),an initial pool of 15,155 SNPs was identified, each possessing genome-wide significance ($P < 5 \times 10^{-8}$) for potential use as IVs. The process of refining this list involved the removal of 15,002 SNPs due to their LD with other genetic variants, along with the exclusion of a duplicate SNP (rs73581564). Additionally, in the phase dedicated to correlating IVs with the outcomes, two SNPs were omitted owing to the lack of corresponding outcome data, and a further duplicate SNP (rs10520176) was eliminated. In the task of synchronizing the exposure and outcome datasets, 26 SNPs were disregarded for their palindromic nature. Consequently, a concise selection of 123 SNPs was finalized for inclusion in the MR analysis. The findings from the IVW analysis suggested a notable positive link between chronotype and the risk of CRC (Odds Ratio: 0.79, 95% CI: 0.67–0.93, P= 5.74E-03), as illustrated in Table 2 and Figure 3. The MR-Egger regression analysis (intercept P=0.81) did not reveal any significant horizontal pleiotropic effects. Furthermore, the MR-PRESSO method identified no outlier SNPs (P = 0.711), as documented in Table 1, and the Cochrane Q test indicated an absence of heterogeneity among the SNPs (Q = 123.72, P = 0.41), also in Table 1. Employing the leave-one-out strategy underscored the resilience of the overall MR findings, even after the sequential exclusion of individual SNPs. Supplementary Table S1 meticulously documents the IV details, affirming no presence of weak instrumental variable bias given that the F statistics for each SNP exceeded 10. The associations persisted after correction for multiple testing.

Sleep duration

In the study led by Dashti et al. (15), an initial collection of 7,924 SNPs, each achieving the threshold for genome-wide significance ($P < 5 \times 10^{-8}$), was gathered. The refinement process, which included a clumping strategy to reduce redundancy and the exclusion of duplicate SNPs, SNPs lacking associated outcome data, and

