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Time is Ripe: Maturation of Metabolomics in Chronobiology

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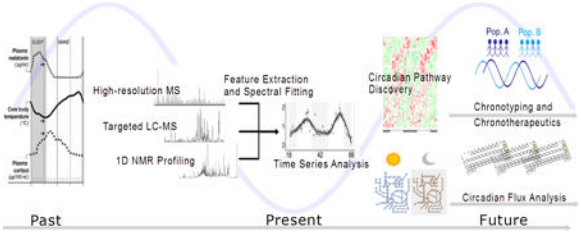
Abstract

Sleep and circadian rhythms studies have recently benefited from metabolomics analyses, uncovering new connections between chronobiology and metabolism. From untargeted mass spectrometry to quantitative nuclear magnetic resonance spectroscopy, a diversity of analytical approaches has been applied for biomarker discovery in the field. In this review we consider advances in the application of metabolomics technologies which have uncovered significant effects of sleep and circadian cycles on several metabolites, namely phosphatidylcholine species, medium-chain carnitines, and aromatic amino acids. Study design and data processing measures essential for detecting rhythmicity in metabolomics data are also discussed. Future developments in these technologies are anticipated vis-à-vis validating early findings, given metabolomics has only recently entered the ring with other systems biology assessments in chronometabolism studies.

Graphical Abstract



Introduction

Daily changes in organismal biology are highly conserved across species throughout evolution. Early scientific work performed on circadian rhythms originated from observations of daily leaf movements in heliotrope plants in 1729[1], opening doors to discoveries of circadian clocks and rest/activity cycles in higher organisms. Exploration of

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temporal changes in metabolism extends back to the earliest days of metabolic discovery, for example the reports from Forsgren that the ratios of bile and glycogen synthesis in rabbits exhibited diurnal variability[2]. The core genetic machinery which produces daily oscillations of transcripts and proteins has since been discovered and consists of transcription-translation feedback loops, driven through a *Bmal-Clock* protein complex in mammals[3]. Analysis of a small number of biomarker metabolites, such as melatonin and cortisol[4], are now commonly used to characterize diurnal patterns in both sleep and circadian research.

Traditionally, sleep and circadian rhythms have been studied by segregated research communities. Sleep studies typically focus on alterations in physiology which correlate with sleep status[5,6], while circadian designs employ higher time resolution sampling to assess oscillatory patterns in physiology[7], considering amplitude, phase, and period metrics. Thus, the sleep field is anchored from a clinical and epidemiological perspective, and the circadian field is driven by molecular discovery of basic clock mechanisms. Metabolism offers a tantalizing connection between these fields as the application of metabolomics technology cuts more broadly and deeply into physiology. An excellent review of key biological findings and interpretations from metabolomics' studies on circadian rhythms has recently been published[8]. Here we discuss how diverse analytical platforms have advanced multiplexed chrono-metabolic biomarker studies. We also discuss future steps and technological developments required for chronobiology metabolomics to truly find the light in translational biology (Figure 1).

Mass spectrometry as an exploratory tool in chronometabolism

Metabolic variation in certain classes of low abundance metabolites such as glucocorticoids[9], catecholamines[10], and bulk lipids[11] has been long-established in both circadian and sleep contexts. It follows then that mass spectrometry (MS) forms an important component in the high-throughput detection of relevant metabolites. Collectively, advancements in complementary MS and nuclear magnetic resonance (NMR) spectroscopy approaches have facilitated recent chronobiology discoveries as will be discussed with a focus on human studies in the next two sections.

The diversity in both front-end separation and MS detection tools, combined with experimental design heterogeneity, has yielded a rich set of putative metabolite markers for further investigation. Figure 2 and Supplemental Table 1 summarize the results from nine key human chronobiology metabolomics studies[12–20] which have identified carnitines, aromatic amino acids, phosphatidylcholines and lysophosphatidylcholines as substantially enriched overlapping metabolite classes that oscillate across circadian time and are perturbed in sleep disruption studies (Figure 2). This evidence would suggest a significant interaction of circadian rhythms and sleep with metabolism through fatty acid metabolism, possibly for both energetic homeostasis via acylcarnitine oxidation and signaling roles that phoshatidylcholines may exert in and across tissues. The aromatic amino acids are both precursors of important neurotransmitters such as serotonin and catecholamines, of relevance to known diurnal changes in cognition and blood pressure respectively.

