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NUTRIENT-REGULATED SKELETAL MUSCLE METABOLISM AND CIRCADIAN CLOCK

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Nutrient-Regulated Skeletal Muscle Metabolism and Circadian Clock
THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Per l'Anna, en Xus i la Montse

Us estimo molt.

POPULAR SCIENCE SUMMARY OF THE THESIS

We are late. We are always late to work, to pick up our kids, to the supermarket, to meet our friends… sometimes, we even feel that our body is late! But how can we control our body schedule? We need then to reset our inner clock. Our inner clock, also known as ***circadian rhythms,*** puts our body *on time*: makes us wake up every morning, be hungry by lunch time, and go to bed at night. If our *clock is out of sync*, that will have a big impact on our body and health.

Scientist have seen that people who work at night, and sleep during the day, have their internal clock shifted. This shift leads to an increased risk of developing obesity, diabetes and cardiovascular diseases. At the same time, people with obesity and diabetes have a disrupted internal clock, which indicates that our clock and our health go hand to hand and affect each other in both ways.

In the studies included in this thesis explore the effects of obesity and type 2 diabetes on our metabolism and internal clock in skeletal muscle cells. **Study I** focused on deciphering the role of a specific type of lipids, lipid aldehydes, on the regulation of insulin sensitivity and glucose homeostasis. This study unveiled, that the lipid aldehyde 4-hydroxy-hexenal (4-HHE) is involved in the development of insulin resistance by forming covalent bindings with proteins, known as adducts. We observed that antioxidant supplementation with glutathione precursor help preventing insulin resistance.

Study II revealed internal clock disturbances in skeletal muscle from men and women with obesity. These disturbances were correlated to changes in plasma lipids and therefore we then investigated the specific effect of the saturated fatty acid palmitate on clock gene expression. **Studies II and III** reveal that palmitate disrupted the expression of core clock genes in the skeletal muscle, and also disrupted the rhythms of circadian genes involved in metabolism and transcription. We observed that palmitate disrupted circadian oscillations in histone acetylation, a key mechanism in the regulation of transcription. The results from studies II and III show that obesity and lipid overload induce circadian disturbances.

Study IV investigates the relationship between type 2 diabetes and circadian disruption in the skeletal muscle. We observed that skeletal muscle cells in culture coming from type 2 diabetic patients presented disrupted circadian gene expression compared to skeletal muscle cells from healthy volunteers. We investigated the mechanisms behind this disruption and discovered that oxidative stress and mitochondrial dysfunction were likely contributors to circadian disruption in type 2 diabetic patients.

Altogether, the studies in this thesis highlight the close relationship between metabolic health and circadian rhythms. Together the results suggest that strategies aiming at re-synchronizing our circadian clock might ameliorate the metabolic disturbances present in the context of obesity and type 2 diabetes.

RESUM EN CATALÀ

La obesitat és un factor de risc pel desenvolupament de malalties cardiometabòliques com la diabetis de tipus 2. La obesitat està associada amb l'emmagatzematge patològic de lípids en teixits com el múscul esquelètic. Aquest emmagatzematge patològic pot causar problemes metabòlics. A més a més, la regulació del metabolisme està lligada a la regulació dels ritmes circadiaris: cicles d'aproximadament 24h que regulen processos bioquímics, fisiològics i de comportament. L'objectiu d'aquesta tesi és estudiar els efectes de l'acumulació de lípids, la obesitat i la diabetis de tipus 2 en la regulació del metabolisme i els ritmes circadiaris en el múscul esquelètic.

En el **primer estudi,** vam investigar els efectes de l'estrès oxidatiu i l'acumulació de l'aldehid lipídic 4-hydroxy-2-hexenal (4-HHE) en el desenvolupament de la resistència a la insulina en el múscul esquelètic. En aquest estudi, vam observar que persones amb diabetis de tipus 2 presenten nivells elevats de 4-HHE en el plasma. Després, vam comprovar que si injectàvem 4-HEE a rates, aquestes desenvolupaven resistència a la insulina. Per tal de descobrir els mecanismes d'acció de 4-HHE, vam subministrar varies dosis a cèl·lules musculars en cultiu. Vam poder observar que 4-HHE reduïa la captació de glucosa i a la vegada disminuïa la funció dels enzims que normalment responen a la insulina. Seguidament, vam constatar que el mecanisme pel qual 4-HHE pertorbava la resposta a la insulina era unint-se a aquests enzims, formant el que s'anomena "adducte", i que si incrementàvem els nivells de glutatió a la cèl·lula, podíem prevenir aquests efectes. En aquest estudi vam concloure que acumulació de 4-HHE en el múscul contribueix en el desenvolupament de resistència a la insulina i diabetis de tipus 2, i per tant podria ser un bon candidat terapèutic.

En el **segon estudi,** vam investigar quin rol tenen la obesitat i a la resistència a la insulina en la regulació dels ritmes circadiaris. En aquest estudi vam revelar que l'expressió dels gens circadiaris en el múscul esquelètic es veu afectada per la obesitat i per la pèrdua de pes. Quan vam correlacionar la expressió dels gens circadiaris amb les característiques clíniques dels participants vam observar que els canvis correlacionaven inversament amb canvis en la quantitat de lípids en el plasma. En cèl·lules musculars vam comprovar que l'expressió dels gens circadiaris oscil·lava en cicles de 24h i vam observar que l'expressió canviava al tractar les cèl·lules amb àcid palmític. D'aquest estudi vam concloure que l'expressió i la funció de gens circadiaris en el múscul esquelètic es veu afectada per la obesitat i més concretament per l'àcid palmític.

En el **tercer estudi**, vam voler explorar més en detall els efectes de l'àcid palmític en l'expressió gènica circadiària del múscul esquelètic. També vam voler examinar l'impacte de l'àcid palmític en l'acetilació de la histona H3 en la lisina 27 (H3K27), com a mecanisme de regulació de la expressió gènica. Vam observar que l'àcid palmític redueix el número de gens circadiaris i afecta la expressió circadiària de gens implicats en la regulació del metabolisme, transport de proteïnes i la regulació de la transcripció. A més a més, vam observar que l'àcid palmític induïa canvis en l'acetilació de la H3K27 en cèl·lules musculars. Aquest estudi

demostra que l'àcid palmític present en la nostra dieta pot causar canvis en el nostre ritme circadiari i causar canvis d'histones.

En el **quart estudi,** vam voler elucidar si canvis en els ritmes circadiaris del múscul esquelètic estan associats a la disfunció metabòlica en els casos de diabetis de tipus 2. Vam observar que el número de gens circadiaris en les cèl·lules musculars dels controls era més elevat que en les cèl·lules dels diabètics de tipus 2. A més, les cèl·lules procedents de diabètics de tipus 2 presentaren un ritme circadiari alterat pel que fa la funció de la mitocòndria. Aleshores vam voler investigar si la mitocòndria tenia un paper important en la regulació dels ritmes circadiaris en el context de la diabetis. Per això, vam induir disfunció mitocondrial en cèl·lules musculars de controls (no diabètics) i vam observar canvis en la expressió circadiària. Per tant, en aquest estudi vam identificar una comunicació bidireccional entre la funció mitocondrial i la expressió de gens circadiaris, i aquests dos processos són disfuncionals en el context de la diabetis.

En resum, els articles inclosos en aquesta tesi remarquen la importància de regular el metabolisme de lípids i glucosa per evitar defectes metabòlics i en els ritmes circadiaris. Els resultats d'aquests estudis també demostren un fort lligam entre la regulació del metabolisme i els ritmes circadiaris, i per tant destaquen la necessitat de desenvolupar nous tractaments on es tinguin en compte els ritmes circadiaris de cada persona, per tal de poder maximitzar els beneficis del tractament. A més a més, el desenvolupament d'estratègies per re-sincronitzar els ritmes circadiaris seria beneficiós per prevenir o evitar la evolució de malalties metabòliques.

ABSTRACT

The prevalence of obesity has tripled in the last four decades, becoming a major risk for the development of cardiometabolic diseases such as type 2 diabetes. Obesity is associated with pathological storage of lipids in skeletal muscle, causing a major disturbance in metabolism. Furthermore, metabolic homeostasis is tightly regulated by the circadian clock. This thesis aims at gaining further insight in skeletal muscle metabolic regulation by lipid accumulation and the circadian clock.

Study I investigated the role of oxidative stress and accumulation of the lipid aldehyde 4-hydroxy-2-hexenal (4-HHE) in the development of skeletal muscle insulin resistance. The results unveil elevated circulating levels of 4-HHE in type 2 diabetic humans and Zucker diabetic fatty rats. Acute intravenous injection of 4-HHE in rats, significantly altered whole-body insulin sensitivity and decreased glucose infusion rate. *In vitro*, 4-HHE impaired insulin-stimulated glucose uptake and signaling and induced carbonylation of cell proteins. Increasing intracellular glutathione pools prevented 4-HHE-induced carbonyl stress and insulin resistance. 4-HHE plays a causal role in the pathophysiology of type 2 diabetes and might constitute a potential therapeutic target to taper oxidative stress-induced insulin resistance.

Study II investigated the role of obesity and systemic factors associated with insulin resistance in the regulation of skeletal muscle clock gene expression. We determined that skeletal muscle clock gene expression was affected by obesity and weight loss. When correlating clock gene expression with clinical characteristics of the participants, we observed invers correlated with plasma lipids. Circadian time-course studies revealed that core clock genes oscillate over time, and expression profiles were altered by palmitate treatment. In conclusion, skeletal muscle clock gene expression and function is altered by obesity, coincident with changes in plasma lipid levels.

Study III explored the effect of the saturated fatty acid palmitate on circadian transcriptomics and examined the impact on histone H3 lysine K27 acetylation (H3K27ac) in primary human skeletal muscle myotubes. Palmitate disrupted transcriptomic rhythmicity in myotubes. Genes that lost or gained rhythmicity after palmitate treatment were involved in metabolic processes, protein translation and transport, and transcriptional regulation. Additionally, histone H3K27ac, a marker of active gene enhancers, was modified by palmitate treatment in myotubes. Our results indicate that dietary saturated fatty acids impart post-transcriptional modifications to histone proteins and regulate circadian transcriptomics.

Study IV elucidated whether altered circadian rhythmicity of clock genes is associated with metabolic dysfunction in T2D. Transcriptional cycling of core clock genes *BMAL1, CLOCK*, and *PER3* was altered in skeletal muscle from individuals with T2D. This was coupled with reduced number and amplitude of cycling genes and disturbed circadian oxygen consumption in T2D myotubes. Mitochondrial associated genes were enriched for rhythmic peaks in NGT, but not T2D, and positively correlated with insulin sensitivity. Mitochondrial disruption altered core-clock gene expression and free-radical production, phenomena that were restored by

resveratrol treatment. The results identify bi-directional communication between mitochondrial function and rhythmic gene expression, processes which are disturbed in diabetes.

LIST OF SCIENTIFIC PAPERS

I. C. O. Soulage, **L. Sardon Puig**, L. Solère, B. Zarrouki, M. Guichardant, M. Lagarde and N. J. Pillon. *Skeletal Muscle Insulin Resistance is Induced by 4-Hydroxy-2-Hexenal, a By-Product of n-3 Fatty Acid Peroxidation*. Diabetologia. 2018 Mar;61(3):688-699.

II. **L. Sardon Puig**, N.J. Pillon, E. Näslund, A. Krook and J.R. Zierath. *Influence of Obesity, Weight Loss, and Free Fatty Acids on Skeletal Muscle Clock Gene Expression.* Am J Physiol Endocrinol Metabolism. 2020 Jan 1;318(1):E1-E10.

III. **L. Sardon Puig**, A. Altintaş, S. Casaní-Galdón, B. M. Gabriel, R. Barrès, A. Conesa, A.V. Chibalin, E. Näslund, A. Krook, N. J. Pillon, J. R. Zierath. *Circadian Transcriptomic and Epigenomic Remodeling with Lipid Overload and Human Obesity*. bioRxiv 2021.02.23.432336; doi: https://doi.org/10.1101/2021.02.23.432336

IV. B.M. Gabriel, A. Altıntaş, J.A.B. Smith, **L. Sardon Puig**, X. Zhang , A.L. Basse , R.C. Laker, H. Gao, Z. Liu, L. Dollet, J.T. Treebak, A. Zorzano, Z. Huo, M. Rydén, J.T. Lanner, K.A. Esser, R. Barrès, N.J. Pillon, A. Krook, J.R. Zierath. *Disrupted Circadian Core-clock Oscillations in Type 2 Diabetes are Linked to Altered Rhythmic Mitochondrial Metabolism*. bioRxiv 2021.02.24.432683; doi: https://doi.org/10.1101/2021.02.24.432683

Publications not included in this thesis:

I. **L. Sardon Puig,** M. Valera-Alberni, C. Cantó, N.J Pillon. *Circadian Rhythms and Mitochondria: Connecting the Dots*. Front Genet. 2018 Oct 8;9:452

II. N. J Pillon, B. M Gabriel, L. Dollet, J. A B Smith, **L. Sardón Puig**, J. Botella, D.J Bishop, A. Krook, J.R Zierath. *Transcriptomic Profiling of Skeletal Muscle Adaptations to Exercise and Inactivity*. Nature Communications. 2020 Jan 24;11(1):470.

III. A. M Abdelmoez, **L. Sardón Puig**, J.A.B. Smith, B. M Gabriel, M. Savikj, L. Dollet, A.V. Chibalin, A. Krook, J.R. Zierath, N.J. Pillon. *Comparative Profiling of Skeletal Muscle Models Reveal Heterogeneity of Transcriptome and Metabolism*. Am J Physiol Cell Physiology 2020 Mar 1;318(3):C615-C626

CONTENTS

LIST OF ABBREVIATIONS

2-HE	2-Hexadecenal
4-HHE	4-Hydroxy-2-Hexenal
4-HNE	4-Hydroxy-2-Nonenal
AGEs	Advanced Glycosylation End-products
ALEs	Advanced Lipoxidation End-products
BMAL1	Aryl Hydrocarbon Receptor Nuclear Translocator Like
BMI	Body Mass Index
BSA	Bovine Serum Albumin
ChIP-seq	Chromatin Immunoprecipitation and Sequencing
CIART	Circadian Associated Repressor of Transcription
CLOCK	Clock Circadian Regulator
CPT1	Carnitine Palmitoyltransferase 1
CRY	Cryptochrome Circadian Regulator
D3T	3H-1,2-Dithiole-3-thione
DAG	Diacylglycerol
DBP	D-Box Binding PAR BZIP Transcription Factor
FA	Fatty Acid
FCCP	Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone
FFA	Free Fatty Acids
GEO	Gene Expression Omnibus
GSH	Reduced Glutathione
H3K27ac	Histone H3 Lysine 27 Acetylation
HATs	Histone Acetyltransferases
HDACs	Histone Deacetylases
HIF1-α	Hypoxia Inducible Factor-1

HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
IRS1	Insulin Receptor Subtrate 1
JmjC	Jumonji C
JNK	c-Jun N-terminal Kinase
K27	Lysine 27
KO	Knockout
LPL	Lipoprotein Lipases
NEFA	Non-Esterified Fatty Acids
NPAS2	Neuronal PAS Domain Protein 2
NR1D1/2	Nuclear Receptor Subfamily 1 Group D Member 1/2
PA	Palmitate
PBS	Phosphate Buffer Saline
PER	Period Circadian Regulator
PGC-1α	Peroxisome Proliferator-Activated Receptor-γ Coactivator-1α
PKB/Akt	Protein Kinase B
PKC β	Protein Kinase C Beta Type
RNA-seq	RNA Sequencing
ROS	Reactive Oxygen Species
RYGB	Roux-en-Y Gastric Bypass
SCN	Suprachiasmatic Nucleus
SDS	Sodium Dodecyl Sulfate
SIRT	Sirtuin
SphK	Sphingosine Kinase
TAG	Triacylglycerols
TEAD1	Transcriptional Enhancer Factor TEF-1

1 INTRODUCTION

As of 2020, the World Health Organization has reported that the prevalence of worldwide obesity has tripled since 1975, affecting now more than 650 million people. Obesity is a major risk factor for the development of several chronic diseases including type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), cardiovascular diseases, and several types of cancers and musculoskeletal disorders. A typical feature of obesity is an increase in circulating free fatty acids (FFAs) concomitant with a reduction in fatty acid clearance, which is associated with ectopic accumulation of lipids in tissues other than the adipose tissue such as liver and skeletal muscle (1-3). This pathological storage of lipids in skeletal muscle can cause metabolic disturbances that contribute to the development of insulin resistance (4). Because skeletal muscle accounts for 60 to 80% of glucose uptake in response to insulin (5-7), an impairment of glucose uptake in skeletal muscle largely contributes to the development of whole-body insulin resistance and type 2 diabetes. Hence, interventions to reduce ectopic accumulation of lipids are key to improve whole-body insulin sensitivity (1).

Metabolic homeostasis is tightly linked to the circadian clock (8, 9), a highly conserved timing system that acts as a master regulator of many physiological processes (9, 10). The circadian clock can be entrained by environmental factors such as light, nutritional intake, physical activity and sleeping behavior (8, 9, 11, 12). Disruption of the circadian clock is linked to detrimental changes in hormone levels that control eating behavior, as well as glucose and lipid metabolism (9). Therefore, a harmonized circadian clock is indispensable for metabolic homeostasis.

The work of this thesis focuses on understanding how skeletal muscle metabolism is regulated by lipids and the circadian clock. The aim is to decipher the interactions between FFA metabolism, insulin resistance and novel circadian mechanisms regulating skeletal muscle metabolism. The results presented in the thesis reveal new strategies aiming at ameliorating and preventing metabolic disturbances.

2 STATE-OF-THE ART

2.1 WHOLE BODY ENERGY HOMEOSTASIS

Energy homeostasis depends on the balance between energy intake and energy expenditure (13). Carbohydrates, lipids and proteins are the main sources of energy, and are known as macronutrients. When the energy intake exceeds energy expenditure, the excess energy is stored. A continuous positive energy balance leads to weight gain, and contributes to the development of metabolic disturbances (13-15). In this section we will review the consequences of impaired glucose and lipid metabolism, as contributors to the metabolic syndrome.

2.1.1 Glucose and insulin sensitivity

In physiological conditions, plasma glucose levels are maintained constant. After food intake, excess glucose is taken up by the cells and broken down to produce energy or is stored in form of glycogen (16). To keep glucose levels in plasma constant, *de novo* production of glucose (gluconeogenesis) or breaking down of stored glycogen (16, 17) is under continuous regulation. Insulin plays a key role controlling postprandial plasma glucose levels. When plasma glucose levels increase, increasing levels of insulin are secreted in order to stimulate glucose uptake in tissues such as skeletal muscle and adipose tissue (16, 17). Insulin also stimulates hepatic glucose uptake, induces glycogen synthesis and suppresses hepatic glucose production (18, 19). Insulin action occurs when insulin binds to its membrane receptor and activates a cascade signal that is transmitted from protein to protein inside the cell, culminating in glucose uptake (20-22). Insulin binds the membrane insulin receptor, that activates the insulin receptor substrate (IRS1/2). In turn, IRS1/2 bind to and activate phosphoinositide 3-kinase (PI3K), leading to activation of protein kinase B (AKT), then activates Akt substrate of 160kDa (AS160). AS160 activates the translocation of Glucose transporter type 4 (GLUT4) to the cell membrane, allowing glucose uptake by the cell (20) (Figure 1). Insulin resistance is

defined by the reduced ability of insulin to induce downstream effects such as glucose uptake and utilization (20), and can occur at many levels within the insulin signaling pathway. Moreover, insulin resistance appears as a consequence of many different factors including increased visceral adiposity and lipotoxicity.

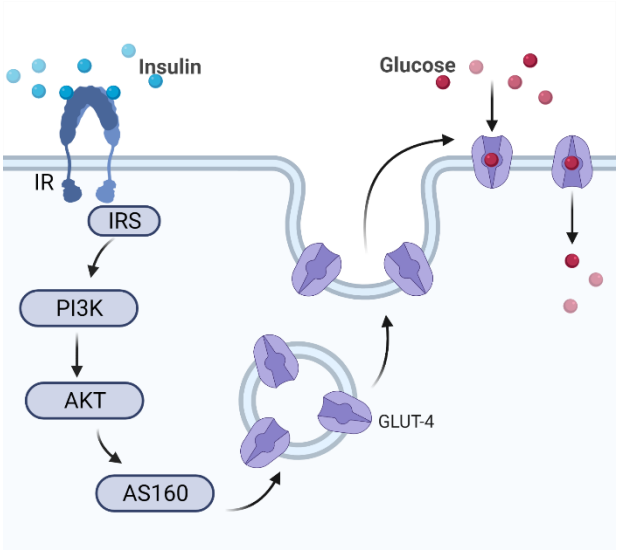

Figure 1. Schematic representation of the insulin signaling pathway in the cell. Insulin binds its membrane receptor, which activates IRS. IRS activates then PI3K and AKT, which will activate AS160. This signaling cascade promotes GLUT4 translocation to the membrane and allows glucose uptake.

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2.1.2 Fatty acid metabolism

Under physiological conditions, lipoprotein lipases (LPL) hydrolyze triglycerides present in circulating chylomicrons and very low-density lipoproteins (VLDL), liberating free fatty acids. Free fatty acids then cross the plasma membrane and enter the cell, where Acyl-CoA synthase adds a CoA thioester to the free fatty acids, forming an Acyl-CoA. The Acyl-CoA cannot cross the cell membrane again. Depending on energy demand and composition of the Acyl-CoA, fatty-acyl CoAs can undergo beta-oxidation, glycerol-lipid formation or sphingolipid and ceramide formation (23) (Figure 2).

