IMPACT OF CIRCADIAN MISALIGNMENT ON EXTRACELLULAR VESICLES IN INACTIVE ULCERATIVE COLITIS

BY

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Malia R. Gasteier

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For my dad, who I know is looking down with the biggest smile.

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LIST OF ABBREVIATIONS

GIT	Gastrointestinal Tract
IBD	Inflammatory bowel disease
UC	Ulcerative colitis
HC	Healthy control
EV	Extracellular vesicle
GPA33	Glycoprotein A33
Tsg101	Tumor susceptibility gene 101
AUC	Area under the curve
SCN	Suprachiasmatic nucleus
PK2	Prokineticin 2
AVP	Arginine vasopressin
VIP	Vasoactive intestinal peptide
TGF_{α}	Transforming growth factor alpha
SCFAs	Short chain fatty acids
2BAs	Secondary bile acids
MVBs	Multivesicular bodies
SWSD	Sleep work shift disorder

ABSTRACT

Introduction

Several bodily functions are regulated by circadian rhythms over the course of a 24-hour oscillation period within living organisms. The central clock is located in the suprachiasmatic nucleus (SCN) and simultaneously orchestrates peripheral circadian clocks in every organ system, including, but not limited to, the gastrointestinal tract (GIT). Recent work has shown evidence that patients with inflammatory bowel diseases (IBD), including ulcerative colitis (UC), have higher levels of circulating extracellular vesicles. Given the already existing relationship between IBD and circadian rhythm, this study aimed to address the potential relationship between circulating extracellular vesicles in inactive UC subjects under circadian misalignment.

Methods

9 subjects, 4 UC and 5 HC were recruited into the study. All UC subjects were inactive (partial Mayo Score ≤ 1) and on stable medications with no flares for the last 3 months. For two weeks prior to entry into the circadian lab all subjects were on a prescribed regular sleep schedule. Subjects were then kept in the circadian lab for 6 days with strict control of their light/dark cycle. All subjects underwent baseline blood draw, occurring every 2 hours over 24 hours. This blood draw protocol was repeated following the 3 days of simulated night shift sleep scheduling. Extracellular vesicles were extracted from each blood sample at both baseline and post shift and utilized in western blots to determine the protein presence of human exosome markers GPA33 and tsg101with antibodies normalized to tubulin. Western blots were then analyzed using ImageJ software, and statistical analysis was performed using R software and GraphPad Prism 9.

Results

It was found that none of the subjects demonstrated clear rhythmic oscillation in exosomes regardless of alignment or misalignment. However, GPA33 showed to be significantly lower in UC compared to HC regardless of alignment or misalignment ($P=0.001$). Area under the curve (AUC) data for GPA33 did not show significance for HC vs. UC or in alignment vs. misalignment ($p>0.05$). Tsg101 was significantly increased in both HC and UC from alignment to misalignment ($p<0.001$). AUC data for tsg101 confirmed significant increase of tsg101 following misalignment in both groups ($p=0.03$).

Discussion

The results of this preliminary study highlight the importance of studying environmental factors that influence circadian timing, including night shift work, which may contribute to flares of IBD. Given the observed increase in total exosome marker presence (tsg101) following circadian misalignment, further investigation and characterization of these extracellular vesicles is warranted on a larger study scale.

CHAPTER ONE

INTRODUCTION

Circadian Rhythm

Throughout the evolution of living organisms, one thing has always been consistent: the sun. The earth's rotation around its axis occurs over 24-hours, with 12 hours of sunlight exposure and 12 hours of darkness. To best maintain homeostasis, organisms evolved internal clocks in the brain that regulate various functions to optimize alertness and sleep relative to light changes in the environment (Reddy et al., 2022). This process became referred to as circadian rhythm, defining any biological or physiological variation that takes place under a 24-hour cycle (Vitaterna et al., 2001). Evidence of circadian rhythm dates back millennia, with notable observations first seen as far back as the 1700's. In 1729, astronomer Jean Jacques d'Ortous de Mairan noted that the mimosa plant, when placed in a light-tight dark room, would unfold its leaves during the day and would close its leaves at night (Huang, 2018). Upon further observation, it was noted that these plants continued their opening and closing leaf patterns even in complete darkness. This suggested that the plants were modulated by something endogenous rather than solely the environment (Dibner & Schibler, 2017). This seemingly simple discovery pioneered what we know as chronobiology today, with much of our biological processes' dependent on the circadian rhythm.

The Molecular Clock

Current research in circadian physiology has confirmed that circadian rhythms are indeed endogenous and are composed of 24 -hour oscillators in the suprachiasmatic nucleus (SCN) which is located in the anterior hypothalamus and is known as the central clock, as well as peripheral clocks in cells and tissues across the body (W. Huang et al., 2011). The peripheral

clocks in the body are alerted via transmitters from the SCN by outputs such as PK2, glutamate, AVP, VIP, and TGF$_{\alpha}$ to name a few (Dibner et al., 2010). However, recent literature has found that peripheral clocks can function independently of the central clock, though they are still controlled by the light-dark cues in the SCN (Richards & Gumz, 2012). Researchers wanted to determine just how circadian timing was expressed and orchestrated, which placed the ball in the court of genetics. The molecular genetics of the SCN were first studied in a *Drosophila melanogaster* model to further understand circadian rhythm at a deeper level (Hastings, 1998). In this *Drosophila* model, it was found that there were alterations of the period (per) gene that changed the periodicity of locomotor activity. It was later discovered that this mechanism was operated via a negative feedback loop, which means that transcription factors were driving their own repressors (Trott & Menet, 2018). This mechanism of negative feedback loops allows for self-sustained circadian oscillations regardless of external influence (Pett et al., 2018). These negative feedback loops at the transcriptional level mediate central clock genes in a diurnal manner that include but are not limited to: *muscle and brain ARNT like-1 (Bmal1), muscle and brain ARNT like-2 (Bmal2), circadian rhythmic motion output cycle stagnates (Clock), cryptochrome 1 (Cry1), cryptochrome2 (Cry2), period 1 (Per1), period 2 (Per2), period 3 (Per3)* and *neurosomal PAS domain protein (Npas2)* (Luo et al., 2021). It has been shown in numerous studies that melatonin is involved as a key player in the regulation of these listed clock genes, and therefore enables the synchronization of central and peripheral clocks (Charrier et al., 2017). Melatonin is the primary hormone produced by the pineal gland in response to dark exposure. It is a derivative of serotonin that is produced in the tryptophan-serotonin biosynthetic pathway as a response to light exposure signaling from the SCN (Vasey et al., 2021). Melatonin is a key player in the orchestration of circadian rhythm, as its serum concentration drastically shifts from

low during the day (10-20 pg/ml) to high at night (80-120 pg-ml), indicating that the regulation of clock genes is also modulated in the same fashion (Tordjman et al., 2017). As melatonin and the SCN are both highly sensitive to light, peripheral clocks in the body are controlled in an analogous way (Pevet & Challet, 2011). This information suggests that the timing of light exposure is incredibly important in its influence on circadian rhythm, which can easily be disrupted by several lifestyle factors in humans that are often centered around light exposure timing.

