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Review

Lipid metabolism around the body clocks

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ABSTRACT

Lipids play important roles in energy metabolism along with diverse aspects of biological membrane structure, signaling and other functions. Perturbations of lipid metabolism are responsible for the development of various pathologies comprising metabolic syndrome, obesity, and type 2 diabetes. Accumulating evidence suggests that circadian oscillators, operative in most cells of our body, coordinate temporal aspects of lipid homeostasis. In this review we summarize current knowledge on the circadian regulation of lipid digestion, absorption, transportation, biosynthesis, catabolism, and storage. Specifically, we focus on the molecular interactions between functional clockwork and biosynthetic pathways of major lipid classes comprising cholesterol, fatty acids, triacylglycerols, glycerophospholipids, glycosphingolipids, and sphingomyelins. A growing body of epidemiological studies associate a socially imposed circadian misalignment common in modern society with growing incidence of metabolic disorders, however the disruption of lipid metabolism rhythms in this connection has only been recently revealed. Here, we highlight recent studies that unravel the mechanistic link between intracellular molecular clocks, lipid homeostasis and development of metabolic diseases based on animal models of clock disruption and on innovative translational studies in humans. We also discuss the perspectives of manipulating circadian oscillators as a potentially powerful approach for preventing and managing metabolic disorders in human patients.

1. Introduction: molecular organization of the mammalian circadian clock

The circadian clock system has been developed by most organisms as a fundamental adaptation mechanism driving periodical oscillations of behavior and physiology in anticipation of the daily geophysical time changes. In mammals, this system encompasses myriads of interconnected oscillators organized in hierarchical structure of master or central pacemaker, situated in the paired suprachiasmatic nuclei (SCN) of the hypothalamus, and slave or peripheral oscillators situated in the organs [1,2]. The SCN clock is synchronized on a daily basis by environmental cues (*Zeitgebers*), with light-dark cycles representing the predominant *Zeitgeber*. Neural and hormonal rhythms emanating from SCN, along with feeding, temperature, oxygen, and metabolite levels synchronize peripheral clocks across the body [3,4]. A fundamental property of the circadian oscillators is their cell autonomy [5]. Whereas molecular oscillators operative in SCN neurons are keeping a phase coherence among them, peripheral clocks have a weaker degree of coupling within the organs, although the coupling strength differs among the tissues [6]. Indeed, the molecular oscillators operative in adjacent α- and β-cells in the pancreatic islets exhibit distinct phases [7,8], while hepatocyte clocks keep synchrony to some extent in the absence of the central clock [9]. The molecular architecture of the core mechanism responsible for driving circadian oscillations in both SCN neurons and in the peripheral tissues relies on transcription-translation (TTFL) and post-transcriptional feedback loops on the expression of core-clock genes (summarized in Fig. 1). The two central components of the molecular clock are the transcription factors Circadian Locomoter Output Cycles Kaput Protein (CLOCK) and Brain and Muscle Aryl hydrocarbon receptor nuclear translocator-like 1 (BMAL1), which form a heterodimeric complex triggering the rhythmic expression of many "clock-controlled genes" (ccgs), outside the core clock machinery. In the

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primary feedback loop, they activate the transcription of the Period (*Per*) and Cryptochrome (*Cry*) genes, encoding the four corepressor proteins PER1, PER2, CRY1 and CRY2 [10]. Once in sufficient concentrations, the PER and CRY1 transcriptional factors heterodimerize and inhibit the CLOCK-BMAL1 complex activity [11,12]. As a result, the lack of CLOCK-BMAL1 activators lead to diminished levels of PER and CRY proteins until they can no longer prevent their own transcription. As a consequence, a new cycle of PER and CRY protein accumulation begins. In a secondary feedback loop, the CLOCK-BMAL1 complex controls the rhythmic expression of the genes encoding the REV-ERBs nuclear hormone receptors (REV-ERBα and REV-ERBβ) and RORα (and RORβ in neurons) [13]. In turn, REV-ERBα and RORα compete for the same ROR DNA-binding elements (RORE) within the *Clock* and *Bmal1* promoter regions, resulting in the repression or activation, of the *Clock* and *Bmal1* transcription respectively (Fig. 1). Post-translational modifications of the core clock proteins, such as phosphorylation, acetylation, ubiquitination/degradation and more, provide an additional level of regulation of the molecular clockwork [14]. Especially, PER and CRY stability is controlled by the casein kinase 1ε/δ (CK1ε/δ). The phosphorylation of the PER and CRY proteins triggers polyubiquitination by E3 ubiquitin ligase complexes, which promotes proteasomal degradation of the proteins.

2. Circadian organization of lipid metabolism

There is increasing evidence that molecular clocks coordinate temporal organization of different aspects of the lipid metabolism, including lipid digestion and absorption in the intestine, transportation, intracellular lipid metabolism, and accumulation (see Fig. 2). In this chapter we summarize the current knowledge on the circadian regulation of different steps of lipid metabolism, and its role in pathology (See Fig. 3.).

2.1. Circadian regulation of lipid digestion and absorption

Dietary lipid digestion and absorption, occurring in the intestinal lumen, represent initial steps of the whole-body lipid metabolism. At this stage, the organism must cope with hydrophobic nature of lipids, which requires their emulsification by bile salts, and the formation of bile salts-containing micelles. Bile acids, the pivotal components of the bile, are produced by the liver in a circadian manner upon strict control

