2023

Chronic Circadian Disruption Increases Risk for Cardiovascular Disease

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2023

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GRADUATE BIOMEDICAL SCIENCES – CELL, MOLECULAR, AND DEVELOPMENTAL BIOLOGY

ABSTRACT

Circadian disruption is a disturbance in biological timing which can occur within or between different organizational levels, ranging from molecular rhythms within specific cells to misalignment of behavioral and environmental cycles. Previous work has shown that changing the timing of food availability is sufficient to lower blood pressure and improve insulin sensitivity in prediabetic males, demonstrating the importance of timing of food intake for cardiovascular health. Findings from our group demonstrated that less than one week of mistimed feeding is sufficient to invert diurnal blood pressure rhythms, although kidney excretory rhythms and kidney function remained aligned with the light-dark cycle. Many shift workers maintain a lifestyle of irregular meal timing for years, even decades, and are at greater risk for cardiovascular and kidney disease. However, the long-term role of mistimed feeding in the development of kidney disease is still not known. Here we show that long-term circadian disruption via food availability results in moderate renal damage in a sex-dependent manner. We found that circadian rhythms in blood pressure are lost as early as four weeks after the start of mistimed food availability in mice. Both male and female mice subjected to this type of circadian disruption developed significant renal cortical fibrosis. Interestingly, males, but not females, also demonstrated

fibrosis in the renal outer medulla. Our results demonstrate the integral role of the peripheral clocks in circadian misalignment. Overall, our studies provide new insights into how meal timing impacts risk of kidney fibrosis independent of overall hypertension. Further, we conclude that disrupted diurnal blood pressure rhythmicity is associated with increased kidney disease risk as observed in humans with non-dipping blood pressure patterns. Our studies suggest that females are better protected against changes in food availability.

Keywords: circadian rhythms, food intake, kidney, physiology, fibrosis, sex differences

DEDICATION

I dedicate this dissertation to God, without whom none of this would have been possible. It is also dedicated to my mother, Emily, and my father, James Sr., who have taught me what unconditional love and support look like and what hard work will yield.

ACKNOWLEDGMENTS

I express my deepest gratitude to my mentor and advisor, Dr. David Pollock. He took a massive gamble on me as a fourth-year student who had left two previous labs and wanted to join his at the start of the COVID-19 pandemic, and he worked hard to help me make up for lost time. Dr. Pollock challenged and encouraged me to think for myself while offering gentle guidance to keep me from letting my curiosity carry me away. His dedication, support, tranquility, and occasional dad jokes restored my enjoyment of science. It reignited my interest in research after a tough couple of years. Given his extensive record of high-achieving trainees, I am honored to join the long list of students that have thrived under his mentorship.

To my committee members: Dr. Carmen De Miguel, Dr. Edward Inscho, Dr. Shannon Bailey, and Dr. Kelly Hyndman, I think of you all as the Marvel Avengers that I got the privilege to assemble. I am appreciative to each of you for your time and effort as members of my committee and your support as I navigated my thesis project. Thank you for your guidance and enthusiastic willingness to serve as my thesis committee members. Your invaluable feedback and advice have played a significant role in shaping me into the renal physiologist that I am today.

I would also like to thank my past and present lab mates: Dr. Anne Ramsey, who always offered a listening ear and positive spirit; Dr. Mary Latimer, whose positivity and infectious energy made my last few months in the lab a pleasure; Tha Luong, who taught me by letting me teach her what I knew in the lab; Dr. Binli Tao, who patiently helped me

get my bearings when I joined the lab, and Dr. Megan Rhoads, who answered all one million of my questions, showed me the ropes and gave me several spirit-lifting pep talks during this journey.

To my community, both in person and online: I could not have done this without you. Before joining Dr. Pollock's lab, I was in the process of dropping out of graduate school. Upon sharing that information, I was inundated with support, encouraging messages, requests to meet and strategize on navigating the hurdle of switching labs, and even offers to continue my Ph.D. at other universities. The community I've found is something that I am boundlessly appreciative of, and hope will be around for graduate students for decades to come.

To my village: Dr. Sade Williams-Clayton, Dr. Maigen Bethea, Dr. Margaret Bell, Dr. Zoya Anderson, Dr. Amber Jones, and Dr. Tayleur White; I love each of you to the moon and back. To have a family of beautiful, intelligent, powerful Black women beside me has single-handedly kept me afloat so many times during this journey. Words cannot express how appreciative I am of how much you have taught me about grad school and about life. Thank you for your support, your love, and your friendship. Thank you for showing me who I am and whose I am. Thank you for the laughs, the tears, and the countless unforgettable memories. I am so fortunate to have met you all, and I count myself immensely blessed to have all of you as sisters for life. None of us were doctors when we met – now we all are. It truly takes a village, and I can't think of a more inspiring group to be able to call mine.

To my mother, Emily – I watched you finish your master's degree while being a mother to three kids and working full-time. I watched you make countless seen and unseen sacrifices to ensure that I could have the best possible life. I cannot thank you enough for that, and I hope this achievement shows you that those sacrifices were not in vain. Though you always expressed that you'd be proud of me with or without my Ph.D., I hope this is among one of the proudest and most fulfilled moments we get to share.

To my father, James – I also watched you complete your master's degree. Watching you walk across the stage in your tam and gown when I was a child showed me that I could one day do the same. I have learned nearly everything I know about working hard and being good from following your example. You taught me that I could do anything or be anything, regardless of what boundaries may lie in my way. You are indeed the World's Greatest Dad, and I'm so glad that God brought you and my mother, and thus you and I together. Anyone who knows me knows that I am most proud of my parents. I hope this achievement brings you even a fraction of the pride you bring me. I love you both so so so so so much.

I would lastly like to thank all of my family and loved ones for their prayers, love, and support. None of you know what I am doing, but you gloat about me anyway. While I may be the first doctor in the family, I certainly will not be the last. I hope I have made and continue to make all of you proud.

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LIST OF ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ADF	Alternate-day fasting
AGT	Angiotensinogen
AMD	Age-related macular degeneration
ANG II	Angiotensin II
AT_1R	Angiotensin II receptor type 1
AT_2R	Angiotensin II receptor type 2
aTRF	Active-period time-restricted feeding
BMAL1	Brain and muscle Arnt-like-1
BP	Blood pressure
CD	Collecting duct
CKD	Chronic kidney disease
CLOCK	Circadian locomotor output cycles kaput
CPAP	Continuous positive airway pressure
CRP	C-reactive protein
CRY	Cryptochrome
CVD	Cardiovascular disease
DCT	Distal convoluted tubule
DOCP	Desoxycorticosterone privalate

ENaC	Epithelial sodium channel
ER	Estrogen receptor
ET-1	Endothelin-1
ET_A	Endothelin receptor A
ET_B	Endothelin receptor B
FASPS	Familial advanced sleep phase disorder
GFR	Glomerular filtration rate
GPER	G-protein estrogen coupled receptor
HS	High salt diet
HFD	High fat diet
IF	Intermittent fasting
iTRF	Inactive-period time-restricted feeding
JGA	Juxtaglomerular apparatus
K^+	Potassium
KSS	Karolinska sleep scale
MAP	Mean arterial pressure
MSNA	Muscle sympathetic nerve activity
Na^+	Sodium
NCC	Sodium-chloride cotransporter
NHE3	Sodium-hydrogen exchanger 3
NKCC2	Sodium-potassium-2 chloride cotransporter
OA^-	Organic anion

OAT	Organic anion transporter
OC^+	Organic cation
OCT	Organic cation transporter
OSA	Obstructive sleep apnea
OVX	Ovariectomy
PA	Primary aldosteronism
PER	Period
PO_4^{3-}	Phosphate
PVT	Psychomotor vigilance test
RAAS	Renin-angiotensin-aldosterone system
RPF	Renal plasma flow
SCN	Suprachiasmatic nucleus
SGLT2	Sodium-glucose cotransporter 2
SNS	Sympathetic nerve activity
TGF	Tubuloglomerular feedback
TRF	Time-restricted feeding
TTFL	Transcription-translation feedback loop
VI	Visual impairment
WNK	With-no-lysine

CHAPTER 1

STATEMENT OF THE PROBLEM

The circadian clock can be thought of as two distinct yet interconnected parts: the central clock, residing in the suprachiasmatic nucleus (SCN) of the hypothalamus of the brain and peripheral clocks that exist in various tissues throughout the body (1). Each of the circadian clock parts have environmental cues (zeitgebers – German: "*zeit*", time; "*geber*", giver) that serve to entrain the clock's rhythms to the Earth's 24-hour light/dark cycle. Light is the most potent zeitgeber and maintains coordination of biological and physiological rhythms via the SCN. Circadian rhythms in peripheral clocks can be entrained by additional zeitgebers such as activity, temperature, and food intake. Timing of food intake has become a topic of interest in recent years because of the interest in what has been referred to as "intermittent fasting." Time-restricted feeding, characterized as feeding periods ≤12-hours without intentional caloric restriction, has been reported to have cardiovascular benefits in both humans and laboratory animal models (2–6). Recent work from our group has also demonstrated the importance of food intake timing for maintenance of diurnal blood pressure rhythms (7).

Artificial light facilitates human activity throughout the 24-hour day, contributing to lifestyles characterized by chronically disrupted circadian rhythms. Circadian disruption can occur on multiple organizational levels, most commonly between behavioral rhythms

and environmental rhythms (8). One common example of circadian disruption is non-daytime shift work, a designation that encompasses nearly 20% of the United States' work force. Non-daytime shift work includes work shifts starting before or at 6 a.m., ending after 6 p.m., or extending overnight (9). It is well reported that these shift workers have an increased incidence of hypertension (10–12).

Nocturnal blood pressure (BP) is an indicator of kidney health – in healthy adults, nighttime BP drops 10-20% from daytime BP, a pattern characterized as "dipping". In adults, a lack of nocturnal dipping (nondipping BP) has been associated with decreased kidney function (13, 14). Shift work can change diurnal BP rhythms from a dipping to a nondipping phenotype, increasing hypertension risk among non-daytime shift workers (15–17). Our group demonstrated that acute circadian disruption via mistimed food intake is sufficient to result in cardiorenal misalignment between blood pressure and kidney function in mice (7). However, this study did not account for the long-term aspect of shift work. Some workers tend to work their respective shifts for months or even decades; long-term shift work can increasingly contribute to cardiovascular disease risk (12). Thus, the goal of my studies was to determine the cardiorenal effects of chronic circadian disruption via timing of food intake via the following objectives:

Aim 1: Test the hypothesis that chronic circadian disruption via mistimed feeding has long-term effects on diurnal blood pressure patterns.

Aim 2: Test the hypothesis that chronic circadian disruption via mistimed feeding induces kidney pathology.

CHAPTER 2

INTRODUCTION

The 24-hour light/dark cycle is based on the Earth's rotation and is the basis of the biological clock. The circadian clock is appropriately named due to its alignment "about the day." This cycle has been a predictable occurrence throughout life on Earth and thus underlies the functions of many organisms. The circadian "clock" is an endogenous system that synchronizes behavior and physiology with the Earth's 24-hour light/dark cycle of the Earth. Under normal circumstances, the environment and behavior are synchronized by this timing system that regulates physiology accordingly. However, when behavior becomes misaligned from the light/dark cycle, such as in rotating shift work, adverse health consequences such as cardiovascular or cardiometabolic disease can arise within or between organ systems. These functions can also become misaligned or dysfunctional in conditions such as hypertension, obesity, and chronic kidney disease (18–21).

Emerging evidence shows that the kidney is negatively impacted by circadian misalignment. It has been shown that nearly 20% of the genes expressed within the kidney are regulated in a circadian pattern (22). These genes include those responsible for electrolyte balance and maintenance of fluid-volume homeostasis. In recent years,

numerous animal models have been used to demonstrate the importance of an intact circadian system and maintenance of alignment between behavior and the light/dark cycle for optimal kidney function; however, there remains much to be discovered regarding how alterations in behavioral and environmental rhythms can contribute to kidney dysfunction and its associated diseases. Here, we discuss the effects of feeding behaviors on circadian dysfunction and its emerging consequences on renal physiology.

BASICS OF CIRCADIAN BIOLOGY

Identification of circadian rhythms goes back to the early 1700s when Jean-Jaques d'Ortous de Mairan, intrigued by the daily opening and closing of the leaves on a mimosa plant, placed the plant into a dark cupboard (23). He observed that, even without access to sunlight, the plant continued to open its leaves during the day and close them at night. Centuries later, Hall, Robash, and Young won the Nobel Prize for their characterization of the molecular clock (24). We now know that nearly every organism on Earth has developed an endogenous timing system that synchronizes with the 24-hour solar day. This system, known as the circadian timing system, exists independently of the light/dark cycle and can be shifted by additional external factors such as temperature and food intake. Given this information, the circadian clock system consists of two distinct yet interconnected parts: the central clock, residing in the suprachiasmatic nucleus (SCN) of the hypothalamus and the peripheral clocks within various tissues throughout the body. Each of these clocks has its own 'zeitgeber' (German: "zeit", time; "geber", giver) or

rhythmically occurring factor that acts as a cue to entrain the circadian rhythms of those clocks.

Light is the main zeitgeber for organisms on Earth, and most organisms have evolved specialized photoreceptive and phototransductive mechanisms for taking in the presence or absence of light. In mammals, light is taken in through the eyes and sends signals to the brain via the optic nerve. In instances such as travel to a different time zone, internal rhythmicity can be reset over time to acclimate to the new time cues. In laboratory animals and humans, it has been revealed that intact animals exhibit an approximately 24-hour circadian rhythm, even without a light/dark cue, suggesting the existence of an autonomous "master circadian oscillator" (25, 26). Later findings determined this master pacemaker to be the SCN, as it was found to be the direct target of retinal fibers and able to maintain rhythmicity in function when isolated in culture (27, 28). Furthermore, additional studies found that disruption of the SCN results in a complete loss of circadian rhythmicity, which could be rescued by implantation of an intact SCN to a mutant animal (29–33). We now regard the SCN as the master circadian oscillator where circadian rhythms are generated and maintained. Signals from the master oscillator can feed forward in the form of neural or hormonal signals to the peripheral clocks, such as the kidneys, to influence behavior, physiology, and metabolism.

Circadian clocks exist outside of the SCN in peripheral tissues and are important for functional control in most every organ system including the kidney, heart, liver, and muscle. These peripheral clocks can exist even within immortalized cell lines such as stem

cells and can be kept ex vivo for long periods (34–37). Circadian gene expression can be measured in these cultured cells and maintain a rhythmic expression when treated with additional nutrients. These peripheral circadian clocks are believed to have tissue-specific roles and differ from the central pacemaker in that they are not directly entrained by light. Rather, peripheral clocks often have additional zeitgebers such as activity, temperature, and food intake.

The Mammalian Molecular Circadian Clock

Most chronobiological studies in biomedical research over the past few decades have focused on determining the molecular basis of the circadian clock with limited studies on how molecular mechanisms impact physiology and disease. Those studies led to the identification of the transcription-translation feedback loop (TTFL) as the core mechanism governing the circadian timing system. This oscillation of positive and negative autoregulatory feedback loops maintains the ~24-hour period of gene expression in keeping with the day/night cycle in mammals. The first molecular circadian clock gene to be identified was Period (*Per*) in *Drosophila*. Three *Per* mutant lines of flies demonstrating different circadian behaviors were identified: one with a shorter period, one with a longer period, and one with no overt periodicity (38). Decades later, human orthologs for Period and additional clock genes were found.

A network of TTFLs maintains the mammalian molecular circadian clock. The core clock components consist of Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain and Muscle ARNT-Like 1 (BMAL1), Periods (PER1, PER2, and PER3), and Cryptochromes

(CRY1 and CRY2). CLOCK and BMAL1 constitute the positive feedback arm of the loop. Both proteins form a heterodimer that binds to the E-box cis-regulatory element of their target genes, which include the *periods* and *cryptochromes*. The negative feedback loop forms when the PERs and CRYs heterodimerize and translocate to the nucleus. CRY binds to the CLOCK-BMAL1 heterodimer to induce transcriptional repression while PER removes CLOCK-BMAL1 from DNA to eliminate the effect of CLOCK-BMAL1. An additional regulatory feedback loop involves transcription of additional clock-controlled genes (CCGs) such as *Rev-Erbs* (Rev-Erbα and Rev-Erbβ) and ROR-responsive elements (*RAR-related orphan receptors*) in the BMAL1 promoter to inhibit or activate the transcription of BMAL1 and increase or decrease the CLOCK-BMAL1 levels, respectively (Figure 1). Although the TTFL should only take a few hours to cycle, this process takes about 24 hours. This is thought to be due to post translational modifications that cause delays in the transcriptional activation and repression process (39–42). Studies within the past 10 years have recognized microRNAs as a novel post-transcriptional regulatory mechanism contributing to maintenance of a 24-hour TTFL by preventing accumulation of clock proteins within the nucleus (43–46).

Core Clock Gene Mutations

Circadian rhythmicity can fall on a phenotypic scale, with individuals considered ‘morning larks’ or ‘early risers’ on one end and ‘night owls’ or ‘late sleepers’ on the other. This scale has come into popularity with the use of the term chronotype. Chronotype or diurnal preference, refers to behavioral patterns indicative of underlying circadian-

governed biological processes. This often represents a preferred sleep and wake time independent of environmental factors in mammals. Observations of changes in diurnal preference across the lifespan have led to an increased interest in the genetic basis for circadian rhythmicity (47, 48). Several human and laboratory animal models have demonstrated the central circadian clock genes' importance in maintaining rhythmicity and determining diurnal preference.

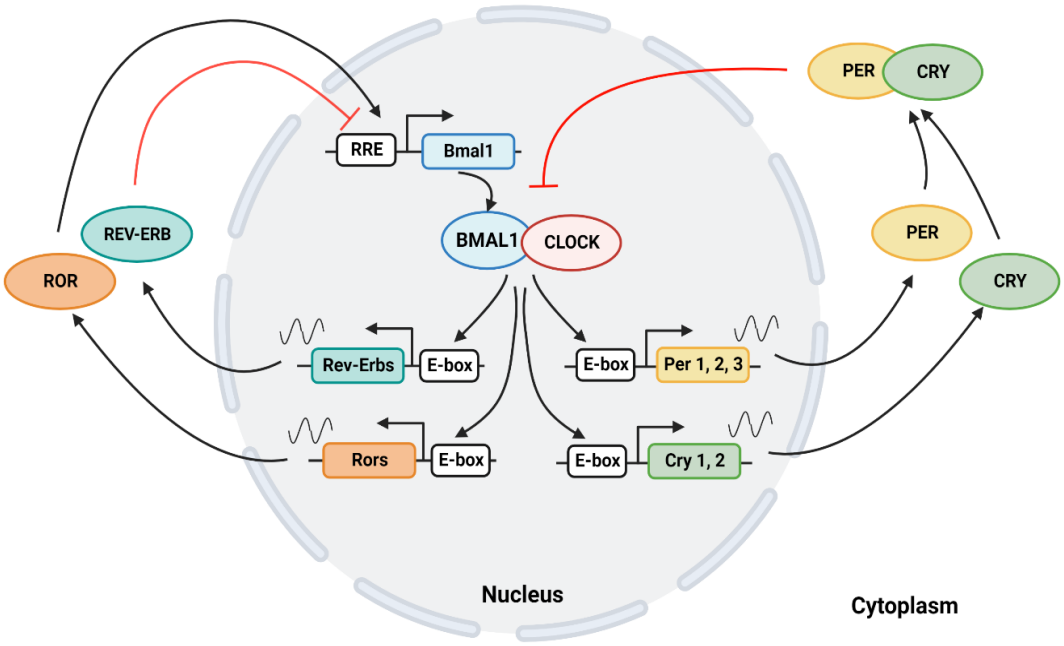


Figure 1: The mammalian molecular circadian clock. The molecular clock is a transcriptional translational feedback loop with positive and negative regulatory arms. The positive arm consists of CLOCK and BMAL1, which heterodimerize to activate transcription of *periods* (PER1-3), *cryptochromes* (CRY1 and 2), and other clock-controlled genes via E-boxes. PER and CRY proteins translocate to the cytoplasm where they heterodimerize to migrate back into the nucleus and repress their own transcription by inhibiting the CLOCK-BMAL1 heterodimer. An additional feedback loop is formed by transcription of *Rev-Erbs* and *Rors* that inhibit or activate transcription of BMAL1, respectively. Figure was created with BioRender.com.

Studies using a candidate-gene approach have associated the 3111T/C polymorphism of the *CLOCK* gene to an evening preference (49–52), although other studies have not seen the same results (53, 54). Interestingly, the *Clock* mutant mouse demonstrated phase delays in body temperature, activity, and wakefulness (55). Daytime preferences have been associated with polymorphisms in *PER1/2* (56, 57). Rare genetic variations in CRY and PER genes have been linked to familial advanced sleep phase syndrome (FASPS), a hereditary form of delayed sleep phase disorder that can cause phase advances of 3-4h in sleep preference and core body temperature (58–60). It is thought that mutations in PER2 may increase heterodimerization of Per2-Cry and speed up the translocation of the dimer into the nucleus, resulting in an accelerated circadian clock (61, 62). Single nucleotide polymorphisms (SNPs) in the *CLOCK* gene have been associated with later sleep times, although this has been disputed in other studies of specific populations (49, 53, 63–65). These differences in findings between populations highlight the difficulty of replicating experiments with rare mutations in humans with regard to extreme phenotype expressions. These studies demonstrate that multiple genes influence diurnal preference and circadian rhythmicity. As chronotypes are complex traits, there are likely even more genes outside of those intricately linked to the clock involved in phenotypic manifestations.

Circadian Misalignment

As there are peripheral clocks within every cell throughout the body, there is an opportunity for misalignment between clocks within these tissues. Circadian misalignment can occur between organ systems and even between specific areas within an organ. A common form of circadian misalignment occurs between behavior and the environment, as is common in those who work rotating or night shifts. Sixteen percent of the US workforce works a non-daytime or rotating shift (Bureau of Labor Statistics, 2019). Longitudinal studies have shown that longer durations of night or rotating shifts can proportionally increase one's likelihood of experiencing poor sleep, having a stroke, and being diagnosed with coronary heart disease or hypertension (66, 67). A 3-year-long study by Skogstad et al. including 65 shift workers and 29 daytime workers found that the number of years as a shift worker are associated with elevated inflammatory markers and increased arterial thickness (68). A 2018 systematic review and meta-analysis combined the findings of 21 studies totaling over 170,000 individuals and found that after 5 years of shift work, cardiovascular disease (CVD) risk increases by about 7% every additional 5 years (11). To demonstrate the cross-cultural effects of circadian misalignment, a recent multi-ethnic study showed that irregular sleep patterns in shift workers contribute to a higher incidence of arteriosclerosis over 5 years compared to those with regular sleeping patterns (69).

Circadian misalignment has been shown to be a risk factor for sterile inflammation in animal models and various human situations (70–72). Additional studies have

demonstrated that even short-term circadian misalignment can elevate inflammatory factors such as C-reactive protein (CRP) (73). Interestingly, the findings from this publication are parallel to those from another study which showed that reproductive-age women doing shift work had a significantly higher serum CRP than day workers; furthermore, this significant increase remained after adjusting for potential confounding variables (74).

More recent studies have focused on whether a relationship exists between sex and the effects of circadian misalignment. Lajoie et al. conducted a cross-sectional study in female hospital employees. When comparing women working two 12-hour days and two 12-hour night shifts followed by 5 days off (forward rotating shift) to those only working day shifts, the forward rotating shift workers reported poorer sleep quality and a higher incidence of metabolic syndrome compared to those working a day shift only (75). A study using data from the Older Finnish Twin Cohort determined that in individuals working shifts, both men and women had an increased risk of mortality due to coronary heart disease, but only men had a slightly increased risk of incident hypertension (76). A 2022 study from Hung et al. used the Framingham Risk Score to estimate the 30-year risk of CVD in 129 male workers from 22-50 years of age, with shifts ranging from permanent day shifts to 5 consecutive night shifts. Workers doing consecutive night shifts had a significantly higher CVD risk estimate than workers on permanent day shifts, underscoring the risk of CVD among workers with circadian misalignments (77). These findings were similar to those seen in the aforementioned Skogstad paper, although the duration of shift work was only about 3 years. Despite strong evidence that longer exposure to night and

rotating shifts can be increasingly harmful, one recent study suggests that ceasing shift work for less than one year is sufficient to decrease arteriosclerosis risk and reduce some inflammatory factors to normal levels (78). More studies will need to determine whether the effects of circadian misalignment due to shift work can be mitigated or reversed by ceasing rotating shifts or stopping shift work altogether.

Overall, there is wide consensus that circadian misalignment increases the risk of cardiovascular disease, particularly over longer periods and regardless of sex, ethnic background, or geographic location. Future studies are needed to determine what factors mediate the link between circadian misalignment and CVD. Additional studies are also needed to determine whether there are sex differences that may mitigate the harm of circadian misalignment.

CIRCADIAN ASPECTS OF KIDNEY FUNCTION

The kidney carefully maintains water and electrolyte homeostasis in the body. Waste products from the blood are excreted from the bladder as urine; as such, the are several steps of filtration and reabsorption of needed nutrients prior to the movement of waste to the urinary bladder. Each human kidney has about 1.2 million nephrons. The nephron is composed of the renal corpuscle, proximal tubule, loop of Henle, distal tubule, and collecting ducts, with each segment contributing to the filtration, reabsorption, and secretion of water, electrolytes, and small solutes. Numerous studies in humans and laboratory research models have shown that there is circadian variation in renal function.

Maintenance of rhythmic excretory function independent of external cues of light, eating, and sleep was demonstrated many decades ago (79). Recent work has also demonstrated that nearly half of the genes in the kidney are regulated by circadian clock proteins. (22, 80, 81). This section will highlight circadian aspects of renal function along the nephron.

Glomerulus

The glomerulus consists of a knot of specialized capillaries situated between two resistance vessels: the afferent and efferent arteriole. To begin filtration, blood enters the glomerulus via the afferent arteriole. The glomerular capillaries are characterized by their high capillary pressure and permeability, facilitating the movement of filtered fluid into the proximal tubule. Certain nutrients and solutes, such as water, sodium (Na^+), glucose, and other small proteins are 'freely permeable' and pass through the filtration barrier easily. Fluid and solutes that are not filtered exit the glomerulus via the efferent arteriole and ultimately returns to the systemic vasculature.

Renal functions such as glomerular filtration rate (GFR), renal plasma flow (RPF), and urinary excretion show circadian rhythms (82–84). Most renal functions follow behavior patterns in diurnal animals, with peaks occurring during phases of increased activity and nutrient intake and nadirs during phases of minimal activity. These phases are often referred to as active and inactive periods, respectively. In humans, the active phase is during the day while in nocturnal animals such as mice and rats, it is during the night. Early studies in adults demonstrated that clearance of inulin and creatinine as measures of GFR and clearance of para-aminohippurate as a measure of RPF both follow diurnal

patterns of activity, reaching their peak during the active period and their nadir during the inactive period (85–88). This led to the hypothesis that clocks within the kidney are involved in regulation of GFR across the 24-hour day (71).

The Firsov group was the first to create a mouse with a podocyte specific knockout of BMAL1, which exhibited a 12h rhythm in GFR as opposed to the 24-hour GFR rhythm observed in control mice (89). The mechanisms underlying the 12h GFR rhythm observed in the knockout animals remain unknown. Results from Ansermet et al. demonstrate an integral role for *Bmal1* and thus the intrinsic circadian clock in glomerular function. There is still much to be discovered about the clock dependent and independent mechanisms underlying glomerular function as GFR appears to be disturbed in those with nephrotic syndrome and diabetic nephropathy (88, 90). These functions may be influenced by zeitgebers or by site-specific expression of clock genes. More work remains to be done to determine where clock genes are expressed within the glomerulus as well as the role of each gene.

Proximal Tubule

In addition to its major role in phosphate reabsorption, the proximal tubule is also responsible for reabsorbing the majority of filtered Na^+ along the nephron. In the first half of the proximal tubule, the Na^+-H^+ antiporter (NHE3) mediates reabsorption of Na^+ (91). Additionally, the Na^+-glucose cotransporter (SGLT2) mediates Na^+ reabsorption alongside glucose (92). A wealth of studies investigated the relationship between the circadian clock and proximal tubule transporters. In one of the first publications to address this

relationship, Wei et al. created a mathematical model predicting that 1) mutations in PER1 or CRY1 would raise Na^+ reabsorption and 2) mutations in BMAL1 or CLOCK would lower Na^+ reabsorption (93). In line with these predictions, studies have shown that *Per1* is a transcriptional regulator of both NHE3 and SGLT1 but not SGLT2 – pharmacological blockade of *PER1* in human proximal tubule cells is sufficient to decrease membrane protein levels of NHE3 and SGLT1, presumably decreasing Na^+ uptake into the cells (94). Male kidney-specific BMAL1 knockout mice demonstrated a decrease in Na^+ retention in response to a potassium (K^+) deficient diet, although the same phenotype was not observed in female kidney-specific BMAL1 knockout mice (95). Tokonami et al. observed a decrease in renal Na^+ channels in kidney specific BMAL1 knockout mice (96). In a whole body BMAL1 knockout rat given an acute salt load, both male and female rats excreted the majority of their salt load within twelve hours (97).

The proximal tubule is the major and final regulatory location for determining levels of phosphate (PO_4^{3-}) – about 75%-85% of phosphate is reabsorbed here. PO_4^{3-} is an important aspect of acid/base balance and thus is crucial for overall health. Many of the transporters involved in PO_4^{3-} transport are members of the solute carrier family, such as SLC34A1 (*Npt2a*), SLC34A3 (*Npt2c*), and SLC20A2 (*PiT-2*) (98–100). Previous publications have detailed the circadian rhythm in mRNA expression of these Na^+-PO_4^{3-} cotransporters (22, 101). Segawa et al. generated a *Npt2a/Npt2c* double knockout mouse model; this model still had some renal PO_4^{3-} absorption, indicating that PiT-2 plays a role in PO_4^{3-} reabsorption in the proximal tubule (102). Interestingly, although PO_4^{3-} reabsorption was not completely abolished, the circadian patterns for plasma and urinary PO_4^{3-} were

significantly blunted. The circadian expression of these three transporters may align so that at least one is always expressed. To date, the transport activity attributed to these transporters has not been measured across the 24-hour day.

