Title: The Impact of Circadian Misalignment on Colonic Barrier Homeostasis in Ulcerative Colitis
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Title: The Impact of Circadian Misalignment on Colonic Barrier Homeostasis in Ulcerative Colitis

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Abstract: Recently there has been compelling evidence that inflammatory bowel disease (IBD) subjects, both Crohn's disease (CD) and Ulcerative Colitis (UC), commonly have disrupted sleep, and disrupted sleep correlates with the risk of disease flare. Sleep/wake cycle, immune function, metabolism and multiple biological processes are all orchestrated by circadian rhythms. Circadian misalignment between the central circadian clock in the brain and environment has been found to contribute to a variety of metabolic and gastrointestinal tract (GIT) diseases. Yet, the prevalence and impact of circadian misalignment on IBD disease activity and GIT mucosal inflammation have not been established. The long-term objective of our research is to investigate the hypothesis that circadian malalignment worsens GIT mucosal inflammation and disease course in IBD. To test this hypothesis, we will conduct a prospective in lab study with human subjects on the impact of circadian misalignment in left sided mild to moderate Ulcerative Colitis. We will perform two circadian measures phase assessments under strictly controlled laboratory conditions at: 1) baseline, and after 2) circadian misalignment after three days of simulated night shifts in both inactive UC patients and healthy control subjects. To test the hypothesis that UC patients (compared to healthy subject controls) are less resilient to circadian misalignment we will assess: time impact of our protocol on circadian rhythms (Aim 1) by the following: phase angle of entrainment [time from dim light melatonin onset to sleep onset] (Aim1a); peripheral circadian rhythms by clock gene expression in colonic tissue from a flexible sigmoidoscopy and in subjects peripheral blood mononuclear cells (Aim1b); and colonic clock gene expression and Per2::Luc activity over 24 hours utilizing an in-vitro model of colonic organoids (Aim1c). Next, we will test the hypothesis that circadian misalignment will increase colonic permeability and mucosal inflammation in UC patients (vs. Controls) (Aim2) through the following: endoscopy score, stool calprotectin, colonic barrier (permeability, AJC proteins), and markers of systemic barrier function and inflammation (Aim 2a); adversely impacting microbial community structure/function (Aim 2b); and use 2D colonic organoid monolayers to explore ex-vivo barrier function by co-culturing with TNF-α/INF-γ (Aim

2c). The results of this innovative proposal will greatly increase our understanding of the important role circadian misalignment may have in UC disease activity and colonic inflammation, and identify new circadian regulated targets for treatment in UC.

Objective: The long-term objective of our research is to investigate the hypothesis that circadian misalignment worsens intestinal mucosal inflammation and disease course in IBD. Key evidence supporting this hypothesis include that disruption of circadian rhythms in the host impacts the intestinal barrier, microbiota composition, and mucosal/systemic immune function, all critical factors in the pathogenesis of IBD. Data from rodents generated by our group and others show that disruption of the light: dark cycle (a model of shift work) or genetic circadian rhythm disruption (of a core clock gene) changes the intestinal microbial community, alters the production of microbial products like short chain fatty acids (SCFAs) resulting in colonic hyperpermeability through disruption of the apical junctional complex (AJC) in colonic IECs, increased mucosal inflammation, culminating in a more pro-inflammatory state in the intestine.11,24-27 In humans, shift work increases susceptibility of the colonic barrier to an injurious agent like alcohol and increases systemic pro-inflammatory cytokines IL-6 and IL-1β28 and that chronic alterations in light:dark cycles in mouse models of IBD increase colitis severity and mortality.29,30 In humans with IBD, our group and others have shown that a later chronotype (Fig. 2A) and high variability in rest-activity rhythms (Fig. 2B) (both markers of circadian misalignment) are associated with worse IBD disease course, increased colonic permeability, alterations in the intestinal microbiota community (dysbiosis), and reduced colonic circadian clock gene expression.5,30 Therefore, the immediate goal of this proposal is to elucidate the mechanisms of colonic inflammation induced by circadian misalignment in UC. This will be the first comprehensive examination of human circadian rhythms in IBD.

Hypothesis: Circadian misalignment worsens intestinal mucosal inflammation and disease course in IBD.

Specific Aims:

- Aim 1: Test the hypothesis that circadian misalignment is more prevalent in UC compared to healthy controls and that UC patients are more susceptible
- Aim 2: Test the hypothesis that circadian misalignment increases colonic permeability and mucosal inflammation through disrupting the AJC and adversely impacting microbial community structure / function in UC.

Background: A.1. IBD is Associated with Sleep Disruption. Crohn's Disease (CD) and Ulcerative Colitis (UC), collectively known as inflammatory bowel disease (IBD), are two devastating chronic diseases of the gastrointestinal tract (GIT). IBD is typically diagnosed at an early age, has no known cure, and has a chronic relapsing and remitting disease course that includes

disabling symptoms during periodic flare ups. IBD affects over 1.5 million individuals in the US with an estimated direct cost of $14 to 31 billion (31, 32). IBD has a heterogeneous disease course that commonly has an aggressive phenotype. Identifying risk factors that can trigger disease flare and aggressive disease course are essential to avoid complications such as hospitalizations and surgery and are critical to improve disease course and patient quality of life. Factors that are associated with a more aggressive disease course include the use of prednisone or inability to wean off prednisone (steroid dependency), early age of diagnosis, hospitalization, need for IBD surgery, and the presence of fistulizing/stricturing perirectal disease in CD (33-35). However, these factors cannot fully explain the disease course in many patients. Recently, there has been an increased awareness of the high prevalence of sleep disruption in IBD. Our group was one of the first to report a high prevalence of sleep disturbances in IBD, (17, 36) and patients with IBD exhibit disrupted sleep (polysomnography) even when their disease is inactive. A subsequent prospective cohort showed that ileo-cecal inflammation in CD correlates with poor sleep.16 In essence, IBD patients in clinical remission with poor sleep had higher subclinical disease activity compared to those IBD patients reporting good sleep.16 Thus, disrupted sleep/wake cycles, an important output of the circadian clock, can be one factor that triggers disease flare and promote progressive disease course; yet the impact of circadian rhythm disruption on disease pathogenesis has not been fully evaluated, which is one of the goals of this application.