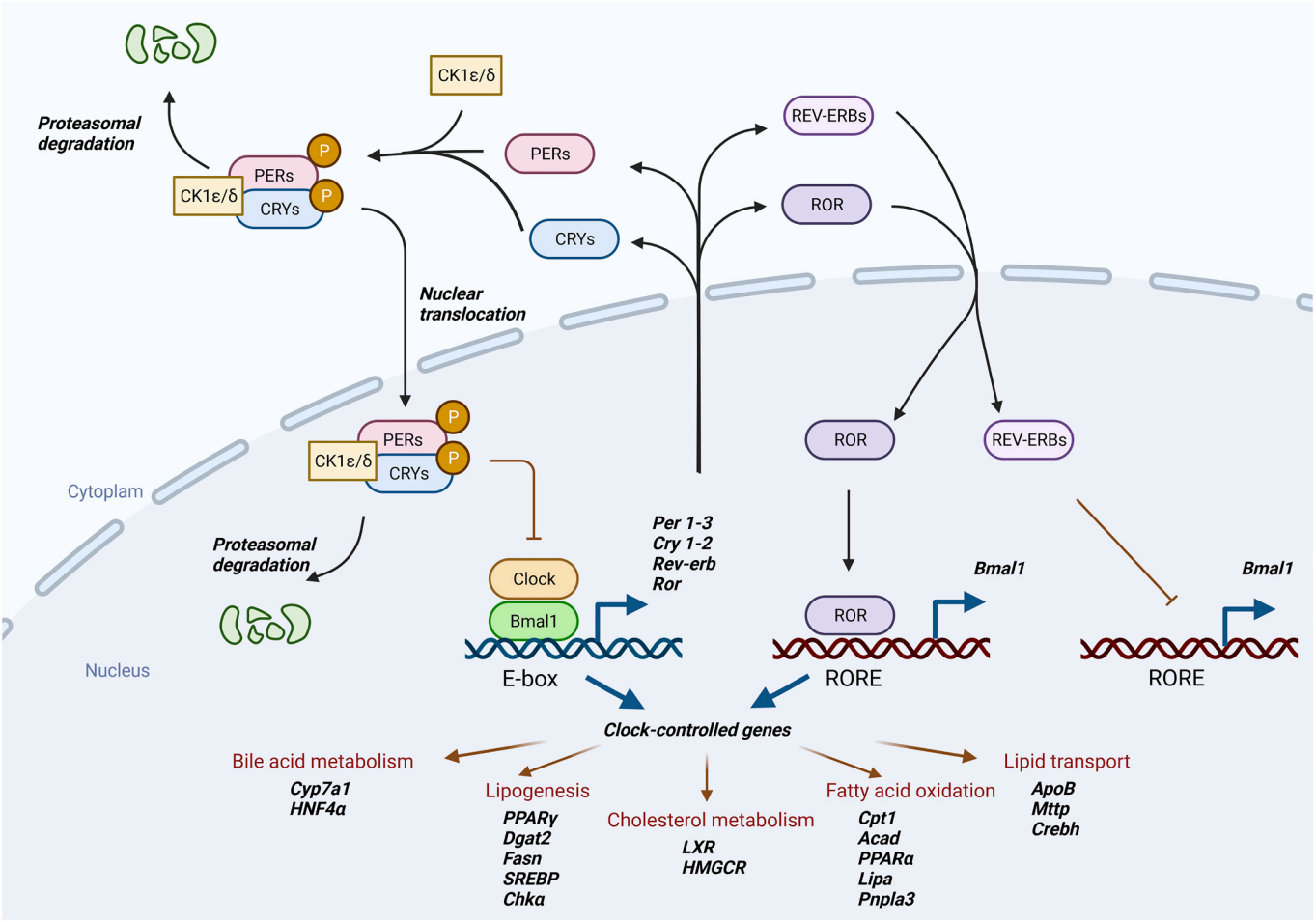

Fig. 1. Molecular makeup of the mammalian circadian oscillator and downstream lipid-related signaling.
The mammalian clock is composed of interconnected transcription-translation feedback loops. CLOCK-BMAL1 complex binds to *E*-box domains on gene promoters, including the genes *Per1/2/3* and *Cry1/2*. PERs and CRYs assemble and repress their own transcription. The main loop is stabilized by the auxiliary loop consisting of REV-ERBα/β and RORα/β/γ. The ROR proteins compete with the REV-ERBs proteins for binding to RORE elements on the *Bmal1* gene promoter, providing both positive (ROR) and negative (REV-ERB) regulation of *Bmal1* and *Clock* transcription. At a post-transcriptional level, the stability of the PER and CRY proteins is regulated by kinases (casein kinase 1ε/δ (CK1ε/δ) and AMP kinase (AMPK)). The phosphorylation of the PER and CRY proteins promote polyubiquitination by E3 ubiquitin ligase complexes, which in turn tag the PER and CRY proteins for degradation by the proteasome. Additionally, core clock proteins regulate the expression of hundreds of genes downstream the circadian feedback loop. These clock output targets are involved in a wide range of physiological processes comprising lipid metabolism. Among these circadian regulated molecules, the genes encoding for the key factors implicated in bile acid and cholesterol metabolism, lipogenesis, fatty acid oxidation, and lipid transport are depicted. See text for further details.