As mammals get phosphate from food, renal phosphate absorption is affected by dietary intake. At least one study has shown that as early as postnatal week 3, the renal phosphate transport system is matured. Studies have varied regarding the effects of high and low phosphorus diets on rats. Early studies have concluded that young rats can adapt their renal phosphate transport capacity to the availability of dietary phosphate (103, 104). At least one study has shown that only exceedingly high phosphate diets have the capacity to affect phosphate homeostasis in rats (105). Adaptive changes in phosphate reabsorption are facilitated by movement of Npt2a, Npt2c, and PiT-2 to and from the apical basement membrane of proximal tubule cells. In instances of high dietary phosphate, these cotransporters are removed from the apical basement membrane; when dietary phosphate is low, the cotransporters are inserted into the apical basement membrane to increase phosphate reabsorption (99). Interestingly, one study has demonstrated that, in a rat model of chronic kidney disease (CKD) with established vascular calcification, diurnal rhythms of circulating phosphate were altered significantly (106). Much remains to be discovered about the mechanisms underlying adaptations to differing dietary phosphates, including the contribution of circadian misalignment to additional risk factors that may affect phosphate reabsorption in a secondary manner.

Proximal tubule cells can also transport organic anions (OA^-s) and organic cations (OC^+s). OC^+s such as creatine, epinephrine/norepinephrine, and morphine are moved by organic cation transporters (OCTs). In the kidneys of a mouse model of cisplatin-induced nephrotoxicity, a circadian oscillation in expression of SLC22A2 (OCT2) was observed (107). OA^-s such as bile salts, oxalate, urate, and amoxicillin are moved by organic anion transporters (OATs). Svetlana et al. developed a nephron specific Bmal1 knockout mouse that showed a significant increase in plasma urea along with a reduced ability for the kidney to secrete furosemide, coinciding with a significant decrease in expression of SLC22A8 (OAT3) (108). Collectively, these results indicate that renal clocks can control a variety of metabolic and homeostatic processes within the proximal tubule.

Loop of Henle

The loop of Henle is composed of three parts: the thin descending limb, thin ascending limb, and the thick ascending limb. Here, about 25% of filtered Na^+ reabsorption occurs, particularly in the thick ascending limb. No water is reabsorbed in the thick ascending limb; however, sodium, potassium, and chloride ions are still actively reabsorbed, mainly regulated by the Na^+-K^+-$2Cl^-$ cotransporter SLC12A1 (NKCC2) (109). Clinical findings have identified that mutations in the gene encoding NKCC2 can result in Bartter syndrome type 1, characterized by decreased blood pressure and increased salt loss (110, 111). Although no work to date has demonstrated a clear circadian rhythm in NKCC2 mRNA expression, some studies have observed circadian expression of proteins that may play a role in diurnal regulation of NKCC2 (112, 113). Interestingly, Hara et al.

found that osmotic pressure within the inner medulla follows circadian oscillations and that diurnal variations in osmotic pressure can be abrogated after loss of BMAL1 in mice (114). Tokonami et al. also utilized a conditional BMAL1 knockout and observed a significant decrease in *Bmal1* expression in the medullary thick ascending limb along with increased urine volume and changes in the circadian rhythm of Na^+ excretion (96). Together, these findings demonstrate a role for the molecular clock in the loop of Henle and collecting duct. However, there is still much to be known about the effect of BMAL1 and other molecular clock genes on thick ascending limb function in general.

Near the end of the thick ascending limb, the nephron passes back between the afferent and efferent arteriole, forming the macula densa. The macula densa is a distinct region of the thick ascending limb that makes up part of the juxtaglomerular apparatus (JGA). The small size of the macula densa makes it difficult to study. The macula densa plays a critical role in mediating the tubuloglomerular feedback (TGF) that regulates afferent arteriolar tone and thus GFR, renal blood flow, and delivery of fluid and electrolytes to the distal parts of the nephron. To date, we are unaware of any studies examining circadian control of macula densa function or TGF.

Distal Convoluted Tubule

The distal convoluted tubule (DCT) is located downstream of the macula densa and is the shortest segment of the nephron. It can be divided into two segments: the early DCT (DCT1) and late DCT (DCT2). DCT cells are unique in that their nuclei are positioned on the apical side of the cell. On the lumen side of DCT cells is a thiazide sensitive NaCl co

transporter SLC12A3 (NCC), which is responsible for nearly all of the Na^+ and Cl^- reabsorption in the DCT. NCC is expressed throughout the DCT, but the DCT2 and collecting duct (CD) also transport Na^+ via the amiloride-sensitive epithelial Na^+ channel (ENaC). Aberrations in NCC function have been associated with marked effects on blood pressure regulation, demonstrating a role for renal tubular Na^+ handling in the development of hypertension (115, 116).

NCC is mainly regulated by a with-no-lysine (WNK) family protein kinase cascade (117). In the early 2000s, mutations in the genes encoding WNK isoforms 1 (WNK1) and 4 (WNK4) were shown to result in Gordon's syndrome, characterized by familial hypertension with increased Na^+ absorption (115, 118). Mice overexpressing *Wnk1* demonstrated increased NCC activation, resulting in increased Na^+ reabsorption and elevated blood pressure (BP) (115, 119). A similar phenotype was observed in mice with mutations in WNK4, revealing a link between the two WNK isoforms and Gordon's syndrome. Mice lacking WNK4 demonstrate a reduction in NCC phosphorylation and thus hypotension, characteristic of Gitelman's syndrome (120–122). These findings indicate that WNK4 is the main isoform regulating NCC function and that the DCT is a critical nephron segment for blood pressure regulation with high therapeutic potential for treatment of hypertension. Results from the past decade suggest that the WNK pathway and NCC are under circadian regulation (123, 124). Susa et al. found no diurnal rhythm of NCC or WNK cascade protein expression in male C57BL/6J mice (124). However, diurnal patterns of NCC phosphorylation were observed; these patterns were diminished with

pharmacological blockade of aldosterone. It appears that diurnal expression of NCC may depend on the expression of additional factors upstream of the WNK signaling cascade.

ENaC facilitates Na^+ reabsorption in the principal cells of the collecting duct. The Gumz and Wingo labs have published findings demonstrating a relationship between the circadian clock and ENaC regulation. It appears that PER1 is highly involved in ENaCα regulation. Gumz et al. showed in 2009 that PER1 regulates ENaC expression and attunes the response to aldosterone in murine inner medullary CD cells (125). Furthermore, in 2010, Gumz et al. found that PER1 and CLOCK directly regulate ENaC transcription via interaction with an E-box response element in its promoter (126). More recent work from the Gumz lab has demonstrated that knockout of *Per1* contributes to a protective lower BP phenotype in salt-sensitive mice but exacerbates hypertension in Dahl salt-sensitive rats, suggesting species differences in circadian regulation of renal Na^+ handling. Though these studies provide a wealth of information on the circadian regulation of electrolyte uptake in the distal convoluted tubule, more studies are needed to provide a clearer understanding of the involvement of renal clocks in regulating this segment of the nephron.

Collecting Duct

The collecting duct (CD) is separated into three distinct sections: the cortical CD, outer medullary CD, and inner medullary CD. Less than 1% of filtered Na^+ is reabsorbed here but this can have profound effects on overall Na^+ balance. In the principal cells of the CD, Na^+ entry is regulated via the epithelial sodium channel (ENaC). This Na^+ channel has

several homologous subunits (α, β, δ, and γ), each of which has been shown to be under circadian regulation (127, 128). In a series of elegant studies utilizing rodent cell lines and PER1 deficient and KO mice, Dr. Michelle Gumz and colleagues found that, in rodent kidney cell lines, aldosterone treatment significantly increased *Per1* mRNA expression. PER1 deficient mice demonstrated a decrease in α*ENaC* mRNA in the medulla; these mice also had increased Na^+ excretion. This study also found that α*ENaC* mRNA expression has a circadian rhythm of expression, which is altered in *Per1* deficient mice (125). This study was pivotal in that it demonstrated that ENaC expression is circadian and proved a direct involvement of PER1 on maintenance of its rhythmic expression. Further work from Gumz demonstrated that PER1 KO mice have decreased α*ENaC* expression in the renal cortex, which is unaffected by aldosterone treatment in rodent cells. The αENaC promoter has an E-box element, which is activated by PER1 (126). Additionally, *Cry2* expression was observed to have an inverse relationship with *Per1* expression, and these two clock genes likely have opposite actions on αENaC in rodent collecting duct cells (129). These studies together widely expanded the understanding in the field of the interactions between the circadian clock and the collecting duct in various models.

NEUROHUMORAL CONTROL OF RENAL FUNCTION

The Renin-Angiotensin-Aldosterone System

The renin-angiotensin-aldosterone system (RAAS) is critical in regulating renal hemodynamics. In response to low blood pressure or low Na^+ levels, renin is released from the juxtaglomerular apparatus of the kidney (130). It is then used to convert angiotensinogen from the liver into angiotensin I. Angiotensin-converting enzyme (ACE) facilitates conversion to angiotensin II (ang II). Ang II causes blood vessel constriction, resulting in increased BP. Ang II also mediates aldosterone secretion from the adrenal gland, which promotes Na^+ and water reabsorption in the kidney, contributing to elevated BP. Chronic activation of the RAAS contributes to hypertension and kidney disease pathophysiology.

Angiotensinogen production in the liver serves as the first step of ang II. Results from mouse studies using microarray analysis in the CircaDB database have shown that the liver has a large number of genes expressed in a circadian pattern (131). Work from our group showed that peripheral zeitgebers such as food intake are sufficient to affect *Per2* expression in the liver in the PER2:Luciferase mouse (7). However, future studies are needed to discern the impact of behavioral factors on angiotensinogen production. Similarly, few studies have examined circulating ang II across a full 24-hour period. One report showed that the normal circadian pattern of circulating ang II in plasma that was absent in children with nocturnal enuresis (132). Whether this occurs in other circadian

type of disorders or patients with cardiovascular and/or renal disease has yet to be investigated from a circadian perspective.

The kidney contains all members of the RAAS, and intrarenal Ang II production was first observed decades ago (133). Ang II has two major receptors, type 1 (AT1R) and type 2 (AT2R). The AT1 receptor is the dominant receptor in the kidney, with expression in the vasculature, glomerulus, and tubules and facilitates vasoconstriction and increased vascular resistance. The AT2 receptor is mainly restricted to the vasculature and proximal tubule. Binding of ANG II to AT2 causes vasodilation and decreased renal fibrosis. It is important to note that two AT1R isoforms exist in rodents – AT1AR and AT1BR. Many of the classical RAAS actions are mediated by AT1Rs. AT2Rs have opposing actions, promoting vasodilation and natriuresis. Numerous studies from the Zhuo group have shown that AT1Rs in the proximal tubule are required to maintain BP homeostasis and for the development of Ang II-induced hypertension (134–137). A well-defined role for AT2Rs in the kidney has yet to be discovered. However, recent studies focused on AT2Rs have focused on producing receptor agonists for the treatment of Ang II-induced hypertension and spontaneous hypertension in rats (138, 139).

Aldosterone is the terminal hormone of the RAAS and comprises ~90% of the mineralocorticoid activity of secretions from the adrenal gland. It is a key regulator of Na^+, potassium, and body fluid homeostasis (140). Aldosterone production and secretion in the adrenal zona glomerulosa is stimulated by increased extracellular ang II and potassium, both of which increase expression of aldosterone synthase (CYP11B2) (141). Aldosterone

increases Na^+ and water reabsorption and potassium excretion via the mineralocorticoid receptor (142).

Plasma aldosterone demonstrates circadian variation, hitting its peak toward the end of the sleeping period and beginning of the waking period in humans (143–145). Several studies provide evidence for direct regulation of aldosterone production by molecular clock genes. CLOCK knockout mice demonstrate decreased BP and disrupted circadian rhythms of plasma aldosterone (146). The Firsov group was the first to generate a kidney-specific molecular clock gene knockout and showed that loss of BMAL1 in renin-producing cells decreased BP and reduced plasma aldosterone levels (96). In recent work, the Gumz lab found that BMAL1 KO in the adrenal zona glomerulosa demonstrated a shortened BP circadian cycle and a delayed peak of BP compared to control mice (147). This finding may be attributable to the RAAS, although aldosterone excretion maintained its diurnal variation. Doi et al. reported that the CRY1/CRY2 dual mutant mouse exhibits salt-sensitive hypertension along with significantly increased plasma aldosterone levels (148). Interestingly, the opposite effect was observed in the PER1 mutant mouse, which showed decreased plasma aldosterone levels compared to wild type mice (129).

Dr. Michelle Gumz and her group have further demonstrated a role for PER1 in regulating the renal effects of aldosterone. Early work in mouse inner medullary collecting duct cells demonstrated that expression of *Per1* is increased in response to aldosterone treatment, demonstrating a direct relationship between aldosterone and the molecular clock (149). Further work began to unravel the relationship between PER1 and

aldosterone, reporting that PER1 regulates expression of αENaC through aldosterone induction (126, 150). This was also shown in vivo, as mice without PER1 excreted more urinary Na^+ than wild-type mice(151). Male PER1 knockout mice treated with high salt and aldosterone analog desoxycorticosterone privalate (HS+DOCP) demonstrated hypertension, disrupted diurnal excretion rhythms, and a nondipping BP phenotype (152). Interestingly, kidney specific knockout of PER1 in mice increased sodium reabsorption and aldosterone levels in the kidney and adrenal glands; these observations were contrary to the PER1 mutant mouse generated by Richards et al. (151, 153). Altogether, these data suggest that PER1 modulates renal sodium retention through regulation of aldosterone levels and regulation of sodium transporters.

Findings from the Framingham Heart Study showed that hypertension incidence increased concurrently with serum aldosterone concentration (154). Primary aldosteronism (PA) has been identified as a risk factor for secondary hypertension. In a 2005 study of approximately 5,500 hypertensive patients, Milliez et al. reported that patients with PA were more likely to experience stroke, heart attack, and atrial fibrillation than patients with essential hypertension (155). PA may be curable with mineralocorticoid receptor blockers such as spironolactone; several studies have investigated the effectiveness of spironolactone treatment in individuals with resistant hypertension. In a cross-sectional study of 42 patients with resistant hypertension, Alvarez-Alvarez et al. found that spironolactone treatment alone was more efficacious in lowering office blood pressure than dual RAAS blockade (156). Much more work remains to be done to define the relationship between aldosterone and patients with hypertension, particularly as it

pertains to potential crosstalk between the mineralocorticoid pathway and neighboring signaling pathways.

Endothelin

Endothelin-1 (ET-1) is a 21-amino acid peptide first identified for its vasoactive capability (100–102). ET-1 functions through two G-protein coupled receptors known as endothelin A (ET_A) and B (ET_B) receptors. ET-1 release from endothelial cells results in a long-lasting vasoconstrictive effect via activation of the ET_A receptor on vascular smooth muscle cells, while activation of the ET_B receptor results in vasodilation via nitric oxide release. In the healthy kidney, ET-1 plays an integral role in controlling blood pressure mainly through the ET_B receptor control of ENaC activity in the collecting duct (157, 158). Tubular production of ET-1 is stimulated by high dietary salt; activation of the ET_B receptor promotes sodium excretion to regulate blood pressure via a NO-mediated pathway (159, 160).

Knockout of the ET_A and ET_B receptors in the collecting duct results in more severe hypertension than a singular ET_B knockout, indicating that ET_A receptors are also involved in ET-1's actions in the collecting duct (161, 162). ET-1 production is increased in salt-sensitive hypertension. In several rat models of experimental hypertension, ET-1 is overexpressed in the endothelium (163, 164) and BP is decreased with treatment of ET-1 antagonists (165–168). Forms of experimental hypertension that respond to endothelin antagonists present with arterial hypertrophy (169, 170). Treatment with endothelin antagonists is able to ameliorate arterial remodeling, indicating a direct role for ET-1 on

blood vessels. In the renal microcirculation, ET-1 is also increased in response to a high salt diet. Work from our group found that Sprague-Dawley rats fed high salt for 1 week had increased expression of the ET_B receptor only, reinforcing that activation of the ET_B receptor maintains sodium balance in the face of high dietary salt intake (171). Additionally, Fellner et al. observed that ET_B receptor blockade restores diminished afferent arteriolar autoregulation in high salt fed Sprague-Dawley rats, suggesting that ET-1 can modulate GFR in response to dietary sodium intake (172).

In 2016, Johnston et al. showed that ET_B deficient male rats had a delayed natriuretic response to a salt load administered at the start of the active period (173). Treatment with an ET_A receptor antagonist ameliorated the delayed natriuretic response. This finding suggests that diurnal natriuresis requires the endothelin system. As a follow up to Johnston's 2016 results, Speed et al. conducted a study to determine whether the endothelin system contributes to circadian disruption of the renal molecular clock in the context of high dietary salt intake (174). In control rats, high salt feeding resulted in a 5.5-hour phase delay of Bmal1 expression in the inner medulla of control rats. However, ET_B deficient rats did not demonstrate any alteration of Bmal1 expression. In 2020, Zhang et al. demonstrated that male collecting duct-specific *Bmal1* KO mice demonstrated a lower blood pressure than control mice. When administered a high salt diet and an ET_B receptor antagonist, there was no change in genotype differences (175).

Work from the Gumz group also shed light on the circadian aspects of the endothelin system, stemming from the finding that both ET-1 and Per1 are upregulated in

response to aldosterone in murine collecting duct cells (149). Global *Per1* KO in male salt-sensitive mice resulted in significantly decreased BP across the day concurrent with an increase in renal ET-1 production (128). Interestingly, global *Per1* KO in C57BL/6J mice resulted in a non-dipping BP phenotype, affected diurnal ratios of sodium and ET-1 excretion, and increased ET-1 mRNA and protein expression (152, 176). More recent work from the Gumz group has shown similar findings in a global *Per1* KO rat, reporting increased renal ET-1 and altered gene expression in the kidney and adrenal glands. Additional findings show that kidney-specific KO of *Per1* in mice increases sodium reabsorption, serum aldosterone, and medullary ET-1 concentration (153). Though there are a variety of findings from these studies, it is evident that *Per1* KO increases ET-1 mRNA and protein. Thus, Per1 can be considered a negative regulator of ET-1.

The role of ET-1 in primary hypertension pathogenesis has not been clearly defined. Plasma ET-1 has been shown to be elevated in patients with essential hypertension, but these findings have not been consistent (177, 178). Early studies found that plasma ET-1 did not differ between hypertensive and normotensive patients or was elevated in patients with severe hypertension with renal involvement (164, 179, 180). Interestingly, more recent studies have reported opposite findings. In 2021, Kostov and Blazhev tested serum ET-1 levels in patients with essential hypertension and normotensive controls. They found that mean levels of ET-1 were significantly higher in hypertensive patients (181). Both prehypertensive and hypertensive adolescents also had higher plasma ET-1 than normotensive children, suggesting that plasma ET-1 may be a diagnostic biomarker for hypertension (182). These studies implicate ET-1 in the

pathogenesis of essential hypertension, but more work may be required to explain the disparities in findings over time. In human primary hypertension, there may be activation of the endothelin system commensurate with hypertension severity.

Renal Sympathetic Nerves

Renal sympathetic efferent nerves are important for regulating renal function, playing a role in the control of GFR, tubular transport, and renin release. The kidney is additionally innervated by sensory afferent nerves that convey information to the brain to modulate sympathetic output. Overactivity of renal sympathetic nerves has been linked to the pathogenesis of hypertension and cardiometabolic disease pathogenesis, although the mechanisms underlying these relationships is not well understood. In 1945, Kottke et al. found that chronic stimulation of renal nerves resulted in hypertension in dogs (183). Decades later, Becker et al. demonstrated that rats lacking functioning ET_B receptors on all tissues except sympathetic nerves (ET_B deficient) developed hypertension with salt-sensitive blood pressure increases (184). Total renal denervation reduced blood pressure in ET_B deficient rats but did not ameliorate the salt-sensitivity of blood pressure. Similar findings were seen by Yoshimoto et al. in Dahl salt-sensitive rats, where renal and lumbar sympathetic nerve activity failed to increase after salt loading, despite an increase in arterial pressure (185). After cessation of salt loading, renal sympathetic nerve activity increased significantly while lumbar sympathetic nerve activity remained the same. These results suggest that sympathetic nerves do indeed contribute to hypertension, but not salt-sensitivity.

Renal sympathetic nerve activity can also contribute to renal inflammation and fibrosis. In 1935, Page et al. found that total renal denervation decreased proteinuria in four of five patients suffering from nephritis (186). Preclinical studies have shown that total renal denervation ameliorates glomerulonephritis and reduces glomerular effects of inflammation (187). Renal nerves may also mediate the trafficking or activation of immune cells in the kidney, contributing to renal inflammation. Work from Xiao et al. revealed that total renal denervation decreases $CD4^+$ and $CD8^+$ T cell accumulation in the kidneys and reduces renal fibrosis in a mouse model of ang II induced hypertension (188). Additionally, findings from the Osborn lab suggest that renal denervation may have direct effects on inflammatory signaling in DOCA-salt induced hypertension (189, 190). Interestingly, renal inflammation can also activate the kidney-brain axis, contributing to chronic activation of the sympathetic nervous system and hypertension pathogenesis.

Many studies have defined the necessity of the sympathetic nervous system for maintaining diurnal blood pressure rhythms (191–193). However, very few studies have investigated diurnal rhythms after renal denervation. Our group has reported that the ET_B deficient rats demonstrate disrupted diurnal blood pressure rhythms and greater 24-hour mean arterial pressure amplitude when fed a high salt diet (184). In the DOCA-salt rat model of hypertension, Banek et al. saw a similar finding in that renal denervation did not affect diurnal blood pressure; however, selective denervation and total denervation reduced the 24-hour amplitude in blood pressure (190). Interestingly, recent work from our group found that long-term environmental circadian disruption dampened diurnal blood pressure rhythms in stroke-prone spontaneously hypertensive rats (194). These

findings expand our understanding of the relationship between sympathetic nerve activity and aspects of cardiorenal health and demonstrate the importance of considering time of day in experiments investigating aspects of CVD.

The effects of renal denervation on circadian blood pressure patterns in humans has been much more difficult to define. After what was perceived to be an initial failure of the SIMPLICITY HTN-3 clinical trial to prove the utility of renal denervation for patients with resistant hypertension, a final follow-up on the trial reported that, after 3 years, patients who underwent renal denervation had larger reductions in blood pressure than sham control patients (195–197). The SPYRAL HTN-ON MED clinical trial evaluated the efficacy of renal denervation in patients taking antihypertensive medications. This trial was one of few to report hourly and diurnal differences in blood pressure and found that patients in the denervated group had improved blood pressure during the night (198). This report supported findings from a 2009 study by Krum et al., demonstrating that blood pressure dipping was improved in patients with a nondipping blood pressure phenotype after denervation (199). Together, these data suggest that renal nerves likely mediate diurnal amplitudes in blood pressure in both preclinical models and clinical subjects. A great deal more work is needed to understand better the contributions of renal sympathetic nerves to the control of blood pressure rhythms.

Circadian Regulation of Blood Pressure

Systemic blood pressure is controlled by a variety of factors ranging from behavioral to neurohumoral factors. Surprisingly, however, the mechanisms responsible for this short term up and down pattern of circadian blood pressure rhythm are not clear. While it is likely that 24-hour oscillations in blood pressure involve many of the same systems that control long-term blood pressure levels, this has not been clarified and could also include more novel mechanisms that may or may not involve renal body fluid regulation. In healthy individuals, there is a 'dip' or decrease in BP of about 10%-20% mmHg during the night. Individuals who do not have this 'dip' in blood pressure are called 'nondippers'. It has been well established that night-time BP during sleep is closely associated with organ damage and can be used to predict cardiovascular health (200, 201). At least one early study has indicated that a nondipping phenotype is a stronger predictor of future cardiovascular events and mortality than standard office blood pressure measurements (202, 203).

CKD has been associated with a nondipping blood pressure pattern, suggesting that altered circadian rhythms play a role in the pathogenesis of cardiovascular disease. Interestingly, at least one study has found that a nondipping phenotype is not associated with end stage renal disease in the CKD in children (CKiD) cohort (204). One key mechanism for regulating systemic blood pressure is the renin-angiotensin-aldosterone system (RAAS). The RAAS is well known to be upregulated in patients with CKD. Clinical trials have shown that RAAS blockade can be beneficial in slowing CKD progression,

suggesting that medications traditionally used for the treatment of hypertension such as ACE inhibitors can provide protection against the loss in renal function that is a hallmark of CKD progression (117, 205, 206). Many patients who are treated with RAAS inhibitors eventually progress to end stage renal disease or die from cardiovascular events. As a response, additional treatment options ranging from high dose ACE inhibitors or angiotensin receptor blocker (ARB) therapies to direct renin inhibition have been tried with varying effects (207, 208).

As the kidneys are a crucial organ involved in blood pressure regulation, hypertension can both be the cause of and effect of kidney damage. In hypertensive patients, there can be a normal diurnal pattern of blood pressure, but the mean arterial pressure is higher. Studies have investigated the relationship between dietary sodium intake and nocturnal dipping behavior and demonstrated that excess sodium can result in target organ damage and salt-sensitive hypertension in both normotensive and hypertensive patients (209–211). Previous studies have demonstrated that the circadian rhythm of sodium excretion is an important factor in the dipping behavior of nighttime blood pressure (212). A vast number of studies using laboratory animals have shown that knockouts of core circadian clock genes exhibit blood pressure phenotypes (Table 1). Prolonged disease or metabolically altered states such as shift work or obesity can lead to renal vasodilation and glomerular hyperfiltration as mechanisms to maintain sodium balance despite increased tubular reabsorption. Indeed, studies on human subjects have demonstrated that daytime urinary sodium excretion can impact nighttime blood pressure and nocturnal dipping (212–214). Though there is a wealth of information from clinical

studies and meta-analyses, there is still much to be done regarding understanding the molecular and physiological mechanisms underlying nocturnal dipping blood pressure phenotypes.

Table 1. Circadian gene knockouts and blood pressure phenotypes in rodent models

Species (Strain)	Model	Type of KO	Blood Pressure Phenotype	Reference
Mouse	*Bmal1 KO*	Global	No circadian rhythmicity, hypertensive	(215, 216)
Mouse	*Bmal1 KO*	Renin producing cells	Lowered blood pressure	(96)
Mouse	*Clock KO*	Global	Hypotensive	(127)
Mouse	*Per2 KO*	Global	Lower diastolic BP, decreased circadian rhythmicity	(217)
Mouse	*Cry1/Cry2 KO*	Global	Salt sensitive hypertension	(148)
Mouse	*Bmal1 KO*	Kidney-specific (cadherin Cre+)	Lower systolic BP in males, no BP phenotype in females	(95)
Mouse	*Per1 KO*	Kidney-specific (cadherin Cre+)	Nondipping hypertension when fed both HS and HS+DOCP	(152, 153, 176)
Mouse	*Bmal1 KO*	Collecting duct cells	Lower 24-hour MAP in males, but not in females	(175)
Mouse	*Bmal1 KO*	Adrenal gland	Shortened BP circadian cycle (~23h), delayed BP peak	(147)
Mouse (129/sv)	*Per1 KO*	Global	Lower 24-hour MAP	(128, 218)
Mouse	*Bmal1 KO*	Global	Loss of diurnal variation and decrease in MAP	(216)
Mouse	*Bmal1 KO*	Endothelial cells	Reduced active period BP	(219)
Mouse	*Bmal1 KO*	Tubular cells	Decrease in systolic BP	(220)
Rat (Dahl SS)	*Per1 KO*	Global	Augmented hypertension after 3 weeks on HS diet	(221)
Rat (Sprague Dawley)	*Bmal1 KO*	Global	Lower MAP	(97)

Sex-Differences in Kidney Function

In the past decade, sex-differences have become a point of focus in the context of many diseases. While some studies suggest that female sex may provide some protective effects in terms of renal pathophysiology, others have argued that it can adversely affect renal disease progressions and outcomes. Studies in both humans and rodents have demonstrated that renal mass and nephron structure is larger in males. Sex differences in renal function have also been investigated in laboratory animal models (222). Renal plasma flow and vascular resistance are higher in females, thus providing a potential explanation for females' lower prevalence of hyperfiltration (223). However, studies have reported higher kidney volume, glomerular volume, and kidney weight in males relative to overall body weight (224, 225). Kidney volume correlates with GFR (226–228). Higher GFR was reported in male living kidney donors until 35 years of age, after which females experienced a steeper decline (229). In a longitudinal study, James et al. reported that males had higher urine creatinine per g body weight, possibly due to increased muscle mass compared to females (230, 231). However, rodent models demonstrate sex differences in renal tubular secretion of creatinine (232, 233).

In a pivotal paper for the field, Veiras et al. found that at baseline, male and female kidneys exhibit sexual dimorphism in transporter mRNA and protein expression that may account for differences in salt handling (234). In recent work, McDonough et al. reported sex differences in renal transporter abundance along the nephron (235). In determining the physiological implications of the observed sex differences in transporters, Soliman et

al. found that male Sprague-Dawley rats exhibit an increased natriuretic response to pharmacological ENaC inhibition (236). This finding is consistent with early findings that amiloride administration increased urine output and sodium excretion in males relative to females (237, 238). Musselman et al. reported higher NKCC2 expression in female Sprague-Dawley rats, which was attenuated by ovariectomy (239). Interestingly, pharmacological blockade of NKCC2 resulted in an increased diuretic and natriuretic response in female Wistar rats, suggesting that NKCC2 likely plays an integral role in sodium reabsorption in females, but not males (240). Blockade of NCC produced a similar sex-specific difference in natriuretic and diuretic response in both mice and rats (241, 242). In 2020, Tahaei et al. utilized 3D imaging of the DCT to find that female mice have an increased NCC presence and a better capability to modulate DCT length in response to diuretics, which could explain the sexual dimorphism in response to NCC blockade (243). In the future, 3D imaging of additional nephron segments may provide insight into the physiological mechanisms driving sex differences in transporter activity and pharmacological responses.

Despite an increased interest in the field in sex-differences, there remain many basic research studies that solely use male animals and kidneys. Studies that have used both male and female animals are focused on the cardio-renoprotective effects of sex hormones (244–246). In females, the focus has been on the classical estrogen receptors (ERs), ER_A and ER_B, and the G protein-coupled estrogen receptor (GPER) as mediators of the effects of estrogens in the kidney [224,225]. Work from our laboratory as well as the Gohar lab has contributed greatly to the understanding of female sex and ovarian

hormones to hypertension, endothelin action, and sodium homeostasis. Recent work from Gohar et al. demonstrated that activation of GPER in the renal medulla facilitates natriuresis in female rats and that GPER mRNA expression is increased in the outer medulla of females, suggesting a sex-specific natriuretic effect of GPER (247).