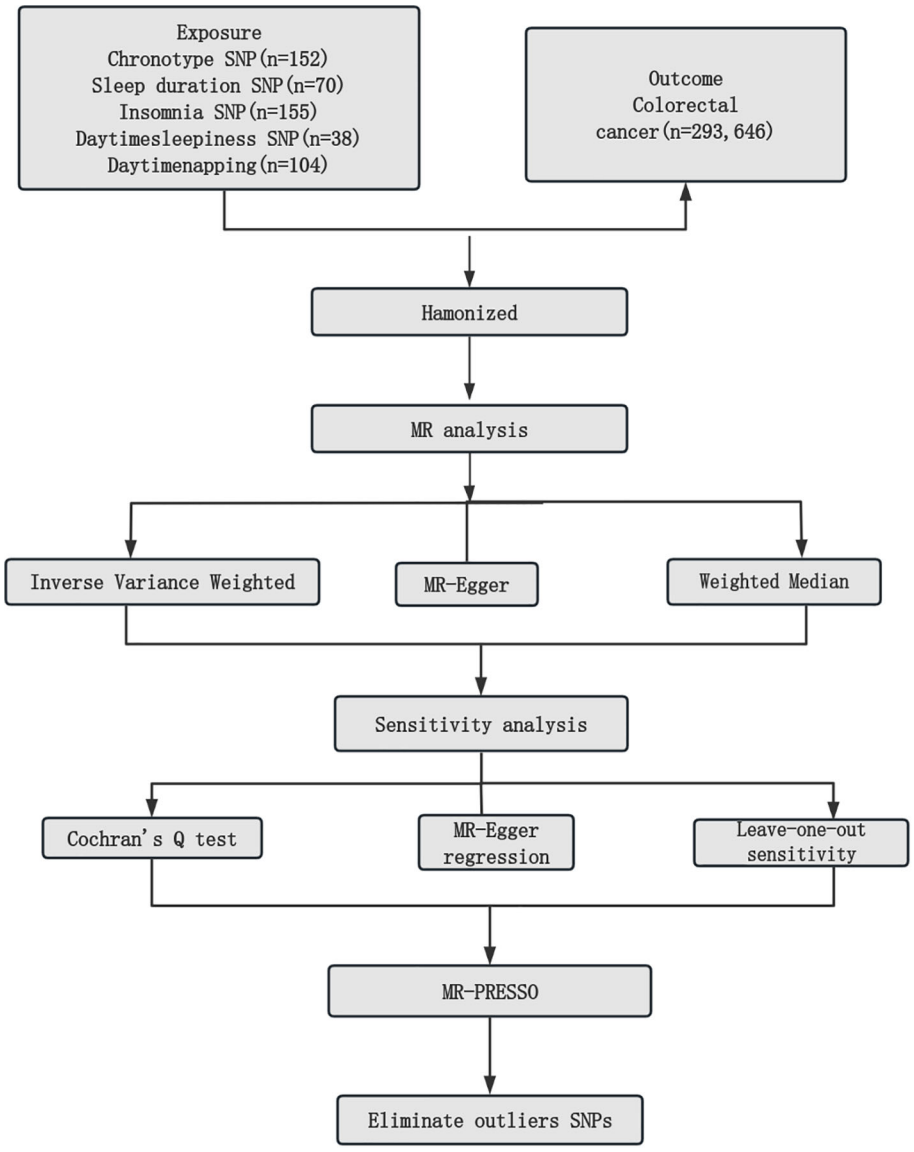

FIGURE 1
Mendelian randomization study workflow. MR, Mendelian randomization; SNPs, single nucleotide polymorphisms; MR-PRESSO, Mendelian Randomization Pleiotropy Residual Sum and Outlier.

palindromic SNPs, ultimately narrowed the pool to 58 SNPs for further investigation. The analysis conducted using the IVW method revealed no statistically significant causal relationship between sleep duration and the risk of CRC (Odds Ratio: 1.01, 95% CI: 0.71–1.43, P = 0.97), as detailed in Table 2 and illustrated in Supplementary Figures S1. Additionally, both the MR-Egger intercept test (intercept P = 0.10) and the MR-PRESSO approach (P = 0.20) found no evidence of horizontal pleiotropy influencing the IV-outcome relationship, as reported in Table 2. The global Q statistic further confirmed the lack of significant heterogeneity among the SNPs (Q=66.27, P = 0.19), as mentioned in Table 2. With F-statistics for all SNPs exceeding 10, the analysis indicated a robust set of instrumental variables, free from the concern of weak instrumental variable bias.

Insomnia

From an initial selection of 228 SNPs known to be linked with insomnia, a rigorous filtering process was applied. This process led to the removal of 73 SNPs due to LD and an additional 2 SNPs were excluded for lacking relevant outcome data. Further refinement was made by eliminating 22 palindromic SNPs during the harmonization of exposure and outcome data. Ultimately, 131 SNPs were deemed suitable for inclusion as IVs in the two-sample MR analysis. The F-statistics from this set of IVs indicated no significant risk of weak instrumental bias, as detailed in Supplementary Table S3. The IVW analysis revealed no statistically significant association between insomnia and the risk of CRC (Odds Ratio: 0.97, 95% CI: 0.89–1.06, P = 0.53), as shown in Table 1 and Supplementary Figure S2.

TABLE 2 MR analysis for the causality of sleep traits with the risk of CRC.

Exposure/Outcome	Nsnp	Methods	*OR* (95%*CI*)	*SE*	*P* value	Horizontal pleiotropy						Heterogeneity		
						MR-Egger regression			MR-PRESSO			Cochran's Q	Q_df	P value
						Egger intercept	*SE*	P value	Global test P value	Global test RSSobs	Outliers			
Chronotype/CRC	152	IVW	0.79 (0.67-0.93)	0.09	5.74E-03	1.37E-03	5.67E-03	0.81	0.42	125.67	_	123.72	121	0.41
		MR Egger	0.74 (0.45-1.23)	0.26	0.25							123.67	120	0.39
		Weighted median	0.77 (0.60-1.00)	0.13	0.04									
Sleep duration/CRC	70	IVW	1.01 (0.71-1.43)	0.18	0.97	-0.02	0.01	0.10	0.20	68.34	_	66.27	57	0.19
		MR Egger	2.89 (0.80-10.50)	0.66	0.11							63.13	56	0.24
		Weighted median	1.24 (0.76-2.03)	0.24	0.37									
Insomnia/CRC	155	IVW	0.97 (0.89-1.06)	0.04	0.53	-8.71E-03	7.13E-03	0.22	0.03	164.44	rs769449 rs910187 rs1580173	162.10	129	0.03
		MR Egger	1.18 (0.86-1.61)	0.16	0.32							160.23	128	0.03
		Weighted median	0.95 (0.84-1.08)	0.06	0.45									
insomnia(after the removal of the outlier)/CRC	155	IVW	0.97 (0.89-1.06)	0.04	0.50	-7.31E-03	6.82E-03	0.29	0.13	146.96	_	144.79	127	0.13
		MR Egger	1.18 (0.86-1.61)	0.15	0.40							143.48	126	0.14
		Weighted median	0.95 (0.84-1.08)	0.06	0.45									
Daytime sleepiness/CRC	38	IVW	0.56 (0.22-1.43)	0.48	0.23	-0.01	0.02	0.48	0.72	28.47	_	26.89	32	0.72
		MR Egger	2.44 (0.04-148.89)	2.10	0.67							26.37	31	0.70