A.2. Circadian Rhythms are Critical to Health. Sleep, rest-wake activity, and multiple biological processes such as metabolism, (37) cell proliferation, (38) and immune activity39 are orchestrated by the circadian clock on a roughly 24h cycle. The discovery of the core molecular machinery of the master or central circadian clock in the suprachiasmatic nucleus (SCN)40 which consists of a transcription and translation feedback loop of positive factors (e.g., Clock, Bmal1) and negative repressors (e.g., Per1, Per2, Cry1, Cry2) has led to a number of key mechanistic discoveries regarding the circadian clock machinery and its functions that are critical for normal physiology and behavior.41-43 Synchronization of internal circadian clock with external environmental cues (e.g., light) is critical for good health,44-47 which is not surprising as the circadian clock is known to control the expression of 10-20% of the genes in the human body.48-50 In addition, peripheral clocks that contain the same core molecular machinery as in the SCN are found in cells of every organ in the body, including colonic IECs in the GIT.51,52 The GIT exhibits diurnal rhythms in many physiologic functions such as motility, intestinal transport, and IEC replication53 all of which are governed by inputs from the SCN as well as other signals that entrain the circadian clock in the GIT such as time of food consumption. Circadian misalignment is a mismatch between the environmental cues (e.g., light) and the central clock in the SCN or a mismatch between the central and peripheral clocks which can occur due to shift work or consumption of a high fat diet, respectively (54, 55). Modern 24h society, with increased nighttime light exposure, light pollution, the increasing

prevalence of shift work, and nighttime eating have made misalignment of our endogenous circadian rhythms more common. There are multiple causes for human circadian misalignment including shift work, long distance travel with jet lag, and perhaps the most common: social jet lag, which is changing the midpoint of sleep more than 2h between work and work-free days due to social obligations.56 The most well-studied model of circadian misalignment, shift work, is now estimated to include 15 million workers in the US.57 Not surprisingly, shift work is associated with a number of GIT disorders and diseases including irritable bowel syndrome,22 peptic ulcer disease,58 and colon cancer.59 Therefore, it is possible that circadian misalignment may be one factor that promotes intestinal inflammation and disease flare in UC.

A.3. IBD is characterized by Intestinal Barrier Dysfunction and Inflammation. The intestinal barrier is the largest interface between the body and the environment and must be selectively permeable to restrict pro-inflammatory microbiota and microbial products but at the same time allow passage of nutrients, electrolytes, and water. Thus, integrity of the intestinal barrier is critical for maintaining good health. Indeed, disrupted intestinal barrier function ("leaky gut"), endotoxemia (i.e., high levels of systemic lipopolysaccharide (LPS)), and subsequent production of pro-inflammatory cytokines and reactive oxygen species are all hallmarks of IBD.60 In fact, fluctuations in intestinal barrier function in IBD is an established predictor of disease flare in CD61-63 and UC.64,65 Several environmental factors can disrupt the intestinal barrier function in IBD like medication,66 alcohol,67,68 and stress69,70 but this only occurs in a subset of individuals suggesting that resiliency of the intestine determines the impact of these environmental factors in chronic inflammatory disorders like IBD. The intestinal barrier consists of the apical junctional complex (AJC), both tight junction (TJ) and adherens junction (AJ) proteins, that links the IECs and seals the paracellular space to prevent translocation of pro-inflammatory bacteria and bacterial components such as LPS into the intestinal mucosa.71 IECs turnover every 4-5 days,72,73 replenished by a small population of intestinal stem cells (ISCs), and IEC regeneration and differentiation are essential to maintain barrier homeostasis.74 It has also been established that alterations in gut microbiota are another hallmark of IBD, and perturbation of the structure of the microbiome can disrupt the intestinal barrier and cause intestinal inflammation in IBD.75 Specifically, in IBD there is a decrease in bacterial diversity76,77 and temporal stability78 with a loss of bacteria in the Firmicutes phylum79 and increase in Proteobacteria.80 It is not understood if alterations in the intestinal microbiota are the cause of or result from increased intestinal permeability and inflammation, but IBD is characterized by both barrier dysfunction and dysbiosis. Disruption of central or peripheral circadian rhythms is sufficient to impact both the intestinal barrier and the microbiota; therefore, it is important to determine if circadian misalignment is sufficient to induce changes in the GIT that promote intestinal inflammation and UC disease flare.

A.4. Circadian Rhythms are Critical to Maintain the Intestinal Barrier. Intestinal barrier homeostasis is mediated by cross-talk between IECs and the intestinal microbiota. This cross-talk occurs via multiple mechanisms including interactions with toll-like receptors (TLRs) on the IECs.81 There are multiple lines of evidence in cell lines, animal models, and human studies that support the scientific premise that disruption of circadian rhythms in the host negatively impacts intestinal barrier integrity: (1) alcohol (0.2%) increases the expression of the circadian clock genes Clock and Per2 in a human IEC line (Caco-2 cells), and knock down of Clock or Per2 gene expression by siRNA prevents alcohol-induced barrier dysfunction,82 (2) chronic disruption of the central clock by either disruption of light:dark cycles or a mutation in the core circadian molecular clock (i.e., Clock mutant mice) increases intestinal permeability and endotoxemia,26 (3) healthy night workers who consume alcohol (0.5g/kg daily for 7 days) have increased colonic permeability compared to healthy day workers consuming alcohol, and colonic permeability inversely correlates with decreased plasma melatonin over 24h,28 (4) our lab25 and others13,27 show that circadian misalignment in the host causes microbial alterations with a decrease in bacteria (Firmicutes) that produce short chain fatty acids (SCFA) like butyrate (Bu),83 and (5) the core clock machinery (Bmal1) regulates IEC cell regeneration and barrier homeostasis.84,85 Taken together, these studies strongly support the concept that circadian misalignment could promote IBD flare via a mechanism involving disrupting the colonic barrier and intestinal microbiota dysbiosis.