Kittikulsuth et al. reported reduced renal ET_B function in male Ang II hypertensive rats; female hypertensive rats had preserved ET receptor function even during chronic Ang II infusion (248). ET_B receptor function was impaired in the renal medulla of male hypertensive rats, resulting in impaired water and sodium excretion, which was preserved in females. In a 2013 follow-up study, Kittikulsuth et al. reported that pharmacological ET_B receptor blockade contributed to a larger, dose-dependent increase in blood pressure than Ang II hypertension alone in female rats only (249). These results indicate that there are sex differences in Ang II mediated ET receptor function, particularly in the renal medulla. Early work from our group showed that the ET_A receptor drives natriuresis in female *ET_B* deficient rats (250). As the inner medullary collecting duct (IMCD) is a major site of ET-1 production and ET_B receptor expression, Jin et al. utilized isolated IMCDs from male and female Sprague-Dawley rats to discover an increased ET_B/ET_A receptor ratio in females only (251). Johnston et al. reported that the ET_B receptor is required for diurnal sodium excretion, particularly in males (173). Ovariectomy (OVX) increases ET_A and ET_B receptor expression in the inner medulla of rats, suggesting that estrogen may regulate ET activity in the kidney (252). Dual inhibition of the ET_A and ET_B receptors in the medulla reduces natriuresis in female rats (253). GPER KO abolished sex differences in ET-1 excretion, demonstrating that GPER is required for increased ET-1 excretion in female, but

not male mice. These findings suggest that there may be crosstalk between GPER and the endothelin system.

Epidemiological studies have found that female sex is related to a slower decline in renal function than males, again suggesting that estrogen has renoprotective effects (254, 255). Menopause or deficiencies in estrogen due to ovariectomy have been shown to exacerbate negative renal pathologies in rats with streptozotocin induced diabetic kidney disease (256). However, these effects can be ameliorated with exogenous estrogen or progesterone replacement (257–259). Interestingly, supplementing male rodents with estrogen improves manifestations of kidney disease (259, 260). In rodent models of renal injury, castration of male rats decreased blood pressure and improved GFR (261, 262). Altogether, these studies suggest that either a) estrogens have renoprotective effects or b) testosterones have a permissive effect with regard to renal injury and may even exacerbate kidney damage.

Sex has an impact on the circadian regulation of physiological functions. Douma et al. showed that female *Per1* KO mice do not develop hypertension in response to HS-DOCP treatment, suggesting an interaction between sex and the molecular circadian clock in the context of driving cardiovascular rhythms. In a kidney specific *Bmal1* KO mouse, Crislip et al. reported that males have a lower systolic BP and a differential expression of electrolyte transporters compared to females (95). Interestingly, the diurnal rhythm of urine excretion was maintained in both sexes. In the *Bmal1* KO rat, mean arterial pressure (MAP) is significantly reduced in both males and females (97). However, male KO rats

demonstrated a loss of diurnal sodium excretion, suggesting that Bmal1 plays a role in circadian control of sodium excretion. Paired with the observed maintenance of diurnal MAP rhythms in males, these findings further suggest that Bmal1 may play different roles in terms of diurnal blood pressure control and sodium homeostasis.

EATING BEHAVIORS

Interest in the health impact of eating behaviors has steadily increased since the 1960s. A poor diet has been identified as the leading cause of non-communicable disease globally (263). There is substantial scientific and sociological evidence showing that the diet is important in terms of preventing and treating acute and chronic illnesses (263–265). Kidney disease affects nearly 10% of the global population and its prevalence is expected to rise due to increases in population age and Western dietary practices. The Western diet is defined as one high in salt and fat and low in fruit and vegetable content. This diet, often high in animal protein, has been linked with the progression of chronic diseases such as CKD. Diets high in animal protein can affect the kidney directly by impeding the ability of the glomerulus to protect itself from barotrauma and hemodynamic injury (266–268). One potential option for patients to reduce the risk of complications and death from cardiovascular disease is to adopt healthy dietary habits. Studies have suggested that healthy dietary patterns such as the Mediterranean diet, the vegetarian diet, and even a vegan diet can improve kidney health (269). In addition to these suggestions, studies in the last decade have investigated the involvement of the

timing of food intake on kidney health, as well. Early studies in rodent models with lesions of the SCN lose diurnal rhythms in activity when fed ad libitum but regain rhythms when given restricted food access (270–272). These seminal findings demonstrated a relationship between timing of food intake and entrainment of circadian rhythms; decades later, this relationship is still not well understood. It is well accepted that circadian patterns of metabolism are likely designed to process foods at specific times of day. However, we still do not fully comprehend the roles of timing of food intake in terms of organ and system-specific effects. This section will expand on the current knowledge regarding different types of food intake timing, diet composition, and their effects on kidney health.

Intermittent Fasting

Intermittent fasting (IF) has increased in popularity over the past 10 years, partly due to clinical findings suggesting that forms of IF may produce significant weight loss and be protective against metabolic diseases (2, 5, 273–275). Intermittent fasting is a term that encompasses different types of diets (Table 2), but generally refers to adopting alternating feeding and fasting periods. Because IF is an umbrella term encompassing several approaches to regulating food intake patterns, its effects appear to be heterogeneous in experimental models and human subjects.

Time-restricted feeding (TRF) is used most often with rodent models, but timing of food availability and restriction range from study to study. Rodents have well defined active and inactive periods, with the majority of activity and food intake occurring during

the dark (lights-off) period. A commonly utilized TRF paradigm includes 12h feeding and fasting periods that span the entirety of the light (inactive, iTRF) or dark (active, aTRF) period, often for multiple weeks (Figure 2). Sims et al. recently utilized aTRF for four weeks in two murine models of hypertension. aTRF decreased systolic BP and improved biomarkers of renal function without affecting body weight or food intake (276). In diabetic (*db/*db) mice with a non-dipping phenotype, aTRF restored nocturnal BP dipping (277). Work from our group demonstrated that in under one week, iTRF inverted circadian MAP rhythms compared to ad libitum fed animals in male C57BL/6J mice. Interestingly, the same paradigm was sufficient to induce a diurnal MAP rhythm in Bmal1 KO mice, suggesting that timing of food intake controls BP rhythms (7).

In male Sprague-Dawley rats fed high salt, one week of iTRF significantly affected the diurnal rhythm of excretion of both sodium and aldosterone, demonstrating that the peripheral zeitgeber of food intake may control sodium balance in the kidney (278). Male Sprague-Dawley rats fed every other day demonstrated a reduced inflammatory response along with a lack of cardiac remodeling compared to sham animals after ischemic injury in the heart (279). Together, these studies provide strong evidence for the importance of timing of food intake in models of aspects of cardiovascular disease (Figure 3). While these feeding schedules are convenient with regard to the diurnal pattern of many animal facilities and rodent behaviors, most do not translate well to the 24-hour day behavioral cycle for many humans. However, they do prove to be useful in determining the broader physiologic effects of eating in or out of sync with normal food intake times. Due to the direct effects of feeding on the liver and gut, many studies regarding fasting/feeding

schemes focus on those organs. Additionally, many studies thus far do not include female animals, so potential sex-differences in response to TRF are still widely unidentified. As many humans undertake various feeding regimens as dietary interventions, much more work has been done as randomized trials in a clinical setting.

Table 2. Types of intermittent fasting with permission schedule

Type of Intermittent Fasting	**Permission**
Alternate day fasting (ADF)	Alternating fasting days (no intake of high energy food and drinks) and feeding days (eating and drinking ad libitum)
Twice-a-week fasting (5:2 fasting)	Fast for two non-consecutive days, eat ad libitum on the remaining five days
Time Restricted Feeding - 14:10 fasting - 16:8 fasting	Food intake within a defined “food window” time - 14h fasting, 10h eating ad libitum - 16h fasting, 8h eating ad libitum
Religious fasting	Fasting undertaken for spiritual or religions reasons, may range from 10-40 days depending on religion

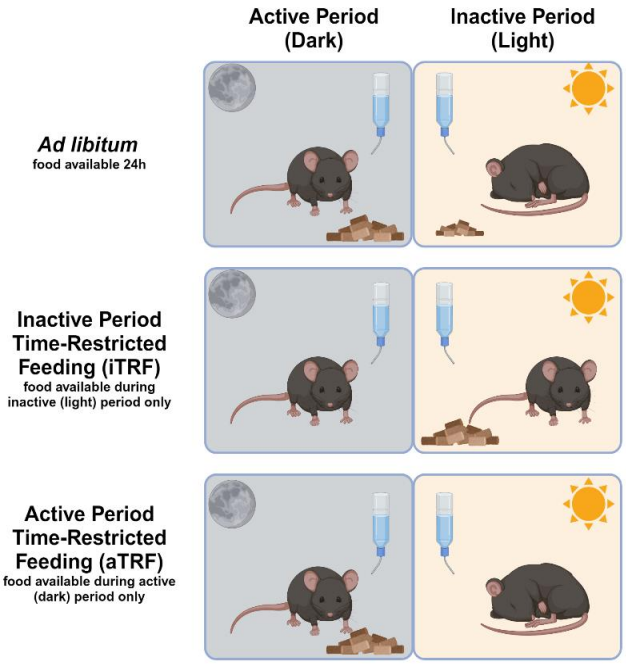


Figure 2: Time-restricted feeding (TRF) schemes in rodent models. Food availability is represented by brown chow pellets. Figure was created with BioRender.com.

Alternate day fasting (ADF) is defined as alternating days of fasting and feeding. On fasting days, individuals eat <25% of their daily caloric requirement. On feeding days, individuals eat normally, as often as desired (*ad libitum)*. Many clinical and randomized controlled trials utilized ADF to investigate the effects of intermittent fasting on cardiovascular disease risk, usually in obese patients. A 2020 meta-analysis of existing clinical trial findings determined that ADF significantly decreased body mass index (BMI), body fat mass, and body weight in obese patients (280). Of the existing studies relating

ADF and cardiovascular disease, the duration of ADF ranges from 8 weeks to 1 year, suggesting that some patients may be able to sustainably adhere to ADF. In 2-3-month stints, ADF appears to decrease body weight, fat mass, and some risk biomarkers of cardiovascular disease such as plasma or serum triacylglycerol and CRP (281, 282). Interestingly, ADF does not appear to decrease low density lipoprotein (LDL), which is associated with long-term risk of cardiovascular mortality (283). In metabolically healthy obese adults, those partaking in ADF had the highest rate of dropout over the course of one year (284). When comparing between ADF and caloric restriction (consuming ~75% of caloric allotment daily), average weight loss was similar. There were also no differences between groups in terms of risk biomarkers of cardiovascular disease, suggesting that ADF does not result in weight loss or cardio protection when compared to daily caloric restriction.

In an effort to assess ADF diet tolerability, Hoddy et al. conducted a study where obese subjects were placed into 1 of 3 ADF groups: ADF-L: lunch (fast day meal during lunchtime), ADF-D: dinner (fast day meal during dinnertime), or ADF-SM: small meals (small fast day meals throughout the day). Interestingly, although body weight decreased similarly in all three groups, systolic blood pressure decreased only in the ADF:SM group (282). This result suggests that, in terms of ADF, timing of food intake can affect blood pressure, a finding that has been observed in other forms of IF. Together, these trials suggest that, for obese patients, ADF may be difficult to adhere to long term. However, the weight loss effects of ADF appear to be more prevalent during shorter-term fasting, which may be more feasible for most adults.

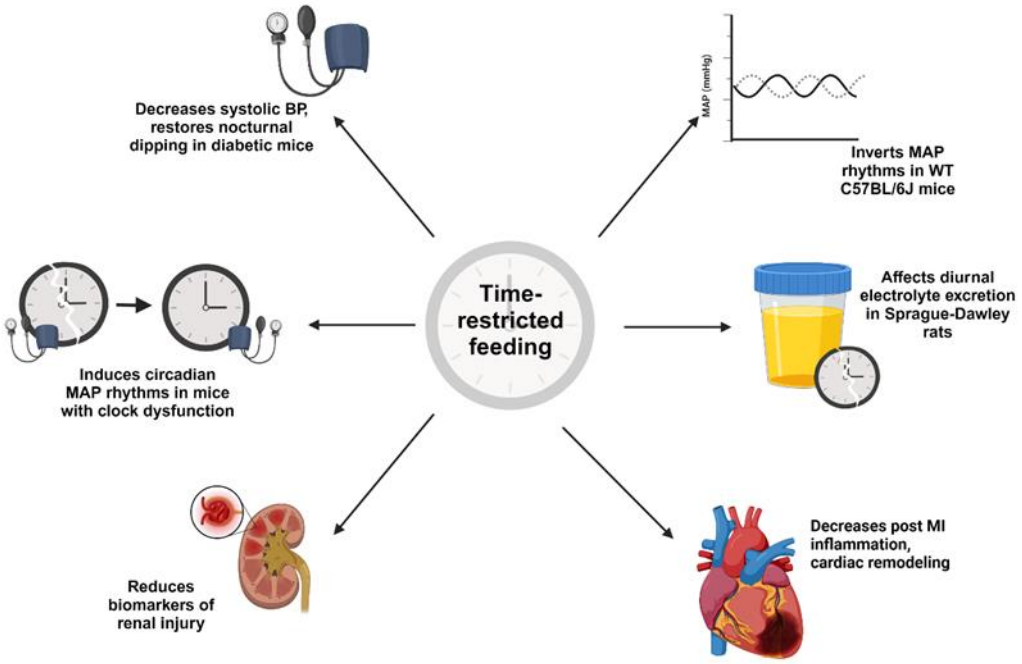


Figure 3: Cardiorenal effects of time-restricted feeding in rodents. Figure was created with BioRender.com.

The 16:8 (16h fasting, 8h eating) is one of the more popular forms of IF as it can be easily incorporated into daily life (Table 2). Surprisingly, there have not been many clinical trials utilizing this feeding scheme. In clinical trials utilizing overweight patients, at least one study has demonstrated that 16:8 IF can reduce body weight, body mass index (BMI) and waist circumference (285, 286). Schroder et al. demonstrated that TRF did not result in significant changes in blood biomarkers of metabolic syndrome but did reduce the 30-year risk of cardiovascular disease in obese patients. 16:8 IF appears to have different effects on resistance trained males, where 8w of IF was demonstrated to decrease fat mass but also lower testosterone and increase adiponectin and leptin (287). After one year, fat mass was still reported to be decreased, along with inflammatory markers and LDL (288). Interestingly, short-term 16:8 TRF did not produce weight loss or significant changes in cardiovascular function in healthy non-obese middle aged and older adults (289). While there is a scarcity of clinical trials using 16:8 in the context of kidney disease or healthy adults, it appears that the feeding scheme may have different effects depending on whether the patient is of older age, overweight, or doing resistance training.

Even fewer clinical trials exist that focus on the effects of 14:10 TRF on cardiovascular disease risk; only two randomized controlled clinical trials of fasting in humans were found. In a 12-week study of 14:10 TRF in patients with metabolic syndrome, patients experienced reduced body weight and BMI. Patients also demonstrated improvements in decreased LDL particle size and decreases in both systolic and diastolic blood pressure (5). Another study investigated the effects of 14:10 TRF on firefighters working 24-hour shifts; firefighters participating in 14:10 TRF demonstrated

significant decreases in body weight and BMI as well as improvements in very-low-density lipoprotein (VLDL), which has been previously found to be significantly associated with cardiovascular disease risk (290, 291). Interestingly, in participants with elevated cardiometabolic disease risk, 14:10 TRF decreased diastolic blood pressure. Although extremely limited, these data suggest that 14:10 TRF can result in weight loss and improvements in risk biomarkers for cardiovascular disease, particularly in those with cardiometabolic disease or elevated risk of cardiometabolic disease.

Another form of intermittent fasting is religious fasting, which can range in time from 7-40 days (Table 2). The most populous and lengthy form of religious fasting occurs during the month of Ramadan, which lasts for 29-30 days and is practiced by millions of people around the world. It is compulsory for all healthy, adult and teenage Muslims and consists of an absolute restriction of food, drink, smoking, and sexual activity from dawn to sunset during the month. While many Muslim children begin to practice Ramadan during their early teenage years, Ramadan fasting can become a concern during the later years of life. It is important to note that the Islamic religion exempts people with significant disease or illness from the obligation of fasting. However, many Muslims have a strong adherence to this religious tradition even during their later years in life. Few studies in the past decade have investigated the cardiovascular risk that Ramadan fasting may pose to older patients; those that exist are observational and/or prospective. Of the studies on the impact of Ramadan fasting in hypertensive patients, sample sizes were modest, ranging from 20-82 participants (292–294). Studies that utilized weight loss as an endpoint observed significant decreases in both body weight and BMI, though there was

rarely a follow up after the fasting period to determine whether weight was recovered. All studies showed a decrease in both systolic and diastolic blood pressure along with HDL, LDL, and VLDL. Studies varied in whether treatment administration was changed, with at least one either a) not changing antihypertensive medications or times of medication or b) changing the timing of medication intake to just before beginning and breaking of the fast. Although extremely limited, the existing data suggest that Ramadan fasting results in modest weight loss and improvements in metabolic parameters.

Yet another eating behavior that does not fall under the umbrella of intermittent fasting is late night eating. Today's 24-hour economy requires working in shifts, including nightshifts. Although shift work originated in the late 18th century, the health effects of working night shifts have only recently become a point of interest. Night shift work is the most common example of circadian disruption (see Section 5. Circadian Disruption and Kidney Dysfunction). Night shift work requires that workers be awake and active between the hours of 10PM and 6AM. As such, night shift workers often eat the majority of their daily caloric intake during the very late night or early morning hours. This behavior can be considered as late-night overeating (LNO), eating a large amount of the daily total energy intake late in the day or during the night (295). Several cross-sectional studies have shown an association between LNO and increased prevalence of aspects of CVD. Late night dinner and bedtime snacking were associated with a higher risk of CKD (odds ratio late night dinner 2.84; 95% CI, 1.40-5.75; odds ratio bedtime snacking 2.87; 95% CI 1.27-6.45) in 341 men (296, 297). A cross-sectional study of 901 Greek men and women without established CVD demonstrated that individuals who consumed >40% of their total daily

energy intake after 7PM had an increased risk of developing carotid plaques (odds ratio 1.70; 95% CI, 1.07-2.68) (295). Furthermore, an epidemiological survey of medical records of nearly 2,000,000 Japanese individuals showed that late night dinner and bedtime snacking increased the incidence of heart attack, stroke, and heart failure (298). One common limitation of these studies is that they do not prove causality between late night eating and CKD or CVD. Thus, it is hard to conclude a cause-and-effect relationship.

Diet Composition

Just as timing of food intake is important, so is diet composition. It is very well established that the nutritional components of one's diet regulate gene expression (299, 300). While timing of food intake has a major influence on peripheral clocks, it is also becoming evident that diet composition can impact the master clock in the SCN (301–304). High-fat diets (HFDs) are established disruptors of circadian rhythms and can result in changes in food intake timing and metabolic perturbations. At least one study directly links HFDs and circadian-controlled secretion of hormones (305). However, many of the experiments investigating the effects of HFD on circadian rhythms have been in the context of obesity or type-2 diabetes (306–308). Hsieh et al. reported alterations in clock gene expression and clock-controlled gene expression in the livers and kidneys of C57BL/6 mice fed a HFD for 11 months (309). This finding demonstrated that diet-induced obesity alters the circadian clock system in mice. A recent study from our group reported salt-sensitive hypertension and disrupted sodium handling in male Sprague-Dawley rats fed a HFD for 8 weeks (310).

Other nutrients have been linked to a role in circadian regulation; at least one study has found that high dietary sodium can affect the molecular clock in the kidney of rats (174). Recent work from our group has begun to investigate the relationship between the molecular clock and electrolyte homeostasis. In 2018, Speed et al. found that ET_B deficient rats fed a high salt diet demonstrated a 5.5-hour phase delay in Bmal1 expression in the renal inner medulla (174). A high-salt diet has also been shown to affect circadian gene expression in mice, also inducing a phase-advance of clock genes in peripheral tissues including the kidney (311). A collecting-duct specific *Bmal1* knockout mouse fed a high salt diet resulted in a significant decrease in male MAP compared to controls, suggesting that collecting duct Bmal1 plays an integral role in BP regulation in male mice (175). In a rat model of circadian disruption via mistimed feeding, Rhoads et al. reported that restricting high salt feeding to the inactive period impaired diurnal excretion of sodium and aldosterone (278). In recent work, Zietara et al. demonstrated that knockout of *Per1* worsens kidney injury and hypertension in salt-sensitive rats, providing further evidence of a relationship between dietary sodium and the circadian clock, particularly in the kidney (221).

In a series of elegant studies, the Gumz group has interrogated the relationship between sodium and the molecular clock in mice. In male *Per1* KO C57BL/6J mice given HS+DOCP, Solocinski et al. reported a significant increase in MAP along with a non-dipping BP phenotype (152). This model was shown to have impaired diurnal excretion of sodium, attributed to inappropriate regulation of sodium handling genes in the distal nephron (176). This finding was recapitulated in a distal-nephron specific *Per1* KO mouse. Building

on these findings, the Gumz group generated a kidney-specific *Per1* KO mouse and found that they demonstrate normal diurnal excretion of sodium in response to DOCA-salt concurrent with increased serum aldosterone and altered adrenal gene expression (153). Together, these studies provide evidence that Per1 is a mediator of circadian BP rhythms via modulation of renal sodium homeostasis.

To date, clinical and epidemiological studies have focused on the effects of high-fat diets on the circadian rhythms of metabolism and gut microbiota as opposed to organ-specific effects. With the increasing proportion of processed foods in the Western diet, future studies may focus on the effects of nutrients such as sugar and phosphates on circadian gene expression in both laboratory animals and clinical trials.

CIRCADIAN DISRUPTION AND KIDNEY DYSFUNCTION

Ramsey and colleagues recently found that long-term environmental circadian disruption intermittently dampened diurnal BP rhythms in stroke-prone spontaneously hypertensive rats (194). These findings expand on the relationship between sympathetic nerve activity and aspects of cardiorenal health and demonstrate the importance of considering time of day in experiments investigating aspects of CVD.

Circadian disruption is a more general term that encompasses aspects of circadian misalignment, desynchrony, social jetlag, and chronodisruption (312). Circadian disruption can occur within or between organizational levels, ranging from desynchrony between peripheral tissue clocks to misalignment between behavior and environment.

This section will focus on evidence from clinical studies and meta-analyses that demonstrate the implication of various types of circadian disruption on risk factors for cardiovascular disease.

Shift Work

In humans, the most common form of circadian disruption results from non-daytime shift work, defined as work outside of normal daytime hours (7am-6pm) (313). Electrical lighting followed by vehicles and mechanization of agriculture all led to globalization in the 21st century, characterized by faster transport and new technology such as telecommunication. Although these technologies resulted in booming progress in various work sectors, they have also contributed to sweeping changes to human routines and circadian rhythms. Artificial indoor lighting allowed for nighttime work and relocation of large companies to different time zones. This relatively modern technology has facilitated the 'round-the-clock' work schedule as companies have to synchronize tasks with the schedule of the other countries that they operate in (314–316). The function of the cardiovascular system is subject to circadian regulation – circadian rhythms have been reported in blood pressure, heart rate, and renal function (7, 317, 318). There is ample evidence demonstrating that disturbances in circadian rhythms are a risk factor for cardiovascular disease. While it is clear that circadian disruption caused by changes in work shifts has incredible effects on the cardiovascular system, few studies have addressed whether tissue specific or systemic circadian rhythms contribute more to the pathophysiology of CVD. Experimental models of circadian disruption via genetic deletion

have provided evidence of a relationship between disruption of circadian rhythms and the cardiovascular system. Mice with a mutated Clock gene demonstrate attenuated CKD-induced fibrosis and cardiac inflammation (319). Loss of proximal tubule Bmal1 results in reduced renal injury and tubulointerstitial fibrosis in a mouse model compared to their wild-type littermates (320). This was attributed to disrupted cellular metabolic homeostasis, particularly through loss of activation of cystathionine β-synthase, a key enzyme for glutathione biosynthesis. The increased fibrogenesis was rescued by Glutathione supplementation. Thus, Bmal1 may exacerbate renal fibrosis in the proximal tubule by inhibiting CBS expression.

A meta-analysis of over 170,000 participants found that CVD event risk increases by 17% among shift workers; after the first five years of shift work, CVD event risk increases by about 7% per additional five years of shift work (11). Circadian misalignment can also augment inflammation markers (321). In a recent study, male and female factory night shift workers were found to have significantly higher mean total leukocytes, neutrophils, monocytes, and lymphocytes (322) The number of years of shift work was associated with increased carotid intima media thickness and pulse wave velocity was significantly increased in shift workers over the course of a 3-year follow up (68, 323). Frank Scheer's group began to delve into potential mediators of the relationship between CVD and shift work in their studies on C-reactive protein (CRP). In a small cohort of healthy male and female shift workers, an acute imposition of circadian misalignment significantly increased CRP, systolic BP, and diastolic BP, suggesting that CRP may mediate the increased inflammation observed in chronic shift workers (70, 73). Following this finding, Nikpour et

al. assayed CRP in reproductive age female shift workers and found that shift workers had significantly greater CRP than daytime workers (74). These findings implicate shift work as a predictor for high CRP and thus a predictor of cardiovascular disease.

Numerous factors unique to shift work may contribute to an increased CVD risk, but it is becoming increasingly evident that the circadian disruption inherent in non-daytime shift work plays an overwhelming role in exacerbating disease risk (324). In one study involving female hospital workers working either daytime or non-daytime shift work schedules, non-daytime shift workers reported poorer sleep duration, daytime dysfunction, and sleep quality (75). Additional studies have demonstrated “complete adjustment” in shift workers, defined by Simon Folkard as low melatonin levels during the work period and peak melatonin occurring 2-3 hours after the onset of the daytime sleep period. Still other workers experience “substantial adjustment”, defined as meeting one of the two requirements for complete adjustment (325). In one study of day versus night workers, nighttime workers demonstrated 15% lower salivary melatonin concentrations compared to day workers on work days, with a 6% suppression in melatonin during the night under artificial light exposure. This suggests that artificial light is sufficient to elicit some change in melatonin production, contributing in part to the circadian disruption common for many non-daytime shift workers (326).

As circadian misalignment requires assessment of circadian phase by core body temperature or additional biomarkers over a period of days, it is difficult to clinically diagnose shift workers with a circadian misalignment disorder. Instead, many studies opt

to utilize subjective surveys of sleep deprivation or function, such as the Karolinska Sleepiness Scale (KSS), the Epworth Sleepiness Scale, or the Psychomotor Vigilance Test (PVT) (327, 328) In a study of 52 intensive care workers on rotating shifts, workers reported increased sleepiness during night shifts compared to day or evening shifts as measured by the KSS and PVT (327). Similar findings were reported in a 2022 study of law enforcement officers working up to seven consecutive night shifts; officers reported increased sleepiness during night shift days but no difference in sleepiness levels between consecutive night shifts (329). These results differ greatly from another study in truck drivers using the KSS, who reported no significant differences in subjective sleepiness between morning, day, and night shift workers (330).

Dietary Factors

BP has a well-established circadian rhythm in both humans and mice. Generally, BP dips during the resting period and increases sharply at the start of the active period (331, 332). Healthy individuals exhibit a 10-20% nocturnal “dip” in BP. People who do not experience this dip are categorized as “non-dippers”. Nocturnal dipping status has been associated with a more rapid decline in kidney function (333–336). Ohkubo et al. reported that for every 5% loss in nocturnal BP dip, there is a 20% increased risk for CVD (14). Another study found that a reduced dipping status and increased BP variability during the day contribute to hypertensive complications (337). Many hypotheses have been posited to explain the circadian rhythmicity of blood pressure. In healthy individuals, sodium excretion reaches its peak during the day and is at a minimum at night. As hypertensive

patients often do not have rhythmic blood pressure, one hypothesis is that nocturnal hypertension may be due to an inability to excrete sodium during the day. Bankir et al. reported that daytime sodium excretion is a determinant of nocturnal BP and dipping phenotype in individuals of African descent (212). Based on the Guytonian concept of pressure natriuresis, it has long been considered that blood pressure determines sodium excretion (338). Thus, the ability of the kidney to eliminate sodium can directly affect blood pressure rhythms, with some species differences.

It is well established that a high salt diet alone may be sufficient to disrupt BP circadian rhythms. Circadian MAP is disrupted in Wistar-Kyoto rats fed high salt; rats exhibit both disrupted MAP rhythms and increased plasma sodium (339). Dahl-Salt sensitive rats fed a high salt diet demonstrate alterations in circadian blood pressure rhythms as well as changes in peak clock gene expression within the kidney (174, 340). Interestingly, there is a significant difference in the MAP of Dahl-S rats on a high vs. low salt diet, further implicating dietary sodium and salt sensitivity as moderators of BP rhythms throughout the day (340). Dahl-S rats fed a high salt diet develop a non-dipper pattern concurrent with proteinuria (341). This suggests that impaired renal function may underlie the non-dipper BP pattern in salt sensitive hypertension. A similar relationship exists in clinical models, as patients with salt-sensitive hypertension are more frequently non-dippers (342, 343). This is attributed to a compensatory pressure-natriuretic mechanism in which patients with increased sodium retention experience increased BP during the night to facilitate excess sodium excretion. In non-dipping patients with

nocturnal hypertension, administration of thiazide diuretics has been shown to improve nocturnal BP patterns and restore diurnal BP rhythms (344, 345).

Feeding is normally coupled to increased locomotor activity. Under normal caloric intake, mice and rats have increased activity and food intake during the dark (active) period. When offered a high caloric diet, there is a resultant decrease in the daytime to nighttime ratio of activity (346–348). Diets high in fat contribute to the onset of obesity, which increases the risk for elevated blood pressure and renal injury. Carroll et al. reported that rabbits fed a high fat diet for 12 weeks demonstrated a loss of diurnal HR and BP rhythms by day 2 of high fat feeding (349). In the Han:SPRD-cy rat model of polycystic kidney disease, 6 weeks of high fat feeding resulted in increased kidney mass, increased fibrosis, and lower creatinine clearance (350). Sanchez-Navarro et al. reported that after 14 weeks, mice fed a moderately high fat diet (45%kCal fat) became obese and demonstrated increased excretion of urinary biomarkers of kidney damage (351). Interestingly, a high fat diet protected against glomerular hypertrophy and albuminuria in hypertensive diabetic mice (352).