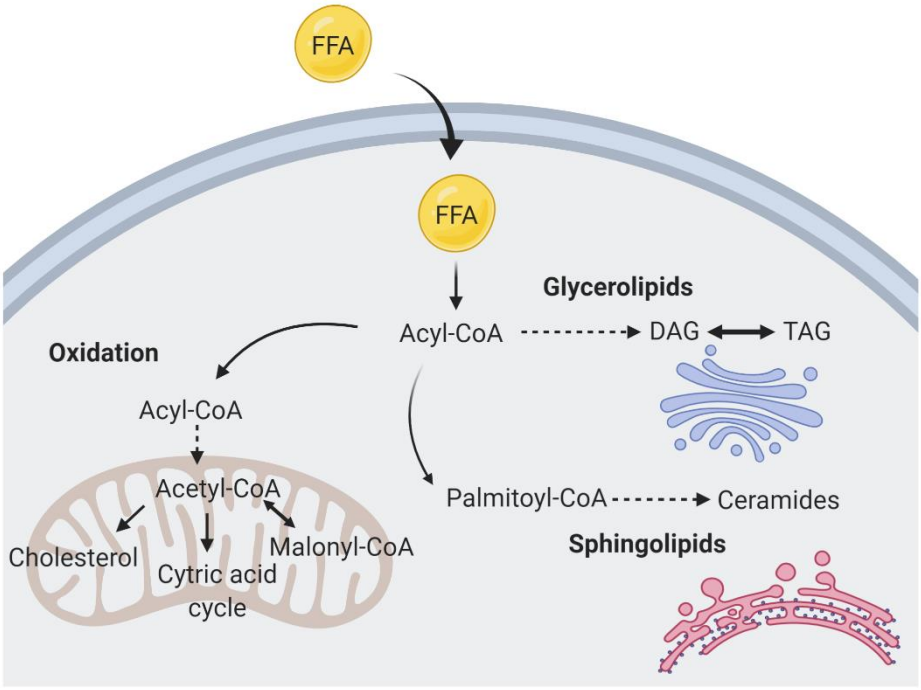

Figure 2. Schematic representation of fatty acid metabolism in the cell. Free fatty acids (FFA) enter the cells and a coenzyme A (CoA) group is added forming an acyl-CoA. Acyl-CoAs can be stored as glycerol-lipids (diacylglycerols (DAG) or triglycerides (TAG)), oxidized in the mitochondria or metabolized into sphingolipids.

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Lipid oversupply has been largely associated with the development of insulin resistance. An increase in circulating plasma lipids occurring during obesity precedes lipid accumulation in skeletal muscle (24, 25). Evidence suggests that this increase in intramyocellular lipid levels may contribute to the development of insulin resistance (24-26). Abnormal lipid accumulation in skeletal muscle could occur due to reduced oxidation of fatty acids, pointing to mitochondrial defects, and/or abnormalities in the mechanisms controlling triglyceride turnover (26, 27). Glycerol-lipid accumulation, increased ceramide formation, and impaired fatty acid oxidation, all contribute to reduced insulin sensitivity in skeletal muscle (28-31).

2.1.2.1 Fatty acid oxidation, oxidative stress and lipid peroxidation

Mitochondrial fatty acid oxidation is a major source of ATP. The rate of lipid oxidation is regulated by carnitine palmitoyltransferase 1 (CPT1), an enzyme responsible for the transport of long chain fatty acids (LCFAs) into the mitochondria. The activity of CPT1 is controlled by

the amount of malonyl-CoA present in the cell (23). Malonyl-CoA is produced from acetyl-CoA derived from beta-oxidation but is also a product of glycolysis. This mechanism allows cross-talk between lipid and glucose metabolism (the Randle cycle), blocking lipid oxidation when cellular energy levels are high (23, 32). However, an increased fatty acid flux into skeletal muscle can lead to a rise in fatty acid oxidation and citric acid cycle activity. A steady increase in citric acid cycle activity contributes to a greater generation of mitochondrial NADH and formation of reactive oxygen species (ROS), resulting in a state of oxidative stress (33-36). Physiological levels of ROS are managed by the cellular antioxidant systems such as superoxide dismutase and glutathione. However, chronic elevation of intracellular fatty acids depletes glutathione pools and antioxidant defenses, promoting pathological oxidative stress (37).

Oxidative stress impairs cell functions via ROS and accumulation of ROS by-products (30). The reaction of lipids with molecular oxygen is known as lipid peroxidation (38). Lipid peroxidation reactions lead to an increase of endogenous production of lipid aldehydes and their derivatives, such as glyoxal, malonic dialdehyde, and 4-HNE (30). These are highly reactive and electrophilic compounds that bind protein molecules and cause covalent modifications, resulting in advanced lipoxidation end products (ALEs) (39). A pathogenic role of ALEs have been described in the development and progression of oxidative-based diseases including diabetes, cardiovascular diseases and neurological disorders (39). By-products of lipid peroxidation including 4-HNE and 4-hydroxy-2-hexenal (4-HHE), induce insulin resistance in skeletal muscle by covalent binding to proteins (40, 41). These protein-adducts result from reactive carbonyl species produced from the reaction between lipid peroxidation by-products and nucleophilic residues of macromolecules. (37, 42).

To ameliorate harmful effects from oxidative stress, antioxidant treatments have been developed. However, such treatments have limited benefit on insulin sensitivity (43);

Therefore, further research on the underlying mechanisms of oxidative stress-induced metabolic dysregulation is required.

2.1.2.2 Sphingolipids and ceramides

During oxidative stress, cells tend to reduce fatty acid oxidation to compensate for elevated ROS levels (33). However, this leads to an increase in intracellular lipids and therefore increased sphingolipid formation, which can induce insulin resistance through different mechanisms. Sphingolipids play a role in intra- and extracellular signaling, traffic of cellular components, as well as structural functions (44). The sphingolipid pathway starts in the endoplasmic reticulum and is completed in the Golgi apparatus. The pathway begins by the condensation of palmitoyl-CoA and serine, and results in a cascade of reactions leading to ceramide and eventually sphingosine-1-phosphate (S1P) formation (23). Accumulation of ceramides and S1P have been associated to metabolic diseases such as arteriosclerosis, diabetes and cancer (45).

Palmitate is the most abundant saturated fatty acid, constitutes 30 to 40 % of circulating free fatty acids (36), and is the principal substrate for ceramide synthesis. Ceramide levels are low under physiological conditions, and increase in response to stimuli such as oxidative stress (46). Accumulation of ceramides alters membrane permeability, promotes oxidative stress and disrupts cellular energy levels (47). Ceramide accumulation has been linked to insulin resistance by inhibition of insulin-stimulated glucose transporter 4 translocation and inhibition of glycogen synthesis (47-49). Ceramides can also reduce activation of signal transduction pathways involved in the regulation of glucose metabolism, including inhibition of Akt and activation c-Jun N-terminal kinase (JNK), which inhibits IRS (31, 50). Ceramides have been associated with a reduction in fatty acid oxidation, increased glycolysis and reduced glycogen synthesis that occur in individuals with type 2 diabetes and obesity, aggravating insulin resistance and overall disturbances in skeletal muscle metabolism (47).

The rate of ceramide generation depends mainly on the availability of long-chain saturated fatty acids (51), and consequently, increased free fatty acids flux in skeletal muscle promotes ceramide synthesis. To avoid the deleterious effects of ceramide accumulation, myocytes increase the degradation and clearance of ceramides. Ceramidase is responsible for the conversion of ceramides into sphingosine. Then sphingosine kinase (SphK) regulates the transition from sphingosine to S1P (52). Increased activity of SphK is linked to reduced tissue inflammation and JNK inhibition (53) and S1P counteracts the effects of ceramides (54), improving the metabolic state of the cells. However, S1P levels are strictly regulated – its degradation occurs either by dephosphorylation or irreversible catabolism into the lipid aldehyde 2-Hexadecenal (2-HE) (55, 56). The role of 2-HE on metabolism has not yet been described, however, 2-HE forms covalent adducts with proteins, DNA and other lipids (55), thereby contributing to the development of insulin resistance.

2.2 THE CIRCADIAN CLOCK

The circadian clock synchronizes biological processes within most living organisms with the rotation of Earth. The mammalian circadian clock regulates 24-hour oscillations of physiological processes such as hormonal secretion, sleep patterns, body temperature and blood pressure. The central clock, situated in the suprachiasmatic nucleus, is responsible for processing light information received by the retina. The central clock coordinates circadian rhythms in the whole organism by sending information to the peripheral tissues through nervous and hormonal signals. Peripheral tissues such as skeletal muscle, adipose tissue and liver, have independent circadian clocks, that follow information from the central clock, but can also be affected by independent cues such as nutrients and physical activity. Peripheral clocks can also send information back to the central clock and therefore influence rhythms in the whole body (57, 58). This mechanism allows the organisms to anticipate daily environmental changes (59).

The molecular machinery of the clock is similar in most cells and works as a transcriptional feedback loop that regulates the expression of many other genes (60-62). The transcription factors Clock Circadian Regulator (CLOCK) and Aryl Hydrocarbon Receptor Nuclear Translocator Like (ARNTL/BMAL1) form heterodimers promoting the expression of the repressor proteins Period Circadian Regulator (PER) and Cryptochrome Circadian Regulator (CRY). PER and CRY dimerize and inhibit CLOCK:BMAL1 activity (57, 60). Similarly, CLOCK:BMAL1 heterodimer also promotes the transcription of D-Box Binding PAR BZIP Transcription Factor (DBP), which enhances the activity of PER:CRY (63). When active, the CLOCK:BMAL1 heterodimer also promotes the expression of the orphan Nuclear Receptor Subfamily 1 Group D Member 1 (NR1D1/REVERBα) and 2 (NR1D2/ REVERBβ), which accumulate and negatively regulate BMAL1 transcription. Circadian associated repressor of

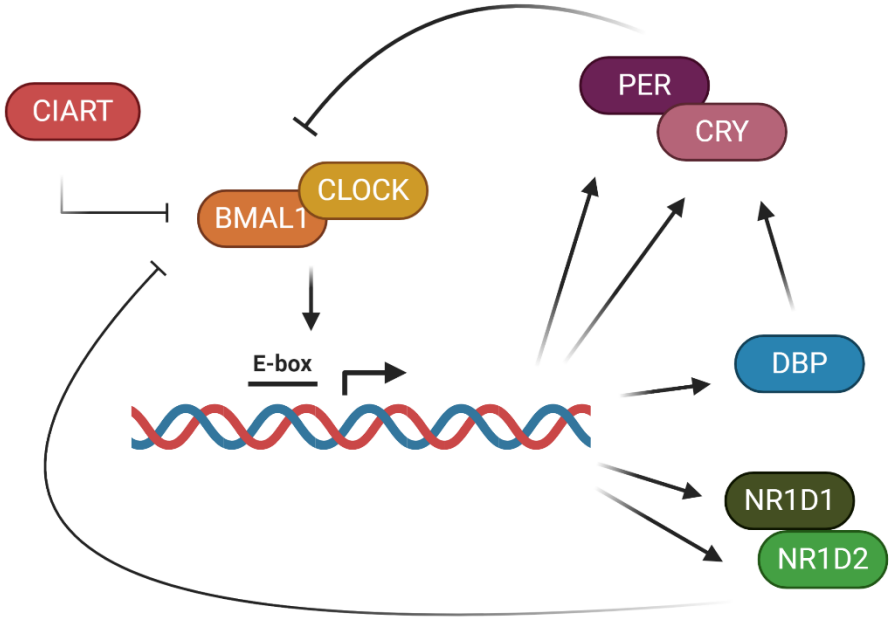

Figure 3. Core clock gene regulation. The molecular clock machinery works as a transcriptional feedback loop. The CLOCK:BMAL1 dimer regulates the expression of the other clock components, which at the same time concomitantly repress CLOCK:BMAL1 activity.

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transcription (CIART) acts independently and inhibits CLOCK:BMAL1 heterodimer transcriptional activity (64). All these elements can be regulated by transcriptional and post-translational modifications, and any modification will translate into changes in the regulation of many metabolic processes and chronobiology (60, 61) (Figure 3).

2.2.1 The circadian clock and metabolism

The circadian clock and metabolic homeostasis are tightly interconnected. Several studies show that alteration of the core clock components impacts metabolic homeostasis. In mice, liver specific BMAL1 depletion induces hypoglycemia, while inactivation of CRY leads to elevated fasting blood glucose (65). CLOCK mutant mice are more prone to hyperphagia, hyperlipidemia and hyperinsulinemia (66), and genetic ablation of either CLOCK or BMAL1 in mice results in loss of diurnal rhythms in plasma triglycerides and glucose levels (67, 68). Several studies show that NR1D1 regulates transcription factors that regulate different processes in a tissue-specific manner. NR1D1 regulates hepatic lipid metabolism (69, 70), UCP1 action in brown adipose tissue (71) and carbohydrate and lipid metabolism in white adipose tissue (72). This handful of data indicates that changes in clock gene expression induces disturbances in metabolism.

Fascinatingly, metabolic alterations also affect the circadian clock. CLOCK gene expression is altered in peripheral blood mononuclear cells of obese men (73) and visceral adipose tissue of obese women (74). Moreover, weight loss induced by 8 weeks of low-calorie diet, restores obesity-induced changes in clock gene expression in subcutaneous adipose tissue from overweight healthy men and women (75). In leukocytes, the expression of core clock genes is significantly lower in men with T2D than in the non-diabetic controls (76, 77). These studies show that metabolic disturbances also regulate the circadian clock. Altogether, literature provides evidence of a bidirectional regulation between circadian rhythms and metabolism.

2.2.2 Exercise and the circadian clock

Exercise training can delay, prevent and ameliorate metabolic disorders (78-80). Regular physical activity improves metabolic regulation, mitochondrial function, transcriptional regulation and intracellular signaling (80). Exercise also plays a role in entraining the circadian clock and may be one strategy to reset the circadian clock and ameliorate the negative effects of disrupted circadian rhythms (81), as well as the negative effects of disrupted metabolism in the circadian clock. Exercise timing has an important role modulating circadian gene transcription: wheel running at the onset of the active phase downregulates PER2 in the suprachiasmatic nucleus in rodents more than exercising at the end of the active phase (82); wheel running in constant darkness leads to downregulation of PER in the suprachiasmatic nucleus of hamsters (83); and induction of HIF1a and clock responsive genes varies depending the timing of exercise in mice (84). In humans, exercise performed in the afternoon has more beneficial effects regulating glucose levels than morning exercise in individuals with type 2 diabetes (85, 86).

Additionally, the circadian clock regulates skeletal muscle function. CLOCK mutant mice containing a truncated CLOCK(Δ19) protein present a reduction in peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and mitochondrial content in the skeletal muscle (87), indicating reduced skeletal muscle function. On the other hand, suppression of CRY1 and CRY2 increases PPARδ expression and improves exercise performance in mice (88). Altogether, these data reveal the importance of timing and frequency of physical activity for the improvement of metabolic disorders as well as circadian homeostasis for skeletal muscle performance.

2.2.3 Diet, the circadian clock and metabolism

Peripheral clocks are sensitive to metabolic cues such as food intake timing and macronutrient composition. In rodents, just one week of high-fat diet affects the length of the circadian period, independently of body weight change (89). Additionally, high-fat diet in mice

leads to changes in clock and clock-controlled gene expression in the liver, hypothalamus and adipose tissue (90). Caloric restriction and fasting also modulate clock gene expression: in rats, caloric restriction induces changes in circadian clock gene expression in the superchiasmatic nucleus independently of meal timing, while fasting blunts more than 80% of circadian transcripts in mouse liver (91). These expression changes might be regulated by changes in energy metabolites. In fact, AMPK activity is regulated according to the energy status of the cells, with higher activity of AMPK during caloric restriction. Elevated AMPK activity induces CRY1 and PER2 degradation (92, 93). NAD/NADH levels also regulate the circadian clock. Elevated levels of the reduced form NADH and NADPH can directly bind CLOCK/BMAL1 heterodimer and NPAS2, increasing their DNA binding activity (94). The oxidized form NAD+ activates SIRT1, which binds CLOCK:BMAL1 heterodimer and leads to PER2 and BMAL1 deacetylation (95, 96). Therefore, calorie intake, macronutrient composition and energy status modulate circadian clock gene expression.

Additionally, timing of food intake impacts the circadian clock and metabolic response: restricted access to food during only the rest phase (light phase) exacerbates diet-induced obesity in mice as compared to pair-fed controls that normally eat during the active phase (dark phase) (97). On the other hand, time-restricted high-fat diet reset clock gene expression and improves the metabolic profile of mice compared to high-fat diet ad libitum controls (98). These studies indicate that timing of food is important for clock and metabolic regulation. In fact, early-time restricted feeding improves insulin sensitivity and oxidative stress in pre-diabetic men (99); A 5h delay on meal time induces changes in the adipose tissue PER2 expression and plasma glucose profile in healthy men (100). Moreover, serum and skeletal muscle metabolome is responsive to timing and composition of the diet (101). Altogether, these studies highlight the importance of the “when” and “what” of the nutritional intake on the regulation of circadian rhythms.

2.3 FATTY ACIDS, THE CIRCADIAN CLOCK AND REGULATION OF TRANSCRIPTION

Lipids are involved in energy storage, signal transduction and serve as a structural component of cell membranes. Additionally, fatty acids regulate gene expression patterns that impact lipid, carbohydrate, and protein metabolism, as well as growth and proliferation. Lipids can interact with the genome in different ways, including targeting several nuclear receptors (PPAR, NFκB, and SREBP), interacting with transcriptional factors and regulating histone modifications (102, 103).

Circadian proteins have also a role in regulation of transcription (104): CLOCK:BMAL1 heterodimer acts as transcription activator complex, while PER:CRY heterodimer and NR1D1 act as transcription repressors (104). Epigenetic modifications, such as DNA methylation and histone modifications, modulate transcription regulation. Histone modifications are under circadian regulation, however, DNA methylation is not (104, 105). Thus, this thesis focuses on histone modifications.

2.3.1 Histone modifications and regulation of transcription.

DNA can present different levels of compaction, regulating transcription activity (Figure 4). The most compacted form of DNA is chromatin; chromatin is formed by nucleosomes, that at the same time consist of a histone protein octamer wrapped by 1.8 turns of DNA (106). The nucleosome is very stable DNA-protein complex, and it possesses dynamic properties that allow transcription to happen (106, 107). The histone octamer is formed by 2 copies of each histone protein: H2A, H2B, H3 and H4 (108, 109). Each histone protein has a region known as "histone fold" and a region known as "histone tail" (109). The "histone folds" are responsible for the formation of the stable octamer, while the "histone tails" interact with the DNA. Histone

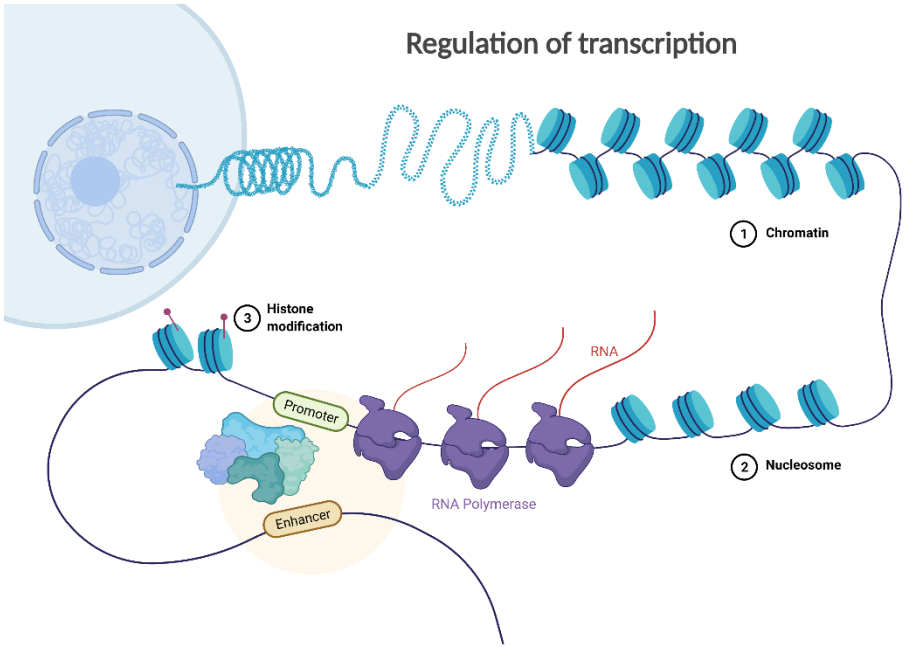

Figure 4. Regulation of transcription. DNA in the cell nucleus presents different levels of compaction and accessibility, which determines the regulation of transcription. **1.** Chromatin is a complex of DNA and protein. Chromatin consists of packed nucleosomes. **2.** Nucleosomes are the basic structural unit of DNA packaging. Eight histone protein subunits are wrapped in 1.8 turns of DNA**. 3.** For the DNA to be accessible for transcription, histone modifications such as acetylation and methylation have to occur.

Adapted from "Regulation of Transcription in Eukaryotic Cells", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates

tails present residues that are target for post-transcriptional modifications such as acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation. These covalent modifications alter the histone-DNA interaction and play a dynamic and integral role in the regulation of gene transcription (108-111) (Figure 4).

2.3.2 Fatty acids and histone modifications

While many histone modifications are responsive to the nutritional status of the cell, histone acetylation is the only modification that can be directly regulated by fatty acids and fatty acid metabolism (102). Histone lysine acetylation plays a fundamental role in transcriptional regulation. Histone acetylation is regulated by the action of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (112). Histone acetylation occurs mainly in the tail of histone proteins H3 and H4. Major acetylation sites include histone H3 lysine 9 (K9), K14,

K18, K23, and K27, and histone H4K5, K8, K12 and K16 (113). Hyperacetylated histones are associated with accessible genomic levels, while deacetylation results in repression and silencing of genes (112, 114). HATs and HDACs are responsive to the cellular metabolic state and thus, coordinate cellular energy and redox status with gene expression and metabolic activity (115). Histone acetylation and fatty acid synthesis compete for the nuclear and cytosolic pool of acetyl-CoA (116, 117), which is mainly produced through fatty acid metabolism (102, 116). Hence, the nutritional state of the cells and fatty acid turnover have a key role in regulation of histone acetylation and transcription.

2.3.3 Circadian rhythms and histone modifications

Chromatin modifications are tightly linked to the circadian clock. Circadian oscillations in chromatin modifications include histone acetylation and deacetylation. For example, histone H3 acetylation is rhythmic in the promoter regions of *Per1* and *Per2* in heart and liver as well as in fibroblasts (118, 119). Additionally, there are also genome-wide rhythms in H3K9 and H3K27 acetylation (120, 121). When it comes to the core clock members, CLOCK protein is a histone acetyltransferase and directly regulates PER1 and DBP transcription (122). Additionally, the CLOCK:BMAL1 heterodimer recruits and modulates the activity of HATs and HDACs (123, 124). The deacetylase SIRT1 is regulated by the cell energy level, and it is associated to CLOCK:BMAL1 heterodimer (96). CRY1, PER and NR1D1 also regulate circadian HDAC activity, including the activity of HDAC1, HDAC2 and HDAC3 (118, 125-127). Collectively, these studies suggest a tight regulation of chromatin acetylation by the core circadian clock.