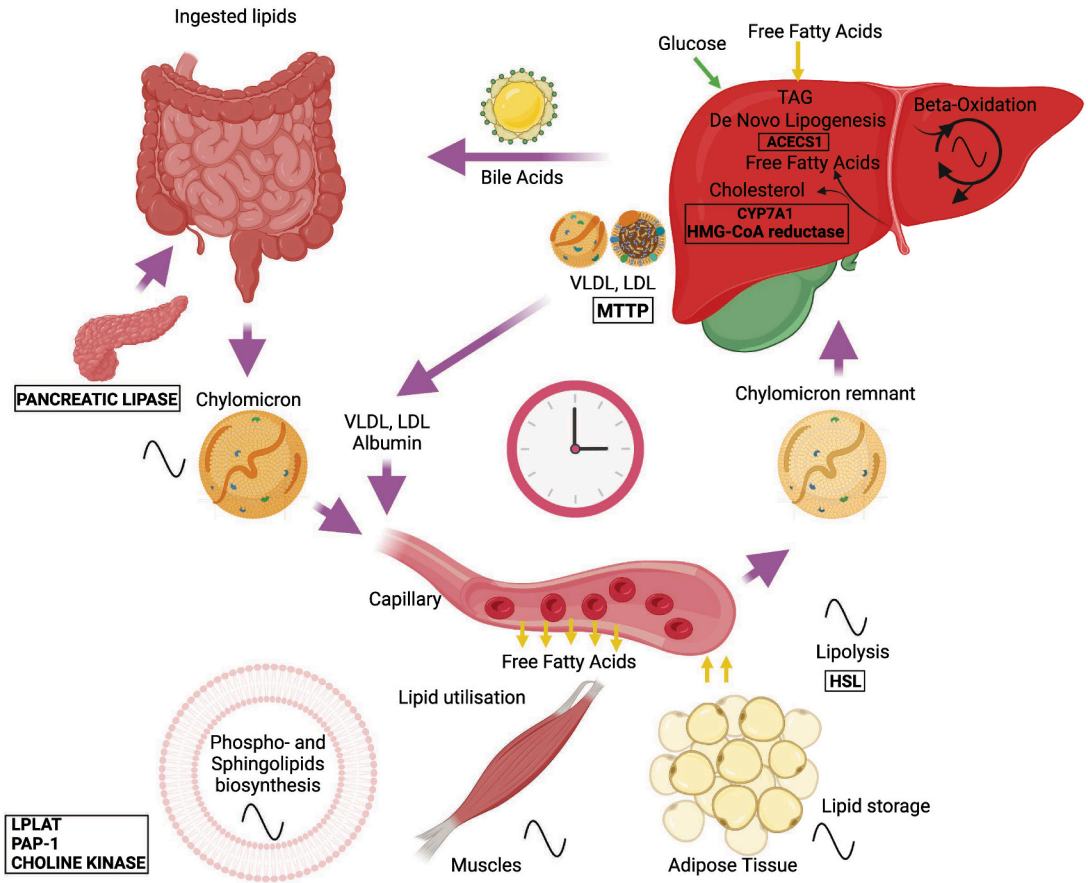

Fig. 2. Circadian organization of lipid metabolism.
Cell-autonomous circadian clocks control different steps of lipid metabolism. Ingested lipids are emulsified with the help of bile acids, broken down in the intestinal lumen and internalized by enterocytes of the small intestine. Triglycerides and cholesterol are repackaged into chylomicrons and travel into the circulatory system. The triglycerides hydrolysed by endothelial lipoprotein lipase result in release of free fatty acids that are taken up by metabolic tissues. The remaining chylomicron remnants are cleared by the liver. Cholesterol and free fatty acids derived from chylomicron remnants are repackaged into low and very low-density lipoproteins (LDL and VLDL), or as well as circulating glucose can be used for *de novo* lipogenesis in liver. Albumin-associated fatty acids, LDL and VLDL particles represent major circulating sources of free fatty acids for metabolic tissues. Once in the cell, free fatty acids are used for energy production *via* beta-oxidation or stored in form of triacylglycerols (TAGs). Cells use free fatty acids or glucose for biosynthesis of phospho- and sphingolipids playing important role in intracellular signal transduction and being principal components of cell membranes. The central clock (indicated by the clock image on the figure) orchestrates the network of cell autonomous oscillators to ensure the temporal organization of the key steps in lipid metabolism (sinusoid signs). Beyond the circadian regulation of transcription of the genes encoding for the key lipid metabolic enzymes (see Fig. 1), circadian regulation of biological activity for some enzymes (indicated in squares) in mice and/or in humans has been recently reported. Those enzymes comprise Pancreatic Lipase; GLP-remodeling enzyme lysophospholipid acyltransferase (LPLAT); GLP-synthesizing enzyme phosphatidate phosphohydrolase 1 (PAP-1); Choline Kinase; Hormone Sensitive Lipase (HSL); cytoplasmic acetyl-CoA synthetase (ACECS1); cholesterol-7alpha-hydroxylase (CYP7A1); beta-hydroxy beta-methylglutaryl-coenzyme A reductase (HMG-CoA reductase); and microsomal triglyceride transfer protein (MTTP).

of the core-clock gene *Rev-erbα*. Indeed, nuclear receptor REV-ERBα drives the rhythmic expression of cholesterol-7alpha-hydroxylase (CYP7A1), the rate-limiting enzyme in the pathway converting cholesterol to bile acids, *via* the stimulation of Liver X Receptor (LXR) by cyclically produced oxysterols [15,16]. In mice, the peak of *Cyp7a1* expression appears at the day-night transition prior to the activity phase, and animals lacking *Rev-erbα* display not only a lower synthesis rate, but also an impaired excretion of bile acids into the bile and feces [17]. Moreover, the liver of clock-mutant mice fed with cholesterol/cholic acids accumulates higher amount of cholesterol due to decreased expression of *Cyp7a1* [18]. In addition, other rhythmically expressed nuclear receptors in the liver such as Hepatic nuclear factor 4 alpha (HNF4α) or Farnesoid X Receptor (FXR), are critical to coordinate transcription of genes involved in the metabolism of bile acids [19–22]. Taken together, these studies imply the roles of circadian clock in regulation of bile formation and emulsification of lipids.

Digestion of emulsified lipids is achieved by the pancreatic lipase secreted to the duodenum. Pioneering physiological studies revealed circadian variability of pancreatic lipase activity that peaked at 9 pm in rats fed *ad libitum*, as well as in the animals that had restricted access to the food during night-time (which is the active/eating phase for nocturnal animals) as measured in the pancreatic homogenates [23]. In mice, the peak of diurnal mRNA expression of pancreatic lipase appeared at the daytime, preceding those for enzyme activity described earlier in rats, and is severely attenuated in whole body clock mutant mice [24]. Although the autonomous molecular clocks were identified in mouse exocrine pancreas [25,26], their regulatory roles in pancreatic enzyme production and secretion remain to be established.

Absorption of the emulsified lipids takes place in the enterocytes in a rhythmic manner. Hydrolysed monoacylglycerols and free fatty acids are taken up by enterocytes from the intestinal lumen for *de novo* synthesis of triacylglycerols (TAGs), followed by their packaging into

chylomicrons representing a transport complex of lipoproteins. Enterocytes possess functional cell-autonomous clocks that are synchronized by SCN and by food intake [25], independently of vagal stimulation [27]. The autonomous nature of enterocyte cellular clocks was demonstrated *in vitro* in cultured organoids from mouse and human intestinal epithelium [25,28]. A recent elegant study by Hong and colleagues provides compelling evidence for cell-autonomous clocks operative in mouse and human intestinal organoids, which orchestrate up to 10% of the enterocyte transcriptome and govern organism-specific, circadian phase-dependent, necrotic cell death responses [29]. These clocks regulate the enterocyte transcriptional landscape, as genes encoding for the key regulators of lipid metabolism, such as microsomal triglyceride transfer protein (*Mttp*), apolipoprotein A IV (*ApoA4)*, apolipoprotein B (*ApoB*), diacylglycerol *O*-acyltransferase 2 (*Dgat2*), fatty acid synthase (*Fasn*), and stearoyl-CoA desaturase-1 (*Scd-1*), exhibited circadian rhythmic expression [30–32] (Fig. 1). By employing an *in situ* loop technique allowing injection of labelled cholesterol directly to the jejunum, it has been demonstrated that the rate of lipid and cholesterol absorption exhibited diurnal rhythmicity in mice, with the highest rate occurring during the active phase [30]. Recent studies in mice lacking the core-clock component *Bmal1* exclusively in intestinal epithelium suggest that the intestinal clock is required for fat absorption, with gut *Bmal1* being protective against development of obesity, hyperlipidemia, and accumulation of fatty acids by the liver [33]. Specifically, BMAL1 induced *E*-box-dependent transactivation of the *Dgat2* gene encoding an essential enzyme for triacylglycerol synthesis and dietary fat absorption. In agreement with this finding, intestinal deficiency of *Rev-erbα*, the transcriptional repressor of *Bmal1* (Fig. 1), enhances dietary fat absorption and increases the likelihood of obesity [33]. Taken together, these data highlight that cell-autonomous clocks operative in the enterocytes play an important role in the lipid absorption in rodents.

2.2. Circadian coupling of lipid transport with lipid metabolism

Plasma lipid concentrations exhibit circadian rhythmicity, with cholesterol and triglycerides showing circadian oscillations with a night peak in rodents [31,34], and in humans [20]. Moreover, genetic disruption of molecular clocks either in *Bmal1* KO or in *Clock* mutant mice abolished diurnal oscillations of triglycerides [30,34]. Circadian metabolome analysis conducted of plasma samples revealed that ~15–20% of all detected circulating metabolites, most notably fatty acids, exhibited circadian rhythmicity in mice [35,36] and in humans [37,38]. Indeed, in-depth MS^{ALL} lipidomics of mouse plasma across two diurnal cycles revealed that within 867 detected lipids, 79% display significant diurnal oscillations [11]. Bilateral lesion of SCN abrogates the diurnal variations of plasma free fatty acid concentration in rats, suggesting a role of central clock in this rhythmicity [39].