Proposed mechanisms contributing to obesity-related hypertension entail crosstalk between renal, metabolic, and neurohumoral pathways. They include overactivation of the sympathetic nervous system (SNS), RAAS stimulation, and increased inflammation resulting in changes in renal function. Some of the physical manifestations of SNS hyperactivation are increased heart rate and renal tubular sodium reabsorption. Direct activation of the SNS has been investigated; muscle sympathetic nerve activity

(MSNA) increases with weight gain and concurrent obesity and hypertension appear to have an additive effect (353, 354). Norepinephrine spillover has also been reported in the kidneys of obese individuals and may contribute to hypertension onset (355, 356). There is a reciprocal relationship between the SNS and the RAAS: SNS overactivity stimulates RAAS activation through increased renin release from the juxtaglomerular apparatus and RAAS activation increases sympathetic tone (357). Increased plasma concentrations of RAAS components have been reported in obese individuals (358–361). Additionally, adipocytes have their own local RAAS and produce angiotensinogen and angiotensin II (362, 363). High fat feeding has been associated with increased oxidative stress, which is a contributor to fibrosis. In a randomized, controlled trial, increased energy expenditure reduced glomerular hyperfiltration and albuminuria in patients with the metabolic syndrome and non-alcoholic fatty liver disease (364). Few clinical trials and meta-analyses have evaluated the effects of oxidative stress induced by diet-induced obesity. In laboratory models, 16 weeks of high fat diet increased renal triglyceride and cholesterol content along with increased oxidative stress and dysfunctional mitochondrial dynamics in tubular cells. Depletion of reactive oxygen species attenuated these effects, suggesting that long term high fat feeding causes kidney injury via increased oxidative stress and aberrant mitochondrial and lipid metabolism (365). Prem and Kurian reported that 16 weeks of high fat diet results in diminished renal function, increased mitochondrial stress, and histological evidence of tubular necrosis and casts in rats with ischemic reperfusion injury (366). Mitochondrial structure is significantly disrupted in glomerular endothelial cells, podocytes, and tubular epithelial cells after 28 weeks of high fat feeding in C57BL/6J

mice. Studies in rodent models suggest that antioxidants may be a treatment option for oxidative stress induced by HFD. Treatment with a mitochondrial-targeting antioxidant restored normal mitochondrial structure, improved glomerular histology, and prevented apoptosis (367). Albrahim et al. recently reported that lycopene protected against increased renal levels of inflammatory markers in rats fed a high fat diet (368).

Vision Impairment

Environmental light serves as the strongest zeitgeber for the circadian system and is integral to keeping biological and physiological rhythms synchronized (369). Light is taken in through the retina and transmitted directly to the SCN via the retinohypothalamic tract. Vision impairment (VI) results in a loss of light perception, contributing to continuous circadian disruption through a failure of light to reach the SCN. Individuals with VI often experience recurring episodes of poor sleep and dysfunctional daytime activity, suggestive of some degree of circadian disruption. Very few studies using experimental models have investigated the relationship between VI and CVD. Mathew et al. reported that C57BL/6J mice on a truncated light/dark cycle (10h light: 10h dark) demonstrated aspects of visual impairment, including a decrease in the overall number of photoreceptors (370). Adults with VI have an increased prevalence of cardiovascular disease as light does not reach the SCN, ultimately preventing entrainment of peripheral clocks. A 2021 study of nearly 1500 middle-aged and elderly adults with VI found that poorer vision positively correlated with coronary heart disease (371). Similar results were reported in a cross-sectional study of nearly 23,000 adults, particularly when paired with

additional poor habits such as smoking and physical inactivity (372). Yet another variable to be considered in CVD risk is age. The leading cause of vision loss in elderly patients is age-related macular degeneration (AMD) (373). Epidemiological findings from as early as the 1970s suggest a cardiovascular risk profile among patients with AMD. The Framingham Eye Study was one of the first to posit that AMD may be indicative of a systemic vascular process influencing CVD development (374). Data from the first National Health and Nutrition Examination Study (NHANES-I) demonstrated a weak association between AMD and vascular diseases (375). An additional case-control study reported a significant positive association between AMD and heart failure in Taiwanese patients (376). A recent cross-sectional study of 200 patients with age-related macular degeneration found an increased risk for vascular disease (377). In a longitudinal nationwide cohort study of over 3,000,000 subjects, AMD with VI was associated with an increased risk of CVD and stroke (378). There is consistent and abundant epidemiologic evidence connecting AMD and CVD. However, future studies investigating the etiology of AMD will require more extensive use of experimental models.

Mental Health

Poor sleep can exacerbate neuropsychiatric disorders, and neuropsychiatric disorders can result in poor sleep. Until recently, there was very little information regarding the relationship between CVD risk and sleep quality. Disruptions in sleep phase have been strongly associated with neuropsychiatric disorders (379–381). Depression has

recently been identified as a nontraditional risk factor for CVD. However, the role of anxiety in CVD risk is not well identified.

Decades ago, it was reported that patients with CVD have a three-fold higher incidence of depression than the general population (382–384). The American Heart Association reported that depression can exacerbate atherosclerosis as well as promote the onset of hypertension and diabetes (385). One potential reason for this is that depression increases the risk of unhealthy lifestyle habits such as smoking, low-quality nutrition, decreased exercise, and poor medication compliance (385). Each of these habits have previously been shown to increase the risk for CVD. While some studies have failed to find an association between depression and CVD outcomes after accounting for confounding factors, more recent studies have found that depression is indeed an independent risk factor for CVD risk and mortality (386–389). A cross-sectional study of nearly 600,000 adults found that poor mental health is associated with premature onset of CVD and poorer cardiovascular health among young adults (390). According to a meta-analysis encompassing nearly 900,000 individuals, those with depression demonstrate a significantly increased risk for coronary heart disease than nondepressed individuals (386). Additionally, patients with depressive symptoms that have cardiac events are more likely to have recurrent events and mortality (391). A meta-analysis of over 25-years of data showed that depression following a heart attack is associated with an increased risk of poorer outcomes within the 2 years after the cardiac event (392).

Recent findings suggest that anxiety symptoms are common in patients with CVD, particularly after a cardiovascular event (393–395). At least one recent study has posited that anxiety may precede hypertension in some cases; general anxiety may lead to hypertensive disorders in pregnant women (396, 397). Anxiety may contribute to morbidity and mortality in CVD patients, an association that is common among a variety of anxiety disorders. A 2021 meta-analysis including over 4,000,000 individuals from 59 studies found a significant association between anxiety and hypertension (393). Stewart et al. reported that chronic moderate-to-severe anxiety was positively associated with a 2-to-4-fold increased risk of CVD in patients with existing coronary heart disease (398). Interestingly, low level anxiety was not associated with an increased CVD morbidity or mortality risk. Anxiety symptoms may have a dose-dependent risk relationship with CVD. In a meta-analysis of 46 cohort studies, Emdin et al. found that anxiety is associated with a significantly increased risk of CVD (399). It is important to note that many studies investigating the relationship between neuropsychiatric disorders and CVD risk vary widely in methodology; thus, the CVD risk associated with anxiety may not be as great as the association with depression. Future work regarding mental health and CVD will need to clarify the contributions of depression and anxiety disorders independently and in conjunction with one another.

Sleep Disorders

Obstructive sleep apnea (OSA) is characterized by repeated pharyngeal collapses during the sleep period, resulting in acute hypoxia and poor sleep quality (400). Individuals with OSA often experience sleep fragmentation, or repeated interruptions of sleep, due to decreased or halted breathing. In a Dutch population-based study of 21,000 healthy men and women, Hoevenaar-Blom et al. found that short sleep and poor sleep quality increase CVD incidence (401). These same findings have been recapitulated in many population-based and cross-sectional studies (402, 403). As OSA is characterized by symptoms resulting in poor sleep quality and decreased sleep duration, there is a logical relationship between OSA and CVD.

OSA is an independent risk factor for CVD, associated with obesity, hypertension, stroke, and heart failure, among other comorbidities. Preclinical studies have demonstrated that intermittent hypoxia is a major contributing mechanism to cardiovascular OSA-associated risks. Experimental models have proved useful in clarifying the vascular and cardiac pathophysiology of OSA. Mice exposed to intermittent hypoxia exhibit cardiac hypertrophy, collagen accumulation, surges in blood pressure, increased aortic wall thickness, and impaired endothelium-dependent relaxation (404–406). The endothelin system has been identified as a major role player in intermittent hypoxia induced hypertension. Compared to control rats, SHR rats exposed to chronic intermittent hypoxia had exacerbated hypertension development and increased ET-1 and ET_A expression (407). Treatment with an ET receptor antagonist ameliorated the BP increase,

indicating that the ET system activation contributes to the cardiovascular phenotype that arises in part as a result of chronic intermittent hypoxia. Patients with OSA have higher plasma ET-1 and higher BP than healthy controls; nocturnal ET-1 and BP demonstrated a linear relationship with OSA severity (408). OSA has been significantly associated with heart failure (409). Findings from the Sleep Heart Health Study showed an increased risk of heart failure and stroke among patients with OSA. Additionally, patients with concurrent OSA and heart failure have more severe deterioration than those without OSA and additionally may be at an increased risk of CVD related mortality (410–414).

The link between OSA and CVD has been demonstrated through clinical trials assessing the utility of continuous positive airway pressure (CPAP). Early studies showed promising results, with CPAP treatments reducing nocturnal BP and restoring diurnal BP rhythms (415, 416). Excitingly, similar BP results were reported by Picard et al. in 2021 (417, 418). More recent trials have reported improved diastolic relaxation in patients with good CPAP adherence (419). Hypoxia is a key factor in the pathophysiology of CKD; thus, the kidney is a target organ of OSA. Increased serum and urinary biomarkers of kidney injury have been reported in patients with OSA, although some studies have reported no difference in urinary levels or some biomarkers (420, 421). Interestingly, components of the RAAS signaling pathway were also observed to be increased in the urine of patients with severe OSA (422). These patients also exhibited blunted renal RAAS activity when challenged with Ang II.

Insomnia is the most common sleep disorder in the United States. It is defined as a perceived difficulty initiating or maintaining sleep by the American Academy of Sleep Medicine (423) awake during the day. Insomnia has been associated with a 45% greater risk of CVD (424). Though the pathogenesis of the relationship between insomnia and CVD is not well defined, SNS overactivation has been suggested as a key contributor. To investigate the relationship between sleep deprivation, SNS activity, and CVD risk factors, Carter et al. utilized the 24-hour total sleep deprivation model. In healthy adults, Carter et al. reported a short-term hypertensive response to total sleep deprivation in both sexes (425). To date, very few studies have directly investigated sympathetic activity via recordings of muscle sympathetic nerve activity (MSNA) in patients with insomnia. In the aforementioned study, MSNA was significantly decreased in males but weakly increased in females (425). Interestingly, resting MRNA did not differ between participants with insomnia and controls in a study that was not adequately powered to assess sex differences (426). In elderly participants, 24-hour total sleep deprivation increased blood pressure and MSNA in postmenopausal women only (427). This pairs well with epidemiological data reporting that hypertension risk is higher in women with sleep deprivation (423, 428, 429). Using a data set consisting of more than 700,000 adults, Grandner et al. recently reported that the association between short sleep duration and hypertension was stronger in women than men (423). There is a need for further experiments to better understand this sex-specific association, especially in postmenopausal women who have a well-documented increased prevalence of CVD comorbidities.

CLINICAL IMPLICATIONS

It is well reported that perturbations in circadian rhythms can affect physiological functions, with increasing data demonstrating that this can be particularly harmful to patients with kidney disease. Circadian disruption can occur on a variety of organizational levels and can involve several organ systems, thus contributing to the prevalence and severity of multiorgan diseases such as cardiovascular disease. By using both basic and clinical/translational research, we are moving toward understanding the physiological mechanisms that link timing of food intake and cardiovascular pathologies. Understanding how sex differences and multi-organ responses affect renal pathophysiology will prove invaluable as the field moves forward. Additionally, future therapies for circadian disruption may involve non-pharmacological treatment options such as chronotherapy, tightly controlled time-restricted feeding, or temporary cessation of activities contributing to circadian disruption.

One topic in the field that has recently gained traction is the mechanisms underlying sex-differences in response to kidney injury onset and severity, particularly in the context of circadian disruption. Studies to address this will likely need to be both longitudinal and somewhat invasive, thus making human subject recruitment difficult. Laboratory animals can be of use in investigating the involvement of sex hormones; the Gohar group has produced data suggesting an emerging relationship between intrarenal ET-1 and GPER. Work from our group as well as the Gumz and Layton labs have demonstrated that there are sex differences in transporter expression that may drive

dimorphisms in response to drugs and dietary challenges. Future work regarding sex differences should consider integrating computational modeling or 3D imaging, as both have proved useful tools that may be applicable to clinical experiments. Moving forward, individuals receiving gender affirming treatment will also need to be considered. In the future, studies may focus on sex differences in biomarker expression, drug therapy efficacy, and how transgender individuals may be affected by hormone therapies or changes in hormonal status. Additionally, studies should take time of day into account, as it appears that there is a multifaceted relationship between sex, the circadian clock, and core renal functions.

Chronotherapy of drug administration (chronopharmacology) may be effective in altering blood pressure control, but results from current literature are inconsistent. At least one meta-analysis of literature found that chronotherapy results in significant decreases in nocturnal systolic and diastolic blood pressure compared to morning dosing regimen drug therapy (430). In key studies, Hermida et al. found that anti-hypertensive drugs were more effective at controlling blood pressure when administered at night [196,197]. These findings have been criticized for weaknesses in experimental design and have not been replicated. The explanation for these differences is not clear but could depend on the medication and dose taken or the severity of the patient's disease. Findings can often be inconsistent between clinical trials on chronotherapy and this treatment option may be better suited to improve personalized medicine. Very few studies examining the timing of medication intake have been randomized trials. Future studies may consider a randomized, blinded, controlled study design to minimize design flaws and

validate findings with greater confidence. The mechanisms underlying chronopharmacology may be also related to the metabolism of the given drug. Chronopharmacology is still a very new and vastly unexplored field, but researchers will likely benefit from validating that existing medications are impacting their functional targets.

Though it gained popularity for its potential to decrease body weight, TRF may prove useful as a treatment option for lifestyles and pathologies lending to circadian disruption. In prediabetic men, restricting food intake to the first 6 hours of the day after waking is sufficient to increase insulin sensitivity and decrease blood pressure (2). In male mice, inverting the availability of food also inverts blood pressure rhythms (7). It appears that peripheral zeitgebers such as food intake do indeed have the ability to modulate blood pressure. In populations such as shift workers that have a well-documented increased risk for CVD, TRF may be a non-pharmacological treatment option to consider. While recent studies have begun to investigate the efficacy of TRF for non-daytime shift workers, many have lacked longevity. One remaining question regarding TRF is whether it is feasible long-term and whether its reported benefits are sustained over months or even years of adherence. While investigating sex-differences in response to TRF schemes is easier to accomplish in experimental models, studies in humans have often lacked sex-diversity, which may limit application of some findings to broader populations.

As there is still much to be known about circadian physiology and how that integrates with renal and cardio physiology, several key questions remain unanswered. (1)

Do particular chronotypes predispose one for the development of CVD? What about unstable sleep phasing such as that occurring in rotating shift workers? (2) What is the involvement of other peripheral clocks or behaviors (i.e., exercise, temperature) on cardiovascular organs? (3) Can non-pharmacological approaches such as time restricted feeding mitigate long-term cardiovascular and cardiorenal effects of circadian misalignment? The current evidence for the utility of TRF is promising but still somewhat ambiguous. Studies using human participants often rely on self-reported outcomes of variables like adherence and sleep, which are subject to bias or false-reporting. This may be difficult to address, as more invasive measures of ensuring adherence to a feeding schedule may negatively impact participation in some studies. There are still additional factors of study design to consider, such as applicability of TRF to different non-daytime shift schedules, rotating shifts, and different kinds of diets, among other variables. More relevant to the field of renal physiology will be experimental and clinical trials to define the efficacy of TRF in varying populations and disease states. With the quickly expanding and extremely integrative field of circadian physiology, it may be reasonable to expect that equally integrative treatment options that include a combination of pharmacological and non-pharmacological approaches will need to be taken.

CHAPTER 3

MATERIALS AND METHODS

Animals

All animal studies were conducted with the approval of the University of Alabama at Birmingham (UAB) Institutional Animal Care and Use Committee in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Male and Female C57BL/6J mice aged 6-8 weeks were purchased from The Jackson Laboratory in Bar Harbor, Maine (stock no. 000664) and used for all studies. Animals were 7-9 weeks old when the food availability challenge began. All mice were given a normal salt diet (0.4% NaCl) in standard chow (NIH-31 Open Formula Mouse/Rat Sterilizable Diet, #7017, Inovtiv, West Lafayette, IN) for the entirety of the study. Temperature and humidity were monitored and kept constant, and animals were housed under a 12h light/12h dark cycle.

Radiotelemetry

All blood pressure and heart rate measurements were conducted using model PA-C10 arterial pressure transmitters (*Data Sciences International*, St. Paul, MN). Transmitters were inserted into the right carotid artery of mice as described previously (431, 432). All animals were given at least 7 days to recover from surgery prior to beginning any recordings. Data were recorded at 2000 samples/second in 2-minute bins every 10

minutes. Telemetry recordings were taken for three consecutive days after 4 and 8 weeks of altered food availability. Telemeters were implanted during weeks 1-3 of controlled feeding based on core surgeon availability.

Controlled Food Availability and Metabolic Cages

A schematic depicting the food availability strategy is available in Figure 4. In brief, inactive period time-restricted feeding (iTRF, 12h food availability starting at ZT0 (7 a.m.) for the entirety of the lights on period), active period time-restricted feeding (aTRF, 12h food availability starting at ZT12 (7 p.m.) for the entirety of the lights off period), and *ad libitum* (ad lib, food availability 24 hours a day) fed C57BL/6J male and female mice were placed into custom automated feeder wheels for the entirety of the altered food availability period. Animals were given one week to acclimate to the feeder wheels prior to the ten-week chronic controlled feeding intervention. The feeder wheels were monitored daily, food was changed weekly, and any problems such as feeder wheel motor errors were detected and rectified quickly.

At the end of the eight-week monitoring period and telemetry recordings, animals were placed into metabolic cages for urine collection (Tecniplast™, *Fisher Scientific*, Waltham, MA). A normal salt gel diet containing agar was used throughout the metabolic cage study to prevent pellet chow from falling into the urine collection tubes (Micro stabilized Rodent Liquid Diet, *TestDiet*, Richmond, IN) (433). Maintaining their controlled feeding schedules, mice were given 2-3 days to acclimate to the metabolic cages and gel diet prior to urine collection for analysis. During collections, urine output, food intake, and

water intake were measured at 7 a.m. and 7 p.m. each day in correspondence with the start of the lights on and lights off periods, respectively.

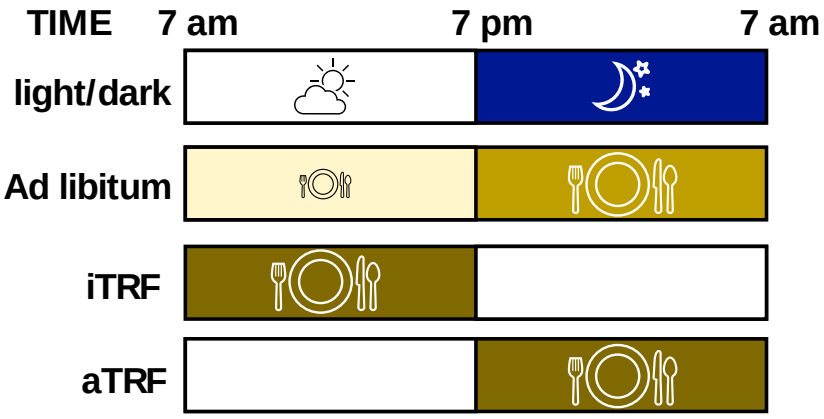


Figure 4. Schematic of food availability over the 12:12 light/dark cycle. aTRF, active/dark period time restricted feeding; iTRF, inactive/light period time restricted feeding

Body Composition

Mice were weighed (MS802S, *Mettler Toledo*, Columbus, OH) weekly to monitor growth and health. During the last week of altered feeding prior to the metabolic cage studies, animals were sent to the UAB Small Animal Phenotyping Core for body composition measurements via quantitative magnetic resonance imaging (QMR). QMR was done using an EchoMRI 3-in-1 Analyzer (*Echo Medical Systems*, Houston, Tx) to provide body weight, lean mass, fat mass, and total water mass for each animal (434).

Aortic Pulse Wave Velocity (PWV)

Aortic PWV was measured by the UAB Comprehensive Cardiovascular Center Mouse Phenotyping Core with a Vevo 3100 ultrasound Doppler probe (435). Anesthesia with isoflurane was maintained in mice while in a supine position on a temperature-controlled platform (37°C). ECG measurements were obtained at the proximal and distal aorta. Aortic PWV was calculated by dividing the distance by the time difference between pulse arrivals as determined by ECG R-peaks.

Urine and Serum Measurements

At the end of the study, terminal blood was collected in 5mL microcentrifuge tubes from experimental mice via cardiac puncture, allowed to clot at room temperature for 1h, and centrifuged for 15min at 3000 rpm (1500 x g) at 4°C. The resulting serum was then aliquoted, snap frozen in liquid nitrogen, and stored at -80°C until analyzed. Serum creatinine measurements were conducted by the UAB O'Brien Center for AKI Research Bioanalytical Core using ion exchange-high-performance liquid chromatography (IEX-HPLC) (436).

Urinary endothelin-1 (ET-1) levels were evaluated by enzyme-linked immunosorbent assay (ELISA) (QuantiGlo ET-1 Kit, Cat no. QET00B, *R&D Systems*, Minneapolis, MN). Aldosterone levels were evaluated in urine by time-resolved fluorescence (7). The reagents and antibodies for the aldosterone ELISA were developed and generously provided by Drs. Elise and Celso Gomez-Sanchez at University of Mississippi Medical Center (437). Briefly, a 96-well plate was coated with mouse

immunoglobulin G (IgG) overnight at 4°C before briefly incubating with goat-anti-mouse IgG. After washing, Aldo A2E11 Integra antibody (1:75000) was added to each well along with standards, samples, and aldo-3CMO-biotin. After incubation and washing, avidin horseradish peroxidase was added to the plate. Chromogenic substrate tetramethylbenzidine was added and the plate was incubated in the dark at room temperature before reading.

Urinary potassium and sodium concentrations were measured via atomic absorption spectrophotometry (iCE™ 3300 AAS, Cat no. 942350033312, *Thermo Scientific*™, Waltham, MA) (438). Urine samples were diluted 1:10000 in atomic absorption diluent (40g lanthanum chloride, 200mL nitric acid in 20L of deionized water). All dilutions were performed in triplicate, and each measurement consisted of the average of three consecutive readings per dilution.

Histological Assessments

Kidneys were fixed overnight in 4% buffered formalin solution at room temperature, transferred to 70% ethanol, and then embedded in paraffin. Tissues were cut along the longitudinal axis into 4µm thick sections and mounted on slides. Tissues were stained with hematoxylin and eosin (H&E) or picrosirius red (PSR) using standard protocols. Tissues stained with PSR were imaged via brightfield microscopy and polarized light (Olympus BX40, *Olympus America*, Center Valley, PA).

Glomerular health and morphology were assessed by analyzing H&E-stained slides and quantifying glomerular area using cellSens imaging software (Olympus Life Sciences,

Olympus America, Center Valley, PA). Under 20x magnification, twenty fields per kidney were examined by a pathologist blinded to experimental groups. Total glomerular area was determined from the outline of the Bowman's capsule. Maximum, minimum, and mean glomerular area according to the number of glomeruli were calculated automatically by the cellSens software. Glomerular area data were reported as the average glomerular area per experimental group.

To determine fibrosis, ten photographs per renal cortex and outer medulla were obtained at 40x magnification with a digital camera (Olympus DP12, *Olympus America*, Center Valley, PA) and examined by a pathologist blinded to experimental groups. Each PSR stained brightfield and polarized image was color thresholded for the presence of fibrosis and quantified using MetaMorph software (*Molecular Devices LLC*, San Jose, CA). Fibrosis data were reported as average percent area covered by PSR staining per experimental group.

For immunohistochemistry, sections were stained with CD3 using anti-CD3 antibody (ab16669, 1:600, Abcam, UK) or anti-F4/80 antibody (MCA497GA, 1:200, BioRad, Hercules, CA). Cortical and outer medullary $CD3^+$ (T cells) and $F4/80^+$ (macrophages) cells were blindly quantified in twenty high-powered microscopic fields (200 µm x 200µm, 40x magnification). Positively stained cells were manually counted by a pathologist blinded to experimental groups. Results are reported as the average cell count per twenty high-powered microscopic fields for all animals in each experimental group.

Statistical Analysis

All results are reported as means ± standard error of the mean (SEM). Specific statistical tests are listed in the legends for each figure. Two-way analysis of variance (ANOVA) was used when analyzing time of day, feeding, and time of day x feeding interaction effects. Generally, two-way ANOVA and post-hoc analysis with Sidak's test for multiple comparisons were utilized to analyze differences between experimental groups when looking at day/night differences. Sidak's test for multiple comparisons allowed for simultaneous pairwise comparisons between groups for all possible combinations of means.

One-way ANOVA was used when solely looking at the effect of feeding. When looking at experimental groups irrespective of time, one-way ANOVA and post-hoc analysis with Tukey's test for multiple comparisons were used. Tukey's test for multiple comparisons was used to assess the significance of differences in experimental groups between pairs of means. All data were tested for normality. Results were considered statistically significant if $p<0.05$. All data were analyzed using GraphPad Prism 10.0.3. for Windows (GraphPad Software, Boston, MA).

CHAPTER 4

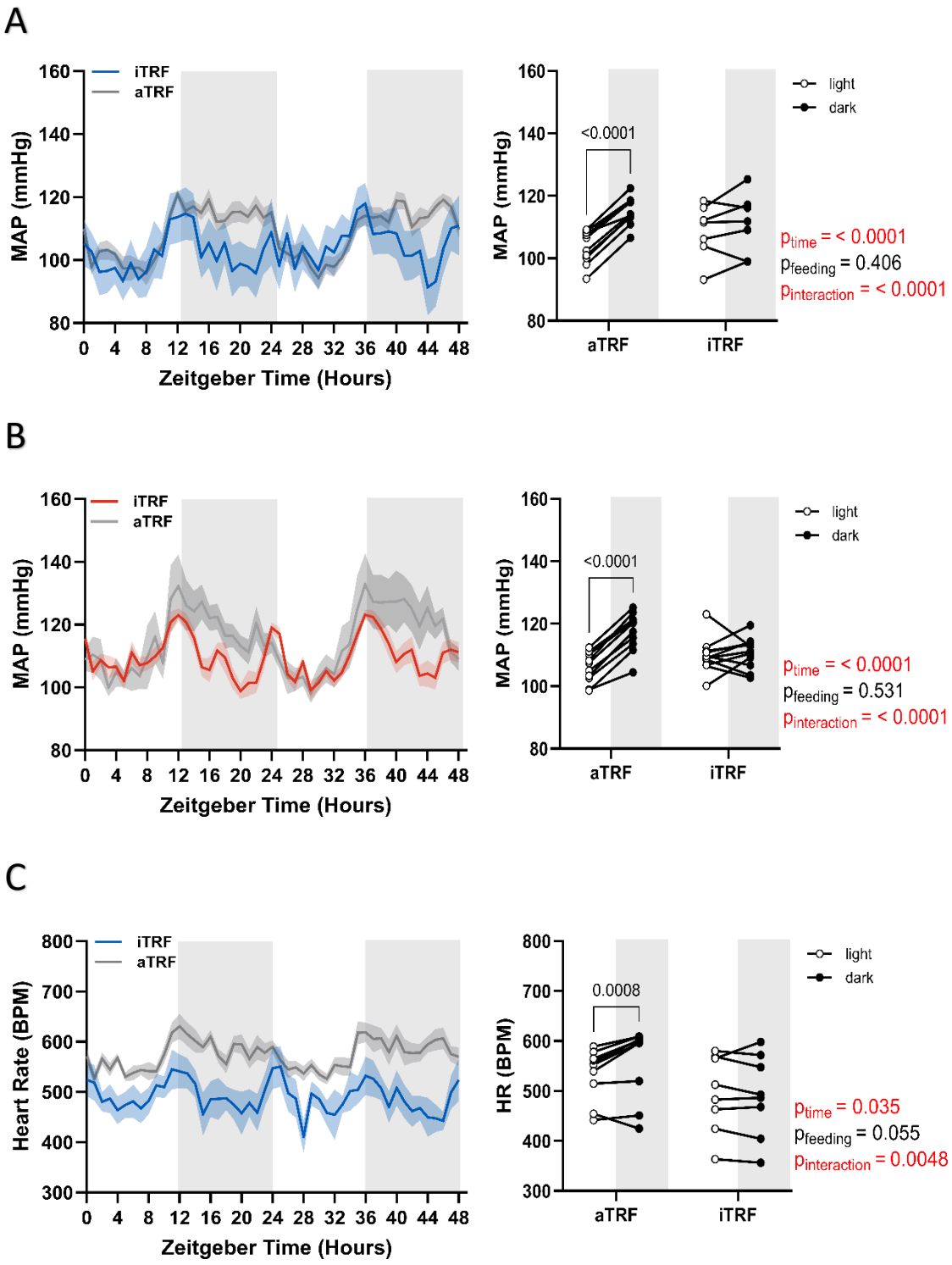
RESULTS

Aim 1: Test the hypothesis that chronic circadian disruption via mistimed feeding has long-term effects on diurnal blood pressure patterns.

Radiotelemetry analysis in mice with chronic circadian disruption via mistimed feeding

Previous work from our lab has demonstrated significant changes in rhythmicity of blood pressure of male wild-type mice in under one week of iTRF (7), and so we used telemetry to assess long-term changes in mean arterial pressure (MAP) control. After 4 weeks of iTRF, male and female mice exhibited a disrupted diurnal pattern in MAP and heart rate (HR) (Figure 5A-D). Interestingly, aTRF male and female mice maintained diurnal patterns in MAP and HR and were reminiscent of patterns seen in ad libitum fed mice (Figure 5A-D) (7, 147, 439, 440) . Diurnal patterns of systolic and diastolic blood pressure were also disrupted in iTRF animals but remained within a normal range, regardless of sex (Appendix Figure 15A-D). Interestingly, both male and female iTRF animals maintained

normal diurnal patterns of activity despite food availability restricted to their inactive period (Figure 5E,F).