Circadian Misalignment

Circadian alignment is the process in which different systems in the body follow a circadian rhythm that is synchronized to the SCN in the brain. The circadian rhythm is controlled by various lifestyle factors such as light and dark exposure, food/drink consumption, exercise/movement, and temperature (Battaglin et al., 2021). However, when these lifestyle factors become disrupted, circadian rhythm becomes misaligned, which is referred to as a circadian rhythm disorder. In modern society, one of the most common alterations is shift work sleep disorder (SWSD). SWSD occurs as a result of altering the sleep wake cycle by shifting the time when one should be sleeping to a time when light, the central clock and social ques are signaling that the individual should be awake (Zee, 2019). Research has found that by flipping the sleep wake cycle in cases like night shift workers, the secretion of melatonin during the night is blocked by light exposure, resulting in the desynchronization of clock genes. This desynchronization has been found to influence numerous diseases such as cancer, cardiovascular disease, and other metabolic disorders (Wang et al., 2011). Because circadian rhythm is expressed in peripheral cells, which have a high presence in the gastrointestinal tract (GIT), it is

important to understand how alterations and misalignment in circadian rhythm impact GIT inflammation and GIT related disease progression.

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is characterized by chronic intestinal inflammation (Zhang, 2014). IBD presents itself in two major forms, which are Crohn's Disease (CD) and ulcerative colitis (UC). Clinically, IBD is characterized by recurring and relapsing episodes of abdominal pain, diarrhea, bloody stool, weight loss and ulceration (Guan, 2019). Research has found that the pathogenesis of IBD likely stems from a culmination of factors including genetics, the environment, the intestinal microbiota composition, and individual immune responses (Guan, 2019). Though the direct causation of IBD is still not fully understood, current data is suggesting that it is linked to an inappropriate and continuing inflammatory response to otherwise commensal microbes in a susceptible host (Khor et al., 2011). This occurs when the innate immune response to intestinal microbes and pathogens is deregulated and there is increased permeability to the epithelial layer within the GIT (Laukoetter et al., 2008). In a healthy individual, the lamina propria in the intestine contains immune cells such as anti-inflammatory and proinflammatory mediators that work in sync to minimize the entry of pathogens. However, in individuals with IBD, the lamina propria is disrupted, resulting in an increase in proinflammatory T-cell subgroups that increase cytokine and chemokine production. This results in a continuous cycle of inflammation, indicative of a highly permeable epithelial layer (Abraham & Cho, 2009). When the GIT becomes permeable, it allows for things like bacteria, harmful metabolites, and other small molecules to "leak" through the gut barrier and into the blood stream, resulting in widespread systemic inflammatory responses (Obrenovich, 2018). The gut barrier controls millions of microorganisms, which consist of bacteria, fungi, and viruses that

each play unique roles in their interactions with host immune systems, and alterations of these microbiota are associated with both forms of IBD (Scaldaferri et al., 2012). The microbiome composition of the GIT can be altered in a variety of ways, with one being circadian misalignment (Mashaqi & Gozal, 2020). Circadian misalignment directly influencing the composition of the microbiome, therefore, directly influences the integrity of the intestinal barrier, resulting in the strong relationship between circadian misalignment and IBD (Swanson et al., 2021).

Ulcerative Colitis

Ulcerative colitis is an idiopathic form of inflammatory bowel disease (IBD) and is characterized by the chronic inflammation of mucosa within the GIT and is specific to the colon and can extend to proximal parts of the large intestine (Abraham & Cho, 2009b). The clinical course of the disease occurs through exacerbation and remission phases. Diagnosis of UC is dependent on clinical presentations, radiological and histological features, and endoscopic evaluation (Conrad et al., 2014).

It is currently estimated that there are 156-291 cases per 100,000 persons each year worldwide, with a higher clinical presence in westernized environments (Lynch & Hsu, 2022). Western lifestyle factors that are involved in the progression of UC are the western diet, medications, and lifestyle factors such as lack of exercise and poor sleep (Du & Ha, 2020). Previous research has found that patients with UC often suffer from abnormal sleep/wake cycles (Ali & Orr, 2014). Sleep disturbance is one of the most common reports of IBD sufferers, with prevalence of 47-82% of patients reporting nonrestorative sleep and night-time sleep awakenings (Rozich et al., 2020). Lab work has also shown longer sleep latency, reduction in stage 3 sleep and more awakenings during sleep utilizing electroencephalographic recordings when compared

to healthy controls (Ranjbaran et al., 2007). These findings, as well as several other studies suggest that there is relationship between the circadian rhythm and exacerbation of disease states in both active and inactive cases of UC.

Gut-Brain Axis

Many people have begun to refer to the gut as the "second brain" in humans, which is being backed by modern research. The GIT and the brain are directly linked via a network known as the gut-brain axis. The GIT is similar to the brain due to the presence of neuronal cells within the submucosal plexus and myenteric plexus, making it similar in connection and communicative abilities (Lebouvier et al., 2009). The gut-brain axis acts as a mechanism of communication between the GIT and the brain via several mechanisms such as the immune system, the enteric nervous system, and microbial metabolites (Cryan et al., 2019). Currently, some of the best described intermediates between the gut and the brain are short-chain fatty acids (SCFAs), secondary bile acids (2BAs) and tryptophan metabolites (Osadchiy et al., 2019). Recent research has found that there are key players in the transportation of these intermediates, with a new interest rising in extracellular vesicles. Understanding the transportation of these intermediates is a key in understanding how they may be involved in the progression of diseases related to the GIT.

Extracellular Vesicles

An extracellular vesicle (EV) is a lipid-bound vesicle that is secreted by cells into the extracellular space. Over the course of evolution, organisms have largely conserved the ability to secrete several types of extracellular vesicles. Subtypes of extracellular vesicles are differentiated by their cargo content, function, biogenesis, release pathways and size (Doyle & Wang, 2019). The subtypes of extracellular vesicles include ectosomes and exosomes. The important subtype

of interest in this research is exosomes due to abundance of accepted exosome marker components, their size, and their process of biogenesis (Isaac et al., 2021). They are typically 30-150nm in size and are secreted primarily by immune cells. They are specialized cargo carriers of several components such as nucleic acids, proteins, and lipids, all of which are essential players in intracellular communication (Chen et al., 2021).