Being water-insoluble, lipids require carriers to circulate in blood. For this purpose, fatty acids utilize albumin. Early studies revealed circadian rhythmicity of serum albumin concentrations [40,41], stemming from circadian secretion of albumin by the liver [42]. Moreover, feeding rhythms are essential to maintain this rhythmicity, and are sufficient to do so even in arrhythmic *Cry1/Cry2* -double knockout mice [42]. In humans, significant diurnal oscillations of albumin were observed in the blood samples from more than half of participants subjected to constant routine conditions, *i.e.* environment devoted of synchronization cues (constant wake in dim light and identical isocaloric meals every hour) [20].

Cholesterol and triglycerides are transported in the circulation in association with proteins, forming lipoprotein particles that play a key role in transport of lipids between intestine, liver, and other peripheral organs. This exchange is mediated by five principal classes of lipoproteins, which differ in size, lipid composition and apolipoprotein content. They can be distinguished by density as chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), high-density lipoproteins (HDL). Circadian oscillations of transported lipids require co-regulation of their carriers. Indeed, plasma LDL levels in rats oscillate across 24 h, with lowest levels observed at the onset of darkness, in an anti-phase to LDL-receptor expression in liver [43]. In humans, the blood concentrations of LDL-cholesterol and HDL-cholesterol showed circadian rhythmicity in over 50% of healthy volunteers on constant routine protocol [20]. Recent studies in mice demonstrated that production of 22:6- and 18:6-containing phosphatidylcholines (PCs) is circadian as result of phosphatidylethanolamine (PE) methylation in the liver, and it is coupled with the production of VLDL and HDL particles respectively [11]. Apolipoprotein B is a primary core component of chylomicrons, VLDL, HDL, and LDL. It is produced in the liver and in the intestine where it interacts with the endoplasmic reticulum with assistance of the MTTP. Expression of *Mttp* transcript is rhythmic in both enterocytes and hepatocytes, and its circadian phase corroborates one of plasma triglycerides [31,32] (Fig. 1). It has been shown that the BMAL1:CLOCK complex directly activates the rhythmic expression of the small heterodimer partner (*Shp*), responsible for the repression of *Mttp* in hepatocytes [32]. Another apolipoprotein (APOA4) was proposed to play a role in the expansion of lipoprotein particles [44]. Recent mechanistic studies suggest that BMAL1 regulates expression of cyclic AMP-responsive element binding protein H (*Crebh*), either by direct binding to its promoter or indirectly, *via* the regulation of *Rev-erbα* [45]. In turn, CREBH drives circadian transcription of *ApoA4*, required for assembly of larger size VLDLs [45]. Altogether, these studies conducted in whole-body arrhythmic mice suggest that molecular clocks modulate both synthesis of primordial lipoproteins, and their packaging into larger particles. Noteworthy, in mice that lack *Bmal1* protein specifically in enterocytes, the expression of *Mttp* and of the genes encoding for other components of chylomicron packaging (*Gpat3*, *Mogat2*, *Dgat1*, *Fasn*, *Cd36*, *Plin3*, *Pparα* and *ApoB*) are unaffected [33], indicating that the MTTP-dependent circadian regulation of primordial lipoprotein production might be tissue-specific.

2.3. Circadian control of intracellular lipid biosynthesis and catabolism

Accumulating evidence suggests that lipid biosynthesis and breakdown are under circadian control. These studies are summarized below by major lipid classes.

2.3.1. Cholesterol

In rodents, the circadian regulation of cholesterol biosynthesis in the liver was suggested a long time ago, with the highest levels during the night and the lowest during the day [46–48]. Moreover, around-the-clock assessment of lathosterol, a circulating marker for body cholesterol synthesis, showed a clear circadian profile of the blood concentration, peaking late at night in 8 human volunteers on a standard diet [49]. Experimental studies in rodents revealed that conversion of both [1-^{14}C]acetate and $^{3}H_2O$ to cholesterol in liver shows circadian rhythm, and correlates with the diurnal activity of beta-hydroxy beta-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) [46,47], the rate-limiting enzyme in cholesterol biosynthesis which irreversibly converts HMG-CoA to mevalonate (Fig. 2). This rhythmicity stems from circadian regulation of the *HMGCR* gene expression at mRNA level [50], which disappeared in *Rev-erbα* KO mice [51]. In rodents, cholesterol catabolism is tightly coupled with bile acid production and relies on circadian expression of *Cyp7a1* which was described above. Unlike rodent studies, the diurnal variation of the bile acid production in humans is phase-independent of cholesterol biosynthesis [49], raising a possibility of inter-species difference in temporal orchestration of cholesterol metabolism.

2.3.2. Fatty acids

Fatty acid synthesis and degradation pathways are also subject to circadian regulation. Concentration of acetyl-CoA, a primary substrate for both palmitate biosynthesis and fatty acid chain elongation, shows

circadian fluctuations in the cytoplasm of mouse embryonic fibroblasts, and in mouse liver [52]. These fluctuations are driven by changes in enzymatic activity of cytoplasmic acetyl-CoA synthetase (AceCS1) *via* rhythmic deacetylation by the NAD^+-dependent Sirtuin-1 (SIRT1)-deacetylase [52] (Fig. 2). Circadian regulation of fatty acid synthase (*Fasn)*, a gene encoding for the multienzyme complex catalyzing the fatty acid synthesis reactions, represents an additional mechanism controlling the synthesis of fatty acids at transcriptional level [51]. Indeed, in addition to feeding cues, expression of *Fasn* is driven by the rhythmic binding of sterol-regulatory-binding protein (SREBP), which is in turn controlled by REV-ERBα accumulation, independently of the feeding regimen [51]. Cells utilize fatty acids as an energy source *via* the beta-oxidation pathway that takes place in mitochondria. A rate-limiting step in oxidation of long-chain fatty acids is formation of acylcarnitine required for their further transport from cytoplasm to mitochondria. It was shown that concentration of carnitine-palmitoyl transferase 1 (CPT1), the enzyme catalyzing acylcarnitine formation, follows a circadian pattern at both mRNA and protein levels [19]. Moreover, circadian proteomics of isolated mitochondria revealed rhythmic concentrations of several enzymes participating in fatty acid oxidation, comprising acyl-CoA dehydrogenase (ACAD) family members (ACAD11 and ACAD9) and hydroxyacyl-CoA dehydrogenase trifunctional enzyme subunit alpha (HADHa). Importantly, daily changes in accumulation of ACAD11, an enzyme catalyzing specifically long-chain fatty acid substrates, correlate with the oscillations in fatty acid oxidation monitored by mitochondrial respiration in the presence of palmitoyl carnitine. This physiological rhythm for ACAD11 accumulation and fatty acid oxidation was lost in animals fed with a high-fat diet (HFD), and in arrhythmic *Per1/Per2* null mice [19]. Strikingly, the rhythm was restored in *Per1/Per2* null mice fed during their active phase, suggesting molecular clocks and feeding cues as two intertwined regulatory mechanisms of beta-oxidation of fatty acids. In non-physiological conditions, diet-induced obesity (DIO) promotes circadian enhancer remodeling in the liver that synergistically boosts circadian oscillations of fatty acid biosynthesis and beta-oxidation. The two processes are linked *via* SREBPs, which directly controls *de novo* lipogenesis and promotes activity of transcription factor PPARα, required for lipid catabolism [53]. Liver transcriptional reprograming of *de novo* lipogenesis genes upon DIO is also directly mediated by HFD-induced rhythmic expression of PPARγ in the gut microbiota [21,22].