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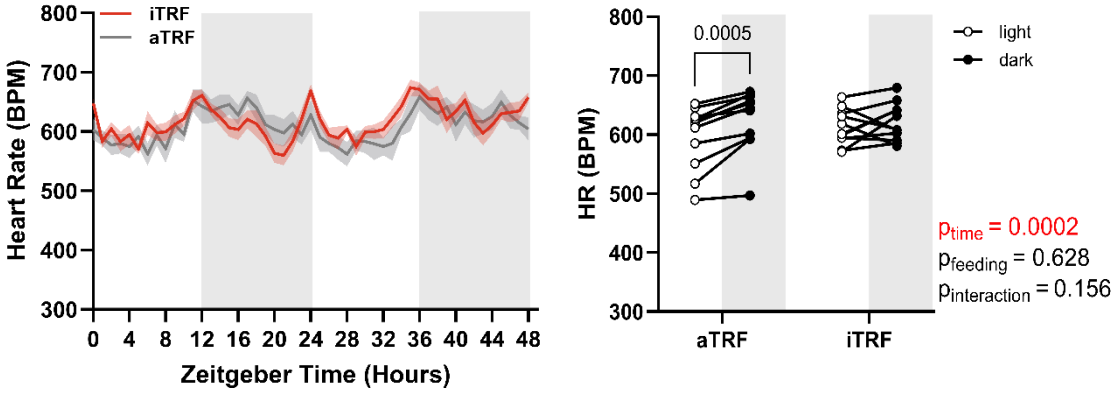
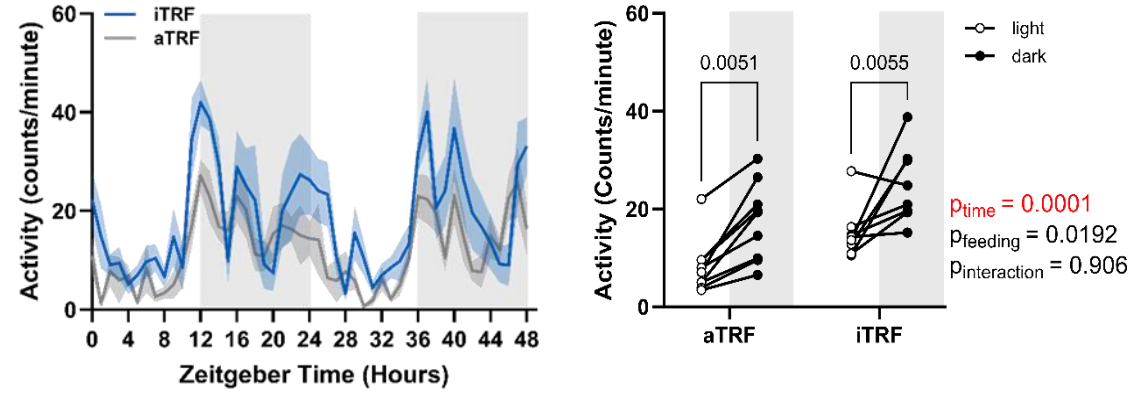

iTRF
aTRF
Heart Rate (BPM)
800
700
600
500
400
300
0 4 8 12 16 20 24 28 32 36 40 44 48
Zeitgeber Time (Hours)
0.0005
HR (BPM)
light
dark
aTRF
iTRF
p_{time} = 0.0002
$p_{feeding}$ = 0.628
$p_{interaction}$ = 0.156

E


iTRF
aTRF
Activity (counts/minute)
60
40
20
0
0 4 8 12 16 20 24 28 32 36 40 44 48
Zeitgeber Time (Hours)
0.0051
0.0055
Activity (Counts/minute)
light
dark
aTRF
iTRF
p_{time} = 0.0001
$p_{feeding}$ = 0.0192
$p_{interaction}$ = 0.906

F

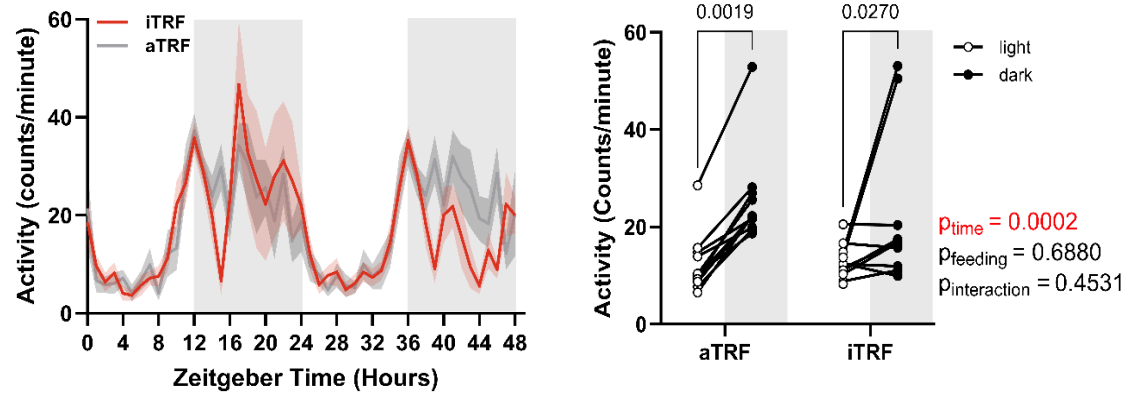
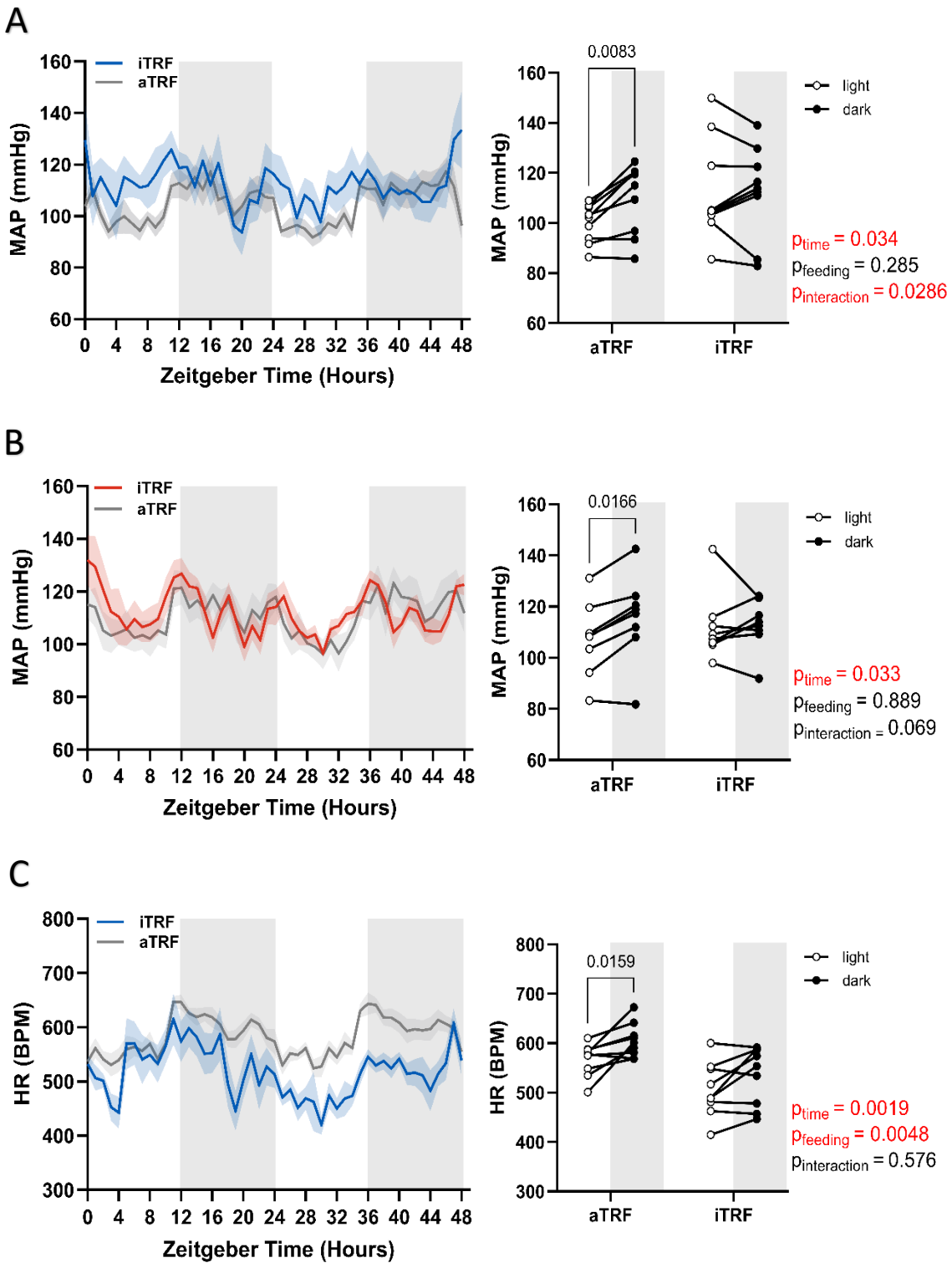
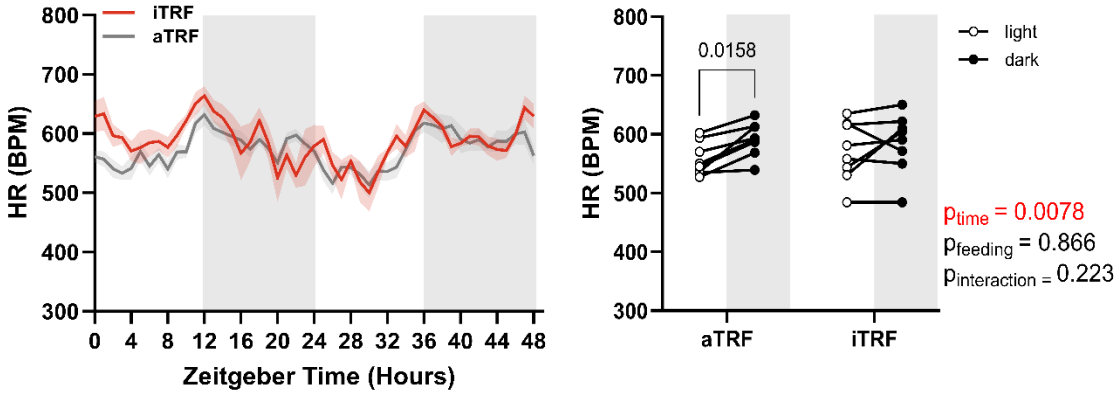

iTRF
aTRF
Activity (counts/minute)
60
40
20
0
0 4 8 12 16 20 24 28 32 36 40 44 48
Zeitgeber Time (Hours)
0.0019
0.0270
Activity (Counts/minute)
light
dark
aTRF
iTRF
p_{time} = 0.0002
$p_{feeding}$ = 0.6880
$p_{interaction}$ = 0.4531

Figure 5. iTRF disrupts diurnal patterns of MAP and HR rhythms after 4 weeks. MAP (A,B), HR (C,D), and activity (E,F) after 4 weeks of aTRF (gray), or iTRF in male (blue) and female (red) mice. Gray shaded boxes indicate dark periods. Data are shown as 48-h trace with shading representing error bars. Day/night averages of the days are also shown. Data are means ± SEM. Feeding schedule, time of day, and feeding schedule x time of day interaction effects were assessed by two-way ANOVA with Šídák's multiple comparisons test. Significant p values are indicated in red with individual comparisons noted in each panel. n = 7-11 mice/group. MAP, mean arterial pressure; HR, heart rate; BPM, beats per minute.

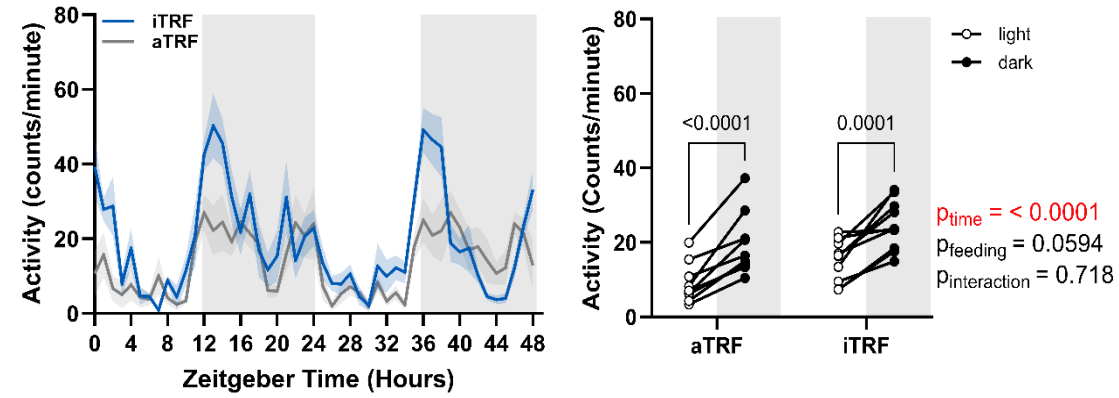
After 8 weeks, MAP and HR in iTRF male and female mice continued to demonstrate a disrupted diurnal pattern. (Figure 6A-D). iTRF appeared to lower HR in males especially during the second 24-hour day of recording, but this effect was not statistically significant (Figure 6C). Female iTRF mice continued to demonstrate disrupted diurnal patterns in HR although the overall beats per minute (BPM) were similar to the aTRF group (Figure 6D). As expected, aTRF mice maintained a diurnal rhythm of MAP and HR at 8 weeks (Figure 6A-D). Interestingly, activity rhythms maintained a diurnal pattern similar to aTRF animals in iTRF males and females although the 48-hour activity rhythms were vastly different between iTRF and aTRF mice (Figure 6E,F).


A
iTRF
aTRF
MAP (mmHg)
Zeitgeber Time (Hours)
0.0083
light
dark
aTRF
iTRF
ptime = 0.034
pfeeding = 0.285
pinteraction = 0.0286
B
iTRF
aTRF
MAP (mmHg)
Zeitgeber Time (Hours)
0.0166
light
dark
aTRF
iTRF
ptime = 0.033
pfeeding = 0.889
pinteraction = 0.069
C
iTRF
aTRF
HR (BPM)
Zeitgeber Time (Hours)
0.0159
light
dark
aTRF
iTRF
ptime = 0.0019
pfeeding = 0.0048
pinteraction = 0.576

D


iTRF
aTRF
HR (BPM)
Zeitgeber Time (Hours)
0.0158
light
dark
aTRF
iTRF
p_{time} = 0.0078
$p_{feeding}$ = 0.866
$p_{interaction}$ = 0.223

E


iTRF
aTRF
Activity (counts/minute)
Zeitgeber Time (Hours)
<0.0001
0.0001
light
dark
aTRF
iTRF
p_{time} = < 0.0001
$p_{feeding}$ = 0.0594
$p_{interaction}$ = 0.718

F

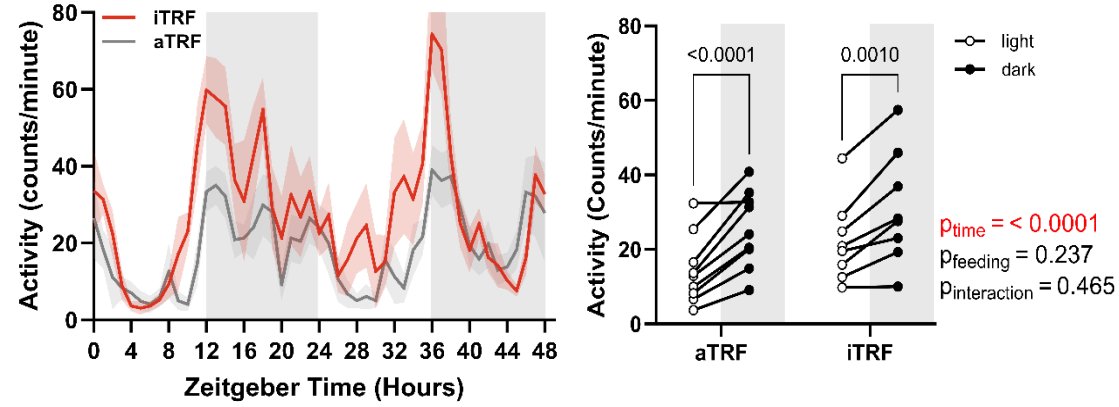
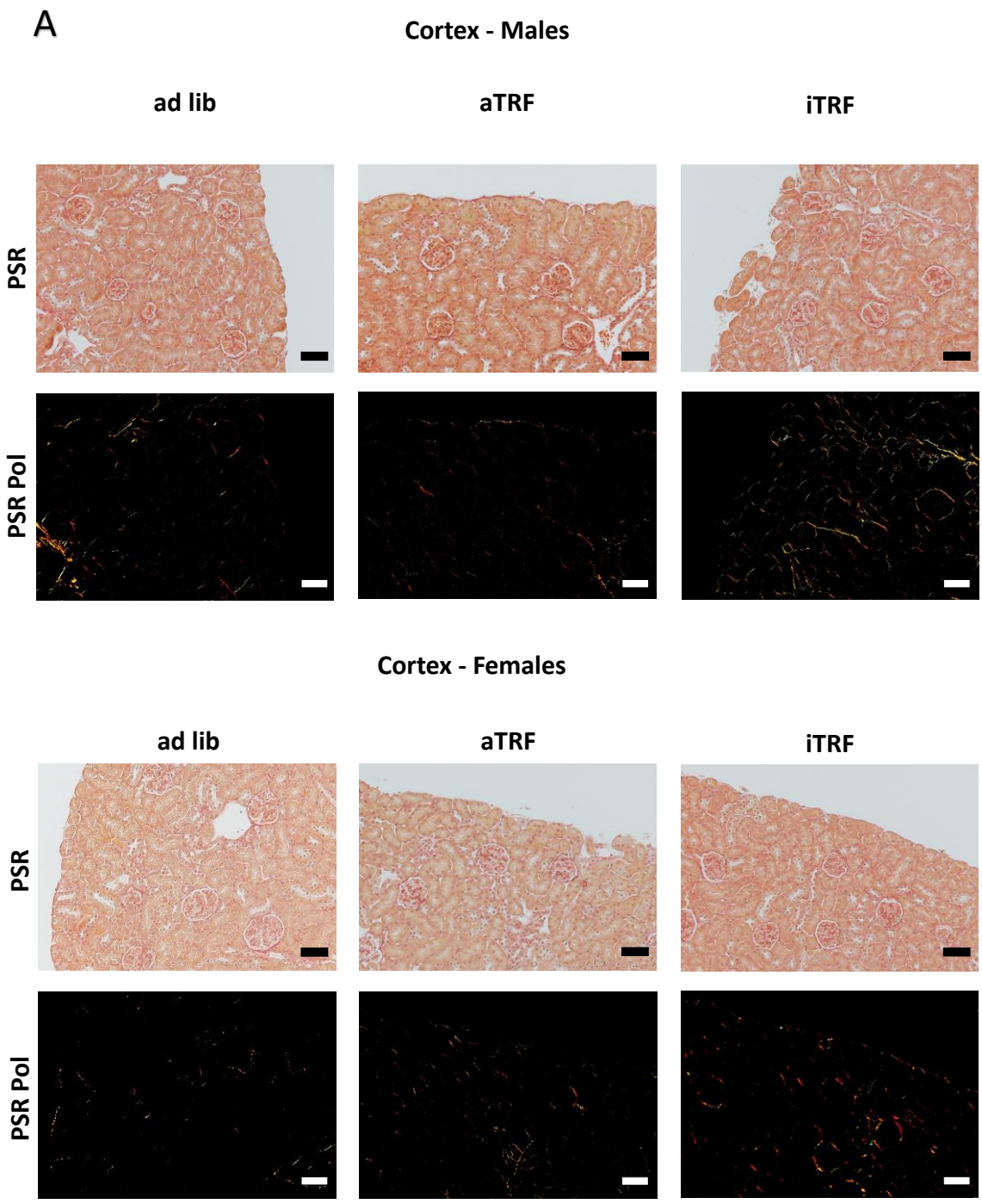

iTRF
aTRF
Activity (counts/minute)
Zeitgeber Time (Hours)
<0.0001
0.0010
light
dark
aTRF
iTRF
p_{time} = < 0.0001
$p_{feeding}$ = 0.237
$p_{interaction}$ = 0.465

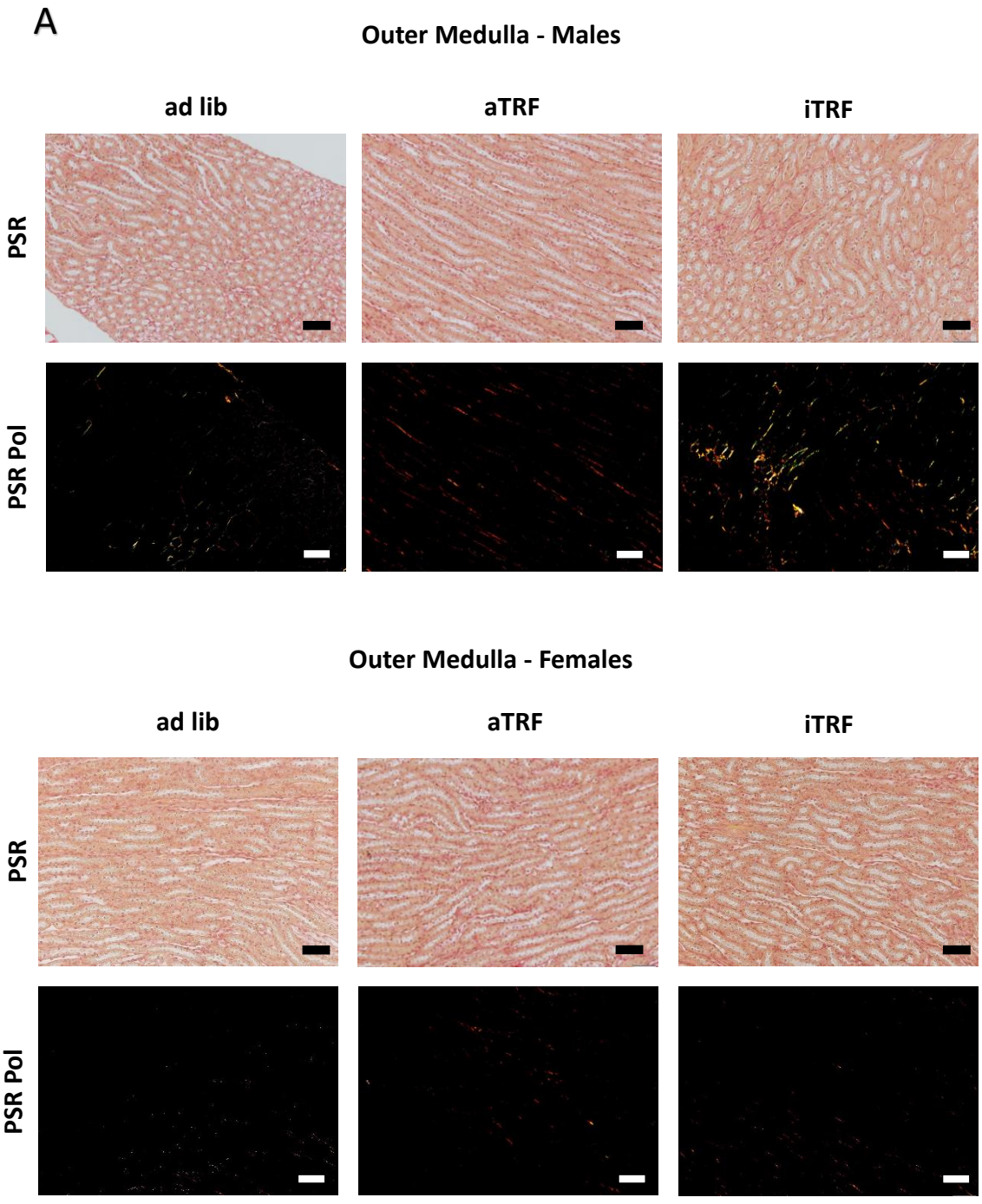
Figure 6. iTRF results in persistent disruption of diurnal patterns of MAP, HR rhythms after 8 weeks. MAP (A,B), HR (C,D), and activity (E,F) after 8 weeks of aTRF (gray) and iTRF in male (blue) and female (red) mice. Gray shaded boxes indicate dark periods. Data are shown as 48-h trace with shading representing error bars. Day/night averages of the days are also shown. Data are means ± SEM. Feeding schedule, time of day, and feeding schedule x time of day interaction effects were determined using two-way ANOVA with Šídák's multiple comparisons test. Significant p values are indicated in red with individual comparisons noted in each panel. n = 7-11 mice/group.

Aim 2: Test the hypothesis that chronic circadian disruption via mistimed feeding induces kidney pathology.

iTRF results in sex-differences in renal fibrosis localization

Male and female aTRF mice showed no cortical or outer medullary fibrosis relative to ad libitum fed mice after chronic circadian disruption vis mistimed feeding (Figure 7A-E). In contrast, cortical fibrosis was observed in both male and female iTRF mice compared to ad libitum mice (Figure 7A-E). Interestingly, only male iTRF animals exhibited outer medullary fibrosis. (Figure 7D). Neither male nor female iTRF mice had increases in serum creatinine (Figure 7F,G), suggesting normal kidney function despite increased renal fibrosis.


A
Cortex - Males
ad lib
aTRF
iTRF
PSR
PSR Pol
Cortex - Females
ad lib
aTRF
iTRF
PSR
PSR Pol


A
Outer Medulla - Males
ad lib
aTRF
iTRF
PSR
PSR Pol
Outer Medulla - Females
ad lib
aTRF
iTRF
PSR
PSR Pol

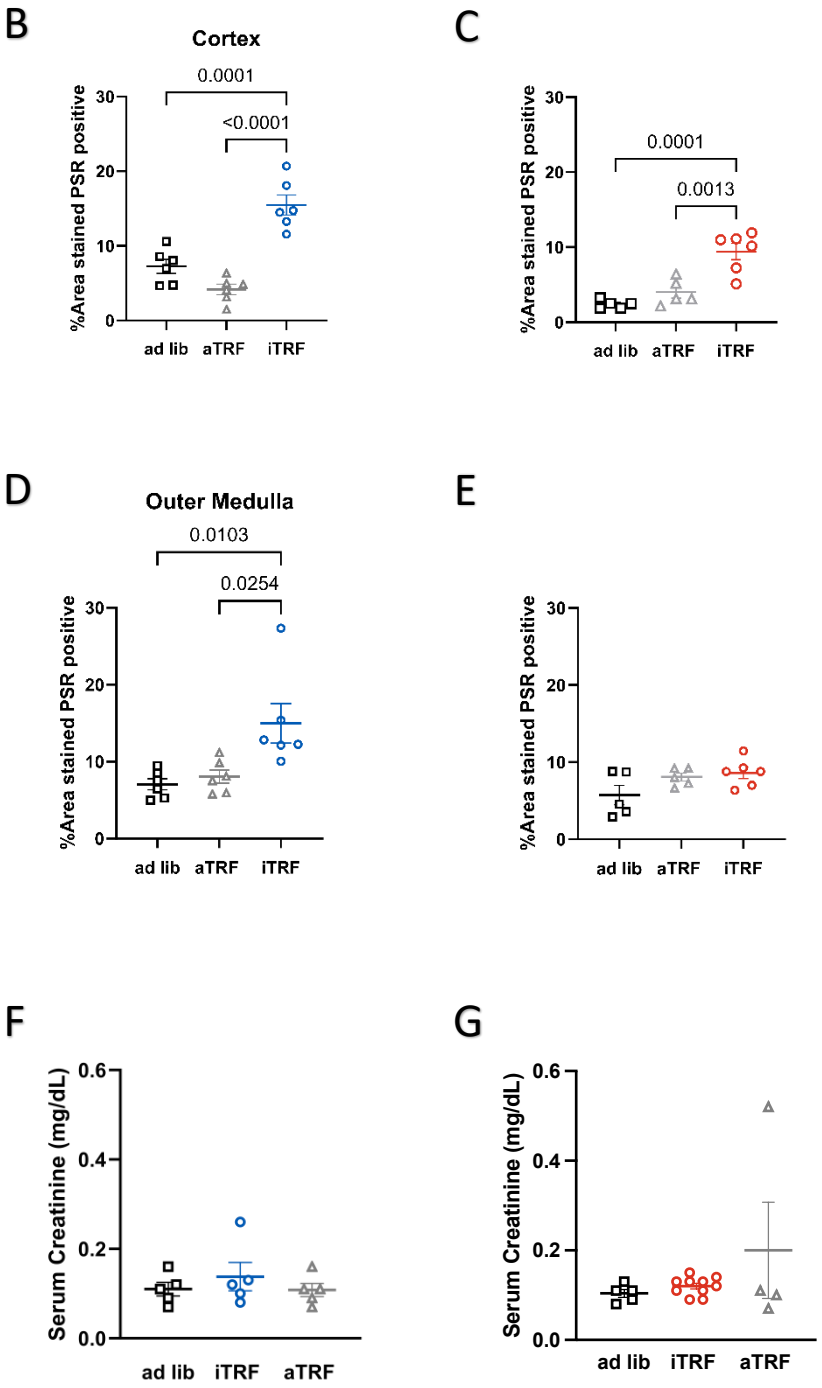
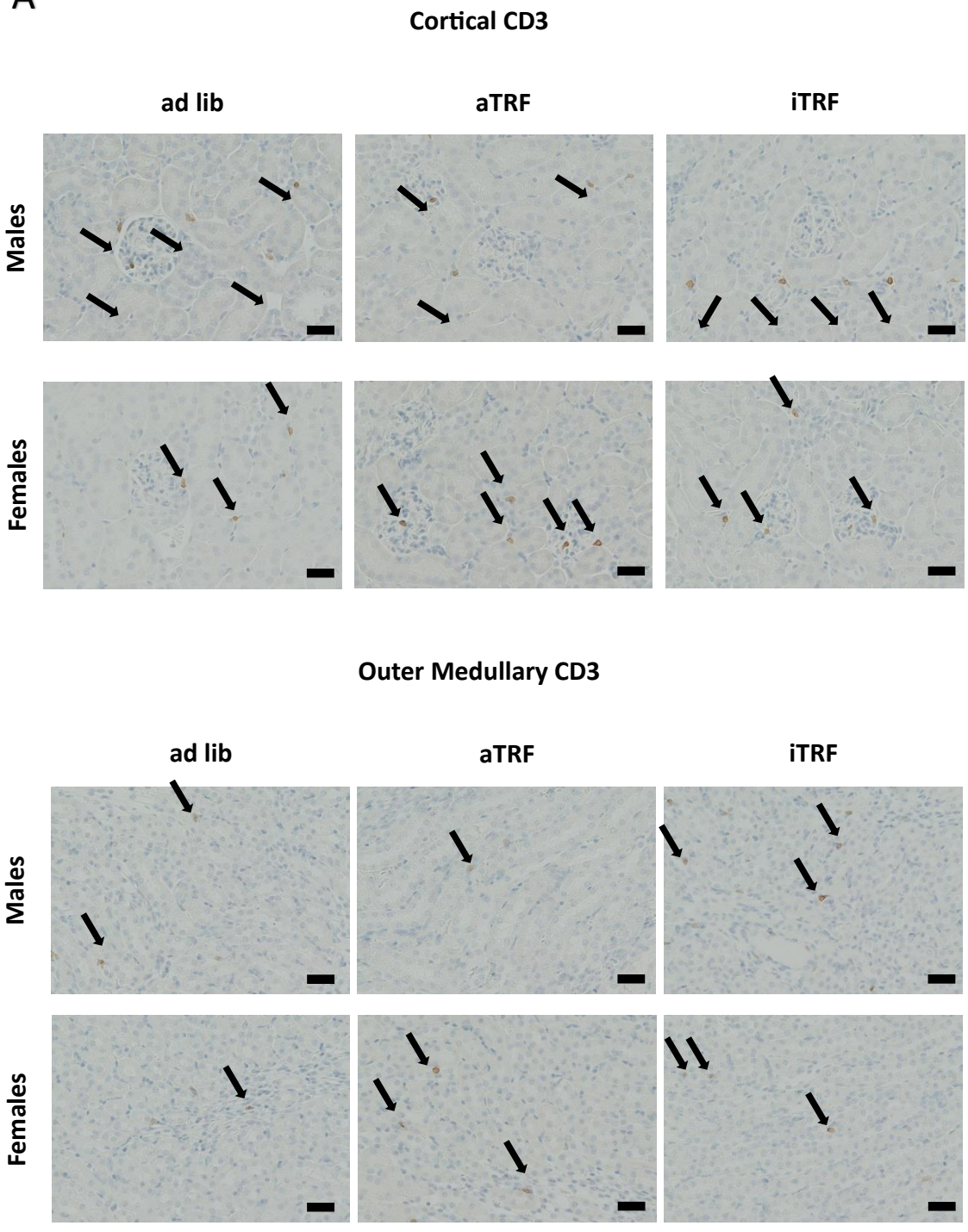