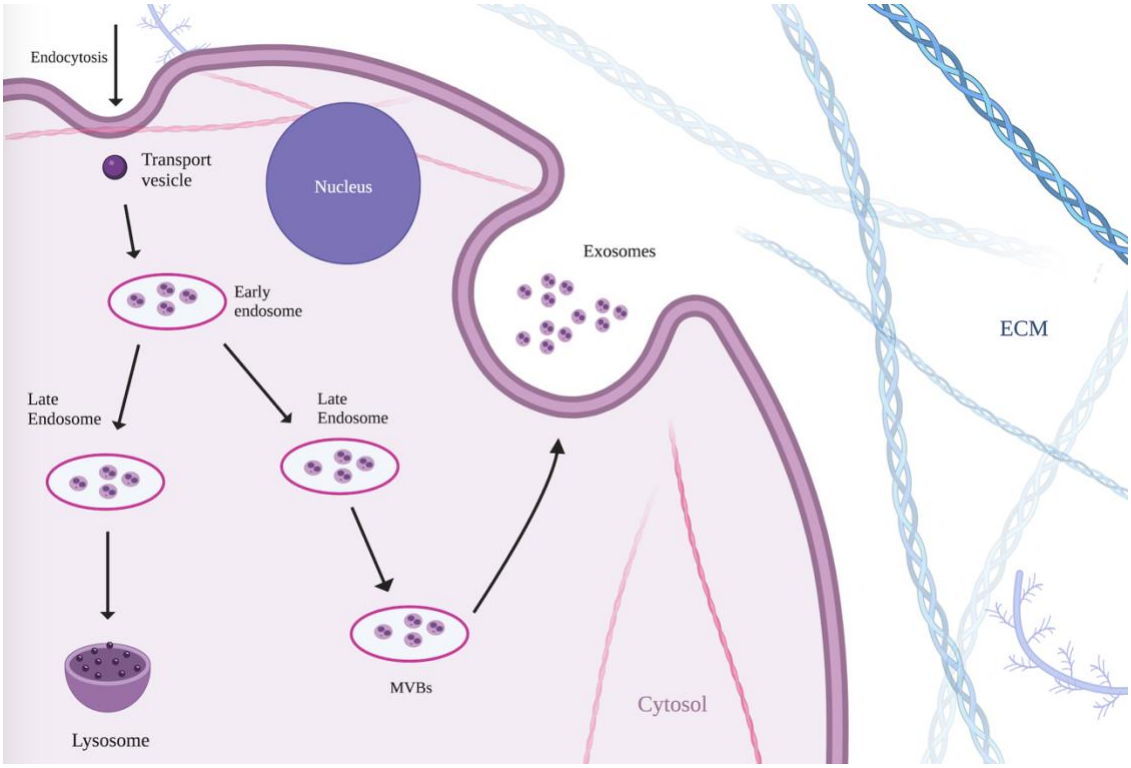


Figure 1. Exosome biogenesis pathway.

The biogenesis of exosomes shown in Figure 1 involves endocytosis of invaginated endosomes from the plasma membrane via transport vesicles (Kalluri & LeBleu, 2020). These then become early-stage endosomes, that eventually mature into late endosomes. Via fusion, late endosomes can form multivesicular endosomal structures, called multivesicular bodies (MVBs),

via the inward invagination of the endosomal membrane, or are discarded to the lysosome (Jella et al., 2018). The endosomes that are converted into MVBs can then partake in an exocytotic process where they are able to fuse with the plasma membrane, allowing for the release of exosomes into the extracellular space (Zhang et al., 2019). The release of these exosomes is what allows for intercellular communication by contact with the cargo of the carrying vesicle via fusion or endocytosis to a recipient cell (Abels & Breakefield, 2016). The ability for exosomes to deliver various forms of bioactive molecules across the body highlights the importance in studying the roles that exosomes play in the progression of physiological processes and disease progression (Frühbeis et al., 2012). The two primary exosome biomarkers of interest for this study are GPA33 and tsg101. GPA33 is short for Glycoprotein A33 and is an intestinal epithelium-specific cell surface marker on exosomes. It is a member of the CTX transmembrane proteins, and it is likely involved in cell-cell adhesion in the gastrointestinal tract (Williams et al., 2015). This performative role of GPA33 allows it to influence the intestinal barrier, with heightened observations of GPA33 found in more than 95% of primary and metastatic tumors in colorectal cancer (Lopes et al., 2020). Because of its role in the intestinal barrier function, it is likely to also play a role in the progression and worsening of IBD. Tsg101, on the other hand, is a more commonly used total marker of exosomes and is short for tumor susceptibility gene 101 protein. Tsg101 is a specific protein associated with the endosomal sorting complex that EVs use for transport. Studies have shown that tsg101 is significantly upregulated in tumor cells in colorectal carcinoma, suggesting that tsg101 is involved in more aggressive tumor metastasis and progression of cancer (Gheytanchi et al., 2021). Similar to GPA33, the role of tsg101 in intestinal inflammation is prominent and likely possesses a relationship to IBD and disease progression.

Research Aims

The synchrony of biological functions to the 24-hour day-night cycle, known as the circadian rhythm, has been known to influence the health and wellbeing of humans and other organisms. In recent years, research has identified a relationship between the circadian rhythm and IBD, a group of chronic inflammatory diseases of the GI tract. More specifically, it has been observed that certain exosomes can be influenced by the circadian rhythm and may in turn have direct impacts on IBD.

The overall purpose of this study is to further explore the relationship between exosomes and UC under circadian misalignment.

1. The first Aim of this study was to determine whether circulating total (tsg101) and gut (GPA33) exosomes display oscillation following night shift simulated circadian misalignment in both healthy controls and inactive ulcerative colitis subjects. It was hypothesized that following the misalignment protocol, subjects with ulcerative colitis would experience a more drastic disruption in exosome oscillation over a 24-hour period than heathy controls.

2. The second Aim of this study was to identify how total and gut specific exosomes in healthy controls and inactive UC subjects change after 3 nights of simulated night shift (circadian misalignment). It was hypothesized that both HC and UC subjects would demonstrate an increase in exosomes after the simulated night shift when compared to baseline.

Western blots were utilized for exosome detection and statistically analyzed for both Aims.