2.4 CONCLUSIONS

Lipid metabolism plays a key role in the development of insulin resistance. Oxidative stress, DAG accumulation, and ceramides contribute to impaired glucose uptake in the skeletal muscle. Lipid aldehydes, by-products of oxidative stress and ceramide metabolism, also interfere with the insulin signaling pathway. Lipids regulate the circadian clock oscillations in

peripheral tissues. Strong evidence points at the circadian clock disturbances as a possible mechanism leading to hyperglycemia, dyslipidemia, insulin resistance, and increased risk of obesity and type 2 diabetes (128, 129). Indeed, increasing evidence suggest the "Circadian Syndrome" is a contributing factor in the development of the metabolic syndrome (9, 11). Additionally, lipid metabolism and circadian clock are important contributors to the regulation of histone acetylation and transcription (102, 122, 130). All these observations show an intimate relationship between nutrients, metabolic homeostasis, and the circadian clock.

The work of this thesis focuses on gaining a better understanding about the effects of oxidative stress on fatty acid metabolism and the development of insulin resistance and other metabolic disturbances in the skeletal muscle. Additionally, this thesis aims at understanding the role of obesity and T2D in the regulation of circadian rhythms in the skeletal muscle. Further studies are aimed at interrogating the factors driving a disrupted circadian clock in skeletal muscle and finding strategies to improve the pathological consequences.

3 RESEARCH AIMS

The overall aim of this thesis is to examine the molecular and cellular response to lipids in skeletal muscle in the context of obesity and type 2 diabetes (T2D). The specific aims of the papers included in the thesis are:

- **Study I:** to determine the role of the lipid aldehyde 4-hydroxy-2-hexenal (4-HHE) in the development of insulin resistance in skeletal muscle.
- **Study II:** to determine the role of obesity, weight loss and plasma lipids in the regulation of skeletal muscle circadian clock.
- **Study III:** to determine the role of obesity and saturated fatty acids in skeletal muscle circadian transcriptomic and epigenomic response to lipid overload.
- **Study IV:** to determine whether circadian control of gene expression and metabolism is altered at the cellular level in skeletal muscle from individuals with T2D.

4 MATERIALS AND METHODS

4.1 ETHICAL PERMITS

Study I was approved by the ethics committee of the University of Lyon (reference D-09-17). Animal experiments performed in study I were under authorisation no.69-266-0501 (INSA-Lyon,DDPP-SV, Direction Départementale de la Protection des Populations Services Vétérinaires du Rhône) according to the guidelines laid down by the French Ministry of Agriculture (no. 2013118) and the European Union Council Directive for the protection of animals (2010/63UE). **Studies II, III** and **IV** were approved by the regional ethics committee of Stockholm and conducted in accordance with the Declaration of Helsinki (2006/225-31/1, 2009/758-31, 2011/2045-31/4, 2012/1955-31/1, 2012/1047-31/2, 2013/647-31/3 and 2016/355-31/4). All participants gave their informed consent prior to enrolment. Animal experiments in **study IV** were conducted in accordance with institutional guidelines for the care and use of laboratory animals as approved by the University of Florida Institutional Animal Care and Use Committee and by the University of Barcelona Committee on Animal Care.

4.2 HUMAN COHORTS

Five different human cohorts were examined in the studies included in this thesis (Table 1). Cohorts were selected depending on study aims and samples availability. Two cohorts included T2D patients and their respective controls: the cohort in study I was part of a collaboration with the University of Lyon and was used to study 4-HHE plasma levels in T2Dindividuals with obesity. A separate cohort was interrogated in study IV to investigate the link between T2D and mitochondrial dysfunction. The other three cohorts included individuals with obesity and their controls: while two cohorts were used to study the sex-dependent effects of obesity and weight loss on clock gene expression in study II, the third cohort was used to study the effect of obesity on H3K27 acetylation.

4.2.1 Cohort for the study of plasma levels of 4-HHE and 4-HNE.

For **study I,** 15 individuals with T2D and 17 healthy volunteers were recruited to measure plasma levels of 4-HHE and 4-HNE. The anthropometric characteristics of the participants and basic clinical chemistry results are shown in Table 1. Blood samples were collected after an overnight fast, and plasma was isolated. Five hundred microlitres of plasma were used to measure 4-HHE and 4-HNE by GC–MS. Plasma samples were also used for the measurement of 4-HHE Michael adducts.

4.2.2 Cohort for the study of the effect of obesity on skeletal muscle circadian clock expression.

For **study II**, two independent cohorts were studied. Clock gene expression was determined in obese (BMI > 30) men (n=18) and women (n=5) before and 6 months after a standard laparoscopic Roux-en-Y gastric bypass (RYGB) performed at Danderyd Hospital, Stockholm, Sweden. Non-obese (BMI < 25) glucose-tolerant age-matched men (n=8) and women (n=6) were included as controls. The RYGB surgery and skeletal muscle biopsy procedure were performed for a previous study as described (131). Non-esterified fatty acids (NEFA) were measured in serum from the male participants using a NEFA-HR(2) Assay (FUJIFILM Wako Chemicals Europe). The anthropometric characteristics of the participants and basic clinical chemistry are shown in Table 1.

4.2.3 Cohort for the study of histone H3K27 acetylation in obesity.

For **study III,** *Vastus lateralis* muscle biopsies were obtained from 7 healthy men to establish primary skeletal muscle cell cultures to determine the effects of palmitate on circadian transcriptomics, histone H3 protein abundance, and H3K27ac. *Vastus lateralis* muscle biopsies were obtained from a cohort of men with normal weight (n=6) or obesity (n=6) as described earlier (131, 132) for immunoblot analysis of H3 protein abundance and H3K27ac. Clinical characteristics and basic clinical chemistry of the men with normal weight or obesity are presented in Table 1.

	Study I		Study II						Study III		Study IV	
	Control	**T2D**	**Control**		**Pre-RYGB**		**Post-RYGB**		**Control**	**Obese**	**Control**	**T2D**
Sex (M/F)	15/2	14/1	(0/6)	(8/0)	(0/5)	(18/0)	(0/5)	(17/0)	(6/0)	(6/0)	(22/0)	(22/0)
Age (yr)	51 (41-57)	66 (55-74)*	30 ± 4	42 ± 2.7	46 ± 5*	42 ± 2.6	46 ± 5*	42 ± 2.6	53 ± 3	48 ± 2	57.6 ± 2.2	61.8 ± 1.5
BMI (kg/m^2)	23.9 ± 2.9	30.8 ± 5 .0*	23.3 ± 1.5	25.8 ± 0.7	41.9 ± 2.0*	39.7 ± 0.7*	30.5 ± 1.8*,†	30 ± 0.7*,†	24.2 ± 0.3	31.1 ± 0.7*	26.8 ± 0.4	27.3 ± 0.5
Body weight (kg)		-	69.9 ± 6.0	82 ± 2.5	118.9 ± 6.2*	128 ± 3.5*	87.4 ± 6.4†	98 ± 3.8*,†	79.8 ± 2.6	103.3 ± 1.6*	83.5 ± 2.2	88.0 ± 1.7
fP-glucose (mmol/l)	5.1 (4.6-5.4)	9.4 (7.5-12.7)*	4.7 ± 0.2	5.4 ± 0.1	5.7 ± 0.4	6.8 ± 0.5	4.8 ± 0.3	5.4 ± 0.2	5.4 ± 0.2	6.5 ± 0.3	5.4 ± 2.2	7.8 ± 0.3*
fP-Insulin (pmol/l)		-	48.8 ± 9.9	50.4 ± 8.9	107.3 ± 24.3	167.0 ± 13.2	60.7 ± 11.7	76.1 ± 20.8	39.1 ± 7.2	116.3 ± 26.8*	48.7 ± 6.6	71.3 ± 7.7*
HOMA-IR		-	1.7 ± 0.4	1.7 ± 0.3	4.5 ± 1.0	7.54 ± 0.9	2.2 ± 0.4	2.7 ± 0.9	1.3 ± 0.2	4.3 ± 1.2*	1.8 ± 0.3	3.6 ± 0.4*
Total cholesterol (mmol/l)	5.81 ± 1.65	3.94 ± 1.11*	4.4 ± 0.5	5.0 ± 0.4	5.8 ± 0.71	4.9 ± 0.2	4.5 ± 0.5	3.8 ± 0.3*,†	4.9 ± 0.2	5.2 ± 0.5	5.3 ± 0.2	4.5 ± 0.2*
HDL Cholesterol (mmol/l)	1.22 ± 0.31	0.88 ± 0.25*	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	1.4 ± 0.2	1.1 ± 0.1	1.3 ± 0.05	1.6 ± 0.3	1.4 ± 0.1	1.3 ± 0.1
LDL Cholesterol (mmol/l)		-	2.5 ± 0.3	3.3 ± 0.3	3.3 ± 0.6	3.3 ± 0.2	2.6 ± 0.6	2.4 ± 0.2†	3.3 ± 0.2	2.9 ± 0.5	3.6 ± 0.2	2.6 ± 0.2*
Triacylglycerols (mmol/l)	1.19 ± 0.60	2.22 ± 0.87*	1.2 ± 0.3	1.2 ± 0.2	1.7 ± 0.4	2.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.1†	-		1.1 ± 0.2	1.2 ± 0.2
fP-NEFA (mmol/l)		-	0.22 ± 0.16	0.36 ± 0.04	0.66 ± 0.07*	0.68 ± 0.06*	0.40 ± 0.11	0.50 ± 0.09	-		0.5 ± 0.0	0.5 ± 0.0

Table 1. Clinical Characteristics of Study Participants. In **study I** values are presented as mean ± SD or median if data were not normally distributed. Statistical significance was determined by the Student's t-test or Mann-Whitney U test. In **study II,** results are mean ± SEM for normal weight and obese women and obese men pre- versus post-RYGB. Differences between normal weight, obese pre-RYGB and post-RYGB were determined using a one-way ANOVA with Sidak's *post hoc* test. In **study III and IV,** results are mean ± SEM. Differences between groups were determined using unpaired Student's *t*-test. *P<0.05 versus control, †P<0.05 versus Pre-RYGB.

4.2.4 Cohorts for the study of skeletal muscle transcriptome in individuals with obesity.

In **study III**, publicly available datasets containing samples from individuals with normal BMI or obesity were collected from the GEO repository and used to study the effect of obesity on the skeletal muscle transcriptome. Six datasets were identified: GSE43760, GSE45745, GSE73034, GSE73078 and GSE135066 (Table 2). Data were downloaded and log2 and quantile transformations were applied when necessary. All datasets were annotated and merged based on ENSEMBL names. Linear model was calculated using empirical Bayes statistics for differential expression with the Limma package (133), blocking for batch effects induced by inter-dataset differences.

4.2.5 Cohorts for the study of skeletal muscle transcriptome in individuals with T2D.

In **study IV**, *Vastus lateralis* muscle biopsies were obtained from 7 men with normal glucose tolerance and 5 men with T2D to establish primary skeletal muscle cell cultures and rhythmic gene expression was determined. Furthermore, we studied the effect of high glucose and high insulin treatment in myotube cultures from men with NGT or T2D. Transcriptomic analysis of *vastus lateralis* muscle biopsies from men with T2D (n=22) or NGT (n=22) was used to study the *in vivo* relevance of the *in vitro* experiments (Table 1).

GEO	*Biopsy*	*Sex*	*Participants (n)*	*Age (mean)*	*BMI (mean)*	*Diagnosis Group*	*PMID*	*Platform*
GSE43760	Vastus Lateralis	F	6	49	22.8	Control	27414688	Affymetrix Human Gene 1.0 ST Array
GSE43760	Vastus Lateralis	F	6	52	34.5	MetS	27414688	Affymetrix Human Gene 1.0 ST Array
GSE45745	Vastus Lateralis	F	6	30	23.3	Control	23583180	Affymetrix Human Genome U219 Array
GSE45745	Vastus Lateralis	F	5	46	41.9	IR	23583180	Affymetrix Human Genome U219 Array
GSE73034	Vastus Lateralis	U	7	55	21.9	Control	28725461	Agilent-014850 Whole Human Genome Microarray 4x44K
GSE73034	Vastus Lateralis	U	7	56	34.1	MetS	28725461	Agilent-014850 Whole Human Genome Microarray 4x44K
GSE73034	Vastus Lateralis	U	7	61	30.2	T2D	28725461	Agilent-014850 Whole Human Genome Microarray 4x44K
GSE73078	Vastus Lateralis	U	10	29	23.4	Control	27437034	Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray 039381
GSE73078	Vastus Lateralis	U	10	40	32.9	MetS	27437034	Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray 039381
GSE135066	Vastus Lateralis	U	14	40	24.5	Control	31519890	Agilent-079407 ArrayXS Human V3 079025

Table 2. Information about the gene arrays included in the meta-analysis. Publicly available transcriptomic studies of skeletal muscle obtained from individuals with obesity and healthy volunteers (control) were collected from the GEO repository. Diagnosis group was based on different clinical measures to assess insulin sensitivity as reported in the published papers. Subjects were classified as insulin sensitive (control), insulin resistant with features of the metabolic syndrome (MetS), insulin resistant based on HOMA-IR (IR), or type 2 diabetic (T2D). Datasets included studies in female individuals (F) or mixed male and female participants (U, undetermined). The number of participants and the average mean and body mass index (BMI) for each study group is reported.

4.3 ANIMAL EXPERIMENTS

In **study I** lean and Zucker diabetic fatty rats were used to measure 4-HHE and 4-HNE levels. At 15 weeks of age, rats were terminated, and blood was collected for plasma isolation. Body weight, blood glucose, insulin and HbA1c were measured for the metabolic characterization of the rats (Table 3). Then, euglycemic–hyperinsulinemic clamps were performed in 3-month-old anaesthetised male Wistar rats as described (134). Rats were fasted overnight, anaesthetised with sodium pentobarbital (35 mg/kg) and infused with 4-HHE (10 mg/kg, 0.1 ml) or DMSO-vehicle via a catheter. A standard 2h clamp was performed by constant infusion of human recombinant insulin and coupled by variable infusion of glucose to maintain blood glucose levels.

Trait	Lean	Obese
Body weight (g)	385 ± 7	729 ± 54*
Fasting blood glucose (mmol/l)	4.44 ± 0.28	25.50 ± 1.33*
Fasting insulin (pmol/l)	168 ± 26	427 ± 52*
HbA_{1c} (%)	4.74 ± 0.28	8.91 ± 0.28*
HbA_{1c} (mmol/mol)	28.3 ± 1.7	73.9 ± 2.3*

Table 3. Characteristics of 15-week-old lean and obese Zucker Diabetic Fatty (ZDF) rats. Values are mean ± SEM for n=5. Statistical significance was determined by the Student t test. *p<0.05

In **study IV**, two different cohorts of mice were studied. The first cohort consisted of adult C57BL/6J male mice and were used to investigate whether BMAL1 and CLOCK DNA binding regulates mitochondrial metabolism in skeletal muscle by performing Chromatin immunoprecipitation (ChIP) and sequencing. Two replicate samples for BMAL1 and CLOCK were used. Each sample required pooling gastrocnemius muscle from 10 adult C57BL/6J male mice (Jackson Labs, Farmington, Connecticut, USA). The mice were entrained to 12L/12D schedule and all tissues were collected at ZT2 and frozen immediately.

The second animal cohort used in **study IV** consisted of *Myo-Cre Opa1*$^{-/-}$ mice generated as reported (135-137) and *Cre OPA1* littermates served as controls**.** OPA knockout (KO) mice

were used to elucidate the effect of mitochondrial disruption in relation to the molecular-clock machinery.

4.4 SKELETAL MUSCLE CELL CULTURE

Obtaining detailed molecular knowledge from *in vivo* models is often complex. Cell cultures are a well-established model in research. The use of cell cultures models allows easier manipulation of experimental conditions and to study the consequences of a specific intervention to a specific group of cells in a controlled environment. Cell cultures introduce an extensive flexibility of treatment, which could not be done *in vivo*. Moreover, collection of samples can be performed much more often. In this thesis, immortalized L6 rat muscle cells (from the American Type Culture Collection) and primary human skeletal muscle cells were used to investigate the effects of lipid aldehydes (4-HNE and 4-HHE), to study the effect of lipid overload on skeletal muscle circadian metabolism (palmitate and oleate), and to study differences between NGT and T2D circadian transcriptomics.

4.4.1 L6 cell culture.

An advantage of using immortalized rat L6 skeletal muscle cells is that their quick proliferation enables rapid data collection. In **study I**, rat L6 cells were used to determine the role of 4-HHE on the development of insulin resistance. L6 cells were cultured in CO_2-ventilated, humidified incubators at 37C, with Minimum Essential Medium Eagle Alpha Modification (Sigma-Aldrich) media containing all necessary nutrients (40). At 80% confluence, myoblasts were differentiated into multinucleated myotubes by serum deprivation. All experiments were performed on fully differentiated myotubes after 4-6 days of serum deprivation.

4.4.2 Primary human skeletal muscle cell culture.

The use of primary skeletal muscle cells was central for **study II**, **study III** and **study IV**. For **studies II** and **III,** *Vastus lateralis* muscle biopsies were obtained from healthy male volunteers (Normal glucose tolerant, NGT) and primary myotube cultures were prepared as described (138). Additionally, for **study IV**, primary cultures were prepared from *vastus lateralis* muscle biopsies from T2D patients. Clinical characteristics from the donors can be found in table 4. Isolated cells were cultured in CO2-ventilated, humidified incubators at 37C, with media containing all necessary nutrients. Cells were regularly tested for mycoplasma by PCR. At 80% confluence, myoblasts were differentiated into multinucleated myotubes that resemble mature skeletal muscle. Although *in vitro* models cannot be used to draw direct conclusions for clinical practice, primary human skeletal muscle cells from several donors introduces the person-to-person genetic differences and therefore improve the translatability of the results obtained *in vitro*.

Trait	NGT	T2D
Age	63 ± 1	60 ± 1
Body weight (kg)	93.6 ± 3.5	89.7 ± 6.2
BMI	28.8 ± 0.8	27.6 ± 1.2
fP-glucose (mmol/l)	5.1 ± 0.2	9.06 ± 0.9*
HOMA-IR	2.5 ± 0.7	2.9 ± 1.1
HbA_{1c} (mmol/l)	30.3 ± 1.5	59.6 ± 5.6*
Triglycerides (mmol/l)	1.4 ± 0.4	1.25 ± 0.3
Cholesterol (mmol/l)	5.8 ± 0.4	3.8 ± 0.4*
HDL (mmol/l)	1.3 ± 0.2	1.2 ± 0.1
LDL (mmol/l)	3.8 ± 0.3	2 ± 0.3*

Table 4. Clinical characteristics of skeletal muscle cells donors. Values are mean ± SEM for n=5. Statistical significance was determined by the Student t test. *$p<0.05$

Primary skeletal muscle cells culture provides a valuable research tool in the study of skeletal muscle development, differentiation, and metabolism. In **study II and III**, primary skeletal muscle cells were derived from individuals with NGT were used to investigate the effect of fatty acids palmitate and oleate on skeletal muscle circadian clock and metabolism. In **study IV,** primary skeletal muscle cells were derived from individuals with NGT or T2D and used to

study the inherent differences in gene expression rhythmicity and investigate the effect of diabetes milieu on skeletal muscle circadian clock and metabolism.

4.5 TREATMENTS AND FUNCTIONAL EXPERIMENTS IN CELLS

4.5.1 4-hydroxy-2hexenal (4-HHE) treatment.

In order to study the effects of 4-HHE on glucose uptake and insulin signaling in skeletal muscle cells, in **study I**, rat L6 cells were serum starved for 4h and then exposed to 5, 10, 50 or 100 μM of 4-HHE for 30 or 60 minutes. Subsequently, L6 cells were incubated for 20 min with 100 nM insulin or 20 μM cytochalasin B. Then, glucose uptake was measured, and protein lysates were collected to study the effect of 4-HHE on insulin signaling and glucose metabolism.

4.5.2 Glucose uptake measurement.

In **study I**, glucose uptake assay was performed as described in (139). In short, glucose uptake was initiated by the addition of 0.1 mM 2-deoxy-D-[^{3}H]-glucose for 5 min at 37°C. Glucose uptake was terminated by three washes in ice-cold phosphate buffer saline (PBS) and solubilization in sodium dodecyl sulfate (SDS). Tritium was detected by liquid scintillation counting and results normalized by protein concentration measured using the Bradford assay. Non-specific glucose uptake was measured in presence of cytochalasin B was subtracted from each determination.

4.5.3 Reduced glutathione and D3T treatment.

Reduced glutathione (GSH) has a major role in aldehyde and ROS detoxification mechanism. In **study I**, GSH was measured after 4-HHE treatment (0 to 100 μM, 30 min) using a commercially available kit from BioVision (Clinisciences, Montrouge, France) and normalized to the protein concentration measured with Bradford assay (Bio-Rad). Prior to 4-HHE treatment, cells were incubated for 24h with or without the GSH precursor D3T (100 μM) to assess the protective role of GSH.

4.5.4 Palmitate, oleate and high glucose and insulin treatment.

For **study II**, the effect palmitate and oleate on the skeletal muscle internal clock was investigated. In **study III,** the focus was the role of palmitate on regulating skeletal muscle circadian transcriptomics. In **study IV,** the circadian differences between skeletal muscle cells from individuals with NGT or T2D were investigated, and the effect of high glucose – high insulin exposure on circadian gene expression was explored. Stock solutions of palmitate (0.4 mM) and oleate (0.4 mM) were prepared in 50% ethanol and then diluted 25 times in a 10.5% BSA solution. BSA in serum-free essential α medium (αMEM) was used as a carrier and control. For **study II**, myotubes were incubated with palmitate, oleate or BSA control for 24h, and then the cells were collected for mRNA analysis. For **study II, III and IV,** myotubes were incubated in low glucose media for 22h prior to experiments. Myotubes were synchronized by serum shock (50% FBS, 2h), washed with PBS, and incubated in low glucose medium containing palmitate (0.4 mM), or BSA (vehicle). To partly mimic a T2D milieu, for **study IV,** a subset of cells were incubated in high glucose and insulin (50 nM insulin, 25 mM glucose) for 22h prior to the serum shock. Media with high glucose and insulin was also added after serum shock. Cultures were collected every 6h for mRNA expression analysis and every 8h for DNA and protein analysis, from 12h to 54h after synchronization (Figure 5).