These studies have primarily used liquid chromatography (LC)-MS metabolomics, although other approaches such as gas chromatography (GC)-MS or flow-injection (FIA)-MS[17] have been also been employed. The GC-MS studies have been part of a targeted commercial platform which combines two forms of LC-MS/MS (LC solvents appropriated for separate positive or negative mode ionizations) with GC-MS[14,16] to identify smaller and more polar metabolites including amino acids, sugars, and steroids. Other human metabolomics studies have instead focused on reverse-phase LC-MS and/or lipidomics, generating unique lists of lipid and other largely nonpolar features which change in sleep and circadian contexts[12,13,15,19]. These lipid profiles have identified subsets of unique circadian phenotypes within a healthy human population[13], as well as facilitated an approach for identifying characteristics of the internal body clock starting with roughly 4000 untargeted features[15]. Another approach combined untargeted quadrupole time-of-flight (qToF)-MS with hydrophilic interaction chromatography (HILIC) as well as reverse-phase lipidomics and high resolution GC-MS to yield a set of unique biomarkers of sleep debt [18], notably including larger lipid species only detected by lipidomics platforms. An exciting extension of circadian metabolomics has recently been performed in real-time breath analysis[21], which has direct clinical implications. While a high enrichment of metabolite features displayed rhythmicity (>36% of features), these masses were not identified as known metabolites. However, as mass resolution, sensitivity, and spectral databases continue to improve, these high-resolution MS analyses can identify metabolites with increased throughput and potential novelty[22].

Recently, metabolomics has yielded more causal links of the circadian clock to targeted metabolite outputs in mice[23,24], and also uncovered unique signaling roles of a specific lipid (PC 18:0/18:1) in regulating diurnal variability in metabolism across tissues[25]. Thus the field currently consists of a collection of untargeted analyses, where larger swaths of metabolites are detected without bias, and targeted analyses, which contain fewer but more confidently identified metabolites. We want to stress that while some of these methods are used in a complementary manner, even two or three collective analyses can only represent a fraction of the entire metabolome, and there exists considerable room for growth in exploratory analyses of the chronobiology metabolome. A total of 328 unique metabolites are significant in the aforementioned human sleep and circadian metabolomics studies (Supplemental Table 1). Encouragingly, 13.4% of these metabolites are found in at least one circadian and one sleep study (Figure 2, Supplemental Table 1). Still, the majority of metabolites found to date are not replicated, highlighting the extent of validation required in both study designs and analytical approaches. Somewhat provocatively, our own work has shown that repeated sleep study protocol and metabolomics analysis yields reproducibility of only about 4.5%, though it is unclear if this inconsistency derives from real biology or technical variability[18]. More quantitative and targeted approaches, including quadrupole-based MS techniques, which benefit from recent enhancements in scanning electronics, and NMR, will further advance the field towards actionable biomarkers in diagnostic and therapeutic settings.

NMR metabolomics as a clinical tool for chronobiology

Robustness, minimal sample processing, straightforward quantification and availability of structural insight are some of the key features of high-resolution NMR spectroscopy relevant to metabolomics. In human biomarker studies, NMR has been used to probe time-of-day variation in saliva metabolic profiles[26] and under different sleep conditions in urine [20]. NMR can be used quantitatively as a rapid and robust primary filter for analyzing circadian oscillations in polar metabolite rich systems. In this way, Giskeødegård et al. have demonstrated that the circadian urine metabotypes of individuals post sleep deprivation are distinct from their well-rested control state (Supplemental Table 1)[20]. In addition, the study also reports distinct excretory metabotypes pre- and post-sleep deprivation. Worth noting is that NMR identified structural isomers in these results, including sugars, which is difficult by LC-MS.