2.3.3. Triacylglycerols

Temporal lipidomic analyses of mouse liver demonstrate that numerous TAG species accumulate in circadian rhythmic manner [11,54]. Concordantly, expression levels of multiple enzymes involved in TAG biosynthesis oscillated with a circadian periodicity, including a rate-limiting enzyme glycerol-3-phosphate acyltransferase (GPAT), as well as subsequent enzymes 1-acylglycerol-3-phosphateacyltransferase (AGPAT), and diacylglycerol acyltransferase (DGAT) [54]. In parallel, TAG catabolism pathways are subject for circadian regulation in liver and in white adipose tissue (WAT). Indeed, expression levels of patatin-like phospholipase domain containing 3 (*Pnpla3*) and of lysosomal acid lipase (*Lipa*), two genes encoding for major hepatic lipases, exhibit circadian oscillations in mice fed *ad libitum* [54]. In WAT, the circadian mobilization of TAGs is mediated by a pair of clock-controlled lipolytic enzymes: TAG lipase and hormone-sensitive lipase [55].

2.3.4. Phospholipids and sphingolipids

Glycerophospholipids (GPLs) and sphingolipids (SLs) represent two major components of cell membranes, with some species serving as second messengers in signal transduction. Large-scale circadian lipidomic analyses in mouse liver revealed that 32% of all GPL and about 2% of SL species exhibit circadian oscillations [11]. The following GPL classes were the most enriched in circadian species: phosphatidylinositols (PI, 5.8%), phosphatidylcholine (PC, 5.5%), phosphatidylethanolamines (PE, 3.9%) [11]. The number of rhythmic lipid species in tissue extracts may be underestimated due to their spatial intra-organ and even intracellular heterogeneity. Indeed, when circadian lipidomic analysis was separately applied to nuclei and mitochondria isolated from the liver cells, rhythmic accumulation of different lipid species was not aligned between these two organelles [19].

Circadian lipidomic analyses in human muscle biopsies revealed that most oscillating lipid species belong to GPLs and SLs lipid classes [9,10]. Noteworthy, the rhythmicity of the lipid metabolites persisted in human skeletal myotubes differentiated *in vitro*, reaching up to 18% of all detected species *[9]*. The major differences in the lipid context between human skeletal muscle tissue and primary myotube cells differentiated *in vitro* was observed for the cardiolipins (CLs), a class of GPLs found exclusively in mitochondria. While CLs were enriched in muscle biopsies, the most abundant CL72:8_C18:2 was non-detectable in primary myotubes [9]. In mouse liver mitochondria, CLs comprise 7.5% of all circadian rhythmic species, reaching 25% upon feeding restricted to the active phase in wild-type (WT), but not in arrhythmic *Per1/Per2* null mice [19], suggesting a role of core-clock machinery in the mitochondrial anticipation of feeding cues. Recent circadian lipidomic study in human pancreatic islets synchronized *in vitro* revealed that only about 5% of detected lipid species exhibit circadian rhythmicity, mainly within PIs and SLs classes [56]. The discrepancy in the percentage of oscillating lipid species between different reports in humans may reflect tissue-specific lipid composition or stem from the high inter-donor variability. Indeed, application of metabolic clustering of lipid species detected in blood plasma, and in skeletal muscles of different subjects suggests that there are different circadian metabolic phenotypes in the general population [9,57].

Several studies suggested the role of functional cell-autonomous clocks in regulating phospholipid metabolism. Biosynthesis of phospholipids, assessed by incorporation of radiolabeled precursors in cultured NIH3T3 mouse fibroblasts synchronized *in vitro*, exhibited a circadian rhythm that was antiphasic to *Per1* oscillations [58,59]. This rhythmicity was abolished in cells bearing compromised circadian clocks *via* downregulation of *Clock*, suggesting that the biosynthesis of phospholipid circadian rhythm in cultured cells depends on the endogenous molecular clock machinery [58]. Concordantly, the number of oscillating GPL and SL species was drastically reduced upon clock-depletion in cultured human myotubes, with the altered rhythmicity in the oscillating lipid species [9]. Moreover, in cultured fibroblasts, the synthesis of GPLs showed circadian rhythm, concordantly with the circadian activity of GLP-synthesizing enzyme phosphatidate phosphohydrolase 1 (PAP-1) and GLP-remodeling enzyme lysophospholipid acyltransferase (LPLAT) (Fig. 2). Interestingly, these two enzymes exhibited antiphasic profiles [59]. Biosynthesis of PC, a fundamental GLP in all eukaryotic cells, also showed circadian oscillations both *in vivo,* and *in vitro* [59,60]. The circadian activity of choline kinase [60], the initial enzyme in a sequence of Kennedy pathway reactions of PC biosynthesis, was shown to be directly regulated by molecular clock *via Bmal1-Rev-erbα-Chkα* axis [60] (Fig. 2).

Being derivates of sphinganine and sphingosine, SLs play an important role in signal transduction and cell-cell interaction. This class includes ceramides, which serve as precursors for two other SL sub-classes - sphingomyelins, and glycosphingolipids. In line with the circadian accumulation of SL species, expression of genes encoding for the enzymes involved in SL biosynthesis showed circadian rhythmic profiles in human skeletal muscles and in pancreatic islets [9,56,61], suggesting rhythmic organization of sphingolipid metabolism. Moreover, *CLOCK* depletion up-regulates expression of *CERK*, *SGPL1* [62] and *UGCG* [9,56,63] transcripts, implying a link between molecular clock and regulation of ceramide synthesis. Indeed, studies in mice revealed two peak high-amplitude oscillations of ceramide concentrations in liver at ZT9 and ZT21 that were completely abolished in *Per1/Per2* null mice [64]. Moreover, the biphasic expression of *CerS2* encoding for an enzyme driving ceramide synthesis, detected in WT mice, and circadian oscillations of neutral and acid sphingomyelinases (*nSMase* and *aSMase*,