(Continued)

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TABLE 2 Continued

Exposure/Outcome	Nsnp	Methods	OR (95%CI)	SE	P value	Horizontal pleiotropy				MR-PRESSO			Heterogeneity		
						MR-Egger regression				Global test P value	Global test RSSobs	Outliers	Cochran's Q	Q_df	P value
						Egger intercept	SE	P value							
		Weighted median	0.85 (0.22-3.31)	0.69	0.82										
Daytime napping/CRC	104	IVW	0.87 (0.53-1.42)	0.25	0.58	-7.13E-03	8.44E-03	0.40		0.27	87.84	–	85.55	78	0.26
		MR Egger	1.78 (0.32-10.09)	0.88	0.51								84.77	77	0.26
		Weighted median	0.87 (0.43-1.75)	0.35	0.69										

*The result of recalculation after removing outliers.MR-PRESSO, MR-Pleiotropy Residual Sum and Outlier method. OR, odds ratio; CI, confidence interval; IVW, inverse-variance weighted. P-value corrected for False Discovery Rate.

Despite this, the global Q statistic pointed to heterogeneity among the results (Q=160.23, P = 0.03). The MR-Egger method did not suggest the presence of horizontal pleiotropy (intercept P=0.22), while the MR-PRESSO method detected it (P = 0.03), identifying rs769449, rs910187, and rs1580173 as outliers. Removal of these outlier SNPs did not alter the findings (IVW, Odds Ratio: 0.97, 95% CI: 0.89–1.06, P = 0.50) from Table 2, but it did resolve the heterogeneity (P=0.14), underscoring the minimal evidence of weak instrument bias.

Daytime sleepiness

In the course of examining the potential link between daytime sleepiness and colorectal cancer risk, our initial screening highlighted 5,657 SNPs significantly correlated ($P < 5 \times 10^{-8}$). A meticulous filtering procedure was then employed to exclude SNPs compromised by LD, resulting in the dismissal of 5,619 SNPs and an additional pair of duplicates. Furthermore, SNPs exhibiting palindromic characteristics were also excluded, leaving a total of 33 SNPs qualified for inclusion in the MR analysis. The subsequent analysis, utilizing IVW models, revealed no substantial link between daytime sleepiness and the likelihood of developing colorectal cancer (Odds Ratio: 0.56, 95% CI: 0.22–1.43, P = 0.23), as detailed in Table 1 and depicted in Supplementary Figure S3. The MR-Egger intercept examination suggested no evidence of horizontal pleiotropy, with P-values exceeding 0.05. Additionally, the application of the MR-PRESSO test (P=0.72) identified no outliers, while the Cochran Q test demonstrated a lack of heterogeneity across the findings (Q=26.89, P=0.72). A comprehensive account of the instrumental variables is available in Supplementary Table S4. The F statistics for these genetic instruments exceeded 10, underscoring their robustness.

Daytime napping

In the study conducted by Dashti et al. (13),an initial selection of 12,211 SNPs, each with a P-value less than 5×10^{-8}, was identified for their significant association with daytime napping habits. Following this, seven SNPs found to be duplicates within the exposure dataset were excluded. Furthermore, a substantial number of variants, specifically 12,107 out of the 12,211, were removed due to LD with other SNPs in the set. Additionally, SNPs lacking corresponding outcome data and those identified as palindromic were also excluded from the analysis. This rigorous screening process resulted in the retention of 79 SNPs for subsequent two-sample MR analysis. The study found no causal relationship between daytime napping and colorectal cancer (CRC), as indicated by an odds ratio (OR) of 0.87 and a 95% confidence interval (CI) of (0.53–1.42), with a P-value of 0.58. Both the MR-Egger regression analysis, which yielded an intercept P-value of 0.40, and the MR-PRESSO method, with a P-value of 0.27, confirmed the lack of horizontal pleiotropy in the instrumental variables related to the examined outcomes. Moreover, the analysis showed no substantial heterogeneity in the link between daytime napping and CRC, with a Q value of 85.55 and a P-value of 0.26. The specifics of the instrumental variables utilized are provided in Supplementary Table S5. In addition, the F statistics for each single