A.5. Circadian Rhythms and IBD. Given that the circadian rhythms of the host can impact colonic barrier integrity, it is likely to also impact chronic inflammation in IBD; however, the impact of circadian misalignment in humans with IBD has not been investigated. Prior work in animal models of IBD by our group29 and others30 show that chronic disruption of central circadian rhythms in the host by shifting light:dark cycles causes significantly worse colitis and increases mortality by Dextran Sodium Sulfate (DSS) (Fig. 1) or by Dextran sodium sulfate/2,4,6-trinitrobenzene sulfonic acid (TNBS). In addition, we recently found that an intestinal specific Bmal1 knock out mice develop worse DSS colitis with increased permeability despite normal central circadian behavior (Fig. 1C&D) suggesting that influencing circadian rhythms in the GIT are sufficient to impact the barrier and mucosal inflammation. Furthermore, a Per2 mutant mouse with increased Per2 activity had constitutively high levels of the tight junction proteins (TJP) occludin and claudin-1 in the colon, is resistant to DSS-induced colitis.86 In humans, genetic analysis found an association between polymorphisms in one of the core circadian clock genes Period 3 (Per3) and increased incidence of Crohn's disease.87 Our group has conducted several studies in humans with IBD to examine the role of circadian rhythms: 1) We examined the association between chronotype (category of circadian timing) and IBD using two validated questionnaires – the Owl and Lark and Munich. We found that a later chronotype is associated with a worse IBD quality of life, IBD subjects on aggressive medications (biologics) had a later midpoint of sleep, and Crohn's subjects with increased social jet lag are more likely to have a

history of fistulizing or stricturing disease (Fig 2A).88 These data suggest that circadian rhythms are associated with IBD disease phenotype. 2) Our examination of endogenous salivary melatonin secretion in four subjects with inactive IBD, revealed that two of the subjects had low melatonin secretion compared to controls and the one had an arrhythmic profile,89 indicating circadian abnormalities are present in IBD patients. 3) We used wrist actigraphy to identify variations or instability in rest-wake activity that correlate to central circadian rhythms in inactive IBD subjects and found that there was less interdaily stability in IBD subjects with an aggressive disease course (history of fistulizing/stricturing disease in CD and history of surgery, steroid dependence, or biologic use in UC and CD) and greater interdaily variability correlated with increased colonic permeability (Figs. 2B, 2C). 4) Examination of stool microbiota in inactive IBD subjects reveals changes in the microbiota that were associated with circadian misalignment. For example, there is a significant decrease in SCFA-producing bacteria (Fig. 2D) and bifidobacterium longum which has been used as a treatment in active UC.90,91 Taken together, these findings support the hypothesis that circadian rhythms influence the intestinal barrier and the intestinal microbiota; however, no human studies have comprehensively studied the central or peripheral circadian rhythms in IBD subjects by the gold standard in the field (DLMO) while examining subclinical inflammation and colonic barrier function, which is one of the goals of our current study.

A.6. Importance of the Proposed Research. There is a clear scientific rationale and crucial need to take the next step and more comprehensively assess central and peripheral circadian rhythms in IBD in order to determine the impact of circadian misalignment in IBD on colonic mucosal inflammation, subclinical systemic inflammation, barrier function, and intestinal microbiota. In the proposed study, we will determine if patients with inactive UC have circadian misalignment of central and/or peripheral circadian rhythms and if they are more susceptible to circadian misalignment after a simulated night shift (Specific Aim 1) and determine if circadian misalignment impacts colonic barrier integrity and intestinal microbiota (Specific Aim 2). We selected UC as a model of IBD because: (1) it always affects the rectum/sigmoid, thus it is relatively easy to access and obtain the required colonic intestinal tissue through sigmoidoscopy, (2) microbiota-directed interventions like fecal microbiota transplant (FMT) appear to be more promising in UC than in CD,92,93 and (3) our data support that circadian misalignment has a substantial impact on the colonic barrier in UC subjects. The knowledge gained from the proposed studies is likely to lead to the identification of new mechanisms of how circadian misalignment promotes chronic inflammation in IBD, and allow for the study of new chronobiological therapeutics in IBD that target circadian misalignment such as bright light therapy, exogenous melatonin, or other molecular targets associated with circadian misalignment induced in IBD.

Research Design: 40 human subjects will be recruited into this proposed study: 20 Healthy Controls and 20 UC subjects with left sided inactive disease (Mayo Score ≤ 2) on stable medications. All subjects (UC and HC) will undergo a full medical history and physical examination including a blood draw for the screening visit. A urine drug screening will take place on the subject's first day at the sleep laboratory. Subjects who wish to participate will have the study explained by the coordinator and will then sign a Rush IRB-approved informed consent. All UC subjects will complete a validated questionnaire regarding disease-specific symptoms (Mayo Score) to determine they are inactive and may be enrolled in the study.

In addition, subjects will provide basic demographic data, UC history if appropriate, UC disease activity status as detailed further above, and the Munich Chronotype questionnaire. All subjects will complete a validated 3-month food frequency questionnaire (FFQ) to collect dietary information (1). 40 participants (Specific Aims 1-2) will be enrolled for the within-subject prospective in-laboratory study protocols. Participants will be young (18-50 y), healthy, non-diabetic, non-obese, Caucasian/African American/Asian and non-Hispanic/Hispanic in ratios consistent with the population of Chicago, and without medication use (excepting oral contraceptives) for HC. HC and UC subjects will be matched according to age ($\pm$ 3 years), sex, race, and BMI ($\pm$ 3 kg/m2). UC subjects will be on stable medications with no recent use of prednisone or antibiotics for the last 3 months. After signing an informed consent, the subject will fill out questionnaires), start a food diary, and sleep diary, and have 2 weeks of wrist actigraphy before their seven-day in lab session in the Clinical Chronobiology Center (CCC). Since participants will be in the sleep lab for 7 days and isolation can cause an increase in depression and anxiety, a normal psychological evaluation is part of the inclusion criteria for this study. This will be determined based on how participants respond on the BDI and STAI questionnaires, which assess depression and anxiety. The study physician will review the questionnaire responses and evaluate if the participant will feel comfortable staying in the lab for 7 days. .

Subject Characteristics: We will recruit (1) 20 HC subjects without any medical illnesses (2) 20 subjects with biopsy proven UC, inactive (Mayo Score ≤ 2) on stable medications and no disease flares for > 3 months. HC and UC subject will be matched according to age ($\pm$ 3 years), sex, race, and BMI ($\pm$ 3 kg/m2).