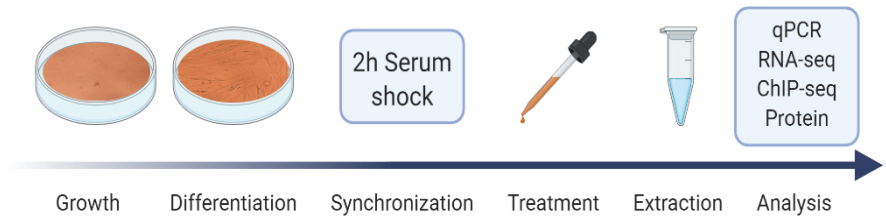

Figure 5. Schematic representation of the timeline of the circadian cell experiments.

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4.5.5 Methods to determine gene expression.

Four different methods were used to study gene expression and the regulation of transcription.

4.5.5.1 Gene arrays.

Gene arrays can generate transcriptomic profiles of thousands of mRNA transcripts at the same time, and subsequently, they have become accessible and easy to analyze. In **study II** publicly available gene array data was used to study changes in clock gene expression in the skeletal muscle of obese women before and after RYGB as compared to normal weight women.

In **study III,** six publicly available datasets were used to study gene expression differences in skeletal muscle from people with obesity or normal weight.

4.5.5.2 Reverse transcription-quantitative PCR (RT-qPCR).

Gene-array data in **study II** was complemented by RT-qPCR. Skeletal muscle clock gene expression of men with obesity before and after RYGB was compared to men with normal weight. RT-qPCR was also used to investigate the effect of palmitate or oleate treatment on clock gene expression in primary skeletal muscle cells. In **study IV,** RT-qPCR was used to study the effects of OPA1 silencing on skeletal muscle cells gene expression.

4.5.5.3 RNA sequencing.

RNA-sequencing allows the quantification of expression of all mRNAs in a tissue simultaneously, without previous knowledge of the target sequences. RNA-seq uses next generation sequencing to reveal the presence and abundance of RNA in a biological sample. In **study III and IV,** changes in primary human skeletal muscle circadian transcriptomics were analyzed by RNA-seq of samples collected every 6h from 12 to 54h after synchronization. RNA quality was assessed using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Aliquots of RNA (1 μg) were processed into RNA sequencing libraries using the Illumina TruSeq Stranded Total RNA with Ribo-Zero Gold protocol (Illumina) as described (140). Briefly, RNA samples underwent fragmentation and sizing, followed by RNA conversion into cDNA. These steps are followed by attachment of adaptors and library quantification. Then, the samples are ready to be sequenced.

4.5.5.4 Chromatin Immunoprecipitation and sequencing (ChIP-seq).

Chromatin immunoprecipitation and sequencing (ChIP-seq) is a powerful tool to identify genome-wide DNA binding sites for transcription factors and other proteins. After performing ChIP protocols, DNA-bound protein is immunoprecipitated using a specific antibody. The bound DNA is co-precipitated, purified, and sequenced. ChIP-seq elucidates insights into gene expression regulation. In **study III**, ChIP-seq of histone H3K27 acetylation (#Ab4729) was performed in DNA samples collected every 8h, from 24 to 48h after synchronization in order to study circadian changes at the enhancer level. The aim of this experiment was to elucidate the role of palmitate on circadian gene expression regulation. In **study IV,** ChIP-seq for BMAL1 (#SAB4300614) and CLOCK (#ab3517) was performed in skeletal muscle from C57BL/6J male mice. The aim of this experiment was to study BMAL1 and CLOCK binding to mitochondrial genes and investigate the role of BMAL1 and CLOCK on the regulation of mitochondrial rhythms in skeletal muscle and the development of insulin resistance. ChIP-seq was performed as described (141), including DNA processing into DNA sequencing libraries.

4.5.6 Methods to determine protein abundance.

To investigate alterations in protein abundance and signalling, Western blot analysis was used in **study I** and **study III.** Western blot is a technique used to measure the abundance of a specific protein and can also be used to estimate protein activity. In this thesis western blot analysis has been used to study the effect of 4-HHE in rat skeletal muscle and L6 cells, to determine the effect of obesity in skeletal muscle biopsies from humans, and reveal the effect of palmitate in primary skeletal muscle cells. To maintain protein integrity and post-translational modifications, samples were lysed in ice-cold buffer containing protease and phosphatase inhibitors. Protein concentration was determined, and samples were diluted to the same final concentration in Laemmli buffer. Protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Criterion XT Bis-Tris Gels (Bio-rad), transferred to PVDF membranes and Ponceau staining was performed to determine total

protein per lane. The membranes were blocked by 7.5% non-fat dried milk in tris-buffered saline- (TBS) Tween 20. Western blot was performed using primary antibodies (1:1000 concentration) in tris-buffered saline (TBS) containing 0.1% bovine serum albumin (BSA) and 0.1% NaN_3. In **study I**, antibodies to Ser473-Akt (#9271), total Akt (#4691), p85 (#4292) and total IRS1 (#2382) from Cell Signaling were used. In **study III,** Bionordika antibodies for Acetyl-Histone H3 Lysine 27 (#8173S), Acetylated-Lysine (#9441S) and Histone H3 (#4499T) were used. Species-appropriate horseradish peroxidase conjugated secondary antibodies were used at a concentration of 1:25,000 in 5% skimmed milk in TBS-Tween. Proteins were visualized by chemiluminescence and the quantification was performed by ImageLab software v. 5.2.1 (BioRad).

4.5.7 Oxygen consumption measurements.

To study mitochondrial function in skeletal muscle cells from derived from individuals with T2D versus NGT**,** we measured oxygen consumption rate (OCR) and extracellular acidification rates (ECAR) in **study IV**. Cells were subjected to a Seahorse XF Mito Stress Test (142). OCR and ECAR were measured in cells from NGT donors (n=5) and T2D donors (n=5) at three time points under unstimulated conditions, then after treatment with 1 µM oligomycin, 2 µM FCCP, and 0.75 µM rotenone + antimycin A. First, oligomycin inhibits ATP synthase (complex V) and reduces the electron flow, resulting in a decrease of OCR. Then, FCCP collapses the proton gradient and disrupts mitochondrial membrane potential. As a result, electron flow through Electron Transport Chain is uninhibited and oxygen consumption by complex IV reaches the maximum. The FCCP-stimulated OCR can then be used to calculate spare respiratory capacity, defined as the difference between maximal respiration and basal respiration. Lastly, rotenone and antimycin A, complex I and complex III inhibitors, block mitochondrial respiration and enable the calculation of nonmitochondrial respiration. OCR and ECAR were then normalized to protein content (BCA assay, ThermoFisher).

4.5.8 Mitochondrial disruption treatments

In **study IV,** we investigated the role of the mitochondrial dysfunction on the regulation of clock gene expression. Skeletal muscle cells from donors with NGT were used to study the effect of FCCP (2 µm), Rotenone a/antimycin a (0.38 µm), and oligomycin (1 µm) on clock gene expression. Cells were synchronized and compounds or vehicle control were added 14h after synchronization. Cells were harvested for RNA after 4h of exposure and gene expression was measured by RT-qPCR.

Primary skeletal muscle cells were also used to study the effects of *OPA1* silencing on clock gene expression. OPA1 is an established regulator of inner-mitochondrial morphology and function in skeletal muscle (143-146). With silencing of OPA1 we aimed at disruption mitochondrial function and study the effects on clock gene expression. After differentiation, cells were transfected as described (147), using 5 µM of either Silencer® Select, Negative Control No. 2 (#4390847) or validated siRNAs against *OPA1* (#s9852), for two separate 5-hr transfection periods separated by ~48 hrs. Gene expression using RT-qPCR and NAD levels using an enzymatic cycling assay (148) were measured in skeletal muscle cells after *OPA1* silencing.

4.6 BIOINFORMATIC ANALYSES

The bioinformatic field develops methods and software for the analysis, understanding and representation of large-scale biological data. Sequencing technologies are becoming more available and the need to develop new tools to process such results is increasing rapidly. Sequencing and bioinformatic tools were used analyzing and understanding the data produced in **studies III** and **IV.**

4.6.1 RNA-seq and ChIP-seq sample processing.

After library preparation, RNA and DNA samples were sequenced using X Ten platform (Illumina) and HiSeq2000, respectively. Raw reads were obtained after sequencing. Quality of RNA-Seq samples was explored using Fastqc software, that estimates the probability that the corresponding base call is incorrect. RNA-seq reads ($\overline{n} \approx 40$ M) from FASTQ files were quality-trimmed using Trim_Galore (v0.4.3). Trimmed reads were aligned using STAR (v2.5.3a) (149) aligner with Ensembl human annotation (GRCh38, release 92) (150) and gene features were counted using FeatureCounts from subread (v1.5.2) package (151) resulting in 27 M uniquely mapped and 20 M assigned reads to genomic features (genes) on average, respectively. The lowly expressed genes were discarded from downstream analysis using filterByExpr function from EdgeR package (152) resulting 18,482 genes. As an apparent batch effect was introduced by participants (Figure S1), this batch effect was removed by using Limma's removeBatchEffect function (153) (Figure 6).

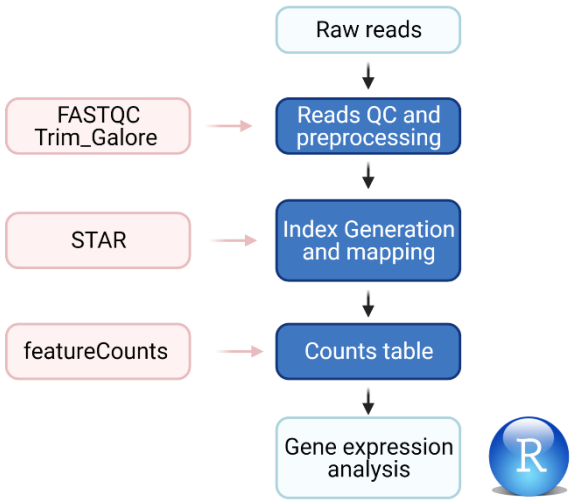

Figure 6. Pipeline for RNA-seq processing

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H3K27ac ChIP-Seq samples quality was also assessed using Fastq software and quality trimmed with Trimmomatic on the same parameters as RNA-Seq data (154). Reads were mapped to *H. sapiens* reference genome using Bowtie2 aligner (155) and Samtools was used for duplicate removal (156). Bam files were then subjected to peak count using MACS software for broad peaks using a q-value of 0.01 and a shift of 147 bp (157). Called peaks were quantified with FeatureCounts and associated to closest genes using RGmatch software, mapping regions

to TSS, promoter of 1st exon (151, 158). The resulting peak matrix was Reads Per Kilobase of transcript, per Million mapped reads (RPKM) normalized and batch corrected using ComBat to correct for sequencing lane bias (159).

BMAL1 and CLOCK ChIP-seq reads were aligned to *Mus musculus* genome assembly GRCm38 (mm 10) using Bowtie2 (160). The biological replicates of the aligned reads were merged for *CLOCK*, *BMAL1*, and input, respectively. Homer software (161) was deployed to perform peak calling for the CLOCK sample and the BMAL1 sample with the input sample as background. The default FDR rate threshold 0.001 was used to detect significant peaks.

4.6.2 Functional analysis using R

Alongside Python, R is the most commonly used programming language for biological data processing. R was used for the analysis of the gene array, RNA-seq and H3K27ac ChIP-seq performed in **study II, III and IV**. Differences in gene expression of gene arrays or RNA-seq, as well as differences in histone acetylation in the ChIP-seq, were determined using the R package Limma (133). Limma uses linear models and allows to identify changes in expression of thousands of genes throughout different conditions. Limma package was particularly useful in study III, allowing to study the effect of palmitate independently of time with its blocking feature.

Rhythmic oscillations of gene expression from the RNA-seq in **study III** and **IV,** as well as rhythmic oscillations in acetylated regions of H3K27ac ChIP-seq in **study III**, were analyzed with the R Package rain (162). Rain uses non-parametric methods to detect rhythmicity in time series. Rhythmicity was determined based on a 24h longitudinal period. Additionally, in **study IV**, the DODR R package (163) was used to detect differential rhythmicity among conditions. Genes with adjusted meta p-value below 0.10 were considered differentially rhythmic

Pathway analysis has become a valuable resource for gaining insight into the biological relevance of the changes in expression observed after a specific intervention (164). Pathway analysis was used in **study III** and **study IV**, to investigate the biological significance of the changes observed in circadian gene expression after palmitate treatment or T2D diagnosis, respectively. In the last decades, many different pathway analysis approaches have been developed. In **study III**, Functional Annotation Clustering analysis were performed using DAVID bioinformatic resources 6.8 (165, 166). The DAVID Functional Annotation Clustering function uses an algorithm to measure relationships among annotation terms. We introduced a list of significant genes (FDR<0.1) and DAVID measured the degree of co-association between gene to group annotation contents from the same or different resources into annotation groups. For each Functional Cluster, an enrichment score is calculated by performing the geometric mean (-log scale) of p-values of terms included in the cluster. For **Study III** analysis, Functional Clusters with an enrichment score greater than 1.3 were selected. The p-values for each individual term were adjusted for multiple testing by FDR.

In **study IV**, pathway enrichment analysis was performed by using both over-representation analysis (ORA) and gene-set enrichment analysis (GSEA). When using ORA, we introduced a list of significant features (e.g circadian genes in T2D cells) and searched for terms that were enriched. For GSEA we used all features and introduced a ranking metric (e.g amplitude log2-fold-changes). The outcome in this case is a list of enriched terms where all features contribute according to their ranks.

Study IV used ORA to investigate which terms are enriched based on the circadian genes specific for each disease and treatment groups (NGT or T2D and BSA or high glucose and insulin). Similarly, genes with significant correlations between insulin resistance (M-value) and basal gene expression were tested for gene ontology (GO) (167) cellular components (CC) enrichment using ORA method. GSEA was used to investigate enriched pathways based on the relative amplitude log2-fold-changes of cycling genes. ClusterProfiler (168) R package was

used to analyze all gene enrichment results and the Reactome database (169) was used for the interpretation of biological processes.

5 RESULTS AND DISCUSSION

5.1 STUDY I: SKELETAL MUSCLE INSULIN RESISTANCE IS INDUCED BY 4-HYDROXY-2-HEXENAL, A BY-PRODUCT OF N-3 FATTY ACID PEROXIDATION

Oxidative stress occurs as a consequence of ROS accumulation and the inability of the tissues to detoxify ROS by-products (34). Oxidative stress is involved in the pathophysiology of insulin resistance and its progression to T2D (33). 4-hydoxy-neonal (4-HNE) and 4-hydroxy-2-hexenal (4-HHE) are lipid aldehydes produced from lipid peroxidation under conditions of oxidative stress. 4-HNE had been previously studied for its role on the development of insulin resistance, however, the role of 4-HHE in the development of insulin resistance was unknown. In **study I** we determined the specific role of 4-HHE in the development of insulin resistance in skeletal muscle.

5.1.1 Increased plasma 4-HHE levels in individuals with type 2 diabetes

Advanced glycation end-products (AGEs) are increased in the circulation of individuals with T2D, the prototypical one being glycated hemoglobin (HbA1c), which is used clinically as a marker of blood glucose control (170). However, advanced lipoxidation end products (ALEs) are not easily measured, and haven't been used as insulin resistance and T2D markers. We determined the plasma levels of free (unbound) 4-HHE and 4-HNE in individuals with

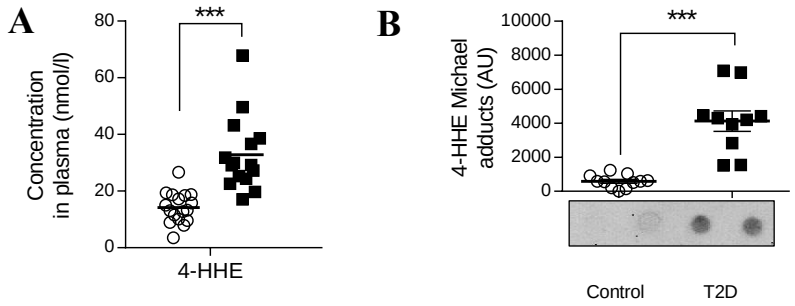


Figure 7. 4-HHE plasma levels are increased in individuals with type 2 diabetes. A. Plasma was collected from type 2 diabetic (T2D) or healthy volunteers (Control) and 4-HHE was measured using GC-MS. **B.** 4-HHE adducts on plasma proteins were measured by dot blot using anti-4-HHE Michael adduct antibody. Data are mean ± SEM, 2-way ANOVA. White circles represent values for NGT, and black squares values for T2D.

T2D or NGT (Table 1). Gas chromatography-mass spectrometry (GC-MS) analysis revealed that 4-HHE levels were increased in individuals with T2D (Figure 7A), while the levels of 4-HNE remained the same between groups (Figure 1A in the article). 4-HHE plasma levels are also increased in chronic kidney disease (171), reinforcing the evidence for lipid aldehyde accumulation in metabolic disease states. One of the properties of lipid aldehydes is their ability to form adducts, by covalently binding to other molecules such as phospholipids, DNA and proteins (172) and therefore interfering with physiological processes. We revealed that individuals with T2D had increased levels of protein adducts (aka Michael adducts) compared to normal glucose tolerant individuals (Figure 7B). This accumulation of protein adducts has been previously reported in T2D and correlates with glycemic control (173, 174).

Proteins containing lipid adducts belong to the group of molecules known as ALEs, which have been largely studied for their role in the development of diabetes, cardiovascular diseases and other metabolic diseases (30, 39, 42). Similarly, AGEs result from nonenzymatic binding of a sugar molecule with a free amino acid group of a protein, and are formed when glucose levels are increased (i.e. state of hyperglycemia) (39, 175). Adducts can modify protein structure and lead to oligomerization, aggregation and malfunction (30), resulting in metabolic impairment. ALEs and AGEs formation are both enhanced by oxidative stress and ROS accumulation. ALEs have a causative role in the development of T2D (175), while AGEs might be responsible of the pathogenesis of long-term complications in diabetes. Our data reinforces the evidence for increased production of lipid aldehydes and formation of protein adducts during the development and state of diabetes.

5.1.2 4-HHE induces insulin resistance *in vivo* and *in vitro*

In order to investigate whether increased 4-HHE plasma levels could be contributing to the development of insulin resistance, we performed an euglycemic hyperinsulinemic clamp in rats given an intravenous bolus of 4-HHE. Infusion of 4-HHE (10 mg/kg) in rats reduced the glucose disposal rate during the clamp (Figure 8A and B), indicating development of insulin

resistance. The clamp technique primarily measures peripheral insulin sensitivity of the liver, adipose and muscle tissue. In individuals with normal glucose tolerance, skeletal muscle is responsible for 80 to 90% of glucose uptake (176) and plays a key role on the regulation of whole-body insulin sensitivity. We therefore investigated the mechanisms of action of 4-HHE on skeletal muscle insulin resistance. We cultured rat L6 skeletal muscle cells and exposed them to 4-HHE for 30 min and observed a dose-dependent reduction in glucose uptake under

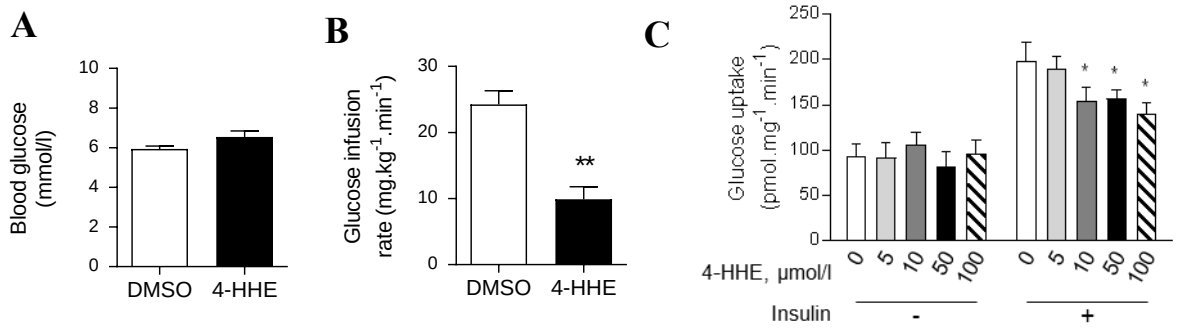


Figure 8. 4-HHE induces insulin resistance in rats and L6 muscle cells. Euglycemic hyperinsulinemic clamps were performed in anesthetized rats infused with 4-HHE (10 mg/kg iv) or vehicle (DMSO) as described in methods. **A.** Mean Plasma glucose during the second hour of the clamp. **B.** Glucose infusion rate calculated during the second hour of clamp. Data are mean ± SEM, n=4, one-way ANOVA **C.** L6 cells were exposed to different concentrations of 4-HHE and insulin-stimulated glucose uptake was measured using ^{3}H 2-deoxy-glucose. Data are mean ± SEM, n=5, one-way ANOVA. *p<0.05

insulin-stimulated conditions (Figure 8C). These data reveal a causative role for 4-HHE on the development of whole-body insulin resistance with specific effect on skeletal muscle glucose uptake. Insulin resistance is the first step towards T2D development and is also responsible of the major complications of diabetes including nephropathy, neuropathy, retinopathy and macro- and microvascular damage (33, 34, 177, 178). Thus, revealing the mechanisms by which 4-HHE induced insulin resistance *in vivo* and *in vitro* is key to define strategies to prevent development of T2D.