B
Cortex
0.0001
<0.0001
%Area stained PSR positive
30
20
10
0
ad lib
aTRF
iTRF
C
0.0001
0.0013
%Area stained PSR positive
30
20
10
0
ad lib
aTRF
iTRF
D
Outer Medulla
0.0103
0.0254
%Area stained PSR positive
30
20
10
0
ad lib
aTRF
iTRF
E
%Area stained PSR positive
30
20
10
0
ad lib
aTRF
iTRF
F
Serum Creatinine (mg/dL)
0.6
0.4
0.2
0.0
ad lib
iTRF
aTRF
G
Serum Creatinine (mg/dL)
0.6
0.4
0.2
0.0
ad lib
iTRF
aTRF

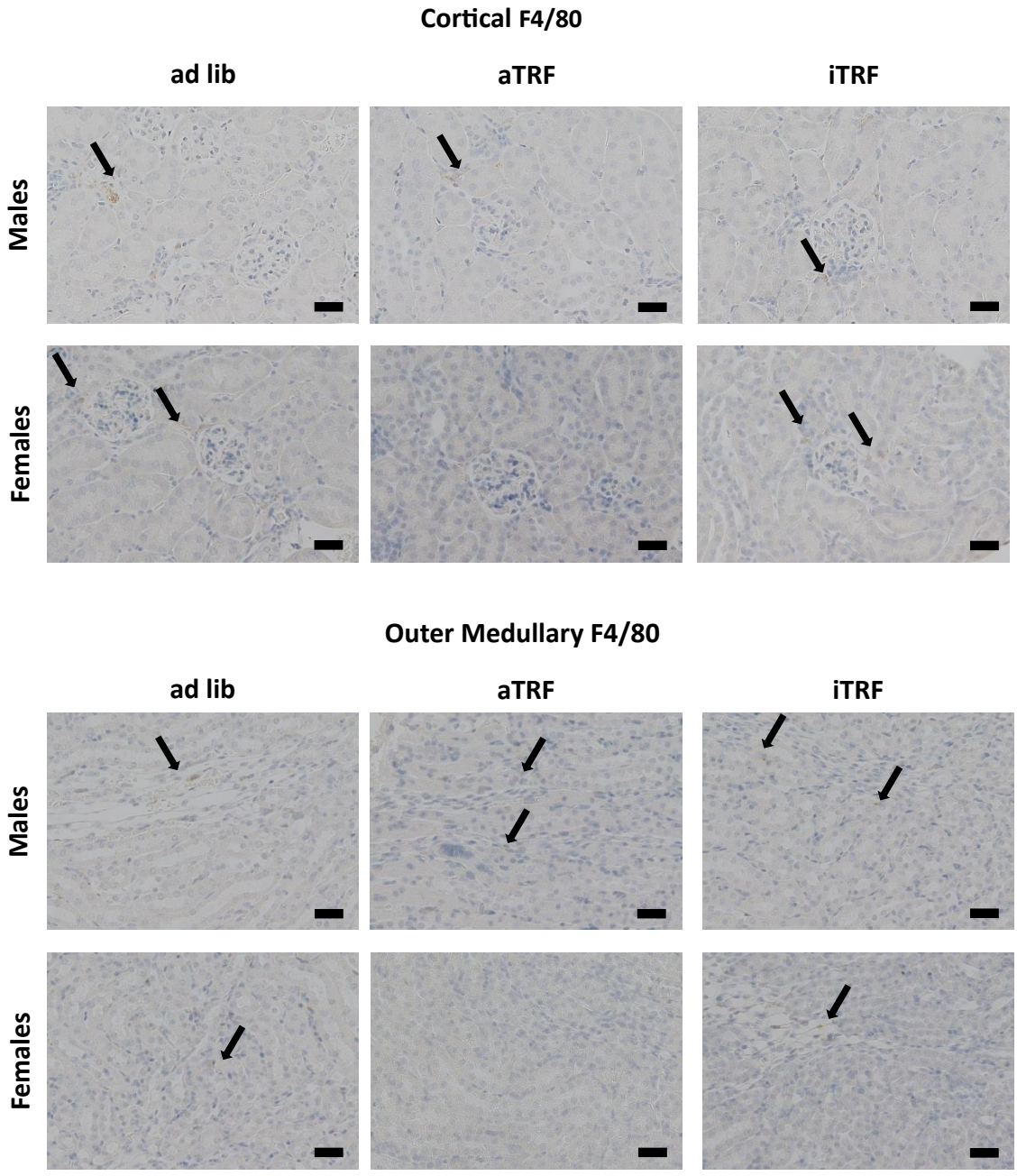
Figure 7. iTRF results in sex-differences in renal fibrosis localization. Representative images of picrosirius red (PSR) staining of the renal cortex and outer medulla from ad libitum (left), aTRF (middle), and iTRF (right) mice after chronic feeding intervention (A) (magnification: 20x, scale bars = 50μm). Top: bright field illumination; bottom: polarization contrast illumination. Semi-quantitative analysis of bright field PSR staining in the renal cortex (B,C) and outer medulla (D,E) from ad libitum (black), aTRF (gray), and iTRF male (blue) and female (red) mice after chronic feeding intervention. Serum creatinine measurements after chronic feeding intervention (F,G). Data are means ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test. Significant p values are indicated. n = 4-10 mice/group.

Sex-differences in immune pathology in iTRF mice

Numerous findings have shown that the immune response plays a key role in fibrogenesis. Of particular note are macrophages, which have been shown to undergo differentiation during fibrogenesis (441, 442) and T-cells, which have less defined, tissue-specific roles in fibrosis onset (443, 444). Because we observed increased fibrosis in iTRF mice, we were interested in determining whether increased T cell and macrophage infiltration may be contributing to the fibrotic phenotype (Figure 8A). There were no differences in the amount of $CD3^+$ T cells in the renal cortex (Figure 8B,C). Interestingly, there was a significant increase in outer medullary $CD3^+$ cells in male iTRF mice that was not observed in female iTRF mice (Figure 8D,E). Histological staining demonstrated no differences in $F4/80^+$ macrophages in the cortex of any mice, regardless of sex or timing of food availability (Figure 8F,G). Unexpectedly, there was a significant decrease in the number of outer medullary $F4/80^+$ cells only in aTRF males (Figure 8H,I).

A


Cortical CD3
ad lib
aTRF
iTRF
Males
Females
Outer Medullary CD3
ad lib
aTRF
iTRF
Males
Females


Cortical F4/80
ad lib
aTRF
iTRF
Males
Females
Outer Medullary F4/80
ad lib
aTRF
iTRF
Males
Females

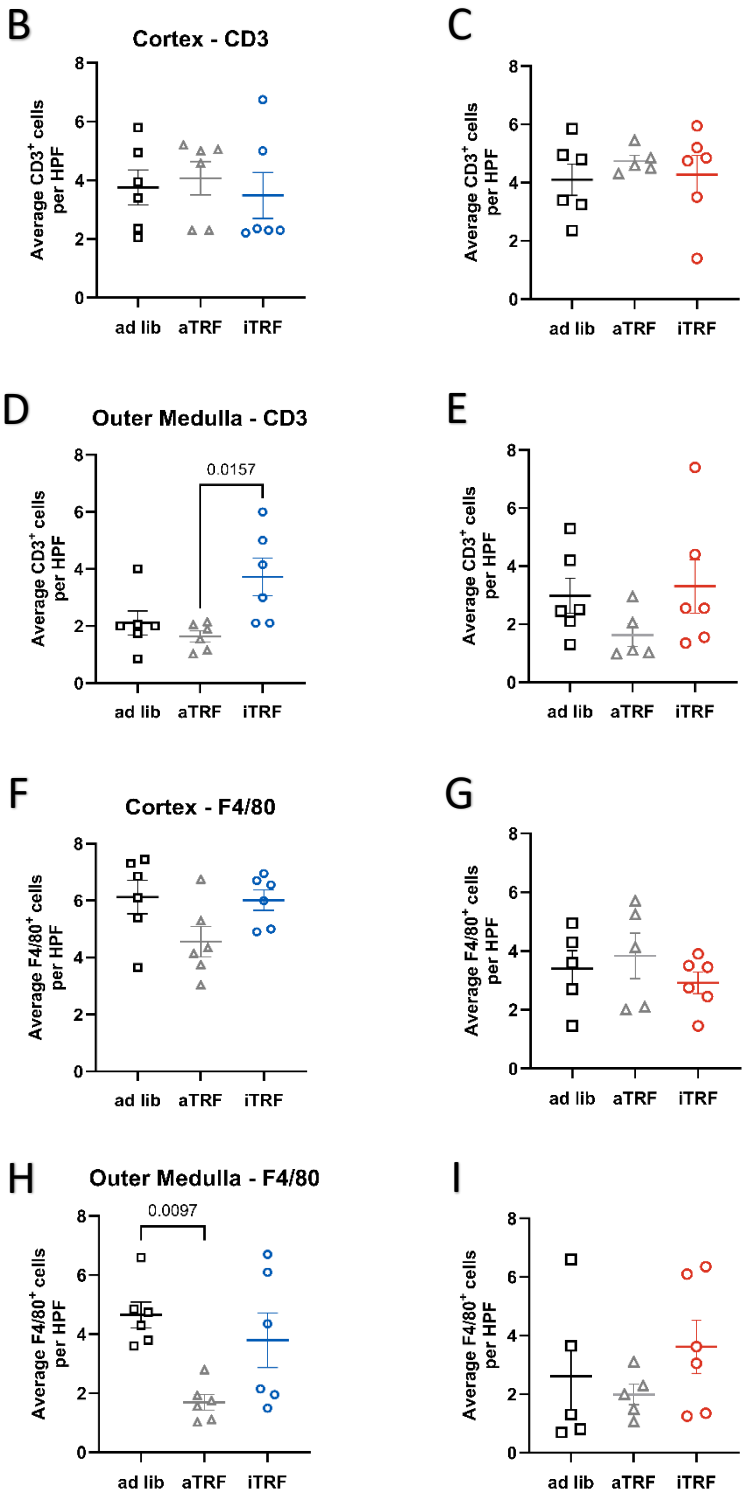

B
Cortex - CD3
Average CD3+ cells per HPF
ad lib
aTRF
iTRF
C
D
Outer Medulla - CD3
0.0157
E
F
Cortex - F4/80
Average F4/80+ cells per HPF
G
H
Outer Medulla - F4/80
0.0097
I

Figure 8. Sex-differences in renal immune pathology in iTRF mice. Representative images of T-cell (CD3) and macrophage (F4/80) staining of the renal cortex and outer medulla from male and female mice after chronic feeding intervention (A) (magnification: 40x, scale bar = 20μm). Semi-quantitative analysis of average cells per high powered field (HPF) stained positive for CD3 in the renal cortex (B,C) and outer medulla (D,E) and stained positive for F4/80 in the renal cortex (F,G) and outer medulla (H,I) in ad libitum (black), aTRF (gray), and iTRF male (blue) and female (red) mice. Data are means ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test. Significant p values are indicated. n = 5-6 mice/group.

iTRF results in decreased glomerular Bowman's space

We also analyzed glomerular morphology after chronic circadian disruption via mistimed feeding (Figure 9A). Under ad libitum and aTRF conditions, glomerular structure remained intact in males and females. However, male and female iTRF mice demonstrated a visibly reduced glomerular Bowman's space (Figure 9A). As larger glomeruli can contribute to decreased Bowman's space, we quantified glomerular area in all experimental groups. Despite visible morphological changes, glomeruli from iTRF mice did not have a larger glomerular area than ad libitum or aTRF mice, regardless of sex (Figure 9B,C).

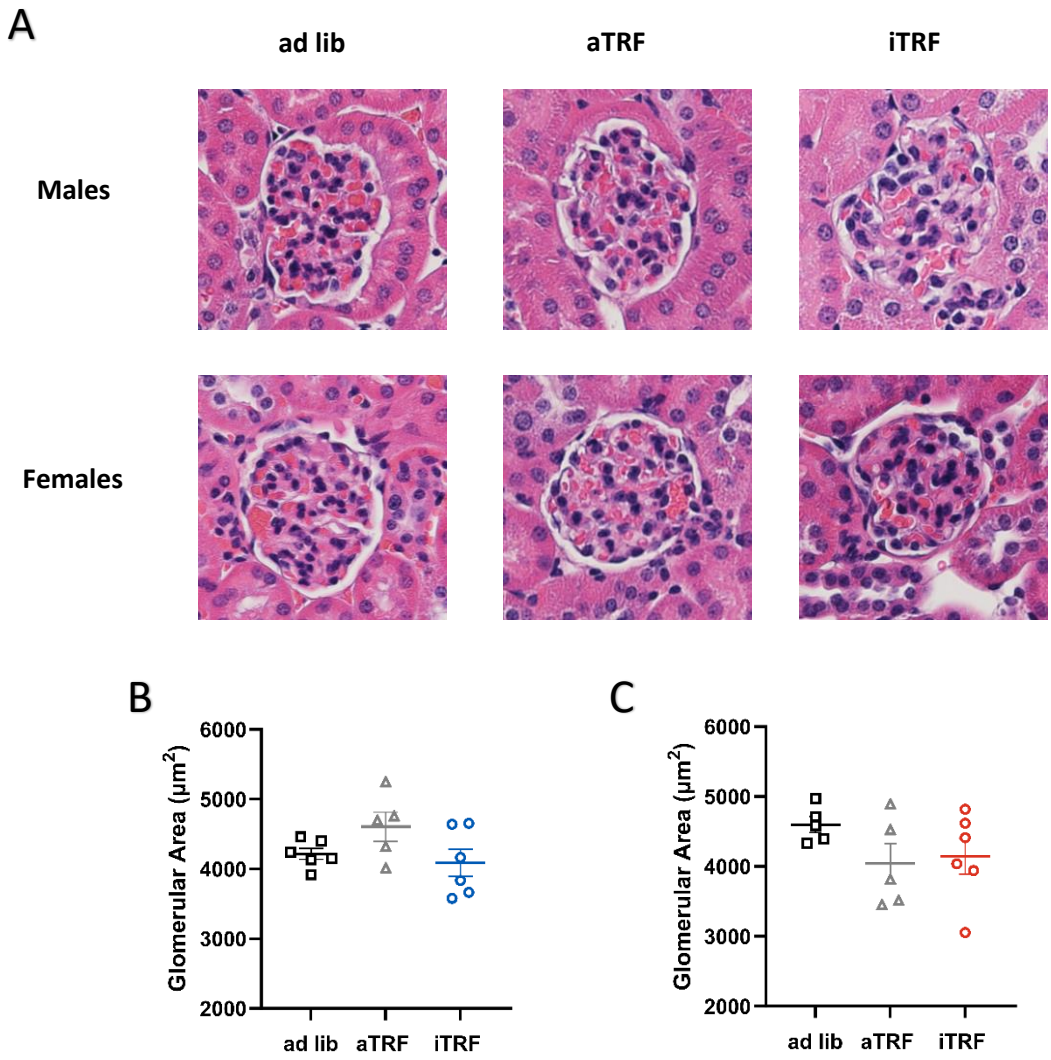
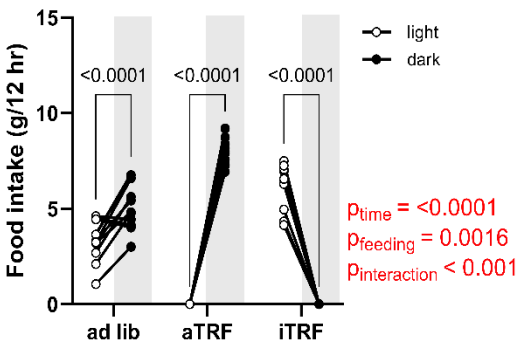


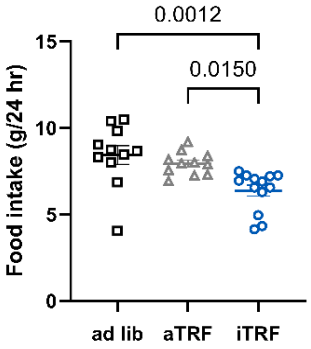
Figure 9. iTRF results in visibly decreased glomerular Bowman's space without change in glomerular area. Representative images of hematoxylin and eosin (H&E) staining of glomeruli from male (top) and female (bottom) mice after chronic feeding intervention (A) (magnification: 40x, scale bars = 20µm). Quantification of glomerular area in ad libitum (black), aTRF (gray), and iTRF male (left, blue) and female (right, red) mice (B). Data are means ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's multiple comparisons test. Significant p values are indicated. n = 5-6 mice/group.

Chronic circadian disruption via mistimed feeding results in decreased daily food intake

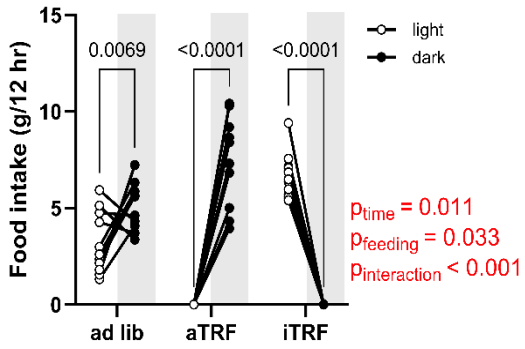
Under ad libitum and aTRF conditions, male and female C57BL/6J mice exhibited similar 24-h food intake after chronic circadian disruption via mistimed feeding. Interestingly, iTRF male and female mice consumed significantly less food during the same time period (Figure 10A,B). 24-h water intake was similar between the three groups, regardless of sex (Figure 10C,D). Ad libitum fed mice exhibited the expected diurnal difference in food and water intake (Figure 10A,B). In both iTRF and aTRF male and female mice, water intake followed food intake being higher in light and dark periods, respectively (Figure 10C,D). Thus, the diurnal rhythm of water intake was inverted for iTRF animals, but remained significantly different between the day and night periods. aTRF and iTRF mice demonstrated a disrupted diurnal pattern of urine output, regardless of sex (Figure 10E,F). 24-hour urine volume was markedly decreased compared to ad libitum male mice, though it failed to reach significance (Figure 10E). For females, both aTRF and iTRF mice excreted significantly less urine than ad libitum animals over a 24-hour period (Figure 10F).

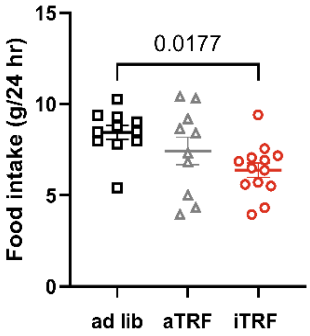
A


Food intake (g/12 hr)
15
10
5
0
<0.0001
<0.0001
<0.0001
ad lib
aTRF
iTRF
light
dark
p_{time} = <0.0001
$p_{feeding}$ = 0.0016
$p_{interaction}$ < 0.001

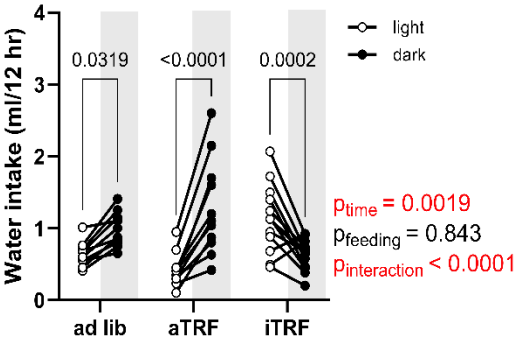

Food intake (g/24 hr)
15
10
5
0
0.0012
0.0150
ad lib
aTRF
iTRF

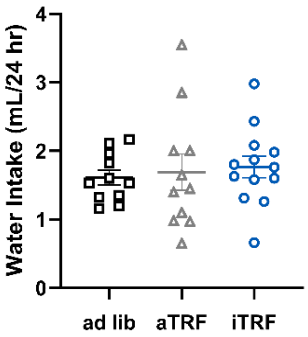
B


Food intake (g/12 hr)
15
10
5
0
0.0069
<0.0001
<0.0001
ad lib
aTRF
iTRF
light
dark
p_{time} = 0.011
$p_{feeding}$ = 0.033
$p_{interaction}$ < 0.001

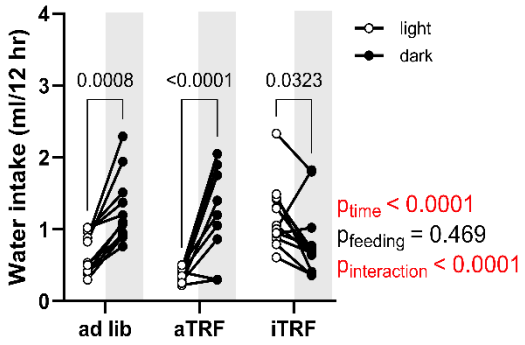

Food intake (g/24 hr)
15
10
5
0
0.0177
ad lib
aTRF
iTRF

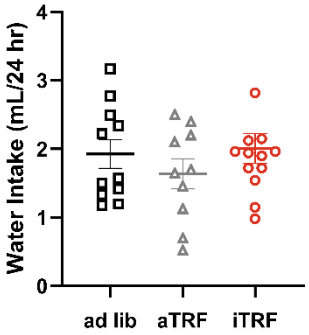
C


Water intake (ml/12 hr)
4
3
2
1
0
0.0319
<0.0001
0.0002
ad lib
aTRF
iTRF
light
dark
p_{time} = 0.0019
$p_{feeding}$ = 0.843
$p_{interaction}$ < 0.0001

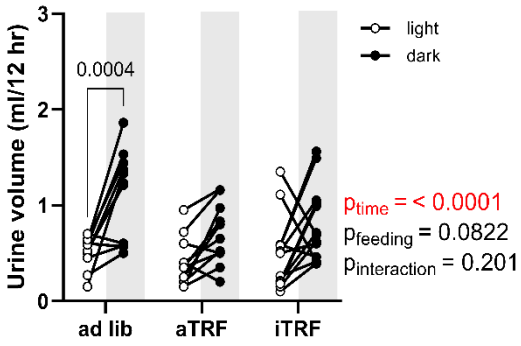

Water Intake (mL/24 hr)
4
3
2
1
0
ad lib
aTRF
iTRF

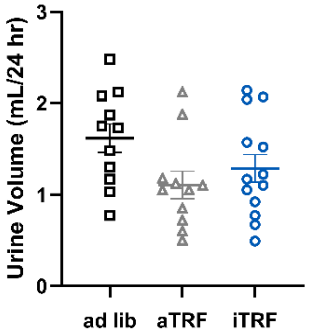
D


Water intake (ml/12 hr)
0.0008
<0.0001
0.0323
light
dark
ptime < 0.0001
pfeeding = 0.469
pinteraction < 0.0001
ad lib
aTRF
iTRF

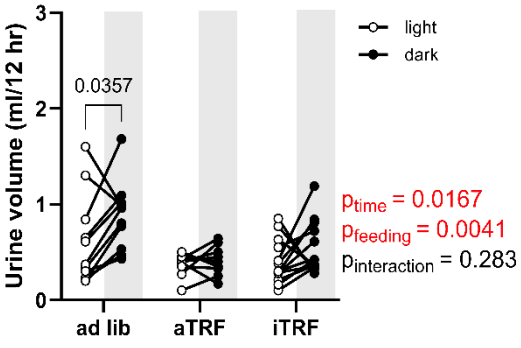

Water Intake (mL/24 hr)
ad lib
aTRF
iTRF

E


Urine volume (ml/12 hr)
0.0004
light
dark
ptime = < 0.0001
pfeeding = 0.0822
pinteraction = 0.201
ad lib
aTRF
iTRF


Urine Volume (mL/24 hr)
ad lib
aTRF
iTRF

F


Urine volume (ml/12 hr)
0.0357
light
dark
ptime = 0.0167
pfeeding = 0.0041
pinteraction = 0.283
ad lib
aTRF
iTRF

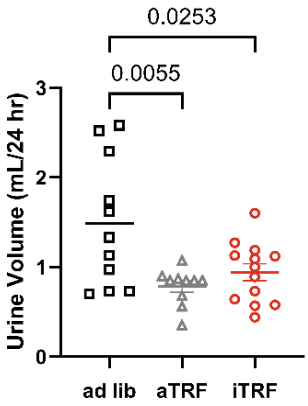
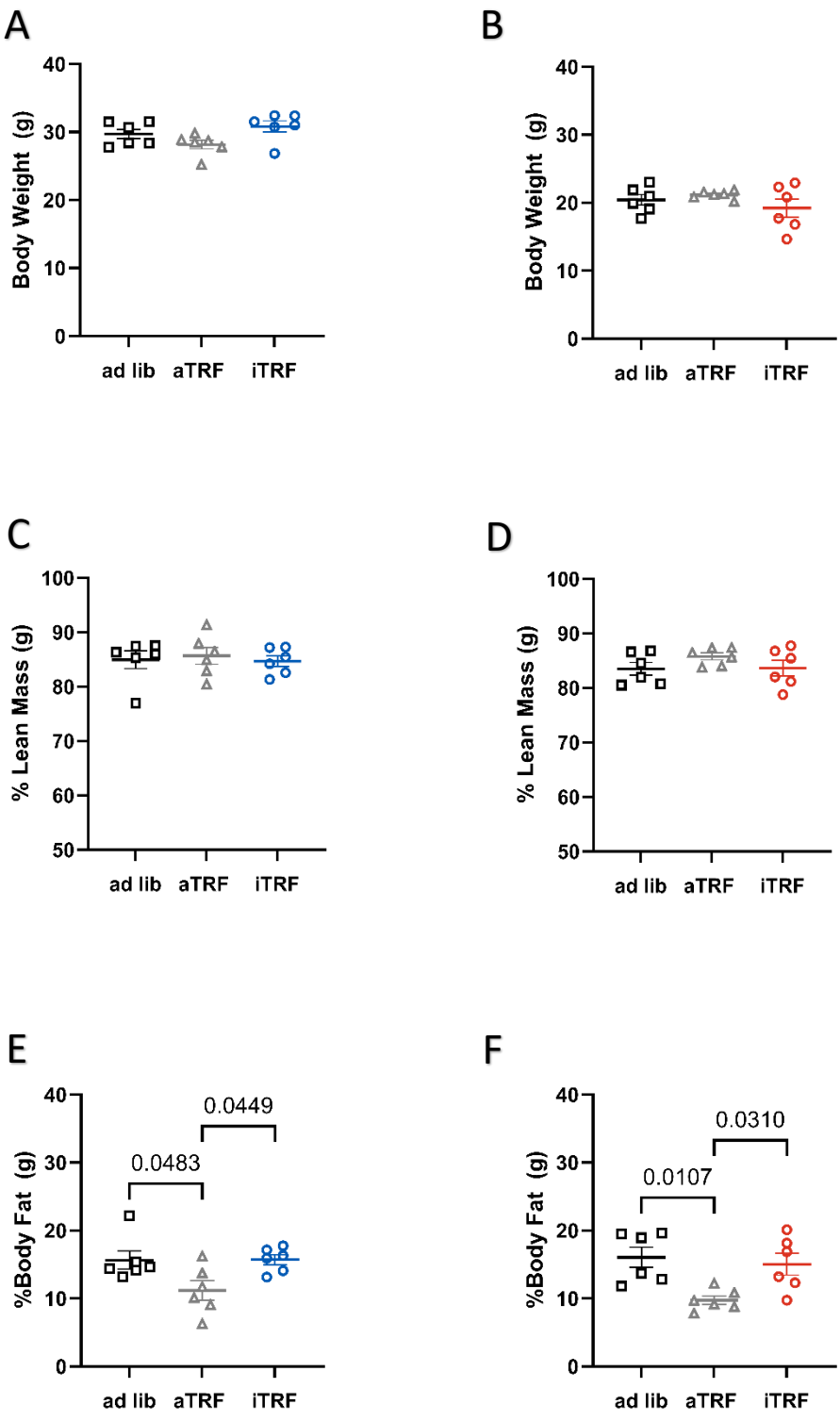

0.0253
0.0055
Urine Volume (mL/24 hr)
ad lib
aTRF
iTRF

Figure 10. Decreased food intake in iTRF mice after 8 weeks. Food intake (A,B), water intake (C,D), and urine volume (E,F) (averages over two 24-hour days) from ad libitum (black), aTRF (gray), and iTRF in male (blue) and female (red) mice. Left panels represent 12-hour light and dark (shaded) time periods. Right panels represent 24-hour totals. Data are means ± SEM. Statistical analysis of 12-hour data was determined by two-way ANOVA with Šídák's test for multiple comparisons. Statistical analysis of 24-hour data was determined by one-way ANOVA with Tukey's test for multiple comparisons. Significant p values are indicated. n = 35 mice/group

Body weight is not affected by decreased food intake in chronic iTRF mice

Given differences in the 24-h food intake in iTRF animals, we assessed whether the observed decrease in food intake affected overall body weight and composition. After chronic circadian disruption via mistimed feeding, body weight and lean mass percentage remained similar between all feeding groups in males and females (Figure 11A-D). Interestingly, both male and female aTRF mice had significantly lower body fat percentage (Figure 11E,F). There were no significant differences in total body water in any of the male groups (Figure 11G). However, females had a modest but statistically significant increase in total body water following aTRF (Figure 11H).