Non-invasive magnetic resonance spectroscopy has been used to demonstrate the relation of human sleep architecture with bioenergetics[27] and alterations in glia[28]. High-resolution NMR can be used to investigate intact cells and tissues and has been used to investigate temporal metabolism of intact red blood cells (RBCs) and hepatocytes, albeit without the time resolution or span needed for a true circadian study[29,30]. Anucleate RBCs are of special interest in this regard as they have functional non-transcriptional molecular clocks based on peroxiredoxin cycling[31]. Furthermore, profiling of tissue samples by High Resolution Magic Angle Spinning (HRMAS)-NMR may shed further light on chronobiology of intact tissues[32].

The major limiting factor in adoption of NMR metabolomics is the lack of sensitivity compared to MS. Using conventional probes, low micromolar concentration of polar metabolites in complex biological mixtures can be reliably quantified using sophisticated profiling techniques[33]. Fortunately, there are recent developments in NMR methods such as micro-coil NMR and hyperpolarization that may improve the detection limit and decrease the acquisition time significantly[34]. A number of important compounds found in human sleep and circadian biomarker studies can be already detected by NMR such as aromatic and branched chain amino acids, histidine, and creatine (Figure 2, Supplemental Table 1), amongst which phenylalanine and tryptophan have already been identified across multiple studies.

The true quantitative nature of NMR remains under-exploited, mainly due to the complex spectral overlap of biological mixtures and peak shift across samples[35]. Multidimensional NMR may be used to deal with the overlap issue, but comes at the expense of the acquisition time and sensitivity. Technically, efforts are ongoing to increase the detection limit and decrease the acquisition time of multidimensional NMR (reviewed in this issue by Giraudeau and co-authors), which has potential for significant contributions to the field of circadian metabolomics. One-dimensional NMR remains the most common method to leverage the quantitative capacity of NMR[33], and can be combined with quantitative approaches to assess metabolite concentrations[36,37,33]. Recently, we have used high temporal resolution (2 hour sampling) NMR-based targeted profiling of culture media and cells from human osteosarcoma (U2 OS) cell lines to demonstrate that circadian rhythms of

glucose and glutamine metabolism are strongly affected by Myc oncogene expression[38]. We have also used quantitative targeted profiling of the secretome of U2 OS cells to show the presence of linear metabolic patterns latent in a typical circadian metabolic experiment, and suggest that detrending of such data prior to circadian analysis is potentially advantageous[39].

Experimental Design and Data Processing Considerations for Time-Dependent Data

The basic aim of circadian analysis is to detect biological variance over a defined time series, therefore separating analytical and biological variation is critical. Concerns of signal-to-noise have been noted in microarray analysis, where 18.3% of transcripts significantly oscillate with a median 1.51 fold change[40]. Encouragingly, the same set of liver samples yields a median 1.98 fold change in over 50% of detected metabolites (unpublished). Regardless of the approach taken, in order to assess true positives in oscillating metabolites, sufficient power must be incorporated into the experiment. For example, sampling every one or two hours greatly increases the probability of detecting a truly oscillating metabolite compared to every four or six hours. Recent work suggests that sampling every 2 hours over 48 hours provides a reasonable compromise between time resolution and resource allocation[41]. The scalability concerns of large sample numbers inherent in proper circadian study designs present key data processing considerations, including the use of proper quality control samples to mitigate unwanted variance due to instrumental drift and removal of spurious metabolic features, which has been reviewed in excellent detail by Dunn et al. for liquid chromatography mass spectrometry (LC-MS) metabolomics[42].

Multiple statistical approaches have been developed for extracting temporal rhythms and compared[43], each with their own strengths and weaknesses. One particularly strong algorithm, JTK_CYCLE[44], is the most popular method in the studies discussed in this review and remains a cutting-edge choice for analysis of rhythmicity. Fortuitously, analyzing waveform data takes advantage of biological phenomena to reduce sampling requirements. Given that these algorithms search for repeating patterns in time-series data, replicates in the number of days offers advantages over replicate time points within a single day [41]. Thus these concerns for experimental scale can be partly mitigated by study design, recent statistical methods, and improved metabolomics instrumentation.