5.1.3 Mechanism of actions of 4-HHE

The insulin signaling cascade leads to glucose uptake and can be regulated at the levels of GLUT4 translocation, protein kinase B (Akt) phosphorylation and/or upstream at the insulin receptor or IRS (179, 180). Treatment of L6 myotubes with increasing concentrations of 4-

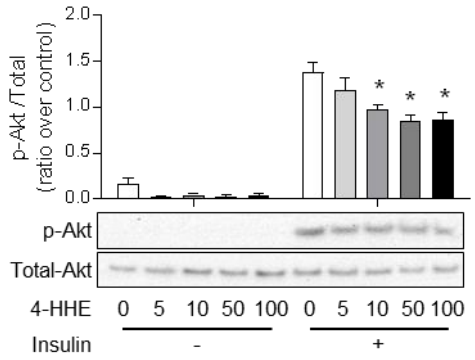


Figure 9. Insulin-induced phosphorylation of Akt is reduced after 4-HHE treatment. L6 cells were exposed to increasing concentrations of 4-HHE. Protein extracts were collected before and after insulin stimulation and analyzed by Western blotting. Data are expressed as means±SEM, n=4. One-way ANOVA *P<0.05

HHE for 30 minutes led to a reduction of Akt phosphorylation by 50% (Figure 9). A previous study demonstrated that in 3T3 adipocytes, the lipid peroxidation by-product 4-HNE disrupts the insulin signalling cascade by directly binding IRS1 and promoting its degradation (181). However, we did not observe any change in the total amount of PKB/Akt and IRS1 proteins in response to 4-HHE, indicating that 4-HHE deleterious effects are not mediated by specific degradation of proteins of the insulin signalling pathway in skeletal muscle cells. Lipid aldehydes, such as 4-HHE and 4-HNE, have high chemical reactivity, and thus form covalent binding with other molecules such as DNA, phospholipids and proteins (172, 182, 183). In fact, incubation of L6 cells with 4-HHE for 30 min was sufficient to induce an increase in Michael adducts formation in a dose dependent manner (Figure 10A). These results are consistent with the increase in protein adducts we observed in human plasma and therefore supports that 4-HHE-induced insulin resistance is due to adduct formation.

Protein adducts can lead to protein dysfunction and impairment of cellular responses by several mechanisms (41, 184, 185). Protein adducts inhibit proteasomal activity, promoting the formation of protein aggregates, pro-inflammatory responses and ER stress (186). Additionally, lipid peroxidation end-products interact with mitochondrial proteins and lead to mitochondrial dysfunction and increased oxidative stress (187). Thus, accumulation of ALEs and protein adducts increases oxidative and ER stress in the cells, promoting insulin resistance. It is plausible that the deleterious effects of 4-HHE on insulin signalling happened through ER stress, although this was not tested in the current study.

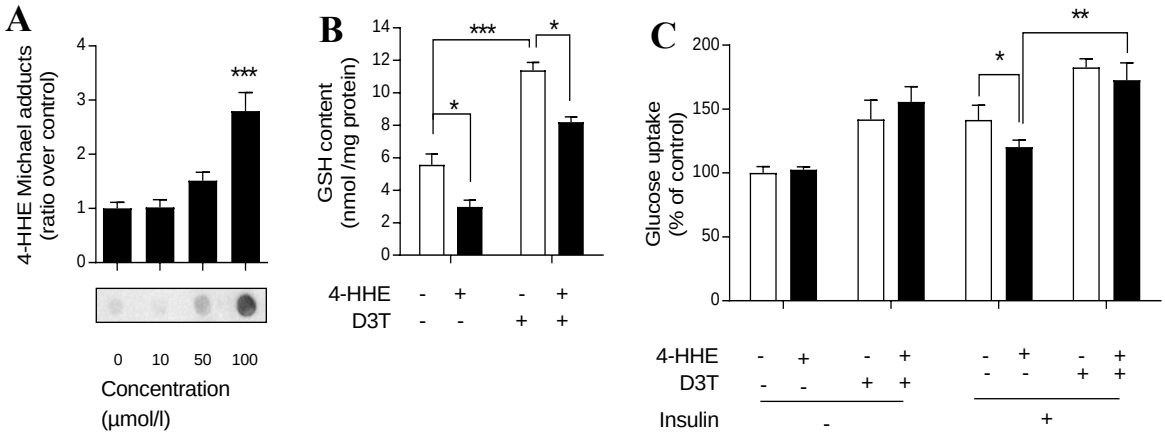


Figure 10. 4-HHE treatment induces Michael adducts formation. A. L6 cells were exposed to 4-HHE for 30 min and adduct formation on proteins was determined by Dot Blot using antibodies against 4-HHE Michael adducts. Mean ± SEM, n=6, 1-way ANOVA. **B.** L6 cells were pre-incubated with 100 µmol/l of D3T for 24 h and then treated with 50 µmol/l 4-HHE for 30 min. Then reduced glutathione was measured. Mean ± SEM, n=3, 2-way ANOVA (D3T vs 4-HHE). **C.** After D3T pre-incubation and 4-HHE exposure, insulin stimulated glucose uptake was measured using ^{3}H-2-deoxy-glucose. Mean ± SEM, n=4, 2-way ANOVA (D3T vs 4-HHE). *p<0.05, **p<0.01, ***p<0.001.

One of the major mechanisms for aldehyde detoxification is their reaction with reduced glutathione (GSH) (188). Lipid aldehydes have high affinity for GSH and in the presence of GSH do not bind to other molecules. Individuals with type 2 diabetic have a severe deficiency of glutathione synthesis due to limited precursor availability (189) and dietary supplementation with GSH precursor amino acids can restore GSH synthesis and reduce oxidative stress in T2D (189). Recapitulating the decreased GSH content in individuals with T2D, treatment of L6 cells

with increasing concentration of 4-HHE also decreased GSH concentration. Increasing GSH pool by treating L6 cells with D3T, a GSH precursor doubled the concentration of GSH in the cells and reduced the amount of Michael adducts formed after 4-HHE treatment (Figure 10B). Moreover, D3T pre-treatment also increased basal glucose uptake and prevented 4-HHE induced insulin resistance (Figure 10C). Concomitantly, our data showed that increasing GSH pools is an effective strategy to prevent the deleterious effects of 4-HHE on skeletal muscle insulin sensitivity. Increasing GSH pools reversed the deleterious effects of 4-HHE on insulin sensitivity, and therefore supports the idea that the lipid aldehyde 4-HHE disrupts insulin sensitivity by forming covalent adducts with key proteins that interfere with the insulin signalling pathway.

5.1.4 Conclusions and future perspectives for study I

In conclusion, in **study I** we demonstrated a causal role for the lipid peroxidation by-product 4-HHE in the development of insulin resistance and T2D through protein adduct formation. These reactive lipid peroxidation by-products represent an interesting therapeutic target to taper insulin resistance. Antioxidant strategies and supplementation might be a good mechanism to prevent and ameliorate the negative effects of oxidative stress to the insulin signalling pathway (43, 190-192). For example, weight loss and physical activity improve antioxidant defences and lower lipid peroxidation (193); antidiabetic medication act as antioxidants indirectly by lowering glucose levels and directly acting as scavengers (191); and vitamin E supplementation reduces oxidative stress and improves glycaemic control (191, 194, 195). Prospective studies have shown vitamin E supplementation might protect against insulin resistance and other cardiometabolic diseases (196, 197). Altogether, our study reinforces the need of developing antioxidant strategies for the prevention of insulin resistance and T2D complications and opens a new horizon for research aiming at more specific treatments reducing protein adduct formation involved in the insulin signalling pathway.

5.2 STUDY II: INFLUENCE OF OBESITY, WEIGHT LOSS, AND FREE FATTY ACIDS ON SKELETAL MUSCLE CLOCK GENE EXPRESSION

According to the World Health Organization, obesity is characterized by a BMI value greater than 30 kg/m^2, while normal weight is defined by a BMI between 18.5 and 24.9 kg/m^2. Obesity is characterized by an increase of circulating fatty acids and ectopic accumulation of lipids in tissues other than the adipose tissue (15, 198). Additionally, weight gain and obesity have been linked to disturbances in the circadian clock (74). The objective of **study II** was to determine the effect of obesity and circulating free fatty acids on the skeletal muscle circadian clock.

5.2.1 Identification of disrupted skeletal muscle internal clock in obesity

The first aim of this study was to investigate whether obesity induces changes in expression of core clock genes compared to normal weigh controls and whether weight loss can restore these changes. Skeletal muscle biopsies were obtained from normal weight controls and patients (women and men) with obesity before and after a standard laparoscopic Roux-en-Y gastric bypass (RYGB). Biopsies were obtained at the same time of the day and after an overnight fast. Microarray and qPCR analysis revealed altered expression of several clock genes in individuals with obesity of both sexes. *CRY1* expression was upregulated and *DBP* downregulated in skeletal muscle from women with obesity compared to normal weight

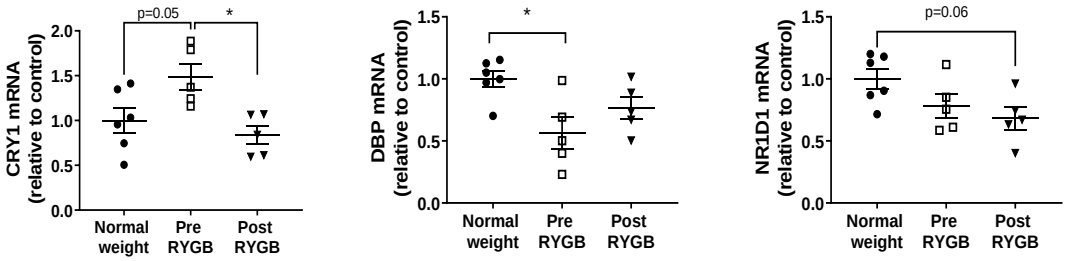


Figure 11. Core clock genes affected by obesity in skeletal muscle biopsies from female subjects. Previously reported Affymetrix Human Genome U219 Array (GSE45745) of skeletal muscle biopsies from women with obesity before and after RYGB (n=5) and normal weight controls (n=6) was used to determine changes in clock gene expression. mRNA expression of CRY1 and DBP were altered in obese women compared to their normal weight controls. Data are mean ± SEM, one-way ANOVA *p<0.05

controls (Figure 11). In men, *CLOCK*, *CRY1*, *CRY2* and *DBP* mRNA was downregulated in skeletal muscle from volunteers with obesity compared to controls (Figure 12). These data show that individuals with obesity have a altered skeletal muscle circadian clock expression.

Similarly, previous studies show that obesity disregulates clock gene expression in other tissues such as adipose tisuue and white blood cells (73, 74). In peripheral mononuclear blood cells, expression of BMAL1, CRY1, CRY2 and PER2 is increased in individuals with obesity (73); while the expression of CLOCK, BMAL1, CRY2 and NR1D1 is increased and expression of PER2 is decreased in visceral adipose tissue of women with obesity compared to their lean controls (74). These studies reveal that clock gene expression is affected by obesity in a tissue-specific manner, with CRY genes the only common core genes affected across these three tissues examined.

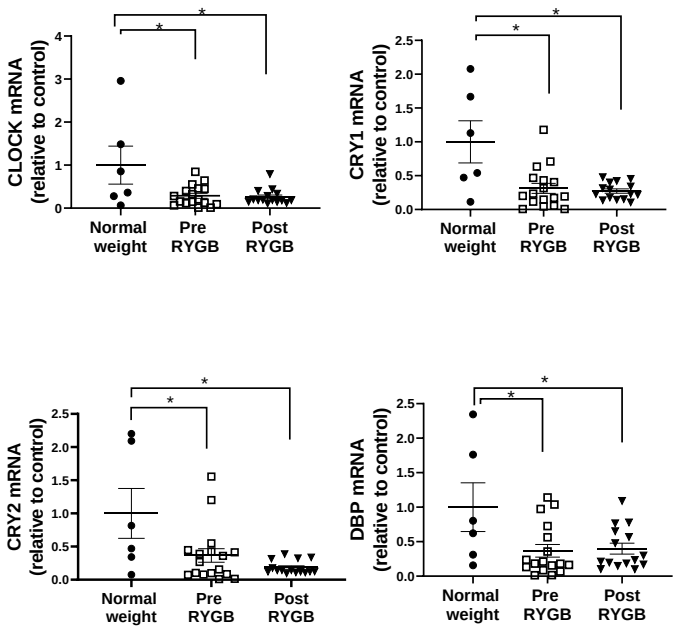


Figure 12. Core clock genes affected by obesity in men skeletal muscle biopsies from male subjects. Skeletal muscle biopsies from men with obesity before (n=17) and after RYGB (n=15) and normal weight controls (n=6) was used to determine changes in clock gene expression. Gene expression of CLOCK, CRY1, CRY2 and DBP was altered in obese men compared to their normal weight controls. Data are mean ± SEM, one-way ANOVA *p<0.05

Additionally, our results also suggest that obesity affect clock gene expression in a sex-dependant manner. In our study, men with obesity had a higher number of clock genes affected and presented an overall decrease in clock gene expression levels. Women in this study had a most robust mRNA expression of the skeletal muscle internal clock, and obesity influenced the expression of fewer clock genes. Our results are in agreement with previous studies showing sex-dependent circadian regulation, for example men and women present differences in body

temperature oscillations and sleep patterns, and respond differently to circadian dysregulation (199). Circadian misalignment induced by shift-work has sex-dependent effects on hunger and satiety (200). Circadian regulation of mental functions is different in men and women: after forced dyssynchrony protocol, women's performance in the morning was more impaired compared to men (201). It is possible that sex-dependant differenes in the regulation of circadian rhythms are due differences in sex hormone secretion and response. We did not investigate sex-driven circadian differences in this study, however, sex differences should be taken into account during the development of strategies aiming at circadian regulation.

We interrogated a third cohort with men and women with obesity that underwent a 3-week very low calorie diet (n=8, 1000kcal/day) for 3 weeks. This cohort was used in a previous study investigating the effects of short-term low calorie diet on skeletal muscle lipid profile and metabolic gene expression (202). The study revealed that 3-week low calorie diet in obese adults alters metabolic gene expression and reduces phosphatidylcholine and triglyceride species in the skeletal muscle (202). Our aim was to investigate whether a shorter intervention and a more subtle weight loss of the subjects would be sufficient to induce changes in skeletal muscle clock gene expression. Only PER2 gene expression was changed among the core clock genes (Figure 13). In this study, participants achieved a 7% body weight loss, while the RYGB cohorts participants achived a 25% body weight loss. Thus, these results indicate that, even though 3-week low calorie diet in adults with obesity improves skeletal muscle lipid metabolism and induces changes in gene expression (202), a greater weight loss is necessary to affect clock gene expression in the skeletal muscle.

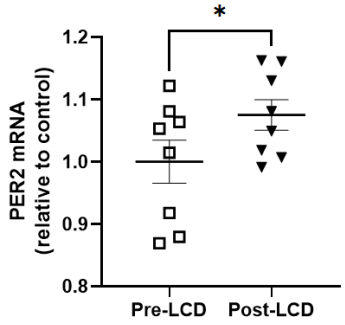


Figure 13. PER2 gene expression is affected by 3-week low calorie diet in skeletal muscle biopsies from obese subjects. Previously reported Affymetrix Human Gene 2.1 ST Array (GSE103682) of skeletal muscle biopsies from subjects with obesity before and after low calorie diet (LCD) (n=8) was used to determine changes in clock gene expression. Only gene expression of PER2 was changed after the LCD. Paired t-test. *p<0.05

5.2.2 Identification of circulating lipids as potential drivers of clock gene disruption

To explore which obesity-associated factors could be driving the changes in clock gene expression observed in skeletal muscle, expression of clock genes from normal weight participants and individuals with obesity pre-RYGB was correlated with body weight, BMI,

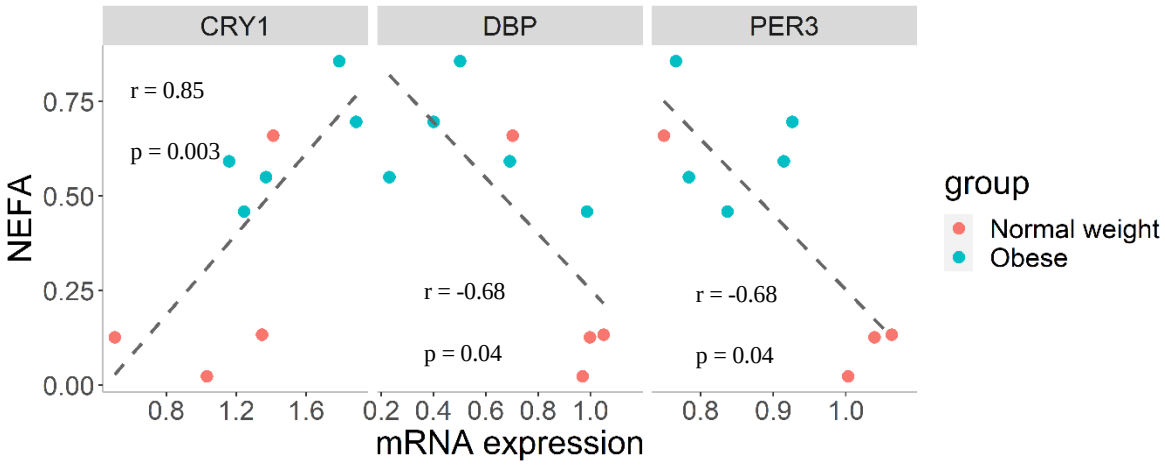


Figure 14. Spearman correlation between CRY1, DBP and PER3 and NEFA levels in women with obesity. Spearman coefficients between clock gene expression and NEFA levels of normal-weight vs. obese pre-RYGB women.

glucose, insulin, HOMA-IR (Homeostatic Model Assessment for Insulin Resistance), cholesterol, LDL, HDL, triglyceride and non-esterified fatty acids (NEFA). Clock gene expression was strongly correlated with NEFA in women, and with cholesterol levels in men. In women, plasma NEFA concentration was inversely correlated with *DBP* and *PER3* mRNA and positively correlated with *CRY1* mRNA (Figure 14). In men, total cholesterol level was inversely correlated with *CRY2*, *DBP*, *NPAS2*, *NR1D1*, *PER1* and *PER3* mRNA (Figure 15). Thus, in both cohorts, changes in plasma lipids levels were correlated with changes in skeletal muscle clock gene expression. Alterations in plasma lipid levels are associated with obesity and can lead to ectopic deposition of lipids in tissues such as skeletal muscle and liver (198), and disturbances in lipid metabolism and accumulation of NEFA in skeletal muscle are associated with the development of insulin resistance (203). Additionally, plama lipid levels oscillate in a circadian manner in humans (101, 204) and are under circadian control: disruption

of the circadian clock by constant light exposure leads to a shift in lipid plasma levels in mice (205). Thus, our analysis underscores a correlation between changes in lipid plasma levels and

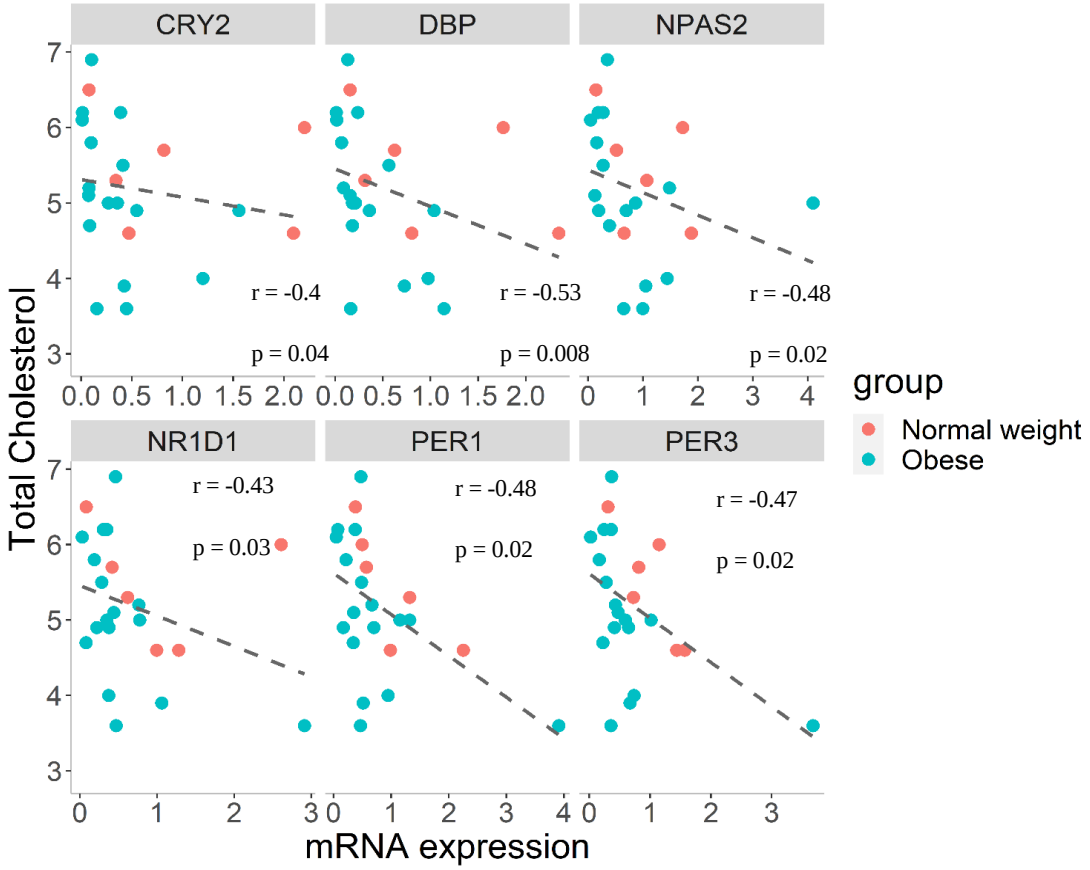


Figure 15. Spearman correlation between CRY2, DBP, NPAS2, NR1D1, PER1, PER3 and total cholesterol levels in men with obesity. Spearman coefficients between clock gene expression and total cholesterol levels of normal-weight vs. obese pre-RYGB men.

changes in clock gene expression. However, whether changes in plasma lipid levels are changing clock gene expression, or clock gene expression is regulating plasma lipid levels is not known. Therefore we investigated the role of plasma lipid levels as candidates that might modify clock gene expression.