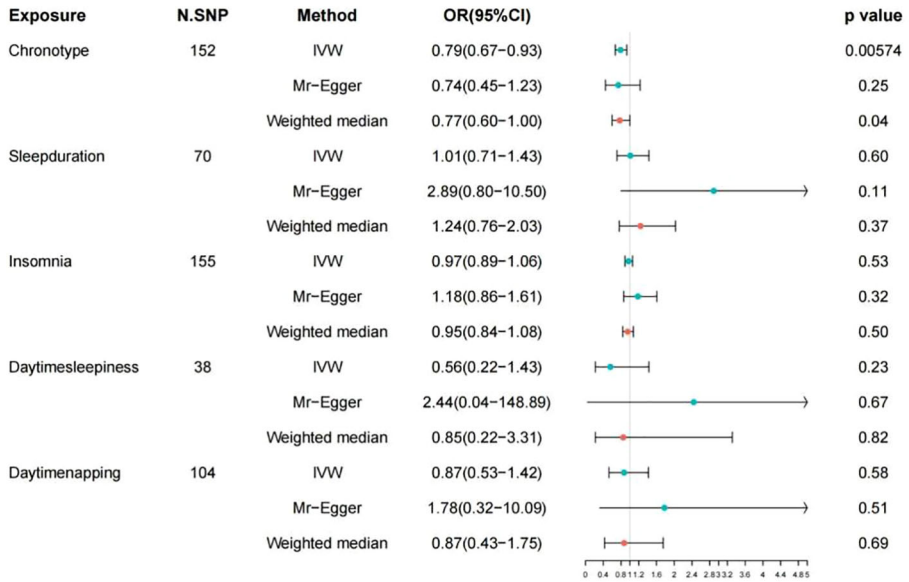

FIGURE 2
Associations of genetic liability to 5 sleep traits with risk of colorectal cancer in FinnGen study. OR, odd ratio; CI, confidence interval.

nucleotide polymorphism (SNP) effectively ruled out any weak instrumental variable bias, as all F statistics were above 10.

Other analyses

In an effort to examine associations between 424 SNPs identified in the primary MR analysis and potential confounding factors, researchers turned to the PhenoScanner database. This detailed review pinpointed five potential confounders: BMI, frequency of alcohol consumption, history of smoking, and overall height, which are elaborated in Supplementary Table S7. By systematically excluding SNPs linked to these confounders with significant genome-wide associations, the integrity of the MR findings, along with the results from sensitivity analyses, remained robust and aligned with earlier reports. These outcomes, reinforcing the consistency of the analyses, are thoroughly documented in Supplementary Table S8 and illustrated across Supplementary Figures S5-S9.

Discussion

This investigation assessed the potential causal links between five sleep-related traits—chronotype, daytime napping, sleep duration, daytime sleepiness, and insomnia—and the risk of CRC. Findings indicated a significant positive correlation between chronotype and CRC incidence. Conversely, analyses did not demonstrate causal associations between CRC risk and other sleep traits such as daytime napping, the length of sleep, levels of daytime sleepiness, or the presence of insomnia.

Poor sleep quality is recognized as a potential contributor to cancer incidence and mortality. Previous research highlighted a correlation between the sleep deficit resulting from shift work and increased risks of type 2 diabetes, coronary heart disease, stroke, and cancer (26). A comprehensive population-based analysis revealed that night shift work elevates prostate cancer risk by disrupting circadian rhythms. Research from multiple regions has established an association between working night shifts and an elevated risk of breast cancer, particularly for tumors that test positive for estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (27, 28). Additionally, there's an established connection between circadian rhythm disruptions and gastrointestinal cancers. These rhythms play crucial roles in regulating cell growth, immune equilibrium, gut barrier function, microbial equilibrium, and metabolic processes. Disorders in circadian rhythms lead to alterations in associated genes (CLOCK, PER, BMAL1) (29). Furthermore, sleep disturbances have been associated with cancer progression (30), with a study by Lin et al. revealing a significantly higher colorectal cancer prevalence among individuals with sleep disorders (31). As the most prevalent sleep disturbance, insomnia's relationship with cancer risk was explored by Lin et al., showing that individuals with insomnia had a notably increased cancer risk, suggesting insomnia could serve as an early indicator of cancer development (32). Research also identified a relationship between insomnia and CRC incidents, where less frequent insomnia corresponded with a reduced CRC risk (33). Findings also indicated that longer sleep durations (8 hours and ≥9 hours) heighten colorectal cancer risk, with men facing a higher risk than women in cases of prolonged sleep (34). Additionally, extended napping has been linked to increased mortality among CRC survivors, with both napping frequency and duration correlating with elevated colorectal cancer risks (27, 35). Current data also point to sleep-disordered breathing and obstructive sleep apnea (OSA) as factors raising cancer incidence. A national cohort study established an association