Future Directions

The comparative analysis of metabolites from sleep and circadian studies presented in Figure 2 raise some important questions to be addressed. Given that medium-chain carnitines, lysophosphatidylcholines, and phosphatidylcholines are clearly enriched amongst metabolite classes, the mechanism driving these changes needs to be defined[45]. For example, lauroylcarnitine is significant in five of the nine studies in Figure 2, but it is unclear what role this molecule may play in chronobiology. These chemical classes are readily observed in LC-MS analyses and may have emerged due to analytical and platform bias of more reverse-phase LC-MS and electrospray ionization analyses used thus far in chronometabolism, however given the unique roles lipids play in biology for energy

production and signaling, these metabolites likely truly sit at the nexus of sleep, circadian rhythms, and metabolism. Deeper and more diverse metabolomics analyses are still needed to elucidate the significance of other metabolite classes, including more specific detection methods for sugars, steroids, metabolites conjugated through biotransformations, and other nonpolar aromatic compounds not detected in most circadian and sleep studies to date. We believe some of these other compound classes are also important players in chronobiology, and will be added to the overlapping list of metabolites in Figure 2 as the field expands. Moving forward, circadian biologists striving for mechanistic elucidation of these overlapping metabolites can benefit from robust LC-MS analyses previously used, while further exploration of chrono-metabolism should focus on diversity in analytical detection methods.

Chronobiology has seen a wave of systems biology data in recent years, at each level of the central dogma[19,46,47]. While tools exist to integrate metabolite and transcription data for pathway mapping[48] including circadian-specific datasets[49], they have little use yet broadly in the field. Rich databases for microarray and RNASeq circadian studies have been developed[50], which opens the door for developing analogous metabolomics databases as the number of these studies continues to grow with ever-improving metabolite identification and high-resolution MS data.

The vast majority of high-throughput chronobiology studies to date have been performed using static metabolite profiling. While advantageous for biomarker detection, the field still lacks mechanistic understanding of the steady-state metabolite changes observed in these studies. Flux analysis has yielded valuable insights into defining the activity of reactions and pathways which contain the metabolites of interest, and exciting new advancements in isotope tracing analysis should clarify the true phenotypic output of the clock. Improved precision and sensitivity in LC-MS metabolomics along with untargeted isotope-based metabolomics platforms[51,52] that are further discussed within this issue by Fabien Lestisse has great potential for unbiased screening of clock kinetics. NMR has long been used for metabolic flux analysis and through such analyses, our group has shown that GABA-transaminase independently regulates metabolic and sleep homeostasis in *Drosophila* neurons[53]. The recent advent of multidimensional NMR technologies coupled to ultrafast acquisition such as nonuniform sampling (NUS) promises to further enhance NMR-based metabolic flux analysis[54]. Targeted MS labeling studies have also been performed in the context of sleep and neurotransmitter modulation in neurons[55,56], and has just recently been applied to interrogate *in vitro* the role of the clock in carcinogenesis[57], which we expect to garner greater use in the near future.

Ultimately, these tools may unearth drug targets which modulate circadian clocks and augment metabolic disease[58]. Furthermore, the new technologies in remote monitoring and wearable technology, combined with sequencing and metabolite data in patients can decipher inter-individual variability in clock outputs and metabolic status, laying the foundation for the dream of precision medicine.