Sample Size Analysis: All power estimates were computed using R package pwr, and we assume a Type I error rate of 0.05 and a 2-sided test for all analyses. Power estimates were derived from the data from my prior K grant. The primary endpoints of this study will be phase angle/DLMO (Aim 1) and colonic permeability (13-24h urinary sucralose) (Aim 2). For Aim 1, we found a ~2h phase advance in DLMO in NW compared to DW at -0.2 (SD=2.1) compared to -4.34 (SD=3.8), respectively. With a sample size of 20 in each group (10 men/10 women), this

provides an 86% power to detect differences in DLMO, but 98% power if sex is pooled. For Aim 2, we found a 40% increase in colonic permeability in night shift works compared to day workers after moderate alcohol consumption at 0.57 compared to 0.11 (SD=0.20). With 20 subjects, this will give us a 84% power to detect a difference in colonic permeability between the two groups for each sex and if sex is pooled with give us a 97% power with a two-sided two-sample t-test at alpha=0.05 level. These conservative estimates provide a good estimate of the power to detect differences in phase angle and colonic permeability between UC patients before and after simulated night shifts. We anticipate we will have nonparametric data and will use the Wilcoxon match-pair test for analysis of colonic permeability, inflammatory cytokines, and markers of endotoxemia. Order effects will be used as a covariate in multivariate analysis with analysis of variance (ANOVA) and linear regression as indicated. Microbiota data analysis is described above. Non-parametric analysis of actigraphy data will be analyzed with the nparACT package. In our periodogram analysis of time series data we will use Fast Fourier Transform Non-linear Least Squares (FFT-NLLS), a nonlinear least-squares algorithm of a times series typically used for luminescence data (149) and analyzed with Biodare software (150). Data will be presented as mean ± SEM for variables that can be considered normally distributed (or median and range for variables not normally distributed). All analyses will use R (v.3.2.5), Biodare 2, and SPSS (SPSS Inc., Chicago, IL).

Inclusion Criteria: *Study will include individuals that are;*

Healthy Controls:

1. M/F, 18-50 y/o, age ± 3y sex, race, and BMI (□ 3 kg/m2) match with UC subject
2. No clinical evidence of any medical illness
3. Normal psychological evaluation based on question responses and negative drug screen (See Below)

Ulcerative Colitis:

1. 1. M/F, 18-50 y/o.
2. 2. Inactive Disease (Mayo Score ≤ 2)
3. 3. Stable medications with no disease flares for the > 3 months
4. 4. Left-sided UC (Montreal E1 or E2)
5. 5. Normal psychological evaluation based on questionnaire responses and negative drug screen (See Below)

Exclusion Criteria: *Study will not include individuals that are;*

Healthy Controls

1. History of drug abuse, gastrointestinal (GI) surgery, GI diseases, or systemic diseases such as renal (creatinine>1.2 mg/dl), liver, cardiac, or diabetes (Hgb-A1c>8%)
2. Antibiotic use within last 12 weeks
3. Shift work in the last 6 months
4. Use of probiotic supplement except yogurt in last 4 weeks.
5. Atypical American diet with daily fiber ≥ 16 grams or daily saturated fat ≤ 11 grams by Food Frequency Questionnaire
6. Chronic use of NSAIDS. A washout period of 3 weeks is needed before the subject could be enrolled into the study.
7. Chronic Alcohol use. A washout period of 3 weeks is needed before the subject could be enrolled into the study.
8. Significant Depression (score ≥ 14 BDI)
9. Significant Anxiety (score ≥ 40 STAI)
10. Regular use of medications that affect intestinal permeability, intestinal motility and/or endogenous melatonin including metoclopramide, NSAIDs, antibiotics, beta blocker, psychotropic medication, hypnotics and exogenous melatonin products during 4 weeks prior to the study
11. People who crossed more than 2 time zones in the previous month
12. Inability to sign an informed consent form.
13. Have children Under 6 Months

Ulcerative Colitis:

1. Patients with other forms of colitis such as Crohn's disease (CD) or indeterminate colitis
2. Patient with active UC (Mayo > 2)
3. Pancolonic UC (colitis past the splenic flexure, Montreal E3))
4. Gastrointestinal surgery
5. Other GI diseases or systemic diseases (cardiac, renal failure, cirrhosis)
6. Shift work in the last 6 months
7. Antibiotic use within last 12 weeks
8. Patients who have used anti-diarrheal agents such as Lomotil or Imodium within 3 days of the study
9. Prednisone use the last 30 days
10. Significant Depression (score ≥ 14 BDI)
11. Significant Anxiety (score ≥ 40 STAI)
12. Use of probiotic supplement in last 4 weeks except yogurt.
13. Intentional change in diet.

14. Chronic use of NSAIDS. A washout period of 3 weeks is needed before the subject could be enrolled into the study. Low dose aspirin is allowed.
15. Chronic Alcohol use. A washout period of 3 weeks is needed before the subject could be enrolled into the study.
16. Have children under 6 months

Subject Recruitment: All Human UC subjects will be recruited at the RUMC Digestive Disease Clinic and the Rush Crohn's and Colitis Center by the PI or Clinical Coordinator. Healthy control subjects will be recruited by established recruiting methods, which we have successfully used over the past ten years, including notices to local organizations and internet, newspaper, and radio advertising. Based on our experience, of the initial calls made by potential subjects, 15% remain interested in the study after they are informed of study procedures. Of these individuals, 70% pass an initial telephone screening (in which questions are asked about medical condition, current medication use and work and travel history). Of those remaining individuals, 33% pass the medical and psychological screening (described below), and of these, 70% complete the study.

In this study we will recruit 40 Human Subjects in the following two groups:

1) Ulcerative Colitis (UC) (n=20).

2) Healthy Controls (HC) (n=20).

Each potential subject will undergo an extensive screening procedure prior to participation in the study. The procedure begins with a telephone screening, involving a series of questions from a preliminary screening questionnaire and answering any questions potential subjects may have about the study. If the original preliminary telephone screening questionnaire reveals no grounds for exclusion (see "Inclusion/Exclusion Criteria" below), then potential subjects are asked to come to our clinic for a physical examination. These patients will all be interviewed by the study Clinical Coordinator and then get a full physical exam by the PI who is a licensed Gastroenterologist. Potential subjects will also complete several questionnaires to determine their suitability for study. Each potential subject is given a tour of the Clinical Chronobiology Center (CCC) in which they will live during the temporal isolation portion of the study. Every attempt will be made to acquaint each prospective subject with all of the procedures involved in this study in order to minimize the possible effect of uncertainty about the experimental procedures on the results. Written informed consent on an RUMC IRB-approved form will be obtained from each subject before his/her study begins. Each subject will be told that they will be free to discontinue participation in the experiment at any time and that the investigators also reserve the right to discontinue the study for medical or other reasons at any time.