5.2.3 Disruption of clock gene expression *in vitro* by the saturated fatty acid palmitate

Palmitate and oleate are two of the most abundant circulating lipids in humans and vary in concentration depending on food intake (101, 206). Palmitate is a saturated fatty acid and has been associated to the development of insulin resistance and other metabolic disturbances in skeletal muscle cells (207-209). On the other hand, the unsaturated fatty acid oleate has been associated to improvement of insulin sensitivity in skeletal muscle (209-212). To test whether lipids could play a causal role in the disruption of skeletal muscle internal clock, primary human myotubes were exposed to palmitate or oleate. Palmitate, but not oleate reduced DBP expression in a manner similar to obesity. In synchronized cells (Figure 16), we observed that *BMAL1*, *CIART*, *CRY2*, *DBP*, *PER1* and *PER3* mRNA expression was altered over time after palmitate treatment, thus indicating that palmitate directly disrupts clock gene expression in skeletal muscle. Our results are consistent with a previous study showing that palmitate altered the expression of the core clock components DBP, NR1D1 and PER2 in mouse hepatocytes

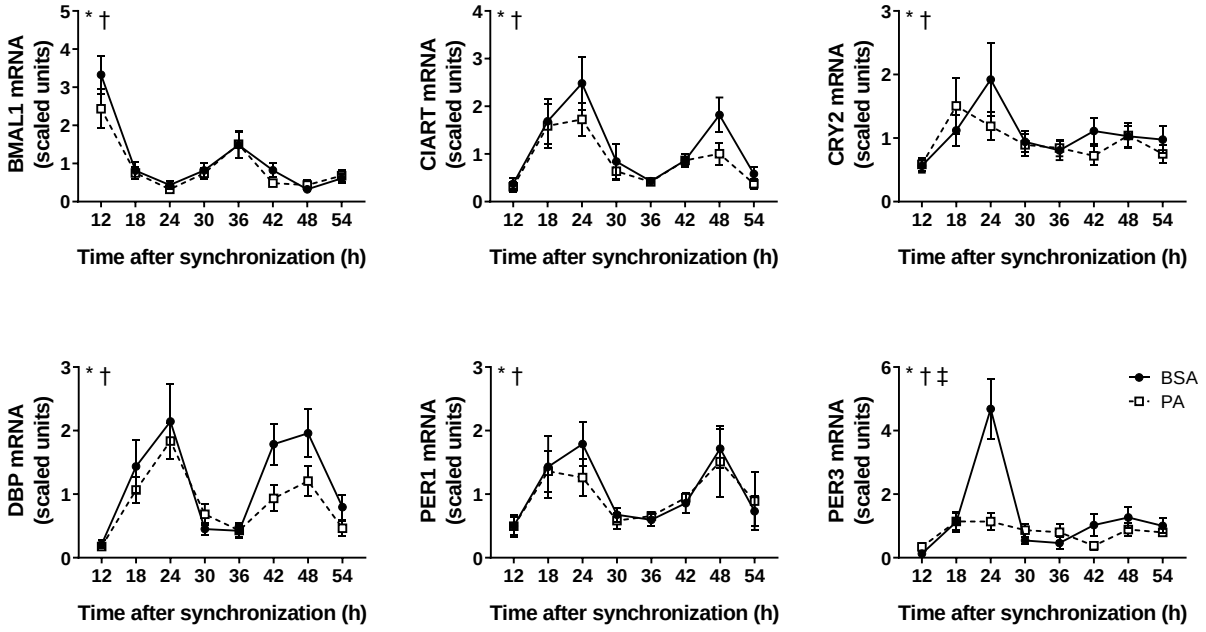


Figure 16. Expression of BMAL1, CIART, DBP, PER1 and PER3 was disrupted after palmitate treatment in human skeletal muscle cells. Human myotubes were synchronized by serum shock and exposed to palmitate (PA) (0.4 mM). Cells were collected every 6 h from 12 to 54 h. mRNA levels were measured by qPCR. Results are mean ± SEM, n=7, two-way ANOVA (time, palmitate), followed by Sidak's *post hoc* analysis. *P≤0.05 effect of time, †P≤0.05 effect of palmitate, ‡P≤0.05 interaction.

(213). Altogether revealing a role for the saturated fatty acid palmitate on the regulation of the circadian clock.

5.2.4 Conclusions and future perspectives for study II

Altogether, **study II** demonstrated that core clock gene expression is disrupted in skeletal muscle of individuals with obesity. Interestingly, obesity affected clock gene expression differently in a sex dependent manner. Previous studies have also shown differential responses to circadian dysregulation (199-201), however, the mechanisms regulating these differences are still unknown. Sex-driven circadian differences could be due differential hormonal signaling and response in men and women. Behavioral differences, such as sleeping and eating patterns, could be also contributing to those differences. Even though in our study we didn't investigate the sex-dependent differences in clock gene expression and response to obesity, it would be interesting to examine these differences.

Obesity is characterized by an increase of circulating lipids in the plasma (214, 215). Moreover, people with obesity present a lower ratio of unsaturated FA to saturated FA (215), and have elevated levels of palmitate in the plasma (26) compared to normal weight controls. In our study, clock disruption in obesity correlated with changes in circulating lipid levels. We showed a consistent dysregulation of the skeletal muscle core clock genes by obesity and weight loss *in vivo* and palmitate *in vitro*.

Our findings are supported by previous studies showing dysregulation of the clock in the context of obesity in other tissues (73, 74) as well as studies showing that palmitate affects the regulation of circadian clocks (213). Supported by these previous reports, our data indicated that modifying dietary regimes and weight loss may be beneficial for sustaining a robust circadian clock and maintain metabolic homeostasis.

5.3 STUDY III: CIRCADIAN TRANSCRIPTOMIC AND EPIGENOMIC RESPONSE TO PALMITATE IN SKELETAL MUSCLE

The saturated fatty acid palmitate is involved in the pathophysiology and metabolic complications observed in the context of obesity (203, 216, 217). In study II we observed that obesity and palmitate disrupted the skeletal muscle circadian rhythms. In **study III** we aimed to further investigate the role of palmitate in the disruption of skeletal muscle core clock genes and its impact to downstream clock-controlled genes. We used advanced sequencing methods and multi-omics integration approaches to study the effects of palmitate in circadian transcriptomics as well as possible mechanisms of action. Usage of multi-omics technologies give us the possibility to investigate biological processes in a more comprehensive manner. In our specific case it allows the integration of epigenetic changes in histone tails to changes in circadian gene transcription, and therefore reveal biological insights that we would not be able to study using other techniques.

5.3.1 Palmitate treatment alters circadian transcriptomics in primary skeletal muscle cells

Transcriptomic analysis of skeletal muscle cells after palmitate (0.4 mM) or BSA-vehicle treatment revealed that palmitate reduced the number of rhythmic genes (Rain analysis, FDR<0.1) (Figure 17A). We observed that the core clock genes *BMAL1*, *CIART*, *DBP*, *CRY1*, *CRY2*, *NR1D1*, *NR1D2*, *PER1*, *PER2* and *PER3*, were rhythmic in both conditions (Figure 17B). However, *NPAS2* and *CLOCK* were only rhythmic in BSA-vehicle-treated cells, not in palmitate-treated cells (Figure 17B).

When comparing rhythmic genes in BSA-vehicle and palmitate treated myotubes, 730 genes were common between the 2 groups. 3206 genes were only rhythmic in BSA-vehicle treated myotubes and showed an enrichment for pathways involved in ribosomal proteins, protein translation and ubiquitination and cell division. This data is supported by the previous

studies and shows that protein translation is under circadian regulation (218, 219): the ribosomal S6 protein kinase 1 (S6K1) rhythmically phosphorylated BMAL1, and induces rhythmic protein synthesis (218).

There were 1490 genes only cycling in palmitate treated cells. These genes were associated with the regulation of transcription, JmjC activity and serine/threonine protein kinase activity. Palmitate-induced changes in transcriptional regulation have been previously described (220). For example, in skeletal muscle cells, adipocytes and hepatocytes, palmitate induces changes in expression of genes involved in ER stress (217, 220-222); A genome-wide analysis in human pancreatic islets showed that palmitate induces changes in the expression of 1860 genes, including genes associated to the regulation of metabolism (223). However, our data indicates that palmitate regulates the expression of genes involved in the regulation of transcription in a circadian manner and therefore palmitate might have an impact into the overall circadian gene expression. Palmitate activation of protein kinases has been reported in skeletal muscle L6 cells and beta cells (224, 225). Additionally, the expression and the activity of protein kinases have been shown to be rhythmic (226). Activation of protein kinases is linked

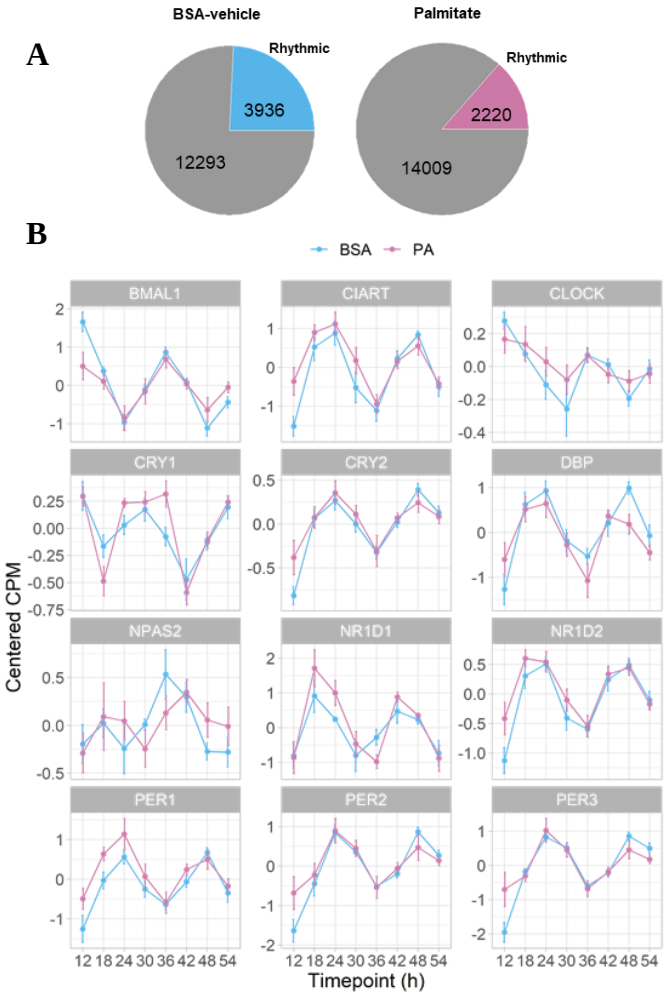


Figure 17. Palmitate attenuates the number of rhythmic transcripts in skeletal muscle cells. A. Proportion of rhythmic genes identified (RAIN analysis, FDR<0.1). **B.** Core clock genes expression in BSA-vehicle and palmitate treated cells. Results are mean ± SEM.

to an increase of fatty acid oxidation and insulin secretion, respectively (224, 225), thus revealing a role of palmitate in the regulation of metabolism. These results indicate that palmitate impacts circadian metabolism of fatty acids and glucose while regulating circadian transcription of genes.

Analysis of the 730 common rhythmic genes revealed an enrichment of clusters involved in circadian regulation as well as regulation of transcription, suggesting a robust regulation of these pathways. Altogether, our results suggest a differential regulation of circadian gene expression between palmitate and BSA-vehicle treated myotubes.

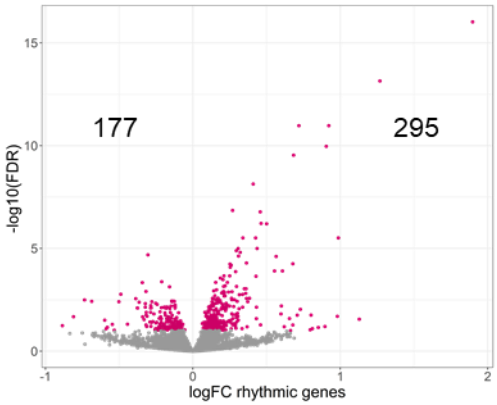


Figure 18. Palmitate induces changes in expression of rhythmic genes. Differential expression (DE) analysis of rhythmic genes after palmitate treatment (Limma, FDR<0.1)

Next, we investigated whether palmitate alters the expression of rhythmic genes. We performed differential expression analysis on 5426 genes that were rhythmic in at least one condition. Our analysis revealed that the expression of 472 genes was altered after palmitate treatment (Limma, FDR<0.1) (Figure 18). Functional Annotation Clustering analysis on the altered genes showed that palmitate affected genes involved in regulation of lipid metabolism and cell growth, concomitant with previously reported changes in gene expression after palmitate treatment in human hepatocytes (220). Functional Annotation Clustering also revealed changes in genes involved in the developments of metabolic disorders. Altogether, our results indicate that palmitate causes metabolic and circadian disturbances in skeletal muscle cells.

5.3.2 Palmitate increases histone H3 lysine 27 acetylation in a rhythmic manner

Palmitate treatment induces changes in the expression of genes involved in metabolism, as well as ER stress and oxidative stress (220). Moreover, in our study, palmitate promoted rhythmicity of genes involved in regulation of transcription in skeletal muscle cells. Histone modifications are important regulators of transcription and can impact gene expression by modulating chromatin structure or recruiting histone modifiers (227). Therefore, we investigated whether changes in histone modifications are associated with altered rhythmicity of genes. Acetylated histone H3 lysine 27 (K27) is a known marker of active enhancers and active transcription (104, 228, 229). Thus, we assessed the relative abundance of total histone H3 and histone H3K27 acetylation over a 48h period in cells incubated in the presence or absence of palmitate. Although, the relative abundance of histone H3 was stable over time and was not affected by palmitate treatment (Figure 19A), histone H3K27ac abundance was affected in a time-dependent manner (Figure 19B). Our first hypothesis was that palmitate increases the total pool of acetyl-CoA in the cells, and that leads to an increased overall acetylation (102). However, we found that total lysine acetylation was decreased after palmitate treatment (Figure 19C), indicating that the increase in H3K27ac was due a more specific increase of acetylation on this site. Representative blots are shown in figure 19D. A possible mechanism could be through an alteration of cell energy levels and sirtuin (SIRT) activity. Sirtuins are NAD^+-dependent HDACs that are sensitive to cell energy levels and metabolism (96). SIRT1 expression and/or activity is reduced in obesity and after palmitate exposure (230, 231). Moreover, SIRT1 activity is under circadian control (96) and SIRT1 ablation in mouse embryonic fibroblasts leads to changes in circadian gene expression (96). The circadian CLOCK:BMAL1 dimer coexists with SIRT1 in a chromatin regulatory complex (96), with CLOCK acting as a histone acetyltransferase (HAT), with high affinity for H3 and H4 (122). A previous study in hepatocytes revealed that palmitate disrupts CLOCK:BMAL1 dimer

formation in a SIRT1 dependent manner (213), indicating that SIRT1 activity is required for maintaining a stable CLOCK:BMAL1 interaction and activity. Thus, in skeletal muscle cells, palmitate may alter energy levels and affect SIRT1 activity, leading to changes in CLOCK:BMAL1 interactions and changes in histone H3K27 acetylation.

5.3.3 Palmitate attenuates rhythmic behavior in H3K27 acetylated regions

Palmitate-treatment altered global H3K27 acetylation levels, consistent with previous evidence that palmitate acetylates enhancer regions regulating lipid metabolism in skeletal muscle and liver cells (229, 232). To explore the overlap between the changes in H3K27ac and changes in circadian transcriptomics, chromatin immunoprecipitation (ChIP) and sequencing

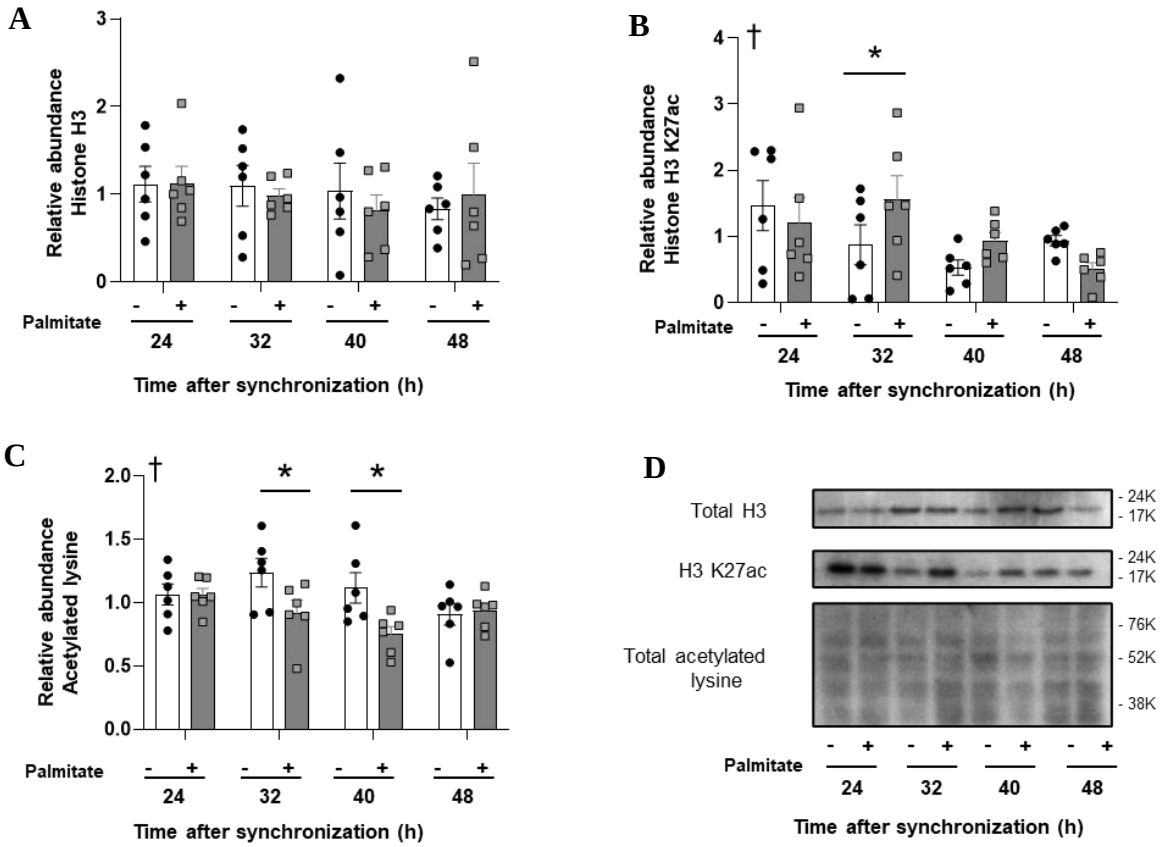


Figure 19. Histone H3K27 acetylation is increased by palmitate treatment in a rhythmic manner. Synchronized primary human skeletal muscle cells (n=6) treated with palmitate (0.4 mM) or BSA-vehicle. Protein lysates were collected every 8h from 24h to 48h after synchronization. Bar plots represent relative protein abundance of **A.** histone H3, **B.** histone H3K27 acetylation (ac), and **C.** total acetylated lysine in palmitate treated samples compared to BSA-vehicle-treated controls. **D.** Representative blots from H3, H3K27ac and total acetylated lysine. Results are mean ± SEM, n=6, paired two-way ANOVA (time, palmitate), followed by Sidak's *post hoc* analysis. *$P \leq 0.05$ effect of palmitate, †$P \leq 0.05$ interaction.

was performed in synchronized skeletal muscle cells treated with palmitate or BSA-vehicle. We selected the 6,132 acetylated regions that were annotated to a gene for downstream analysis. We identified 1,018 rhythmic regions in BSA-vehicle treated samples, compared to 850 rhythmic regions in palmitate treated samples (Figure 20). In total, there were 1,639 rhythmic acetylated regions from which 229 were common among the 2 groups. Differential acetylation analysis revealed 708 acetylated regions affected by palmitate treatment (Limma, FDR<0.1).

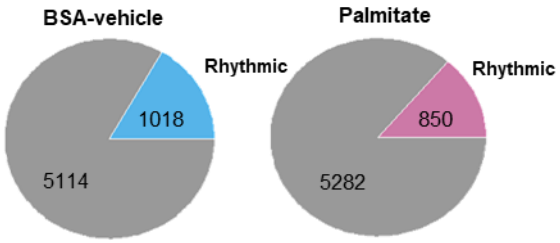


Figure 20. Palmitate reduced the number of rhythmic regions in skeletal muscle cells. Proportion of rhythmic regions identified (RAIN analysis, FDR<0.1).

To determine whether changes in rhythmicity of H3K27 acetylated regions are responsible for the changes observed in transcript profiles, we examined the overlap between rhythmic H3K27 acetylated regions and rhythmic transcript profiles. When comparing the transcriptomic and the acetylation analysis (Figure 21), we found 169 unique features cycling commonly in BSA-vehicle treated myotubes (RNA and H3K27ac) and 48 unique features for palmitate treated myotubes (Figure 21). We identified 15 features commonly cycling between the four groups (Figure 21). According to cluster enrichment analysis, BSA-vehicle treated cycling features were involved in DNA repair pathways, while palmitate features belong to protein phosphorylation and angiogenesis pathways.

Histone H3K27 acetylation seems to regulate rhythmicity of a limited number of transcripts in control and palmitate treated myotubes. Therefore, it is likely that regulation of transcript oscillations is accompanied by parallel changes in other histone marks, DNA methylation, mRNA stability and/or post-transcriptional RNA processing (233). All these processes influence gene expression and can contribute to transcript rhythmicity.

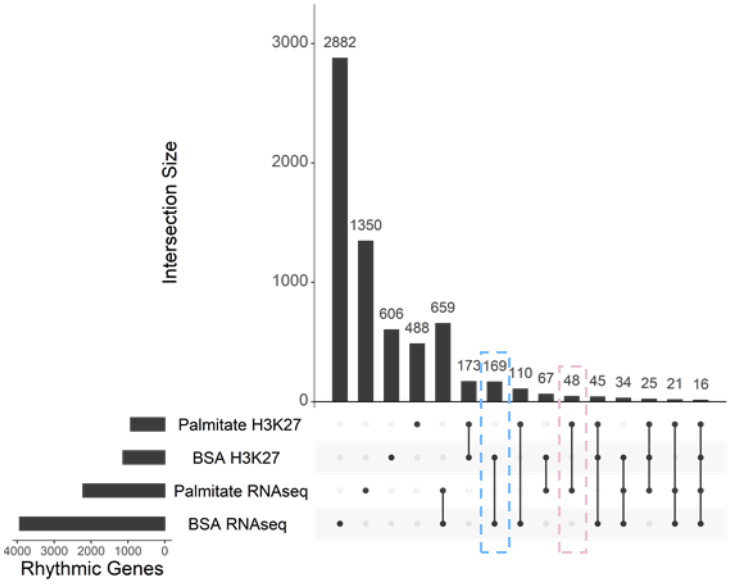


Figure 21. Changes in enhancer rhythmic acetylation leads to changes in rhythmic transcriptomics. Overlap of rhythmic genes (RNA-seq) and genes associated to rhythmic regions (H3K27ac ChIP-seq) in BSA-vehicle-treated and palmitate-treated cells

5.3.4 Obesity increases histone H3K27 acetylation in skeletal muscle

In **study II**, we reported that clock gene expression changes after palmitate treatment were concurrent with the changes in skeletal muscle biopsies from men and women with obesity (234). We explored whether changes in H3K27ac observed after palmitate treatment also occur in skeletal muscle biopsies of individuals with obesity. Alterations in H3K27 acetylation have been previously reported in the context of aging (169), with increased expression of genes regulating extracellular matrix structure and organizations. However, the specific role of this histone site in the context of obesity is still unexplored. In our study, abundance of H3K27ac was unaltered in skeletal muscle biopsies from men with obesity compared to their normal weight controls (Figure 22).