Conclusions

Metabolomics has greatly elucidated the chronobiology-metabolism connection, with the advent of broader and deeper metabolome analyses and rhythmicity detection algorithms. Now equipped with a set of promising metabolite hits, further validation is required to understand mechanistically the interplay of circadian clocks, sleep, and metabolism before leveraging this information for therapeutic purposes. Exciting advancements in MS metabolite profiling and new NMR acquisition techniques will clarify metabolic kinetics through flux analysis and provide a deeper systems-level understanding of metabolic networks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Metabolism is a nexus of sleep and circadian processes as observed by metabolomics
- An overlapping set of metabolites is emerging from sleep and circadian studies
- These studies have unique data processing, design and statistical considerations
- Validation and increased coverage are required for robust chrono-metabolism markers
- Future mechanistic studies will be needed to complement biomarkers

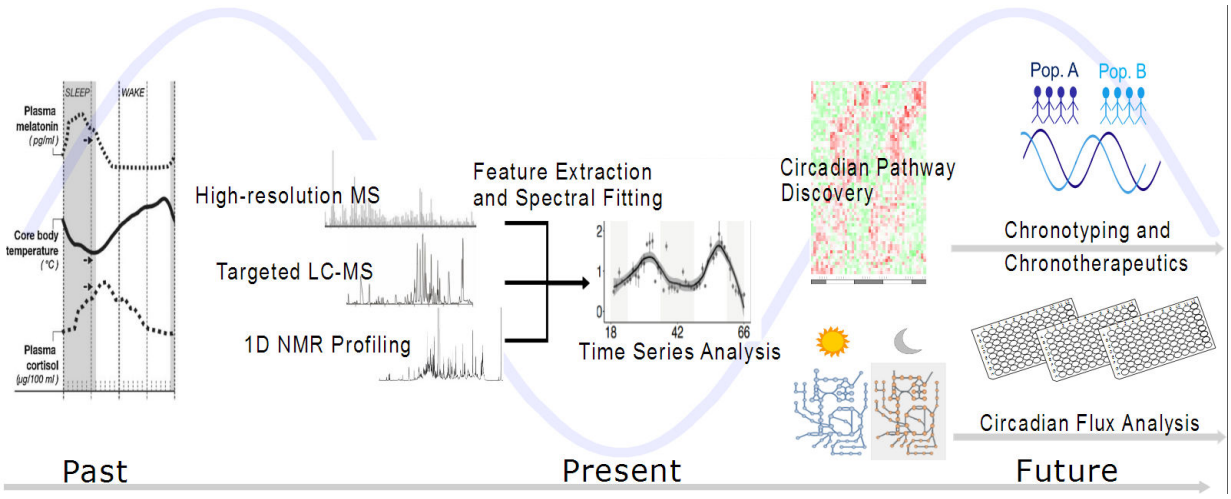

Figure 1.

Timeline of metabolomics in chronometabolic studies, both current approaches and future directions.

Source: Melatonin and cortisol data reprinted with permission from reference [4].

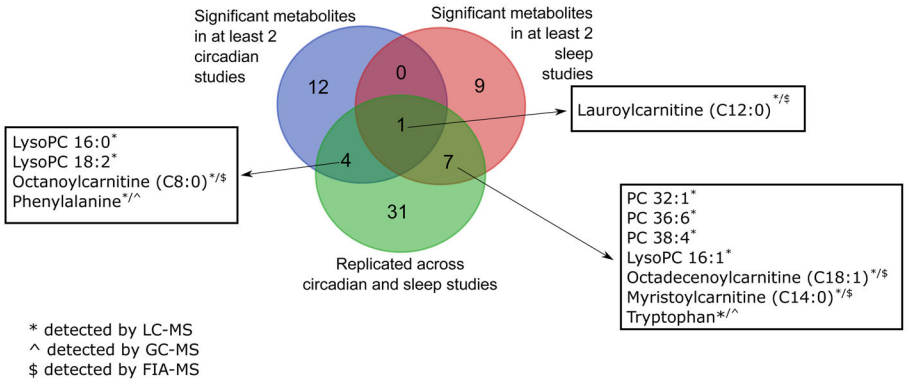

Figure 2.

Number of significant metabolites replicated across at least two circadian (blue) or sleep (red) studies and overlapping in at least one circadian and sleep study (green). Any overlaps across the three groups implies these metabolites are found in at least three of the nine considered papers.