CHAPTER TWO

MATERIALS AND METHODS

Subject Demographics and Clinical Characteristics

9 total subjects were utilized in this study, 5 healthy controls (HC) and 4 with inactive UC (Mayo Score ≤ 2). Subjects enrolled into this study underwent a full medical history and physical examination during a pre-study screening visit.

Inclusion criteria for HC subjects: (1) M/F, (2) 18-50 y/o, (3) ± 3y sex, race, and BMI (3 kg/m2) match with UC subject, (4) no clinical evidence of any medical illness, and (5) have normal psychological evaluation based on question responses and negative drug screen. Inclusion criteria for UC subjects: (1) M/F, (2) 18-50 y/o, (3) inactive disease (Mayo Score ≤ 2), (4) stable medications with no disease flares for the > 3 months, (5) left-sided UC (Montreal E1 or E2), and (6) have normal psychological evaluation based on question responses and negative drug screen. Baseline demographics for enrolled subjects are shown in Table 1.

HC participants were excluded from enrollment if they met any of the following exclusion criteria: (1) History of drug abuse, (2) gastrointestinal (GI) surgery, (3) GI diseases, or (4) systemic diseases such as (a) renal (creatine>1.2 mg/dl), (b) liver, (c) cardiac or (d) diabetes, (5) antibiotic usage within the last 12 weeks, (6) use of probiotic supplements except for yogurt in the last 4 weeks, (7) shift work in the last 6 months, (8) atypical American diet with daily fiber ≥ 16 grams or daily saturated fat ≤ 11 grams by food frequency questionnaire, (9) chronic use of NSAIDS unless a 3-week washout period is done, (10) chronic alcohol use unless a 3 week washout period is done, (11) significant depression (score ≥ 14 BDI), (12) significant anxiety (score ≥ 40 STAI), (13) regular use of medications that impact intestinal integrity such as (a) NSAIDS, (b) antibiotics, (c) beta blockers, (d) psychotropic medications, (e) hypnotics and (f)

exogenous melatonin products during 4 weeks prior to study, (14) people who crossed more than 2 time zones in the previous month, (15) inability to sign an informed consent form, (16) have children under 6 m/o. UC participants were excluded from enrollment if they met any of the following exclusion criteria: (1) patients with other forms of colitis such as Crohn's disease, (2) patients with active UC (Mayo Score ≥ 2), (3) pancolonic UC (colitis past the splenic flexure, Montreal E3), (4) gastrointestinal surgery, (5) other GI or systemic diseases, (6) shift work in the last 6 months, antibiotic usage within the last 12 weeks, (7) patients who have used anti-diarrheal agents within 3 days of the study, (8) prednisone use in last 30 days, (9) significant depression (score ≥ 14 BDI), (10) significant anxiety (score ≥ 40 STAI), (11) use of probiotic supplements except for yogurt in the last 4 weeks, (12) intentional change in diet, (13) chronic use of NSAIDS unless a 3-week washout period is done, (14) chronic use of alcohol unless a 3-week washout period is done, (15) have children under 6 m/o.

Table 1. Demographics of subjects at enrollment.

Disease Type	**UC**	**HC**
Number of participants	4	5
Age Range (years)	21-41	19-35
Gender		
Male	1/4, 25%	4/5, 80%
Female	3/4, 75%	1/5, 20%
Race		
African American	2/4, 50%	1/5, 20%
Hispanic or Latino	1/4, 25%	1/5, 20%
Caucasian	1/4, 25%	2/5, 40%
Other	0/4, 20%	1/5, 20%
UC- Ulcerative colitis; HC- Healthy control		

All subjects enrolled in the study provided written informed consent at the time of recruitment, and all study procedures were approved by the Rush University Medical Center Institutional Review Board. The study was registered on: Clinicaltrials.gov #NCT05180279.

Study Design

9 subjects, 4 UC and 5 HC were recruited into the study. For two weeks prior to entry into the circadian lab all subjects were on a prescribed regular sleep schedule. All experimental procedures took place over a 7-day lab evaluation, with strict control over their light/dark cycle exposure. For this specific study, day 1 was an allotted adaptation day to allow for lab environment acclimation. All subjects underwent baseline blood draw, occurring every 2 hours over 24 hours on test day 1. This blood draw protocol was repeated following the 3 days of simulated night shift sleep scheduling on test day 2, totaling 26 blood samples per subject (Figure 1). The samples were then centrifuged down to obtain plasma, exosomes were extracted, and western blots were performed to observe protein density across time points in HC vs UC and alignment vs misalignment. Primary antibodies utilized were GPA33 and tsg101. Tubulin was selected as the normalization protein as it is a housekeeping protein that has minimal hour-to-hour oscillation to optimize data efficacy.