A
Body Weight (g)
40
30
20
10
0
ad lib
aTRF
iTRF
B
Body Weight (g)
40
30
20
10
0
ad lib
aTRF
iTRF
C
% Lean Mass (g)
100
90
80
70
60
50
ad lib
aTRF
iTRF
D
% Lean Mass (g)
100
90
80
70
60
50
ad lib
aTRF
iTRF
E
%Body Fat (g)
0.0449
0.0483
40
30
20
10
0
ad lib
aTRF
iTRF
F
%Body Fat (g)
0.0310
0.0107
40
30
20
10
0
ad lib
aTRF
iTRF

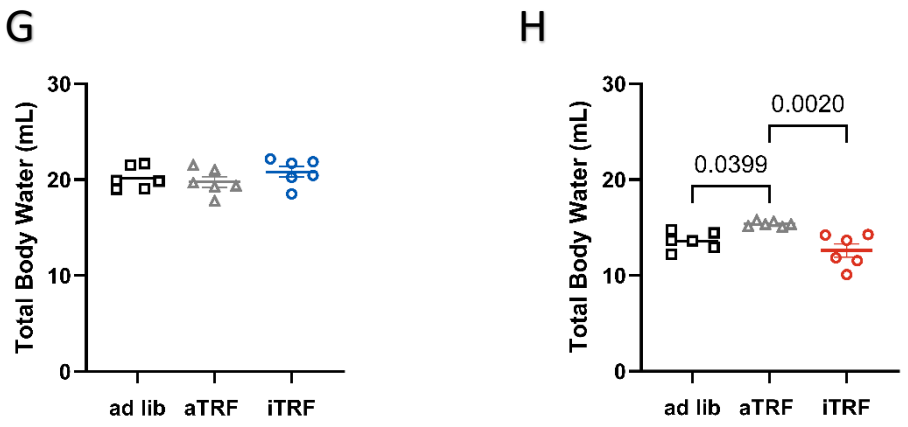


Figure 11. No change in body weight in chronic iTRF mice relative to ad lib or aTRF mice. Body weight (A,B), lean mass percentage (C,D), body fat percentage (E,F), and total body water (G,H) for male (left, blue) and female (right, red) mice after chronic circadian disruption via mistimed feeding. Body fat and lean mass are reported as percentage of total body weight. Data are means ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's test for multiple comparisons. Significant p values are indicated. n = 6 mice/group

Chronic iTRF results in sex-differences in increased arterial stiffness

Increases in arterial stiffness have been reported in laboratory models of circadian disruption as well as in shift workers (445, 446). To that end, we measured pulse wave velocity (PWV) in AL, iTRF, and aTRF mice. Interestingly, only male iTRF mice demonstrated a significant increase in PWV (Figure 12A). Female mice did not exhibit a change in PWV, regardless of timing of food intake (Figure 12B).

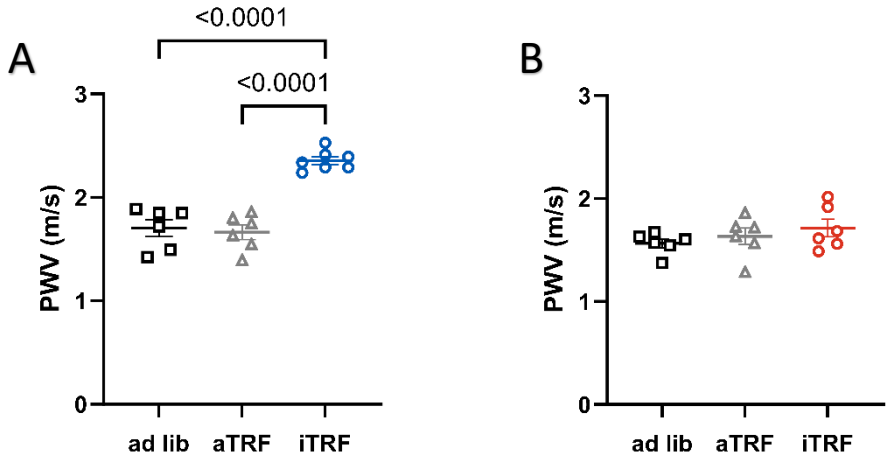
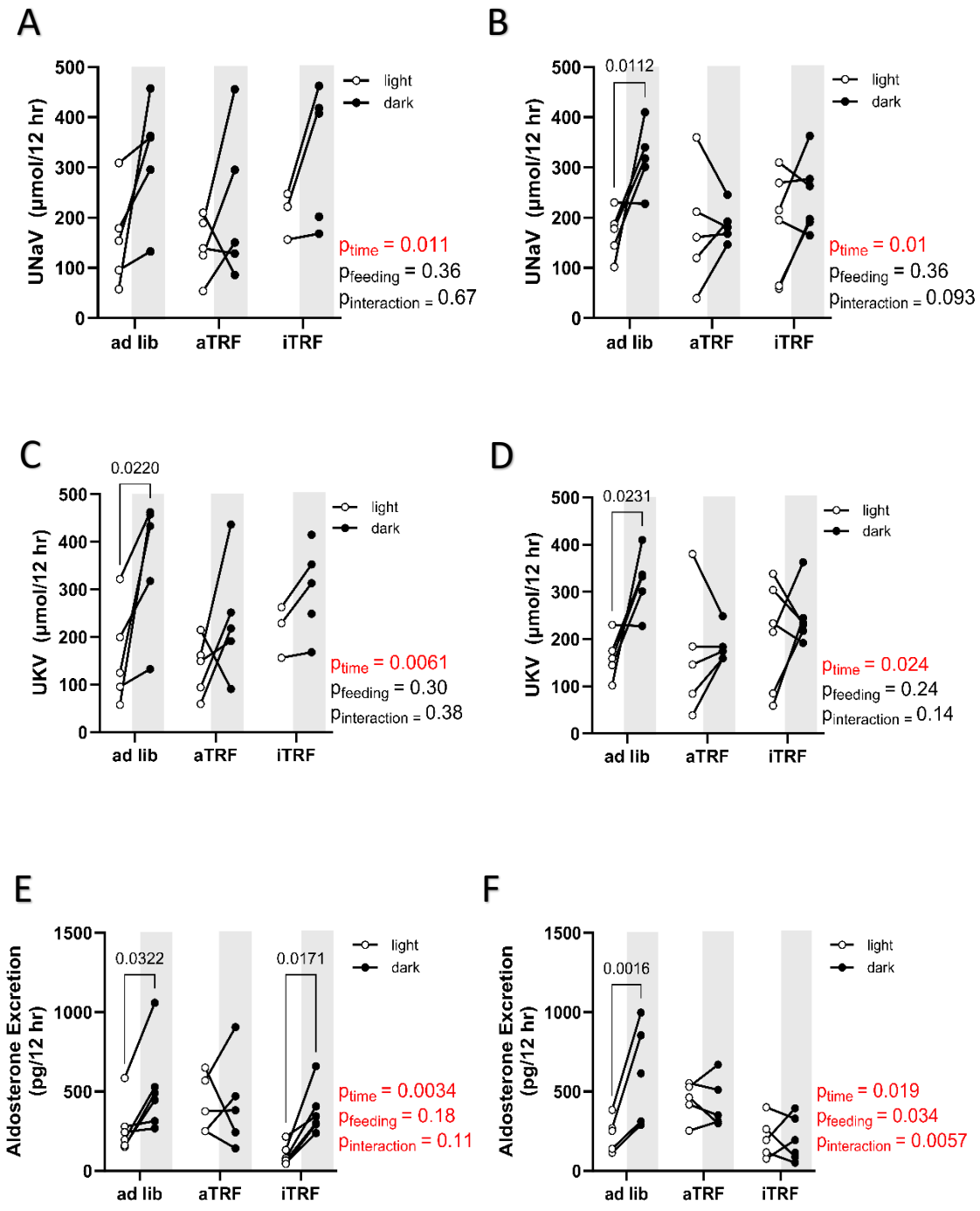


Figure 12. Sex-differences in arterial stiffness in chronic iTRF mice. Pulse wave velocity (PWV) for male (A) and female (B) mice after chronic circadian disruption via mistimed feeding. Data are means ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's test for multiple comparisons. Significant p values are indicated. n = 6 mice/group

Chronic iTRF and aTRF mice have disrupted diurnal patterns of urine excretion

In contrast to prior experiments demonstrating that male mice maintain diurnal rhythms in urine volume after one week of iTRF (7), 8 weeks of iTRF resulted in disrupted diurnal urine excretion patterns in both male and female mice (Figure 10E,F). Surprisingly, the diurnal pattern of excretion in aTRF mice was not similar to that what was observed in ad libitum fed mice. Male and female iTRF and aTRF mice also demonstrated a disrupted diurnal pattern in urinary excretion of sodium or potassium (Figure 13A-D). As expected, ad libitum fed mice displayed a strong diurnal rhythm in urinary sodium and potassium.

While female aTRF and female and male iTRF mice exhibited disrupted diurnal patterns of aldosterone and ET-1 excretion, male aTRF animals maintained a diurnal pattern similar to that of ad libitum fed mice (Figure 13 E-H). Although ET-1 excretion in ad libitum mice appeared to demonstrate the expected diurnal difference, it failed to reach significance.


A
UNaV (µmol/12 hr)
500
400
300
200
100
0
ad lib
aTRF
iTRF
light
dark
ptime = 0.011
pfeeding = 0.36
pinteraction = 0.67
B
0.0112
UNaV (µmol/12 hr)
ptime = 0.01
pfeeding = 0.36
pinteraction = 0.093
C
0.0220
UKV (µmol/12 hr)
ptime = 0.0061
pfeeding = 0.30
pinteraction = 0.38
D
0.0231
UKV (µmol/12 hr)
ptime = 0.024
pfeeding = 0.24
pinteraction = 0.14
E
Aldosterone Excretion (pg/12 hr)
1500
1000
500
0
0.0322
0.0171
ptime = 0.0034
pfeeding = 0.18
pinteraction = 0.11
F
0.0016
Aldosterone Excretion (pg/12 hr)
ptime = 0.019
pfeeding = 0.034
pinteraction = 0.0057

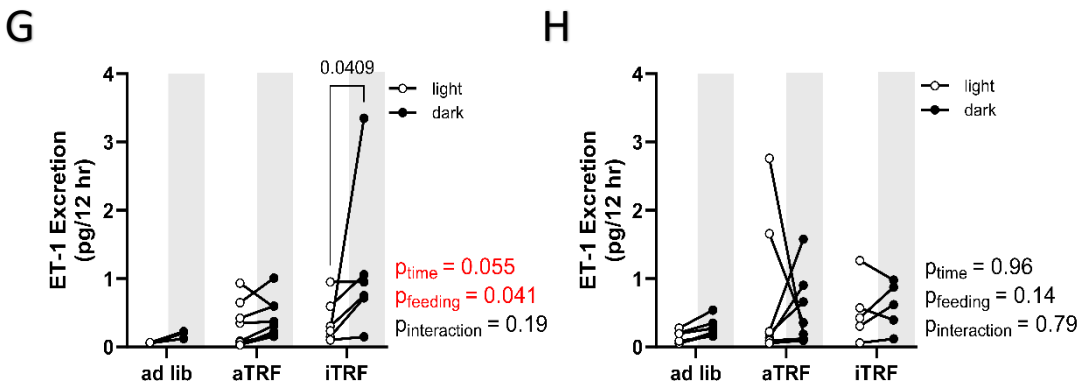


Figure 13. iTRF and aTRF mice have disrupted patterns of urine excretion. Urine excretion rates of sodium (A,B), potassium (C,D), aldosterone (E,F), and endothelin-1 (G,H) after chronic circadian disruption via mistimed feeding in ad libitum, iTRF, and aTRF male (left) and female (right) mice. Data are presented as 12-hour light and dark (shaded) time periods. Data are means ± SEM. Statistical analysis of 12-hour data was determined by mixed-methods ANOVA with Šídák's test for multiple comparisons. Significant p values are indicated. n = 4-6 mice/ group. UNaV, urinary sodium excretion; UKV, urinary potassium excretion; ET-1, endothelin-1

CHAPTER 5

DISCUSSION

The central goal of this dissertation was to determine the cardiorenal effects of long-term circadian disruption via mistimed feeding (iTRF). This work was meant to build upon previous findings from our group that just one week of iTRF inverted blood pressure (BP) rhythms in male C57BL/6J mice (7) and findings that misaligned meal timing is associated with risk of cardiovascular and kidney disease (447, 448). We initially evaluated the effects of a chronic circadian disruption on BP rhythms in male and female mice. It is well reported that BP rhythmicity can be indicative of organ health and predictive of cardiovascular and renal outcomes (449, 450). In our experiment, we observed disrupted diurnal BP patterns as early as 4 weeks after beginning the iTRF protocol in both sexes. This BP phenotype is similar to a recent preliminary study from Toffoli et al., who showed that male and female night shift workers lose their nocturnal dip in systolic and diastolic BP (17). Thus, chronic iTRF in mice is sufficient to affect BP patterns long-term.

The kidney is crucial to BP control as it functions to regulate extracellular fluid volume homeostasis. Night shift workers are at an increased risk for CVD compared to daytime workers, and disruptions in circadian rhythms have been identified as a contributing factor to that risk (10, 451). Given such an intriguing BP phenotype in our acute and long-term iTRF models, we became interested in the cardiorenal effects of

chronic iTRF in our mice. Findings from these experiments drew us to conclude that mistimed feeding is a unique risk factor contributing to CVD risk. These results provide a useful model for investigating the effects of circadian disruption on peripheral clocks independent of the central clock. These findings also provide a trove of new knowledge for the cardiorenal physiology field.

Aim 1: Test the hypothesis that chronic circadian disruption via mistimed feeding has long-term effects on blood pressure rhythms.

Disruption of BP rhythms has been identified as a risk factor for cardiovascular and renal outcomes, though the molecular mechanisms underlying regulation of BP rhythms remain unclear. Previous work from our group has demonstrated the importance of timing of food intake for modulating BP rhythms (7). In under one week, iTRF was sufficient to invert mean arterial pressure (MAP) rhythms relative to ad libitum fed male C57BL/6J mice even when maintained in a normal 12/12 light/dark cycle. Additional findings demonstrated a sex-difference in circadian control of BP rhythms and renal function (173). We designed the current study to build upon previous work by determining the effect of chronic iTRF on both sexes. We initially hypothesized that the inverted BP rhythms previously reported would be maintained after chronic iTRF. Interestingly, we observed that male and female mice exhibited disrupted diurnal MAP patterns as early as 4 weeks and persisting through 8 weeks after beginning iTRF. This BP pattern in our mice, fed a normal chow diet, recapitulates what others have observed in murine models with global

and tissue-specific molecular clock gene knockouts in terms of an altered BP phenotype (152, 215, 216). Hou et al. demonstrated the benefit of restricting food availability to the active period (aTRF) by showing that 8-hour or 10-hour aTRF protected against the development of non-dipping BP in *db/db* mice and restored BP dipping in previously non-dipping mice (277). We show in our experiment that aTRF mice exhibit a BP phenotype similar to what has previously been reported in ad libitum fed mice in terms of BP dipping, further demonstrating that it is the mistiming of food intake driving the BP phenotype in iTRF animals (95, 147). It is important to note that the disrupted diurnal BP pattern in iTRF mice is not influenced by a light zeitgeber, as lights remained on their normal 12:12 light/dark cycle for all groups. Thus, our findings demonstrate the powerful effect of the peripheral zeitgeber of food intake timing on controlling BP rhythms.

Similar to BP rhythms, iTRF animals exhibited a disrupted circadian HR rhythm after 8 weeks in mice of both sexes. Prior studies have shown that the central clock in the suprachiasmatic nucleus (SCN) as well as a functional molecular clock in the myocardium are indispensable for maintaining heart rate (HR) circadian rhythms in mice (286, 452–454). The heart is also a peripheral clock and can be disrupted through changes in both environmental and behavioral factors. The circadian clock in the heart is disrupted during hypertension-induced hypertrophy and ischemia/reperfusion injury (455, 456). However, few if any studies have assessed whether cardiac metabolism is affected in these disease states. Thus, one potential mechanism underlying the disruption of HR rhythms in iTRF mice could be a misalignment between the central and cardiac molecular clocks caused by a food dependent hormonal or metabolic mechanism. This model could prove useful

for future studies investigating the relationship between the circadian clock and cardiac metabolic rhythms and processes.

Interestingly, overall HR was increased in female mice relative to males. This finding is reminiscent of findings from a recent report that female C57BL/6J mice exhibit a significantly higher HR compared to males even on an ad libitum diet (457). It is important to consider that the autonomic nervous system plays a critical role in regulating heart rate. In clinical studies, heart rate variability is the standard non-invasive assessment of autonomic control of the heart. At least one study has reported gender differences in resilience strategies among law enforcement officers (458). Others have seen sex differences in heart rate variability, but report that females may have increased parasympathetic nervous system activity despite increased heart rate (459, 460). Thus, there may be differing pathophysiology of cardiovascular disease by sex. Females may have an initially higher stress response to mistimed feeding that reduces with time whereas males may be better able to acutely modulate to circadian stressors.

In healthy individuals, BP follows a well-defined circadian rhythm wherein it increases during periods of high activity and decreases during the resting period. We expected to see disrupted diurnal activity patterns reflective of BP patterns in iTRF mice. Interestingly, male and female iTRF mice maintained normal diurnal activity patterns for the duration of the study even through food availability was restricted to the 12-hour lights on period. Maintenance of diurnal activity patterns in short-term iTRF has been reported previously (461, 462). It is known in the field that the SCN regulates circadian

rhythms in behavioral activity (463). Our findings confirm that activity does indeed follow the light cycle and thus the central clock, independent of food intake timing. One aspect of our model that requires further investigation is the apparent increase in activity in iTRF animals during the transition from light to dark when food availability is removed. It is unclear whether this is a persistent effect driven by light cues or the change in food availability. Nonetheless, food restriction to the light period imposes a temporal conflict with the activity phase maintained by the SCN. This likely causes peripheral and central clocks to become uncoupled given the different zeitgebers controlling central and peripheral clocks, in other words, a multifaceted circadian misalignment.

Aim 2: Test the hypothesis that chronic circadian disruption via mistimed feeding induces kidney pathology.

Shift work has been identified as a risk factor contributing to cardiovascular disease onset (464). As shift workers tend to maintain their positions for years or even decades, we became interested in investigating how long-term CD via mistimed feeding affected the cardiac and renal morphology and physiology in our mice.

Interstitial fibrosis is a key morphological change in the tubulointerstitial compartment. Increased renal fibrosis has been reported in other models of circadian disruption such as obesity (465–468) and genetic deletion of molecular clock genes (319, 320, 469).

In our experiment, we observed significant cortical fibrosis in male and female iTRF mice consistent with a sex-independent mechanism. However, we did see protection from fibrosis in the renal medulla of female mice relative to males consistent with a wide range of studies showing that premenopausal females tend to be protected from manifestations of renal injury (470–472). These sex differences suggest multiple mechanisms may be responsible for the development of fibrosis in distinct regions of the kidney.

There are a number of possible mechanisms underlying kidney fibrosis, but increasing evidence suggests that renal inflammation is a key player in renal fibrogenesis and progression. Clinical studies have found that shift workers have increased immune activity, suggesting that survey of inflammatory markers may be an early indicator of CVD in shift workers (473, 474). Both the innate and adaptive immune systems can contribute to the pathogenesis of kidney injury and cardiovascular disease (475–477).

Macrophages have a wide variety of phenotypes and functions, making them a multifaceted player in renal disease pathogenesis. In previous years, macrophages have emerged as a master player in kidney fibrosis. Accumulation of renal macrophages correlates with the severity of fibrosis in humans and rodent models (478, 479). Macrophages are initially recruited to the glomerulus and tubulointerstitium to initiate the innate immune response. Continued renal damage can result in continuing macrophage infiltration and irreversible fibrosis. Many studies have drawn a relationship between the circadian system and the proinflammatory macrophage response in humans and rodent models (480, 481). In our study, we observed no increases in cortical or outer

medullary macrophages in our mice based on histological staining for F4/80. Interestingly, there was a significant decrease of renal macrophages in aTRF males compared to ad libitum males, but macrophage content was still within a relatively normal range for mice. Based on our findings, it appears that after 8 weeks, macrophages are not present in significant numbers in the renal cortex or outer medulla in male or female iTRF mice. There remains the possibility that specific subsets of macrophages may be changed that are not detected with our analytical method.

Innate immune cells such as macrophages can perpetuate early renal damage by recruitment of adaptive immune cells, such as T cells (443, 475, 482). Most studies investigating the role of T cells in kidney injury focus on CD4 (T helper cells). However, at least one study suggests that CD3 (T cells) increase after ischemia and contribute to renal fibrogenesis (444). Loef et al. found that night shift workers had a higher mean number of plasma T cells compared to daytime workers, suggesting that chronic circadian disruption may influence immune status (473). In our model, iTRF males demonstrated a significant increase in T cells in the outer medulla. In contrast, we saw no significant increases in CD3 T cells in the renal cortex or outer medulla of female iTRF mice. This is interesting in the context of the renal fibrosis data, as it suggests that there may be a differential adaptive immune response driving the sex-difference in fibrosis in iTRF mice. It is possible that early in the course of iTRF there was an innate immune response that passed, as T cell infiltration can occur secondarily to macrophage involvement.

It is important to note that the images taken from this experiment are representative of one snapshot in time and do not differentiate between resident and infiltrating immune cells. It is also possible that we missed a time period during which a mass infiltration of immune cells could have occurred. Given that fibrosis is well established after 8 weeks of iTRF, it stands to reason that there could have been a more substantial immune response that already occurred. Future experiments with this model could involve flow cytometry across the iTRF timeline to better define the immune response in our model.

The renal medulla is often the first area of the kidney to be injured in a variety of kidney disorders including hypertension. In spontaneously hypertensive rats, tubulointerstitial changes and lesions are often confined to the medullary space initially before expanding to the outer cortical region; control kidneys in rats treated with angiotensin II demonstrate initial profibrotic changes in the medulla (483). Interestingly, none of the chronic mistimed feeding animals became hypertensive by week 8. It will be of interest to determine whether the observed fibrosis can be reversed by returning iTRF animals to an aTRF or ad libitum feeding schedule. Given that C57BL/6J mice are notoriously resistant to the development of renal fibrosis, (484–486) our findings demonstrate a useful model for investigating mechanisms underlying fibrosis in wild-type, normal chow-fed C57BL/6J mice.

In the glomerular compartment, one of the major morphological modifications related to renal pathology is mesangial expansion. C57BL/6J mice are hearty against

histological manifestations of injury, but we did see a visible decrease in Bowman's space in iTRF mice, regardless of sex. Upon quantitative analysis of the glomerular area, we found no difference in the size of the glomeruli between experimental groups or between sexes. Thus, it appears that there is no glomerular enlargement contributing to the visibly decreased Bowman's space. However, we did not assess whether alternative causes related to structural alterations in glomerular capillaries including podocyte structure may be contributing to the observed morphology in iTRF mice.

The visibly decreased Bowman's space observed in the glomeruli of iTRF mice was not expected without changes in overall MAP and may highlight the importance of maintaining rhythmic MAP as being equally important as the overall level of BP. Clinical studies have reported a strong association of CKD risk in patients with non-dipping BPs (487, 488). Our data suggest that morphological changes in the kidney may underlie CKD onset and progression. To truly understand the physiological implications of our findings, a future study will need to assess glomerular filtration rate, glomerular permeability, and albuminuria in iTRF mice.

Consistent with a change in overall metabolism during mistimed feeding is our observation that body weights were not different among groups even though the iTRF group consumed less food and so fewer calories. This self-imposed mild caloric restriction in iTRF animals was observed in both males and females. In contrast, iTRF for 5-6 days was reported not to have any effect on caloric intake over a 24-hour period (7). Recent studies have reported that caloric restriction and alteration of food intake timing can

result in remodeling of the gut microbiome (12, 59–61). These changes can occur quite rapidly in humans and laboratory animals in order to better adapt to sudden changes in diet composition (59). Changes in gut bacteria composition have been associated with regulation of fat storage (63, 64). This may in part explain why iTRF animals maintain body weight and body fat percentage despite decreased overall food intake. An interesting future study may investigate if there are changes in gut microbiota to facilitate absorption of select nutrients in an effort to maintain body weight despite decreased food intake in iTRF mice. Our findings in aTRF animals are consistent with the literature; TRF has been reported to result in decreased body fat with preservation of lean mass in healthy individuals (65–67). As our focus was on the cardiorenal effects of iTRF, we did not investigate potential mechanisms underlying changes in body composition in any animals. While the benefits of caloric restriction on metabolic health and lifespan are well-established, future studies will be needed to determine whether these benefits are maintained in the context of circadian disruption via mistimed feeding.

An additional potential explanation underlying the caloric restriction in iTRF mice is that peripheral zeitgebers do not override the light/dark cycle. iTRF animals may, after increased dark period activity, eat their fill during the first few hours of the light period and then enter their sedentary period as usual. These animals may be maintaining their normal activity rhythm by depriving themselves of some calories in exchange for maintaining their light/dark cycle of activity. Interestingly, despite their decreased 24-h food intake, iTRF animals maintain body weight and body composition similar to that of ad libitum fed animals. This matches previous findings that rats fed exclusively during the

light period demonstrate weight gain and adiposity similar to that of ad libitum animals (489). However, daytime fed mice on a high fat diet gained more weight compared to ad libitum fed mice animals (490). As the most common cause for mistimed feeding in humans, shift work is linked to increased body weight through several pathways, including disruptions in hormones and metabolism (491, 492). Additionally, individuals with late meal timings often find lower success in terms of dietary weight loss interventions (493). Collectively these findings suggest that the timing of food intake alone may contribute to increased body weight observed in some shift workers.

PWV is a measure of arterial stiffness and a predictor of cardiovascular disease risk. Increased PWV is also a predominant risk factor for chronic kidney disease (494). Excitingly, we found that only male iTRF animals exhibited a significant increase in PWV. This increase in PWV could be a result of increased endothelial dysfunction, resulting in increased inflammation and collagen deposition. Interestingly, there was no discernible increase in aortic fibrosis between any of our groups or between sexes based on our histological analysis. However, we have preliminary data suggesting that aortic wall thickness is increased in iTRF compared to ad libitum-fed mice which could account for the elevated PWV (Appendix Figure 18). Future studies in this model will need to investigate the effects of chronic TRF on vascular wall remodeling as well as endothelial function that may impact PWV independent of fibrosis.

We originally expected that iTRF males would become hypertensive. Thus, our findings appear to have captured changes in kidney fibrosis that are unrelated to

elevations in BP. The intake of food at a time when the body is not anticipating a nutrient load likely leads to what we could consider a type of stress on organ-specific metabolism. Furthermore, we observed increased PWV early in the absence of hypertension and even fibrosis that again, may be due to misaligned metabolism that allows for expansion of the vascular wall (495). Although none of our animals became hypertensive during this experiment, extension of the number of days of iTRF may capture the onset of hypertension in iTRF male mice. It is also possible that the movement back and forth between iTRF and ad libitum that many shift workers experience may exacerbate the response to circadian misalignment caused by our model of controlled feeding. As arterial stiffness is widely regarded as a surrogate marker of cardiovascular disease, our findings suggest that female mice may be better able to modulate to changes in timing of food intake. Additionally, males may be at an increased risk of developing CVD when food intake is mistimed with relation to the environment.

BP can be impacted by a variety of behavioral and neurohumoral factors, many of which have circadian rhythms. Zhang et al. previously showed that acute iTRF results in a misalignment of cardiorenal function – MAP rhythms are inverted relative to ad libitum fed male mice, but kidney excretory rhythms remain aligned with the light/dark cycle (7). We report that after eight weeks, chronic iTRF and aTRF mice demonstrate abrogated diurnal excretory patterns regardless of sex. This finding suggests that while the renal molecular clock can initially maintain functional synchrony with the central clock, a long-term circadian disruption induced by a peripheral zeitgeber such as food intake may be sufficient to affect peripheral tissues independent of the light cycle. In our chronic model

of iTRF, we observed disrupted diurnal patterns of excretion of aldosterone, endothelin-1 (ET-1), sodium, and potassium. This finding contrasted with previously published data in the acute iTRF model that showed maintenance of kidney excretory rhythms (7). While Zhang et al. observed maintained excretory rhythms despite inverted MAP rhythms in iTRF males, we observed disrupted diurnal excretory patterns that matched the disrupted diurnal MAP patterns in iTRF animals, regardless of sex. This finding suggests that while the renal molecular clock may remain in synchrony with the central clock during acute circadian disruption, chronic circadian disruption is likely sufficient to cause circadian misalignment between the kidney and the central clock.