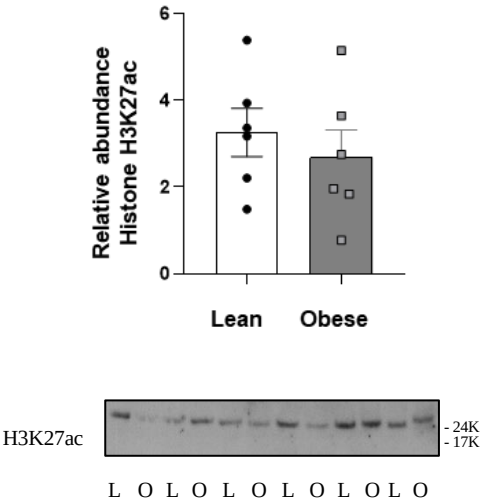


Figure 22. H3K27ac in skeletal muscle biopsies from men with normal weight or obesity. Skeletal muscle biopsies were obtained from men with normal weight (Lean; n=6) or obesity (Obese; n=6). Protein lysates were collected and relative abundance of histone H3K27ac was measured. Results are mean ± SEM. *P<0.05. Student's unpaired *t*-test was used.

A meta-analysis of 5 datasets containing transcriptomic data from skeletal muscle biopsies from individuals with normal BMI or obesity (BMI>30 kg/m^2) collected from the GEO database (Table 2), revealed that obesity affected the expression of 406 genes (FDR<0.1) in human skeletal muscle, with 14 and 12 of these showing changes in mRNA expression and H3K27ac, respectively in palmitate-treated primary myotubes. Only one gene, Transcriptional enhancer factor TEF-1 (TEAD1), was influenced by both human obesity in mRNA level and palmitate-treatment at the mRNA and H3K27 acetylation level. TEAD1 is a ubiquitous transcriptional enhancer factor and its protein transactivates a wide variety of genes. These results suggest differential response to obesity and palmitate exposure and indicate that the changes observed in skeletal muscle cells after palmitate treatment are induce by an acute lipid overload, rather than obesity per se.

5.3.5 Conclusions and future perspective for study III

In summary, the saturated fatty acid palmitate disrupts circadian transcriptomics and histone H3K27 acetylation in primary human myotubes. In this study we investigated the specific role of H3K27 acetylation on the regulation of circadian transcriptomics after palmitate treatment. However, we uncovered that H3K27 acetylation could be responsible for the regulation of a limited number of genes, and that several mechanisms must be involved in the circadian regulation of transcriptional changes. Our results provide a link between

nutrient overload, disruptions of circadian rhythms, and metabolic pathways. Increased histone H3K27 acetylation in palmitate-treated primary human myotubes suggests a specific role for this histone mark in the transcriptional changes that occur in peripheral tissues in response to lipid-overload. Constant disruption of circadian rhythms in skeletal muscle due to lipid overload may lead to genomic changes that influence metabolism. Thus, dietary or therapeutic modulation of lipid levels, a cornerstone in the treatment of metabolic disorders, may prevent circadian misalignment in peripheral tissues.

5.4 STUDY IV: DISRUPTED CIRCADIAN CORE-CLOCK OSCILLATIONS IN TYPE 2 DIABETES ARE LINKED TO ALTERED RHYTHMIC IN MITOCHONDRIAL METABOLISM

Obesity and excess lipids led to disrupted skeletal muscle internal clock in studies II and III. Obesity and excess lipids are risk factors for the development of other metabolic diseases such as T2D. It is known that glycemic control is under circadian regulation (235, 236) and there is an association between circadian misalignment and the development of T2D: night shift workers have increased risk of developing T2D (237, 238), and forced desynchrony protocol with 28h days reduces whole body glucose tolerance (239). The aim of **study IV** was to investigate whether skeletal muscle circadian rhythms from individuals with T2D are inherently different from the rhythms of people with normal glucose tolerance and study the mechanisms that could be underlying those differences.

5.4.1 Intrinsically dysregulated circadian rhythm of gene expression in T2D myotubes

We performed a transcriptomic analysis on skeletal muscle cells from T2D and NGT donors over time. Our analysis revealed that cells from T2D presented a reduced number of rhythmic genes compared to cells from NGT donors (Rain analysis, FDR<0.1) (Figure 23A). These circadian differences between rhythmic gene expression in skeletal muscle from NGTs and T2Ds occurred despite the robust manipulations that the cells undergo. Our hypothesis was that these differences could due a distinctive genetic background of the donors and/or epigenetic alterations triggered by chronic exposure to the hyperglycemic and hyperinsulinemic milieu present in individuals with T2D. Indeed, chronic exposure of myotubes *in vitro* to high glucose and insulin (50 nM insulin, 25 mM glucose) to mimic a T2D milieu (240) reduced the number of cycling genes in both T2D and NGT groups (Data not

shown, Figure 1C in the article), indicating an effect *per se* to long term exposure to a T2D milieu.

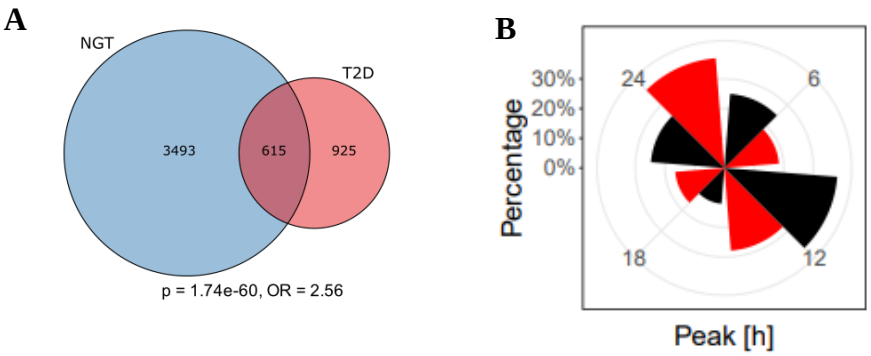


Figure 23. Intrinsically dysregulated circadian rhythm of gene expression in skeletal muscle cells from individuals with T2D. A. Venn diagram showing the overlapping rhythmic genes between NGT and T2D. Overlapping rhythmic genes were significantly enriched (Fisher's exact test, p = 1.74e-60; Odds-ratio, OR = 2.56, background = 18,483). **B**. Percentage of circadian genes at each peak time for control treatments (black=NGT, red=T2D).

Peak times of cycling genes in NGT and T2D were also altered (Figure 23B): T2D cells had the highest number of cycling genes displaying peaks at 24 hours, while NGT had the highest number of cycling genes displaying peaks at 12 hours. Additionally, the mean log2 amplitude of cycling genes was lower in T2D compared to NGT (p=2.2e-16, two-sided Kolmogorov-Smirnov test). Altogether, our results showed that T2D myotubes have reduced total number of cycling genes, altered peak time and reduced circadian amplitude, and therefore have an altered skeletal muscle circadian clock.

Among the core clock genes, PER3 and BMAL1 were differentially rhythmic between NGT and T2D (FDR<0.1); *NPAS2* displayed different peak times between NGT and T2D and *CLOCK* was only cycling in NGTs (FDR<0.1) (Figure 24). These results reinforce the association between misalignment of the circadian clock and T2D.

5.4.2 Ablated rhythmic mitochondrial metabolism in T2D

To investigate the causes of disrupted circadian transcriptomics in T2D myotubes, we performed gene enrichment analysis (ORA) for each condition and gene peak time. Our

analysis revealed that several mitochondrial GO cellular components ("*Mitochondrial matrix*", "*Mitochondrial inner membrane*", and "*Mitochondrial protein complex*") were enriched for cycling genes at peak time 24 hours (ZT24) in NGT cells, but not in any other condition.

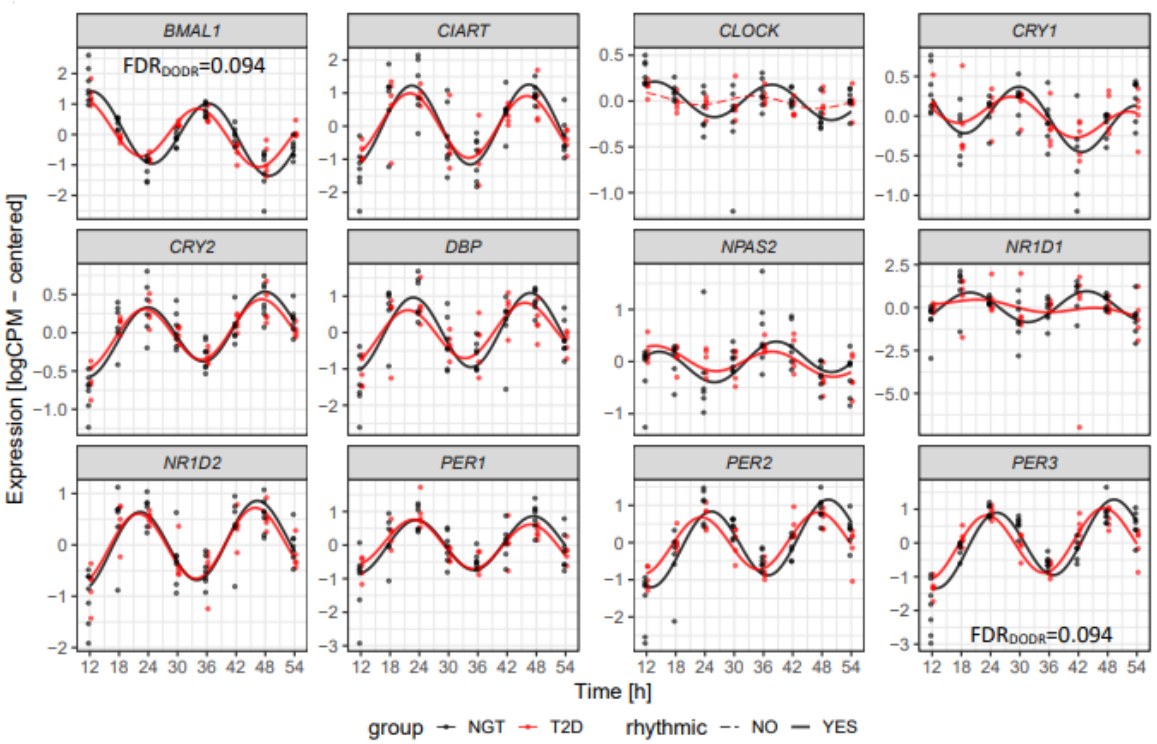
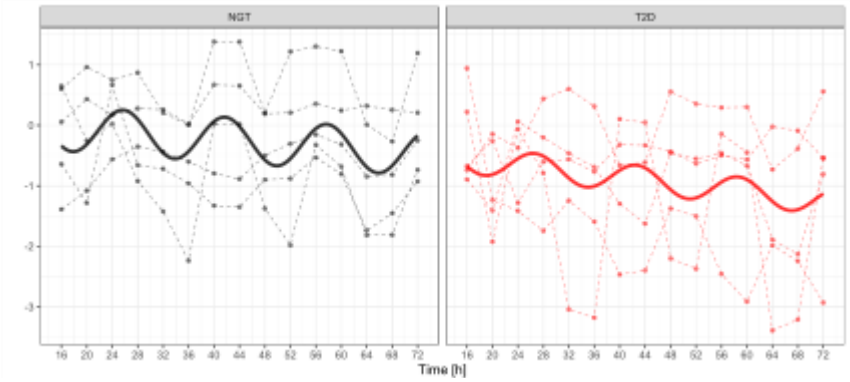


Figure 24. Core clock gene expression in T2D and NGTs. Circadian rhythmicity of core clock genes. Red=T2D, Black=NGT. Lines show the harmonic regression fits and solid line indicates circadian rhythmicity (FDR<0.10). Dashed lines indicate the absence of circadian rhythm (FDR>0.1). Time points are hours post-synchronization.

Mitochondrial diurnal rhythms in oxidative capacity and oxygen metabolism have been demonstrated in several tissues, including skeletal muscle *in vivo* (241, 242). Additionally, mitochondrial function and dynamics is disrupted in T2D (243-245). We therefore investigated whether synchronized myotubes from donors with T2Ds displayed different circadian oscillations of OCR as a measurement of mitochondrial metabolism. We observed that NGT cells presented cycling basal OCR (RAIN P=0.015), while T2Ds cells didn't (RAIN P = 0.451) (Figure 25). Moreover, we observed differential rhythmicity of basal OCR between NGT and T2D cells (DODR, P = 4.08e-9) (Figure 25). Our results strengthen the idea of "cell memory" and are consistent with previous data demonstrating that induced pluripotent stem cells from donors with T2D that were differentiated into myoblasts had multiple defects as compared to

healthy subjects, including reduced insulin-stimulated glucose uptake and reduced mitochondrial oxidation (246).



Analysis	p value
DODR NGTvsT2D	4.08E-09
RAIN NGT	0.015
RAIN T2D	0.451

Figure 25. Ablated rhythmic mitochondrial metabolism in T2D. Relative oxygen consumption rate of synchronized myotube cultures from donors with NGT (black) versus T2D (red), as measured by Seahorse XF Analyzer (Agilent) for n=5 individuals in both groups.

To test the *in vivo* clinical relevance of our findings in NGT and T2D myotubes, we performed a transcriptomic analysis on *vastus lateralis* muscle biopsies obtained from men with NGT (n=22) or T2D (n=22) at a single timepoint (Figure 26A). Additionally, a hyperinsulinemic-euglycemic clamp was performed to determine whole-body insulin sensitivity (M-value). As expected, insulin sensitivity was greater in men with NGT as compared to T2D (P<0.0001, Figure 26B). Spearman's rank correlation was used to study the

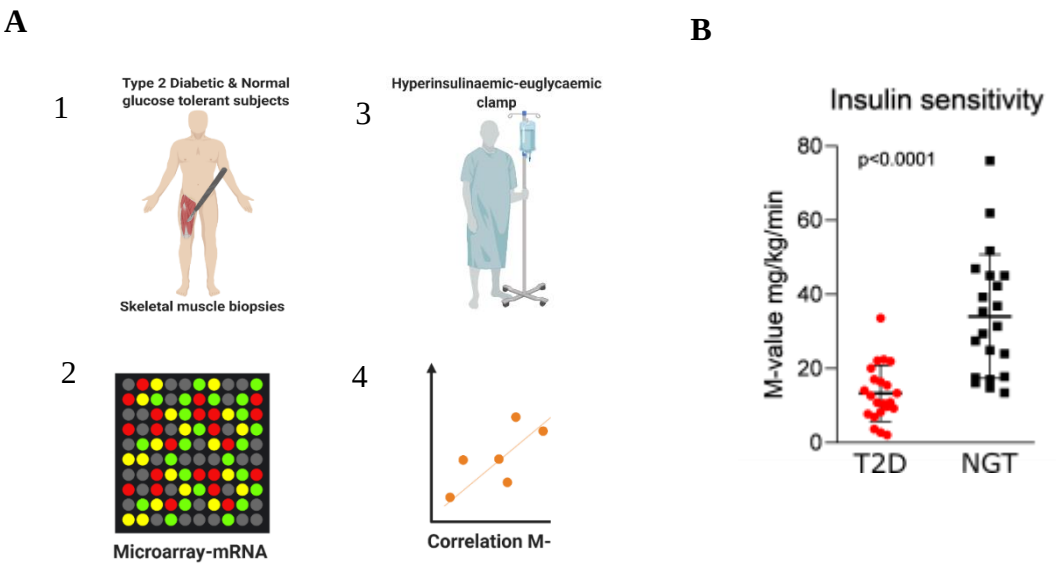


Figure 26. Insulin sensitivity assessment. A. Schematic of experimental design **B.** Insulin sensitivity (M-value) of T2D (n=22, red) and NGT (n=22, black). Student's t-test

relationship between basal skeletal muscle gene expression and insulin sensitivity across the cohort. GSEA revealed that most of the enriched GO:CC were related to the mitochondria (FDR < 0.10). These results are consistent with previous studies showing that T2D is associated with reduced skeletal muscle mitochondrial function and metabolic inflexibility (247, 248). The GO:CC *"Mitochondrial inner membrane"*, and "*Mitochondrial matrix*" were common between this analysis and the analysis performed in skeletal muscle cells. Therefore, our results point at mitochondria as a cellular location implicated in both the regulation of insulin sensitivity and associated with impaired cycling behaviors in skeletal myotubes.

5.4.3 Inner mitochondrial membrane and core clock expression

Mitochondria are involved in essential functions such as ATP production, Ca^{2+} regulation, ROS production and scavenging, as well as regulation of apoptosis (249). Therefore, mitochondrial disruption is largely associated with cell malfunction. To test whether functionality of the inner mitochondrial membrane was responsible for the changes of clock gene expression observed T2D skeletal muscle cells, we treated myotubes from NGT donors with different compounds targeting mitochondria. We used FCCP – interferes with proton gradient and disrupts ATP synthesis –, Oligomycin – inhibits complex V –, and a mixture of Rotenone and Antimycin A (Rot/AA) – inhibit complex I and III. After serum shock myotubes were incubated with these compounds for 4h before collection (ZT14-

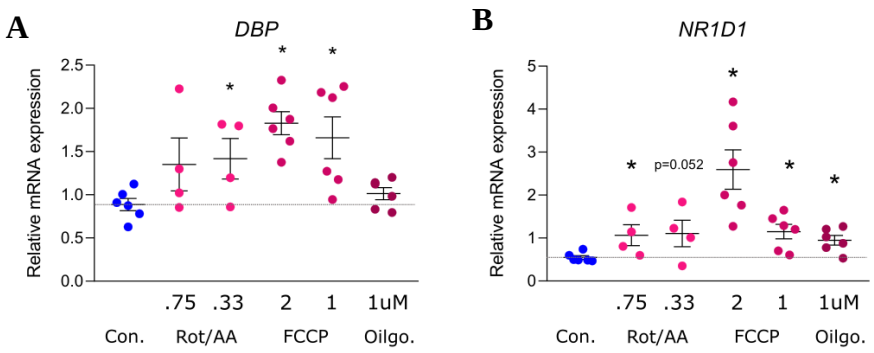


Figure 27. Pharmacological disruption of the inner mitochondrial membrane. mRNA expression of molecular clock-associated gene **A.** DBP and **B.** NR1D1 in myotubes from NGT donors (n=4-6 donors). One-way ANOVA, *=p<0.05 compared to vehicle control (Con.).

ZT18). mRNA expression of core clock genes exposed to inhibition of mitochondrial complex I and III (Rot/AA) as well as changes in proton gradient (FCCP) increased mRNA expression of *DBP* as compared to vehicle-treated myotubes (Figure 27A). Additionally, changes in proton gradient (FCCP) and inhibition complex V (Oligomycin) increased mRNA expression of *NR1D1* as compared to vehicle control-treated myotubes (Figure 27B). These data provide evidence that manipulation of mitochondrial function alters clock-associated gene expression in primary human myotubes.

Optic Atrophy Protein 1 (OPA1) is an established regulator of inner-mitochondrial morphology and function in skeletal muscle (60, 143-146). OPA1 is required for fusion of the inner mitochondrial membrane, cristae remodeling and respiratory chain complexes assembly (250). *OPA1* did not display cycling mRNA in myotubes from NGT or T2D donors. However, *OMA1* and *HIGD2A*, responsible for the cleavage and stabilization of OPA1 protein (251, 252), respectively, displayed cycling mRNA patterns in myotube cultures from NGT, but not T2D donors. To further elucidate the effect of mitochondrial disruption in relation to the molecular-clock machinery, skeletal muscle-specific *Opa1*$^{-/-}$ mouse model was used. Molecular-clock genes *Clock, Bmal1, Nr1d1* and *Per3* (Figure 28) were decreased in *Opa1*$^{-/-}$ mice, suggesting retrograde signaling from OPA1 to the molecular-clock.

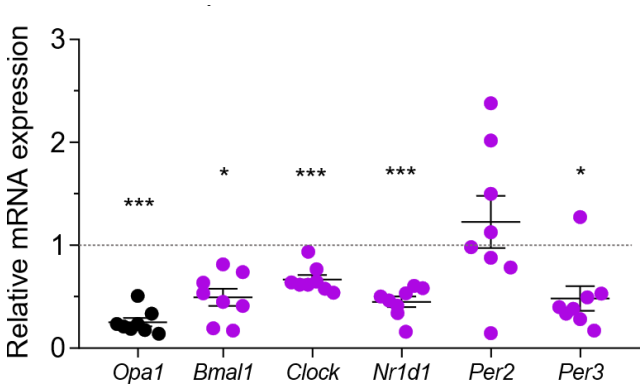


Figure 28. OPA1 mKO alters core clock gene expression. *OPA1* and Clock gene expression (*Bmal1, Clock, Nr1d1, Per2, Per3*) from *Opa1 MKO-Opa1* skeletal muscle *Opa1*$^{-/-}$ mice (n=8) relative to control (n=7). One-way ANOVA, *=p<0.5.