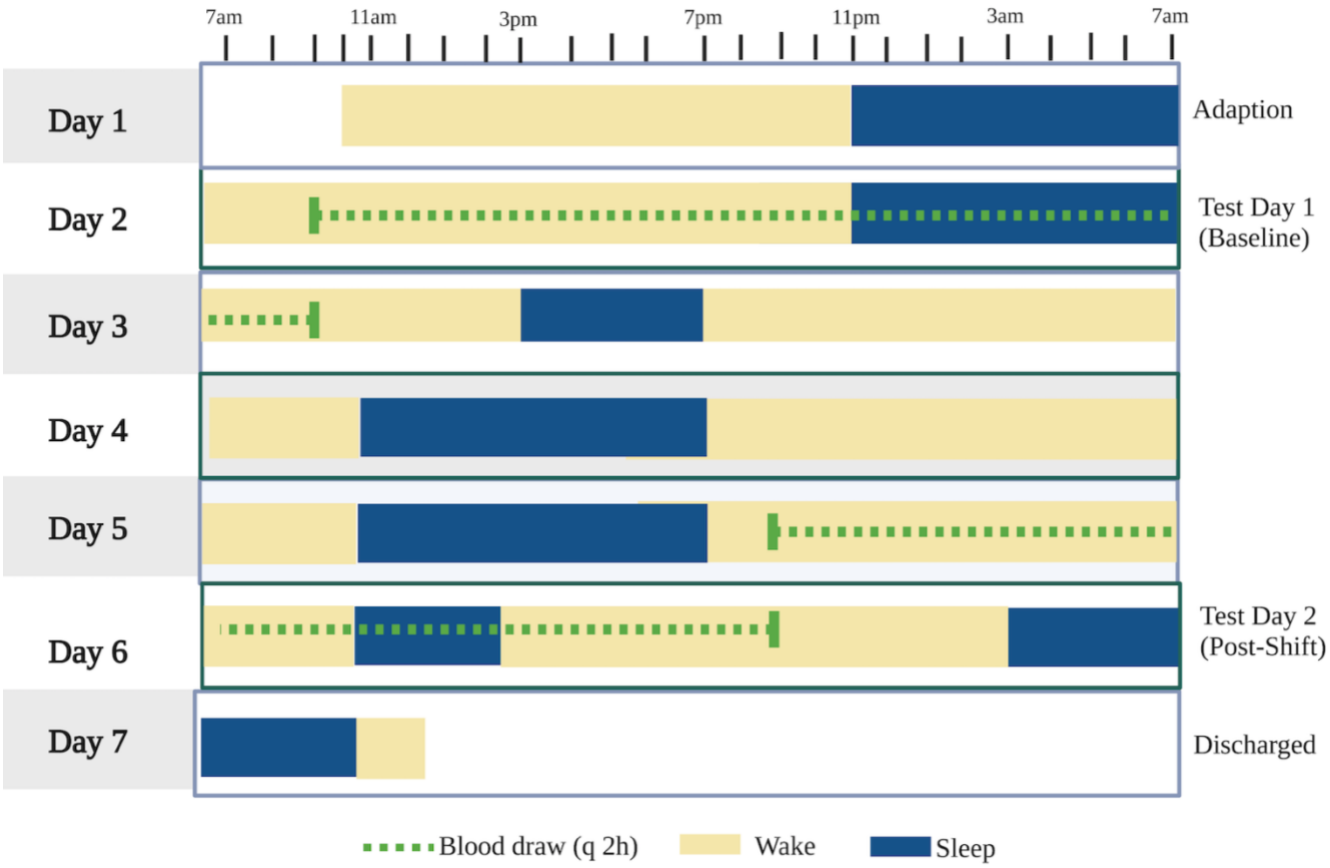


Figure 2. Circadian misalignment and blood draw protocol. The protocol includes a seven-lab day stay with an adaptation period, followed by a baseline blood draw every 2 hours over 24 hours (Test Day 1). This is followed by three days of simulated night shifts with a repeat blood draw every 2 hours over 24 hours (Test Day 2). Created with Biorender.com.

Isolation of Extracellular Vesicles

Extracellular vesicles were extracted from 250 µL of plasma via centrifugation at 4°C 15000 x g for 15 minutes to remove cellular components. The supernatant was then filtrated using a microporous membrane filter (Nalgene, Filters- Thermo Scientific 725-2520). ExoQuick precipitation kit (EXOQ20A-1) was added to the filtrate and stored at 4°C for 30 minutes. The samples were then centrifuged at 4°C at 3000 x g for 10 minutes to collect exosome pellets. The

exosome pellets were resuspended in a cocktail of RIPA buffer (include details) protease inhibitor cocktail (include details) and phosphatase inhibitor cocktail (include details). Samples were stored at -20°C for optimal exosome preservation.

Western Blot Analysis

Equal amounts of protein concentrations were quantified and normalized to the α/β-Tubulin band. Extracted exosome samples (50 ug) were boiled at 90ºC for 5 minutes with 2x Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA). Samples were electrophoresed on 12.5% tris-HCl gels and transferred to a nitrocellulose membrane (GE Healthcare Limited, Buckinghamshire, UK). Non-specific binding sites were blocked for 1 hour at room temperature [2.5% BSA and 2.5% non-fat dry milk [all in tris-buffered saline and Tween-20(TBS-T)]. Membranes were incubated overnight at 4ºC with primary antibody [GPA33: 1:500, Invitrogen ab108938; tsg 101: 1,1000, Santa Cruz Biotechnology sc-7964; α/β-Tubulin: 1:1,000, Cell Signaling 2148 (all in TBS-T)]. Membranes were incubated in HRP-conjugated anti-rabbit or anti-mouse secondary antibody (1:2,000) for 1 hour at room temperature. Chemiluminescent substrate (ECL, GE Healthcare) was applied to the membrane for protein visualization using autoradiography film (HyBlot CL, Denville Scientific, Metuchen, NJ). As a final step, the autoradiography films were processed and scanned for statistical analysis.

Statistical Analysis

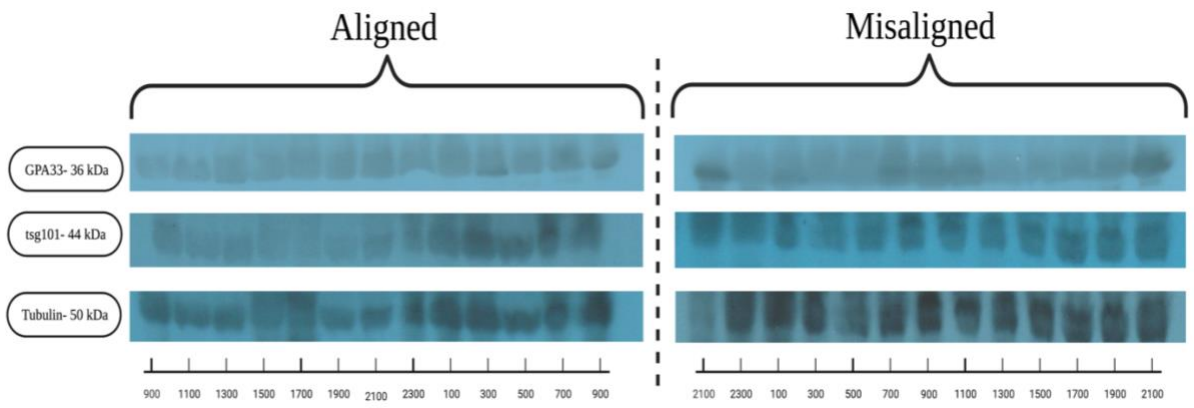
All data were analyzed using grouped two-way analysis of variance (ANOVA) where appropriate. Differences were considered significant at $p < 0.05$. All western blots were measured with ImageJ software (NIH, Bethesda, MD and all data analyses were performed using GraphPad Prism 9 (La Jolla, CA) and R software Cosinor package (Version 3.8.1).

CHAPTER THREE

RESULTS

To assess the relationship between exosomes and circadian misalignment, western blots were utilized to observe human exosome marker protein densities across 24 hours at baseline, and 24 hours after 3-night shift simulated sleep/wake cycles for all subjects. Images of western blots for HC (Figure 3a) and western blots for UC (Figure 3b) are shown for both alignment and misalignment.