Additionally, it remains unknown whether chronic circadian disruption via mistimed feeding affects GFR. Given that renal excretory rhythms of hormones that maintain electrolyte homeostasis and select electrolytes are lost, it is reasonable to posit that diurnal patterns of GFR may also be affected.

PERSPECTIVE

In the past decade, time-restricted feeding (TRF) has gained popularity as a tool for weight loss. TRF emphasizes timing food intake in alignment with circadian rhythms, leading some investigators to consider it a chrono-nutritional strategy. Consuming calories during the active phase as opposed to the inactive phase is associated with improvements in cardiometabolic health (2, 496). Many of the benefits of TRF are independent of reductions in body weight, but some studies have seen body weight reductions in both humans and laboratory animals (497). Recent studies from our laboratory demonstrated that in less than one week, restricting food access to the inactive period in mice is sufficient to result in inverted BP rhythms and cardiorenal misalignment.

Perturbations of circadian rhythms have been associated with an increased prevalence of cardiovascular disease. It is well established that BP is under circadian control. The Fernandez group has demonstrated in many publications that hypertension and disturbed BP circadian rhythm contribute to cardiovascular disease risk and kidney damage (498–500). Recent studies from our laboratory demonstrated that in less than one week, inducing circadian disruption by restricting food access to the inactive period in mice is sufficient to re-entrain BP rhythms and cause, cardiorenal misalignment, even in global Bmal1 knockout mice (7). The work in this thesis builds on the previous work by extending the circadian disruption model to 8-weeks and determining its effects on the cardiorenal system.

Our studies demonstrated that circadian disruption via mistimed feeding results in disrupted BP rhythms, decreased food intake without weight reduction, and sex differences in multi-organ fibrosis. The mechanisms behind these results remain unknown but create opportunities for this model to be used for further investigations. There are a variety of speculative mechanisms underlying the disruption of BP rhythms, ranging from aberrations in the hypothalamic-pituitary-adrenal axis to increases in sympathetic nervous tone (501, 502). One surprising finding was the decrease in food intake without weight reduction. Given ad libitum access to food during their feeding periods, iTRF mice appear to self-impose a caloric restriction after eight weeks. This is even more interesting when considering previous data from the Takahashi lab, which demonstrated that mice under caloric restriction self-impose a temporal restriction of food intake but do not lose weight compared to ad libitum fed mice (461). The study revealed that iTRF animals rapidly eat during the first hour of food access and again toward the end of the feeding period. Other studies have also determined that mistimed eating can increase body weight and cardiovascular disease risk (173, 497). Taken together, it appears that although iTRF animals are eating significantly less than their ad libitum or aTRF counterparts, the weight gain associated with mistimed food intake is sufficient to counteract any weight loss that might result from caloric restriction. This may be explained by increased storage of adipose tissue in other organs such as the liver or kidneys or by remodeling of the gut microbiota to facilitate increased uptake of specific nutrients. This has been observed in humans undertaking intermittent fasting schemes, such as those that fast for Ramadan (503, 504).

The biological variable of time is arguably one of the most important variables to be considered in biomedical research. Any research that is meant to translate into a treatment for cardiovascular diseases in humans must consider that at least 25% of the US workforce participates in non-daytime shift work. Thus, experiments must be designed in a way that considers the complexity of circadian rhythms in gene expression and timing of environmental cues. The circadian system is much more complex than the four 'core' clock genes and in vivo experiments to determine the effects of nearly any physiological or metabolic aspect must take this into consideration. The circadian system is made of the central and peripheral clocks, residing within the SCN and tissues and organs, respectively.

There were a number of strengths to this study. One strength is the inclusion of both male and female animals. While this study may have progressed further if using just males, there are a number of exciting phenotypes with sex differences that would have gone undiscovered. With the nephrology and circadian physiology fields emphasizing the consideration of biological sex in experimental design, the findings from this study demonstrate the importance of the variable of sex. Another strength to the study is its length. C57BL/6J mice are notoriously hearty and, as such, often do not display overt phenotypes unless treated with a pharmacological agent or placed under long-term protocols such as a high fat diet. Although acute iTRF elicited a cardiorenal phenotype in one week, the model used in this study is a chronic example of circadian disruption via mistimed feeding (7). As such, there are a number of strong histological phenotypes that arise after 10 weeks. All of the animals in this study were fed a normal salt, normal fat

chow, which demonstrates the importance of timing of food intake rather than adding the variable of diet composition. One limitation of this study is the length of the feeding and fasting periods. Although laboratory mice are diurnal and have tightly regulated 'active' and 'inactive' periods, the restriction of food to the entire 12-hour lights on or lights off period is not highly translational to human food intake patterns.

Our studies have reported a sex difference in outer medullary fibrosis and arterial stiffness, suggesting that circadian disruption via mistimed feeding poses a lesser cardiovascular risk to females than males. However, both sexes demonstrated disrupted diurnal MAP patterns, which may be causative or preliminary to the observed fibrosis. While the results of this project contribute a wealth of new knowledge to the cardiorenal field, there are still countless unknown physiological and molecular mechanisms behind the observed phenotypes of the iTRF and aTRF models. As today's society relies more and more heavily on non-daytime shift workers, a comprehensive understanding of the effects of circadian misalignment on peripheral tissues is sure to prove invaluable in the years and decades to come.

It has been previously proposed that in mammals, food processing and anticipation of food intake is the main purpose of circadian gene expression in peripheral clocks (462). The rhythmicity of gene expression is output from peripheral zeitgeibers that, under normal circumstances, are synchronized and governed by the central pacemaker in the SCN (Figure 15A). However, when food availability is in a long-term temporal conflict with the activity rhythm dictated by the SCN, peripheral clocks become uncoupled from the SCN and are more strongly entrained by peripheral zeitgeibers such

as food intake (Figure 15B). The misalignment of eating patterns with light and even activity patterns increase several risk factors for cardiovascular and kidney disease given the disrupted BP patterns and renal fibrosis observed in the current study. These findings support the idea that behavioral modifications including meal timing may be able to reduce risk of cardiovascular disease in shift workers. Of note, we observed that this risk appears greater in males compared to females. Further, the disconnect between caloric intake and body weight further supports risk of a metabolic issue in individuals that eat during late/night hours and provides further rationale for more studies in shift workers and others engaged in “atypical” meal timing.

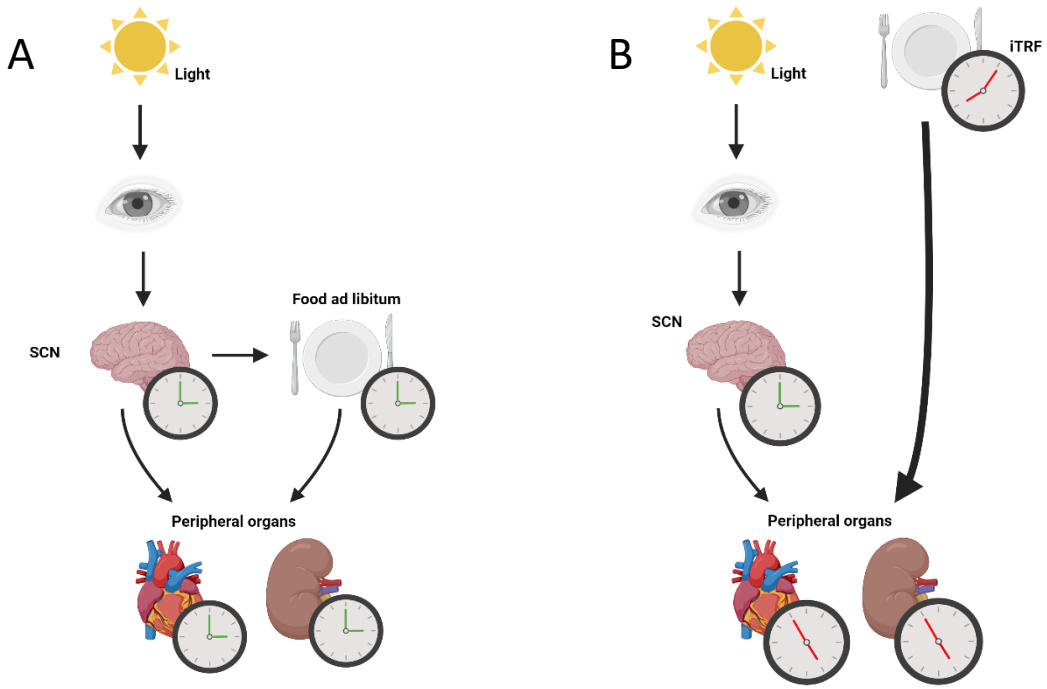


Figure 14. Hypothetical model of the entrainment of peripheral clocks. When food is available ad libitum or during the normal activity phase, the SCN governs synchronization of the peripheral tissue oscillators (A). When food is only available during the inactivity phase, timing of food intake acts as a dominant zeitgeber on the oscillators within peripheral tissues (B). Adapted from "Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus" by Damiola et al., 2000, *Genes and Development*, 14:2954-2961. Adapted with permission. Figure created with BioRender.

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APPENDIX A:

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

MEMORANDUM

DATE: 14-Feb-2023

TO: Pollock, David

FROM: Shannon M. Bailey

Shannon M. Bailey, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 14-Feb-2023.

Protocol PI:	Pollock, David
Title:	Integrating Novel Mechanisms Controlling Sodium Excretion and Blood Pressure: Project 1- Circadian Control of Sodium Excretion
Sponsor:	National Heart, Lung, and Blood Institute/NIH/DHHS
Animal Project Number (APN):	IACUC-20721

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

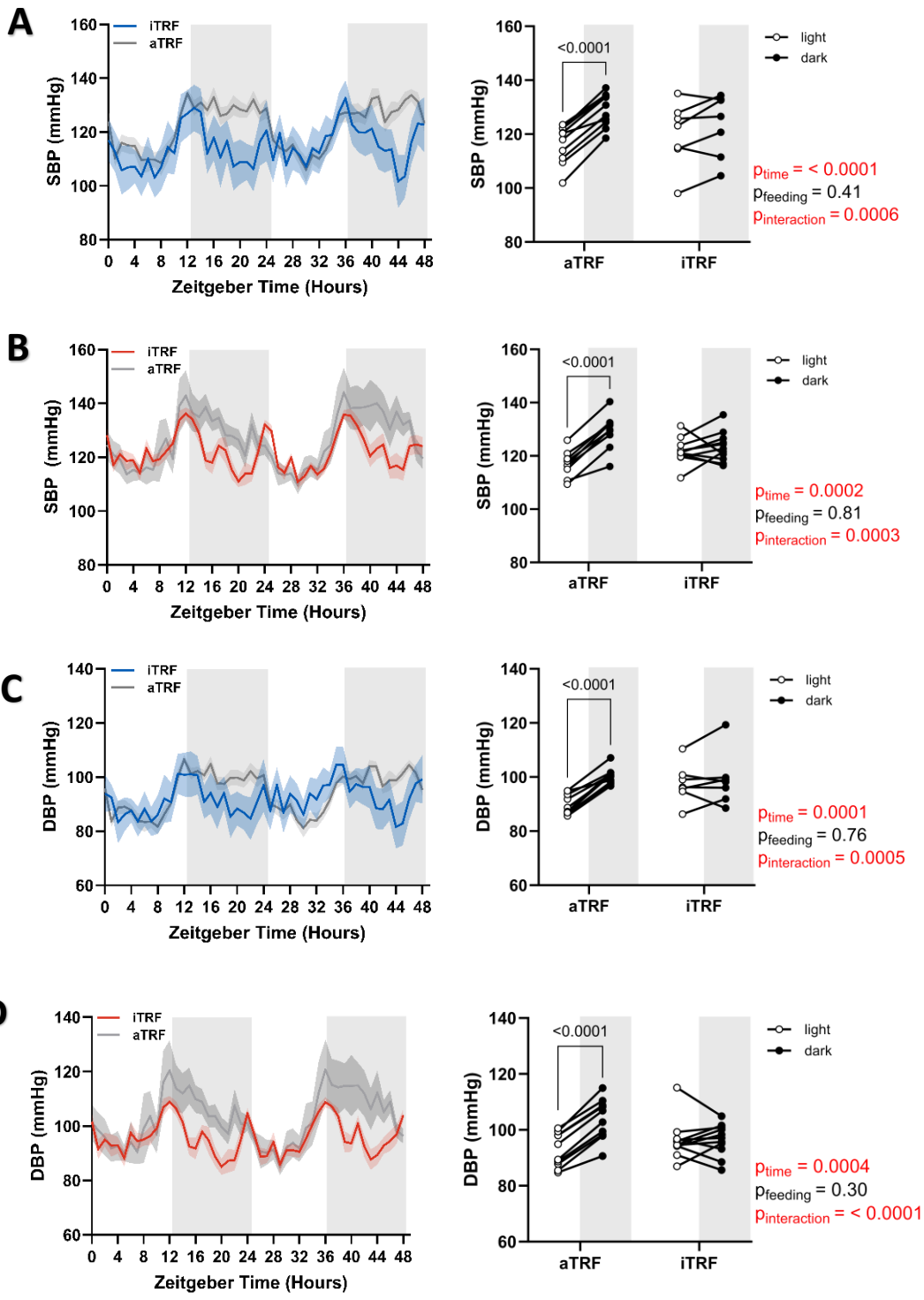
This protocol is due for full review by 13-Feb-2026.

Institutional Animal Care and Use Committee (IACUC)

403 Community Health on 19th | 933 19th Street South

APPENDIX B:

SYSTOLIC AND DIASTOLIC BLOOD PRESSURE


A
iTRF
aTRF
SBP (mmHg)
Zeitgeber Time (Hours)
<0.0001
light
dark
aTRF
iTRF
p_{time} = < 0.0001
$p_{feeding}$ = 0.41
$p_{interaction}$ = 0.0006
B
p_{time} = 0.0002
$p_{feeding}$ = 0.81
$p_{interaction}$ = 0.0003
C
DBP (mmHg)
p_{time} = 0.0001
$p_{feeding}$ = 0.76
$p_{interaction}$ = 0.0005
D
p_{time} = 0.0004
$p_{feeding}$ = 0.30
$p_{interaction}$ = < 0.0001

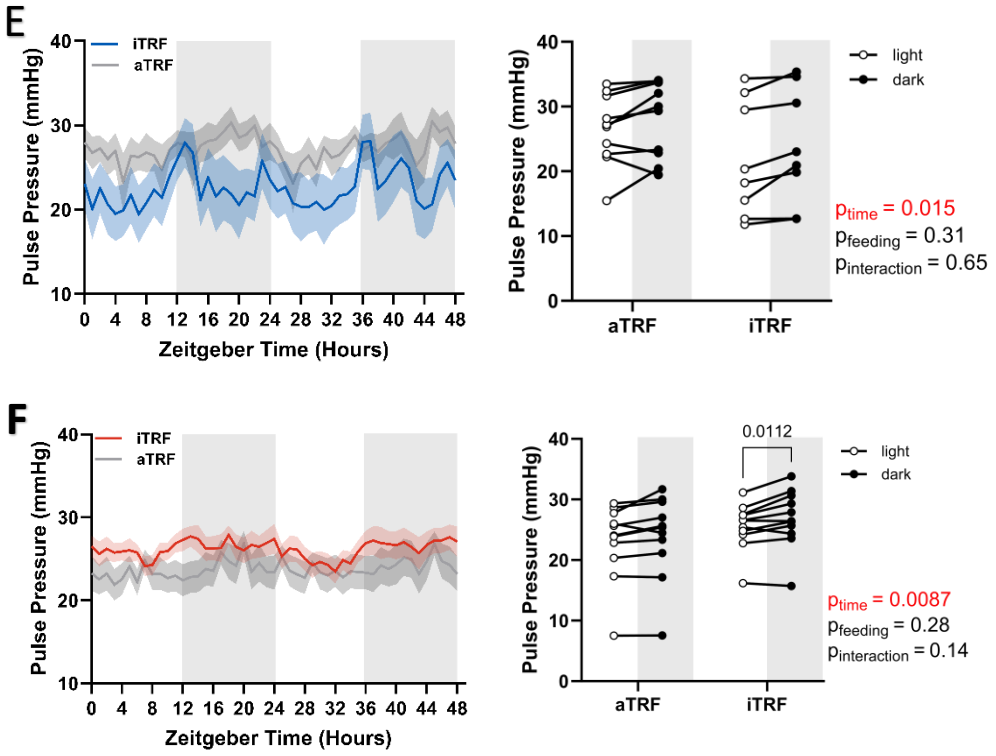
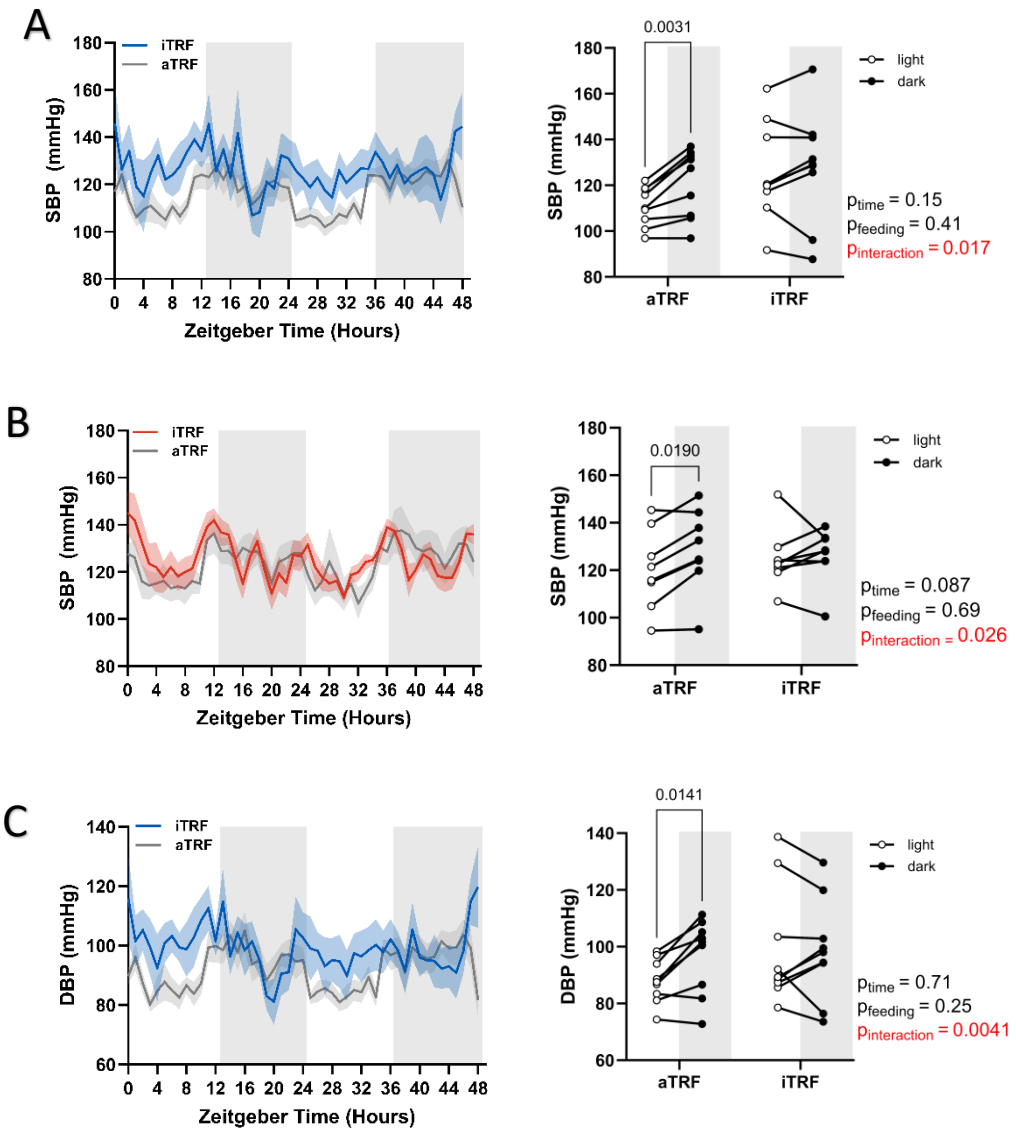


Figure 15. Disruption of diurnal systolic and diastolic BP patterns after 4 weeks of iTRF. Systolic BP (A,B), diastolic BP (C,D), and pulse pressure (E,F) during days 29 and 30 of aTRF (gray) or iTRF in male (blue) and female (red) mice. Gray shaded boxes indicate dark periods. Data are shown as 48-h trace with shading representing error bars. Day/night averages of the days are also shown. Data are means ± SEM. Feeding schedule, time of day, and feeding schedule x time of day interaction effects were assessed by two-way ANOVA with Šídák's multiple comparisons test. Significant p values are indicated in red with individual comparisons noted in each panel. n = 7-11 mice/group. SBP, systolic blood pressure; DBP, diastolic blood pressure.


A
iTRF
aTRF
SBP (mmHg)
Zeitgeber Time (Hours)
0.0031
light
dark
aTRF
iTRF
p_{time} = 0.15
$p_{feeding}$ = 0.41
$p_{interaction}$ = 0.017
B
0.0190
p_{time} = 0.087
$p_{feeding}$ = 0.69
$p_{interaction}$ = 0.026
C
DBP (mmHg)
0.0141
p_{time} = 0.71
$p_{feeding}$ = 0.25
$p_{interaction}$ = 0.0041

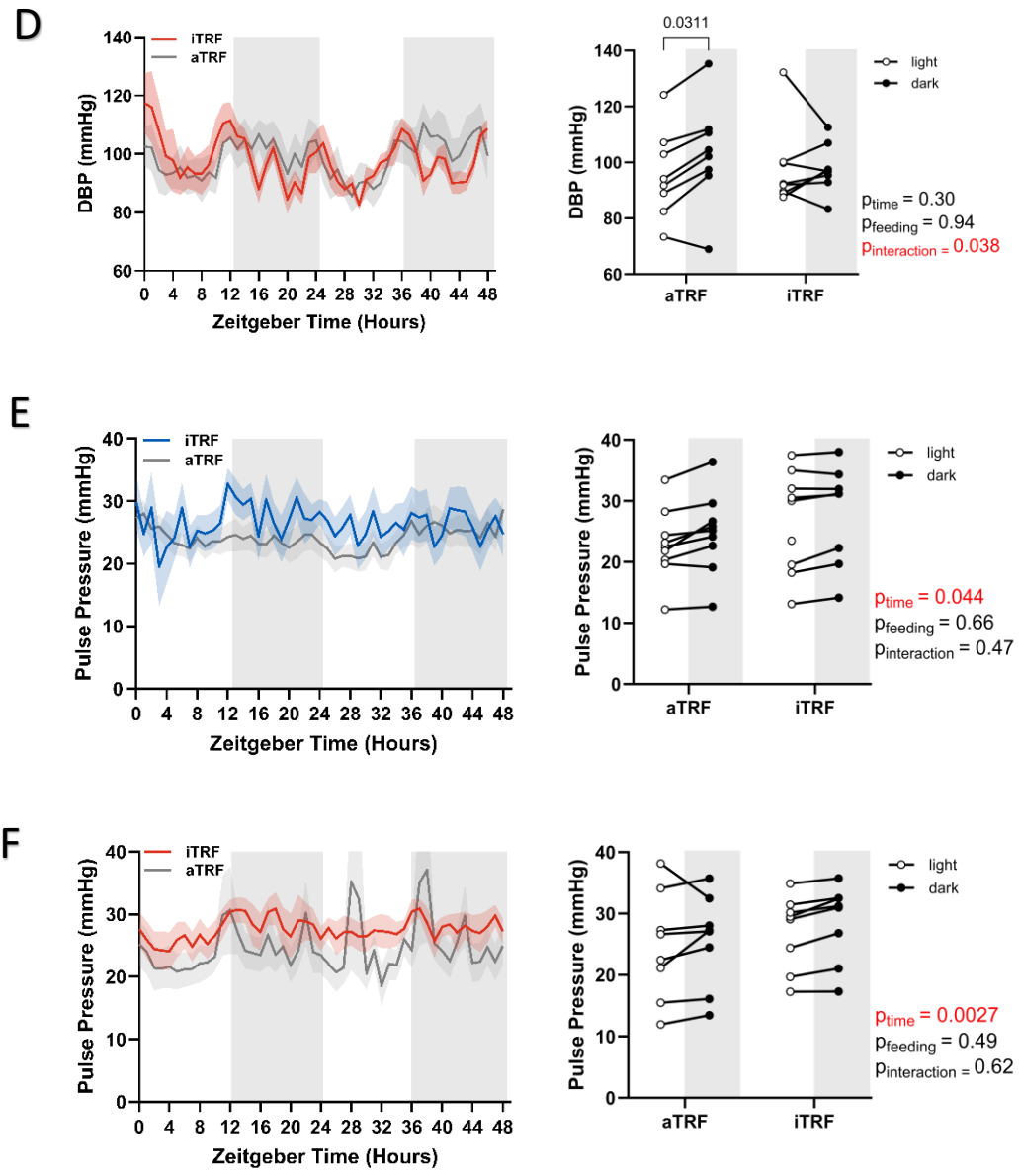


Figure 16. Persistent disruption of diurnal systolic and diastolic BP patterns after 4 weeks of iTRF. SBP (A,B), DBP (C,D), and pulse pressure (E,F) during days 59 and 60 of aTRF (gray) or iTRF in male (blue) and female (red) mice. Gray shaded boxes indicate dark periods. Data are shown as 48-h trace with shading representing error bars. Day/night averages of the days are also shown. Data are means ± SEM. Feeding schedule, time of day, and feeding schedule x time of day interaction effects were assessed by two-way ANOVA with Šídák's multiple comparisons test. Significant p values are indicated in red with individual comparisons noted in each panel. n = 7-11 mice/group.

APPENDIX C:

PRELIMINARY: AORTIC FIBROSIS AND WALL THICKNESS

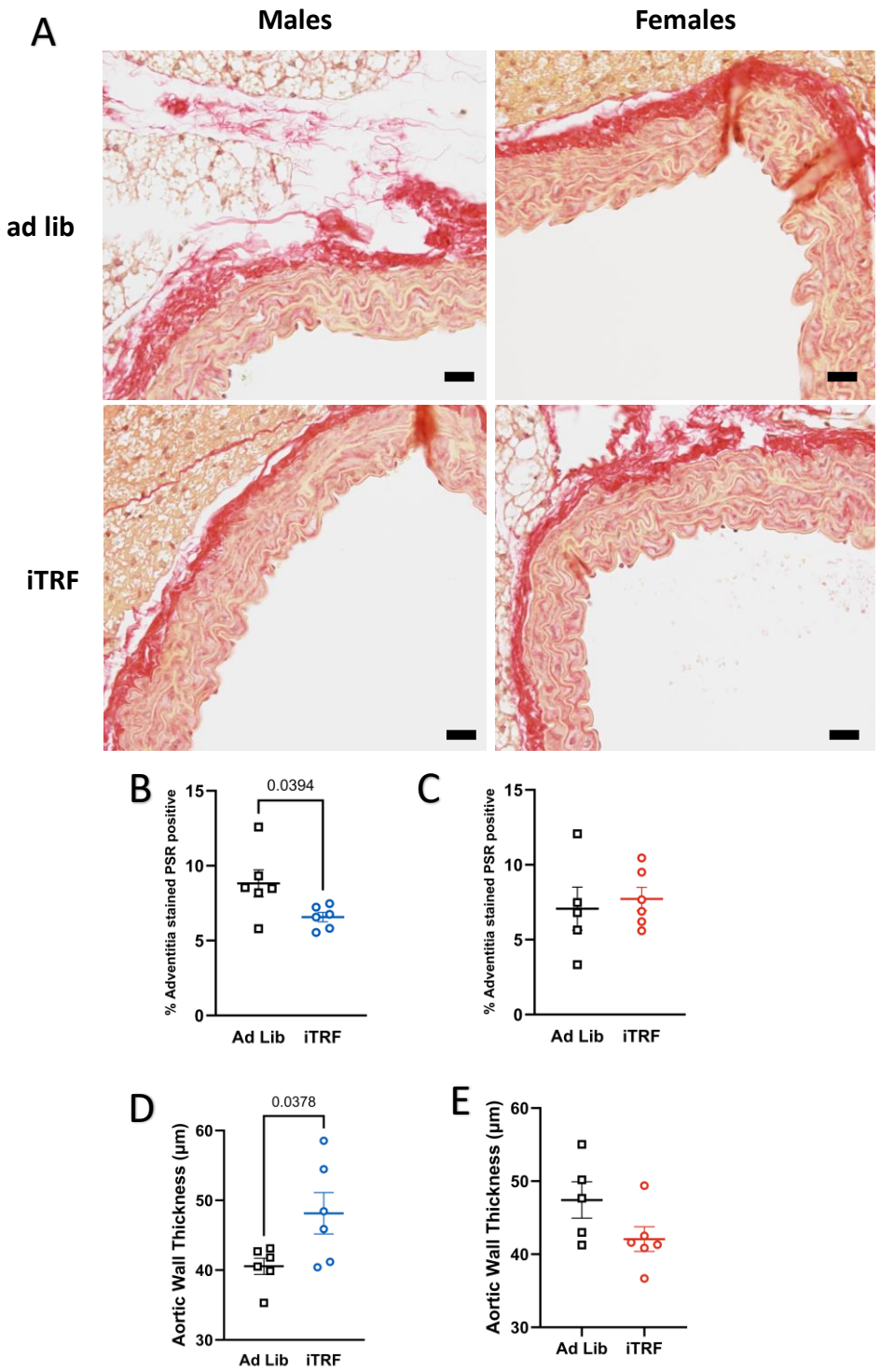

A
Males
Females
ad lib
iTRF
B
0.0394
% Adventitia stained PSR positive
15
10
5
0
Ad Lib
iTRF
C
% Adventitia stained PSR positive
15
10
5
0
Ad Lib
iTRF
D
0.0378
Aortic Wall Thickness (µm)
60
50
40
30
Ad Lib
iTRF
E
Aortic Wall Thickness (µm)
60
50
40
30
Ad Lib
iTRF

Figure 17. Sex differences in aortic fibrosis. Representative images of picrosirius red staining of aortas from male (left) and female (right) mice after ten weeks of feeding intervention (A) (magnification: 40x, scale bar = 20μm). Quantification of aortic fibrosis represented as percent of total image field stained positive for PSR in male (B) and female (C) mice. Quantification of aortic wall thickness in male (D) and female (E) mice. Data are means ± SEM. Student's t-tests for unpaired data were used in B-E. n = 5-6 mice/group.