Opa1 ablation in skeletal muscle reduced life span, impaired normal growth and caused an inflammatory myopathy in mice (135), making it difficult to draw conclusions regarding

the direct role of OPA1 in the regulation of skeletal muscle clock. Thus, we turned to our *in vitro* model of myotube cultures. OPA1 silencing in skeletal muscle cells allowed an in-depth study of how specific disruption of the inner mitochondrial membrane would affect clock

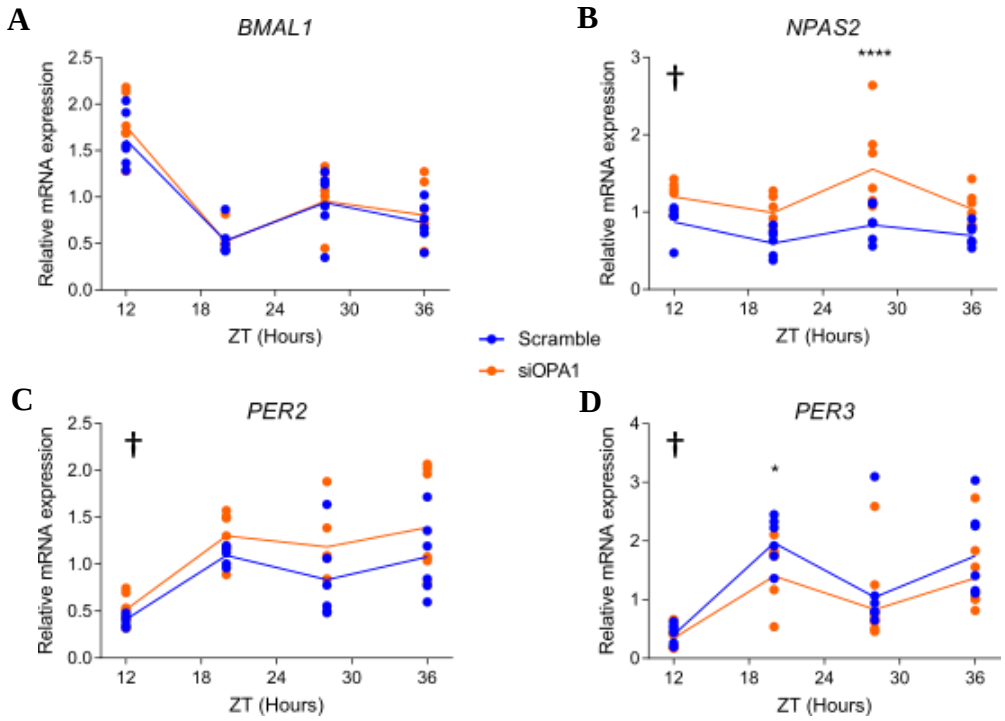


Figure 29. siOPA1 alters core clock gene expression in skeletal muscle cells. Primary human skeletal muscle cells treated with siRNA targeting *OPA1* (n=6). Core-clock genes *BMAL1* (A), *NPAS2* (B), *PER2* (C), and *PER3* (D). †$p<0.05$ overall difference *siOPA1 vs.* scramble. *$p<0.05$ *siOPA1 vs.* Scramble at time point (2-way ANOVA).

gene expression over time. In synchronized primary human skeletal myotubes, siRNA-targeted reduction of *OPA1*, resulted in unchanged BMAL1 (Figure 29A) expression, while *NPAS2* (Figure 29B), *PER2* (Figure 29C), and *PER3* (Figure 29D) had altered *mRNA* expression. Collectively, these data suggest that manipulating inner-mitochondrial membrane function results in altered mRNA expression of clock genes.

In order to corroborate that the effects observed in the core clock expression were caused by disruption of the mitochondrial inner membrane, we explored clock gene expression after the disruption of the outer mitochondrial membrane. Mitofusin 2 (Mfn2) is a GTPase essential for mitochondrial fusion, which regulates mitochondrial dynamics and

mitochondrial function (253). Moreover, Mfn2 expression in the skeletal muscle negatively correlates with body weight and insulin resistance (254). Therefore, we investigated the consequences of Mfn2 ablation in the skeletal muscle on expression of clock genes. In our study, core clock gene expression was unaltered in skeletal muscle-specific Mfn2$^{-/-}$ mouse model and siRNA targeted reduction of MFN2 in skeletal muscle cells (Figure 30). These results highlight a specific role for the inner mitochondrial membrane on the regulation of circadian rhythms in skeletal muscle.

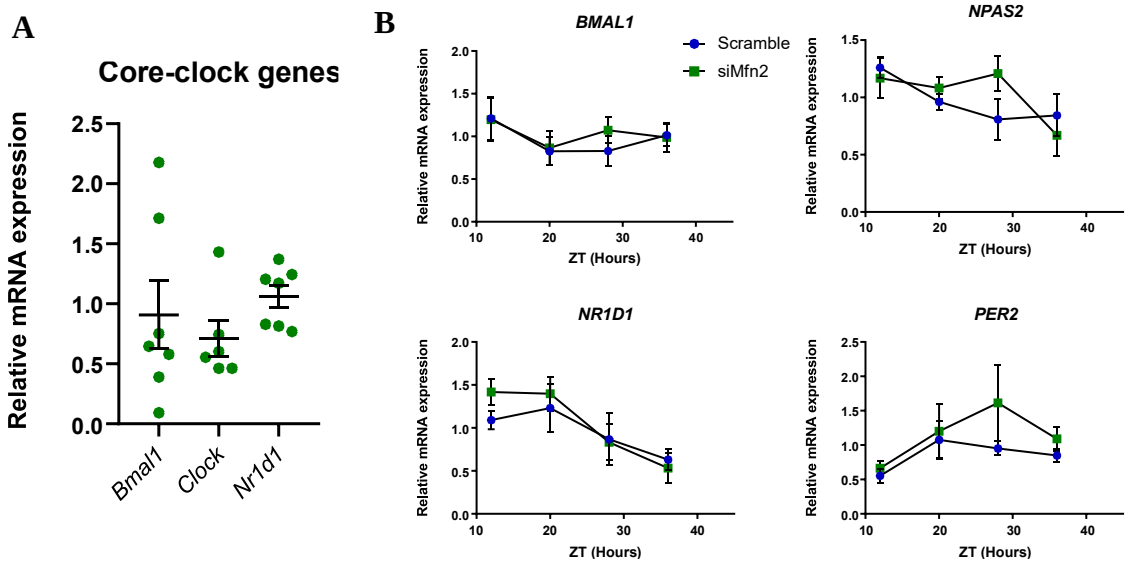


Figure 30. Effects of MFN2 on core-clock gene. **A.** Tibialis anterior from Mfn2loxP/loxP (WT) and skeletal muscle specific Mfn2-/- mice (Mfn2 MKO) mice (n=7 per genotype) was obtained and total RNA was extracted for clock gene analysis. **B.** Primary human skeletal muscle cells were treated either with scramble-siRNA or siMFN2-siRNA targeting MFN2 (n=6), and clock gene expression was studied. Results are mean±SEM, two-way ANOVA.

5.4.4 siRNA depletion of OPA1 increases mitochondrial reactive oxygen species

Prolonged hyperglycemic in T2D results in an increased production of ROS (255), mainly produced by mitochondria, leading to a state of oxidative stress. To reveal whether there is a connection between the inner-mitochondrial-membrane, changes in core-clock expression and oxidative stress, we explored the circadian behavior of known antioxidant genes in our RNA-sequencing data. Interestingly, Hypoxia Inducible Factor-1 (HIF1-α) mRNA displayed circadian rhythms in the myotube cultures from the NGT but not the T2D donors (Figure 31A).

HIF1-α plays a key role in molecular signaling from the core-clock to mitochondria and vice-versa in skeletal muscle (84). Moreover, several mitochondrial genes with antioxidant activity such as *GPX1*, *GPX3* and, *GPX4*, were circadian in NGT cells, but not in T2D. NADH/NAD^+ ratio has an important role on the regulation of ROS production and clearance (256). A high NADH/NAD^+ ratio in the mitochondrial matrix will result in an increased ROS production (257). NAD^+ levels reflect mitochondrial metabolism and its abundance display a circadian rhythm (258). In our dataset, several genes involved in NADH/NAD^+ were circadian in NGT, but not T2D (*NARPT*, *NAMPT*, *NADSYN1*), while *NNT* was circadian in T2D but not NGT. Altogether, these results suggest a dysregulation of antioxidant mechanisms and NADH/NAD^+ metabolism in T2D myotubes.

We investigated changes in NAD^+ and ROS levels in skeletal muscle cells after disruption of the mitochondria by OPA1 silencing. Control cells presented differences in NAD^+ levels over time, while NAD^+ levels from cultures treated with siRNA targeting OPA1 did not differ over time (Figure 31B). However, NAD^+ concentration was unaltered between siOPA1- and SCR-treated myotubes (Figure 31B). We assessed mitochondrial ROS concentration using live-cell microscopy and measured MitoSOX fluorescence and observed that ROS levels were increased in OPA1-depleted myotubes compared to the controls (Figure 31C). In order to investigate whether increased ROS was responsible of the changes in clock gene expression after OPA1 depletion, we treated the skeletal muscle cells with the antioxidant resveratrol. Among other actions, resveratrol reduces HIF1-α activity, increases SIRT1 activity and suppresses ROS production (259). Resveratrol reduced mitochondrial ROS levels in OPA1-depleted myotubes and rescued the OPA1-mediated changes in *NPAS2* expression (Figure 31C

and 31D). Our results suggest that OPA1 regulation of mitochondrial ROS levels constitute a feedback loop permitting bi-directional control of circadian metabolism in skeletal muscle.

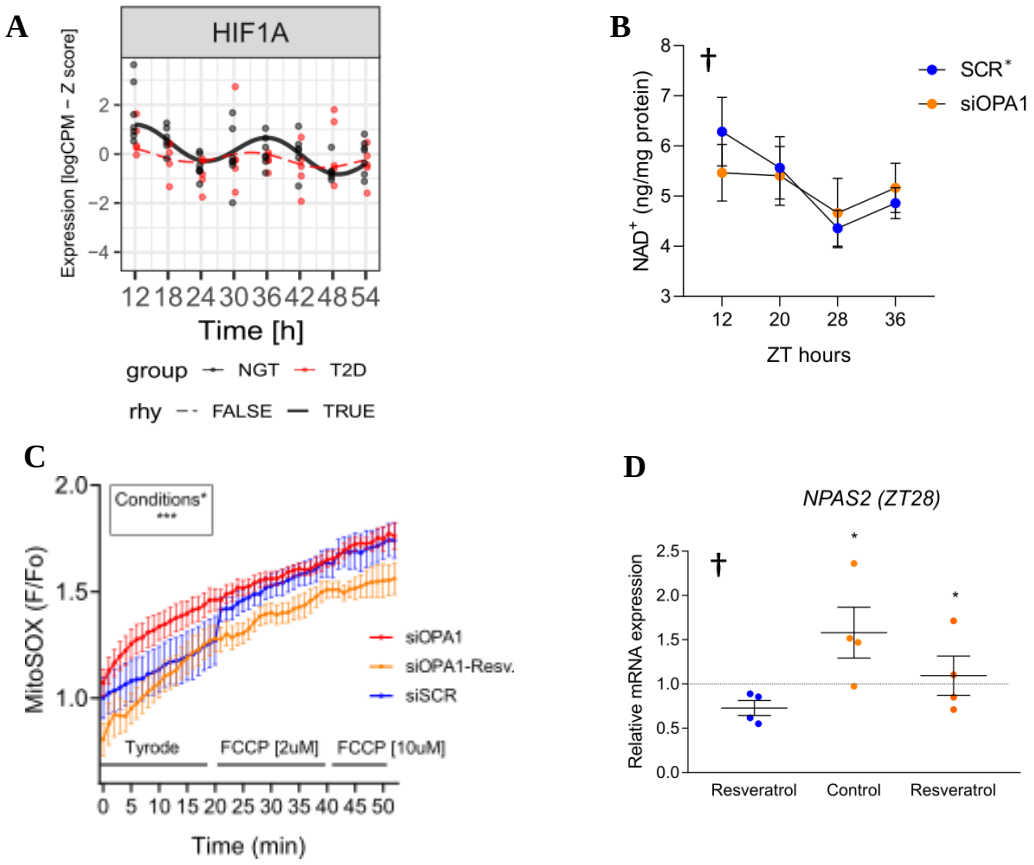


Figure 31. Reduction of oxidative stress restores clock gene expression. A HIF1α mRNA expression in NGT or T2D (n=7, n= 5). Solid line indicates circadian (adj.p<0.1) genes **B.** NAD^+ concentration in synchronized primary human skeletal muscle cells (n=6) treated with either scramble (blue) or siRNA targeting *OPA1* (orange), two-way ANOVA, †overall variance of time, *<0.05 difference between timepoints **C.** Live-cell microscopy measuring MitoSOX fluorescence, indicative of mitochondrial ROS, in human skeletal muscle cells treated with scramble-siRNA, siOPA1-siRNA or siOPA1-siRNA + resveratrol (n=6). ***=p<0.0001. **D.** NPAS2 mRNA expression in synchronized skeletal muscle cells (n=4) treated with either scramble (blue) or siRNA targeting *OPA1* (orange) with and without resveratrol (20uM) treatment.

5.4.5 Conclusions and future perspective for study IV

Our study shows disturbances in rhythmicity of gene expression and metabolism in skeletal muscle cells of individuals with T2D. Of importance, this dysregulation remains in skeletal muscle cells in culture, in the absence of systemic factors, hormones, nutritional clues or direct regulation of the central clock or other peripheral clocks. Our results suggest an important role for the inner mitochondrial membrane in the regulation of circadian rhythms

and metabolism in the context of T2D. The dysregulation of circadian metabolism in skeletal muscle of people with T2D underscores the need to take circadian biology into account and consider approaches in chrono-medicine (260) when prescribing pharmacological therapy.

6 SUMMARY AND CONCLUSIONS

Altogether, this thesis explored how metabolic diseases, namely T2D and obesity, affect skeletal muscle metabolism and circadian rhythms. **Studies I, II and IV** showed that people with obesity and T2D present impaired skeletal muscle metabolism and circadian clock. In **studies I, II and III** we revealed the role of lipids and lipid overload on the development of metabolic and circadian disturbances on the skeletal muscle (Figure 32).

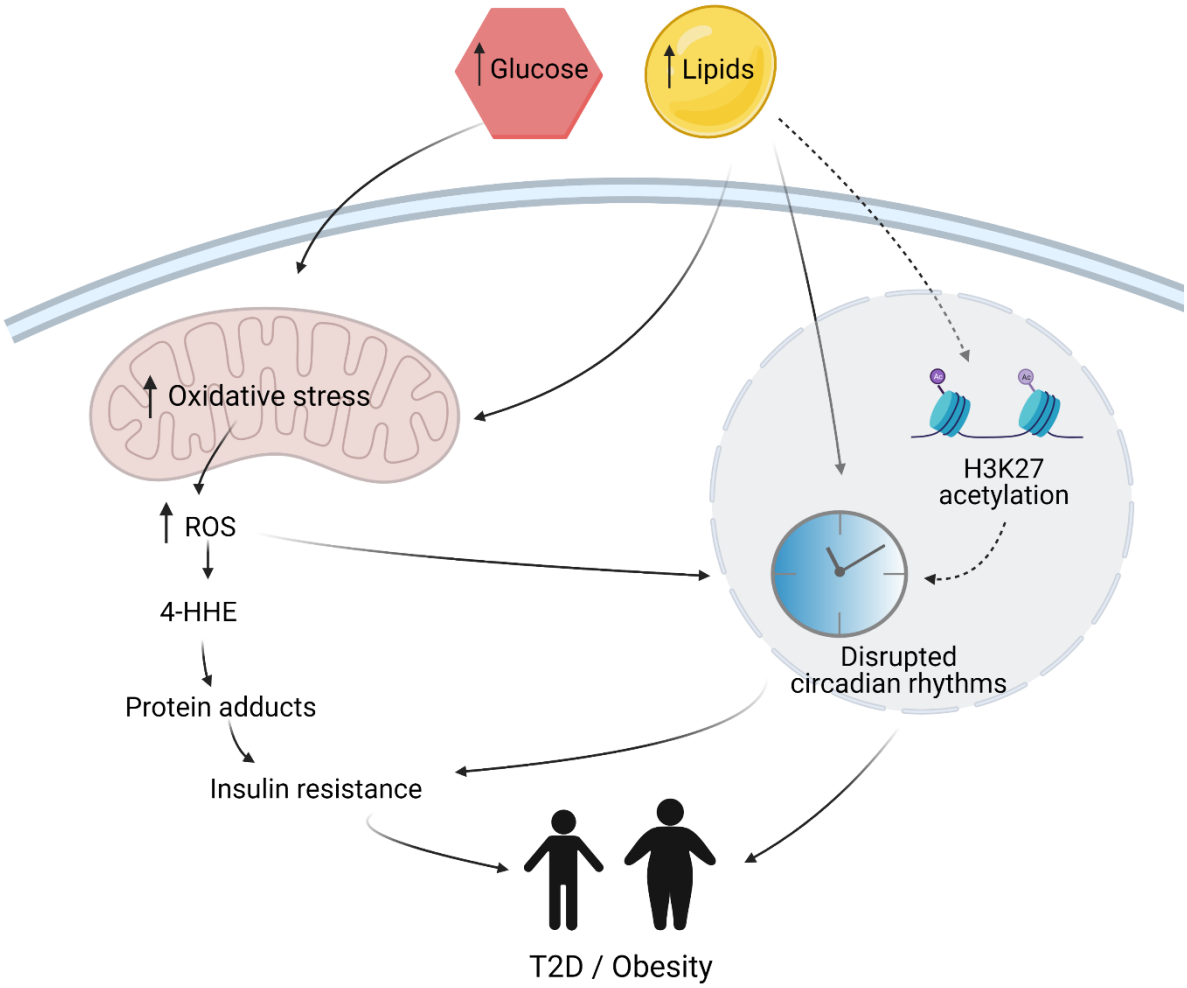


Figure 32. Schematic representation of the results from this thesis

Created with BioRender.com.

Increased lipid and glucose plasma levels promote skeletal muscle mitochondrial dysfunction, increase ROS production and induce oxidative stress (30, 249, 261). In **study I**, we demonstrated that oxidative stress and increase of ROS lead to the accumulation of the lipid

aldehyde 4-hydroxy-2-hexenal (4-HHE), promoting protein adduct formation which interfere with the insulin signaling pathway and induce insulin resistance in the skeletal muscle. Our results supports previous studies showing a causative role of oxidative stress and lipid aldehydes in the development of metabolic disturbances such as insulin resistance (30, 41, 171, 181, 262), and contributes to this body of knowledge by showing the specific role of 4-HHE.

Oxidative stress and mitochondrial dysfunction are also linked to circadian rhythms (263). Melatonin is often used as a marker of circadian rhythms, and it is known to have antioxidant properties, such as the regulation of intracellular glutathione and lipid peroxidation (264-266); Additionally, SIRT1, known to regulate clock gene expression via deacetylation of BMAL1 and PER2 (95, 96, 213), initiates several effectors against oxidative stress, including p53 and FOXO (267). NAD^+/NADH availability regulates SIRT1 activity (96), and at the same time regulates CLOCK:BMAL DNA binding (94, 261), linking the redox state of NAD^+/NADH to circadian rhythms. In **study IV,** we elucidated that increased oxidative stress in T2D patients was responsible for changes in the skeletal muscle circadian gene expression.

Furthermore, increased plasma lipid levels in obesity correlate with changes of core clock gene expression in skeletal muscle in a sex-dependent manner in **study II**. The saturated fatty acid palmitate disrupts skeletal muscle circadian clock and clock-controlled genes. Lipid overload with palmitate leads to changes in overall H3K27 acetylation and circadian acetylation of enhancers in **study III**. Our results contribute to previous studies showing that obesity and high fat diet lead to the disruption of circadian rhythms (73, 74, 98, 206, 268, 269) and describes the specific role of the saturated fatty acid palmitate in the regulation of circadian gene expression.

Overall, increased levels of circulating glucose and ectopic accumulation of saturated fatty acids in the skeletal muscle contribute to the development metabolic diseases such as T2D and obesity (270). Increased circulating fatty acids and glucose lead to metabolic dysregulation

through mitochondrial dysfunction and circadian rhythms disturbances. Our results provide new links between metabolic and circadian pathways, with ROS, histones, and saturated fatty acids at the crossroads of metabolism and circadian rhythms. Our results highlight the importance of regulating lipid and glucose metabolism to prevent metabolic and circadian disturbances.

7 POINTS OF PERSPECTIVE

This thesis investigated dysregulation of skeletal muscle circadian rhythms in the context of obesity and T2D metabolic complications. While many different mechanisms contribute to the development of metabolic diseases and circadian disturbances, our results contribute to the evidence that obesity and palmitate disrupt circadian rhythms in the skeletal muscle. Palmitate also disrupted circadian H3K27 acetylation, however, these changes did not fully account for all observed changes at the gene expression level. Therefore, other mechanisms regulating skeletal muscle circadian rhythms gene expression in the context of obesity and palmitate exposure remain unknown.

Besides, further investigation on sex-dependent differences in circadian response to obesity is required. In our studies we reported sex-dependent differences in core clock gene expression in skeletal muscle in men and women with obesity. Additionally, previous studies have reported sex-dependent differences in the circadian response to sleep deprivation and regulation of metabolism (199-201). However, studies about sex-driven differences in the regulation of circadian rhythms as well as metabolism are often underpowered (271, 272). Even though the NIH and European Union developed regulations to increase inclusion of women in clinical research (273), the analysis of these studies often fail to analyze the results based on sex (271). Moreover, most preclinical research is done in male animals (274, 275). In fact, all animal models included in the studies of this thesis were male, and the primary human skeletal muscle cells were only from male donors. Sex-driven differences in response to stimuli, treatments and interventions should become a common practice in research of all fields.

The causal link between T2D and circadian rhythms disruption remains to be fully elucidated. A better understanding of the cause-and-effect relationship between these two processes is crucial for developing strategies to re-synchronize the circadian rhythms and suppress the pathophysiology of T2D. Eating or exercising at certain times of the days might be beneficial for the circadian regulation of glucose homeostasis.

Chrono-pharmacology has been suggested as a good approach for improving the treatment of many diseases, based on selecting the optimal time of the day for a medical intervention, drug treatment and even food consumption and exercise (85, 276-278). This approach may be challenging to put into practice due to individual variations in optimal treatment timing. For example, sex differences in circadian regulation should be considered, as well as individual chronotype and morning-evening preference (279). In order to apply a chrono-pharmacological treatment to a person an accurate system to define a person's chronotype needs to be developed and taken into account for a personalized medicine.

While chrono-pharmacology may be a few steps away, there are simpler measures we can take to improve our circadian and metabolic homeostasis. Our studies show that avoiding overconsumption of saturated fatty acid might prevent skeletal muscle circadian impairment, as well as reduce oxidative stress and its negative effects on glucose metabolism. Other studies have shown that sleeping patterns (237, 239, 280-282), artificial light exposure in the evenings (125, 283, 284), food composition and timing (9, 97, 99, 100) and exercising schedules (81, 84, 85, 87, 88) have a great impact circadian rhythms. Thus, implementation of optimal routines for exercise, sleeping patterns and food consumption will have a positive impact on our health and well-being and should be complemented with further development of personalized medicine.

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