A



**Healthy Control*

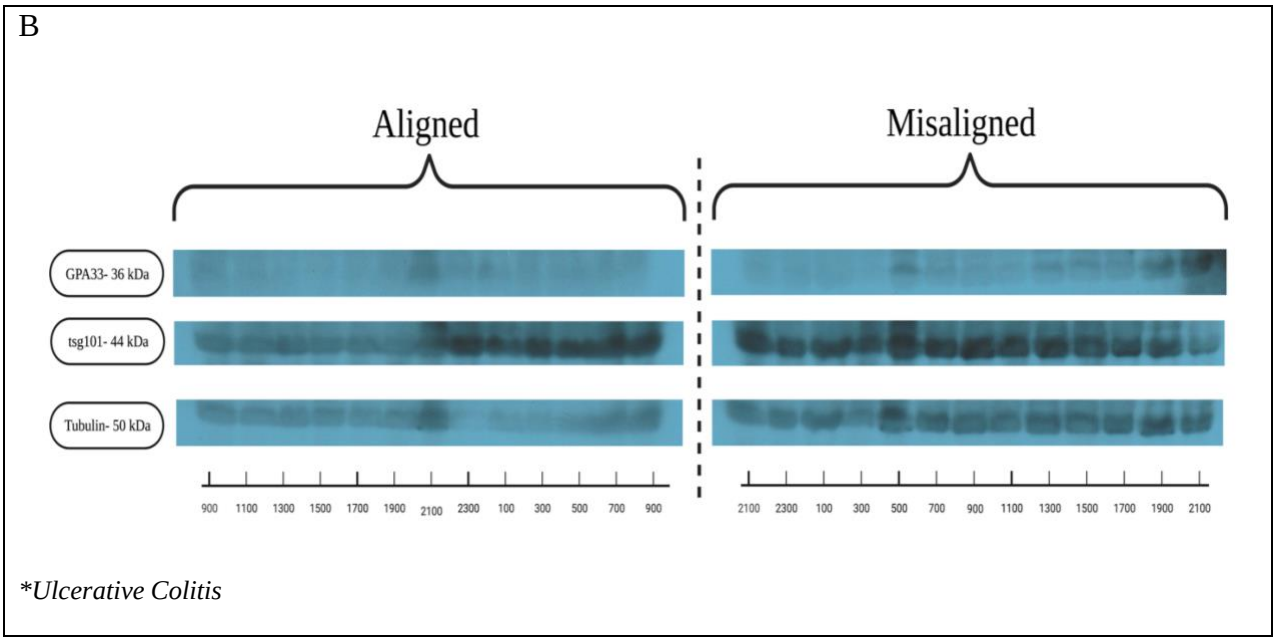
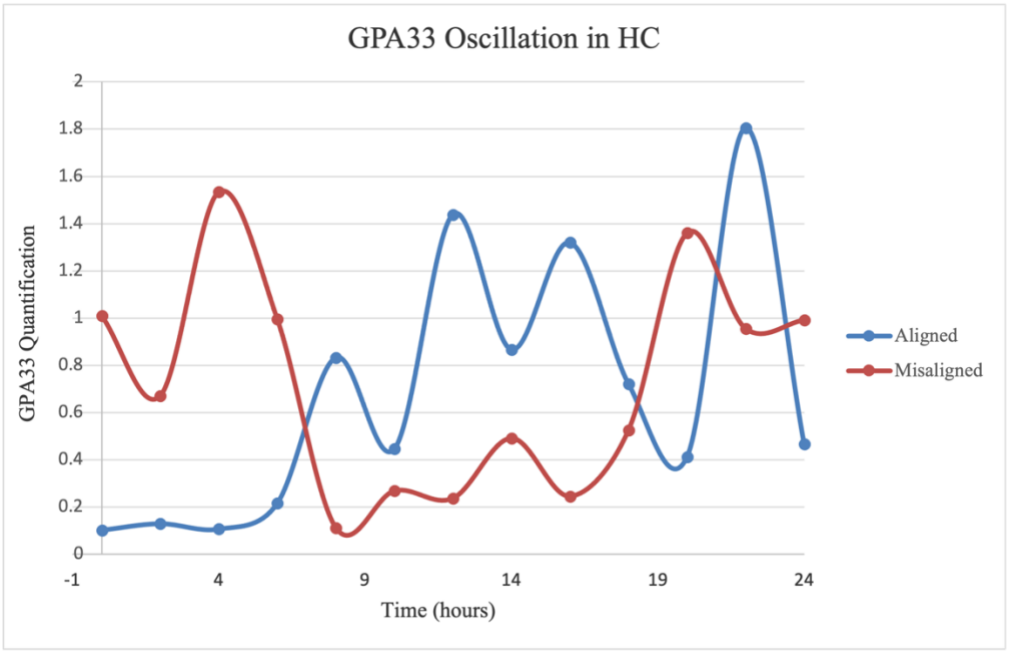


Figure 3. Western Blot Imaging. (A) Healthy control aligned vs. misaligned bands representing GPA33 (36 kDA) and tsg101 (44kDa) normalized to tubulin (50 kDa) at all collected time points. (B) Ulcerative colitis aligned vs. misaligned bands representing GPA33 (36 kDA) and tsg101 (44kDa) normalized to tubulin (50 kDa) at all collected time points.

Western blot data was also analyzed to determine if the oscillations occurring over every two hours followed circadian rhythmic trends. GPA33 alignment and misalignment, nor tsg101 alignment or misalignment in the model were rhythmic (Figure 4a-b), which was consistent across all subjects.