Early Withdrawal: Participants may withdraw at any time.

Procedures: After consenting for the study all subjects: 1) Will be interviewed by a study coordinator and fill out detailed questionnaires so that we can collect demographic data, medical history, data on disease status and research questionnaires; 2) Will supply blood and urine samples (to assess the inclusion/exclusion criteria and to obtain baseline biological markers; 3) Will undergo an exam by PI Dr. Ali Keshavarzian to assess the inclusion/exclusion criteria and to obtain baseline UC disease characteristics and have initial blood drawn/urine sample. 4) After interviews and exam and blood/urine analysis, as part of the screening process, eligible patients will be asked to continue participation and will be re-informed about the study.

All experimental procedures will commence after admission to the CCC for a 7 day in-lab evaluation. The sleep episode will be the same as that maintained during the 2 weeks prior to the in-lab phase assessment. On Test Day 1 (TD1, Baseline alignment), at 0900h all subjects will have a unprepped flexible sigmoidoscopy (FS) with rectal biopsy collection (19 biopsies, ~15 cm from the anal verge), after which intestinal permeability will be assessed (24h urine collection after ingestion of a sugar cocktail: sucrose, mannitol, lactulose, sucralose103), and every 2 hour blood sampling during/surrounding standardized meals (primary and secondary outcomes for Specific Aims 1 & 2). During the last 8h of the phase assessment, subjects will be exposed to a background illumination ~3 lux to assess the endogenous circadian phase through hourly plasma melatonin (dim light melatonin onset, DLMO). On Protocol Day 3, subject behavioral cycles will be shifted by 12h, and this schedule will be maintained until Protocol Day 7. Following the short 4h sleep episode on Day 3, subjects will have 3 “night shift” days (Days 3-5). The 12h shift on Day 3 will be achieved by including an 8h wake episode and a 4h sleep opportunity, thereby maintaining the same sleep opportunity-to-wake ratio (1:2). The rationale for the 12h inversion of the sleep-wake timing is that we previously found that the influence of circadian misalignment on physiological variables are maximal when the sleep/wake and fasting/feeding cycle was shifted by ~12h relative to the endogenous circadian timing system.19,101 On Test Day 2 (TD2, Circadian misalignment), all subjects will have all the identical tests conducted as described on TD1; however, the FS and start of repeated measures will be at 2100 h – matched to the sleep/wake cycle and not clock time to isolate the impact of circadian misalignment. 24 hours prior to each flexible sigmoidoscopy, stool will be collected from subjects. Discharge from the lab will be on day 7 after an 8h sleep period. We will test for order effects (see Statistics).

Blood draw. Blood (30 cc) will be drawn in endotoxin free tube and serum then immediately separated and stored in -80ºC freezer until measurement for Serum endotoxin (END). Blood will also be drawn for CBC, comprehensive biochemical profile [CMP that includes

liver function test], and PT. At first blood draw on Day 2 and last blood draw on Day 7, an additional 10 ml will be collected from patients on biologics, to evaluate antibody levels.

All subjects will complete an IBDQ, FFQ, BDI, STAI, PSQ, Munich Chronotype, IRLS, Demographic Questionnaire, Mayo Score, CSD-C (Consensus Sleep Diary), and Berlin Questionnaire. All information collected from subjects will be used for research purposes only. All the procedures in the study (blood draw, stool samples, urine samples, sigmoidoscopy with biopsies and questionnaires) are well-established and considered routine tests in clinical practice (including flexible sigmoidoscopy).

Diet Assessments: Meal habits will be recorded during screening. All subjects must eat breakfast (>200 kcal) at least 5 days a week, and subjects unusual diets by FFQ (vegan or gluten free) will be excluded due to impact on microbiota. For two weeks prior to each inpatient laboratory stay, all subjects will undergo ambulatory monitoring of their general activity using a small wrist activity monitor for ensuring compliance with the sleep-wake schedule. In addition, subjects will be provided meals for the last 3 days before admission and asked to keep regular meal schedules. Medical and psychiatric condition summary. Volunteers must be ambulatory and have no major visual or auditory handicaps. Medical suitability will be determined by clinical history, physical examination, and clinical biochemical screening tests of blood and urine, past or present psychopathology. Finally, a clinical interview will be carried out on all potential subjects to determine their suitability for the study and their ability to tolerate the conditions of the simulated night shifts environment (with regular contact with research staff and technicians). All volunteers selected must be drug-free with only moderate or no use of caffeine, alcohol, or other compounds by history, which will be verified during the screening process by comprehensive toxic analysis. Caffeine, alcohol, or other compounds (including herbal medications/remedies) will not be permitted for three weeks immediately prior to admission to the Clinical Chronobiology Center (CCC), and a urinary comprehensive toxic analysis will be carried out upon admission to ensure compliance. Prescribed medications are also prohibited with some exceptions.

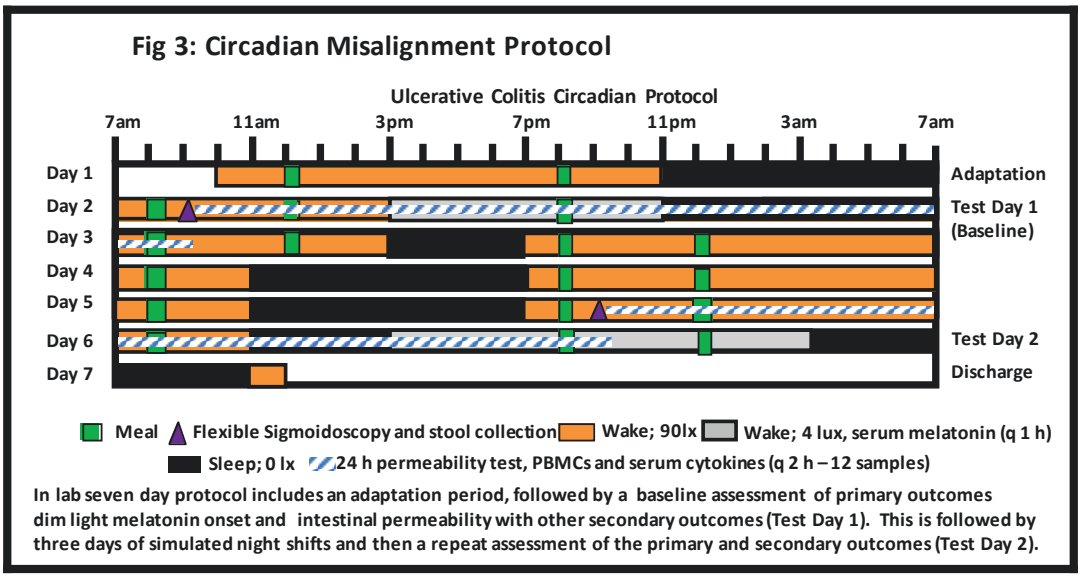
Sleep Assessment: We will use a structured sleep questionnaire, the Pittsburgh Sleep Quality index, to access sleep habits. In addition, a Beck's depression index will be completed to evaluate a common confounder in sleep, depression. 2 weeks before participating in the circadian lab assessment, subjects completed sleep logs and wear a waterproof wrist actigraphy monitor (Actiwatch-L, Spectrum, Bend OR).