respectively) encoding for enzymes involved in hydrolysis of sphingomyelin, were perturbed in *Per1/Per2* null mice [64]. The interaction between molecular clocks and SL seems to be bidirectional, since decrease in SL levels by myriocin treatment resulted in shortening of circadian period length and phase advance in human islets [56]. Moreover, disruption of SL metabolism by myriocin or by UGCG inhibitor PDMP decreased membrane fluidity in human pancreatic islet cells and impaired insulin secretion, recapitulating changes observed in T2D or clock-compromised islets [56]. A recent study applying matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) technique for single-cell lipidomic analysis revealed that distinct SL composition controls different functional states of human dermal fibroblasts [65], allowing coordinated response to extrinsic cues and cell-cell communication. Indeed, overexpression of either trihexosylceramide synthase (GM3S) or globoside synthase (Gb4S), the enzymes driving alternative sphingolipid-processing pathways, resulted in clear functional separation of human dermal fibroblast into papillary or reticular states respectively, as measured by single-cell RNA-sequencing. [65]. Collectively, these studies suggest that SL metabolism is regulated by circadian clockwork *in vivo* and *in vitro* and imply that SLs may in turn regulate circadian rhythms. Further studies would be required to dissect the full complexity of reciprocal interaction between clock machinery and SL metabolism pathways.

3. Interplay between circadian lipids and metabolic disorders

The rate of obesity and type 2 diabetes (T2D) has drastically increased over the last several decades in our modern society. A large body of epidemiological studies reported association between the desynchrony between internal circadian rhythms and external cues, a phenomenon dubbed circadian misalignment emblematic for our 24/7 society, and growing incidence of metabolic disorders (reviewed in [66–71] and summarized in Fig. 3). Moreover, recent studies showed disruption of functional clocks in different human tissues upon obesity

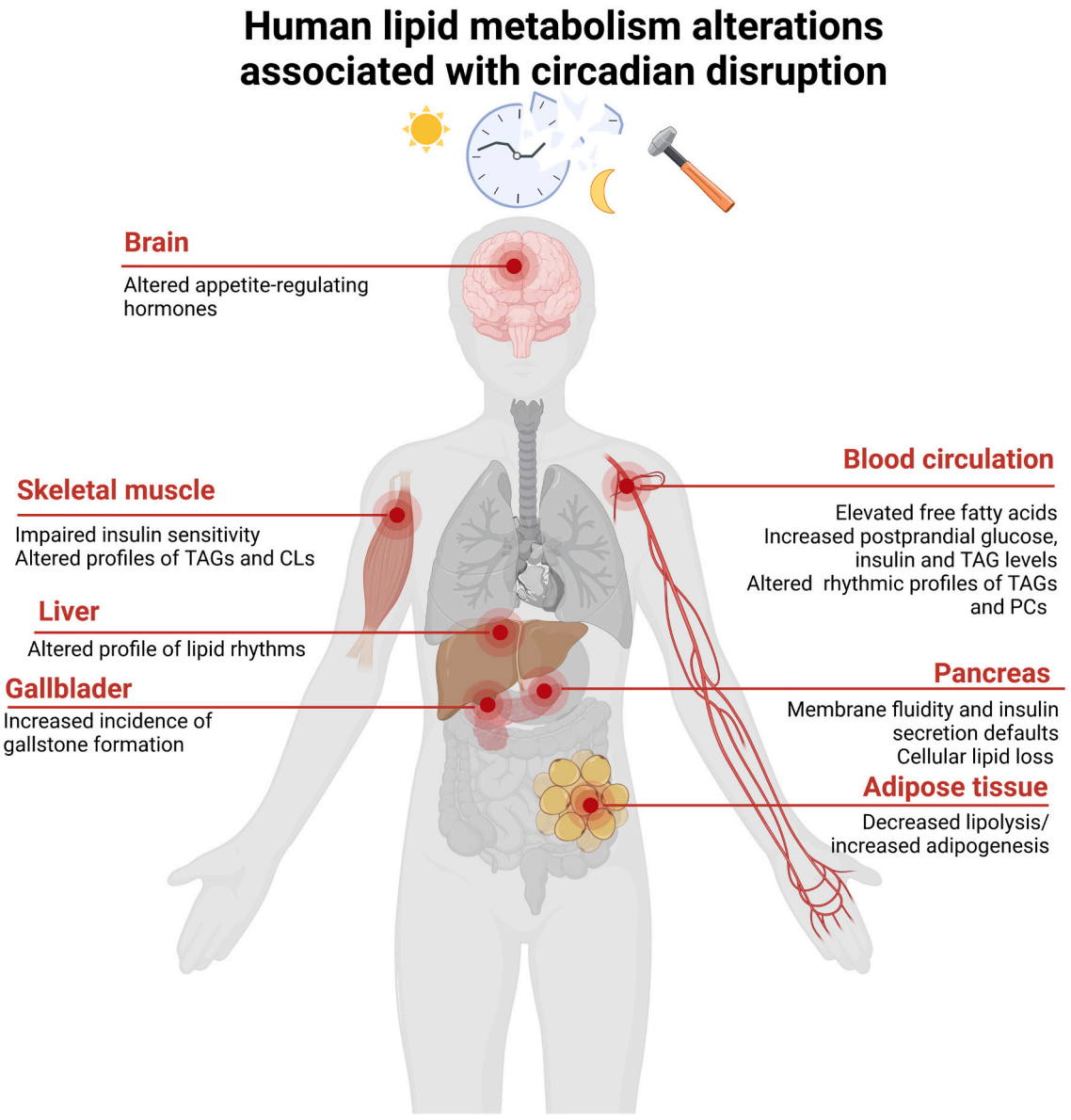

Fig. 3. Impact of circadian disruption on human lipid metabolism.
Circadian disruption is associated with altered lipid metabolism in different peripheral organs. Here we report the main findings generated by human studies addressing the impact of *in vivo* circadian misalignment or *in vitro CLOCK* depletion on lipid metabolism - related pathways. The reported alterations of the human temporal lipid landscape may contribute to the development of obesity, T2D, atherosclerosis, steatohepatitis and additional cardiometabolic diseases.

and T2D [72–76]. In turn, genetic mouse models of clock disruption, along with newly developed approaches for studies of clock perturbation induced in human primary cultures, paved the way for deciphering the mechanistic link between intracellular molecular clocks and development of metabolic diseases, which we summarize in this chapter.