A



B

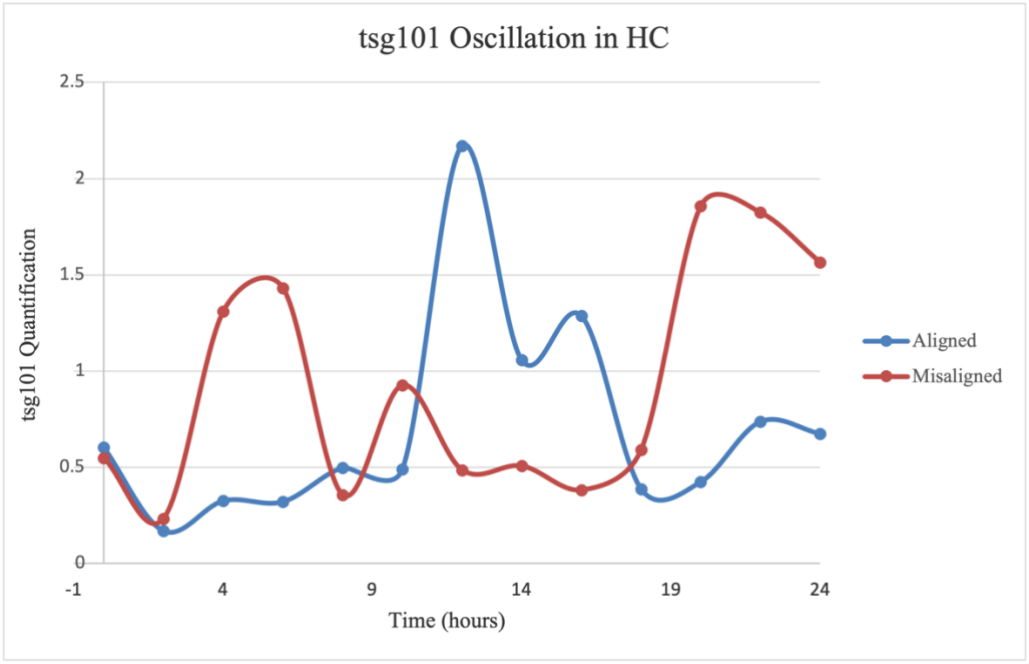


Figure 4. Observed trends in rhythmicity for GPA33 and tsg101. (A) GPA33 rhythmicity of a HC subject. (B) GPA33 rhythmicity of a HC subject.

Analysis of each western blot was done utilizing ImageJ software for measurement and quantification and further analyzed in GraphPad Prism 9. Differences were considered significant at $p < 0.05$, though less statistically significant findings were still reported. Two-way analysis of variance (ANOVA) was utilized to examine the influence of healthy condition vs. ulcerative colitis condition under alignment or misalignment type. When analyzing GPA33, it was found that there was a significantly lower amount of GPA33 present in UC at both baseline and after misalignment when compared to HC. However, there was no significant difference when comparing alignment vs misalignment types (Figure 5a). Area under the curve was measured for each western band peak and analyzed separately in a two-way ANOVA. In this analysis, there was no significant differences for neither condition nor type in GPA33 (Figure 5b).

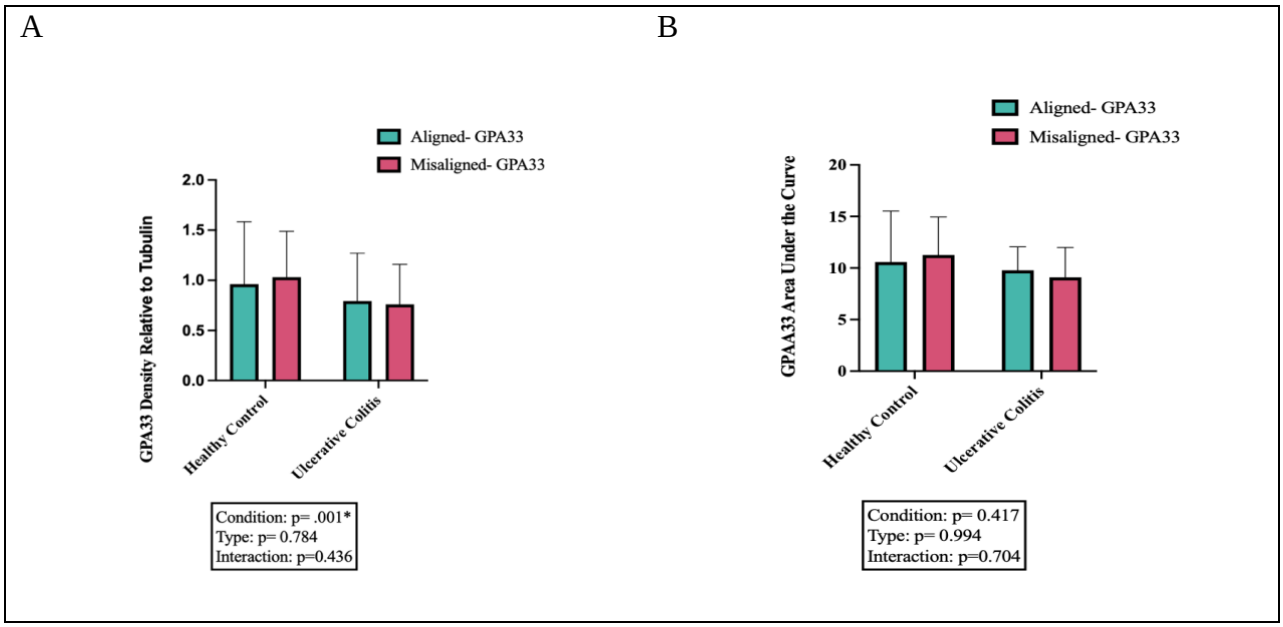


Figure 5. Western blot analysis of GPA33 in HC and UC subjects comparing alignment vs. misalignment. (A) GPA33 density relative to tubulin. (B) Area under the curve analysis of GPA3.

Similar to GPA33, tsg101 band density was analyzed utilizing a two-way ANOVA. Tsg101 significantly increased in both HC and UC subjects from alignment to misalignment, but tsg101 density in HC and UC did not have a significant difference between each other (Figure 6a). Area under the curve analysis of tsg101 showed a significance in both HC and UC from alignment to misalignment, but HC and UC were not significantly different when compared against each other (Figure 6b).