Phlebotomy: There may be some discomfort or bruising on the initial insertion of the catheter into a vein, but wearing the catheter should not be painful. Occasionally, mild discomfort may occur from the catheterin the vein. If this happens, it can be repositioned or removed, asking the subject's permission before any subsequent reinsertion. To help keep

the venipuncture site clean, we may ask permission to shave the forearm hair of the subject before insertion of the IV. There is a rare possibility of developing a small blood clot, inflammation, or local infection around the vein where the catheter is inserted, or in rare cases a generalized infection spread through the bloodstream as a result of the IV catheter. Occasionally, there is a black and blue mark at the site of the IV insertion, which may last a couple of weeks; and, rarely, a small scar may remain permanently at the venipuncture site.

Saliva Collection: Participants will be asked to produce a saliva sample on days 2-6 at 7 am and 7 pm. They will be provided a conical tube to spit into. A minimum of 2 ml is needed. To minimize contamination, participants will be instructed to not brush their teeth, use mouthwash, eat, or drink anything (except water) at least 1 hour prior to sampling. The tube of saliva will then be placed into a centrifuge and then aliquoted into 1.5 mL cryovials. One cryovial will have 500ul of saliva + 500ul of Zymo DNA/RNA 2x solution. The second cryovial will be 500ul of saliva flash frozen. These samples will be used for analysis of inflammatory cytokines and 16S sequencing. Core temperature device: On Day 2 and 5 participants will be given a Core device, a non-medical device that will measure their body core temperature. Body temperature will be used to measure the central circadian rhythm. The device will be programmed with the participant's study unique ID and it will be secured on their chest via medical grade adhesives when they get their catheter inserted on day 2 and 5. After activation, the device automatically collects and transmits core temperature data to our database. Once the day is completed the device will be removed from the participant's chest, the adhesive patches will be tossed out and the device will be disinfected.

Flexible Sigmoidoscopy: At two points in the study subjects will have an unprepped limited flexible sigmoidoscopy by Dr. Keshavarzian and sigmoid mucosal samples will be collected:19 endoscopic pinch biopsy samples will be taken and used for: (a) two formalin-fixed and paraffin embedded for histology, (b) two OCT fixed for immunohistochemistry of AJC proteins, (c) two RNA Later for RT-PCR of AJC protein mRNA expression, (d) five snap frozen in liquid nitrogen for Western blot assessment of AJC proteins, (e) two snap frozen in liquid nitrogen for microbiota interrogation, and (f) six fresh samples to generate colonoids. Frozen samples will be transferred from liquid nitrogen tank to -80ºC freezer within 8-12 hours.



Data Collection: All source data will be collected via a paper subject folder and/or a REDCap survey. Subject folders will be coded and stored in a locked cabinet within a locked research room only study staff will have access to. Data from the paper charts will be entered into the REDCap project for the study for electronic storage. The REDCap project is password protected on the Rush server and will only be accessible by IRB approved study staff.

Biospecimen Collection & Storage: All biospecimens collected for the purposes of this study will be coded and stored in the Rush Gastroenterology Research Laboratory. Only IRB approved study staff will have access to these samples.

Data Analysis Methods: 40 human subjects will be recruited into this proposed study: 20 Healthy Controls and 20 left sided UC subjects with inactive disease (Mayo Score ≤ 2) on stable mild to moderate medications (aminosalicylates, 5-ASA) . All subjects (UC and HC) will be interviewed by the PI and will undergo a full medical history and physical examination including blood draw and urine collection. The PI is a licensed physician Gastroenterologist who specializes in the management of inflammatory bowel disease, to determine their eligibility and co-morbidities. Subjects who wish to participate will have the study explained by the coordinator and PI and will then sign a Rush IRB-approved informed consent. All UC subjects will complete a validated questionnaire regarding disease specific symptoms (Mayo Score) to determine they are inactive and may be enrolled in the study.

In addition, subjects will provide basic demographic data, UC history if appropriate, UC disease activity status as detailed further above, and Munich Chronotype questionnaire. All subjects will complete a validated 3 month food frequency questionnaires (FFQ) to collect dietary information (149). 40 participants (Specific Aims 1-2) will be enrolled for the within-subject prospective in-laboratory study protocols. Participants will be young (18-50 y), healthy,

non-diabetic, non-obese, Caucasian/African American/Asian and non-Hispanic/Hispanic in ratios consistent with the population of Chicago, and without medication use (excepting oral contraceptives) for HC. HC and UC subject will be matched according to age ($\pm$ 3 years), sex, race, and BMI ($\pm$ 3 kg/m2). UC subjects will have only left sided disease, be on stable medications with no recent use of prednisone or antibiotics for the last 3 months. After signing an informed consent, subjects will fill out questionnaires start a food diary, and have 3 weeks of wrist actigraphy prior to their seven day in lab session in the Clinical Chronobiology Center (CCC). Since the participants will be in the sleep lab for 7 days, the questionnaire responses will be assessed for evidence of psychopathology, which is part of the exclusion criteria. Dr. Sharon Jedel, Ph.D., Associate Psychologist, will review the questionnaire responses before the participant finishes the screening. Participants who show some evidence of psychopathology on the questionnaires will undergo a structured interview (SCID-R) (~30 min) to confirm if they are ineligible.

Subject Characteristics: We will recruit (1) 20 HC subjects without any medical illnesses (2) 20 subjects with biopsy proven left sided UC, inactive (Mayo Score ≤ 2) on stable medications and no disease flares for > 3 months. HC and UC subject will be matched according to age ($\pm$ 3 years), sex, race, and BMI ($\pm$ 3 kg/m2).