3.1. Lessons from genetic models of clock dysfunction

As already discussed above, the circadian clocks, notably those operative in metabolically active tissues, play a pivotal role in temporal orchestration of lipid homeostasis. Not surprisingly, disruption of circadian rhythmicity is associated with dyslipidemia, steatohepatitis and obesity in various whole-body clock mutant mouse models [34,77,78]. Recently, a mechanistic link has been established between clock disruption and the development of nonalcoholic fatty liver disease (NAFLD) [79]. On one hand fatty acid uptake is increased in *Clock* mutant (*Clkδ19/δ19*) animals *via* increased levels of hypoxia-inducible factor 1α (HIF1α) protein. On the other hand, CLOCK KO mice exhibit altered temporal expression of adipogenesis and proliferation markers in white adipose tissue [80]. Concordantly, loss of function of nuclear receptors REV-ERBα and REV-ERBβ results in profound defects of lipid metabolism, including dysregulation of WAT lipogenesis, a marked increase in liver TAGs, and severe hepatic steatosis [81–83]. Moreover, mice bearing an adipocyte-specific deletion of *Bmal1* showed increased adipose tissue mass and develop obesity, primarily because of greater food consumption during the rest phase (daytime for mice), as compared to wild-type counterparts [84]. In mouse adipocyte precursor cells, BMAL1, together with PER3, were identified as key regulators of adipogenesis *via* direct control of the pro-adipogenic gene Kruppel-Like factor 15 (*Klf15*) [24]. In contrast, intestine-specific deletion of *Bmal1* limited dietary fat absorption and protected mice from DIO and hyperlipidemia developments [33]. Similarly, specific deletion of *Rev-erbα* only in adipocytes did not result in dysregulation of lipid synthesis and storage programs [85]. Finally, whole-body deletion of the core clock component *RORα* or *Cry1* protected against DIO [86,87].

In *Drosophila*, loss of function of the clock gene *period* affects the levels of DAGs and acylcarnitines, with the later exhibiting daily oscillations in WT flies [88]. Taken together, these studies demonstrate that functional molecular circadian clocks are necessary for proper lipid metabolic function across different species and across tissues within the organism.

3.2. Altered feeding cycles and nutritional challenges in mouse studies

Circadian energy metabolism is altered when feeding patterns are out-of-sync with the internal circadian clock, *i.e.* when food is consumed during the usual rest phase. Indeed, the restriction of food access to the active phase (night-time feeding for mice) has been shown to protect animals from developing metabolic disorders as compared to mice fed *ad libitum* or during daytime, even when the food was enriched in fat (HFD) [89–93]. Recent data from the J. Bass group indicated that the protection from DIO supplied by the time-restricted feeding during the active period is mediated by enhancing adipocyte thermogenesis [93]. In contrast, food inversion in mice uncouples circadian rhythms of clock gene expression in metabolic organs from the SCN rhythms [94,95], leading to perturbed temporal expression profiles of clock-controlled genes involved in metabolic regulation that are discordant with the rhythms of rest-activity cycle in these animals [96–100]. Moreover, a recent study revealed that mice fed with a lithogenic diet (1.25% cholesterol and 0.5% cholic acid), known to promote the formation of calculi, during the sleep phase only (ZT0-ZT12), showed increased gallbladder volume, hyperbilirubinemia, and impaired circadian cholesterol metabolism resulting in increased incidence of gallstone formation [28], providing a link between circadian misalignment, impaired digestion of lipids, and high risk of gallstones formation.

The connection between circadian rhythms and feeding is reciprocal. Indeed, a fat-enriched diet (HFD) on its own leads to attenuation of diurnal rhythms in feeding and locomotor activity, as well as in hormonal secretion, concomitant with a circadian amplitude decrease and period lengthening of core-clock gene expression in peripheral organs (*e.g.* liver and WAT) [101,102]. Mouse liver transcriptome analyses conducted around-the-clock revealed that HFD significantly impacted the circadian clock gene expression oscillation [22]. Interestingly, mRNA and protein analyses of mouse WAT identified loss of transcriptional rhythm of the *Clock* gene in mice fed with HFD, whereas the Clock protein levels kept circadian rhythm [103], with the latter phenotype being reversed upon low-fat diet (LFD). In this line, caloric restriction (CR), known to extend mouse life span, prevents reduction in circadian amplitude of gene expression with age [25,26]. However, the Takahashi group recently showed that the highest degree of life-span extension is reached when food was consumed during the active phase [25]. Several key genes related to lipid metabolism, such as Apoa4, Hmgcr, Lepr and Lpin1, were dysregulated in old animals [25,27]. Interestingly, the expression levels of certain genes that were up-regulated in mice fed *ad libitum* with aging, remained low in the liver of animals that were subjected to CR, irrespectively of feeding time [25].

3.3. Impact of chronic circadian misalignment on human lipid metabolism

Disruption of circadian rhythms due to shift work or social jet lag contributes to metabolic dysregulation, however the disruption of lipid metabolism rhythms in this conjunction has only recently been addressed (Fig. 3). A controlled laboratory study in human subjects, conducted by Patrick Schrauwen and colleagues, reported that even a short-term 12 h day-night shift for 3 consecutive days led to misalignment of the core molecular clocks and impaired insulin sensitivity in skeletal muscle, that were accompanied by changes in fat metabolism as well as by elevated plasma free fatty acid levels [104]. In a follow-up study, the authors investigated the impact of circadian misalignment on the skeletal muscle lipidome and discovered that TAGs were the most abundant lipid species altered upon misalignment [105]. In addition, they observed decreased levels in muscle cardiolipins (CLs) that were independent of the daytime, whereas PCs kept their morning-evening pattern that was comparable with the aligned period. These findings highlight that simulated shift work rearranges the circadian organization of human skeletal muscle lipidome in a lipid class-specific manner. Such disruption of temporal orchestration of the lipid metabolism associated with the circadian misalignment may take part in the development of insulin resistance.

Using a similar study design, a recent untargeted lipidomic analysis of blood plasma samples indicated that mean TAG levels were increased after only 3 days of simulated nightshift schedule relative to dayshift [106]. Moreover, TAGs containing saturated fatty acids that are associated with elevated cardiovascular risk [2–5], such as 16:0 and 14:0, displayed dampened circadian rhythms after the nightshift as compared to the day-shift condition. In contrast, this protocol resulted in a decreased mean abundance and phase advance of the glycerophospholipids [106]. Since saturated and monounsaturated glycerophospholipids are negatively associated with cardiovascular death [6–8], this work strengthens the connection between disruption of internal circadian rhythms and elevated cardiovascular risk that has been found epidemiologically in night-shift workers [107].