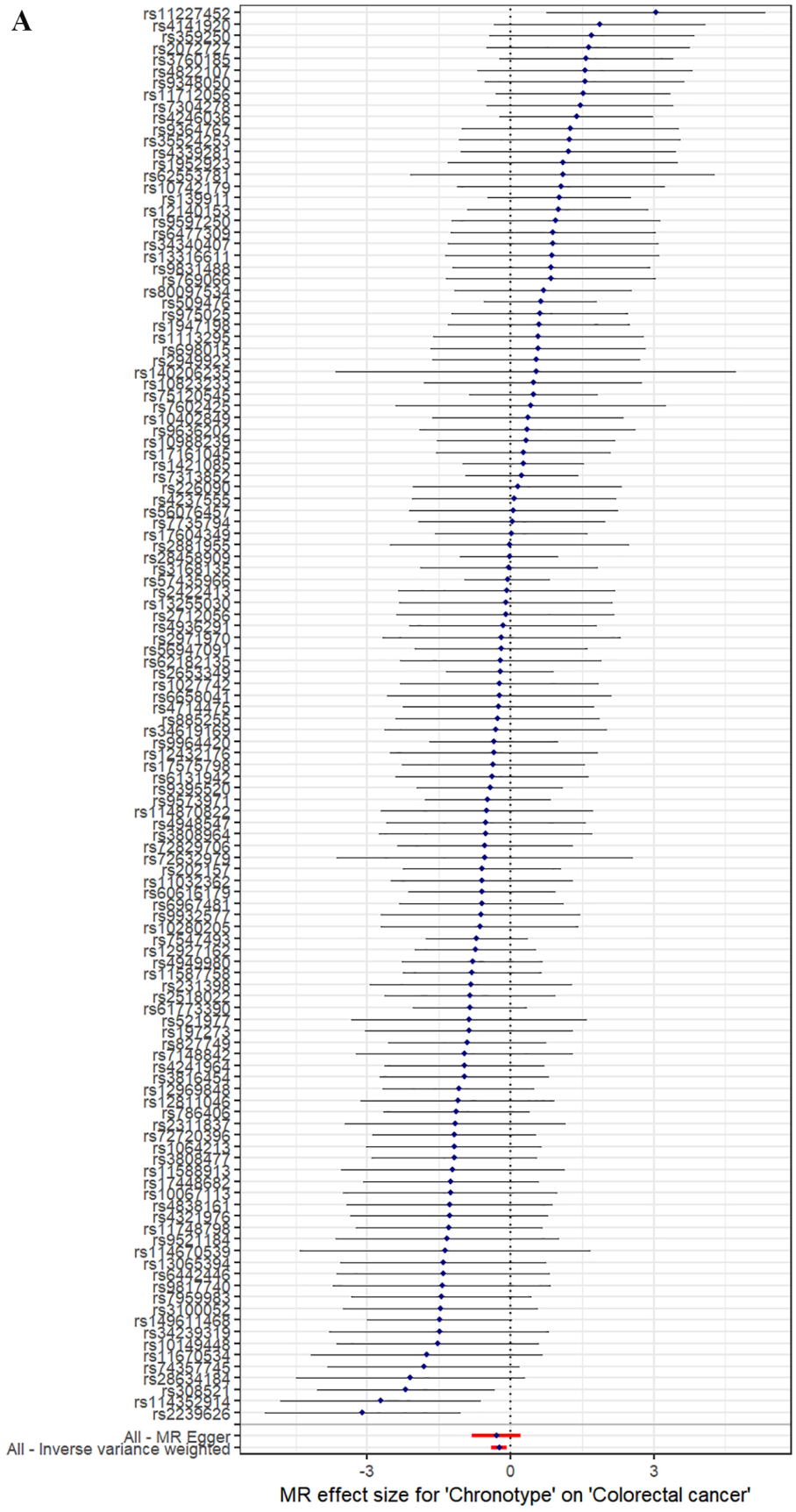

FIGURE 3 (Continued)