Study Outcomes:

Primary outcome measures:

- **Aim 1** – Circadian Misalignment of central clock: Phase Angle will be measured as the difference between circadian clock (dim light melatonin onset) and behavior cycle (sleep onset)
- **Aim 2** – Barrier Function: Mayo Endoscopic Score and Intestinal permeability will be measured including 12-24-h urinary sucralose (primarily representing colonic permeability)

Secondary outcome measures:

- **Aim 1** – Circadian Misalignment of peripheral clocks: Clock gene expression (CLOCK, BMAL) in PBMCs during each phase assessment and in colonic biopsies
- **Aim 2** – Barrier Function: Colonic mucosal AJC proteins (E-cadherin, occludin, claudin2,3,4), stool calprotectin and mucosal and stool microbiota composition and function. Studies will include serum markers of intestinal permeability and bacterial translocation [lipopolysaccharide (LPS) and LPS-binding protein (LBP)]; serum zonulin and intestinal fatty acid protein; immune changes (serum interleukin-6 as proinflammatory marker and soluble CD14 as marker of macrophage/monocyte

activation); and stool and serum metabolomics: short-chain fatty acid (SCFA) and Trimethylamine N-oxide (TMAO) and TMA.

Exploratory Outcome Measures:

- **Aim 1** – Colonic clock gene expression and Per2::Luc activity in colonic organoids over 24 hours in both groups and each condition
- **Aim 2** – RNA seq of colonic organoids every 2 hours for over 24 hours to identify key molecular and inflammatory pathways under circadian regulation
- **Aim 3**: Use body temperature to measure central circadian rhythm to see if it correlates with melatonin levels at baseline and after misalignment

Figure 1. Outcome Measures

Aims	Outcome Measure	Test	Assessment	Statistical Test
Aim 1	Primary	Phase Angle of entrainment (DLMO – sleep onset)	Baseline Misalignment	ANOVA
	Secondary	Clock gene expression in PBMCs	Baseline Misalignment	Cosinor Analysis, Mixed Model ANOVA
	Secondary	Urinary Melatonin	Baseline Misalignment	Wilcoxon signed-rank test
	Exploratory	Clock gene expression and Per2::LUC in Colonic Organoids	Baseline Misalignment	Cosinor Analysis, FFT-NLLS
Aim 2	Primary	Intestinal Permeability (i.e. 12-24 h Urinary Sucralose)	Baseline Misalignment	Mann-Whitney U test
	Secondary	Mayo Score (Endoscopy)	Baseline Misalignment	Wilcoxon signed-rank test
	Secondary	Stool Calprotectin	Baseline Misalignment	Wilcoxon signed-rank test
	Secondary	Serum cytokines (IL-6, TNF-α)	Baseline Misalignment	Repeated measures ANOVA
	Secondary	Fecal Microbiota	Baseline Misalignment	Principal Coordinate Analysis
	Secondary	Serum LBP, LPS, zonulin, sCD14, metabolomics	Baseline Misalignment	Repeated measures ANOVA
	Exploratory	TER in 2D Colonic	Baseline	ANOVA

		Organoids with *ex-vivo* TNF-α/INF-γ	Misalignment	
	Exploratory	RNA seq of Colonic Organoids	Baseline	JTK Cycle
			Misalignment	

Safety and Adverse Events: We have identified five key potential risks to human subjects in this study:

1. Risk to Confidentiality and Record-Keeping of patient personal health information (PHI).
2. Risk of potential side effects associated with Phlebotomy (blood draw).
3. Risk of potential side effects associated with Sigmoidoscopy and Colonic Biopsy.
4. Risk of potential side effects associated with completion of the Questionnaires.
5. Risk of potential side effects associated with in lab study

Minimizing Risk:

1. **Maintenance of Confidentiality and Record-Keeping.** Subject confidentiality will be protected by maintaining all records of participation in this research project under a double lock and kept confidential as required by law. Electronic records are within the RUMC firewall and password protected. Data of subjects will only be associated with a coded identification number, having had all identifying information removed. Patient PHI information will be available only to the PI and clinical coordinators.
2. **Minimize the risk of potential side effects associated with Phlebotomy.** Blood will be drawn over two 24 hour periods. The PI has significant experience with this protocol from previous studies. There may be some discomfort or bruising on initial insertion of the catheter into a vein, but wearing the catheter should not be painful. Occasionally, mild discomfort may occur from the tube in the vein. If this happens, it can be repositioned or removed, asking the subject's permission before any subsequent reinsertion. To help keep the venipuncture site clean, we may ask permission to shave the forearm hair of the subject prior to insertion of the IV. There is a rare possibility of developing a small blood clot, inflammation, or local infection around the vein where the catheter is inserted, or in rare cases a generalized infection spread through the bloodstream as a result of the IV catheter. Occasionally, there is a black and blue mark at the site of the IV insertion, which may last a couple of weeks; and, rarely, a small scar may remain permanently at the venipuncture site. A standard laboratory protocol for surveillance for heparin-induced thrombocytopenia will be followed during the protocol. This consists of monitoring the subject's platelet levels at baseline and every 2 days, when signs of any heparin-induced thrombocytopenia would be expected to appear.

There has not yet been a case of this in our laboratory in over 8 years of operation. Prior to insertion of the IV catheter, the skin around the insertion site will be washed with a Betadine solution followed by alcohol. An anti-bacterial ointment will be applied to the IV catheter insertion site prior to dressing the site. Hypoallergenic tape will be used on the IV dressing to minimize the development of contact dermatitis. The IV catheter insertion site will be checked every 24-36 hours for signs of infection by removing the dressing and cleaning the site with alcohol. The IV catheter dressing, the IV bag/bottle, the IV tubing and manifold and the antibacterial ointment will be changed every 24-36 hours to minimize the potential for infection. The IV catheter insertion will be done while the subject is supine, and a staff member will remain in the suite with the subject for at least 10 minutes after the insertion to ensure that the subject does not feel light-headed or dizzy. The subject's vital signs will be checked at least once per day. This will include pulse rate, respiratory rate, systolic and diastolic blood pressure.