Kent and colleagues investigated the phase-resetting effects of a combined light and food stimulus on circulating lipid rhythms and clinical markers of hepatic function in a cohort of 16 healthy volunteers [20]. They observed that the liver lipid and protein rhythms have shifted according to a phase-response curve, but with magnitude and direction of shifts that differed from the centrally controlled melatonin rhythm. Another recent study attempted to unravel the importance of meal timing (morning *versus* afternoon) and composition (carbohydrate-*versus* fat-rich) on diurnal plasma lipid metabolites [108]. Lipid patterns were intricately regulated by both the time of day and meal

composition. Among the analyzed lipid metabolites, one third of the species exhibited different postprandial responses in the morning and in the afternoon. Consistently, previous studies proposed that night eating resulted in increased postprandial glucose, insulin and TAG levels in comparison to daytime meals [109,110]. In this line, two recent cross-over randomized trials proposed that subjects displayed increased hunger when they either skipped breakfast [111], or shifted high calorie intake from morning to evening [112] while consuming equivalent amount of calories. To understand why late meal consumption resulted in increased 24-h ghrelin:leptin ratio, Vujović et al. measured gene expression in WAT in a subset of study participants [111]. They observed that late meals altered lipid metabolism pathways by decreasing lipid catabolism and increasing lipid synthesis [111], thus providing a potential mechanism linking the timing of food consumption with weight gain and increased risk of obesity. Although further studies are required to confirm this association, such findings strongly suggest that eating during resting circadian phase leads not only to circadian clock dysregulation, but also to perturbed metabolic rhythms in general, and lipid homeostasis in particular.

In this context, alignment of the eating patterns with circadian sleep-wake cycles represents a potentially powerful strategy to overcome the incidence of metabolic diseases [91,113,114]. In a cross-over trial, the serum and skeletal muscle diurnal metabolomes of overweight/obese men were interrogated in response to a time restricted eating (TRE) intervention *versus* extended feeding [115]. The authors observed that TRE increased the number of oscillating serum and skeletal muscle metabolites without modification of the muscle core clock gene expression. Interestingly, the majority of the rhythmic serum metabolites measured were lipids, with fatty acid metabolites being enriched upon TRE. In this line, a meta-analysis of 17 TRE randomized controlled trials concluded that the TRE has beneficial effects on the lipid spectrum of overweight participants, in particular decreased levels of TAGs, total cholesterol and LDL [116]. However, these modifications are not always consistent among different studies, likely due the high variability in the intermittent fasting protocol employed, the intervention durations, patient's baseline lipid levels, or the timing of the blood samples taken throughout the intermittent fasting studies.

3.4. Alteration of the human circadian lipidome in metabolic diseases

Alterations of the circadian organization of the transcript and protein landscape have been reported for various human pathologies such as metabolic, immune, cardiovascular diseases and cancer [117–119]. However, the connection between development of metabolic diseases and changes in the circadian organization of lipid homeostasis have not been yet thoroughly examined in humans.

Total LDL and HDL-Cholesterol/TAG serum levels measured by conventional methods and collected at 7 time points over 24 h in T2D patients and in non-diabetic control individuals did not exhibit significant variations across the day in either group [120]. In contrast, when daily variations of individual metabolites in plasma were assessed by targeted LC-MS metabolomics in a similar cohort (T2D, overweight/obese non-diabetic individuals, and lean non-diabetic controls), significant temporal metabolite changes were observed in all study groups [121], further highlighting the importance to measure individual lipid metabolites rather than total lipoprotein and cholesterol content [122]. Reciprocally, the impact of lipid overload, experimentally tested *in vitro* on palmitate-treated human myotubes, resulted in the reprogramming of the circadian genes and pathways involved in lipid metabolism [123]. Interestingly, most metabolites that exhibited significant circadian rhythmicity were involved in biological pathways associated with the onset or progression of T2D. In turn, T2D-associated serum lipid changes encompassed lyso-, diacyl- and ether-phospholipids [124]. Although the accumulation peak of most of these rhythmic metabolites was not significantly altered upon high BMI or T2D, 6 metabolites, including diacyl- and lyso-PCs, were identified with both robust 24-h rhythms and significant concentration differences between T2D and control groups [121]. These temporal differences highlight the importance of controlling time of day of the blood sampling for diagnostic and research purposes.

Study of the human circadian lipidome in peripheral organs is highly challenging due to the need of repetitive tissue sampling, which is even more complicated upon pathological conditions. However, we could recently report the first temporal lipidomic profiling of human pancreatic islets derived from non-diabetic and T2D donors [56]. This analysis revealed both global and temporal alterations in phospho- and sphingolipids, associated with decreased cellular membrane fluidity in T2D islets [56]. Importantly, when we artificially disrupted the non-diabetic islet clocks employing siRNA-mediated clock perturbation, we could recapitulate the membrane fluidity and insulin secretion defects observed in the T2D islets, suggesting a key role of the pancreatic islet clocks in T2D pathophysiology with respect to perturbed lipid homeostasis [56]. Interestingly, an impaired circadian clock was recently associated with cytoplasmic lipid loss characterizing the pathological transformation of pancreatic stellate cells (PSCs) during chronic pancreatitis development [125].

4. Conclusions and perspectives

Intracellular oscillators, organized in a complex circadian clock system, orchestrate nearly all aspects of our body metabolism, comprising lipid homeostasis. This article highlights an involvement of circadian regulatory mechanisms in lipid digestion, absorption, transport, biosynthesis, catabolism, and accumulation. It summarizes the roles of cell-autonomous clock components in regulation of intracellular metabolism of cholesterol, fatty acids, triacylglycerols, glycosphingolipids, and sphingomyelins. In turn, lipid species, such as sphingomyelins [56], may modulate circadian clockwork, thus making this interaction bidirectional, although the exact mechanism still needs to be unravelled. Moreover, circadian dysfunction has been recognised as a pathogenic component for metabolic disorders, including metabolic syndrome, obesity and T2D. Recently, the alterations of molecular clockwork upon T2D and obesity has been identified in human islets [56,75], skeletal muscle [72,73], white adipose tissue [74] and skin fibroblasts [76]. Hence, the molecular oscillators may be considered as novel therapeutic targets for prevention and treatment of such disorders *via* timely scheduled exercise, light exposure, meal timing, or clock modulators.

Technical advances in metabolomics and lipidomics approaches provided new information on daily oscillations of a plethora of lipid species, thus generating circadian metabolome and lipidome databases in different mouse and human tissues. Beyond the circadian regulation of genes encoding for key metabolic enzymes, accumulating data reveal the diurnal activity of functional enzymatic complexes that represent an important aspect of circadian regulation of lipid metabolism [1,68] (Fig. 2). These data should be considered for translational research on development of novel diagnostic and prognostic lipid biomarkers of different metabolic disorders. Noteworthy, temporal lipid composition may vary not only between different tissues [11] or intracellular compartments [19], but also between the individuals, rising a concept of different circadian metabolic phenotypes in the general population [9,57]. Together with large-scale circadian proteomics and transcriptomics analysis, diurnal metabolomics and lipidomics pave the way towards personalized medicine. Furthermore, circadian biology is becoming a critical parameter for improving drug efficiency and diminishing drug toxicity [126]. Importantly, organ- and even cell-specific responses of the clocks to different signals [127,128], and possibly to pharmaceutical agents, may introduce inter-organ circadian clock misalignment thus contributing to potential alteration of whole-body metabolism, including metabolism of lipids.

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