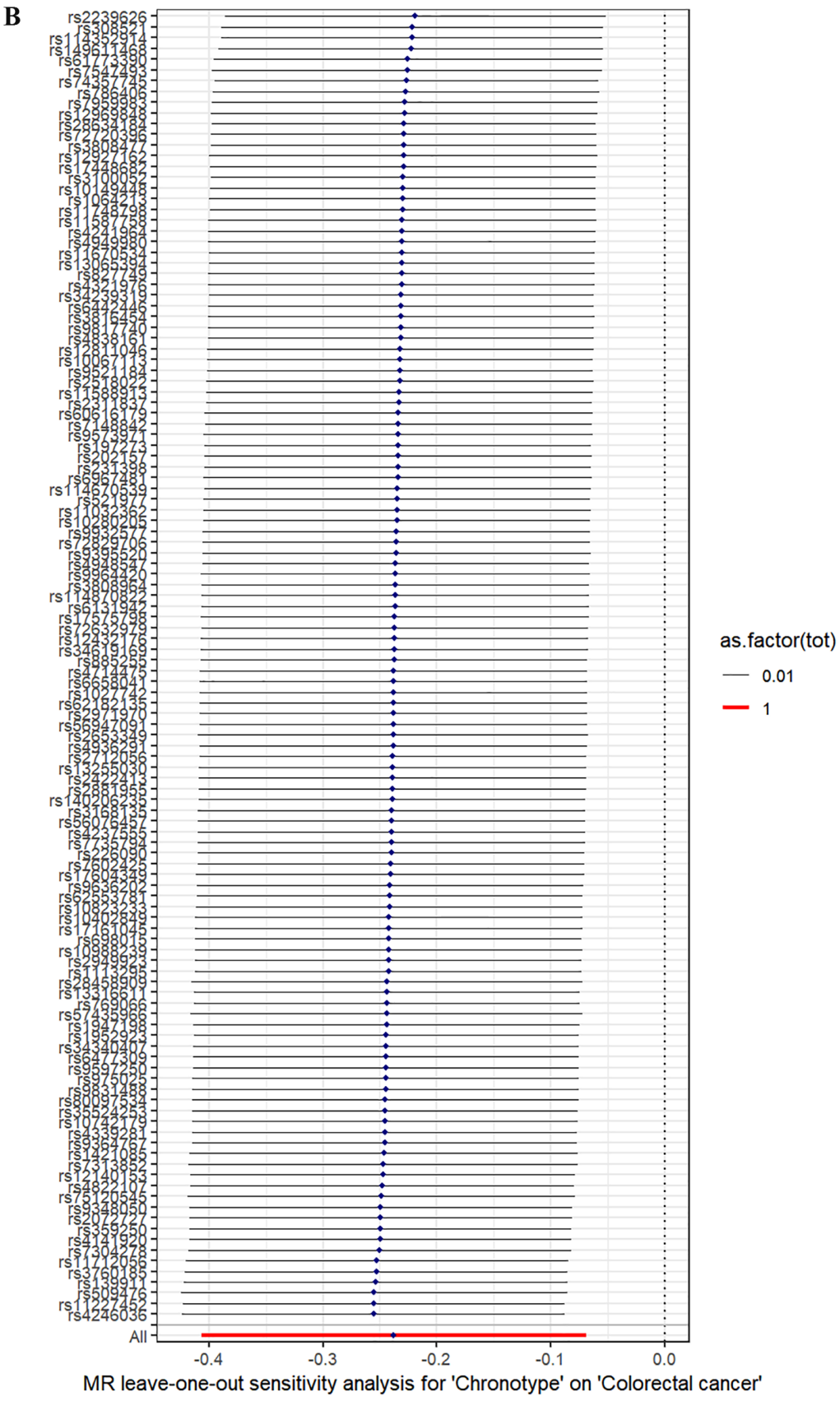

FIGURE 3 (Continued)