3. **Minimize the risk of potential side effects associated with Sigmoidoscopy.** The primary risk for most subjects undergoing sigmoidoscopy and biopsy is minimal discomfort during sigmoidoscopic examination. Risks include discomfort from air being added into the colon, bloating much like gas pain, possible irritation and a small amount of blood loss. On extremely rare occasions (1 in 17,000), the procedure can cause a tear or hole in the lining of the colon or significant bleeding. This may require surgery to repair. The PI (GS), who will perform all sigmoidoscopic examinations, is an experienced gastroenterologist with extensive experience in endoscopy over the last 10+ years. He has never experienced any subject complications while performing thousands of sigmoidoscopies. Furthermore, these sigmoidoscopic examinations will be limited to the most distal 15 to 20cm of the rectum and sigmoid rather than the typical, routine examination that requires advancing the scope to 45 to 60 cm (this shorter advancement of the scope is adequate to determine whether there is active disease and to obtain biopsies). Thus, discomfort and risk will be markedly lower than routine testing. Thus, unlike in routine sigmoidoscopy, we will do the procedure without any colon preparation (eliminate discomfort associated with laxative) and to a more limited extent. This limited procedure is not associated with any significant discomfort (no need to negotiate colon flexures that is the main source of discomfort associated with endoscopic procedures) and significantly decreases (and in fact eliminates) the tiny risk of perforation (< 1/10,000) associated with routine sigmoidoscopy. This opinion is based on our lab's 30+ years of experience performing limited sigmoidoscopy in research subjects in our sections and also based on personal experience—the PI (GS) has been a "healthy control" subject in several prior studies and has undergone this limited sigmoidoscopy and biopsy. The biopsy procedure is routine and painless with a tiny risk (< 1/10,000) of significant bleeding. The PI has obtained pinch mucosal biopsy

specimens from the colon from thousands of subjects including as many as 18-24 biopsies on many occasions. He has not had any complications or bleeding in any of his research subjects over the past 10+ years. The expertise and experience of the PI and research team should significantly minimize the potential complications including bleeding and perforation. Plan to deal with complications. Subjects with possible complications will be seen by the PI immediately (within a few hours), regardless of the time of day. The PI has full access to the GI Suite 24/7. These subjects will be evaluated, and if necessary, will undergo additional testing, such as CBC and KUB. If there is evidence of bleeding or perforation, the subject will be admitted to the hospital for observation and additional therapy. As stated above, we do not expect any complications such as bleeding or perforation. Subjects will have blood tests, including prothrombin time and platelet count, to avoid biopsy procedures in those with a bleeding disorder.

4. **Minimize the risk of potential side effects associated with completion of the Questionnaires.** We have taken steps to reduce the risk of discomfort associated with answering medical/personal questions by keeping an individual's responses completely confidential (including not sharing an individual's responses with their physician), allowing participants to leave blank any questions that may make them uncomfortable on any of the forms, and providing brief assessment/counseling by the Division clinical psychologist or PI for any subject who becomes distressed during their participation in the study. If a participant appears to have difficulty reading, they can choose to have the questionnaires read to them.
5. **Minimize the risk of potential side effects associated with in lab stay.** The subject may become sleepy during some segments of the study. The subject will be asked to remain awake during the entirety of their scheduled wake times. Should the subject feel that he/she is unable to remain awake, he/she is free to withdraw his/her consent to participate in this experiment and then go to sleep. At the end of the study, the subject may have difficulty sleeping and waking at their usual times. It may take several days to readjust to their regular routine. This experience is similar to jet lag, and may be associated with upset stomach, insomnia, irritability, or excessive daytime sleepiness. The protocol is designed so that changes in circadian phase are minimized. Subjects may not sleep as well in the laboratory as they do when at home. This may be exacerbated during the simulated night shift when subjects remain continuously awake. Nevertheless, short term partial sleep loss has never been shown to be directly deleterious to health although sleep loss can cause drowsiness and increase the risk of accidents. While dangerous accidents are unlikely in the controlled environment of the laboratory, we have less control when subjects leave the laboratory. Thus, we will ensure that the subject fully appreciates this risk and they will be given the opportunity

to have recovery sleep after completion of the protocol before leaving the laboratory. Taxi rides are arranged so that subjects can return home safely.

6. **Minimize the risk of potential psychological stress and suitability of in lab stay.** Subjects with any psychiatric history will be excluded from the study. This includes individuals with a history of psychiatric illnesses or psychiatric disorders will be excluded such as alcoholism, drug dependency, major mood disorders such as major depression and manic depressive illness, schizophrenic disorders, anxiety disorders including panic disorder, generalized anxiety disorder, post-traumatic stress disorder, obsessive compulsive disorder, agoraphobia, claustrophobia, paranoid personality disorder, schizoid personality disorder, schizotypal personality disorder, borderline personality disorder, and antisocial personality disorder. Participants will complete questionnaires prior to their in-person stay in our sleep lab, which the study physician will review. If a participant feels distress during their stay, the study physician will be called. Participants may leave at any point if they feel psychological stress.

Safety Monitoring Board: Although the study physician and coordinators will evaluate a participant's comfort level prior to the participant staying in the sleep lab and during their in-person stay, the study will have a Data Safety and Monitoring Board. The board will convene every six months to evaluate the study data for participant safety, study conduct, and progress, and make recommendations if applicable. This will ensure that the study participant's safety is always being evaluated and improved.

Cost to Participants: There is no additional cost to participants in this study. All study activities will be covered by the study grant.

Payment to Participants: Participants may receive up to a total reimbursement of $2,000 for completing all study activities. Each participant will receive up to $1500 reimbursement for the cost of travel and time invested in the study along with an additional $500 compensation incentive for completing both in-laboratory protocols. Participants will receive $700 after completion of the Day 2 study activities, $500 after completion of Day 3 and Day 5 activities, and the remaining $800 once all study activities have been completed and the actigraphy watch has been returned to study staff.

Data Management: Data and Biospecimens will be securely stored on-site at Rush University Medical Center for up to 6 years post study closure. Participants will be offered to participate in the department's tissue repository long-term storage of their data and biospecimens for the purposes of future use. For any participants who do not consent to the tissue repository, their biospecimens will be destroyed and their data deleted 6 years post-study closure.

Confidentiality: Subject confidentiality will be protected by maintaining all records of participation in this research project under a double lock and kept confidential as required by law. Electronic records are within the RUMC firewall and password protected. Data of subjects will only be associated with a coded identification number, having had all identifying information removed. Patient PHI information will be available only to the PI and clinical coordinators.

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