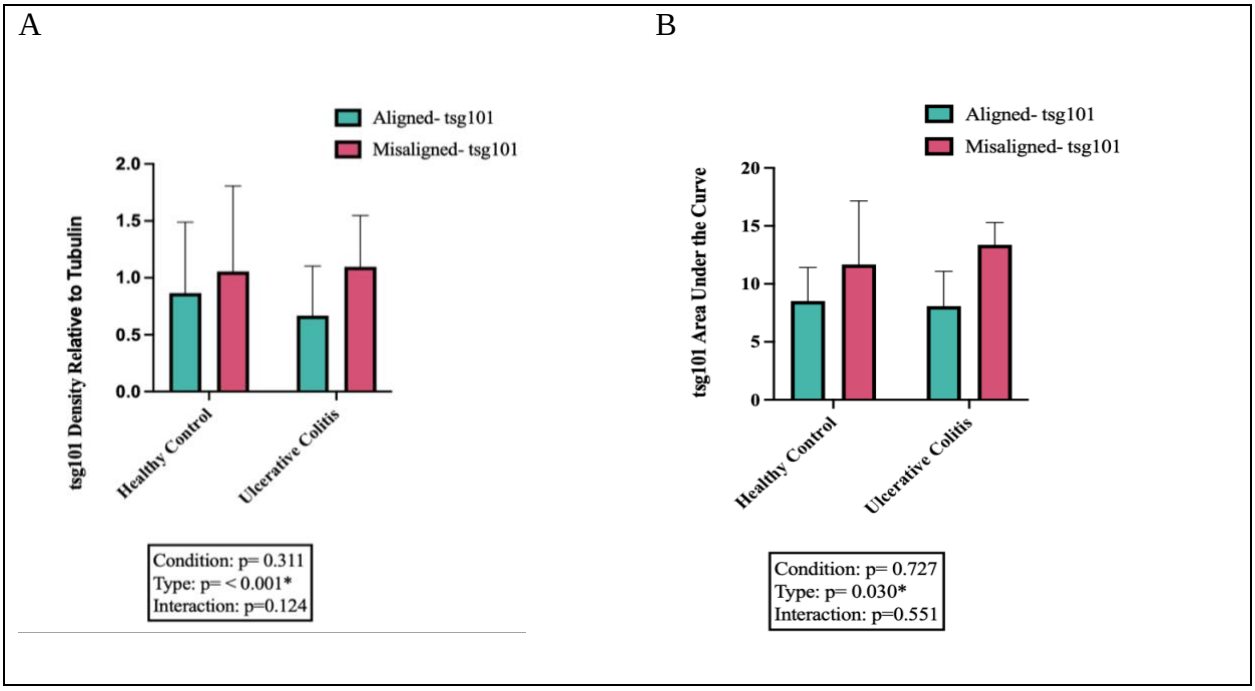


Figure 6. Western blot analysis of tsg101 in HC and UC subjects comparing alignment vs. misalignment. (A) tsg101 density relative to tubulin showed significant difference in GPA. (B) Area under the curve analysis of tsg101.

CHAPTER FOUR

DISCUSSION

Research has established a profound relationship between circadian misalignment and IBD. However, with new studies looking at the role that EVs play in various diseases, the relationship that EVs may with IBD circadian misalignment has not been addressed. This study aimed to observe the potential impact of circadian misalignment on EVs in inactive UC patients, particularly looking at two human exosome markers. To induce circadian misalignment, subjects were on a prescribed regular sleep schedule monitored via wrist actigraphy watches for 2 weeks prior to the lab stay. During this lab stay, blood was collected every 2 hours over 24 hours on test day 1, 3 days of simulated night shift sleep/wake cycles were induced, followed by another blood collection every 2 hours over 24 hours on test day 2. It was hypothesized for Aim 1 that under circadian misalignment, UC subjects would experience a more drastic exosome oscillation over a 24-hour period than healthy controls. Aim 2 hypothesized that both HC and UC subjects would demonstrate an increase in exosomes after the simulated night shift when compared to baseline.

The results of Aim 1 did not follow a rhythmic trend which was consistent between HC and UC, as well as in alignment vs misalignment. This suggests that while exosomes are changing from baseline to post shift, there is not a clear oscillation pattern taking place hour to hour over a 24-hour cycle, rejecting our hypothesis. This rejection may be attributed to the large quantity of data points utilized in this study, as previous studies have shown that exosomes tend to follow circadian oscillation. However, most existing studies have only used 4-6 time points over 24-hours on average, while this study had 13 samples over 24-hours. This suggests that exosomes may be more sporadically modulated rather than under a circadian oscillation pattern.

To interpret the results for Aim 2, bands were first visually observed and measured, followed by analysis under a two-way ANOVA. The visualization of the bands shown in Figure 3 correspond directly to analysis Figures 5 and 6. The statistical tests ran found that GPA33 was significantly lower in UC compared to HC, but that alignment and misalignment did not have significant impacts. However, though we know exosomes are involved directly in cell-cell communication, they may be involved in more mechanisms that are not solely harmful to the subject, suggesting that UC having a lower presence of GPA33 may not be a harmful finding for HC subjects. AUC data ran on GPA33 found that neither condition group nor type of influence made a significant difference in GPA33 levels suggesting that circadian misalignment did not significantly change the presence of GPA33 in circulation for HC nor UC subjects. However, levels of tsg101 significantly increased in both HC and UC subjects from alignment to misalignment and was confirmed with AUC analysis. Since tsg101 is not specific to the intestine and is an overall exosome marker, this finding is incredibly important. Because exosomes are primarily secreted by immune cells, this rise in total exosome marker tsg101 suggests that there is an influx of information signaling taking place in individuals following circadian misalignment after just 3 days of sleep shift. While it is still not fully understood whether exosomes only communicate “bad” intercellular cargo, there is still a clear difference that is worth further examination. If the increase in circulating exosomes is increasing the communication of inflammatory molecules, it could very well play a role in the exacerbation of IBD, specifically UC in this study.

It is important to note that this study did have limitations. First, the sample size was small at 9 total subjects, 5 HC and 4 UC that may limit the ability to detect significant differences. Though this study did show some significant findings in the relationship between exosomes and

circadian misalignment in IBD, larger scale studies are warranted to determine long term clinical efficacy. Secondly, exosomes are incredibly complex to isolate and ensure purified samples. More intricate isolation technique, proteomics and further analysis are required to ensure the pure presence of exosomes, though this study provides preliminary data that there are changes in overall extracellular vesicles that is worth further investigation. And lastly, multiple quality control western runs should be completed, as western blots are very tedious and are prone to technical error. This would ensure that findings are accurate and that observed trends are not due to error. The results of this study provide the preliminary basis for the likelihood that extracellular vesicles, and potentially human exosomes are influenced by circadian misalignment in patients with inactive UC and further investigation into this relationship is warranted.

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Vita

Malia Rose Gasteier was born and raised in Chicago, Illinois alongside her siblings, Ashli, Kyle, Aurora, and Noah. Before attending Rush University, she completed her first degree at the University of St. Francis where she earned a Bachelor of Science in biology and a minor in chemistry in 2021.

During her time in graduate school at Rush University, Gasteier conducted her thesis research in the Rush Center for Integrated Microbiome and Chronobiology Research Data and Biorepository working on her own research as well as assisting in several other center projects. While attending Rush, she became a student intern for Rush Graduate College operating their science communication sector and documenting large scale events such as the Graduate Research Retreat and the Center for Emerging Infectious Diseases. She was also elected as the marketing director for TEDxRushU and was a chair committee member for the 2023 event. During this time, she simultaneously completed the Entrepreneurship for Biomedicine training program at Washington University School of Medicine and was a member of the National Association of Science Writers.

Gasteier will move forward within the science communication sector, specifically in scientific and medical writing. Her goal is to engage the public in science, and to make the work scientists do less intimidating to our communities. She hopes to help close the gap of information, before someday returning to school for her doctorate.