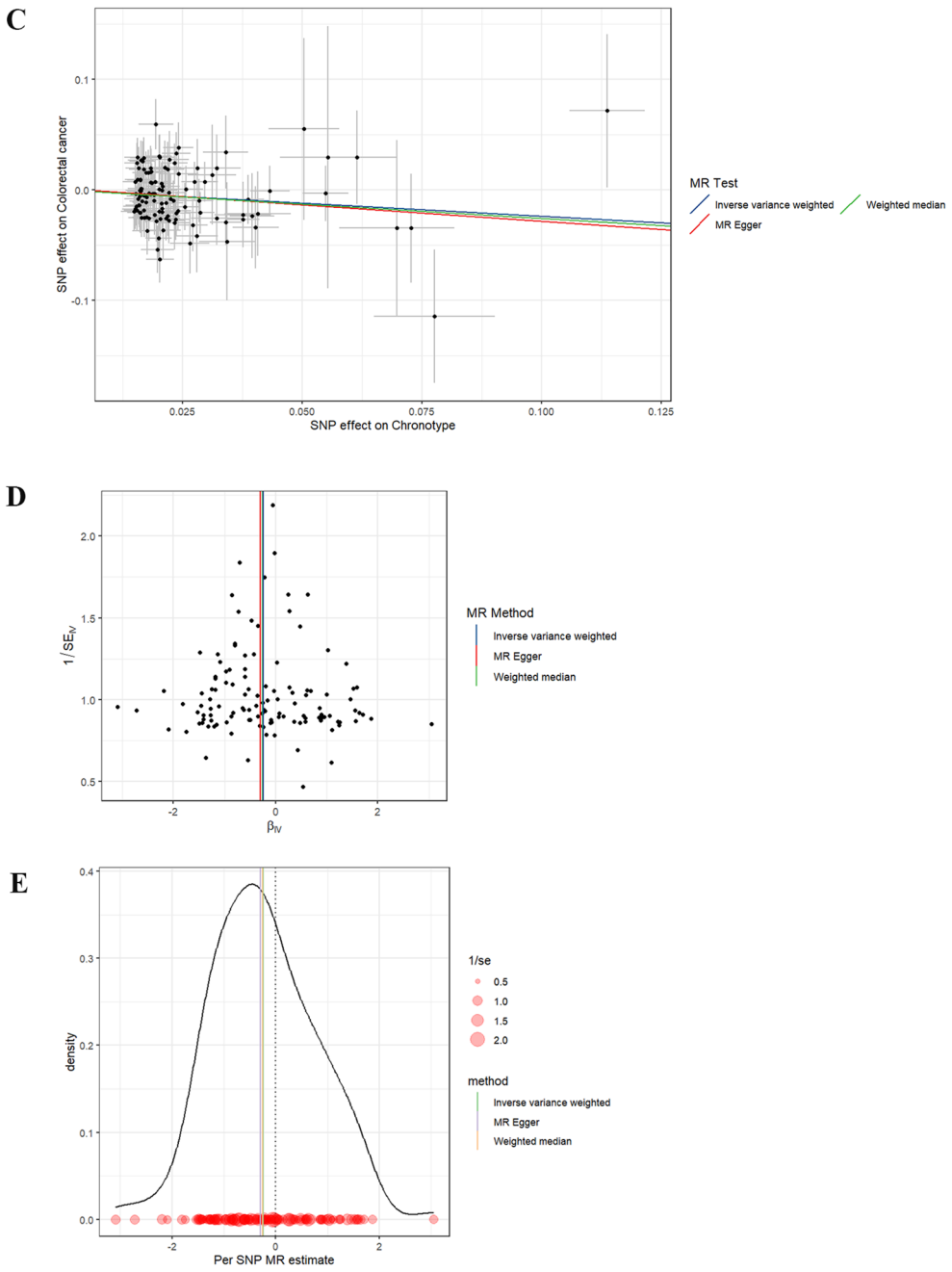

FIGURE 3 (Continued)
(A) Forest plot. The vertical axis represents the number assigned to each SNP, while the horizontal axis represents the confidence interval. **(B)** Leave-one-out sensitivity analysis. Circles indicate MR estimates for chronotype on CRC using IVW fixed effect method if each single nucleotide polymorphism was omitted in turn. The bars indicate the CI. **(C)** Scatter plot. The x-axis represents the effect of SNPs on exposure, and the y-axis represents the effect of SNPs on outcomes. The slope is less than 0, indicating that the exposure factor is a favorable factor for the outcome. **(D)** funnel plot showing the extent of heterogeneity among the individual Wald ratio estimates. **(E)** Density plot. The abscissa indicates the range of SNPs, the ordinate represents the probability at the corresponding SNP value point.

between obstructive sleep apnea and CRC (36). Chronotype, determined by biological circadian and sleep-wake rhythms, and influenced by work and social stress, may contribute to cancer development if evening chronotypes disrupt circadian rhythms due to misalignment with individual lifestyle behaviors (37).

The linkage between sleep disruptions and tumor development encompasses various molecular mechanisms. Primarily, sleep disturbances in individuals with cancer trigger an inflammatory response. These disruptions are recognized for initiating oxidative stress and systemic inflammation, which culminate in endothelial dysfunction and reduced oxygen supply to tissues. Such states provoke changes in sympathetic nervous system activity, immune responses, and the regulation of genes involved in cancer development (38). Additionally, melatonin, which governs sleep-

wake cycles, plays a critical role in the carcinogenic impact associated with sleep disorders (39). This hormone has been shown to inhibit tumor expansion through multiple mechanisms. Specifically, melatonin promotes apoptosis in cancer cells, curtails their proliferation, and influences angiogenesis and metastasis. It also adjusts immune responses, impacts oncogenic signaling pathways, and offers antioxidative benefits (40). Disruptions in sleep patterns interfere with the normal release of melatonin.

Melatonin has been identified as a significant factor in preventing and slowing down the progression of CRC by inhibiting tumor cell proliferation and promoting cell death. Research has highlighted melatonin's role in CRC prevention and treatment through its influence on lipid metabolism and the composition of the gut microbiome (41). According to Kvietkauskas et al., optimal levels of melatonin and glycine can diminish the growth of CRC liver metastases by exhibiting antiangiogenic properties (42). Therefore, melatonin emerges as a potential adjunctive therapy for advanced CRC. Moreover, the relationship between sleep disturbances and cancer progression is further explained through the lens of various hormones such as growth hormones, prolactin, dopamine, estrogen, leptin, and ghrelin. Ghrelin, in particular, is implicated in tumor advancement and reduced survival rates (43), while leptin is known to stimulate the production of pro-inflammatory cytokines TNF-α and IL-6, thereby facilitating cancer cell proliferation (44). Earlier research indicates that insufficient or suboptimal sleep can compromise immune functionality by inhibiting the release of hormones critical for immune stimulation, such as growth hormone, prolactin, and dopamine (45). The complex interplay between the HPA axis and the sympathetic nervous system is crucial for the maintenance of regular sleep patterns, which subsequently influence immune system responses. In our research, we could not confirm direct associations between daytime napping, feelings of sleepiness during the day, insomnia, sleep duration, and the incidence of colorectal cancer (CRC). Nonetheless, it is plausible that these sleep behaviors could have an indirect effect on the risk of developing CRC via the mechanisms mentioned above.

Our research offers several distinct advantages. At the forefront, it is the first of its kind to delve into the genetic relationships between a comprehensive spectrum of sleep patterns and CRC risk. This approach not only addresses the limitations present in prior observational and cross-sectional studies but also significantly enhances the breadth of research within this domain. To ensure the robustness of our findings, we meticulously selected genetic instruments from a vast pool of published genetic associations, utilizing large-scale GWAS to minimize the influence of potential weak instrument bias. Moreover, through detailed sensitivity and heterogeneity analyses, and a methodical evaluation of possible confounders, the study upholds the integrity and reliability of its conclusions. A pivotal aspect of our methodology was the use of the MR-PRESSO technique, which effectively identified and eliminated any potential bias in our Mendelian Randomization results due to the pleiotropic effects associated with sleep traits, further solidifying the validity of our findings.

While our research provides valuable insights, it also encounters certain limitations. Primarily, the dataset predominantly originates from European GWAS, involving participants from the UK Biobank who are generally more educated and healthier. This raises questions about the applicability of our findings across diverse populations. There might have been an overlap among participants, with daytime sleepiness potentially encompassing daytime napping, which, along with overlapping samples in the two-sample MR analysis, could have exaggerated the outcomes. Secondly, the reliance on self-reported questionnaires for gathering sleep-related data might have introduced biases. Future endeavors could benefit from incorporating device-measured sleep parameters. Thirdly, the absence of large-scale studies focused on specific age groups or genders precluded an in-depth analysis of these variables. Additionally, our application of a two-sample MR analysis, based on two extensive GWAS datasets, presupposes a linear association between sleep traits and colorectal cancer risk, not accounting for possible non-linear dynamics or stratified effects. Lastly, the study did not explore other sleep-related factors such as snoring, OSA, and overall sleep quality that may influence colorectal cancer risk. These aspects will be addressed as reliable data become available.

Conclusion

In conclusion, our MR findings suggest that an individual's chronotype has a contributory role in the development of CRC and propose that modifying sleep habits could serve as a preventive measure against CRC. While the study acknowledges the possibility of minor effects that it could not exclude, it underscores the need for more comprehensive MR analyses or extensive RCTs in the future to verify the influence of sleep traits on CRC risk. Concurrently, ongoing research aims to unravel the underlying biological mechanisms linking sleep-related characteristics with CRC.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

XM: Data curation, Writing – original draft, Writing – review & editing. EF: Data curation, Formal analysis, Writing – review & editing. DL: Data curation, Formal analysis, Writing – review & editing. YY: Software, Writing – review & editing. SL: Resources, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2025.1416243/full#supplementary-material

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